Sperm Protamine and Chromatin Integrity: As Biomarkers of Sperm Quality and Assisted Conception Outcome

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

By

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“In the Name of ALLAH, the most Beneficent, the most Merciful”

Dedicated to my Mother, Father (late)

and Family

For their endless love, patience, kindness, and support

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Riffat Bibi

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LIST OF ABBREVIATIONS

ACTH	Adenocorticotropin hormone
AgRP	Agouti-related peptide
ALH	Amplitude of the lateral head displacement
ART	Assisted reproductive techniques
ATZs	Astheno-teratozoospermic
BMI	Body mass index
CART	Cocaine and amphetamine-regulated transcript
CASA	Computer-assisted sperm analysis
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
H	Head
FSH	Follicle-stimulating hormone
GH	Growth hormone
GnRH	Gonadotrophin releasing hormone
hCG	Human chorionic gonadotrophin
HMT	Head midpiece tail
HT	Head tail
LH	Luteinizing hormone
LIN	Linearity
M	Midpiece
MCH	Melanin concentrating hormone
mRNA	Messenger ribonucleic acid
MSH	Melanocyte stimulating hormone
MT	Midpiece tail
NON-OBST-AZOOs	Obstructive azoospermia
NPY	Neuropeptide-y
HM	Head midpiece
OATZs	Oligo-astheno-teratozoospermics
OBST-AZOOs	Obstructive azoospermia

OZs	Oligozoospermics
PZs	Polyzoospermics
SDI	Sperm deformity index
T	Testosterone
TRH	Thyrotropin-releasing hormone
TZI	Teratozoospermic index
TZs	Teratozoospermics
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
DFI	DNA fragmentation index
MFI	Male factor subfertility
SCD	Sperm chromatin dispersion
TB	Toluidine blue
CMA3	Chromomycin A3
FR	Fertilization rate
CLBR	Cumulative live birth rate
TUNEL	Terminal transferase dUTP nick-end labeling
ROS	Reactive oxygen species
PGS	Pre-implantation genetic screening
HCG	Human chorionic gonadotropin
HOS	Hypo-osmotic swelling test
EG_A	Embryo grade A
SCSA	Sperm chromatin structure assay
CR	Cleavage rate
TNMS	Total motile sperms
BR	Blastocyst rate
IR	Implantation rate
ET	Embryo transferred
TEC	Total egg collected

2PN	Two pronuclei
MII	Metaphase two oocyte
ROC	Receiver operating characteristic
AUC	The area under the curve
OR	Odds ratios
AO	Acridine orange test
ORT	Oocyte retrieve
CI	Confidence interval
COH	Controlled ovarian hyper stimulation
OPU	Oocyte pick-up
CMI	Chromatin maturity index
IVF	*Invitro* fertilization
ICSI	Intra cytoplasmic sperm injection
AMH	Anti-Mullarian hormone
WHO	World health organization
MACS	Magnetic activated cell selection
DGC	Density gradient centrifugation
S-up	Swim-up
SDF	Sperm DNA fragmentation
N	Normozoospermia
MMF	Moderate male factor
SMF	Sever male factor
MFI	Male factor subfertility

GENERAL ABSTRACT

Background: Subfertility affects one out of every seven couples worldwide. After a year, about 25% of couples had no children despite the unprotected sexual activity, 15% got medical advice, and fewer than 5% were still unable to conceive despite their best efforts. In over half of the cases, the underlying etiology is attributed solely to men. Standard sperm analysis is the most essential laboratory investigation for men. Semen analysis, frequently indicates oligozoospermia (low sperm count), asthenozoospermia (poor sperm motility), or morphologically aberrant spermatozoa. In contrast, oligoasthenoteratozoospermia is the condition in which all of these anomalies coexist. The World Health Organization's (WHO) 2010 standard semen analysis, which is used to evaluate treatment options and the chance of spontaneous conception, considers sperm count, motility, and morphology. The use of assisted reproductive technology (ART) to treat subfertility has dramatically increased in recent years. The introduction of these novel procedures, particularly intra-cytoplasmic sperm injection (ICSI), has resulted in a demand for more effective male fertility potential investigation tools. Most of these novel diagnostic approaches focus on the male gamete's genetic integrity.

The growing concern about the possible transmission of genetic diseases through ICSI, where natural control mechanisms during spermatocyte interaction are bypassed, potentially resulting in cytogenetic aberration, foetal deformities, and postnatal anomalies in babies born through ICSI. Even though ART has reached its maximal level of efficacy, the "take-home baby" rate has remained stable for several years. One of the explanations could be a lack of male diagnosis and treatment. DNA damage can be caused by abnormal chromatin folding during spermatogenesis, and defective apoptosis just before ejaculation causes abnormal ROS production in the ejaculate, and extra-testicular factors such as age, body mass index, lifestyle, hormonal factors, hyperthermia, and in-vitro semen handling. Spermatozoa preparation technique and storage temperature post preparation affect sperm quality, chromatin integrity, ability to fertilize the oocyte, post-fertilization-embryo cleavage rate, embryo quality, blastocyst formation, and implantation rate.

Aim: The study aims to explore the role of protamines in the condensation of sperm chromatin and the impact of abnormal protamine expression on chromatin integrity.

Additionally, the research aims to assess the effectiveness of utilizing protamine and chromatin integrity as predictors of assisted conception outcomes, including fertilization rates, embryo development, and pregnancy rates.

Objective: Current study was designed for the thorough analysis of the molecular and biochemical markers for male subfertility

1. Compare semen parameters, sperm viability, oxidative stress markers, and reproductive hormone levels in fertile and subfertile men.
2. To estimate sperm DNA fragmentation and chromatin damage in fertile and subfertile men, and to assess the clinical value of sperm chromatin structure assay (SCSA), sperm chromatin dispersion assay (SCD), toluidine blue (TB) and acridine orange testing (AOT).
3. To evaluate the impact of high DNA fragmentation and chromatin decondensation on the outcome of standard IVF and ICSI.
4. To evaluate the impact of sperm preparation methods on sperm DNA fragmentation, chromatin condensation, and IVF, ICSI and PGT outcomes.

Materials and methods: A total of 753 couples were recruited for the study 146 fertile/control (without any history of fertility problems), and 607 male factor /subfertile (with a history of subfertility) were recruited. A couple of details history and relevant information were collected from each subject with informed consent. Semen and blood from the male partner were obtained for further analysis.

Each semen sample was further split into aliquots: 1). standard semen analysis, vitality, reactive oxygen species, sperm DNA damage, and chromatin abnormalities, 2). sperm processing to inseminate oocytes through ICSI/IVF/PGT. According to WHO 2010 standards, sperm counts, motility, and morphology were examined. Sperm vitality was tested utilizing the hypo osmatic test (HOS) and eosin nigrosin stain test. Reactive oxygen species (ROS), thiobarbituric acid-reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POD) levels were analyzed through a spectrophotometer. Male and female partner serum follicular stimulation hormone (FSH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH) levels were

determined, in men's testosterone (T) levels and female's estradiol (E_2), progesterone (P_4) and anti-mullerian hormone levels (AMH) were measured, through spectrophotometer.

DNA fragmentation index (DFI) in spermatozoa was analyzed by sperm chromatin dispersion (SCD), sperm chromatin structure assay (SCSA), and acridine orange testing (AO). Chromomycin A3 (CMA3+) chromatin maturity index (CMI) and toluidine blue (TB+) staining to measure sperm chromatin condensation (maturity) in a male subject.

In an experiment, the sperm chromatin integrity in the categories of fertile and subfertile patients was measured in two BMI groups (normal weight and overweight). The potential effects of a father's high BMI on fertilisation, embryo quality, the overall live birth rate, and birth weight were also examined, as well as the correlation matrix.

Another aspect of the investigation involved measuring the chromatin integrity of sperm from viable and subfertile men that had been cryopreserved and prepared using different techniques such as swim-up (S-UP), density gradient centrifugation (DGC) and magnetic-activated cell sorting (MACS). In a different experiment, the success of assisted conception was examined between two groups of sperm DNA damage (20% and >20%). Sperm, protamine, or chromatin condensation (30 and > 30%), as well as female features and hormonal parameters that affect ART results, were compared across the board in the last set of studies. The impact of sperm preparation and storage methods as well as sperm chromatin integrity on the success of assisted conception was also evaluated.

To test for the statistically significant differences between the two groups and across all of them, ANOVA with post hoc tests (such as Tukey) was performed. To assess differences in means, a spearman correlation analysis was used between the various parameters. For those outcomes that were connected to one or more examined parameters, prediction models were built. To look for continuous outcome variables, a logistic regression model was used. The statistical package for social sciences (SPSS) 20 IBM was used for all statistical analyses. The Hosmer-Lemeshow goodness-of-fit test was used to determine the model's dependability. A p-value of <0.05 was considered to be statistically significant.

Results: Several parameters including male and female age, BMI subfertility duration, socioeconomic status, the weight of the child and in men semen volume, PH, WBC/HPF,

liquefication time (min) were comparable between the groups, while reduced sperm concentration $x10^6$ ($p=0.002$), progressive motility ($p<0.01$) and sperm morphology ($p<0.001$) were elevated in MMF and SMF subfertile couples as compared to control group. CASA parameters VSL, VCL, VAP, and ALH were reduced in MMF and SMF. The sperm membrane integrity (HOS) percentage of fertile subjects measured through HOS was 86.50±0.96%, whereas in MMF was 69.55±0.73%, similarly, SMF had membrane integrity of 63.07±1.21%. Sperm vitality (eosin) in subfertile men was 71.74±1.26 and in MMF and SMF were 48.85±1.18 and 49.69±1.25 respectively. There was a significant difference in SOD ($P<0.001$) and POD ($P<0.001$) levels in a fertile male group compared to fertile control. SMF patients had significant ($P<0.001$) lower levels of SOD, POD, CAT, and higher levels of ROS and TBRAS compared to MMF and fertile male patients. A strong correlation was found between oxidative stress (ROS) with BMI, impaired semen parameters, and HOS. Comparable hormonal (FSH, LH, prolactin) levels ($p>0.05$) while a significant decrease in serum testosterone concentration was found in MMF and SMF male patients compared to fertile control subjects.

Sperm DNA fragmentation and chromatin damage of fertile men was significantly ($p\leq0.05$) lesser than in subfertile men categories i.e., moderate male factor (MMF) and severe male factor (SMF), sperm DNA damage correlated with subnormal semen parameters concentration, motility, morphology, sperm velocity. There are raised ROS levels in semen of subfertile men compared to fertile men and a positive correlation between oxidative stress markers and sperm DNA damage in fertile and subfertile men was observed. Sperm chromatin integrity by CMA3+ and TB+ showed a significant ($p<0.05$) increase in chromatin decondensation in SMF and MMF subfertile men. An increase in sperm DNA fragmentation above 20 % value of SCSA, SCD, and AO affects the men's fertility outcome in MMF and SMF men. Three methods to determine sperm DNA fragmentation were SCSA, SCD and AO and SCD had the same prognostic value as SCSA to evaluate sperm DNA fragmentation. The intra-assay variation in DFI, measured by SCSA was more closed values measured using the SCD technique than AO. Higher than 20% DNA fragmentation and higher than 30 % chromatin decondensation affect the fertilization rate and cleavage rate but had no effect on pregnancy and live birth rate after

standard IVF and ICSI. DNA fragmentation and chromatin maturity can predict fertilization, cleavage, and but not aneuploidy and live birth rates in ART.

Paternal BMI correlates significantly ($p<0.05$) negatively with semen parameters (concentration, motility, morphology, and vitality), DNA fragmentation, and chromatin maturity. The analysis of the percentage of spermatozoa with chromatin maturity (CMA3+) and chromatin integrity (TB+) was reduced significantly in overweight men ($p<0.01$) compared with a reference group. Increase in paternal BMI correlate with the increase in sperm chromatin damage (SCD r= 0.282, TB+ r= 0.144, $p<0.05$), immaturity (CMA3+, r=0.79, $p<0.05$) and oxidative stress (ROS) (r=0.282, $p<0.001$). Peri-fertilization effects were an increase in oocytes fertilization in couples with overweight men (FR =67%) compared with normal-weight men (FR=74%), similarly, paternal overweight correlates with poor fertilization(r=-0.187, $p<0.01$), after multiple regression paternal weight remain predictor of successful fertilization. During the developmental stage, the number of embryo in cleavage was higher in normal-weight men, while day 3 (D3) embryos, percent good quality embryo D3, and blastocyst formation rate were comparable between the groups. A negative correlation was found between implantation rates and paternal BMI (–r=0.110, $p<0.01$). The paternal overweight group (2952.14±511.64gm) had increased neonatal birth weight (within normal range) when compared with the reference group (2577.24±324.94gm, $p<0.001$) following assisted reproductive technology (ART). The cumulative live birth rate (CLBR) was higher ($p<0.05$) than normal weight men compared to the paternal overweight group. In CLBR per embryo transfer and per FR used was the difference between groups statistically significant ($p<0.05$). We found paternal overweight BMI > 24.5 kg/m^2 had a reduced fertilization rate with an OR of 1.98(CI 95% 1.323-2.967, p=0.001). After controlling for several potential confounders, the multiple linear regression model revealed a positive relationship between paternal BMI with fertilisation rate and CLBR (weight within normal range). The present study demonstrated the impact of paternal overweight on male reproductive health, as these patients with overweight had a higher percentage of immature sperm (CMA3+) with impaired chromatin integrity (SCD, TB+) in their semen and had decreased fertilization rate, CLBR following assisted reproductive treatments. The present study supports that paternal overweight should be regarded as one of the predictors for fertilization, CLBR and useful for counseling, to

consider body mass index not only in women but also for men, in couples opting for ART treatment, and warrant a poor reproductive outcome in overweight men. Male patients with old age (>40) had a higher percentage of immature sperm (CMA3+) with impaired chromatin integrity (SCD, TB+) in their semen. Female characteristics were comparable between all groups. Female age and BMI kg/m^2 significantly negatively correlated with the number of the oocyte, fertilization rate, cleavage rate and pregnancy rate.

DGC-MACS technique along with the classic sperm preparation (DGC, SU, DGC-SU) methods, significantly ($p<0.05$) improved semen parameters. DGC-MACS sperm preparation methods, then other methods of sperm preparation (DGC, SU, DGC-SU) and significantly ($p<0.05$) better yield mature sperm concentration with intact sperm chromatin integrity. Paternal overweight impacts fertilization, embryo quality, live birth rate, and birth weight. In four semen preparation groups i.e., DGC, SU, DGC-SU, and DGC-MACS, in male subjects with teratozoospermia and men with increased (≥ 20%) and normal value (< 20%) SDF a threshold value determine, all four preparation techniques had significant ($p<0.05$) improved number of spermatozoa with and after DGC-MACS a significant ($p<0.05$) improvement in mature and intact DNA, condensed chromatin, viability. DGC-MACS sperm preparation techniques had significant ($p<0.05$) ICSI cycles success in improving the percentage of fertilization, cleavage rate, pregnancy rate and non-significant improvement in the live birth rate after DGC-MACS

Conclusion: Fertility is linked to sperm quality, and standard sperm analysis is insufficient for predicting male subfertility. Testing for sperm DNA integrity and chromatin condensation are helpful prognostic tools for IVF/ICSI patients. Abnormal sperm morphology can reduce fertilizations and increase failed implantation, and oxidative stress and morphology assessment may be helpful for treating male subfertility.

Paternal overweight and age are factors contributing to the global drop in male fecundity, and less than 40 years of age and normal weight prognostic for better ART success in terms of fertilization.

Sperm preparation method for better quality sperm separation and post-24-hour quality sperm isolation was combining the density gradient centrifugation with MACS. DGC-MACS preparation technique is a safe and cost-effective method to improve assisted

reproduction outcomes. Further studies are necessary to validate these findings and clarify the issue of male subfertility.

Testing for sperm DNA integrity and sperm chromatin condensation are prognostic tools for IVF/ICSI patients, and sperm quality parameters are one of the factors contributing to male fertility. Male age and BMI measurement should be considered in couples opting for ART treatment, and younger age and weight loss before undergoing in-vitro fertilization procedures can improve quality of sperm and increase fertilization rate. Further studies and meta-analyses are necessary to validate these findings and clarify the issue of male subfertility.

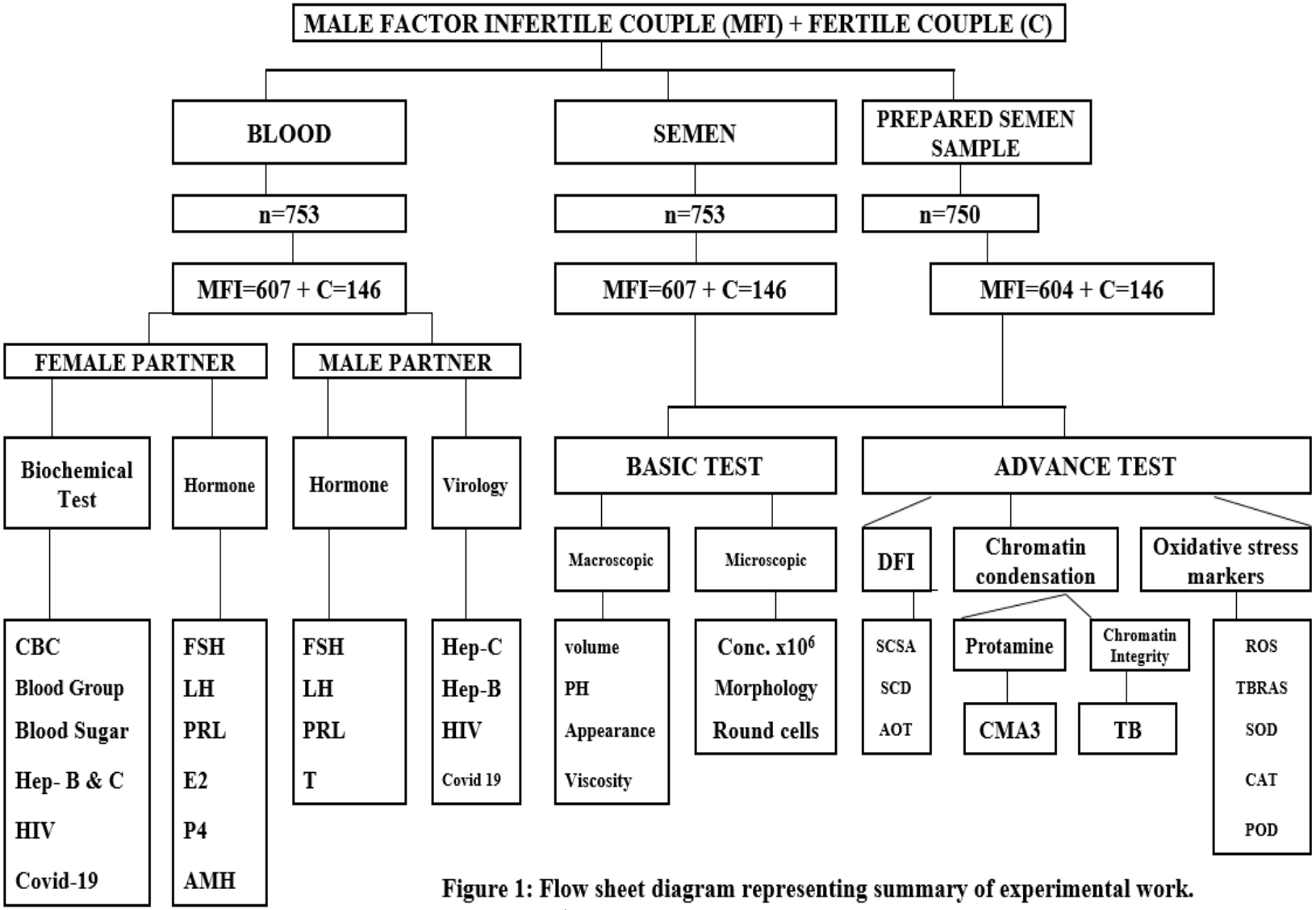
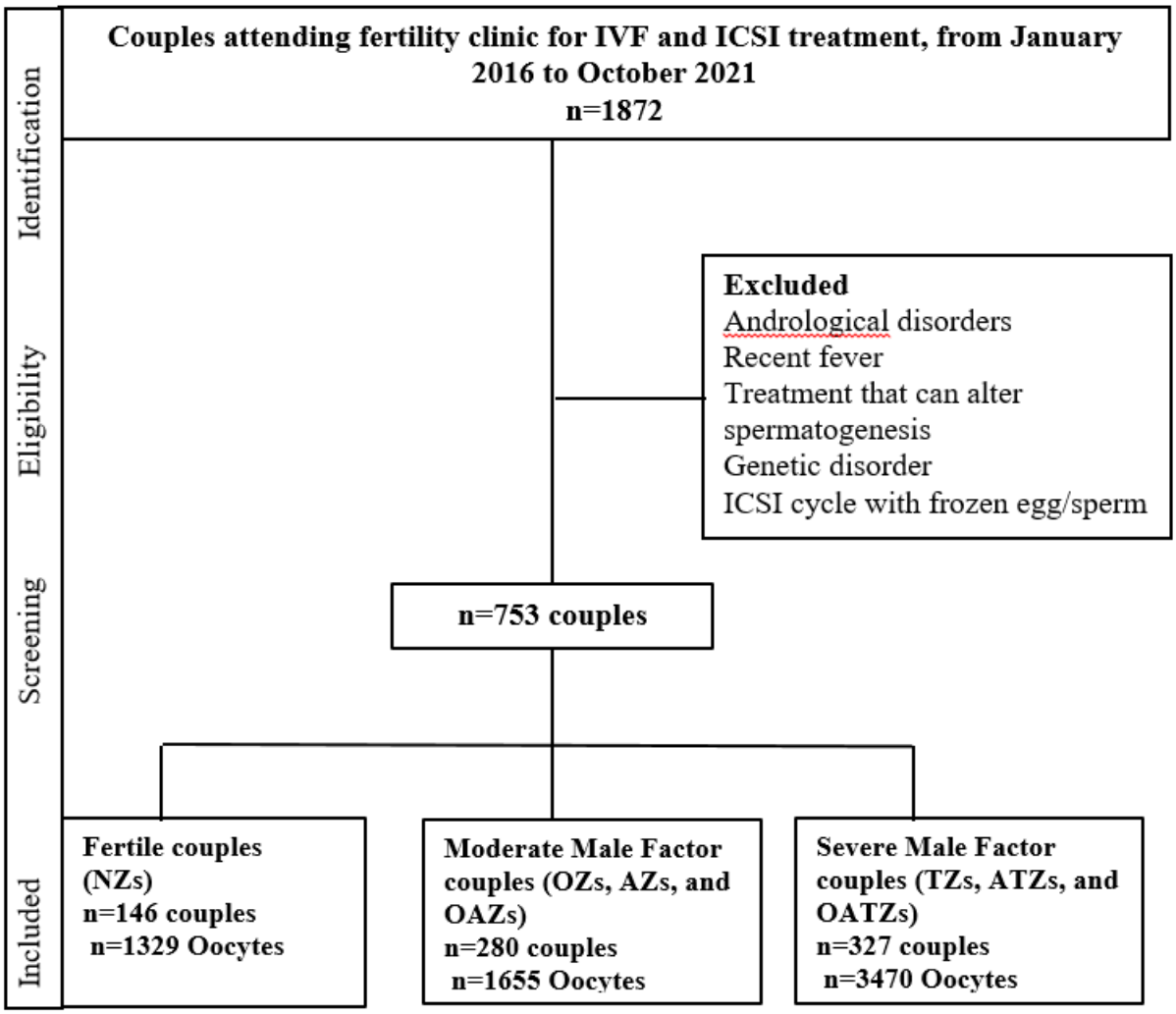


Figure 1: Flow sheet diagram representing summary of experimental work.



ICSI/IVF + PGT-A

PRIMARY OUTCOME	**SECONDARY OUTCOME**
Fertilization rate	Positive clinical pregnancy
Cleavage rate	Implantation rate
Blastocyst rate	Cumulative live birth rate
Euploid & aneuploid embryos	Neonatal birth weight

Figure 2. Schematic recruitment in the study and outcome measures.

GENERAL INTRODUCTION

Introduction

1. Male subfertility

1.1. Definition and prevalence

Subfertility is a medical and social issue, both in terms of magnitude and impact on well-being. Subfertility is the inability of a couple to conceive or become pregnant following a year of consistent, unprotected sexual activity (Zegers-Hochschild *et al.*, 2017; Stanaway *et al.*, 2018). As a result of the apparent increased prevalence of subfertility, the World Health Organization (WHO) has recognized subfertility as a reproductive system disease since 2010 (Topp *et al.*, 2015). It is estimated that 4-17 percent of couples seek medical treatment for subfertility. Finally, 3-4 percent of all couples are childless involuntarily at the end of their reproductive life phase. Subfertility is a developing problem that affects all cultures and societies around the world. It affects at least one out of every six couples and approximately 10% -15% of all adult married couples (Mascarenhas *et al.*, 2012). Gynecologists in most parts of the developing world have difficulty investigating subfertile couples because of the widespread belief that the cause of subfertility is female, and because males rarely present themselves for investigation, making it difficult to determine the true contribution of the malefactor to subfertility. However, estimating the exact prevalence is difficult, and more epidemiological studies are needed. One issue is that subfertility is extremely diverse and usually complex, with numerous male and female factors potentially contributing. Approximately 15% of all couples of reproductive age are experiencing subfertility issues, with malefactors accounting for more than a quarter of all subfertility cases. Subfertility is caused by male factors in about one-third of cases, by both male and female factors in another two-third, and by female factors in the remaining. The reason why a couple experiences fertility issues is unknown in 10–20% of cases. (Ombelet *et al.*, 2008; Zegers-Hochschild *et al.*, 2017).

Despite high fertility rates, subfertility rates in low-income countries range from 22 percent in South Asia to 29 percent in some Sub Saharan African countries (Agarwal *et al.*, 2015)

Figure 3. Pakistan is one of the world-populated countries and has a population growth rate of around 2%, and a higher rate of subfertility (21.9%) the prevalence of subfertility is 21.9 percent, with 3.9 percent being primary subfertility and 18.0 percent being secondary subfertility (Zegers-Hochschild *et al.*, 2017; Ahmed *et al.*, 2020). This implies direct affected country's married population with this problem is more than one-fifth (Figure 3 & 4). However, the high population growth rate subjugates this fact, according to statistics on married Pakistani females, with average 6.5 numbers of children per female (Ahmed *et al.*, 2020).

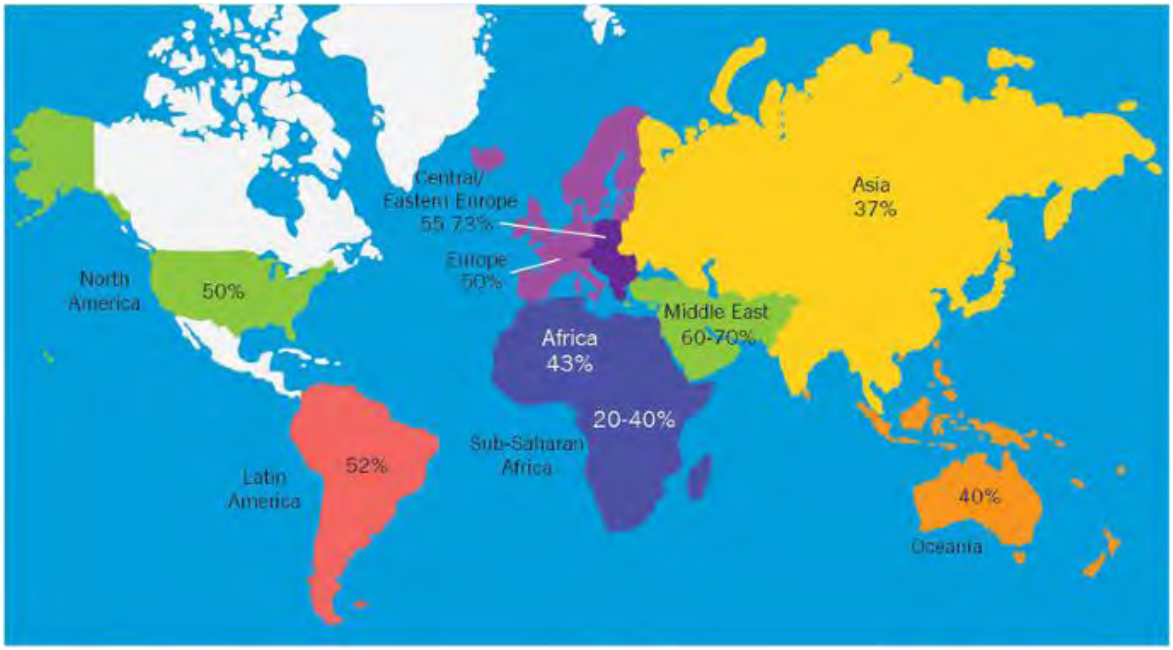


Figure 3. A map of the world with rates of subfertility cases in each of the studied regions—North America, Latin America, Africa, Europe, Central/Eastern Europe, the Middle East, Asia, and Oceania—attributable to male factors. Incidence of subfertility worldwide (Agarwal *et al.*, 2015).

Subfertility is a condition with psychological, economic, and medical implications resulting in trauma, and stress, particularly in a social set-up like the third world, with a strong emphasis on childbearing (Mascarenhas *et al.*, 2012). In the past decade, treatment of male subfertility accomplished far-reaching avenues with semen analysis has advanced up to sperm function tests, surgical anastomoses, and sperm obtained directly from

epididymis/testes (Barratt *et al.*, 2017). It has been reported that of the subjects who underwent the semen analysis, more than half had abnormal semen parameters (Leung *et al.*, 2018). These contradictory findings underline the requirement for more dependable metrics to assess the seriousness of this problem.

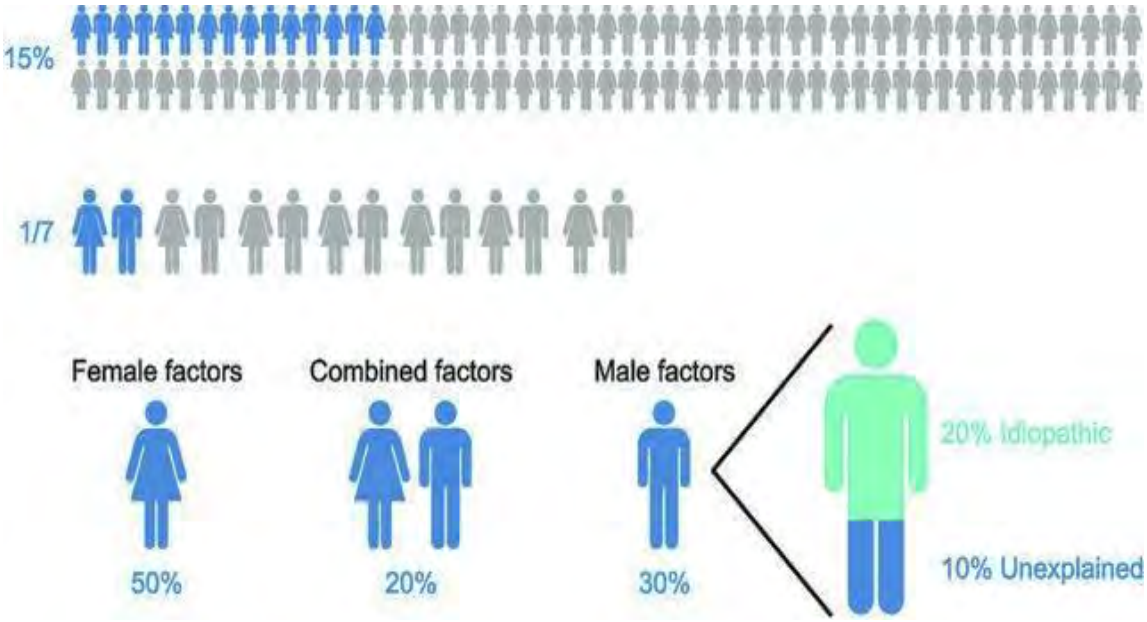


Figure 4. Incidence of subfertility (Agarwal *et al.*, 2015). The figure is based on a study, which looked at the incidence of subfertility in a large sample of couples from around the world. The most common cause of subfertility is female factors. This is followed by male factor infertility, combination of male and female factor infertility, idiopathic and unexplained infertility.

2. Evaluation of male subfertility

The evaluation of the male factor included a careful history, clinical examination, and 2-3 semen samples performed at intervals of three months. (Pizzol *et al.*, 2014).

The quality of human sperm has traditionally been determined by microscopic examination and biochemical testing in the diagnosis of male subfertility. Conventional semen analysis includes measuring seminal volume, pH, sperm concentration, motility, and morphology have been published by the world health organization (WHO) for anthology laboratories (WHO, 1987; 1992; 1999, 2010) that was accepted as a source of a standard approach for

human semen analysis on a global scale (WHO 2010; Table 1). Complete sperm analysis is one of the most effective and important tests in andrology (Cooper *et al.*, 2010).

Table 1: The first five editions of the WHO laboratory manual for the evaluation and processing of human semen and sperm cervical mucus contact show the progression of normal semen parameters from 1980 to 2010 in each case

Semen characteristics	WHO 1980	WH0 1987	WH0 1992	WH0 1999	WH0 2010[1]
Volume (mL)	ND	>2	>2	>2	1.5
Sperm count (10^6/mL)	20-200	>20	>20	>20	15
Total sperm count (10^6)	ND	>40	>40	>40	39
Total motility (% motile)	>60	>50	>50	>50	40
Progressive motility[2]	>2[3]	>25%	>25%(grade a)	>25%(grade a)	32%(a + b)
Vitality (% alive)	ND	>50	>75	>75	58
Morphology (% normal forms)	80.5	>50	>30[4]	(14)[5]	4[6]
Leukocyte count (10^6/mL)	<4.7	<1.0	< 1.0	c 1.0	< 1.0

[1] lower reference limits generated from the lower fifth centile value. [2]grade a= rapid progressive motility (>25um/s): b=slow/sluggish progressive motility (5-25um/s): normal =50% motility (grades a +b) or 25% progressive motility (grade a) within 60 min of ejaculation; [3] forward progressive (scale 0-3); [4] arbitrary value; [5]value not defined but strict criterion is suggested; [6] strict (kygerberg) criterion; ND= not defined.

2.1. Computer-Aided Sperm Analysis (CASA)

Subfertile couples' expectations of having their baby have dramatically increased since the emergence of computer-assisted methods of human semen analysis in in-vitro fertilization and other auxiliary technologies. Semen analysis as an essential diagnostic tool for male reproductive health evaluation can be done either manually which depends on personal expertise with inter and intra-lab variation. Whereas with the introduction of computer-

aided sperm analysis (CASA) in the 1980s, this test became more accurate, exact, and objective in terms of sperm concentration and motility. The examination of sperm motility can be done with fewer errors if an objective CASA is used instead of microscope slides (O'Meara *et al.*, 2022). In addition, the CASA divides motility into three categories: average path velocity (VAP), straight-line velocity (VSL), and curvilinear velocity (VCL). The entire distance that the sperm head can travel in an observation period is called curvilinear velocity (m/sec). Straight-line velocity (VSL) (m/sec) is a measurement of the sperm's straight-line cover distance. The distance traveled by spermatozoa in a general direction is measured in average path velocity (VAP) (m/sec). The oscillation of the actual path around the average path is measured by the wobble, which is given as VAP/VCL. The size of a sperm head's lateral displacement around its average course is measured in the amplitude of the lateral head displacement (ALH) (m/sec). Linearity (LIN) is a measure of a curved path's linearity, which is computed by dividing VSL by VCL (Finelli *et al.*, 2021).

2.2. Reproductive Hormones:

During normal spermatogenesis, normal sperm are generated, along with normal luteinizing hormone (LH) and follicle-stimulating hormone (FSH) activities. Spermatogenesis control through the hypothalamic pituitary testicular axis (HPT axis). Two distinctive axes operate under the HPT axis one is the hypothalamic hypophysial seminiferous tubular play an important function in spermatogenesis (the creation and maturation of spermatozoa) and the second is the hypothalamic hypophysial leydig cell axis helps steroidogenesis (the synthesis and secretion of testosterone), hence testis serves as both a reproductive and endocrine organ (Hikim and Swerdloff, 1999). Hormonal regulation of spermatogenesis is a complicated interplay between anatomical parts of the testis and the neurological activity in the brain's hypothalamic-pituitary axis that begins before birth and continues through puberty and maturity (Zhao *et al.*, 2020). LH regulates steroidogenesis in the leydig cell of the testis, while Follicle-stimulating hormone (FSH) regulates gametogenesis in the seminiferous epithelium, which is regulated by hypothalamic gonadotropin-releasing hormone (GnRH) (Holtermann Entwistle *et al.*, 2022).

3. Etiology of male fertility:

Numerous conditions, such as premature ejaculation, genetic disorders, such as cystic fibrosis, structural problems, such as testicle blockage or obstruction of the ejaculatory tract, harm to any part of the male reproductive system, or inflammation of the epididymis, prostate, or seminal vesicles, can all result in subfertility (Hofny *et al.*, 2010; Agarwal *et al.*, 2019). Sixty percent of instances caused by subfertility happen when there are issues with sperm production and function, as well as aberrant LH and FSH levels. Genetic flaws, developmental issues, cryptorchidism, radiation, chemotherapy, infections, and varicoceles are all possible causes of non-obstructive subfertility (Pizzol *et al.*, 2014; Tendayi, 2020). Male subfertility can also be caused by a variety of other causes, such as obesity or wearing tight underwear that raises the temperature of the scrotum, cigarette smoking, alcohol misuse, anabolic steroid abuse, or increased exposure to environmental hazards such as pesticides and radiation Figure 5. However, more than 30% of male subfertility with low semen parameter values is idiopathic, meaning it has no known cause (Tendayi, 2020; Amor *et al.*, 2021; Amor *et al.*, 2019; Agarwal *et al.*, 2017; Agarwal *et al.*, 2019).

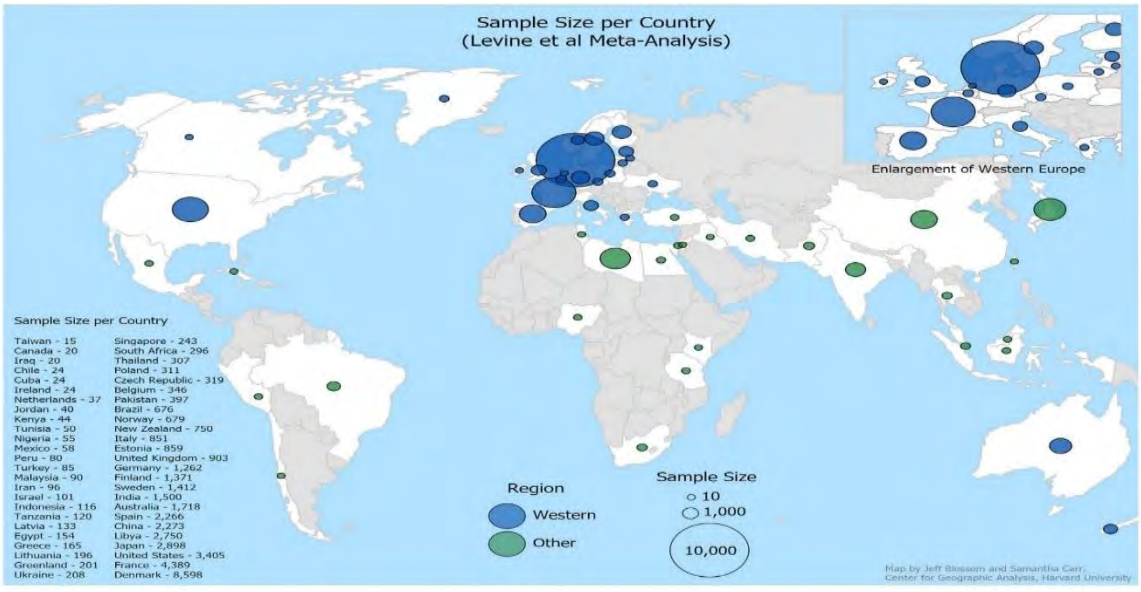


Figure 5. The concern over alleged patterns of diminishing human sperm count is called into doubt by the article "A Bio-variability Framework for Understanding Global Sperm Count Trends." (Barratt *et al.*, 2017)

4. Diagnosis of male subfertility

Distinguishing between fertile and subfertile men is based on standard threshold values for each of the semen parameters. An ejaculation should yield semen parameters above these standard thresholds to be classified as normal fertile (WHO, 2010). Any values below their corresponding threshold, the patient will be classified according to different pathological conditions depending on the affected parameters, as illustrated in Table 2.

Table 2: Semen parameters according to WHO 2010 and pathological conditions related to standard threshold values

Pathological condition	Standard threshold
Oligozoospermia sperm	concentration less than $15x10^6$ /ml; Total sperm count less than $39x10^6$
Asthenozoospermia	Progressive motility less than 32%
Teratozoospermia	Normal morphology sperm less than 4%
Oligo-astheno-teratozoospermia	Presence of the above three conditions
Azoospermia	Absence of sperm in the ejaculate
Cryptozoospermia	Absence of sperm in the fresh ejaculate, but found in the centrifuged pellet
Aspermia	Absence of ejaculate
Necrospermia	Spermatozoa in the ejaculated semen are dead
Leukospermia	The number of white blood cells in the ejaculate is more than normal levels

Using a light microscope to analyze basic parameters of 100 to 200 sperm as described by WHO standards, the predictive value of WHO result could be reduced, by intra-technician observation and lack of training could lead to higher variation in the result of two semen

sample results of the same individual from two different laboratories or reported by two technicians of the same laboratory (WHO, 2010).

Consequently, fertile men showed abnormal semen analysis result, secondly it only highlight standard quantity parameters and does not predict the quality of sperms.

The introduction of new tests including viability, sperm antibodies, inflammation, and total motile sperm concentration, and assisted conception clinic's laboratories made it a routine to assess these parameters pre and post-sperms preparation (Topp *et al.*, 2015). Still, the predictive value of these conventional standard parameters for sperm fertilization potential is limited.

4.1. Spermatogenesis:

Spermatogenesis is cell proliferation and differentiation involves specific genes which make the process conserved in most species. Spermatogenesis in humans and mammals is a complex and unique process that includes multistep which takes place in the testis, resulting in the production of male gametes named the spermatozoa.

Male subfertility is caused by defective spermatogenesis, which is essential to the development and maintenance of male reproduction. Leydig cells, myoid cells, and Sertoli cells are only a few of the somatic cells that make up the testis' microenvironment or niche, which is crucial for controlling healthy spermatogenesis (Hunter *et al.*, 2012) Figure 6.

Leydig cells are a significant part of the testicular stroma, and seminiferous tubules' peritubular myoid cells are one of their main cell types. The Sertoli cells HOS this process by producing and contributing different types of proteins and enzymes.

Diploid primordial germ cells, the initial cell in process of spermatogenesis is already present at birth, and at puberty form diploid spermatogonial cells (Cannarella *et al.*, 2020). Spermatogonial cells go through mitosis and produce primary spermatocytes. Primary spermatocytes undergo meiosis I and generate two secondary spermatocytes with the same DNA content. The second meiotic division produces haploid spermatids.

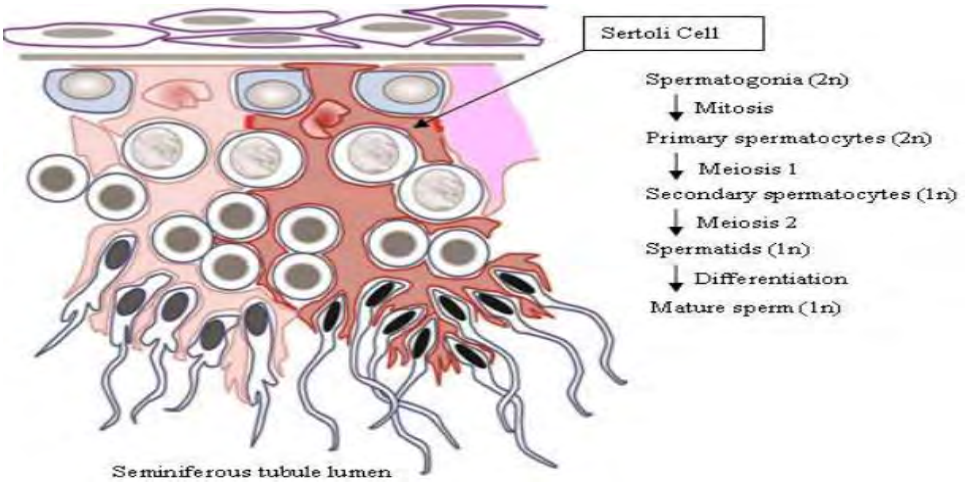


Figure 6. Diagrammatic Representation of spermatogenesis (Hunter *et al.*, 2012). The diagram shows the different stages of meiosis, which is the process by which the sperm cells are produced. Meiosis is a two-step process that reduces the number of chromosomes in the sperm cells from 23 pairs to 23 single chromosomes.

Spermatid undergoes physiological, biochemical, and morphological modifications and transforms from round to elongated spermatid including nuclear content condensation, trimmed off cytoplasmic bridges, Golgi apparatus modified into the acrosomal cap, flagellum formation, and final differentiation into spermatozoa, the whole process completed in seventy-four days. Spermatogonia produce 16 primary spermatocytes at the time of puberty, each spermatocyte after meiosis produces four spermatids, and spermatogonium at the end produces 64 spermatocytes. By end of puberty, the human testis produces 200 million sperm per day (Hunter *et al.*, 2012 ; Qian *et al.*, 2020; Nakano *et al.*, 2021).

With the completion of spermiogenesis, the spermatozoa dropped into the lumen of the seminiferous tubule along with the addition of enzymatic secretion (containing fructose, prostaglandins, fibrinolytic, fibrinogens, calmodulin, and coagulating proteins) from seminal vesicles, testis, prostate, vas deferens, and accessory glands. Spermatozoa are then transported to epididymis with seminal plasma, where they further attain maturation, mobility, and fertilization capacity Figure 6. Mature spermatozoa are highly specialized cells that carry and protect paternal chromatin in the male and female genital tract. They

ensure the transfer of the intact paternal chromatin to the next generation after fertilizing the oocyte (Cannarella *et al.*, 2020).

5. Etiology of Sperm DNA damage:

Sperm DNA damage could incur during spermatogenesis or transportation and possibly in vivo or in-vitro. Epididymis and ejaculate have higher DNA-damaged sperm proportion compared to testicular spermatozoa present in a tight association with Sertoli cells that play a protective role against exogenous factors. During prophase I of meiosis I genetic recombination results after crossing over and chromatic exchange between sister chromosomes (Dada *et al.*, 2012).

The programmed function of nucleases cause DSB in sperm DNA and enzyme ligase repair damaged DNA under strict checkpoints to prove or disprove meiosis. An error in this process may result in fragmented DNA in mature spermatozoa. Histone to protamine replacement during spermatogenesis is another point where DNA damage could incur. A normal sperm cell differentiation program includes round and elongated spermatid DNA toroid formation, which causes transient torsion stress on chromatin and relief necessitates DNA break (Timermans *et al.*, 2020). This step favor replacement of histone with transition protein and ultimately with protamine under the function of topoisomerase II endonuclease. Defect during this exchange incline DNA break which may suggest incomplete, immature, or anomaly during spermatogenesis (Cannarella *et al.*, 2020).

5.1. Sperm Chromatin remodeling:

Somatic cell chromatin differ from sperm chromatin in milieu. The somatic chromatin nucleosome composed of DNA wrapped around the octamer core of histones gives it the appearance of a bead string. In sperm chromatin, during the final maturation of spermatid major event was the replacement of the somatic histones with transition nuclear protein (Agarwal and Said, 2003) as shown in Figure 7. In mitotic germ cells, DNA methylation occurs, resulting in paternal-specific imprints. Phosphorylation occurs in meiotic cells and aids in recombination and the formation of XY bodies. XY body formation involves ubiquitylation, sumoylation, and incorporation of the H2AZ and H3.3 variants.

Hyperacetylation occurs during spermiogenesis to aid in the histone protamine transition (Dada *et al.*, 2012).

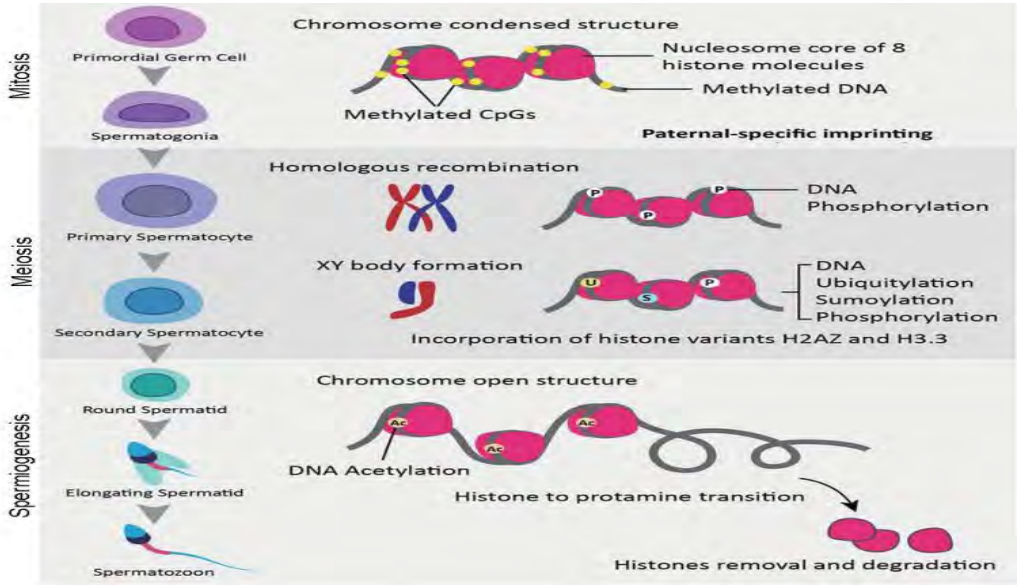


Figure 7. Epigenetic modifications occur during spermatogenesis (Dada *et al.*, 2012). In sperm chromatin, during the final maturation of spermatid major event was the replacement of the somatic histones with transition nuclear protein and final with protamine, this packaging leads to sperm nuclear chromatin compaction six times more condense and stabilizes compared to somatic chromatin.

Toroids are doughnut-shaped packaging of sperm chromatin, these toroids are cross-linked by disulfide bridges formed at cysteine residue of protamine by sulfhydryl oxidation. Finally, these bond assembly in protamine makes the nuclear DNA transcription and translation complete shutdown, and this packaging leads to sperm nuclear chromatin compaction six times more condense and stabilizes compared to somatic chromatin as shown in Figure.7 (Dada *et al.*, 2012). There are two variants of human protamine, protamine 1 (P1) and protamine 2 (P2), and in subfertile men these protamines are expressed in a ratio equal to 1 (Amor *et al.*, 2019; Nemati *et al.*, 2020).

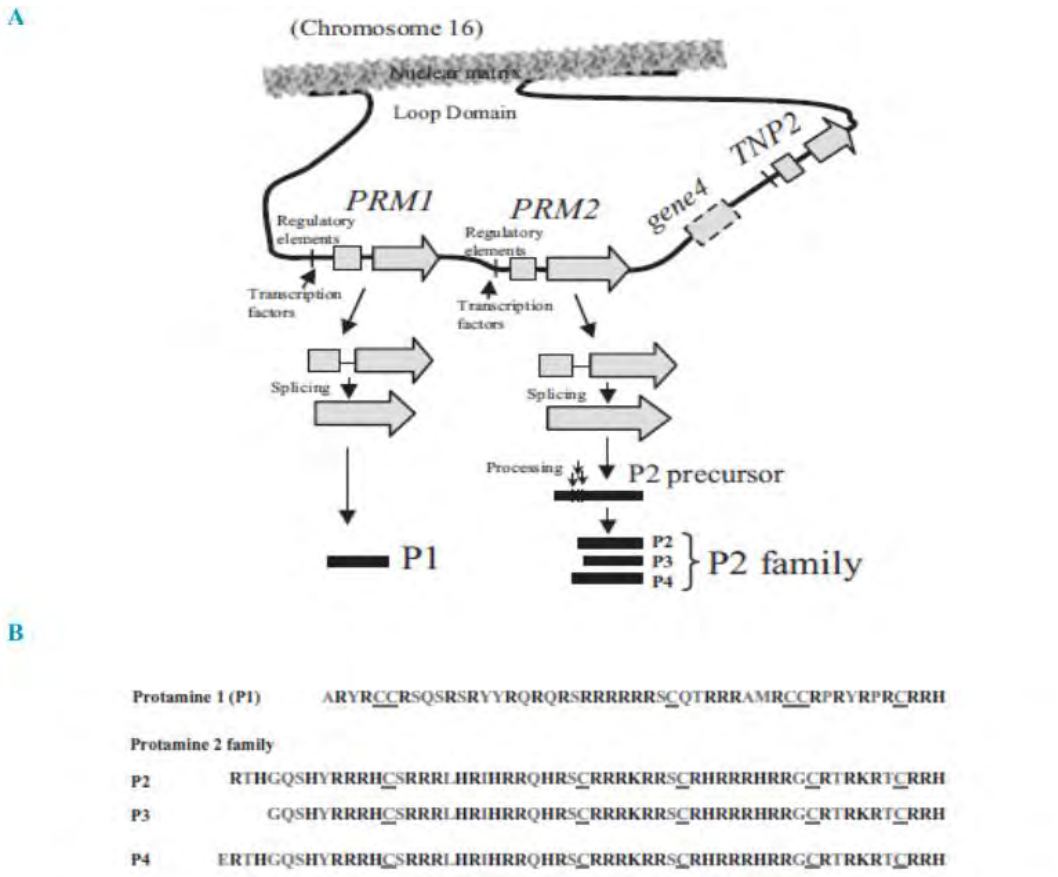


Figure 8. A loop domain-like genomic organisation of the protamine genes (protamine 1 and 2) on chromosome 16. The transition protein 2 gene, a sequence from gene 4, and the protamine 2 families are all found in the genomic sequences of the loop domain (PRM 2 consists of protamine 2, 3, and 4 components). Adopted from (Oliva, 2006).

Translation of genes encoding protamine (PRM1) and protamine 2 (PRM2) varies in different species of mammals. Gene encoding protamine is located on chromosome 16 at 16p13.3 in humans. P1 and P2 both have common arginine residues from about 55-79% of amino acid, which bounds the negatively charged DNA (Nemati *et al.*, 2020) Figure 8.

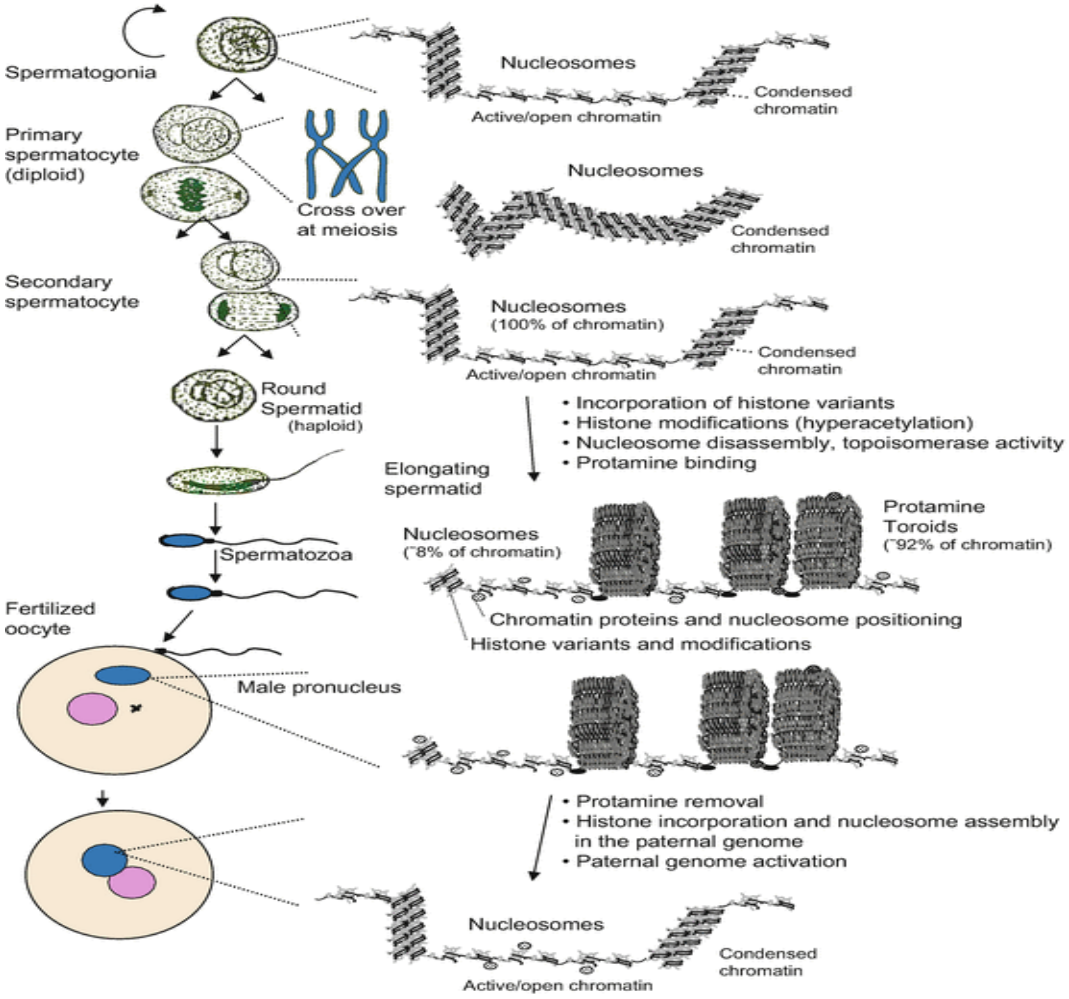


Figure 9. Histones replacement by protamine. Diagrammatic illustration of the major alteration that occurs during spermiogenesis, in which the nucleo histone (top) in the form of the nucleosome as somatic cells undergoes several modifications resulting in nucleoprotein complex, in addition to the toroid loops model. Adapted from (Nemati *et al.*, 2020).

Protamine 3 (gene 4) contains aspartic and glutamic acid repeating units similar to clusters of arginine and lysine units in the DNA binding domains of protamine. The sequence and size of the amino acid P3 are similar to P2. The high content of negatively charged amino acids is involved in some other functions and is not involved in DNA condensation (Aoki *et al.*, 2006; Aoki *et al.*, 2005) Figure 9. Protamine genes have a unique sequence that helps in transcription regulation including; i). cAMP response element (CRE) sequence is highly

conserved and resides in positions -57 to -48. ii). P1 and P2 gene transcription up and down-regulation is directed through the binding of other transacting proteins with promoter regulation sequences for the P1 and P2 genes. iii). Large methylated domain, which facilitates nuclear matrix attachment and potentiation of P1 P2 and TP2 gene locus in round spermatid, and matrix attachment region (MAR) contains alanine (Ala) repeats that is the site of methylation before P1 P2 and TP2 genes locus. iv). the protamine gene with TATA box facilitates transcription factor binding and hence initiates transcription (Nemati *et al.*, 2020).

5.2. Oxidative stress

Sperm DNA integrity is critical for safe haploid paternal genomic message delivery to Oocyte and a unique diploid next-generation result after successful fertilization (Xie *et al.*, 2018). The origin of sperm DNA damage is not fully understood. Standard semen analysis is not sufficient for sperm quality assessment one of the biological tools is sperm DNA damage, a marker of male reproductive health and fertility. Sperm DNA fragmentation is referred to as backbone breakage it could be single-strand nick (SSB) and double DNA strand break (DSB) caused by intrinsic or extrinsic factors including nucleases, free radicles, mutagens, and deficient protamination. Single strand DNA breaks result when 8 OH guanine and 8 OH 20 deoxyguanosine (8 OHdG) are formed after the oxidative attack of hydroxyl radical and ionizing radiation. Hydroxyl radical activates sperm caspases and endonucleases that may induce double-strand sperm DNA break (DSB) (Agarwal *et al.*, 2019). DSB becomes lethal when genomic error becomes extensive and irreparable that leading to cell death. Fertilization with a normal genomic oocyte that can repair some paternal genetic errors, became irreversible and incompatible with resultant zygote development when the paternal genome has extensive damage (Aitken *et al.*, 1998; Said *et al.*, 2012; Aitken and De Iuliis, 2007) (Figure 10).

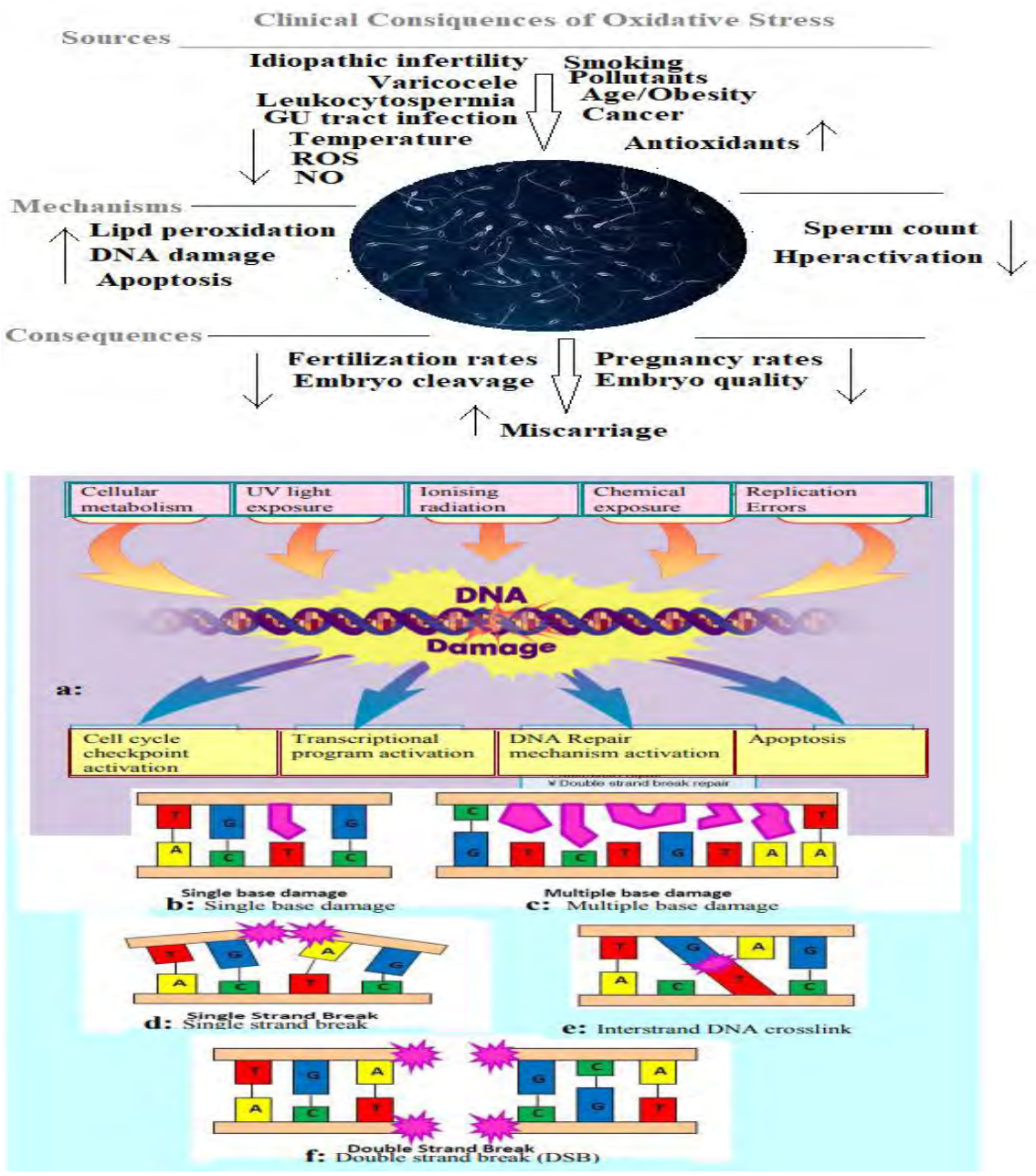


Figure 10. Association of increasing reactive Oxygen species (ROS) production with subfertility and DNA damage (Said *et al.*, 2012)

5.3. Abortive apoptosis

Eukaryotic cell death happens to those with genomic DNA damage, one of the hallmarks of programmed cell death. Apoptosis is essential that destroys 75% of spermatogenic cells

to support the limited capacity of the Sertoli compartment capacity. Spermatozoa of subfertile males during spermatogenesis contain improperly packaged DNA of 50 to 70%, which leads to irregular strand breaks that make the denatured sperm DNA more sensitive to programmed cell death (Gavrieli *et al.*, 1992). Escape of this process is named abortive apoptosis, where the apoptosis is initiated but due to some hidden factors leads to the survival of fragmented DNA spermatozoa in semen of subfertile men. DNA backbone of a sugar-phosphate and purine and pyrimidine is altered by faulty endogenous endonuclease activity causing DNA nicks. Another reason could be lacking cell surface protein Fas and associated ligands involved in apoptosis (Alkhayal *et al.*, 2013). Fas ligament binds Fas receptor which activates caspase enzymes activation and phagocytosis engulf defective sperm cell. Inoperative Fas-ligand increased the number of damaged spermatozoa escaping from apoptosis and later accumulating into the ejaculate (Asgari *et al.*, 2019). Abortive apoptosis appears to be the reason for DNA break at the round spermatid stage, apoptosis becomes irreversible during the spermatogonia phase and in the spermatogenesis, initial stages, and ultimately Sertoli cells digested these cells (Engel *et al.*, 2018).

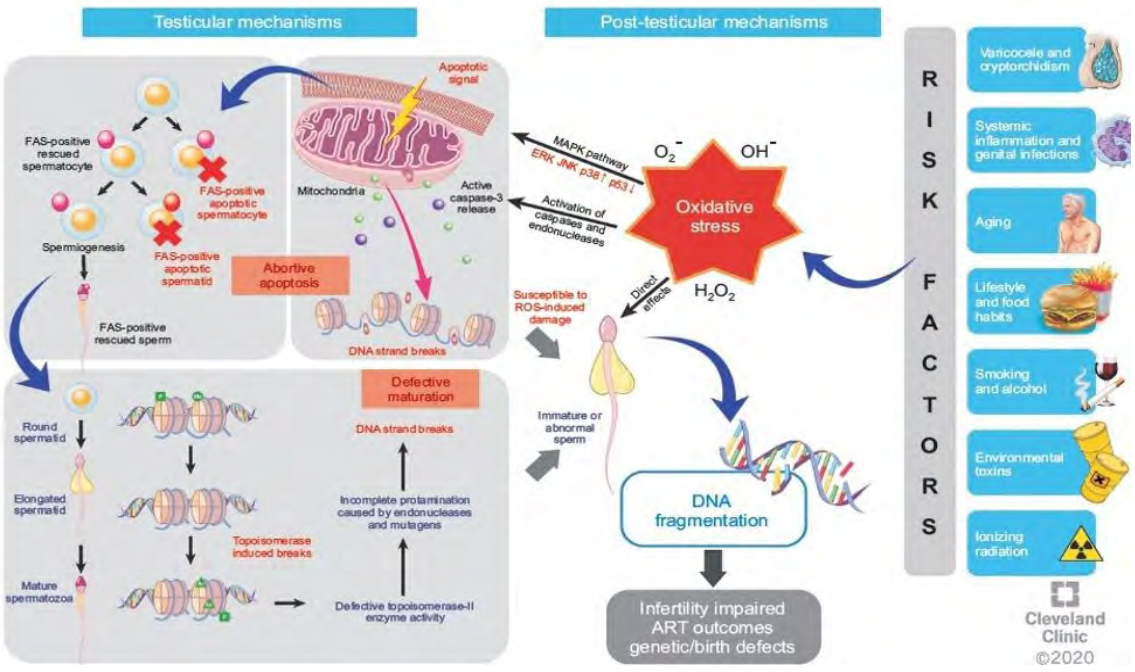


Figure 11. Intrinsic and extrinsic origin of chromatin damage (Agarwal, 2003).

5.4. Extrinsic Origin

The extrinsic factor (Figure 11) that causes sperm DNA damage includes; Post testicular oxidative trauma, bacterial infection, age, abstinence, the temperature of testes, varicocele, chemical exposure, cryopreservation, and sperm preparation techniques for ART (Zeqiraj *et al.*, 2018).

5.4.1. Post testicular oxidative trauma

Immature sperm movement from seminiferous tubules to the epididymis and after ejaculation can produce higher ROS levels which cause mature sperm DNA damage. Sperm DNA damage could be directly or indirectly mediated through the activation of caspases and endonucleases. Another reason for higher ROS level production is co centrifugation of immature and mature sperms (Hayashi *et al.*, 2007). Similarly, in the epididymis, epithelial cells induce ROS damage through hydroxyl radical or nitric oxide or caspases and endonucleases. Ejaculated sperm DNA fragmentation consider dynamic with a decrease in DNA damage with the longevity of time after ejaculation. Semen collection methods also influence the extent and percentage of DNA damage (Qian *et al.*, 2020).

5.4.2. Bacterial infection

Leukocytes present in ejaculate act as immune surveillance with phagocytosis of abnormal sperms (Eini *et al.*, 2021). This is a correlation between increased ROS levels and DNA damage with genital tract inflammation and leukocytespermia (Romany *et al.*, 2017).

At testes and epididymis level protective cover given by seminal plasmas antioxidants against leukocyte and increase leukocyte quantity breach this protection and increase oxidative stress which leads to DNA damage. This DNA damage is most common in epididymitis and prostatitis conditions of male genital infections. Genitourinary infections of *Chlamydia trachomatis* and *Mycoplasma* generate DNA damage and influence standard semen parameters that lead to decreased fertility potential (Castellini *et al.*, 2020).

5.4.3. Abstinence

Semen pH, viability, morphology, motility, or sperm DNA damage did not change with abstinence time while the sperm number and volume correlate positively with abstinence days. One of the studies suggests a negative correlation of chromatin quality with short abstinence, while another study found SDF lower levels with 24h to 3h ejaculation than 3 to 5 days (Levine *et al.*, 2017).

5.4.4. Temperature Stress

Testicular and epididymal hyperthermia cause chromatin damage similarly occupational heat exposure and febrile status are also shown to compromise sperm DNA (Wu *et al.*, 2020). Pregnancy rates drop and DNA strand breaks increase as a result of paternal heat stress (Ilacqua *et al.*, 2018).

5.4.5. Varicocele

Men with varicocele, which impacts roughly 15-20% of men overall and one-fourth of those who receive ART treatment, experience decreased semen quality and production. Male patients with varicocele had greater concentrations of sperm ROS, spermatozoa with increased DNA fragmentation index, residual cytoplasmic droplets, and premature chromatin. Scrotal hyperthermia, apoptosis, hormonal imbalance, hypoxia, hypo-perfusion, and toxic metabolites backflow are potential causes of damage caused by varicocele. Sperm DNA damage and ROS production could be reversed after the varicocele repair (Tavalaee *et al.*, 2015).

5.4.6. Chemical exposure

Exposure to air pollutants and occupational hazards including industrial chemicals like toluene, xylene, herbicides, pesticides, and organochlorines significantly stimulate DNA damage (Qian *et al.*, 2020).

Cocaine or marijuana reduces the quantity and quality of sperm including DNA strand breaks due to increased apoptosis. 200 exogenous chemicals, only a few chemicals can cause sperm DNA damage (Iftikhar *et al.*, 2021).

5.4.7. Cryopreservation

Cryopreservation of sperm in males is commonly practiced in those undergoing cancer therapies, vasectomy or surgical subfertility, and assisted reproduction treatment (Glander and Schaller, 1999). Lower temperature maintains intact sperm DNA of stallions, rabbits, dogs, and bulls, for a longer time between 5ºC and 15ºC than at a higher temperature of 20 or 37ºC (Qian *et al.*, 2020). While elephant semen at 37 ºC has stable DNA integrity.

