# RELATIONSHIP BETWEEN SEMEN CHARACTERISTICS, OXIDATIVE STRESS AND SPERM DNA FRAGMENTATION IN HEALTHY FERTILE AND SUBFERTILE MEN WITH VARICOCELE

*A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE THESIS REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY*

**BY**

*Dr AROZIA AKRAM*

**DEPARTMENT OF ANIMAL SCIENCES**
**FACULTY OF BIOLOGICAL SCIENCES**
**QUAID-I-AZAM UNIVERSITY**
**ISLAMABAD, PAKISTAN**
**2013**

# RELATIONSHIP BETWEEN SEMEN CHARACTERISTICS, OXIDATIVE STRESS AND SPERM DNA FRAGMENTATION IN HEALTHY FERTILE AND SUBFERTILE MEN WITH VARICOCELE

**BY**

*Dr AROZIA AKRAM*

**DEPARTMENT OF ANIMAL SCIENCES**
**FACULTY OF BIOLOGICAL SCIENCES**
**QUAID-I-AZAM UNIVERSITY**
**ISLAMABAD, PAKISTAN**
**2013**

## CERTIFICATE

**This thesis by Dr Arozia Akram is accepted in its present form by the Department of Biological Sciences as satisfying the thesis requirement for the degree of Doctor of Philosophy in Reproductive Endocrinology.**

**Supervisor:** ____________________

**External Examination:** ____________________

____________________

**Chairman:** ____________________

**Dated:** ________________

*It is not where you are in life*

*It is who you have by your side that matters*

**DEDICATED**

**TO**

**MY IDEAL, LOVING HUSBAND, WING COMMANDER**

**MOAZZAM AND**

**MY WONDERFUL SONS, ABUZAR AND ZARAHMER,**

**WITHOUT WHOSE SUPPORT I WOULD HAVE NOT**

**REACHED THIS FAR**

ALSO TO

MY WONDERFUL PARENTS, WHO PRAYED FOR ME WITH EVERY BREATH OF THEIRS

AND TO

MY ROLE MODEL, MY INSPIRATION, PROFESSOR DR AKHTAR SOHAIL CHUGHTAI, WHO ALWAYS STOOD BY ME THROUGH THICK AND THIN

***In the name of Allah, the beneficient, the Merciful***

**None but Allah is worthy of worship**

**Mohammad is His messenger**

# CONTENTS


| | |
|---|---|
| **List of Abbreviations** | **i** |
| **List of Tables** | **ii-iii** |
| **List of Figures** | **iv-v** |
| **Abstract** | **1-3** |
| **Introduction** | **4-45** |
| **Study Plan** | **46** |
| **Methodology** | **47-71** |
| **Results** | **72-136** |
| **Discussion** | **137-164** |
| **References** | **165-217** |

# ACKNOWLEDGEMENTS

My sincerest and humblest thanks to Almighty Allah, who bestowed upon me the courage and knowledge and enabled me to work out this huge project. I offer my gratitude and thanks to our Holy Prophet, Hazrat Mohammad (peace be upon him), who is a beacon of guidance for the entire universe.

“Success is not measured by what you accomplish but by the opposition you have encountered and the courage with which you have maintained the struggle against overwhelming odds”, this is the philosophy of my life. My time spent during my PhD research can be concluded in one simple sentence, “The strength to withstand anything”.

Special thanks to my respected teacher, an inspiration, **Professor Dr Mohammad Shahab (PhD), Department of Neuroendocrinology, Quaid-i-Azam University, Islamabad**, **Pakistan**, for standing by me at all times and giving me his valuable guidance and generous advice throughout my PhD tenure.

Thank you to my advisor, **Dr Sarwat Jahan (PhD), Associate Professor, Reproductive Physiology/Endocrinology Laboratory, Quaid-i-Azam University, Islamabad**, **Pakistan**, for giving me the opportunity to explore all new possibilities in research.

I was blessed to be part of the best team ever at **Center for Reproductive Medicine, Cleveland Clinic, Ohio, USA,** that comprised of a remarkable group of highly skilled professionals whose basic faith lay in strong teamwork, under the perfect leadership **of my co-advisor, Professor Dr Ashok Agarwal (PhD), Director, Center for Reproductive Medicine/Andrology Laboratory, Cleveland Clinic Hospital**. I would like to express my sincerest gratitude to Dr Agarwal, for being highly polished and skilled professional with exceptional leadership qualities. He was undoubtedly the best team leader I know so far. A strong believer of teamwork, Dr Agarwal’s team was all amalgamated like pearls in a string. Thank you Dr Agarwal for believing in my capabilities and letting me accomplish all my dreams.

Very special thanks to **Dr Rakesh Sharma**, **Project Staff & Coordinator, Center for Reproductive Medicine/Andrology Laboratory, Cleveland Clinic, Ohio, USA**. As my main research co-ordinator, he had the patience to stand by me, at a stretch of hours, just making sure that I grasped all techniques proficiently by the end of the day. His belief in my success persevered me to work even harder.

I would like to express my gratitude to **Nelly Pitteloud (MD), Associate Professor, Reproductive Endocrine Unit, Massachusetts General Hospital/ Harvard Medical School, Boston, Massachusetts.** Thank you for making me strong by teaching me how to achieve real success by battling the odds and winning.

I am also grateful to **Dr Ali Ahmady (PhD), Director IVF Laboratories, University Hospitals/Case Western Reserve University, Cleveland, Ohio, USA,** for training me in Assisted Reproductive Techniques.

A special thank you to **Dr Salman Ahmad Imtiaz, Associate Professor, Assisted Conception Unit, Services Institute of Medical Sciences, Lahore, Pakistan,** and his wonderful team members, Shaeen and Rabbani(late), for supporting and guiding me in sample handling and data collection.

I am greatly indebted to the **Higher Education Commission of Pakistan,** for providing me the financial support for my project. I am also highly grateful to **Mr Jehanzeb Khan, Project Director, International Research Support Initiative Program, Higher Education Commission of Pakistan**, for his persistent help at every step of achieving my scholarship.

Sometimes in life, you find a special friend, who makes you believe that there is good in the world and convinces you that there really is an unlocked door just waiting for you to open it. Your forever friend gets you through the hard times, the sad times and the confused times .Thank you, **Dr Zarrin Fatima Rizvi**, for being an angel for me. I can never repay you for all the love, care and support that you gave me all through the difficult times.

“Family is not about blood, it is about willing to hold your hand when you need it the most”. Although working independently and doing research under my own initiative was daunting at first but the staff and my fellow colleagues because of their very supportive and cooperative attitude, allayed any doubts that I had. Bundles of thanks to my colleagues and best friends forever, Dr Omar Chughtai, Dr Disha Mankame, Dr Akshat Paliwal, Dr Rachna Dubey, Dr Sohaib Hasan, Dr Muhammad Taimoor, Dr Bushra Anwar, Inam Mohammad, Dr Fatima Kaleem, Dr Shoaib Rizvi, Dr Zeeshan Haider, Ambreen Aamir, Wing Commander Aamir Shabbir, Franciele Lunardi,Carolina Sellitto, Mark Pattenden, Debbie Monteleone Garlak, Lisa Sullivan, Dr Atif Zafar, Dr Afia Hasnain, Dr Stefan du Pleissis, Dr Rajinder Singh, Dr Pooja Singh, Dr Elena Gianetti, Dr Luiz Carvalho, Dr Saleem Bani Hani, Dr Afroze Adeel, Dr Gohar, Farah Gohar, Dr Zia-ur-Rahman Khan, Dr Sameera Zia, Brigadier Dr Zulfiqar Baig, Dr Shakeel

Ahmad, Dr Fasiha Ijlal, Aparna Thiyagarajan (late), Patrick Parker, Hyder Syed, Usama Qayyum, Dr Shahzad Irfan, Dr Sababa Khan, Riffat Gillani, Aneela Imran, Sarah Umer, Annie Asim and Group Captain Asim for their persistent co-operation and support.

A special thank you to my wonderful students, Dr Hassan Ejaz, Dr Saher Siddiqi, Dr Ahmar Hameed Bajwa, Dr Naim Muhammad, Dr Mariam Nadeem, Anthony Kashou, Garrett Hack, Nathan Martin Reynolds, Tahir Beydola and David Benjamin and Mary Francispillai.

Thank you to the most wonderful brother-in- law, Babur Niaz Rabbani and his magnificient wife, Mahwish Babur for supporting me in all my endeavors. My humble gratitude to my siblings, Dr Abhishek Deshpande, Sobia Haroon, Ayesha Ali, Ali Saleem Malik, Ahmad Shoaib Akram, Afroze Shoaib, Dr Ahmad Junaid Akram, Momna Junaid Akram and Umair Sarwar for their appreciation, encouragement and prayers that enabled me to achieve this task. Thank you to my uncles, Sarwar Farooq, Arif Farooq and Asad Farooq and aunts, Shahida Arshad, Attiya Abid, Asia Haq, Sabiha Arshad and Tayyaba Sarwar, and all my wonderful cousins, for all their prayers and wishes.

I am also thankful to all those difficult people in my life, for they, through their negative reinforcement, have enabled me today, to be what I wanted to be.

***Dr Arozia Akram***

# ABBREVIATIONS

| | |
|---|---|
| **ADP** | Adenine Dinucleotide Phosphate |
| **BMI** | Basal Metabolic Index |
| **cAMP** | Cyclic Adenosine Monophosphate |
| **ds** | Double Stranded |
| **EN** | Eosin Nigrosin |
| **FasL** | Fas Ligand |
| **GSH** | Glutathione |
| **$G_6$PDH** | Glucose 6 Phosphate Dehydrogenase |
| **LPO** | Lipid Peroxidation |
| **MPF** | Maturation Promoting Factor |
| **mM** | Micromoles |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate |
| **OS** | Oxidative Stress |
| **PBS** | Phosphate Buffer Saline |
| **PKA** | Protein Kinase A |
| **PUFA** | Polyunstaurated Fatty Acid |
| **ROO** | Peroxyl |
| **ROS** | Reactive Oxygen Species |
| **ss** | Single Stranded |
| **SOD** | Superoxide Dismutase |
| **TAC** | Total Antioxidant Capacity |
| **TNF-alpha** | Tumor Necrosis Factor Alpha |
| **TUNEL** | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| **VAR** | Varicocele |
| **WBC** | White Blood Cells |
| **WHO** | World Health Organization |

# LIST OF TABLES

**Table 1: Mean age, height, body weight and body mass index of healthy fertile controls, varicocele negative and varicocele positive subfertile patients in the study population**

**Table 2: Mean semen volume, sperm concentration, motility and morphology of healthy fertile controls and subfertile male subjects, with and without varicocele**

**Table 3: Mean morphological sperm defects of fertile controls and subfertile varicocele negative and positive male subjects**

**Table 4: Mean of leukocytospermia between varicocele negative and varicocele positive subfertile subjects in the study population**

**Table 5: Correlation of leukocytospermia (Endtz Positive Test) with semen characteristics in the varicocele negative and varicocele positive subfertile subjects**

**Table 6: Correlation of leukocytospermia (Endtz Positive Test) with % WHO abnormal sperm morphology in the varicocele negative and varicocele positive subfertile subjects**

**Table 7: Mean of reactive oxygen species of healthy fertile controls, varicocele negative and varicocele positive subfertile subjects in the overall study population**

**Table 8: Mean ± SEM of total antioxidant capacity of healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

**Table 9: Mean of sperm DNA fragmentation by TUNEL Assay of healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

**Table 10: Correlation of reactive oxygen species (Log (ROS+1)) and total antioxidant capacity (TAC) in healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

**Table 11: Correlation of reactive oxygen species (Log (ROS+1)) and sperm DNA fragmentation by TUNEL Assay in healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

**Table 12: Correlation of reactive oxygen species (Log (ROS+1)) with semen characteristics in healthy fertile controls**

**Table 13: Correlation of reactive oxygen species ((Log (ROS+1)) with semen characteristics in varicocele negative subfertile subjects**

**Table 14: Correlation of reactive oxygen species (Log (ROS+1)) with semen characteristics in varicocele positive subfertile subjects**

**Table 15: Correlation of total antioxidant capacity (TAC) with semen characteristics in healthy fertile controls**

**Table 16: Correlation of total antioxidant capacity (TAC) with semen characteristics in varicocele negative subfertile subjects**

**Table 17: Correlation of total antioxidant capacity (TAC) with semen characteristics in varicocele positive subjects**

**Table 18: Correlation of sperm DNA fragmentation with semen characteristics in healthy fertile controls**

**Table 19: Correlation of sperm DNA fragmentation with semen characteristics in varicocele negative subfertile subjects**

**Table 20: Correlation of sperm DNA fragmentation with semen characteristics in varicocele positive subfertile subjects**

**Table 21: Correlation of Leukocytospermia (Endtz Positive Test) with oxidative stress markers in the varicocele negative and varicocele positive subfertile subjects in the study poulation**

**Table 22: Mean of semen characteristics of subfertile subjects according to the level of varicocele grading**

**Table 23: Mean ± SEM of reactive oxygen species levels in Grade I, II and III subfertile varicocele subjects**

**Table 24: Mean ± SEM of total antioxidant capacity (TAC) levels in Grade I, II and III subfertile varicocele subjects**

**Table 25: Mean ± SEM of TUNEL+ sperms in Grade I, II and III subfertile varicocele subjects**

**Table 26: Correlation of reactive oxygen species (Log (ROS+1)) and total anti oxidant levels (TAC) in Grade I, II and III varicocele positive subfertile subjects**

**Table 27: Correlation of reactive oxygen species Log (ROS +1) and TUNEL$^{+ve}$ sperm cells in grade I, II and III varicocele positive subfertile subjects**

# LIST OF FIGURES

**Figure1: Comparison of mean sperm concentrations (million/mL) of healthy fertile controls and subfertile male subjects, with and without varicocele**

**Figure 2: Comparison of mean percentage sperm motility of healthy fertile controls and subfertile male subjects, with and without varicocele**

**Figure 3: Comparison of mean percentage normal sperm morphology (WHO Criteria) of healthy fertile controls and subfertile male subjects, with and without varicocele**

**Figure 4: Comparison of mean percentage normal sperm morphology (Strict Criteria) of healthy fertile controls and subfertile male subjects, with and without varicocele**

**Figure 5: Mean ± SEM of reactive oxygen species levels in healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

**Figure 6: Mean ± SEM of total antioxidant capacity of healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

**Figure7: Mean ± SEM of sperm DNA fragmentation in healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

**Figure 8a: Non- significant correlation of reactive oxygen species (Log (ROS+1)) and total antioxidant capacity (TAC) in healthy fertile controls**

**Figure 8b: Non- significant correlation of reactive oxygen species (Log (ROS+1)) and total antioxidant capacity (TAC) in varicocele negative subfertile subjects**

**Figure 8c: Significant correlation of reactive oxygen species (Log (ROS+1)) and total antioxidant capacity (TAC) in varicocele positive subfertile subjects**

**Figure 9a: Correlation of LOG (ROS+1) and TUNEL$^{+ve}$ sperm cells in healthy fertile controls**

**Figure 9b: Correlation of LOG (ROS+1) and TUNEL$^{+ve}$ sperm cells in varicocele negative subfertile subjects**

**Figure 9c: Correlation of LOG (ROS+1) and TUNEL$^{+ve}$ sperm cells in varicocele positive subfertile subjects**

**Figure 10: Correlation of total antioxidant capacity (TAC) with percentage sperm motility in healthy fertile controls**

**Figure 11: Correlation of total antioxidant capacity (TAC) with Strict's normal sperm morphology in varicocele positive subjects**

**Figure 12: Correlation of sperm DNA fragmentation with percentage sperm motility in varicocele negative subfertile subjects**

**Figure 13: Correlation of sperm DNA fragmentation with percentage sperm morphology according to Strict Criteria in varicocele negative subfertile subjects**

**Figure 14a: Correlation of sperm DNA fragmentation with sperm concentration (million/mL) in varicocele positive subfertile subjects**

**Figure 14b: Correlation of sperm DNA fragmentation with percentage sperm motility (%) in varicocele positive subfertile subjects**

**Figure 14c: Correlation of sperm DNA fragmentation with WHO sperm morphology (%) in varicocele positive subfertile subjects**

**Figure14d: Correlation of Sperm DNA fragmentation with sperm morphology (%) according to Strict Criteria in varicocele positive subfertile subjects**

**Figure 15a: Correlation of reactive oxygen species (Log (ROS+1)) and total anti oxidant capacity (TAC) in grade I varicocele positive subfertile subjects**

**Figure 15b: Correlation of reactive oxygen species (Log (ROS+1)) and total anti oxidant capacity (TAC) in grade II varicocele positive subfertile subjects**

**Figure 15c: Correlation of reactive oxygen species (Log (ROS+1)) and total anti oxidant levels (TAC) in grade III varicocele positive subfertile subjects**

**Figure 16a: Correlation of reactive oxygen species (Log (ROS +1)) and TUNEL$^{+ve}$ sperm cells in grade I varicocele positive subfertile subjects**

**Figure 16b: Correlation of reactive oxygen species (Log (ROS +1)) and TUNEL$^{+ve}$ sperm cells in grade II varicocele positive subfertile subjects**

**Figure 16c: Correlation of reactive oxygen species (Log (ROS +1)) and TUNEL$^{+ve}$ sperm cells in grade III varicocele positive subfertile subjects**

# ABSTRACT

Varicocele, vascular lesions of the pampiniform plexus, is one of the most controversial issues in the field of andrology, regarding its diagnosis and management. Earlier studies suggest that males with varicocele are more at risk of spermatogenic dysfunction hence affecting fertility. The mechanism by which varicocele affect fertility and spermatogenesis is still unknown. An inherent challenge to aerobic life of spermatozoa, the cells responsible for propagation of the species, is oxygen toxicity. Reactive oxygen species (ROS) in the semen have both, physiological and pathological role in male fertility. Increased oxidative stress affects fertility by causing damage to sperm membranes, proteins, and DNA. Seminal oxidative stress is thus emerging as an essential step in the diagnosis and prognosis of subfertile males approaching the infertility clinics. Seminal plasma is endowed with an array of free radical scavengers called antioxidants that protect the spermatozoa against oxidative stress. Hence antioxidants are essential for the survival and functioning of spermatozoa. Varicocele has been found to be associated with increased production of ROS in the semen along with decreased antioxidant levels in the seminal plasma, suggesting that spermatogenic dysfunction in varicocele males maybe in part related to oxidative stress. Oxidative stress also affects the genomic integrity of the spermatozoa by causing both, single and double stranded DNA breaks as seen in the ejaculates of sub fertile males.

The initial evaluation of a male coming for subfertility treatment includes a detailed history, a thorough physical examination, hormonal evaluation and semen analyses. Since a normal spermiogram does not give a definitive diagnosis about sperm function, specialized sperm function tests have been developed to test different aspects of sperm function. The conventional parameters of semen including sperm concentration, motility, and morphology are insufficient for evaluation of reproductive potential. Standardization of protocols for assessment of reactive oxygen species levels and the extent of sperm DNA fragmentation in the subfertile males is very important in clinical practice and may help develop new therapeutic strategies and treatment regimes.

To explain the pathophysiological mechanisms of sperm dysfunction that might account for the subfertility, the aim of the study was to evaluate

(i) semen quality (ii) levels of reactive oxygen species (ROS) in the semen (iii) the total antioxidant capacity (TAC) of the seminal plasma (iv) the extent of sperm DNA fragmentation (v) the relationship between the seminal parameters, levels of reactive oxygen species, total antioxidant capacity and sperm nuclear DNA fragmentation in males coming for subfertility evaluation, diagnosed clinically with or without varicocele.

My study population consisted of 115 normal healthy fertile males, 121 sub fertile males diagnosed with varicocele on ultrasonography, while 66 males were sub fertile but had no varicocele. The varicocele subfertile group was further categorized into grade I, II, III, based on physical examination. After a detailed history and physical examination, semen analysis was performed using the WHO, 1999, Criteria. Semen volume, percentage sperm motility, normal and abnormal sperm morphology, both by WHO 1999, Criteria and Strict morphology was assessed. There was no significant difference ($p>0.05$) in the time of abstinence and seminal volumes in controls, varicocele negative and varicocele positive subjects. Varicocele positive subjects showed ($p<0.001$) the lowest mean sperm concentration, percentage sperm motility and percentage normal sperm morphology as compared to the varicocele negative subfertile subjects and healthy fertile controls. According to the WHO, 1999, Criteria of sperm morphology, both varicocele negative and positive subfertile subjects had a significant difference ($p<0.001$) in tapered sperm morphology as compared to healthy fertile controls. Varicocele positive and negative subfertile subjects showed a significantly higher ($p<0.001$) amorphous sperm morphology as compared with the controls. A significant difference ($p<0.001$) was seen in small sperm morphology between the varicocele positive subjects and fertile controls. Megalo sperm defects were significantly ($p<0.001$) more in the varicocele positive subjects than the varicocele negative and healthy fertile controls. No significant difference ($p>0.05$) was observed in bicephalic sperm defects in all the three sub-groups. A significantly higher percentage of ($p<0.001$) tail defects were seen in the varicocele negative and varicocele positive subjects as compared to the controls. Significantly raised ($p<0.001$) head defects were also seen in the varicocele negative and varicocele positive subjects as compared to the healthy fertile controls. A significantly raised ($p<0.001$) levels of ROS and sperm DNA fragmentation, with significantly ($p<0.001$) decreased total anti-oxidant capacity levels were seen in the varicocele negative and varicocele

positive subjects as compared to the healthy fertile controls. ROS levels showed a significant ($p<0.05$) negative correlation with TAC levels in the varicocele positive subjects. However, a significant ($p<0.05$) positive correlation was seen between ROS and sperm DNA fragmentation in healthy fertile controls as compared with the subfertile groups. A significant negative correlation ($p<0.05$) was seen between TAC and percentage sperm motility in healthy fertile controls, whereas the varicocele positive subjects showed a significant positive correlation between TAC and normal sperm morphology as assessed by Strict Criteria.

Sperm DNA fragmentation showed a significant ($p<0.05$) negative correlation between percentage sperm motility and normal sperm morphology in varicocele negative subfertile subjects. However, varicocele positive subfertile subjects showed significant ($p\leq0.001$) negative correlations of sperm DNA fragmentation with sperm concentration, motility and normal sperm morphology. Leukocytospermia also showed no significant correlation ($p>0.05$) with semen parameters as well as with oxidative stress markers.

In another set of observations, semen quality and oxidative stress parameters was studied in male subjects having different grades (I, II, III) of varicocele. A significantly ($p<0.01$) decreased sperm motility was seen in grade 1 varicocele subjects as compared to grade II and III varicocele. A significantly ($\leq0.01$) increased megalo sperm defects were observed in grade I and III as compared with subjects having grade II varicocele. No significant differences ($p>0.05$) were seen in the ROS, TAC and sperm DNA fragmentation in subfertile subjects having different grades of varicocele. Also no significant correlation of ROS with TAC and sperm DNA fragmentation by TUNEL Assay was seen in different grades of varicocele.

In conclusion the present study suggests

- Subfertility, especially with a varicocele leads to a deterioration in semen quality
- Increased ROS levels maybe a causative factor for increased sperm DNA fragmentation, as seen in the sub-fertile group

# INTRODUCTION

For the survival of human race, fertility and reproduction have been the core issues since pre-historic ancient times (Bhasin, 2007). A state of complete mental, social and physical well-being of both male and female reproductive tract is called Reproductive Health (Cocuzza and Agarwal, 2007). Infertility is a term that describes a couple who has failed to conceive or has been unable to induce pregnancy within one year of regular unprotected intercourse in the fertile phase of menstrual cycle (Evers, 2002; Rowe *et al*, 1993). Subfertility describes any form of decreased fertility with a prolonged time of unwanted non-conception, in couples unsuccessfully trying to conceive (Jenkins, 2004; Gnoth *et al*, 2005). Of the total infertile couples who seek evaluation for infertility, 30-50% of infertility is accounted by male factor (McLachlan and de Kretser, 2001). Sperm dysfunction is the commonest defined cause of infertility, affecting one out of six couples (Bhasin, 2007).

## SPERM FUNCTION TESTING

**S**emen analysis, the cornerstone of assessment of male factor infertility, not only evaluates the spermatozoa but also the seminal plasma and non-sperm cells (Samplaski *et al*, 2010). An ideal sperm helps in (i) diagnosis of a specific spermatozoal dysfunction; (ii) prediction of fertilization or pregnancy rates and (iii) indication of specific therapies for alleviation of the identified dysfunctional spermatozoa (Muller, 2000). Assessment of seminal parameters is helpful in investigation of male factor infertility, genital tract infections and pathologies (Comhaire and Vermeulen, 1995; Silber, 2000). Semen analysis is useful in evaluation of adverse effects of drugs, environmental pollutants and chemical products affecting fertility of males (Sharpe, 2000; Bonde and Storgaard, 2002). A wide variety of semen parameters measured by the semen analysis, are an indicator of the semen quality and can reduce the number of variables evaluated (Aitken *et al*, 1982; Carrell, 2000; Krause 1995; De Jonge, 1999). Considering that a history of infertility is the major reason for semen analysis, it is necessary that methodologies employed for semen analysis should be standardized. Practicing a routine semen analysis is the first step towards determination of the influence of genital pathophysiology on the

reproductive capacity of male, even then certain parameters might not be of any clinical significance (World Health Organization, 1999).

Microscopic examination of concentration, motility and morphology has been used as the routine parameters of human spermatozoa. The clinicians base their initial diagnosis upon the fundamental information provided by analysis of these indicators (Barratt *et al*, 2010). However, the conventional semen analysis provides very limited diagnostic and prognostic information about sperm function (Samplaski *et al*, 2010). A general and obvious cause of male infertility is definitely impaired sperm function (Pasqualotto *et al*, 2008).

Analysis of seminal parameters also helps in providing important clinical information regarding spermatogenesis, the functional competence of spermatozoa and also the secretory pattern of accessory genital glands. It is well-documented that accuracy of semen analysis, standardization of proper methodologies and procedures and reference values, all contribute towards the quality control in laboratory practice. Currently, the World Health Organization (WHO) has standardized basic requirements of semen analysis providing guidelines that describe several diagnostic procedures and andrology techniques for evaluation of semen quality that have gained worldwide acceptance as guidance to standardized methodology for human semen analysis and help in assessment of semen parameters, like, concentration, motility and morphology (WHO, 1987; 1992; 1999). Therefore, a basic semen analysis takes help of semen parameters towards formulation of a diagnostic work-up in accordance with the clinical evaluation of a male presenting for subfertility (Andrade-Rocha, 2003; WHO, 1999).

## SEMEN PARAMETERS

### Sperm Density

Total sperm number is defined as the total number of spermatozoa in the entire ejaculate and is obtained by multiplying the sperm concentration by the seminal volume (WHO, 2010).

**Sperm Concentration**

Sperm concentration is referred to as the number of spermatozoa per unit volume of the semen and is an indicator of the number of the spermatozoa ejaculated and the volume of the fluid that dilutes them. This determines the amount of spermatozoa present in the semen specimen and is expressed in sperms/milliliter (mL). It is further sub-divided as follows: (Andrade-Rocha, 2003).

**Polyzoospermia:** is defined as the presence of a sperm concentration between 250-350 x $10^6$ /mL but with normal sperm parameters - motility, morphology and viability. In sperm counts greater than 350 x $10^6$ /mL, polyzoospermia may be associated with asthenozoospermia and/or teratozoospermia (Andrade-Rocha, 1994). Polyazoospermia is considered a pathological finding not only because of an overproduction of spermatozoa, but also for its association with decreased reproductive performance as a result of dysfunctional acrosomal membrane (To¨pfer-Petersen *et al*, 1987), chromosomal abnormalities (Chan *et al*, 1986) and decreased ATP content (Calamera *et al*, 1987).

**Normozoospermia:** Although the sperm count is between 20-250 x $10^6$ /mL, several disorders, e.g, leukocytospermia, antisperm antibodies, abnormal functional activity of the seminal vesicles and the prostate gland, genital tract infections varicocele, can cause impairment of semen quality in normozoospermic males, leading to asthenozoospermia and /or teratozoospermia (Andrade-Rocha, 2003).

**Oligozoopsermia:** Sperm counts may vary between 10-20 x $10^6$ /mL in mild, 5-10 x $10^6$ /mL in moderate and $< 5 \times 10^6$ /mL in severe oligozoospermia (Andrade-Rocha, 2003). Functional disturbances of the testis, e.g endocrine disorders varicocele and as well as factors of nontesticular origin, e.g drug toxicity, environmental pollutants, mumps orchitis, radiation and exposure to chemical products all are involved in the causation of mild and moderate oligozoospermia (Merino *et al*,1995; Forti and Krausz,1998). Severe oligozoospermia is associated with genetic abnormalities, such as Y chromosome microdeletions (Dohle *et al*, 2002). Oligozoospermia is associated with abnormal sperm morphology and decreased sperm motility, hence deteriorating semen quality and its fertilization capacity. However, these males have the natural ability to fertilize naturally, even in severe oligozoopsermic conditions (Matorras *et al*, 1996).

**Azoospermia**: Differential diagnosis of azoospermia is based on physical examination of the male, testicular biopsy, endocrine evaluation and genetic screening. Azoospermia is classified into two types, for diagnostic purposes, (i) **non-obstructive** or **secretory** resulting as a cause of extreme testicular failure and (ii) **obstructive** or **excretory** caused by occlusion of the testis, epididymis and excretory ducts, hence preventing the release of spermatozoa in the seminal ejaculate (Kolettis, 2002). Microdeletions of the Y chromosome may also be involved in the pathogenesis of azoopsermia (Dohle *et al*, 2002). Congenital bilateral absence of the vas deferens and the seminal vesicles as a result of cystic fibrosis gene mutation is a special case of azoospermia. Inspite of its rarity, this pathology is easily identifiable by the presence of elevated levels of prostatic biomarkers, absence of seminal vesicle markers, a seminal ph $<7.0$ and a seminal volume of $\leq$ 1.0mL (Daudin *et al*, 2000).

## Sperm Motility

Spermatozoa do not exhibit progressive motility, on reaching the caput of the epididymis from the seminiferous tubules and the rete testis. Once exposed to the microenvironment of epidiymis, occurance of molecular changes in the spermatozoa lead to increased capacity of forward progressive and sustained motility. Mature and motile sperm remain stored within the cauda epididymis, in a quiescent phase and release at ejaculation after acquiring an instantaneous burst of vigorous activity (Cooper, 1996; Moore, 1998). Analysis of sperm motility gives information on epididymal function. Sperm motility depends on the quality of the spermatozoa produced and hence related directly with testicular function. The secretions by the prostate also influence the sperm and seminal vesicles. Therefore, sperm motility is affected by functional disorders of the genitalia (Andrade-Rocha, 1994).

## Sperm Morphology

This important parameter of semen evaluates the quality of the sperm and is divided into the following criteria:

**(i)The WHO Criteria**

World Health Organization, (1999), Criteria describes the percentage of normal oval sperm heads, as well as a variety of sperm defects present in the semen. Assessment of sperm morphology using this criterion thoroughly evaluates the sperm head, midpiece and tail defects, indicating abnormal spermatogenesis and associated seminal pathologies (Moench and Holt, 1931; Hartman *et al*, 1964; Zamboni, 1987; Bartoov *et al*, 1980).

**(ii)The Tygerberg Strict Criteria**

The Tygerberg Strict Criteria (Kruger *et al*, 1986) defines the sperm morphology by evaluation of the acrosomal status of the sperm membrane. According to this criterion, spermatozoa having an oval head and a well-defined acrosome covering 40-70% of the sperm head are classified as being normal. Semen having > 14% spermatozoa with normal morphology present a good prognosis for in vivo as well as in vitro fertilization. Values ranging from 4-14% also indicate good prognosis but a decreased rate of fertilization than semen with more number of normal spermatozoa. Value of normal spermatozoa <4% indicates poor prognosis (Andrade-Rocha, 2003).

**Leukocytospermia**

Leukocytospermia, defined as >1 x 10 million WBC/mL is correlated negatively with different parameters of sperm function, especially with impaired sperm motility and morphology, acrosomal membrane damage and sperm tail defects Presence of leukocytes in the epididymis, seminal vesicles, urethra and prostate is a physiological process required for elimination of abnormal germ cells from the seminal ejaculate (Aziz *et al*, 2004; Wolf, 1995). High leukocyte content causes an increased generation of toxic metabolites exceeding the neutralizing capacity of antioxidants present in the seminal plasma, leading to generation of oxidative stress (Andrade-Rocha, 2003). Quantification of seminal leukocytes constitutes an important part of the standard semen analysis, but it may be difficult to see them under the light microscope. The Endtz test stains for peroxidase within the polymorphonuclear granulocytes, distinguishing them from immature germ cells (Shekarriz *et al*, 1995).

The diagnosis of leukocytospermia has been done by immunohistochemical, cytochemical and morphological techniques (Wolff *et al*, 1992; Jochum *et al*, 1986). The Endtz Test has been recommended by the WHO for determination of WBCs in the semen and is based on the peroxidase activity of polymorphonuclear leukocytes (WHO, 1987). It is a simple, cost-effective test, but the only limitation is the lack of lymphocyte detection in the semen. However, neutrophils and macrophages are the main peroxidase positive cells which are important in diagnosis as they are the source of reactive oxygen species by phagocytosis (Wolff *et al*, 1992; Agarwal *et al*, 1994).

**Variability in semen analysis**

According to a consensus, the most important step in the investigation of male infertility is the basic semen analysis that has been interpreted using the World Health Organization (WHO) Criteria (Said *et al*, 2011). For standardization and consistency in the laboratory procedures, WHO has been publishing manuals for the examination of human semen and semen-cervical mucus interaction. These manuals identify exclusion criteria, such as time of spermatogenic cycle, temperature, abstinence and patient health corresponding with the spermatogenic cycles. The manuals have been regularly updated since, 1980, 1987, 1992 and 1999, (Lewis 2007). The consistency in results in different laboratories is because of addition of normal reference values from these WHO manuals. However, it is important to note that values were identified in healthy fertile men rather than men who were at the verge of subfertility. However, the data was obtained from laboratories who used different methodologies for semen analysis. This heterogeneity further decreased the clinical significance of the standard reference values established by the WHO (Alvarez *et al*, 2003, Jorgensen *et al*, 2001) because although these reference values were from men who had fathered children, yet these studies lacked actual reference ranges and limits. The lack of consensus between different laboratories, these reference values were considered either too high or too low, further subdividing a group of fertile men as subfertile (Barratt *et al*, 1988, Chia *et al*, 1998, Gao *et al*, 2007, 2008). In addition to treating the subfertile men, fertile men with a low semen quality may also be investigated and treated subsequently (Lemcke *et al*, 1997, Bostofte *et al*, 1972).

The data has recently been modified in men, based on the evaluation of one thousand nine hundred and fifty three men whose partners conceived within a period of one year. These men had 1.5mL semen volume, 39 million/ejaculate total sperm count, 15million/mL sperm concentration, 58% vitality, 40% total motility with 32% total progressive motility and 4% normal sperm morphology (Cooper *et al*, 2010). Previous data indicates subtle variations existing in semen characteristics from different geographic areas as well as between samples from the same individual (Alvarez *et al*, 2003, Jorgensen *et al*, 2001). However, there is still controversy about certain aspects of the 2010 WHO manual. Eliasson *et al*, (2010), has recommended evaluation of sperm morphology and progressive motility. This evaluation of sperm count, motility and morphology could be improved by applying standardized procedures training workshops and thorough quality control schemes (Franken *et al*, 2003, 2006, 2007). Assessment of sperm membrane integrity is important for dead spermatozoa or alive spermatozoa with <40% progressive motility. Additionally a one-step eosin-nigrosin staining may also be required, especially in cases where ICSI is recommended (Bjorndahl, 2003).

**Contemporary methods of semen analysis**

Currently methods employed for evaluation of human semen vary substantially, ranging from those recommended by the World Health Organization (WHO), to advanced automated technology, for example, Computer Aided Sperm Analyzer (CASA), for characteristics of sperm motility, morphology and analysis of other physical and biochemical parameters (Boyle *et al*, 1992; Barratt *et al*, 1993; Macleod and Irvine, 1995). Computer aided sperm analyzers (CASA) has been developed which uses digital image analysis for automated analysis of the semen (Mortimer, 2000). CASA provides a rapid measurement of individual "classical" sperm parameters, for example, sperm count and motility (Krause, 1995) and allowing determination of sperm motion characteristics called "kinematics" that cannot be determined under light microscopy ( Davis and Katz, 1993; Boyle *et al*, 1992). The application of this technology has been challenged due to errors in its setup and detection of specific objects (Davis and Katz, 1993). The only advantage of CASA is that this system, through serial digital images, plots the movements of the sperm head, showing motion kinetics of the sperm that are not possibly

assessed through routine microscopy (Kay and Robertson, 1998). Several studies have emphasized the clinical value of sperm kinetics in diagnosis of males with unexplained infertility and prediction of in vivo and in vitro fertilization rates (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996; Peedicayil *et al*, 1997; Shibahara *et al*, 2004). Despite the clinical importance of assessment of sperm kinetics, it is still believed that individual sperm motility parameters hold little importance (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996). The difference in various models of CASA instruments and difference in their setup have made it impossible to reach to a conclusion (Sukcharoen *et al*, 1995). A comparison of the measurements obtained by using CASA with those obtained by manual semen analysis may show discrepancy because of the difference in methodologies (ESHRE, 2000). The use of CASA has been criticized because of difficulties in operating the equipment (Boone *et al*, 2000; Carrell, 2000; Oehninger *et al*, 2000) and because of difficulties in achieving optimum set-up procedures (Davis and Katz, 1993; Mortimer, 1994; Clements *et al*, 1995). Semen analysis with automated equipment may help in avoiding biases and intra- and inter- laboratory variability using the manual methodologies (Barosso *et al*, 1999). However, these computerized systems still have problems in their development and hence not recommended for routine use (Wang *et al*, 1991; Kruger *et al*, 1995; Davis *et al*, 1992; Davis and Gravance, 1994).

The most widely used semen parameter is sperm count. Males having a sperm count less than 20 million spermatozoa/mL are categorized as “subfertile”, while males with a sperm count less than 5 million spermatozoa/mL are considered “infertile”. Similarly, semen samples having less than 14% of normal sperm morphology, according to Strict criteria are “subfertile” while, males containing less than 5% sperms with normal morphology are categorized as “severely impaired” and recommended for donor insemination (Agarwal *et al*, 2003). Criticism on the reported predicted values of sperm count is a result of day-to-day variation in the sperm concentration (Huszar *et al*, 1988a, b).

Sperm function testing seems to have lost significance in the era of assisted reproductive technology. Most couples seeking infertility treatment are opting for inexpensive and less

invasive solutions that would increase the success rate for a spontaneous pregnancy or pregnancy through assisted reproductive treatment strategy (Muller, 2000). Despite the development of several sperm function tests, very few are being adopted in the clinical settings as none of them has been proved to be a reliable predictor of the fertility status of the male (Agarwal *et al*, 2008a).

The sperm-cervical mucus penetration test (SMTP), which measures the ability of the spermatozoa to swim in the cervical mucus has a different reference range, hence creating a discrepancy in interpretation of results (Kremer and Jager, 1992).

The presence of antisperm antibodies (ASA) has a negative effect on human fertility (Naz and Menge, 1994). However, the testing for antisperm antibodies still remains controversial because of the variability in application of different techniques as well as interpretation of results. Previously used methods are all obsolete, with only the mixed antiglobulin reaction (MAR) test and the immunobead test (IBT) being used to detect the presence of ASA (World Health Organization, 1999). Hence, it can be said that ASA testing plays a limited role in diagnosis of cases of unexplained fertility or severe asthenozoospermia (Agarwal and Said, 2011).

The European Society of Human Reproduction and Embryology (ESHRE) and World Health Organization (WHO), have included Sperm-Binding Assays and Induced Acrosome Reaction as additive tests helping in prediction of fertilization outcomes as well as diagnostic applicability in the clinical settings (ESHRE, 1996; Oehninger *et al*, 2000). Esterhuizan *et al*, 2000, pointed out that evaluation of the sperm morphology; especially the acrosomal configuration gives a good estimate of the fertilizing ability of the sperm. (Menkveld *et al*, 2003), concluded that the acrosomal status is a reflection of the fertilizing ability of the sperm. Determining the acrosome reaction is important for the diagnostic and therapeutic strategies in infertile couples opting treatment through assisted reproductive technology (Franken *et al*, 1997).The human sperm-oocyte interaction in vitro assay, first described by Overstreet and Hembree, (1976), was developed for evaluation of zona penetration and outlined procedures for the hemi-zona assay (Burkman *et al*, 1988) and intact zona pellucida binding test (Liu and Baker, 1992).

The Hypo-Osmotic Swelling Test determines the function of the sperm plasma membrane and is indicated in infertile patients having very few or no motile sperm in the semen (Jeyendran *et al*, 1984). This assay is generally used for assessment of sperm viability, immotile cilia syndrome (Peeraer *et al*, 2004).or severe asthenozoospermia (Franken and Oehinger, 2012).

The results of zona-free hamster oocyte sperm penetration (SPA) assay, which examines the ability of spermatozoa for capacitation and used for prediction of the likelihood of spontaneous pregnancy in vivo as well as successful fertilization using IVF, are not considered meaningful because of the false positive and false negative rates (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996).

**Limitations of semen analysis**

Although, manual semen analysis, using light microscopy, is an easy test to perform, accuracy in technique is important for accurate interpretation of results (Keel and Webster, 1990; Mortimer, 1990). However, manual analysis is prone to inter and intra-observer technical variations (Keel and Webster, 1990). International standardization of the essential sperm parameters – count, motility and normal sperm morphology, organization of international training workshops and establishment of external quality control plans will improve semen analysis (Franken *et al*, 2003; Franken and Kruger, 2006; Franken and Dada, 2007). Adequate training of technicians is necessary for consistency of results within a given laboratory (Barosso *et al*, 1999). This interlaboratory variation maybe the result of different factors (i) different methodology of preparing the semen and seminal smears (ii) difference in interpretation of results (iii) experience of the technician (Coetzee *et al*, 1999).

Semen analysis has several limitations for epidemiological studies of male fertility (Macleod, 1979; Bonde, 2010). More importantly, the selection criteria, recruitment and sample collection, preparation and processing of the samples, uniformity in protocols, quality assurance and well- trained personnel, should be the same for the study group as well as for the reference group (Bonde, 1996; Jørgensen *et al*, 2001; Andersen *et al*, 2000). Nevertheless, there are advantages also, for example, such studies allow the study

of male fertility independent of any attempts made for obtaining a pregnancy, establishing a relationship between semen quality and fertility (Macleod, 1979; Bonde, 2010).

Hence, to date the manual semen analysis is still considered the most reliable method for assessment of sperm parameters-count, motility and morphology (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996). An ideal sperm function test should have the following clinical steps (i) diagnosis of the specific cause of spermatozoal dysfunction (ii) fertilization and pregnancy rate predictability (iii) therapeutic strategy for the specific spermatozoal dysfunction (Muller, 2000). Addition of advanced sperm function test to routine semen analysis might eventually be useful in the clinical settings, but more information is still needed to determine whether these test will help in predicting the fertility potential (Samplaski *et al*, 2010).

## VARICOCELE

### Etiology

Varicocele, the elongated and tortuous spermatic veins, are the most frequent abnormal physical finding in males presenting for evaluation of infertility (Al-Ali *et al*, 2010).
Presence of varicocele dates back to the 1$^{st}$ century A.C, when the Greek physician Celsus reported that “the veins over the testicles are twisted, which makes that testicle swollen than its counterpart, appearing as if its nutrition has become defective” (Kantartzi et al, 2007; Tulloch, 1952). Varicocele is defined as abnormal tortuosity and dilatation of the pampiniform venous plexus within the spermatic cord, occurring most frequently on the left side. Varicocele occurs approximately 15-20% in the general population, 19-41% in men with primary infertility and 69-94% in men with secondary infertility (Jarow, 2001; Witt and Lipshultz, 1993; Gorelick and Goldstein, 1993., Vivas-Acevedo *et al*, 2010; Meacham *et al*, 1994; Greenberg, 1977). Thirty–five percent to 50% of males with primary infertility and 81% of males with secondary infertility are diagnosed with varicocele (Deepinder *et al*, 2008; Fretz and Sandlow, 2002).

Anatomical variations, congenital and/or acquired valvular defects, venous reflux secondary to venous obstruction are the factors involved in the controversial etiology of varicocele. According to one theory, varicocele occurs as a result of anatomical difference between the right and left spermatic veins. The right spermatic vein inserts directly into the inferior vena cava at an acute angle whereas the left spermatic vein inserts into the left renal vein at a right angle. This disparity leads to increased hydrostatic pressure in the left spermatic vein. This hydrostatic pressure is transferred to the pampiniform plexus causing their dilatation. A second theory suggests that there is regression of blood due to dysfunctional valves of internal spermatic veins. Finally, a third theory suggests that varicocele occurs due to partial obstruction of the left spermatic vein as a result of compression of the left renal vein between the superior mesenteric artery and aorta (Naughton *et al*, 2001). Pooling of blood in the pampiniform plexus as a result of valvular dysfunction occurs more likely in the left spermatic vein than in the right because of the normal anatomical asymmetry (Biyani *et al*, 2009). The left testicular vein is longer than the right testicular vein and enters the left renal vein at a right angle. The "nutcracker effect", produced as a result of compression of the left renal vein between the descending aorta and the superior mesenteric artery leads to increased left renal vein compression causing retrograde blood flow down the internal spermatic veins and cremasteric veins. This explains the reason why varicocele is more common on the left side than on the right (Agarwal *et al*, 2009). The right varicocele is considered rare maybe associated with abnormal drainage into the right renal vein, congenital malformation, e.g. situs inversus or a compressive retroperitoneal tumor (Vivas-Acevedo *et al*, 2010; Wilms *et al*, 1988). However, due to the use of modern diagnostic techniques, such as conventional or Colored Doppler ultrasound of the scrotum, the increased frequency of bilateral localization has been documented in more recent studies (Kantartzi *et al*, 2007; Das *et al*, 1999; Lund *et al*, 1999; Lemack *et al*, 1998).

**VAR GRADING**

Varicocele has been categorized into three grades on the basis of severity and the physical findings (Vivas-Acevedo *et al*, 2010); **Grade 1** or **small varicocele:** invisible but palpable on Valsalva maneuver (straining), **Grade II** or **medium varicocele:** visible

and palpable distension (without Valsalva) of sperm cord structures on upright examination at room temperature, **Grade III** or **large varicocele:** visible through scrotal skin, on clinical examination when standing upright at room temperature (Jarow, 2001; Redmon *et al*, 2002; WHO, 1992).

**Pathophysiology**

The pathophysiology of varicocele is controversial, with theories ranging from congenital/acquired valve defects, venous obstruction and anatomical variations. It has also been proposed that factors like changes in testicular blood flow and venous pressure, retrograde flow of adrenal/renal products, hyperthermia, hormonal dysfunction, autoimmunity, Leydig cell dysfunction and apoptosis maybe involved in pathogenesis of varicocele (Vivas-Acevedo *et al*, 2010; Allamaneni *et al*, 2004; Naughton *et al*, 2001; Agarwal *et al*, 2009; Shiraishi *et al*, 2009). Factors, such as increased intratesticular pressure, increased testicular temperature, hypoxia due to attentuation of blood, hormonal profile abnormalities (Fuzisawa *et al*, 1989; Comhaire, 1991; Sweeney *et al*, 1995; Wright *et al*, 1997). Insufficiency of the internal spermatic valves and malfunctioning of valves of external spermatic and cremasteric veins leads to reflux of warm blood from the abdominal cavity into the scrotum, causing an increased intratesticular temperature (Goldstein and Eid, 1989; Chehval and Purcell, 1992).Chronic vasoconstriction caused as a result of increased concentration of toxic metabolites, such as, catecholamines from the adrenal gland lead to dysfunction of the spermatic epithelium. The mechanism by which increased intratesticular temperature in varciocele affects spermatogenesis is not clearly understood. One of the theories suggests that it is the thermal damage of the sperm DNA and the proteins present in the nucleus of the spermatic tubular cells and/or Leydig cells which alters spermatogenesis (Naughton *et al*, 2001; Fuzisawa *et al*, 1989). It is also speculated that oxidative stress, caused either by increased reactive oxygen species levels or decreased levels of antioxidants may also cause spermatogenic dysfunction (Hendin *et al*, 1999).