5.4.8. Sperm preparation for ART

Sperm preparation for in-vitro insemination washed out antioxidant defense of sperm (SOD, CAT, GPx) to combat oxidative stress and seminal plasma protein (semenogelin I and II) that inhibits sperm viability and progression (Nadalini *et al.*, 2014). Culture media contain human serum albumin, carbohydrates, amino acid, buffer, and antibiotics with reduced oxidative protection even remaining less in those few culture media that added ETDA and taurine (Amorini *et al.*, 2021; Said and Land, 2011).

The use of assisted reproductive methods (ARTs) has emerged as the preferred method of treating both male and female subfertility during the last 20 years due to advancements in the management of subfertility. However, the current success rates of the operations are still insufficient. The growth of an embryo following the fertilisation of oocytes with low-quality sperm from subfertile men is one of the primary factors affecting the success of ICSI. Failure of the ART process keeps on triggering the urge to refine sperm separation procedures and new sperm selection tools to boost the outcome; embryo quality, implantation, and pregnancy (Karimi Zarchi *et al.*, 2020). Getting a significant number of viable, mobile, and properly functioning spermatozoa that can fertilise the female oocyte is the primary goal of sperm preparation techniques (Hernandez-Silva *et al.*, 2021). Currently, density gradient centrifugation (DGC) and swim-up are commonly applied to collect a pool of sperm with excellent motility and adequate morphology primarily based on spinning and migration of the sperm, and these approaches are based on selecting sperm with higher motility characteristics without taking their molecular properties into account (Tavalaee *et al.*, 2012).

The impact of the aforementioned techniques has not been evaluated enough on invisible anomalies such as DNA stability, sperm chromatin condensation, cell wall maturity, extracellular matrix, apoptosis and apoptosis-like appearance, and assisted reproductive technology (ART) outcome (Zhang *et al.*, 2011). Both DCG and swim-up procedures are not designed to effectively select sperm without DNA damage, intact chromatin, and non-apoptotic sperm (Chen and Bongso, 1999; Bibi *et al.*, 2022). Likewise, in intra cytoplasmic sperm-injection cycles (ICSI), the choice of active sperm is made based on the embryologist's primary preferences for its motility and better morphological characteristics, thus ICSI circumvents the natural defense barriers and allows for fertilization with DNA-damaged sperm. As depicted in Figure 12, the upper left inset shows the significant mutations to the sperm DNA brought on by base oxidative damage, such as the 8-OHdG residue, strand breaks, and changes to epigenetic markers. (Said and Land, 2011). Additionally, it displays the genomic locations with less genomic compaction within the nucleosomal organization namely, short DNA linkers bridging protamine donuts and histone solenoids within protamine-containing toroidal donuts—where such modifications are more likely to occur. The oocyte repair ability, which is responsible for fixing the paternal DNA, is shown in the top right insert (Figure 12). The lower left images show a coordinated development, while the bottom right panel exhibits some of the conventional repercussions of oocyte errors to mend the paternal DNA alterations (Champroux *et al.*, 2016).

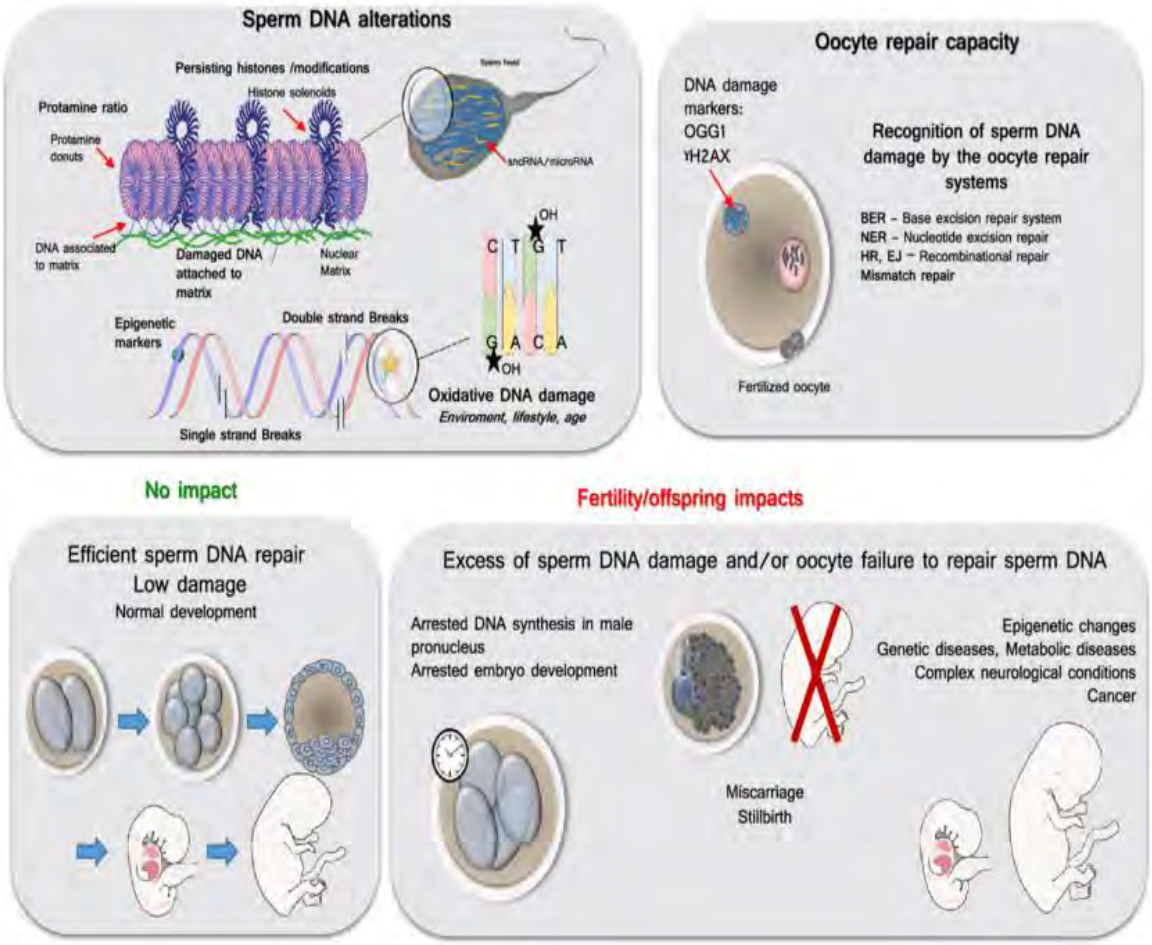


Figure 12. Some features of sperm DNA damage and their potential implications if not corrected are depicted schematically (Champroux *et al.*, 2016)

The apoptotic process is one of the spermatozoa's molecular characteristics that is connected to male fertility, and it has drawn a lot of interest nowadays. Numerous studies have demonstrated the significance of systemic cell death (apoptosis) in sperm, which may explain why assisted reproduction results in a lower rate of pregnancy and implantation. Common apoptosis indicators like caspase activation reduced mitochondrial membrane potential (MMP), and phosphatidylserine (PS) translocation to the cell surface have been observed in the ejaculated human sperms (Said *et al.*, 2007). However, the utilization of magnetic-activated cell sorting (MACS) to ascertain the transfer of phosphatidylserine (PS) to the cell layer leaflet during initial apoptosis with annexin-V linkage appears to be the most promising method (Said *et al.*, 2007; Grutzkau and Radbruch, 2010). The data

demonstrated the usefulness of MACS improves sperm viability, and maturation, and lowering sperm chromosomal abnormalities and apoptosis when performed in conjunction with standard techniques of sperm processing employing gradient centrifugation (DGC) and swimming (SU) procedures (Gil *et al.*, 2013). Similarly, some authors found an increase in pregnancy rate but not in implantation and pregnancy rates, while other researchers found increased pregnancy, fetal quality, pregnancy, and live birth rates (Romany *et al.*, 2017). However, some authors did not notice a significant difference between MACS and conventional semen selection methods for reproductive outcomes (Stimpfel *et al.*, 2018). Such variability can be attributed to the large variance with the low number of patients included in these studies (Martinez *et al.*, 2018; Pacheco *et al.*, 2020; Degheidy *et al.*, 2015). Most studies have not looked at the affectivity of MACS in subfertile with normal and increased SDF levels and the relative efficiency of the different semen preparation processing procedures for improving sperm DNA quality, chromatin maturity along with ART outcome (Berteli *et al.*, 2017; Gil Julia *et al.*, 2021; Cakar *et al.*, 2016; Ziarati *et al.*, 2019; Nadalini *et al.*, 2014).

5.4.9. Age

There are controversial finding on male age affect fertility capacity and semen parameters with fragmented DNA spermatozoa. Spermatogenesis continues throughout human male life but some stud suggests the quality and fertility deteriorate. Decrease apoptosis, double-strand DNA break decline in a healthy sperm cell selection (Evenson *et al.*, 2020). It has been reported that in male partners opting for semen analysis; over 50% of men presented with abnormal semen parameters. It is estimated that the prevalence of male subfertility between the age of 15 to 50 years was up to 6% (Agarwal *et al.*, 2015; Barratt *et al.*, 2017; Bahamondes and Makuch, 2014). In recent years' advancing age becomes a key factor contributing to debility in reproductive health indices in both sexes. Old males have augmented estrogen levels, due to the amplification of aromatase; through a negative response loop, men display indications of hypogonadotropic hypogonadism. Along with increased osmotic damage, lipotoxicity, and irregularity in adipokine absorption, the hormonal imbalance directly harms the gonads, downstream reproductive system, and

the embryo. It is generally well accepted that reproductive function highly correlates with the degree of adiposity, age, nutrition, or metabolic condition related to food intake in human medicine (Rybar *et al.*, 2011; Zahid *et al.*, 2015). Paternal age >40 years were associated with reduced semen quality (Prikhodko *et al.*, 2020). Furthermore, infection, immunological factors, trauma or surgical insult to the male reproductive organs, and exposure to toxic chemicals or other materials are all known acquired factors that contribute to male subfertility. (Ombelet *et al.*, 2008; Leung *et al.*, 2018; Barratt *et al.*, 2017). Similarly, a correlation was found between men's age and semen quality found even after adjustment for reproductive hormones. Semen analysis is a routine and simple method for assessing male fertility status. However, alone it is not sufficient to predict assisted reproductive outcomes (Chohan, 2006; Davidson *et al.*, 2015). The relationships between age, semen characteristics, male reproductive hormones, sperm DNA fragmentation, chromatin structure, and ART outcome have been inconsistently correlated, according to numerous studies and meta-analyses. In-vitro fertilisation (IVF) and intracytoplasmic insemination (ICSI), notably ICSI, which gets over the natural defense mechanisms and allow for fertilisation with DNA-damaged sperm that have been linked to reduced pregnancy rates and pregnancy loss. Consequently, there are growing worries about the health of the children who are produced. (Sermondade *et al.*, 2013; Keltz *et al.*, 2010; Barratt *et al.*, 2017). Besides, other factors, age would be the leading cause of lower pregnancy rate and failure of reproductive outcomes. Therefore, the overall health and normal age of parents should be considered in couples as an important concern in attaining successful reproductive outcomes.

5.4.10. Body mass index

Overweight men had reduced sperm concentration, sperm number, total motile sperm count, and semen volume (Linabery *et al.*, 2013; Jahan *et al.*, 2011). Similarly, lack of physical activity has been shown to harm fertility but endurance exercise has also correlated with negative sperm quality (Hofny *et al.*, 2010). Overweight men were 2.5 times more likely to have reduced volume (Campbell *et al.*, 2015). Increased BMI has a negative correlation with sperm concentration, motility, and morphology (Craig *et al.*,

2017; Bibi *et al.*, 2022). BMI < 16.5 and >30 were associated with reduced semen quality (Leisegang *et al.*, 2021). Even after accounting for reproductive hormones, a clear correlation between BMI and semen parameters was identified (Maghsoumi-Norouzabad *et al.*, 2020).

5.4.11. Other factors

Additionally, medical, environmental, and lifestyle theoretically cause the degradation of sperm DNA (Babakhanzadeh *et al.*, 2020). Common other factors include; electromagnetic radiation, mobile phone, hormone imbalance, cryptorchidism, nutritional status, and folate in semen associated with sperm DNA integrity (Agarwal *et al.*, 2017; Skakkebaek *et al.*, 2006; Ilacqua *et al.*, 2018). The present study was designed to evaluate demographic, biochemical, and molecular risk variables in subfertile men based on the literature evaluation mentioned above. Contradictory results highlight the need for more robust methodologies to investigate the prevalence of this issue, it may be accounted for by the overall lack of knowledge on the specific nature of the molecular cause for such sperm issues. Considering the potent role of chromatin integrity and maturity role in men's fertility and assisted conception success, the present study was directed to study the spermatozoa chromatin integrity and maturity male factor subfertility and normal healthy control in the Pakistani population to improve the reproductive health of men. Moreover, information gathered from current data could be used by doctors/clinical practitioners and researchers which could be of great benefaction for them to improve the management of subfertile couple's treatment.

5. Aims

The aim of present study was to study the spermatozoa chromatin integrity and maturity effect on male reproductive health of men in the Pakistani population and to determine its effect on assisted reproduction outcome.

6. Objectives

As male factor subfertility is poorly understood and there is no gold standard for the diagnosis of success of fertility treatment for such couples with male factor subfertility.

Prognostic indicators can forecast the trajectory and severity of the disease. Various clinical, epidemiological and genetic risk factors should be identified before it threatens both men's fertility and ART treatment outcome. Pakistan is an economically developing country in Asia, studies have been conducted on male subfertility but there are many gaps to be filled. The reasons causing spermatozoa to lose their ability to fertilise are not well understood or well supported by credible data. It is important to identify the men who are more likely to be at high risk of moderate/severe male factor subfertility so that they can benefit from intervention. Determining the prognostic value of various demographic, clinical, biochemical, and molecular indicators in the etiology of subfertility in Pakistani men was the focus of the current study, which was aimed to do so prospectively.

The current study was designed for the comprehensive assessment of genetic and molecular risk factors in male subfertility

- To determine the concentration, motility, morphology, and sperm velocity of normal and abnormal semen parameter values in fertile and subfertile men (CASA parameters).
- To calculate the changes in the levels of male blood serum reproductive hormones, oxidative stress indicators, and sperm viability in studied male groups.
- To assess the clinical value of Sperm chromatin structure assay (SCSA), sperm chromatin dispersion assay (SCD) and acridine orange stating (AO) in the diagnosis of male subfertility.
- To determine the correlation between subnormal semen standard parameters (concentration, motility, morphology, sperm velocity) and quality parameters (sperm chromatin integrity and protamine).
- To assess the intra-assay variation in DNA fragmentation index (DFI), as measured by SCSA, SCD and AO to evaluate the clinical utility of these tests.
- To evaluate sperm chromatin integrity by chromomycin A3 (CMA3) staining and (toluidine blue) TB staining.

- To estimate the impact of high DNA fragmentation and chromatin decondensation on the outcome of standard IVF and ICSI to develop a tool for optimizing the *invitro* fertilization outcome.
- To investigate the correlation of paternal body mass index (BMI) on semen parameters (concentration, motility, morphology, and vitality), DNA fragmentation, and chromatin maturity and to investigate the correlation matrix of the possible impact of paternal high BMI on fertilization, embryo quality, live birth rate, and birth weight.
- Aimed to investigate the correlation of paternal age on semen parameters (concentration, motility, morphology, and vitality), oxidative stress, hormonal levels, sperm chromatin dispersion and chromatin compaction markers.
- Evaluated sperm preparation methods, to find the best method of sperm preparation and to evaluate comprehensively the impact of multiple semen separation techniques and storage methods on, sperm DNA fragmentation and chromatin condensation.
- To evaluate the magnetic activated cell selection (MACS) technique with the classic sperm preparation methods, to find the ideal method of sperm selection and tried to build its relationship with improving semen quality parameters.
- To determine the impact of four semen preparation groups i.e., density gradient centrifugation (DGC), swim-up (SU), DGC-SU, and DGC-MACS, in male subjects with teratozoospermic men a threshold value determines, to improve the number of spermatozoa with mature and intact DNA, condensed chromatin, and better viability.
- To assess the effect of sperm preparation techniques on the ICSI cycle's success, percentage of fertilization, cleavage rate, pregnancy rate and live birth rate.

CHAPTER 1

Analysis of demographic and semen standard and quality parameters in subfertile and fertile Pakistani men

ABSTRACT

A key goal of reproduction research is to identify factors that indicate the success of assisted reproductive technologies (ARTs). Gamete quality is critical for producing high-quality embryos and increasing the success of ARTs. The objective of the present study was to evaluate the effect of male factor subfertility on assisted conception (IVF/ICSI) outcomes. The present study included male factor subfertile couples undergoing conventional *in-vitro* fertilization (IVF) and intra cytoplasmic insemination (ICSI). It is important to identify men at high risk of subfertility. The use of biomarkers in the diagnosis of male fertility would allow appropriate diagnosis of male factor surveillance in ART treatment and outcome. The present study elaborated on demographic, clinical and biochemical risk components for subfertile men in the Pakistani population. A total of 753 couples were included in this study, 607 with male factor subfertility subgrouped into couples with moderate male factor (MMF) 280 and couples with severe male factor (SMF) 327, while 146 with normal (N) men. History, blood samples from both partners and semen were collected from men subject with informed consent after diagnosis. Data were analyzed and laboratory tests were performed. Data were statistically analyzed by one-way ANOVA, Chi-square test and the odds ratio. Several parameters including male and female age ($p>0.05$), BMI ($p>0.05$), subfertility duration ($p>0.05$), socioeconomic status ($p>0.05$), and in men semen volume, pH, WBC/HPF, liquefication time ($p<0.05$), while reduced sperm concentration $x10^6$ ($p=0.002$), progressive motility ($p<0.01$) and sperm morphology ($p <0.001$) were elevated in MMF and SMF subfertile couples as compared to control group. CASA parameters VSL, VCL, VAP and ALH were reduced in MMF and SMF, Sperm membrane integrity (HOS) percentage of fertile subjects measured through HOS was 86.50±0.96%, whereas in MMF was 69.55±0.73%, similarly, SMF had membrane integrity of 63.07±1.21%. Sperm vitality (Eosin) in subfertile men was 71.74±1.26 and in MMF and SMF was 48.85±1.18 and 49.69±1.25 respectively. There were significant differences in SOD ($P<0.001$) and POD ($P<0.001$) levels in the fertile male group as compared to the fertile control. SMF patients had significant ($P<0.001$) lower levels of SOD, POD, CAT, and higher levels of ROS and TBRAS compared to MMF and fertile male patients. A strong correlation was found between oxidative stress (ROS) with BMI, impaired semen parameters and HOS. Comparable hormonal (FSH, LH, Prolactin) levels

($p>0.05$) while a significant decrease in serum testosterone concentration was found in MMF and SMF male patients compared to fertile control subjects. To summarize, the current investigation highlighted the significance of medical and demographic risk factors in the diagnosis of sexual dysfunction. Sperm anomalies were more consistently detected in subfertile men with MMF and SMF compared to N control individuals and should be regarded as negative predictors of fertilization and clinical pregnancy and should be considered in the counseling, diagnostic and therapeutic interventions to improve the reproductive health of men in Pakistan.

INTRODUCTION

One in seven couples worldwide experience subfertility, with 25% of those failing to conceive in a year, 15% seeking medical attention for the condition, and less than 5% still being unable to have children despite every effort they make. In more than half of these cases, the underlying cause is solely attributed to the male individual. (Mascarenhas *et al.*, 2012). The most essential laboratory investigation for men is standard sperm analysis. Semen analysis is used in the diagnosis of male factor, which is frequently indicated by oligozoospermia (Os), asthenozoospermia (OAs), or the presence of teratozoospermia (Candela *et al.*, 2021). These abnormalities can also occur concurrently, and the combination of all of them is known as oligoasthenoteratozoospermia (OATs) (Ombelet *et al.*, 2008). Men were categorized into moderate and severe factors According to WHO 2010 standard semen analysis includes sperm count, motility, and morphology which help in determining the treatment options and chance of spontaneous conception. Moderate factor men were with a single abnormal finding of the semen analysis or by a total motile sperm count between 5–20 × 10^6/mL with normal morphology <4%, Sever factor men were with low sperm count, >15x10^6/ml, low sperm motility >32 % , total motile sperm count less than 5 × 10^6/mL and low sperm morphology >4% (Smith *et al.*, 2007).

Assisted reproductive technology (ART) has become increasingly important in the treatment of subfertility in recent decades. The introduction of these novel procedures, particularly intracytoplasmic sperm injection (ICSI), has resulted in a demand for more effective male fertility potential investigation tools (Leung *et al.*, 2018). A substantial portion of these novel diagnostic approaches focuses on the male gamete's genetic integrity. This interest has grown due to the increasing concern over the potential genetic transmission of diseases through ICSI, wherein the natural control mechanisms during spermatocyte interaction are bypassed, potentially resulting in congenital anomalies, genetic abnormalities, and developmental defects in ICSI-born /infants (Agarwal *et al.*, 2015). Even though ART has reached its maximal level of efficacy, the "take-home baby" rate has remained stable for several years. One of the explanations could be a lack of male diagnosis and can be caused by abnormal spermiogenesis, faulty apoptosis before ejaculation, or excessive ROS generation in the ejaculate, and extra-testicular factors such

as age, body mass index, lifestyle, hormonal factors, hyperthermia and invitro semen handling (Zhao *et al.*, 2020; Kort *et al.*, 2006).

As male subfertility is poorly understood and there is no gold standard for its diagnosis. The search for predictive factors to forecast the course and extent of the disease is currently underway. Various clinical and epidemiological risk factors should be identified, Pakistan is an economically developing country in Asia, studies have been done on male factor subfertility but there are many gaps to be filled. It is important to identify the men who are more likely to be at high risk of subfertility so that they can benefit from intervention. Therefore, the goal of the current study was to evaluate the predictive value of various demographic, health-related, and potential biomarkers in the etiology of male subfertility in the Pakistani population.

MATERIALS AND METHODS

Subjects

This prospective study included 753 couples who underwent IVF/ICSI procedures out of which 604 couples were involved in the ICSI/IVF program at Fertility and Genetic services, Islamabad, Pakistan, from April 2016 to October 2021. The study population involves fertile 146 and 607 subfertile men.

Ethical Compliance

The institutional review board of Quaid-i-Azam University authorized the research proposal, and the ethics committee of the SKMC Islamabad Pakistan awarded its approval. The subject's detailed information (brief medical history, including male and female ages, male body mass index (BMI), period of subfertility, primary or secondary subfertility, and information about earlier spontaneous abortions -related data) was obtained through a questionnaire, asking for the appropriately structured question by face to face interview.

Inclusion and exclusion criteria

A complete physical evaluation was performed, including an assessment of scrotal size to rule out cryptorchidism and malformations of the external genitalia; a Doppler assessment to rule out varicoceles; an immunobead binding evaluation to rule out the existence of anti-sperm immune cells; and genetic fingerprinting to rule out the chronic illnesses such as liver/renal disease, patients who are extremely obese, patients who have hyperglycemia were excluded. There was no subfertility factor in the female partner of the couple included in this study. The semen sample was subjected to analysis for seminal characteristics and the blood sample was drawn for hormonal determination. Fertile males were those without any history of fertility problems and within one year of unprotected intercourse, their partners had spontaneous pregnancy. The fertile and subfertile couples were recruited from assisted conception unit-fertility genetics services (Salma & Kafeel Medical Services) Islamabad.

Measurement of Body Mass Index (BMI)

Height was measured by using Stadiometer and weight by a weighing machine according to the criteria. (Ma *et al.*, 2020). The height of men and women of both groups was recorded in meters and weight in kilograms during their visit to the hospital and BMI was calculated in Kg/m^2 from height and weight by the following formula:

$$BMI = \frac{\text{weight in kilogram}}{\text{height in meters}^2}$$

Semen Collection

Participants provided semen samples that were collected by masturbation into a sterile wide-mouthed, dry, clean, and non-sperm toxic plastic container (labeled corresponding to the patient's name and date) in a private room near the semen laboratory. Subjects were asked to provide a sample after a recommended period of 2-6 days of sexual abstinence.

Semen Analysis

A semen sample was analyzed according to the World Health Organization's guidelines 2010.

Physical Parameters/Characteristics

As soon as the sample was received, the time of specimen produced and the last emission day was recorded. Samples were allowed to liquefy at room temperature. Liquefication was noted after at 37ºC for 30 minutes. Once fully liquefied, all samples were examined for their color, consistency, volume, and pH.

Microscopic Parameters

In seminal plasma pus cells (at a magnification of 400x: high power field/HPF), particular debris and sperm agglutination were analyzed using a phase-contrast microscope.

Sperm Morphology

For morphological analysis semen samples were diluted (1:5) with the diluting fluid. Using a dry, clean glass slide, the people's clinical code was written on it. A 5-10µl drop of semen

was placed on the slide and a smear was prepared using the edge of the other slide. For a thorough sperm morphological study, stained slides were manually examined after the smear had been air-dried and fixed in ethyl alcohol. At least one hundred sperms were counted, and their head, midpiece, and tail defects were noted. Percentages were then calculated based on these data.

Abnormality Index

The sperm deformity index (SDI), (1996) is used to assess the entire spermatozoon using strict criteria and is classified several times if and over one abnormality persists. The index value is determined by taking into account both normal and abnormal sperm cells and evaluating the average number of deformities per sperm. Teratozoospermic index (TZI) is calculated as described by WHO, (1992, 1999); Menkveld and Kruger, (1996). The number of abnormalities present per abnormal spermatozoon is represented by TZI. The TZI value ranged from 1.00 (each abnormal sperm cell has only one deformity) to 3.00. (Each spermatozoon has head, midpiece and tail defects). According to WHO criteria, a TZI value greater than 1.6 is linked to lower pregnancy rates in unmanaged infertile couples, and an SDI of 1.6 is the cutoff point for in vitro fertilisation failure. For Index values following observations and calculations for each sample were also measured:

Number of normal spermatozoa

Number of spermatozoa with defect

Number of spermatozoa with head defect

Number of spermatozoa with midpiece defect

Number of spermatozoa with tail defect

$$\text{Sperm deformity index (SDI)} = \frac{\text{Total number of defect}}{\text{Number of spermatozoa counted}}$$

$$\text{Teratozoospermic index (TZI)} = \frac{\text{Total number of defect}}{\text{Number of spermatozoa with defect}}$$

Oxidant Activity

A UV Spectrophotometer (Agilent 8453) was used to measure the event of oxidative stress (reactive oxygen compounds and thiobarbituric-acid reaction products) and antioxidant enzymes (catalase, sodium (Na) per-oxidase, and guaiacol per-oxidase) in the sperm plasma of control and subfertile male participants.

Superoxide dismutase assay

Utilizing the protocol, SOD activity was determined as described by Kakkar *et al.* (1984).

Procedure detail

To achieve this, 0.3 ml of sample, 1.2 ml of sodium pyrophosphate buffer (0.052 mM; pH 7.0), 0.1 ml of phenazine methosulphate (186 μM), and 0.2 ml of NADH were combined. To stop the reaction and record 560 nm readings, 1 ml of glacial acetic acid was added to the mixture after 1 minute. Units per milligram of protein are used to express results.

Guaiacol peroxidase assay

The activity of guaiacol peroxidase (POD) was assessed using the technique of Chance and Maehly (1955).

Procedure detail

To conduct the reaction, 0.1 ml of homogenate was mixed with 0.3 ml of 40 mM H2O2, 2.5 ml of 50 mM phosphate buffer (pH = 5.0), and 0.1 ml of 20 mM guaiacol. At 470 nm, changes in absorbance were observed after one minute. One unit of POD activity was defined as an absorbance change of 0.01 units in 1 minute.

Reactive oxygen species assay

Reactive oxygen species (ROS) estimation followed the guidelines of Hayashi et al. (2007).

Procedure detail

Dissolving 4.1 g of sodium acetate in 500 ml of distilled water yielded 0.1 M sodium acetate buffer. The pH was kept constant at 4.8. A second solution was made by adding 50 mg of ferrous sulphate (FeSO4) to 10 mg of sodium acetate buffer after dissolving 10 mg

of N, N-Diethyl-Paraphenylenediamine Sulfate Salt (DEPPD) in 100 ml of sodium acetate buffer. Incubation took place for 20 minutes at room temperature in the dark after a 1:2 mixture of the two solutions. The absorbance was checked at 505 nm using a Smart Spec TM plus Spectrophotometer after 20 µl of the solution mixture, 1.2 ml of buffer, and 20µl of the sample were taken in a cuvette. There were three readings per sample per 15 seconds.

Lipid peroxidation by thiobarbituric acid assay

Using the procedure of Wright *et al.,* 1981, thiobarbituric acid (TBA) was used to determine the amount of malondialdehyde in plasma.

Procedure detail

One milliliter of the reaction solution was made up of 0.58 milliliters of phosphate buffer (0.1 M) with a pH value of 7.4 and 0.02 milliliters of ferric chloride (FeCl3) of 100mM. The final mixture was incubated for 1 hour at 37 °C in a water bath before the reaction was stopped with 1 ml of 10% trichloroacetic acid. Following the addition of 1 ml of 0.67% thiobarbituric acid at 95 °C boiling water, all of the tubes were kept there for 20 minutes before being transferred to the crushed ice bath. Following that, the sample was centrifuged for 15 minutes at 25000 rpm, and 535 nm readings from the spectrophotometer were recorded against a reagent blank.

CAT assay

The method of Chance and Maehly was modified slightly to ascertain CAT's activities (Chance and Maehly, 1955).

Procedure detail

A cuvette was filled with 0.1 ml of the sample, 2.5 ml of 50 mM phosphate buffer (pH 5.0), and 0.4 ml of 5.9 mM H2O2 to measure the levels of CAT in the samples. After one minute, the solution's absorbance at 240 nm wavelength was measured. The change in absorbance at 0.01 units for a minute was regarded as one unit of CAT activity.

Assessment of sperm vitality

Sperm vitality Test –Eosin Nigrosin Stain:

Sperm vitality was assessed in wet mount smears after supravital staining with aqueous Eosin-Nigrosin as follows. One drop of semen was mixed on a slide with one drop of 0.5% aqueous yellowish eosin solution and one drop of nigrosin (10% in distilled water) and covered with a cover slip. After 1–2 min the spermatozoa stained red (dead spermatozoa) can be distinguished from the unstained spermatozoa (live spermatozoa). Nigrosine was used as a counter-stain to facilitate visualization of the unstained live spermatozoa. On each slide, 100 spermatozoa from each semen sample were evaluated.

Assessment of sperm membrane integrity (Hypo osmotic swelling –Test; (HOS-Test):

Hypo-osmotic swelling test (HOS-test) was used for the assessment of membrane integrity of spermatozoa. A 100 µl sample of sperm suspension was added to 1 ml of hypoosmotic solution (equal parts of 150 mOsmol fructose and 150 mOsmol sodium citrate solutions), followed by 60 min incubation at 37 C. After incubation, a minimum of 200 spermatozoa were examined per slide under a light microscope and the percentage of spermatozoa that showed typical tail abnormalities (curly tail) indicative of swelling were calculated in Figure 13.

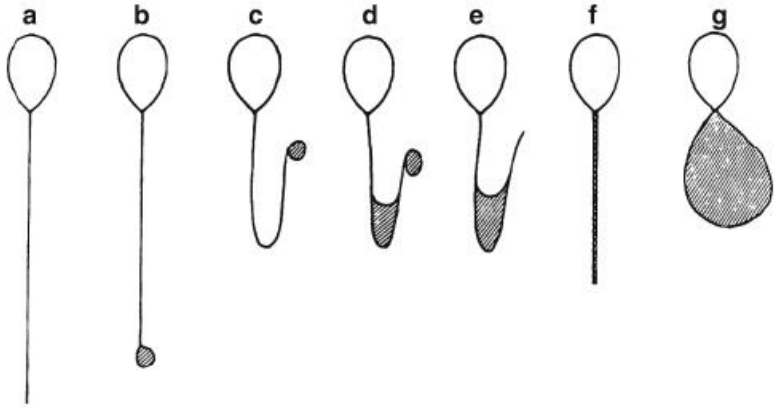


Figure 13. Various morphological changes in human spermatozoa exposed to hypo-osmatic stress are depicted schematically. (a) Unaltered morphology sperm (b-g) sperm with various types of tail swelling indicated by the hatched area

Hormonal Analysis

Serum was separated from centrifuged blood using a micropipette and stored in eppendorf tubes that were properly labeled and kept at 2-8oC until hormones were analysed. The error of repeatedly freezing and thawing the samples was avoided. Hormones estimated for each group of fertile and subfertile subjects were LH, FSH and testosterone.

Protein/Peptide Hormones (LH and FSH)

Principle of LH Assay

For the in vitro quantitative measurement of luteinizing hormone in human serum and plasma, use the LH Immunoassay. The Elecsys and Cobas immunoassay analyzers are intended for use with the electrochemiluminescence immunoassay, or "ECLIA." principles of a sandwich. The assay took 18 minutes in total to complete.

20 µL of the sample, a monoclonal LH-specific antibody that has been biotinylated, and a monoclonal LH-specific antibody that has been labeled with a ruthenium complex form a sandwich complex during the first incubation.

Second incubation: The complex is bound to the solid phase by the interaction of biotin and streptavidin after the addition of microparticles coated in streptavidin.

The measuring cell is aspirated with the reaction mixture inside, and the microparticles are then magnetically drawn to the electrode's surface. Then, using ProCell/ProCell M, unbound substances are eliminated.

A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode. Results are obtained by comparing an instrument-specific calibration curve produced by two-point calibration with a master curve made available by the reagent barcode or e-barcode.

The range of measurement is 0.100 to 200 mIU/mL. (defined by the lower detection limit and the maximum of the master curve). The reported value for values below the lower detection limit is 0.100 mIU/mL. Values that are above the range of measurement are reported as > 200 mIU/mL. The minimum detectable level is 0.100 mIU/mL. The lowest measurable analyte level that can be distinguished from zero is represented by the lower detection limit. The value is two standard deviations above the lowest standard (master calibrator, standard, 1 + 2 repeatability study, n = 21).

Principle of FSH Assay:

Utilizing the Elecsys FSH Immunoassay, the follicle-stimulating hormone in human serum and plasma can be determined quantitatively in vitro.

The electrochemiluminescence immunoassay, also known as an "ECLIA," is designed to be used with immunoassay analyzers made by Elecsys and Cobas.

Sandwich theory. 18 minutes were allotted for the assay.

During the first incubation, a sandwich complex is formed when 40 µL of the sample, two monoclonal antibodies that are specific for FSH and biotinylated with one another.

The complex is subsequently bound to the solid phase by the interaction of biotin and streptavidin following the addition of microparticles coated with streptavidin.

The measurement cell receives the reaction mixture by aspiration, and the microparticles are then magnetically attracted to the electrode's surface. ProCell/ProCell M is then used to remove any remaining unbound substances.

A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

Results are obtained by comparing an instrument-specific calibration curve produced by two-point calibration with a master curve made available by the reagent barcode or e-barcode.

The range of measurement is 0.100 to 200 mIU/mL. (Defined by the lower detection limit and the maximum of the master curve). The reported value for values below the lower

detection limit is 0.100 mIU/mL. Values that are above the range of measurement are reported as > 200 mIU/mL. The test has a lower detection limit of 0.100 mIU/mL. The lowest measurable analyse level that can be distinguished from zero is represented by the lower detection limit. The value of two standard deviations above the lowest standard is used to calculate it (master calibrator, standard 1 + 2 SD, repeatability study, n = 21).

Principle of Prolactin Assay

Prolactin in human serum and plasma can be determined quantitatively in vitro using the Elecsys Prolactin II Immunoassay. The Elecsys and Cobas e immunoassay analyzers are intended for use with the electrochemiluminescence immunoassay, or "ECLIA."

Sandwich theory. 18 minutes were allotted for the assay.

First incubation: A first complex is formed when 10 uL of sample and a monoclonal prolactin-specific antibody are biotinylated.

Second incubation: Following the addition of streptavidin-coated microparticles and a monoclonal prolactin-specific antibody labeled with a ruthenium complex, a sandwich complex is formed and is bound to the solid phase by the interaction of biotin and streptavidin.

The measuring cell is aspirated with the reaction mixture inside, and the microparticles are then magnetically drawn to the electrode's surface. ProCell/ProCell M is then used to remove any remaining unbound substances.

A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

Results are obtained by comparing an instrument-specific calibration curve produced by two-point calibration with a master curve made available by the reagent barcode or e-barcode.

The instructions in the instruction manual for the hormone assay procedure were strictly followed.

Each sample's analyte concentration is automatically determined by the analyzer (either in µIU/mL, ng/mL, or mIU/L).

Conversion factors: ng/mL x 21.2 = µIU/mL (mIU/L) and mIU/L x 0.047 = ng/mL
The range for measurement is 0.0470–470 ng/mL or 1.00–10000 IU/mL. (Defined by the lower detection limit and the maximum of the master curve). Less than 1 IU/mL or 0.0470 ng/mL are reported for values below the lower detection limit. Over the measurement range values
Values above the measuring range are reported as > 10000 µIU/mL or > 470 ng/mL (or up to 100000 µIU/mL or 4700 ng/mL for 10-fold diluted samples).
. Over the measuring range, values are reported as > 10000 IU/mL or > 470 ng/mL (or up to 100000 IU/mL or 4700 ng/mL for tenfold diluted samples).

1.00 µIU/mL (0.047 ng/mL) is the lower detection limit.

The smallest analyte level that can be separated from zero is represented by the lower detection limit. According to the repeatability study's formula (master calibrator, standard 1 + 2 SD, n = 21), it is calculated as the value that is two standard deviations higher than the lowest standard.

STEROID HORMONE (Testosterone)

Principle of Testosterone Assay

For the in vitro quantitative determination of testosterone in human serum and plasma, use the Elecsys Testosterone II Immunoassay. For use on elecsys and Cobas e immunoassay analyzers, the electrochemiluminescence immunoassay, or "ECLIA," is designed.

Competition theory. 18 minutes were allotted for the assay.

First incubation: 20 µL of the sample is incubated with a monoclonal antibody that is specific for testosterone and has been biotinylated. The sample analyte binds to the labeled antibody's binding sites (depending on its concentration).

The complex is bound to the solid phase through an interaction between biotin and streptavidin following the addition of streptavidin-coated microparticles and a testosterone derivate labeled with a ruthenium complex.

ProCell/ProCell M is then used to remove any remaining unbound substances. A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

By using a master curve provided by the reagent barcode or e-barcode and a calibration curve created specifically for the instrument by two-point calibration, results are obtained.

The range of measurement is 0.025–15.0 ng/mL or 0.087–52.0 nmol/L. (defined by the limit of detection and the maximum of the master curve). Values that are less than the detection threshold are reported as 0.025 ng/mL or 0.087 nmol/L. Values that are above the measurement range are indicated by the notation > 15.0 ng/mL or > 52.0 nmol/L.

0.012 ng/mL or 0.042 nmol/L is the limit of the blank.

0.025 ng/mL or 0.087 nmol/L is the limit of detection.

0.120 ng/mL or 0.416 nmol/L is the limit of quantitation.

According to the CLSI (clinical and laboratory standards institute) EP17A specifications, the limit of blank and limit of detection were established.

The outcome of functional sensitivity testing was used to determine the limit of quantitation. The maximum value for a blank is the 95th percentile value from n measurements of analyte-free samples across multiple independent series. The concentration below which analyte-free samples are found with a 95% probability is known as the limit of a blank.

The standard deviation of low concentration samples and the limit of blank is used to calculate the limit of detection. The lowest analyte concentration that can be detected is the limit of detection, which is a value that is greater than the limit of the blank with a 95% probability.

The lowest analysis concentration that can be consistently measured with an intermediate precision CV of less than 20% is the limit of quantitation (functional sensitivity). A low testosterone sample concentration was used to make the determination.

Statistical Analysis

For all statistical calculations, we made use of the Statistical Package for the Social Sciences (IBM SPSS software, version 20 and Graph pad prism version 5). The distribution of all parameters was normal. The data were reported as the mean standard error for the descriptive analysis of the results (SE) and expressed as mean±SEM. Spearman's analysis was also utilized to show correlations between the various parameters. ANOVA was used to assess differences in means ($p< 0.05$). For those outcomes that were connected to one or more examined parameters, prediction models were built. Logistic regression was used to look for predictors that were significantly linked to ART outcomes. The Hosmer-Lemeshow goodness-of-fit test was used to determine the model's prediction's dependability

RESULTS

Demographic characteristics

The study enrolled the participation of 753 couples in total. Of the total sample, fertile normal health of males was 20% (n=146) who participated as the control in our study while subfertile male patients were 80% (n=607). The subfertile patients were split into various categories, and the percentage of these subjects has been summarized in Figure 14. Mild male factor (MMF) subfertile group (n=280) was defined as male participants with a single abnormal finding of the semen analysis or by a total motile sperm count between 5–20 × 10^6/mL with normal morphology <4%, [asthenozoosprmia (AZs), Oligozoospermia (OZs), oligo-asthenozoospermia (OAZs)], whereas the server male factor (SMF) subfertility (n=327) group includes men with were with low sperm count, >15x106/ml, low sperm motility >32 %, total motile sperm count less than 5 × 10^6/mL and low sperm morphology >4% [teratozoopsermia (TZs), astheno-teratozoospermia (ATZs), Oligo-teratozoospermia (OTZs), and Oligo-astheno-teratozoospermia (OATZs)] Moderate factor men (MMF) were with Sever factor men (SMF).

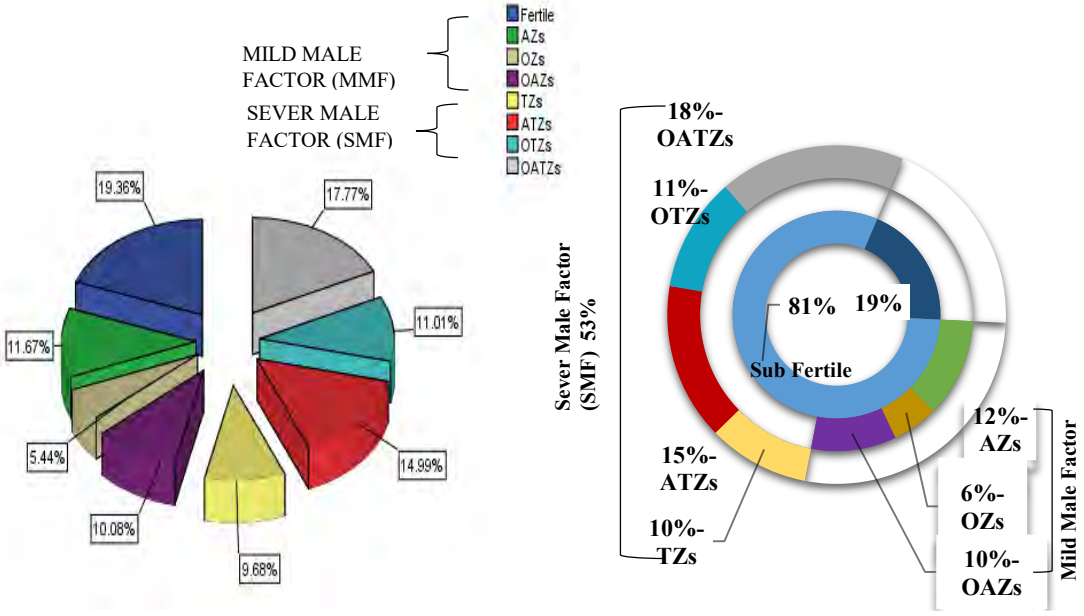


Figure 14. Pie chart showing the percentage of fertile and subfertile male subjects and subgroups in the studied population.

Characteristics of study male population

Characteristics of the study population are summarized in Table 3. Demographic results of the current study revealed no significant (P>0.05) difference in mean±SEM.

Age (Years)

Mean±SEM age of the total studied male subjects was 38.82±0.1 years. Mean±SEM age of fertile males was 38.08±0.6 years. The mean±SEM age of the MMF was 38.82±0.53 and SMF was 39.09±0.37and total subfertile males (both MMF and SMF) (data not shown in table) was 39.0±0.31 years. There is no statistical difference between Mean±SEM ages of fertile control and MMF and SMF male subjects as shown in Table 3. & Figure. 16a.

Body mass index (BMI)

Mean±SEM BMI of fertile male subjects was 24.90±0.3 Kg/m^2 and the total subfertile (including both SMF and MMF) (data not shown in the table) males had a BMI of 25.19±0.14 Kg/m^2. Mean±SEM BMI of the whole studied male population was 25.16±0.2. There is no significant difference in the BMI of fertile control and MMF and SMF male subjects.

Mean±SEM weight and height of fertile and subfertile male subjects were shown in Table 3. and Figure. 16a. There was a non-significant (P>0.05) difference in mean±SEM body weight and height among fertile and both categories of subfertile male subjects.

Subfertility duration

There is no statistical difference between Mean±SEM subfertility duration of fertile control and MMF and SMF male subjects as shown in Table 3. & Figure. 16b.

Socio-Economic Status

The socio-economic characteristics of these fertile and subfertile male subjects had no difference in all studied subjects (Figure. 15a).

Living together

There was no difference between couples living together or husbands working abroad or both partners staying away from each other due to some other reasons (Figure. 15b).

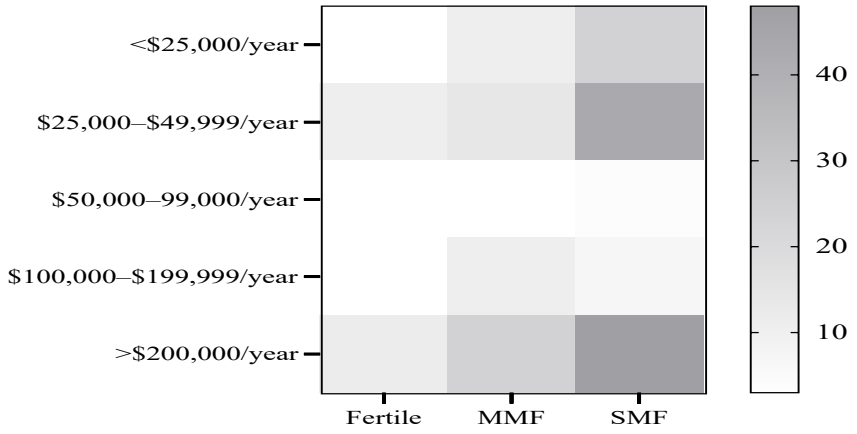
Table 3: Mean±SEM characteristics of the male subject included in the study

Parameters	Fertile (NZs) (146)	MMF (OZs, AZs, OAZs) (280)	SMF (TZs, ATZs,OTZs, OATZs) (327)	Total (753)
Age (Years)	38.08±0.6	38.82±0.53	39.09±0.37	38.82±0.1
Height (cm)	177.5±1.3	176.28±0.92	180.38±0.8	178.7±0.6
Weight (Kg)	92.37±7.5	90.74±5.81	73.30±4.11	81.60±3.1
BMI (kg/m2)	24.90±0.3	25.16±0.26	25.25±0.16	25.16±0.2
Subfertility duration (Years)	7.21±0.6	8.27±0.53	10.27±0.39	9.53±0.3

Values represent Mean±SEM;

a= Fertile vs MMF and SMF; b=MMF vs SMF, P<0.001 ***, P<0.01**, P<0.05*

(a)



(b)

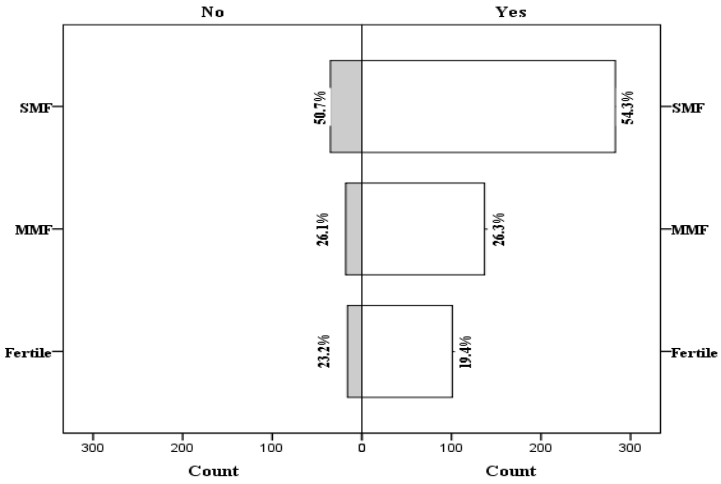
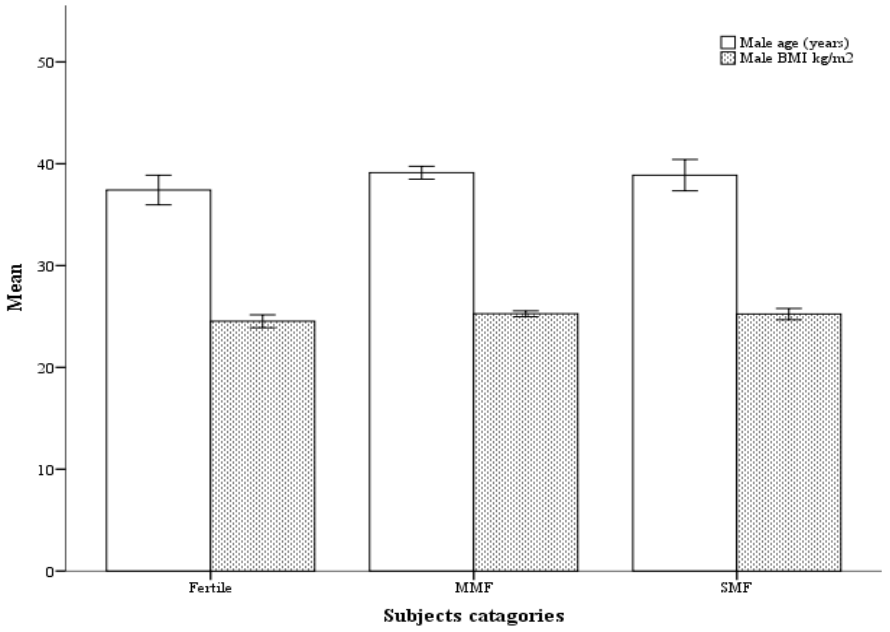


Figure 15. Mean (a) annual income of couples and (b) Couples living together included in the study

(a)



(b)

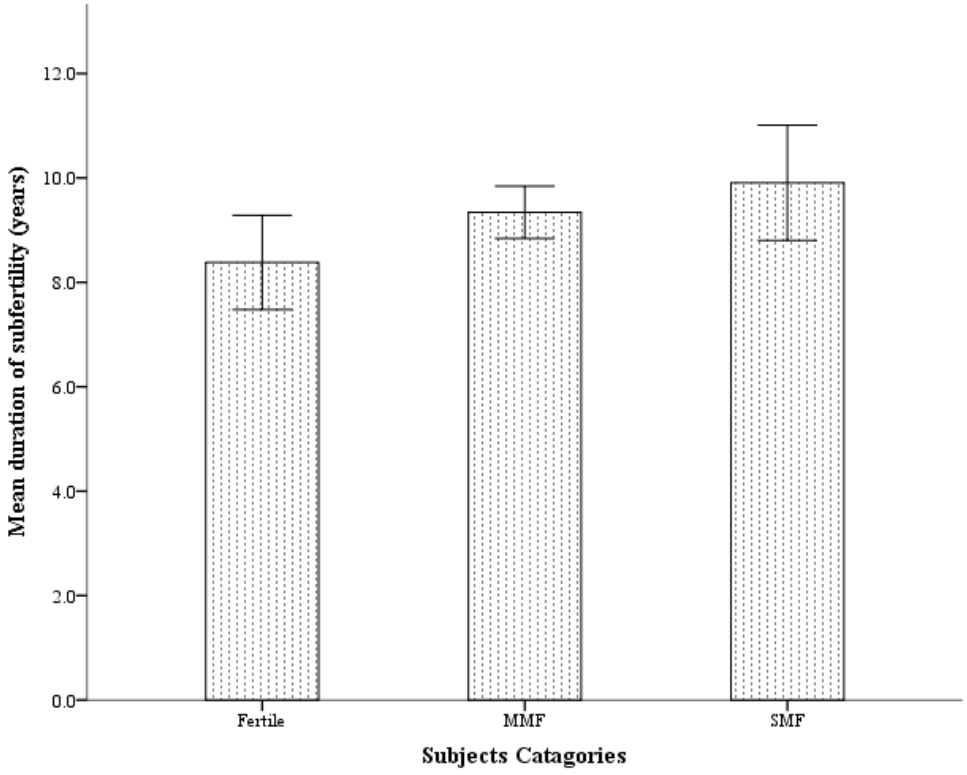


Figure 16. Mean (a) Age (years), BMI kg/m^2 and (b) subfertility duration (years) in studied groups

a= Fertile vs MMF and SMF; b=MMF vs SMF

P<0.001 ***, P<0.01**, P<0.05*

Semen Characteristics of Study Male Population:

Semen Volume (ml)

Mean±SEM semen volumes of fertile and subfertile male subjects were shown in Table 4. The semen volume of fertile men was 3.94±0.13ml. Mean±SEM semen volume of subfertile patients with MMF was 4.05±0.13ml and with SMF was 3.69±0.08ml. There was a non-significant (P>0.05) difference in mean semen volume of fertile and different categories of subfertile male patients.

pH

Mean±SEM pH values of different categories of subfertile patient groups showed no statistically significant (P>0.05) when compared to fertile male subjects (Table 4).

Last emission and Liquefication time

There was a non-significant (P>0.05) difference in the last emission and liquefication time between fertile subjects and subfertile categories (Table 4).

White blood cells (WBCs)

In fertile male subjects, the Mean±SEM of white blood cells (WBCs) counts in high power fields (HPF) was 3.52±0.17. No significant (P>0.05) difference was found in WBC count within fertile male subjects and subfertile male patients (Table 4).

Sperm Count (million/ml)

Mean±SEM sperm count in fertile men was 121.21±5.3 million/ml, whereas the mean±SEM sperm count of subfertile MMF patients was 46.9±2.5 million/ml and in SMF was 34.12±1.48 million/ml (Table 4). There was a significant (P<0.001) decrease in mean sperm count in MMF and SMF compare to fertile. SMF showed a significant (P<0.001) decrease in sperm count compared to MMF male subjects.

Sperm motility

Significant (P>0.05) difference was noticed in sperm motility in fertile control subjects than that of MMF and SMF male subjects. MMF and SMF male patients showed a significantly (P<0.001) lower percentage of rapid linear progressive and forward

progressive motile sperm when compared to the values of fertile. MMF and SMF male patients had a significantly (P<0.001) higher percentage of slow progressive motile, Local motile and immotile sperm than fertile control male patients. SMF male subjects had significant (P<0.001) low levels of rapid linear progressive and forward progressive motile sperm, and a significantly (P<0.001) higher percentage of slow progressive motile, Local motile and immotile sperm than in MMF (Table 4).

Sperm morphology

A significant (P<0.001) decrease was found in the percentage of morphological normal-form sperm in SMF male patients when compared to fertile and MMF male subjects. Mean±SEM percentage of morphologically normal spermatozoa was 4.96±0.13% in fertile male subjects, while subfertile male patients' morphological normal spermatozoa mean±SEM was 5.04±0.08 and 2.23±0.04 in MMF and SMF respectively (Table 4). Morphologically abnormal sperm mean percentage was significantly (P<0.001) higher in SMF male patients than in fertile and MMF male subjects.

Abnormal sperm categories

In fertile males mean percentage of sperm with head defect, head midpiece (HM), head-midpiece-tail (HMT), midpiece defect (MP), sperm with tail defect, midpiece tail (MT) defect per hundred spermatozoa were significantly (P<0.001) higher in SMF male patients than in fertile and MMF male subjects. Mean±SEM percentage of sperm with head-tail (HT) defect per hundred spermatozoa showed no significant change was observed between the studied groups as shown in Table 4.

Sperm Abnormality index

Mean±SEM sperm deformity index (SDI) of the fertile male was 0.99±0.03 and in subfertile male patients, SDI was 1.08±0.03 (MMF) and 1.30±0.04 (SMF) respectively. Mean±SEM SDI was significantly (P<0.001) high in MMF and SMF than in the fertile subjects. In SMF mean SDI was significantly (P<0.001) increased compared to fertile and MMF male subjects.

The fertile male had a mean±SEM teratozoospermic index (TZI) of 1.65±0.04 and it in the subfertile male patient was 1.98±0.0 and 1.70±0.04 in MMF and SMF. It was observed that a highly significant ($P<0.001$) increase in TZI of MMF male patients compared to fertile and SMF. A significant increase in TZI was also found in SMF male patients than in the fertile control ($P<0.001$) in the contrary a significant ($P<0.01$) decrease in TZI was found in SMF male patients compared to MMF (Table 4).

Table 4: Comparison of conventional sperm parameters in the different studied groups.

	Fertile (NZs) (146)	MMF (OZs, AZs, OAZs) (280)	SMF (TZs, ATZs, OATZs) (327)
Volume (ml)	3.94±0.13	4.05±0.13	3.69±0.08
pH	8.00±0.00	8.11±0.11	7.99±0.01
WBC/HPF	3.52±0.17	3.80±0.41	2.87±0.10
Liquefication time (minutes)	31.13±0.45	32.9±0.83	31.43±0.46
Count x10^6	121.21±5.3	46.9±2.5^{a***}	34.12±1.48^{ab***}
Fast forward progressive (%)	29.04±0.90	11.89±0.99^{a***}	11.74±0.50^{ab***}
Slow Forward progressive (%)	22.95±0.87	24.24±0.94^{a***}	16.98±0.82^{a***}
Forward Motility (%)	51.99±1.0	35.78±1.95^{a***}	25.00±1.48^{ab***}
Non-progressive Motility (%)	22.71±0.95	6.59±0.41^{a***}	11.74±0.43^{ab***}
Immotile (%)	24.62±1.12	57.56±0.94^{a***}	45.64±1.6^{ab***}
Normal Morphology %	4.96±0.13	5.04±0.08	2.23±0.04^{ab***}
Abnormal Morphology %	94.27±0.26	94.96±0.08	97.77±0.04^{ab***}
Head Defects %	13.76±0.42	18.28±0.40^{a***}	17.39±0.54^{a***}
Head-Midpiece defects %	5.66±0.48	7.88±0.32^{a***}	9.00±0.30^{ab***}
Head Tail defects %	5.15±0.49	5.19±0.36	4.97±0.23
Mid-piece defects %	6.90±0.41	7.78±0.32	5.86±0.28^{b***}
Mid piece Tail defects %	6.72±0.39	8.15±0.38^{a***}	6.39±0.27^{b***}
Head Midpiece Tail defects %	9.03±0.64	14.12±0.62^{a***}	19.82±0.75^{ab***}
TZI	1.65±0.04	1.98±0.0^{a***}	1.70±0.04^{ab***}
SDI	0.99±0.03	1.08±0.03^{a***}	1.30±0.04^{ab***}

Values represent Mean±SEM;

a= Fertile vs MMF and SMF; b=MMF vs SMF, P<0.001 ***, P<0.01**, P<0.05*

Computer-assisted sperm analysis (CASA)

Sperm velocity measurements; Straight-line velocity (VSL µm/sec)

Mean±SEM VSL of fertile and subfertile male subjects is illustrated in Table 5 and in Figure. 17. It was observed that MMF and SMF male subjects' sperms had a significant ($P<0.001$) decrease in VSL compared to fertile male subjects. Similarly, sperms of MMF male patient's had significantly ($P<0.05$) higher VSL than SMF male patients.

Average path velocity (VAP µm/sec) and curvilinear velocity (VCL µm/sec)

There was a significant ($P<0.05$) decrease in VAP of MMF male patients' sperm compared to fertile male subjects (Table 5. Figure. 17). Fertile male subjects had significant ($P<0.05$) higher VAP than SMF male patients. Sperms VCL showed a significant decrease in MMF ($P<0.001$) and SMF ($P<0.05$) compared to fertile male subjects.

Local motile (%) and Circular swimmer

MMF male patients had a significantly ($P<0.001$) low percentage of local motile sperm compared to local motile sperm of fertile male subjects. No significant difference was observed in the number of circular swimmer sperm compared between fertile, SMF and SMF (Table 5; Figure. 18).

The amplitude of lateral head displacement (ALH µm/sec)

Mean±SEM ALH of fertile and subfertile male subjects was shown in Table 5. It was observed that there was no appreciable ($P>0.05$) difference in ALH, WOB and LIN of fertile and all subfertile male subject categories (Table 5).

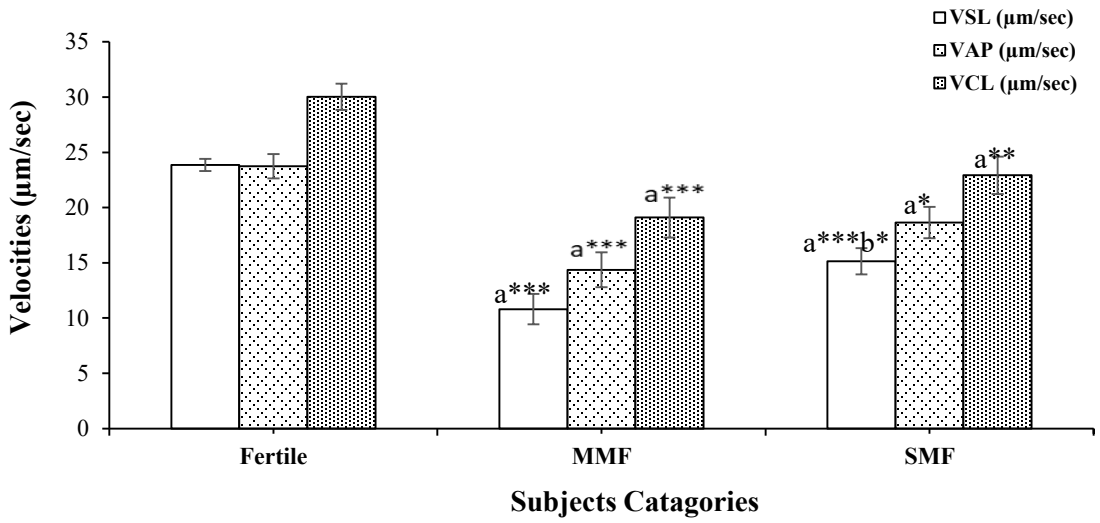


Figure 17. Mean±SEM sperm velocities (µm/sec) were measured through CASA of fertile and subfertile subjects i.e., MMF and SMF.

a= Fertile vs MMF and SMF; b=MMF vs SMF, P<0.001 ***, P<0.01**, P<0.05*

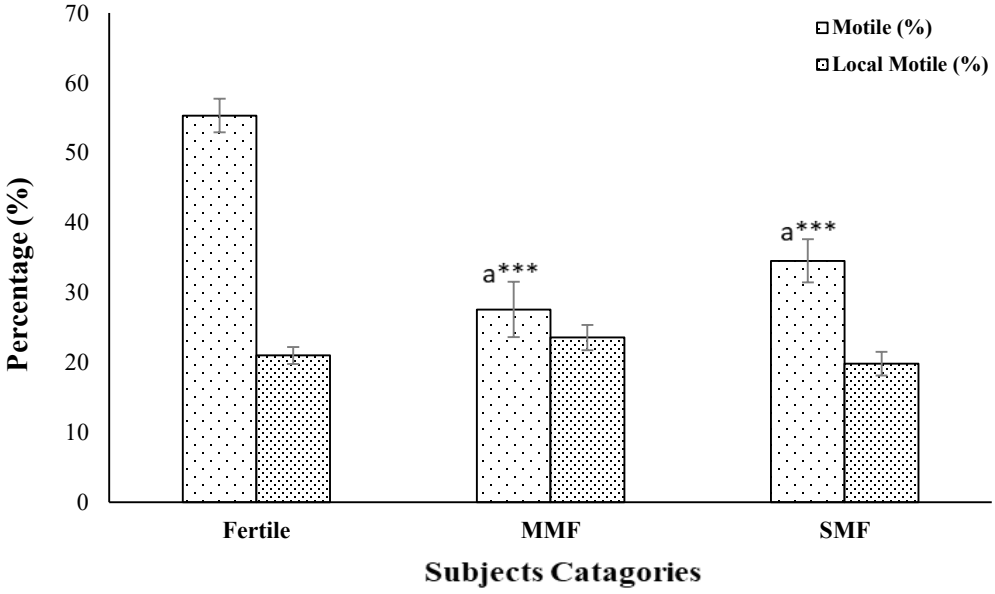


Figure 18. Mean±SEM sperm percent motility was measured through CASA in fertile and subfertile subjects i.e., MMF and SMF.

a= Fertile vs MMF and SMF; b=MMF vs SMF

P<0.001 ***, P<0.01**, P<0.05*

Table 5: Mean Computer-assisted sperm analysis of fertile and subfertile men

CASA parameters	Fertile (31)	MMF (30)	SMF (62)
Straight line velocity µm/sec (VSL)	23.87±0.55	10.80±1.38[a***]	15.15±1.19[a***b*]
Average path velocity µm/sec (VAP)	23.74±1.11	14.37±1.59[a***]	18.65±1.42[a*]
Curvilinear velocity µm/sec (VCL)	30.03±1.20	19.10±1.82[a***]	22.92±1.70[a**]
Motile (%)	55.32±2.40	27.57±3.96[a***]	34.53±3.08
Local Motile (%)	21.00±1.81	23.57±1.95	19.81±1.60
Wobble (WOB)	0.97±0.03	1.00±0.00	0.97±0.02
Amplitude of lateral head displacement µm/sec (ALH)	0.90±0.05	1.00±0.15	0.73±0.06
Circular swimmer (%)	0.16±0.08	0.23±0.08	0.27±0.08
Linearity (LIN)	0.94±0.04	0.63±0.09[a***]	0.84±0.05[a*]

Values represent mean± SEM and values in parentheses represent the number of subjects

a= Fertile vs MMF and SMF; b=MMF vs SMF

P<0.001 ***, P<0.01**, P<0.05*

SPERM VITALITY

Membrane Integrity (Hypo-Osmotic Test; HOS) and Sperm Vitality (Eosin Test):

The sperm membrane integrity percentage of fertile subjects measured through Hos was 86.50±0.96%, whereas MMF was 69.55±0.73%, similarly, SMF had membrane integrity of 63.07±1.21%. Sperm vitality in fertile men was 71.74±1.26 and in MMF and SMF 48.85±1.18 and 49.69±1.25 respectively.

Data showed a highly significant ($p<0.001$) decrease in sperm membrane integrity (% eosin) percentage of subfertile MMF and SMF patients than fertile male subjects (Table 6). MMF male patients had a significant ($P<0.05$) increase percentage of sperm membrane integrity compared to SMF. Sperm vitality (Eosine) percentage was significantly higher in fertile male subjects than in MMF ($P<0.001$) and SMF ($P<0.01$) male patients.

OXIDATIVE STRESS MARKERS

Antioxidant enzymes, i.e., Catalase (CAT), Superoxide Dismutase (SOD), Guaiacol peroxidase (POD) and oxidative stress marker, i.e., reactive oxygen species (ROS) and Lipid Peroxidation (LPO), was estimated in semen sample of the fertile and subfertile male subjects.

SOD, CAT and POD:

Antioxidant enzymes including superoxide dismutase, SOD; catalase, CAT and Guaiacol peroxidase (POD) were evaluated in all groups (Table 8). There was a significant difference in SOD ($P<0.001$) and POD ($P<0.001$) levels in the fertile male group compared to the fertile control. SMF patients had a significantly ($P<0.001$) lower level of SOD and POD compared to MMF and fertile male patients (Table 7: Figure.19).

Estimation of reactive oxygen species (ROS), and Lipid Peroxidation (TBRAS):

A significant increase in ROS and lipid peroxidation (thiobarbituric acid reactive species) were observed in SMF ($P<0.001$) as compared to the fertile control and MMF. There were significantly ($P<0.05$) reduced levels of ROS in MMF compared to fertile (Table 7: Figure. 19).

Table 6: Sperm membrane integrity and vitality of fertile and subfertile subjects.

	Fertile (NZs) (n=146)	Mild Male Factor (OZs, AZs, OAZs) (n=280)	Sever Male Factor (TZs, ATZs, OATZs) (n=327)
Membrane integrity (Hypo Osmatic Swelling) %	86.50±0.96	69.55±0.73^{a***}	63.07±1.21^{ab***}
Sperm vitality (Eosin) %	71.74±1.26	48.85±1.18 ^{a***}	49.69±1.25 ^{a***}

Values represent Mean±SEM

a= Fertile vs MMF and SMF; b=MMF vs SMF

*=P<0.05, **= P<0.01, ***=P<0.00

Table 7: Oxidative stress markers in semen sample of the studied population

	Fertile (NZs) (n=146)	Mild Male Factor (OZs, AZs, OAZs) (n=280)	Sever Male Factor (TZs, ATZs, OATZs) (n=327)
CAT (u/ml)	9.66±0.08	9.50±0.04	9.18±0.05
SOD (U/mg)	13.67±0.24	13.29±0.18^{a***}	11.92±0.11^{ab***}
POD (U/min)	10.09±0.03	11.0±.03 ^{a***}	10.31±.04 ^{ab***}
Lipid peroxidation (nM/ml)	28.05±0.12	28.23±0.04	29.47±0.10^{ab***}
ROS (nmol)	1.29±0.07	1.05±0.02^{a*}	2.43±0.05^{ab***}

Values expressed as Mean±SEM

a= Fertile vs MMF and SMF

b=MMF vs SMF

*=P<0.05, **= P<0.01, ***=P<0.001

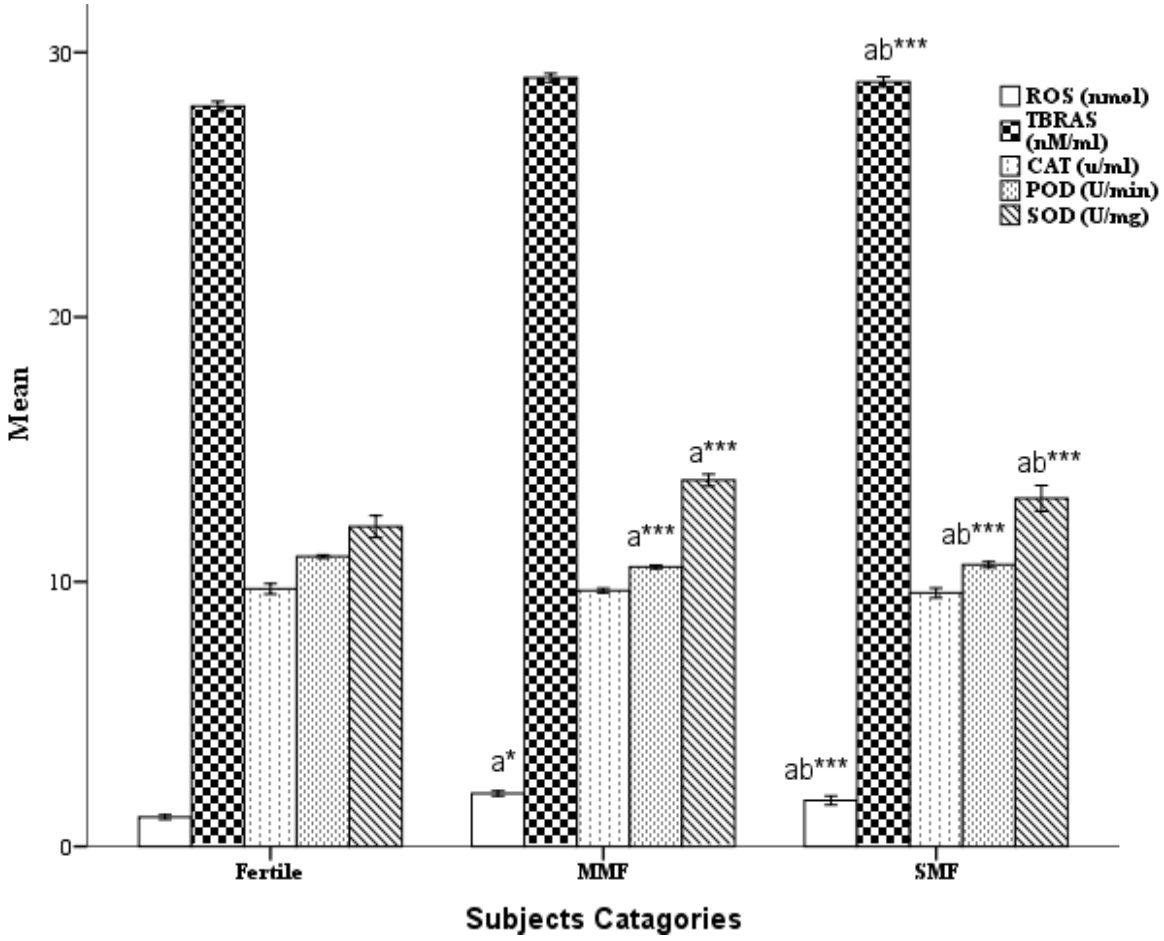


Figure 19. Mean±SEM concentration of oxidative species including; reactive oxygen species (ROS) nmol, Lipid peroxidation (TBRAS) nM/ml and antioxidant levels including; superoxide dismutase (SOD) u/ml, catalase (CAT) and guaiacol peroxidase (POD), in semen samples of the studied population.

a= Fertile vs MMF and SMF; b=MMF vs SMF

P<0.001 ***, P<0.01**, P<0.05*

Correlation between ROS, age, BMI and semen parameters

We found no correlation between ROS and age (r=0.023, p=0.533) while a positive correlation was found between increased ROS with male BMI (r=0.123 p=0.001). However a negative correlation between ROS and normal morphology (r=-0.137) was negative significant (p=0.000), while a positive correlation was observed between ROS with TZI (r=0.570, p=0.00) and with SDI (r=0.717 p=0.001), a negative correlation was observed between ROS and immotile sperm r=0.199, p=0.0001 progressive motility (AB) r=-0.187, p=0.0001 and membrane integrity (HOS) (r= -0.328, p=0.0001) (Table 8).

While increased age correlated positively (r=0.252) significantly (p=0.0001) with BMI. whereas no correlation was observed between men's age and normal morphology (r=0.002, p=0.954), similarly no correlation was observed between age with TZI (r=-0.062, p=0.119) and a weak negative correlation with SDI (r=-0.096 p=0.015), a positive correlation was observed between age and progressive motility (AB) and membrane integrity (HOS) (r= -0.171 and r=-0.216, p=0.0001).

A negative correlation was observed between BMI and normal morphology (r=-0.86, p=0.02) while a positive correlation was observed between BMI with TZI and SDI (r=0.147 and r=0.125, p=0.0001), and a negative correlation was observed between BMI and progressive motility (AB) and membrane integrity (HOS) (r= -0.333 and r=-0.176, p=0.0001).

Table 8: Correlation between ROS, Age, BMI and semen parameters in the studied population

	Spearman's	ROS	Age	BMI
Men Age	r=	.023		.252**
	p=	.533		.000
BMI	r=	.123**	.252**	
	p=	.001	.000	
Normal	r=	-.137**	.002	-.086*
	p=	.000	.954	.020
TZI	r=	.570**	-.062	.147**
	p=	.000	.119	.000
SDI	r=	.717**	-.096*	.125**
	p=	.000	.015	.002
AB	r=	-.187**	.171**	-.333**
	p=	.000	.000	.000
IMMOTILE	r=	.199**	.228**	.309**
	p=	.000	.000	.000
HOS	r=	-.328**	.216**	-.176**
	p=	.000	.000	.000

ROS, Reactive oxygen species; BMI, body mass index, TZI, teratozoospermia index; SDI, Sperm deformity index; AB, progressive motility, HOS, hypo-osmotic swelling test.

REPRODUCTIVE HORMONE:

The hormonal concentration of Prolactin (ng/ml), LH (mIU/ml), FSH, and testosterone level in blood serum were evaluated in fertile and subfertile male subjects.

Prolactin (ng/ml):

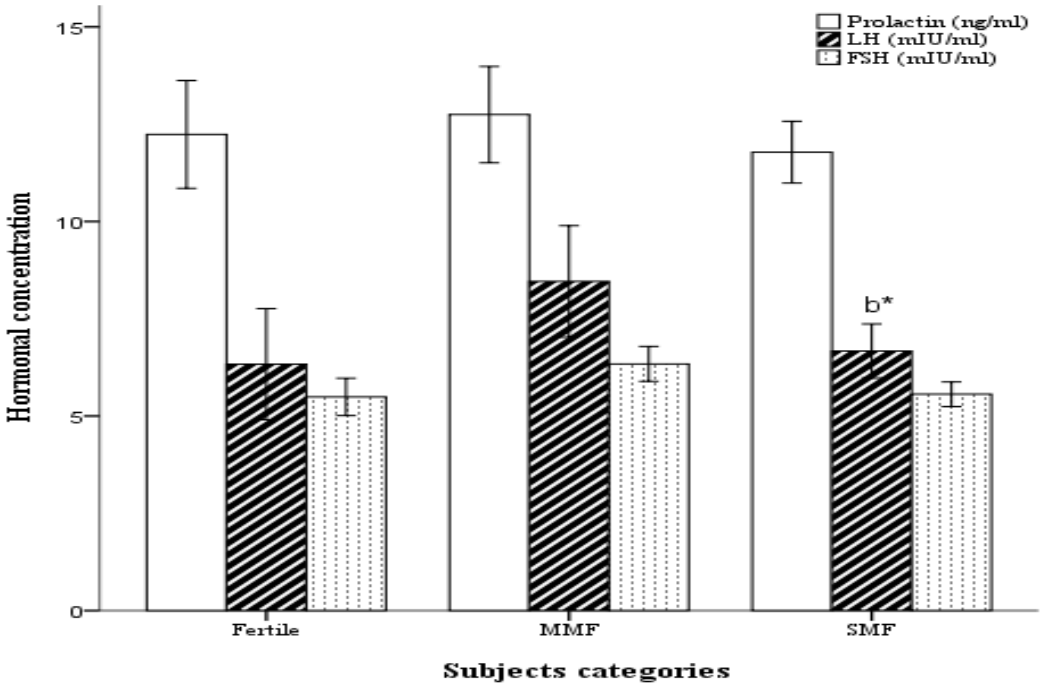
Mean±SEM serum concentration of prolactin in the fertile male subject was 12.23±0.69ng/ml, whereas subfertile MMF and SMF men had 12.70±0.61 ng/ml and 11.78±0.39 ng/ml respectively. There was no statistically significant ($P>0.05$) difference found between fertile male subjects and other categories of subfertile male patients.

LH and FSH (mIU/ml)

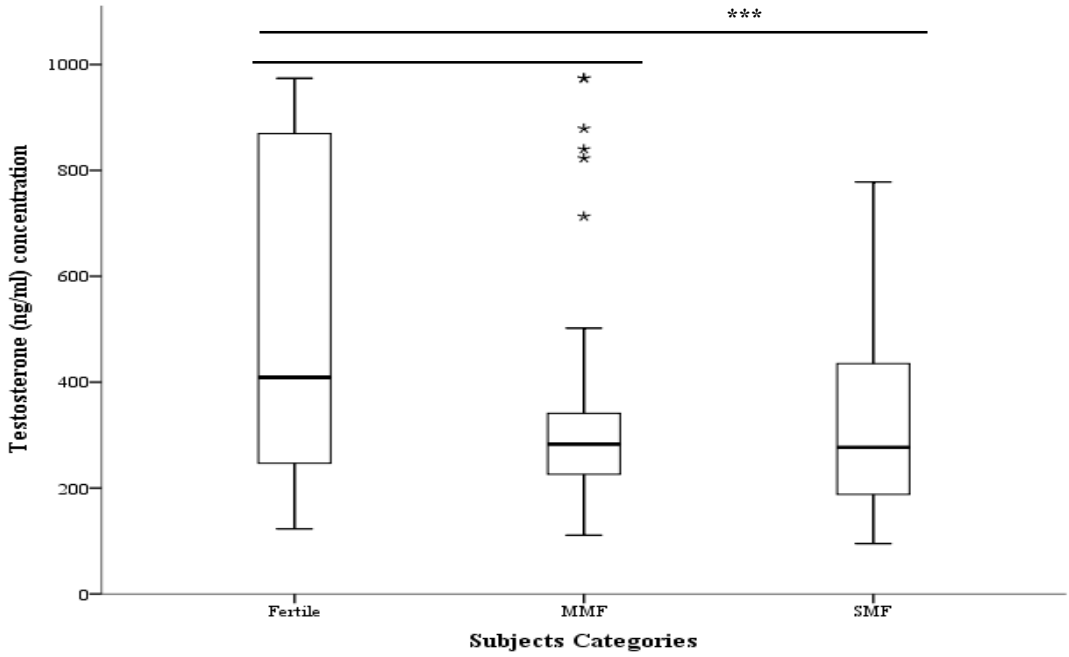
Mean±SEM serum LH concentration in fertile male subjects was 6.46±0.72mIU/ml, whereas MMF and SMF subfertile male patients had mean±SEM LH concentration of 8.46±0.72mIU/ml and 6.75±0.35mIU/ml respectively. Mean±SEM serum FSH concentration was 5.64±0.28mIU/ml in fertile male subjects, whereas mean±SEM serum FSH concentration was 6.32±0.22mIU/ml and 5.66±0.17mIU/ml in MMF and SMF subfertile male respectively. A significant ($P<0.05$) increase in LH concentration was found in MMF male patients compared to SMF male patients. There was a non-significant ($P>0.05$) difference in serum LH and FSH concentration (mIU/ml) in fertile and subfertile categories of SMF and MMF male subjects (Table 9; Figure.20a).

Testosterone (ng/ml)

Testosterone (T) concentration in fertile male subjects was 505.98±26.86 ng/ml, while subfertile male patients had mean±SEM T concentration of 319.71±10.62ng/ml and 336.0±10.63ng/ml in MMF and SMF respectively. A significant decrease in serum testosterone concentration was found in MMF and SMF male patients compared to fertile control subjects (Tablet 9; Figure. 20b).



(a)



(b)

Figure 20. Mean±SEM of (a); Prolactin (ng/ml), LH (mIU/ml), FSH (mIU/ml) and (b); Boxplot Testosterone (ng/ml) levels of fertile and subfertile subjects i.e., MMF and SMF.

a= Fertile vs MMF and SMF; b=MMF vs SMF

*=P<0.05, **= P<0.01, ***=P<0.001

Table 9: Serum levels of Prolactin (ng/ml), LH (mIU/ml), FSH (mIU/ml) and Testosterone (ng/ml) levels of fertile and subfertile subjects

	Fertile (NZs) 146	**Mild Male Factor (OZs, AZs, OAZs) 280**	**Sever Male Factor (TZs, ATZs, OATZs) 327**
Prolactin (ng/ml)	12.23±0.69	12.70±0.61	11.78±0.39
FSH (mIU/ml)	5.64±0.28	6.32±0.22	5.66±0.17
LH (mIU/ml)	6.46±0.72	8.46±0.72	6.75±0.35[b*]
Testosterone (ng/ml)	505.98±26.86	319.71±10.62[a***]	336.00±10.63[ab***]

Values represent Mean±SEM

a= Fertile vs MMF and SMF; b=MMF vs SMF

*=P<0.05, **= P<0.01, ***=P<0.001

DISCUSSION

Male factor subfertility is gaining attraction because of declining semen quality, and sperm standard parameters of healthy young men. Subfertility remains a major health concern worldwide, malefactors contribute to more than half of all subfertility cases (Barratt *et al.*, 2017; Levine *et al.*, 2017). Male factor subfertility is rising in Pakistan and around the globe and it's become challenging because of therapeutic modality. Male subfertility is challenging because oxidative stress, apoptosis, and chromatin damage contribute significantly to its complex pathophysiology (Ahmed *et al.*, 2020). The male fertility problems and coexistence of imbalance are associated with the alteration of semen parameters. This study has determined that fertile and subfertile men link with semen characteristics. We found a higher prevalence of oligoasteno teratozoospermia than astheno teratozoosprmia in severe male factor subfertility as reported before by (Dada *et al.*, 2012; Leung *et al.*, 2018; Salas-Huetos and Aston, 2021).

Semen standard parameters in male factor subfertile are similar as reported before. Semen volume, pH, and WBC levels showed no difference in fertile and subfertile men. There is no significant difference in BMI and age in fertile and male factor subfertile men (MacDonald *et al.*, 2010). There is equal distribution of socioeconomic background of all studied groups. The minimum duration of subfertility was one year and the maximum was 18 years. The evaluation, management, and therapeutic interventions of male factor subfertility can be aided by identifying the various risk factors and assessing particular markers. Sperm quality is related to human fertility, and healthy sperm characteristics, such as sperm amount, pH, liquefication time, sperm concentration, sperm motility, sperm morphological characteristics, and CASA variables, were examined in fertile men (Finelli *et al.*, 2021). The length of abstinence was considerably correlated with total motile count and sperm concentration in the semen. The duration of abstinence and the semen parameters showed no difference. Subfertile men were classified depending on the classic sperm characteristics. In comparison to male subjects who were fertile and had normal sperm variables, subfertile men had reduced sperm counts, progressive sperm, and morphological characteristics. The present study identified a correlation between standard semen parameters of normozoospermic and male factor subfertile subjects. Sperm

concentration is lowest in severe malefactors and lowers in mild male factors when compared to the fertile subject. Current data showed a declining trend in sperm count in Asian males which is similar to US and European countries. This may be attributed to lifestyle, smoking, food habits, fertilizer, gossypol, and other pesticides (Mascarenhas *et al.*, 2012). Sperm motility and total motile sperm had prognostic ability to distinguish between male factor subfertility and normal fertile men. Reduced sperm count, motility and morphology are linked with poor fertilization because of slow capacitation and acrosome reaction. WHO 2010 revised criteria illustrated reference value of sperm morphology that 96% of spermatozoa are morphologically abnormal in normal fertile men. We found a similar value in fertile normal men (WHO, 2010). Although morphology classification has mostly been used to predict fertility success in recent years, it is often overlooked that this assessment might help researchers better understand why certain men's spermatozoa have a lower functional capacity (For instance, those who lack the ability to fertilise due to an increased prevalence of acrosome deficiencies or those who are partially or entirely immobile as a direct consequence of tail or midpiece abnormalities). In the long run, greater assessments are anticipated, by providing details on spermatogenesis and other processes involving the male reproductive system, it is possible to identify variables that impact male reproductive health. Sperm morphological defects of head, tail and mid-piece of subfertile men had higher percentage of these defects in their sperm and TZI and SDI in semen sample. Sperm morphological defects are higher in patients with severe male factor (Candela *et al.*, 2021; Delbes *et al.*, 2013).

Globally, genuine adhering to WHO criteria for semen evaluation has been insufficient, leading to lower precision and constrained inter-center generalisability of findings. Although WHO guides were published from 1980 to 2010, the concern of lack of homogeneity has long been acknowledged. It is believed, however, that the findings of the previous investigation accurately reflect WHO method imprecision in a typical busy laboratory (Cooper *et al.*, 2010; Topp *et al.*, 2015). The CASA method proved to be more precise than the WHO method. CASA parameters are deranged in subfertile male subjects as reported in previous studies as predictors of fertility (Finelli *et al.*, 2021; O'Meara *et al.*, 2022). We're aware that the ability to "identify" a quickly moving sperm is a source of contention. It's vital to remember that determining the exact velocity of each spermatozoon

isn't necessary to distinguish between slow and quick spermatozoa; CASA is the only way to do so when compared to the WHO technique, CASA is preferable in terms of precision.

Male reproductive hormones are involved in the initiation and maintenance of reproductive function, as well as sperm quality. The subfertile men had deranged reproductive hormones as a well-established fact that these hormones directly or indirectly regulate spermatogenesis, sperm function and semen quality by interacting with the reproductive axis of males. Endocrine levels are routinely assessed in cases with low sperm count or absence of spermatozoa in ejaculate, erectile dysfunction, hypothermia, or hypogonadism. Basic reproductive hormonal assessment includes serum FSH, LH, and testosterone, and may also include prolactin, estradiol, or inhibin (MacDonald *et al.*, 2010; Zhao *et al.*, 2020). Reproductive hormone concentration influence male reproductive health as evident in different anthological diseases associated with semen deformities. There is a shred of strong evidence that male reproductive hormone concentration had a strong association with fertility problems. In male reproductive hormones influence the reproductive axis which leads to different andrological diseases due to low semen quality i.e., sperm motility, CASA motility parameters and morphology, and quantity i.e., sperm concentration. Sperm VSL, VAP and VCL were compromised in male factor subfertility due to low motility. To develop and sustain testicular function, FSH and LH work together; LH controls testosterone synthesis in Leydig cells, while FSH controls spermiogenesis and spermatogenesis (Tendayi, 2020; MacDonald *et al.*, 2010). It was shown that high levels of FSH and LH in azoospermia and oligozoospermia encourage Leydig and Sertoli cells to produce and secrete testosterone in proportionate amounts, hence enhancing spermatogenesis (Tendayi, 2020). Due to the poor participation rates in semen quality studies, proposed using male reproductive hormones to predict semen quality in epidemiology investigations. Treatment with hormone replacement medication, which can promote tube activity, may be useful for subfertile men, and hormonal assessment should predict response to treatment (Hofny *et al.*, 2010).

The hypo-osmotic swelling test, on the other hand, appears to be a potential means of identifying living spermatozoa for ICSI. Under hypo-osmotic conditions, only live sperm cells with a chemically and physically intact membrane experience tail swelling due to

water influx. We found a lower level of viable sperm in subfertile men compared to fertile men. Even though various organizations have looked into the link between the HOS and other sperm parameters, as well as the HOS's predictive usefulness for IVF success, there are surprisingly few data on comparisons of the HOS and other dye-exclusion tests in the literature. Although the current investigation found a HOS and the eosin Y test are strongly associated, which corresponds with the findings of (Takahashi *et al.*, 1990). The uniformity in the disparity between the HOS and the eosin Y tests, at least for asthenozoospermic patient samples, can be used to explain the extremely substantial differences in the comparisons between the eosin Y and the hypo-osmotic solutions, as shown by the paired t-test (Holmes *et al.*, 2019). It's difficult to explain the disparities in findings between the two exams. Because spermatozoa with a blunt tail tip were deemed non-viable, lowering the likelihood that they would contain false-positive data, the HOS produced better results, which cannot be attributed to an overestimation of favorably reacting cells. Only observably enlarged cells were deemed viable. They are not responsible for either the divergence or the differences between the two primary viability tests. While eosin Y exclusion only requires structural integrity of the cell membrane, response to hypoosmotic conditions necessitates both structural and functional integrity of the lipid bilayer (Gashi *et al.*, 2021). Given this disparity, one would predict eosin Y to have larger percentage viability than the HOS, which contradicts our data. In cases of full asthenozoospermia brought on by electron microscopic abnormalities, it is not yet clear whether using the HOS will lead to appropriate fertilisation and cleavage. Compared to motile spermatozoa, immotile spermatozoa more frequently have centriolar deficiencies (Stangera *et al.*, 2010). The growth of the embryo may be decelerated, interrupted, or ceased if an immotile spermatozoon is injected. This happens because aberrations of the mitotic spindle are even more prevalent in these instances (Gashi *et al.*, 2021).

Is it well known that ROS production in human sperm is necessary for tyrosine phosphorylation associated with capacitation for normal fertilization (Agarwal *et al.*, 2019). In contrast to healthy controls, the current research compared the ROS levels in a male patient with subnormal semen parameters. Similarly, many studies showed that increased ROS activity than antioxidant leads to oxidative stress. It can cause compromised semen quality which leads to male subfertility. Due to the creation of lipid peroxides, which

damage the polyunsaturated fatty acid (PUFA)-rich outer membrane of the sperm and are thought to have an impact on the sperm's genetic integrity, fatty acids in the cell membrane are attacked by free radicals. ROS can also affect the cytoskeleton assembly, mitochondria, genetic replication, transcription, and translation of the sperm axoneme. Increased ROS level is a major concern in subfertile men, and ROS level in male factor subfertile men is still higher in raw and washed semen. Increased ROS levels indicated that semen lack adequate antioxidant levels or that ROS was being overproduced. Studies showed that *in-vitro* exposure to ROS causes modification in base, causes DNA dimers, genomic defects, frameshift mutations, chromosomal aberrations, and frameshift genetic changes in the spermatozoa genetic material. There is a contradictory result on ROS levels correlation with age and in this study, we found no correlation between ROS with age. While a positive association was found between paternal overweight and ROS production (Amorini *et al.*, 2021). To shield spermatozoa from ROS, the defensive antioxidant system in sperm is mainly composed of both enzymatic and nonenzymatic substances that interact with one another. SOD, CAT, and GPx are the three primary antioxidant enzymes. The superoxide anion is neutralised by CAT and GPx, while SOD eliminates the hydrogen peroxide that was produced by SOD. GPx is a catalytic agent for the reduction of organic peroxides (Walczak–Jedrzejowska *et al.*, 2012). The bioavailability of reduced glutathione (GSH), which GPx oxidises to its disulfide form, is necessary (GSSG). GR, which needs NADPH, is necessary for GSH recycling from GSSG. One of the most significant sources of NADPH is glucose-6-phosphate dehydrogenase (G6PD), an enzyme that breaks down glucose-6-phosphate into phospho-6-glucono-lactone in the pentose phosphate pathway (Amorini *et al.*, 2021) discovered that in human spermatozoa, the pentose phosphate pathway responds dynamically to oxidative stress and functions, along with GPx and GR, as a functionally effective antioxidant defense in in-vitro studied. (Espinoza *et al.*, 2009) a greater role for the pentose phosphate pathway than for glycolysis in preserving the motility of goat sperm. In the present study, no relationship between sperm motility and the activity of SOD, CAT, GPx, or GR was discovered. G6PD activity was still significantly lower in the LM group than in the HM group. In the LM group, the MDA level was noticeably higher. The existence of additional pathways connecting G6PD activation and oxidative stress is suggested by such findings. Peroxiredoxins (PRDXs), according to Fernandez and

O'Flaherty (2018), are perhaps essential elements of such a system. Because of the cysteine (Cys) residue at their active sites, this family of antioxidant enzymes is found in all subcellular compartments of human spermatozoa and can scavenge a wide range of ROS. PRDXs become inactive when the Cys thiol group is oxidised because it creates a disulfide link. The reduction of oxidised PRDXs is carried out by the thioredoxin- (TRX) thioredoxin reductase (TRD), which needs NADPH produced by G6PD, similarly to glutathione (Aitken *et al.*, 1998). This series of events demonstrates how declining G6PD activity can cause an increase in MDA levels, even if GPx and GR activity remains constant. However, the study's small sample size is a major drawback. On the other hand, it was discovered that G6PD deficiency does not increase sperm susceptibility to oxidative stress caused by hydrogen peroxide. The researchers proposed different strategies for NADPH synthesis, including nicotinamide nucleotide transhydrogenase, isocitrate dehydrogenase and glutamate dehydrogenase, NADPH-linked malic enzyme, and NADH kinase (Said *et al.*, 2012).

Sperm morphological defects especially one with cytoplasmic droplets were higher in subfertile men with higher reactive oxygen activity than in control. The present study results are following the earlier studies which suggested that the generation of ROS was determined to be higher in sperm with abnormal morphology. Immature spermatozoa produce the primary free radical superoxide anion (Aitken *et al.*, 1998). The abundant glucose 6 phosphate dehydrogenase in retained cytoplasmic residue in sperm mediates the activation of the NADPH system and may involve in the higher production of ROS (Hayashi *et al.*, 2007). Mitochondrial genomic mutation or alternation through a vicious cycle also contributes to increased ROS production. The increased ROS levels in subfertile men than fertile men indicates that oxidative stress early detection and immediate treatment with antioxidants help in improving the pregnancy rate and also improve sperm DNA irreversible damage (Amorini *et al.*, 2021).

Conclusion

Male fertility is intricately linked with the quality of their semen, which is evaluated based on several parameters, including semen volume, pH, liquefaction time, sperm count, motility, morphology, and computer-aided sperm analysis (CASA) parameters. While there is some debate around the precise measurement of sperm velocity, CASA is considered a more precise method of assessing the speed of sperm compared to the WHO manual. In cases of total necrozoospermia, the fertilization rate of immotile spermatozoa is quite low, and injecting non-viable sperm can result in reduced positive outcomes.

Findings of the present study showed that oxidative stress markers (ROS, POD, CAT, TBRAS) can play a causative role in male subfertility so, it is important to give proper antioxidant therapy to improve fertility. A proper workup for oxidative stress and sperm morphology assessment could be helpful to select suitable antioxidant remedies to treat male subfertility as it's well understood that abnormal sperm morphology is reported to reduce successful fertilization with increased miscarriage after embryo transfer.

CHAPTER 2

Sperm Protamine (CMA3) and Chromatin Integrity (SCD, SCSA, TB Tests) Estimation, As A Biomarkers Of Sperm Quality

ABSTRACT

A key goal of reproduction research is to identify factors that indicate the effectiveness of assisted reproductive technologies (ARTs) in terms of a positive outcome. Gamete quality is critical for producing high-quality embryos and increasing the success of ARTs. The purpose of this study was to assess the connection between sperm chromatin quality and sperm cell DNA fragmentation. The three categories of male factor subfertile patients (MFI) were normozoospermic (N), severe male factor (SMF), and mild male factor (MFI) (MMF). Sperm chromatin dispersion (SCD), sperm chromatin structure assay (SCSA), Acridine Orange (AO), chromatin maturation index (CMI), including CMA3 to measure protamine content, and toluidine blue (TB) to measure chromatin condensation was used to study the DNA fragmentation index (DFI) in spermatozoa. CMI was verified using protamine content, chromomycin A3 (CMA3), and chromatin condensation TB, which were significantly lower in N compared to subfertile groups, while DFI was measured using SCD, SCSA, and AO (MMF and SMF). SCD, SCSA and AO levels are significantly high in MMF and SMF compared to N control men. A comparison of different methods was done and it found that SCD correlates highly ($p<0.01$) with SCSA and CMA3 and TB has comparable ($p<0.01$) results. Furthermore, the sperm chromatin maturation index (CMI) is checked by chromomycin A3 (CMA3) and toluidine blue or aniline blue (AB)-stain sperm chromatin maturation assay (SCMA) tests. The present study reveals that sperm chromatin maturity and DNA fragmentation are important risk factors in male factor subfertility. Studies showed that SCD and CMA3 are easy and less expensive methods for the dedication of sperm DNA fragmentation. Moreover, chromatin integrity markers (CMA3 and SCD) could be valuable features and might be useful in understanding the cause of an adverse outcome and providing probable clinical evidence of male factor subfertility and as a new method for male factors screening and prediction for clinicians and doctors. Such risk factors should be considered in the counseling, diagnostic and therapeutic interventions to improve the reproductive health of men in Pakistan.

INTRODUCTION

Subfertility is defined as no conception despite one year of unprotected attempts (Zegers-Hochschild *et al.*, 2017). One initial investigation aimed to find a cause of subfertility is the evaluation of semen samples to rule out malefactors; which are attributed to 40-50% of subfertility (Stanaway *et al.*, 2018). Conventional semen analysis includes sperm count, motility, and morphology; however, these parameters do not strictly predict fertilization potential (Dada *et al.*, 2012). Sperm abnormal chromatin and DNA fragmentation assessment are hidden anomalies frequent in subfertile men (Amor *et al.*, 2019). Subsequently, routine sperm parameters alone do not enable the identification of a substantial proportion of subfertile men. Reactive oxygen species (ROS) correlate with sperm DNA damage and might also be associated with human subfertility (Zeqiraj *et al.*, 2018). The presence of damaged DNA may also result in faulty nuclear remodeling caused by faulty protamine deposition during spermiogenesis. One of the features of spermatozoa protamines is that they will be concerned with the safety of the genetic code. In humans, protamines replace approximately 85% of the histones during the process of spermatogenesis (Oliva, 2006). Incomplete protamination could render the spermatozoa greater at risk of attacks with the aid of using endogenous or exogenous agents, including free radicals, mutagen (Hayashi *et al.*, 2007), and nucleases (Agarwal *et al.*, 2019) as shown in Table 10.

Assessment of sperm DNA integrity:

There are numerous assays developed to determine the extent of sperm DNA damage. These techniques differ based on underline phenomena and specific aspects of DNA damage to detect. There is no one standardized method for sperm DNA integrity measurement (Hammadeh *et al.*, 2001). The interpretation of results is complicated because of heterogeneity in the sperm population, with a non-coding region or intron DNA break and DNA damage repaired post-fertilization. Sperm deoxyribonucleic acid fragmentation index is a commonly used technique involving methods such as Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, Comet assay, sperm chromatin structure assay (SCSA), acridine Orange test, and sperm chromatin dispersion (SCD) assay (Chohan *et al.*, 2006). Despite all the controversial findings on the utility of

sperm chromatin assessment role in clinical practice, still have diagnostic and prognostic value to assess male fertility potential (Agarwal and Said, 2003).

Table 10: Risk factors causing sperm DNA damage in men

STUDIES	OBSERVATION /FINDINGS
Male age	
(Alshahrani *et al.*, 2014)	Men with age above 40 years had higher DFI.
(Brahem *et al.*, 2011); (Luetjens *et al.*, 2002)	Increased risk in spermatozoa diploidy/aneuploidy of aged men.
Diet and lifestyle	
(Harlev A, 2015)	Smoking induces sperm DNA damage
(Cui *et al.*, 2016)	Increases sperm DNA damage induced due to smoking activates the checkpoint kinase 1 (Chk1) which in cause S and G2 checkpoint arrest.
(Akang EN, 2017)	Alcohol consumption increase DFI.
(De Iuliis *et al.*, 2009)	Alcohol intake induces the sperm mitochondria to generate more H_2O_2, which raises DFI.
Obesity	
(Dupont and Plummer, 1990)	Lower sperm motility and a higher risk of sperm DNA damage.
(Dupont *et al.*, 2013)	Sperm DNA integrity is adversely affected by OS brought on by obesity.
Environmental toxicants	
(Jeng, 2014); (McPherson and Lane, 2015)	DFI and endocrine disrupting chemical exposure are positively correlated.
(Meeker *et al.*, 2010)	High DFI is caused by exposure to bisphenol-A.
Chemo/Radio therapy	
(Ahmad and Agarwal, 2017);(Smit *et al.*, 2010)	DFI and radiation therapy have a positive correlation with deteriorating sperm quality.
(Agarwal *et al.*, 2008)	Men exposed to radiation from cell phones, Wi-Fi, and other radioactive sources have poor sperm DNA integrity.
Infections and testicular trauma	
(Gallegos *et al.*, 2008)	DFI is elevated in mycoplasma and chlamydia infections.
(Erenpreiss *et al.*, 2002)	Sperm DNA damage is very prevalent in leukocytospermic patients.
(Wang *et al.*, 2012)	High DFI is related to varicocele because ROS are produced in excess.

Sperm Chromatin structure assay (SCSA):

This technique was first introduced by Evenson in the 1980s and it measured sperm DNA denaturation induction by diluting the sperm in buffer and later in a low PH solution that allows binding of acridine orange (AO), a fluorescent dye, and the sample analyzed by

flow cytometry (Evenson, 1999). Metachromatic properties of sperm DNA binding with AO, in the case of spermatozoa with intact double-strand DNA intercalation with AO that emits green fluorescence, while sperm with fragmented allows single-strand binding and red fluorescence emits. These fluorescent signals are emitted by individual cells and detected by a photomultiplier that analyzes red and green fluorescent signals (Liffner *et al.*, 2019). The fluorescent patterns are expressed as dot plots with green fluorescence on the y-axis and red on the x-axis. Denatured DNA sperm signals lie on the right side of the main population at 45^{o} angle descent. Increased DNA stainability expressed on the top end is a population of immature sperm with compacted sperm DNA. HDS is high DNA stainability calculated as the ratio between the cells starts at 75% green fluorescent scale and total population number (Evenson *et al.*, 2020). HDS increased values correlate with low fertilization capacity and increase time to conceive after a natural try of timely intercourse, intrauterine insemination, and in vitro fertilization. Seminal plasma interferes with the acid denaturation step, particularly with a low sperm count that can decrease acquisition time that limitation could be overcome by lowering cell numbers to 1500-3000. Incomplete acid denaturation cause underestimation of DNA damage. The value obtained is expressed as sperm DNA fragmentation index (DFI), calculated by dividing the red fluorescence by total fluorescence (Chohan *et al.*, 2006). Each sample was analyzed as a duplicate and the mean values of the results were used to calculate DFI (Table 11).

Acridine orange test (AO):

The principle is the same as SCSA but this technique is cheap and simpler as rather than flow cytometry, it employs a fluorescent microscope (Tejada *et al.*, 1984). Limitations of AO are the rapid fading, vague colors, visual estimation, and heterogeneous staining that may lead to overestimation (Talebi *et al.*, 2013; Chohan *et al.*, 2006).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay:

These methods imply the fluorescent labeling of the nucleotide at the 3’ end of a broken DNA strand with the DNA repair enzyme terminal deoxynucleotidyl transferase (TdT) (Gavrieli *et al.*, 1992). TUNEL provides a percentage of spermatozoa and does not quantify individual sperm DNA fragmentation based on fluorescence intensity(Sergerie *et al.*,

2005). This intensity can either be measured through a microscope or flow cytometer. TUNEL positive indicates DNA fragmentation which does not predict apoptosis.

Table 11: Histochemical procedures used different staining methods or fluorochromes procedures to assess indirectly sperm chromatin structure.

Technique	Reference	Measured damage	Detection method	Sample required	Advantages	Limitations
Sperm chromatin structure assay (SCSA) 	(Evenson, 1999))	Percentage of sperm with or without fragmented DNA	Flow cytometry 	1 or 2 × 10^6 sperm	Less time-consuming than the other techniques 2-DNA fragmentation index (DFI) determined by SCSA is a stable parameter over time	Does not determine the extent of DNA damage in individual sperm
Acridine orange test (AOT) 	(Tejada *et al.*, 1984)	Percentage of sperm containing SSB or DSB	Fluorescence microscopy	0.1- 0.5 ml of the semen sample. 300 cells are analyzed	Inexpensive and easy to perform	1. Rapidly fading fluorescence 2. Non-specific color 3. Heterogeneous slide staining
TdT-mediated dUTP nick-end labeling (TUNEL) 	(Gavrieli *et al.*, 1992)	Percentage of sperm containing SSB and/or DSB	Flow cytometry/ fluorescence microscopy 	50 µl of sperm suspension for ART 500 cells sample is observed	1. Flow cytometry increases the sensitivity and reproducibility of the assay 2. Sperm DNA fragmentation determined by TUNEL is a stable parameter over time	1. Estimates the number of cells with DNA damage without quantifying the level of damage 2. Light microscopy decreases the efficiency of the assay 3- Expensive
Sperm chromatin dispersion (SCD) 	(Fernandez *et al.*, 2005)	Percentage of sperm with or without fragmented DNA	Fluorescence microscopy	0.2ml of the semen sample	Inexpensive and easy to perform	1. New technique and its clinical importance are not proven yet 2. Not accurate in determining the extent of DNA damage
Comet assay 	(Ostling and Johanson, 1984)	1-DSB under neutral conditions 2-SSB and DSB and alkali labile sites under alkaline conditions	Fluorescence microscopy	1000 to 5000 sperm	1-Very sensitive technique 2- Detects DNA damage in individual cells quantitatively 3- Few cells are required	Alkali labile sites, which are abundant in mammalian cells, can cause overestimation when an alkaline Comet assay is used.

DNA-dense packaging limits the enzyme binding with sperm and reduces the accuracy of the technique. The sensitivity and reproducibility of the assay were reduced when intensity was measured through a microscope rather than flow cytometry. The increased cost of assay makes its application reduced clinically (Cissen *et al.*, 2016).

Sperm Chromatin Dispersion (SCD)/ Halosperm assay:

This assay was introduced by Fernandez et al. and is also known as the halosperm assay (Fernandez *et al.*, 2005). This assay is based on the principle in which intact sperm are immersed in a slide coated with low melting agarose matrix, and sperm DNA denatured with low PH solution then sperm membrane and nuclear proteins remove with lysis buffer. Then slides stained with DAPI (4',6 diamidino 2 phenylindoles) peripheral halo formed because dispersed DNA loops in nucleotides required removal of part or all DNA binding proteins (histone and Protamines) with a central core. Visualized under a fluorescent microscope. Nuclear halo formation most likely requires intact chromatin and nuclear matrix where halos are comprised of 46kb DNA loops that are held linked to MARs in base and spread out as naked or relaxed DNA parts. Neat and washed samples both can be used for this test (Fernandez *et al.*, 2005). Fragmented sperm chromatin formed small halos/nondispersed while highly condensed chromatin form large/ distinct halos. Fragmented DNA failed to produce halos and subfertile men had a DFI value of 30%.

Comet assay/ single cell gel electrophoresis (SCGE):

Comet assay can be performed with fresh and a minimum of 5,000 sperm is required, patient with oligozoospermia (low sperm count) comet assay can detect sperm DNA fragmentation (Ostling and Johanson, 1984). Lysed spermatozoa embedded in agar and intact, high molecular weight, intact DNA under electric field migrate slowly and remain in sperm head while denatured fragmented DNA migrates out and forms a comet (Timermans *et al.*, 2020). Sperm DNA was stained using fluorescent dye SYBR green and visualized under a fluorescent microscope, 200 to 300 sperm cells have measured in the length of the tail to measure the extent of DNA fragmentation. DNA denaturation is not required in Neutral comet assay which makes it more sensitive to measure double-strand break. Acidic or alkaline comet measures double and single-strand DNA breaks (Simon *et al.*, 2017).

INDIRECT HISTOCHEMICAL DETECTION OF CHROMATIN/DNA INTEGRITY:

Histochemical procedures used different staining methods or fluorochromes procedures to assess indirectly sperm chromatin structure Table 12.

Aniline blue staining:

Protamine rich in arginine and cysteine residue presents abundantly in mature spermatozoa nucleus, while nuclear content of immature sperm has lower protamine content and abundant lysine-rich histone. Mature spermatozoa remain unstained with protamine does not react with dye while immature sperm stain blue because of rich lysine histone content which has a greater affinity with acidic AB dye. Increased blue-stained sperm indicates uncondensed chromatin. As the technique needs a bright field microscope to make it less expensive with results correlated with AO. The only prominent drawback of the test is heterogeneous slide staining (Kim *et al.*, 2013).

Toluidine blue staining:

Toluidine blue is a basic thiazine metachromatic dye in nature and binds acidic components of phosphate residue of immature nuclei DNA with damaged uncondensed chromatin. TB is semi-soluble in water and alcohol. TB has three isoforms; ortho toluidine, para toluidine, and meta toluidine. Fragmented DNA of immature nuclei provides observed using a light microscope, metachromatic shift from light blue to purple-violet color. This test provides the structure and packaging of DNA (Erenpreiss *et al.*, 2004).

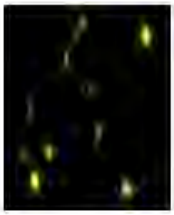
Chromomycin A3 (CMA3) assay:

Guanine cytosine-specific fluorochrome CMA3 dye *Insitu* competes with protamine in the DNA binding site and staining with CMA3 correlates with protamine deficiency. Highly CMA3 fluorescence test indicated low DNA protamination and poor chromatin packaging. CMA3 stain high in spermatozoa of subfertile men.

CMA3 staining is used as a discriminant of IVF success and failure with 73 % sensitivity and 75% specificity. Positive CMA3 higher levels (>30%) indicate decondensation failure and reduced fertilization rate. Sperm motility, morphology, and concentration have a

negative correlation with CMA3 staining. Globozoospermic men had higher CMA3-positive sperm and sperm DNA damage (Amor *et al.*, 2021; Oliva, 2006).

Table 12: Sperm nuclear maturity assessment by histochemical procedures

Assay	Parameter	Method of analysis	Advantages and limitations
Acid aniline blue (AAB) 	Nuclear maturity (DNA protein composition)	Optical microscopy	Advantage: simple and inexpensive use of bright field microscopy limitations: Heterogeneous slide staining.
Toluidine blue staining (TB) 	Nuclear maturity (DNA protein composition)	Optical microscopy	Advantage: Simple and inexpensive use of an ordinary microscope. Stained smears can be used for morphological assessment limitations: Reproducibility as a limited number of cells scored.
Chromomycin A3 (CMA3) 	Nuclear maturity (DNA protein composition)	Fluorescence microscopy	Advantage: Reliable, negative correlation with sperm concentration, motility, and normal morphology. Sensitivity and specificity are comparable with AAB stain (75% and 82% vs. 60% and 91%, respectively) limitations: Observer subjectivity.

Sperm chromatin condensation and male fertility

Sperm chromatin condensation helps the paternal germ cells in many ways; i). The condensed paternal genome makes the spermatozoa hydrodynamic and lighter which

facilitates its faster movement and efficiently fertilizes the oocyte. ii). Spermatid is devoted to transcription machinery and proteins involved in this process, it allows the paternal message reprogramming easier for oocytes post insemination. iii). Loss of epigenetic information and imprinting during spermatogenesis could influence the paternal genome reactivation post-fertilization (Aoki *et al.*, 2006). Although insemination with damaged DNA results in fertilization this causes defective embryonic growth, implantation failure, and miscarriage or fetal deformities. Subfertile men had sperm more sensitive to chromatin damage by agents like peroxide (H_2O_2), alcohol consumption, smoking, age, obesity, and radiation. The fertilization optional decrease with an increase in the proportion of sperms with >30% DNA damage measured using SCSA (Liffner *et al.*, 2019).

Biologically and clinically, male fertility has been extensively studied, but, unfortunately, right now, the etiology of this subfertility is not understood. Numerous clinical tests for male factor subfertility are introduced and studies and meta-analyses have been conducted to clarify the mechanisms, but many questions remain unanswered. The current study set out to shed light on the relationship between male subfertility susceptibility and chromatin maturity and integrity, as well as their effects on Pakistani men's capacity to conceive. A higher rate of miscarriages is caused by increased DNA damage from altered protamine expression, which is also linked to poor semen quality, a reduced ability to fertilize, poor embryo growth, and unsuccessful implantation. Sperm DNA damage and chromosomal integrity unveil anomalies in idiopathic subfertile men with normal sperm parameters. It is unclear how chromatin aberration and increased DNA damage affect the likelihood of conception.

MATERIALS AND METHODS

Subjects

This prospective study included 753 couples who underwent IVF/ICSI procedures out of which 604 couples were involved in the ICSI/IVF program at Fertility and Genetic services, Islamabad, Pakistan, from April 2016 to October 2021. The study population involves fertile 146 and 607 subfertile men.

Ethical Compliance

The institutional review board of Quaid-i-Azam University authorised the research proposal, and the ethics committee of the SKMC Islamabad Pakistan awarded its approval. The subject's detailed information (brief medical history, including male and female ages, male body mass index (BMI), period of subfertility, primary/ secondary subfertility, and information about earlier spontaneous abortions -related data) was obtained through a questionnaire, asking for the appropriately structured question by face to face interview.

Inclusion and exclusion criteria

A complete physical evaluation was performed, including an assessment of scrotal size to rule out cryptorchidism and malformations of the external genitalia; a doppler assessment to rule out varicoceles; an immunobead binding evaluation to rule out the existence of anti-sperm immune cells; and genetic fingerprinting to rule out the chronic illnesses such as liver/renal disease, patients who are extremely obese, patients who have hyperglycemia were excluded.

There was no subfertility factor in the female partner of the couple included in this study. The semen sample was subjected to analysis for seminal characteristics and the blood sample was drawn for hormonal determination. Fertile males were those without any history of fertility problems and within one year of unprotected intercourse, their partners had spontaneous pregnancy. The fertile and subfertile couples were recruited from assisted conception unit-fertility genetics services (Salma & Kafeel Medical Services) Islamabad.

Semen Collection

Semen samples were obtained through masturbation and placed in sterile, wide-mouthed, dry, clean, and non-sperm toxic plastic containers (labeled with the patient's name and date), in an isolated room close to the andrology laboratory. After being advised not less than 2 to no more than 6 days of sexual abstinence.

Sperm DNA Fragmentation Tests

HALO Sperm or Sperm Chromatin Dispersion (SCD)

Test principle

The difference in response provided by the nuclei of spermatozoa with fragmented DNA compared to those with their DNA intactness forms the foundation of the SpermFunc® DNAf test. Controlled DNA denaturation combined with nuclear protein extraction results in partially deproteinized nucleoids where the DNA loops enlarge to form chromatin dispersion halos. However, the dispersion halo of the spermatozoa nucleoids with fragmented DNA is either absent or barely detectable. When comparing the results of the SCD test and the spermatozoon structure test, researchers are looking at their agreement. Chromatin (SCSA), a correlation coefficient was obtained intraclass R: 0.85. The percentage of the average of the differences in the fragmentation indices was 2.16 in favor of test SCD.

Instruments

1. The instrument used was a general optical microscope

2. Other devices used:

1) 2 ~ 8 ℃pharmaceutical refrigerator 2) -20 ℃refrigerator

3) 37 ℃constant temperature water bath 4) 80 ℃dried heater or water bath

5) Low-speed centrifuge

3. Other materials

1) Semen collecting device

2) 1μl ~ 5μl adjustable micropipettes and some disposable tips

3) 20μl ~ 200μl adjustable micropipettes and some disposable tips

4) 100μl ~ 1000μl adjustable micropipettes and some disposable tips

5) Forceps or hemostats

6) Slide barrels

7) Qualitative filter paper

8) Disposable gloves

9) Distilled water

10) Semen liquefication reagent

11) Ethanol 70 %, 90 % and 100 %

Operational Process

Preparation of reagents

1) We put the tube that contains gel with a low melting point in the dried heater or incubation bath and incubated it for 20 minutes at 80℃ until the gel dissolved completely. Then we transferred the tube to a place at 37 ℃ for use. (After transferring from 80 ℃ to 37 ℃, it gel was balanced for at least 5 min before use.)

2) The room temperature was adjusted to 20~28 ℃ before testing.

2. Preparation of specimen

1) We diluted the semen sample in a culture medium or PBS or NS to a concentration of $5\sim10\times10^6$ /ml before testing.

2) The samples that were not completed testing were stored in SCD preservative reagent for testing in the future. The method was as follows: dispense 100μl of semen to a tube that contains 300μl of SCD preservative reagent, mixed thoroughly. It was very important to do so) and were kept at -20 ℃ or -80 ℃. Before testing, the stored samples were balanced at 37 ℃, then count the sperms and diluted the semen sample by NS to a

concentration of $5\sim10\times10^6$/ml.

The volume of semen (100µl): volume of SCD preservative reagent (300µl) =1:3, we didn't increase the proportion of semen optionally (but the proportion of semen can be reduced).

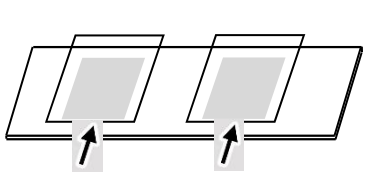
3. Processing

1) After dispensing 60µl of semen samples whose concentration was $5\sim10\times10^6$ /ml to the tube of dissolved gel (we made sure that, this step was run at 37 ℃), the gel was mixed thoroughly. Then incubated at 37 ºC for use in the following steps.

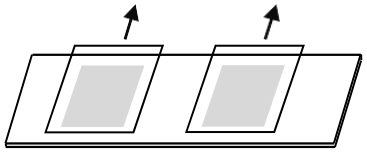
2) We placed the pre-coated slide in the fridge at 2 ~ 8 ℃ for 5 minutes, then immediately dispensed 30µl of sperm suspension made by step 1 to the wells of the pre-coated slide at 37 ℃.

3) Then slide cover was put over the wells of the pre-coated slide quickly and gently (without pressing the slide cover and to avoid any air bubbles). Then the gel made solidified by leaving the slide at 2~ 8 ºC refrigerator for 5 minutes.

4) Then the slide cover was removed carefully. Then we pushed the slide cover toward one side gently until the one end of the slide cover went beyond the pre-coated slide a little (slide 1). Then the edges of the slide cover were pinched and removed horizontally and carefully (slide 2). The slide cover should cling to the pre-coated slide plane instead of pinching it upwards during removal.



Slide 1



Slide 2

5) We introduced the pre-coated slide vertically into a slide barrel containing solution A. Then incubated at room temperature (20 ~ 28 ℃) for 7 minutes accurately.

6) Then we picked the pre-coated slide up. Cleared the fluid remaining on the back

and the side of the slide with filter paper (not to touch the well on the slide). Then introduced the slide vertically in another slide barrel of solution B. Then incubated for 25 minutes accurately at room temperature (20 ~ 28 ℃).

7) Then the pre-coated slide picked up and the fluid remaining was cleared on the back and the side of the slide with filter paper (not to touch the well on the slide). Then the slide was put horizontally into the tray filled with enough distilled water for 5 minutes. Renewed water once or twice during this step.

8) Then the pre-coated slide picked up, and the fluid was cleared remaining on the back and the side of the slide with filter paper (not to touch the well on the slide). Then the slide was introduced vertically in another slide barrel of ethanol 70% for 2 minutes.

9) The pre-coated slide was picked up and the fluid remaining on the back and the side of the slide was cleared with filter paper (not to touch the well on the slide). Then the slide was introduced vertically in another slide barrel of ethanol 90% for 2 minutes.

10) Then the pre-coated slide picked up and the fluid remaining was cleared on the back and the side of the slide with filter paper (not to touch the well on the slide). Then the slide was vertically introduced in another slide barrel of ethanol 100% for 2 minutes.

11) Then the pre-coated slide was dried completely in nature. Then 15 drops to 20 drops of the wright's stain were dispensed on the pre-coated slide, and then 30 drops to 40 drops of the wright's butter were dispensed slowly for staining. (If 15 drops of the wright's stain were dispensed, the wright's butter would be dispensed 30 drops, and blew the mixture softly with the bulb pipet pump on the slide, and didn't destroy the surface tension made by the staining. Fifteen minutes later, rinsed the slide softly with distilled water)

12) Then the slide was air-dried naturally. We observe 500 spermatozoa under the general optical microscope with a 40× field lens and count the sperms with fragmented DNA.

4. Observation and Calculation

1) Then to identify the DNA fragmented sperm: the head of sperm only produces small halos or not, and the thickness of the halos on one side was less than 1/3 of the smallest

diameter of the head of sperm, the sketch maps are as follows (Figure. 21).

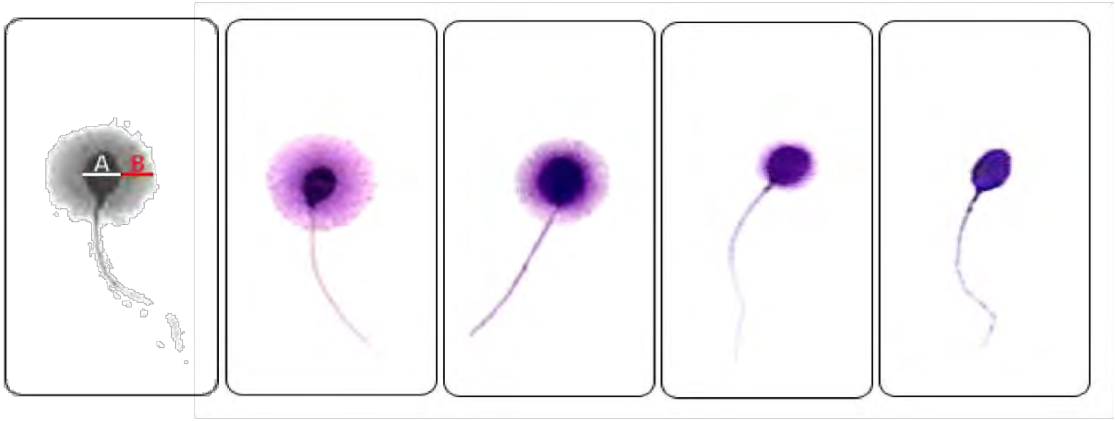


Figure 21. (A) represents the smallest diameter in the sperm head, (B) represents the thickness of the unilateral sperm halo, and B ≤1/3 A indicates the sperm with fragmented DNA. 2-4. represent the sperm without fragmented DNA. 5. Represent the sperm with fragmented DNA.

Formula

% sperms with fragmented DNA= No of sperms with fragmented DNA divided by the whole number of sperms observed × 100%

Percentage of sperms with intact DNA = 1- % of sperms with fragmented DNA

While counting 500 sperms, the number of sperms with fragmented DNA is 75

% sperms with fragmented DNA = 75 ÷ 500 × 100% = 15%

% sperms with intact DNA = 1-15% = 85%.

*The normal reference value: the percentage of sperms with fragmented DNA < 25%.

Sperm Chromatin Structure Assay (SCSA):

Main compositions:

Reagent A	1 vial	2ml	sodium chloride, Bis-Tris, Bis-Tris-HCl
Reagent B	1 vial	3ml	sodium chloride, Tween 20

Reagent C (lyophilized powder) 1 vial 6.5 ml Lyophilized powder of acridine orange, dissolved to 6.5 ml/ vial before use. Lysophilized powder dissolved solution 1 vial 8ml sodium chloride, Glycine, Sucrose, ProClin 300

A flow cytometer (488nm Luminescence) was used to run the test

We Prepared the lyophilized powder dissolved solution into the vial of reagent C, mixing thoroughly.

First, we tested the sperm concentration. Calculated the semen volume required for the best and made the final concentration of sperm in reagent A as 2-3 $x10^6$/ml.

Calculation method: while the sperm concentration of the initial sample was M x 10^6/ml, the required volume of sperm (ul) is 150 / M. For example, if the sperm density was $30x10^6$/ml the required volume of semen was 150/30=5ul. We added the calculated semen volume into the special vial of the flow cytometer and then added 50ul of reagent A, mixing gently.

Add with 100ul of solution B, mixing gently, and react for 30 seconds. We added 300ul of solution C immediately, mixing gently. And we tested the sample on the cytometer (480nm luminescence) and evaluated at least 5000 sperms.

Acridine Orange assay

Principle

AO is a dye that intercalates with cell de-oxy and ribose-genomic nucleic acid (RNA and DNA) and fluoresces to emit different colors, making it easy to differentiate cellular organelles. Acridine molecules and nucleic acid base pairs interact electrostatically, which is how the binding happens. By quantifying the metachromatic shift of AO fluorescence from green (native DNA) to red (reactive oxygen species), it assesses the vulnerability of sperm nuclear DNA to acid-induced denaturation in situ. While binding to single-stranded DNA as an aggregate, the fluorochrome AO intercalates into double-stranded DNA as a monomer. Native DNA is bound to the monomeric AO, which fluoresces green, while relaxed or denatured DNA is bound to the aggregated AO, which fluoresces red (Figure 22.1c).

Technique

The AO assay can be used for fluorescence microscopy, Carnoy's fixative, which contains methanol and acetic acid in a 1:3 ratio, is used to fix thick semen smears for at least two hours. Slides are gently rinsed with deionized water after being stained in AO for five minutes. For the estimates of the numbers of sperm with green and red fluorescence to be precise, at least 200 cells should be counted. Spermatozoa that fluoresce in a range of yellow-orange to red are thought to have damaged DNA, while those that fluoresce in a spectrum of green are thought to have normal DNA content.

The DNA fragmentation index (DFI) = the ratio of (yellow to red)/(green plus yellow to red) fluorescence.

Advantages

A biologically reliable test for sperm DNA quality is the AO assay. Because of the low intra-assay variability (less than 5%), the method is very repeatable. The AO assay exhibits a strong positive correlation with other single-stranded DNA evaluation methods. Figure 22b).

Toluidin Blue (TB)

Principle

Tolonium chloride, also known as TB, binds specifically to the acidic components of the tissue and is a basic thiazine metachromatic dye. Alcohol and water both partially dissolve it. Alternatively known as methylamine or amino toluene, the dye represents three isoforms: ortho-toluidine, para toluidine, and meta-toluidin. It has a high binding affinity for phosphate residues of sperm DNA in immature nuclei and provides a metachromatic shift from light blue to a purple–violet color. This stain is a sensitive structural probe for DNA structure and packaging.

Technique

Based on the metachromasia concept, TB dye can absorb light at spectral lines and change color without altering its chemical makeup. Apart from being air-dried in the room, semen smears also were fixed in newly prepared 96% ethanol-acetone (1:1) at 4°C for at least half

an hour, hydrolyzed in 0.1 N HCl at 4°C for 5 minutes, and washed three times in purified water for approximately two mins each. Smears were also stained for 5 minutes with 0.05percent TB. 50percent citrate phosphate makes up the staining buffer (McIlvain buffer, pH 3.5).

Fixed preparations are dehydrated two times for 3 minutes each at 37°C in tertiary butanol and one time for three minutes each in xylene before being incorporated in DPX (a mixture of di-styrene, a plasticizer, and xylene). Sperm heads with unbroken chromatin stain light blue, while those with defected chromatin stain violet (purple). Light microscopy is used to examine the results of the TB staining (Figure 22.1a).

Chromomycin A3 (CMA3)

Principle of staining method

Guanine-cytosine-specific fluorochrome CMA3 is a protamine deficiency indicator of sperm DNA indirectly because it reveals poorly packaged chromatin in spermatozoa. CMA3 staining is only identified in GC-rich segments and is thought to stay competitive with protamines for adhesion to DNA's hinge region. As a result, high CMA3 luminescence in spermatozoa is a good indication of a low protamination state.

Technique

Semen smears slides were being settled in a 3:1 solution of methanol and glacial acetic acid at 4°C for twenty minutes before actually air-drying at room temperature for 20 minutes. A 100 L CMA3 solution is added to the slides for twenty minutes. The CMA3 solution is composed up of 0.25 mg/mL CMA3 in McIlvain's buffer (pH 7.0) with 10 mmol/L MgCl2. The films are washed in a buffer before getting mounted in a 1:1 v/v PBS-glycerol solution. After that, these same slides are kept at 4°C 24 hrs. A fluorescent microscope is used to assess luminescence. On every slide, 200 sperm cells are assessed at probability sampling.

CMA3 immunofluorescence is tested by separating sperm cells that stain bright yellow (CMA3+) versus those that light-color a dull yellow (CMA3-).

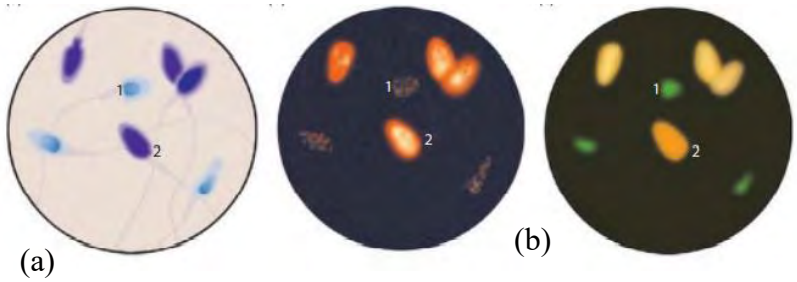


Figure 22. (a) Toluidine blue staining, (1) mature sperm heads are light blue, and (2) immature sperm heads are violet. (b) Acridine orange (AO) staining, (1) native DNA to fluoresce green (2) denatured DNA fluoresces red

STATISTICAL ANALYSIS

Data were presented using mean and SEM. One-way ANOVA and Tukey's group's comparison tests were employed to compare male participants who were fertile and all subfertile, and the Statistical Package for the Social Sciences was used for the statistical studies (IBM SPSS software, version 20). Statistics were considered significant for P values under 0.05. For those outcomes that showed a link to one or more assessed parameters, prediction models were built. The Hosmer-Lemeshow goodness-of-fit test was statistically used to determine whether the model's predictions were reliable. To demonstrate the effectiveness of the process, Bland-Altman plots are used.

RESULTS

Demographic characteristics:

The study enrolled the participation of 753 couples in total. Of the total sample, fertile normal healthy males were 20% (n=146) who participated as the control in our study while subfertile male patients were 80% (n=607). The subfertile patients were split into various categories. The mild male factor (MMF) subfertile group (n=280) was defined as male participants with a single abnormal finding of the semen analysis or by a total motile sperm count between 5–20 × 10^6/mL with normal morphology <4%, whereas the server male factor (SMF) subfertility (n=327) group includes men with were with low sperm count, >15x10^6/ml, low sperm motility >32 %, total motile sperm count less than 5 × 10^6/mL and low sperm morphology >4%.

Sperm Chromatin Integrity Assay

Acridine orange test (AO):

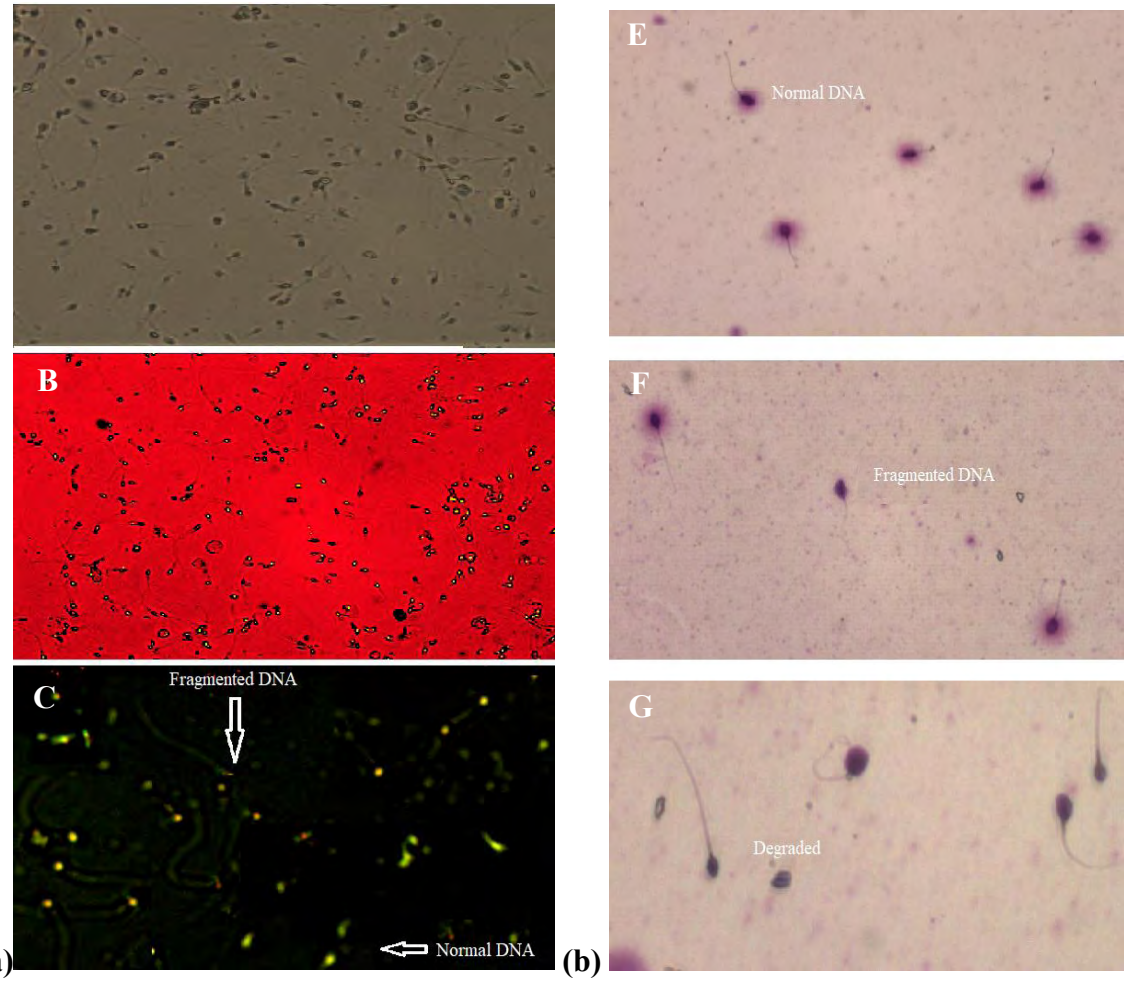
The staining pattern of the Acridine orange test of sperm DNA from fertile and subfertile men was evaluated for SDF. Mean±SEM sperm DNA fragmentation among subfertile male patients was 25.36±1.01 % and the percentage of sperm DNA damage in subfertile categories of MMF and SMF was 22.23±0.49 and 28.78±0.71 respectively SMF had significantly ($p<0.01$) higher levels of SDF compared to fertile subjects and MMF subfertile patients (Figure. 23a & 24).

Sperm chromatin dispersion assay (SCD, HaloSperm):

The mean DNA fragmentation levels ± SEM of the fertile controls was 9.6±0.87% and MMF and SMF were reported as 13.6±0.52% and 32.05±0.46% respectively. The fragmentation levels of the controls had significantly ($P<0.001$) lower SDF compared to MMF and SMF while MMF had significantly ($P<0.001$) lower SDF compared to SMF male subjects (Figure. 23b & 24).

Sperm chromatin structure analysis (SCSA):

The mean sperm DNA fragmentation index (DFI) obtained from SCSA in SMF fertile subjects had the lowest mean percentage DFI compared to fertile male subjects. MMF had a significantly ($P<0.001$) lower DNA fragmentation index compared to SMF (Figures. 24 & 25).



a) (b)

Figure 23. (a) Acridine orange stained sperm cells B&C were fluorescent microscope and A was bright field microscope view of a related area of analysis (b) Sperm assessed by Halosperm test (image taken from the bright field microscope). Sperm without DNA fragmentation: sperm with a big halo (E) and sperm with a medium halo. Sperm with DNA fragmentation: sperm with a small halo (F) and sperm without a halo and degraded sperm (G).

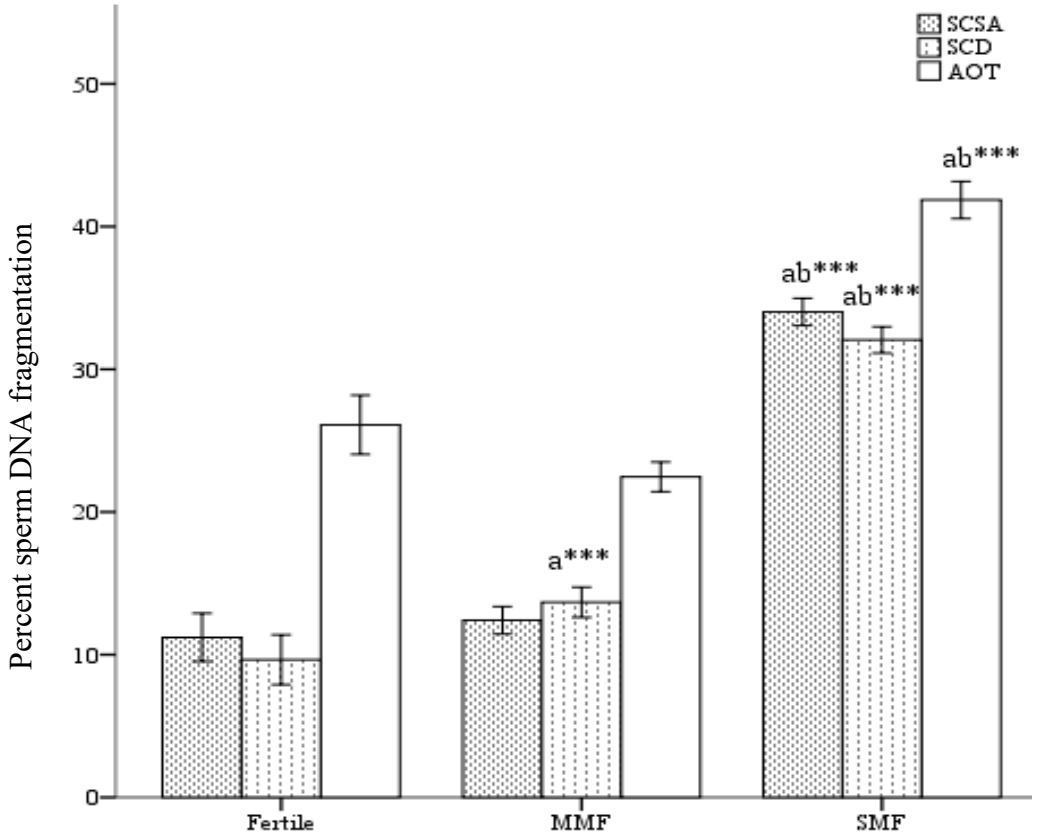
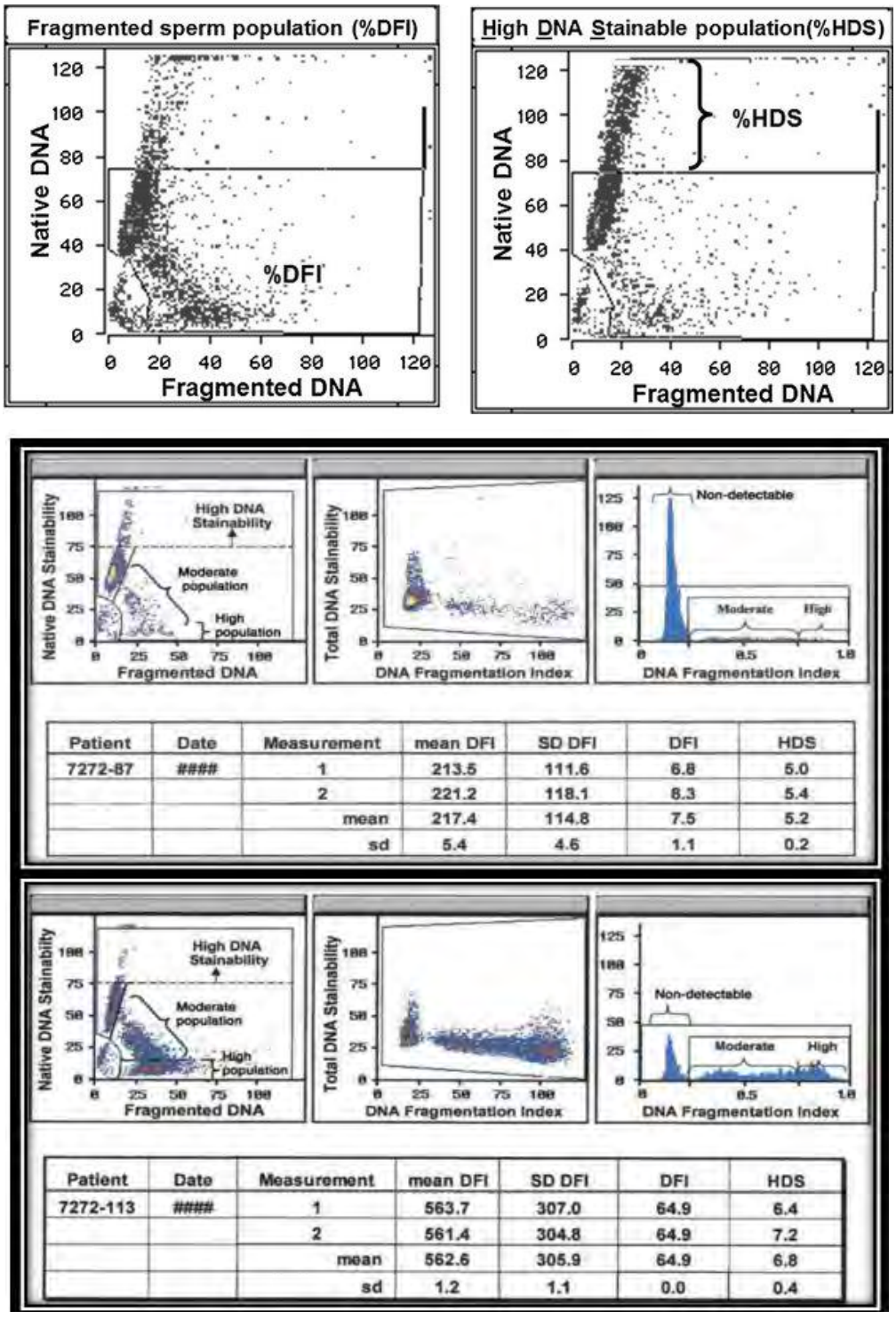


Figure 24. Sperm DNA fragmentation percentage (measured utilizing three methods) in different categories of male patients of the studied population

a= Fertile vs MMF and SMF; b=MMF vs SMF

*=P<0.05, **= P<0.01, ***=P<0.001



Patient	Date	Measurement	mean DFI	SD DFI	DFI	HDS
7272-87	####	1	213.5	111.6	6.8	5.0
		2	221.2	118.1	8.3	5.4
		mean	217.4	114.8	7.5	5.2
		sd	5.4	4.6	1.1	0.2

Patient	Date	Measurement	mean DFI	SD DFI	DFI	HDS
7272-113	####	1	563.7	307.0	64.9	6.4
		2	561.4	304.8	64.9	7.2
		mean	562.6	305.9	64.9	6.8
		sd	1.2	1.1	0.0	0.4

Figure 25. Spermatozoa DNA Structure analysis by SCSA flow cytometry. (a) dot plot made from a sample of fertile men's semen (b) Sperm DNA fragmentation variations in subfertile men; native DNA is shown on the Y-axis and fragmented DNA is shown on the X-axis.