**Effect of varicocele on seminal parameters**

The speculation that varicocele causes male infertility is based on the fact that varicocele has an increased incidence, its correlation with decreased semen parameters and reduced testicular size and finally from the fact that correction of varicocele leads to an improvement of semen parameters and pregnancy rates (Kantartzi *et al*, 2007). Researchers are debating on the extent of varicoceles effect on sperm characteristics, as earlier studies have shown that sperm parameters seen in varicocele patients vary from normozoospermia to mild or moderate asthenozoospermia, teratozoospermia and asthenoteratzoospermia. Sperm concentration is not affected initially, but later all three sperm parameters- concentration, motility and morphology deteriorate, even causing azoospermia in a few cases (Papadimas and Mantalenakis, 1983).

## FREE RADICALS

A group of highly reactive atoms or ions or chemical molecules, having one or more unpaired electron and oxidatively modifying stable biomolecules by stealing their electrons, making it to become a free radical itself, hence beginning a chain reaction leading to disruption of living cells(Warren *et al*, 1987; Sikka, 2001). Free radicals play a very important role in the pathophysiology of human spermatozoa. The mechanism of regulation of ROS is unclear but it may involve tyrosine phosphorylation of sperm proteins. The processes of reduction-oxidation play a vital role in acquisition of sperm fertilizing ability (Agarwal *et al*, 2003; Sikka, 1996; Sharma *et al*, 1999). However, uncontrolled and excessive generation of ROS leads to oxidative stress, resulting in impaired viability, decreased sperm motility and increased defects of the mid-piece (de Lamirande and Gagnon, 1995; Aitken *et al*, 1989).

Human spermatozoa are very susceptible to ROS attack because of a high content of polyunsaturated fatty acids within the plasma membrane (Aitken *et al*, 1989; Jones *et al*, 1979; Sharma and Agarwal A. 1996). To protect the spermatozoa against free radical toxicity, the seminal plasma possess an antioxidant system comprising of high and low molecular weight factors (Kovalski *et al*, 1992). An indicator of oxidative stress is the imbalance between total antioxidant capacity (TAC) and production of ROS in the

seminal fluid and is correlated with male infertility (Pasqualotto *et al*, 2000). Infertile men have an impaired seminal plasma antioxidant capacity than fertile men, suggesting an association between male infertility and total antioxidant capacity (Lewis *et al*, 1997; Sharma *et al*, 1999).

**Generation of reactive oxygen species in human semen - sources of origin**

Leukocytes and morphologically defective spermatozoa are the main source of the reactive oxygen species alongwith immature spermatozoa (Kessopoulou *et al*, 1992). The mature and immature spermatozoa are a potential source of production of ROS in the human semen. Generation of ROS in the semen is also from other different sources like, epithelial cells, morphologically defective spermatozoa and leukocytes being the two major sources of production (Kessopoulou *et al*, 1992; Aitken RJ., 1995, Garrido *et al*, 2004). ROS includes both radical (hydroxyl ion, nitric oxide and superoxide) and non-radical (hydrogen peroxide, lipid peroxide, singlet oxygen and ozone) oxygen derivatives (Agarwal and Prabakaran 2005). All these derivatives participate in a cascade of reactions leading to the formation of free radicals which damage organic substrates later (Sikka, 2001).

**Mechanism of generation of reactive oxygen species by the spermatozoa**

**Cytoplasmic Droplets**

A negative correlation between ROS production by spermatozoa and the poor sperm quality is a clear indication suggesting that human spermatozoa produce oxidants (Aitken *et al*, 1992; Hendin *et al*, 1999; Gil-Guzman *et al*, 2001; Gomez *et al*, 1998). Presence of excessive residual cytoplasm in the form of a cytoplasmic droplet is the main link between poor semen quality and increased production of ROS. Impaired spermatogenesis leads to defective extrusion of cytoplasm. The resulting spermatozoa released from the germinal epithelium during spermiation carry surplus residual cytoplasm and are immature and functionally defective (Huszar *et al*, 1997). ROS production by the residual cytoplasm is mediated by the cytosolic glucose-6-phosphate de-hydrogenase ($G_6PD$). $G_6PD$ by controlling the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH), mediates the rate of glucose flux in the hexose monophosphate

shunt. NADPH with the help of an enzyme system, NADPH-oxidase, acts as a source of electrons for the production of ROS in spermatozoa (Aitken *et al*., 1997). ROS generation by the spermatozoa occurs through two pathways (1) at the sperm plasma membrane level through the NADPH-oxidase system and (2). At the level of the mitochondria through NADH-dependant oxidoreductase (diphorase) system (Aitken *et al*, 1992). The mitochondrial system is the major source of ROS, mainly the superoxide anion ($O_2^-$) in spermatozoa of infertile men. The superoxide anion, in presence of transition metals, like iron and copper and the enzyme, superoxide dismutase, leads to generation of hydroxyl radical. This pernicious hydroxyl radical is an extremely powerful initiator of the lipid peroxidation cascade, leading to a deterioration of sperm functions (Plante *et al*, 1994; Agarwal *et al*, 1994).

**Polymorphonuclear Leukocytes**

Polymorphonuclear leukocytes, representing 50-60% of all seminal leukocytes are the major source of ROS generation in the semen (Aitken and West 1990; Ochsendorf, 1999; Shekarriz *et al*, 1995; Fedder *et al*, 1993; Wolff and Anderson 1988). Leukocytospermia, according to the WHO is the presence of peroxidase-positive leukocytes in concentrations $>1 \times 10^6$ per milliliter of semen (WHO,1999). Activation of leukocytes by stimuli, like inflammations and infections leads to an increased production of NADPH. NADPH, in turn, triggers activation of the leukocyte myeloperoxidase system further leading to a respiratory burst, subsequently resulting in an increased production of ROS (Pasqualotto *et al*, 2000; Blake *et al*, 1987).

**Mechanism of ROS generation in spermatozoa**

Generation of ROS by human spermatozoa at physiological levels promotes tyrosine phosphorylation events associated with sperm capacitation. Enhancement of tyrosine phosphorylation status of human spermatozoa by ROS depends partly on the ability of hydrogen peroxide to suppress tyrosine phosphatase activity and partly on the ability of ROS molecules to stimulate cAMP generation by adenylyl cyclase. Generation of cyclic adenosine monophosphate (cAMP) via this mechanism, then stimulates tyrosine phosphorylation via a Protein Kinase A (PKA) dependant mechanism involving an

intermediary tyrosine kinase (Rivlin *et al*, 2004; Aitken *et al*, 1998; Baker *et al*, 2005). This redox control of tyrosine phosphorylation during sperm capacitation involves a low, steady state level of ROS production. However, if for any reason, the physiological rate of ROS generation increases within the spermatozoa or by the infilterating leukocytres, a state of oxidative stress is induced (Aitken, 1999).

**Physiological role of ROS on male fertility**

Free radicals have both, a physiological and pathological role in males. Physiologically, reactive oxygen species are involved in capacitation, acrosome reaction and hyperactivation. Pathologically, reactive oxygen species induce sperm DNA damage and lipid peroxidation causing an alteration in sperm plasma membrane permeability, affecting sperm morphology and impairing the fertility status (Jones *et al*, 1979; Saleh and Agarwal A. 2002; Agarwal and Said 2003).

Earlier, it was mostly believed that ROS have detrimental effects on sperm functions, but now there is increasing evidence that very low concentrations of ROS are involved in signal transduction pathways (de Lamirande *et al*, 1997). Low levels of ROS are essential for fertilization, capacitation, hyperactivation, motility and acrosome reaction (Agarwal *et al*, 2004; Griveau and Lannou, 1997). An increase in levels of cAMP occurs because of increased levels of intracellular calcium, tyrosine kinase and ROS, resulting in hyperactivation while the sperm is capacitating. These capacitated hyperactive motile spermatozoa undergo physiological acrosome reaction, thereby acquiring the ability to fertilize (Aitken 1995; Visconti *et al*, 1995). Reactive oxygen species like hydrogen peroxide, nitric oxide and superoxide anion, all promote sperm capacitation and acrosome reaction (Griveau *et al*, 1995; Zini *et al*, 1995).

**OXIDATIVE STRESS**

A condition associated with an increased rate of cellular damage induced by oxygen and its free radicals, known as reactive oxygen species is called Oxidative Stress. This oxidative process can be enhanced by various stress related conditions like, infections, physical injury, aging, exposure to toxins, acquired immunodeficiency syndrome, chronic disease states and exposure to different types of food, all leading to cellular damage. The

most prevalent cause of male infertility is defective sperm function (Aitken and Clarkson, 1987). Of the many causes of male infertility, the most extensively studied in the recent years is oxidative stress (OS). Like any other aerobic cell, spermatozoa are constantly facing the "oxygen-paradox" (Sies H 1993), i.e. where oxygen is necessary to support life, its metabolites, e.g ROS can alter cell fuctions and endanger the survival of the cell (de Lamirande and Gagnon 1995). Oxidative stress and its role in male infertility was first appreciated in 1943 by a Scottish andrologist John Macleod who demonstrated that under anaerobic conditions, catalase could support the motility of human spermatozoa (MacLeod J, 1943; Baker and Aitken, 2005).Oxidative stress induced by excessive generation of ROS has damaging effects on the sperm, hence influencing the fertilization process (Sikka *et al*, 1995; Sikka, 1996).

**Oxidative Stress and pathological mechanisms of ROS injury**

**Leukocytospermia and oxidative stress**

An association exists between presence of leukocytes in the semen (Aitken *et al*, 1992; Wolff and Anderson, 1988). Leukocytes produced during infection and inflammation in the prostatic and seminal vesicle secretions are the extracellular source of ROS. By release of soluble products by the leukocytes or by direct cell-cell contact of spermatozoa, the leukocytes may also stimulate spermatozoa to produce ROS (Ochsendorf FR, 1999; Shekarriz *et al*, 1995; Plante *et al*, 1994). Leukocytes produce ROS when the concentration of leukocytes in the seminal plasma is abnormally high or seminal plasma is removed during sperm preparation for assisted reproduction (Potts and Pasqualotto 2003; Agarwal *et al*, 2006). Presence of increased interleukin-8 and decreased superoxide dismutase activity in semen depicts an association between a defective ROS scavenging modulated by proinflammatory cytokines, suggesting an active pro-inflammatory response (Rajasekaran *et al*, 1995; 1996). Significant oxidative stress to spermatozoa is seen in the semen of infertile patients due to the leukocytes, as a result of increased production of these pro-inflammatory chemokines (Sikka *et al*, 1995).

**Lipid Peroxidation of spermatozoa**

Oxidative stress targets all the components of the sperm cell including sugars, proteins, lipids and nucleic acids The most susceptible macromolecules present in the sperm plasma membrane are lipids in the form of polyunsaturated fatty acids (PUFA), containing two carbon-carbon double bonds (Makker *et al*, 2009). . Oxidative deterioration of PUFA is called lipid peroxidation (Halliwell, 1989). Lipid peroxidation of the spermatozoal plasma membrane also causes morphological defects of the midpiece with deleterious effects on acrosome reaction and sperm capacitation (de Lamirande and Gagnon I, II, 1992). The key mechanism of the ROS-induced sperm damage leading to infertility is lipid peroxidation (Alvarez *et al*, 1987). This manifestation of oxygen activation is commonly of two types (a) enzymatic LPO (NADPH and ADP dependant) and (b) non-enzymatic LPO (Sikka *et al*, 1995). The most significant effect of LPO is a disruption of all the cellular transport processes, perturbations of ionic and metabolite gradients and derangement of signal transduction. Calcium homeostasis is further affected as a result of removal of LPO metabolites by increased activity of glutathione peroxidase (Lenzi *et al*, 1994).

**Reactive oxygen species and sperm toxicity**

A potential source of ROS in the human ejaculate is the presence of morphologically abnormal spermatozoa and leukocytes, incurring oxidative damage subsequently leading to a loss of sperm function. The extent of damage depends upon the time and duration of exposure of the ejaculate to ROS and other external factors, like, temperature, ions, proteins, oxygen tension and ROS scavengers (Agarwal and Saleh 2002). Apoptosis plays a very important in elimination of abnormal spermatozoa (Sinha *et al*, 1999). Disruption of the inner and outer mitochondrial membranes occur leading to activation of caspases and apoptosis by inducing the release of cytochrome C-protein (Lee *et al*, 1997). A decreased sperm count is seen as a consequence of increased ROS levels (Sikka, 2004). Spermatozoa, being rich in PUFA are very susceptible to ROS attack resulting in damage to axonemes, depletion of ATP, axonemal protein phosphorylation and a decrease in sperm motility (de Lamirande and Gagnon, I, II 1992). Another hypothesis for impairment of ROS induced reduction in sperm motility is a decrease in the antioxidant

defenses of the spermatozoa because of inhibition of $G_6$PDH enzyme as a result diffusion of $H_2O_2$ across the plasma membranes (Griveau *et al*, 1995).

**Varicocele and oxidative stress**

Oxidative stress in the varicocele patients maybe the result of altered testicular microenvironment and hemodynamics. Spermatogenic dysfunction could be the result of various compensatory mechanisms trying to maintain spermatogenesis in varicocele patients. These compensatory mechanisms may cause up regulation or down regulation of various molecular mechanisms and pathways involved in generation of free radicals (Agarwal, *et al*, 2009). Although it has already been that oxidative stress in varicocele patients leads to impairment of semen parameters, yet the etiology of raised oxidative stress levels is still unclear (French *et al*, 2008).

**Evaluation of oxidative stress**

A balance between creation and destruction i.e a balance between pro-oxidants and antioxidants is called the balance of ROS. Positive oxidative stress status is defined as any shift in this balance towards formation of more pro-oxidants or less antioxidant (catalase, glutathione peroxidase, reductase, SOD and GSH). This imbalance induces oxidative stress in the semen and is the major cause of idiopathic infertility in males. An inherent decreased expression of antioxidant enzymes or increased oxidative stress because of increased ROS production cause decreased lipid peroxidation, affecting sperm viability, and decreasing sperm motility (Sikka *et al*, 1995).

**Mechanisms to combat ROS - Antioxidants**

It is now well- known that generation of reactive oxygen species from the spermatozoa plays a very important role in the pathophysiology of male infertility. Abundance of polyunsaturated fatty acids in the plasma membrane makes the spermatozoa more vulnerable to oxidative stress, resulting in sperm dysfunction (Aitken, 1994; Aitken *et al*, 1991; Iwasaki and Gagnon 1992; Zalata *et al*, 1995; Zini *et al*, 1993; Aitken and Clarkson, 1987). A very effective protective environment against oxidative stress is provided by an anti-oxidant system in the seminal plasma (Aitken, 1999). Anti-oxidants

are compounds and reactions which oppose or suppress the actions of ROS. The enzymatic antioxidants, catalase and superoxide dismutase (SOD) with its two isozymes play a very important role in scavenging the ROS. Catalase converts hydrogen peroxide to oxygen and water, while SOD converts superoxide anion into oxygen and hydrogen peroxide. SOD protects spermatozoa by neutralizing lipid peroxidation and spontaneous oxygen toxicity (Alvarez *et al*, 1987). SOD and catalase decrease lipid peroxidation by removing the superoxide anion generated by NADPH-oxidase in neutrophils (Aitken *et al*, 1995). Another most important enzymatic anti-oxidant is glutathione peroxidase which removes peroxyl (ROO) from hydrogen peroxide as well as other peroxides (Calvin *et al*, 1981). The non-enzymatic antioxidants present in the seminal plasma are Vitamin E, pyruvate, urate, ascorbate, glutathione, Vitamin A, albumin, ubiquitol, taurine and hypotaurine. Vitamin C and E, beta-carotenes, carotenoids and falvonoids constitute the dietary antioxidants (Sies 1993; Ford and Whittington 1998; Tarín *et al*, 1998; Greco *et al*, 2005; Hughes *et al*, 1998).Vitamin E and C protect spermatozoa against plasma membrane damage while carotenoids (beta carotene) and ubiquinols decrease lipid derived free radicals by quenching the singlet oxygen, thus reducing the detrimental effects on sperm lipid peroxidation (Sikka, 1996). Antioxidants play a very crucial role in maintaining normal sperm function: they prevent sperm DNA damage, protect normal spermatozoa from ROS generated by abnormal spermatozoa, prevent damage to spermatozoa because of cryopreservation, block premature sperm maturation, improve sperm quality in smokers and play a vital role in outcome of assisted reproductive techniques (Agarwal *et al*, 2004; Lewin and Lavon 1997; Lenzi *et al*, 2004; Wroblewski *et al*, 2003; Ford and Whittington 1998; Tarín *et al*, 1998; Donnelly *et al*, 1999; Greco *et al*, 2005; Hughes *et al*, 1998). The sperm plasma membrane integrity is also maintained by metal chelators like, cerulopasmin, lactoferrin and transferrin (Wroblewski *et al*, 2003; Sanocka *et al*, 2004). It is the lack of a strong defense mechanism against ROS which renders the spermatozoa susceptible to peroxidative damage (Smith *et al*, 1996). The total antioxidant capacity of seminal plasma is the sum of enzymatic (e.g superoxide dismutase, glutathione peroxidase and catalase) and nonenzymatic (e.g taurine, hypotaurine, pyruvate, ascorbate, urate, glutathione and vitamin E (Donnelly *et al*, 1999; Saleh and Agarwal, 2002; Alvarez and Storey, 1989; Benzie and Strain, 1996; Kampa *et*

*al*, 2002; Sharma and Agarwal 1996; Siciliano *et al*, 2001). Low levels of the total antioxidant capacity of the seminal plasma play a major role in male infertility (Fingerova *et al*, 2007; Smith *et al*, 1996; Lewis *et al*, 1997). It is important to accurately measure the seminal TAC so that it becomes a reliable and easy to use tool for diagnosis and evaluation of male infertility (Said *et al*, 2003).

**SPERM DNA FRAGMENTATION**

Male factor infertility is a very important health issue regarding increased miscarriage rates and abnormalities in the offspring. It is now well known that spermatozoa of subfertile men have a large number of structural and functional defects (Aitken *et al*, 1991; Liu and Baker 1994; Mortimer *et al*, 1986). These defects maybe a result of occupational or environmental exposure to physical and chemical agents (Friedler, 1996). Routine clinical evaluation of an infertile male is usually revolving around routine semen analysis; seminal pH, sperm count, motility, morphology and the presence of anti-sperm antibodies (Morris *et al*, 2002). Nevertheless, about 15% of the men seeking infertility evaluation still present with normal semen parameters (Guzick *et al*, 1998). Therefore the assessment of the functional parameters of sperm e.g acrosomal integrity, vitality and viability, enzymatic activities and chromatin are recommended because no single parameter can be said to be have a definitive diagnostic value for infertility evaluation (Guzick *et al*, 1998; Nasr-Esfahani 2004.,2006; Tavalee, 2007). Many of these analyses may describe the functional status of the testis and sperm, yet they may not focus on the integrity of the male genome present in the sperm head (Morris *et al*, 2002). As the sperm cell functions to transmit the male genome to the oocyte, integrity of this DNA is an important requirement for normal embryo development, resulting in a successful pregnancy later (Fernandez *et al*, 2009.). One of the major reasons for post-fertilization failure is abnormalities in the genome of the male (Morris *et al*, 2002). A close relationship exists between infertility and DNA anomalies, such as sperm maturation defects, single and double stranded DNA breaks, defective chromatin structure and aneuploidies (Aravindan *et al*, 1997). Tests assessing the quality of the sperm should be able to identify the fertilization ability of the sperm, their ability to reach the oocyte and activation of embryo growth (Agarwal and Allamaneni, 2005). Diagnosis of sperm DNA

integrity has become extremely important in the era of assisted reproductive techniques for a consistently high reproductive efficiency and hence prove to be a better diagnostic and prognostic marker than routine semen analysis (Shamsi *et al*, 2008).

**Origin of sperm DNA fragmentation:**

Various extra and intra-testicular factors contribute to etiology of sperm chromatin damage (Tavalee *et al*, 2008**).** Extratesticular factors such as cigarette smoking (Gaur *et al*, 2007), paternal age (Schmid *et al*, 2007), infections and inflammations of the genital tract (Ozmen *et al*, 2007), drugs, hormonal factors, varicocele and increased scrotal temperature (Ståhl *et al*, 2006; Nasr-Esfahani *et al*, 2009). Intratesticular damage to DNA may occur because of alterations in packaging of sperm chromatin (Zini and Libman 2006), excessive production of reactive oxygen species in the seminal ejaculate (Aitken and Krausz 2001) and pre-ejaculatory abortive apoptosis (Moustafa *et al*, 2004; Sakkas *et al*, 2003).

**Mechanisms of sperm DNA fragmentation**

**Defective sperm chromatin packaging**

Functioning as an extremely specialized cell, a mature sperm is designed to transmit the haploid set of chromosomes to the offspring. Spermatogenesis is a complex process constituting male germ cell proliferation by a mitotic division followed by two meiotic divisions for a haploid set of genome, eventually differentiating into haploid spermatids through the process of spermiogenesis (Fernandez *et al*, 2009; Tavalee *et al*, 2008). The highly organized, compact and condense unique chromatin structure of mammalian sperm, allows protection and easy transport of the paternal genome through both the female and male reproductive tracts and hence its delivery to the oocyte in a good condition (Ward and Coffey, 1991). A series of modifications take place during spermiogenesis, during which histones are lost and relaced by transition proteins and protamines (Dadoune, 1995; Kierszenbaum, 2001; Lee and Cho 1999). A toroid, the basic packaging unit of sperm chromatin, is formed by dense condensation of DNA strands by protamines and further compacted by the intermolecular and intramolecular disulfide cross-links between cysteine residues present in protamines (Kosower *et al*,

1992). When compared to other mammals, retention of large sized histones makes the human sperm chromatin less compact (Bench *et al*, 1996; Gatewood *et al*, 1987). Protamines also play an essential role in egg-sperm fertilization, thus any abnormality in the levels of protamines may cause damage to sperm DNA and hence impairing fertilization (Fraser, 2004). A high histone to protamine ratio has been reported in sperm chromatin of infertile men (Oliva, 2006; Steger *et al*, 2000). This altered histone to protamine ratio, called abnormal packing, increases the susceptibility of the poorly compacted chromatin to external stresses (Tavalaee *et al*, 2009; Zini and Libman 2006). To relieve the torsional stress a result of supercoiling of sperm chromatin, Topoisomerase II enzyme creates temporary nicks in DNA and repairs them just before spermiogenesis and ejaculation is completed (Laberge and Boissonneault. 2005; Marcon and Boissonneault 2004; McPherson and Longo 1993; Muratori *et al*, 2006). It has also been reported earlier that injected or penetrated oocytes that fail to progress to the pronuclear stage, contain either sperms with premature condensation or a condensed sperm. This phenomenon is related to the effect of the maturation promoting factor (MPF) in the metaphase II oocyte on the sperms with excess histones or the sperms deficient in protamines (Sakkas *et al*, 1999).

**Apoptosis**

Apoptosis or programmed cell death plays a very important role in spermatogenesis (Blanco-Rodríguez and Martínez-García 1996; Angelopoulou and Karayiannis, 2000). As the Sertoli cells are able to support only a limited number of germ cells in the testis, overproduction of germ cells is prevented through apoptosis (Rodriguez *et al*, 1997; Sinha Hikim and Swerdloff, 1999). Also clonal expansion of those germ cells consisting of damaged DNA is also prevented by apoptosis. Defective remodelling of the spermatozoal cytoplasm may lead to "abortive apoptosis", a phenomenon in which the ejaculate contains defective sperm cells that have escaped the apoptotic pathway. Apoptotic markers like fas are involved in abortive apoptosis (Sakkas *et al*, 1999). Fas ligand (FasL), secreted by the Sertoli cells, interacts with the Fas protein present in the germ cell surface and triggers the apoptotic pathway (Lee *et al*, 1997). A large percentage of sperms showing Fas expression are seen in the ejaculate of men with poor seminal

parameters (Sakkas *et al*, 1999), suggesting that such sperms with Fas expression and chromain damage had started to undergo abortive apoptosis but escaped the apoptotic pathway subsequently (Sakkas *et al*, 2003). Specific proteinases called "caspases" may also play a role in regulation of apoptosis in the human seminiferous epithelium. These caspases exist as inactive pre-enzymes and get activated in response to ligand- binding of cell-surface death receptors such as Tumor Necrosis Factor (TNF-alpha ) and Fas, eventually resulting in death of the cell (Ozmen *et al*, 2007; Tarozzi *et al*, 2007).

**Correlation between sperm DNA fragmentation, ROS and semen quality**

An essential prerequisite for successful fertilization and embryo development is intact sperm DNA (Moustafa *et al*, 2004). Alterations in the spermatogenic cycle lead to production and release of abnormal immature spermatozoa. These immature spermatozoa have defects in chromatin packaging, altered protamination, excessive production of ROS and increased DNA damage (Balhorn *et al*, 1988; Bianchi *et al*, 1996; de Yebra *et al*, 1998; Evenson *et al*, 2000; Gorczyca *et al*, 1993; Manicardi *et al*, 1995; Sailer *et al*, 1995). Spermatozoa of men undergoing infertility evaluation have a limited antioxidant defense system while presenting unsaturated fatty acids and DNA as substrates for free radical attack (Aitken *et al*, 1989; Aitken *et al*, 1991; Alvarez *et al*, 1987; Koppers *et al*, 2010). During normal physiological conditions, the main biological source of superoxide anion radicals is mitochondrial respiration (Saleh and Agarwal, 2002). Electron leakage from sperm mitochondria, act as the basic source for activation of pro-apotòtic molecules, apoptosis and DNA fragmentation (Koppers *et al*, 2008; 2010). Maintainance of the reproductive potential of men is assessed by the quality of sperm DNA (Agarwal and Allamaneni, 2004). A higher percentage of DNA fragmentation and poor semen quality is seen in spermatozoa of infertile men as compared to normal fertile men (Lopes *et al* 1998). Abnormal sperm DNA could be a leading cause of infertility with men having with normal spermiograms (Alvarez, 2003). Evaluation of sperm DNA integrity in addition to routine semen analysis could be a good indicator of spermatozoal quality (Agarwal and Allamaneni, 2004).

**Mechanism of sperm DNA damage by leukocytospermia**

Leukocytes are found in almost every human ejaculate (el-Demiry *et al*, 1987). Leukocytes play a very important role in phagocytic clearance and immunosurveillance of abnormal spermatozoa (Tomlinson *et al*, 1992). Presence of leukocytes in the seminal plasma is an indicator of poor semen quality (Wolff *et al*, 1990). Cytokines like interleukin (IL)-6 and IL-8 recruit and activate leucocytes during inflammatory conditions (Evenson *et al*, 2002; Shamsi *et al*, 2008). These activated leucocytes generate large amounts of ROS leading to sperm DNA damage (Comhaire *et al*, 1999; Reichart *et al*, 2000).

**Link between antioxidant and sperm DNA fragmentation**

The antioxidants in the seminal plasma and the characteristic tight packaging of the sperm DNA are the two most important factors that protect the spermatozoa from oxidative stress. ROS causes DNA damage in the form of base pair modifications, frame-shift mutations, deletions, and chromosomal re-arrangements, single and double stranded DNA breaks and DNA cross-links (Kemal *et al*, 2000; Aitken and Krausz , 2001). A better predictor of the outcome of spontaneous pregnancy and assisted reproductive techniques than the traditional sperm parameters is the evaluation of DNA damage of the sperm (Zini *et al*, 2001; Evenson *et al*, 1991; Nallella *et al*, 2006; Borini *et al*, 2006). Both single and double-stranded DNA fragmentation can be measured by an assay called the Terminal deoxynucleotidyl transferase dUTP nick end labeling Assay (TUNEL) (Zini and Sigman 2009; Zini *et al*, 2008). In patients presenting with idiopathic infertility or an issue of oxidative stress, TUNEL test with Flow Cytometry can be offered to establish the sperm DNA integrity (Sharma *et al*, 2010).

**New generation of diagnostic tests for infertility: Review of advanced sperm function tests**

Advanced semen tests have been developed for evaluation of sperm function. These tests now have a better defined role in the diagnosis and treatment of male infertility (Samplaski *et al*, 2011). Some of these advanced sperm function tests are expensive and laborious and should only be applied only when there is a suspicion of abnormality.

Routine semen analysis should be considered as the baseline test used in evaluation of male infertility. Direct tests for measurements of oxidative stress include, total, extracellular, intracellular ROS levels in the semen and total antioxidant capacity, while the indirect tests include, evaluation of lipid (malondialdehyde), protein (carbonyl) oxidation products and oxidized DNA (8-hydroxy-2-deoxyguanosine) (Hamada *et al*, 2013).

Various assays have been introduced for assessment of ROS, which now is considered to be an important and useful tool for infertility evaluation (Deepinder *et al*, 2008).

**Malondialdehyde Measurement**

Malondialdehyde, a byproduct of thiobarbituric acid reactive substances (TBARS) assay is one of the tests used for measurement of oxidative stress (Agarwal *et al*, 2012).

**Nitro Blue Tetrazolium Reduction**

Nitro Blue Tetrazolium Reduction is a highly sensitive, simple, easily available and inexpensive test for assessment of role of leukocytes and spermatozoa in production of seminal ROS (Esfandiari *et al*, 2003).

**Cytochrome-c Reduction**

Cytochrome-c reduction is the standard procedure applied for detection of extracellular release of superoxide from cells or tissue extracts in in vitro assays. Due to its limited access, Cytochrome c is not able to measure superoxide formation within the intact cells (Elferink, 1984).

**Electron Spin Resonance Spectroscopy**

Electron Spin Resonance Spectroscopy, a simple and least ambiguous technique, detects the free radicals with the use of magnetic properties of unpaired electrons. However, because of lack of standardization, scientists are putting in more effort to develop this technique for assessment of in vivo generation of radicals and the oxidation-reduction status (Weber, 1990).

### Thiobarbituric acid-reactive substances

Thiobarbituric acid-reactive substances or isoprostane methodology, is another widely employed assay for measurement of lipid peroxidation levels induced by ROS, it still lacks specificity (Yagi, 1998; Roberts and Morrow, 2000).

### Xylenol Orange Assay

An easy, rapid, inexpensive, sensitive and fully automated assay has been proposed recently called the Xylenol Orange Assay, but merits further investigation due to lack of comparative data, threshold values and standardization of protocol (Erel, 2005).

### Chemiluminescent Signals – The Chemiluminescence Assay

The apparent strong correlation between raised oxidative stress levels and reduced fertility makes measurement of ROS, a useful tool in evaluation of subfertile males (Sharma *et al*, 1999). Inclusion of ROS measurement in clinical practice is variable, primarily because of the lack of standardization of ROS methodology and development of range of reference values (Athayde *et al*, 2007). To date, insufficient evidence is there for defining elevated ROS levels as a cause or an effect of abnormal semen parameters and damage to the sperm However, a recent study by Agarwal *et al*, (2006), reported high levels of ROS as an independent marker of male factor infertility in leukocytospermic semen samples, after adjusting the sperm parameters (Agarwal *et al*, 2006). This finding suggests role of ROS in causation of male factor infertility and encourages the use of ROS measurement as a diagnostic tool in clinical settings (Deepinder *et al*, 2008).

One of the most widely used methods of ROS quantifying both intracellular as well as extracellular is the Chemiluminescence Assay (Makker *et al*, 2009). This technique has been developed for assessment of oxidative stress to spermatozoa. It is the most commonly used technique, quantifying both, intracellular and extracellular ROS (Agarwal *et al*, 2004; Sharma and Agarwal, 1996). This assay is performed on fresh and neat semen samples, with a minimum sperm concentration of greater than one million sperms per milliliter (Agarwal *et al*, 2004). Chemiluminescence assays, using highly sensitive probes, for example, lucigenin or luminol have relatively well-established threshold values for both the fertile and infertile men (Athayde *et al*, 2007; Ochsendorf *et*

*al*, 1994; Williams and Ford, 2005). However, auto-oxidation of lucigenin leads to production of superoxide anions itself, making it less ideal for use in oxidative stress measuring assays (Liochev and Fridovich, 1997). Levels of ROS are assessed by measuring a sensitive probe, Luminol-dependant chemiluminescence in a luminometer (Makker *et al*, 2009). Depending upon the type of probe used (Luminol [5-amino-2,3-dihydro-1,4-phthalazinedione; also, 3-aminophthalic hydrazide] and Lucigenin [N, N$^{/}$ -dimethyl-9,9$^{/-}$biacridinium dinitrate]), this method differentiates between superoxide and hydrogen peroxide generation by the spermatozoa. It involves generation of a signal, by the stressed spermatozoa, in the presence of redox sensitive probes, e.g lucigenin and luminol. The intensity of the signal produced is negatively associated with sperm function, reflecting the fertilization potential of human spermatozoa in vivo and in vitro (Aitken *et al*, 1991, 1989; Gomez *et al*, 1998; Said *et al*, 2004).

The main confusing factor is the presence of leukocytes in the semen sample as the assay cannot differentiate the exact source of ROS production, whether ROS is generated from the leukocytes or the spermatozoa. Determination of the exact source of ROS is important because of the differing clinical implications of leukocytospermia and pathologic conditions in which spermatozoa are a source of ROS generation themselves. Factors interfering with chemiluminescence measurement include, sample volume, sperm count (atleast >1 x $10^6$ /mL), concentration of reactants, reagent injection, temperature control, background luminescence and instrument sensitivity (Agarwal *et al*, 2004; Sharma *et al*, 2001).

The main limitation of this assay is that it is unable to differentiate ROS production from the leukocytic infiltration in the semen or from the spermatozoa themselves. Factors, for example, volume of the semen sample, concentration of reactants, variations in temperature, sensitivity of the instrument and luminescence in the background are multiple factors interfering with the accuracy of the results. Different types of luminometers are available commercially for example, single, double or multiple tube luminometers that are cheap, sensitive and suitable to be included in the andrology laboratories (Agarwal *et al*, 2004).

Basal levels of ROS are important for the physiological functions of spermatozoa (Desai *et al*, 2008). Using, Berthold Luminometer, Autolumat LB 953; BadWild, Germany, Desai *et al*, (2009) suggested a physiologic ROS levels of <0.0188 x $10^6$ counted photons per minute (cpm)/20 x $10^6$ sperm, whereas our study used < 20 Relative Light Units /second/x $10^6$ sperms as a physiologic cut-off value of ROS value, using Berthold Luminometer, LKB Autoplus 953, Berthold Technologies, (Oak Ridge, TN, USA).

**Assessment of Total Antioxidant Capacity of the seminal plasma**

Seminal antioxidants, both enzymatic and non-enzymatic, as well as the total antioxidant capacity (TAC) have also been used as a direct method of measurement of oxidative stress. Earlier studies have shown decreased levels of TAC and some non-enzymatic antioxidants (Nallela *et al*, 2004; Sharma *et al*, 1999; Pasqualotto *et al*, 2000; 2001; 2008; Smith *et al*, 2006; Saleh *et al*, 2003; Giulini *et al*, 2009; Mancini *et al*, 2007). The enzymatic antioxidants, superoxide dismutase (SOD), present both, within the sperm itself and the seminal plasma and catalase cause inactivation of the peroxide and superoxide radicals (Mennella and Jones, 1980; Zini *et al*, 1993). Some studies have reported minor decrease in seminal plasma SOD levels in infertile men, (Alkan *et al*, 1997; Sanocka *et al*, 1997), while others have not (Miesel *et al*, 1997; Zini *et al*, 2000; Hsieh *et al*, 2002).

The total antioxidant capacity of the seminal plasma can be measured in various ways (Tremellen, 2008). Different techniques, for example, Phycoerythrin fluorescence based assay (Miller *et al*, 1993), oxygen radical absorption capacity (Cao and Prior, 1998) and ferric reducing ability of plasma (Benzie and Strain, 1996). The most widely used method for assessment of seminal TAC is the Enhanced Chemiluminescence Assay (Miller *et al*, 1993). This Assay, although accurate for measurement of TAC in the seminal plasma, is still laborious, time consuming and needs expensive instruments. The colorimetric method is currently gaining acceptance for measurement of total antioxidant capacity in the seminal plasma (Said *et al*, 2003). This method inexpensive, simple and rapid, has gained popularity as an alternative to the Enhanced Chemiluminescence Assay (Miller *et al*, 1993).

Measurements of seminal enzymatic antioxidants, for example, catalase was markedly decreased in infertile men with varicocele while levels of superoxide dismutase were either increased, decreased or unchanged (Mostafa *et al*, 2009; 2012; Sakamoto *et al*, 2008; Abd-Elmoaty *et al*, 2010; Hurtado de Catalfo *et al*, 2007). However, Jeulin *et al*, (1989); Alkan *et al*, (1997); Miesel *et al*, (1997); Sanocka *et al*, (1997); Zini *et al*, (2000), did not find any link between reduced catalase activity in the seminal plasma and male infertility. Glutathione peroxidase (GPX) protects against oxidative stress by inhibiting the lipid peroxidation (Twigg *et al*, 1998). A link between male infertility and decreased GPX has been reported by Giannattasio *et al*, (2002) in the seminal plasma and by Garrido *et al*, (2004b) within the spermatozoa.

The non-enzymatic antioxidants-Vitamin E, Vitamin C, alpha-tocopherol, albumin, glutathione, amino acids, carnitine, urate, flavonoids, prostasomes and carotenoids act by directly neutralizing the activity of the free radicals (Tremellen, 2008). A number of researchers have reported a significant decrease in the seminal plasma non-enzymatic antioxidant activity in the infertile men compared with the fertile men (Fraga *et al*, 1991; 1996; Smith *et al*, 1996; Therond *et al*, 1996; Lewis *et al*, 1997; Gurbuz *et al*, 2003; Koca *et al*, 2003; Mostafa *et al*, 2006; Song *et al*, 2006).

**The Total Antioxidant Capacity (TAC) Assay**

Assessment of antioxidant status is done by measuring the total antioxidant capacity (Kambayashi *et al*, 2009). The total antioxidant capacity of the seminal plasma can be measured by different methods; the ferric reducing ability of plasma (FRAP) (Benzie and Strain, 1996), the total radical trapping antioxidant potential (TRAP) (Wayner *et al*,1985), the oxygen radical absorbance capacity (ORAC) (Cao *et al*, 1993) and the Trolox equivalent antioxidant capacity (TEAC) (Miller *et al*, 1993). Each assay involves the generation of a different radical, measuring a range of end points by acting through various mechanisms (Rice-Evans CA, 2000; Miller and Rice-Evans 1996). Two types of approaches have been used in these assays: 1). The Inhibition Assays in which the extent of scavenging by electron or hydrogen ion donation of a pre-formed free radical is compared to Trolox, a standard antioxidant compound is the marker of antioxidant activity and 2). Other assays involving generation of a radical by addition of an

antioxidant (Miller and Rice-Evans, 1997). The disadvantage with most of these assays was that it took more time to measure TAC as most of the samples could not be treated simultaneously. Therefore an efficient TAC assay that could measure many samples simultaneously, using a 96 well plate, was developed (Kambayashi *et al*, 2009). A more practical and easy approach is to measure the total available antioxidant protection in the seminal plasma, called as seminal TAC (Said *et al*, 2003). Therefore TAC measurement can be used as a quick in-office diagnostic and prognostic tool for evaluation of male infertility patients (Mahfouz *et al*, 2009).

**Contemporary methods for assessment of sperm DNA fragmentation**

Sperm genomic integrity has been studied extensively in the past decade as a major cause of male infertility (Sakkas *et al*, 1999). The focus on sperm chromatin abnormalities has been intensified because of the transmission of genetic diseases through assisted reproductive technology. Evidence suggests existence of a negative correlation between sperm nuclear DNA fragmentation and fertility potential of the spermatozoa, both in vivo as well as in vitro (Sun *et al*, 1997; Spano *et al*, 2000). This emphasizes upon the fact that integrity of the sperm genome should be considered an important criteria for assessment of a spermatozoon's fertility (Amann, 1989).

Despite the apparent conflict amongst scientists about measurement of sperm's genomic integrity in the clinical laboratories as a routine assay, assessment of DNA damage on a commercial basis has markedly increased. Thus, it is clear that development of advanced sperm function tests is a requirement for assessment of male fertility potential (Barratt *et al*, 2010). However, standardization of protocols is a prerequisite for production of strong clinical data and comparison of results between different laboratories. There are still widespread differences in various protocols used for DNA assessment. This lack in standardization of methodologies leads to inappropriate negative and positive controls (Pacey *et al*, 2010).

For assessment of the diagnostic and predictive value of DNA integrity, high quality clinical data for comparison is required. Despite the number of studies done on assessment of DNA integrity in the past, there is still a lack of large prospective clinical

trials. Secondly, there is so much difference in the standardization of protocols that it is impossible to clearly define the nature of DNA damage. Also, some of these advanced tests cannot be applied to the clinical settings because of being expensive, labor intensive and toxic reagents. High quality data should be published continuously and a meta-analysis done for clinical evaluation of DNA damage (Barratt *et al*, 2010).

It has always been a challenge for researchers to find the most accurate technique for quantification of DNA fragmentation in spermatozoa and its association with fertility. Various techniques have been devised to study the spermatozoal genomic integrity. Spermatozoal chromatin defects are identified by staining with chromomycin A3, toluidine blue and aniline blue. Spermatozoal DNA integrity can be assessed by techniques like COMET Assay, Sperm Chromatin Structure Assay (SCSA), Acridine Orange (AO) Staining, In-situ Nick Translation (INST) and Terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL). Fluorescence In Situ Hybridization (FISH) detects the chromosomal aneuploidies in spermatozoa. PCR microdeletion analysis detects genetic abnormalities as Yq microdeletions in infertile men with non-obstructive azoospermia and severe oligozoospermia. (Shamsi *et al*, 2008; Dada *et al*, 2006, 2007). Multiple assays being used for assessment of the sperm's chromatin integrity have their own advantages and limitations. The choice of applying a specific assay depends upon multiple factors e.g cost effectiveness, laboratory facilities and well-trained lab personnel (Agarwal and Said, 2004).

**Comet Assay**

A very sensitive technique for determination of sperm DNA damage, measuring the heterogeneity and response of individual cells within a cell population (Bajpayee *et al*, 2002). The assay is based on the principle that smaller fragments of DNA migrate at a faster rate towards the anode in an electromagnetic field as compared to larger non-fragmented DNA (Klaude *et al*, 1996; Irvine *et al*, 2000). DNA fragmentation of the spermatozoa can be quantified microscopically by measuring the length of the comet in two to three hundred individual cells (Agarwal *et al*, 2003). This assay is simple and sensitive requiring only a small number of cells and detects non uniform responses within

a mixed population of cells (Shamsi *et al*, 2008). However, residual RNA can create a background during the analysis, overestimating the DNA damage. Presence of proteins can also hamper the movements of DNA fragments during electrophoresis, underestimating the DNA damage. Entanglement of the migrating filaments and overlapping comet tails may also mask the effects of the Comet Assay. This assay is not suitable for clinical use because of high inter-laboratory variations (Olive *et al*, 1992; 2001). Also, this assay is preferred for assessment of DNA damage in cryopreserved sperms (Duty *et al*, 2002).

**Sperm Chromatin Dispersion Test (SCD)**

This test is based on the principle that sperms with DNA fragmentation fail to produce a characteristic halo, when mixed with aqueous agarose after denaturation by acids and removal of nuclear proteins (Fernandez *et al*, 2005). This test is fast and simple and the percentage of dispersed or non-dispersed (seen as small halos or none at all) nuclei of the spermatozoa are visible to the naked eye, but since this test has been introduced recently, very little is known about its clinical applicability and limitations (Fernández *et al*, 2003).

**Sperm Chromatin Structure Assay (SCSA)**

SCSA reveals the DNA fragmentation index (% DFI). This technique assesses the extent of denaturation of DNA after treatment with acid or heat by measuring the metachromatic shift from green fluorescence (double stranded DNA) to red fluorescence (single stranded DNA). However, this technique only measures the percentage of spermatozoa with dispersed or non-dispersed nuclei and hence is unable to provide information about the extent of spermatozoal DNA damage (Frazer, 2005). Assessment of DNA fragmentation by SCSA has already been extensively standardized and performed by SCSA Diagnostics, Brookings, SD, laboratory but other laboratories have not yet standardized this assay (Zini and Sigman, 2009; Zini *et al*, 2008).

**Acridine Orange Test (AOT)**

A simplified version of SCSA, microscopically exhibiting sperms with double stranded DNA as having green fluorescence and single stranded DNA with red fluorescence (Spano *et al*, 1999). Although this test has been used by some laboratories for

improvement in infertility evaluations (Hoshi *et al*, 1996), this test is less sensitive and remains controversial in evaluation of male infertility because of indistinct colors and rapidly fading fluorescence of the spermatozoa under the microscope (Duran *et al*, 1998).

**In situ Nick Translation (ISNT)**

In situ nick translation (ISNT) assesses the presence of endogenous nicks in the DNA. As this test has low sensitivity compared to other assays measuring DNA damage, its utility in male infertility evaluation also remains controversial (Evenson *et al*, 2006). The only advantage of this assay being that ISNT identifies an appreciable and variable level of endogenous DNA in the spermatozoa (Shamsi *et al*, 2008). However, ISNT is used for single stranded DNA breaks only in contrast to TUNEL which involves both single and double stranded DNA (Irvine *et al*, 2000).The clinical significance of ISNT Assay is extremely limited due to the lack of sensitivity in comparison with other assays (Twigg *et al*, 1998a) and also because no correlation has yet been proved with fertility outcome while conducting studies in-vivo (Irvine *et al*, 2000).

**Terminal dUTP Nick – End Labeling (TUNEL) Assay**

This technique, first described by Gorczyca *et al*, (1993) is a direct quantification of DNA breaks in mammalian spermatozoa. A signal is created by incorporation of biotinylated deoxyuridine (dUTP) to 3'OH end of single and double stranded DNA breaks and catalyzed by recombinant terminal deoxynucleotiyl transferase (TdT) enzyme. Flow cytometry, light and fluorescent microscopy is used to detect the incorporated labeled nucleotides. Fragmented DNA is seen as brightly fluorescing under fluorescent microscope (Frazer, 2005; Lopes *et al*, 1998). TUNEL assay detects both single as well as double stranded DNA breaks and gives an estimate of the number of cells within a population of sperm cells with fragmented DNA. However, TUNEL assay is unable to quantify the degree of DNA damage within the cell (Frazer 2005).

TUNEL Assay measures DNA fragmentation in poor quality spermatozoa. Failure of chromatin condensation in abnormal spermatozoa increases the sensitivity towards more damage to the DNA. This defective DNA might have a correlation with defective

spermatogenesis hence impairing fertility. Presence of such damaged DNA in morphologically normal as well as abnormal spermatozoa has led to the need of development of such highly sensitive assays that can quantify sperm DNA fragmentation (Shamsi *et al*, 2008).

**Comparison of TUNEL Assay with other techniques applied for assessment of sperm DNA fragmentation**

The TUNEL Assay is widely used in male infertility research which needs assessment of the sperm chromatin integrity and related DNA abnormalities (Sun *et al*, 1997; Duran *et al*, 2002; Carell *et al*, 2003; Muratori *et al*, 2003; Benchaib *et al*, 2003). Although, expensive, the flow cytometric assessment of the DNA damage is more sophisticated, reliable and accurate. The interobserver variability was found to be <7%, while the intraobserver variability was < 8% (Barroso *et al*, 2000).

Considering sensitivity of these assays measuring spermatozoal DNA damage, COMET Assay might be one of the best by showing sensitivity to detection of single stranded by reversing supercoiled double stranded DNA into single strands by using alkaline lysis buffer. More sensitivity is seen by removal of protamines that impede migration of DNA through agarose by Protaminase K. However, minor changes in the standard protocol by inclusion of antioxidants in electrophoresis buffer, alterations in timings of electrophoresis, changes in pH can all affect the sensitivity of the assay. Artificial damage to DNA induced at the alkali labile sites within the strand of DNA might overestimate true breakage of DNA in the spermatozoa thus jeopardizing the sensitivity of the assay (Singh *et al*, 1996).

In situ nick translation (ISNT) not only measures DNA damage but also assesses the efficiency of compacted DNA. This assay shows a relationship between DNA damage and semen quality by measuring the condensation of chromatin hence reflecting the quality of ongoing processes of spermatogenesis. The major drawback with this assay is that it measures less than ten percent of labeled spermatozoa (Manicardi *et al*, 1995; Shamsi *et al*, 2008).