COMPARISON CHROMATIN INTEGRITY ASSAY; SCSA AND SCD & SCSA AND AO:

All three methods have equal mean values while with AO the range was wide and the upper limit was 54.3%, the slides need to be read as soon they get stained, as the stain fades off quickly which leads to over calculation (Table 13).

SCD vs SCSA

Male patients were positively (r= 0.96, p=0.001) and strongly linked with both the semen chromatin dispersion obtained by the SCD assay (HaloSperm) as well as the sperm chromatin structure in the SCSA assay (DFI). A Bland-Altman plot was used to verify these results (Table 14: Figures 26a &b).

The measurements of DNA damage were remarkably consistent. SCD has a systematic offset of about 1.01% and tends to underestimate DNA damage in comparison to SCSA. The results from these two approaches were considered to be extremely highly concordant because of the inter-method ICC, which was 0.95.

In sperm from subfertile men and fertile financial, the SCSA and SCD revealed amounts of DNA fragmentation. SCSA and SCD were discovered to have a strong significant association (r=0.95, p=0.04) for sperm Strand breaks in semen from subfertile and fertile men.

AO vs SCSA

In male patients who were fertile or subfertile, the significant proportion of Strand breaks estimated by the AO roughly equivalent to an assessment of the SCSA assay (DFI) was highly associated (r = 0.56, p 0.05). These findings were verified by a Bland-Altman plot analysis (Table 14. Figure 27a&b).

Contextual fertile and subfertile men demonstrated a link (r=0.584; P=0.01) between SCSA and AO for Strand breaks in sperm.

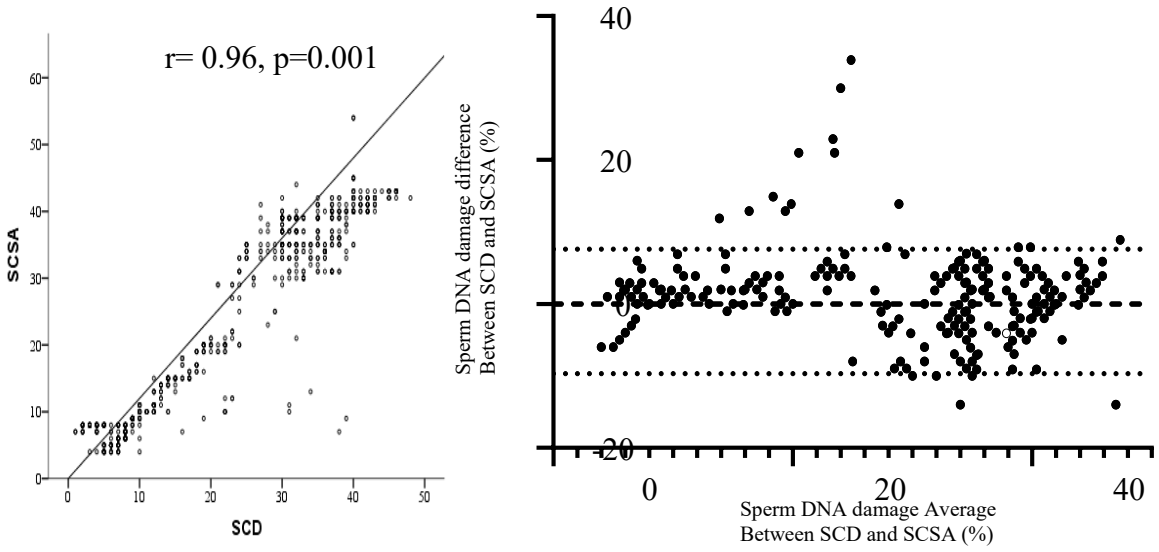


Figure 26. (a) Scatterplot SCD and SCSA (b) Bland–Altman plots to illustrate SCD versus SCSA effectiveness in measuring Sperm DNA fragmentation in sperm

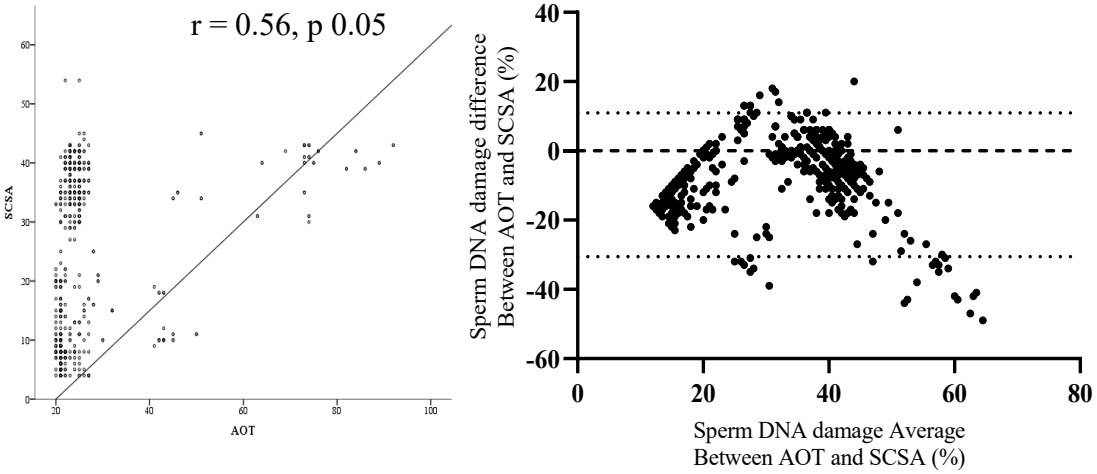


Figure 27. (a) Scatterplot AO and SCSA (b) Bland–Altman plots to illustrate AO versus SCSA effectiveness in measuring Sperm DNA fragmentation in sperm

Table 13: Percentage of sperm DNA strand break measured utilizing three methods

	DFI	95% confidence interval difference	
	Mean±SEM	Lower	Upper
Acridine orange test (AO)	26.3±30.46	25.43	54.3
Sperm chromatin dispersion assay (SCD)	22.71±0.50	21.73	18.5
Sperm chromatin structure analysis (SCSA)	23.72±0.52	22.69	24.74

DFI, DNA fragmentation index.

*=P<0.05, **= P<0.01, ***=P<0.00

Table 14: Assessment of reliability of percentages of DNA fragmentation

	Mean difference (SD)	ICC	Limit of agreement	CV	p-Value
Between **AO and SCSA**	-9.81 (15.98)	0.30	-30.54,10.92	27.24	0.00
Between **SCD and SCSA**	-1.01 (4.4)	0.95	-9.7, 7.6	23.68	0.04

Inter-method reliability and bland Altman between AO, SCD and the SCSA assay

ICC: Intra-class Correlation Coefficient

CV: Covariance

p-value: of the signed-rank test

Sperm DNA fragmentation categories:

According to the three SCSA DFI groupings of fewer than or equal to 15 percent DFI, significantly larger than 15 percent to less than 30% DFI, and exceeding or comparable to 30 percent DFI, Figure 28 displays the distribution of spermatozoa dispersion data for subfertile men. Apart from AO, all DFI categories displayed comparable degrees of DNA epigenetic changes under SCSA and SCD. Troubles like muddled colors, rapid fading, and uneven dyeing of slides were observed earlier in the ongoing investigation.

Standard semen parameters and DNA damage correlation

Semen properties such as sperm concentration, motility, morphology, membrane integrity, vitality, and straight-line velocity were identified to have a significant negative relation with sperm DNA strand breaks (based on SCSA, SCD, and AO diagnostic testing). Table 15 demonstrate the findings of the correlation analyses.

Reproductive hormonal and spermatozoa DNA damage

A significant inverse relationship with both serum testosterone (ng/ml) levels and sperm DNA damage was discovered (SCSA, SCD and AO). Spermatozoa DNA fragmentation was not significantly correlated with plasma levels of prolactin (ng/ml), LH (mIU/ml), or FSH (mIU/ml) (Table 16).

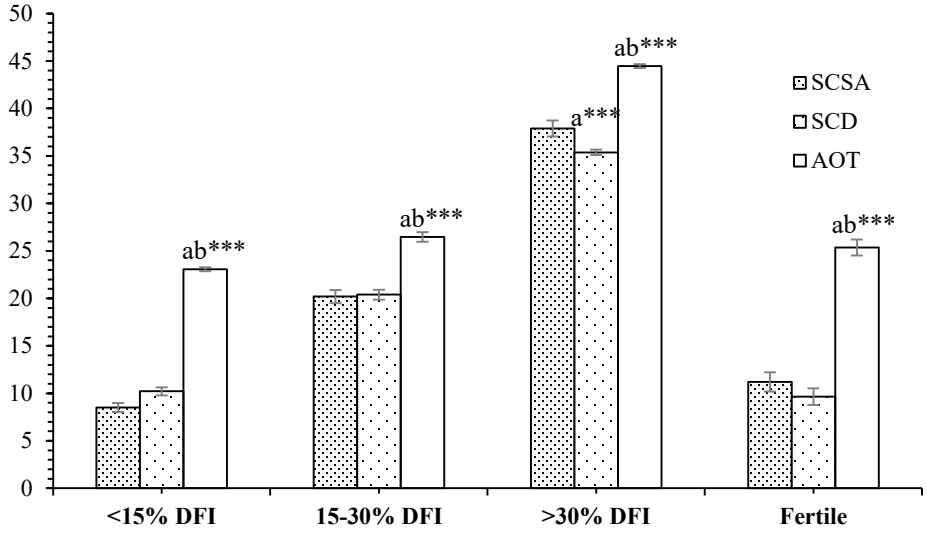


Figure 28. Comparison of DNA fragmentation in sperm of men according to SCSA percent DNA fragmentation index (%DFI) values.

a= Fertile vs MMF and SMF; b=MMF vs SMF, *=P<0.05, **= P<0.01, ***=P<0.001

Table 15: Correlations between Sperm DNA Damage and Sperm Characteristics

Sperm characteristics	SCSA		SCD		AO	
	[a]*SSC*	*p-Value*	*SSC*	*p-Value*	*SSC*	*p-Value*
Sperm concentration x10^6	-.500**	0.00	-.480**	0.00	-.393**	0.00
Motility %	-.298**	0.00	-.304**	0.00	-.368**	0.00
Straight line velocity µm/sec (VSL)	-.255**	0.00	-.298**	0.00	-0.09	0.33
Normal morphology %	-.687**	0.00	-.678**	0.00	-.707**	0.00
Sperm vitality (eosin)	-.282**	0.00	-.296**	0.00	-.256**	0.00
Membrane integrity (Hos)	-.294**	0.00	-.314**	0.00	-.250**	0.00

[a]*SCC:* Spearman Correlation Coefficient

Table 16: Correlations between sperm DNA damage and reproductive hormones

Hormonal profile	SCSA		SCD		AO	
	[a]*SSC*	*p-Value*	*SSC*	*p-Value*	*SSC*	*p-Value*
Prolactin (ng/ml)	0.04	0.30	0.05	0.05	0.17	0.20
FSH (mIU/ml)	0.03	0.52	0.00	0.95	-0.02	0.69
LH (mIU/ml)	0.13	0.00	0.11	0.01	0.07	0.07
Testosterone (ng/ml)	-.261**	0.00	-.212**	0.00	-.176**	0.00

[a]*SCC* Spearman Correlation Coefficient

Oxidative stress marker's effect on DNA damage

The link between both sperm DNA fragmentation and the oxidative stress marker (Table 17). In comparison to men with normal semen parameters, DNA fragmentation was significantly higher in subfertile spermatozoa.

When likened to CAT and the positive control, the relationship between sperm DNA fragmentation and SOD, ROS, and TBRAS was also noticeably stronger (p=0.05). Furthermore, a simple linear regression study found that there is a significant positive relationship between ROS and lipid peroxidase (TBRAS) and sperm cells DNA fragmentation in subfertile male subjects. (Table 17; Figure. 29).

Spermatozoa DNA fragmentation was correlated with ROS (r=0.535, p=0.0001) in normal health fertile male subjects, and it was also correlated with SOD and lipid peroxidase (TBRAS) (r=0.283 and 0.432, p=0.001). While in subfertile male subjects sperm increase in DNA fragmentation was correlated with increase in ROS (r=0.701, p=0.0001), SOD (r=0.209, p=0.0001) and lipid peroxidase (TBRAS) (r=0.601, p=0.0001) levels. In fertile and subfertile subjects, there was no relationship between CAT and sperm DNA fragmentation.

DFI and conventional parameters predictive value:

The ROC curve was used to describe how well sperm DFI and semen analysis (concentration, progressive sperm, and morphological characteristics) could identify male subfertility (Figure 30). AUC of semen DFI seemed to be 0.927 (95% CI 0.876, 0.978), which was higher than the concentration, progressive sperm, and TNMS of laboratory testing (AUC of 0.891; 95% CI 0.849-0.933), which were all lower than 0.873 (95% CI 0.833-0.912). (Table 18). At a cut-off of 27%, the test's specificity and sensitivity for sperm DFI were recorded highest (Table 18; Figure. 30).

Table 17: Correlations between sperm DNA damage and oxidative stress markers

		ROS		SOD/POD		CAT		TBRAS	
	N	SCC[a]	p-Value	SCC	p-Value	SCC	p-Value	SCC	p-Value
DFI %		**.535****	**.000**	.283**	.001	-.134	.108	.432**	.000
	MFI	**.701****	**.000**	.209**	.001	.023	.567	.601**	.000

[a]*SCC* Spearman Correlation Coefficient

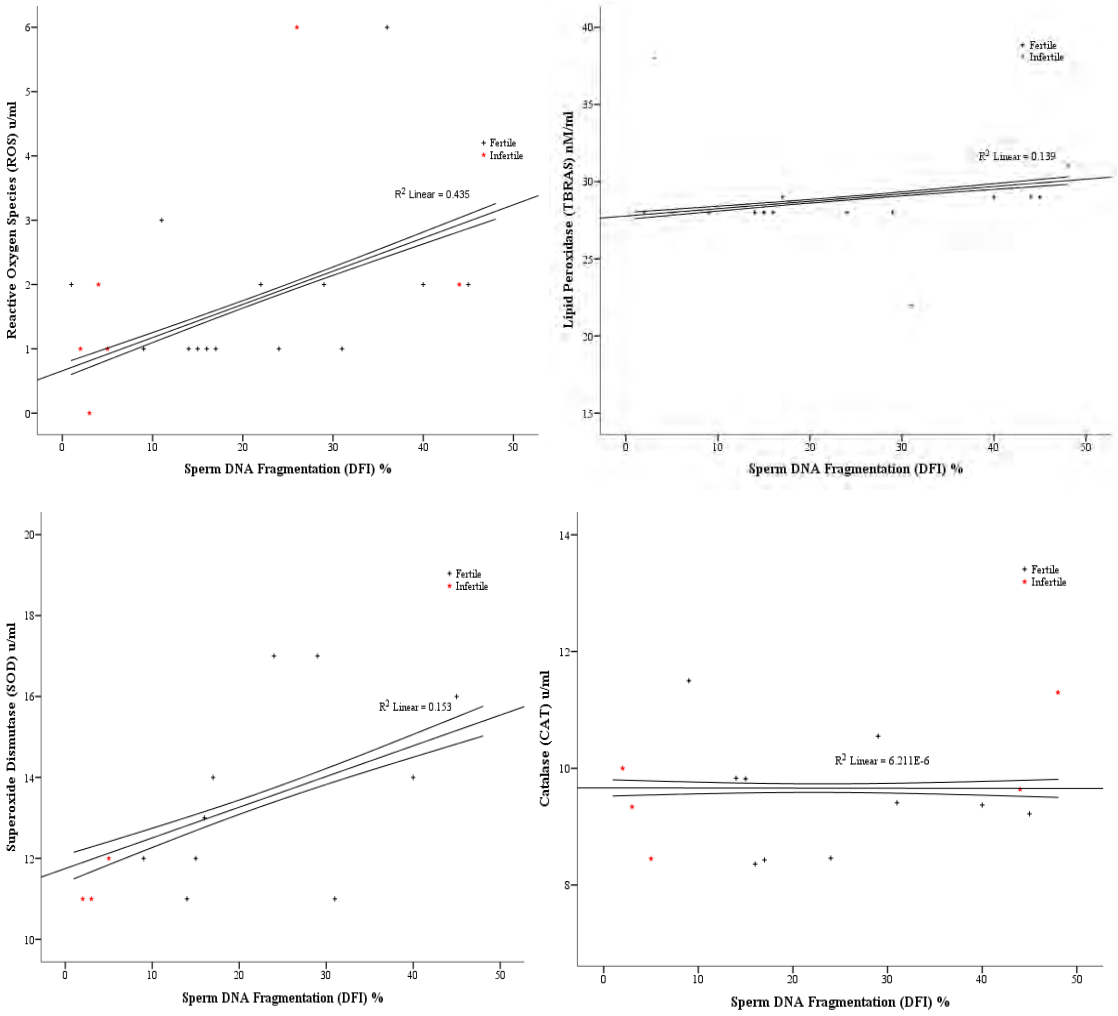
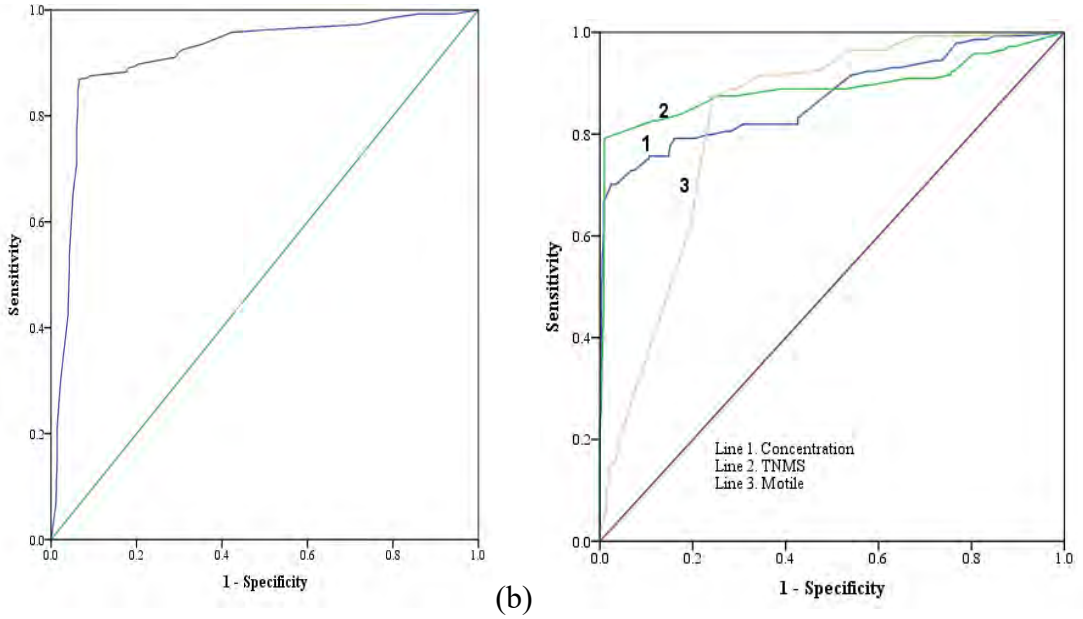


Figure 29. Correlations and simple linear regression analysis of sperm DNA damage with oxidative stress markers in semen samples of fertile and subfertile subjects.

Table 18: AUC comparison of sperm DFI and semen parameters according to the fertile and subfertile men

Test Result Variable(s)	AUC	SE	p-Value	95% CI
DNA fragmentation Index (DFI) %	0.927	0.02	0.00	(0.876-0.978)
Sperm concentration x10^6/ml	0.873	0.02	0.00	(0.833-0.912)
Progressive motility %	0.830	0.01	0.03	(0.798-0.862)
Total motile sperm (%)	0.891	0.02	0.00	(0.849-0.933)

AUC area under the curve, CI confidence interval



(a) (b)

Figure 30. ROC curve of (a) semen DFI versus (b) sperm analysis in male subfertility. When particularly in comparison to sperm DFI, the three semen characteristics of concentration, total motile sperm (TNMS), and motility have fairly low as predicted in AUCs

Sperms Chromatin Condensation

Percent spermatozoa protamination:

The evaluation of sperm protamine insufficiency used toluidine blue staining (TB+) and chromomycin A3 (CMA3+) indirectly.

Chromomycin A3 (CMA3+) Staining

The percentage of CMA3-positive sperm cells in the SMF group as showed significantly was substantially different compare to control group and MMF (35.66±0.38% vs. 20.19±0.6% and 21.28±0.4% $p<0.001$). Box plot measured the percentage of protamine deficient sperm (CMA3+) subfertile subjects were significantly low compared to and subfertile categories (Figure. 31 b&e).

Toluidine blue staining

In contrast to controls and MMF, patients with SMF had considerably more mean semen TB positive sperm cells. Additionally, the findings demonstrate that MMF sperm samples had significantly (P 0.05) more TB-positive sperm cells than the control group did. Box plots also showed similar results (Figure 31 a&d).

Correlation between semen parameters and Chromatin Condensation (protamination):

The table 19 shows a negative significant ($P<0.05$) correlation between sperm count, motility, morphology and vitality (Hos) with positive CMA3+ and TB+ staining in all groups. No correlation was found between VSL, CMA3+ and TB+ (Table 19).

Correlation between chromatin condensation (protamination) and Testosterone level.

There was a significant ($P<0.05$) negative correlation was found between testosterone level and chromatin condensation (TB+) and chromatin integrity (CMA3+) as shown in Table 19.

Correlation %sperm protamination and DNA fragmentation

A significant (P<0.05) positive correlation was found between sperm chromatin condensation (TB+, CMA3+) and chromatin integrity (SCD and SCSA) as shown in Figure 32 a-d.

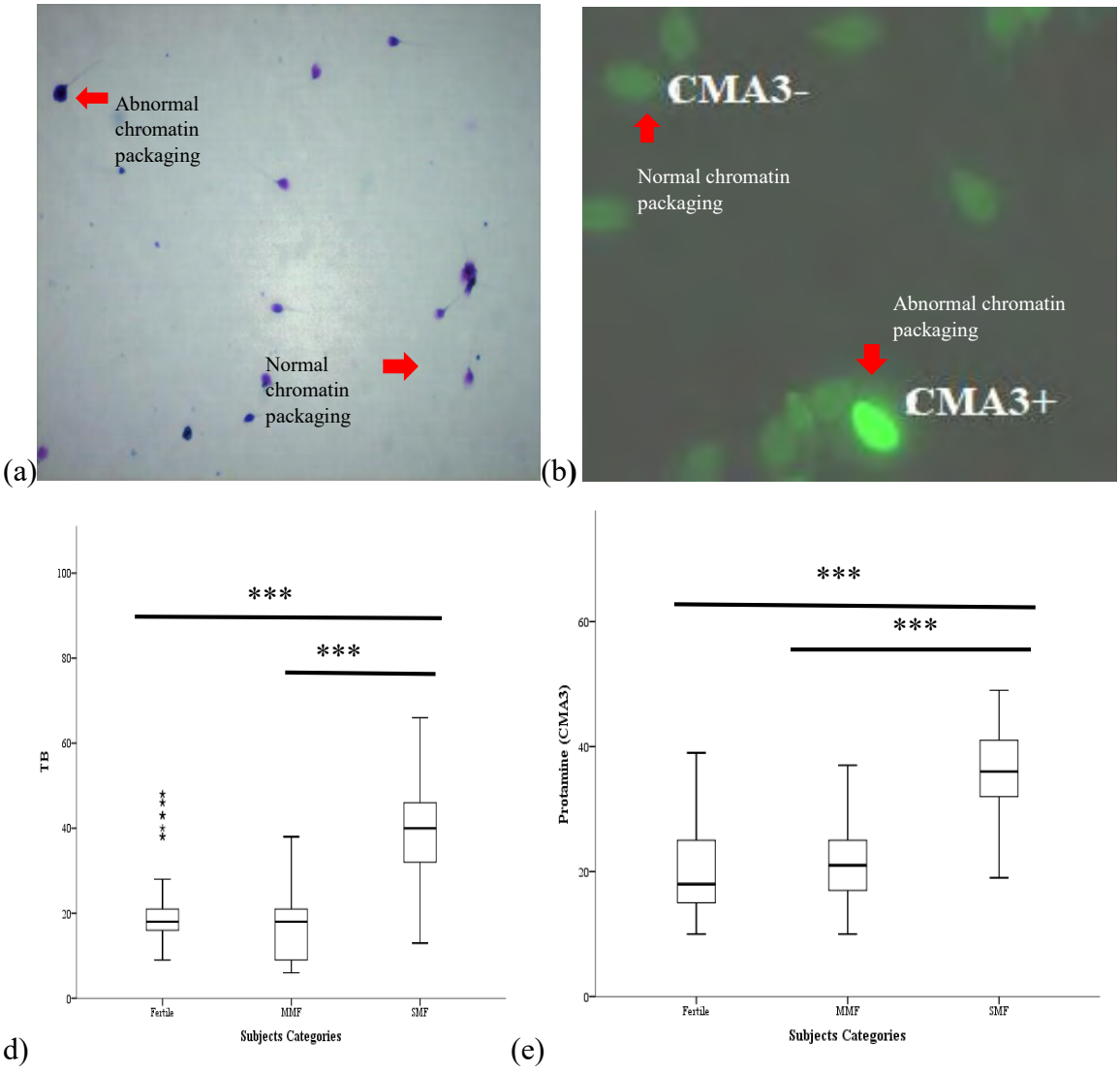


Figure 31. (a) Sperm toluidine blue (TB) stain (b) staining with CMA3; lower panel: Box plot representing (d) TB positive and (e) sperm CMA3 positive measure of Protamine deficiency sperm of the fertile and subfertile categories of male studied population *=P<0.05, **= P<0.01, ***=P<0.001

Table 19: Sperm percent protamine concentration (TB and CMA3 staining) correlations with sperm standard parameters, VSL, membrane integrity and testosterone level.

Characteristics	Chromatin-TB+		Protamin-CMA3+	
	[a]*SSC*	*p-Value*	[a]*SSC*	*p-Value*
Sperm concentration x10^6	-.500**	0.00	-.405**	0.00
Motility %	-.092*	0.02	-.106**	0.01
Straight line velocity µm/sec (VSL)	-0.06	0.52	-0.15	0.09
Normal morphology %	-.665**	0.00	-.673**	0.00
Membrane integrity (Hos) %	-.253**	0.00	-.296**	0.00
Testosterone (ng/ml)	-.209**	0.00	-.166**	0.00

[a]*SCC:* Spearman Correlation Coefficient

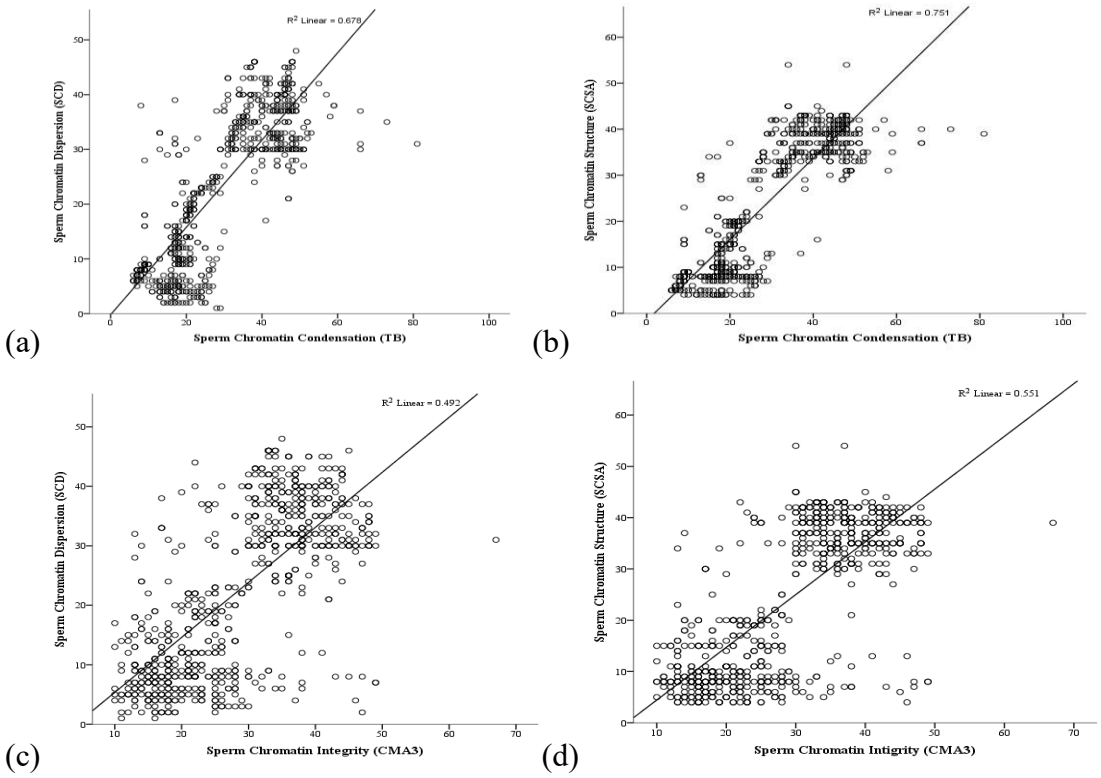


Figure 32. Correlation analysis between the percentage of sperm percent protamine deficiency measured by percent TB+ and Chromomycin A3 (CMA3+) stain with sperm chromatin dispersion (SCD) (a & c) and sperm chromatin structure (SCSA) (b & d).

Comparison of sperm conventional and quality parameters in all patients according to Chromatin Condensation

Using the chromomycin (CMA3) staining method, the DNA condensation (protamination) of the sperm was quantified. The patients were divided into two groups based on the findings of this test, according to the value of CMA3 positivity, as defined by (Zandemami *et al.*, 2012)

- Group of condensed chromatin (CMA3 positive ≤31%, $n = 416$)
- Group of non-condensed chromatin (CMA3 positive >31%, $n = 337$).

The analysis of various standard and quality sperm parameters between the two chromatin condensation groups is shown in Table 20.

Comparing the non-condensed chromatin group to the condensed chromatin group revealed that sperm concentration, progressive motility, morphologically normal spermatozoa, sperm with membrane integrity, and viable sperm ratio were all substantially lower in the non-condensed chromatin group. ($p<0.0001$), whereas and the CMA3 positive stain value (lesser protamine content) was higher among the non-condensed chromatin group in comparison with the group of condensed chromatin ($p < .0001$).

Oxidative stress markers (ROS, SOD, lipid peroxidase) levels were significantly low in the condensed chromatin group. Similarly, the more viable and membrane intact sperm were in the chromatin condensed group, and there was more sperm DNA damage in the chromatin noncondensed group compared to the condensed group.

Mean±SEM serum testosterone level in the sperm chromatin condensed group is higher than in the non-condensed group (Table 20).

Table 20: Comparison of studied parameters between the two groups of condensed chromatin and not condensed chromatin (CMA3 staining)

	Condensed chromatin (<31%)	non-condensed chromatin (>31%)	p-value
Male Age (years)	38.58±0.37	39.12±0.40	0.33
Male BMI Kg/m^2	25.04±0.17	25.30±0.18	0.28
Sperm concentration (10^6 sperm/ml)	69.40±2.95	36.07±1.70***	0.00
Progressive motile	37.79±0.94	27.47±1.18***	0.00
Normal morphology (%)	4.57±0.08	2.23±0.05***	0.00
Sperm vitality (eosin)	57.62±1.01	48.96±1.36***	0.00
Membrane integrity (Hos)	75.30±0.75	62.10±1.34***	0.00
CAT (u/ml)	9.66±0.05	9.66±0.06	0.96
SOD (U/ml)	13.19±0.12	11.95±0.13***	0.00
POD (U/min)	10.92±0.02	10.26±0.03***	0.00
Lipid peroxidase (nM/ml)	28.38±0.07	29.44±0.10***	0.00
ROS (U/ml)	1.27±0.04	2.52±0.05***	0.00
AO	26.08±0.55	42.72±0.72***	0.00
SCD	14.14±0.52	33.27±0.47***	0.00
SCSA	14.22±0.52	35.44±0.45***	0.00
Testosterone (ng/ml)	386.49±12.49	336.60±11.20**	0.004

Values expressed as Mean±SEM.

*=P<0.05, **= P<0.01, ***=P<0.001

DISCUSSION

Male factor subfertility is gaining attraction because of declining semen quality, and sperm standard parameters of healthy young men. Male factor subfertility is rising around the globe and it's become challenging because of therapeutic modality (Agarwal *et al.*, 2015). The quality of human semen has conventionally been assessed by microscopic and biochemical examination to evaluate sexual dysfunction. This test gives the clinician the base of initial diagnosis and groups them into normal, moderate/severe factors of male subfertility. However, none of these tests address sperm quality and function to predict the fertility outcome after treatment (Smith *et al.*, 2007). The goal of the current study was to determine whether the male factor contributes to unsuccessful IVF and ICSI attempts and to add each or even more assessments to predict the outcome of ART in an attempt to enhance success rates, reduce anxiety, improve counseling, and lower costs. In the current study, we discovered that male factor subfertile men (MMF and SMF) had broken sperm chromatin integrity as a result of poor sperm parameters, which had adverse effects on sperm quality. Immature chromatin and DNA fragmentation alterations predominated in subnormal semen samples (Nemati *et al.*, 2020). Moreover, we found a higher percentage of spermatozoa with abnormal chromatin maturity and DNA damage in a subfertile group (MMF and SMF) compared with controls as suggested previously (Sun *et al.*, 2018). In addition to the higher level of DFI in the percentage of SCD, and SCSA in subfertile groups (MMF and SMF) with poor standard semen parameters when compared with normal control, and observation were in agreement with the reports of several studies (Talebi *et al.*, 2013; Dehghanpour *et al.*, 2020; Evenson, 2016). SCD test is intended to assess chromatin decondensation, which could be related to DNA damage but is not measuring directly the DNA breaks. The SCD test could potentially be used as a prospective substitute for a more accurate evaluation of DNA integrity using a straightforward diagnostic laboratory setup, given the close relationship between the results of SCD and SCSA (Turner *et al.*, 2020; Evenson, 1999). Exploring the relationship between SCD and pregnancy rate, the ROC curve analysis results showed that sperm DNA damage assessment was a reliable indicator of fertility achievements for infertile couples. As initially disclosed, the cut-off value for increased precision was 20 percent sperm DNA fragmentation (Alkhayal *et al.*, 2013).

Subfertile men have a higher proportion of sperm cells with denatured DNA compared to normal men, making SCSA a more popular method for determining the degree of sperm DNA strand breaks because SCD is not a strong predictor of DNA damage. Protamines are characterized by a reduction of nucleoprotein histones during sperm production, and they are then inserted into the hinge region of double-stranded DNA to create a DNA-Protamine complex (Amor *et al.*, 2019; Sergerie *et al.*, 2005). Male subfertility may result from abnormalities in the interpretation of each type of nucleoprotein specific to sperm cells, which changes the chromatin condensation of sperm (Aoki *et al.*, 2006). In the present study, we found a significant increase in CMI levels in moderate (Os, As, TZs) and severe male (OA, NOA, and OAT-S) subfertile patients compared with normozoospermic. Our data are in agreement with previous experimental results showing that normal semen samples prevailed in low chromatin and DNA fragmentation alteration (Cannarella *et al.*, 2020). Moreover, the percentage of spermatozoa with abnormal CMI and DFI was significantly higher in a subfertile group compared with controls as suggested previously (Gosálvez *et al.*, 2014; McDowell *et al.*, 2014). Halosperm assessment, a cost-effective and simpler test than the more widely used SCSA and TUNEL, makes for greater measurement of DNA integrity using just optical microscopy, which is popular in most laboratory facilities (Cissen *et al.*, 2016); (Chohan *et al.*, 2006; Evenson *et al.*, 2020).

In addition to the higher level of DFI in the percentage of SCD, SCSA cells in subfertile groups compared with normal control, this observation is in agreement with the reports of several studies (Liffner *et al.*, 2019; Zeqiraj *et al.*, 2018; Zheng *et al.*, 2018; Timermans *et al.*, 2020). Using SCSA, subfertile men have a higher percentage of spermatozoa with denatured DNA compared with normal men, suggesting that subfertility is associated with poor sperm DNA integrity (Chen *et al.*, 2020). In addition to the importance of sperm DFI assessment, the present study showed that a higher percentage of sperm CMI (checked through chromatin condensation and abnormal spermatozoa protamination) has been shown to play an essential part in sexual function, embryogenesis, repeated implantation failure, and pregnancy outcome (Saylan and Erimsah, 2019; Turner *et al.*, 2020; Alkhayal *et al.*, 2013).

Since DNA damage can be brought on by both internal factors, such as early apoptosis, aberrant rearrangement, and the resulting protamine (P1/P2 ratio) inequities, and external factors, such as storage conditions or cryopreservation, it is unclear about what operational consequences of sperm DFI and CMI (Tahmasbpour *et al.*, 2014; Asgari *et al.*, 2019; Hernandez-Silva *et al.*, 2021). ROS and oxidative damage are often serious explanations for declining sperm quality; preventing this antioxidant therapy could play a task (Amorini *et al.*, 2021). With the increasing number of sperm component faults, hardly some treatment options are available. The general lack of information regarding the precise biochemical nature of the cause of such sperm defects may help to explain this. The factors causing spermatozoa to lose their ability to fertilise are not well understood or well supported by reliable data.

Conclusion:

Male factor subfertility is a growing concern due to declining semen quality and sperm standard parameters. Traditional semen analysis methods are insufficient in predicting fertility outcomes after treatment. Assessing chromatin integrity and DNA fragmentation is crucial to predict success rates of assisted reproductive techniques. SCD test can substitute for more accurate evaluation of DNA integrity and sperm DNA damage assessment is a reliable indicator of fertility achievements for infertile couples. Although several tests for DNA damage and chromatin condensation were used in the present study some required high precision equipment (SCSA) and others have less predictive value (AO, TB staining), we found that SCD to assess DFI and CMA3+ staining to evaluate CM are inexpensively and precisely examine chromatin status and considered as applicable tools in ART.

CHAPTER 3

Sperm protamine, chromatin integrity, paternal overweight, and age influence assisted reproductive techniques outcome

ABSTRACT

A key goal of reproduction research is to identify factors that indicate the success of assisted reproductive technologies (ARTs). Gamete quality is critical for producing high-quality embryos and increasing the success of ARTs. This study aimed to evaluate the relationship between sperm chromatin quality and DNA fragmentation of spermatozoa with embryo aneuploidy. The present study aimed to evaluate the effect of sperm chromatin quality and DNA fragmentation of spermatozoa on assisted conception (IVF/ICSI) outcomes. Low and middle-income countries are facing a rapid increase in age, obesity, and overweight burden, particularly in urban settings. Being overweight in men is associated with subfertility and a higher risk to have a low sperm count or no sperm in their ejaculate. Despite potential limitations, this is one of few studies conducted to determine the potential risk of paternal overweight on sperm epigenetics and assisted conception outcome including fertilization, embryo quality, cleavage rate, reduce blastocyst development, implantation and cumulative birth rate.

The research took place at the Salma Kafeel Medical Centre in Islamabad, Pakistan, one of the fertility treatments facility. From January 2016 to July 2019, a total of 1872 couples who underwent 1st ovulatory stimulation were included. 753 married people underwent a complete ART process, which includes intracytoplasmic insemination (ICSI), which includes fresh transfer, during the follow-up period from January 2016 to October 2021. Couples grouped as the one with male factor subfertility (MFI) were grouped into normozoospermic (N), severe male factor (SMF) and mild male factor (MMF) subfertility. DNA fragmentation index (DFI) in spermatozoa was analyzed by sperm chromatin dispersion (SCD), sperm chromatin structure assay (SCSA), Acridine Orange (AO), chromatin maturation index (CMI) including CMA3+ to measure protamination and Toluidine Blue (TB) to measure chromatin condensation. DFI was measured by SCD, SCSA and AO while CMI was checked through protamination chromomycin A3 (CMA3+) and chromatin condensation TB+. Analysis was also run according to paternal body mass index (BMI) <24.5-20 kg/m^2 served as a reference group while the male patient with BMI >24.5-30kg/m^2 were considered to be overweight. couple with four metaphases (MII) oocyte, their male partner's semen samples were collected and prepared on the day of

oocyte collection and assessed for volume, concentration, motility, and morphology, and sperm chromatin integrity was measured by sperm chromatin dispersion assay (SCD), toluidine blue (TB) staining and Chromomycin A3 staining (CMA3). The influence of paternal BMI on embryonic development was assessed, which was classified into; peri fertilization effect (fertilization rate, FR), early/late embryonic development (cleavage rate, CR and blastocyst rate-BR), implantation stage (positive beta hCG), cumulative live birth (CLBR) stage (deliver of at least one live birth- with neonatal birth weight) effect were documented. Patients were separated into three groups according to their ages: group 1: male age equal to or less than 30 years (n=90), group 2: age between 31 to 40 years (n=330), and group 3: age above 40 years (n=330).

Embryo aneuploidy was significantly higher in male factor subfertile groups (MMF and SMF) compared to N. A positive correlation was observed between fertilization rate (FR) and live birth rate (LBR) with sperm count, motility, vitality, and a negative correlation between sperm morphology, sperm DFI, (SCD, SCSA and AO) sperm CMI (CMA3+ and TB+). No correlation was observed between embryo aneuploidy and sperm DFI and CMI. Spermatozoa DFI and CMI are associated with low fertilization and live birth rate and predict $\geq$50% embryo aneuploidy with accuracy. Embryo aneuploidy was comparable after IVF and ICSI treatment. A negative correlation between fertilization rate (FR) was observed with sperm DNA fragmentation, (SCD, and SCSA) and sperm chromatin decondensation (CMA3+ and TB+) after IVF treatment. While a negative correlation was observed between clinical pregnancy rate (CPR) after ICSI with sperm DNA fragmentation, (SCD) and sperm chromatin de-condensation (CMA3+). No correlation was observed between embryo aneuploidy and sperm DNA fragmentation and chromatin de-condensation.

The analysis of the percentage of spermatozoa with chromatin maturity (CMA3+) and chromatin integrity (TB+) was reduced significantly in overweight men ($p<0.01$) compared with a reference group. Increase in paternal BMI correlate with the increase in sperm chromatin damage (SCD $r= 0.282$, TB+ $r= 0.114$, $p<0.05$), immaturity (CMA3+, $r=0.79$, $p<0.001$) and oxidative stress (ROS) ($r=0.282$, $p<0.001$). Peril-fertilization effects were an increase in oocytes fertilization in couples with overweight men (FR =67%) compared with

normal-weight men (FR=74.8%), similarly, paternal overweight correlates with poor fertilization (r=-0.187, p<0.01), after multiple regression paternal weight remain predictor of successful fertilization. During the developmental stage, the number of embryos in cleavage was higher in normal-weight men, while day 3 (D3) embryos, percent good quality embryo D3, and blastocyst formation rate were comparable between the groups. No correlation was found between implantation rates and paternal BMI (r=0.42, p=0.25). The paternal overweight group (2952.14±53.64gm) had increased neonatal birth weight (within normal range) when compared with the reference group (2577.24±30.94gm, p<0.001) following assisted reproductive technology (ART). CLBR was higher (p<0.05) than normal weight men compared to the paternal overweight group. CLBR per embryo transfer and FR used was the difference between groups statistically significant (p<0.05).

We found paternal overweight BMI > 24.5 kg/m^2 had a reduced fertilization rate with an OR of 1.98(CI 95% 1.323-2.967, p=0.001). The multiple linear regression analysis of paternal BMI showed a positive association with fertilization rate and CLBR (weight within normal range) after adjustment for several potential cofounders. The present study demonstrated the impact of paternal overweight on male reproductive health, as these patients had a higher percentage of immature sperm (CMA3+) with impaired chromatin integrity (SCD, TB) in their semen and had decreased fertilization rate, CLBR following assisted reproductive treatments. The present study supports that paternal overweight should be regarded as one of the predictors for fertilization, CLBR, and useful for counseling, to consider body mass index not only in women but also for men, in couples opting for ART treatment, and warrant a poor reproductive outcome in overweight men.

Conventional semen parameters, Reactive oxygen species (ROS), SOD, POD, CAT, and TABRS did not statistically differ with increasing male age or between different age groups, but there was a significant inverse relationship between sperm DNA damage and increasing male age. When compared younger men (30 years) to males >40 years showed higher levels of sperm DNA damage (p<0.01). The concentration of LH, FSH and testosterone levels were comparable between the groups. While a significant (p<0.05) increase in chromatin immaturity was observed in the old age group (>40years). A positive (p<0.05) association was observed between advanced male age and sperm chromatin

dispersion (SCD) and decondensation (CMA3). Despite potential limitations, this is one of the studies with extensive information on the potential risk of paternal age on sperm epigenetics. The present study demonstrated the impact of male age on male reproductive health, as these patients had a higher percentage of sperm chromatin damage (halosperm-SCD) in their semen. Sperm DNA damage evaluation will help in the evaluation and diagnosis of the underlying cause of poor fertility and can help the clinician in selecting the right treatment option. Female age and BMI kg/m^2 significantly negatively correlated to fertilization rate, cleavage rate and outcome rate.

Sperm chromatin condensation abnormalities were more frequently seen in couples who face ART (IVF/ICSI) fertilization failure and unsuccessful implantation despite successful embryo transfer, which should be regarded as negative predictors of fertilization and clinical pregnancy. The couples undergoing Invitro-fertilization (IVF) or intracytoplasmic injection (ICSI) with male factor subfertility including impaired semen parameters, sperm DNA and chromatin quality should be considered as a useful tool for evaluation and prediction of assisted reproduction techniques (ART) success (FR, LBR) and could be regarded in the future as an indication for preimplantation genetic testing for aneuploidy.

INTRODUCTION

Subfertility is defined as no conception despite one year of unprotected attempts(Skakkebaek *et al.*, 2006). One initial investigation aimed to find a cause of subfertility is the evaluation of semen samples to rule out malefactors; which are attributed to 40-50% of subfertility (Cooper *et al.*, 2010). Conventional semen analysis includes sperm count, motility, and morphology; however, these parameters do not strictly predict fertilization potential (Cissen *et al.*, 2016; Ozmen *et al.*, 2007; Wdowiak *et al.*, 2015). Sperm abnormal chromatin and DNA fragmentation assessment are hidden anomalies frequent in subfertile men (Sakkas and Alvarez, 2010). Subsequently, routine sperm parameters alone do not enable the identification of a substantial proportion of subfertile men. Reactive oxygen species (ROS) correlate with sperm DNA damage and might also be associated with human subfertility (Ribas-Maynou *et al.*, 2020; Sabeti *et al.*, 2016). The presence of damaged DNA may also result in faulty nuclear remodeling caused by faulty protamine deposition during spermiogenesis. One of the features of spermatozoa protamines is that they will be concerned with the safety of the genetic code. In humans, protamines replace approximately 85% of the histones during the process of spermatogenesis (Niederberger, 2005; Rathke *et al.*, 2014). Incomplete protamination could render the spermatozoa greater at risk of attacks with the aid of using endogenous or exogenous agents, including free radicals, mutagens (Alvarez *et al.*, 2002; Irvine *et al.*, 2000), and nucleases (Carreira *et al.*, 2015; Szczygiel and Ward, 2020). (Amor *et al.*, 2019; Mangoli *et al.*, 2018).

Sperm chromatin condensation and male fertility

Sperm chromatin condensation helps the paternal germ cells in many ways; i). The condensed paternal genome makes the spermatozoa hydrodynamic and lighter which facilitates its faster movement and efficiently fertilizes the oocyte. ii). Spermatid is devoted to transcription machinery and proteins involved in this process, it allows the paternal message reprogramming easier for oocytes post insemination. iii). Loss of epigenetic information and imprinting during spermatogenesis could influence the paternal genome reactivation post-fertilization. Although insemination with damaged DNA results in fertilization this causes defective embryonic growth, implantation failure, and miscarriage

or fetal deformities. Subfertile men had sperm more sensitive to chromatin damage by agents like peroxide (H_2O_2), alcohol consumption, smoking, age, obesity, and radiation. The fertilization optional decrease with an increase in the proportion of sperms with >30% DNA damage measured using SCSA. Altered protamine expression reasons multiplied DNA damage that's related to bad semen quality, decreased fertilization capability, poor embryo development, recurrent implantation failure, and a higher miscarriage rate. Sperm DNA damage is a hidden anomaly of idiopathic subfertile men with normal standard semen parameters. The higher DNA damage correlates significantly with minimal chance for natural conception.

Assisted conception and chromatin assessment:

The introduction of Assisted conception techniques (ART) with invasive reproductive technologies are subzonal insemination (SUZI), gametic intrafallopian transfer (GIFT) or superovulation, intrauterine insemination (IUI) and intra cytoplasm sperm injection (ICSI) have greatly enhanced the expectation of subfertile couples about having their baby. The probability of conception after IUI and in-vitro fertilization reduce when sperm chromatin damage exceeds. The effect of sperm chromatin damage on the outcome is controversial and some studies showed no association between sperm chromatin damage on IVF/ICSI outcome and some showed a significant negative correlation between sperm chromatin damage and embryo grade, blastocyst formation, and outcome. Some meta-analyses showed sperm chromatin integrity has a significant effect on pregnancy outcomes using routine IVF and no effect on outcomes after ICSI. This suggests that assessment of sperm DNA integrity help to predict the IUI and IVF outcome and patients with higher sperm chromatin should be counseled to consider assisted conception as a treatment choice. Successful pregnancy after IVF and ICSI is possible when DFI is less than 27% measured utilizing SCSA, however, studies are contradicting these results. The miscarriage rate is higher in patients with high sperm chromatin damage. Spontaneous apportion after assisted conception also increases when sperm chromatin non condensed and testing DNA integrity should be assessed to predict the miscarriage rate. The proportion of sperm DNA damage was found to be higher in couples with recurrent miscarriage, and implantation failure. No linkage was found between sperm chromatin abnormalities with offspring anomalies.

Aitken suggests sperm DNA damage can result when an oocyte failed in an attempt to repair DNA after fertilization giving rise to mutation. These induced mutations result in childhood cancer or a higher risk of imprinting errors in offspring.

Sperm contributes haploid genome to embryonic genetic information at the time of fertilization. The frequency of meiotic issues in offspring with numerical and structural chromosomal abnormalities showed a higher prevalence in subfertile men compared to the fertile population (Mazzilli *et al.*, 2017). Furthermore, subfertile men with a higher incidence of DNA fragmentation correlate with poor sperm quality and a higher degree of chromosomal anomalies (Amor *et al.*, 2019). Sperm with a higher DNA fragmentation rate and impaired sperm chromatin will increase the prevalence of chromosomal aneuploidy, low pregnancy, and implantation after ICSI (Cissen *et al.*, 2016; Irvine *et al.*, 2000; Ozmen *et al.*, 2007). From the information obtained so far from the literature available on preimplantation genetic diagnosis for aneuploidy, the severe male factor abnormalities could make contributions to a higher prevalence of chromosomally abnormal embryos even if the female age is under 36 years (Pagidas, Ying, and Keefe 2008). Sperm chromatin maturity assessment is based on sperm nuclear protein stains (toluidine blue) and chromomycin A3 (CMA3) staining for defective-protamination (Lolis *et al.*, 1996). CMA3 competes with protamines for binding to the DNA minor groove, indicating the state of protamination indirectly.

Different methods to check sperm chromatin integrity are accomplished by evaluation of sperm chromatin fragmentation (DFI) utilizing sperm chromatin structure analysis (SCSA), Sperm Nucleus DNA integrity Kit, terminal transferase dUTP nick-end labeling (TUNEL), the alkaline/acidic comet assay, and sperm chromosomal aberration by in situ hybridization (FISH). Furthermore, the sperm chromatin condensation s checked by sperm protamine deficiency chromomycin A3 (CMA3) staining and toluidine blue or aniline blue (AB)-stain sperm. In intracytoplasmic sperm injection (ICSI) patients with better oocytes, Alvarez Sedó et al. (Sedó *et al.*, 2017) found that sperm DNA aberration was inversely linked with blastulation and successful outcome rates. *In vitro* fertilization, effects studied by Zheng et al. (Zheng *et al.*, 2018) discovered that sperm DNA damage had a detrimental effect on cleavage stage embryogenesis, blastulation, and successful outcome. High sperm

DFI was found to have reduced embryogenesis and implantation, as well as increased spontaneous abortion, after assisted reproduction as shown in meta-analyses (Deng *et al.*, 2019). After the preimplantation genetic screening, sperm DNA damage was not linked to blastocyst aneuploidy, morphokinetic, or pregnancy (Green *et al.*, 2020). A meta-analysis suggests that sperm DNA damage reduced successful implantation after ART (Simon *et al.*, 2017), and another study found that clinical or neonatal outcomes of ICSI cycles were unaffected by sperm DFI (Chen *et al.*, 2020). Rizvi et al (Razavi *et al.*, 2003) found sperm protamination effect on fertilization in ICSI. Kyarydis et al (Karydis *et al.*, 2005) indicate spermatozoa with abnormal chromatin condensation do not have a role in ART outcome. Marchiani and colleagues (Marchiani *et al.*, 2017) found CMA3+ and fertilization rate as two independent factors. According to the literature cited above, still inconclusive regarding the sperm chromatin damage effect on embryogenesis and successful outcome.

Semen analysis is a routine and simple method for assessing male fertility status. However, alone it is not sufficient to predict assisted reproductive outcomes (Chohan, 2006; Davidson *et al.*, 2015). With the development of new predictive tools to identify male fertility potential. Sperm deoxyribonucleic acid fragmentation index is a commonly used technique involving methods such as Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, Comet assay, Sperm chromatin structure assay (SCSA), acridine Orange test, and sperm chromatin dispersion (SCD) assay (Tímermans *et al.*, 2020; Chohan, 2006). Identification of DFI through SCD assays is cheaper yet equally reliable when compared with TUNEL assay which is expensive and utilizes advanced equipment (Zeqiraj *et al.*, 2018; Agarwal, 2003; Agarwal *et al.*, 2017).

Paternal BMI and age

Multiple cross-sectional studies and meta-analyses have found inconsistent results of varying correlations between BMI, semen parameters, male reproductive hormones, Sperm DNA fragmentation, chromatin structure, and ART outcome (Linabery *et al.*, 2013; Davidson *et al.*, 2015). A systematic review of the literature demonstrates that sperm DNA damage is associated with lower pregnancy rates and pregnancy loss after assisted conception techniques employing in-vitro fertilization (IVF) and Intracytoplasmic insemination (ICSI) (Bandel *et al.*, 2015; Zahid *et al.*, 2015; Macdonald *et al.*, 2013;

Sermondade *et al.*, 2013), particularly ICSI where it circumvents the natural defense barriers and allows for fertilization with DNA damaged sperm. Therefore, increasing concerns regarding the health outcomes for the resulting offspring (Sermondade *et al.*, 2013; Keltz *et al.*, 2010; Barratt *et al.*, 2017). Besides, other factors, obesity would be the leading cause of lower pregnancy rate and failure of reproductive outcomes. Therefore, the overall health and normal BMI of parents should be considered and of important concern in attaining reproductive outcomes. We aimed to investigate the correlation of paternal BMI on semen parameters (concentration, motility, morphology, and vitality), DNA fragmentation, and chromatin maturity. Furthermore, we investigated the correlation matrix of the possible impact of paternal high BMI on fertilization, embryo quality, live birth rate, and birth weight.

Subfertility is a global reproductive health issue faced by 10-15% of couples of reproductive ages. An estimated 3.5–16.7% of couples are affected in developing countries and 6.9–9.3% of couples are affected in developed countries (Ombelet *et al.*, 2008; Barratt *et al.*, 2017). Fertility specialists in third-world countries face major difficulty during the investigation of subfertile couples. Due to the limiting social beliefs that the cause of subfertility lies in the female. As a consequence of which male partners hardly present themselves for investigation making it difficult to access the true cause of subfertility (Agarwal *et al.*, 2015; Topp *et al.*, 2015). Male factor subfertility prevails in approximately 25% of all such couples (Barratt *et al.*, 2017; Asgari *et al.*, 2019). It has been reported that in male partners opting for semen analysis; over 50% of men presented with abnormal semen parameters. It is estimated that the prevalence of male subfertility between the ages of 15 to 50 years was up to 6% (Agarwal *et al.*, 2015; Barratt *et al.*, 2017; Bahamondes and Makuch, 2014). Obesity has recently emerged as a major contributor to debility in reproductive health indices in both sexes, as excess energy alters the reproductive system's regulatory mechanisms. Obese people have higher estrogen levels due to the amplification of aromatase in adipose tissue; through a negative response loop, men show signs of hypogonadotropic hypogonadism, and these hormonal fluctuations, in addition to increased oxidative stress, lipotoxicity, and instabilities in adipokine absorption, directly harm the gonads, peripheral reproductive organs, and the embryo (Mintziori *et al.*, 2020). It is generally well accepted that reproductive function highly correlates with the

degree of adiposity, nutrition, or metabolic condition related to food intake in human medicine (Campbell *et al.*, 2015; Zahid *et al.*, 2015). Paternal BMI Kg/m^2 < 16.5 (underweight) and >30 (obesit**y)** were associated with reduced semen quality (Raad *et al.*, 2019; Campbell *et al.*, 2015; Leisegang *et al.*, 2021). Moreover, known acquired factors that contribute to male subfertility include infection, immunological factors, trauma or surgical insult to the male reproductive organs, and exposure to toxic chemicals or other materials (Ombelet *et al.*, 2008; Leung *et al.*, 2018; Barratt *et al.*, 2017). Similarly, a direct association was found between men's BMI kg/m^2 and semen quality found even after adjustment for reproductive hormones (Maghsoumi-Norouzabad *et al.*, 2020).

The present study aimed to evaluate the impact of sperm DNA fragmentation and chromatin condensation, paternal BMI and age on fertilization rate, pregnancy rate aneuploidy, and live birth rates. Focusing on semen samples from male partners of subfertile couples undergoing ART treatments, we examined chromatin maturity status (by CMA3 and TB staining) and DNA fragmentation (using SCSA, and SCD), to see if these male molecular markers could predict ART success rate, and their relationship with ART outcomes as the test in combination while accounting for many confounding factors could alter the statistical study.

MATERIALS AND METHODS

Subjects

This prospective study included 753 couples who underwent IVF/ICSI procedures out of which 604 couples were involved in the ICSI/IVF program at Fertility and Genetic services, Islamabad, Pakistan, from April 2016 to October 2021. The study population involves fertile 146 and 607 subfertile men.

Ethical Compliance

The institutional review board of Quaid-i-Azam University authorised the research proposal, and the ethics committee of the SKMC Islamabad Pakistan awarded its approval. The subject's detailed information (brief medical history, including male and female ages, male body mass index (BMI), period of subfertility, primary/ secondary subfertility, and information about earlier spontaneous abortions -related data) was obtained through a questionnaire, asking for the appropriately structured question by face to face interview.

Inclusion and exclusion criteria

A complete physical evaluation was performed, including an assessment of scrotal size to rule out cryptorchidism and malformations of the external genitalia; a doppler assessment to rule out varicoceles; an immunobead binding evaluation to rule out the existence of anti-sperm immune cells; and genetic fingerprinting to rule out the chronic illnesses such as liver/renal disease, patients who are extremely obese, patients who have hyperglycemia were excluded.

There was no subfertility factor in the female partner of the couple included in this study. The semen sample was subjected to analysis for seminal characteristics and the blood sample was drawn for hormonal determination. Fertile males were those without any history of fertility problems and within one year of unprotected intercourse, their partners had spontaneous pregnancy. The fertile and subfertile couples were recruited from assisted conception unit-fertility genetics services (Salma & Kafeel Medical Services) Islamabad.

Male patients were divided into three groups

Normozoospermic (N) n= 146, men with all semen parameters within normal limits

Moderate male factor (MMF) n= 280, men with a single abnormal finding of the semen analysis or by a total motile sperm count between 5–20 × 10^6/mL with normal morphology <4% (WHO 2010)

Sever male factor (SMF) n= 327 with low sperm count, >15x106/ml, low sperm motility >32 % , total motile sperm count less than 5 × 10^6/mL and low sperm morphology >4%.

A total of 753 couples reached to embryo transfer stage, out of which 570 couples were treated with ICSI, 107 with IVF and both ICSI and IVF were 76. Reasons for postponed embryo transfer; risk of ovarian hyperstimulation syndrome, elevated progesterone levels (>1.5ng/ml), and insufficient endometrium on the trigger day. The pregnancy rate and live birth rate were assessed only for fresh embryo transfer to prevent potential confounding bias.

Ovarian stimulation, IVF, ICSI, and Embryo Development

After a long protocol with mid-luteal phase long-acting gonadotropin-releasing hormone analogs (triptorelin, Decapeptly, Ipsen Pharma) followed by an exogenous individual dose of recombinant follicle stimulation hormone r-FSH (Gonal-F, Merk Serono- Germany) were used to induce multiple follicular growths, with starting dose ranging from 150 to 225 IU, according to age, body mass index, antral follicular count, AMH level and response to previous stimulation. The stimulation was titer according to ovarian response (estradiol level and ultrasound every 2 days, till at least two follicles, reached 17mm diameter. Finally, At 34–36 hours following delivery of human chorionic gonadotrophin u-HCG (IVF-C, LG Lifesciences), oocytes were harvested transvaginally by ultrasound guidance, under general anesthesia sedation, and cultured in human tubal fluid supplemented with 5% human serum albumin (HSA) in a 5% CO_2 humidified gas environment at 37°C. Depending on sperm indices and couples' reproductive histories, oocytes were inseminated using conventional IVF with cumulus oocytes were incubated with 60,000 spermatozoa/oocyte in in-vitro fertilization supplemented with HSA -IVF-plus medium (Vitrolife Goteborg-Sweden) or for ICSI, OLYMPUS IX51/71/81/53/73/83 microscope assembled with INTEGRA Ti microinjector was used. Oocytes were assessed at 16–18

hours after insemination based on the presence of two pronuclei. Individually fertilized oocytes were sequentially cultivated in G1/G2 Plus (Vitrolife Goteborg-Sweden) and incubated in MIRI multiroom incubator (Esco Medical-and scored 40, 62, 88, and 112 hours after insemination. Additionally, the percentage and type of fragmentation, as well as the number and shape of blastomeres and nuclei, were all counted (Magli *et al.*, 2007). Developmental embryos with at least four cells and no more than 40% fragmentation were individually managed in HEPES-buffered media layered with pre-equilibrated mineral oil for the biopsy procedure at 62–64 hours after insemination. A 0.2 µm zona pellucida breach was made, and a nucleated blastomere was delicately aspirated with a refined glass needle into the perivitelline space. Embryos were properly washed and transferred to blastocyst growth medium after blastomere biopsy. Biopsied samples were placed in 0.2 ml PCR tubes containing 2microliter PBS and were shipped to igenomix Dubai where the Next-generation sequencing NGS platform was validated in previous studies(Pagidas, Ying, and Keefe 2008; Coates et al. 2017) and commercially available in the market, (Resproseq, Life-Thermofisher-USA) analysis for pre-implantation genetic testing for aneuploidy (PGT-A) was performed. The presence of two pairs, one set, or three or more sets of the tested chromosomes, respectively, was used to define euploidy, haploidy, and polyploidy. Monosomy and trisomy were described as the occurrence of an abnormal number of copies of one or two chromosomes, respectively.

Semen Preparation

A semen sample was collected by masturbation on the day of oocyte aspiration after 2-5 days of abstinence and left to liquefy at 37°C for 30 minutes before analysis. According to WHO 2010 standards, sperm counts, motility, and morphology were examined, To summarise, sperm number was determined using an upgraded Neubauer chamber after proper dilution, motility was determined using a Leica microscope DM300 scoring at least 100 spermatozoa/slide, and morphology was determined using Diff-Quick staining Sperm vitality using a hypo-osmotic swelling test (HOS), briefly 1 ml of 150 mOsm hypoosmotic swelling solutions a mixture of sodium citrate (25 mmol/l) and fructose (75 mmol/l) was added to 0.1 ml of semen and waited for 30 minutes at 37^{o}C. Samples with and without HOS treatment were put on a glass slide with a glass coverslip and evaluated (Takahashi

et al., 1990) and reactive oxygen species (ROS) was estimated (Hayashi *et al.*, 2007). For the semen preparation, 138 samples were processed with a swim-up procedure, for swim-up the semen sample was used without centrifugation, and the sample, 0.5-1.0 ml, was layered gently under 0.5 ml of G-Mops plus (Vitrolife, Gothenburg, Sweden) and incubated for 1 h at 37ºC and 612 samples processed using density gradient centrifugation (DGC), for DGC sperm Grade (Vitrolife, Gothenburg, Sweden) was diluted in medium G-Mpos Plus (Vitrolife, Gothenburg, Sweden) to generate dilutions of 45 and 90 percent density and in 15ml falcon tubes, two 90 percent and 45 percent columns were created by layering 1-1.5 mL of each solution, commencing at the bottom with the 90 percent fraction. 1 mL of neat sample stratified as the top layer of columns and centrifuged at 300 g for 15 minutes, and the pellet was collected after centrifugation and washed once at 350 g for 10 minutes and the final fraction includes only the topmost 0.25ml fraction which was collected gently into a new tube.

After preparation, the acquired fraction was tested for sperm count and motility and then maintained at 37 °C in the same medium for 15 minutes before being utilized to inseminate the oocytes. Each sample was split into two aliquots: one for sperm DNA damage (SCD, SCSA), and chromatin abnormalities (CMA3+, TB+), and the other for inseminating oocytes through ICSI or IVF.

Paternal BMI and Assisted conception outcome

Informed consent was taken from the study participants. 750 couples reached to embryo transfer stage. Reasons for postponed embryo transfer include; the risk of ovarian hyperstimulation syndrome, elevated progesterone levels (>1.5ng/ml), and insufficient endometrium on the trigger day. Pregnancy rate and live birth rate were assessed only for fresh embryo transfer to prevent potential confounding bias while 307 patients followed up till delivery, and during that time we analyzed cumulative pregnancy outcomes among couples, with or without achievement of live birth, and outcomes were calculated among couples were analyzed (Figure 30).

Paternal age and sperm quality markers

The final total number of couples was 750 with couples based on male partner age patients were divided into three groups 1: ≤ 30 years (n=90), group 2: 31-40 years (n=330), and group 3: >40 years (n=330). The average age of the female partners was equal to or less than 35 years and BMI less than 24.5 kg/m^2, while the male partner's average age was 38 years old and average BMI was 25.6 kg/m^2.

Male sperm samples were obtained, and their volume, concentration, motility, and morphology were evaluated. Sperm chromatin dispersion assay (SCD), chromatin integrity-toluidine blue (TB) staining, and sperm chromatin compaction-Chromomycin A3 staining were used to measure the sperm's chromatin integrity (CMA3+). In all patients, we assessed sperm chromatin integrity (TB+), sperm DNA damage (Halosperm-SCD), chromatin maturity (CMA3 staining), seminal ROS, SOD, POD, CAT, and TABRS (chemiluminescence assay).

Sample size calculation

The prevalence of subfertility in the Pakistani population is 4%(Ahmed *et al.*, 2020) and the sample size was calculated by using the formula;

$$n= (z^2 \text{ x } p \text{ x } 1\text{-}q)/E^2$$

The sample size was calculated with a confidence interval of 95% and a precision of 1.5% (error) (Dupont and Plummer, 1990; Abdullah *et al.*, 2020). This sample was inflated by 20% to non-responders (failure to give consent, medical and personal dropouts) and the adjusted sample size was approximately 750 patients.

Statistical analysis

Data were presented using mean and SEM. One-way ANOVA and Tukey's group's comparison tests were employed to compare male participants who were fertile and all subfertile, and the Statistical Package for the Social Sciences was used for the statistical studies (IBM SPSS software, version 20). Statistics were considered significant for P values under 0.05. For those outcomes that showed a link to one or more assessed parameters, prediction models were built. The Hosmer-Lemeshow goodness-of-fit test was

statistically used to determine whether the model's predictions were reliable. Logistic regression was used to look for predictors that were significantly linked to ART outcomes. To test the accuracy, sensitivity, and specificity of the paternal parameters in predicting the results of ART, as well as to determine the cutoff values for these parameters, we used receiver operating characteristic (ROC) curve analysis. Subgroup analyses were carried out using either the traditional IVF or the ICSI. To examine the relationship between paternal demographic characteristics with fertilisation rate while controlling for potential confounding variables such as maternal age, maternal BMI, paternal age, subfertility duration, and sperm chromatin integrity assay, a multiple linear regression analysis was conducted. A 95% confidence interval was calculated for the adjusted odds ratios (OR) using logistic regression (CI). To confirm the results and control for potential confounders in the computation of CLBR a cox regression was performed. A p-value of <0.05 was considered to be statistically significant. All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) 20 IBM (Armonk, NY).

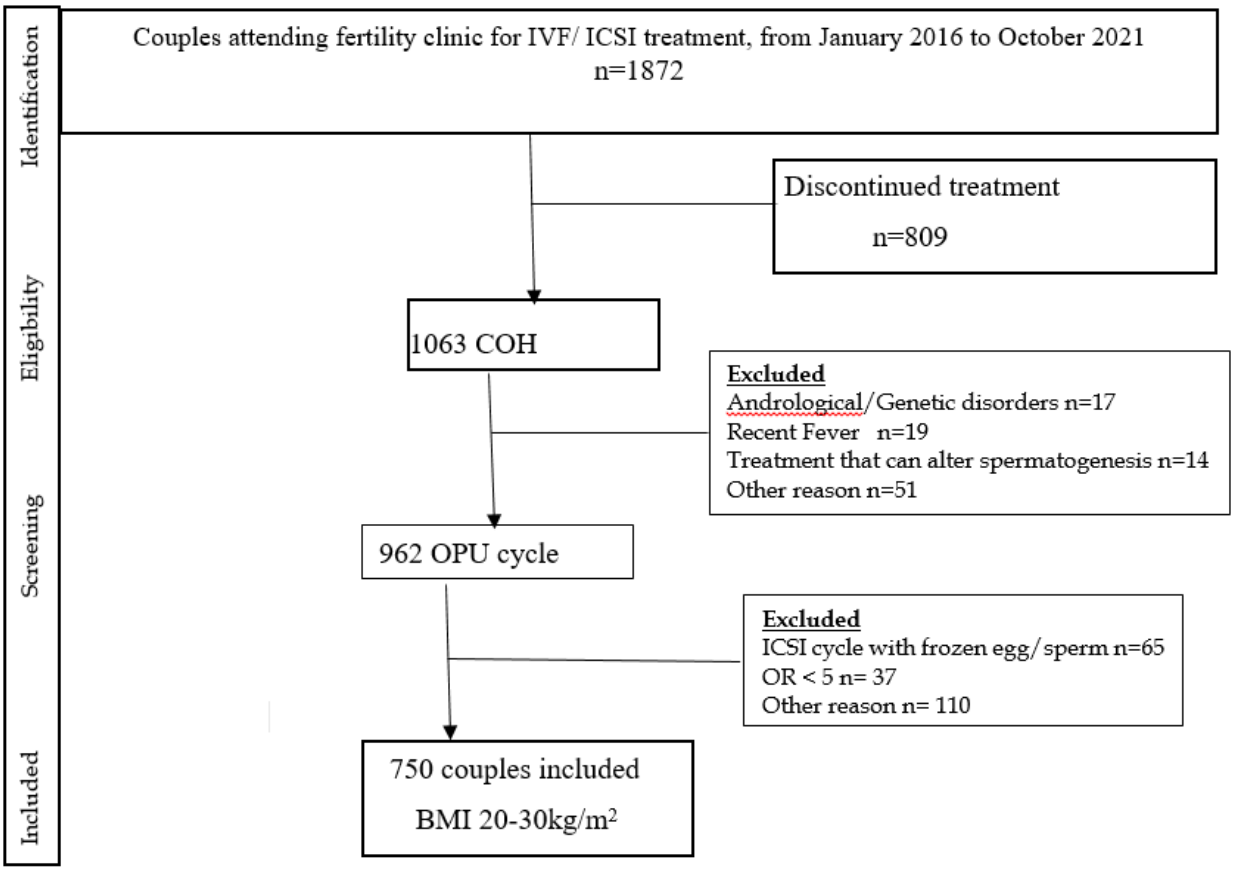


Figure 33. Schematic recruitment in the study. COH controlled ovarian hyperstimulation; OR, oocyte retrieve; OPU, oocyte pick-up; EQ_A embryo with A quality.

RESULTS

Assisted Conception Treatment and outcome:

Demographic characteristics:

The demographic characteristics of the couple are summarized in the table 21. according to the in-vitro insemination technique. There was a higher percentage of the couple with primary subfertility (65%) and most couples stayed together (88%). Most of the patients had no significant family history. Similarly, there was the most couple with higher annual income and were mostly self-employed (Table 21). Female partner characteristics had been summarized in and there is no significant ($p<0.05$) difference in age, BMI, endometrial thickness, and reproductive hormonal levels (Table 22). Male partner age, BMI, and reproductive hormonal levels were comparable between the insemination groups and subfertile and fertile male subject categories as illustrated in Table 23.

Neat Sperm conventional and quality parameters

The neat sperm characteristics were significant differences ($p <0.05$) between the three insemination groups. Semen sample used for intra-cytoplasmic insemination had a significantly ($p<0.05$) lesser percentage of total motile sperm with more ROS production, more sperm DNA fragmentation (SCD, SCSA, AO) and higher protamine deficiency (CMA3+), Similarly the sperm sample used for ICSI had lesser chromatin condensation (TB+) compared to IVF and IVF/ ICSI insemination procedure (Table 24).

Prepared Sperm conventional and quality parameters

The prepared sperm characteristics were comparable between the three insemination groups. The semen sample used for intra-cytoplasmic insemination had a significantly ($p<0.05$) lesser percentage of total motile sperm with more ROS production, more sperm DNA fragmentation (SCD, SCSA, AO) and higher protamine deficiency (CMA3+). Similarly, the sperm sample used for ICSI had lesser chromatin condensation (TB) compared to IVF and IVF/ ICSI insemination procedures as shown in Table 25.

Table 21: Demographic characteristics of couple according to the procedure used to fertilize oocytes

		IVF (107)	ICSI (570)	IVF/ICSI (76)	Total (753)
Subfertility	**P. Subfertility**	8.50%	54.30%	2.10%	64.90%
	Sec. Subfertility	6.50%	27.00%	1.60%	35.10%
Parity	**0**	11.30%	62.20%	2.70%	76.10%
	1	2.00%	9.30%	0.40%	11.70%
	2	0.80%	3.90%	0.50%	5.20%
	>3	0.90%	6.00%	0.10%	7.00%
Abortion	**0**	10.40%	66.50%	2.80%	79.70%
	1	2.90%	8.60%	0.50%	12.10%
	2	1.30%	2.70%	0.10%	4.10%
	Recurrent abortion	0.40%	3.5%	0.30%	4.10%
Living together	**Yes**	13.40%	72.00%	2.90%	88.30%
	No	1.70%	9.70%	0.30%	11.70%
Family history	**Na**	6.50%	41.20%	1.70%	49.40%
	Hypertension (HTN)	0.90%	2.30%	0.30%	3.50%
	Diabetes mellitus (DM)	1.30%	5.60%	0.30%	7.20%
	HTN/DM	6.00%	32.10%	1.30%	39.40%
	Thalassemia Minor	0.10%	0.10%		0.30%
	Others	0.10%		0.10%	0.30%
Annual Income	**<$25,000/year**	1.30%	15.00%	0.40%	16.70%
	$25,000–$49,999/year	3.40%	25.60%	0.90%	29.90%
	$50,000–$99,000/year	0.40%	4.30%		4.70%
	$100,000–$99,999/year	5.10%	25.60%	1.30%	32.10%
	>$99,999/year	3.80%	12.40%	0.40%	16.70%
Profession	**Working abroad**	1.00%	5.30%		6.20%
	Army/Police	1.40%	5.30%	0.50%	7.20%
	Banker		1.90%		1.90%
	Businessman	1.40%	25.80%	1.00%	28.20%
	Govt. Job	2.90%	16.30%		19.10%
	Civil Servant	1.00%	5.30%	0.50%	6.70%
	Doctor	3.30%	9.10%	0.50%	12.90%
	Driver/labor/jobless	0.50%	10.00%	0.50%	11.00%
	Property dealer	0.50%	2.40%		2.90%
	Land Lord	1.40%	2.40%		3.80%

Table 22: Baseline characteristics of female patients according to the procedure used to fertilize oocytes

	IVF (107)	ICSI (570)	IVF/ICSI (76)	Total (753)
Female Age years	32.17±0.51	32.99±0.24	34.42±1.05	32.91±0.21
Female BMI Kg/m^2	27.79±0.37	28.63±0.16	28.71±0.66	28.51±0.14
Hemoglobin %	12.00±0.15	11.96±0.07	12.22±0.38	11.98±0.06
Endometrial thickness cm	1.05±0.04	1.05±0.02	1.11±0.08	1.05±0.02
Female FSH mIU/ml	8.24±0.72	7.28±0.27	7.38±0.65	7.44±0.25
Female LH mIU/ml	8.71±1.10	8.33±0.45	10.00±2.06	8.46±0.41
Female Prolactin ng/ml	19.77±1.34	21.96±0.73	18.44±2.31	21.46±0.63
Female Estradiol pg/ml	172.7±33.6	301.1±28.1	227.6±60.2	277.1±23.3
AntiMullarianHormone ng/ml	4.67±0.61	3.50±0.16	3.12±0.40	3.63±0.15
Thyroid Stimulation Hormone	2.12±0.19	2.37±0.16	1.52±0.21	2.29±0.13

Values expressed as Mean±SEM. Values in parentheses represent the number of ART cycles. a= IVF vs ICSI and IVF/ICSI; b=ICSI vs IVF/ICSI

*=P<0.05, **= P<0.01, ***=P<0.001

Table 23: Baseline characteristics of male patients according to the procedure used to fertilize oocytes

	IVF (107)	ICSI (570)	IVF/ICSI (76)	Total (753)
age years	37.79±0.73	39.00±0.30	39.11±1.45	38.82±0.27
BMI Kg/m^2	24.80±0.33	25.24±0.13	24.75±0.56	25.16±0.12
FSH mIU/ml	5.33±0.25	5.97±0.14	5.79±0.59	5.85±0.12
LH mIU/ml	6.00±0.80	7.52±0.35	6.19±0.76	7.20±0.31
Prolactin ng/ml	11.85±0.74	12.20±0.34	12.39±1.10	12.15±0.30
Testosterone ng/ml	518.89±27.26	324.69±7.93	456.61±45.87	363.48±8.54

Values expressed as Mean±SEM. Values in parentheses represent the number of ART cycles. a= IVF vs ICSI and IVF/ICSI; b=ICSI vs IVF/ICSI

*=P<0.05, **= P<0.01, ***=P<0.001

Table 24: Neat semen conventional and quality parameters of male patient grouped according to the procedure used to fertilize oocytes

Neat Sample	IVF (107)	ICSI (570)	IVF/ICSI (76)	Total (753)
TNMS %	65.16±1.27	37.8±1.05^{ab**}	60.54±2.9	44.48±0.9
Sperm vitality (Eosin) %	67.83±0.94	49.1±1.02^{ab**}	66.79±1.6	53.75±0.8
Membrane integrity (Hos) %	82.60±0.71	65.06±0.94	81.18±1.4	69.39±0.7
CAT (u/ml)	9.65±0.08	9.66±0.04	9.67±0.20	9.66±0.04
SOD (U/ml)	13.14±0.22	13.62±0.11	12.64±0.4	13.48±0.1
Lipid peroxidase (nM/ml)	28.22±0.10	29.05±0.08	28.50±0.1	28.86±0.1
ROS (U/ml)	1.44±0.06	1.96±0.05^{ab**}	1.46±0.15	1.83±0.04
Acridine orange test (AO)	26.46±0.69	35.7±0.65^{ab**}	29.07±2.1	33.53±0.5
Sperm chromatin dispersion (SCD)	15.97±1.12	24.8±0.53^{ab**}	17.25±2.6	22.71±0.5
Sperm chromatin structure analysis (SCSA)	17.31±1.13	25.8±0.58^{ab**}	18.36±2.7	23.7±0.52
Protamine deficiency - CMA3+	25.08±0.76	29.8±0.43^{ab**}	26.89±2.0	28.7±0.38
Chromatin Condensation-TB+	21.59±0.97	30.9±0.58^{ab**}	24.1±2.90	28.7±0.51

Values expressed as Mean±SEM. Values in parentheses represent the number of ART cycles.

a= IVF vs ICSI and IVF/ICSI; b=ICSI vs IVF/ICSI

*=P<0.05, **= P<0.01, ***=P<0.001

Table 25: Semen post preparation conventional and quality parameters of male patient according to the procedure used to fertilize oocytes

Prepared Sample	IVF (107)	ICSI (570)	IVF/ICSI (76)	Total (753)
TNMS %	76.41±3.26	41.28±1.92	71.25±7.16	50.50±1.72
Membrane integrity	82.23±0.83	63.91±1.08	81.18±1.47	69.03±0.86
ROS (U/ml)	0.49±0.05	0.94±0.04	0.61±0.15	0.84±0.03
DFI-SCD	13.18±0.36	13.77±0.18	13.61±0.93	13.64±0.16
Protamine -CMA3+	28.43±0.87	27.97±0.43	29.21±2.09	28.11±0.38

Values expressed as Mean±SEM. Values in parenthesis represent the number of ART cycles.

a= IVF vs ICSI and IVF/ICSI; b=ICSI vs IVF/ICSI

*=P<0.05, **= P<0.01, ***=P<0.001

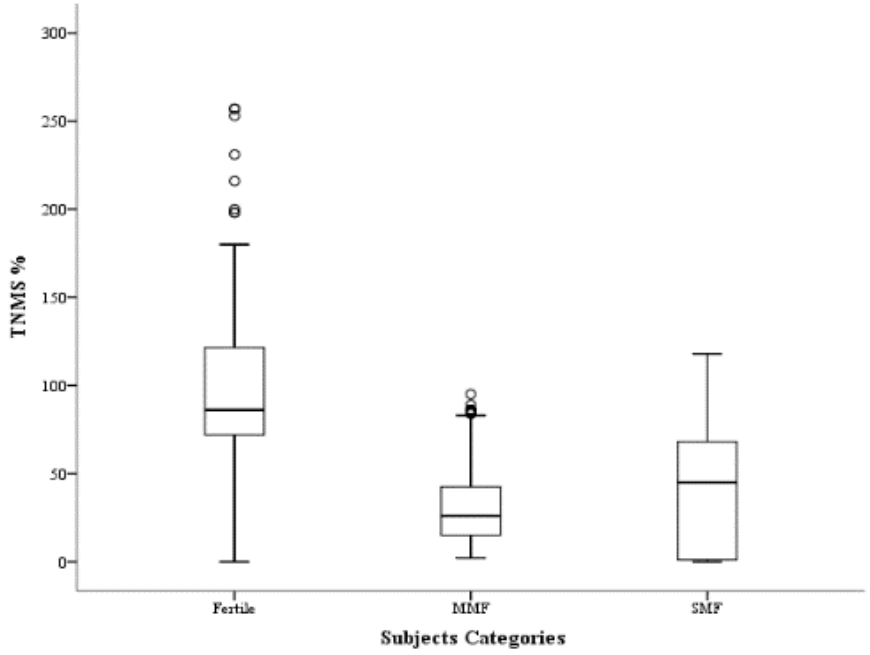
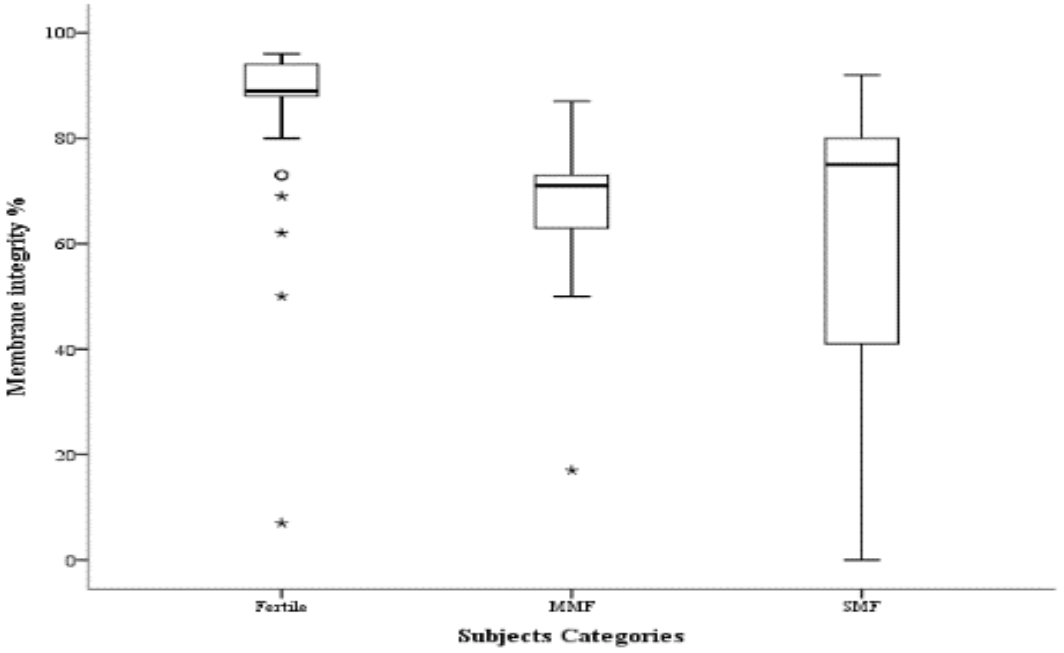
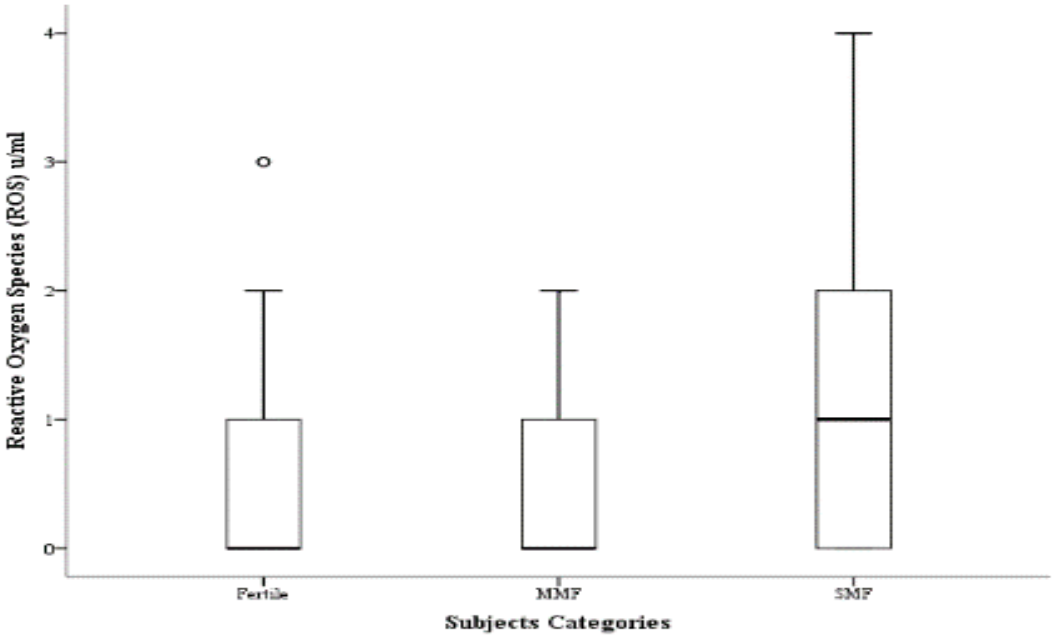


Figure 34. Prepared semen (total motile sperm) TNMS percentage in spermatozoa of male patients who underwent ART procedure based on fertile and subfertile categories

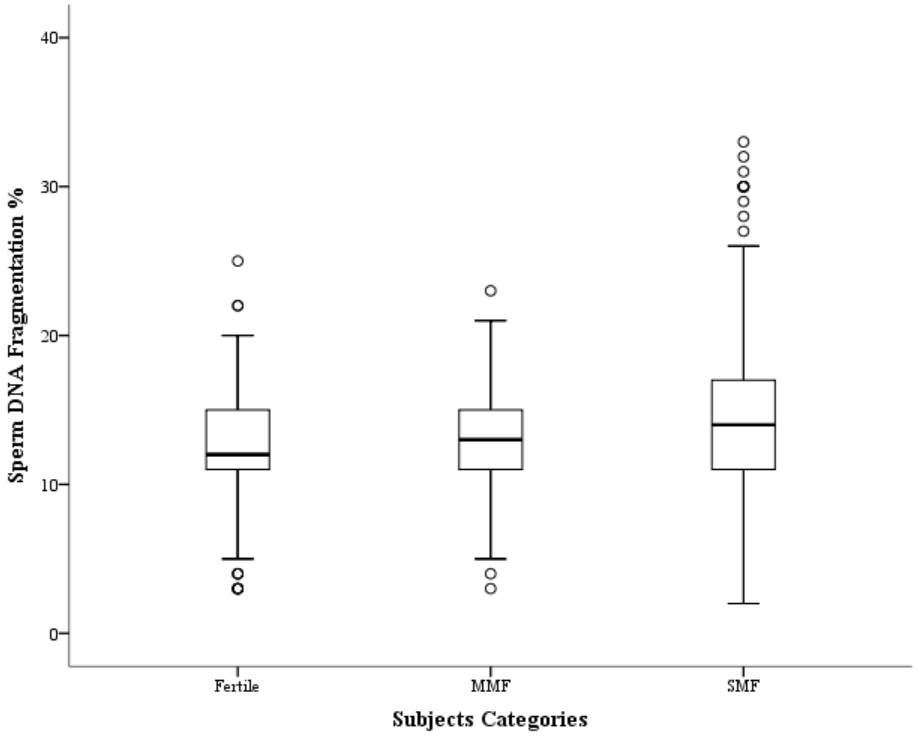


(a)

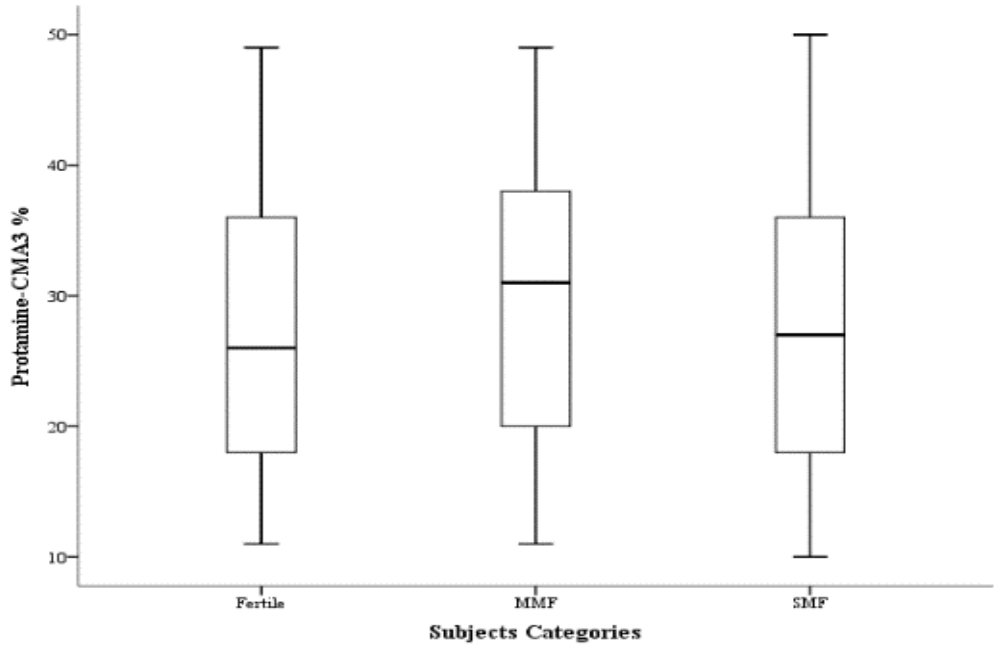


(b)

Figure 35. Post preparation (a) sperm membrane integrity (b) ROS percentage in spermatozoa of male patients who underwent ART procedure based on fertile and subfertile categories

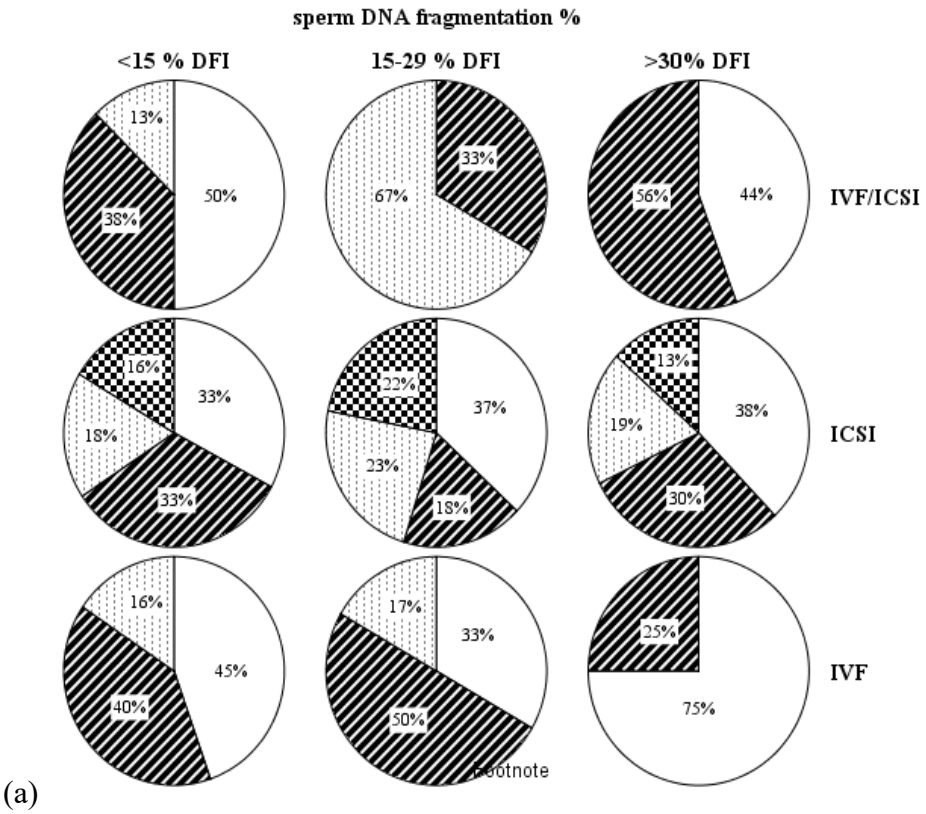


(a)

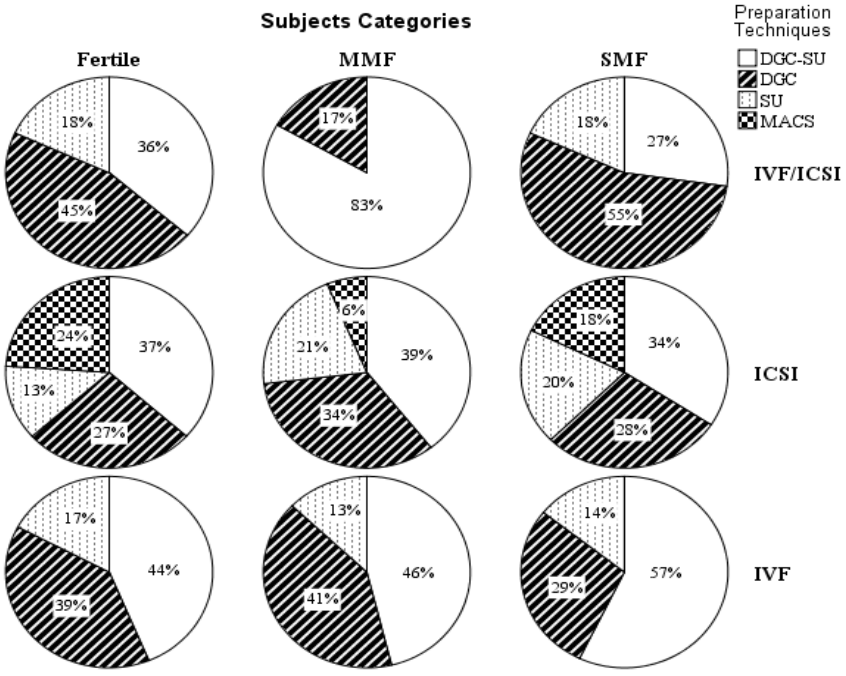


(b)

Figure 36. Post preparation (a) sperm DNA fragmentation (b) protamine (CMA3+) percentage in spermatozoa of male patients who underwent ART procedure based on fertile and subfertile categories



(a)



(b)

Figure 37. Percentage of patients grouped according to (a) Sperm DNA damage categories, (b) fertile and subfertile (MMF and SMF) categories Sperm preparation technique with IVF, ICSI and IVF/ICSI procedure

The fertile (NZs) control semen sample's conventional and quality parameters including TNMS, ROS, sperm DNA fragmentation, chromatin condensation and protamine deficiency were significant ($p<0.05$) different when compared to subfertile (MMF and SMF) categories.

Patient distribution:

The distribution of all participants into fertile and subfertile i.e., MMF and SMF categories and further distributed into groups according to the procedure used to fertilize oocytes, semen preparation techniques and sperm DNA fragmentation level (Figure 37a &b)

Assisted Conception Stimulation Cycle:

Couples were grouped into fertile and subfertile subject subgroups into moderate and severe malefactors that had a comparable mean number of oocytes collected, days of stimulation, total gonadotropin dose, estradiol and progesterone level on hCG trigger day (Table 26).

The details of categories based on insemination cycles of conventional *in-vitro* fertilization and intra-cytoplasmic single sperm insemination and sibling oocyte dividing into both insemination procedures are summarized in Table 27.

Table 26: Assisted conception cycle details of fertile and subfertile categories according to the procedure used to fertilize oocytes

	IVF (107)	ICSI (570)	IVF/ICSI (76)	Total (753)
Total number of Oocyte collected	1329	1655	3470	6454
Mean Oocyte collected	10.0±0.7	8.4±0.5	9.3±0.3	9.2±0.3
Total Gonadotropin dose	2498±128	2687±134	3241±310	2932±1.0
Stimulation days	15.6±0.3	15.0±0.2	15.0±0.2	15.1±0.1
Estradiol level on hCG day	2121±196	1920±138	2370±294	2195±164
Progesterone level on hCG day	194±16	234±18	213±26	216±14

Values expressed as Mean±SEM. Values in parentheses represent the number of an ART cycle.

a= IVF vs ICSI and IVF/ICSI; b=ICSI vs IVF/ICSI,*=P<0.05, **= P<0.01, ***=P<0.001

Table 27: Assisted conception cycle details of patients grouped according to the procedure used to fertilize oocytes

	IVF (107)	ICSI (570)	IVF/ICSI (76)	Total (753)
Total number of oocytes	1088	4993	373	6454
Mean oocyte collected	7.11±0.45	9.57±0.30	13.32±1.34	9.18±0.26
Total Gonadotropin dose	2308±125	3142±225	2965±334	2932±165
Stimulation days	15.76±0.20	14.89±0.15	16.04±0.79	15.14±0.12
Estradiol level on hCG day	1867±158	2282±219	2462±154	2195±1.64
Progesterone level on hCG day	227±14	213±20	178±34	216±14

Values expressed as Mean±SEM. Values in parentheses represent the number of an ART cycle.

a= IVF vs ICSI and IVF/ICSI; b=ICSI vs IVF/ICSI

*=P<0.05, **= P<0.01, ***=P<0.001

Insemination procedures influence on art outcome

Peri-fertilization effect

The percentage of degenerated egg cells was considerably higher after ICSI (10.7% vs. 4.3%; P=0.00), but this is not unreasonable given the nature of the process used to inseminate oocytes. The total of divided COCs (oocytes) was equivalent between the groups of ICSI versus traditional IVF groups (Table 28).

Embryo development effect

In the present investigation, it was revealed that the percentage of blastocysts among embryos that were cultured until day 5/6 was significantly higher in a traditional IVF group (53.47% vs. 42.65%; p = 0.00). Furthermore, the number of good-quality cleavage stage embryos (grade one) was similar (2.99±0.35 vs. 2.74±0.12; p >0.05). Interestingly, if the quality of the blastocysts were higher in the conventional IVF.

Implantation and pregnancy outcome

Even though there was a trend in favor of traditional IVF, statistically significant differences were not seen between the differences analysed cycles for the outcome of ETs in terms of clinical pregnancy rate (for day 3 and day 5 ETs combined). As shown in Table 28, it was also possible to compare the live birth rate per CPR between the treatment groups.

Clinical live birth rate and neonatal weight

181 couples were followed till delivery during the study time and there was no significant difference observed between neonatal weights in all insemination groups as described in Table 28 (i.e., the ICSI vs. conventional IVF and ICSI/IVF groups).

Table 28: The Findings of ART Cycles Including Number of Oocytes And Embryos, Based On according to the procedure used to fertilize oocytes

	IVF	ICSI	IVF/ICSI	Total
	6.81±0.53	7.68±0.23	10.4±7.2	7.66±0.21
Mature oocyte-MII	(107)	(570)	(76)	(753)
Mature oocyte %	98.14±0.75	91.90±0.74	88.21±3.08	92.82±0.61
Zygote-II PN	5.44±0.41	5.17±0.17	7.29±0.67	5.30±0.15
	(102)	(553)	(76)	(683)
	82.67±2.04	69.43±1.01	73.00±2.84	71.55±0.90
Fertilization rate/MII %	(102)	(553)	(28)	(683)
Total embryo on day 3	4.15±0.37	3.96±0.13	4.96±0.35	4.03±0.12
	(102)	(551)	(28)	(681)
Grade-I embryo	2.99±0.35	2.74±0.12	3.74±0.31	2.82±0.11
	(93)	(480)	(23)	(596)
Cleavage rate %	81.37±2.70	83.85±1.00	74.89±3.99	83.11±0.92
	(102)	(551)	(28)	(681)
Blastocyst rate %	52.47±2.22	42.65±1.66	36.57±4.66	43.15±1.46
	(19)	(171)	(14)	(204)
	3.02±0.08	3.09±0.06	3.77±0.25	3.11±0.05
Embryo Transfer day	(101)	(546)	(28)	(675)
Embryo Transfer (ET)	2.32±0.07	2.06±0.04	2.36±0.19	2.11±0.03
Clinical pregnancy rate	24.02±2.43	26.54±1.49	21.71±6.40	25.96±1.29
(CPR)/ET %	(101)	(546)	(28)	(675)
	40±6.67	30.61±2.95	40±16.33	32.58±2.67
Live birth rate/CPR %	(55)	(245)	(10)	(310)
Birth weight Gms	2388±380	2405±299	2225±266	2341±321 (181)

Values expressed as Mean±SEM. Values in parentheses represent number of ART cycle, a= IVF vs ICSI and IVF/ICSI; b=ICSI vs IVF/ICSI,*=P<0.05, **= P<0.01, ***=P<0.001

Sperm preparation techniques influence on art outcome

Peri-fertilization effect

The number of divided COCs (oocytes) was similar between the groups and the mature oocyte's number was not statistically different in all (i.e., the MACS vs DGC, SU, DGC-SU) techniques compared. There was a non-significant difference in percent mature oocytes MII between the groups. The fertilization rate was better in the MACS technique but did not reach to statistically significant difference and was comparable to all other techniques (Figure 38a).

Embryos development effect

The number of D3 embryos obtained and their grade was higher in MACS technique groups, and the same was in all cases in the percentage of blastocyst formation rate. The number of good-quality embryos on day three was similar ($p > 0.05$). There was no difference in the number of embryos transferred between the groups' as shown in Figure 38a&b.

Implantation and clinical pregnancy outcome

The outcome of ETs in terms of clinical pregnancies rate (for day 3 and day 5 ETs together) was significantly ($p<0.00$) higher in MACS when compared with the other semen preparation techniques used for treatments ($P > 0.05$). Live birth rate per CPR was also compared able between the treatment groups, statistically significant was not observed between differences analyzed cycles Figure 39a.

Cumulative live birth rate and Neonatal/Birth Weight:

Couples followed till delivery during the study time and there was no significant difference was observed between neonatal weights in all semen preparation groups (MACS vs DGC, SU, DGC-SU) as shown in Figure 39b.

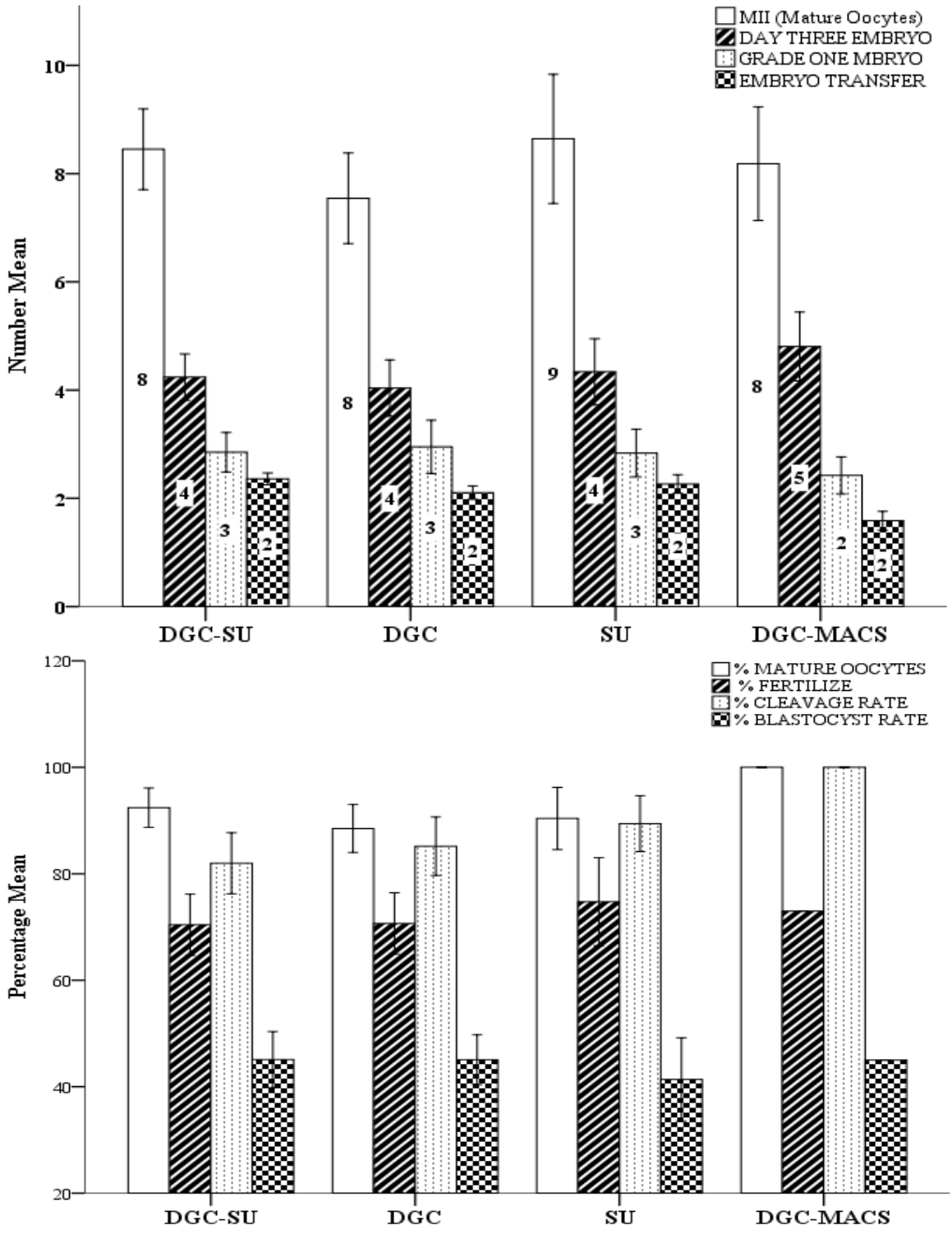
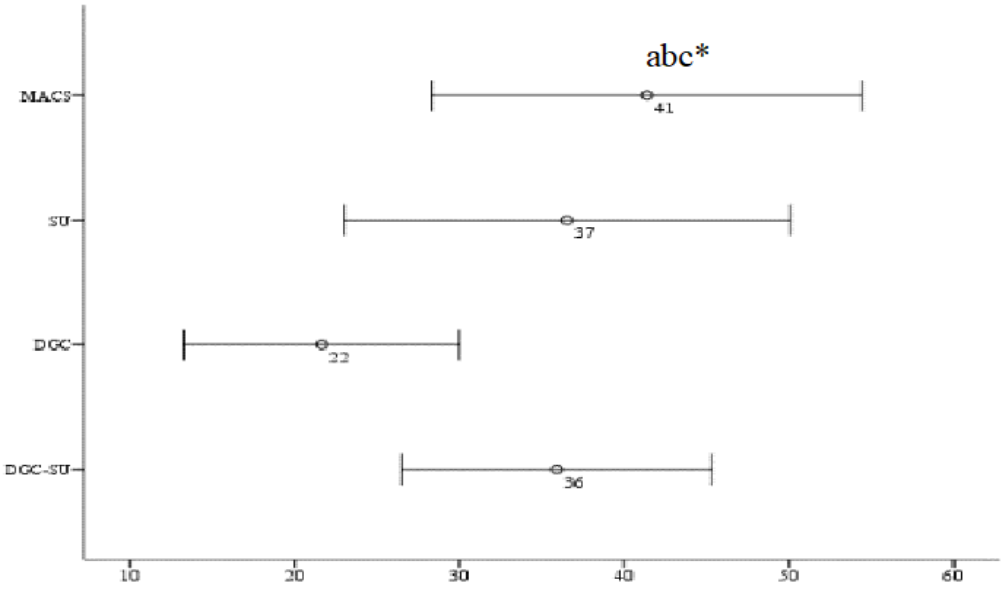
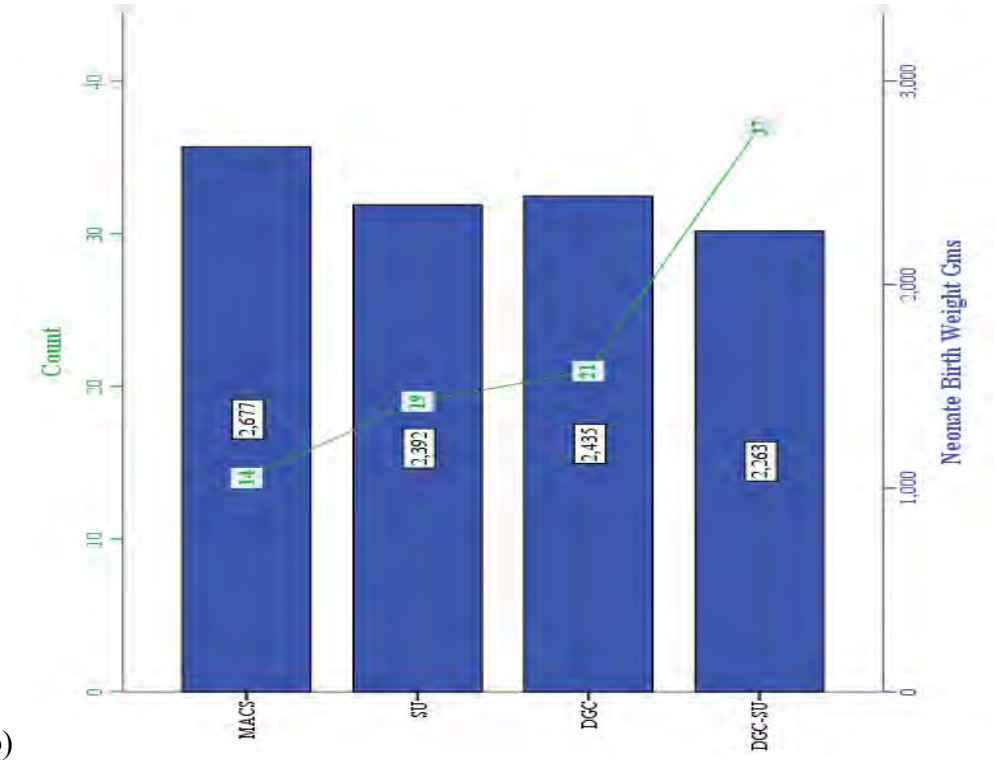


Figure 38. Influence of different sperm preparation techniques on assisted conception outcome (a) number of MII (mature Oocyte), number of day three embryos, number of grade 1 embryos and number of embryos transferred (b) percent mature oocyte, fertilization rate, cleavage rate and blastocyst rate.



(a)



(b)

Figure 39. Influence of different sperm preparation techniques on assisted conception outcome (a) clinical pregnancy rate (b) live birth rate and neonatal birth weight

a= SU vs DGC, DGC-SU, MACS

b= DGC vs DGC-SU, MACS

c= DGC-SU vs MACS

Sperm DNA fragmentation (DFI) influences on art outcome

Peri-fertilization effect

The number of divided COCs (oocytes) equally between the groups and the mature oocyte's number was not statistically different (i.e., the >15% DFI vs 15-30% DFI vs >30% DFI) groups. There was no significant difference in percent mature oocytes MII between the groups.

Fertilization rate was significantly ($p<0.05$) lower in >30% DFI when compared to the other two groups (>15% DFI and 15-30% DFI) (Figure 40).

Embryo developmental effect

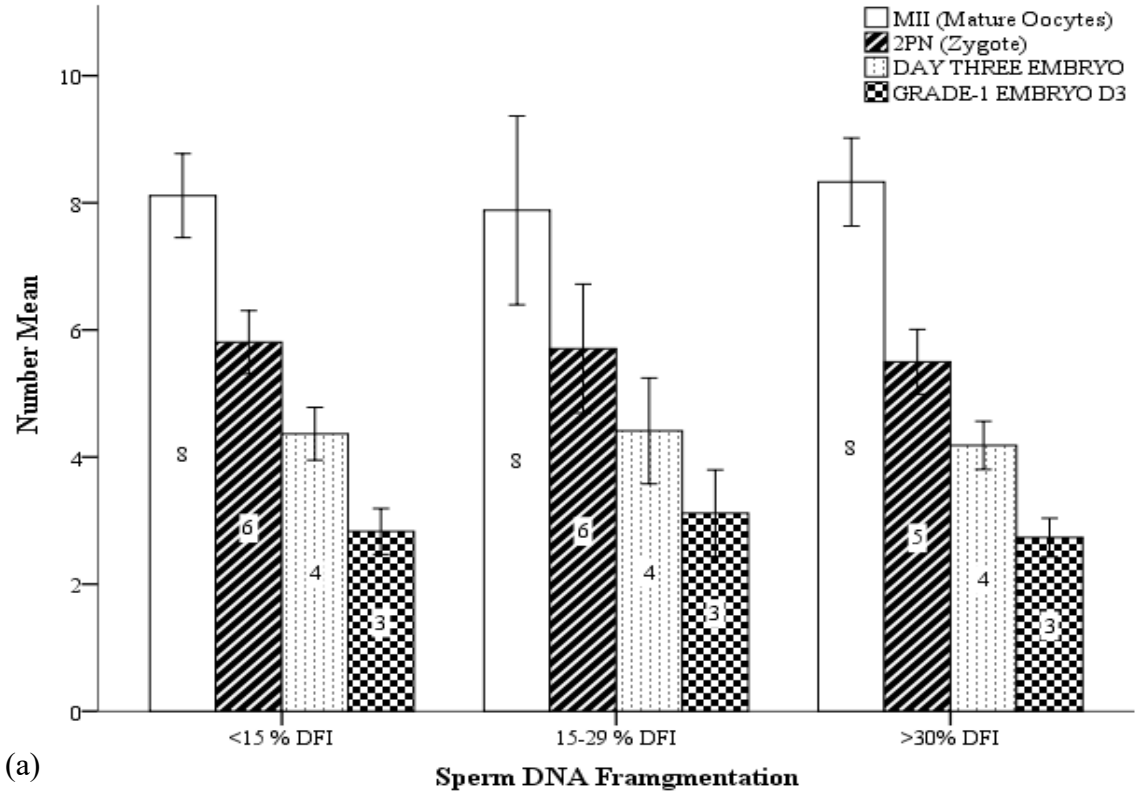
The number of cleaved embryos obtained and their quality were similar among groups, and the same was in the case of a blastocyst. The number of good-quality embryos on day three was similar ($p >0.05$). There was no difference in the number of embryos transferred between the groups (Figure 41).

Implantation and clinical pregnancy outcome

The outcome of ETs in terms of clinical pregnancy rate showed no difference between the treatments ($P >0.05$). Live birth rate per CPR was also compared able between the treatment groups, although there was a trend in favor of the >15% DFI, but statistically significant were not observed between differences in analyzed cycles (Figure 42a).

Cumulative live birth and Neonatal/Birth Weight

181 couples followed till delivery during the study time and there was no significant difference was observed between neonatal weights in all groups (i.e., >15% DFI vs 15-30% DFI vs >30% DFI) as shown in Figure 42b.



(a)

Figure 40. Assisted conception outcome parameters (includes number of MII (mature Oocyte), day three embryo, number of grade 1 embryo) in subjects grouped according to DFI categories

(b)

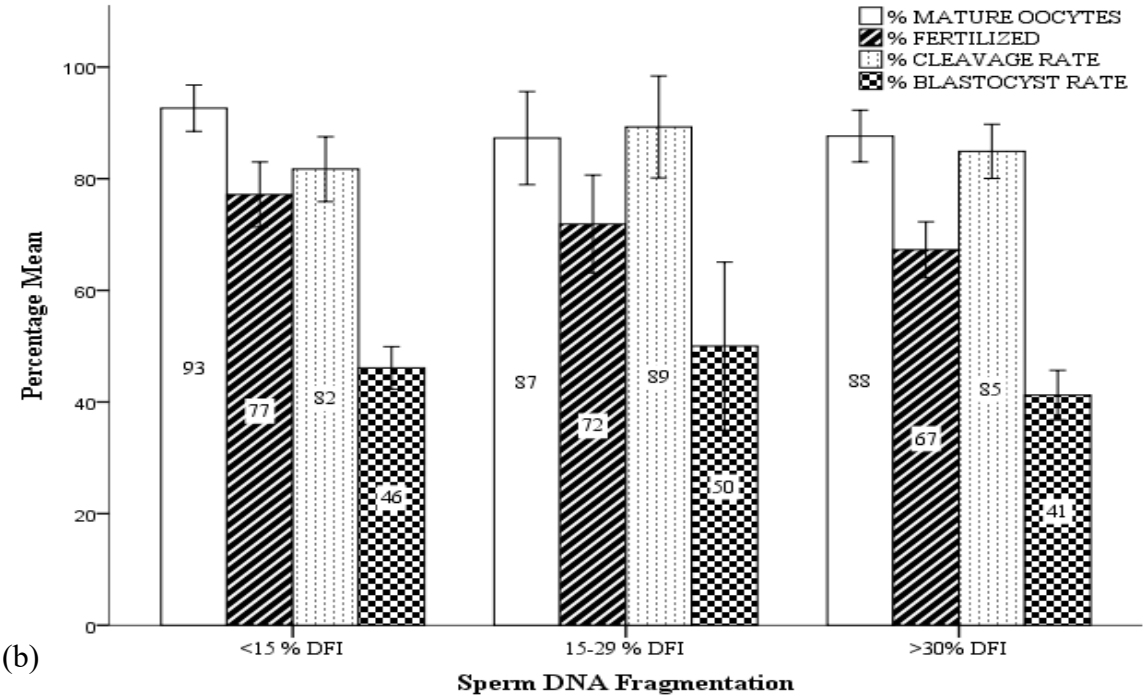
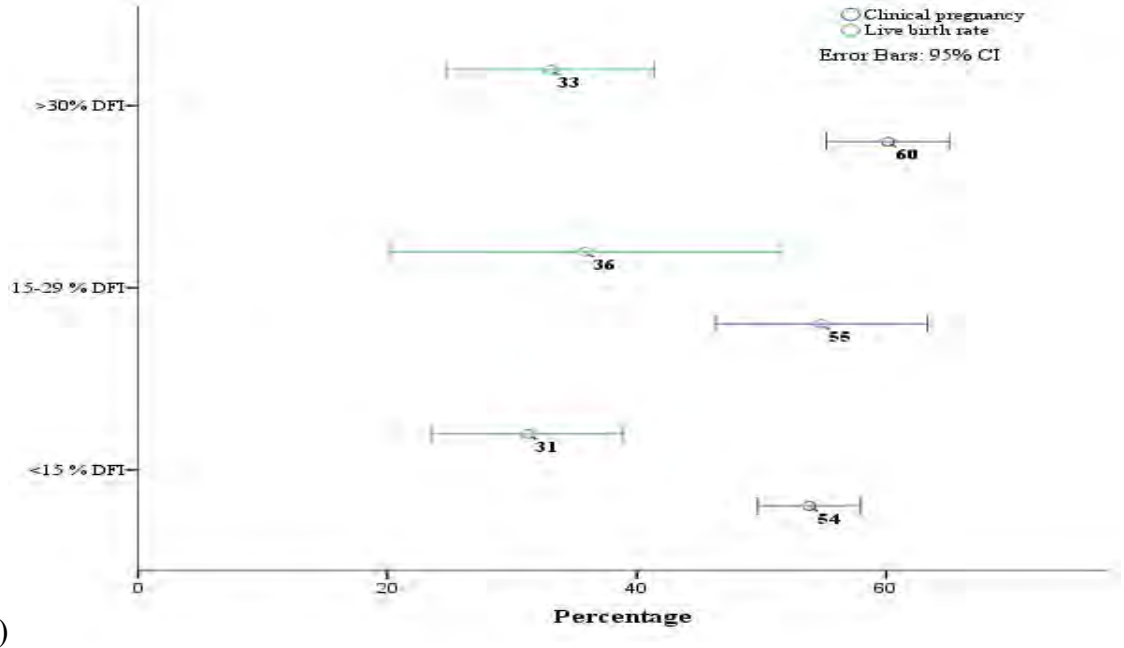
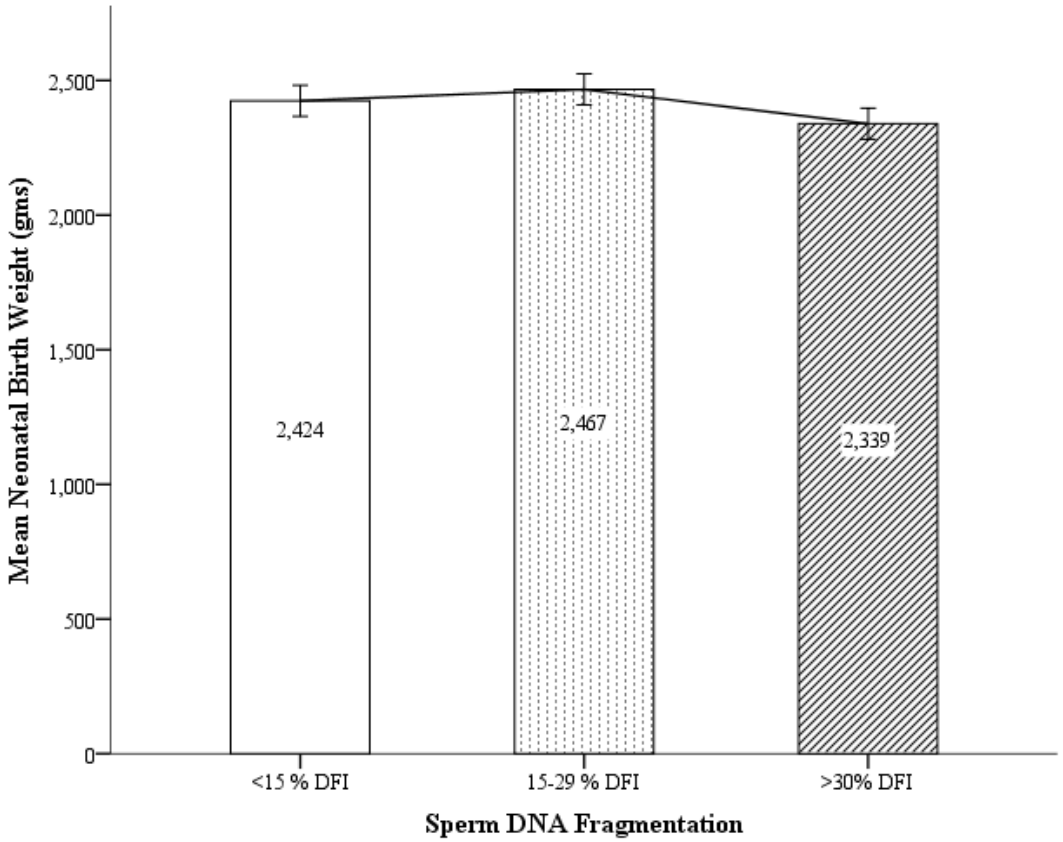


Figure 41. Assisted conception outcome parameters including percent mature oocyte, fertilization rate, cleavage rate and blastocyst rate in subjects grouped according to DFI categories.



(a)



(b)

Figure 42. Assisted conception outcome parameters according to DFI categories (a) clinical pregnancy and live birth rate (b) neonatal birth weight

Subfertility categories influence on art outcome

Peri-fertilization effect

The COCs (oocytes) were divided equally between the groups and the mature oocyte's number was not statistically different (i.e., the NZs vs MMF, SMF groups; 7.41±0.50 vs. 7.55±0.40 and 7.81±0.28). There was no significant difference in percent mature oocytes MII between the groups.

Table 29 showed Fertilization rate was significantly lower in SMF when compared to the other two groups (NZs and MMF).

Embryo development effect

The number of D3 embryos obtained and their quality was similar among groups, and the same was in the case of the blastocyst. The number of good-quality embryos on day three was similar ($p > 0.05$) (Table 29). There was no difference in the number of embryos transferred between the groups.

Implantation and clinical pregnancy outcome

The clinical pregnancies rate (for day 3 and day 5 ETs together) did not show a change between the treatments (34.82% vs. 22.94%, 24.47% $P < 0.05$). Live birth rate per CPR was also compared able between the treatment groups, although there was a trend in favor of the fertile subjects, statistical significance was not observed between differences in analyzed cycles (Table 29).

Cumulative live birth rate and Neonatal/Birth Weight

181 couples followed till delivery during the study time and there was no significant difference was observed between neonatal weights in all groups (i.e., the SMF vs. NZs and MMF groups) as shown in Table. 29.

Table 29: The outcome of the ICSI/IVF cycles in terms of oocytes and embryos according to sperm parameters.

	Control (NZs)	MMF (OZs, AZs, OAZs)	SMF (TZs, ATZs, OATZs)	Total (753)
Mature oocyte-MII	7.41±0.50 (134)	7.55±0.40 (244)	7.81±0.28 (375)	7.66±0.21 (753)
Mature oocyte %	91.71±1.4	94.98±0.98	91.95±0.92	92.82±0.6
TWO PN	5.86±0.40 (128)	5.32±0.28 (189)	5.09±0.21 (366)	5.30±0.15 (683)
Fertilization rate %	79.03±1.7	73.12±1.67	68.13±1.28	71.55±0.9
Total embryo on day 3	4.54±0.33 (127)	3.96±0.23 (188)	3.88±0.16 (681)	4.03±0.12 (681)
Grade one embryo	3.35±0.36 (115)	2.74±0.20 (168)	2.67±0.13 (313)	2.82±0.11 (596)
Cleavage rate %	83.06±2.2 (127)	81.93±1.84 (188)	83.73±1.23 (681)	83.11±0.9 (681)
Blastocyst rate %	47.46±3.3 (41)	43.88±2.70 (41)	41.45±1.98 (122)	43.15±1.5 (204)
Embryo Transfer day	3.15±0.10 (89)	3.00±0.09 (159)	3.17±0.08 (236)	3.11±0.05 (484)
Embryo Transfer	2.05±0.08	2.20±0.06	2.09±0.04	2.11±0.03
Clinical pregnancy rate %	34.82±3.2 (125)	22.94±2.16 (188)	24.47±1.79 (362)	25.96±1.3 (675)
Live birth rate %	34.25±5.6	29.07±4.93	33.77±3.86	32.58±2.7

Values represent Mean±SEM. Values in parentheses represent the number of ART cycles. a= Fertile vs MMF and SMF; b=MMF vs SMF,*=P<0.05, **= P<0.01, ***=P<0.001

Male factor subfertility influences Sperm DNA fragmentation and ART cycle outcome

The results showed that there is no significant difference in mean male age and female age, among the patients, included in this study. The means of sperm concentration, progressive motility, immotile, normal sperm morphology, vitality and ROS level of all investigated patients were comparable ($p>0.05$) between IVF and ICSI treatments. Similarly, the mean percentage of sperm DNA damage by SCD (22±0.7, 22±0.76) and SCSA levels (24±0.8, 24±0.8) were comparable ($p>0.05$) (not shown in table) in patients undergoing ICSI and IVF treatment. While N control subjects had significantly lower SCD and SCSA levels compared to MMF ($p<0.05$) and SMF ($p<0.01$) in both IVF and ICSI treatment groups Table 30. Spermatozoa DNA damage measured by SCD and SCSA was significantly correlated ($r=.793$, $p=0.0001$).

The three male factor subfertile groups were homogeneous in mean stimulation days, gonadotropins dose, and level of estradiol and progesterone on HCG day (data not shown). The control N subject had a fertilization rate significantly higher after ICSI ($p=0.04$) and IVF ($p=0.001$) compared to SMF groups while the percent fertilization rate in MMF was lower ($p<0.05$) compared to the N control group after ICSI. The live birth rate was not statistically significant but higher in the control (N) group after the IVF cycle (Table 30). Correlations between the percentage of DNA damage measured with SCD and SCSA with ART outcome are reported in Table 31. A negative correlation was found between SCD ($r=-0.199$, $p=0.001$, $n=290$) and SCSA ($r=-0.193$, $p=0.002$, $n=290$) with fertilization rate after IVF. SCD correlate ($r=-0.112$, $p=0.02$ $n= 570$) negatively with clinical pregnancy rate after ICSI. SCD or SCSA, no correlation was observed with other ART outcomes Table 31. SCD value to predict clinical pregnancy rate ROC analysis was performed, at a threshold of 20%, SCD predicted to attain positive pregnancy. The clinical pregnancy rate after ICSI was significantly reduced in MMF and SMF compared to N (Figure 43). We found no correlation between SCD and SCSA and pre-implantation genetic testing for aneuploidy, including euploidy, haploidy, polyploidy, monosomy, trisomy, haploidy, polyploidy and gnonosomal aneuploidy (Table 31). Applying linear logistic regression we

found that SCD (data not shown) or SCSA did not predict the euploid embryo in all-male categories after IVF and ICSI.

Correlation between Chromatin maturity and ART outcome

Sperm chromatin immaturity revealed by CMA3+ and TB+ in the ICSI treatment group were 28± 0.5 and 27±0.7, $p<0.05$, while in the IVF treatment group were 29± 0.6 and 29±0.8, $p>0.05$ (not shown in table). However, N control men had a significantly ($p<0.05$) lower mean percentage of CMA3+ and TB+ levels compared to MMF and SMF subjects in both IVF and ICSI treatment groups. Spermatozoa DNA damage measured by CMA3+ and TB+ were significantly correlated ($r=0.709$, $p=0.0001$, $n=750$). TB+ levels were significantly ($p<0.05$) higher in SMF after ICSI and IVF (41±0.7, 41±0.7) compared to N (18±0.6, 20±1.3) and MMF (19±0.7, 19±0.8) subjects. Mean percent CMA3+ levels were significantly ($p<0.05$) higher in both ICSI and IVF in subjects with MMF (24 ±0.6, 23 ±0.7) and SMF (36±0.5, 38±0.5) when compared to normal control subjects (18±0.6, 19±1.1).

Fertilization rate ($r=-.191$, $p=0.002$, $n=183$) after IVF including IVF-ICSI and clinical pregnancy rate ($r=-.129$, $p=0.02$, $n=570$) after ICSI correlated negatively with CMA3+. No correlation was observed with other ART outcomes, including live birth rate as illustrated in Table 31. A ROC curve analysis was performed to determine the threshold of CMA3+ and fertilization rate and we found 30% CMA3+ specificity (Figure 45). We found lower fertilization after IVF in SMF and MMF compared to N control when chromatin maturity was high (CMA3+ higher than 30%) (Figure 44). While no correlation was found between aneuploidy and sperm chromatin maturity (CMA3+, TB+) (Table 32).

Table 30: Summary of ART cycle outcomes

	ICSI				IVF (IVF/ICSI)			
Variables	N	MMF	SMF	p-Value	N	MMF	SMF	p-Value
No of cycle	49	136	275	570	100	70	120	183
Mean MII	8.56±1.1	8.1±0.6	9±0.5	0.4	9.9±0.8	7.6±0.5	9±0.6	0.4
Fertilization rate (%)	78±4.5	**72±2.3***	**62±2.2***	**0.001**	74±2.9	74±2.1	**68±2.2***	0.04
Cleavage rate %	85±3.1	82±2.2	84±1.4	0.65	82±2.8	83±3	81±2.3	089
Embryo A quality	3.3±0.5	2.6±0.24	2.7±0.16	0.38	3.3±0.4	3±0.7	**2.1±0.16***	0.01
Blastocyst formation rate%	52±6.5	48±5.3	40±3.3	0.23	35±6.4	35.5±6.5	33±3.4	0.99
Euploid rate %	65±5.1	56±3.3	59±2.6	0.46	64±4.3	60±3.9	54±3.8	0.54
LBR (%)	46±0.5	38±0.3	36±0.2	0.27	36±0.1	40±0.04	34±0.04	0.65

Values represent Mean±SEM; ICSI, Intracytoplasmic sperm insemination; IVF, in-vitro fertilization; N, normozoospermic; MMF, Moderate Male Factor; SMF, Sever Male Factor, MII; metaphase II oocyte, LBR; live birth rate. P<0.001 ***, P<0.01**, P<0.05*

Table 31: Correlation between sperm parameters and sperm chromatin tests after IVF /ICSI cycle with clinical outcomes in the studied group

		ICSI				IVF (IVF/ICSI)			
	SC	**SCSA**	**SCD**	**CMA3+**	**TB+**	**SCSA**	**SCD**	**CMA3+**	**TB+**
FR	r=	-.023	-.078	-.027	-.056	**-.193****	**-.199****	**-.191****	**-.238****
	p=	.671	.153	.624	.304	**.002**	**.001**	**.002**	**.000**
	n=	570	570	570	570	183	183	183	183
CR	r=	.032	.073	.042	.009	.039	.018	-.117	-.009
	p=	.511	.138	.398	.860	.530	.773	.060	.888
	n=	416	416	416	416	262	262	262	262
EQ_A	r=	-.071	-.029	.112	.075	.075	.053	-.106	-.107
	p=	.173	.583	.060	.152	.267	.431	.112	.112
	n=	379	379	379	379	224	224	224	224
BR	r=	.049	-.105	-.122	.014	.141	.152	.124	-.172
	p=	.663	.349	.275	.899	.387	.348	.445	.288
	n=	182	182	182	182	140	140	140	140
Euploidy	r=	-.014	-.100	-.023	-.017	.066	-.016	.077	.062
embryo %	p=	.795	.068	.677	.752	.295	.803	.219	.325
	n=	254	254	254	254	231	231	231	231
	r=	-.081	**-.112***	**-.129***	-.073	-.074	-.082	-.011	-.071
CPR	p=	.116	**.028**	**.012**	.157	.208	.167	.854	.230
	n=	370	370	370	370	243	243	243	243
	r=	-.079	-.039	-.007	-.024	-.067	-.031	-.081	-.112
LBR	p=	.108	.426	.883	.631	.296	.634	.206	.080
	n=	369	369	369	369	242	242	242	242

FR; fertilization rate, CR; cleavage rate, EQ_A; Embryo A quality, BR; blastulation rate, CRP; cumulative pregnancy rate. P<0.001 ***, P<0.01**, P<0.05*

Table 32: Pre-implantation genetic screening (PGS) analysis of embryos in different categories of subfertile male patients.

	ICSI					IVF				
PGT-A	N 38	MMF 99	SMF 117	Total 254	p-Value	N 73	MMF 31	SMF 127	Total 231	p-Value
Aneuploidy %	49.1	61.7	60.5	59.3	0.15	62.03	55.3	56.3	57.3	0.43
Gonosomal aneuploidy %	15.6	18.06	11.4	14.67	0.45	22.8	17.06	14.21	16.67	0.55
Monosomies %	15.6	24.7	20.1	21.07	0.46	19.6	17.4	21.7	19.91	0.71
Trisomies (%)	20.25	23.94	21	21.98	0.89	32.5	20.37	18.8	21.74	0.34
Haploidy and polyploidy (%)	7.5	12.06	9.1	9.9	0.67	9.9	10.84	9.04	9.79	0.89
Normal XY	21.8	14.59	12.75	15.07	0.37	10.9	18.8	15.89	15.89	0.44
Normal XX	21.25	24.76	21.65	27.2	0.36	21.25	24.76	21.65	27.2	0.84
Complex abnormalities (%)	12.8	10	18.3	14.2	0.54	10.8	6.11	16.96	12.26	0.23
No information	15.7	18.5	16.8	17.27	0.9	17	14.16	19.43	16.91	0.39

Values represented as Mean percentage, ICSI, Intracytoplasmic sperm insemination; IVF, in-vitro fertilization; N, normozoospermic; MMF, Moderate Male Factor; SMF, Sever Male Factor. P<0.001 ***, P<0.01**, P<0.05*

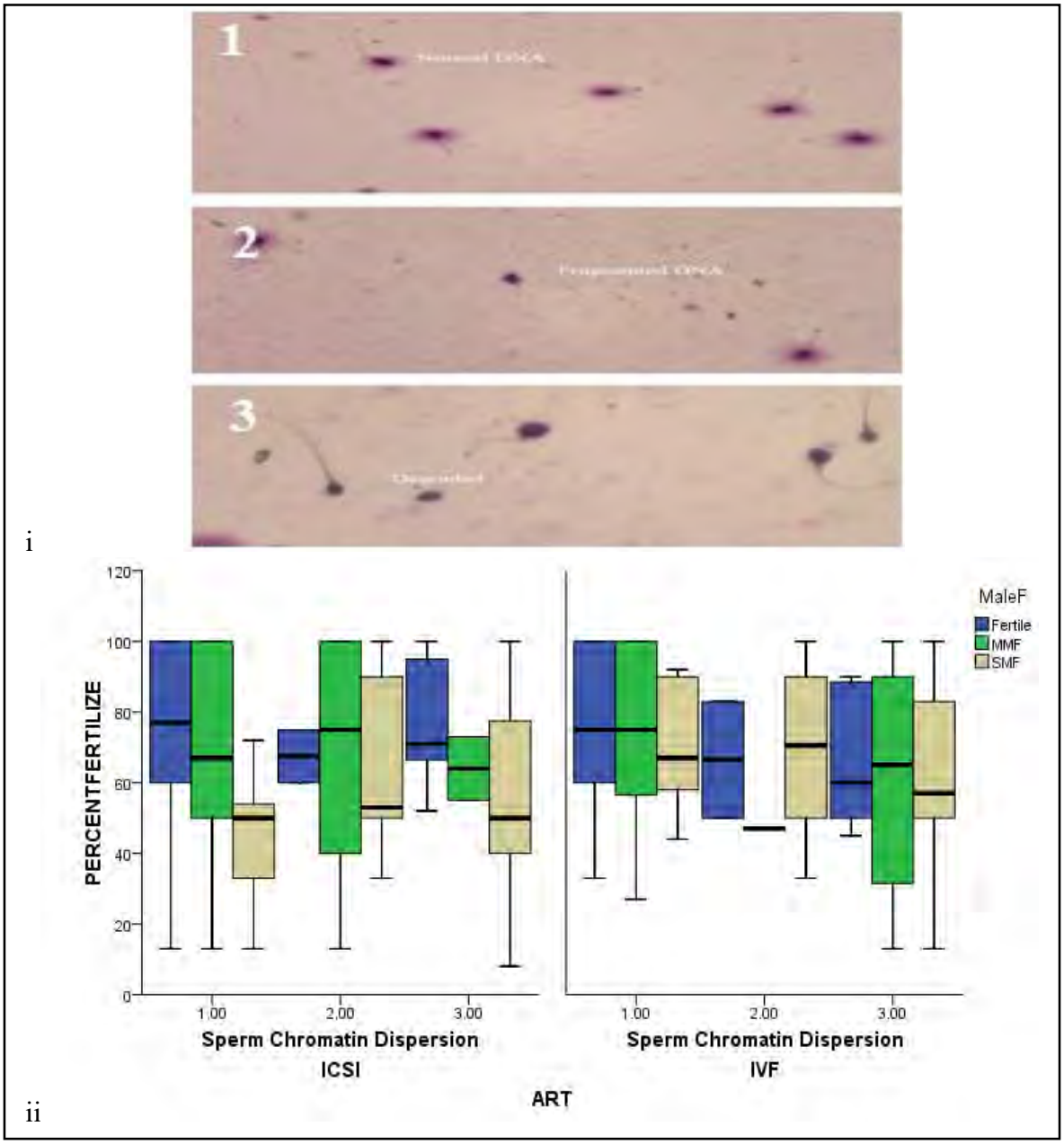
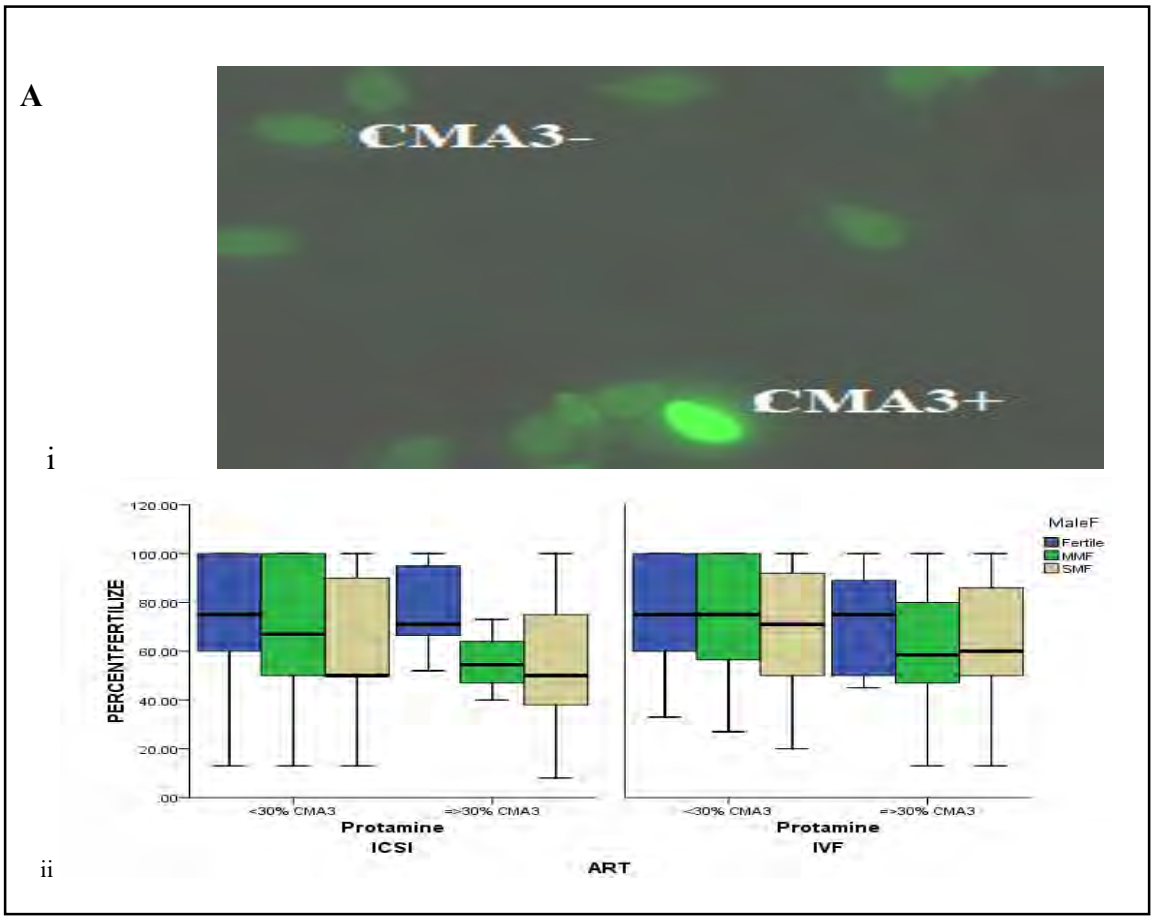


Figure 43. (i) Sperm assessed by Halosperm test (image taken from the bright field microscope). 1; Sperm without DNA fragmentation, sperm with big halo and sperm with medium halo, 2; Sperm with DNA fragmentation: sperm with small halo, and 3; sperm without halo and degraded sperm. (ii) Cluster box plot showing a decrease in fertilization rate with an increase in DNA damage (1=<15% SCD, 2=15-29% SCD, and 3= >30% SCD) in sperm of male partner in male factor subfertile couples, undergoing IVF and ICSI. Values are mean, n=613. N; normozoospermic, MMF; Moderate Male Factor, SMF; Sever Male Factor. P<0.001 ***, P<0.01**, P<0.05*, P<0.001 ***.