In order to detect the DNA breaks, Sperm Chromatin Dispersion Test (SCD) and Comet Assay need denaturation of DNA as an initial step in the procedure, under acidic and alkaline conditions. In contrast, TUNEL measures both single stranded and double stranded DNA breaks at a neutral pH. TUNEL Assay is a direct method measuring actual breaks in the DNA strands, whereas SCSA measures indirect spermatozoal DNA damage (Sakkas *et al*, 2010).

The streptavidin labeled enzyme HRP used in TUNEL Assay for the substrate hydrogen peroxide provides specificity and accuracy without any interference in the background. Integrity of spermatozoal DNA plays a pivotal role in spermatogenesis and during the fertilization process later. Low quality of sperm DNA maybe one of the main factors contributing towards impaired fertilizing ability, emphasizing upon the importance of evaluation of DNA integrity as a requirement for evaluation of male fertility potential (Shamsi *et al*, 2008).

For the first time, Sharma *et al*, (2010) reported a detailed standardized protocol of the TUNEL Assay and also established reference ranges for DNA fragmentation, both for healthy fertile controls and sub fertile men. Although this assay is more labor intensive, is simple and easy to handle, hence applied to our study, considering it to play a pivotal role in the diagnosis and prognosis of sub fertile men opting for treatment through assisted reproductive technology. Thus, the aim of including this technique in our study was to assess its clinical applicability as a routine test in evaluation of males with idiopathic infertility or cases where oxidative stress plays a major role.

TUNEL scores individual sperm cells as positive or negative fragmentation depending upon their intensity of fluorescence upon flow cytometry and does not assess direct quantification of breaks in the DNA strands (Boe-Hansen *et al*, 2005). TUNEL Assay can simultaneously detect single and double stranded DNA breaks in contrast to Comet Assay which requires different protocols for assessment of both types of DNA stands breaks (Frazer, 2005).

During the recent years, the importance of genomic integrity of the sperm towards fertilization and pregnancy outcome has led to the introduction of these advanced tests, for example, the SCSA (Evenson *et al*, 2002), TUNEL Assay (Gorczyca *et al*, 1993) and COMET Assay (Ostling and Johanson, 1984), in the clinical diagnostic settings. The question at this point is the applicability of which test in the diagnostic evaluation of these sub fertile men. This certainly depends upon the technical expertise and cost effectiveness of the test in an andrology laboratory. Standardization of protocols of the procedure is also an important issue, as this would give the predictive value of the test being applied (Henkel *et al*, 2010). Although , the SCSA, evaluated and used in the clinical settings by (Evenson *et al*, 2002), has been considered as a "gold standard" by scientists like Chohan *et al*, (2006), others like Boe-Hansen *et al*, (2005), pointed out lack of standardization of procedure protocol, hence affecting the results. On the other hand, Makhlouf and Niederberger, (2006), claim lack of clinical verification of these DNA tests. While the SCSA, similar to the Acridine Orange (AO) Test (Tejada *et al*, 1984), needs a DNA denaturation step for detection of breaks in the DNA strands, the TUNEL Assay (Fraga *et al*, 1991), COMET Assay (Ostling and Johanson, 1984) and (In situ Nick Translation (INST) (Gorcyza *et al*, 1993) do not require this initial denaturation of DNA for measurement of single and double strands of DNA (Henkel *et al*, 2010). Hence, it is clearly seen that the SCSA has susceptibility towards denaturation and detects the "potential" DNA, whereas the TUNEL Assay detects the "real" DNA damage (Alvarez, 2005; Henkel, 2007). This is also one of the reasons explaining why the threshold for TUNEL Assay are lower than those of SCSA and that tests measuring "real" DNA damage have higher predictive values than those detecting only "potential" DNA damage (Alvarez, 2005).

Further efforts are required to distinguish between tests applied for determination of different aspects of the same parameter, not only considering different methodologies but also regarding linguistically, differentiating between DNA "fragmentation" and DNA "damage". However, these different techniques measure different aspects of damage to the DNA, for example, "potential", "real" DNA damage or DNA strand breaks, sperm DNA denaturation, and abnormalities in sperm chromatin condensation (Henkel *et al*,

2007), the World Health Organization should develop a standard, accurate, reliable and cheap technique for assessment of DNA that can be applied in the andrology laboratories, even in the under developed countries (Henkel *et al*, 2010).

**Flow Cytometry**

Flow Cytometry measures the intensity of fluorescence of compounds that are oxidized by ROS (Bass *et al*, 1983). This assay identifies dysfunctional spermatozoa because of the presence of ROS intracellularly. This technique has the advantage of measuring the intracellular production of ROS in viable parts of the spermatozoal population (Guthrie *et al*, 2006).Flow Cytometry is a technique used to measure the fluorescent intensity of compounds oxidized by ROS. Fluorescent probes, e.g 2,7-dichlorodihydrofluorescein diacetate, dihydrorhodamine 1,2,3 and dihydroethidine can be oxidized by generation of ROS within the cell, making them highly fluorescent . Quantification of this fluorescence is a measure of the rate and quantity of production of ROS (Bass *et al*, 1983). This assay identifies dysfunctional sperm populations because of intracellular ROS generation and measures intracellular ROS exclusively in that viable portion of the sperm population, in contrast to other methods which measure ROS generated only extracellularly or in a small proportion of nonviable spermatozoa (Guthire and Welch, 2006).

As it is well recognized now that abnormal sperm function is one of the major causes of infertility, although in the past two decades, major advances in this field have been achieved, knowledge of the biochemical and molecular basis of this condition is still limited (Burkman *et al*, 1988., Liu and Baker, 1992). Indeed our understanding of normal human spermatozoa physiology is still lacking (Franken and Oehinger, 2012). Some couples seeking evaluation for infertility are categorized as having unexplained or idiopathic infertility due to the inability of any detectable abnormality by standard investigations (Moghissi and Wallach,1983; Alkan *et al*, 1997). The traditional semen analysis used for evaluation of male subfertility provides limited information about spermatozoa function. Better predictors of the fertilizing potential than traditional semen parameters assessed by routine semen analysis are the specialized sperm function tests (Katsuki *et al*, 2005). A standard routine semen analysis performed by an experienced

person assists the clinician in diagnosing and then prescribing the therapy of choice for that specific sperm disorder (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996). Application of advanced sperm function tests is useful for determination of specific spermatozoal defects. Newer sperm function tests may be useful clinically and could help in the diagnosis of unexplained infertility (Samplaski *et al*, 2010). Research studies conducted in the last ten years have supported the association of increased production of ROS with abnormal seminal parameters. Inclusion of evaluation of ROS production in the semen may help in development of new therapeutic regimes and improve pregnancy rate through assisted reproductive techniques (Deepinder *et al*, 2008).

**Aims and Hypothesis**

Earlier studies on male infertility have included individual or a couple of sperm characteristics in clinical and bench research. The basic objective of the present study was to conduct a comprehensive assessment of males coming to the Andrology Clinic for subfertility evaluation, starting from basic semen analysis to advanced sperm function tests for estimation of oxidative stress and sperm nuclear DNA fragmentation. Secondly, to evaluate whether oxidative stress and sperm DNA fragmentation cause any deterioration in semen quality of subfertile males or not.

**Specific Aim 1:**

To determine changes in oxidative stress markers - ROS, TAC and sperm DNA fragmentation in healthy fertile controls and sub fertile men with/without varicocele

**Hypothesis**:

Oxidative stress will result in an increased expression of ROS and sperm DNA damage while decreasing TAC in subfertile varicocele positive men as compared to varicocele negative infertile controls

**Rationale:**

Oxidative stress releases oxygen free radicals which result in increased reactive oxygen species, decreases total anti-oxidant capacity and causes increased sperm DNA fragmentation.

**Specific Aim 2:**

To compare the changes produced by oxidative stress in sperm parameters - concentration, motility and morphology in healthy fertile donors and infertile men with/without varicocele

**Hypothesis:**

Oxidative stress will result in decreased sperm concentration, motility and changes in morphology.

**Rationale:**

Oxidative stress induced by oxygen free radicals has been shown to affect semen quality by altering sperm parameters

**Specific Aim 3:**

To determine alterations in oxidative stress markers with increasing severity of varicocele positive sub fertile men

To determine the effect of grading of varicocele on semen quality and oxidative stress in subfertile men

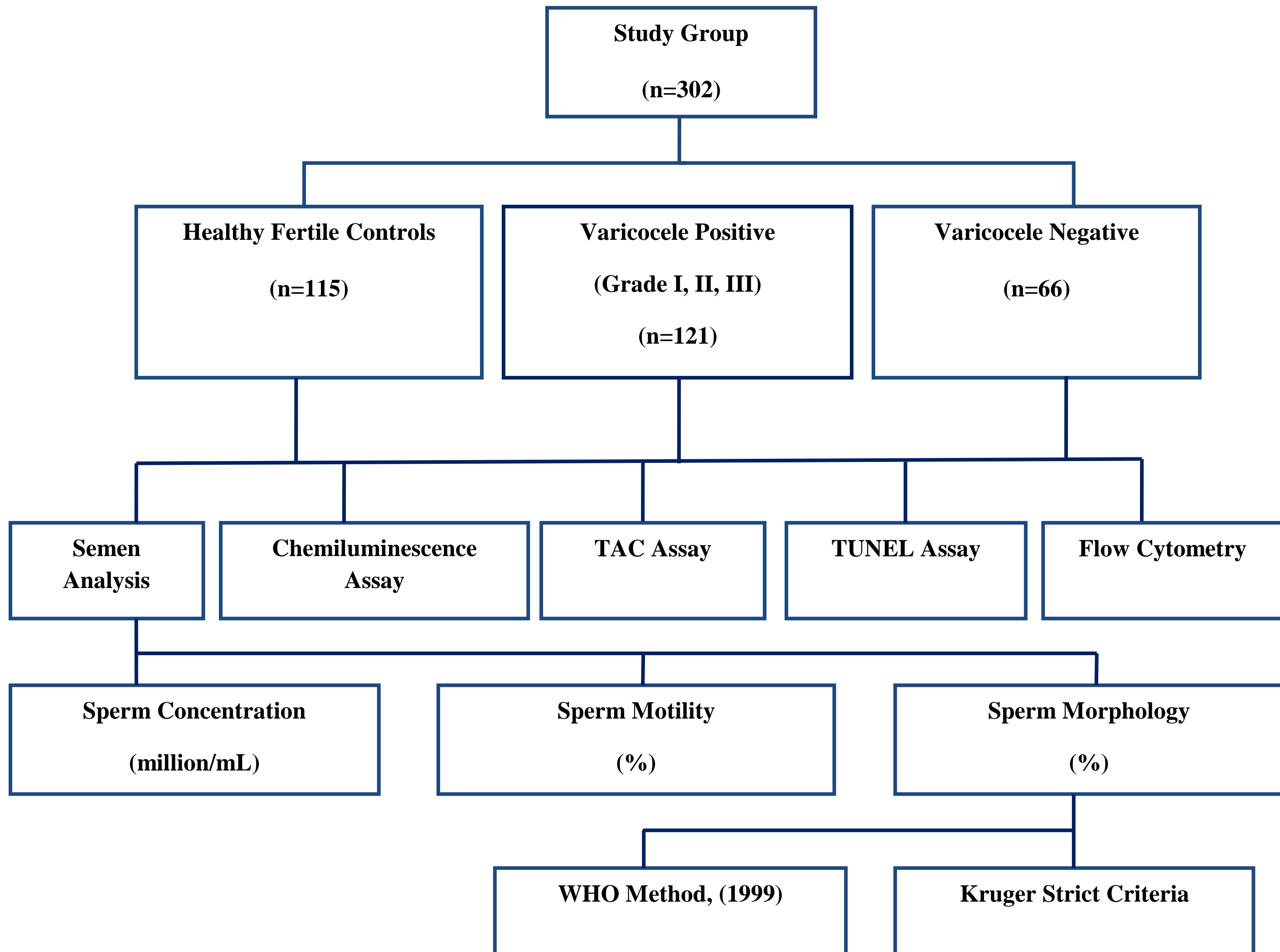
**Hypothesis:**

The greatest increase in oxidative stress markers will be observed in men with severe varicocele.

**Rationale:**

Men with increasing severity of varicocele have significantly greater testicular damage leading to increased oxidative stress and defective sperm function.

# STUDY PLAN



# METHDOLOGY

The study was approved by the Institutional Review Board of the Cleveland Clinic, Ohio, USA. An informed consent for participation in the study was obtained from each subject. Our study population consisted of 302 males. Of these, 115 were healthy and fertile 187 males were seeking evaluation and treatment for subfertility. The sub fertile group was further sub-divided into two groups -121 sub fertile males who were diagnosed with varicocele on ultrasound and 66 males who were sub fertile but having no varicocele. The fertile group comprised of healthy males who had a normal semen analysis and married males whose female partners had a spontaneous pregnancy within one year of unprotected intercourse or were pregnant at the time of this study. Subfertile males presented to the andrology laboratory for evaluation.

## CLINICAL DIAGNOSIS OF VARICOCELE

A thorough history and detailed history of the every subject was taken. A physical examination of all the male subjects of the study group was performed by an infertility specialist, in the standing position, with and without Valsalva maneuver. Varicocele was clinically graded as:

- Grade I (small sized, palpable with Valsalva)
- Grade II (medium sized, palpable without Valsalva)
- Grade III (large sized, visible through scrotal skin)

### Investigation of varicocele by Color Doppler ultrasound

Ultrasonography was performed in the Department of Radiology, Cleveland Clinic Hospital, Ohio, USA. Clinical diagnosis of varicocele was confirmed by scrotal Doppler ultrasonography. The patients were evaluated both in the supine as well as the upright position, with and without Valsalva maneuver for a thorough investigation of the fluxes in the seminal cord veins. A prolonged venous reflux or augmentation, seen as a venous rush during Valsalva, confirmed the diagnosis. The ultrasound study of the scrotum was performed with high frequency linear probes. Blood vessels were first studied in grey scale and then with colored Doppler and pulse Doppler. Color Doppler ultrasound was

calibrated for the correct detection of fluxes as a slow flow (7.5kHz). Varicocele, as seen by real-time ultrasound appeared as a hollow, tubular structure that became larger during Valsalva maneuver. Presence of enlarged venous structures was evaluated with the B-mode. A radiologic diagnosis of varicocele was made when one or more spermatic veins had a maximal diameter of >3mm. Retrograde flow was seen either at rest or under Valsalva maneuver.

Semen specimens of all the fertile and sub fertile subjects were collected in the collection room at the Clinical Andrology Laboratory or brought from home in Semen Home Shipping Kits, after a period of 48 to 72 hours of sexual abstinence. The name of the subject, period of abstinence, date, time and location of collection were recorded on the proforma accompanying each semen analysis. Samples were obtained by masturbation and ejaculated into a clean, wide-mouthed glass or plastic container. Lubricants were not used to facilitate semen collection. Coitus interruptus was not acceptable as a means of collection because of the possibility of losing the first portion of the ejaculate, as this part usually contains the highest concentration of spermatozoa. Also, with coitus interruptus there was a chance of cellular and bacteriological contamination of the sample along with the acid pH of the vaginal fluid that might have adversely affected the sperm motility. Samples were protected from extremes of temperature (not less than $20^0$C and not more than $40^0$C) if they were transported to the laboratory. In case of semen collection in the collection room of the Andrology laboratory the subjects were counseled to follow specific instructions as using gloves, applying scrub sani-cloth tissue* (germicidal disposable cloth) on the surfaces of the room including sofas, sink, sink faucets, door handle and magazine covers, to avoid contamination.

## MANUAL SEMEN ANALYSIS

As the first step in the diagnosis of male infertility evaluation, semen analysis was performed manually. Semen samples were analyzed according to the World Health Organization's guidelines (WHO, 1999) and Strict Tygerberg Criteria (1988). The

recommended standard semen parameters and nomenclature according to the WHO Manual (1999) and Strict Criteria (1988) are as following:

| **Semen Parameters** | **Standard Values** |
| --- | --- |
| **Semen Analysis WHO Criteria, 1999** | |
| Ejaculate Volume | 2 - 5mL |
| pH | 7.2-7.8 |
| Sperm Concentration | ≥20 million/mL |
| Sperm Motility | ≥50 % |
| Normal Sperm Morphology | ≥30 % |
| **Strict Criteria of Sperm Morphology** | |
| Normal Sperm Morphology | ≥14 % |

# CUT-OFF VALUES

| Seminal Parameters | Healthy Fertile Controls |
|---|---|
| **Semen Volume (mL)** | 2-5 |
| **Sperm count (mill/mL)** | ≥ 20 |
| **Motility (%)** | ≥ 50 |
| **Normal Sperm Morphology, WHO (1999), Criteria (%)** | ≥ 30 |
| **Normal Sperm Morphology, Strict Criteria, (%)** | ≥ 14 |
| **ROS (RLU/sec x $10^6$ sperms)** | < 20 |
| **TAC (mM)** | >2000 |
| **TUNEL (%)** | < 19 |
| **Leukocytospermia (million/mL)** | 0.0 - 0.1 |

**Equipments and materials**

- Tyrode's Buffer
- Disposable Microcell chamber or/Makler chamber
- Disposable Pasteur pipettes
- Graduated centrifuge tube
- Glass slides and cover slips
- Eppendorf pipettes (5µL, 25µL, 50µL)
- 2mL conical beakers
- Litmus paper (Range 4.0-8.0)

**Procedure**

The freshly collected semen specimens were allowed to liquefy for approximately 20 minutes at 37°C prior to analysis. Once the semen was fully liquefied, all samples were examined for color, consistency, volume and pH

## MACROSCOPIC EXAMINATION

**A. Appearance**

The semen sample was first evaluated by simple inspection at room temperature. A normal sample had a gray-opalescent appearance, was homogeneous and liquefied within 20-60 minutes at $37^0$C. In some cases, if complete liquefaction did not occur within 60 minutes, the Viscosity Treatment System (VTS) was used.

**Viscosity Treatment System**

**Purpose**

This proteolytic enzyme treatment system was used when a freshly collected semen specimen obtained by masturbation failed to undergo liquefaction after 30 minutes of incubation at 37°C. Its use was intended to assist in sample preparation prior to analysis.

**Reagents**

A. Semen VTS Kit; (Conception Technologies, La Jolla, California).

B. 10 vials of proteolytic enzyme containing 5 mg alpha-chymotrypsin per vial.

C. 10 vials of protein neutralizer containing 50 mg human serum albumin, fraction V per vial.

(Note:These reagents were stored at -20°C until use)

**Procedure**

A VTS vial was removed from the freezer and the contents (proteolytic enzyme) were added to the semen specimen. The specimen and powder were then mixed thoroughly. The specimens were incubated at 37°C till they liquefied completely. The specimens were ready for analysis once liquefaction had occurred.

**Note:**If analysis is not performed immediately, the enzyme was neutralized by adding a small amount of sperm washing media (0.5mL).

On naked eye examination, the sample appeared clear if the sperm concentration was too low and appeared brown when red blood cells were present in the ejaculate. The samples were well mixed in the original container and examined within one hour of ejaculation and liquefaction.

**B. Volume -** The volume of the ejaculate was measured either with a graduated disposable sterile pipette or by aspirating the entire specimen into a graduated tube.

**C. pH -** A pH measurement was performed on all specimens using pH paper. A drop of semen was spread evenly onto the pH paper (range 6.4-8.0). After 30 seconds, the color of the impregnated zone was uniform and comparable with calibration strip for assessment of the correct pH. If pH exceeded 7.8, infection was suspected while azoospermia, dysgenesis of the vas deferens or epididymis was suspected if the pH was less than 7.0 in a sample.

(Note: Whatever type of pH paper is used for this analysis, its accuracy was checked against known standards before use in routine semen analysis).

**MICROSCOPIC EXAMINATION**

The specimen was mixed thoroughly prior to analysis.

**A. Agglutination -** Sperms may normally adhere to cells as debris. The presence of agglutination was suggestive of the existence of an immunological cause of infertility

**B. Undifferentiated round cells** (extraneous cells) - The presence of round cells (immature germ cells, white blood cells) were counted in a drop of semen specimen loaded on a Microcell, and observed under 20x phase objective of the microscope. Round

cell concentration was counted in 100 squares multiplied by correction factor. The results were reported as $10^6$/mL.

**C. Percent Motility -** The motility percentage was determined by a drop of specimen loaded on a Microcell chamber, and observed under 20x phase objective of the microscope. The number of motile sperms were counted per total number of sperms.

**D. Bacteria -** presence of bacteria was also noted.

**Morphology Smear**

A seminal smear was made from the fresh ejaculate to determine the sperm form and shapes. A thin smear was prepared, evenly distributed with a second slide and air dried for an hour. The slides were stained with Diff-Quik staining system.

**Guidelines for Sample Processing:**

Field selection was done manually to avoid clumps and debris. A minimum of five to a maximum of ten fields were analyzed. Low density samples (<20 x $10^6$/ mL) were analyzed manually under phase contrast 20x objective for concentration readings. For high density samples, a 1:1 dilution was made using Tyrodes Buffer (e.g. 20μL Tyrodes + 20μL of patient semen specimen). If the sample read >100 million/mL then it was diluted accordingly with Tyrodes. The dilutions were made in duplicate and analysis was done on each one of them to avoid discrepancy.

**Caution**: Since the semen samples present a possible biohazard as they may contain harmful viruses, e.g. hepatitis, herpes and AIDS, they were handled using universal precaution (gloves, disposable jackets, face shields).

## ENDTZ TEST

### (LEUKOCYTOSPERMIA QUANTITATION)

**Equipment and Materials**

A. **Preparation of stock solution**

1. Ethanol- 25 mL of 96%.
2. Benzidine - 0.0625 gin (Sigma catalog #B3503)
3. Distilled water -25 mL

All these chemicals were mixed in a clean 100 mL bottle. A clear and yellow solution was formed. The bottle was covered with aluminum foil and stored in the dark. A fresh stock solution was prepared if the stored solution got darker in color or formed a cloudy precipitate.

(**Caution:** Benzidine is carcinogenic and was handled carefully. Wear gloves while weighing and face mask may be worn to avoid accidental inhalation. The expired Endtz test solution is discarded in concentrated Clorox).

B. **Preparation of working solution**

Four mL of stock solution was mixed with 50µL of 3% $H_2O_2$ (Sigma, Catalog #H1009) in a 10 mL tube (30% stock $H_2O_2$ was diluted ten times). Cover the bottle with aluminum foil and store in dark. Prepare fresh working solution from stock every week and discard old solutions.

C. Tyrodes buffer (Sigma, Catalog #T2397).

D. Makler counting chamber

E. Microcentrifuge tubes

F. Eppendorf pipette (5µL, 20 µL, 40 µL) tips

**Procedure**

Twenty µL of liquified semen specimen was taken in a microcentrifuge tube and 20 µL of Tyrodes solution and 40µL of working benzidine solution were added to it. The solution was mixed and allowed to sit at room temperature for 5 minutes. A Makler counting chamber was loaded with 5µL of the above solution and observed under 10x bright-field objective lens. All granulocytes stained dark brown in color with retention of their round shape. The cells were counted in all the 100 squares of the Makler grid. The numbers of WBCs were calculated by multiplying total number of cells by 4 to correct for dilution factor. The total WBC number was then $10^5$/mL of semen. This number was corrected to million/mL by dividing it by ten.

**Endtz calculations**

WBC x 4 (dilution factor) = $10^5$/mL semen. $10^5$/mL semen was divided by 10 to give the result in $10^6$/mL semen (million/ml). Results were reported as million/mL Endtz positive cells. Normal concentration of white blood cells in semen was taken as < 0.1 X $10^6$/mL.

**Reference Range**

**Normal Value:** WBCs within a range of **0.0 – 0.1 X $10^6$/mL** was considered normal

**Panic Value**: Any Endtz Test greater than **0.2 x $10^6$ mL** was taken as Endtz positive and informed to the ordering physician immediately.

## REACTIVE OXYGEN SPECIES (ROS) MEASUREMENT

### Equipment and Materials

A. Dimethyl Sulfoxide (DMSO; Catalog #D8779, Sigma Chemical Co., St. Louis, MO)

B. Dulbecco's Phosphate Buffered Saline Solution 1X (PBS-1X; Catalog #9235, Irvine Scientific, Santa Ana, CA)

C. Luminol (5-amino-2,3dehydro-1,4 phthalazinedione; Catalog #A8511, Sigma Chemical Co., St. Louis, MO)

D. Luminometer (Model: LKB 953, Wallac Inc., Gaithersburg, MD)

D. Centrifuge

E. Computer-Assisted Semen Analyzer (CASA)

F. Disposable polystyrene tubes with caps (15 mL)

G. Eppendorf pipettes (5 µL, 10 µL)

H. Pipettes (1 mL, 2 mL, 10 mL)

I. Disposable MicroCell Slides

J. Polystyrene round bottom tubes (6mL)

### Reagent Preparation

**100 mM Stock Luminol Solution:** 177.09 mg of Luminol was weighed-out and added to 10 mL of DMSO solution in a polystyrene tube. The tube was covered with aluminum

foil due to the light sensitivity of the luminol and stored at room temperature in the dark until the expiration date.

**5 mM of Working Luminol Solution:** 20 µL luminol stock solution was mixed with 380 µL DMSO in a foil covered polystyrene tube. The solution was made fresh prior to use and stored in the dark at room temperature until needed.

**DMSO solution**: was provided ready to use and stored at room temperature until expiration date.

**ROS Determination**

This procedure was performed in a dark room. Six mL falcon tubes were taken in triplicates and labeled as: Tube 1-3 as Blank, Tubes 4-6 as Negative Control, Tubes 7-9 as Patient Samples and Tubes 10 -12 as Positive Control
The following reagents were added as indicated below.

| Labeled Tubes | PBS – 1 x (uL) | Specimen Volume (uL) | Probe Luminol (5mM) (uL) | Hydrogen Peroxide -(ul) |
|---|---|---|---|---|
| **Blank** | 400 µL | — | | - |
| **Negative Control** | 400 µL | — | 10 µL | - |
| **Patient Sample** | — | 400 µL | 10 µL | - |
| **Positive Control** | 400uL | | 10 µL | 50 uL |

**Note**: To avoid contamination, all pipette tips were changed after each addition of luminol.
Before adding luminol to the aliquots, the luminometer and the computer attached to it, was set first.

**Instrument set up**

The luminometer and the computer were turn on. From the desktop, 'Berthold tube' master icon was clicked to start the program. From the 'Setup menu' 'Measurement Definition' and then 'New Measurement' were selected. At the command, 'Measurement Name" by clicking "Measurement Definition", the initial information - Initials, Date, Analysis and Measurement, was entered which appeared as 'AM061872ROSsp' on the 'Tool bar' .Next, 'Luminometer Measurement' protocol was clicked and 'Rep. assay' was chosen from the drop menu. Each 'Parameters' was defined as follows:

Read time 1 sec

Background read time 0 sec.

Total time 900 sec.

Cycle time 30 sec

Delay 'Inj M read (s)' 0 sec.

'Injector M (μL)' 0 sec

'Temperature ($^oC$)' 37$^oC$

'Temperature control (0 = OFF) 1 = ON

Finally 'Save' was pressed to save all the entered information. Next from the 'Setup' menu select 'Assay Definition' and then 'New Assay' were selected: The following command was given 'Assay Name' (Initials, Date, Analysis, Assay, e.g. AM061872ROSsp) and 'OK' was clicked. 'Measurement Method' was clicked further and from the drop down menu the measurement 'AM061872ROSsp' was selected. From 'Column Menu' all other parameters were clicked to hide except the following:

Sample ID

Status

RLU mean

Read date

Read time

Next, 'Normal' was selected from the 'Sample Type' menu and 'OK' pressed. From the command 'New', 'Workload' was clicked and 'OK' pressed. The 'Work Load' (Date, Initial, Sample or experiment ID) was saved in 'Work Load' file. The name of the file appeared in the 'Title Bar'. The specimens were ready to be analyzed.

**Analyzing the samples**

All the aliquots in the tubes were gently vortexed to mix the solution uniformly. The labeled tubes were placed appropriately in the luminometer in the following order: Blank, Negative Control, Patient Samples and Positive Controls. The lid of the luminometer was closed and ‘Start’ was clicked for the luminometer to scan for the number of tubes placed to avoid any discrepancy if any of the tubes were left from any of the previous procedures. ‘Next’ was clicked after scanning of the tubes. ‘Assay Type’ was selected, file name (AM061872ROSsp) typed and ‘Finish’ was clicked finally. The Excel spreadsheet opened and measurement of tubes started. Once the measurements finished, the data was saved in a folder and prints taken out later for calculations.

**Calculations for ROS**

Average of negative control (triplicate readings)

Average of patient sample (triplicate readings)

Corrected Value = Patient sample average – Negative control average = x RLU/sec

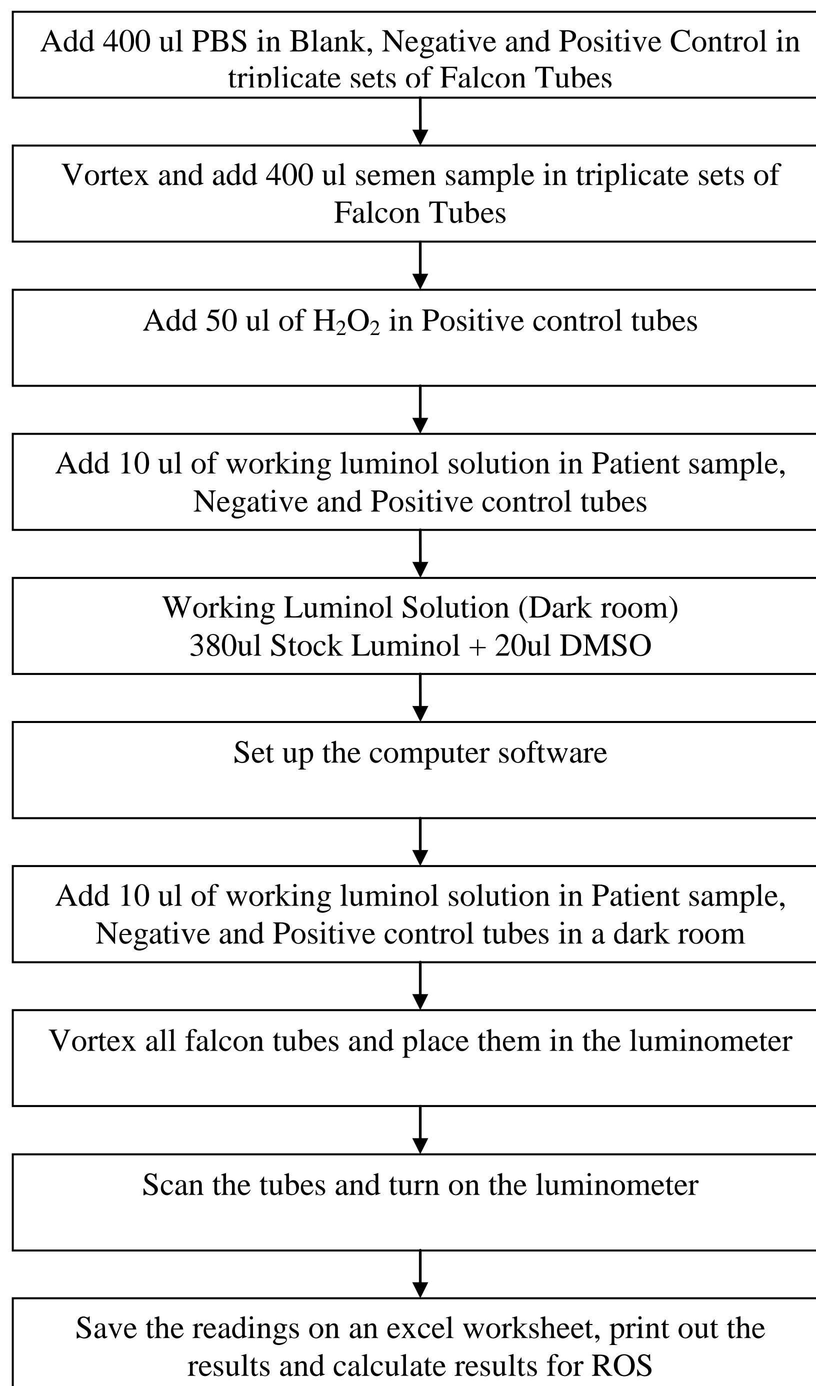
$$\text{Corrected ROS value} = \frac{x}{\text{Sperm Count}} = y \text{ RLU/sec/ } 10^6 \text{ sperm}$$

**Reference Range**

**Normal range:** <20 RLU/sec/x $10^6$ sperm

**Critical Values:** ≥20 RLU/sec/x $10^6$ sperm

# Steps involved in Chemiluminescence Assay



## TUNEL and TAC SAMPLE PREPARATION

Falcon tubes were labeled as patients name, clinic number, date and assay name for TUNEL and cryovials for TAC Assay. For TUNEL assay, the sperm concentration was measured and the volume of the semen set to five million sperms/mL

- Actual sperm count needed for TUNEL Assay = **5 million sperms/ml**

- Sample sperm count = x million (do the manual sperm count manually)

- To prepare 5 million = $\mathbf{\frac{5}{X} \times 1000}$

$$= \mathbf{\frac{5000}{X} = Y\ ul}$$

e. g $\frac{5000}{20\ \text{(million/ml)}}$

Therefore, Y ul of semen was added to each of the four TUNEL labeled centrifuge tubes and centrifuged at 1600 rpm for 6 minutes. The seminal plasma supernatant was gently pipetted out from the centrifuged tubes and added to TAC labeled cryovials and frozen at – $70^0$ C.To the TUNEL tubes, 1000ul of Paraformaldehyde was added over the sperm pellet in each tube to make a total volume of 1ml , vortexed and kept at -$4^0$C for 2-4 hours. After keeping for 2-4 hours, the tubes were taken out from -$4^0$C and centrifuged at 1600 rpm for 6 minutes. The supernatant was discarded with the help of a pipette, taking care not to disturb the pellet at the bottom of the tube. The pellet was then resuspended in 70% ice cold Ethanol, vortexed and frozen at -$20^0$ C to be analyzed later.

## Antioxidant measurement in seminal plasma by TAC ASSAY

### Equipment and materials

A. Antioxidant assay kit (Cat # 709001; Cayman Chemical, Ann Arbor, Michigan).

B. ELx800™ Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, Vermont).

C. Pipettes (20, 200 and 100 microL)

D. Pipette tips (20, 200 and 100 microL)

E. Multichannel pipettes (8 channel, 30-300microL)

F. Aluminium foil

G. Microfuge tubes

H. De-ionized water

I. Polystyrene centrifuge tubes (50 and 15 mL)

J. Round bottom tubes (12 x 75 mm)

### Preparation of assay reagents

All reagents were equilibrated at room temperature before beginning the assay and prepared according to the manufacturer's instructions provided with the assay kit.

**A. Antioxidant assay buffer (10X) (vial # 1)**

Three mL of assay buffer concentrate was diluted with 27 mL of HPLC- grade water in a 50mL conical tube. (The reconstituted vial remains stable for six months when stored at $4^{o}$C).

**B. Chromogen (vial # 2)**

Chromogen (containing ABTS) was reconstituted with 6 mL of water and vortexed. It was sufficient for 40 wells. (The reconstituted vial remains stable for 24h at $4^{o}$C).

**C. Metmyoglobin (vial # 3)**

Lyophilized powder was reconstituted with 600microlitre of assay buffer and vortexed. Once reconstituted, it was sufficient for 60 wells. (The reconstituted reagent remains stable for one month when stored at $-20^{o}$C.

**D. Trolox (vial # 4)**

This vial contains the standard Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid). The lyophilized powder was reconstituted in the bottle with 1mL of water and mixed thoroughly. This was used to prepare the standard curve. The reconstituted vial remains stable for 24h at $4^{o}$C.

**E. Hydrogen Peroxide (vial # 5)**

This vial contains 8.82 M solution of hydrogen peroxide. Ten µL of hydrogen peroxide reagent was dilute with 990 µL of water. Further dilution was done by removing 20microliters and diluting with 3.98mL of water to give 441 µM working solution. (The working solution remains stable for 4h at room temperature).

**Specimen preparation**

The frozen seminal plasma was brought to room temperature and centrifuged at high speed for 5 min. The clear seminal plasma was removed and each sample was diluted 1:10 (10 µL sample + 90 µL assay buffer) in a microfuge tube. The vials were labeled for correct identification. The plate template was used to note the sample being added to each well in duplicate (standard and test samples)

(**Note:**Any errors during pipetting could be be highlighted on the template for any discrepancy in the final results).

**TAC determination**

The standards were prepared in seven clean tubes and marked as A-G for identification. The amount of reconstituted Trolox and Assay Buffer were added to each tube as shown in Table.

| Tube | Reconstituted Trolox (µL) | Assay Buffer (µL) | Final Concentration (mM Trolox) |
|---|---|---|---|
| A | 0 | 1000 | 0 |
| B | 30 | 970 | 0.044 |
| C | 60 | 940 | 0.088 |
| D | 90 | 910 | 0.135 |
| E | 120 | 880 | 0.18 |
| F | 150 | 850 | 0.225 |
| G | 220 | 780 | 0.330 |

For the assay the following reagents were added:

A. 10 µL of trolox standard (tubes A-G) or sample in duplicate + 10 µL of metmyoglobin + 150 µL of chromogen per well were added.

**Note:** A multichannel pipette was used to pipette chromogen.

B. To initiate the reaction, 40 µL hydrogen peroxide working solution was added using multichannel pipette

**Note:** This step was completed as quickly as possible (within 1 minute).

C. The plate was covered with the plate cover and incubated on a shaker for 5 min at room temperature.

D. The cover was removed and absorbance read at 750 nm using a plate reader.

**Calculation of TAC results**

A. The average absorbance of each standard and sample was calculated.

B. The antioxidant concentration of the samples was calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation.

$$\text{Antioxidant } (\mu M) = \frac{\text{Unknown average absorbance} - \text{Y intercept}}{\text{Slope}} \times \text{dilution} \times 1000$$

**Reference Range**

**Normal values**: ≥2000 µM Trolox

**Panic Value**: <2000 µM Trolox

**Steps for setting the ELISA plate reader for the various Pro and antioxidant assays**

1. Login, click ok for password
2. Click on KC Junior - click ok
3. Screen will appear with 2 window options on the top of the screen- select whichever assay to be done, for e.g. TAC follicular fluid or Glutathione or LPO etc.
4. In the center of screen 5 boxes will be present
5. Click on Modify protocol
6. A window will appear with protocol definition – click on Read method from the top icons
7. A screen will appear with the following

Read method type - select End point

Primary wavelength - select the required wavelength by scrolling on the arrow on the window for e.g. 750nm for TAC

Put number of plates as - 1

Plate geometry click on - 8x12, Click ok

8. Click on Template from the top icons
9. Click on well IDs

select either standard /clear/ samples whichever information is to be put by clicking on the window arrow below the plate configuration

Replicate window, next to the above - select 2 for duplicate or 3 for triplicate

Direction - select across, so the duplicate wells will be placed next to each other or if the duplicate wells are to be placed one below the other like for eg. For blank wells in LPO assay select vertical

Map direction- select down and click ok

10. Click on Read plate

Window will appear asking for plate ID, enter the plate ID like the name of the assay, date, your name, time etc and the plate description too in the respective windows

Click on Read plate

11. A window will appear asking to keep the plate on the plate carrier.

After placing the plate on the carrier click on ok

12. The plate will be read and then the raw data will be displayed on the plate

Template

13. Go to plate icon on the upper left hand corner of the screen and click on save plate then click on print results. Select all the pages.

14. Then again click on plate icon and click on export data to Microsoft excel spread sheet

The Microsoft excel spread sheet will be displayed with the raw data etc, save it to your folder .

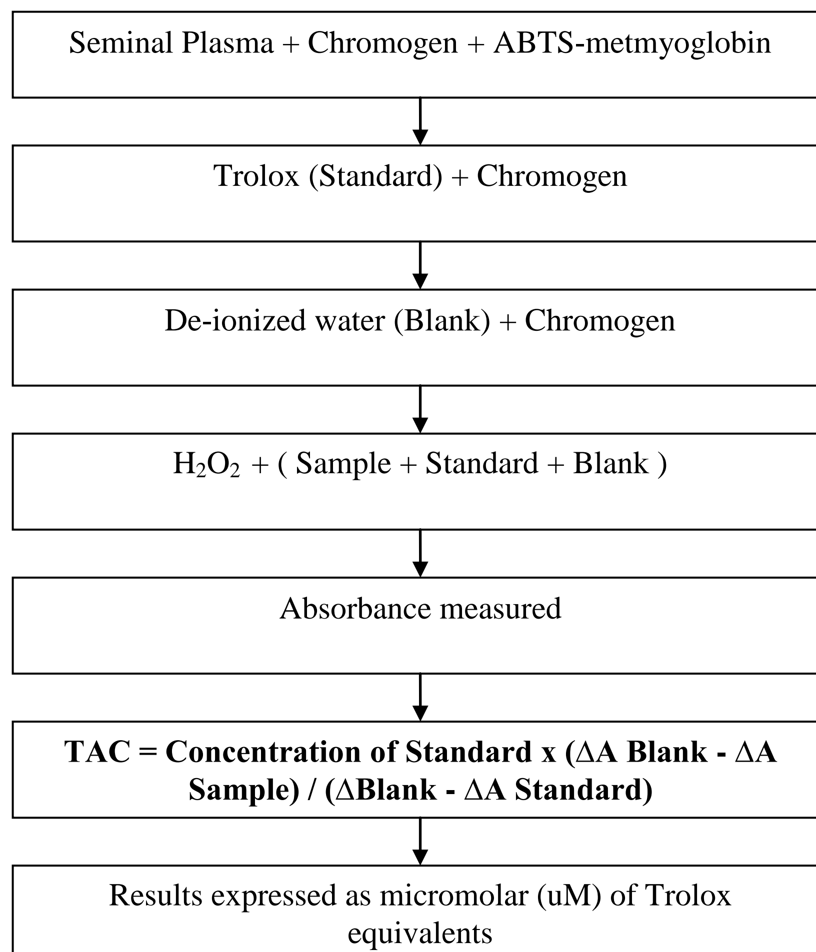
15. Click on exit the excel spread sheet

16. Click on close the plate

17. Window will appear asking, do you want to save the particular assay which was done.

Click on ok or yes and then log out.

# Steps involved in TAC Assay



## Measurement of DNA damage in spermatozoa by TUNEL ASSAY

A. APO-DIRECT™ kit (BD Pharmingen, Catalog % 556381)

B. Ethanol pipettes

C. Pipette tips (200µL and 1000µL)

D. Microcell counting chamber

E. 3.7% paraformaldehyde in PBS

F. Microfuge ependorf tubes

G. Flow cytometer

**Sample preparation**

A. Following liquefaction, semen specimens for volume, sperm concentration, total cell count, motility, and morphology was evaluated.

B. Five µL of the sample was aliquoted and loaded on a Microcell slide chamber (Conception Technologies, San Diego, CA) for manual evaluation of concentration and motility. The concentration of sperm in the sample was adjusted to 2-5 x $10^6$/mL.

C. Using a cryomarker, one 5mL tube was labeled. Label specimen 1 with the patient name, identification number, and date

**Preparation of paraformaldehyde:**

A. To 10.0 mL of formaldehyde (37%) 90.0 mL of PBS (pH 7.4) was added.

B. Check the concentration of sperm in the sample. Adjust the volume to give 2-5X$10^6$/mL. The samples were spun and seminal plasma removed. Add 1.0 mL of 3.7% paraformaldehyde.

C. The cell suspension was placed on ice for 30-60 minutes/ overnight.

D. Store cells in 1 mL of ice-cold 70% (v/v) ethanol at –20˚C until use. Cells can be stored at –20˚C several days before use.

(**Note:** The samples can be processed from A-G, batched and shipped).

**Staining Protocol**

A. The vials containing the positive (6552LZ) and negative (6553LZ) control cells were swirled for resuspension of cells. 2 mL aliquots (approximately 1 million cells/mL) of the suspensions of control cells were removed and placed in 12 x 75 mm centrifuge tubes. The control cell suspensions were centrifuged for 5 minutes at 300 x g or 1600 RPM and 70% (volume/volume) ethanol removed by aspiration, without disturbing the cell pellet.

B. One mL of Wash Buffer (6548AZ) was added to both, the control and sample tubes for resuspension of the cells. The tubes were centrifuged again and the supernatant was removed by aspiration.

C. The Wash Buffer treatment was repeated.

D. All tubes of the control cell pellets were resuspended in 50 µL of the **Staining Solution** (prepared as described below).

**Staining Solution (single assay)**

| Staining Solution | 1 Assay | 6 Assays | 12 Assays |
|---|---|---|---|
| Reaction buffer (green cap) | 10.00 µL | 60.00 µL | 120.00 µL |
| TdT Enzyme (yellow cap) | 0.75 µL | 4.50 µL | 9.00 µL |
| FITC-dUTP (orange cap) | 8.00 µL | 48.00 µL | 96.00 µL |
| Distilled $H_2O$ | 32.25 µL | 193.5 µL | 387.00 µL |
| **Total volume** | **51.00 µL** | **306.00 µL** | **612.00 µL** |

(**Note:**The appropriate volume of Staining Solution to prepare for a variable number of assays is based upon multiples of the component volumes needed for 1 assay. Mix only enough Staining Solution to complete the number of assays prepared per session. The Staining Solution is active for approximately 24 hr at 4°C).

F. Incubate the sperm in the Staining Solution for 60 min at 37˚C. The reaction can also be carried out at room temperature overnight for the control cells. For test samples, the 60 min incubation time at 37˚C may need to be adjusted to longer periods of time.

G. At the end of the incubation time, add 1.0 mL of Rinse Buffer (6550AZ) (Red cap) to each tube and centrifuge each tube at 300 x g for 5 min. Remove the supernatant by aspiration.

H. Repeat the cell rinsing with 1.0 mL of the Rinse Buffer, centrifuge, and remove the supernatant by aspiration.

I. Resuspend the cell pellet in 0.5 ml of the PI/ RNase Staining Buffer (6551AZ). (**Note:**If the cell density is low, decrease the amount of PI/ RNase Staining Buffer to 0.3 mL).

J. Incubate the cells in the dark for 30 min at RT.

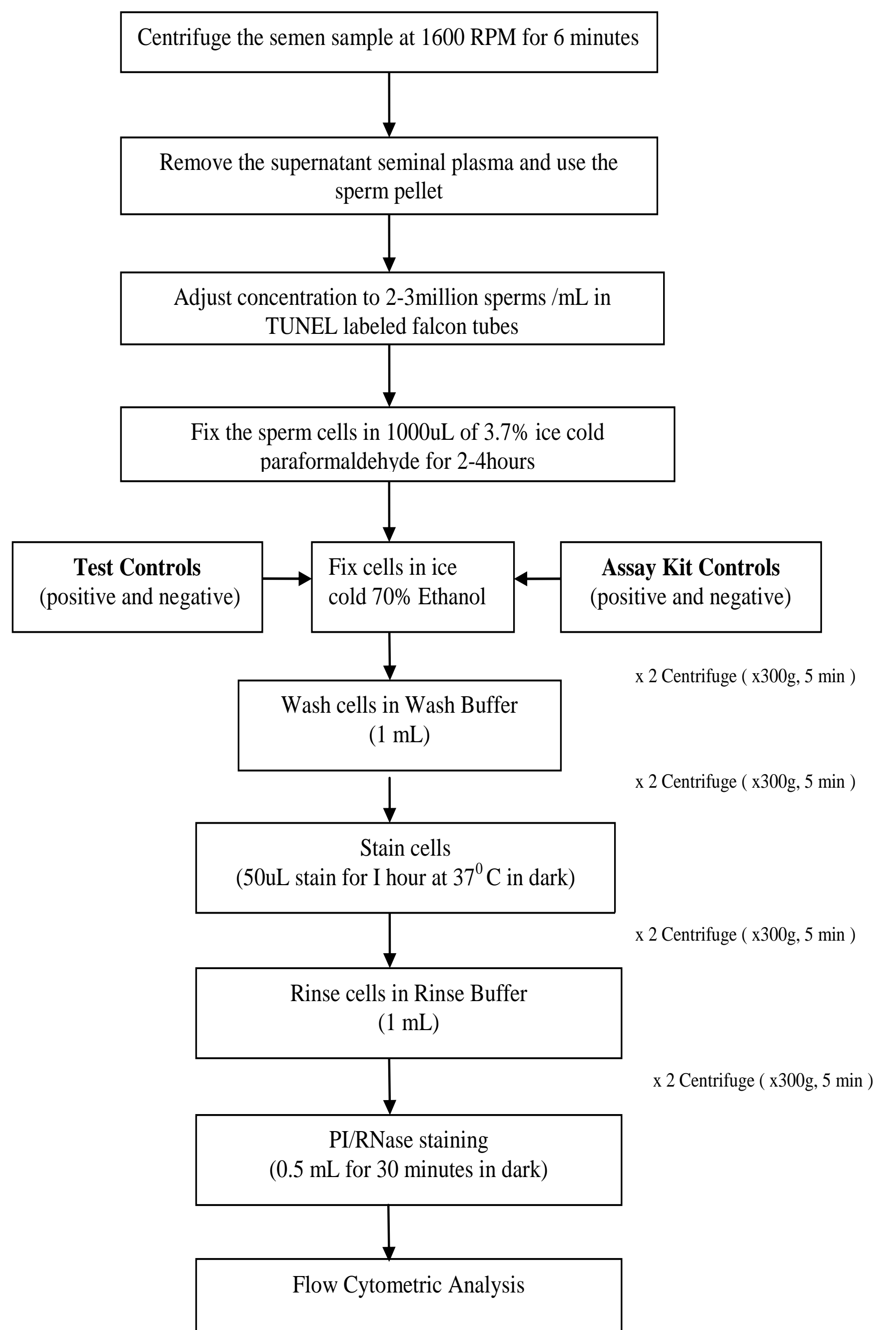
K. Analyze the cells in PI/ RNase solution by flow cytometry. (**Note:**The cells must be analyzed within 3 hr of staining. Cells may begin to deteriorate if left overnight before analysis).