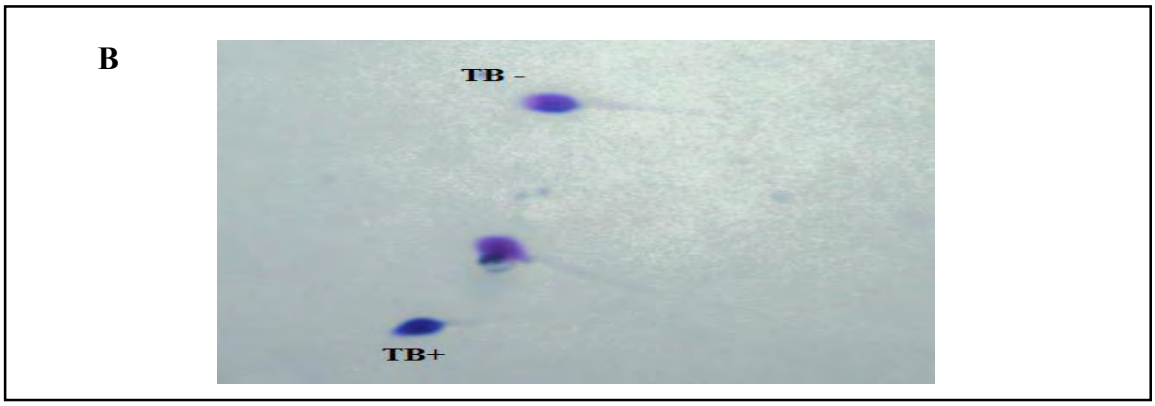


Figure 44. A. (i) Illustrative human sperm cells stained with CMA3, Fluorescent dull green CMA3- Normal condensed sperm chromatin; whereas fluorescent bright green CMA3+ non-condensed sperm chromatin (CMA$_3$ positive) (2) (GrX100). (ii) Cluster Box plot showing a decrease in fertilization rate with an increase in chromatin immaturity in sperm for male factor subfertile couples, undergoing IVF and ICSI. Values are mean, n=750. B. Toluidine blue staining method for sperm chromatin evaluation. Light blue sperm heads show normal chromatin and dark blue sperm heads show damaged chromatin PFR; Percent fertilization rate, AUC; Area under the curve; CI; confidence interval, N; normozoospermic, MMF; Moderate Male Factor, SMF; Sever Male Factor. P<0.001 ***, P<0.01**, P<0.05*, P<0.001 ***.

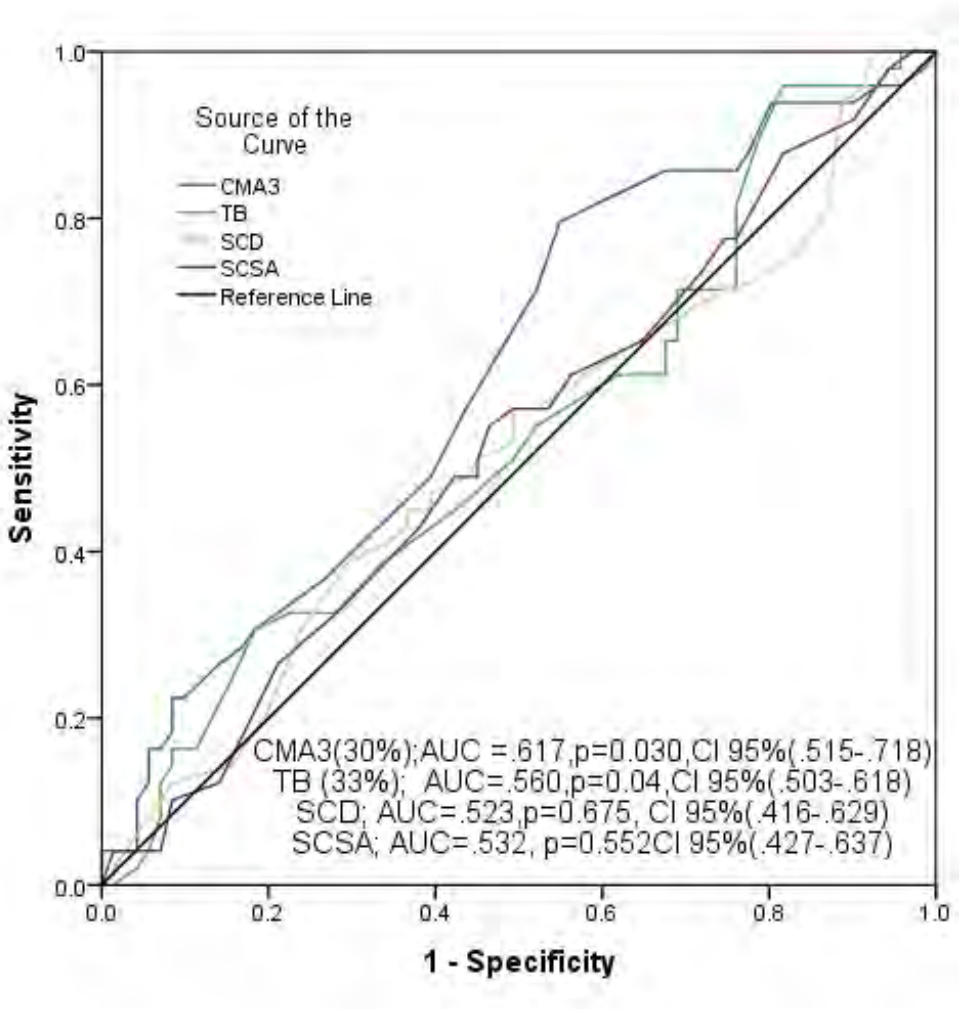


Figure 45. Receiver operating characteristic curve of the probability of model of successful pregnancy outcome in SMF couples after ART treatment. AUC: area under the ROC curve; CI: confidence interval

Sperm chromatin integrity influences clinical pregnancy outcome:

Sperm chromatin integrity and condensation correlate with ART outcome. Subfertile men who did not achieve pregnancy with ART had sperm chromatin dispersion-SCD percentage of 32.85±0.54 and sperm chromatin structure-SCSA was 35.06±0.55% with significant ($p<0.05$ and $p<0.002$) higher level than in those with successful implantation (SCD, 30.7±0.8 and SCSA, 32.2±0.9) (data not shown in table). Comparing the chromatin condensation (CMA3+) percentage in subfertile men who attained pregnancy did not reach a statistically significant level from those with implantation failure, although there was a trend in favor of sperm with lesser protamination deficiency-CMA3 (chromatin condensation) Figure 46. There was a better sperm percentage of chromatin condensation and chromatin integrity in men undergoing IVF treatment and those with normal semen conventional parameters. ICSI treatment cycle men had a higher percentage of sperm with deficient sperm chromatin quality. Men with better chromatin quality with a higher number of COC and a higher percentage of metaphase II, the sibling oocytes were split into ICSI and IVF clinical pregnancy rate was compared with other insemination treatment groups of IVF and ICSI.

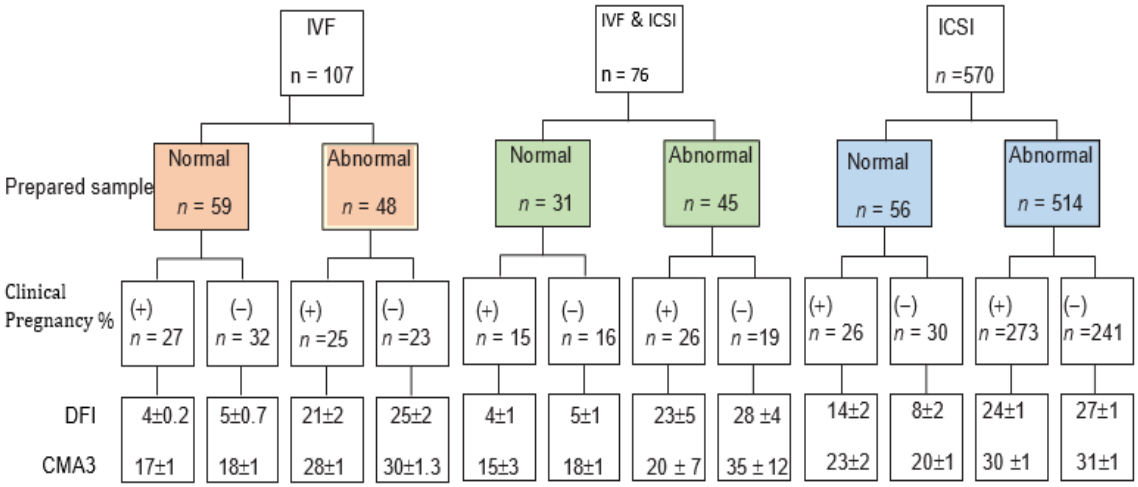


Figure 46. The study's flow diagram displays the number of subjects in each group and the results of clinical pregnancies. Values represent; n=number, percentage of DNA fragmentation, Reactive oxygen species, total motile sperm and chromatin condensation expressed as mean±SEM. IVF, *in vitro* fertilization; ICSI, intra-cytoplasmic sperm injection; IVF & ICSI.

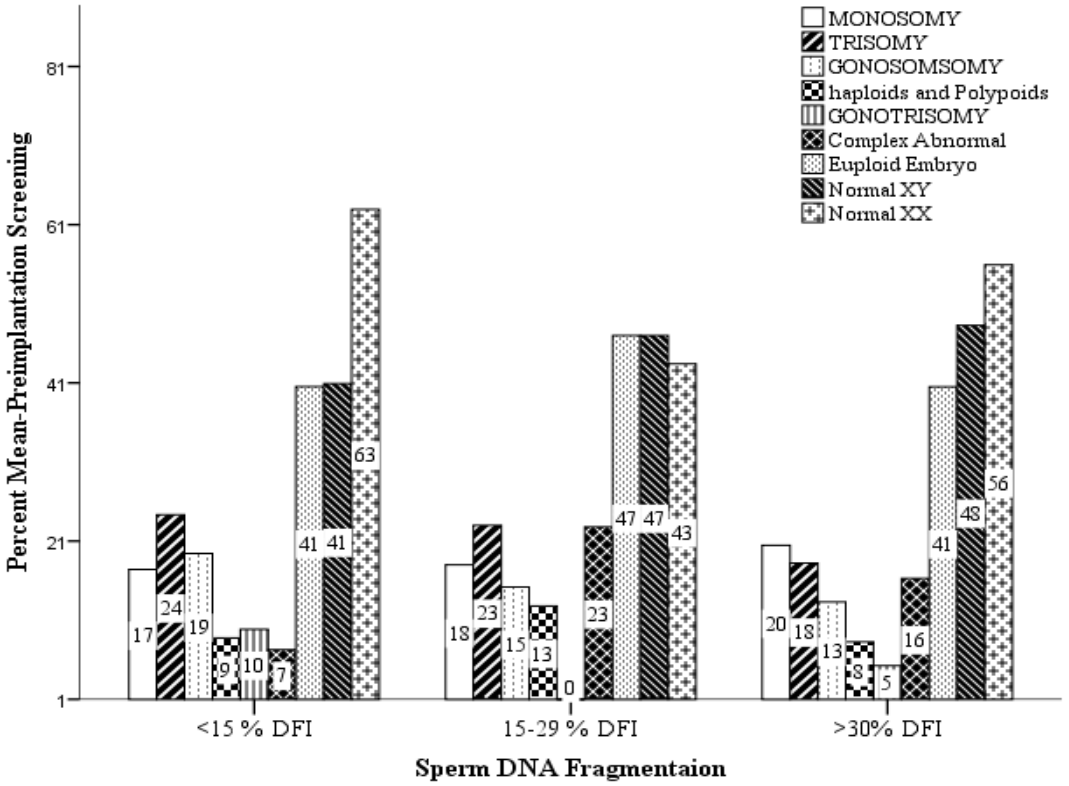
PRE-IMPLANTATION GENETIC TESTING FOR ANEUPLOIDY (PGT-A)

Sperm DNA fragmentation index (DFI) effect on PGT-A:

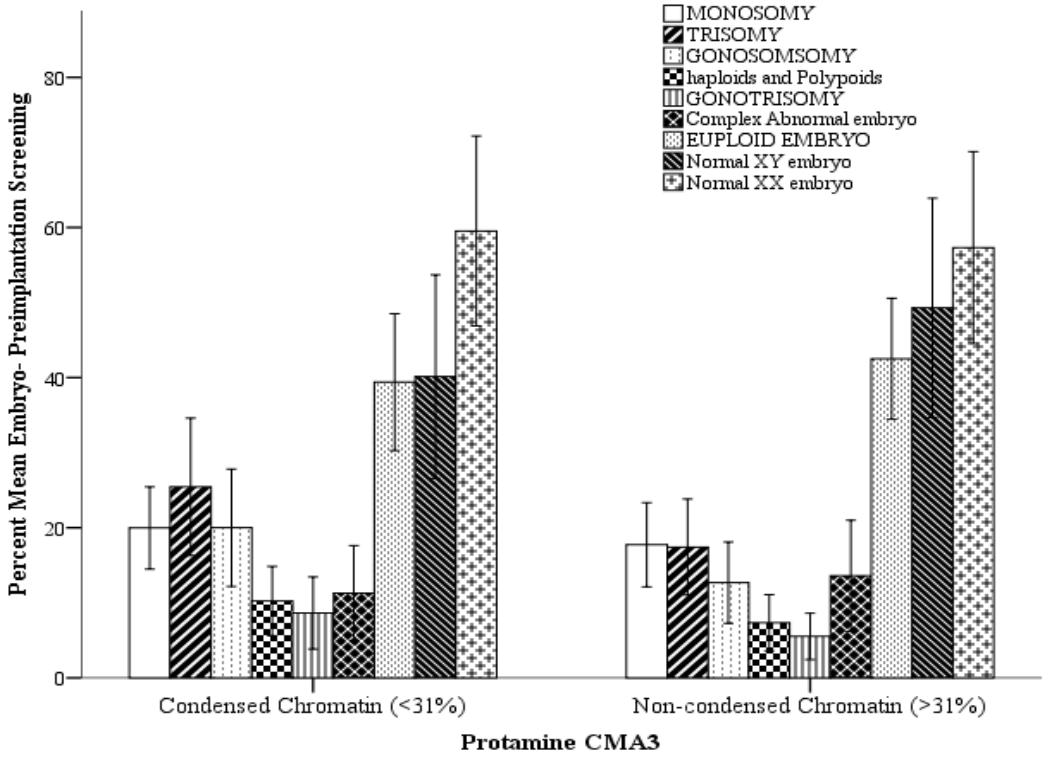
Sperm DNA fragmentation index (DFI) is categorized into three categories (<15% DFI, 15-30% DFI, >30% DFI) and there was no significant difference in euploid, aneuploid, chromosomal haploid, polyploid, monosomy, gonosomy, complex abnormal, normal XX and XY embryos number in all categories Fig 47a.

Sperm Protamination (CMA3+) effect on PGT-A:

Euploid embryos number were compared able from subfertile men categories based on sperm protamine deficit (CMA3+). Chromosomal haploid, polyploid, monosomy, gonosomy, complex abnormal, normal XX and XY embryos number were similar between condense chromatin and non-condensed chromatin sperm men Figure 47b.



(a)



(b)

Figure 47. Effect of Sperm chromatin quality on embryos with pre-implantation genetic screening for Aneuploid (PGT-A) (a) Sperm DNA fragmentation Index-DFI and (b) chromatin condensation-CMA3+

Regression Analyses: Embryos Euploid:

Binary logistic regression analyses were conducted to identify the effect of TNMS (p=0.00) and sperm chromatin quality marker AO (p=0.04), SCD (p=0.01), SCSA (p=0.00) and CMA3+ (p=0.01) on euploid embryos while there is no effect of TB, sperm membrane integrity- Hos and sperm vitality on the percentage of Euploid embryos in normozoospermic (NZs) men. While MMF and SMF regression analyses were conducted no effect had been identified in TNMS, sperm membrane integrity-Hos, sperm vitality and sperm chromatin quality marker (AO, SCD, SCSA, TB+ and CMA3+) on euploid embryos as shown in Table 33.

Table 33: Regression analysis to identify the effect of sperm chromatin quality parameters on euploid embryos in control and MMF and SMF

NZs (Control)	**AOR (95% C.I Lower-Upper), p-Value**
TNMS %	1.02(1.01-1.03), **0.00**
Membrane integrity (Hos) %	0.90(0.78-1.03), 0.12
Sperm vitality (Eosin) %	1.02(0.93-1.12), 0.64
Acridine orange test-AO	0.78(0.61-0.98), **0.04**
Sperm chromatin dispersion-SCD	0.83(0.72-0.96), **0.01**
Sperm chromatin structure-SCSA	1.38(1.15-1.66), **0.00**
Chromatin Condensation-TB	0.91(0.82-1.02), 0.10
Protamine deficiency-CMA3+	1.13(1.04-1.24), **0.01**
MMF & SMF	**AOR (95% C.I Lower-Upper), p-Value**
TNMS %	1.00(0.99-1.01), 0.89
Membrane integrity (Hos) %	1.00(0.98-1.02), 0.92
Sperm vitality (Eosin) %	1.00(0.98-1.03), 0.93
Acridine orange test-AO	0.93(0.85-1.01), 0.08
Sperm chromatin dispersion-SCD	0.98(0.93-1.04), 0.48
Sperm chromatin structure-SCSA	1.03(0.97-1.09), 0.28
Chromatin Condensation-TB	1.07(0.98-1.16), 0.12
Protamine deficiency-CMA3+	1.00(0.97-1.04), 0.84

Paternal BMI and assisted conception outcome

Fertilization was achieved by conventional IVF (n=107) or ICSI (n=570) and IVF+ICSI (n=76). A total of 1416 oocytes were fertilized using IVF and 3816 oocytes were fertilized by ICSI. Of all enrolled 1063 couples, subfertility information was available who underwent their first ovarian stimulations. The percentage of patients under IVF was normal weight male (24.1%), overweight men (17.6%), while for ICSI was normal weight male (23.2%), Overweight men (34.4%). 750 men there was at least one embryo available for transfer after ART treatment (Figure. 33), thus 21% were considered lost to follow-up. No significant differences were seen in the general characteristics of men who were included in the study as compared to those who were excluded or lost to follow-up.

Demographic parameters

The mean paternal age of the studied subjects was 38.8±0.25 and paternal BMI was 25.6±0.1 kg/m^2 (Table 34), the average weight (kg) and height (cm) were significantly higher in the overweight men group ($p<0.001$). There was no significant difference in demographic parameters analyzed among the normal BMI <24.5 kg/m^2 and overweight BMI ≥24.5 kg/m^2 patient groups.

Paternal BMI and Semen standard parameters

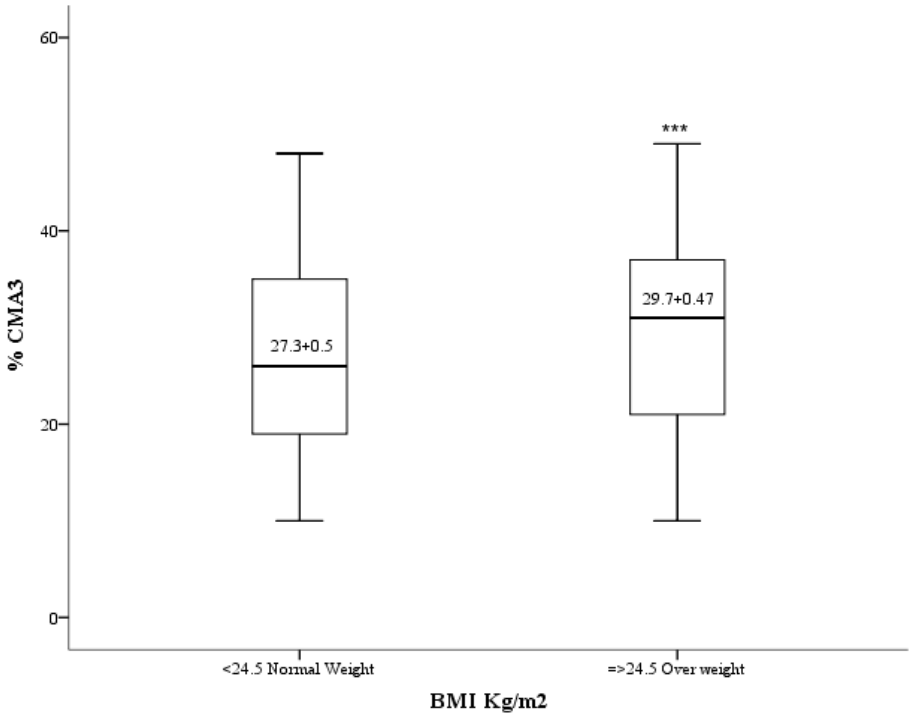
The mean semen volume was 3.69±0.06ml. The mean liquefication time was 31.56±0.39 minutes, WBC was 4.52±0.29 per HPF and pH was 7.97±0.01. The mean sperm count of the subjects was 62.01±2.23 x10^6/ml. The total motile sperm (TNMS) mean was different between the group and overweight men had a significantly ($p=0.002$) lower number of TNMS compared to normal-weight men (Table 35). The mean of morphologically normal sperms was 3.5±0.65% in the studied male population. The mean terato-zoospermic index (TZI) was 1.81±0.2 and the Sperm deformity index (SDI) was 1.17±0.2 in the studied subjects (Table 35). Sperm concentration, motility, and morphology were comparable between the normal weight and overweight men. Mean levels of ROS were significantly ($p=0.00$) higher in overweight (BMI≥24.5 kg/m^2) men than in normal-weight (BMI <24.5 kg/m^2) men (Table 35), while no significant ($p>0.05$) difference in HOS levels was between the two groups (53.3±0.8 and 53.9±0.9).

Table 34: Age, weight, height, body mass index (BMI), married for, wife age, liquefication time, volume, pH and WBC in normal weight (BMI <24.5 kg/m^2) and overweight (BMI ≥ 24.5kg/m^2) and whole studied population

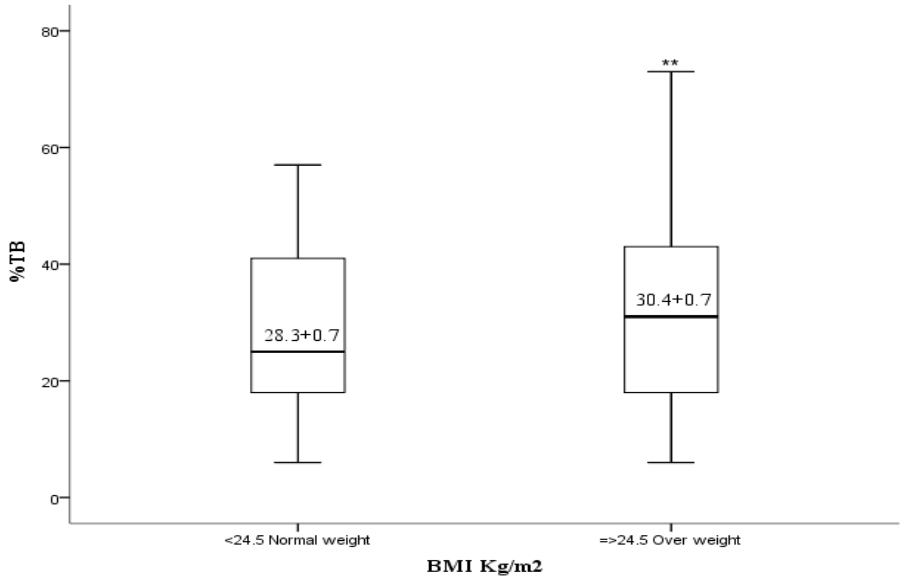
	Normal weight (BMI <24.5 kg/m^2) (n=347)	Overweight (BMI ≥24.5 kg/m^2) (n=403)	Whole studied population (n=750)	p-Value
Paternal Age (Years)	38.7±0.26	38.9±0.24	38.8±0.25	0.68
Weight (KG)	72.5±0.4	84.07±0.3**	77.8±0.4	<0.001
Height (cm)	75.49±4.3	95.06±5.07**	86.93±3.31	<0.001
Paternal BMI(Kg/m^2)	22.3±0.15	27.6±0.3**	25.6±0.1	<0.001
Married for (Years)	9.1±0.8	9.4±0.6	9.10±0.7	0.52
Maternal age (Years)	33.1±0.27	33.1±0.29	32.9±0.28	0.33
Maternal BMI(Kg/m^2)				
SEMEN PARAMETERS				
Liquefication time (Minutes)	31.75±0.71	31.98±0.46	31.56±0.39	0.06
Volume (ml)	3.85±0.09	3.81±0.1	3.69±0.06	0.39
PH	8±0.0	7.9±0.0	7.97±0.01	0.27
WBC/HPF	4.65±0.32	4.35±0.56	4.52±0.29	0.25

Values represent mean± SEM

Values in parentheses represent the number of subjects



(a)



(b)

Figure 48. Sperm quality parameters in overweight and normal weight men. (a) Chromomycin A3 staining (CMA3+) in the sperms of normal weight and overweight men. (b) toluidine blue staining (TB+) in the sperms of normal weight and overweight men. (Statistically significant *p<0.05, **p<0.01, ***p<0.001)

Table 35. Semen parameters analysis in normal weight (BMI >24.5 kg/m^2) and overweight (BMI ≤ 24.5kg/m^2) men and whole studied population

Sperm Parameters	Normal weight (BMI <24.5 kg/m^2) (347)	Overweight (BMI≥24.5 kg/m^2) (403)	Whole studied population (750)	p-Value
Concentration x10^6	62.89±3.30	61.47±2.22	62.01±2.23	0.83
Morphology Normal	3.6 ±0.99	3.5±0.85	3.5±0.65	0.24
TZI	1.81±0.3	1.82±0.3	1.81±0.2	0.67
SDI	1.13±0.3	1.19±0.3	1.17±0.2	0.56
TNMS	11.44±1.5	9.32±0.9	10.75±0.9	0.002
ROS	1.68±0.6	1.9±0.6	1.79±0.4	<0.001
HOS	53.9±0.9	53.3±0.8	53.7±0.9	0.81
DFI-SCD	22.91±1.23	28.74±1.04	25.38±3.80	<0.001

Values represent mean± SEM; TZI, terato-zoospermic index; SDI, sperm deformity index; TNMS, total motile sperm; ROS, reactive oxygen species; HOS, hypo-osmotic swelling test-sperm vitality; DFI-SCD, sperm DNA fragmentation index – measured by sperm chromatin dispersion assay, values in parentheses represent the number of subjects.

Table 36: Correlation between the paternal BMI (kg/m2), CMA3+, TB+, DFI, HOS, and ROS with ART outcome

		PBMI	CMA3+	TB+	DFI-SCD	HOS	ROS
Paternal BMI kg/m^2	r=		0.79**	0.114*	0.282**	0.012	0.282**
	p=		<0.001	.030	<0.001	0.742	<0.001
FR	r=	-0.187**	-0.043	-0.031	-0.062	0.070	-0.148**
	p=	<0.001	0.261	0.422	0.107	.068	<0.001
D3 Embr	r	-0.019	0.075	-0.049	-0.038	0.051	0.021
	p	0.70	0.593	0.13	0.45	0.301	0.59
EG_A	r=	0.052	0.08	0.021	0.030	-0.01	0.06
	p=	0.325	0.1	0.693	0.577	0.78	0.259
CR	r=	-0.110**	.009	.024	-0.005	-0.023	0.042
	p=	0.004	0.814	0.526	0.897	0.556	0.277
BR	r=	0.01	0.07	0.08	0.09	0.23	-0.039
	p=	0.96	0.59	0.64	0.49	0.15	0.7
Euploid	r=	0.061	0.060	-0.014	0.007	-0.004	0.045
	p=	0.142	0.143	0.740	0.858	0.923	0.279
ET	r=	-0.052	0.011	0.015	-0.003	0.105**	0.005
	p=	0.157	0.763	0.684	0.939	0.004	0.882
IR	r=	0.42	0.008	0.05	0.027	0.016	-0.062
	p=	0.25	0.83	0.17	0.45	0.654	0.09
CLBR	r=	-0.38**	0.002	0.025	-0.09	-0.03	-0.52
	p=	<0.001	0.445	0.97	0.16	0.59	0.44

BMI, Body mass index; FR, fertilization rate; D3 embryo (day three embryos), EG_A, embryo grade A, CR cleavage rate; BR, blastocyst rate, ET, number of embryos transferred; IR, implantation rate; CLBR, Cumulative live birth rate. Treatment statistically significant *p=0.05, **p=0.01, ***p<0.001

Sperm chromatin integrity parameters

Overweight men had a higher percentage of DNA Fragmentation SCD (28.74±1.04) ($p=0.00$) compared to normal-weight (22.91±1.23) men (Table 35). Mean Levels of CMA3 in overweight men were 29.7±9.8 which was significantly ($p=0.000$) increased than normal-weight men at 27.3±0.5 (Figure 41a), similarly higher ($p=0.03$) TB levels were observed in overweight men at 30.4±0.7 when compared with normal-weight men 28.3±0.7 (Figure 51b). Increase in paternal BMI correlate positively with impaired spermatozoa chromatin integrity markers; SCD and TB ($r=0.282$ and $r=0.114$, $p<0.05$) and higher percentage of immature sperm-CMA3($r=0.79$, $p<0.001$) in ejaculate.

Peri-fertilization stage

When each subfertility category was categorized into normal and overweight men, the peri-fertilization defects (failed fertilization) were positively associated with the male overweight group, and the observed difference was statistically significant (1201/3292, 40% and 687 of 2188 means 31%, $p<0.001$). A negative significant correlation was found between paternal BMI and percent fertilization rate (FR) $r=-0.187^{**}$, $p<0.001$ Table 36. A total of 1461 oocytes were inseminated by IVF, 1354 (92.7%) oocytes successfully formed normal pronuclear (2Pn) formation, while 107 (7.3%) oocytes failed to fertilize. Of 3395 oocytes inseminated by ICSI, 546 (16%) oocytes failed to fertilize, while 2849 (83.9%) formed normal 2Pn. When cases were categorized into BMI categories couples with normal weight had higher fertilization percentages after IVF 878/925 (94.9%) than overweight men 475/531(89.5% $p<0.05$). No significant ($p>0.05$) difference in ART parameters i.e., total egg collected (TEC), metaphase two (MII) and two pronuclei (2PN) between the overweight (BMI>24.5 kg/m^2) men than normal weight (BMI $\leq$24.5 kg/m^2) men. A significant decrease in percent fertilization rate (PFR) and increase (within normal range) between the overweight (BMI>24.5 kg/m^2) men and normal weight (BMI $\leq$24.5 kg/m^2) men (Table 37).

Successful fertilization percentage by ICSI in normal-weight men had 1218/1514 (80.4%) significantly better than in overweight men had 1631/2079 (78.5%, $p<0.001$) Table 38. Multiple linear regression analyses showed that despite correction of potential cofounders (paternal age, maternal age, maternal BMI, subfertility duration, sperm chromatin integrity

markers (SCD, CMA3, TB), a negative association remained between fertilization rate and paternal BMI (β = -1.32, SE = 0.317; p<.001).

Early/late embryonic development

Early embryo development includes cleavage rate, day three (D3) embryo, embryo grade A (EG_A) and late embryo developmental stages of blastocyst formation, euploid embryo and number of embryos transferred we found no significant (p>0.05) difference between two BMI groups (normal weight vs overweight) (Table 37) and both insemination groups (IVF vs ICSI). We found a significant negative correlation between paternal BMI with cleavage rate (r=-0.11, p=0.004), while no correlation was found between paternal BMI and day three embryo, embryo grade A (EG_A), blastocyst rate, euploid embryo and number of embryos Table 36.

Implantation stage

The implantation rate was 37% in the normal-weight men group and 35% in the overweight men group and there was no difference in both groups. Subcategories according to insemination (IVF vs ICSI) showed no significant difference. Implantation rate (IR) r=-0.42, p=0.25we found no correlation with paternal BMI (Table 37).

Outcomes: Cumulative Live Birth Rates

In paternal overweight group had a 20.1% cumulative birth rate (CLBR) compared to the reference group 31.2% (p>0.02) Table 37, we found a significant increase (within normal range) in neonatal birth weight in the paternal overweight group. Subcategories of insemination (IVF vs ICSI) showed no significant difference in CLBR Table 38. While a paternal BMI had a significant negative impact on CLBR (r=-0.38, p<0.001). A negative association remains between paternal BMI and CLBR (β = -2.84, SE = 0.82; p<0.001). Moreover maternal BMI (p=0.03) and maternal age (p<0.001) were independent predictors of CLBR.

Paternal BMI to predict successful fertilization

Percent fertilization higher than 50% was observed when the mean BMI was 24 kg/m^2 and less than 50% fertilization rate was observed at a mean BMI of 25 kg/m^2 (Figure 49a). A

ROC for paternal BMI for 50% fertilization was determined (Figure 49b). We found that the attainment of a good fertilization rate (>50%) was predicted with a specificity of 70% and sensitivity of 47% at a BMI $\leq$24.5 kg/m^2. By binary logistic regression analyses, we identify that the probability of attaining a>50% fertilization rate was higher when paternal BMI is $\leq$24 kg/m^2 with OR of 1.98 (CI 95% 1.323-2.967, p=0.001) for all couples. We found an association between paternal BMI with CMA3+ and fertilization rate (Figures 51 a & b).

The plotted Kaplan–Meier curves (Figure 50 a&b), with Cox regression found a negative association between the paternal overweight (HR =5.12, p = 0.05) and the CLBR per ET (Figure 50a). Both curves were statistically significant and had a statistically significant negative association between CLBR per percent fertilization Oocyte (2Pn) and paternal overweight (X^2 = 5.39, p < 0.05) which was consistent with the result obtained in the univariate analysis as illustrated in Figure 50b.

Table 37: Assisted reproduction parameters and Embryo development outcomes in normal-weight (BMI >24.5 kg/m^2) and overweight (BMI ≤ 24.5kg/m^2) and the whole studied population.

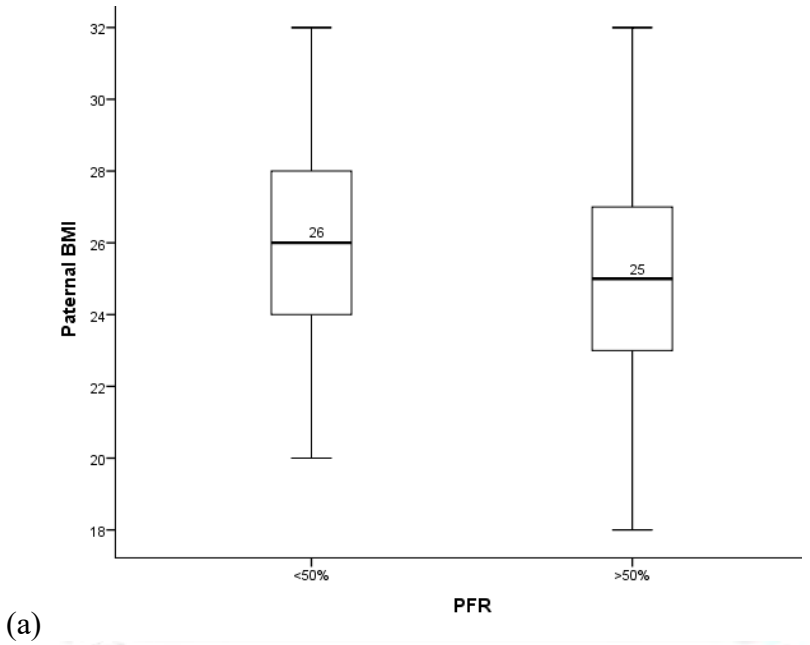
Parameters	normal weight (BMI <24.5 kg/m^2) (347)	Overweight (BMI ≥24.5 kg/m^2) (403)	Whole studied population (750)	P-Value
TEC	8.9±6.4 (2471)	9.2±7.0 (3942)	9.1±6.7 (6413)	0.59
MII	7.7±0.5 (2188)	7.8±0.6 (3292)	7.7±0.6 (5480)	0.83
2PN	5.3±0.7 (1501)	5.2±0.2 (2091)	5.2±0.4 (3592)	0.84
FR	74.8±0.25	67±0.2	70.2±0.8	0.001
D3 embryos	3.8±0.7	4.1±0.4	4.0±0.2	0.31
EG_A	2.8±0.9	2.7±0.4	2.7±0.6	0.89
CR	82.4±0.24	83.5±0.23	83.1±0.24	0.56
BR	42.5±0.24	39±0.25	40.7±0.24	0.42
Aneuploidy (%)	39.1±2.1	38.1±2.0	38.1±1.4	0.45
IR (%)	131/347 (37%)	143/403 (35%)	274/750 (36%)	0.51
CLBR	31.2±3.4	20.1±3.2	25.2±3.4	0.02
Birth weight (gm)	2577.24±30.94	2952.14±53.64	2752.5±36.98	<0.001

Values represent mean± SEM; TEC Total egg collected, MII metaphase two oocytes, 2PN, two pronuclei: FR, fertilization rate; D3 embryo, day three embryos; EG_A, embryo grade A; CR cleavage rate; BR, blastocyst rate, IR, Implantation rate; ET, number of embryos transferred; CLBR cumulative live birth rate., BMI, Body mass index; BR, blastocyst rate, ET, number of embryo transferred; Treatment statistically significant *p=0.05, **p=0.01, ***p<0.001

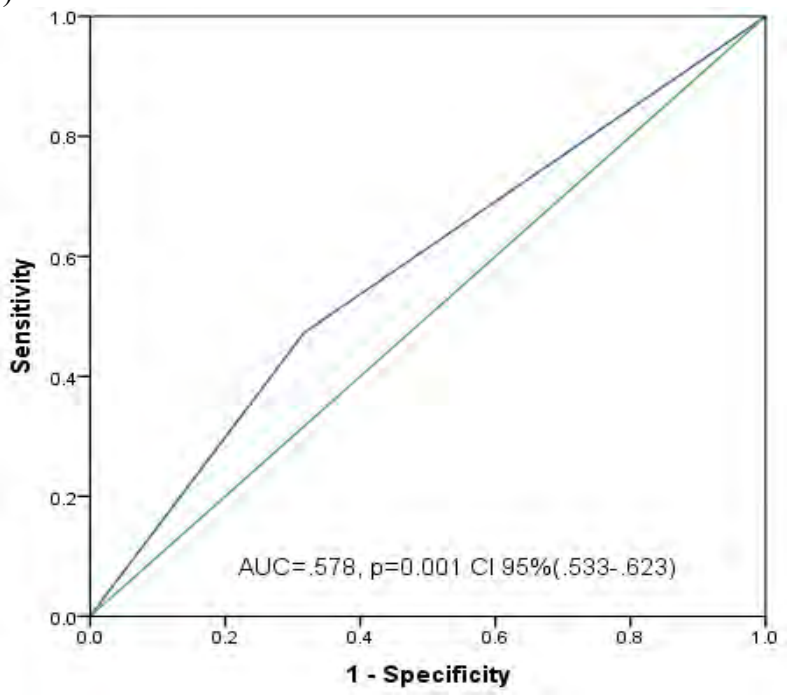
Table 38: Fertilization rate and embryo development after IVF and ICSI

	IVF				ICSI			
	Cohort (318)	**Normal weight men**	**Overweight men**	**P-value**	**Cohort (432)**	**Normal weight men**	**Overweight men**	**p-value**
FR	77.0±3.3 (1354)	77.8±2.3 (878)	72.5±1.4 (475)	0.04	68.7±2.7 (2849)	72.9±2.7 (1218)	65.8±2.3 (1631)	0.01
D3 Embryo	4.0±3.2 (696)	3.9±3.0 (432)	4.2±3.5 (264)	0.93	3.9±3.1 (1604)	4.2±3.5 (689)	3.7±2.9 (915)	0.16
EG_A	2.8±2.8 (425)	2.8±3.0 (278)	2.8±2.5 (147)	0.89	2.9±2.8 (1027)	2.8±2.9 (406)	2.9±2.7 (621)	0.73
CR	80.1±2.0 (1409)	80.0±2.8 (9040)	80.2±2.7 (505)	0.56	82.9±2.8 (3423)	84.1±3.1 (1395)	82.1±2.3 (2028)	0.42
BR	38.4±1.1 (844)	42.1±1.5 (589)	31.9±1.2 (255)	0.9	43.0±2.7 (2277)	39.3±2.2 (903)	45.8±2.9 (1374)	0.38
CLBR	26.7±3.6	28.7±3.8	27.6±3.7	0.76	40.5±4.2	29.4±4.3	36.7±4.3	0.39

BMI, Body mass index; FR, fertilization rate; D3 embryo (day three embryos), EG_A, embryo grade A, CR cleavage rate; BR, blastocyst rate.

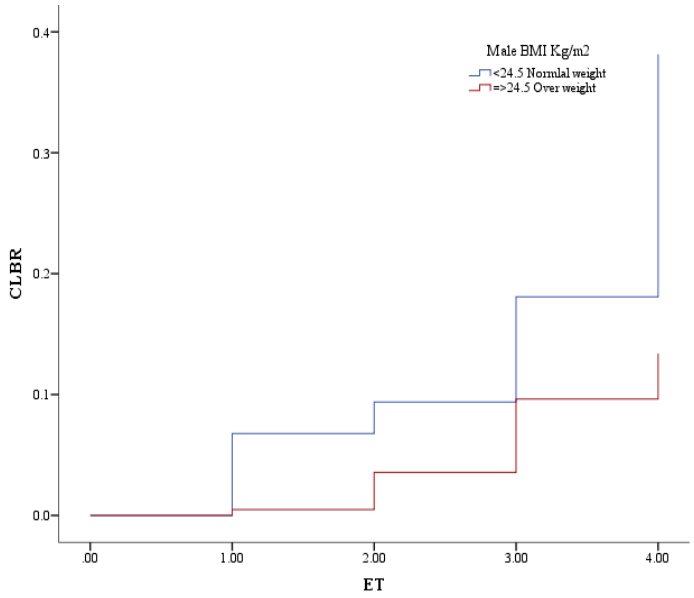


(a)

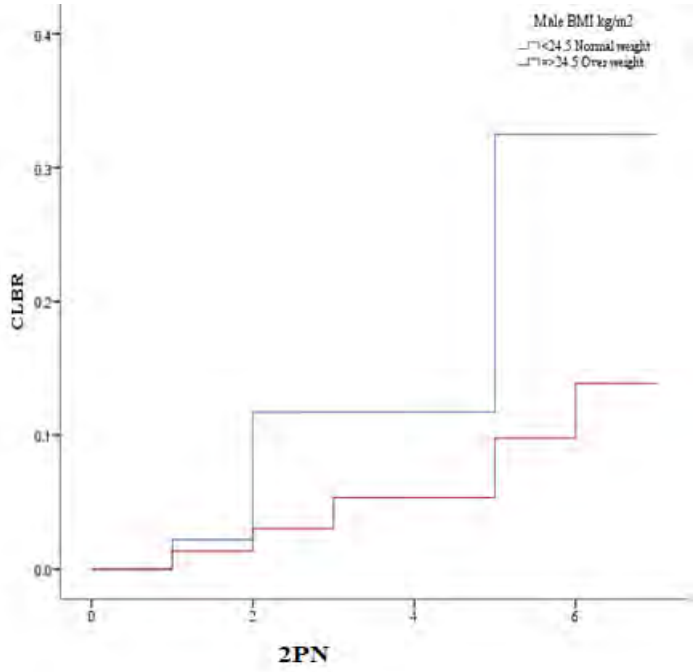


(b)

Figure 49. (a) Box figure illustrates the paternal BMI in groups with PFRs of less than 50% (n=202) and more than 50% (n=548). (b) Receiver operating characteristic curve shows how well the paternal BMI (24), which is a predictor of fertility, performs in predicting the achievement of a minimum fertility rate.

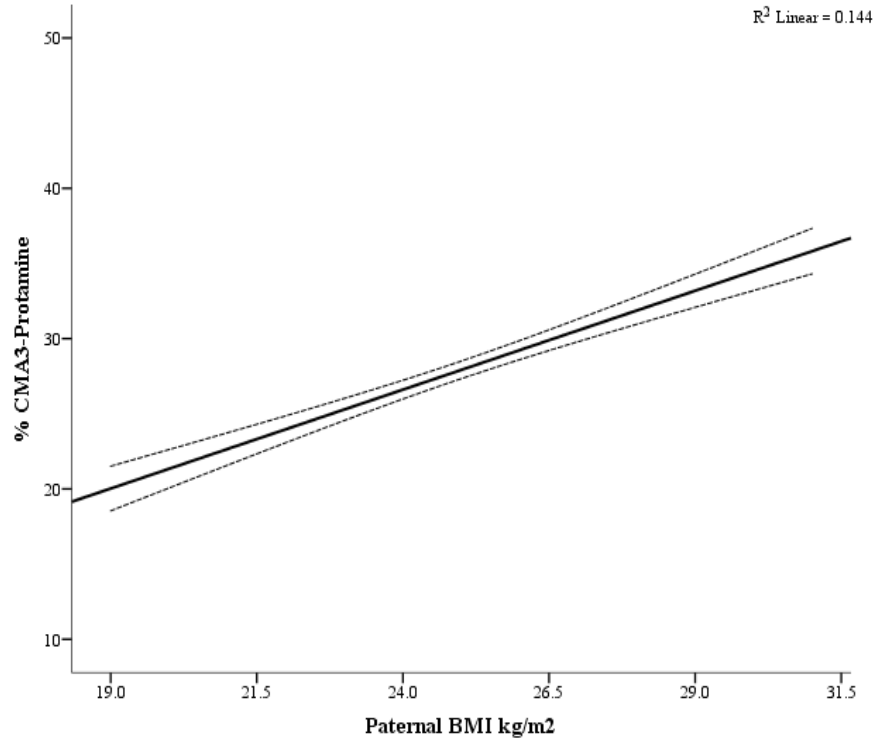


(a)

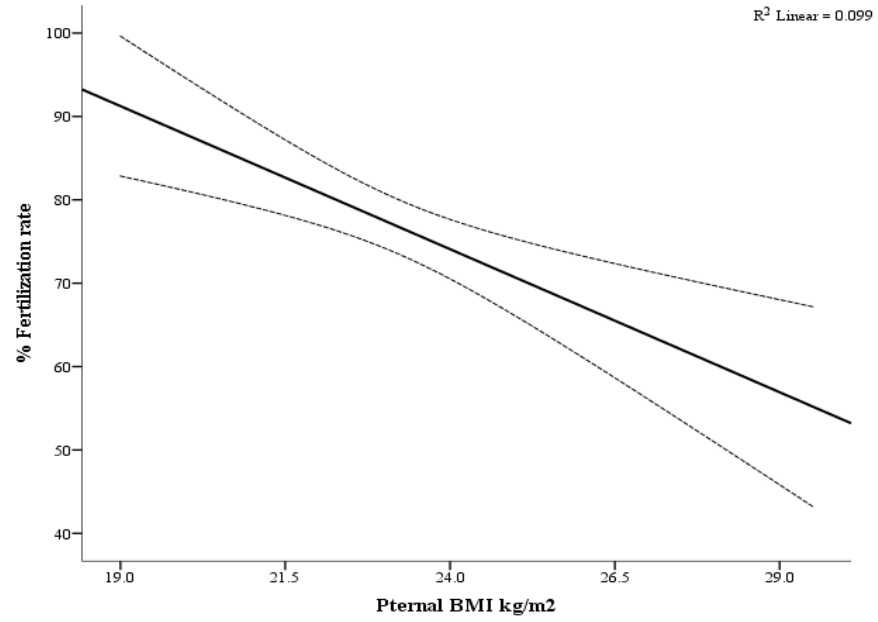


(b)

Figure 50. An unadjusted analysis of reproductive outcomes in ART cycles for cumulative live birth rate (CLBR) (a) CLBR per ET and (b) CLBR per 2PN, respectively.



(a)



(b)

Figure 51. Scatterplot charts showing the relationship of (a) CMA3+, (b) Fertilization rate with paternal BMI. Graph of the association using linear regression lines and a 95% confidence interval of paternal BMI with CMA3+ and fertilization rate

ANALYSIS OF SPERM CHROMATIN PACKAGING AND REPRODUCTIVE BIOMARKER TO EVALUATE THE CONSEQUENCE OF ADVANCED MALE AGE

When compared to individuals who were omitted from the study or lost to follow-up, there were no appreciable variations in the general characteristics of the men who were included in the study.

3.1 Demographic parameters

The mean paternal age of the studied subjects was 38.8±0.25 and paternal BMI was 25.6±0.1 kg/m2. There was no significant ($p>0.05$) difference in male and female BMI, and female partner age and BMI when analyzed among the patient groups. The overall duration of subfertility there was a significant difference between the different age groups.

3.2 Paternal age and Semen standard parameters

The mean± standard error from the mean (SEM) for various semen parameters was comparable between the groups (Table 39). No significant differences were seen in conventional semen parameters, including concentration, normal morphology, total motile sperms (TNMS), and HOS were similar between all age groups.

3.3 Biochemical and hormonal analysis

There was no statistical difference in the mean levels of ROS, POD, SOD, MDA and hormonal levels (FSH, LH, Prolactin, Testosterone levels) in all age groups (Table 40).

3.4 Sperm chromatin integrity parameters

Old age men (>40 years) had a higher percent of DNA Fragmentation SCD (26.6±0.6, $p=0.001$) compared to >30 years age (23.2±0.88) men (Table 41, Figure 52a). While percent DNA fragmentation remained comparable between old age men >40 years and 30-40 years age group (25.1±0.4, $p>0.05$). Mean levels of CMA3 in old age men >40 years were 30±0.71which was significantly ($p=0.04$) increased than men age >30 years 26.6±1.03 (Table 40, Figure 55b), similarly no significant but increased TB levels were observed in >40years aged men (28.65±1.14) when compared with other two age groups (<30 years and 30-40 years) men 26.71±1.83 and 26.47±0.86 (Table 41). Increase in

paternal age correlate positively with spermatozoa chromatin dispersion; SCD (r=0.124, p=0.001) (Figure 52a) and higher percentage impaired sperm chromatin compaction-CMA3 (r=0.1, p=0.009) in ejaculate (Figure 52b). Linear regression was used to compare paternal age with CMA3 and SCD. There was significant positive linear association between paternal age with CMA3 (β = 0.169, t=2.63, CI 95% (0.042 to 0.295); p=0.009) and SCD (β = 0.195, t=3.42, CI 95% (0.08 to 0.307); p=0.001) (Figure 53 a & b).

Peri-fertilization effect

The number of divided COCs (oocytes) was similar between the groups and the number of mature oocyte's number was not statistically different in the three age groups. There was no significant difference in percent mature oocytes MII between the groups.

The fertilization rate was comparable compared with other groups (Figure 54).

Embryo developmental effect

The number of day three embryos obtained and grade was comparable in groups, and the same was in the case of the blastocyst. The number of grade-one embryos in the cleavage stage was similar (p >0.05). While the number of embryos transferred between the groups was the same (Figure 54).

Paternal age effect on PGT-A:

Paternal age is categorized into three categories (<30, 30-40, >40) and no significant difference in euploid, aneuploid, chromosomal haploid, polyploid, monosomy, gonosomy, complex abnormal, normal XX and XY embryos number in all categories Figure 55.

Table 39: Demographic characteristics of couples included in the study group according to age

	<30 years (n=90)	30-40 years (n=330)	>40 years (n=330)
Male age (year)	28.06±0.30	36.24±0.18	45.40±0.32
Male BMI kg/m^2	22.79±0.23	23.03±0.11	22.71±0.15
Female age (year)	27.46±0.66	32.35±0.35	35.62±0.45
Female BMI kg/m^2	26.89±0.40	27.16±0.19	26.75±0.26
Subfertility Duration (year)	5.03±0.36	8.40±0.31*	12.50±0.55**

Values represented as Mean ±SEM; Statistically significant *p<0.05, **p<0.01, ***p<0.001

Table 40: The effects of male age on semen parameters, biochemical profile and reproductive hormones concentration in studied grouped according to age

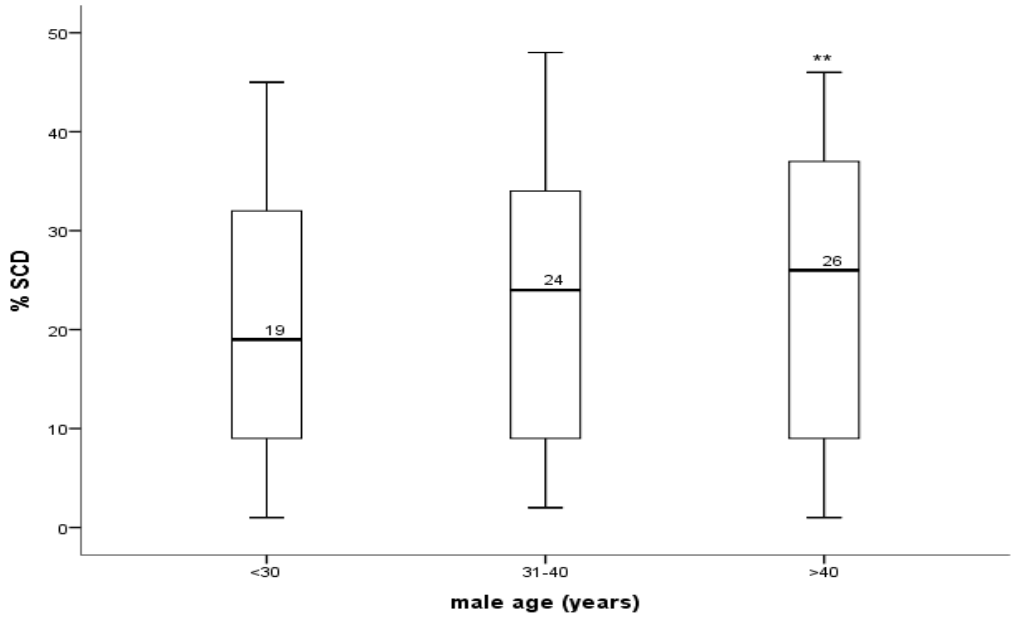
	Below 30 years (n=90)	30 to 40 years (n=330)	Above 40 years (n=330)
Semen Volume (ml)	4.03±0.18	3.74±0.09	3.93±0.14
pH	8±0.00	8.00±0.01	8.15±0.15
Liquefaction time (min)	31.85±0.93	33.36±0.97	31.07±0.39
WBC/HPF	3.32±0.26	3.01±0.13	3.02±0.18
Concentration x10^6/ml	62.02±8.13	56.97±3.29	53.32±4.05
Normal morphology %	4.00±0.20	3.63±0.12	3.47±0.16
TNMS %	50.42±3.20	44.82±1.64	44.45±2.08
Viability (HOS) %	73.32±2.47	70.13±1.28	70.49±1.57
ROS (umol/min)	1.60±0.12	1.71±0.07	1.72±0.08
SOD (U/min)	13.73±0.35	13.42±0.17	13.47±0.20
POD (nmole)	10.84±0.08	10.63±0.05	10.66±0.05
CAT (g/dl)	9.71±0.14	9.62±0.06	9.62±0.09
MDA (nmol/ml)	28.56±0.24	28.82±0.11	28.86±0.18
FSH (mIU/ml)	6.08±0.40	5.50±0.19	5.91±0.35
LH (mIU/ml)	8.10±1.35	6.75±0.52	8.67±1.00
Prolactin (mIU/ml)	10.84±1.12	11.34±0.52	13.13±0.72
Testosterone (ng/ml)	382.25±31.82	365.55±15.63	394.80±19.55

Values represented as Mean ±SEM; Statistically significant *p<0.05, **p<0.01, ***p<0.001

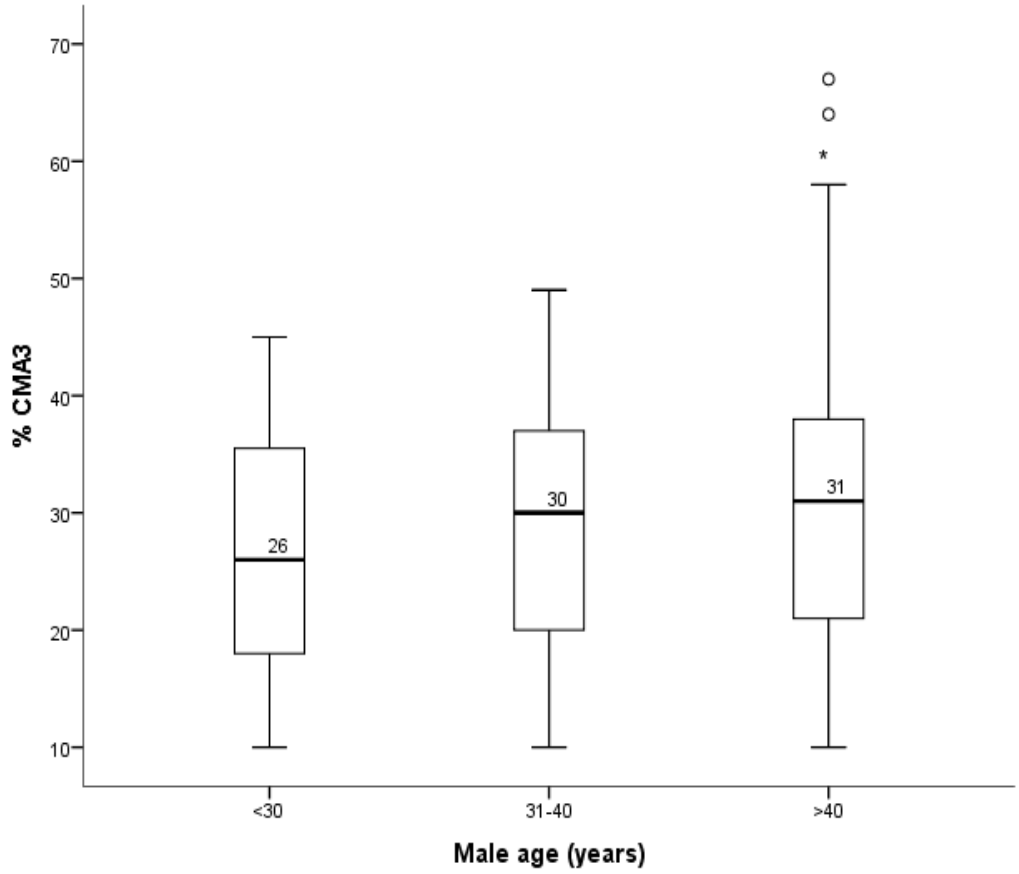
Table 41: Male age influence sperm chromatin dispersion (SCD), chromatin integrity (TB) and chromatin compaction (CMA3) in studied groups

	Below 30 years (n=90)	30 to 40 years (n=330)	Above 40 years (n=330)
Sperm chromatin dispersion-SCD %	23.2±0.88	25.1±0.4	26.6±0.6**
Chromatin integrity-TB %	26.71±1.83	26.47±0.86	28.65±1.14
Chromatin compaction- CMA3 %	26.6±1.03	29.04±0.50	30±0.71*

Values represented as Mean ±SEM; Statistically significant *p<0.05, **p<0.01, ***p<0.001

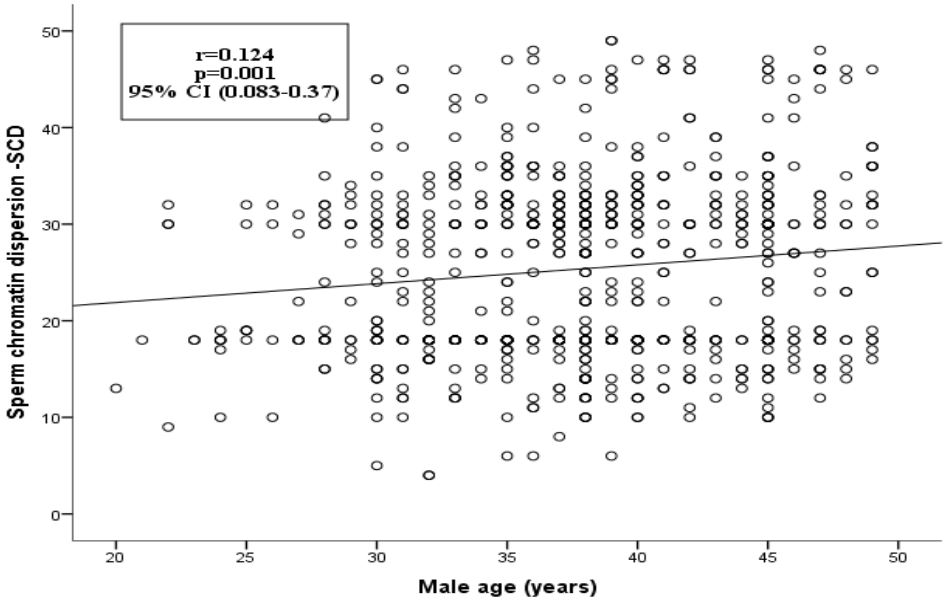


(a)

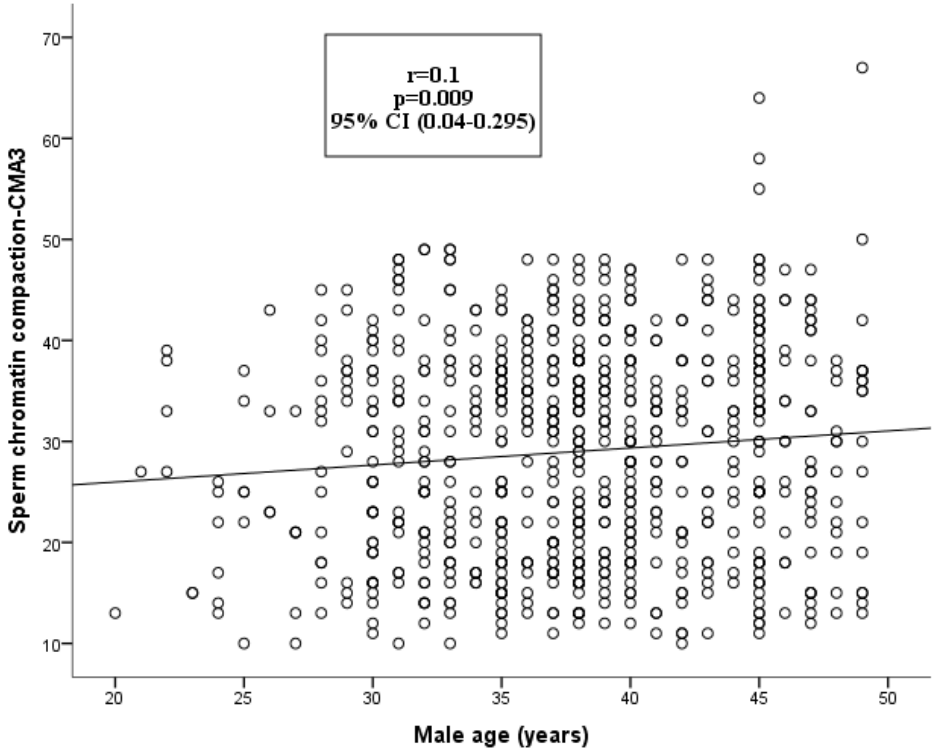


(b)

Figure 52. Percentage of (a) sperm chromatin dispersion (SCD) and (b) sperm protamine (CMA3+) content in sperm of males in different age groups

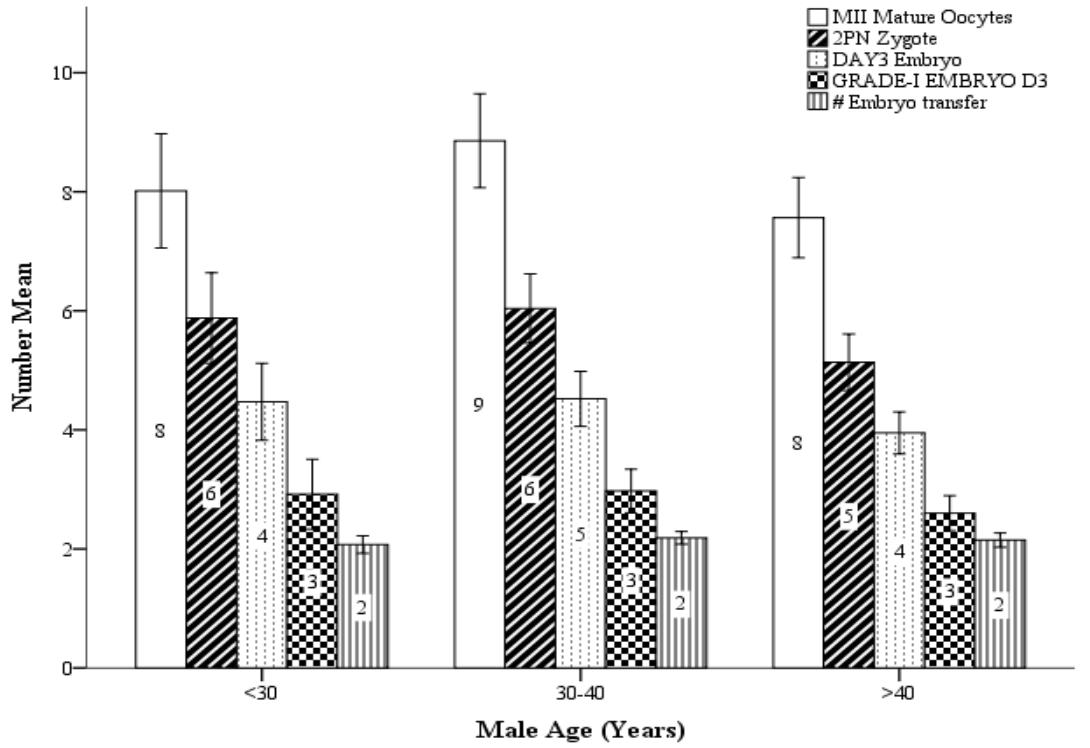


(a)

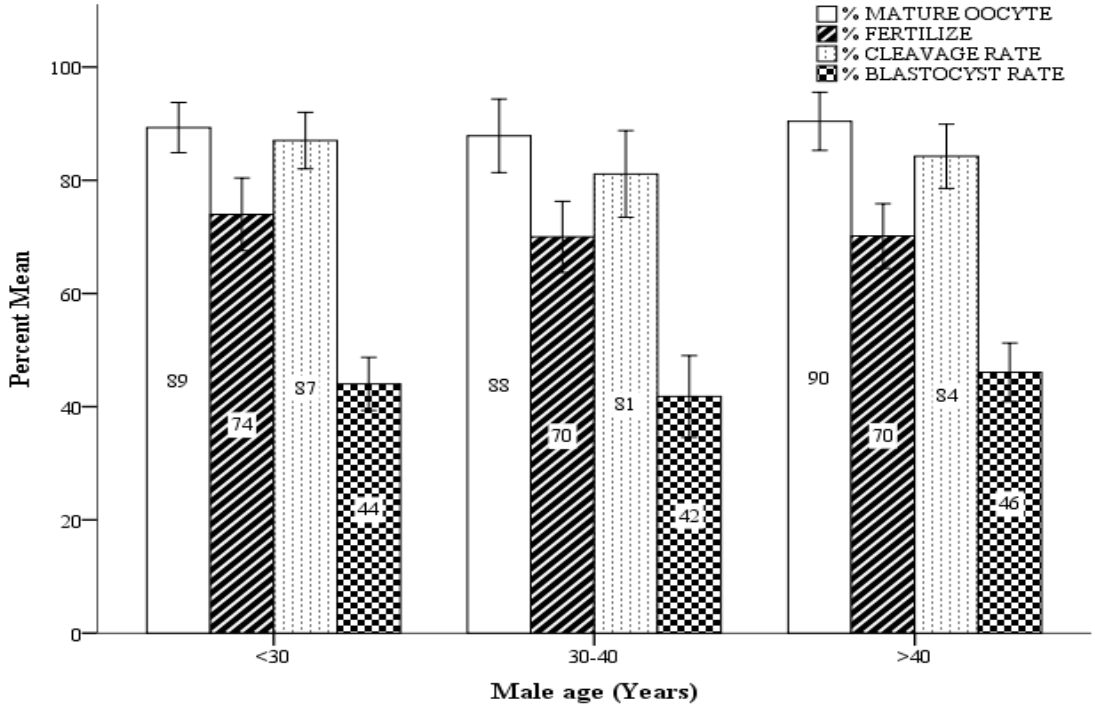


(b)

Figure 53. The relationship of sperm chromatin dispersion and sperm chromatin compaction (CMA3) to male age (a) scatterplots correlation lines depicting the association between sperm chromatin dispersion (SCD) and male age with (b) sperm chromatin compaction/protamine (CMA3) and male age



(a)



(b)

Figure 54. Assisted conception outcome parameters according to male age categories mean number of (a) MII, 2PN (Zygote), Day three (D3) embryo, grade-I D3 embryo, number of embryo transfer (b) mean percentage of maturation rate, fertilization rate, cleavage rate, and blastocyst rate

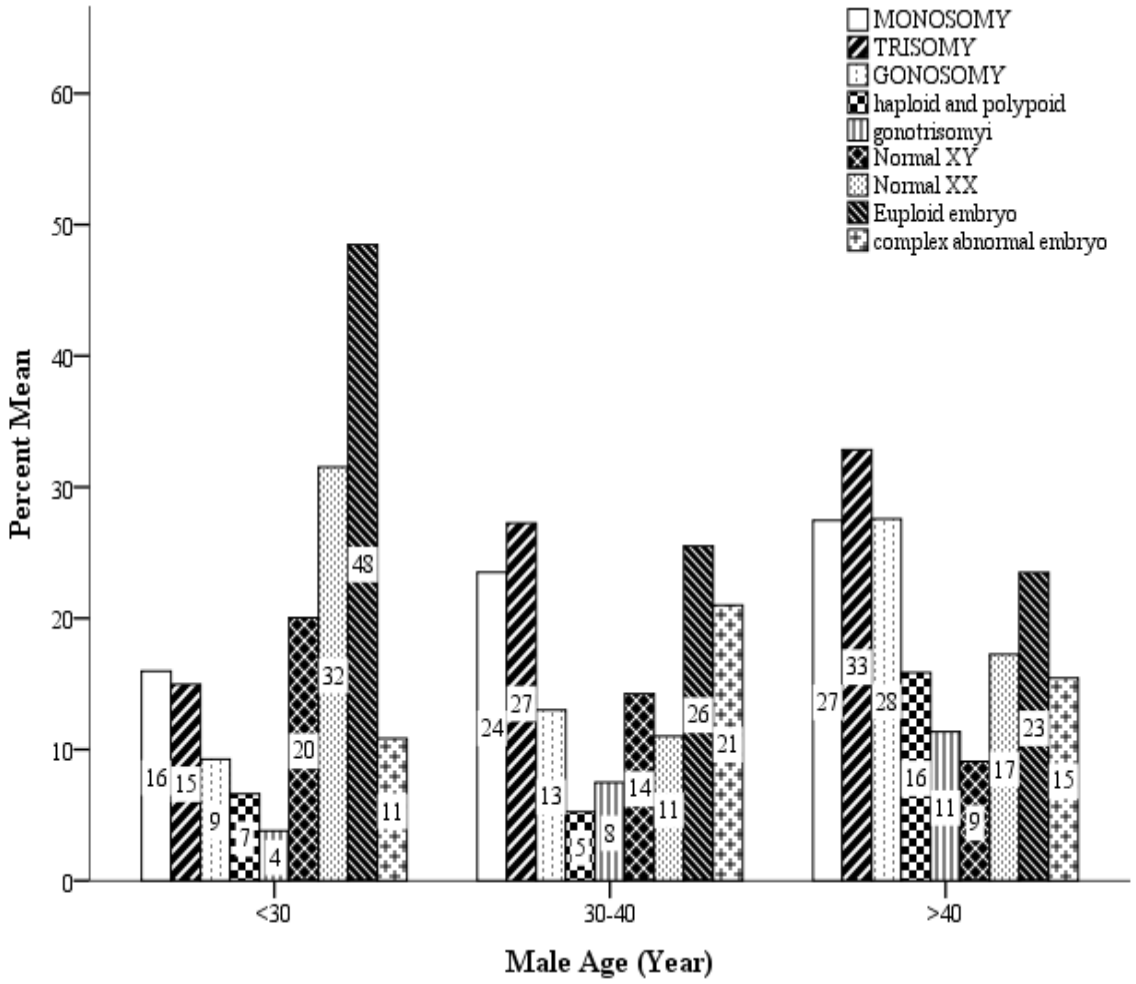


Figure 55. Effect of paternal age on embryos with pre-implantation genetic screening for Aneuploid (PGT-A)

FEMALE CHARACTERISTICS AND ART OUTCOME

Correlation of female characteristics and ART outcome:

There was a negative significant negative ($p < 0.05$) correlations found between female age and number of cumulus-oocyte complex (COCs), number of metaphase II (MII), the zygote (II-PN), total embryos D3 and day of embryo transfer D3/5. Also, Female BMI was correlated negatively ($p < 0.05$) with COCs, blastocyst rate, and the number of embryos transferred D3/5.