**Reference Range**

**Normal range: ≤19%** DNA damage

**Panic values: >19%** DNA damage

# Steps involved in TUNEL Assay



## Flow Cytometric Analysis

The percentage of DNA damaged spermatozoa cells was calculated on a FACScan Flow Cytometer (Becton Dickinson). For each assay, a minimum of 10,000 spermatozoa were examined for DNA damage, at a flow rate of <100 cells/second. For exclusion of aggregates and debris, the sperms were gated using $90^0$ and forward-angle light scatter. Using an argon laser at 15mW, with a wavelength of 488nm, red fluorescence (580-630nm), produced by propidium iodide (PI) was analyzed in the FL-2 channel. The mean fluorescence and PI-positive cells were calculated on a 1023-channel scale and analyzed using the flow cytometer software FlowJo, version 6.4.2 (FlowJo, LLC, Ashland, OR), (Sharma et al, 2013; Sharma and Agarwal, 2011).

## Statistical Analysis

Using, SPSS Statistics 20, data were expressed as mean ± SEM. To compare fertile and all subfertile male categories, Independent Sample T-Test was applied. Pearson's Correlation Coefficient was used to find out the correlational study.

# RESULTS

Our study included a total of 302, both fertile and subfertile groups. Hundred and fifteen men were normal healthy fertile men who participated as controls in our study. The subfertile group was further sub-divided into two categories: 66 subfertile males were diagnosed as having no varicocele while 121 subfertile males were diagnosed with varicocele on ultrasonography. Of the 121 (64.7%) subfertile males with varicocele, all had varicocele on the left side of the testis. 67 (55.3%) of the subjects had grade I, 35 (28.9%) of the varicocele positive subjects were diagnosed with grade 2 and 19 (15.7%) with left sided grade 3 varicocele. None of the subfertile male subjects evaluated in our study had varicocele in the right testis. Leukocytospermia was excluded in the healthy fertile control group.

Results of vital signs of the males are shown in Table 1

**Age (years)**

Mean ± SEM age of fertile males was 35.10±0.52, whereas the mean ± SEM age of varicocele negative males was 36.32±0.94 while that of varicocele positive males was 35.65±0.51 respectively.

**Height (cm)**

The mean ± SEM height (cms) of fertile males was 178.84±0.73, whereas the mean ± SEM height of varicocele negative males was 179.75±0.94, while height of varicocele positive males was 179.79±0.67.

**Weight (kg)**

The mean ± SEM weight in kg, of fertile males were 93.42±1.84, whereas the mean ± SEM weight of varicocele negative males was 91.12±2.72, while weight of varicocele positive males were 96.79±1.76.

**Body Mass Index (kg/m$^2$)**

The body mass index (BMI) in kg/m$^2$ of healthy fertile controls was 29.08±0.49, whereas the mean ± SEM BMI of varicocele negative males was 28.04±0.71, while BMI of varicocele positive males were 29.83±0.46.

**Table 1: Mean age, height, body weight and body mass index of healthy fertile controls, varicocele negative and varicocele positive subfertile patients in the study population**

| Basic Characteristics | Controls (115) | VAR – (66) | VAR + (121) |
|---|---|---|---|
| **Age (years)** | 35.10±0.52 | 36.32±0.94 | 35.65±0.51 |
| **Height (cm)** | 178.84±0.73 | 179.75±0.94 | 179.79±0.67 |
| **Body Weight (kg)** | 93.42±1.84 | 91.12±2.72 | 96.79±1.76 |
| **BMI ($kg/m^2$)** | 29.08±0.49 | 28.04±0.71 | 29.83±0.46 |

Values in parenthesis represent number of subjects
All values are expressed as mean ± SEM

The semen characteristics of fertile and subfertile male subjects are shown in Table 2, figures 1, 2, 3, 4

**Abstinence (days)**

Mean ± SEM of fertile and subfertile male subjects was 3.60±0.13 in healthy fertile controls, 3.88±0.21 in varicocele negative and 3.90±0.16 in varicocele positive. No significant difference (P>0.05) was seen between the three study groups.

**Semen Volume (mL)**

Mean ± SEM semen volumes of fertile and subfertile male subjects was 3.34±0.11 in healthy fertile controls, 3.34±0.18 in varicocele negative and 3.40±0.15 in varicocele positive. No significant difference (P>0.05) was seen between the three study groups.

**Sperm Concentration (million/mL)**

The mean ± SEM sperm concentration in healthy fertile controls was 81.18±5.05, 44.30±7.45 in varicocele negative and 40.28±4.76 in varicocele positive male subjects. Varicocele negative and positive male subjects showed a significant (p<0.001) decrease in sperm concentrations as compared to healthy controls.

**Sperm motility (%)**

The mean ± SEM percentage sperm motility of healthy fertile controls was 64.84±1.04, 41.83±1.82 in varicocele negative and 40.12±1.61 in varicocele positive male subjects. A significant (p<0.001) decrease in % sperm motility was seen in varicocele negative and positive male subjects as compared to healthy controls.

**WHO normal sperm morphology (%)**

Mean ± SEM percentage of normal sperm morphology according to the WHO criteria, (1999), was 38.46±0.66 in controls, 7.26±0.59 in varicocele negative and 6.98±0.57in varicocele positive. A significant (p<0.001) decrease in percentage of morphologically normal form sperms was seen in varicocele negative and positive male subjects as compared to healthy controls.

**Kruger Strict Criteria for normal sperm morphology (%)**

Mean ± SEM percentage of normal sperm morphology according to the Strict Criteria, was 14.47±0.06 in controls, 2.70±0.23 in varicocele negative and 2.68±0.24 in varicocele positive subjects. Mean percentage normal sperm morphology was significantly lower

($p<0.001$) in varicocele negative and positive male subjects as compared to healthy fertile controls.

**Table 2: Mean semen volume, sperm concentration, motility and morphology of healthy fertile controls and subfertile male subjects, with and without varicocele**

| Semen Characteristics | Controls (115) | VAR – (66) | VAR + (121) | p1 value | p2 value | p3 value |
|---|---|---|---|---|---|---|
| **Abstinence (days)** | 3.60±0.13 | 3.88±0.21 | 3.90±0.16 | 0.23 | 0.93 | 0.14 |
| **Volume (mL)** | 3.34±0.11 | 3.34±0.18 | 3.40±0.15 | 0.98 | 0.81 | 0.75 |
| **Concentration (million/mL)** | 81.18±5.05 | 44.30±7.45 | 40.28±4.76 | 0.000*** | 0.63 | 0.000*** |
| **Motility (%)** | 64.84±1.04 | 41.83±1.82 | 40.12±1.61 | 0.000*** | 0.50 | 0.000*** |
| **WHO Normal Morphology (%)** | 38.46±0.66 | 7.26±0.59 | 6.98±0.57 | 0.000*** | 0.75 | 0.000*** |
| **Strict Normal Morphology (%)** | 14.47±0.06 | 2.70±0.23 | 2.68±0.24 | 0.000*** | 0.95 | 0.000*** |

Values in parenthesis represent number of subjects. All values are expressed as mean ± SEM.
P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive
p= 0.05*, p =0.01**, p =0.001***

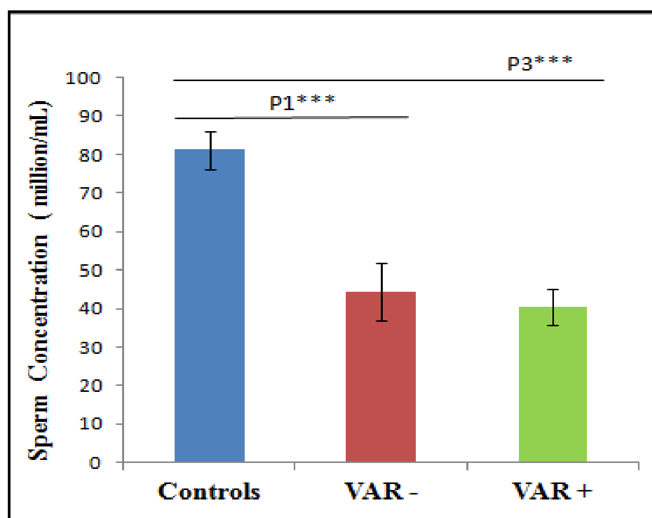



**Figure1: Comparison of mean sperm concentrations (million/mL) of healthy fertile controls and subfertile male subjects, with and without varicocele**

P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VA R + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive

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

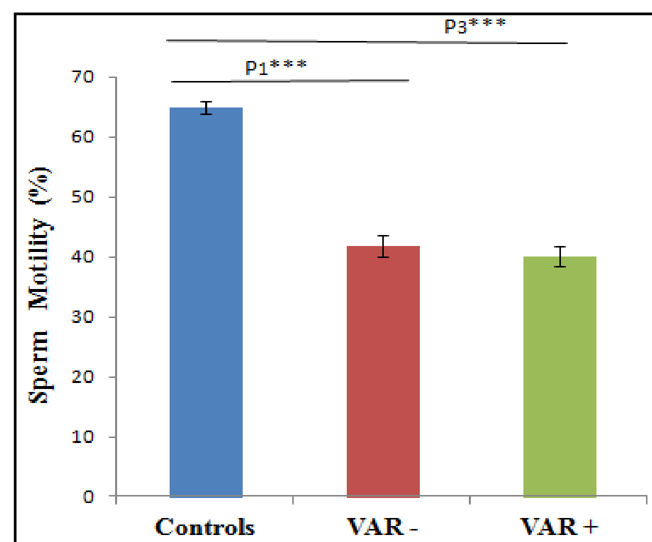



**Figure 2: Comparison of mean percentage sperm motility of healthy fertile controls and subfertile male subjects, with and without varicocele**

P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VA R + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive

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

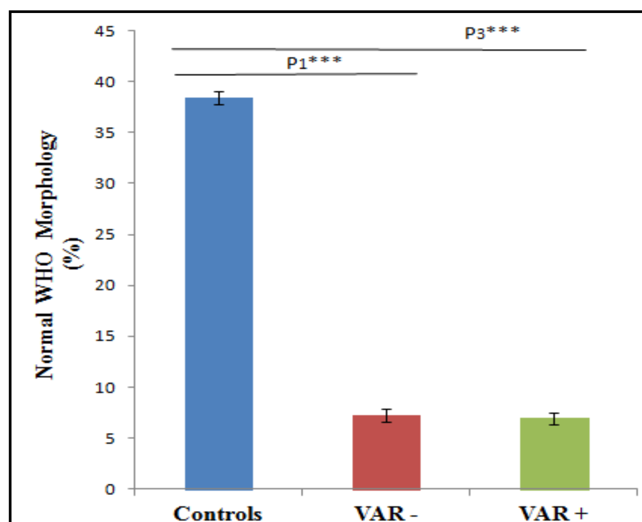



**Figure 3: Comparison of mean percentage normal sperm morphology (WHO Criteria) of healthy fertile controls and subfertile male subjects, with and without varicocele**

P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VA R + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive

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

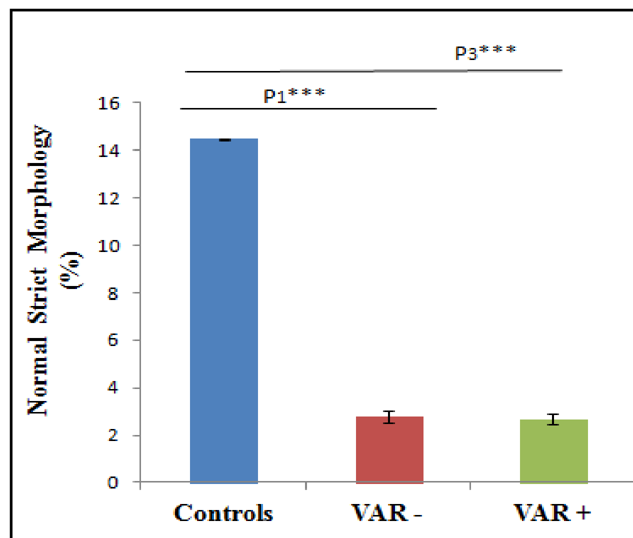



**Figure 4: Comparison of mean percentage normal sperm morphology (Strict Criteria) of healthy fertile controls and subfertile male subjects, with and without varicocele**

P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VA R + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive

p= 0.05*, P =0.01**, P =0.001***

Abnormal sperm categories (%) of fertile controls, varicocele negative and varicocele positive subfertile men are shown in Table 3

According to the WHO, (1999), criteria for sperm morphology, the mean ± SEM percentage of tapered sperms was 11.63±0.50 in controls, 8.33±0.99 in varicocele negative and 7.55±0.66 in varicocele positive male subjects. The sperms of healthy controls showed a significant difference in the tapered morphologies of varicocele negative ($p=0.001$) and varicocele positive ($p<0.001$) male subjects. Mean overall ± SEM percentage of amorphous sperms in controls was 34.00±0.86, 63.17±1.57 in varicocele negative and 65.12±1.03 in varicocele positive subjects. A highly significant increase ($p<0.001$) in amorphous morphology defect was seen in both the varicocele negative and varicocele positive male subjects as compared to the controls. Fertile male subjects had an overall small sperm defect of 7.49±0.39 while a mean ± SEM of 5.17±0.62 was seen in varicocele positive subjects, showing a significant difference ($p<0.001$) between the controls and varicocele positive subjects. Fertile controls had a mean ± SEM 1.90±0.10 percentage of megalo sperms, 2.61±0.83 in varicocele negative and 2.55±0.20 in varicocele positive subjects. A significant difference was seen ($p<0.05$) between the megalo sperms varicocele negative and positive subfertile men. The mean ± SEM bicephalic sperm morphology of fertile controls was 1.56±0.13 , 2.50±1.27 in varicocele negative and 1.47±0.14 in varicocele positive subjects, with no considerable difference ($p>0.05$) seen between the three groups. The fertile controls had mean ± SEM 6.63±0.41 of tail defects, 15.70±1.35 in varicocele negative and 17.42±1.00 in varicocele positive subjects. The fertile controls showed a significant decrease ($p<0.05$) in tail defects when compared to the varicocele negative and positive group. The fertile controls subjects head defects, as assessed by Strict Criteria of sperm morphology, had a mean ± SEM of 85.53±0.06, 97.30±0.23 35 in varicocele negative and 97.32±0.24 in varicocele positive subjects with a highly significant difference ($p<0.001$) seen when the varicocele negative and positive groups were compared with healthy fertile controls .

**Table 3: Mean morphological sperm defects of fertile controls and subfertile varicocele negative and positive male subjects**

| Abnormal Sperm Morphology (%) | Controls (115) | VAR – (66) | VAR + (121) | p1 value | p2 value | p3 value |
|---|---|---|---|---|---|---|
| **Abnormal sperm morphology categories according to WHO, (1999), Criteria** | | | | | | |
| **Tapered Sperms** | 11.63±0.50 | 8.33±0.99 | 7.55±0.66 | 0.001*** | 0.50 | 0.000*** |
| **Amorphous Sperms** | 34.00±0.86 | 63.17±1.57 | 65.12±1.03 | 0.000*** | 0.28 | 0.000*** |
| **Small Sperms** | 7.49±0.39 | 5.17±0.62 | 4.32±0.35 | 0.002 | 0.20 | 0.000*** |
| **Megalo Sperms** | 1.90±0.10 | 2.61±0.83 | 2.55±0.20 | 0.18 | 0.93 | 0.002* |
| **Bicephalic Sperms** | 1.56±0.13 | 2.50±1.27 | 1.47±0.14 | 0.33 | 0.35 | 0.65 |
| **Tail Defects** | 6.63±0.41 | 15.70±1.35 | 17.42±1.00 | 0.000*** | 0.30 | 0.000*** |
| **Kruger Strict Criteria** | | | | | | |
| **Head Defects** | 85.53±0.06 | 97.30±0.23 | 97.32±0.24 | 0.000*** | 0.95 | 0.000*** |

Values in parenthesis represent number of subjects.
All values are expressed as mean ± SE; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group.
VAR - denotes varicocele negative; VAR + denotes varicocele positive.
p= 0.05*, p=0.01**, p=0.001***

The presence of white blood cells and leukocytospermia, as determined by the Endtz Test, is explained in Table 4

The white blood cells (WBCs) mean ± SEM was 1.24±0.14 in varicocele negative and 1.14±0.15 in varicocele positive male subjects. There was no significant ($p>0.05$) difference seen between the two groups. Mean ± SEM of leukocytospermia in varicocele negative was 0.13±0.03 and 0.16±0.04 in varicocele positive male subjects. No significant difference ($p>0.05$) was seen between the two subfertile subgroups.

**Table 4: Mean of leukocytospermia between varicocele negative and varicocele positive subfertile subjects in the study population**

| Round Cells (million/mL) | VAR – (66) | VAR + (121) | p value |
|---|---|---|---|
| **Undifferentiated Cells (million/mL)** | 1.24±0.14 | 1.14±0.15 | 0.66 |
| **Endtz Test - Leukocytospermia (million/mL)** | 0.13±0.03 | 0.16±0.04 | 0.64 |

Values represent mean ± SEM; values in parenthesis represent number of subjects
p= 0.05*, p=0.01**, p=0.001***

Correlation of leukocytospermia (Endtz Positive Test) with semen characteristics in varicocele negative and varicocele positive subfertile subjects is shown in Table 5.

The seminal volume of our subfertile study group showed a non-significant negative correlation (r=-.138; p=0.060) with leukocytospermia. A non-significant positive correlation was seen between sperm concentration (r=0.046; p=0.528) and leukocytospermia. A non-significant positive correlation was also seen between sperm percentage motility (r=0.040; p= 0.583) and leukocytospermia. A non-significant positive correlation was observed between leukocytospermia and sperm morphology, both as assessed by the WHO, 1999, Criteria (p=0.047; r= 0.522) and Kruger Strict Criteria (r =0.047; p = 0.527).

**Table 5: Correlation of leukocytospermia (Endtz Positive Test) with semen characteristics in the varicocele negative and varicocele positive subfertile subjects**

| Semen Parameters | r value | p value |
|---|---|---|
| **Semen Volume (mL)** | - 0.138 | 0.060 |
| **Sperm Concentration (million/mL)** | 0.046 | 0.528 |
| **Sperm Motility (%)** | 0.040 | 0.583 |
| **WHO Normal Sperm Morphology (%)** | 0.047 | 0.522 |
| **Strict Normal Sperm Morphology (%)** | 0.047 | 0.527 |

Note: values represent Pearson r and the P values. Parameters having a positive Pearson r value represent a positive correlation, while parameters with a negative Pearson r value represent a negative correlation
* Correlation is significant at the 0.05 level (2 tailed)
** Correlation is significant at the 0.01 level (2 tailed)
*** Correlation is significant at the 0.01 level (2 tailed)

Table 6 table explains the correlation of leukocytospermia and sperm morphological defects in the subfertile group, with and without varicocele. Sperms with tapered morphology showed a non-significant negative correlation (r = -0.108; p =0.192) with leukocytospermia. A non-significant negative correlation (r= -0.039; p=0.596) was seen between amorphous sperms and leukocytospermia. A non-significant positive correlation was seen between small sperms (r=0.027; p=0.774) and leukocytospermia. Non-significant negative correlations were seen in megalo sperms (r= -0.056; p=0.585), bicephalic sperms (r = -0.114; p =0.527). However, a non-significant positive correlation was seen between leukocytospermia and tail defects (r = 0.036; p = 0.632). A non-significant negative correlation was seen between leukocytospermia and head defects (r= -0.047; p=0.527) when assessed by Kruger Strict Criteria of sperm morphology

**Table 6: Correlation of leukocytospermia (Endtz Positive Test) with % WHO abnormal sperm morphology in the varicocele negative and varicocele positive subfertile subjects**

| Morphological Sperm Defects | r value | p value |
|---|---|---|
| **Tapered sperms (%)** | -0.108 | 0.192 |
| **Amorphous sperms (%)** | -0.039 | 0.596 |
| **Small sperms (%)** | 0.027 | 0.774 |
| **Megalo sperms (%)** | -0.056 | 0.585 |
| **Bicephalic sperms (%)** | -0.114 | 0.527 |
| **Tail Defects sperms (%)** | 0.036 | 0.632 |
| **Strict Criteria Head Defects (%)** | -0.047 | 0.527 |

Note: values represent Pearson r and the P values. Parameters having a positive Pearson r value represent a positive correlation, while parameters with a negative Pearson r value represent a negative correlation
* Correlation is significant at the 0.05 level (2 tailed)
** Correlation is significant at the 0.01 level (2 tailed)
*** Correlation is significant at the 0.001 level (2 tailed)

Table 7, figure 5, gives a detail of the mean ± SEM reactive oxygen species (ROS) levels in the controls, varicocele negative and varicocele positive groups.

Mean ± SEM of the ROS levels (million/mL) was 14.90±0.21 in healthy fertile control controls, 212.91±24.50 in the varicocele negative group and 239.44±19.94 in the varicocele positive group. A highly significant (P<0.001) increased ROS levels were seen, both, between the varicocele negative group and varicocele positive as compared to the fertile controls (p<0.001). The mean ± SEM of Log (ROS+1) was 1.19±0.00 in healthy fertile controls, 2.13±0.05 in the varicocele negative group and 2.16±0.04 in the varicocele positive group A highly significant difference (p<0.001) in the ROS levels was seen between the varicocele negative group and healthy fertile controls and also in varicocele positive and the fertile controls (p<0.001).

**Table 7: Mean of ROS of healthy fertile controls, varicocele negative and varicocele positive subfertile subjects in the overall study population**

| Oxidative Stress Markers | Controls (115) | VAR – (66) | VAR + (121) | p1 value | p2 value | p3 value |
|---|---|---|---|---|---|---|
| ROS (million/mL/x$10^6$ sperms) | 14.90±0.21 | 212.91±24.50 | 239.44±19.94 | $0.000^{***}$ | 0.41 | $0.000^{***}$ |
| Log (ROS+1) | 1.19±0.00 | 2.13±0.05 | 2.16±0.04 | $0.000^{***}$ | 0.63 | $0.000^{***}$ |

Values in parenthesis represent number of subjects. All values are expressed as mean ± SEM;
P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive .
$p= 0.05^{*}$, $p=0.01^{**}$, $p=0.001^{***}$

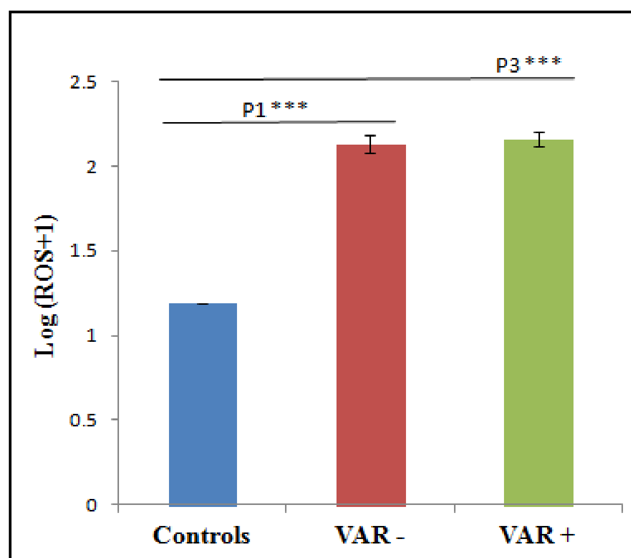



**Figure 5: Mean ± SEM of ROS levels in healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

P1 = P between controls and VAR – group; P3 = P between controls and VAR + group. $p= 0.05^{*}$, $p=0.01^{**}$, $p=0.001^{***}$

Table 8, figure 6 explains the means of the TAC levels of controls, varicocele negative and varicocele positive subjects in the study

The mean ± SEM of the TAC levels (mM) were found to be 2476.98±28.64 in healthy fertile controls, 2226.94±73.83 in the varicocele negative group and 2188.86±61.14 in the varicocele positive group. A highly significant difference ($p<0.001$) was seen between the varicocele negative group and healthy fertile control group. It was also seen that the varicocele positive group and healthy fertile controls had a highly significant difference ($p<0.001$) in their seminal TAC values.

**Table 8: Mean ± SEM of TAC levels of healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

| Oxidative Stress Markers | Controls (115) | VAR – (66) | VAR + (121) | p1 value | p2 value | p3 value |
|---|---|---|---|---|---|---|
| **TAC** (mM) | 2476.98±28.64 | 2226.94±73.83 | 2188.86±61.14 | 0.000*** | 0.70 | 0.000*** |

All values are expressed as mean ± SEM;
P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group.
VAR - denotes varicocele negative; VAR + denotes varicocele positive. p= 0.05*, p=0.01**, p=0.001***

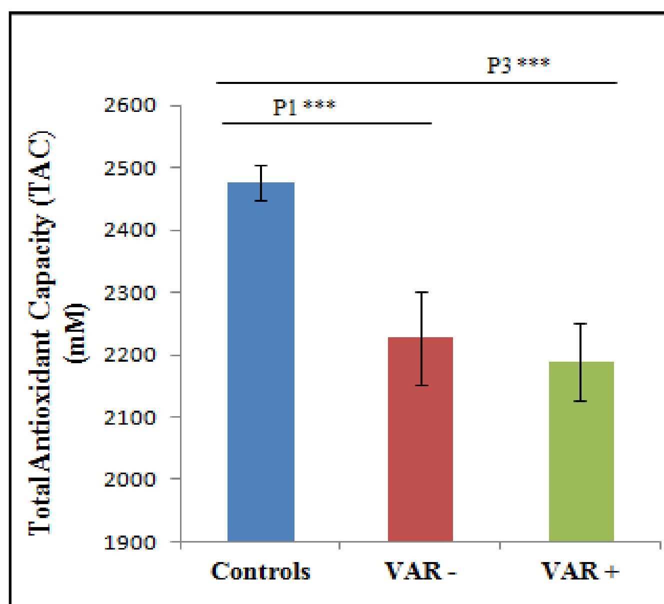



**Figure 6: Mean ± SEM of TAC levels of healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.
P= 0.05*, P=0.01**, P=0.001***

Table 9, figure 7, gives a detail about the sperm DNA fragmentation via the TUNEL Assay

The mean ± SEM of percentage TUNEL$^{+ve}$ sperm cells were found to be 14.87±0.20 in healthy fertile controls, 22.91±1.80 in the varicocele negative group and 25.84±1.42 in the varicocele positive group. A highly significant difference ($p<0.001$) was seen between the varicocele negative group and healthy fertile control group. The varicocele positive group, also had a highly significant difference ($p<0.001$) in their % of fragmented sperm DNA as compared to the healthy fertile controls.

**Table 9 Mean of sperm DNA fragmentation by TUNEL Assay of healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

| Oxidative Stress Markers | Controls (115) | VAR – (66) | VAR + (121) | p1 value | p2 value | p3 value |
|---|---|---|---|---|---|---|
| TUNEL$^{+ve}$ sperm cells (%) | 14.87±0.20 | 22.91±1.80 | 25.84±1.42 | 0.000*** | 0.21 | 0.000*** |

All values are expressed as mean ± SEM;
P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR+ group.
VAR - denotes varicocele negative; VAR + denotes varicocele positive.
p= 0.05*, p=0.01**, p=0.001***

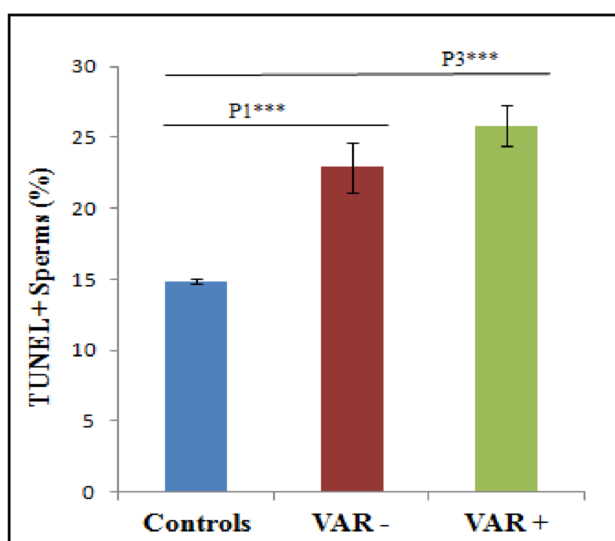



**Figure7: Mean ± SEM of sperm DNA fragmentation in healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.
p= 0.05*, p=0.01**, p=0.001***

Table 10, figures 8 a, b, c, d show correlation of the reactive oxygen species (ROS) and total antioxidant capacity (TAC) in the study population. The healthy fertile controls, figure 8a, show a non -significant (r= 0.019; p=0.840) correlation between the reactive oxygen species (Log (ROS+1) and total antioxidant capacity (TAC) levels. The varicocele negative subjects, figure 8b, also have a non -significant (r= 0.088; p=0.484) correlation between the reactive oxygen species (Log (ROS+1) and total anti-oxidant (TAC) levels. Figure 8c shows a significant negative (r= -0.218; p <0.05) correlation between the reactive oxygen species (Log (ROS+1)) and total antioxidant (TAC) levels.

**Table 10: Correlation of reactive oxygen species (Log (ROS+1)) and total antioxidant capacity (TAC) in healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

| Log (ROS+1) | Total Anti-oxidant Capacity (TAC) (micromoles) | |
|---|---|---|
| | r value | p value |
| **Healthy Fertile Controls** | 0.019 | 0.840 |
| **Varicocele Negative** | 0.088 | 0.484 |
| **Varicocele Positive** | - 0.218 | 0.016* |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

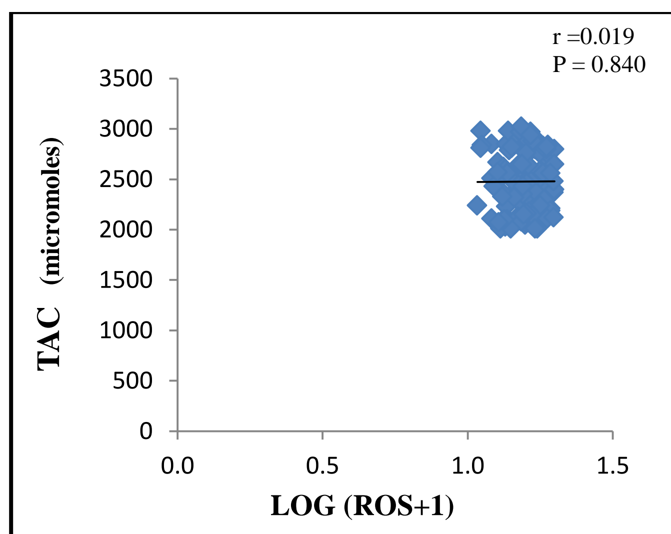



**Figure 8a: Non- significant correlation of reactive oxygen species (Log (ROS+1)) and total antioxidant capacity (TAC) in healthy fertile controls**

All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.

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

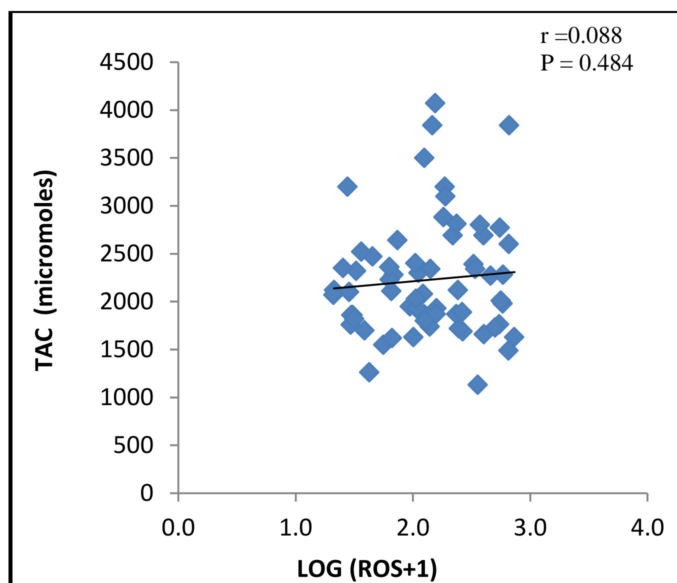



**Figure 8b: Non- significant correlation of reactive oxygen species (Log (ROS+1)) and total antioxidant capacity (TAC) in varicocele negative subfertile subjects**

All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.

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

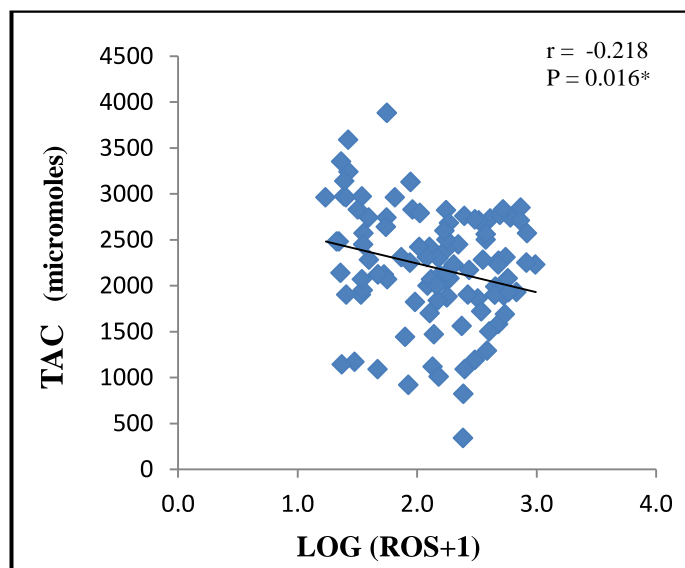



**Figure 8c: Significant correlation of reactive oxygen species (Log (ROS+1)) and total antioxidant capacity (TAC) in varicocele positive subfertile subjects**

All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.

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

Table 11, figure 9 a, b, c, show correlation of ROS and sperm DNA fragmentation in healthy fertile controls, varicocele negative and varicocele positive subfertile subjects

A significant positive (r= 0.189; p<0.05) correlation was seen between the reactive oxygen species levels (Log (ROS+1)) and sperm DNA fragmentation ($TUNEL^+$ sperm cells) in healthy fertile controls. Figure 9b shows a non-significant negative (r= -0.107; p=0.393) correlation was seen between the reactive oxygen species levels (Log (ROS+1) and sperm DNA fragmentation ($TUNEL^+$ sperm cells) in varicocele negative subjects. Figure 9c shows a non-significant positive (r= 0.175; p=0.055) correlation was seen between the reactive oxygen species levels (Log (ROS+1)) and sperm DNA fragmentation ($TUNEL^+$ sperm cells) in varicocele positive subfertile subjects.

**Table 11: Correlation of reactive oxygen species (Log (ROS+1)) and sperm DNA fragmentation by TUNEL Assay in healthy fertile controls, varicocele negative and varicocele positive subfertile subjects**

| LOG (ROS+1) | TUNEL + Sperm Cells (%) | |
|---|---|---|
| | **r value** | **P value** |
| **Healthy Fertile Controls** | 0.189 | 0.043* |
| **Varicocele Negative** | - 0.107 | 0.393 |
| **Varicocele Positive** | 0.175 | 0.055 |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

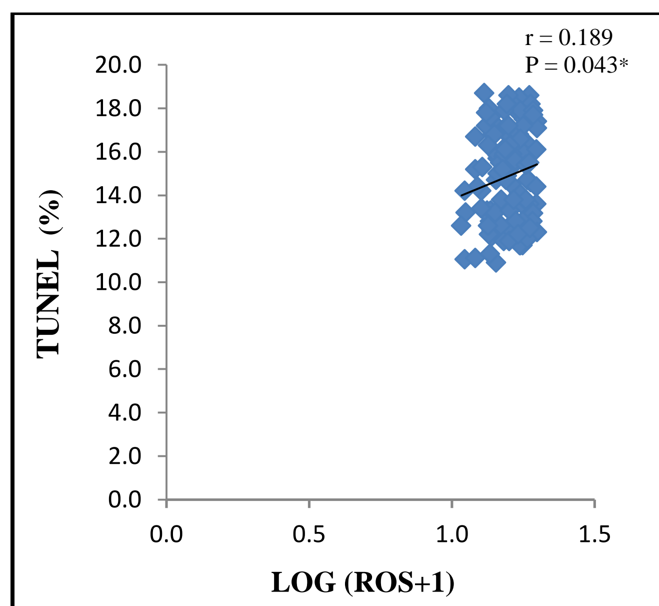



**Figure 9a: Correlation of LOG (ROS+1) and TUNEL$^{+ve}$ sperm cells in healthy fertile controls**

All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.
p= 0.05*, p=0.01**, p=0.001***

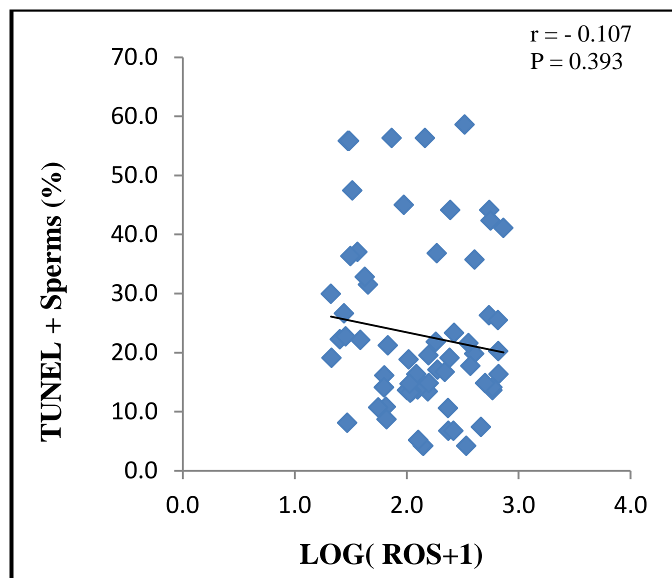



**Figure 9b: Correlation of LOG (ROS+1) and TUNEL$^{+ve}$ sperm cells in varicocele negative subfertile subjects** All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.

p= 0.05$^{*}$, p=0.01$^{**}$, p=0.001$^{***}$

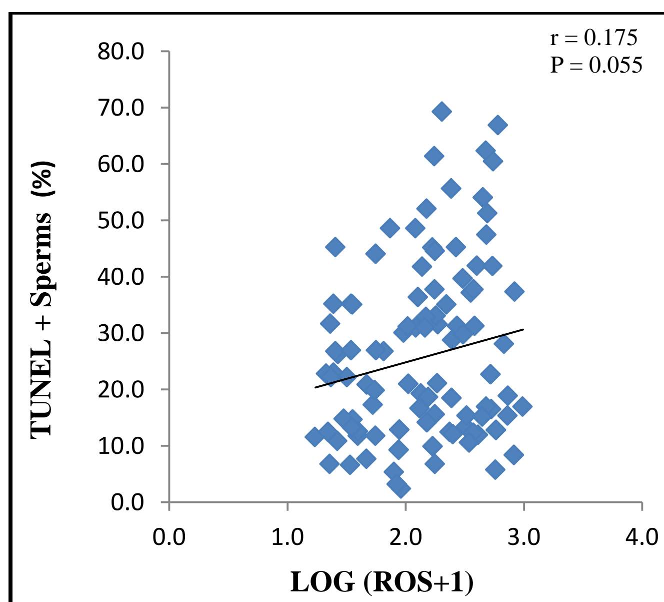



**Figure 9c: Correlation of LOG (ROS+1) and TUNEL$^{+ve}$ sperm cells in varicocele positive subfertile subjects**

All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.

p= 0.05$^{*}$, p=0.01$^{**}$, p=0.001$^{***}$

Table 12 explains the correlation of ROS with semen characteristics in the healthy fertile control group.

A non-significant positive (r=0.039; p=0.682) correlation was seen between reactive oxygen species (Log (ROS+1)) and the sperm concentrations in healthy fertile controls. The percentage sperm motility also showed a non-significant (r= -0.068; p=0.472) negative correlation between reactive oxygen species (Log (ROS+1)) in healthy fertile controls. The healthy fertile controls showed a non-significant (r= -0.147; p=0.118) negative correlation when between normal sperm morphology, as assessed by WHO method, and reactive oxygen species (Log (ROS+1)). A non-significant positive (r= 0.066; p=0.482) correlation was seen between reactive oxygen species (Log (ROS+1)) and the normal sperm morphology, as assessed by Strict Criteria, in healthy fertile controls.

**Table 12: Correlation of reactive oxygen species (Log (ROS+1)) with semen characteristics in healthy fertile controls**

| Semen Characteristics | r value | p value |
|---|---|---|
| **Sperm Concentration (million/mL)** | 0.039 | 0.682 |
| **Sperm Motility (%)** | -0.068 | 0.472 |
| **Sperm WHO Morphology (%)** | -0.147 | 0.118 |
| **Sperm Strict Morphology (%)** | 0.066 | 0.482 |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

Table 13 shows correlation of ROS with semen characteristics in varicocele negative subfertile subjects.

A non-significant negative (r= -0.152; p=0.222) correlation was seen between reactive oxygen species and (Log (ROS+1)) and the sperm concentrations in varicocele negative subfertile subjects. The percentage sperm motility also showed a non-significant (r= 0.095; p=0.450) positive correlation between reactive oxygen species and Log (ROS+1) in varicocele negative subfertile subjects. The varicocele negative subfertile subjects showed a non-significant (r= 0.070; p=0.577) positive correlation between normal sperm morphology, as assessed by WHO method, and reactive oxygen species (Log (ROS+1)). A non-significant positive (r= 0.015; p=0.905) correlation between reactive oxygen species (Log (ROS+1)) and the normal sperm morphology, as assessed by Strict Criteria, in varicocele negative subfertile subjects.

**Table 13: Correlation of reactive oxygen species ((Log (ROS+1)) with semen characteristics in varicocele negative subfertile subjects**

| **Semen Characteristics** | **r value** | **p value** |
|---|---|---|
| **Sperm Concentration (million/mL)** | -0.152 | 0.222 |
| **Sperm Motility (%)** | 0.095 | 0.450 |
| **Sperm WHO Morphology (%)** | 0.070 | 0.577 |
| **Sperm Strict Morphology (%)** | 0.015 | 0.905 |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

Table 14 shows correlation of ROS with semen characteristics in varicocele positive subfertile subjects.

A non-significant positive (r= 0.023; p=0.805) correlation was seen between reactive oxygen species (Log (ROS+1)) and the sperm concentrations in varicocele positive subfertile subjects. Varicocele positive subfertile subjects showed a non-significant (r= 0.000; p=0.995) positive correlation between the percentage sperm motility and reactive oxygen species (Log (ROS+1)). The varicocele positive subfertile subjects showed a non-significant (r= 0.122; p=0.183) positive correlation when between normal sperm morphology, as assessed by WHO method, and reactive oxygen species (Log (ROS+1)). A non-significant positive (r= 0.117; p=0.200) correlation was seen between reactive oxygen species (and Log (ROS+1)) and the normal sperm morphology, as assessed by Strict Criteria, in varicocele positive subfertile subjects.

**Table 14: Correlation of reactive oxygen species (Log (ROS+1)) with semen characteristics in varicocele positive subfertile subjects**

| Semen Characteristics | r value | p value |
|---|---|---|
| **Sperm Concentration (million/mL)** | 0.023 | 0.805 |
| **Sperm Motility (%)** | 0.000*** | 0.995 |
| **Sperm WHO Morphology (%)** | 0.122 | 0.183 |
| **Sperm Strict Morphology (%)** | 0.117 | 0.200 |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

Table 15, figure 10, explains the correlation of total antioxidant capacity (TAC) with semen characteristics in healthy fertile controls

A non-significant negative (r= -0.029; p=0.759) correlation was seen between total antioxidant capacity (TAC) and the sperm concentrations in healthy fertile controls. The percentage sperm motility also showed a significant (r= -0.233; p=0.012) negative correlation between total anti oxidant capacity (TAC) in healthy fertile controls. The healthy fertile controls showed a non-significant (r= 0.131; p=0.162) positive correlation between normal sperm morphology, as assessed by WHO method, and total anti oxidant capacity (TAC). A non-significant positive (r= 0.159; p=0.089) correlation was seen between total anti oxidant capacity (TAC) and the normal sperm morphology, as assessed by Strict Criteria, in healthy fertile controls.

**Table 15: Correlation of total antioxidant capacity (TAC) with semen characteristics in healthy fertile controls**

| Semen Characteristics | r value | p value |
|---|---|---|
| **Sperm Concentration (million/mL)** | -0.029 | 0.759 |
| **Sperm Motility (%)** | -0.233 | 0.012** |
| **Sperm WHO Morphology (%)** | 0.131 | 0.162 |
| **Sperm Strict Morphology (%)** | 0.159 | 0.089 |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

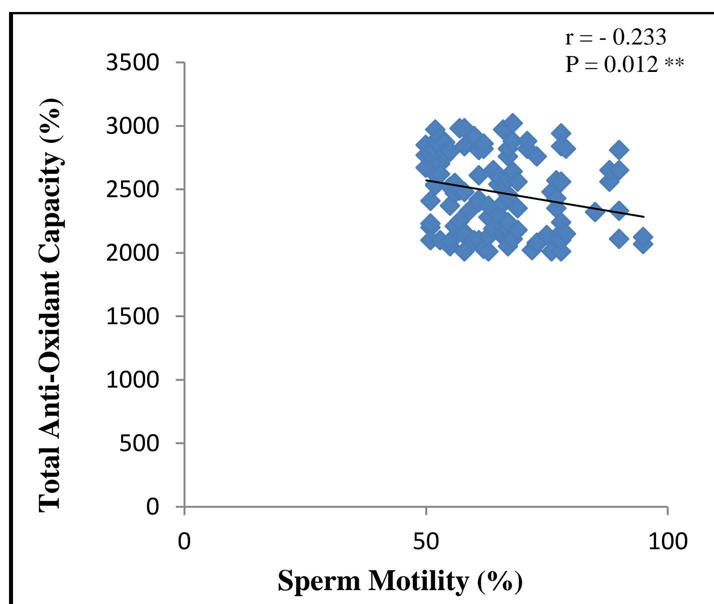



**Figure 10: Correlation of total antioxidant capacity (TAC) with percentage sperm motility in healthy fertile controls**
All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.
p= 0.05*, p=0.01**, p=0.001***

Table 16 shows correlation of TAC with semen characteristics in varicocele negative subfertile subjects.

A non-significant negative (r= -0.041; p=0.745) correlation between total antioxidant capacity (TAC) and the sperm concentrations was seen in varicocele negative subfertile subjects. Varicocele negative subfertile subjects showed a non-significant (r= -0.071; p=0.570) negative correlation between total anti-oxidant capacity (TAC) and the percentage sperm motility. The varicocele negative subfertile subjects showed a non-significant (r= -0.192; p=0.122) negative correlation between normal sperm morphology, as assessed by WHO method, and total anti oxidant capacity (TAC). A non-significant negative (r= -0.113; p=0.367) correlation was seen between total antioxidant capacity (TAC) and the normal sperm morphology, as assessed by Strict Criteria, in varicocele negative subfertile subjects.

**Table 16: Correlation of total antioxidant capacity (TAC) with semen characteristics in varicocele negative subfertile subjects**

| Semen Characteristics | r value | p value |
|---|---|---|
| **Sperm Concentration (million/mL)** | -0.041 | 0.745 |
| **Sperm Motility (%)** | -0.071 | 0.570 |
| **Sperm WHO Morphology (%)** | -0.192 | 0.122 |
| **Sperm Strict Morphology (%)** | -0.113 | 0.367 |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

Table 17, figure 11, explains the correlation of total antioxidant capacity (TAC) with semen characteristics in varicocele positive subjects.

A non-significant negative (r= -0.117; p=0.199) correlation was seen between total anti-oxidant capacity (TAC) and the sperm concentrations in varicocele positive subfertile subjects. Varicocele positive subfertile subjects showed a non-significant (r= 0.056; p=0.542) positive correlation between the percentage sperm motility and between total anti-oxidant capacity (TAC). The varicocele positive subfertile subjects showed a non-significant (r= 0.109; p=0.233) positive correlation when between normal sperm morphology, as assessed by WHO method, and total antioxidant capacity (TAC). A significant positive (r= 0.192; p=0.035) correlation between total antioxidant capacity (TAC) and the normal sperm morphology, as assessed by Strict Criteria, in varicocele positive subfertile subjects.

**Table 17: Correlation of total antioxidant capacity (TAC) with semen characteristics in varicocele positive subjects**

| Semen Characteristics | r value | p value |
|---|---|---|
| Sperm Concentration (million/mL) | -0.117 | 0.199 |
| Sperm Motility (%) | 0.056 | 0.542 |
| Sperm WHO Morphology (%) | 0.109 | 0.233 |
| Sperm Strict Morphology (%) | 0.192 | 0.035* |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

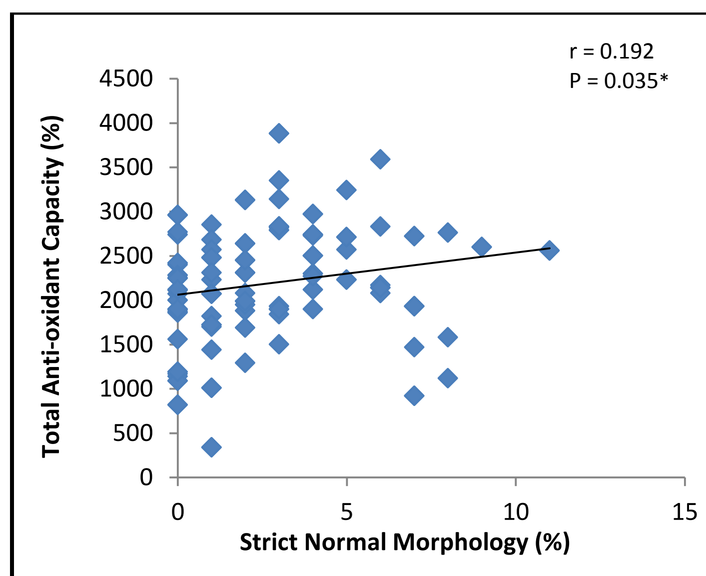



**Figure 11: Correlation of total antioxidant capacity (TAC) with Strict's normal sperm morphology in varicocele positive subjects**

All values are expressed as mean ± SEM; P1 = P between controls and VAR – group; P2 = P between VAR- and VAR + group; P3 = P between controls and VAR + group. VAR - denotes varicocele negative; VAR + denotes varicocele positive.
p= 0.05*, p=0.01**, p=0.001***

Correlations between sperm DNA fragmentation with semen characteristics in healthy fertile controls is shown in Table 18.

A non-significant positive (r= 0.020; p=0.830) correlation was seen sperm DNA fragmentation and the sperm concentrations in healthy fertile controls. Healthy fertile controls showed a non-significant (r= 0.177; p=0.058) positive correlation between percentage sperm motility and sperm DNA fragmentation. The healthy fertile controls showed a non-significant (r= -0.012; p=0.898) negative correlation when between normal sperm morphology, as assessed by WHO method, and sperm DNA fragmentation. A non-significant negative (r= -0.088; p=0.351) correlation was seen between sperm DNA fragmentation and the normal sperm morphology, as assessed by Strict Criteria, in healthy fertile controls.

**Table 17: Correlation of sperm DNA fragmentation with semen characteristics in healthy fertile controls**

| Semen Characteristics | r value | p value |
|---|---|---|
| **Sperm Concentration (million/mL)** | 0.020 | 0.830 |
| **Sperm Motility (%)** | 0.177 | 0.058 |
| **Sperm WHO Morphology (%)** | -0.012 | 0.898 |
| **Sperm Strict Morphology (%)** | -0.088 | 0.351 |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

Table 19, Figure 12, 13, explains the correlation of sperm DNA fragmentation with semen characteristics in varicocele negative subfertile subjects

A non-significant positive (r= 0.162; p=0.194) correlation was seen between sperm DNA fragmentation and the sperm concentrations in varicocele negative subfertile subjects. Varicocele negative subfertile subjects showed a significant (r= -0.408; p=0.001) negative correlation between sperm DNA fragmentation and the percentage sperm motility. The varicocele negative subfertile subjects showed a non-significant (r= -0.226; p=0.068) negative correlation between normal sperm morphology as assessed by WHO method and sperm DNA fragmentation A significant negative (r= -0.264; p=0.032) correlation was seen between sperm DNA fragmentation and the normal sperm morphology as assessed by Strict Criteria in varicocele negative subfertile subjects.

**Table 19: Correlation of sperm DNA fragmentation with semen characteristics in varicocele negative subfertile subjects**

| Semen Characteristics | r value | p value |
|---|---|---|
| Sperm Concentration (million/mL) | 0.162 | 0.194 |
| Sperm Motility (%) | -0.408 | 0.001*** |
| Sperm WHO Morphology (%) | -0.226 | 0.068 |
| Sperm Strict Morphology (%) | -0.264 | 0.032* |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

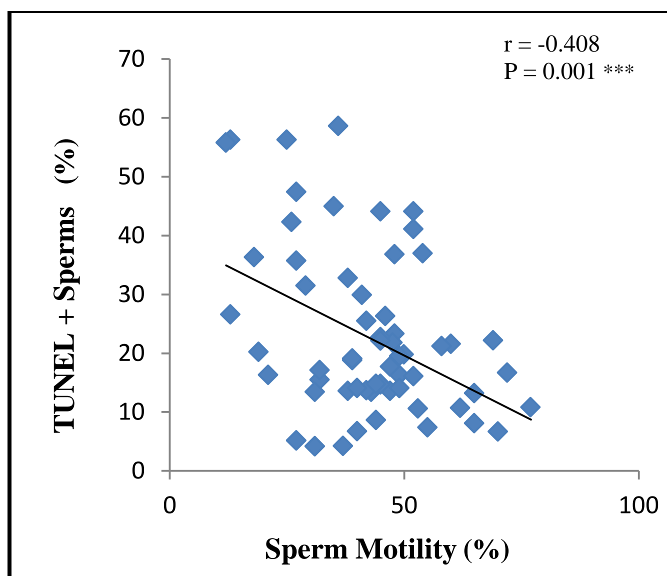



**Figure 12: Correlation of sperm DNA fragmentation with percentage sperm motility in varicocele negative subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

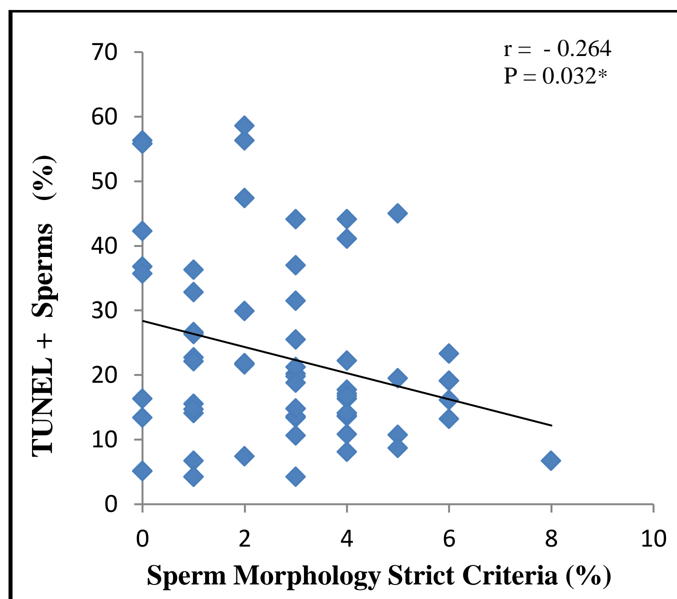



**Figure 13: Correlation of sperm DNA fragmentation with percentage sperm morphology according to Strict Criteria in varicocele negative subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

Table 20, figures 14a, b, c, d, give a detail of the correlation of sperm DNA fragmentation with semen characteristics in varicocele positive subfertile subjects

A significant negative (r= -0.310; p=0.001) correlation was seen between sperm DNA fragmentation and the sperm concentrations in varicocele positive subfertile subjects. Varicocele positive subfertile subjects also showed a significant (r= -0.328; p=0.000) negative correlation between the percentage sperm motility and sperm DNA fragmentation. The varicocele positive subfertile subjects showed a significant (r= -0.221; p=0.015) negative correlation between normal sperm morphology as assessed by WHO method and sperm DNA fragmentation. A significant negative (r= -0.180; p=0.049) correlation was seen between sperm DNA fragmentation and the normal sperm morphology as assessed by Strict Criteria in varicocele positive subfertile subjects.

**Table 20: Correlation of sperm DNA fragmentation with semen characteristics in varicocele positive subfertile subjects**

| Semen Characteristics | r value | p value |
|---|---|---|
| **Sperm Concentration (million/mL)** | -0.310 | 0.001*** |
| **Sperm Motility (%)** | -0.328 | 0.000*** |
| **Sperm WHO Morphology (%)** | -0.221 | 0.015** |
| **Sperm Strict Morphology (%)** | -0.180 | 0.049*** |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

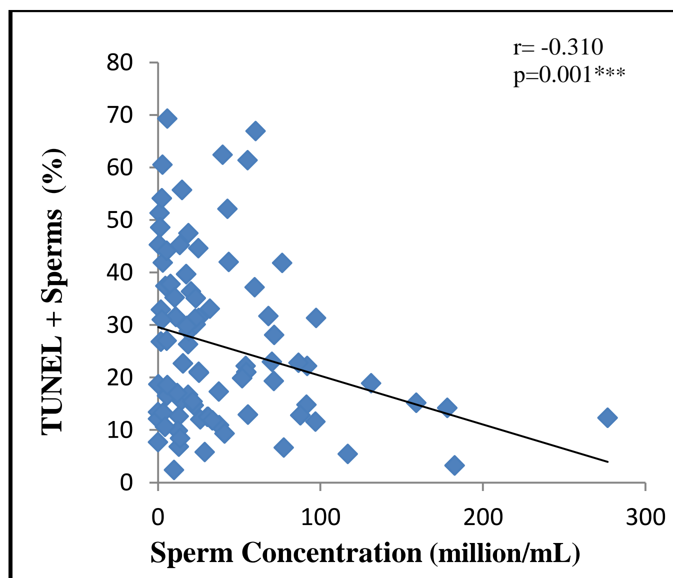



**Figure 14a: Correlation of sperm DNA fragmentation with sperm concentration (million/mL) in varicocele positive subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

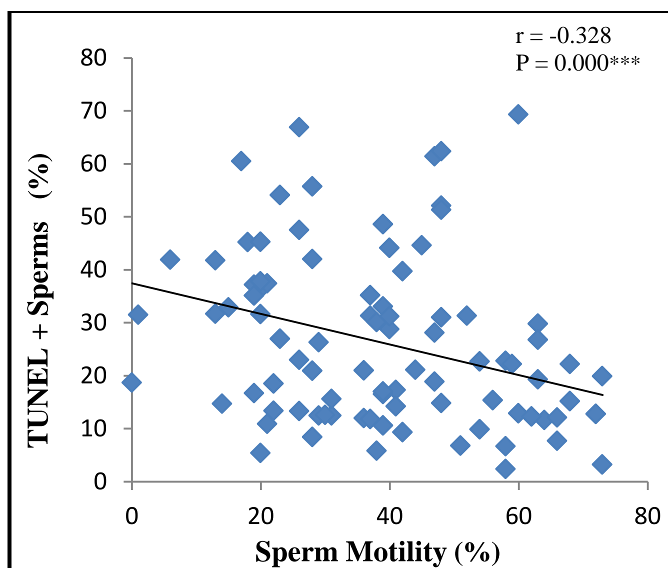



**Figure 14b: Correlation of sperm DNA fragmentation with percentage sperm motility (%) in varicocele positive subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

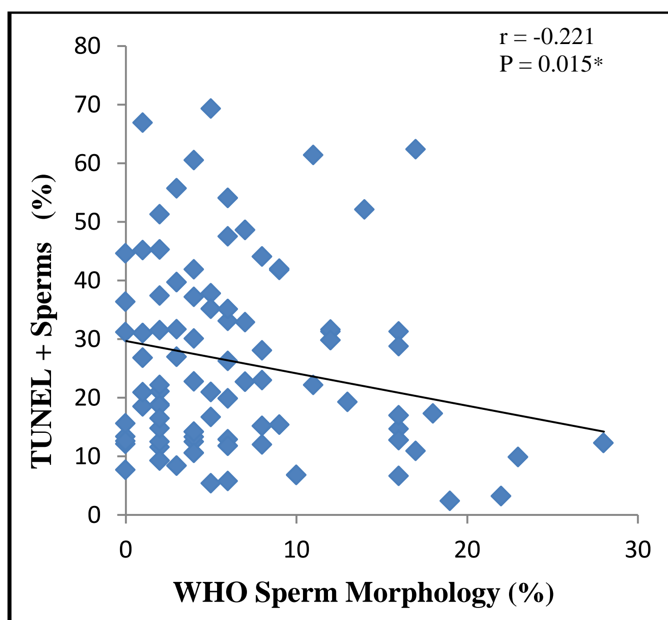



**Figure 14c: Correlation of sperm DNA fragmentation with WHO sperm morphology (%) in varicocele positive subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

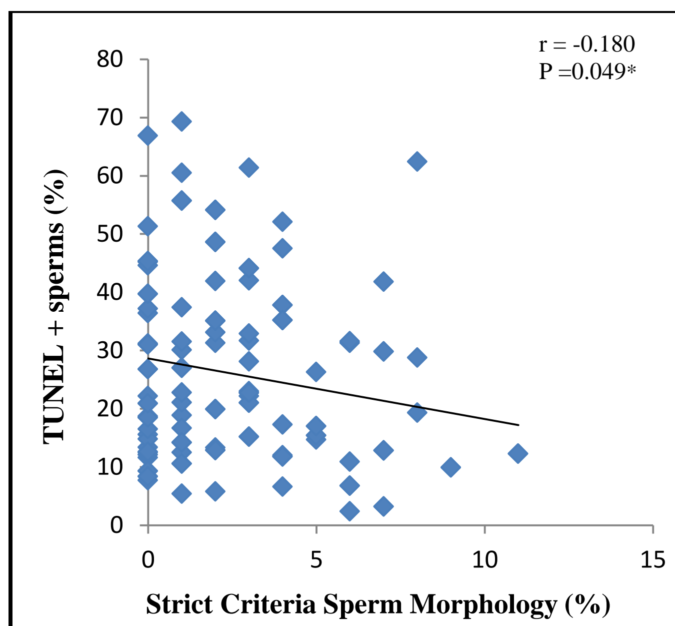



**Figure14d: Correlation of Sperm DNA fragmentation with sperm morphology (%) according to Strict Criteria in varicocele positive subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

Table 21 explains the correlation of leukocytospermia and oxidative stress markers in the subfertile group, with and without varicocele.

A non-significant positive correlation was seen between leukocytospermia and ROS (r=0.057; p=0.435) and between log transformation of ROS, Log (ROS+1) (r=0.082; p=0.262). A significant negative correlation was seen between leukocytospermia and the total anti–oxidant capacity (r= -0.151; p=0.039*) in the subfertile group, with and without varicocele. A non-significant negative correlation was seen between leukocytospermia and the sperm DNA fragmentation (r= -0.003; p=0.963) in the subfertile group, with and without varicocele.

**Table 21: Correlation of Leukocytospermia (Endtz Positive Test) with oxidative stress markers in the varicocele negative and varicocele positive subfertile subjects in the study population**

| Oxidative Stress Markers | r value | p value |
|---|---|---|
| **ROS** (million/mL/x$10^6$ sperms) | 0.057 | 0.435 |
| **Log (ROS+1)** | 0.082 | 0.262 |
| **TAC** (mM) | -0.151 | 0.039* |
| **TUNEL$^+$sperms** (%) | -0.003 | 0.963 |

Note: values represent Pearson r and the P values. Parameters having a positive Pearson r value represent a positive correlation, while parameters with a negative Pearson r value represent a negative correlation
* Correlation is significant at the 0.05 level (2 tailed)
** Correlation is significant at the 0.01 level (2 tailed)
*** Correlation is significant at the 0.001 level (2 tailed)

Table 22 gives a detail of the means of semen characteristics of subfertile subjects according to the level of varicocele grading.

Mean ± SEM of fertile and subfertile male subjects was 3.79±0.18 in healthy fertile controls, 3.63±0.21 in varicocele negative and 4.84±0.66 in varicocele positive. No significant difference (P>0.05) was seen between the three study groups.

Mean ± SEM of the semen volume (mL) was 3.21±0.199, in grade I, 3.67±0.35 in grade II and 3.58±0.26 in grade III varicocele positive group. There was no significant difference (p>0.05) seen in the mean semen volumes of grade I grade II and grade III varicocele subjects. Mean ± SEM of sperm concentrations of grade I was 45.00±7.35, grade II was 28.54±5.89 and grade III was 45.24±11.09 Different varicocele grades showed no significant difference (p>0.05) in the sperm concentrations. The sperm percentage motility had mean ± SEM 36.28±2.12 in grade I, 41.80±3.03 in grade II and 50.58±3.38 in grade III. grade I and II varicocele when compared with each other showed no significant difference (p>0.05) in the percentage sperm motility. Grade II and III varicocele, when compared with each other, also showed no significant difference (p>0.05) in the percentage sperm motility. However, a significant increase (P<0.05) was seen in sperm motility in grade III when compared with grade I varicocele group. Mean ± SEM percentage of normal sperm morphology, as assessed by WHO 1999 Criteria, in grade I was 7.43±0.82, grade II was 6.11±0.93 and grade III was 7.00±1.35 varicoceles, with no significant difference (P>0.05) between the three grades of varicocele. The sperms with tapered heads had a mean ± SEM of 7.62±1.01 in grade I, 6.32±0.65 in grade II and 9.33±1.77 in grade III varicocele, with no significant difference (P>0.05) seen between the three levels of varicocele grading. The spermatozoa in grade I varicocele had a mean ± SEM of 62.58±1.54, grade II had 69.26±1.41 and grade III had 66.42±2.13 amorphous morphology. A significant difference (P<0.05) was seen in amorphous sperm morphology between grade I and grade II varicocele group However, no significant difference (P>0.05) was seen between grade II and III and when grade I and III varicocele groups were compared. No significant difference (p>0.05) was seen in sperms with small morphology in grade I with a mean ± SEM of 4.84±0.51, grade II (3.74±0.62) and grade III (3.55±0.62) varicocele subjects. No significant difference (P>0.05) was seen in megalo sperm morphology in grade I (2.46±0.22) and grade II (1.83±0.38)

varicocele groups. However, a significant difference ($P < 0.05$) was seen between the mean ± SEM of varicocele grade II and grade III (3.89±0.58) subjects having megalo sperm morphology. Also, a significant difference ($P = 0.01$) was seen between the mean ± SEM of varicocele grade I and grade III subjects having megalo sperm morphology.

According to Strict Criteria of sperm morphology, no significant difference was seen between the mean ± SEM of normal oval heads of grade I with a mean ± SEM of (3.04±0.35), grade II (2.26±0.42) and grade III (2.16±0.52). Also the mean ± SEM of head defects according to the Strict Criteria of sperm morphology in grade I (96.96±0.35), grade II (97.74±0.42) and grade III (97.84±0.52) were also seen to be not statistically different ($P > 0.05$).

**Table 22: Mean of semen characteristics of subfertile subjects according to the level of varicocele grading**

| Semen Characteristics | Varicocele Grades | | | p1 value | p2 value | p3 value |
|---|---|---|---|---|---|---|
| | G I (67) | G II (35) | G III (19) | | | |
| **Volume(mL)** | 3.21±0.19 | 3.67±0.35 | 3.58±0.26 | 0.21 | 0.86 | 0.35 |
| **Concentration (million/mL)** | 45.00±7.35 | 28.54±5.89 | 45.24±11.09 | 0.13 | 0.14 | 0.98 |
| **Motility (%)** | 36.28±2.12 | 41.80±3.03 | 50.58±3.38 | 0.13 | 0.07 | 0.002* |
| **WHO Morphology (%)** | | | | | | |
| **Oval Heads** | 7.43±0.82 | 6.11±0.93 | 7.00±1.35 | 0.32 | 0.58 | 0.80 |
| **Tapered sperms** | 7.62±1.01 | 6.32±0.65 | 9.33±1.77 | 0.40 | 0.69 | 0.42 |
| **Amorphous sperms** | 62.58±1.54 | 69.26±1.41 | 66.42±2.13 | 0.006* | 0.25 | 0.22 |
| **Small sperms** | 4.84±0.51 | 3.74±0.62 | 3.55±0.62 | 0.19 | 0.84 | 0.23 |
| **Megalo sperms** | 2.46±0.22 | 1.83±0.38 | 3.89±0.58 | 0.17 | 0.007* | 0.01* |
| **Bicephalic sperms** | 1.70±0.21 | 1.29±0.18 | 1.00±0.00 | 0.18 | 0.45 | 0.18 |
| **Tail Defects** | 18.79±1.47 | 14.91±1.39 | 17.00±2.48 | 0.09 | 0.43 | 0.58 |
| **Strict Morphology (%)** | | | | | | |
| **Normal Heads** | 3.04±0.35 | 2.26±0.42 | 2.16±0.52 | 0.17 | 0.88 | 0.22 |
| **Abnormal Heads** | 96.96±0.35 | 97.74±0.42 | 97.84±0.52 | 0.17 | 0.88 | 0.22 |

Values in parenthesis represent number of subjects. G, I, II, III indicates the level of varicocele grading
All values are expressed as mean ± SEM;
P1 = P between GI and GII varicocele group; P2 = P between GII and GIII varicocele group; P3 = P between GI and GIII varicocele group. VAR + denotes varicocele positive.
P= 0.05*, p=0.01**, p=0.001***

Mean ± SEM ROS levels (million/mL/x$10^6$ sperms) in grades I, II and III varicocele positive subjects are shown in Table 23.

Mean ± SEM ROS levels in grade I varicocele positive subjects were 230.34±28.20, in grade II were 253.51±38.91 and grade III had 245.59±35.94.When ROS was converted to its Log transformation, the mean ± SEM Log (ROS+1) levels in grade I varicocele positive subjects were 2.15±0.05, in grade II were 2.15±0.08 and grade III had 2.25±0.09. A non-significant difference ($P>0.05$) was seen between grade I, II and III.

**Table 23: Mean ± SEM of reactive oxygen species levels in varicocele grade I, II and III subfertile subjects**

| Oxidative Stress Markers | Varicocele Grades | | | p1 value | p2 value | p3 value |
|---|---|---|---|---|---|---|
| | G I (67) | G II (35) | G III (19) | | | |
| ROS (million/mL/x10$^6$ sperms) | 230.34±28.20 | 253.51±38.91 | 245.59±35.94 | 0.63 | 0.89 | 0.78 |
| Log (ROS+1) | 2.15±0.05 | 2.15±0.08 | 2.25±0.09 | 0.94 | 0.50 | 0.39 |

Values in parenthesis represent number of subjects. G, I, II, III indicates the level of varicocele grading
All values are expressed as mean ± SEM;
P1 = P between GI and GII varicocele group; P2 = P between GII and GIII varicocele group; P3 = P between GI and GIII varicocele group. VAR + denotes varicocele positive.
P= 0.05*, p=0.01**, p=0.001***

Mean ± SEM TAC levels (mM) in grade I, II and III varicocele positive subjects are shown in Table 24.

The mean ± SEM of the TAC levels (mM) were found to be 2265.72±84.31 in grade I varicocele positive subjects, 2138.83±101.95 in grade II and 2010.00±165.66 in the grade III varicocele positive group. No significant difference ($p>0.05$) was seen in the total anti oxidant levels between the three grades of varicocele positive groups.

**Table 24: Mean ± SEM of total antioxidant capacity (TAC) levels in varicocele grade I, II and III subfertile subjects**

| Oxidative Stress Markers | Varicocele Grades | | | p1 value | p2 value | p3 value |
|---|---|---|---|---|---|---|
| | G I (67) | G II (35) | G III (19) | | | |
| TAC (mM) | 2265.72±84.31 | 2138.83±101.95 | 2010.00±165.66 | 0.36 | 0.48 | 0.16 |

Values in parenthesis represent number of subjects. G, I, II, III indicates the level of varicocele grading
All values are expressed as mean ± SEM;
P1 = P between GI and GII varicocele group; P2 = P between GII and GIII varicocele group; P3 = P between GI and GIII varicocele group. VAR + denotes varicocele positive.
P= 0.05*, p=0.01**, p=0.001***

Table 25 explains the mean ± SEM sperm DNA fragmentation by the TUNEL Assay in varicocele positive grade I, II and III subjects

The mean ± SEM of the $TUNEL^{+ve}$ sperm cells levels (%) were 24.66±1.67in grade I varicocele positive subjects, 28.32±2.73 in grade II and 25.44±4.77 in the grade III varicocele positive group. A non- significant difference ($p>0.05$) was seen in the sperm DNA damage between the three grades of varicocele positive subjects.

**Table 25: Mean ± SEM of TUNEL+ sperms in varicocele grade I, II and III subfertile varicocele subjects**

| Oxidative Stress Markers | Varicocele Grades | | | p1 value | p2 value | p3 value |
|---|---|---|---|---|---|---|
| | G I (67) | G II (35) | G III (19) | | | |
| TUNEL$^{+}$sperms (%) | 24.66±1.67 | 28.32±2.73 | 25.44±4.77 | 0.23 | 0.57 | 0.84 |

Values in parenthesis represent number of subjects. G, I, II, III indicates the level of varicocele grading
All values are expressed as mean± SEM;
P1 = P between GI and GII varicocele group; P2 = P between GII and GIII varicocele group; P3 = P between GI and GIII varicocele group. VAR + denotes varicocele positive.
P= 0.05*, p=0.01**, p=0.001***

Table 26, figures 15a, b, c explains correlations between the reactive oxygen species levels (Log (ROS+1) and total anti-oxidant (TAC) levels in grade I, II and III varicocele positive subfertile subjects. A non-significant correlation (r= -0.208; p=0.091) correlation (figure 10a) was seen between the reactive oxygen species levels (Log (ROS+1)) and total anti-oxidant (TAC) levels in grade I varicocele positive subfertile subjects. . Figure 10b shows a non-significant correlation (r= -0.122; p=0.486) correlation was seen between the reactive oxygen species levels (Log (ROS+1) and total anti-oxidant (TAC) levels in grade II varicocele positive subfertile subjects. A non-significant correlation (r= -0.410; p=0.081) correlation (figure 10c) was seen between the reactive oxygen species levels (Log (ROS+1)) and total anti-oxidant (TAC) levels in grade III varicocele positive subfertile subjects.

**Table 26: Correlation of reactive oxygen species (Log (ROS+1)) and total anti oxidant levels (TAC) in varicocele positive grade I, II and III subfertile subjects**

| LOG (ROS+1) | Total Anti-oxidant Capacity (TAC) (micromoles) | |
|---|---|---|
| | r value | p value |
| **Grade I** | -0.208 | 0.091 |
| **Grade II** | -0.122 | 0.486 |
| **Grade III** | -0.410 | 0.081 |

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

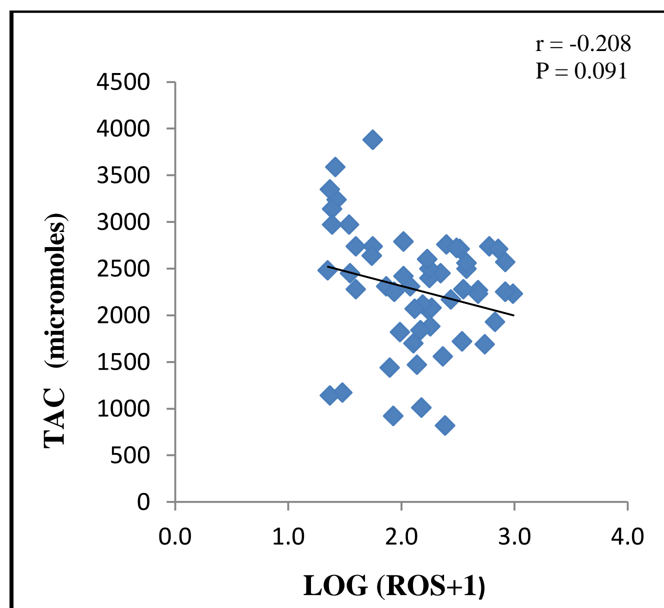



**Figure 15a: Correlation of reactive oxygen species (Log (ROS+1)) and total anti oxidant capacity (TAC) in varicocele positive grade I subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

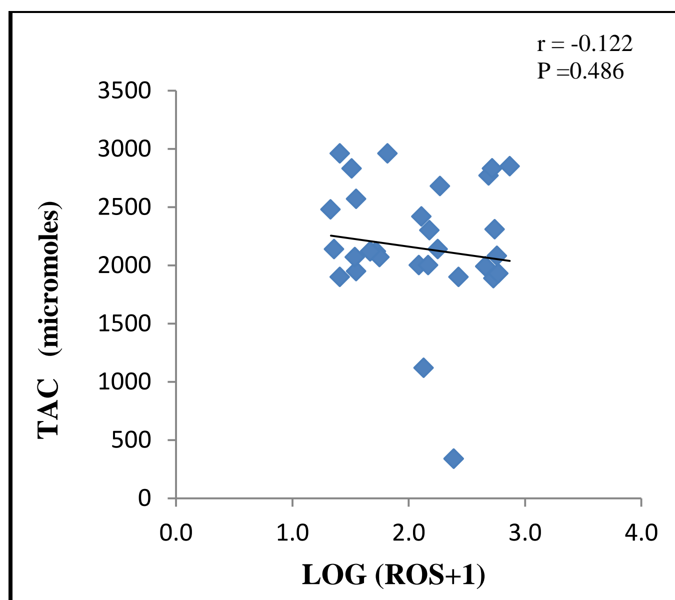



**Figure 15b: Correlation of reactive oxygen species (Log (ROS+1)) and total anti oxidant capacity (TAC) in varicocele positive grade II subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

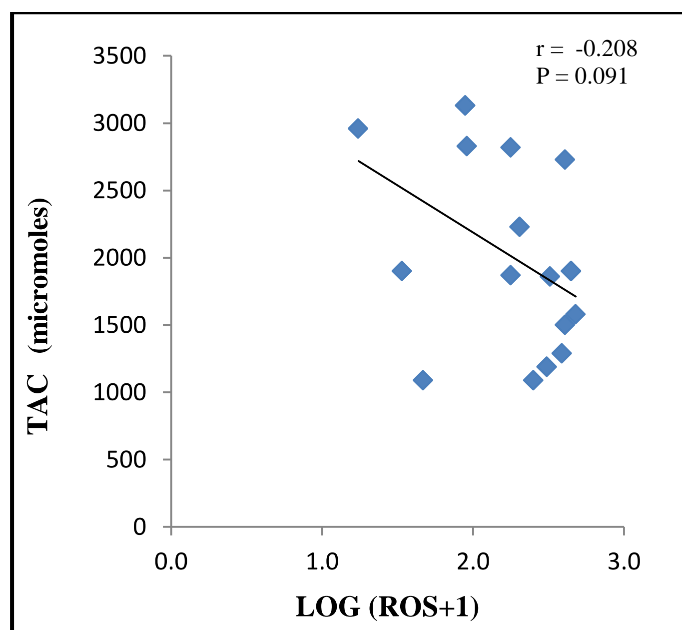



**Figure 15c: Correlation of reactive oxygen species (Log (ROS+1)) and total anti oxidant levels (TAC) in varicocele grade positive III subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed

Table 27, figures 16 a, b, c shows a non-significant positive (r= 0.077; p=0.534) correlation was seen between the reactive oxygen species levels (Log (ROS+1) and sperm DNA fragmentation (TUNEL$^{+}$ sperm cells) in grade I varicocele positive subfertile subjects. A non –significant positive (r= 0.193; p=0.267) correlation was seen between the reactive oxygen species levels (Log (ROS+1) and sperm DNA fragmentation (TUNEL$^{+}$ sperm cells) in grade II varicocele positive subfertile subjects as explained in figure 11b. A non –significant positive (r= 0.423; p=0.071) correlation was seen between the reactive oxygen species levels (Log (ROS+1) and sperm DNA fragmentation (TUNEL$^{+}$ sperm cells) in grade III varicocele positive subfertile subjects (figure 11c).

**Table 27: Correlation of reactive oxygen species Log (ROS +1) and TUNEL$^{+ve}$ sperm cells in varicocele positive grade I, II and III subfertile subjects**

| LOG (ROS+1) | TUNEL $^{+}$ Sperm Cells (%) | |
|---|---|---|
| | r value | p value |
| **Grade I** | 0.077 | 0.534 |
| **Grade II** | 0.193 | 0.267 |
| **Grade III** | 0.423 | 0.071 |

$^{***}$ Correlation is significant at the 0.001 level (2-tailed)
$^{**}$ Correlation is significant at the 0.01 level (2-tailed)
$^{*}$ Correlation is significant at the 0.05 level (2-tailed)

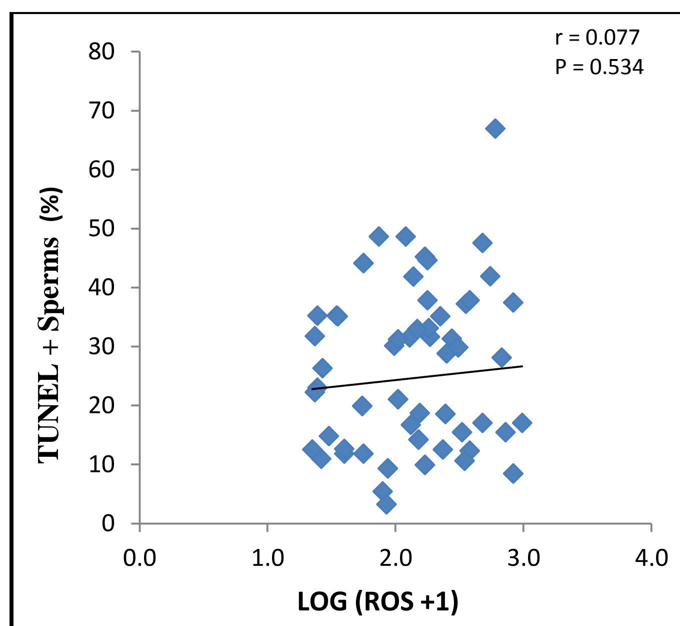



**Figure 16a: Correlation of reactive oxygen species (Log (ROS +1)) and TUNEL$^{+ve}$ sperm cells in varicocele positive grade I subfertile subjects**

$^{***}$ Correlation is significant at the 0.001 level (2-tailed)
$^{**}$ Correlation is significant at the 0.01 level (2-tailed)
$^{*}$ Correlation is significant at the 0.05 level (2-tailed)

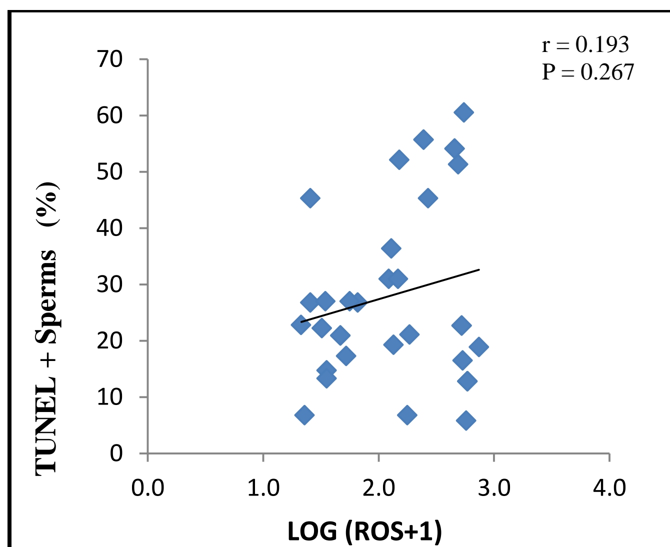



**Figure 16b: Correlation of reactive oxygen species (Log (ROS +1)) and TUNEL$^{+ve}$ sperm cells in varicocele positive grade II subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

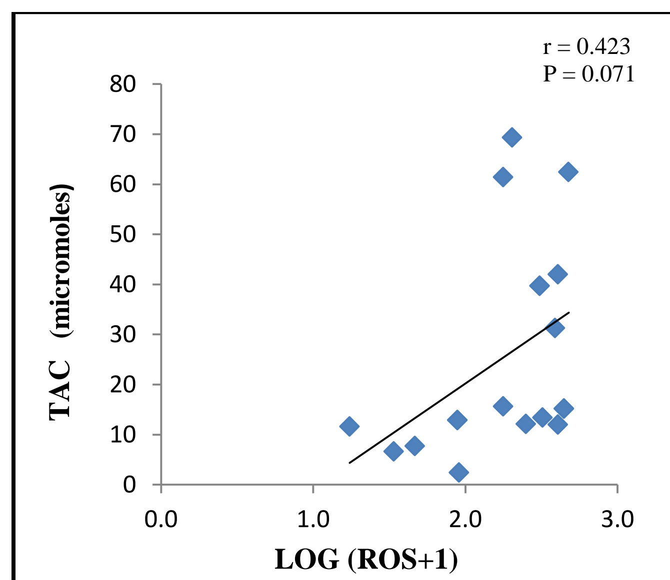



**Figure 16c: Correlation of reactive oxygen species (Log (ROS +1)) and TUNEL$^{+ve}$ sperm cells in varicocele positive grade III subfertile subjects**

*** Correlation is significant at the 0.001 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

# DISCUSSION

Standard semen analysis, under light microscopy, is widely used as the basic step in the laboratories for the initial evaluation of males coming for infertility evaluation. While semen analysis is essential for evaluation of infertility and diagnosis of disease severity, it has its limitations in diagnostic and prognostic aspects (Lewis, 2007). Diagnosis of sperm dysfunction by standard semen analysis is difficult because of spermatozoa being highly specialized cells, expressing diverse biological properties for achievement of fertilization (Carell, 2000; De Jonge, 1999; Aitken *et al*, 1982).

Varicocele is one of the most controversial subjects in male infertility (Silber, 2001) but other causes, such as anti-sperm antibodies, hormonal imbalance could also contribute to infertility (WHO, 1992).Varicocele is of particular interest from the andrological point of view not only for its prevalence (19-41%) in males but also for having its role in infertility (Manicini *et al*, 2012; Naughton *et al*, 2001, Turner and Lysiak, 2008). The link between varicocele and male factor infertility has been postulated since the time of Celsius, who observed testicular hypotrophy in varicocele patients (Baccetti *et al*, 2006).

Despite the high frequency of varicocele in subfertile men and the documented fact that varicoceles cause spermatogenic dysfunction, the exact mechanism of this negative effect has still not yet been resolved (Saleh *et al*, 2003). However, it is said that varicoceles can affect all sperm parameters including sperm count, motility and morphology (Al-Ali *et al*, 2010; Naughton *et al*, 2001). Various studies have shown that there is a significant overlap between fertile and infertile men with regard to sperm concentration, motility and morphology showing that semen analysis does not always correlate with fecundity (Guzick *et al*, 2001). In contrast there are studies in which the standard semen analysis was unable to detect some functional deficiency necessary for fertilization, in men presenting for subfertility (Van der Steeg *et al*, 2011).

The present study has enlightened the important relationship between the semen quality, as assessed by the conventional semen parameters in the three different groups of fertile and subfertile male subjects.

The healthy fertile male subjects in our study, had normal semen parameters (semen volume, sperm count, percentage motility and percentage sperm morphology, as were already been reported by Ombelet *et al*, (1997); Aziz *et al*, (2006); Ahmad *et al*, (2007); Menkveld *et al*, (2001;) and Guzick *et al*, (2001).The subfertile subjects having varicocele and without varicocele were sub categorized based on semen parameters and oxidative stress parameters, as already studied by Agarwal *et al*, (2006); Sharma *et al*, (1999); Cocuzza *et al*, (2008); Allamaneni *et al*, 2004; Weese *et al*, (1999) and Pasqualotto *et al*, (2000, 2001, 2008).

In the present study, no significant difference (P >0.05) was seen in the semen volumes between the healthy fertile controls and the subfertile group, which was similar to the findings reported by Hendin *et al*, (1999). Our findings are in contrast with the study conducted by Blumer *et al*, (2008) where an increase was seen in ejaculate volumes of varicocele subfertile subjects.

Sperms concentration (million/mL) in all the three study groups was within the normal range, with a comparably significant (P<0.001) decrease seen in the varicocele positive and negative sub-groups, as compared to healthy fertile controls. A large scale study by WHO also showed a significantly decreased sperm concentration in infertile men with varicocele, compared to men with idiopathic infertility (WHO, 1992).

Sperm motility is a critical indicator of semen quality and the fertility potential (Nallella *et al*, 2005; Ahmad *et al*, 2007). Spermatozoa produce energy in the form of ATP by oxidative phosphorylation which is necessary for motility of the sperm (Blummer *et al*, 2008; Amann, 1989; O'Connell *et al*, 2002). Depletion of enzymes in the spermatozoal tail that are involved in anaerobic glycolysis result in decreased generation of energy production hence causing a decrease in sperm motility (Victorino *et al*, 2006). Decreased sperm motility may be a result of decreased axonemal phosphorylation of proteins, inhibiting glucose-6-phosphate dehydrogenase enzymatic activity causing sperm immobilization (Maneesh and Jayalekshmi, 2006). A significant decrease (P <0.001) was seen in the percentage sperm motility in the varicocele positive and negative subfertile subjects as compared to healthy fertile control subjects. Our results coincide with the

findings of Vivas-Acevedo *et al*, (2010), who also found diminished sperm motility in subfertile males with varicocele. However our results are in contrast to the findings of Saleh *et al*, (2002) who found no significant difference in the percentage sperm motility between a sub-set of infertile men and healthy fertile controls.

Detailed assessment of sperm abnormalities is a useful biomarker of the different external factors qualitatively affecting human spermatogenesis (Auger *et al*, 2001).Morphology of the spermatozoa is the end result of highly complex process of cellular modifications occurring during the process of spermiogenesis (Rowe *et al*, 1993; WHO, 1999). Sperm morphology is considered as the best predictor of fertility by some clinicians (Menkveld *et al*, 2001; Nallella *et al*, 2006). Human spermatozoa, even in healthy fertile men, display a variety of sperm abnormalities because of marked homogeneity (Chia *et al*, 1998).It has always been an important part of semen analysis to evaluate the percentage of morphologically normal sperm as well as the determination of various morphological defects (Rowe *et al*, 1993; WHO, 1999). Holstein and Roosen-Runge (1981), demonstrated that varicocele affects sperm morphology by impairing differentiation of spermatozoa in the early spermatid stage. In our study, comparison of sperm normal and abnormal forms was assessed, both according to the WHO, (1999), method and Strict Criteria, in the three study groups. We found a significant ($P<0.001$) decrease in the normal sperm forms in varicocele negative and varicocele positive subfertile subjects as compared to healthy fertile control subjects. Our findings are in accordance with Vazquez-Levin *et al*, (1997) and Schatte *et al,* (1998), who found a reduced number of morphologically normal forms, assessed by Strict Criteria, in patients with varicocele. Our findings differ from the observation of Lund and Larsen, (1998), who found no difference in the normal sperm forms and tail defects, but only in the amorphous from those males having a varicocele and from controls, while our study found significant differences between the tapered, amorphous, small, megalo and tail defects in varicocele subfertile males as compared to normal fertile controls. Our results also differ from the findings of Saleh *et al*, (2002), who also found no significant difference in percentage sperm morphology between healthy fertile controls and a group of infertile subjects.

Defective spermatogenesis, characterized by spermatozoa carrying surplus residual cytoplasm is one of the major causes of defective sperm morphology or teratozoospermia (Agarwal and Said, 2005). Comparison of the abnormal sperm categories in our study according to the WHO method, showed a significant difference (P<0.001) in the tapered, amorphous, small and tail defective morphologies in the varicocele positive group as compared to the healthy fertile group. Head defects as assessed by Strict Criteria showed a significant difference in both the varicocele negative and positive sub groups as compared to the healthy controls. These findings are in accordance with the studies conducted by MacLeod (1965), who saw an increased number of elongated tapered sperm heads and amorphous sperms. However, a study conducted by Rodrigues-Rigau *et al*, (1981), found no significant difference in any pattern of sperm morphology in men with and without varicocele.

Taking into account all sperm parameters, our study showed a significant decrease in sperm parameters, percentage motility and normal morphology in the subfertile group, with or without varicocele, compared to the healthy fertile control group. However, the sperm concentration, in our study, was within the normal range in all the three groups. A study conducted by Villanueva-Diaz *et al*, (1999) and Chehval and Purcell, (1992), also reported a significant decrease in semen parameters in infertile men with varicocele, presenting for subfertility evaluation. Our study is in contrast to the studies conducted by Redmon *et al*, (2002) and Lund and Larsen, (1998), who observed that semen quality and sperm characteristics were not affected in infertile men, with and without varicocele.

Despite the high frequency of varicocele in the infertile population, the association between varicocele and impaired semen quality still remains controversial (Blumer *et al*, 2012; Hauser *et al*, 2001). Pasqualotto *et al*, (2005), observed decreased sperm concentration and progressive motility in varicocele infertile men compared with fertile men with and without varicocele. In contrast, Naftulin *et al*, (1991), observed no difference between the semen parameters in infertile men with or without varicocele. Another study conducted by Lund and Larsen, (1998), on seventy seven men during a period of 8 years, also found no significant deterioration in the semen parameters of men with varicocele.

Thus it maybe speculated that varicocele sperm quality, causes a negative effect on fertility in some but not in all men. On the other hand, it may also be speculated that varicocele does not affect fertility, but may simply co-exist in some men with idiopathic infertility having abnormal semen parameters (Kantartzi *et al*, 2007).

The epididymis, prostate, seminal vesicles and bulbourethral glands, together, contribute to the formation of the seminal fluid (Vivas-Acevedo *et al*, 2010). Prostate and epididymis are considered as the major sources of seminal leukocytes (Simbini *et al*, 1998). Hughes *et al*, (1981), identified three different types of leukocytes, capable of phagocytizing spermatozoa: (i) polymorphonuclear cells about 10-12 um in diameter, (ii) large macrophages, about 30um which are capable of engulfing numerous spermatozoa and (iii) smaller macrophages/monocytes having a 10-12um diameter (Hughes *et al*, 1981). Leukocytes present generally in most ejaculates, play an important role in phagocytic clearance and immunosurveillance of abnormal spermatozoa (Tomlinson *et al*, 1992). Genital tract infection is confirmed by the presence of an increased concentration of leukocytes in the semen and has an association with an increased immature germ cell concentration (Sigman and Lopes, 1993). With increasing prevalence of leukocytopsermia amongst infertile males, it can be questioned as to whether is there a correlation exists between seminal leukocytes and semen quality (Lackner *et al*, 2010). Clinical and experimental data indicate that presence of WBCs may significantly affect sperm function (Wolff *et al*, 1990; Berger *et al*, 1982; Kovalski, 1992). We found no significant difference ($P>0.05$) between leukocytospermia in our subfertile sub group. In our present study, a negative correlation was seen between leukocytospermia and semen volume. However, the sperm count, motility and normal sperm morphology, also showed non-significant positive correlations with leukocytospermia in the sub fertile, varicocele negative and varicocele positive sub-groups. Non-significant negative correlations were seen between the tapered ($P>0.05$), amorphous, small and megalo sperm morphologies whereas a non-significant positive correlation ($P<0.05$), was seen in the sperm head defects and sperm tail defects. Aziz *et al*, (2004), also reported a positive correlation between leukocytospermia and sperm tail defects, an observation similar to our findings. Our results are also in accordance with the study conducted by Ziyyat *et al*, (2008,) who observed a decrease in normal sperm motility and morphology in semen samples

exceeding a threshold of 1 x $10^6$ leukocytes/mL. Ford, (2004), Agarwal *et al*, (2003); Henkel *et al*, (2005) Sharma *et al*, (2001) and Aziz *et al,* (2004) also reported a negative impact of leukocytes on semen quality. However, the observations of Aziz *et al*, (2004), Tomlinson *et al*, (1992) and (Kiessling *et al*, 1995, reported a positive correlation between increasing leukocyte counts in the semen and an increase in all types of sperm deformities, especially sperm tail defects. Hence, large numbers of contaminating leukocytes are indicative of poor semen quality and have been implicated as a possible cause of male infertility (Van der Ven *et al*, 1987; Wolff *et al*, 1990). Thus, these results imply that leukocytes might have both negative as well as positive effects on specific semen parameters (Lackner *et al*, 2010).