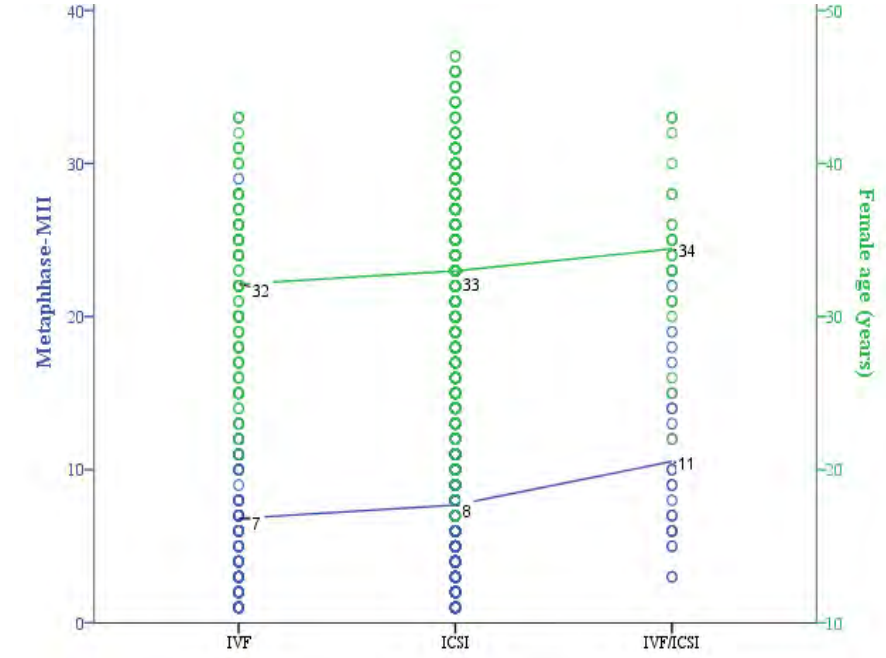
Female serum anti-mullerian hormone (AMH) levels were correlated positively high ($p < 0.01$) with COCs, number of metaphase II (MII), the zygote (II-PN), total embryos D3, cleavage rate and number of embryos transferred D3/5.

Female serum FSH levels correlate negatively ($p < 0.05$) with COCs, number of metaphase II (MII), the zygote (II-PN), total embryos D3, and Grade I embryos D3.

LH levels significantly ($p<0.05$) negative correlation with blastocyst formation rate. The grade I embryos and day of embryo transfer D3/5 were also significantly related ($p < 0.05$) to serum prolactin level and estradiol correlate significantly ($p<0.05$) with percent mature oocyte. (Table 42, Figure 56 a&b).

The mean number of metaphase oocytes-MII was 11 and significantly high in IVF/ICSI compared to IVF (mean=7) and ICSI group (mean=8) when plotted against female age (years) and female BMI (kg/m^2).

(a)



(b)

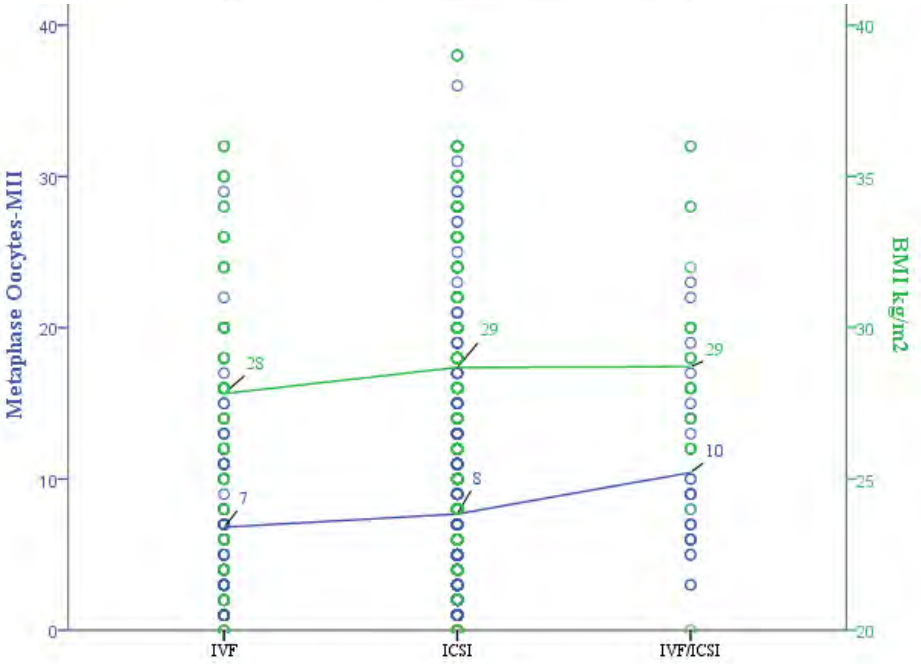


Figure 56. Influence of (a) Female age-years (b) Female BMI–Kg/m^2 on the number of metaphase oocytes (MII) collected according to procedure utilized for insemination i.e., IVF, ICSI and IVF/ICSI.

Table 42: Correlation of female parameters with assisted conception outcome.

		Female Age	Female BMI	AMH	FSH	LH	Prolactin	Estradiol	TSH
COCs	*SSC*	**-.174****	**.075***	**.334****	**-.154****	.067	-.054	.030	-.037
	p	**.000**	**.046**	**.000**	**.000**	.107	.206	.517	.413
Metaphase II-MII	*SSC*	**-.180****	.013	**.339****	**-.166****	.042	-.049	-.012	-.028
	p	**.000**	.735	**.000**	**.000**	.319	.246	.802	.525
% MII Oocytes	*SSC*	.046	-.045	-.055	.019	-.037	-.069	**.133****	.024
	p	.257	.267	.216	.656	.383	.105	**.005**	.588
Zygote -II-PN	*SSC*	**-.161****	.012	**.273****	**-.118****	.014	-.053	-.018	-.024
	p	**.000**	.749	**.000**	**.005**	.743	.222	.704	.596
Fertilization rate	*SSC*	-.011	-.007	-.042	**.136****	-.012	.003	-.058	-.029
	p	.783	.857	.356	**.001**	.783	.952	.224	.520
ET day 3/5	*SSC*	**-.097***	-.020	.038	-.072	-.046	**-.100***	-.022	.019
	p	**.033**	.658	.455	.123	.330	**.036**	.677	.699
Grade I embryos D3	*SSC*	-.058	.074	.096	**-.091***	-.016	**-.156****	-.003	.025
	p	.156	.071	.054	**.047**	.732	**.001**	.959	.616
Total embryos D3	*SSC*	**-.103****	.036	**.208****	**-.094***	.039	-.067	.035	-.016
	p	**.008**	.344	**.000**	**.027**	.358	.124	.463	.731
Cleavage rate	*SSC*	**.092***	.048	**-.184****	.069	.045	.007	.072	.045
	p	**.017**	.210	**.000**	.103	.296	.869	.135	.322
Blastocyst rate	*SSC*	-.047	**-.144***	.037	-.011	**-.174***	.062	-.042	-.009
	p	.502	**.039**	.656	.896	**.034**	.453	.661	.911
Embryos transferred	*SSC*	-.054	**-.106****	**.171****	.028	-.045	-.047	-.010	.003
	p	.165	**.006**	**.000**	.514	.299	.280	.836	.950
Clinical pregnancy rate	*SSC*	-.032	.012	-.024	-.091	.102	-.065	.016	-.130
	p	.567	.828	.730	.144	.101	.305	.820	.057
cumulative live birth	*SSC*	-.004	.138	.003	-.163	-.082	-.032	.029	-.037
	p	.968	.167	.983	.169	.492	.790	.822	.757
Birth weight gm	*SSC*	.050	-.176	.130	-.005	-.006	.188	-.026	.107
	p	.670	.126	.339	.970	.958	.117	.373	.844

[a]*SCC:* Spearman Correlation Coefficient.
*p=0.05, **p=0.01, ***p<0.001

DISCUSSION

Male factor subfertility is gaining attraction because of declining semen quality, and sperm standard parameters of healthy young men. Male factor subfertility is rising around the globe and it's become challenging because of therapeutic modality. The diagnosis of male subfertility has relied primarily on microscopic analysis and biochemical analysis to assess the human sperm quality to provide the physician with the basis of initial assessment and factions them into normal, moderate/severe factors of male subfertility. However, none of these tests address sperm quality and function to predict the fertility outcome after treatment. The present research conducted to evaluate male factors contributing to futile IVF and ICSI attempts to find the hidden reasons and to add one or more tests to predict the ART outcome and to improve implantation, reduce oxidative damage, evidence counseling, and reduce costs. Sperm chromatin alteration had detrimental effects on sperm quality in preset data we found the sperm chromatin integrity compromised in male factor subfertile men (MMF and SMF) due to impaired semen parameters.

Using SCSA is more popular to measure sperm DNA damage, as SCD is the measure of DNA damage indirectly, subfertile men's sperm had denatured DNA percentage higher than normal men, so poor sperm DNA integrity could cause subfertility or unsuccessful implantation (Green *et al.*, 2020).

The present study showed that increased sperm CMI (checked through chromatin condensation TB and abnormal spermatozoa protamination CMA3) influences male fertility, early embryonic development, and recurrent implantation failure and pregnancy outcome (Lara-Cerrillo *et al.*, 2021). We predict the probability of CMA3 level to determine fertilization rate, sperm chromatin maturity at 30% sperm CMA3+ level was predictive of successful fertilization with greater sensitivity (Ferrigno *et al.*, 2021).

The in-vitro oocyte fertilization after IVF and embryogenesis in SMF compared to MMF and N subjects did not have any change in cleavage rate and blastocyst development (Hammadeh *et al.*, 2005). It is because a significantly lower pregnancy rate was observed in the current study among couples with malefactors (SMF and MMF) compare to N after ICSI as suggested by other researchers as well (Engel *et al.*, 2018). We found with ROC

30% CMA3+ level and TB+ 33% as a threshold in SMF to predict the successful pregnancy achievement after ART treatment. Thus deficient protamination causes poor fertilization rate and clinical pregnancy rate (Magli *et al.*, 2007).

Lack of protamination of sperm chromatin causes abnormal sperm DNA packing and sperm DNA vulnerable to external stresses. Sperm chromatin condensation defect should be considered a sperm abnormality, higher level of DNA fragmentation and chromatin immaturity, in male factor subfertility, known to harm embryo kinetics, fertilization, and implantation potential and advisable to treat sperm quality and function parameters before starting Intracytoplasmic sperm insemination (ICSI) / *Invitro* fertilization (IVF). At the time of sperm mechanical insemination, the chromatin composition and DNA damage of microinjected sperm are unknown. The methods to evaluate sperm DNA fragmentation using SCD and chromatin maturity by CMA3 are less invasive and cheaper compared to SCSA and TUNEL. Hence, these advantages make sperm chromatin structure testing by SCD (to measure DNA damage) and CMA3 (to measure chromatin maturity) assessment an important examination that aids men's fertility potential analysis, and before putting the patient through assisted conception procedure.

Present work indicates that an increase in DFI and chromatin decondensation did not correlate with embryonic aneuploidy, complex abnormalities, trisomies, and gonosomal aneuploidy this could be due to the contributed maternal genome potential to halt the further embryogenesis of aneuploidy embryos before its genome take part or embryogenesis became ceased when paternal genome contribute aneuploidies. (Green *et al.*, 2020). We found no association of SMF with an increase of gonsomal-trisomies in embryos compared with subjects with normozoospermic. SMF and MMF interestingly in the subfertile couple is depended on sperm quality and sperm DNA damage and chromatin maturity and did not affect the genetic quality (aneuploidy) of the embryo produced, maternal genome and machinery (oocyte) contribute to the repair of paternal chromatin abnormalities but this ability is limited and depends on the extent of damage, as evidenced in previously reported study by, we concluded SMF had a decreasing number of euploid embryos (65% euploidy rate with N, 59% in MMF, and 54% in SMF), biopsy on day three embryos. Several studies showed the frequency of aneuploidy higher in the case of a severe

male factor compared with the normal male population. DNA damage in gametes can lead to problems in offspring, DNA correction at the time of early embryogenesis and after conception is crucial for preventing DNA damage from being passed down to future generations (Gat *et al.*, 2017). Because sperm, unlike oocytes, lack DNA repair capabilities, repairing sperm DNA aberration in the early stage after conception is crucial. Damage to sperm DNA in zygotes can result in miss-rejoining, chromosomal assembly, and acentric fragment formation if not repaired appropriately. As a result, sperm DNA damage repair before the zygote's division is important (Linan *et al.*, 2018).

Sperm quality markers did not correlate with the live birth rate. It's important to remember that there is a variety of factors that are crucial for successful implantation and normal fetus development, which might not relate to the fact that ARTs were used to achieve clinical pregnancy. To our knowledge, this is the first study to include male factor subfertility including normal, moderate/severe male factor subfertility and sperm quality characteristics including DNA fragmentation and chromatin condensation are some of the available contributing factors in the prediction of ART outcomes. Our study highlighted the impact of men's overweight on impaired sperm quality and outcomes following ART treatment. It is demonstrated that an increase in paternal weight harmed the integrity of sperm chromatin due to elevated reactive oxygen species generation. Our analyses found no influence of paternal BMI on sperm morphology and concentration, while, overweight men had lower motility compared to normal-weight men. Moreover, we found that there have been no statistically significant raises in the spermatozoa deformities index (SDI) in overweight. A percentage of normal motile sperm with altered chromatin in overweight men statistically more in comparison to normal-weight men was observed, which include weight problems. Paternal BMI had a significant negative association with ROS, DFI, CMA3, and TB levels. The paternal weight harmed the integrity of sperm chromatin and its condensation, which represents a higher percentage of immature sperm that could be due to elevated reactive oxygen species generation. These effects are following the findings of previous data suggesting weight gain to be related to higher sperm DNA damage and ROS (Anifandis *et al.*, 2013; Raad *et al.*, 2019; Chohan, 2006; Zeqiraj *et al.*, 2018). Therefore, we can also additionally conclude that being overweight harmed motile spermatozoa molecular components. An increase in the paternal BMI could lead to

impaired sperm chromatin integrity making spermatozoa's genetic material vulnerable to the external environment insult, as it is understood that sperm chromatin condensation is critical to the protective role in woman's reproductive tract and additionally to prevent manipuated epigenetic impact at some point of the pre-implantation period. Similarly, we observed increase in paternal BMI causes low fertilization and clinical pregnancy rate after the ART cycle. Hence, chromatin integrity is correlated with negative reproductive consequences of low fertilization rates, poor embryogenesis, assisted reproductive technology failure, and abortion (Ma *et al.*, 2020; Takagi *et al.*, 2019).

In the present study, we found increased normal neonatal (within normal range) birth weight in the paternal overweight group compared to normal weight. The outcomes of this examination indicated that paternal BMI has an impartial effect on the birth weight of neonates after ART cycles. In ART conception cycles, current information regarding the impact of increased paternal BMI on neonatal birth weight has shown conflicting results (Magnus *et al.*, 2018; Anderson *et al.*, 2018; Magnus, 2001). Meta-analyses concluded that ART cycles are related to poor postnatal consequences with low birth weight (Anderson *et al.*, 2018; Oldereid *et al.*, 2018; Magnus *et al.*, 2018).

The epigenetic modifications in paternal genome reprogramming remain at some point in embryogenesis, neonatal and postnatal pheno & genotype. Paternal exposures to environmental insult that could be due to diet, lifestyle, and different exposures during spermatogenesis, can result in irreversible epigenetic and phenotypic modifications and the following generation expressed its consequences. Adipocyte altered microRNAs expression in sperm leads to a histone modification and DNA methylation, embryogenesis genetic promoter with histone H3 occupancy leads to formation lysine four on histone H3 (H3K4me1) that is similar to the theory that leptin gene incorporates into the neonates of the obese father (Ma *et al.*, 2020; Linabery *et al.*, 2013; Davidson *et al.*, 2015; Terashima *et al.*, 2015). Our study highlighted the impact of men's age on impaired sperm quality and sperm chromatin dispersion and compaction. Our analyses found no influence of male age on sperm morphology, motility and concentration. It may be attributed to the patient enrollment in the present study as these are patients seeking fertility help. Moreover, we looked into the relationship between male age and oxidative stress levels. ROS production

and levels of antioxidant enzymeimbalance result inmpaired male fertility potential. There are contradictory results on the relationship between levels of ROS production in semen with advanced male age. Few studies found a positive relationship (Leisegang *et al.*, 2017), while otherss found no relationship between male age and ROS higher production (Nikitaras *et al.*, 2021; Darbandi *et al.*, 2019; Alshahrani *et al.*, 2014). The present study found no link between male age with ROS production and no difference in ROS levels in all age groups.

Paternal age and reproductive hormone levels association analysis was done. Circulating androgen hormones linked directly with sperm quality parameters and reproductive hormones imbalance leads to impaired spermatogenesis and leads to male poor sexual health (Pizzol *et al.*, 2014; Zhao *et al.*, 2020). The current study found no correlation between male age and reproductive hormone concentration (Alshahrani *et al.*, 2014). In the current study, we discovered a link between sperm DNA damage and increasing male age (SCD)(Gu *et al.*, 2018). The current findings are consistent with other studies that came to the same conclusion after using various sperm damage measuring tools, such as the sperm chromatin structure assay, single cell gel electrophoresis (COMET), and TUNEL assay (Winkle *et al.*, 2009; Evenson *et al.*, 2020). A study found a link between paternal age and sperm DNA damage in oligoasthenoteratozoospermia (OAT) (Plastira *et al.*, 2007), but not in the control group (Fainberg and Kashanian, 2019; Belloc *et al.*, 2014). While some studies found no difference in sperm DNA damage with increasing male age (Darbandi *et al.*, 2019), others did (Rubes *et al.*, 2021; Rybar *et al.*, 2011). Paternal age harmed sperm chromatin integrity and condensation, resulting in a higher percentage of immature sperm CMA3 (Rybar *et al.*, 2011; Deenadayal Mettler *et al.*, 2020). There is very little literature on the influence of advanced male age on sperm chromatin packaging in humans, despite previous data suggesting that advancing human male age is associated with increased sperm chromatin damage. In the current study, older men have a higher risk of sperm DNA damage and chromatin de-condensation than younger men. These findings are consistent with previous studies that have shown decreased CMA3 staining in older age (Deenadayal Mettler *et al.*, 2020). The increase in CMA3 staining would most likely be explained by a decrease in protamination or a problem with protamines caused by low thiol levels. This would raise the histone to protamine ratio, which is responsible for male subfertility.

Immature spermatozoa shedding from the seminiferous tubes and abnormal protamine dephosphorylation are two alternative hypotheses for the etiology. As a result, we can conclude that having older fathers harms the molecules that make up motile spermatozoa. Given that sperm DNA is well protected due to chromatin condensation, which is required during sperm transit in the female reproductive system, and that epigenetic reprogramming can be manipulated at some point during the pre-implantation period, an increase in paternal age could result in impaired sperm chromatin integrity, making spermatozoa's genetic material vulnerable to external environment insult. It is believed that highly-hierarchical epigenetic changes occur in the paternal genome after fertilisation, which include the dissolution of the sperm nuclear envelope, decondensation of the genetic material via the breakage of disulfide bridges among protamines, substitution of maternal histones for male protamines, and genetic material rearrangement.

In summary, our examination established paternal overweight and age as independent risk components for sperm DNA damage, and chromatin condensation and impacts the reproductive health at pre and post-embryological stages of development. The normal weight of female and male partners before in-vitro fertilization is sagacious to increase the quality of gametes, fertilization rate and ART outcome. This finding needs to be confirmed through future large prospective studies. Some limitations linked with paternal BMI study are a less number of couples, and not included female factors in the analysis to determine the true influence of the sperm characteristics on ART outcome, as females mask the sperm contribution to embryo development. The age-related data is limited in a way that it does not include other confounding factors, such as familial histories and other old age diseases. A follow-up cohort research of older and younger male ART patients is advisable to examine the impact of paternal increasing age on semen chromatin packaging. Moreover, future studies should focus on the association between paternal overweight and advanced age in subfertile couples with idiopathic subfertility, because still debatable to consider paternal weight and age can be used for subfertility prognosis. This study has the limitation that examination has insufficient statistics against maternal parameters, diet, gestational diabetes, term or preterm delivery, mode of delivery, etc. Warranting limitation of the study a future cohort study of overweight and normal-weight, old age and young ART patients

is recommended to analyze the effect of maternal and paternal weight gain and advancing age on pre and postnatal parameters after assisted conception.

Conclusion

Despite potential limitations, this is one of few studies with extensive information on the potential risk of paternal overweight and advanced age on sperm epigenetics and fertility outcomes. Our record also provides evidence that paternal overweight is one of the factors contributing to subfertility. We found normal weight as a predictor of better ART success in terms of fertilization, and cumulative live birth rate (CLBR). Consideration of paternal BMI measurement in couples opting for ART treatment and recommendation to lose weight before undergoing in-vitro fertilization procedures to improve quality of sperm, and oocyte, better fertilization rate and CLBR post-in-vitro fertilization. The current study also shows that, in comparison to younger patients, older individuals have decreased concentration of spermatozoa with normal morphology. Similarly, older men had worse chromatin integrity of spermatozoa as shown by toluidine blue (TB+) staining, considerably greater levels of immaturity (CMA3+), and a higher percentage of DNA Fragmentation % SCD. One of the elements affecting fertility is the age of the male partner. As a result, it is recommended that subfertile couples seek ART at a younger age.

In summary, our study demonstrated that proper protamine content and sperm chromatin integrity are important for successful fertilization and outcome in couples with male factor subfertility. Our data suggest paternal age and BMI are independent risk components for sperm DNA damage, and chromatin condensation and impacts reproductive health that could alter the pre and post-embryological stages of development. This finding needs to be confirmed through future large prospective studies. Female age and BMI kg/m^2 significantly negatively correlated to fertilization rate, cleavage rate and outcome rate. The sperm chromatin maturity and DNA integrity assessment included routine semen analysis for couples with male factor subfertility and could be considered before ART.

CHAPTER 4

Comparison of Magnetic Activated Cell Sorting (MACS) With Conventional Sperm Preparation Techniques for the Improvement of Semen Quality and Assisted Reproductive Outcome

ABSTRACT

Sperm separation plays a critical role in assisted reproductive technology, based on migration, density gradient centrifugation, and filtration and a properly selected sperm could help in increasing assisted reproductive outcomes.

The current study aimed to assess the prognostic value of four sperm selection techniques i.e., density gradient centrifugation (DGC), Swim-up (SU), DGC-SU, or DGC followed by magnetic-activated sperm selection (DGC-MACS), on s spermatozoa functional parameters in whole studied couples (n=753).

In another set of experiments subfertile couples, underwent an exclusively intracytoplasmic sperm injection (ICSI) procedure, with an isolated teratozoospermia in the male partner (n=385). Semen samples were prepared by one of the sperm preparation techniques in selected couples and sperm characteristics were assessed following the preparation of spermatozoa. Percent improvement in normal mature spermatozoa percentage, fertilization, cleavage, pregnancy and live birth rate were assessed in couples with isolated impaired semen parameter's male partner.

The normal morphology of spermatozoa, spermatozoa DNA fragmentation (SDF), and chromatin (CMA3) content with DGC-MACS preparation were better compared to the other three. Embryo cleavage, clinical pregnancy, and implantation were improved, in the DGC-MACS than in the other tested techniques. A better percentage of the live birth rate was observed in the DGC-MACS group. Sperm selection through the DGC-MACS preparation technique enhances cleavage, pregnancy, and implantation percentage in infertile couples with teratozoospermia men undergoing assisted reproductive technique (ART).

DGC-MACS technique helps in the selection of an increased percentage of normal viable and mature sperm with intact chromatin integrity. Similarly, non-apoptotic spermatozoa selection in teratozoospermia utilizing the DGC-MACS technique is safe for improving the assisted reproductive technique success rate.

INTRODUCTION

During the last two decades' advancement within the management of subfertility, the appliance of ART has become a therapeutic option for handling male and female subfertility (Skakkebaek *et al.*, 2006; Simopoulou *et al.*, 2016). Nonetheless, the current success rates of ART remain subnormal (Okun *et al.*, 2014). The development of an embryo following the fertilization of oocytes with bad-quality sperm from subfertile males is one of the most important factors of ICSI success. (Engel *et al.*, 2018; Esteves *et al.*, 2011; Lewis, 2007). Failure of the ART process keeps on triggering the urge to refine sperm separation procedures and new sperm selection tools to boost the outcome; embryo quality, implantation, and pregnancy (McDowell *et al.*, 2014; Lewis, 2007; Candela *et al.*, 2021; Hernandez-Silva *et al.*, 2021; Shi *et al.*, 2021). The semen preparation technique's primary goal enhances the quantity of live, fast swimming, mature compacted chromatin and functional sperm able to normally fertilize the oocyte. DGC and swim-up for sperm selection are the most frequently used techniques, routinely employed for sperm enrichment with fast swimming and improved morphology which depends on centrifugation and migration of the sperm (McDowell *et al.*, 2014; Ayad *et al.*, 2021). These methods rely on selecting sperm with higher motility while ignoring their molecular characteristics. The impact of the aforementioned techniques have not been evaluated enough on invisible anomalies i.e, apoptosis and programmed cell death-like phenomena, DNA damage, sperm chromatin, membrane maturation, ultrastructure, and assisted reproductive technique (ART) outcome (Sharma *et al.*, 2015; Tavalaee *et al.*, 2017). Both DCG and swim-up procedures are not designed to effectively select sperm without DNA damage, intact chromatin, and non-apoptotic sperm. In addition, the subsequent selection of active sperm in ICSI is the primary selection by an embryologist in terms of sperm motility and best morphology, thus ICSI circumvents natural barriers of defense which lead to the fertilization of oocytes with chromatin defective sperm (Simopoulou *et al.*, 2016).Apoptosis process molecular features of spermatozoa which are linked with male reproduction, attained importance in current years (Štiavnická *et al.*, 2017; Chen *et al.*, 2006). Numerous studies have shown the importance of systemic cell death (apoptosis) in

sperm, which may be linked with a lower rate of pregnancy and implantation with assisted reproduction (Mahfouz *et al.*, 2009; Engel *et al.*, 2018; Tavalaee *et al.*, 2015). The Ejaculated human sperms have been shown to exhibit phosphatidylserine (PS) translocation or externalization which is involved in two processes 1) in apoptosis and 2) during fusion of two membranes, i.e. during the process of capacitation or/and at the time of fertilization involves acrosome reaction and sperm/oocyte binding (Romany *et al.*, 2014; Espinoza *et al.*, 2009; Tavalaee *et al.*, 2012). In necrotic cells, membrane integrity disruption cause PS accessible and serve as a trigger to initiate apoptosis. MACS a process for sperm selection simply and effectively utilize nanobeads, nanoparticles, and magnetic beads that serve as conjugated proteins or antibodies to select sperm cell. Annexin-V binding has a high affinity to PS in sperms cells with disrupted membrane integrity, which is one of the reasons for sperm's low fertilization capacity (Fry *et al.*, 1992; Dirican *et al.*, 2008; Tavalaee *et al.*, 2012; Iftikhar *et al.*, 2021). This method is practiced along with the traditional methods of sperm selection using the gradient centrifugation (DGC) and swim-up (SU) methods (Pacheco *et al.*, 2020; Baldini *et al.*, 2021). The MACS along with DGC and SU improves sperm viability, and maturation, and reduces aneuploidy in sperm and apoptosis (Cakar *et al.*, 2016). A contradictory finding has been found about the use of MACS in ART and its success rate(Gil *et al.*, 2013). Subfertility is a medical and social issue, both in terms of magnitude and impact on well-being (Ombelet *et al.*, 2008). Male factor subfertility is attributed to 40-50% of subfertility (Leung *et al.*, 2018; Rumbold *et al.*, 2019). With the introduction of assisted reproductive techniques of in-vitro fertilization and intra-cytoplasmic insemination, subfertility treatment success depends on the quality of both gametes, fertilization, zygote formation, development, and implantation potential (McDowell *et al.*, 2014; Lepine *et al.*, 2019; Cheles *et al.*, 2020). According to WHO 2010 standard semen analysis includes sperm count, motility, and morphology which help in determining the treatment options and chance of spontaneous conception (Topp *et al.*, 2015; Cooper *et al.*, 2010). Mature human sperm nuclei are tight-packed due to histone 85-90% percent replacement with positively charged protamine (Amor *et al.*, 2019; Rosenborg, 1990; Xie *et al.*, 2018). Compacted, hydrodynamic, and organized sperm chromatin is essential for epigenetic regulation and protection of the sperm DNA from external insult. Routine semen assessment doesn’t include sperm chromatin assessment

(Gosálvez *et al.*, 2014). Chromomycin A3 (CMA3) staining method used for the evaluation of chromatin condensation highly CMA3 fluorescence test indicated low DNA protamination and poor chromatin packaging (Ahmed *et al.*, 2018). CMA3 stain was high in spermatozoa of subfertile men (Amor *et al.*, 2019). Spermatozoa selection has shown a correlation with fertilization potential, implantation, and pregnancy outcome after assisted conception(Ahmed *et al.*, 2018). Most researchers are working on the improvement of sperm selection techniques, while most selection techniques are mainly based on the selection of motile sperms with intact chromatin (Chohan *et al.*, 2006; Rosenborg *et al.*, 1990). Recent data suggest that an improved assisted reproduction success rate could be achievable through the separation of spermatozoa with increased chromatin condensation and intact DNA (Timermans *et al.*, 2020). While poor chromatin condensation incident measurement seems helpful in choosing the appropriate technique between in-vitro fertilization and Intracytoplasmic injection (Gosálvez *et al.*, 2014; Chohan *et al.*, 2006). Multiple techniques have been used to measure the extent of sperm DFI of the raw and prepared semen sample (Pizzol *et al.*, 2014; Hammadeh *et al.*, 2001; Chohan *et al.*, 2006; Agarwal *et al.*, 2017; Agarwal and Said, 2003). SCD assay based on DNA de-condensation which relates to the observation of sperm nucleoids with DNA fragmented chromatin formed small halos/nondispersed while highly condensed chromatin form large/ distinct halos (Liffner *et al.*, 2019; Fernandez *et al.*, 2005). Direct observation of halo indicating DFI after staining and evaluation could be done under a bright-field or fluorescent microscope (Liffner *et al.*, 2019; Zeqiraj *et al.*, 2018; Fernandez *et al.*, 2005). Many previous studies have shown better quality yield comparing two common methods of swim-up and density gradient centrifugation (Zhang *et al.*, 2011; Chen and Bongso, 1999). Data on separation of intact chromatin sperms utilizing different techniques have been shown inconclusive results, with some studies reported after processing decreased levels of sperm DNA damage, while other studies show varying levels of fragmentation depending on the separation technique (Twigg *et al.*, 1998; Saylan and Erimsah, 2019; Karimi Zarchi *et al.*, 2020; Said and Land, 2011). Few of the previous studies includes magnetic-activated cell sorting (MACS) and *invitro* sperm storage at room temperature or 37 degrees centigrade for 24 hours and to find cheap, effective, and short time storage methods(Nadalini *et al.*, 2014; Said *et al.*, 2008; Pacheco *et al.*, 2020). However, few

studies did not notice a significant difference between MACS and DGC/SU semen preparation methods for fertility outcomes. This variability can be attributed to large variance with the less participant in studies (Said and Land, 2011; Romany *et al.*, 2017; Romany *et al.*, 2014). Previous literature did not look at the effectiveness of MACS in subfertile with normal and increased SDF levels and the relative efficiency of the different semen preparation processing procedures for improving sperm DNA quality, chromatin maturity along with ART outcome. Based on these inconclusive results about the use of the MACS approach to ART, an ideal sperm preparation method for sperm selection is yet to be determined. The sperm chromatin dispersion assay (SCD) to measure SDF and CMA3 to determine chromatin integrity, we evaluated the MACS technique with the classic sperm preparation methods, to find the ideal method of sperm selection and tried to build its relationship with improving semen parameters.

To our knowledge this is the first study in which four groups were analyzed i.e., DGC, SU, DGC-SU, and DGC-MACS, in male subjects with teratozoospermia and men with increased (≥ 20%) and normal value (< 20%) SDF a threshold value determined before (Sergerie *et al.*, 2005), to determine the impact of different sperm selection techniques in improving the number of spermatozoa with mature and intact DNA, condensed chromatin, viability, and to further assess the of the effect of sperm preparation techniques on the ICSI cycles success, percentage of fertilization, cleavage rate, pregnancy rate and live birth rate. By using the sperm chromatin dispersion assay (SCD) to measure sperm DNA fragmentation and CMA3 to determine chromatin structure, we evaluated sperm preparation methods, to find the best method of sperm preparation and to evaluate comprehensively the impact of multiple semen separation techniques on sperm DNA fragmentation and chromatin condensation.

MATERIALS AND METHODS

Subjects

This prospective study included 753 couples who underwent IVF/ICSI procedures out of which 604 couples were involved in the ICSI/IVF program at Fertility and Genetic services, Islamabad, Pakistan, from April 2016 to October 2021. The study population involves fertile 146 and 607 subfertile men.

Ethical Compliance

The institutional review board of Quaid-i-Azam University authorised the research proposal, and the ethics committee of the SKMC Islamabad Pakistan awarded its approval. The subject's detailed information (brief medical history, including male and female ages, male body mass index (BMI), period of subfertility, primary/ secondary subfertility, and information about earlier spontaneous abortions -related data) was obtained through a questionnaire, asking for the appropriately structured question by face to face interview.

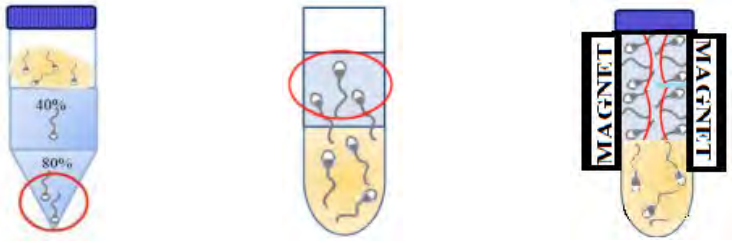
Inclusion and exclusion criteria

A complete physical evaluation was performed, including an assessment of scrotal size to rule out cryptorchidism and malformations of the external genitalia; a doppler assessment to rule out varicoceles; an immunobead binding evaluation to rule out the existence of anti-sperm immune cells; and genetic fingerprinting to rule out the chronic illnesses such as liver/renal disease, patients who are extremely obese, patients who have hyperglycemia were excluded.

There was no subfertility factor in the female partner of the couple included in this study. The semen sample was subjected to analysis for seminal characteristics and the blood sample was drawn for hormonal determination. Fertile males were those without any history of fertility problems and within one year of unprotected intercourse, their partners had spontaneous pregnancy. The fertile and subfertile couples were recruited from assisted conception unit-fertility genetics services (Salma & Kafeel Medical Services) Islamabad.

Experimental Design

Patients' semen samples (n=385) were divided into four groups. Group 1; DGC semen preparation techniques. Group 2; Swim-up SU. Group 3; DGC followed by SU. Group 4; DGC-MACS the direction of sperm movement was illustrated in Figure 57. As a standard clinical practice, the MACS selection is mostly recommended in a clinic for subfertile men with low sperm count, poor sperm morphology, and higher SDF >20%, the reason for the basic characteristics of the MACS group participants differ from other groups.



Density gradient **Swim-up** **MACS**

Figure 57. Preparation methods for sperm selection and selected sperm sample tested for total motile sperm (TNMS), ROS, HOS, SCD, CMA3+ and TB+

Sperm Selection Technique Density-gradient centrifugation (DGC) technique

Sperm Grade (Vitrolife, Gothenburg, Sweden) was diluted in medium G-Mpos Plus (Vitrolife, Gothenburg, Sweden) to generate dilutions of 45 and 90 percent density for density gradient centrifugation (DGC). In 15ml falcon tubes, two 90 percent and 45 percent columns were created by layering 1-1.5 mL of each solution, commencing at the bottom with the 90 percent fraction. 1 mL of neat sample stratified as the top layer of columns and centrifuged at 300 g for 15 minutes. The pellet was collected after centrifugation and washed once at 350 g for 10 minutes.

Swim-up (SU) technique

For the semen swim-up procedure, the semen sample was used without centrifugation, sample, 0.5-1.0 ml, was layered gently under 0.5 ml of G-Mops plus (Vitrolife,

Gothenburg, Sweden) and incubated for 1 h at 37ºC. The final fraction includes only the topmost 0.25ml fraction which was collected gently into a new tube.

DGC-SU technique

For the DGC-SU procedure, the sperm sample is prepared first with the DGC technique as mentioned above section. The final 0.25ml pellet was layered gently under 0.5 ml of G-Mops plus *(*Vitrolife*, Gothenburg,* Sweden) and incubated for 1 h at 37ºC. The final fraction includes only the topmost 0.25ml fraction which was collected gently into a new tube.

DGC-MACS technique

The samples were subjected to a non-apoptotic selection technique using the MACS ART Annexin V Reagent (Madison, CT, USA) and following the manufacturer's instructions. Cells obtained after density gradient centrifugation were centrifuged at 300 g for 5 minutes. The cells were resuspended in 90 ml of the binding buffer after the supernatant was removed. The sperm suspension was then incubated for another 20 minutes and then was put into another column where got attached to the magnet after being washed with 2 mL of binding buffer (Madison, CT, USA). The apoptotic sperm were maintained in the separation column, while the non-apoptotic sperm in the negative fraction was passed collected in a tube after going through the column. Finally, after discarding the apoptotic sperm fraction, the non-apoptotic sperm fraction was centrifuged. Each prepared fraction was divided into 2 aliquots and used for the analysis of sperm parameters, vitality, reactive oxygen species, and sperm DNA damage as chromatin condensation after a fraction was used in ICSI.

***Invitro* fertilization (ICSI)**

After a long protocol as per clinic policy and hormone analogs stimulation, patients were given exogenous gonadotropins (Gonal-F RFF; EMD Serono inc., Rockland, MA) to stimulate their ovaries. At 34–36 h after human chorionic gonadotrophin (HCG) (IVF-C LG Chem Life Sciences)administration, oocytes were collected transvaginally under ultrasound guidance and cultured in fertilization medium (G1/G2 Vitro life Gothenburg, Sweden)with 5% human serum albumin (HSA) in a 5% CO2/O2 humidified tri gas

atmosphere at 37°C. ICSI was used to inseminate the oocytes. Oocytes were scored 16–18 hours post-ICSI based on the appearance of two PNs. Individually fertilized oocytes were cultured and scored 40, 62, 88, and 112 hours after insemination. The embryos on day 3 were graded based on percentage and type of fragmentation, as well as the number and morphology of nuclei and blastomeres

Statistical Analysis

For the statistical analyses, we used the Statistical Package for the Social Sciences (IBM SPSS software, version 20). Differences between treatments were analyzed using the analysis of variance statistics and Turkey's test for means. Student t-test was applied between two groups analysis. Data are presented as mean±SEM. Wilcoxon signed-rank test was used to access the significance of difference post preparation non-parametric Friedman test for paired samples was applied to assess the efficacy of the preparation group over others. The reliability of the prediction produced by the model was statistically tested by the Hosmer-Lemeshow goodness-of-fit test.

RESULTS

Demographic characteristics of fertile and subfertile male categories

There was no significant difference in demographic characteristics (mean male age, body mass index (BMI), and duration of subfertility, of all four sperm preparation groups DGC, SU, DGC-SU, and DGC-MACS (Table 43) in the whole studied (subgrouped as a fertile and mild male factor (MMF) and severe male factor (SMF) couples.

Neat semen sperm standard and quality parameters

Sperm count, normal morphology, total motile sperms (TNMS) in the neat ejaculated semen, the levels of white blood cells (WBC), reactive oxygen species (ROS), sperm parameters viability (HOS %), percent sperm DNA fragmentation (SCD) levels, and percent protamination (CMA3) levels were comparable between all study groups ($p>0.05$)

Post sperm selection technique improves standard and quality parameters

Assessment of the efficacy of DGC-MACS in improving semen parameters post preparation was compared with all three preparation techniques (DGC, SU, DGC-SU) in improving the mean percentages of sperm ROS, HOS, SCD and protamine shown in Table 43. There was a non-significant improvement in HOS% and a significant ($p<0.00$) decrease in ROS% ($p<0.00$), SDF % ($p<0.00$), and CMA3% ($p<0.00$) in DGC-MACS prepared sperms when compared with the other sperms preparation techniques (Table 43). A significant ($P<0.01$) improvement in percent total motile sperm (TNMS) Figure 45 after DGC-MACS (76±26%) was obtained compared to DGC; 67±23%, SU; 68±28%, and DGC-SU; 64±26%. The CMA3 levels (immature sperm concentration) after preparation in DGC-MACS were 21.9±7 and significantly ($p=0.00$) lower compared to DGC; 27.5±10, SU; 28.1±11, DGC-SU; 27.3±10.4, as shown in Figure 58 a & b. in the whole studied fertile and subfertile (MMF and SMF) couples.

Influence of sperm preparation technique in fertile and subfertile male categories:

All preparation techniques yielded significantly ($P<0.05$) higher proportions of motile and morphologically normal spermatozoa when compared with the neat sample in fertile and subfertile male categories. A significantly higher number of total motile sperm were

obtained after MACS compared to DGC, SU and DGC-SU techniques in the fertile and SMF subjects. MMF and SMF male patients had significantly (P<0.00) reduced total motile sperm compared to fertile subjects after all preparation techniques.

Assessment of ROS and vitality:

Compared to the neat sample significantly (P<0.05) reduced ROS with improved vitality were observed after all preparation in SMF, MMF and fertile male subjects. After all preparation methods, there was a significant (p<0.05) reduction in ROS and improvement in sperm vitality was observed in all subjects (Table 43). Compared to the fertile subjects there was a significant (P<0.05) higher level of ROS and less viable sperm after preparation in SMF and MMF. After MACS preparation significantly (P<0.05) improved less ROS with more viable spermatozoa obtained in SMF compared to DGC, SU and DGC-SU techniques.

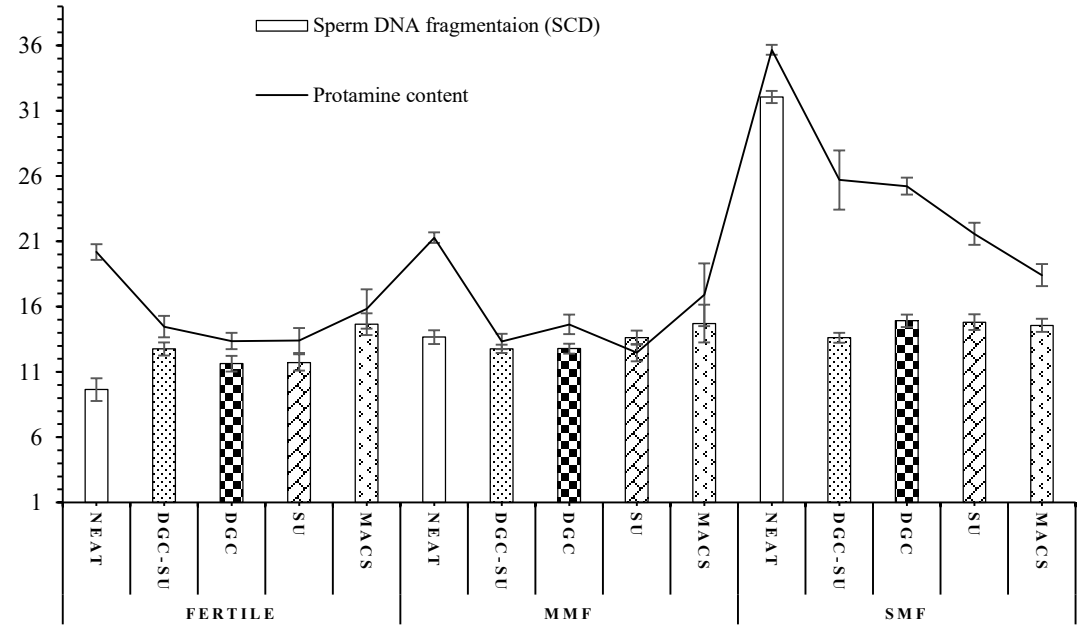
Sperm DNA fragmentation and chromatin condensation (protamine):

Compared to fresh sperm samples percent sperm protamine (CMA3) concentration significantly improved after all preparation of MACS, DGC, SU and DGC-SU groups in the fertile (NZS) and SMF subfertile case (Table 43). Sperm DNA fragmentation (SCD) significantly (p<0.05) reduced after preparation compared to fresh in SMF. The DFI (SCD) and protamine (CMA3) concentration was found to be comparable between SU, DGC, DGC-SU and MACS, and also among the Fertile and MMF groups. DNA fragmentation assessment was improved after the MACS sperm separation technique (Table 43: Figure 58b) in SMF, and a significant (p<0.05) difference was noted among the MACS and SU, DGC, and DGC-SU groups.

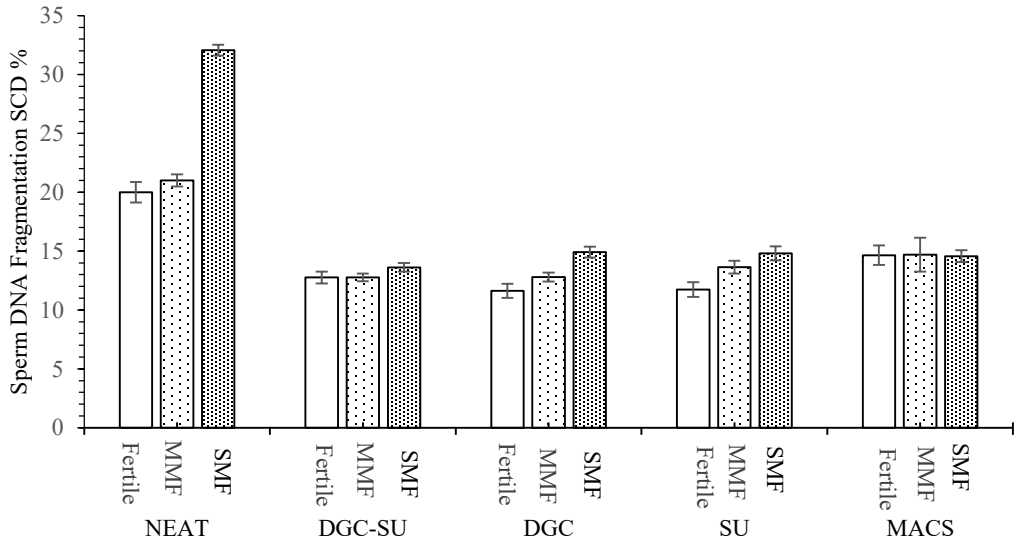
Table 43: Semen quality parameters of the samples analyzed before and after the SU, DGC, DGC-SU and MACS techniques were employed to improve sperm quality in fertile and subfertile male subjects

			DGC-SU (n=284)	DGC (n=237)	SU (n=138)	DGC-MACS (n=94)	Total (n=753)
Fertile (146)	TNMS	Neat	71±3	78±2	79±3	40±2^{d***}	71±2
		Prep	64±4	78±4	78±4	82±4^{d***}	73±2
	SCD	Neat	9±1	6±1	6±2	24±3	10±1
		Prep	13±1	12±1^{d**}	12±1^{d*}	15±1^{c**}	12±0
	Protamine	Neat	20±1	19±1	18±1	25±2	20±1
		Prep	27±1^{c**}	27±1^{c*}	21±1	22±2^{c*}	25±0
	ROS	Neat	1.3±0.1	1.2±0.1	1.1±0.1	1.8±0.2	1.3±0.1
		Prep	0.4±0.1^{c**}	0.3±0.1^{c**}	0.3±0.1^{c**}	0.4±0.1^{c**}	0.3±0^{c**}
	Vitality	Neat	72±2	75±2	76±1	75±5	72±1
		Prep	88±2^{c**}	90±1^{c**}	90±1^{c**}	89±1^{c**}	89±1
MMF (280)	TNMS	Neat	44±2	39±2	44±2	37±7^{d***}	42±1
		Prep	38±3^{a***}	29±3^{a***}	40±5^{a***}	36±7^{a*}	35±2 ^{a***}
	SCD	Neat	13±1	13±1	14±1	24±4	14±1
		Prep	13±0	13±0	14±1	15±1^{c***}	13±0
	Protamine	Neat	21±1	21±1	20±1	26±2	21±0
		Prep	25±1	26±1	27±1	23±2^{a*}	26±0
	ROS	Neat	1.0±0.0	1.0±0.0	1.0±0.0	1.6±0.3	1.1±0
		Prep	0.5±0.1^{c***}	0.5±0.1^{c***}	0.4±0.1^{ac***}	0.7±0.2^{ac***}	0.5±0
	Vitality	Neat	50±2	45±2	48±3	63±6	49±1
		Prep	71±1	67±1^{a***}	70±2^{a***}	73±1	69±1^{a***}
SMF (327)	TNMS	Neat	35±3	41±3	29±3	36±3	35±2
		Prep	38±3^{abd***}	46±3^{abd***}	28±4^{ad***}	78±8^{a*bc**}	40±2^{a***}
	SCD	Neat	33±1	33±1	32±1	32±1	32±0
		Prep	14±0^{c***}	15±0^{abc***}	15±1^{ac***}	15±1^{c***}	14±0^{abc**}
	Protamine	Neat	38±0^{ab***}	37±1^{ab***}	36±1^{ab***}	27±1	36±0
		Prep	33±2^{c**}	31±1^{c**}	32±1^{c**}	22±1^{c*}	29±1
	ROS	Neat	2.6±0.1	2.5±0.1	2.6±0.1	1.8±0.1	2.4±0.1
		Prep	1.4±0.1^{abcd***}	1.2±0.1^{abcd***}	1.3±0.1^{abcd***}	0.6±0^{abc***}	1.2±0.1^{abc***}
	Vitality	Neat	48±2	54±2	43±3	53±2	50±1
		Prep	59±2^{abcd***}	67±2^{abc***d*}	53±3^{abc***}	86±0^{b***c**}	61±1^{ab***}

Values expressed as Mean±SEM. Values in parentheses represent the number of subjects. a= Fertile vs MMF and SMF; b=MMF vs SMF; c= Neat vs Prep; d= MACS vs SU,DGC, DGC-SU *=P<0.05, **= P<0.01, ***=P<0.001



(a)



(b)

Figure 58. (a) Sperm chromatin integrity (SCD) and maturity (Protamine content-CMA3) and (b) Sperm DNA fragmentation before after semen preparation techniques in fertile and subfertile male subjects.

Comparison of MACS with other conventional sperm preparation techniques in isolated teratozoospermia

Demographic characteristics:

The demographic characteristics of the couple are summarized in Table 44. According to the sperm preparation technique. There was a higher percentage of the couple with primary subfertility (65%) and most couples stayed together (88%). Most of the patients had no significant family history. Similarly, there were the most couples with higher annual income and mostly self-employed. Female partner characteristics had been summarized in Table 44. and there is no significant ($p<0.05$) difference in age, BMI, endometrial thickness, and reproductive hormonal levels. Male partner age, BMI, and reproductive hormonal levels were comparable between the preparation groups and subfertile and fertile male subject's categories.

Neat Sperm conventional and quality parameters

The neat sperm characteristics were significant differences ($p <0.05$) between the three insemination groups (Table 45). Semen sample used for intra-cytoplasmic insemination had a significantly ($p<0.05$) lesser percentage of total motile sperm with more ROS production, more sperm DNA fragmentation (SCD, SCSA, AO) and higher protamine deficiency (CMA3+) and chromatin damage (TB+).

Prepared Sperm conventional and quality parameters

The prepared sperm characteristics were comparable between the three insemination groups. Semen sample used for intra-cytoplasmic insemination had a significantly ($p<0.05$) lesser percentage of total motile sperm with more ROS production, more sperm DNA fragmentation (SCD, SCSA, AO) and higher protamine deficiency (CMA3), Table 45.

Table 44: **Male Age, male body mass index (BMI), female age, and anti-mullarian hormone (AMH) in the whole studied population**

	DGC-SU (n=100)	DGC (n=99)	SU (n=92)	DGC-MACS (n=94)	P-Value
Male Age (Year)	34.89±3.9	34.90±4.50	34.9±646	39.07±8.59	0.93
Male BMI (Kg/m2)	25.34±3.46	25.25±3.1	24.5±3.02	25.39±2.98	0.09
Subfertility duration(Year)	9.32±5.38	9.54±5.57	9.24±6.42	10.34±6.16	0.19
Female age(Year)	30.7±5.31	30.86±5.61	31.8±6.08	33.01±5.42	0.28
AMH	3.98±2.94	4±3.70	3.30±3.30	3.6±3.50	0.56
Total Gonadotropin dose	2529±1536	2539±1878	2925±1755	2625±1834	0.92
Stimulation days	15±3	15±2	14.5±2.8	15±2	0.89
Estradiol level on day of HCG	1974±1316	1791±1076	2040±1408	1804±1601	0.38

Values represent mean± SD

Table 45: Sperm Count, volume, WBC/HPF, TNMS (total motile sperms), Normal % (normal morphology), ROS% (reactive oxygen species), HOS % (Hypo osmatic test), SDF% (sperm DNA fragmentation), and CMA3% levels in teratozoospermia male subjects

	DGC-SU (n=100)	DGC (n=99)	SU (n=92)	DGC-MACS (n=94)	P-Value
COUNT mx10^6	22.39±2.76	27.33±9.18	40.00±23.9	30.17±9.44	0.26
Volume (ml)	3.65±1.59	3.95±1.97	3.93±1.52	3.63±1.65	0.33
WBC/HPF	2.89±1.96	2.91±1.79	3.16±1.78	4.49±7.45	0.78
TNMS %	32.25±0.7	43.9±2.5	44.7±2.65	37.40±2.13	0.07
Normal %	2.8±0.68	2.58±0.65	2.4±0.81	2.70±0.93	0.36
HOS %	39.25±27.13	49.09±25.31	51.13±25.9	54.65±19.90	0.79
ROS %	23.86±1.4	29.7±1.91	29.78±1.18	28.95±1.10	1.0
SDF %	25.15±11.8	20.9±13.2	23.1±12.6	25.3±11.7	0.68
CMA3 %	27.5±10.3	28.7±10.7	29.3±103	29.6±12.4	0.45

Values represent mean± SD

Effect of sperm preparation techniques on quality sperm selection, in teratozoospermia men with normal and increased sperm DNA fragmentation

The SDF% of neat semen samples we compared with prepared sperm in all four groups in a cohort of SDF with < 20 and ≥ 20%, showed significant ($p<0.01$) improvement in mature sperm selection (CMA3) post preparation after all semen preparation methods when compared with pre preparations presented in Table 47. This improvement was more after DGC-MACS ($z=-4.92$, $p<0.00$) and ($z=-6.4$, $p<0.00$).

Similarly, CMA3 values in cohort with SDF levels ≥20 % MACS; showed 18.4±5 level, while DGC; 30.53 ±10.1, SU; 27.7±12, DGC-SU; 30.1±10.6 a significant ($p=0.00$) low levels after DGC-MACS preparation, whereas in SDF ≥ 20 group DGC-MACS; 24.5±8, DGC; 28.9±10.8, SU; 28.4±10.4, DGC-SU; 26.06±9.98 no significant difference ($p=0.06$) found. DGC-MACS <20% group showed a significant reduction in the population of chromatin de-condensed (CMA3) immature sperms as shown in Figures 59 & 60.

Effect of sperm selection techniques on assisted reproductive technology cycle parameters in teratozoospermia

The ART cycle parameters and outcome of sperm preparation (DGC, SU, DGC-SU, and DGC-MACS) techniques are summarized in Figure 61. It is a significant ($p<0.05$) increase in cleavage rate in the DGC MACS group. All other parameters are comparable between all preparation groups.

Effect of sperm selection techniques on assisted reproductive technology cycle parameters in teratozoospermia men with normal and increased sperm DNA fragmentation

DGC-MACS group has significantly ($p<0.01$) improved the cleavage rate in both cohorts where SDF was < 20 % and ≥ 20%. While there is no difference in the live birth rate in both cohorts where SDF was < 20 % and ≥ 20% Table 48.

Effect of sperm selection techniques on assisted reproductive technology cycle outcome

Pregnancy rate (DGC-MACS; 52.5±45, DGC; 44.4±49, SU; 35.8±48, DGC-SU; 36±48) was significantly ($p<0.00$) higher in the DGC-MACS group and implantation rate was non significantly better in the DGC-MACS group compared to all other preparation groups as shown in Table 48 and Figure 61.

Table 46: ROS% (reactive oxygen species), HOS % (Hypo osmatic test), TNMS (total normal motile sperms), SDF% (sperm DNA fragmentation), and CMA3% levels in the whole studied population after preparation

	DGC-SU (N=100)	DGC (N=99)	SU (N=92)	DGC-MACS (N=94)	P-Value
HOS %	65.8±25.8	66.0±23.3	65.1±24.7	73.8±17.5	0.253
ROS %	1.4±1.0	1.1±1.02	1.0±1.0	0.53±0.5[abc]	0.00
TNMS%	64±26	67±23	68±28	76±26 [abc]	0.01
SDF %	14.2±3.5	14.7±4.7	14.5±3.9	12.3±4.7[bc]	0.01
CMA3 %	27.5±10.1	29.7±10.48	28.1±11.1	21.9±7.4[abc]	0.00

Values represent mean± SD

Table 47: Results of Wilcoxon signed-rank tests comparing the proportion of SDF (sperm DNA fragmentation) sperm cells found in neat semen samples (T0) and after separation (T1) in the four processing groups populations with < 20 and ≥ 20% SDF.