In the highly complex relationship between leukocytes and semen parameters, a number of factors, such as proinflammatory cytokines and/or ROS are involved (Fraczek and Kurpisz, 2007). The negative impact of leukocytospermia on semen quality has been attributed to the presence of harmful ROS (Ford, 2004; Agarwal *et al*, 2003; Henkel *et al*, 2005). Our subfertile group showed a non-significant ($P>0.05$) positive correlation between ROS and leukocyte count above one million/mL which co-ordinates with the findings of Aitken *et al*, (1995a, b) who also found a highly significant correlation of ROS generation with seminal leukocyte concentrations. Shekkariz *et al*, (1995) also found a significant correlation between a positive Endtz Test and a positive chemiluminescence response for ROS in whole semen samples.

A large number of antioxidant defense mechanisms in the seminal plasma can readily reduce any negative impact of ROS by scavenger mechanisms (Saleh *et al*, 2002). Our study found a significant ($P<0.05$) negative relationship of TAC with leukocytospermia which co-ordinates with the finding of Aitken and Baker, (1995) and Sharma *et al*, (2001) who concluded that the major culprits for decreased antioxidant capacity of human semen are not the spermatozoa but the infiltrating leukocytes. However, although the seminal plasma is richly endowed with antioxidants that protect the spermatozoa from oxidative stress and DNA damage, their major role in vivo is still debatable (Twigg *et al*, 1998; Potts *et al*, 2000).

Leukocytospermia may cause sperm DNA fragmentation in a cascade-like manner, whose mechanism of action might be occurring through oxidative stress (Erenpreiss *et al*, 2002). Infertile men with leukocytospermia and high levels of ROS have a high incidence of sperm DNA fragmentation. A 25% increase in seminal ROS may be associated with a 10% increase in sperm DNA fragmentation (Mahfouz *et al*, 2009). Our study showed a non–significant (P>0.05) negative correlation of leukocytospermia with sperm DNA fragmentation in contrast to the study of Mahfouz *et al*, (2009), who observed a positive correlation between seminal ROS, leukocytospermia and sperm DNA fragmentation.

Recently, the excessive production of ROS in the male reproductive tract has become a real concern (Moein *et al*, 2007). Oxidative stress is now considered a key factor in the etiology of male infertility, regardless of its origin, seminal or testicular, with a well-documented negative impact on fertility (Smith *et al*, 2006). Increased oxidative stress has been implicated to be one of the major causes of male infertility. (Sharma *et al*, 1999; Pasqualotto *et al*, 2000). ROS is involved physiologically in regulation of sperm function, but has toxic effects on sperm function and fertility (Stephen and Chandra, 1998).

ROS can have detrimental effects on the sperm function though peroxidation of unsaturated fatty acids within the sperm plasma membrane (de Lamirande and Gagnon, 1995; Aitken *et al*, 1993). Existence of a correlation between various sperm parameters and oxidative stress is shown by an association of higher ROS levels leading to poor sperm count, morphology and motility (Agarwal *et al*, 1994). In the recent years, oxidative stress (OS) has been studied extensively due to its effect on the fertility status. Evidence now suggests that in 30-40% of cases, ROS-mediated damage to sperm is a significant contributing pathology (Iwasaki and Gagnon, 1992; Zini *et al*, 1993; Ochsendorf, 1999; Shekarriz *et al*, 1995; Agarwal *et al*, 2006). Both leukocytes and spermatozoa are a source of ROS in the seminal plasma, under normal physiological conditions. ROS production is also highest in immature spermatozoa from males with abnormal semen parameters, especially abnormal sperm morphology, mid-piece and tail defects (Aziz *et al*, 2004).

The decreased sperm concentration is attributed, by some researchers to the high germ cell apoptosis in males with varicocele (Marmar, 2001). We did not observe a decrease in sperm concentration with increasing levels of ROS. Our results are in contrast to the study conducted by Shekarriz *et al*, (1995) who observed a significant decrease in ROS levels at lower sperm concentrations.

Free radicals have already been proved to play a causative role in sperm dysfunction through lipid peroxidation within the sperm plasma membrane (Aitken *et al*, 1993). In the presence of elevated ROS levels, the spermatozoa are susceptible to peroxidation due to a high content of polyunsaturated fatty acids within the plasma membrane (Aitken *et al*, 1989). This lipid peroxidation causes damage to the spermatozoal plasma membrane affecting its fluidity and motility (Kasperczyk *et al*, 2008; Venkatesh *et al*, 2009). Our subfertile varicocele group had decreased sperm motility with elevated ROS levels. This is in concordance with the studies conducted by de Lamirande and Gagnon (1992) and Aitken, (1989), who also found loss of sperm motility with the production of ROS. Decreased motility is attributed either to the presence of antisperm antibodies or elevated levels of reactive oxygen species (Marmar, 2001).

While a decrease in the sperm parameters - count, motility and normal sperm morphology is seen with increased oxidative stress, decreased sperm motility is the best marker for oxidative stress in routine semen analysis, Aitken and Baker, (1995); Aitken *et al*, (1995 b,c); Whittington *et al*, (1999); Keskes-Ammar *et al*, (2003); Kao *et al*, (2007). de Lamirande and Gagnon, (1992, I, II), have also shown previously that most sensitive indicator of oxidative stress is the sperm motility, as increased levels of ROS cause inhibition of enzymes involved in oxidative phosphorylation and glycolytic pathways, decreasing the generation of ATP. These studies clearly suggest that increased production of ROS leads to decreased sperm motility (Blumer *et al*, 2012). An inverse relationship between the percentage of motile spermatozoa and levels of ROS was also reported earlier by Iwasaki and Gagnon, (1992). These studies also indicate a direct effect of ROS on sperm axonemes, causing damage to axonemal proteins leading to inhibition of motility (de Lamirande and Gagnon 1992). Therefore defective sperm function maybe a consequence of elevated reactive oxygen species levels (Hendin *et al*, 1999).

Regarding sperm morphology in subfertile varicocele subjects, our results are in accordance with the findings of MacLeod, (1965), who found the "stress pattern" as the classic morphologic finding which included increased numbers of elongated tapered sperm heads and amorphous spermatozoa associated with varicocele.

In recent years, sperm morphology has also been evaluated by "Strict Criteria" which identifies normal forms of sperms (Kruger *et al*, 1986). Our results are in accordance with previous studies conducted by Vasquea-Levin *et al*, (1997), and Schatte *et al*, (1998), who also indicated reduced numbers of morphologically normal forms, as assessed by "Strict Criteria". Our finding of increasing defective sperm morphology and elevated ROS levels are in accordance with previous studies by Gomez *et al*, (1996); Fischer and Aitken, (1997) and Said *et al*, (2005) who also reported that teratozoospermic sperms produce increased amounts of ROS as compared with normal forms of sperms.

Elevated ROS levels have been implicated in reduced fertility in patients with varicocele (Smith *et al*, 2006). In our study a significant increase ($P<0.001$) was seen in the ROS values in the subfertile subjects, with and without varicocele, as compared to healthy fertile controls. Similar findings were seen in previous studies by Smith *et al*, (2006), Barbieri *et al*, (1999), Hendin *et al*, (1999), Pasqualotto *et al*, (2000) and Agarwal *et al*, (2003), who have also demonstrated higher ROS concentrations in semen samples from men with varicocele, suggesting a link between oxidative stress and sperm dysfunction.

Several earlier studies conducted by Hendin *et al*, (1999); Pasqualotto *et al*, (2008); Mostafa *et al*, (2009) and Shiraishi *et al*, (2010) have shown increased oxidative stress markers in fertile men with varicocele compared with fertile men without varicocele. However, Cocuzza *et al*, (2008), found comparable results in both 33 fertile men with and without varicocele compared with 81 healthy fertile controls. It still remains unclear how these fertile men with increased oxidative stress markers retain their fertility. The efficient equilibrium between oxidants and antioxidants of these fertile men maybe the underlying mechanism for neutralization of the elevated ROS production in the presence of a varicocele. However, the mechanism of this preservation of fertility in fertile men with varicocele, having raised oxidative stress markers is still unknown. A number of

other studies were also conducted to compare the oxidative stress markers-total ROS in the semen in fertile and infertile men with varicocele (Hamada *et al*, 2013). Mostafa *et al*, (1999), observed increased levels of antioxidants- catalase, superoxide dismutase, glutathione peroxidase, Vitamin C and E, with decreased levels of malondialdehyde and hydrogen peroxide in fertile men with varicocele than in the infertile varicocele group (Hamada *et al*, 2013). Shirashi *et al*, (2010), found increased levels of 4-hydroxynonenal-modified proteins in infertile men with varicocele compared with their fertile group. Conversely, Pasqualotto *et al*, (2008) and Hendin *et al*, (1999), using the same experimental design, concluded higher ROS and lower TAC in fertile men with varicocele with, no difference in ROS and TAC in the infertile men with varicocele. However, due to the small size of these studies and use of different oxidative stress markers, there was no significant difference (P=0.3) seen, despite the raised ROS levels in infertile men with varicocele than in fertile men with varicocele.

Controlled trials assessing ROS have shown increased levels in infertile varicocele men as compared to healthy fertile controls (Nallela, *et al* 2004; Hendin *et al*, 1999; Sharma *et al*, 1999; Pasqualotto *et al*, 2000, 2001, 2008; Allamaneni *et al*, 2004; Smith *et al*, 2006; Dada *et al*, 2010; Mostafa *et al*, 2009). High seminal levels of nitric oxide, nitric oxide synthase, hydrogen peroxide and extracellular superoxide in the semen, have also been seen in varicocele infertile men than fertile men (Wu *et al*, 2009; Sakamoto *et al*, 2008; Mehraban *et al*, 2005; Xu *et al*, 2008; Abd-Elmoaty *et al*, 2010; Mostafa *et al*, 2012; Mazzilli *et al*, 1994).

The pathophysiology of varicocele has been studied extensively to clarify the underlying mechanisms of varicocele induced male infertility. Several studies have been conducted to see the association between oxidative stress and male infertility seen in varicocele men. Using different methodologies, these studies have measured oxidative stress markers in semen of varicocele men and compared them with levels of these markers in healthy fertile men or infertile men with idiopathic infertility (Hamada *et al*, 2013).

A study conducted on the Australian population by Tunc *et al*, (2009), ROS levels were four times higher in the infertile group compared to the controls, using the nitro blue

tetrazolium assay. However, Venkatesh *et al*, (2009), found a 150 fold increase in ROS levels in the infertile men compared to healthy fertile controls. ROS levels may play an important role in the prognosis in males diagnosed with idiopathic infertility as suggested by Yumura *et al*, (2009), who followed the patients from their first visit onwards and found significantly raised levels of ROS in the non-pregnant group compared with the pregnant ones.

ROS levels may vary in different populations (Venkatesh *et al*, 2009). Many studies on the US population have already shown significantly raised levels of ROS in the infertile men compared to healthy fertile controls (Pasqualotto *et al*, 2000, 2008; Agarwal *et al*, 2006). Another Brazilian study by Cocuzza *et al*, (2008), hypothesized a positive correlation between increased ROS levels with age, suggesting decreased chance of fatherhood with advancing age. Similar results were seen in an Iranian population by Moin *et al*, (2007), suggesting increased ROS levels in the infertile group. A Canadian study observed increased ROS levels in the semen of 40% of infertile men (Iwasaki & Gagnon, 1992). However, the study done on the Indian population saw raised ROS levels in the entire study population (Venkatesh *et al*, 2009).

Of the indirect methods used for assessment of oxidative stress, the malondialdehyde (MDA) assay was the preferred method. 6 out of 8 studies demonstrated high malondialdehyde levels in infertile men with varicocele compared to healthy fertile men (Mostafa *et al*, 2009, 2012; Wu *et al*, 2009; Abd-Elmoaty *et al*, 2010; Yesilli *et al*, 2005; Hurtado de Catalfo *et al*, 2007; Blumer *et al*, 2012; Akyol *et al*, 2001). Measurement of sperm or seminal plasma MDA levels with thiobarbituric acid assay is also the most widely used test for assessment of lipid peroxidation of the sperm plasma membrane (Tremellen, 2008). Since the MDA levels are very low in the spermatozoa, it is measured with the help of high-pressure liquid chromatography (HPLC) (Shang *et al*, 2004; Li *et al*, 2004) or with the help of spectrophotometry or iron-based promoters (Aitken *et al*, 1993). Elevated levels of MDA were seen both in the sperm and the seminal plasma, with increased ROS production, in infertile men compared with normozoopsermic controls (Nakamura *et al*, 2002; Hsieh *et al*, 2006; Sanocka *et al*, 1997; Tavilani *et al*, 2005). Other direct tests for assessment of lipid peroxidation, for example, the c11-BODIPY

assay (Kao *et al*, 2007; Aitken *et al*, 2007) and 8-Iso-PGF2alpha (Khosrowbeygi and Zarghami, 2007) are not yet commonly used.

Seminal plasma and spermatozoa possess a protective system constituting chain-breaking antioxidants to blunt the effect of free radical chain reactions (Meucci *et al*, 2003). Hence it can be said that total antioxidants in the seminal plasma are the most protective defensive mechanism available to spermatozoa against ROS (Mahfouz *et al*, 2009). Characterization of oxidative stress levels by TAC depends upon the extracellular (neutrophils) or intracellular (abnormal spermatozoa) generation of ROS (Sharma *et al*, 1999). Only one- third of ROS generated by abnormal spermatozoa with retention of residual cytoplasm as a result of defective spermatogenesis is released extracellularly (Aitken and Fischer, 1994; Plante *et al*, 1994). The usual ROS scavengers, superoxide dismutase and catalase are very effective in protection against generation of ROS extracellularly, with the TAC levels reflecting the extent of oxidative stress However, TAC levels may not be able to differentiate the extent of oxidative stress where the source of cytotoxic oxygen radicals is intracellular (Sharma *et al*, 1999; Aitken *et al*, 1992; Gomez *et al*, 1996).The antioxidants may react directly with the reactive radicals causing their destruction or neutralizing them into a less active, less dangerous state (DeFeudis *et al*, 2003; Lu *et al*, 2009). Previous studies have shown that the presence of ROS in sperms of infertile subjects is associated with lower levels of chain-breaking antioxidants in the seminal plasma. These antioxidants trap ROS directly to prevent amplification and subsequent oxidative damage to the sperm, reducing the capacity to recycle antioxidants in sperm plasma membranes. Hence these sperms are more susceptible to peroxidative damage (Lewis *et al*, 1997). This low seminal antioxidant status has previously been shown to be related to male infertility (Agarwal *et al*, 2004, 2005, 2006; Said *et al*, 2003). Hence, the cellular damage because of oxidative stress is a result of overwhelming of antioxidant defense mechanisms because of excessive production of ROS (Fingerova *et al*, 2007).

The varicocele positive and negative group, in our study, had a comparable decrease ($P<0.001$) in the TAC levels as compared to healthy fertile controls. A previous study conducted by Elmoaty *et al*, (2010), found reduced antioxidant levels and raised levels of

oxidants in the semen of infertile men with varicocele. Barbieri *et al*, (1999), also found a similar systemic decrease in antioxidant defenses in subfertile men with varicocele. It has already been demonstrated that infertile males possess low levels of antioxidants, thus exhibiting a lower total antioxidant capacity (Sharma, 1999) in the seminal plasma.

Seminal fluid is a very important source of ROS scavengers - antioxidants, protecting spermatozoa from oxidative insult (Zini *et al*, 1993; Jeulin *et al*, 1989; Gagnon *et al*, 1991). However, overproduction of ROS overwhelms seminal antioxidant defenses, leading to oxidative stress (Agarwal *et al*, 2003; Sikka, 2001). Antioxidant enzymes are generally intracellular and since spermatozoa have little cytoplasmic fluid, there is virtually little antioxidant capacity because of no capacity for protein synthesis (Zini *et al*, 1993).

Our present study showed comparable seminal plasma TAC levels in the subfertile group than healthy fertile controls. We also observed a significant ($P<0.05$) negative correlation between reactive oxygen species production and total antioxidant capacity of the seminal plasma in varicocele positive subfertile subjects. However a non-significant ($P>0.05$) positive correlation was seen between reactive oxygen species production and total antioxidant capacity in healthy fertile controls and the varicocele negative subfertile group. Many previous studies have suggested an association between decreased TAC and male infertility by proposing that an impaired seminal plasma antioxidant capacity maybe a cause of the increased sensitivity to oxidative damage of spermatozoa in varicocele men (Smith *et al*, 2006; Lewis *et al*, 1995). Previous study conducted by Smith *et al*, (1996), and Barbieri *et al*, (1999), also found a significant reduction in seminal antioxidant levels in patients with varicocele. However, our observations are in contrast to the study done by Hendin *et al*, (1999), who found correlation between ROS and TAC in the seminal plasma of varicocele men.

Agarwal *et al*, (2006), conducted a meta-analysis to evaluate alterations in the levels of ROS and antioxidants in infertile men with varicocele, by combining several publications from previous literature. The aim was also to assess the role of oxidative stress in the pathophysiology of varicocele related infertility by previously conducted studies of

Sharma *et al*, (1999), Hendin *et al*, (1999), Pasqualotto *et al*, (2000) and Saleh *et al*, (2003). A total of one hundred and eighteen patients and seventy-six healthy fertile controls were included in the meta- analysis. The overall estimate showed that the infertile patients had significantly higher levels of ROS than healthy fertile controls with a mean difference of 0.73 ($P<0.0001$). This came out with a ROS concentration of $4.37x10^4$ cpm (counted photons per minute)/$20x10^6$ spermatozoa/mL on a linear scale. The TAC values in this meta-analysis showed significantly decreased levels of TAC in infertile varicocele patients as compared with controls. Overall, the whole varicocele group had 386 fewer trolox equivalents than healthy fertile controls ($P<0.00001$). The results of this meta-analysis indicated increased oxidative stress (increased ROS, $P=0.0001$) and decreased TAC concentrations ($P<0.00001$) in infertile patients with varicocele. The decreased TAC concentrations maybe a consequence of increased utilization of antioxidants to combat the increased oxidative stress (Mostafa *et al*, 2001).

For now, it seems that assessment of ROS and TAC alone is not sufficient enough to assess if varicocele is the underlying cause of testicular damage. More studies including specific markers involved in ROS-induced testicular damage, for example, 4-hydroxynonenal – modified proteins might be more conclusive. Other strategies, for example, slowing the apoptosis of the germ cells, preventing the aggregation of the oxidized proteins and decreasing the signal transduction pathway of ROS, may help terminate the pathological effects of oxidative stress (Hamada *et al*, 2013).

The developmental normality of the embryo is one of the most important prognostic factors measuring the reproductive competence of the male. Such paternal effects are mediated by epigenetic and genetic changes in the sperm DNA (Lewis, 2007). Maintainance of genetic integrity in the male germ line has a major impact on conception, progress of pregnancy and the well-being of the progeny (Aitken, 1999; Baker and Aitken, 2005). A number of studies have proposed that presence of fragmented sperm DNA maybe the result of impaired chromatin packaging, indicating an apoptotic process (Sakkas *et al*, 2002). Apoptosis, ensures the selection of sperm cells with undamaged DNA during spermatogenesis, leading to production of spermatozoa that can be supported by the Sertoli cells (Smith *et al*, 2006; Lee *et al*, 1997). Testicular

dysfunction as a result of raised intratesticular temperatures in varicocele patients maybe another reason for causing sperm DNA damage as this raised temperature directly leads to DNA fragmentation (Fujisawa *et al*, 1988; Turner, 2001).

Sperm DNA damage is now considered as a more useful diagnostic tool in the clinical assessment of semen quality (Zini *et al*, 2001; Loft *et al*, 2003; Holt, 2005). A landmark publication in the 1980s by Evenson *et al*, (1980), proposed that assessment of DNA integrity in spermatozoa, maybe a useful and potentially independent marker of the fertility status, both in humans and animals (Barratt *et al*, 2010; Evenson *et al*, 1980). Availability of new therapeutic options as a result of the increasing prevalence of male infertility makes it necessary to challenge the usefulness of tests that measure sperm DNA integrity (Sergerie *et al*, 2005). A sperm DNA integrity assay not only predicts fertilization but also helps clinicians in their treatment regimens (Perreault *et al*, 2003).
Our study showed highly significant ($P<0.001$) increased sperm DNA fragmentation in the subfertile subjects, more so in the varicocele positive sub-group. Similar findings were previously reported by Smith *et al*, (2006), who showed increased levels of sperm DNA fragmentation, using TUNEL Assay, in patients with clinical varicocele compared to normozoospermic controls. Our results are also in concordance with an earlier study conducted by Saleh *et al*, (2003), where significantly high levels of ROS and raised levels of sperm nuclear DNA damage in infertile patients with varicocele were also observed, suggesting that oxidative stress may play a very important role in causing sperm DNA fragmentation. However, our TAC levels were within the normal range in all the three groups, compared to the study conducted by Saleh et al (2003), who showed decreased TAC levels in infertile patients with varicocele.

Several factors, including heat stress (Mieusset and Bujan, 1995; Wright *et al*, 1997), exposure to toxic agents (Sinha and Swerdloff, 1995; Benoff *et al*,1997, 2004), testicular hypoxia (Hsu *et al*, *1994*; Li *et al*, 1999), androgen deprivation (WHO, 1992; Fujisawa *et al*, 1994) and increased oxidative stress (Hendin *et al*, 1999) are associated with varicocele and may induce pathways that lead to DNA damage and apoptosis (Smith *et al*, 2006).In the ejaculates of infertile men, oxidative stress has been seen to affect the genomic integrity of the sperm by causing single and double-strand DNA breaks (Fraga

*et al*, 1996; Irvine *et al*, 2000; Saleh and Agarwal, 2002; Wang *et al*,2003; Moustafa *et al*, 2004).

Our group of subfertile subjects, with and without varicocele, have demonstrated significantly high levels of oxidative stress as seen by elevated levels of ROS and increased sperm DNA fragmentation. These results are in concordance with previous studies conducted by Saleh *et al*, (2003); Wang *et al*, (2003); Hendin *et al*, (1999) suggesting a strong relationship between oxidative stress and sperm DNA damage. Sperm DNA fragmentation is a matter of debate as a prognostic factor of a male's fertility potential (Alvarez *et al*, 2003). The determination of sperm DNA integrity maybe important as an adjunct to standard semen analysis (Wu *et al*, 2009). In assisted reproductive clinics, the main objective of a good DNA integrity test is identification of the proportion of men contributing to the infertility problem of couples Sergerie *et al*, (2005); Sun *et al*, (1997), suggested sperm DNA fragmentation as an independent measure of sperm quality that might have better prognostic and diagnostic capabilities compared to standard semen. Kemal *et al*, (2000), reported that ROS significantly increases DNA damage by modification of all bases leading to production of DNA cross-links, frame-shift mutations, deletions and base-free sites. Another contributing factor could be a significant decrease of antioxidants in the seminal plasma (Barbieri *et al*, 2001; Chen *et al*, 2001).

The observations in our study were a significant increase ($P<0.001$) in the sperm DNA fragmentation in the subfertile population, both having varicocele or without varicocele, as compared to the healthy fertile control group are in concordance with previous studies conducted by Saleh *et al*, (2003), who found higher DNA fragmentation in infertile patients with varicocele than healthy fertile controls. This DNA fragmentation in varicocele patients maybe a result of increased intratesticular temperature, affecting both the testicular function and nuclear DNA (Fujisawa *et al*, 1988; Turner, 2001).In contrast, Ku *et al*, (2005), demonstrated high levels of sperm nuclear DNA fragmentation, as assessed by TUNEL Assay, but with no changes in sperm quality as assessed by classic semen analysis.

We found non-significant (P>0.05) correlation of semen characteristics with sperm DNA fragmentation in healthy fertile controls. However, a significant (P=0.001) negative correlation was observed between % sperm motility and sperm DNA fragmentation in varicocele negative subfertile group, in contrast to Morris *et al*, (2002), who observed a positive association of DNA damage with sperm motility. Also a significant (P<0.05) negative correlation was observed between normal Strict sperm morphology and sperm DNA fragmentation in varicocele negative subfertile subjects. A highly significant (P=0.001) correlation was observed between sperm concentration, percentage sperm motility and percentage sperm morphology and sperm DNA fragmentation in the varicocele positive subfertile group. Our results are in accordance with the studies conducted by evidence by Irvine *et al*, (2000) and Sun *et al*, (1997) who evidenced an important relationship between DNA fragmentation and semen quality and found a negative relationship between semen quality (sperm concentration, motility and morphology) and presence of DNA fragmentation in spermatozoa. Blumer *et al*, (2008) also reported a strong negative correlation between sperm morphology and high DNA fragmentation in the varicocele group as compared to healthy men. However, this correlation was not found by Hughes *et al*, (1996), who observed a significant difference of sperm DNA damage both in control and patient population. Kodama *et al*, (1997) also found significantly higher levels of oxidative base damage in spermatozoa of infertile patients compared with those of controls, supporting the possible significance of oxidative DNA damage in the male germ line. Twigg *et al*, (1998) also observed that spermatozoa exposed to exogenous ROS or stimulated to produce endogenous ROS also showed higher rates of DNA fragmentation as compared to untreated control samples.

The nature of the nuclear DNA fragmentation needs further investigative studies as this DNA fragmentation may be the result of prolonged exposure to factors damaging the nuclear DNA (Enciso *et al*, 2006). Enhanced oxidative stress, both as a result of increased production of ROS and decreased antioxidant capacity have been reported previously by Barbieri *et al*, (1999) and Hendin *et al*, (1999). ROS and TAC are well known factors inducing DNA fragmentation both in vivo and in vitro (Agarwal *et al*, 2003). ROS may be released by the dilated spermatic vens into the seminiferous tubules by the retained cytoplasmic droplets in immature spermatozoa (Ollero *et al*, 2001) and

seem to be frequent in semen samples from varicocele infertile men (Zini *et al*, 2000). Chronic oxidative stress, in vivo, along with proteases and cytokines, chronic infections or inflammatory conditions, may damage not only the DNA but also the nuclear proteins (Enciso *et al*, 2006). It has been reported earlier by Molina *et al*, (2001), that infertile men with varicocele have spermatozoa with less condensed chromatin, allowing ROS to have a better access to the spermatozoal nuclei, producing greater DNA damage. In the recent years, concern has increased about toxic effect of production of high levels of ROS on the sperm quality and function (Saleh and Agarwal, 2002). Presence of high levels of ROS in semen of infertile men has been previously reported between 25% to 40% by Padron *et al*, (1997). Strong evidence suggests that increased ROS causes the occurrence of single and double stranded DNA breaks, as seen in spermatozoa of infertile men (Fraga *et al*, 1996; Kodama *et al*, 1997; Sun *et al*, 1997; Aitken and Krausz, 2001). Additionally, spermatozoa of infertile males are also more susceptible to DNA damaging agents, for example, hydrogen peroxide and irradiation (McKelvey-Martin *et al*, 1997). However, antioxidants, such as, alpha-tocopherols and ascorbic acid provide significant protection against these deleterious effects (Donnelly *et al*, 1999).

Several significant correlations comparing different parameters, for example, the DNA Fragmentation Index (DFI%) using SCSA and TUNEL Assay , have already been described by Henkel *et al*, (2010), Zini *et al*, (2001), Erenpreiss *et al*, (2004) and Chohan *et al*, (2006). Correlations have also been described with Sperm Chromatin Dispersion Test (Henkel *et al*, 2010), COMET Assay (Chohan *et al*, 2006) and Toluidine Blue Image Cytometry Test (Erenpreiss *et al*, 2004). However, existence of a strong correlation between two parameters is merely an indicator that the two parameters are somehow related to each other, directly or indirectly (Henkel *et al*, 2010).

Using the COMET Assay for assessment of DNA fragmentation, Hughes *et al*, (1996) found an association between impaired semen quality and significantly increased DNA damage in a patient population compared with normozoopsermic controls. Irvine *et al*, (1999), found a negative correlation between semen quality and sperm DNA damage using COMET Assay, In situ Nick Translation on decondensed and condensed

spermatozoa. Similar negative correlation was observed by Sun *et al*, (1997), between sperm motility, morphology and concentration and DNA strand breaks in spermatozoa of two hundred and eighty-five men seeking infertility evaluation. This relationship was not found by Hughes *et al*, (1996) who observed DNA fragmentation in spermatozoa of healthy fertile men and asthenozoospermic infertile men using the modified single cell get electrophoresis assay, as a result of differences in the selection of their study groups. Irvine *et al*, (2000), has also shown the damaging effects of oxidants, for example, hydrogen peroxide on DNA integrity, using the ISNT methodology.

Existence of increased DNA fragmentation in spermatozoa of infertile men has raised the clinical importance of detecting this damage. Three different techniques-COMET Assay and ISNT, condensed and ISNT decondensed method were applied by Irvine *et al*, (2000), for detection of human spermatozoal DNA strand breaks. Using the Comet Assay, this study was able to distinguish between the central trends in DNA damage between the patients and normal fertile control population, but did not show the same levels of correlations as were observed by using the ISNT-condensed method. The high correlations between semen quality and DNA damage, seen in this study by applying the ISNT-condensed method indicates that this method measures more than just DNA damage by assessment of DNA compaction. Thus it can be said that the ISNT methodology measures condensed chromatin, reflecting the quality of the ongoing processes of sperm maturation and differentiation. However, this procedure labels only ten percent of the spermatozoa, even in the infertile group, giving a very narrow dynamic range.

Applying the SCSA methodology, a previous study conducted by Saleh *et al*, (2003), observed a significant increase in DNA fragmentation in infertile men with varicocele. Similarly, Smith *et al*, (2006), using the SCSA and TUNEL Assays, also observed increased levels of DNA fragmentation in spermatozoa of varicocele men, both with normal and abnormal semen parameters. Applying the SCSA method, Smith *et al*, (2006), also found negative correlations between DFI% and sperm concentration (r= -0.25; P<0.01), percentage sperm motility (r=- .38; P<0.01), normal sperm morphology (r= -0.23; P<0.01) and a positive correlation (r= 0.17;P<0.05) between DNA

Fragmentation Index and head defects. This finding of DNA fragmentation in varicocele men having normal seminal parameters is an indication that there is no guarantee spermatozoal genomic integrity in varicocele men with normal sperm parameters (Smith *et al*, 2006). A positive correlation seen between ROS and DFI in Smith *et al*, (2006), study indicated an association between DNA damage and oxidative stress in the semen.

A suitable DNA assessment assay not only predicts the fertilization failure but also helps clinicians in choosing the best therapeutic strategy for the patient (Perreault *et al*, 2003). Spermatozoal DNA fragmentation not only measures the quality of the sperm but also helps in diagnosis and prognosis than routine semen analysis (Sun *et al*, 1997). DNA fragmentation of spermatozoa is still a matter of debate considering it as a prognostic factor for the fertility status of a man. TUNEL Assay, not only is a measure of sperm quality but could complement tests used in assisted reproductive techniques for evaluation of sperm functions (Sergerie *et al*, 2005).

Previous studies have shown a greater percentage of DNA fragmentation in the semen samples of men with varicocele than healthy fertile men (Enciso *et al*, 2006). Saleh *et al*, (2003), reported a mean DNA fragmentation of 25% in spermatozoa of infertile men with varicocele, while Chen *et al*, (2004), 23% DNA fragmentation using the TUNEL Assay. Our results, using the TUNEL Assay, suggest mean DNA fragmentation of 25.84% in semen samples of varicocele positive men. Enciso *et al*, (2006) and Saleh *et al*, (2003), found no significant difference in the frequency of spermatozoa with DNA fragmentation between the varicocele negative and varicocele positive groups, suggesting that there is no definite range of frequency of DNA fragmentation in spermatozoa of varicocele men. Using TUNEL Assay, Sergerie *et al*, (2005), observed mean DNA fragmentation as 40.9±14.3% in the infertile group and 13.1±7.3% in fertile men. Our study showed a DNA fragmentation of 22.91±1.80% in the varicocele negative subfertile group and 14.87±0.20% in healthy fertile controls, using the TUNEL Assay.

TUNEL labeling can be detected with the help of fluorescence microscopy or flow cytometry. Studies on neat semen samples from similar group of men, for DNA fragmentation, using either of the two techniques have shown that TUNEL fluorescence

seen by microscopy is much lower than that observed by flow cytometry (Muratori *et al*, 2010). Studies conducted by Zini *et al*, (2001); Younglai *et al*, (2001); Chohan *et al*, (2006); Dominguez-Fandos *et al*, (2007); Avendan˜o *et al*, (2009a), using fluorescence microscopy for detection of sperm DNA fragmentation in the semen of sub fertile men, reported half of that compared to studies conducted by Sergerie *et al*, (2005); Sepaniak *et al*, (2006); Torregrosa *et al*, (2006); Domı´nguez-Fandos *et al*, (2007) and Varum *et al*, (2007), who used flow cytometry (16.32% versus 32.08%). A comparative study using semen samples from the same subjects, between fluorescence microscopy and flow cytometry , showed that flow cytometry yielded greater (Domı´nguez-Fandos *et al*, 2007) and lower (Cohen-Bacrie *et al*, 2009]) measures than those seen by fluorescence microscopy. This discrepancy might be because of the different threshold criteria used for positive and negative events during flow cytometric data analysis for TUNEL (Cohen-Bacrie *et al*, 2009;Domı´nguez-Fandos *et al*, 2007).

Another test for direct evaluation of oxidative damage in sperm DNA is the measurement oxidized deoxynucleoside, 8-oxo-7,8-dihydro 20 deoxyguanosine (8-OHdG) in the sperm itself or seminal plasma by HPLC (Fraga *et al*, 1991; Loft *et al*, 2003), enzyme-linked immunoabsorbent assay -ELISA (Nakamura *et al*, 2002) or using immunoflurorescence for direct measurement within sperm (Kao *et al*, 2007). Since a number of prospective studies have reported an inverse correlation between natural conception and spermatozoal 8-OHdG levels (Loft *et al*, 2003), measurement of this direct oxidative stress marker in the sperm seems to be important in the andrological evaluation.

Previous evidence suggests that DNA integrity of the spermatozoa is a better indicator of spermatogenesis and male fertility status than the traditional semen analysis (Spano *et al*, 2000; Zini *et al*, 2001). Although several earlier studies have shown defective chromatin packaging and abortive apoptosis as the primary reason of DNA damage in men with varicocele, oxidative stress seems be the major cause of varicocele induced spermatozoal sperm dysfunction (Sakkas *et al*, 2002; Fujisawa and Ishikawa, 2003).

Saleh *et al*, (2003), observed increased DNA fragmentation in sperms of infertile men with varicocele, while Bertolla *et al*, (2006), observed the same findings in an adolescent

study population. Smith *et al*, (2006), observed high levels of sperm DNA fragmentation in varicocele patients having normal semen parameters.

Using different DNA evaluation techniques, earlier studies conducted by various scientists have indicated increased DNA fragmentation in patients having abnormal semen parameters- sperm count, percentage motility and morphology. (Agarwal and Said, 2003). Saleh *et al*, (2003), using the SCSA technique, found a highly significant negative correlation between DNA fragmentation and sperm count ($r= -0.31$, $p=0.001$), % motility ($r=-0.47$, $P<0.0001$) and morphology ($r=-0.04$, $p<0.0001$). Irvine *et al*, (2000) and Chan *et al*, (2001), both observed a highly significant negative correlation between DNA fragmentation and sperm count ($r= -0.54$, $P=0.001$), % motility ($r=-0.37$, $P=0.026$) and morphology ($r=-0.37$, $P=0.026$), using the COMET Assay. Irvine *et al* (2000), in the same study, and Tomlinson *et al*, (2003) using INST Assay, also described a highly significant negative correlation between DNA fragmentation and sperm count ($r= -0.66$, $P<0.0001$), % motility ($r=-0.38$, $P=0.016$) and morphology ($r= -0.38$, $P=0.016$). Blumer *et al*, (2012), also found altered spermatogenesis with very few spermatozoa having intact DNA, using COMET Assay. This study also demonstrated a negative correlation between percentage sperm morphology and DNA fragmentation ($r= -0.450$) in the varicocele group as compared with the non-varicocele group. A negative correlation was also seen between grade A varicocele and increased sperm DNA fragmentation ($r= -0.538$) as compared to the non-varicocele men. It could be expected that semen sample of varicocele men having a high sperm count, high percentage sperm motility and lower percentage of abnormal morphology would present with decreased DNA fragmentation. This could be explained by the fact that seminal oxidative stress is presented in a greater intensity in varicocele men. Thus, while in men without a varicocele, increased ROS production would affect the spermatozoal function at different times, increased oxidative stress and decreased antioxidant capacity in men with varicocele would produce more generalized effect on spermatozoal cell membrane, affecting spermatogenesis (Hendin *et al*, 1999; Alvarez *et al*, 1987). Increased production of ROS from morphologically abnormal spermatozoa is an indication that it is this ROS and decreased antioxidant mechanisms that lead spermatozoal dysfunction (Engel *et al*, 1999).

The relationship between sperm quality and DNA fragmentation, especially in subfertile males with abnormal semen characteristics including leukocytospermia, is complex and depicts a cascade-like manner 1) an inverse relationship between DNA damage and sperm quality, especially affecting sperm concentration, 2) increased leukocytospermia affecting sperm genomic integrity, particularly in sperms having moderate to poor morphology and 3) damaged sperm DNA decreasing the number of cells in the semen thus worsening its quality (Erenpreiss *et al*, 2002). The lack of correlation, in our study, between certain sperm characteristics and severity of varicocele in subfertile patients, maybe because of the impaired fertilizing capacity of sperm due to the direct effects of elevated ROS levels, without affecting the routine seminal parameters (Agarwal *et al*, 2004).

Two possible mechanisms leading to increased DNA fragmentation in varicocele patients are apoptosis and oxidative stress Sperms undergo incomplete apoptosis during their transition from spermatocytes to spermatids. These cells continue to mature further and hence apoptotic DNA fragmentation is seen in these ejaculated sperms (Bertolla *et al*, 2006; Sakkas *et al*, 2002).

Oxidative stress is the other major culprit in the origin of sperm DNA damage (Aitken, 2006).Oxidative stress has been shown to affect sperm DNA integrity, causing high frequencies of single and double-strand breaks in sperm DNA. Exposure to spermatozoa to artificially produced ROS significantly increases DNA damage through modification of bases and by production of base-free sites, deletions, frame-shift mutations and DNA cross-linkages (Irvine *et al*, 2000).

A significant positive correlation ($P<0.05$) was observed between reactive oxygen species (Log (ROS+1)) levels and sperm DNA fragmentation in our healthy fertile group. This damage to sperm DNA in normozoospermic men may account to an inherent defect of sperm chromatin packaging as a result of nuclear remodeling during spermiogenesis (Barone *et al*, 1994). However, the exact explanation of such phenomenon warrants further investigation. Varicocele negative sub-group showed a non-significant ($p>0.05$) negative correlation ($P>0.05$) between reactive oxygen species (Log (ROS+1)) levels and sperm DNA fragmentation while the varicocele positive sub-group showed a non-

significant (p>0.05) positive correlation of ROS with sperm DNA fragmentation. Since varicocele leads to increased production of ROS, both in the testes as well as in the seminal plasma, it could be speculated that sperm DNA fragmentation seen in varicocele men is caused by oxidative stress derived from overproduction of ROS (Smith *et al*, 2006; Türkyilmaz *et al*, 2004).

One of the most controversial subjects in male infertility is varicocele (Silber, 2001). Varicocele leads to venous stasis of the testis, causing heat stress, hypoxia and accumulation of toxic metabolites. As a result of this hypoxia in the testis, there is an increased production of ROS and a decreased concentration of antioxidant enzymes without renewal of blood because of venous stasis. Oxidative stress generation as a result of this imbalance between ROS production and antioxidant protection causes lipid peroxidation which affects the sperm chromatin and induces DNA fragmentation in the sperm (Blumer *et al*, 2008; Smith *et al*, 2006; Hendin *et al*, 1999; Aitken and Krausz, 2001).

Occasionally, occurance of varicocele can be only on the right side or bilaterally in both testes, however, the right varicocele is considered rare (Wilms *et al*, 1988). Our study included 187 subfertile subjects of whom, 121 (64.7%) were diagnosed with a unilateral left varicocele. Of these, 55.3% were diagnosed with grade I, 28.9% had grade II varicocele and 15.7% had grade III varicocele. Our study coordinates with a previous study conducted by Agarwal *et al*, (2004), who also found a unilateral left varicocele in subjects undergoing infertility evaluation. However the patient number in that study was smaller as compared to our study. Various studies conducted by Al-Ali, (2010); Saleh *et al,* (2003); Cocuzza and Agarwal (2007); Vivas Acevedo *et al*, (2010) and Zini *et al* (1998), have studied the grading of varicocele and impact on semen quality and oxidative stress markers.

It has always been a controversial issue to positively correlate varicocele grade to the degree of spermatogenic dysfunction (Al-Ali *et al*, 2010). Various study groups tried to correlate different clinical parameters with the presence and severity of varicocele (Zedan *et al*, (2009); Pasqualotto *et al*, (2005); Ishikawa and Fujisawa, (2005**)**. A body of data

has indicated impairment of important sperm parameters in subfertile patients with varicocele (Agarwal *et al*, 2009; Naughton *et al*, 2001; Marmar, 2001). This deterioration of semen quality is expected in long-standing varicocele as exposure of the testis to prolonged abnormal intratesticular environment enhances abnormal pathophysiological mechanisms within the testis, leading to decreased semen quality (Al-Ali *et al*, 2010). This fact was observed by Sirashi *et al*, (2010), who found out, that patients with long-standing grade III varicocele complained of elevated scrotal temperature, hence giving a clear explanation for the reduced semen quality in grade III varicocele. In contrast some investigators reported no change in semen quality depending upon the varicocele grade (Zargoshi, 2007).

We found no significant difference ($p>0.05$) in the sperm concentrations in all the three grades of varicocele. Similar observations were seen by Vivas Acevedo *et al*, (2010) and Zargoshi, (2007), who also found no significant difference in the semen volumes and sperm concentrations between subfertile subjects with various grades of varicocele. Shiraishi *et al*, (2010), also reported no significant difference in the sperm concentration between various grades of varicocele. A significant ($P<0.01$) decrease in percentage sperm motility was observed in grade I as compared to grade III subfertile varicocele subjects. Zargoshi, (2007) also reported that higher grades of varicocele are not associated with any pronounced deleterious effects on sperm concentration and percentage sperm motility. However, Mori *et al*, (2008), found decreased sperm motility in grade III compared with grade I. Smith *et al*, (2006), observed diminished sperm motility in all grades of varicocele, possibly due to deterioration of sperm plasma membrane as a result of increased reactive oxygen species, resulting in alteration of flow of ions necessary for normal sperm motility, while Vivas-Acevedo Giovanny *et al*, (2010), reported diminished sperm motility in grade III varicocele than grade I and II group. There was a non- significant ($P>0.05$) decrease in normal sperm morphology, as assessed both by the WHO sperm morphology criteria as well as by Strict Criteria, in all, grade I, II and III varicocele subjects, Except for a significant ($P\leq0.01$) increase in megalo sperm defects seen in grade III varicocele subjects as compared with grade I and II, we observed no significant ($P>0.05$) difference in normal as well as abnormal sperm morphology as assessed both by the WHO, (1999), criteria and Strict's criteria of sperm

morphology. These findings are similar with the findings of Vivas-Acevedo Giovanny *et al*, 2010, who found defective sperm morphology in grade III varicocele than grade I and II group. Defective sperm morphology, as seen in grade III varicoceles, occurs as a result of endothelial dysfunction because of an enhanced effect of vasoconstrictor substances (Yildiz *et al*, 2003). Zargoshi *et al*, (2007) also found no association between higher grades of varicocele and more pronounced effects on sperm concentration and percentage motility. However, in our study, the percentage sperm motility was significantly decreased in grade I varicocele positive sub-group as compared to grade III. Our findings differ from the findings of Villanueva-Diaz *et al*, (1999), who found no correlation of decreased sperm parameters and varicocele grade and Diamond *et al*, (2007) also reported no correlation of semen quality with varicocele grade.

All subfertile subjects with I, II and III grades, had elevated ROS levels, with no significant difference ($P>0.05$) between the three groups. Our findings are consistent with the observations of Allamaneni *et al*, (2004), who also found raised ROS levels with increasing severity of the varicocele grade in subfertile subjects. Previous studies conducted by Abd-Elmoaty *et al*, (2010) and Koksal *et al*, (2000), all reported significantly raised levels of seminal oxidants in subfertile subjects with grade 3 varicocele as compared to those with grade 1 varicocele while our study showed increased levels of ROS in all the three grades of varicocele with no statistical significance. However, previous studies by Cocuzza *et al*, (2008), did not find any significant difference in ROS levels with varicocele grade and hence contradict our findings.

In our study, there was no significant difference ($P>0.05$) seen in the total antioxidant capacity (TAC) levels between the three grades of varicocele.Our results differ from previous studies conducted by Abd-Elmoaty *et al*, (2010) and Koksal *et al*, (2000), who reported decreased levels of antioxidants in subfertile subjects with grade 3 varicocele as compared to those with grade I varicocele.

Recent studies have demonstrated a significantly higher proportion of sperm cells with fragmented DNA in the varicocele samples than samples from fertile men (Saleh *et al*,

2003; Chen *et al*, 2004; Encisco *et al*, 2006). DNA fragmentation may thus be a consequence of triggering of an apoptotic-like process by the ROS overproduction or a direct expression of this damage (Encisco *et al*, 2006). Our results show non- significant (P>0.05) increased DNA fragmentation in all subfertile subjects having different grades (I, II or III) of varicocele. Our results are in accordance with the findings of Saleh *et al*, (2003), who also found significantly higher levels of sperm DNA damage in infertile men.

Our study found non-significant negative correlation between reactive oxygen species (ROS) and total antioxidant capacity (TAC) in all subfertile subjects having different grades (I, II or III) of varicocele. Our results are in contrast with the study conducted by Hendin *et al*, (1999), who found no significant (P>0.05) correlation between reactive oxygen species (ROS) and total antioxidant capacity (TAC) in all subfertile varicocele subjects. This absence of correlation may account to the anatomical relationship between the structures (prostate and seminal vesicles) producing the seminal plasma and varicocele. Varicocele may be directly influenced by temperature and testicular blood flow whereas seminal plasma producing structures might have no effect upon them (Hendin *et al*, 1999). Another possibility maybe a variation in the source of generation of the total antioxidant species, whether the antioxidants are non-enzymatic and primarily extracellular or enzymatic or intracellular (Miesel *et al*, 1997; Kurpisz *et al*, 1996).

In the hypospermatogenic testes of varicocele men**,** germ cell apoptosis is initiated**,** but several cells may escape this apoptotic process and continue maturing, leading to poor sperm quality and nuclear DNA fragmentation (Sakkas *et al*, 1999; Said *et al*, 2004). DNA fragmentation commonly seen in the spermatozoa of subfertile men is mediated by high levels of ROS. This oxidative sperm DNA damage is closely associated with impaired spermatogenic function and male infertility (Sun *et al*, 1997). ROS alters DNA integrity by peroxidation of polyunsaturated fatty acids in the lipid bilayer of the sperm plasma membrane, thus altering the sperm maturation, spermatogenesis, capacitation, acrosome reaction and sperm-oocyte fusion (Sanocka *et al*, 2004). We observed non-significant positive correlations between reactive oxygen species (ROS) and sperm DNA fragmentation, as assessed by the TUNEL Assay, in all the three grades of varicocele

subfertile subjects. Our results are similar to the findings of Saleh *et al*, (2003), who also observed a positive correlation between increased ROS and significantly increased spermatozoal DNA fragmentation in infertile men with various grades of varicocele. Several other studies by Blumer *et al*, (2012); Bertolla *et al*, (2006) and Smith *et al*, (2006), have also shown an association between varicocele and increased sperm DNA fragmentation and observed increased DNA fragmentation in sperms of infertile men with varicocele. The finding of elevated seminal oxidative stress indicates that oxidative stress might be playing a major role in the pathogenesis of sperm DNA fragmentation in subfertile men with and without varicocele. However, further studies are required to rule out this possibility.

## CONCLUSION

Varicocele maybe one of the biggest causes of male subfertility, yet its pathophysiology still remains unclear. Our study indicates that subfertile subjects, with varicocele, had a significant increase in oxidative stress as seen by increased levels of reactive oxygen species, possibly as a result of poor quality spermatozoa. However, the total antioxidant capacity showed a comparable decrease in all the three study groups. The antioxidant values represent the balance between synthesis and utilization and could be influenced by different modulatory factors. A significant increase in sperm DNA fragmentation in patients with varicocele might indicate the involvement of seminal oxidative stress in its pathogenesis. This sperm DNA fragmentation, independent of its cause, might be a reason of affecting the quality of ejaculated spermatozoa, especially in varicocele subfertile males. Semen analysis, the first conventional step in evaluation of male sub fertility, provides little information about spermatozoal function. Specialized sperm function tests are needed for determination of specific defects of human sperm physiology. Even though it is clear that oxidative stress is involved in the pathogenesis of varicocele yet different aspects of varicocele still need to be investigated.

## REFERENCES


- Abd-Elmoaty M. A., Saleh, R., Sharma, R. & Agarwal, A. 2010.I ncreased levels of oxidants and reduced antioxidants in semen of infertile men with varicocele. *Fertil Steril* 94, 1531–1534
- Agarwal A & Said TM. (2003a). Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update* 9:331‑45
- Agarwal A and Said TM. 2011. Interpretation of Basic Semen Analysis and Advanced Semen Testing. *Current Clinical Urology: Male Infertility: Problems and Solutions* Springer Science+Business Media, LLC
- Agarwal A, Allamaneni SS, Said TM. 2004. Chemiluminescence technique for measuring reactive oxygen species. *Reprod Biomed Online* 9(4):466-8
- Agarwal A, Allamaneni SS. 2004. Role of free radicals in female reproductive diseases and assisted reproduction. *Reprod Biomed Online* 9(3):338-47
- Agarwal A, Allamaneni SS. 2004. The effect of sperm DNA damage on assisted reproduction outcomes. A review. *Minerva Ginecol* 56(3):235-45
- Agarwal A, Allamaneni SS. 2005. Sperm DNA damage assessment: a test whose time has come. *Fertil Steril* 84(4):850-3
- Agarwal A, Bragais FM, Sabanegh E. 2008a. Assessing sperm function. *Urol Clin North Am* 35:157–171
- Agarwal A, Hamada A, Esteves SC. 2012. Insight into oxidative stress in varicocele-associated male infertility: part 1. *Nat Rev Urol* 9(12):678-90
- Agarwal A, Hamamah S and Shekkariz M. 1994. Reactive oxygen species and fertilizing capacity of spermatozoa. *Contracept Fert Sex* 22: 327-330
- Agarwal A, Ikemoto I, Loughlin KR. 1994. Effect of sperm washing on levels of reactive oxygen species in semen. *Arch Androl* 33(3):157-62
- Agarwal A, Nallella KP, Allamaneni SS, Said TM. 2004. Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod Biomed Online* 8(6):616-27

- Agarwal A, Prabakaran S, Allamaneni SS. 2006. Relationship between oxidative stress, varicocele and infertility: a meta-analysis. *Reprod Biomed Online* 12(5):630-3
- Agarwal A, Said T M. 2011. Interpretation of Basic Semen Analysis and Advanced Semen Testing In *Current Clinical Urology: Male Infertility: Problems and Solutions* Springer Science+Business Media, LLC
- Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG. 2006. Oxidative stress in an assisted reproductive techniques setting. *Fertil Steril* 86(3):503-12
- Agarwal A, Said TM. 2003. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update* 9(4):331-45
- Agarwal A, Said TM. 2005. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. *BJU Int* 95(4):503-7
- Agarwal A, Saleh RA, Bedaiwy MA. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 79: 829–843
- Agarwal A, Saleh RA. 2002. Role of oxidants in male infertility: rationale, significance, and treatment. *Urol Clin North Am* 29(4):817-27
- Agarwal A, Sharma RK, Desai NR, Prabakaran S, Tavares A, Sabanegh E. 2009. Role of oxidative stress in pathogenesis of varicocele and infertility. *Urology* 73(3):461-9
- Agarwal A, Sharma RK, Nallella KP, Thomas AJ Jr, Alvarez JG, Sikka SC. 2006. Reactive oxygen species as an independent marker of male factor infertility. *Fertil Steril* 86(4):878-85
- Agarwal A, Sharma RK, Nelson DR. 2003. New semen quality scores developed by principal component analysis of semen characteristics. *J Androl* 24(3):343-52
- Agarwal A, Sushil P and Shyam A. 2006. What an andrologist / urologist should know about free radicals and why. *Urology* 67: 2-8
- Agarwal A. 2004. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod* 19:129–138

- Agarwal A. and Prabhakaran S.A. 2005. Mechanism, measurement and prevention of oxidative stress in male reproductive physiology. *Ind. J. Exp. Biol* 43: 963-974
- Agarwal, Ashok, and Tamer M. Said. 2004. Sperm chromatin assessment. *Textbook of ART* 2nd ed. Taylor & Francis Group, London: UK 93-106
- Ahmad L, Jalali S, Shami SA, Akram Z. Arch Androl. 2007. Sperm preparation: DNA damage by comet assay in normo- and teratozoospermics. Arch Androl 53(6):325-38
- Aitken J, Fisher H. 1994. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays* 16(4):259-67
- Aitken RJ and Clarkson JS: 1987. Cellular basis of defective sperm function and its association with the generation of reactive oxygen species by human spermatozoa. *Journal of Reproduction and Fertility* 81:459-469
- Aitken RJ, Baker HW. 1995. Seminal leukocytes: passengers, terrorists or good samaritans? *Hum Reprod* 10(7):1736-9
- Aitken RJ, Best FS, Richardson DW, Djahanbakhch O, Mortimer D, Templeton AA, Lees MM. 1982. An analysis of sperm function in cases of unexplained infertility: conventional criteria, movement characteristics, and fertilizing capacity. *Fertil Steril* 38 (2):212-21
- Aitken RJ, Best FS, Warner P, Templeton A. 1984. A prospective study of the relationship between semen quality and fertility in cases of unexplained infertility. *J Androl* 5: 297–303
- Aitken RJ, Buckingham D, West K, Wu FC, Zikopoulos K, Richardson DW. 1992. Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. *J Reprod Fertil* 94(2):451-62
- Aitken RJ, Buckingham D. 1992. Enhanced detection of reactive oxygen species produced by human spermatozoa with 7-dimethyl amino-naphthalin-1, 2-dicarbonic acid hydrazide. *Int J Androl* 15(3):211-9

- Aitken RJ, Buckingham DW, Brindle J, Gomez E, Baker HW, Irvine DS. 1995. Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. *Hum Reprod* 10(8):2061-71
- Aitken RJ, Buckingham DW, West KM. 1992. Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. *J Cell Physiol* 151(3):466-77
- Aitken RJ, Clarkson JS, Fishel S. 1989. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* 41(1):183-97
- Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS, Wu FC. 1989. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J Androl* 10(3):214-20
- Aitken RJ, Clarkson JS. 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 81(2):459-69
- Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, Irvine S. 1997. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev* 47(4):468-82
- Aitken RJ, Harkiss D, Buckingham D. 1993.Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *J Reprod Fertil* 98:257–265
- Aitken RJ, Harkiss D, Buckingham DW. 1993. Analysis of lipid peroxidation mechanisms in human spermatozoa. *Mol Reprod Dev* 35(3):302-15
- Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS: 1998. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. *J Cell Sci* 111:645-656

- Aitken RJ, Irvine DS, Wu FC. 1991. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am. J. Obstet. Gynecol* 164: 542–51
- Aitken RJ, Krausz C. 2001. Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 122(4):497-506
- Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M. 1995. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J Cell Sci* 108(5):2017-25
- Aitken RJ, West KM. 1990. Analysis of the relationship between reactive oxygen species production and leucocyte infiltration in fractions of human semen separated on Percoll gradients. *Int J Androl* 13(6):433-51
- Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA. 2007. Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. *Mol Hum Reprod* 13:203–211
- Aitken RJ. 1994. A free radical theory of male infertility. *Reprod Fertil Dev* 6(1):19-23
- Aitken RJ. 1995. Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev* 7(4):659-68
- Aitken RJ. 2006. Sperm function tests and fertility. *Int J Androl* 29(1):69-75
- Aitken RJ: 1999. The human spermatozoon – a cell in crisis? The Amoroso Lecture. *J Reprod Fertil* 115:1-7
- Akyol O, Ozbek E, Uz E, Koçak I. 2001. Malondialdehyde level and total superoxide dismutase activity in seminal fluid from patients with varicocele. *Clin Exp Med* 1: 67–68
- Al-Ali BM, Marszalek M, Shamloul R, Pummer K, Trummer H. 2010. Clinical parameters and semen analysis in 716 Austrian patients with varicocele. *Urology* 75(5):1069-73
- Alkan I, Simsek F, Haklar G, Kervancioglu E, Ozveri H, Yalcin S, Akdas A. 1997. Reactive oxygen species production by the spermatozoa of patients with

idiopathic infertility: relationship to seminal plasma antioxidants. *J Urol* 157:140–143

- Allamaneni SS, Naughton CK, Sharma RK, Thomas AJ Jr, Agarwal A. 2004. Increased seminal reactive oxygen species levels in patients with varicoceles correlate with varicocele grade but not with testis size. *Fertil Steril* 82:1684-1686
- Alvarez C, Castilla JA, Martinez L, Ramirez JP, Vergara F, Gaforio JJ. 2003. Biological variation of seminal parameters in healthy subjects. *Hum Reprod* 18:2082–2088
- Alvarez JG, Storey BT. 1989. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res* 23(1):77-90
- Alvarez JG, Touchstone JC, Blasco L, Storey BT. 1987. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. *J Androl* 8(5):338-48
- Alvarez JG. 2003. DNA fragmentation in human spermatozoa: significance in the diagnosis and treatment of infertility. *Minerva Ginecol* 55(3):233-9
- Alvarez JG. 2005. The predictive value of sperm chromatin structure assay. *Hum Reprod* 20:2365–2367
- Amann RP. 1989. Can the fertility potential of a seminal sample be predicted accurately? *J Androl* 10:89–98
- Andersen AG, Jensen TK, Carlsen E, Jørgensen N, Andersson AM, Krarup T, Keiding N, Skakkebaek NE. 2000. High frequency of sub-optimal semen quality in an unselected population of young men. *Hum Reprod* Feb;15(2):366-72
- Andrade-Rocha FT. 1994. Study on the general characteristics of the spermatozoa in polyzoospermic semen. *Rev Bras Ginec Obstet* 16:77–79
- Andrade-Rocha FT. 2003. Semen analysis in laboratory practice: an overview of routine tests. *J Clin Lab Anal* 17(6):247-58
- Angelopoulou R, Karayiannis V. 2000. Proliferation and apoptosis of germ cells. *Arch Hellen Med* 17(5):477-490

- Aravindan GR, Bjordahl J, Jost LK, Evenson DP. 1997. Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Exp Cell Res* 236(1):231-7
- Athayde KS, Cocuzza M, Agarwal A, Krajcir N, Lucon AM, Srougi M, Hallak J. 2007. Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. *J Androl* 28(4):613-20
- Auger J, Eustache F, Andersen AG, Irvine DS, Jørgensen N, Skakkebaek NE, Suominen J, Toppari J, Vierula M, Jouannet P. 2001. Sperm morphological defects related to environment, lifestyle and medical history of 1001 male partners of pregnant women from four European cities. *Hum Reprod* 16(12):2710-7
- Avendaño C, Franchi A, Duran H, Oehninger S. 2010. DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. *Fertil Steril* 94(2):549-57
- Aziz N, Agarwal A, Lewis-Jones I, Sharma R, Thomas AJ Jr. 2004. Novel associations between specific sperm morphological defects and leukocytospermia. *Fertil Steril* 82:621-627
- Aziz N, Agarwal A, Nallella KP, Thomas AJ Jr. 2006. Relationship between epidemiological features and aetiology of male infertility as diagnosed by a comprehensive infertility service provider. *Reprod Biomed Online* 12(2):209-14
- Aziz N, Saleh R, Sharma R, Lewis-Jones I, Esfandiari N, Thomas AJ, Agarwal A. 2004. Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertil Steril* 81:349–54
- Baccetti BM, Bruni E, Capitani S, Collodel G, Mancini S, Piomboni P, Moretti E. 2006. Studies on varicocele III: ultrastructural sperm evaluation and 18, X and Y aneuploidies. *J Androl* 27(1):94-101
- Bajpayee M, Dhavan A, Devendera P, Pandey AK, Mathur N, Seth PK. 2002. Gender-related differences in basal DNA damage in lymphocytes of a healthy

Indian population using the alkaline comet assay. *Mutat Res Genet Toxicol Environ Mutagen* 520:83-91

- Baker MA, Aitken RJ. 2005. Reactive oxygen species in spermatozoa: methods for monitoring and significance for the origins of genetic disease and infertility. *Reprod Biol Endocrinol* 29:3-67
- Balhorn R, Reed S, Tanphaichitr N. 1988. Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. *Experientia* 44(1):52-5
- Barbieri ER, Hidalgo ME, Venegas A, Smith R, Lissi EA. 1999. Varicocele-associated decrease in antioxidant defenses. *J Androl* 20(6):713-7
- Barone JG, De Lara J, Cummings KB, Ward WS. 1994. DNA organization in human spermatozoa. *J Androl* 15(2):139-44
- Barratt CL, Aitken RJ, Björndahl L, Carrell DT, de Boer P, Kvist U, Lewis SE, Perreault SD, Perry MJ, Ramos L, Robaire B, Ward S, Zini A. 2010. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications-a position report. *Hum Reprod* 25(4):824-38
- Barratt CL, De Jonge CJ. 2010. Clinical relevance of sperm DNA assessment: an update. *Fertil Steril* 94(6):1958-9
- Barratt CL, Dunphy BC, Thomas EJ, Cooke ID. 1988. Semen characteristics of 49 fertile males. *Andrologia* 20: 264–9
- Barratt CLR, Tomlinson MJ, Cooke ID. 1993. Prognostic significance of computerized motility analysis for in vivo fertility. *Fertil Steril* 60: 520–525
- Barroso G, Mercan R, Ozgur K, Morshedi M, Kolm P, Coetzee K, Kruger T, Oehninger S. 1999. Intra- and inter-laboratory variability in the assessment of sperm morphology by strict criteria: impact of semen preparation, staining techniques and manual versus computerized analysis. *Hum Reprod* 14(8):2036-40
- Barroso G, Morshedi M, Oehninger S. 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 15:1338–44

- Bartoov B, Eltes F, Weissenberg R, Lunenfeld B. 1980. Morphological characterization of abnormal human spermatozoa using transmission electron microscopy. *Arch Androl* 5:305–322
- Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* 130(4):1910-7
- Bench GS, Friz AM, Corzett MH, Morse DH, Balhorn R. 1996. DNA and total protamine masses in individual sperm from fertile mammalian subjects. *Cytometry* 23(4):263-71
- Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H, Guérin JF. 2003. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod* 18(5):1023-8
- Benoff S and Gilbert BR. 2001. Varicocele and male infertility: part I. Preface. Human Reprod Update 7(1):47-54
- Benzie IF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 239(1):70-6
- Berger RE, Karp LE, Williamson RA, Koehler J, Moore DE, Holmes KK. 1982. The relationship of pyospermia and seminal fluid bacteriology to sperm function as reflected in the sperm penetration assay. *Fertil Steril* 37(4):557-64
- Bertolla RP, Cedenho AP, Hassun Filho PA, Lima SB, Ortiz V, Srougi M. 2006. Sperm nuclear DNA fragmentation in adolescents with varicocele. *Fertil Steril* 85(3):625-8
- Bhasin S. Approach to the infertile man. 2007. *J Clin Endocrinol Metab* 92(6):1995-2004
- Bianchi PG, Manicardi GC, Urner F, Campana A, Sakkas D. 1996. Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa. *Mol Hum Reprod* 2(3):139-44
- Biyani CS, Cartledge J, Janetschek G. 2009. Varicocele. *Clin Evid* pii: 1806

- Bjorndahl L, So¨derlund I, Kvist U. 2003. Evaluation of the one step eosin nigrosin staining technique for human sperm vitality assessment. *Hum Reprod* 18: 813–6
- Blake DR, Allen RE, Lunec J. 1987. Free radicals in biological systems-a review orientated to inflammatory processes. *Br Med Bull* 43(2):371-85
- Blanco-Rodríguez J, Martínez-García C. 1996. Spontaneous germ cell death in the testis of the adult rat takes the form of apoptosis: re-evaluation of cell types that exhibit the ability to die during spermatogenesis. *Cell Prolif* 29(1):13-31
- Blom E. 1950. A one-minute live-dead sperm stain by means of Eosin-Nigrosin. *Fertil Steril* 1: 176-177
- Blumer CG, Fariello RM, Restelli AE, Spaine DM, Bertolla RP, Cedenho AP. 2008. Sperm nuclear DNA fragmentation and mitochondrial activity in men with varicocele. *Fertil Steril* 90(5):1716-22
- Blumer CG, Restelli AE, Giudice PT, Soler TB, Fraietta R, Nichi M, Bertolla RP, Cedenho AP. 2012. Effect of varicocele on sperm function and semen oxidative stress. *BJU Int* 109: 259–265
- Boe-Hansen GB, Ersboll AK, Christensen P. 2005. Variability and laboratory factors affecting the sperm chromatin structure assay in human semen. *J Androl* 26:360‑8
- Bonde JP, Ernst E, Jensen TK, Scheike T, Giwercman A, Olsen J, Skakkebaek NE. 1998. Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. *Lancet* 352: 1172–7
- Bonde JP, Giwercman A, Ernst E. 1996. Identifying environmental risk to male reproductive function by occupational sperm studies: logistics and design options. *Occup Environ Med* 53: 511–9
- Bonde JP, Storgaard L. 2002. How work-place conditions, environment toxicants and lifestyle affect male reproductive function. *Int J Androl* 25:262–268
- Boone WR, Jones JM, Shapiro SS. 2000. Using videotaped specimens to test quality control in a computer-assisted semen analysis system. *Fertil Steril* 73:636–640

- Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, Coticchio G. 2006. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. *Hum Reprod* 21(11):2876-81
- Bostofte E, Serup J, Rebbe H. 1983. Has the fertility of Danish men declined through the years in terms of semen quality? A comparison of semen qualities between 1952 and 1972. *Int J Fertil* 28: 91–95
- Boyle CA, Khoury MJ, Katz DF, Annest JL, Kresnow M-J, DeStefano F,Schrader S. 1992. The relation of computer-based measures of sperm morphology and motility to male infertility. *Epidemiology* 3:239–246
- Burkman LJ, Coddington CC, Franken DR, Krugen TF, Rosenwaks Z, Hogen GD. 1988. The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril* 49(4):688-97
- Calamera JC, Giovenco P, Brugo S, Dondero F, Nicholson RF. 1987. Adenosine 5-triphosphate (ATP) content and acrosine activity in polyzoospermic subjects. *Andrologi*a 1 9:460–463
- Calvin HI, Cooper GW, Wallace EW. 1981. Evidence that selenium in rat sperm is associated with a cysteine-rich structural proteins of the mitochondrial capsule. *Gamete Res* 4. 139-145
- Cao G and Prior RL: 1998. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin Chem* 44: 1309
- Cao G, Alessio HM, Cutler RG. 1993. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radic Biol Med* 14(3):303-11
- Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, Campbell B. 2003. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl* 49(1):49-55
- Carrell DT. 2000. Semen analysis at the turn of the century: an evaluation of potential uses of new sperm function assays. *Arch Androl* 44(1):65-75

- Chan JP, Corselli JU, Patton WC, Jacobson JD, Chan SR, King A. 2001. A simple comet assay for archived sperm correlates DNA fragmentation to reduced hyperactivation and penetration of zona-free hamster oocyte. *Fertil Steril* 75:186-192

- Chan SYW, Tang LCH, Tang GWK, Ho PC, Wang C. 1986. Spermatozoal fertilizing capacity in polyzoospermia: a preliminary study. *Andrologia* 18:208–213
- Chehval MJ, Purcell MH. 1992. Varicocelectomy: incidence of external vein involvement in the clinical varicocele. *Urology* 39: 573-575
- Chehvall MJ and Purcell MH. 1992. Deterioration of semen parameters over time in men with untreated varicocele: evidence of progressive testicular damage. *Fertil Steril* 57:174-177
- Chen C, Lee S, Chen D, Chien H, Chen I, Chu Y, Liu J, Chen W, Wu G. 2004. Apoptosis and kinematics of ejaculated spermatozoa in patients with varicocele. *J Androl* 25:348–353

- Chen SS, Chang LS, Wei YH. 2001. Oxidative damage to proteins and decrease of antioxidant capacity in patients with varicocele. *Free Radic Biol Med* 30(11):1328-34
- Chia SE, Tay SK, Lim ST. 1998. What constitutes a normal seminal analysis? Semen parameters of 243 fertile men. *Hum Reprod* 13(12):3394-8
- Chohan KR, Griffin JT., Lafromboise M, De Jonge CJ, Carrell, DT. 2006. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *J Androl* 27:53–59
- Clements S, Cooke ID, Barratt CL. 1995. Implementing comprehensive quality controls in the andrology laboratory. *Hum Reprod* 10:2096–2106
- Cocuzza M, Agarwal A. 2007. Nonsurgical treatment of male infertility: specific and empiric therapy. *Biologics* 1(3):259-69
- Cocuzza M, Athayde KS, Agarwal A, Pagani R, Sikka SC, Lucon AM, Srougi M, Hallak J. 2008. Impact of clinical varicocele and testis size on seminal reactive

oxygen species levels in a fertile population: A prospective controlled study. *Fertil Steril* 90:1103-1108

- Cocuzza M, Athayde KS, Agarwal A, Sharma R, Pagani R, Lucon AM, Srougi M, Hallak J 2008. Age-related increase of reactive oxygen species in neat semen in healthy fertile men. *Urology* 71:490–494
- Coetzee K, Kruger TF, Lombard CJ, Shaughnessy D, Oehninger S, Ozgür K, Pomeroy KO, Muller CH. 1999. Assessment of interlaboratory and intralaboratory sperm morphology readings with the use of a Hamilton Thorne Research integrated visual optical system semen analyzer. *Fertil Steril* 71(1):80-4
- Cohen-Bacrie P, Belloc S, Me´ne´ zo Y J, Clement P, Hamidi J, Benkhalifa, M. 2009. Correlation between DNA damage and sperm parameters: a prospective study of 1,633 patients. Fertil Steril 91:1801–1805
- Comhaire F, Vermeulen L. 1995. Human semen analysis. *Hum Reprod Update* 1:343–362
- Comhaire F. 1991. The pathogenesis of epididymo-testicular dysfunction in varicocele: factors other than temperature. *Adv Exp Med Biol* 286: 281-287
- Comhaire FH, Mahmoud AM, Depuydt CE, Zalata AA, Christophe AB. 1999. Mechanisms and effects of male genital tract infection on sperm quality and fertilizing potential: the andrologist's viewpoint. *Hum Reprod Update* 5(5):393-8
- Consensus Workshop on Advanced Diagnostic Andrology Techniques. 1996. ESHRE (European Society of Human Reproduction and Embryology) Andrology Special Interest Group. *Hum Reprod* 11:1463–1479
- Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT, Vogelsong KM. 2010. World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 16(3):231-45
- Cooper TG. 1996. Epididymis and sperm function. *Andrologia* 28 Suppl 1:57-9
- Dada R, Gupta NP, Kucheria K. 2006. Cytogenetic and molecular analysis of male infertility: y chromosome deletion during nonobstructive azoospermia and severe oligozoospermia. *Cell Biochem Biophys* 44:171-8

- Dada R, Kumar R, Kumar R, Sharma RK, Gupta NP, Kucheria K. 2007. High incidence of Yq microdeletions in semen is better parameter than blood DNA. *Asian J Androl* 7 :720-2
- Dada R, Shamsi MB, Venkatesh S, Gupta NP, Kumar R. 2010. Attenuation of oxidative stress & DNA damage in varicocelectomy: implications in infertility management. Indian *J Med Res* 132:728–730
- Dadoune JP. 1995. The nuclear status of human sperm cells. *Micron* 26(4):323-45
- Das KM, Prasad K, Szmigielski W, Noorani N. 1999. Intratesticular varicocele: evaluation using conventional and Doppler sonography. *AJR Am J Roentgenol* 173:1079–1083
- Daudin M, Bieth E, Bujan L, Massat G, Pontonnier F, Mieusset R. 2000. Congenital bilateral absence of the vas deferens: clinical characteristics, biological parameters, cystic fibrosis transmembrane conductance regulator gene mutations, and implications for genetic counseling. *Fertil Steril* 74(6):1164-74
- Davis RO, Bain DE, Siemers RJ, Thal DM, Andrew JB, Gravance CG. 1992. Accuracy and precision of the CellForm-Human automated sperm morphometry instrument. *Fertil Steril* 58(4):763-9
- Davis RO, Gravance CG. 1994. Consistency of sperm morphology classification methods. *J Androl* 15(1):83-91
- Davis RO, Katz DF. Operational standards for CASA instruments. 1993. *J Androl* 14:385–394
- De Jonge C. 1999. Attributes of fertile spermatozoa: an update. *J Androl* 20(4):463-73
- de Lamirande E, Gagnon C. 1992. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. *J Androl* 13(5):368-78
- de Lamirande E, Gagnon C. 1992. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J Androl* 13(5):379-86

- de Lamirande E, Gagnon C. 1995. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. *Hum Reprod* 10 Suppl 1:15-21
- de Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. I: 1992. effects on the motility of intact spermatozoa and on sperm axonemes. *J Androl* 13:368–78
- de Lamirande E, Jiang H, Zini A, Kodama H and Gagnon C. 1997. Reactive oxygen species and sperm physiology. *Rev Reprod* 2: 48-54
- de Yebra L, Ballescá JL, Vanrell JA, Corzett M, Balhorn R, Oliva R. 1998. Detection of P2 precursors in the sperm cells of infertile patients who have reduced protamine P2 levels. *Fertil Steril* 69(4):755-9
- DeadEnd (TM) Fluorometric TUNEL System Technical Bulletin, TB235 (Promega Technical Bulletin). Available from: www. Promega.com, accessed on March 2007
- Deepinder F, Cocuzza M, Agarwal A. 2008. Should seminal oxidative stress measurement be offered routinely to men presenting for infertility evaluation? *Endocr Pract* 14(4):484-91
- DeFeudis FV, Papadopoulos V, Drieu K. 2003. Ginkgo biloba extracts and cancer: a research area in its infancy. *Fundam Clin Pharmacol* 17(4):405-17
- Desai N, Sharma R, Makker K, Sabanegh E, Agarwal A. 2009. Physiologic and pathologic levels of reactive oxygen species in neat semen of infertile men. *Fertil Steril* 92(5):1626-31
- Diamond DA, Zurakowski D, Bauer SB, Borer JG, Peters CA, Cilento BG Jr, Paltiel HJ, Rosoklija I, Retik AB. 2007. Relationship of varicocele grade and testicular hypotrophy to semen parameters in adolescents. *J Urol* 178:1584-8
- Dohle GR, Halley DJ, Van Hemel JO, van den Ouwel AM, Pieters MH, Weber RF, Govaerts LC. 2002. Genetic risk factors in infertile men with severe oligozoospermia and azoospermia. *Hum Reprod* 17:13–16

- Dominguez-Fandos D, Camejo MI, Ballesca JL, Oliva R. 2007. Human sperm DNA fragmentation: correlation of TUNEL results as assessed by flow cytometry and optical microscopy. *Cytometry A* 71:1011–1018

- Donnelly ET, McClure N, Lewis SE. 1999. Antioxidant supplementation in vitro does not improve human sperm motility. *Fertil Steril* 72(3):484-95
- Donnelly ET, McClure N, Lewis SE. 1999. The effects of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis* 14: 505-512

- Dougherty KA, Emilson LB, Cockett AT, Urry RL. 1975. A comparison of subjective measurements of human sperm motility and viability with two live-dead staining techniques. *Fertil Steril* 26 (7):700-3
- Duran EH, Gurgan T, Gunalp S, Enginsu ME, Yarali H, Ayhan A. 1998. A logistic regression model including DNA status and morphology of spermatozoa for prediction of fertilization in vitro. *Hum Reprod* 13:1235-9
- Duran EH, Morshedi M, Taylor S, Oehninger S. 2002. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum Reprod* 17(12):3122-8
- Duty SM, Singh NP, Ryan L, Chen Z, Lewis C, Huang T, Hauser R. 2002. Reliability of the comet assay in cryopreserved human sperm. *Hum Reprod* 17(5):1274-80

- el-Demiry MI, Hargreave TB, Busuttil A, Elton R, James K, Chisholm GD. 1987. Immunocompetent cells in human testis in health and disease. *Fertil Steril* 48(3):470-9
- Elferink JG. 1984. Measurement of the metabolic burst in human neutrophils: a comparison between cytochrome c and NBT reduction. *Res Commun Chem Pathol Pharmacol* 43:339-342

- Eliason R and Treichel L. 1971. Supravital staining of human spermatozoa. *Fertil Steril* 22(2):134-7

- Eliasson R. 2010. Semen analysis with regard to sperm number, sperm morphology and functional aspects. *Asian J Androl* 12: 26–32
- Ellekilde Bonde JP. 2010. Semen analysis from an epidemiologic perspective. *Asian J Androl* 12(1):91-4
- Enciso M, López-Fernández C, Fernández JL, García P, Gosálbez A, Gosálvez J. 2006. A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy. *Theriogenology* 65(2):308-16
- Enciso M, Muriel L, Fernández JL, Goyanes V, Segrelles E, Marcos M, Montejo JM, Ardoy M, Pacheco A, Gosálvez J. 2006. Infertile men with varicocele show a high relative proportion of sperm cells with intense nuclear damage level, evidenced by the sperm chromatin dispersion test. *J Androl* 27(1):106-11
- Endtz AW. 1974. A rapid staining method for differentiating granulocytes from "germinal cells" in Papanicolaou-stained semen. *Acta Cytologica* 18(1):2-7
- Engel S, Schreiner T, Petzoldt R. 1999. Lipid peroxidation in human spermatozoa and maintenance of progressive sperm motility. *Andrologia* 31: 17–22
- Erel O. 2005. A new automated colorimetric method for measuring total oxidant status. *Clin Biochem* 38:1103-1111
- Erenpreiss J, Hlevicka S, Zalkalns J, Erenpreisa J. 2002. Effect of leukocytospermia on sperm DNA integrity: a negative effect in abnormal semen samples. *J Androl* 23(5):717-23
- Erenpreiss J, Jepson K, Giwercman A, Tsarev I, Erenpreisa J, Spano M .2004. Toluidine blue cytometry test for sperm DNA conformation: comparison with the flow cytometric sperm chromatin structure and TUNEL assays. *Hum Reprod* 19:2277–2282
- Esfandiari N, Sharma RK, Saleh RA, Thomas AJ Jr, Agarwal A. 2003. Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. *J Andro*l 24:862-870
- ESHRE Andrology Special Interest Group. Consensus Workshop on Advanced Diagnostic Andrology Techniques. 1996. *Hum Reprod* 11: 1463–79

- ESHRE Capri Workshop Group. 2000. Optimal use of infertility diagnostic tests and treatments. *Hum Reprod* 15:723–732
- Esterhuizen AD, Franken DR, Lourens JG, Prinsloo E, van Rooyen LH. 2000. Sperm chromatin packaging as an indicator of in vitro fertilisation rates. *Hum Reprod* 15: 657–61
- Evenson DP, Darzynkiewicz Z, Melamed MR. 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 210:1131–1133
- Evenson DP, Jost LK, Baer RK, Turner TW, Schrader SM. 1991. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod Toxicol* 5(2):115-25
- Evenson DP, Jost LK, Corzett M, Balhorn R. 2000. Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study. *J Androl* 21(5):739-46
- Evenson DP, Larson KL, Jost LK. 2002. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl* 23(1):25-43
- Evenson DP, Wixon R. 2006. Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology* 65:979‑91
- Evers JL. 2002. Female subfertility. *Lancet* 360:151–159
- Fedder J, Askjaer SA, Hjort T. 1993. Nonspermatozoal cells in semen: relationship to other semen parameters and fertility status of the couple. *Arch Androl* 31(2):95-103
- Fernandez JL, de la Calle JFV, Tamayo M, Cajigal D, Agarwal A, Gosalvez J. 2009. Sperm DNA integrity and male infertility: current perspectives. *Arch Med Sci* 5(1A):S 55-S62
- Fernandez JL, Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M, LaFromboise M, De Jonge C. 2005. Halosperm is an easy, available, and cost-effective alternative for determining sperm DNA fragmentation. *Fertil Steril* 84:860

- Fernández JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. 2003. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *J Androl* 24(1):59-66
- Fernandez, J., Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M, LaFromboise M, De Jonge C. 2005. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* 84: 833-42
- Fingerova H, Novotny J, Barborik J, Brezinova J, Svobodova M, Krskova M, Oborna I. 2007. Antioxidant capacity of seminal plasma measured by TAS Randox. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 151(1):37-40
- Fischer H and Aitken R. 1997. Comparative analysis of the ability of precursor germ cells and epididymal spermatozoa to generate reactive oxygen metabolites. *J Exp Zool* 277:390-400
- Ford WC, Whittington K. 1998. Antioxidant treatment for male subfertility: a promise that remains unfulfilled. *Hum Reprod* 13(6):1416-9
- Ford WC. 2004. Regulation of sperm function by reactive oxygen species. *Hum Reprod Update* 10(5):387-99
- Fraczek M, Kurpisz M. 2007. Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa. *J Androl* 28(2):325-33
- Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. 1991. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci USA* 88:11003–11006
- Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM and Ames BN. 1996. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res* 351:199–203
- Franken DR, Bastiaan HS, Kidson A, Wranz P, Habenicht UF. 1997. Zona pellucida mediated acrosome reaction and sperm morphology. *Andrologia* 29: 311–7
- Franken DR, Dada OA. 2007. Does training assist medical laboratory scientists with better evaluation of sperm morphology. *Afr J Reprod Health* 11: 3–8

- Franken DR, Kruger TF. 2006. Lessons learned from a sperm morphology quality control programme. *Andrologia* 3: 225–9
- Franken DR, Menkveld R, Kruger TF, Sekadde-Kigondu C, Lombard C. 2003. Monitoring technologist reading skills in a sperm morphology quality control program. *Fertil Steril* 79: 1637–43
- Franken DR, Oehninger S. 2012. Semen analysis and sperm function testing. *Asian J Androl* 14(1):6-13
- Fraser L. 2004. Structural damage to nuclear DNA in mammalian spermatozoa: its evaluation techniques and relationship with male infertility. *Pol J Vet Sci* 7(4):311-21
- French DB, Desai NR, Agarwal A. 2008. Varicocele repair: does it still have a role in infertility treatment? *Curr Opin Obstet Gynecol* 20(3):269-74
- Fretz PC, Sandlow JI. 2002. Varicocele: current concepts in pathophysiology, diagnosis, and treatment. *Urol Clin North Am* 29(4):921-37
- Friedler G. 1996. Paternal exposures: impact on reproductive and developmental outcome. An overview. *Pharmacol Biochem Behav* 55(4):691-700
- Fujisawa M, Hayashi A, Imanishi O, Tanaka H, Okada H, Matsumoto O and Kamidono S .1994. The significance of gonadotropin-releasing hormone test for predicting fertility after varicocelectomy. *Fertil Steril* 61:779–782
- Fujisawa M, Ishikawa T. 2003. Soluble forms of Fas and Fas ligand concentrations in the seminal plasma of infertile men with varicocele. *J Urol* 170: 2363–5
- Fujisawa M, Yoshida S, Matsumoto O, Kojima K, Kamidono S. 1988. Deoxyribonucleic acid polymerase activity in the testes of infertile men with varicocele. *Fertil Steril* 50(5):795-800
- Fuzisawa M, Yoshida S, Kojima K, Kamidono S. 1989. Biochemical changes in testicular varicocele. *Archs Androl* 22: 149-159
- Gao J, Gao ES, Walker M, Yang Q, Wu JQ, Zhu QX, Wen SW. 2008. Reference values of semen parameters for healthy Chinese men. *Urol Int* 81(3):256-62

- Gao J, Gao ES, Yang Q, Walker M, Wu JQ, Zhou WJ, Wen SW. 2007. Semen quality in a residential, geographic and age representative sample of healthy Chinese men. *Hum Reprod* 22(2):477-84
- Garrido N, Meseguer M, Alvarez J, Simon C, Pellicer A, Remohi J. 2004b.Relationship among standard semen parameters, glutathione peroxidase/glutathione reductase activity, and mRNA expression and reduced glutathione content in ejaculated spermatozoa from fertile and infertile men. *Fertil Steril* 82:1059–1066
- Garrido N, Meseguer M, Simon C, Pellicer A, Remohi J. 2004. Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility. *Asian J Androl* 6(1):59-65
- Gatewood JM, Cook GR, Balhorn R, Bradbury EM, Schmid CW. 1987. Sequence-specific packaging of DNA in human sperm chromatin. *Science*. 236(4804):962-4
- Gaur DS, Talekar M, Pathak VP. 2007. Effect of cigarette smoking on semen quality of infertile men. *Singapore Med J.* 48(2):119-23
- Giannattasio A, De Rosa M, Smeraglia R, Zarrilli S, Cimmino A, Di Rosario B, Ruggiero R, Colao A, Lombardi G. 2002. Glutathione peroxidase (GPX) activity in seminal plasma of healthy and infertile males. *J Endocrinol Invest* 25:983–986
- Gil-Guzman E, Ollero M, Lopez MC, Sharma RK, Alvarez JG, Thomas AJ Jr, Agarwal A. 2001. Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. *Hum Reprod* 16(9):1922-30
- Giulini S, Sblendorio V, Xella S, La Marca A, Palmieri B, Volpe A. 2009. Seminal plasma total antioxidant capacity and semen parameters in patients with varicocele. *Reprod Biomed Online* 18(5):617-21
- Gnoth C, Godehardt E, Frank-Herrmann P, Friol K, Tigges J, Freundl G. 2005. Definition and prevalence of subfertility and infertility. *Hum Reprod* 20(5):1144-7
- Goldstein M, Eid JF. 1989. Elevation of intratesticular and scrotal skin surface temperature in men with varicocele. *J Urol* 142: 743-745

- Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. 1996. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl* 17(3):276-87
- Gomez E, Irvine DS, Aitken RJ. 1998. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. *Int J Androl* 21(2):81-94
- Gorczyca W, Gong J, Darzynkiewicz Z. 1993. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 53(8):1945-51
- Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z. 1993. Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. *Exp Cell Res* 207(1):202-5
- Gorelick JI, Goldstein M. 1993. Loss of fertility in men with varicocele. *Fertil Steril* 59(3):613-6
- Govaerts LC. 2002. Genetic risk factors in infertile men with severe oligozoospermia and azoospermia. *Hum Reprod* 17:13–16
- Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. 2005. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* 26(3):349-53
- Greenberg SH. 1977. Varicocele and male fertility. *Fertil Steril* 28(7):699-706
- Griveau JF, Dumont E, Renard P, Callegari JP, Le Lannou D. 1995. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *J Reprod Fertil* 103(1):17-26
- Griveau JF, Le Lannou D. 1997. Reactive oxygen species and human spermatozoa: physiology and pathology. *Int J Androl* 20(2):61-9
- Gurbuz B, Yalti S, Ficicioglu C, Zehir K. 2003.Relationship between semen quality and seminal plasma total carnitine in infertile men. *J Obstet Gynaecol* 23:653–656

- Guthrie HD, Welch GR. 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J Anim Sci* 84(8):2089-2100
- Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf MP, Hill JA, Xu D, Vogel DL. 2001. Sperm morphology, motility, and concentration in fertile and infertile men National Cooperative Reproductive Medicine Network. *N Engl J Med* 345(19):1388-93
- Guzick DS, Sullivan MW, Adamson GD, Cedars MI, Falk RJ, Peterson EP, Steinkampf MP. 1998. Efficacy of treatment for unexplained infertility. *Fertil Steril* 70(2):207-13
- Halliwell B. 1989. Tell me about free radicals, doctor: a review. *J R Soc Med* 82(12):747-52
- Hamada A, Esteves SC, Agarwal A. 2013. Insight into oxidative stress in varicocele-associated male infertility: part 2. *Nat Rev Urol* 10(1):26-37
- Hartman CG, Schoenfeld C, Copeland E. 1964. Individualism in semen picture of infertile men. *Fertil Steril* 15:231–253
- Hauser R, Paz G, Botchan A, Yogev L, Yavetz H. 2001. Varicocele: effect on sperm functions. *Hum Reprod Update* 7: 482–5
- Hendin BN, Kolettis PN, Sharma RK, Thomas AJ Jr , Agarwal A. 1999.Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *J Urol* 161:1831–1834
- Henkel R, Hoogendijk CF, Bouic PJ, Kruger TF. 2010. TUNEL assay and SCSA determine different aspects of sperm DNA damage. *Andrologia* 42(5):305-13
- Henkel R, Kierspel E, Stalf T, Mehnert C, Menkveld R, Tinneberg HR, Schill WB, Kruger TF. 2005. Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in nonleukocytospermic patients. *Fertil Steril* 83:635-642

- Henkel R. 2007. DNA fragmentation and its influence on fertilization and pregnancy outcome. In: Oehninger SC, Kruger TF (eds). *Male Infertility: Diagnosis and Treatment. Parthenon Publishing, USA*, 277–290
- Holstein AF, Roosen-Runge EC. 1981. Atlas of Human Spermatogenesis. Berlin, Germany: Grosse-Verlag 224 p ill; 31 cm
- Holt WV. Is quality assurance in semen analysis still really necessary? 2005. A spermatologist's viewpoint. *Hum Reprod* 20(11):2983-6
- Hoshi, K., Katayose, H., Yanagida, K., Kimura, Y. and Sato, A. 1996. The relationship between acridine orange fuorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil Steril* 66: 634-639
- Hsieh YY, Chang CC, Lin CS. 2006.Seminal malondialdehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration and motility. *Int J Biol Sci* 2:23–29
- Hsieh YY, Sun YL, Chang CC, Lee YS, Tsai HD, Lin CS. 2002.Superoxide dismutase activities of spermatozoa and seminal plasma are not correlated with male infertility. *J Clin Lab Anal* 16:127–131
- Hsu HS, Chang LS, Chen MT and Wei YH. 1994. Decreased blood flow and defective energy metabolism in the varicocele-bearing testicles of rats. *Eur Urol* 25:71–75
- Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. 1996. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol Hum Reprod* 2(8):613-9
- Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. 1998. The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. *Hum Reprod* 13(5):1240-7
- Hughes L, Ryder TA, Mckensie ML, Pryse-Davies J, Stedronska J and Hendry WF. 1981. The use of transmission electron microscopy to study non-spermatozoal cells in semen. Oligozoospermia—*Recent Progress in Andrology*. Raven Press, New York pp. 65-75

- Hurtado de Catalfo GE, Ranieri-Casilla A, Marra FA, de Alaniz MJ, Marra CA. 2007.Oxidative stress biomarkers and hormonal profile in human patients undergoing varicocelectomy. *Int J Androl* 30:519–530
- Huszar G, Corrales M, Vigue L. 1988a. Correlation between sperm creatine phosphokinase activity and sperm concentrations in normospermic and oligospermic men. *Gamete Res* 19:67–75
- Huszar G, Sbracia M, Vigue L, Miller DJ, Shur BD. 1997. Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane beta 1,4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. *Biol Reprod* 56(4):1020-4
- Huszar G, Vigue L, Corrales M. 1988b. Sperm creatine phosphokinase activity as a measure of sperm quality in normospermic, variable spermic, and oligospermic men. *Biol Reprod* 38:1061–1066
- Irvine DS, Twigg JP, Garden EL, Fulton N, Milne PA, Ailken RJ. 2000. DNA integrity in human spermatozoa; relationship with semen quality. *J Androl* 21:33-44
- Ishikawa T, Fujisawa M. 2005. Effect of age and grade on surgery for patients with varicocele. *Urology* 65(4):768-72
- Iwasaki A, Gagnon C. 1992. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 57(2):409-416
- Jarow JP. 2001. Effects of varicocele on male fertility. *Hum Reprod Update* 7(1):59-64
- Jenkins J, Daya S, Kremer J, Balasch J, Barratt C, Cooke I, Lawford-Davies J, De Sutter P, Suikari AM, Neulen J, Nygren K. 2004. European Classification of Infertility Taskforce (ECIT) response to Habbema et al., 'Towards less confusing terminology in reproductive medicine: a proposal'. *Hum Reprod* 19:2687–2688
- Jeulin C, Soufir JC, Weber P, Laval-Martin D, Calvayrac R. 1989. Catalase activity in human spermatozoa and seminal plasma. *Gamete Res* 24:185–196

- Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 70(1):219-28
- Jochum M, Pabst W, Schill WB. 1986. Granulocyte elastase as a sensitive diagnostic parameter of silent male genital tract inflammation. *Andrologia* 18(4):413-9
- Jones R, Mann T, Sherins R. 1979. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril* 31(5):531-7
- Jørgensen N, Andersen AG, Eustache F, Irvine DS, Suominen J, Petersen JH, Andersen AN, Auger J, Cawood EH, Horte A, Jensen TK, Jouannet P, Keiding N, Vierula M, Toppari J, Skakkebaek NE. 2001. Regional differences in semen quality in Europe. *Hum Reprod* 16(5):1012-9
- Kambayashi Y, Binh NT, W Asakura H, Hibino Y, Hitomi Y, Nakamura H, Ogino K. 2009. Efficient assay for total antioxidant capacity in human plasma using a 96-well microplate. *J Clin Biochem Nutr* 44(1):46-51
- Kampa M, Nistikaki A, Tsaousis V, Maliaraki N, Notas G, Castanas E. 2002. A new automated method for the determination of the Total Antioxidant Capacity (TAC) of human plasma, based on the crocin bleaching assay. *BMC Clin Pathol* 2(1):3
- Kantartzi PD, Goulis ChD, Goulis GD, Papadimas I. 2007. Male infertility and varicocele: myths and reality. *Hippokratia* 11(3):99-104
- Kao SH, Chao HT, Chen HW, Hwang TI, Liao TL, Wei YH. 2008. Increase of oxidative stress in human sperm with lower motility. *Fertil Steril* 89(5):1183-90
- Kasperczyk A, Kasperczyk S, Horak S, Ostalowska A, Grucka-Mamczar E, Romuk E, Olejek A, Birkner E. 2008. Assessment of semen function and lipid peroxidation among lead exposed men. *Toxicol Appl Pharmacol* 228:378–384

- Katsuki T, Hara T, Ueda K, Tanaka J, Ohama K. 2005. Prediction of outcomes of assisted reproduction treatment using the calcium ionophore-induced acrosome reaction. *Hum Reprod* 20: 469-75
- Kay VJ, Robertson L. 1998.Hyperactivated motility of human spermatozoa: a review of physiological function and application in assisted reproduction. *Hum Reprod Update* 4:776–786
- Keel B, Webster B. 1990. The standard semen analysis. In Webster B (ed.) CRC *Handbook of the Laboratory Diagnosis and Treatment of Infertility*. Boca Raton, FL: CRC Press, 27–69
- Kemal Duru N, Morshedi M, Oehninger S. 2000. Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. *Fertil Steril* 74(6):1200-7
- Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H, Hammami S, Zghal K, Fki H, Damak J, Bahloul A. 2003.Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Arch Androl* 49:83–94
- Kessopoulou E, Tomlinson MJ, Barratt CL, Bolton AE, Cooke ID. 1992. Origin of reactive oxygen species in human semen: spermatozoa or leucocytes? *J Reprod Fertil* 94(2):463-70
- Khosrowbeygi A, Zarghami N. 2007.Levels of oxidative stress biomarkers in seminal plasma and their relationship with seminal parameters. BMC *Clin Pathol* 7:6
- Kierszenbaum AL. 2001. Transition nuclear proteins during spermiogenesis: unrepaired DNA breaks not allowed. *Mol Reprod Dev* 58(4):357-8
- Kiessling AA, Lamparelli N, Yin HZ, Seibel MM, Eyre RC.1995. Semen leukocytes: friends or foes? *Fertil Steril* 64:196-198
- Klaude M, Eriksson S, Nygrer J, Ahnstrom G. 1996. The comet assay: mechanism and technical considerations. *Mutat Res* 363: 89-96