	CMA3	DGC-MACS	DGC-SU	DGC	SU
SDF<20%	T0-T1	Z =-4.981[b], P=0.000	Z =-3.450[b], P=0.001	Z =-4.806[b], p=0.000	Z =-4.716 [b], p=0.01
SDF≥20%	T0-T1	Z = -6.455, P=0.000	Z=-5.691, P=0.000	Z=-3.660, p=0.000	Z = -3.244 [b], p=0.001

Wilcoxon Signed Ranks Test

Table 48: MII (Oocyte metaphase two), 2PN (2 pronuclei), fertilization rate, D3 EMB (Day three embryo), Cleavage rate, number (N) of embryo transferred, in the four processing groups populations with < 20 and ≥ 20% SDF in the studied population Values represent mean± SEM. MACS vs SU, DGC, DGC-SU

	SDF ≤20 %					SDF >20 %				
	DGC (36)	SU (42)	DGC-SU (32)	DGC-MASC (39)	P-Value	DGC (64)	SU (50)	DGC-SU (55)	DGC-MASC (55)	P-Value
MII	8.2+6.1	8.1+6.3	7.1+4.8	8+5	0.8	7.3+7.1	9+5.6	8.5+56	8.6+5.8	0.7
2PN (Fertilization rate %)	5+4.4 (66)	5.7+4.5 (73)	5.1+4.3 (69)	5.7+4 (70)	0.5	5.1+4.8 (73)	6.1+4.4 (74)	5.3+3.9 (68)	5.8+4.2 (58)	0.7
D3 EMB (Cleavage rate %)	3.4+3.1 (79)	4.4+3.6 (81)	4.3+4.02 (85)	5.1+3.4 (93)	0.3	4.08+4.4 (85)	4.0+3.1 (75)	3.6+2.6 (77)	4.5+2.7 (87)[c]	0.1
No Embryo transferred	2.3±0.7	2.4±0.7	2.4±0.88	1.4±0.75	0.7	1.8±0.7	2.3±0.76	2.1±0.86	1.6±0.8[bc]	0.00
implantation rate %	21±42.5	35.7±49	19±40	36±45	0.2	35±48	28±46	34±48	38±42	0.9

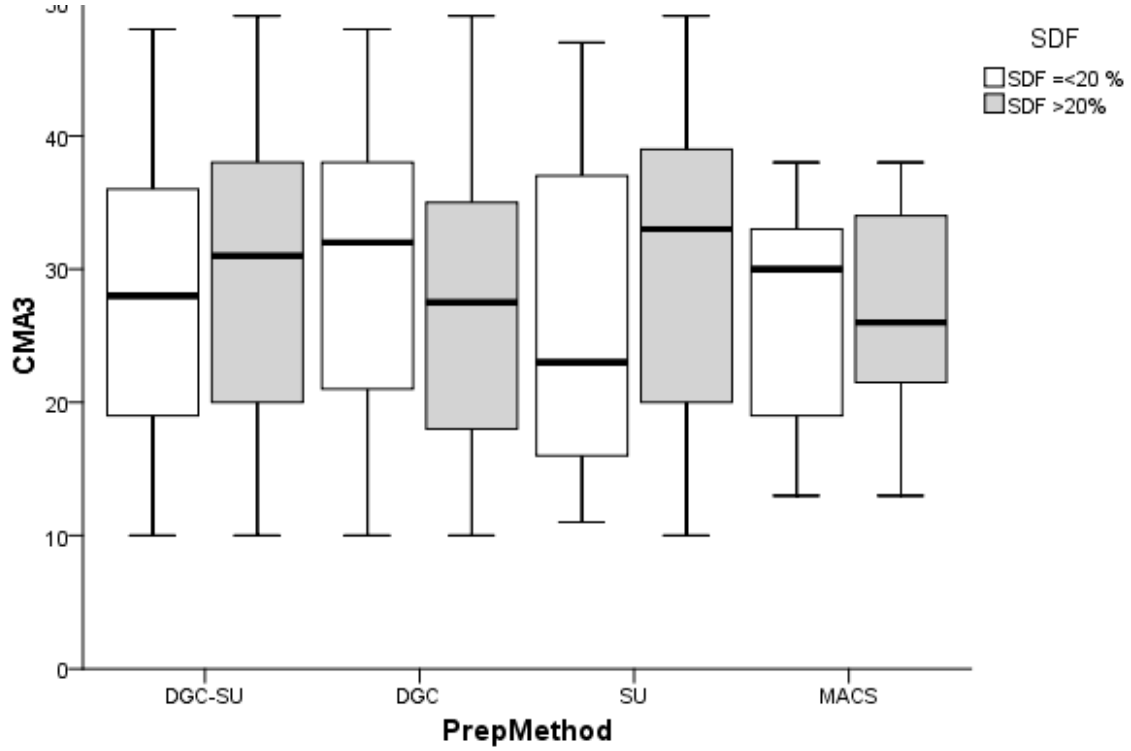
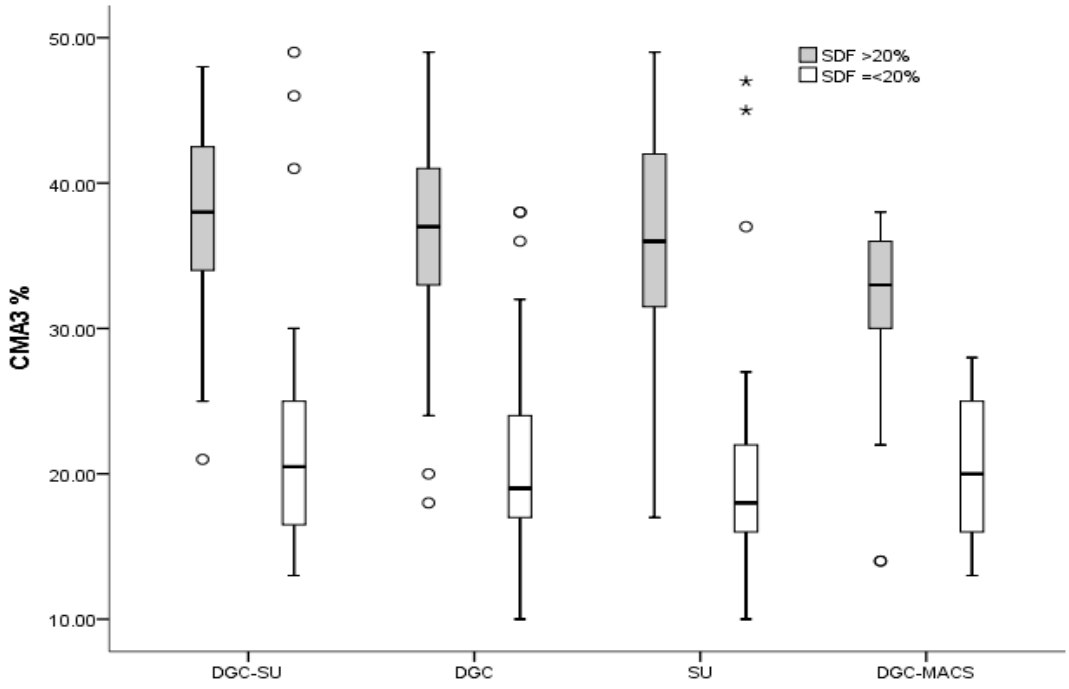
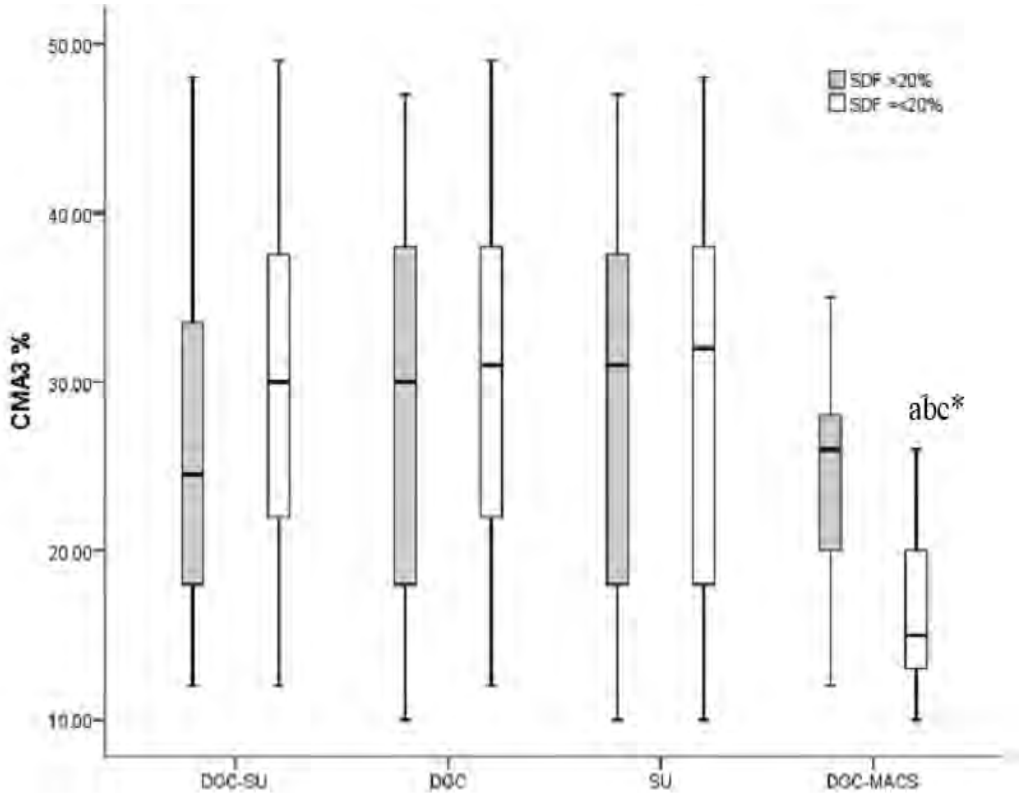


Figure 59. Values of CMA3+ before and after selection by DGC-SU, DGC, SU, and MACS all individual semen samples with < 20 and ≥ 20% SDF



(a)



(b)

Figure 60. Values of CMA3 (a) before and (b) after selection by DGC-SU, DGC, SU, and DGC-MACS all semen samples with $\leq$ 20 and $\geq$ 20% SDF

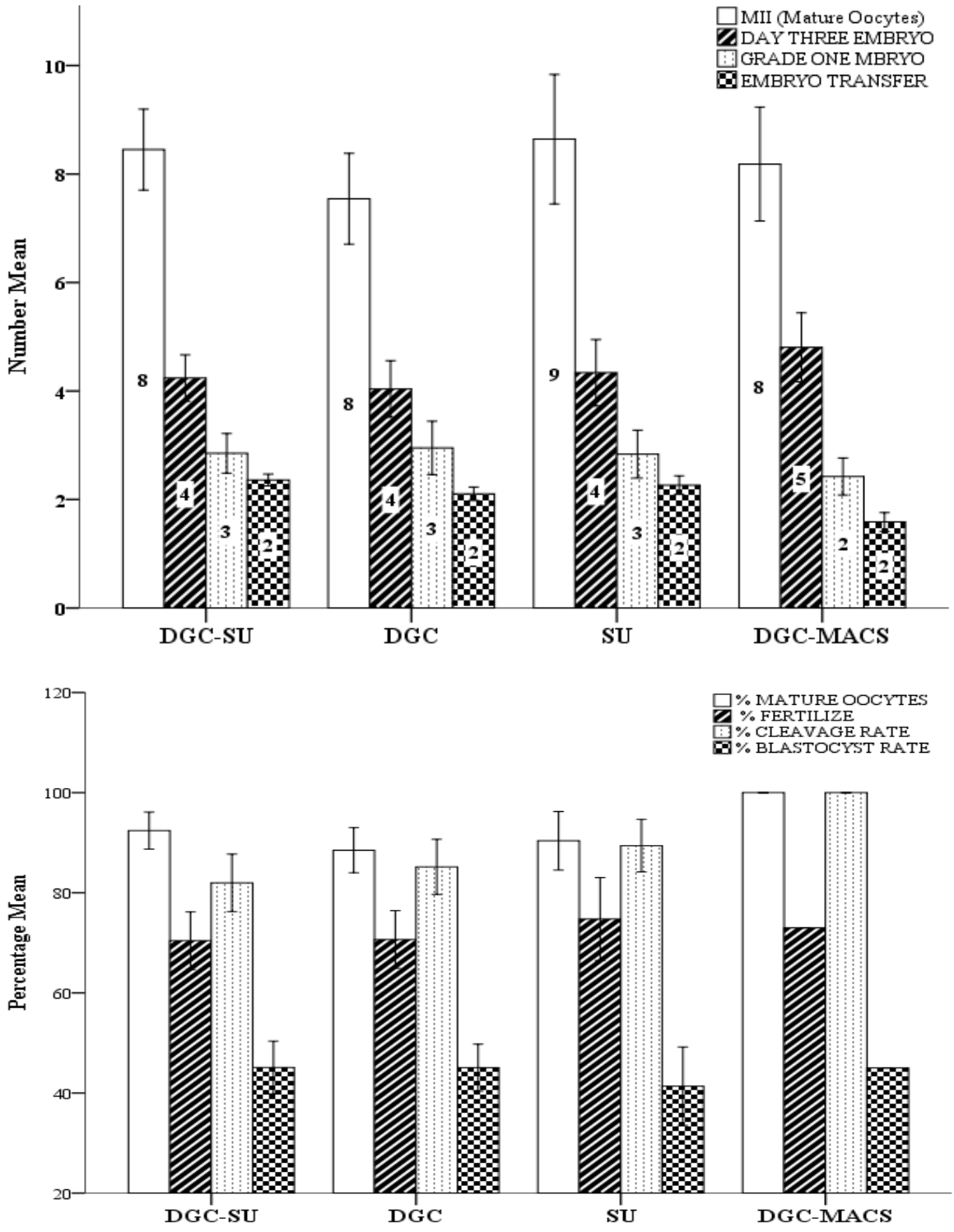


Figure 61. Assisted conception outcome parameters mean number of (a) MII, 2PN (Zygote), Da three (D3) embryo, grade-I D3 embryo, number of embryo transfer (b) mean percentage of maturation rate, fertilization rate, cleavage rate, and blastocyst rate in the four processing groups populations in the teratozoospermic men

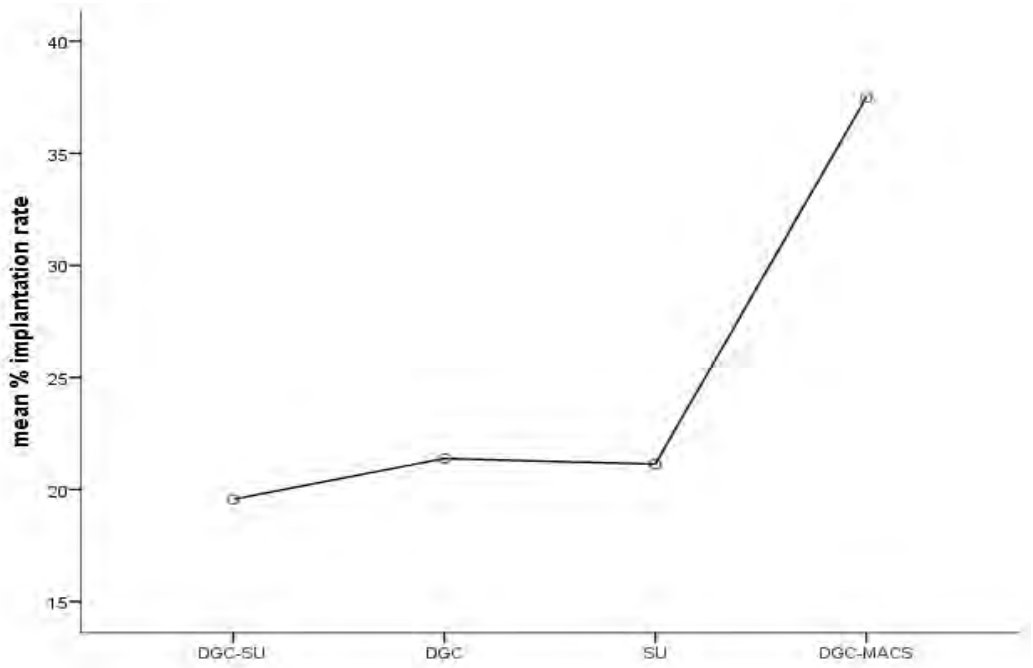
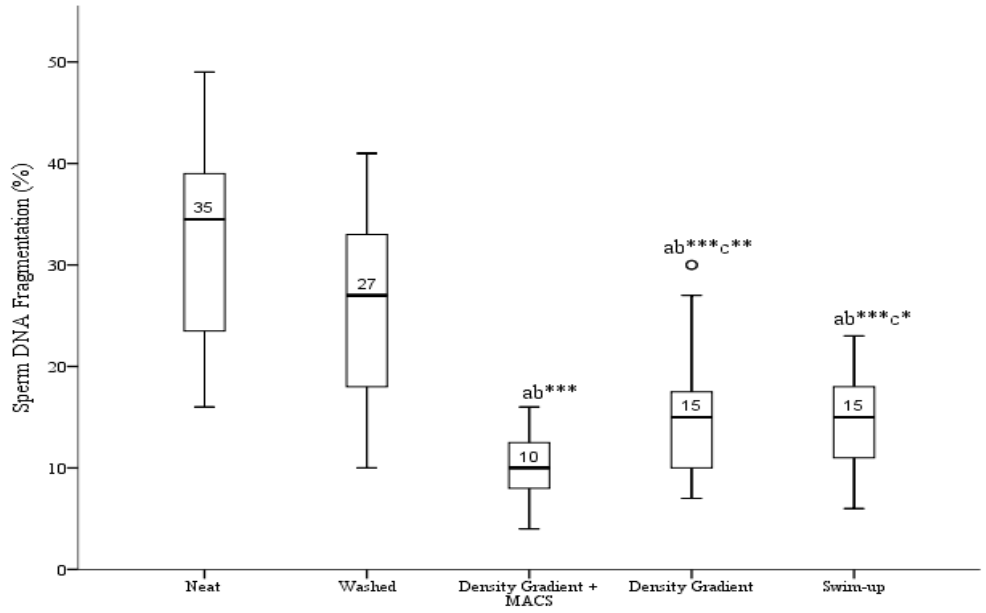


Figure 62. Values of mean clinical implantation rate after selection by DGC-SU, DGC, SU, and DGC-MACS all individual

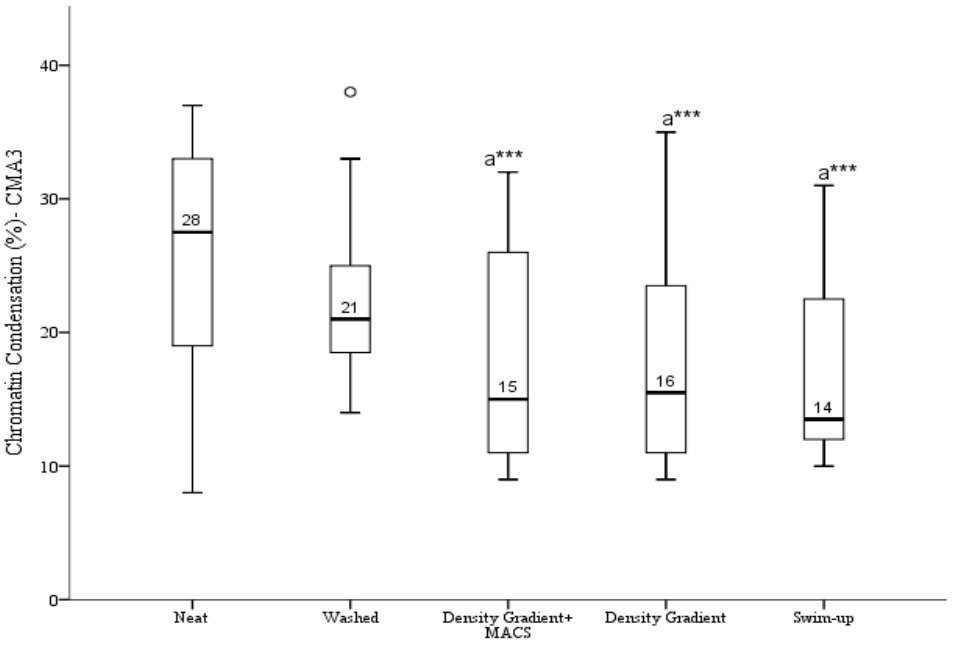
Experiment II

Effect of Different Sperm Separation Techniques and Storage Temperature On Sperm Chromatin Integrity:

The neat sample had a percent sperm chromatin dispersion level of 16.6±0.7 and a level of chromatin condensation (CMA3) percent level of 30.2±1.1. DNA fragmentation soon after sperm preparation techniques showed significantly ($p<0.05$) lower after swim-up (14.9±0.60), density gradient (15.1±0.95), and density gradient with MACS (10.2±0.5) than neat (32.5±1.4) and washed (25.3±1.3), density gradient plus MACS had lower DNA fragmentation level than swim-up ($p<0.05$) and density gradient ($p<0.01$). Sperm condensation percent levels in the neat sample were (28%) and immediately after processing of washed sample (21%) was lower compared to swim-up (14%), density gradient (16%), and density gradient +MACS (15%) (Figure 63). Sperm DNA fragmentation level after 24 hours at room temperature (RT) and 37ºC was recorded for each preparation and there were significantly ($p<0.05$) lower levels of percent DNA fragmentation in density gradient+ MACS preparation after both incubation RT (24%) and 37ºC (14%) compared to washed (35%) at RT and (35%) at 37ºC. while there was no significant difference between washed levels and Swim-up DNA fragmentation level at RT (36%) and (25%) 37ºC, and density gradient at RT (30%) while there was significantly less DNA damage level in DGC fraction (15%) at 24 hours incubation at 37ºC compared to washed and swim-up. While in all preparation there was a significant ($p<0.05$) lesser DNA fragmentation level at 37ºC after 24hour when compared to RT for 24h Figure 64a. Chromatin condensation levels after 24hr at RT and 37ºC were comparable in all preparation methods Figure 64b. Normal motile sperm (NMS) recovered after density gradient was significantly better than all processing techniques shown in Figure 65.



(a)

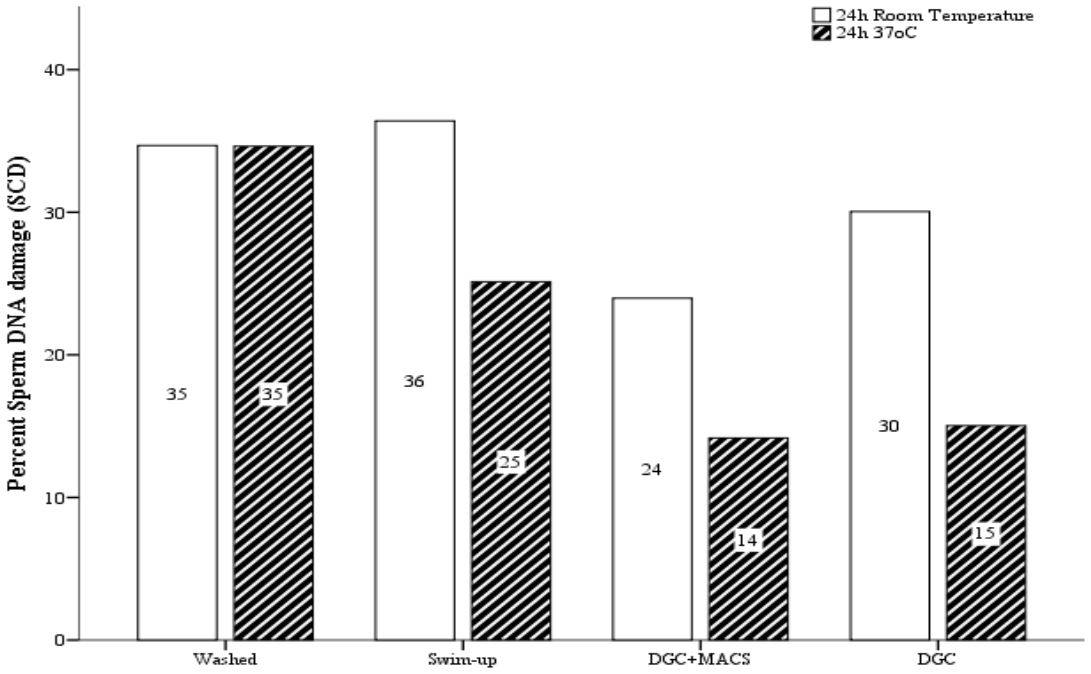


(b)

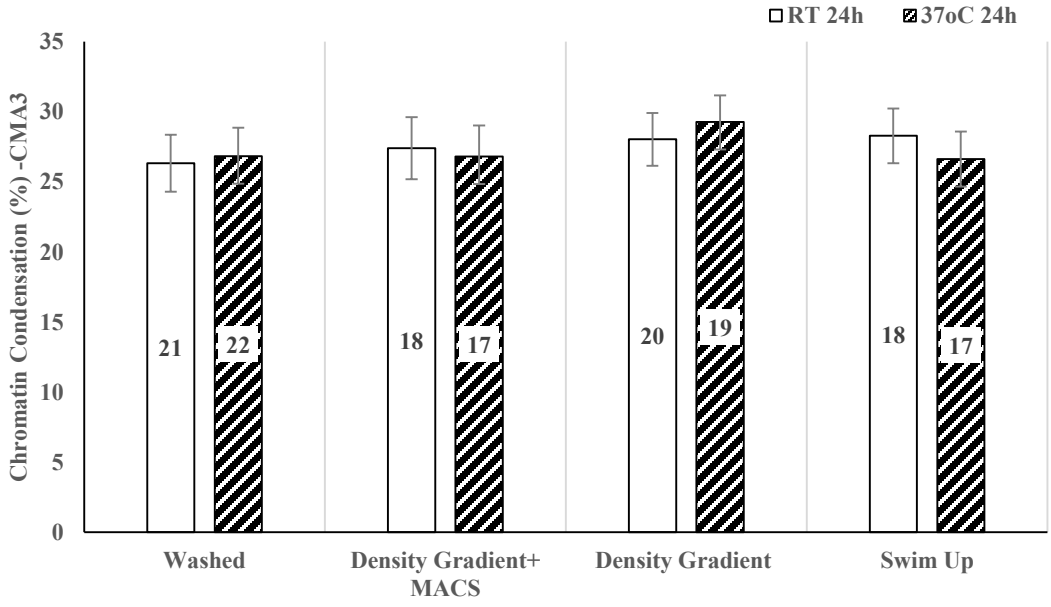
Figure 63. Levels of a percent (a) DNA fragmentation and, (b) chromatin condensation following preparation of semen by different separation methods

a=Neat vs washed, swim-up, density gradient, and density gradient +MACS; b= washed vs swim-up, density gradient, and density gradient +MACS; c= density gradient +MACS vs swim-up, density gradient.

*=p<0.05,** p<0.01, ***p<0.001



(a)



(b)

Figure 64. Percent (a) DNA fragmentation and, (b) sperm chromatin condensation (CMA3) after 24 hours at Room temperature and at 37ºC post semen processing

*=p<0.05,** p<0.01, ***p<0.001

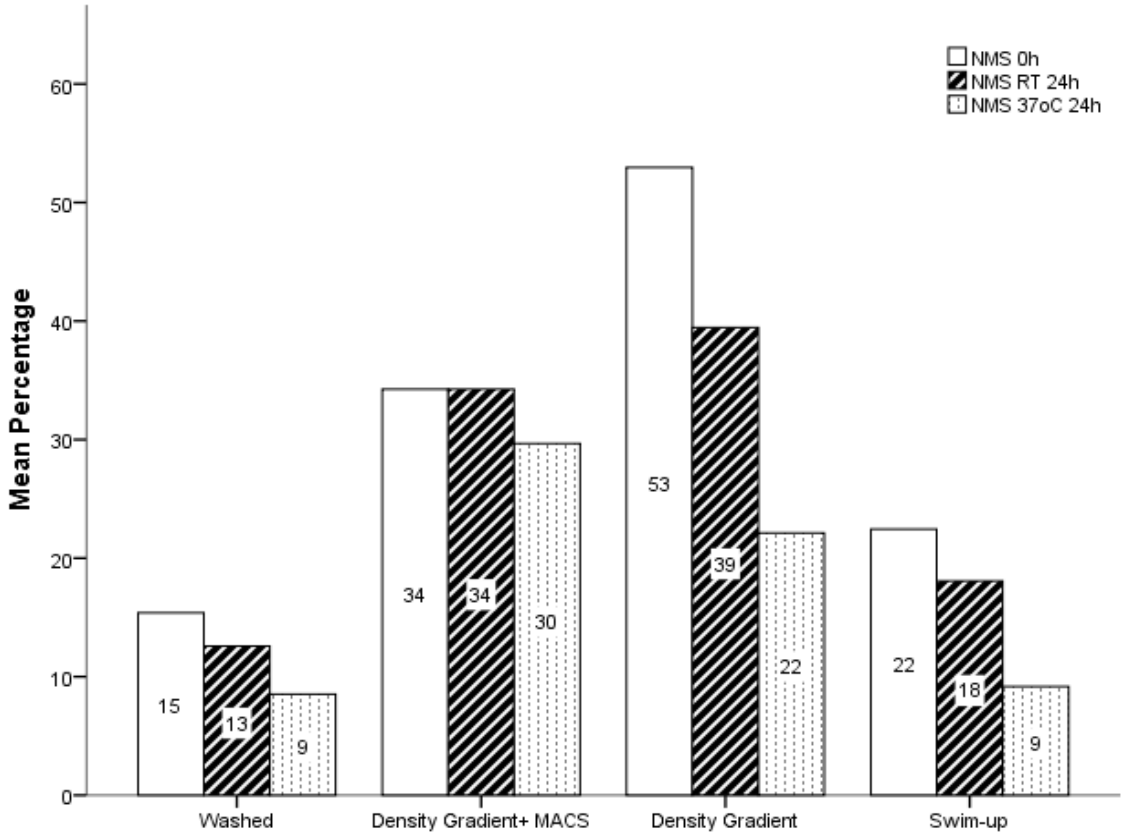


Figure 65. The percentage of normal progressively motile spermatozoa after 0 hours and 24 hours at Room temperature and at 37^{o}C post semen processing

DISCUSSION

During the last two decades, there was an increased number of assisted reproductive techniques used for the treatment of subfertile couples (Leung *et al.*, 2018). Despite advancement in the ART field, success and live birth rates remain relatively low, and to improve the success of ICSI, (Simopoulou *et al.*, 2016) one possibility could be improving the quality of spermatozoa (Štiavnická *et al.*, 2017). The selection of ideal spermatozoa and selection of that sperm based on viability and normal morphology still have a chance for selection of sperm with chromatin damage or immature spermatozoa which may lead to abnormal embryogenesis post-ICSI and failed implantation (Amor *et al.*, 2019; Sharma *et al.*, 2015; Dehghanpour *et al.*, 2020). Our data showed a better selection of sperm with normal morphology and motility post preparation of is DGC-MACS technique. The molecular content of DGC-MACS selected sperm showed mature sperm of condensed chromatin with lesser SDF along with a lesser ROS level and better vitality (HOS). Recently apoptosis has been given much attention because of its vital role in reproduction (Dirican *et al.*, 2008; J.Schaller, 1999; Swerdloff, 1999; Glander and Schaller, 1999; Hikim and Swerdloff, 1999). Apoptosis of germinal cells is known to play an important role in normal spermatogenesis. As a result, the altered apoptotic process is closely linked to sperm abnormalities and male subfertility (Aitken and 2007). Magnetic cell sorting utilizing annexin V-conjugated microbeads can label and separate sperm with PS translocation due to disrupting membrane integration at the molecular level, which is an apoptotic symptom (Dirican *et al.*, 2008; Degheidy *et al.*, 2015; Grunewald *et al.*, 2009; Tavalaee *et al.*, 2012).

Very limited studies are available on the evaluation of whether DGC-MACS improves reproductive outcomes, which are determined in terms of the oocyte number, embryos, and transfer procedures to achieve childbirth (Said and Land, 2011; Said *et al.*, 2007). Our findings reveal that adopting the DGC-MACS approach for preparing human spermatozoa can result in increased cleavage and chemical pregnancy rates, as well as a tendency toward improved implantation rates. (Dirican *et al.*, 2008; Ziarati *et al.*, 2019). In the present study, we divided the men's groups into a cohort with SDF normal and with higher SDF ranges to assess the true advantage of the DGC-MACS. We observed no significant benefit

of choosing DGC-MACS between cohorts of SDF with a cutoff of 20% (Sergerie *et al.*, 2005). Thus, evaluating the actual scientific effect of the increased proportion of nonapoptotic sperm semen samples from unselected men is not properly established and needs similar studies. (Grunewald *et al.*, 2009). DGC-MACS sperm selection is presently provided to patients in very specific cases and is not used as a preferred method, in cases with men have increased SDF or numerous failed ART attempts without an obvious female cause (Dirican *et al.*, 2008; Bucar *et al.*, 2015; Gil *et al.*, 2013; Sharma *et al.*, 2015; Iftikhar *et al.*, 2021; Gil Juliá *et al.*, 2021; Aydos and Aydos, 2021). Given the controversy surrounding the introduction of add-ons into clinical practice without proper safety and thorough reviews, clinicians must have reliable records as a result of careful research designed, both prospective (RCTs) and retrospective, utilizing proper statistical methods, proper designing, and unbiased date to ensure patients receive fertility treatment option tailored to their needs and preferences (Pacheco *et al.*, 2020; Romany *et al.*, 2017; Gil *et al.*, 2013).

Traditional swim-up or one-step wash should not be used to separate neat semen with increased oxidative stress due to the presence of infection or immature and damaged sperm. . The cellular content of the ejaculate is driven to the pellet, allowing infection and defective spermatozoa to mix with mature spermatozoa and cause harm (Romany *et al.*, 2017; Simopoulou *et al.*, 2016). DGC holds great promise for isolating mature, infection-free spermatozoa, whereas DGC-MACS has more swift and easy sorting technology in high concentrations (McDowell *et al.*, 2014; Cakar *et al.*, 2016). Infection and morphologically defective cells can be identified and eliminated from suspensions using paramagnetic microbeads (Akerlöf *et al.*, 1987). When defective spermatozoa are removed, oxidative stress is reduced in important and healthy cells. (Agarwal *et al.*, 2019). As a result, less oxidative stress in the ejaculate following DGC-MACS selection could be another possible explanation for the increased cleavage rates found after DGC-MACS separation in this investigation (Dirican *et al.*, 2008; Ziarati *et al.*, 2019). Our research, on the other hand, revealed the impact of magnetic passage on oxidative stress reduction (Romany *et al.*, 2014; Degheidy *et al.*, 2015; Grunewald *et al.*, 2009; Esbert *et al.*, 2017). Our findings show a higher cleavage rate, as well as a trend toward higher pregnancy and implantation, could be attributed to the removal of genetically defective cells due to

apoptotic properties (Dirican *et al.*, 2008). DGC-MACS preparation of spermatozoa thus result in the selection for balanced gametes and improved outcome rates. There was no difference in the live birth rate across all groups and cohorts (Degheidy *et al.*, 2015; Cakar *et al.*, 2016; Martinez *et al.*, 2018; Grützkau and Radbruch, 2010). Semen storage could be a useful tool for many fertility centers where they outsource sperm chromatin integrity testing. Lesser damage to chromatin is the target to choose the best short-term storage method for shipment. Our results showed that analysis performed within 4 hours skewed chances to overestimate the chromatin damage as reported in previous studies, and that sperm DNA damage was directly linked with time delay at a rate of 1% increase per hour (Jackson *et al.*, 2010). This short-term storage could also be beneficial for in-vitro insemination procedures where there was delayed oocyte retrieval or in-vitro maturation of metaphase I oocytes or rescue ICSI on the following day. In the present study, we found for short-term storage wet ice storage is the as simple and cheapest method as reported before (Jackson *et al.*, 2010; Said and Land, 2011; Zhang *et al.*, 2011). While in the case of long-term storage snap-frozen and slow cryopreservation are the best methods that do not increase the damage to sperm chromatin structure (Aboulmaouahib *et al.*, 2016; Thijssen *et al.*, 2014).

The quality of sperm prepared after different separation techniques influences *in-vitro* insemination methods (IUI, IVF, or ICSI) outcome (Saylan and Erimsah, 2019; Baldini *et al.*, 2021; Lara-Cerrillo *et al.*, 2021; Aydos and Aydos, 2021). The choice for separation techniques is to recover a higher proportion of normal motile spermatozoa with intact chromatin, which makes it more appropriate for physiological methods of fertility treatment, such as IUI or IVF (Lepine *et al.*, 2019; McDowell *et al.*, 2014; Zini *et al.*, 2008). Spermatozoa preparation technique and storage temperature post preparation affect sperm quality, chromatin integrity, ability to fertilize the oocyte, post-fertilization-embryo cleavage rate, embryo quality, blastocyst formation, and implantation rate (McDowell *et al.*, 2014; Rumbold *et al.*, 2019; Amor *et al.*, 2019; Sun *et al.*, 2018). The negative effect has been shown by many previous studies which are of great concern for the zygote health and an increased incidence of imprinting disorders of Angelman's syndrome and Beckwith-Wiedemann syndrome could result after the successful fertilization of fragmented DNA spermatozoa (Hammadeh *et al.*, 2001; Zini *et al.*, 2008; Gil Julia *et al.*, 2021; Zheng *et al.*,

2018). The percentage of sperm DNA damage assessment using SCD is comparable to the result obtained after SCSA and TUNEL assay (Fernandez *et al.*, 2005). Many previous data support quality spermatozoa recovery with density gradient centrifugation and MACS, but these techniques are practically not possible in severe oligo–asthenozoospermia and oligo-teratozoospermia conditions (Lepine *et al.*, 2019; Stimpfel *et al.*, 2018; Pacheco *et al.*, 2020; Lee *et al.*, 2010; Tavalaee *et al.*, 2012; Delbes *et al.*, 2013; Esbert *et al.*, 2017). The present study evaluated the effectiveness of four different sperm preparation methods on human sperm motility, morphology, and chromatin integrity at different temperatures and time intervals post-separation. We found that separation techniques of density gradient centrifugation combined with MACS and temperature does influence the spermatozoa chromatin quality and normal spermatozoa recovery (Said *et al.*, 2008). The percentage of sperm with chromatin condensation and normal motility depends on preparation methods and incubation temperature as well as the incubation time (Karimi Zarchi *et al.*, 2020). One explanation of in-vitro damage to sperm quality and chromatin integrity is the production of ROS (Agarwal *et al.*, 2019). Post preparation Immotile, immature, and morphology-poor spermatozoa could be the source of ROS generation. Lipid peroxidation increase induces a reduction of sperm motility, and 8-hydroxy 2 deoxy-guanosine cause sperm DNA damage, ROS-induced DNA fragmentation makes chromatin remodeling failure, especially during spermatogenesis (Agarwal *et al.*, 2019; Xie *et al.*, 2018; Qian *et al.*, 2020). The sperm chromatin condensation and packaging evaluation through different staining methods, CMA3 staining in the present study showed no significant increase in chromatin decondensation post preparation incubation both at room temperature and at 37ºC (Ahmed *et al.*, 2018). Sperm DNA damage increased at 37ºC has been reported by, two studies that concluded the same increase in sperm fragmented DNA both at room temperature and 37ºC after 24h. A recent study reported a reduction in the incidence of biochemical pregnancy (7.3%) and clinical pregnancy (7.7%) with every 1-hour delay in ICSI time post 40hours post HCG trigger. Few recent studies focused on short time (4 h) incubation and only consider one type of preparation technique of swim-up or density gradient centrifugation (Amor *et al.*, 2019; Timermans *et al.*, 2020; Peer *et al.*, 2007; Hernandez-Silva *et al.*, 2021; Schuffner *et al.*, 2002; Jackson *et al.*, 2010; Nabi *et al.*, 2014; Ahmed *et al.*, 2018; Pacheco *et al.*, 2020).

Despite these promising results, it's worth noting that the number of patients studied in previous data including the current study is small, and most studies are underpowered to conclude ART outcomes. To better understand this process and determine the technique's genuine value further studies are needed. Before sophisticated sperm selection procedures are extensively employed in ART, procedure safety and efficacy should be thoroughly investigated.

Conclusion:

DGC-MACS preparation technique is a method of choice for improving the percentage of mature normal, viable sperms with condensed chromatin and intact genetic integrity, which is safe to enhance assisted reproduction outcomes. The findings of the present study show that the DGC-MACS preparation technique is a method of choice to improve the percentage of mature, normal and viable sperm with condensed chromatin and intact genetic integrity in patients with teratozoospermia, and it shows that it is safe and improves assisted reproduction outcomes. Further research involving a larger TZs patient population and more applications could yield more comprehensive information about the ART results. However further studies should be performed to examine embryogenesis and follow up for defects in the offspring, their development and reproductive health should be considered before the adoption of sophisticated sperm selection methods in ART. While sperm preparation method for better quality sperm separation and post 24-hour quality sperm isolation was combining the density gradient centrifugation with MACS. Separation of the higher proportion of quality sperm of genetic integrity may influence fertility assessment and treatment outcomes.

GENERAL DISCUSSION

Male factor subfertility is gaining popularity as sperm quality and standard parameters of healthy young men decline. Male factors contribute to more than half of all subfertility cases, which remains a health concern globally. Male factor subfertility is on the rise in Pakistan and around the world, and treatment options are limited. Male subfertility is difficult to treat because oxidative stress, apoptosis, and chromatin damage all play a role in its complex pathophysiology (Dada *et al.*, 2012; Cooper *et al.*, 2010). The subfertile male subjects were categorized based on semen parameters as were categorized by previous data (Delbes *et al.*, 2013; Candela *et al.*, 2021; Dehghanpour *et al.*, 2020; Karimi Zarchi *et al.*, 2020; Kumar *et al.*, 2011).

Subfertile men were classified based on standard sperm parameters. Subfertile MMF had low sperm counts as well as low motility and motility categories (rapid progressive motile, slow progressive motile, and local motile), whereas subfertile SMF had decreased motility, motility categories, morphology and total sperm counts. As previously reported, motile sperm count is a predictor of fertility outcome, and the percentage of motile spermatozoa was less in MMF and SMF subfertile subjects compared to fertile subjects. (Smith *et al.*, 2007). Low sperm motility and motility categories (rapid progressive motile, slow progressive motile, and local motile) and increased immotile sperms percentage were observed in SMF subfertile males, which is the fact that motility is important for sperm quality and fertility potential. The current study discovered a connection between standard sperm parameters in normozoospermic and male factor subfertile subjects. Current data show a declining trend in sperm count in studied males, which is similar to that seen in Asia, the United States and Europe. This could be attributed to lifestyle factors such as smoking, eating habits, fertilizer, gossypol, and other pesticides (Delbes *et al.*, 2013; Karimi Zarchi *et al.*, 2020). Studies on sperm morphology investigated morphology as the best predictor of fertility (Karimi Zarchi *et al.*, 2020; Candela *et al.*, 2021). Subfertile SMF male patients in the current study had a low percentage of normal morphology sperms (without the head, midpiece, or tail defects) and a high percentage of abnormal morphology sperms (with head, midpiece, or tail defects). These findings support previous research that found a significantly higher percentage of abnormal morphology sperm in subfertile males.

(Engel *et al.*, 2018). In the present study, SMF and MMF male patients had sperm deformity index (SDI) and teratozoospermic index (TZI) significantly high compared to fertile, (Candela *et al.*, 2021) a significantly low sperm SDI in the fertile compared to subfertile was observed by candela et al 2021. As studied earlier, sperm vitality (eosin-Y) and membrane integrity (HOS) assays showed a correlation with male subfertility (Stangera *et al.*, 2010; Takahashi *et al.*, 1990; La Vignera *et al.*, 2012). In men, the oxidative stress relates to fertility problems was well-known, although there was no conclusive evidence for oxidative stress markers SOD, POD, TBRAS and ROS influence the male reproductive axis and especially with subfertility associated with impaired semen quality (sperm chromatin integrity) and quantity (count, sperm motility and CASA parameters of motility) (Said *et al.*, 2012). Therefore the present study highlighted the association of sperm quality makers with conventional semen parameters in subfertile males with oxidative stress markers concentration and sperm parameters and a possible correlation between male reproductive hormones, which might suggest a direct interaction between spermatogenesis, and oxidative stress. Similarly, Testosterone (T) concentration was significantly lower in subfertile men compared to fertile, likewise, T concentration was non-significantly high in SMF patients. Fertile and other subfertile categories MMF did not show any significant variation in T concentration. Previous studies demonstrated a reduction in the amount of androgen with a degree of increase in fat mass in fertile and subfertile males (Zhao *et al.*, 2020; MacDonald *et al.*, 2010). T levels in normal-weight (BMI<24) fertile and all categories of subfertile (MMF) males were comparable. Significant low levels of testosterone were detected in overweight subfertile male patients (SMF), negative impact by the paracrine inhibitory effect of leptin on Leydig cells a site of testosterone production, also lipolysis increase when testosterone interaction with adipocytes androgen binding receptor (Maghsoumi-Norouzabad *et al.*, 2020). Serum levels of FSH and LH were found to be considerably higher in SMF, while LH and FSH evaluation results in MMF were marginally higher, reflecting testicular dysfunction and changing the usual feedback relationship between the testis and hypothalamus (Hofny *et al.*, 2010). An increased gonadotropin concentration and a marked variation from normal levels are linked to male subfertility. The previous report was supported by non-significant increases in LH levels in MMF and SMF patients compared to fertile (Zhao *et al.*, 2020).

It was widely documented that patients with NON-OBST-AZOOs, OATZs, and OZs had elevated serum FSH levels. Currently, SMF patients had higher gonadotropin (LH and FSH) concentrations and lower T concentrations; these results were consistent with subfertile men's significantly higher gonadotropin levels and lower T concentrations when compared to the control population (Macdonald *et al.*, 2013).

Sperm protamine (CMA3) and chromatin integrity markers (SCD, SCSA, TB)

The quality of human semen has traditionally been assessed microscopically and biochemically to diagnose male subfertility. This test provides the clinician with the foundation for an initial diagnosis and categorizes the results into normal, moderate, and severe male subfertility variables. However, none of these tests examine sperm quality and function to forecast the success of treatment on fertility (Smith *et al.*, 2007). Sperm chromatin modification harmed sperm quality. We observed that sperm chromatin integrity was compromised in male factor subfertile couples (MMF and SMF) due to impaired sperm parameters. Our findings are consistent with previous research that found immature chromatin and DNA fragmentation in subnormal sperm samples (Nemati *et al.*, 2020). In addition, as previously suggested, we found a greater proportion of spermatozoa in a subfertile group (MMF and SMF) compared to controls with aberrant chromatin maturity and DNA damage (Sun *et al.*, 2018). The higher level of DFI in the proportion of SCD and SCSA in subfertile groups (MMF and SMF) with poor standard semen characteristics as compared to normal control, as well as his observation, is in line with the findings of other studies. (Talebi *et al.*, 2013; Dehghanpour *et al.*, 2020; Evenson, 2016). SCD test is intended to assess chromatin decondensation, which could be related to DNA damage but is not measuring directly the DNA breaks. As a result of the strong relationship between the SCD and SCSA values, it is possible to view the SCD test as a potential replacement for a straightforward diagnostic laboratory setup that would allow for a more accurate evaluation of DNA integrity (Turner *et al.*, 2020; Evenson, 1999). With enhanced sensitivity, the cut-off value was chosen at 20% sperm DNA fragmentation, and ROC curve analysis confirms that SCD might be one of the predictors of pregnancy rate. The sperm DNA damage assessment was found to be an effective predictor of successful conception in infertile couples (Alkhayal *et al.*, 2013).

SCSA is more frequently employed to evaluate the degree of sperm DNA fragmentation because SCD does not directly detect DNA damage. Compared to normal males, subfertile men have a larger proportion of spermatozoa with denatured DNA (Amor *et al.*, 2019; Sergerie *et al.*, 2005). Male subfertility may result from abnormalities of sperm-specific nucleoprotein, which change the genetic packaging of sperm (Aoki *et al.*, 2006). In the current investigation, we demonstrated that people with normozoospermia had considerably lower CMI levels than male subfertile patients with moderate (Os, As, TZs) and severe (OA, NOA, and OAT-S) subfertility. Lesser DNA fragmentation and chromatin de-condensation percentage in normal sperm samples (Cannarella *et al.*, 2020). In addition, as was already reported, a subfertile group had a significantly higher proportion of spermatozoa with aberrant CMI and DFI than controls (Gosálvez *et al.*, 2014; McDowell *et al.*, 2014). The SCD test is a prospective replacement that permits a more precise evaluation of DNA integrity using simply a light microscope, which is common in typical laboratories. SCD is less expensive and logistically simpler (Chohan *et al.*, 2006; Evenson *et al.*, 2020).

The percentage of SCD and SCSA cells in subfertile groups was higher than in normal controls, indicating a higher degree of DFI, which is consistent with earlier research findings (Liffner *et al.*, 2019; Zeqiraj *et al.*, 2018; Zheng *et al.*, 2018; Timermans *et al.*, 2020). Subfertility is associated with problems with sperm DNA integrity because subfertile men have a larger percentage of spermatozoa with denatured DNA than fertile ones (Chen *et al.*, 2020). A higher percentage of sperm CMI (as determined by chromatin condensation and abnormal spermatozoa protamination) has been demonstrated to play an important role in male fertility, early embryonic development, recurrent implantation failure, and pregnancy outcome, in addition to the significance of sperm DFI assessment (Saylan and Erimsah, 2019; Turner *et al.*, 2020; Alkhayal *et al.*, 2013).

Assisted reproduction outcome

The present study was conducted to check whether the male factor contributes to futile ART and to add one or more tests to predict the ART outcome. Sperm chromatin alteration had detrimental effects on sperm quality in preset stud we found the sperm chromatin integrity compromised in male factor subfertile men (MMF and SMF) due to impaired

semen parameters. Using SCSA is more popular for sperm DNA fragmentation, as SCD is an indirect measure. Subfertile men have a higher denatured DNA sperm than normal men, so poor sperm DNA integrity could result in subfertility and conception failure (Green *et al.*, 2020).

Sperm CMI (as determined by chromatin condensation and abnormal spermatozoa protamination) increased percentage is crucial for male fertility, early embryonic development, recurrent implantation failure, and pregnancy outcome in addition to the significance of sperm DFI assessment (Lara-Cerrillo *et al.*, 2021). The findings of the ROC curve analysis demonstrated that 30% sperm CMA3 level for fertilization had greater sensitivity, as was previously reported (Ferrigno *et al.*, 2021).

The current study also revealed that, in the early stages of in-vitro embryogenesis, blastocyst development, and cleavage rate are comparable in a male patient with SMF compared to MMF and fertile subjects, however a difference in oocyte fertilisation was observed following IVF (Hammadeh *et al.*, 2005). It is because a significantly lower pregnancy rate was observed in the current study among couples with male factors (SMF and MMF) compare to N after ICSI as suggested earlier (Engel *et al.*, 2018). ROC 30% CMA3 level and TB 33% as a threshold in SMF to predict the successful pregnancy achievement after ART treatment. As a result, protamines play a crucial role in the fertilisation process, and a lack of protamination lowers both the rate of clinical pregnancy and the rate of fertilisation (Magli *et al.*, 2007).

As a result of aberrant sperm DNA packing and increased sperm DNA sensitivity to environmental stresses, the current findings suggest that chromatin protamine deficiency causes these problems. Because it impacts embryo dynamics, fertilisation, and implantation potential, defective sperm chromatin condensation needs to be regarded as a sperm anomaly in male factor subfertility. DNA fragmentation is increased as a result of it as well. To improve sperm quality and function before starting intracytoplasmic sperm insemination (ICSI) or in vitro fertilisation, it is strongly advocated (IVF). During ICSI, the chromatin composition and DNA damage of the microinjected sperm remain unknown. SCD and CMA3 procedures are less invasive and more affordable than SCSA and TUNEL for evaluating sperm DNA fragmentation and chromatin maturity. These advantages make

testing the chromatin structure of sperm using the SCD (to detect DNA damage) and CMA3 (to measure chromatin maturity) approaches useful tools to support standard men's analysis in identifying male factor subfertility and before attempting assisted conception. In the current study, it was observed that chromatin decondensation or DFI was not associated with embryonic trisomies, complex abnormalities, gonosomal aneuploidy, or aneuploidy. It's possible that sperm-derived aneuploidies induce an early halt to embryo development, or that oocytes can restrict aneuploidy embryos from developing further before embryonic genome activation. (Green *et al.*, 2020). When compared to normozoospermic patients, we found no association between SMF and an increase in sex chromosomal abnormalities in embryos. SMF and MMF in the subfertile couple are interestingly dependent on sperm quality, sperm DNA damage, and chromatin maturity and did not affect the genetic quality (aneuploidy) of the produced embryo. This is because the oocyte has the competency to repair paternal chromatin abnormalities, but this capability is constrained depending on the severity of the damage, as evidenced in previously reported (Gat *et al.*, 2017). Because sperm, unlike oocytes, lack DNA repair capabilities, repairing sperm DNA damage in the fetus after conception is crucial. Damage to sperm DNA in zygotes can result in miss-rejoining, chromosomal aberration, and/or the formation of acentric fragments if not repaired appropriately (Linan *et al.*, 2018).

Paternal BMI influences sperm chromatin integrity and ART outcome

Our research focused on male overweight influencing sperm quality and treatment outcomes after ART. By increasing the formation of ROS, paternal overweight compromises the sperm chromatin. Our results showed that sperm progression was lower in overweight men than in normal-weight men, but paternal BMI had no effect on sperm morphology or concentration. Additionally, overweight individuals had similar spermatozoa deformities index (SDI) as normal weight men. Men who were overweight had statistically considerably more normal motile sperm with altered chromatin than men who were of normal weight, demonstrating their vulnerability to biological insults like adiposity. In this condition, paternal BMI is significantly inversely correlated with ROS, DFI, CMA3, and TB levels. A higher percentage of immature sperm was produced as a result of the paternal weight compromising the sperm's chromatin's integrity and causing

its decondensation, which may have enhanced the production of reactive oxygen species. These results confirm earlier studies that connected weight gain to elevated levels of ROS and sperm DNA damage (Anifandis *et al.*, 2013; Raad *et al.*, 2019; Chohan, 2006; Zeqiraj *et al.*, 2018; Agarwal *et al.*, 2017). As a result, we may also draw the additional conclusion that fathers' weight harms the quality markers of the motile spermatozoa. An increase in the paternal BMI could result in impaired sperm chromatin integrity making spermatozoa's genetic material vulnerable to external environment insult. Similar to this, we found that following an ART cycle, a rise in paternal BMI is associated with a decrease in the rate of clinical pregnancy and fertilisation. Therefore, poor fertility outcomes such as low fertilisation rates, poor embryo quality, recurrent failures of assisted reproductive technology attempts, and miscarriages are associated with chromatin condensation and DNA integrity (Ma *et al.*, 2020; Takagi *et al.*, 2019).

In the current study, we observed that the paternal overweight group had a higher normal newborn birth weight (within the normal range) than the normal weight group. The findings of this study show that the paternal BMI has no effect on neonatal birth weight after ART cycles. Recent studies on the impact of increased paternal BMI on newborn birth weight during ART conception cycles have yielded contradictory results (Magnus *et al.*, 2018; Anderson *et al.*, 2018; Magnus, 2001). ART cycles are constantly related to a decreased risk of neonatal consequences with low birth weight (Anderson *et al.*, 2018; Oldereid *et al.*, 2018; Magnus *et al.*, 2018),

Spermatozoa likely go through epigenetic modifications as a result of which the paternal genome programs the neonatal phenotype, even though the specific mechanisms by which paternal BMI affects infant outcomes are unknown. At some stage during embryonic development, some epigenetic markers from male gametogenesis might still be present. Long-lasting epigenetic modifications and phenotypic impacts in the progeny may be the result of environmental exposures during spermatogenesis, including food, lifestyle, and other variables. However, couples experiencing assisted reproduction cannot infer the results of experimental studies (Ma *et al.*, 2020; Linabery *et al.*, 2013; Davidson *et al.*, 2015; Terashima *et al.*, 2015).

Advanced male age effect Sperm chromatin packaging and reproductive biomarker

Our research highlighted the effect of men's age on sperm quality markers, chromatin dispersion, and compaction. Male age did not affect sperm morphology, motility, or concentration, according to our findings. It could be attributed to the current study's patient enrollment, as these are patients seeking fertility assistance. Furthermore, we investigated the link between male age and oxidative stress levels. Male fertility potential is impaired due to an imbalance in ROS production and antioxidant enzyme levels. There are conflicting findings regarding the relationship between levels of ROS production in sperm and advanced male age (Sigman, 2020; Luo *et al.*, 2022), while others found no relationship (Evans *et al.*, 2021; Alshahrani *et al.*, 2014, 2014). The current study found no link between male age and ROS production. Paternal age and reproductive hormone levels association analysis was done. Circulating androgen hormones linked directly with sperm quality parameters and reproductive hormones imbalance leads to impaired spermatogenesis and leads to male poor sexual health (Zhao *et al.*, 2020)

Sperm preparation techniques and storage temperature

Despite advancement in the ART field, success and live birth rates remain relatively low, and to improve the success of ICSI, (Simopoulou *et al.*, 2016) one possibility could be improving the quality of spermatozoa (Štiavnická *et al.*, 2017). The selection of ideal spermatozoa and selection of that sperm based on viability and normal morphology still have a chance for selection of sperm with chromatin damage or immature spermatozoa which may lead to abnormal embryogenesis post-ICSI and failed implantation (Amor *et al.*, 2019; Sharma *et al.*, 2015; Dehghanpour *et al.*, 2020). Our data showed a better selection of sperm with normal morphology and motility post preparation of is DGC-MACS technique. The molecular content of DGC-MACS selected sperm showed mature sperm of condensed chromatin with lesser SDF along with a lesser ROS level and better vitality (HOS). Recently apoptosis has been given much attention because of its vital role in reproduction (Dirican *et al.*, 2008; J.Schaller, 1999; Swerdloff, 1999; Glander and Schaller, 1999; Hikim and Swerdloff, 1999). Apoptosis of germinal cells is known to play an important role in normal spermatogenesis. As a result, the altered apoptotic process is closely linked to sperm abnormalities and male subfertility. (Aitken and 2007). Magnetic

cell sorting utilizing annexin V-conjugated microbeads can label and separate sperm with PS translocation due to disrupting membrane integration at the molecular level, which is an apoptotic symptom (Dirican *et al.*, 2008; Degheidy *et al.*, 2015; Grunewald *et al.*, 2009; Tavalaee *et al.*, 2012).

Very limited studies are available on the evaluation of whether DGC-MACS improves reproductive outcomes, which are determined in terms of the oocyte number, embryos, and transfer procedures to achieve childbirth (Said and Land, 2011; Said *et al.*, 2007). Our findings reveal that adopting the DGC-MACS approach for preparing human spermatozoa can be increased cleavage and chemical pregnancy rates, as well as a tendency toward improved implantation rates (Dirican *et al.*, 2008; Ziarati *et al.*, 2019). In the present study, we divided the men's groups into a cohort with SDF normal and with higher SDF ranges to assess the true advantage of the DGC-MACS. We observed no significant benefit of choosing DGC-MACS between cohorts of SDF with a cutoff of 20% (Sergerie *et al.*, 2005). Thus, evaluating the actual scientific effect of the increased proportion of apoptotic sperm semen samples from unselected men is not properly established and needs similar studies (Grunewald *et al.*, 2009). DGC-MACS sperm selection is presently provided to patients in very specific cases and not used a preferred method, in cases with men who have increased SDF or numerous failed ART attempts without an obvious female cause (Dirican *et al.*, 2008; Bucar *et al.*, 2015; Gil *et al.*, 2013; Sharma *et al.*, 2015; Iftikhar *et al.*, 2021; Gil Juliá *et al.*, 2021; Aydos and Aydos, 2021). Proper safety and thorough reviews, clinicians must have reliable records as a result of careful research designed, both prospective (RCTs) and retrospective, utilizing proper statistical methods, proper designing, and unbiased data to ensure patients receive fertility treatment options tailored to their needs and preferences (Pacheco *et al.*, 2020; Romany *et al.*, 2017; Gil *et al.*, 2013).

Traditional swim-up or one-step wash should not be used to separate neat semen with increased oxidative stress due to the presence of infection or immature and damaged sperm (Eva Akerolf *et al.*, 1987). The cellular content of the ejaculate is driven to the pellet, allowing infection and defective spermatozoa to mix with mature spermatozoa and cause harm (Romany *et al.*, 2017; Simopoulou *et al.*, 2016). DGC holds great promise for isolating mature, infection-free spermatozoa, whereas DGC-MACS has more swift and

easy sorting technology in high concentrations (McDowell *et al.*, 2014; Cakar *et al.*, 2016). Infection and morphologically defective cells can be identified and eliminated from suspensions using paramagnetic microbeads (Eva Akerolf *et al.*, 1987). When defective spermatozoa are removed, oxidative stress is reduced in important and healthy cells (Agarwal *et al.*, 2019). As a result, less oxidative stress in the ejaculate following DGC-MACS selection could be another possible explanation for the increased cleavage rates found after DGC-MACS separation in this investigation (Dirican *et al.*, 2008; Ziarati *et al.*, 2019). Our research, on the other hand, revealed the impact of magnetic passage on oxidative stress reduction (Romany *et al.*, 2014; Degheidy *et al.*, 2015; Grunewald *et al.*, 2009; Esbert *et al.*, 2017). Our findings show a higher cleavage rate, as well as a trend toward higher pregnancy and implantation, which could be attributed to the removal of genetically defective cells due to apoptotic properties (Dirican *et al.*, 2008). DGC-MACS preparation of spermatozoa thus results in the selection of balanced gametes and improved outcome rates. There was no difference in the live birth rate across all groups and cohorts (Degheidy *et al.*, 2015; Cakar *et al.*, 2016; Martinez *et al.*, 2018; Grützkau and Radbruch, 2010). Semen storage could be a useful tool for many fertility centers where they outsource sperm chromatin integrity testing. Lesser damage to chromatin is the target to choose the best short-term storage method for shipment. Our results showed that analysis performed within 4 hours skewed chances to overestimate the chromatin damage as reported in previous studies, and that sperm DNA damage was directly linked with time delay at a rate of 1% increase per hour (Jackson *et al.*, 2010; Said and Land, 2011; Zhang *et al.*, 2011). While cryopreservation is the best method that does not increase the damage to sperm chromatin structure (Aboulmaouahib *et al.*, 2016; Thijssen *et al.*, 2014).

The quality of sperm prepared after different separation techniques influences *in-vitro* insemination methods (IUI, IVF, or ICSI) outcome (Saylan and Erimsah, 2019; Baldini *et al.*, 2021; Lara-Cerrillo *et al.*, 2021; Aydos and Aydos, 2021). The choice for separation techniques is to recover a higher proportion of normal motile spermatozoa with intact chromatin, which makes it more appropriate for physiological methods of fertility treatment, such as IUI or IVF (Lepine *et al.*, 2019; McDowell *et al.*, 2014; Zini *et al.*, 2008). Spermatozoa preparation technique and storage temperature post preparation affect sperm quality, chromatin integrity, ability to fertilize the oocyte, post-fertilization-embryo

cleavage rate, embryo quality, blastocyst formation, and implantation rate (McDowell *et al.*, 2014; Rumbold *et al.*, 2019; Amor *et al.*, 2019; Sun *et al.*, 2018). The negative effect has been shown by many previous studies which are of great concern for the zygote health and an increased incidence of imprinting disorders of Angelman's syndrome and Beckwith-Wiedemann syndrome could result after the successful fertilization of fragmented DNA spermatozoa (Hammadeh *et al.*, 2001; Zini *et al.*, 2008; Gil Julia *et al.*, 2021; Zheng *et al.*, 2018). The percentage of sperm DNA damage assessment using SCD is comparable to the result obtained after SCSA and TUNEL assay(Fernandez *et al.*, 2005). Many previous data support quality spermatozoa recovery with density gradient centrifugation and MACS, but these techniques are practically not possible in severe oligo–asthenozoospermia and oligo-teratozoospermia conditions (Lepine *et al.*, 2019; Stimpfel *et al.*, 2018; Pacheco *et al.*, 2020; Lee *et al.*, 2010; Tavalaee *et al.*, 2012; Delbes *et al.*, 2013; Esbert *et al.*, 2017). The present study evaluated the effectiveness of four different sperm preparation methods on human sperm motility, morphology, and chromatin integrity at different temperatures and time intervals post-separation. We found that separation techniques of density gradient centrifugation combined with MACS and temperature does influence the spermatozoa chromatin quality and normal spermatozoa recovery(Said *et al.*, 2008). The percentage of sperm with chromatin condensation and normal motility depends on preparation methods and incubation temperature as well as the incubation time (Karimi Zarchi *et al.*, 2020). One explanation of in-vitro damage to sperm quality and chromatin integrity is the production of ROS (Agarwal *et al.*, 2019). Post preparation Immotile, immature, and morphology-poor spermatozoa could be the source of ROS generation. Lipid peroxidation increase induces a reduction of sperm motility, and 8-hydroxy 2 deoxy-guanosine cause sperm DNA damage, ROS-induced DNA fragmentation makes chromatin remodeling failure, especially during spermatogenesis (Agarwal *et al.*, 2019; Xie *et al.*, 2018; Qian *et al.*, 2020). The sperm chromatin condensation and packaging evaluation through different staining methods, CMA3 staining in the present study showed no significant increase in chromatin decondensation post preparation incubation both at room temperature and at 37ºC (Ahmed *et al.*, 2018). Sperm DNA damage increased at 37ºC has been reported by, two studies that concluded the same increase in sperm fragmented DNA both at room temperature and 37ºC after 24h. A recent study reported a reduction in the incidence of

biochemical pregnancy (7.3%) and clinical pregnancy (7.7%) with every 1-hour delay in ICSI time post 40hours post HCG trigger. Few recent studies focused on short time (4 h) incubation and only consider one type of preparation technique of swim-up or density gradient centrifugation (Amor *et al.*, 2019; Timermans *et al.*, 2020; Peer *et al.*, 2007; Hernandez-Silva *et al.*, 2021; Schuffner *et al.*, 2002; Jackson *et al.*, 2010; Nabi *et al.*, 2014; Ahmed *et al.*, 2018; Pacheco *et al.*, 2020).

The present study has some limitations such as less number of couples, and the exclusion of female factors in the analysis to determine the true influence of the sperm characteristics on ART outcome, as females mask the sperm contribution to embryo development. Moreover, future studies should focus on the association between DNA fragmentation, chromatin maturity, and semen quality, especially in male factor subfertile couples, because there is clinical concern regarding whether these tests can be used for diagnosis and prognosis of subfertility.

GENERAL CONCLUSION

The current study concluded the following points based on the prospective evaluation of several demographic, clinical, biological, and quality markers in the pathophysiology of male factor subfertile couples in Pakistan:

- The quality of sperm is a significant factor that affects reproductive success in humans. As abnormal sperm morphology is linked to reduced rates of successful fertilization and increased miscarriage rates following embryo transfer.
- Current data suggested that male subfertility can be caused by various factors such as oxidative stress, elevated sex steroids, and reproductive hormonal levels. These factors can lead to impaired sperm viability and membrane integrity, resulting in sperm dysfunction and reduced fertility potential.
- Alterations of spermatozoa protamination (as index of chromatin maturation) and sperm DNA fragmentation (chromatin integrity index) are associated with impaired male reproduction in the Pakistani population. Sperm chromatin dispersion assay, to assess sperm DNA fragmentation, and CMA3 staining, to evaluate protamine content, are inexpensive tools to precisely examine chromatin status and, thereby, improve ART outcomes.
- Moreover, our study highlighted the impact of men's BMI and age on impaired sperm quality and sperm chromatin dispersion and compaction, which can result in impaired male fertility potential. Overall, our study emphasizes the importance of maintaining a healthy weight and lifestyle to improve male fertility potential and reproductive outcomes.
- DGC-MACS preparation technique is a safe and effective method for improving the percentage of mature, normal, and viable sperm with condensed chromatin and intact genetic integrity in patients with teratozoospermia. Combining the density gradient centrifugation with MACS can result in the separation of a higher proportion of quality sperm with genetic integrity, which may significantly influence fertility assessment and treatment outcomes.

FUTURE PERSPECTIVES

In the current study on spermatozoa chromatin integrity and the role of protamine in susceptibility to male factors, subfertile couples have been investigated comprehensively in the Pakistani population. However, further studies are required as several gaps remained unfilled and questions remained unanswered that need to be investigated.

- For the majority of the risk factors for subfertility, a proper diagnosis could help better management, which is why couples' thorough investigations and more focused ones are required to ensure the timely diagnosis and management and to establish female or male factors of subfertility, which might help in decision for fertility treatment to achieve the ultimate goal of successful normal live birth.
- It is necessary to carry out further research on the epigenetic aspects affecting male fertility, which can help medical professionals understand the numerous mechanisms and causes of poor pregnancy outcomes.
- The use of artificial intelligence, deep learning, and machine learning could help in urologic oncology and study of subfertility treatment.
- Spermatozoa protamine content (chromatin maturation) and chromatin integration testing in male partner’s spermatozoa specifically in those couples with idiopathic subfertility but have reduced fertilization rate, poor embryo quality and implantation failure could be used as a biomarker to predict reproductive health in Pakistani couples.
- To assess the antioxidants and micronutrient therapy as medical treatment and its role in male patient with reduced sperm quality and chromatin maturity in Pakistani subfertile couples.
- It is necessary to conduct large sample-sized studies in the future to elucidate the association of sperm protamine and chromatin integrity with the severity of male factor subfertility.

- Proper management of paternal BMI prior to ART and to assess the effect of weight reduction in improvement of reproductive health and assisted therapy outcome
- To assess other sperm storage and sperm preparation (IMSI/ PICSI/spermbots etc.) technique weather could help in reducing sperm chromatin damage and assure the improvement in ART outcomes.
- Designing specific treatment approaches may be aided by identifying important structural and functional characteristics of the spermatozoa regulated by specific proteins and genes.
- Among others, genomics, transcriptomics, epigenomics, proteomics, metabolomics, reactomics, pharmacogenomics, and bioinformatics are particularly relevant "spermomics"/multiomics technologies in the assessment of sperm cells and seminal fluids and could enhance our understanding of the molecular events driving spermatogenesis and spermiogenesis in fertile versus subfertile men. These approaches provide unprecedented power of data analysis, visualization, interpretation, and compilation.
- With the large population selection and multicenter approach could help understanding the etiology of male factor subfertility and the role played by the different intrinsic and extrinsic factors in developed and developing countries should be a research priority to improve male reproductive health and reproductive outcome after assisted therapies.
- Since the pathophysiology of male infertility is still obscure, it is worthwhile to combine the advanced approaches, especially high-throughput multiomics technologies and big data tools, into comprehensive and large-scale strategies, along with lifestyle choices and environmental factors, in order to develop diagnostic clues, management avenues, and promising therapeutic options towards precision male infertility therapeutics and diagnostics.

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ANNEXURE I

QUESTIONNAIRE

Serial no: _____ Sample no: _____

Name of patient: _____ Date of sample collection: _____

Age of patient: _____ (year) Time of sample collection: _____

Weight of patient: _____ (Kg) Address/Area: _____

Height of patient: _____ (m-cm) _____

Present Occupation: _____ Former Occupation: _____

SUBFERTILITY STATUS

Date of marriage: _____ Age of wife at marriage: _____

Age of patient at marriage: _____ Present age of wife: _____

Number of children: _____

Date of birth of first baby: _____

Date of birth of last baby: _____

Socioeconomic condition (monthly income): _____

MEDICAL HISTORY

Tuberculosis: _____

Mumps: _____

Inguinal Hernia: _____

Radiation: _____

Injury/Operation: _____

Diabetes: _____

Obesity: _____

Liver/Renal diseases: _____

Hepatitis: _____

Exercise: _____

Hypertension (HTN): _____

M/C of wife:

Regular:_____Irregular:_____

Hepatitis: _____

Any other disease to wife:

_____

_____

_____

_____

Signature of Researcher

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ORIGINAL ARTICLE

ANDROLOGIA WILEY

Protamines and DNA integrity as a biomarkers of sperm quality and assisted conception outcome

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Abstract

Present research aim was to identify functional tests in semen associated with DNA damage and chromatin maturity (protamination) which predict the outcome in assisted reproduction. Couples were grouped according to male partner semen parameters, into normozoospermia (NZs), severe male factor (SMF) and mild male factor (MMF). DNA fragmentation index (DFI) in spermatozoa was analysed by sperms chromatin dispersion (SCD), sperm chromatin structure assay (SCSA) and acridine orange testing (AOT). Chromomycin A3 (CMA3) and toluidine blue (TB) staining to measure sperm chromatin maturity (CM). DFI and chromatin decondensation were significantly lower in N compared to male factor categories (MMF and SMF). Aneuploidy embryos were significantly higher in couples with male factor infertility (MMF and SMF). A positive correlation was observed between fertilization rate (FR) and live birth rate (LBR) with sperm concentration, motility, vitality, normal sperm morphology and negative correlation between sperm DFI and sperm CM. No correlation was observed between embryo aneuploidy and sperm DFI or CM. Lower percentage of spermatozoa chromatin integrity are associated with low fertilization and live birth rate. Male factor infertility, due to impaired semen parameters and chromatin defects could be regarded in future as an indication of IVF/ICSI, and predictor of assisted reproductive techniques outcome.

KEYWORDS
art outcome, sperm chromatin, sperm dna damage, sperm protamination

1 | INTRODUCTION

Infertility is defined as no conception despite 1 year of unprotected attempts (Skakkebaek et al., 2006). One initial investigation aimed to find a cause of infertility is the evaluation of semen samples to rule out male factors, which are attributed to 40%–50% of infertility (Cooper et al., 2010). Routine semen analysis includes sperm count, motility and morphology; however, they do not strictly predict fertilization potential (Cissen et al., 2016; Ozmen et al., 2007; Wdowiak et al., 2015). Sperm abnormal chromatin and DNA fragmentation assessment are hidden anomalies frequent in infertile men (Sakkas & Alvarez, 2010). Subsequently, routine sperm parameters alone do not enable the identification of a substantial proportion of infertile men. Reactive oxygen species (ROS) might also be associated with human infertility and oxidative stress, which is implicated in the aetiology of sperm dysfunction and male infertility (Ribas-Maynou et al., Jul. 2020; Sabeti et al., 2016). In humans, protamines replace approximately 85% of the histones during the process of spermatogenesis (Niederberger, 2005; Rathke et al., 2014).

Damaged sperm DNA also arises from problems in nuclear remodelling through stages of spermatogenesis, resulting directly from altered sperm protamination (Rathke et al., 2014; Sakkas

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Article

Analyzing the Differential Impact of Semen Preparation Methods on the Outcomes of Assisted Reproductive Techniques

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Abstract: Sperm separation plays a critical role in assisted reproductive technology. Based on migration, density gradient centrifugation and filtration, a properly selected sperm could help in increasing assisted reproductive outcomes in teratozoospermia (TZs). The current study aimed to assess the prognostic value of four sperm selection techniques: density gradient centrifugation (DGC), swim-up (SU), DGC-SU and DGC followed by magnetic-activated cell sorting (DGC-MACS). These were evaluated using spermatozoa functional parameters. A total of 385 infertile couples underwent the procedure of intracytoplasmic sperm injection (ICSI), with an isolated teratozoospermia in the male partner. Semen samples were prepared by using one of the mentioned sperm preparation techniques. The improvements in the percentage of normal mature spermatozoa, rate of fertilization, cleavage, pregnancy and the number of live births were assessed. The normal morphology, spermatozoa DNA fragmentation (SDF) and chromatin maturity checked by using chromomycin A3 (CMA3) with DGC-MACS preparation were better compared to the other three methods. Embryo cleavage, clinical pregnancy and implantation were better improved in the DGC-MACS than in the other tested techniques. The DGC-MACS technique helped in the selection of an increased percentage of normal viable and mature sperm with intact chromatin integrity in patients with teratozoospermia.

Keywords: magnetic-activated cell sorting; assisted reproductive technique; sperm DNA fragmentation; density gradient centrifugation

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1. Introduction

During the past two decades of advances in infertility treatment, the use of ART emerged as a therapeutic option for managing male and female subfertility [1,2]. However, the current success rates of assisted reproductive technology (ART) remain relatively low [3]. The development of an embryo after the fertilization of oocytes with poor-quality sperm from infertile men is one of the most important factors in intracytoplasmic sperm injection's (ICSI) success [4–6]. Teratozoospermia (TZs) is defined as a percentage of spermatozoa with normal shape under the lesser reference limit. The cutoff values for normality varied greatly in recent decades to 4% [7]. One recent study provided clear evidence that apoptotic alterations are closely correlated to abnormal sperm morphology and DNA damage [8]. Sperm DNA fragmentation (SDF) involves single- or double-stranded (ss or ds) breaks in sperm DNA and can be caused by extrinsic factors (i.e., heat exposure, smoking, environmental pollutants and chemotherapeutics) as well as intrinsic factors (i.e., defective germ cell maturation, abortive apoptosis and oxidative stress

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Analysis of sperm chromatin packaging and reproductive biomarker to evaluate the consequence of advanced male age

Riffat Bibi[1*], Sarwat Jahan[1], Salma Kafeel Qureshi[2], Suhail Razak[3*], Tayyaba Afsar[3], Ali Almajwal[3], Mashal Kafeel Qureshi[2], Mohammad Eid Hammadeh[4] and Houda Amor[4]

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In this study, the semen parameters, sperm chromatin integrity, antioxidant enzyme levels, and reproductive hormone levels of subfertile male subjects from Pakistan were assessed in relation to their age. Data on the demographic characteristics of the 750 study participants, including their general health, body mass index (BMI), and reproductive status, were collected from subfertile men from Pakistan. Semen and blood were collected to determine standard semen parameters, sperm chromatin dispersion (Halosperm-SCD), sperm chromatin integrity using toluidine blue (TB) staining, sperm chromatin maturity using chromomycin A3 (CMA3+) staining, and reproductive hormone (FSH, LH, prolactin and testosterone levels). The patients were divided into three groups according to their age: Group 1 included male subjects aged 30 years or less (n = 90), Group 2 included male subjects between the ages of 31 and 40 years (n = 330), and Group 3 included male subjects over 40 years of age (n = 330). Conventional semen parameters, reactive oxygen species (ROS), superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), and lipid peroxidation (MDA) did not statistically ($p > 0.05$) differ with increasing male age or between different age groups. When compared to younger men (<30 years), sperm SCD (23.2 $\pm$ 0.88%) was significantly (p = 0.01) lower as compared to male patients aged >40 years (26.6 $\pm$ 0.6%). The concentration of LH, FSH, and testosterone levels were comparable between the groups ($p > 0.05$), while a significant (p = 0.04) increase in sperm chromatin immaturity CMA3+ (30 $\pm$ 0.71%) was observed in the old age group (>40 years) compared to the <30-year group (26.6 $\pm$ 1.03%). A positive association was observed between advanced male age and sperm chromatin dispersion (SCD) (r = 0.124, p = 0.001) and decondensation (CMA3+) (r = 0.1, p = 0.009). Despite potential limitations, this study has been carried out with extensive information on the potential risk of male age on sperm integrity. The present study demonstrated

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RESEARCH Open Access

The influence of paternal overweight on sperm chromatin integrity, fertilization rate and pregnancy outcome among males attending fertility clinic for IVF/ICSI treatment

Riffat Bibi[1], Sarwat Jahan[1], Tayyaba Afsar[2], Ali Almajwal[2], Mohammad Eid Hammadeh[3], Nawaf W. Alruwaili[2], Suhail Razak[2*] and Houda Amor[3]

Abstract

Background: Low and middle-income countries are facing a rapid increase in obesity and overweight burden, particularly in urban settings. Being overweight in men is associated with infertility and a higher risk to have a low sperm count or no sperm in their ejaculate. Despite potential limitations, this is one of few studies conducted to determine the potential risk of paternal overweight on sperm standard parameters, sperm chromatin integrity and assisted conception outcome including fertilization, embryo quality, cleavage rate, reduce blastocyst development, implantation, and cumulative live birth rate (CLBR).

Methods: A cross-sectional study of 750 infertile couples undergoing assisted reproduction technique at a single reproductive medicine center of Salma Kafeel Medical Centre Islamabad. Sperm from men undergoing ART were analyzed for chromatin integrity using sperm chromatin dispersion assay (SCD), Chromomycin A3 staining (CMA3), and toluidine blue (TB) staining, while other semen parameters were assessed on same day includes; standard semen parameters, reactive oxygen species (ROS), sperm deformity index (SDI), teratozoospermic index (TZI), and hypo-osmatic swelling test (HOST). Paternal body mass index (BMI) < 24.5–20 kg/m^2 served as the reference group, while the male patients with BMI > 24.5-30 kg/m^2 were considered to be overweight.

Results: In the analysis of the percentage of spermatozoa with chromatin maturity (CMA3) and chromatin integrity (TB) was reduced significantly in overweight men ($p < 0.01$) compared with a reference group. Increase in paternal BMI correlate with the increase in sperm chromatin damage (SCD $r = 0.282$, TB $r = 0.144$, $p < 0.05$), immaturity (CMA3, $r = 0.79$, $p < 0.05$) and oxidative stress (ROS) ($r = 0.282$, $p < 0.001$). Peri-fertilization effects were increased in oocytes fertilization in couples with overweight men (FR = 67%) compared with normal-weight men (FR = 74.8%), similarly, after univariant regression paternal weight remain predictor of sperm chromatin maturity, successful fertilization and CLBR. In the embryo, developmental stage number of the embryo in cleavage was higher in normal weight men, while day 3 (D3) embryos, percent good quality embryo D3, and blastocyst formation rate were compared able between the groups. The paternal overweight group had significant ($p < 0.001$) increased neonatal birth weight (2952.14 ± 53.64gm; within normal range) when compared with the reference group (2577.24 ± 30.94gm) following

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Sperm Protamine and Chromatin Integrity: As Biomarkers of Sperm Quality and Assisted Conception Outcome by Riffat Bibi

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