- Kobayashi H, Gil-Guzman E, Mahran AM, Rakesh, Nelson DR, Thomas AJ Jr, Agarwal A. 2001. Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. *J Androl* 22(4):568-74
- Koca Y, Ozdal OL, Celik M, Unal S, Balaban N. 2003.Antioxidant activity of seminal plasma in fertile and infertile men. *Arch Androl* 49:355–359
- Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. 1997. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 68(3):519-24
- Köksal IT, Tefekli A, Usta M, Erol H, Abbasoglu S, Kadioglu A. 2000. The role of reactive oxygen species in testicular dysfunction associated with varicocele. *BJU Int* 86(4):549-52
- Kolettis PN. 2002. The evaluation and management of the azoospermic patient. *J Androl* 23(3):293-305
- Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. 2008. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* 93(8):3199-207
- Koppers AJ, Garg ML, Aitken RJ. 2010. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radic Biol Med* 48(1):112-9
- Kosower NS, Katayose H, Yanagimachi R. 1992. Thiol-disulfide status and acridine orange fluorescence of mammalian sperm nuclei. *J Androl* 13(4):342-8
- Kovalski NN, de Lamirande E, Gagnon C. 1992. Reactive oxygen species generated by human neutrophils inhibit sperm motility: protective effect of seminal plasma and scavengers. *Fertil Steril* 58(4):809-16
- Krause W. 1995. Computer-assisted semen analysis systems: comparison with routine evaluation and prognostic value in male fertility and assisted reproduction. *Hum Reprod* 10(1):60-6
- Kremer J, Jager S. 1992. The significance of antisperm antibodies for sperm-cervical mucus interaction. *Hum Reprod* 7:781–784

- Kruger TF, du Toit TC, Franken DR, Menkveld R, Lombard CJ. 1995. Sperm morphology: assessing the agreement between the manual method (strict criteria) and the sperm morphology analyzer IVOS *Fertil Steril* 63(1):134-4
- Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, van Zyl JA,Smith K. 1986.Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil Steril* 46:1118–1123
- Ku JH, Shim HB, Kim SW, Paick JS. 2005. The role of apoptosis in the pathogenesis of varicocele. *BJU Int* 96(7):1092-6
- Kurpisz M, Miesel R, Sanocka D, Jedrzejczak P. 1996. Seminal plasma can be a predictive factor for male infertility. *Hum Reprod* 11(6):1223-6
- Laberge RM, Boissonneault G. 2005. On the nature and origin of DNA strand breaks in elongating spermatids. *Biol Reprod* 73(2):289-96
- Lackner JE, Agarwal A, Mahfouz R, du Plessis SS, Schatzl G. 2010. The association between leukocytes and sperm quality is concentration dependent. *Reprod Biol Endocrinol* 8:12
- Lee CH, Cho YH. 1999. Aspects of mammalian spermatogenesis: electrophoretical analysis of protamines in mammalian species. *Mol Cells* 9(5):556-9
- Lee J, Richburg JH, Younkin SC and Boekelheide K. 1997. The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology 138:2081–2088
- Lemack GE, Uzzo RG, Schlegel PN, Goldstein M. 1998. Microsurgical repair of the adolescent varicocele. *J Urol* 160: 179- 181
- Lemcke B, Behre HM, Nieschlag E. 1997. Frequently subnormal semen profiles of normal volunteers recruited over 17 years. *Int J Androl* 20:144–52
- Lenzi A, Picardo M, Gandini L, Lombardo F, Terminali O, Passi S, Dondero F. 1994. Glutathione treatment of dyspermia: effect on the lipoperoxidation process. *Hum Reprod* 9(11):2044-50
- Lenzi A, Sgrò P, Salacone P, Paoli D, Gilio B, Lombardo F, Santulli M, Agarwal A, Gandini L. 2004. A placebo-controlled double-blind randomized trial of the use of combined l-carnitine and l-acetyl-carnitine treatment in men with asthenozoospermia. *Fertil Steril* 81(6):1578-84

- Lewin A, Lavon H. 1997. The effect of coenzyme Q10 on sperm motility and function. *Mol Aspects Med* 18 Suppl:S213-9
- Lewis SE, Sterling ES, Young IS, Thompson W. 1997. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertil Steril* 67(1):142-7
- Lewis SE. 2007. Is sperm evaluation useful in predicting human fertility? *Reproduction* 134(1):31-40
- Li H, Dubocq F, Jiang Y, Tiguert R, Gheiler EL and Dhabuwala CB. 1999. Effect of surgically induced varicocele on testicular blood flow and Sertoli cell function. *Urology* 53:1258–1262
- Li K, Shang X, Chen Y. 2004. High-performance liquid chromatographic detection of lipid peroxidation in human seminal plasma and its application to male infertility. *Clin Chim Acta* 346:199–203
- Liochev SI, Fridovich I. 1997. Lucigenin (Bis-N-methylacridinium) as a mediator of superoxide anion production. *Archives Biochem Biophysics* 337:115–120
- Liu DY, Baker HW. 1992. Morphology of spermatozoa bound to the zona pellucida of human oocytes that failed to fertilize in vitro. *J Reprod Fertil* 94:71–84
- Liu DY, Baker HW. 1994. Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm-zona pellucida penetration and reduced fertilization in vitro. *Hum Reprod* 9(9):1694-700
- Loft S, Kold-Jensen T, Hjollund NH, Giwercman A, Gyllemborg J, Ernst E, Olsen J, Scheike T, Poulsen HE, Bonde JP. 2003. Oxidative DNA damage in human sperm influences time to pregnancy. *Hum Reprod* 18(6):1265-72
- Lopes S, Jurisicova A, Sun JG, Casper RF. 1998. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Hum Reprod* 13(4):896-900
- Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF. 1998. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples

and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril* 69(3):528-32

- Lü JM, Lin PH, Yao Q, Chen C. 2010. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *J Cell Mol Med* 14(4):840-60
- Lund L, Larsen SB. 1998. A follow-up study of semen quality and fertility in men with varicocele testis and in control subjects. *Br J Urol* 82(5):682-6
- Macleod IC, Irvine DS. 1995. The predictive value of computer-assisted semen analysis in the context of donor insemination programme. *Hum Reprod* 10:580–586
- Macleod J, Wang Y. 1979. Male fertility potential in terms of semen quality: a review of the past, a study of the present. *Fertil Steril* 31: 103–16
- MacLeod J. 1965.Seminal cytology in presence of varicocele. Fertil Steril 16:735
- MacLeod J: 1943. The role of oxygen in the metabolism and motility of human spermatozoa. *Am J Physiol* 138:512-518
- Mahfouz R, Sharma R, Sharma D, Sabanegh E, Agarwal A. 2009. Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma. *Fertil Steril* 91(3):805-11
- Makhlouf AA, Niederberger C. 2006. DNA integrity tests in clinical practice: it is not a simple matter of black and white (or red and green). *J Androl* 27:316–323
- Makker K, Agarwal A, Sharma R. 2009. Oxidative stress & male infertility. *Indian J Med Res* 129(4):357-67
- Mancini A, Festa R, Raimondo S, Silvestrini A, Giacchi E, Littarru GP, Pontecorvi A, Meucci E. 2012. Biochemical alterations in semen of varicocele patients: a review of the literature. *Adv Urol* 2012:903931
- Mancini A, Milardi D, Bianchi A, Festa R, Silvestrini A, De Marinis L, Pontecorvi A, Meucci E. 2007. Increased total antioxidant capacity in seminal plasma of varicocele patients: a multivariate analysis. *Arch Androl* 53(1):37-42

- Maneesh M and Jayalekshmi. 2006. Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. *Indian Journal of Clinical Biochemistry* 21(2):80-89
- Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, Sakkas D. 1995. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biol Reprod* 52(4):864-7
- Marcon L, Boissonneault G. 2004. Transient DNA strand breaks during mouse and human spermiogenesis new insights in stage specificity and link to chromatin remodeling. *Biol Reprod* 70(4):910-8
- Marmar JL. Varicocele and male infertility: Part II. 2001. The pathophysiology of varicoceles in the light of current molecular and genetic information. *Hum Reprod Update* 7: 461-472
- Matorras R, Diez J, Corcostegui B, Gutiérrez de Terán G, García JM, Pijoan JI,
- Mazzilli F, Rossi T, Marchesini M, Ronconi C, Dondero F. 1994.Superoxide anion in human semen related to seminal parameters and clinical aspects. *Fertil Steril* 62: 862–868
- McKelvey-Martin VJ, Melia N, Walsh IK, Johnston SR, Hughes CM, Lewis SEM, Thompson W. 1997. Two potential clinical applications of the alkaline single-cell gel electrophoresis assay: (1) human bladder washings and transitional cell carcinoma of the bladder: and (2) human sperm and male infertility. *Mutat Res* 375:93-104
- McLachlan RI, de Kretser DM. 2001. Male infertility: the case for continued research. *Med J Aust* 174(3):116-7
- McPherson S, Longo FJ. 1993. Chromatin structure-function alterations during mammalian spermatogenesis: DNA nicking and repair in elongating spermatids. *Eur J Histochem* 37(2):109-28
- Meacham RB, Townsend RR, Rademacher D, Drose JA. 1994. The incidence of varicoceles in the general population when evaluated by physical examination, gray scale sonography and color Doppler sonography. *J Urol* 151(6):1535-8

- Mehraban D, Ansari M, Keyhan H, Sedighi Gilani M, Naderi G, Esfehani F. 2005. Comparison of nitric oxide concentration in seminal fluid between infertile patients with and without varicocele and normal fertile men. *Urol J* 2(2):106-10
- Menkveld R, Rhemrev JP, Franken DR, Vermeiden JP, Kruger TF. 1996. Acrosomal morphology as a novel criterion for male fertility diagnosis: relation with acrosin activity, morphology (strict criteria), and fertilization in vitro. *Fertil Steril* 65: 637–44
- Menkveld R, Wong WY, Lombard CJ, Wetzels AM, Thomas CM, Merkus HM, Steegers-Theunissen RP. 2001. Semen parameters, including WHO and strict criteria morphology, in a fertile and subfertile population: an effort towards standardization of in-vivo thresholds. *Hum Reprod* (6):1165-71
- Mennella M, Jones R. 1980. Properties of spermatozoal superoxide dismutase and lack of involvement of superoxides in metal-ion-catalyzed lipid peroxidation and reactions in semen. *Biochem J* 191:289–297
- Merino Ruiz MC, De Leon Cervantes MG, Garcia Flores RF. 1995. Male sterility and its association with genital disease and environmental factors. *Ginecol Obstet Mex* 63:427–431
- Meucci E, Milardi D, Mordente A, Martorana GE, Giacchi E, De Marinis L, Mancini A. 2003. Total antioxidant capacity in patients with varicoceles. *Fertil Steril* 79 Suppl 3:1577-83
- Miesel R, Jedrzejczak P, Sanocka D, Kurpisz MK. 1997. Severe antioxidase deficiency in human semen samples with pathological spermiogram parameters. *Andrologia* 29(2):77-83
- Mieusset R and Bujan L. 1995. Testicular heating and its possible contributions to male infertility: a review. *Int J Androl* 18:169–184
- Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci (Lond)* 84(4):407-12
- Miller NJ, Rice-Evans CA. 1996. Spectrophotometric determination of antioxidant activity. *Redox Report* 2:161-171

- Miller NJ, Rice-Evans CA. 1997. Factors influencing the antioxidant activity determined by the ABTS + radical cation assay. Free Radic Res. 1997 Mar; 26(3):195-9. Erratum in: *Free Radic Res* 26(6):594
- Moench GL, Holt H. 1931. Sperm morphology in relation to fertility. *Am J Obstet Gynecol* 22:199–210
- Moghissi KS, Wallach EE. 1983. Unexplained infertility. *Fertil Steril* 39(1):5-21
- Moein MR, Dehghani VO, Tabibnejad N, Vahidi S. 2007. Reactive Oxygen Species (ROS) level in seminal plasma of infertile men and healthy donors. *Iran J Reprod Med* 5(2): 51–55
- Molina J, Castilla JA, Castano JL, Fontes J, Mendoza N, Martinez L. 2001.Chromatin status in human ejaculated spermatozoa from infertile patients and relationship to seminal parameters. *Hum Reprod* 16: 534–539
- Moore HD. 1998. Contribution of epididymal factors to sperm maturation and storage. *Andrologia* 30(4-5):233-9
- Mori MM, Bertolla RP, Fraietta R, Ortiz V, Cedenho AP. 2008. Does varicocele grade determine extent of alteration to spermatogenesis in adolescents? *Fertil Steril* 90(5):1769-73
- Morris ID, Ilott S, Dixon L, Brison DR. 2002. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Hum Reprod* 17(4):990-8
- Mortimer D, 1994. Practical Laboratory Andrology. New York, NY: *Oxford University Press*
- Mortimer D, Pandya IJ, Sawers RS. 1986. Relationship between human sperm motility characteristics and sperm penetration into human cervical mucus in vitro. *J Reprod Fertil* 78(1):93-102
- Mortimer D. 1990. Objective analysis of sperm motility and kinematics. In: Keel BA, Webster BW, eds. CRC Handbook of the Laboratory Diagnosis and Treatment of Infertility. Boston, Mass: *CRC Press*; 97–133
- Mortimer ST. 2000.CASA-practical aspects. *J Androl* 21: 515–524

- Mostafa T, Anis T, El Nashar A, Imam H, Osman I. 2012. Seminal plasma reactive oxygen species-antioxidants relationship with varicocele grade. *Andrologia* 44:66–69
- Mostafa T, Anis T, Imam H, El-Nashar AR, Osman IA. 2009. Seminal reactive oxygen species-antioxidant relationship in fertile males with and without varicocele. *Andrologia* 41: 125–129
- Mostafa T, Anis TH, El-Nashar A, Imam H, Othman IA. 2001. Varicocelectomy reduces reactive oxygen species levels and increases antioxidant activity of seminal plasma from infertile men with varicocele. *Int J Androl* 24(5):261-5
- Mostafa T, Tawadrous G, Roaia MM, Amer MK, Kader RA, Aziz A. 2006. Effect of smoking on seminal plasma ascorbic acid in infertile and fertile males. *Andrologia* 38:221–224
- Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ Jr, Agarwal A. 2004. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod* 19(1):129-38
- Muller CH. 2000. Rationale, interpretation, andrology lab corner validation, and uses of sperm function tests. *J Androl* 21:10–30
- Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G, Baldi E. 2003. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *J Androl* 24(2):253-62
- Muratori M, Marchiani S, Maggi M, Forti G, Baldi E. 2006. Origin and biological significance of DNA fragmentation in human spermatozoa. *Front Biosci* 11:1491-9
- Muratori M, Tamburrino L, Marchiani S, Guido C, Forti G, Baldi E. 2010. Critical aspects of detection of sperm DNA fragmentation by TUNEL/flow cytometry. *Syst Biol Reprod Med* 56(4):277-85
- Naftulin BN, Samuels SJ, Hellstrom WJ, Lewis EL, Overstreet JW. 1991. Semen quality in varicocele patients is characterized by tapered sperm cells. *Fertil Steril* 56: 149–51

- Nakamura H, Kimura T, Nakajima A, Shimoya K, Takemura M, Hashimoto K, Isaka S, Azuma C, Koyama M, Murata Y. 2002.Detection of oxidative stress in seminal plasma and fractionated sperm from subfertile male patients. *Eur J Obstet Gynecol Reprod Biol* 105:155–160
- Nallella KP, Allamaneni SS, Pasqualotto FF, Sharma RK, Thomas AJ Jr, Agarwal A. 2004. Relationship of interleukin‑6 with semen characteristics and oxidative stress in patients with varicocele. *Urology* 64: 1010–1013
- Nallella KP, Sharma RK, Aziz N, Agarwal A. 2006. Significance of sperm characteristics in the evaluation of male infertility. *Fertil Steril* 85(3):629-34
- Nasr-Esfahani MH, Abasi H, Razavi S, Ashrafi S, Tavalaee M. 2009. Varicocelectomy: semen parameters and protamine deficiency. *Int J Androl* 32(2):115-2
- Nasr-Esfahani MH, Razavi S, Mozdarani H, Mardani M, Azvagi H. 2004. Relationship between protamine deficiency with fertilization rate and incidence of sperm premature chromosomal condensation post-ICSI. *Andrologia* 36(3):95-100
- Nasr-Esfahani MH, Razavi S, Vaezi GH, Shiravi AH, Tavalee M. 2006. Effect of acrosomal activity and morphology on fertilization post ICSI. *Med J Reprod & Infertil* 7: 217-224
- Naughton CK, Nangia AK, Agarwal A. 2001. Pathophysiology of varicoceles in male infertility. *Hum Reprod Update* 7(5):473-81
- Naz RK, Menge AC. 1994. Antisperm antibodies: origin, regulation, and sperm reactivity in human infertility. *Fertil Steril* 61:1001–1013
- O¨stling O, Johanson KJ. 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun* 123:291–298
- O'Connell M, McClure N & Lewis SE. 2002. A comparison of mitochondrial and nuclear DNA status in testicular sperm from fertile men and those with obstructive azoospermia. *Hum Reprod* 17:1571–7
- Ochsendorf FR. 1999. Infections in the male genital tract and reactive oxygen species. *Hum Reprod Update* 5(5):399-420

- Oehninger S, Franken DR, Sayed E, Barroso G, Kolm P. 2000. Sperm function assays and their predictive value for fertilization outcome in IVF therapy: a meta-analysis. *Hum Reprod Update* 6:160–168
- Oliva R. 2006. Protamines and male infertility. *Hum Reprod Update* 12(4):417-35
- Olive PL, Durand RE, Banath JP, Johnson PJ. 2001. Analysis of DNA damage in individual cells. *Methods Cell Biol* 64:235-49
- Olive PL, Wlodek D, Durand RE, Banath JP. 1992. Factors influencing DNA migration from individual cells subjected to gel electrophoresis. *Exp Cell Res* 198:259-67
- Ollero M, Gil-Guzma´n E, Sharma RK, Lo´pez MC, Larson KL, Evenson DP, Agarwal A, Thomas AJ, Alvarez JG. 2001. Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. *Hum Reprod* 16:1912–1921
- Ombelet W, Bosmans E, Janssen M, Cox A, Vlasselaer J, Gyselaers W, Vandeput H, Gielen J, Pollet H, Maes M, Steeno O, Kruger T. 1997. Semen parameters in a fertile versus subfertile population: a need for change in the interpretation of semen testing. *Hum Reprod* 12(5):987-93
- Overstreet JW, Hembree WC. 1976. Penetration of zona pellucida of non-living human oocytes by human spermatozoa in vitro. *Fertil Steril* 27: 815–31
- Ozmen B, Koutlaki N, Youssry M, Diedrich K, Al-Hasani S. 2007. DNA damage of human spermatozoa in assisted reproduction: origins, diagnosis, impacts and safety. *Reprod Biomed Online* 14(3):384-95
- Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, Thomas AJ, Jr. Glander HJ, Agarwal A. 2004. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol Reprod* 71:1828-37
- Pacey AA. 2010. Quality assurance and quality control in the laboratory andrology. *Asian J Androl* 12:21–25

- Padron OF, Brackett NL, Sharma RK, Kohn S, Lynn CM, Thomas AJ Jr and Agarwal A. 1997. Seminal reactive oxygen species, sperm motility and morphology in men with spinal cord injury. *Fertil Steril* 67 (6):1115-1120
- Papadimas J, Mantalenakis S. 1983.Hormonal profile in infertile men. *Arch Androl* 11: 73-80
- Pasqualotto FF, Lucon AM, de Góes PM, Sobreiro BP, Hallak J, Pasqualotto EB, Arap S. 2005. Semen profile, testicular volume, and hormonal levels in infertile patients with varicoceles compared with fertile men with and without varicoceles. *Fertil Steril* 83(1):74-7
- Pasqualotto FF, Sharma RK, Kobayashi H, Nelson DR, Thomas AJ Jr, Agarwal A. 2001. Oxidative stress in normospermic men undergoing infertility evaluation. *J Androl* 22(2):316-22
- Pasqualotto FF, Sharma RK, Nelson DR, Thmas AJ, Agarwal A. 2000. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. *Fertil Steril* 73:459-464
- Pasqualotto FF, Sharma RK, Pasqualotto EB, Agarwal A. 2008. Poor semen quality and ROS-TAC scores in patients with idiopathic infertility. *Urol Int* 81(3):263-70
- Pasqualotto FF, Sundaram A, Sharma RK, Borges E Jr, Pasqualotto EB, Agarwal A. 2008. Semen quality and oxidative stress scores in fertile and infertile patients with varicocele. *Fertil Steril* 89(3):602-7
- Peedicayil J, Deendayal M, Sadasivan G, Shivaji S. 1997. Assessment of hyperactivation, acrosome reaction and motility characteristics of spermatozoa from semen of men of proven fertility and unexplained infertility. *Andrologia* 29:209–218
- Peeraer K, Nijs M, Raick D, Ombelet W, Dunphy BC. 2004. Pregnancy after ICSI with ejaculated immotile spermatozoa from a patient with immotile cilia syndrome: a case report and review of the literature. *Reprod Biomed Online* 9: 659–63

- Perreault SD, Aitken RJ, Baker HW, Evenson DP, Huszar G, Irvine DS, Morris ID, Morris RA, Robbins WA, Sakkas D, Spano M, Wyrobek AJ. 2003. Integrating new tests of sperm genetic integrity into semen analysis: breakout group discussion. *Adv Exp Med Biol* 518:253–268
- Plante M, de Lamirande E, Gagnon C. 1994. Reactive oxygen species released by activated neutrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. *Fertil Steril* 62(2):387-93
- Potts JM, Pasqualotto FF. 2003. Seminal oxidative stress in patients with chronic prostatitis. *Andrologia* 35(5):304-8
- Potts RJ, Notarianni LJ, Jefferies TM. 2000. Seminal plasma reduces exogenous oxidative damage to human sperm, determined by the measurement of DNA strand breaks and lipid peroxidation. *Mutat Res* 447(2):249-56
- Rajasekaran M, Hellstrom W, Sikka S. 1996. Quantitative assessment of cytokines (GRO alpha and IL-10) in human seminal plasma during genitourinary inflammation. *Am J Reprod Immunol* 36(2):90-5
- Rajasekaran M, Hellstrom WJ, Naz RK, Sikka SC. 1995. Oxidative stress and interleukins in seminal plasma during leukocytospermia. *Fertil Steril* 64(1):166-71
- Redmon JB, Carey P, Pryor JL. 2002. Varicocele-the most common cause of male factor infertility? *Hum Reprod Update* 8: 53-58
- Reichart M, Kahane I, Bartoov B. 2000. In vivo and in vitro impairment of human and ram sperm nuclear chromatin integrity by sexually transmitted Ureaplasma urealyticum infection. *Biol Reprod* 63(4):1041-8
- Rice-Evans CA. 2000. Measurement of total antioxidant activity as a marker of antioxidant status in vivo: procedures and limitations. *Free Radic Res* 33 Suppl:S59-66
- Rivlin J, Mendel J, Rubinstein S, Etkovitz N, Breitbart H. 2004. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. *Biol Reprod* 70:518-522
- Roberts LJ, Morrow JD. 2000. Measurement of F (2)-isoprostanes as an index of oxidative stress in vivo. *Free Radic Biol Med* 28:505-513

- Rodrigues-Rigau LJ, Smith KD and Steinberger E. 1981. Varicocele and morphology of spermatozoa. *Fertil Steril* 35:54-57
- Rodriguez I, Ody C, Araki K, Garcia I, Vassalli P. 1997. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J* 16(9):2262-70
- Rodríguez-Escudero FJ. 1996. Spontaneous pregnancy in couples waiting for artificial insemination donor because of severe male infertility. *Eur J Obstet Gynecol Reprod Biol* 70:175–178
- Rowe PJ, Comhaire FH, Hargreave TB and Mellows HJ. 1993. WHO manual for the standardized investigation and diagnosis of infertile couple, Cambridge United Kingdom: *Cambridge University Press* 3:33-39
- Said TM, Agarwal A, Sharma RK, Mascha E, Sikka SC, Thomas AJ Jr. 2004. Human sperm superoxide anion generation and correlation with semen quality in patients with male infertility. *Fertil Steril* 82: 871–7
- Said TM, Agarwal A, Sharma RK, Thomas AJ Jr, Sikka SC. 2005. Impact of sperm morphology on DNA damage caused by oxidative stress induced by beta-nicotinamide adenine dinucleotide phosphate. *Fertil Steril* 83:95-103
- Said TM, Aziz N, Sharma RK, Lewis-Jones I, Thomas AJ, Jr., Agarwal A. 2005. Novel association between sperm deformity index and oxidative stress-induced DNA damage in infertile male patients. *Asian J Androl* 7:121-6
- Said TM, Kattal N, Sharma RK, Sikka SC, Thomas AJ Jr, Mascha E, Agarwal A. 2003. Enhanced chemiluminescence assay vs colorimetric assay for measurement of the total antioxidant capacity of human seminal plasma. *J Androl* 24(5):676-80
- Sailer BL, Jost LK, Evenson DP. 1995. Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J Androl* 16(1):80-7
- Sakamoto, Y., Ishikawa, T., Kondo, Y., Yamaguchi, K. & Fujisawa, M. 2008.The assessment of oxidative stress in infertile patients with varicocele. *BJU Int* 101:1547–1552
- Sakkas D, Alvarez JG. 2010. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 93:1027‑36

- Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. 1999. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 4(1):31-7
- Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizarro D. 2002. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod* 66: 1061–7
- Sakkas D, Seli E, Bizzaro D, Tarozzi N, Manicardi GC. 2003. Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reprod Biomed Online* 7(4):428-32
- Saleh RA and Agarwal A. 2002. Oxidative stress and male infertility: from research bench to clinical practice. *J Androl* 23:737–752
- Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, Nelson DR and Thomas AJ. 2003b. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 79 (3):1597–1605
- Saleh RA, Agarwal A, Sharma RK, Said TM, Sikka SC, Thomas AJ Jr. 2003. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. *Fertil Steril* 80(6):1431-6
- Samplaski MK, Agarwal A, Sharma R, Sabanegh E. 2010 New generation of diagnostic tests for infertility: review of specialized semen tests. *Int J Urol* 17(10):839-47
- Sanocka D, Jedrzejczak P, Szumala-Kaekol A, Fraczek M, Kurpisz M. 2003.Male genital tract inflammation: The role of selected interleukins in regulation of pro-oxidant and antioxidant enzymatic substances in seminal plasma. *J Androl* 24:448–455
- Sanocka D, Kurpisz M. 2004. Reactive oxygen species and sperm cells. *Reprod Biol Endocrinol* 2:12
- Sanocka D, Miesel R, Jedrzejczak P, Chelmonska-Soyta AC, Kurpisz M. 1997.Effect of reactive oxygen species and the activity of antioxidant systems on human semen; association with male infertility. *Int J Androl* 20:255–264

- Schatte EC, Hirshberg SJ, Fallick ML, Lipschultz LI, Kim ED. 1998.Varicocelectomy improves sperm strict morphology and motility. *J Urol* 160:1338–1340
- Schmid TE, Eskenazi B, Baumgartner A, Marchetti F, Young S, Weldon R, Anderson D, Wyrobek AJ. 2007. The effects of male age on sperm DNA damage in healthy non-smokers. *Hum Reprod* 22(1):180-7
- Sepaniak S, Forges T, Gerard H, Foliguet B, Bene MC, Monnier-Barbarino P. 2006. The influence of cigarette smoking on human sperm quality and DNA fragmentation. *Toxicology* 223:54–60
- Sergerie M, Laforest G, Bujan L, Bissonnette F, Bleau G. 2005. Sperm DNA fragmentation: threshold value in male fertility. *Hum Reprod* 20(12):3446-51
- Shamsi MB, Kumar R, Dada R. 2008. Evaluation of nuclear DNA damage in human spermatozoa in men opting for assisted reproduction. *Indian J Med Res* 127(2):115-23
- Shang XJ, Li K, Ye ZQ, Chen YG, Yu X, Huang YF. 2004. Analysis of lipid peroxidative levels in seminal plasma of infertile men by high-performance liquid chromatography. *Arch Androl* 50:411–416
- Sharma RK and Agarwal A. 1996. Role of reactive oxygen species in male infertility. *Urology* 48(6):835-50
- Sharma RK, Pasqualotto AE, Nelson DR, Thomas AJ Jr, Agarwal A. 2001. Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. *J Androl* 22(4):575-83
- Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr, Agarwal A. 1999. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Hum Reprod* 14(11):2801-7
- Sharma RK, Sabanegh E, Mahfouz R, Gupta S, Thiyagarajan A, Agarwal A. 2010. TUNEL as a Test for Sperm DNA Damage in the Evaluation of Male Infertility. *Urology* 76(6):1380-6
- Sharma RK, Agarwal A. 2011. Laboratory evaluation of sperm chromatin-TUNEL assay In *Sperm Chromatin: Biological and Clinical Applications in Male*

*Infertility and Assisted Reproduction*, Editors: Armand Zini and Ashok Agarwal. Springer Science + Business Media, Chapter 14: 201-216.

- Sharma R, Masaki J, Agarwal A. 2013. Sperm DNA fragmentation analysis using the TUNEL assay. *Methods Mol Biol*. 927:121-36
- Sharpe RM. 2000. Environment, lifestyle and male infertility. *Baillieres Best Pract Res Clin Endocrinol Metab* 14:489–503
- Shekarriz M, Thomas AJ Jr, and Agarwal A: 1995. Incidence and level of seminal reactive oxygen species in normal men. *Urology* 45:103-7
- Shekarriz, M., A.J. Thomas, Jr., and A. Agarwal. 1995. Effects of time and sperm concentration on reactive oxygen species formation in human semen. *Arch Androl* 34(2):69-75
- Shekarriz, Sharma RK, Thomas AJ Jr., and Agarwal A: 1995. A method of human centrifugation to minimize the iotrogenic sperm injuries caused by reactive oxygen secies. *Eur Urol* 28: 31-35
- Shekarriz, Sharma RK, Thomas AJ Jr., and Agarwal A: 1995. Positive myleoperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. *Journal of Assisted Reproduction & Genetics* 12:70-4
- Shibahara H, Obara H, Ayustawati, Hirano Y, Suzuki T, Ohno A, Takamizawa S, Suzuki M. 2004. Prediction of pregnancy by intrauterine insemination using CASA estimates and strict criteria in patients with male factor infertility. *Int J Androl* 27(2):63-8
- Shiraishi K, Takihara H, Matsuyama H. 2010. Elevated scrotal temperature, but not varicocele grade, reflects testicular oxidative stress-mediated apoptosis. *World J Urol* 28(3):359-64
- Shiraishi K, Takihara H, Naito K. 2009. Testicular volume, scrotal temperature, and oxidative stress in fertile men with left varicocele. *Fertil Steril* 91(4):1388-91
- Siciliano L, Tarantino P, Longobardi F, Rago V, De Stefano C, Carpino A. 2001. Impaired seminal antioxidant capacity in human semen with hyperviscosity or oligoasthenozoospermia. *J Androl* 22(5):798-803
- Sies H. 1993. Strategies of antioxidant defense. *Eur J Biochem* 215(2):213-9

- Sigman, M. and Lopes, L. 1993. The correlation between round cells and white blood cells in the semen. *J Urol* 388: 573-574
- Sikka SC, Rajasekaran M, Hellstrom WJ. 1995. Role of oxidative stress and antioxidants in male infertility. *J Androl* 16(6):464-8
- Sikka SC. 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Front Biosci* 1:78-86
- Sikka SC. 2001. Relative impact of oxidative stress on male reproductive function. *Curr Med Chem* 8(7):851-62
- Sikka SC. 2004. Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. *J Androl* 25(1):5-18
- Silber SJ. 2000. Evaluation and treatment of male infertility. *Clin Obstet Gynecol* 43:854–888
- Silber SJ. 2001. The varicocele dilemma. *Hum Reprod Update* 7(1):70-7
- Simbini T, Umapathy E, Jacobus E, Tendaupenyu G, Mbizvo MT. Study on the origin of seminal leucocytes using split ejaculate technique and the effect of leucocytospermia on sperm characteristics. *Urol Int* 61(2):95-100
- Singh NP. 1996. Microgel electrophoresis of DNA. In: Pfeifer GP, editor, Technologies for detection of DNA damage and mutations. New York: *Plenum Press* 3-24
- Sinha Hikim AP, Swerdloff RS. 1999. Hormonal and genetic control of germ cell apoptosis in the testis. *Rev Reprod* 4(1):38-47
- Smith K. 1986. Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil Steril* 46:1118–1123
- Smith R, Kaune H, Parodi D Madariaga M, Rios R, Morales I, Castro A. 2006. Increased sperm DNA damage in patients with varicocele: relationship with seminal oxidative stress. *Hum Reprod* 21: 986–93
- Smith R, Vantman D, Ponce J, Escobar J, Lissi E. 1996. Total antioxidant capacity of human seminal plasma. *Hum Reprod* 11(8):1655-60
- Song GJ, Norkus EP, Lewis V. 2006.Relationship between seminal ascorbic acid and sperm DNA integrity in infertile men. I*nt J Androl* 29:569–575

- Spanò M, Bonde JP, Hjøllund HI, Kolstad HA, Cordelli E, Leter G. 2000. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. *Fertil Steril* 73(1):43-50
- Spano M, Cordelli E, Leter G, Lombardo F, Lenzi A, Gandini L. 1999. Nuclear chromatin variations in human spermatozoa undergoing swim up and cryopreservation evaluated by flow cytometric sperm chromatin structure assay. *Mol Hum Reprod* 5:29-37
- Ståhl O, Eberhard J, Jepson K, Spano M, Cwikiel M, Cavallin-Ståhl E, Giwercman A. 2006. Sperm DNA integrity in testicular cancer patients. *Hum Reprod* 21(12):3199-205
- Steger K, Pauls K, Klonisch T, Franke FE, Bergmann M. 2000. Expression of protamine-1 and -2 mRNA during human spermiogenesis. *Mol Hum Reprod* 6(3):219-25
- Stephen EH, Chandra A. 1998. Updated projections of infertility in the United States: 1995-2025. *Fertil Steril* 70(1):30-4
- Sukcharoen N, Keith J, Irvine DS, Aitken RJ. 1995. Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using in-vitro fertilization as a functional end-point. *Hum Reprod* 10:2928–2937
- Sun J, Jurisicova A, Casper R. 1997. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biol Reprod* 56: 602–7
- Sweeney TE, Rozum JS, Gore RW. 1995. Alteration of testicular microvascular pressures during venous pressure elevation. *Am J Physiol* 269: 37-45
- Tarín JJ, Brines J, Cano A. 1998. Antioxidants may protect against infertility. *Hum Reprod* 13(6):1415-6
- Tarozzi N, Bizzaro D, Flamigni C, Borini A. 2007. Clinical relevance of sperm DNA damage in assisted reproduction. *Reprod Biomed Online* 14(6):746-57
- Tavalaee M, Razavi S, Nasr-Esfahani MH. 2009. Influence of sperm chromatin anomalies on assisted reproductive technology outcome. *Fertil Steril* 91(4):1119-26

- Tavalee M, Nasr-Esfahani MH, Deemeh MR. 2008. Etiology and Evaluation of sperm chromatin anomalies. *Intl J of Fertil and Steril* 2(1):1-8
- Tavalee M, Razavi S, Nasr-Esfahani MH. 2007. Effects of spermiogenesis defects on fertilization and pregnancy rates in IVF patients. *Yakhteh* 9:103-110
- Tavilani H, Doosti M, Saeidi H. 2005. Malondialdehyde levels in sperm and seminal plasma of asthenozoospermic and its relationship with semen parameters. *Clin Chim Acta* 356:199–203
- Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S.1984. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil Steril* 42: 87–91
- The Practice Committee of the American Society for Reproductive Medicine. Report on varicocele and infertility. 2008. *Fertil Steril* 90: 247-249
- Therond P, Auger J, Legrand A, Jouannet P. 1996. alpha-Tocopherol in human spermatozoa and seminal plasma: relationships with motility, antioxidant enzymes and leukocytes. *Mol Hum Reprod* 2:739–744
- To¨pfer-Petersen E, Vo¨lcker C, Heissler E, Schill WB. 1987. Absence of acrosome reaction in polyzoospermia. *Andrologia* 19: 225–228
- Tomlinson MJ, Moffatt O, Manicardi GC, Bizzaro D, Afnan M, Sakkas, D. 2001. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. *Hum Reprod* 16: 2160-2165
- Tomlinson MJ, White A, Barratt CL, Bolton AE, Cooke ID. 1992. The removal of morphologically abnormal sperm forms by phagocytes: a positive role for seminal leukocytes? *Hum Reprod* 7(4):517-22
- Torregrosa N, Domı´nguez-Fandos D, Camejo MI, Shirley CR, Meistrich ML, Ballesca, JL, Oliva R. 2006. Protamine 2 precursors, protamine 1/protamine 2 ratio, DNA integrity and other sperm parameters in infertile patients. *Hum Reprod* 21:2084–2089

- Tremellen K. 2008. Oxidative stress and male infertility--a clinical perspective. *Hum Reprod Update* 14(3):243-58

- Tulloch WS. 1952. A consideration of sterility factors in the light of subsequent pregnancies. *Edinburgh Med J* 59: 29-34
- Tunc O, Thompson J, Tremellen K. 2010. Development of the NBT assay as a marker of sperm oxidative stress. *Int J Androl* 33(1):13-21

- Türkyilmaz Z, Gülen S, Sönmez K, Karabulut R, Dinçer S, Can Başaklar A, Kale N. 2004. Increased nitric oxide is accompanied by lipid oxidation in adolescent varicocele. *Int J Androl* 27(3):183-7
- Turner TT, Lysiak JJ. 2008. Oxidative stress: a common factor in testicular dysfunction. *J Androl* 29(5):488-98
- Turner TT. 2001. The study of varicocele through the use of animal models. *Hum Reprod Update* 7(1):78-84
- Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. 1998. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod* 13:1429–1436

- Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. 1998a. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Mol Hum Reprod* 4(5):439-45
- Twigg JP, Irvine DS, Aitken RJ. 1998. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum Reprod* 13(7):1864-71
- van der Steeg JW, Steures P, Eijkemans MJ, F Habbema JD, Hompes PG, Kremer JA, van der Leeuw-Harmsen L, Bossuyt PM, Repping S, Silber SJ, Mol BW, van der Veen F. 2011. Collaborative Effort for Clinical Evaluation in Reproductive Medicine Study Group. Role of semen analysis in subfertile couples. *Fertil Steril* 95(3):1013-9

- Van der Ven HH, Jeyendran RS, Perez-Pelaez M, Al-Hasani S, Diedrich K, Krebs D. 1987. Leucospermia and the fertilizing capacity of spermatozoa. Eur J Obstet *Gynecol Reprod Biol* 24(1):49-52
- Varum S, Bento C, Sousa AP, Gomes-Santos CS, Henriques P, Almeida-Santos T, Teodósio C, Paiva A, Ramalho-Santos J. 2007. Characterization of human sperm populations using conventional parameters, surface ubiquitination, and apoptotic markers. *Fertil Steril* 87(3):572-83
- Vazquez-Levin MH, Friedmann P, Goldberg SI, Medley NE, Nagler HM. 1997. Response of routine semen analysis and critical assessment of sperm morphology by Kruger classification to therapeutic varicocelectomy. *J Urol* 158:1804–1807
- Venkatesh S, Riyaz AM, Shamsi MB, Kumar R, Gupta NP, Mittal S, Malhotra N, Sharma RK, Agarwal A, Dada R. 2009. Clinical significance of reactive oxygen species in semen of infertile Indian men. *Andrologia* 41(4):251-6
- Victorino AB, Restelli AE, Bertolla RP, Spaine DM, Cedenho AP, Ortiz V. 2006 Mitochondrial activity in patients with asthenozoospermia**.** *Atlanta: American Urological Association Annual Meeting*
- Villanueva-Diaz CA, Vega-Hernandez EA, Diaz-Perez MA, Echavarria-Sanchez M, Karchmer-Krivitsky S. 1999. Sperm dysfunction in subfertile patients with varicocele and marginal semen analysis. *Andrologia* 31(5):263-7
- Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS. 1995. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* 121(4):1139-50
- Vivas-Acevedo G, Lozano JR, Camejo MI. 2010. Effect of varicocele grade and age on seminal parameters. *Urol Int* 85(2):194-9
- Wang C, Leung A, Tsoi WL, Leung J, Ng V, Lee KF, Chan SY. 1991. Computer-assisted assessment of human sperm morphology: usefulness in predicting fertilizing capacity of human spermatozoa. *Fertil Steril* 55(5):989-93

- Wang X, Sharma RK, Sikka SC, Thomas AJ Jr, Falcone T and Agarwal A. 2003. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil Steril 50(3): 531-5
- Ward WS, Coffey DS. 1991. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod* 44(4):569-74
- Warren JS, Johnson KJ, Ward PA. 1987. Oxygen radicals in cell injury and cell death. *Pathol Immunopathol Res* 6(5-6):301-15
- Wayner DD, Burton GW, Ingold KU, Locke S. 1985. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins. *FEBS Lett* 187(1):33-7
- Weber GF. 1990. The measurement of oxygen-derived free radicals and related substances in medicine*. J Clin Chem Clin Biochem* 28:569-603
- Weese DL, Peaster ML, Himsl KK, Leach GE, Lad PM, Zimmern PE. 1993. Stimulated reactive oxygen species generation in the spermatozoa of infertile men. *J Urol* 149(1):64-7
- Whittington K, Harrison SC, Williams KM, Day JL, McLaughlin EA, Hull MG, Ford WC. 1999. Reactive oxygen species (ROS) production and the outcome of diagnostic tests of sperm function. *Int J Androl* 22:236–242
- Williams AC, Ford WC. 2005. Relationship between reactive oxygen species production and lipid peroxidation in human sperm suspensions and their association with sperm function. *Fertil Steril* 83:929–936
- Wilms G, Oyen R, Casselman J, Peene P, Steeno O, Baert AL. 1988. Solitary or predominantly right-sided varicocele: a possible sign of situs inversus. *Urol Radiol* 9(4):243-6
- Witt MA, Lipshultz LI. 1993. Varicocele: a progressive or static lesion? *Urology* 42(5):541-3
- Wolff H. 1995. The biologic significance of white blood cells in semen. *Fertil Steril* 63:1143–1157

- Wolff H, Anderson DJ. 1988. Immunohistologic characterization and quantitation of leukocyte subpopulations in human semen. *Fertil Steril* 49(3):497-504
- Wolff H, Panhans A, Zebhauser M, Meurer M. 1992. Comparison of three methods to detect white blood cells in semen: leukocyte esterase dipstick test, granulocyte elastase enzyme immunoassay, and peroxidase cytochemistry. *Fertil Steril* 58(6):1260-2
- Wolff H, Politch JA, Martinez A, Haimovici F, Hill JA, Anderson DJ. 1990. Leukocytospermia is associated with poor semen quality. *Fertile Steril* 53(3):528-36
- World Health Organization. 1992. The influence of varicocele on parameters of fertility in a large group of men presenting to infertility clinics. *Fertil Steril* 57: 1289-1293
- World Health Organization. 1987. WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. 2nd ed Cambridge: the Press Syndicate of the University of Cambridge
- World Health Organization. 1992. WHO Laboratory Manual for examination of human semen and sperm-cervical mucus interaction 3rd edition. Cambridge, United Kingdom:Cambridge University Press
- World Health Organization. 1993. WHO Laboratory Manual for Examination of Human Semen and Sperm-cervical Mucus Interaction. 4th edition, Cambridge University Press, Cambridge, UK
- World Health Organization. 1999. WHO Laboratory Manual for Examination of Human Semen and Semen–Cervical Mucus Interaction. 4th ed. Cambridge: Cambridge University Press
- World Health Organization. 2010. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: World Health Organization
- Wright EJ, Young GP and Goldstein M. 1997. Reduction in testicular temperature after varicocelectomy in infertile men. *Urology* 50:257–259
- Wroblewski N, Schill WB, Henkel R. 2003. Metal chelators change the human sperm motility pattern. *Fertil Steril* 79 (3):1584-9

- Wu GJ, Chang FW, Lee SS, Cheng YY, Chen CH, Chen IC. 2009. Apoptosis-related phenotype of ejaculated spermatozoa in patients with varicocele. *Fertil Steril* 91(3):831-7
- Wu Q, Xing J, Xue W, Sun J, Wang X, Jin X. 2009. Influence of polymorphism of glutathione S-transferase T1 on Chinese infertile patients with varicocele. *Fertil Steril* 91(3):960-2
- Xu Y, Xu QY, Yang BH, Zhu XM, Peng YF. 2008.Relationship of nitric oxide and nitric oxide synthase with varicocele infertility [Chinese]. Zhonghua Nan Ke Xue 14: 414–417
- Yagi K. 1998.Simple assay for the level of total lipid peroxides in serum or plasma. *Methods Mol Biol* 108:101-106
- Yesilli C, Mungan G, Seçkiner I, Akduman B, Açikgöz S, Altan K, Mungan A. 2005.Effect of varicocelectomy on sperm creatine kinase, HspA2 chaperone protein (creatine kinase-M type), LDH, LDH-X, and lipid peroxidation product levels in infertile men with varicocele. *Urology* 66: 610–615
- Yildiz O, Gul H, Ozgok Y, Onguru O, Kilciler M, Aydin A, Isimer A, Harmankaya AC. 2003. Increased vasoconstrictor reactivity and decreased endothelial function in high grade varicocele; functional and morphological study. *Urol Res* 31(5):323-8
- Younglai EV, Holt D, Brown P, Jurisicova A, Casper RF. 2001. Sperm swim-up techniques and DNA fragmentation. *Hum Reprod* 16:1950–1953
- Yumura Y, Iwasaki A, Saito K, Ogawa T, Hirokawa M. 2009. Effect of reactive oxygen species in semen on the pregnancy of infertile couples. *Int J Urol* 16:202–207
- Zalata A, Hafez T, Comhaire F. 1995. Evaluation of the role of reactive oxygen species in male infertility. *Hum Reprod* 10(6):1444-51
- Zalata A, Hafez T, Mahmoud A, Comhaire F. 1995. Relationship between resazurin reduction test, reactive oxygen species generation, and gamma-glutamyltransferase. *Hum Reprod* 10(5):1136-40

- Zamboni L. 1987.The ultrastructural pathology of the spermatozoon as a cause of infertility: the role of electron microscopy in the evaluation of semen quality. *Fertil Steril* 48:711–734
- Zargooshi J. 2007. Sperm count and sperm motility in incidental high-grade varicocele. *Fertil Steril* 88(5):1470-3
- Zedan H, El-Mekhlafi AW, El-Noweihi AM, Abd El-Azim NE, Mostafa T. 2009. Soluble Fas and gonadal hormones in infertile men with varicocele. *Fertil Steril* 91(2):420-4
- Zini A, Bielecki R, Phang D, Zenzes MT. 2001. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 75: 674–7
- Zini A, Boman JM, Belzile E, Ciampi A. 2008. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. *Hum Reprod* 23(12):2663-8
- Zini A, de Lamirande E, Gagnon C. 1993. Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. *Int J Androl* 16(3):183-8
- Zini A, De Lamirande E, Gagnon C. 1995. Low levels of nitric oxide promote human sperm capacitation in vitro. *J Androl* 16(5):424-31
- Zini A, Defreitas G, Freeman M, Hechter S, Jarvi K. 2000. Varicocele is associated with abnormal retention of cytoplasmic droplets by human spermatozoa. *Fertil Steril* 74:461–464
- Zini A, Garrels K, Phang D. 2000.Antioxidant activity in the semen of fertile and infertile men. *Urology* 55:922–926
- Zini A, Libman J. 2006. Sperm DNA damage: importance in the era of assisted reproduction. *Curr Opin Urol* 16(6):428-34
- Zini A, Sigman M. 2009. Are tests of sperm DNA damage clinically useful? Pros and cons. *J Androl* 30(3):219-29

- Ziyyat A, Barraud-Lange V, Sifer C, Ducot B, Wolf JP, Soufir JC: 2008. Paradoxical increase of sperm motility and seminal carnitine associated with moderate leukocytospermia in infertile patients. *Fertil Steril* 90:2257-2263