COMPARATIVE STUDY OF INTRACYTOPLASMIC SPERM INSEMINATION OUTCOME, THROUGH SPERM RETRIEVAL PROCEDURES IN MEN WITH AZOOSPERMIA

By

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CERTIFICATE

This is to certify that this dissertation submitted by Shazia Ali is accepted in its present form by the Faculty of Biological Sciences (Animal Sciences), Quaid-i-Azam University, Islamabad, as satisfying requirements for the degree of Master of Philosophy (Reproductive Physiology).

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"Nature is now here accustomed more openly to display her secret mysteries then in case where she shows traces of her working apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of nature by careful investigation of the cases of rare forms of diseases. For it has been found in almost all things, that what they contain of useful or applicable nature is hardly perceived unless we are deprived of them, or they become deranged in some way"

To Holy Prophet (Peace be upon him)

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ABSTRACT

Azoospermia is complete absence of sperms from the ejaculate. It is present in about 1% of all men and 10 -15% of infertile men. The main concern is to find success rate of ICSI procedure in which sperms are either retrieved through Percutaneous Epididymal Sperm Aspirate (Pesa) and Testicular Biopsy so, that the patients suffering from azoospermia can benefit from the study and will know which is the treatment of choice for them. In this retrospective study, the infertile couples were divided into two categories firstly, a total of 105 subjects undergoing ICSI with motile and immotile sperms retrieved through Pesa and Biopsy. Secondly, 102 subjects on the basis of female age were divided into older and younger age group and they also underwent the procedure of ICSI with sperms retrieved through Pesa and Biopsy. The subjects who underwent the ICSI procedure with motile sperms retrieved through Biopsy had highly significant ($P<0.001$) cleavage rate, pregnancy rate and live birth rate. Significantly higher ($P<0.05$) pregnancy rate and live birth rate was observed in the female of older age group undergoing ICSI with sperms retrieved through Biopsy. Therefore, the motility of the sperms of male partner and good ovarian reserve of the female partner do play an important role in the success of the Intracytoplasmic sperm injection procedure.

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ABBREVIATIONS

2-Pronuclear	2-PN
Abortion rate	AR
Cleavage rate	CR
Deoxyribonucleic acid	DNA
DNA Fragmentation Index	DFI
Fertilization rate	FR
Fine needle aspirate	FNA
Follicular Stimulating Hormone	FSH
Human chorionic gonadotropin	HCG
In vitro fertilization	IVF
International Unit	IU
Intracytoplasmic Sperm Injection	ICSI
Live birth rate	VS
Microsurgical Epididymal sperm aspirate	MESA
Non – Obstructive Azoospermia	NOA
Obstructive azoospermia	OA
Oocyte maturity rate	OMR
Percutaneous sperm aspiration	PESA
Percutaneous testicular sperm aspiration	TESA
Polyvinylpyrrolidine	PVP
Testicular sperm extraction	TESE
World Health Organization	WHO

INTRODUCTION

A healthy young couple in their mid-twenties has only 20-25% chance of establishing a pregnancy in each cycle and thus, a range of factors, each with different extent of genetic control, may influence their chances. Infertility is defined as the inability to conceive after one year of regular unprotected intercourse and accounts for one in six couples wishing to start a family. Infertility can be due to hormonal imbalance, related to age, exercise, obesity or infectious disease, it can be immunological, psychological,can result from surgery or blockage, or be associated with defined abnormalities in the gametes for example (abnormal semen parameters). The most common cause of infertility is unexplained and this accounts for about 20% of couples (Uehara et al., 2001).

Infertile couples should be investigated after their inability to conceive for a duration of twelve or months unless there is a medical history and physical findings that dictate earlier evaluation and treatment (American Society for Reproductive Medicine, 2004).

A male factor is solely responsible in about 20 percent of infertile couples and contributory in another 30 - 40 % (Thonneau et al.,1991). Azoospermia, defined as complete absence of sperm from the ejaculate, is present in about one percent of all men and 10 -15 % of infertile men (Jarrow et al .,1989).

Common causes of obstructive azoospermia (OA) include previous vasectomy, congenital absence of vas defrens and postinfective epididymitis as well as rare causes such as youngs syndrome, testicular trauma and retrograde ejaculation. In this group of patients sperm retrieval is successful in almost 100% of cases. The second group of azoospermic men are those with Non-obstructive azoospermia (NOA) cause, characterized by impaired spermatogenesis, ranging from varying degree of maturation arrest to sertoli cell – only syndrome. Clinically they often have testis of decreased volume and raised Follicular stimulating hormone (FSH) levels. The common causes of NOA include Klinefelters syndrome, iatrogenic (e.g radiotherapy), torsion, mumps, orchitis and cryptochidism. Sperm retrieval in this group is effective in approximately, 50% of cases and has been correlated with testicular histology (Tounaye et al., 1997, De Croo et al., 2000, Sousa et al., 2002).

A limited evaluation of both partners is important before reaching a final decision on the management of the couple with infertility (American Urology Association 2004).

An evaluation should be done before one year if male infertility risk factors such as history of bilateral cryptochidism are known to be present, secondly female infertility risk factors including advanced female age (over 35-years) are suspected, thirdly if the couple questions the male partners fertility potential. The initial screening evaluation of the male partner of an infertile couple should include, at a minimum, a reproductive history and two semen analyses. If possible, the two semen analysis should be separated by a time period of one month. The reproductive history should include coital frequency and timing, duration of infertility and prior fertility, childhood illnesses and developmental history, systemic medical illnesses (e.g. diabetes mellitus and upper respiratory diseases) and prior surgeries, sexual history including sexually transmitted diseases and gonadal toxin exposure including heat. If a man has history of previous fertility, this does not exclude the possibility that he can not acquire a new, secondary male infertility factor. Men with secondary infertility should be evaluated in the same way as men who have never initiated a pregnancy (primary infertility) (American Society For Reproductive Medicine, American Urological Association 2004).

If a male infertility factor is present it is always defined by the finding of an abnormal semen analysis, although other male factors may play a role even when the semen analysis is normal. Semen Analysis is the cornerstone of the laboratory evaluation of the infertile male and it helps to define the severity of the male factor causing infertility. Standard instructions have been published by the World Health Organization (WHO). These instructions include a defined period of abstinence of two to three days. Semen can be collected by masturbation or by intercourse using special semen collection condoms that do not contain substances detrimental to sperm. These specimen are collected at home or at the laboratory. The specimen should be kept at room or body temperature during the transport and examined within one hour of collection (World Health Organization 1999). Semen analysis provides information on semen volume as well as sperm concentration, motility and morphology. Sperm morphology assessment by strict criteria is used to identify couples who have a poor chance of fertilization with standard in vitro – fertilization

(IVF) or a better chance of fertilization with Intracytoplasmic sperm injection ICSI (Carson et al., 1999).

Most fertility clinics evaluate semen samples simply by conventional analysis, which does not ensure the absence of male factor problem. Men with abnormal semen parameters are at a significantly greater risk of having high levels of DNA Fragmentation (DNA fragmentation index is a highly accurate, repeatable measure of DNA quality that is proportionate to the level of DNA strand breaks in sperm). It is the best multiple regression model, including count and motility and it predicted that only 21% and 15% percent of the variation in DNA fragmentation Index and nuclear chromatin (DNA and protein) structure of these immature sperm is abnormal, with a characteristically high level of DNA stainability. When all conventional semen parameters were normal, 18% of the men still had ≥30% DFI and fell into high – risk category for poor blastocyst development and failure to initiate an ongoing pregnancy. Equally important, 39% of men with abnormal semen parameters did not have high levels of DNA fragmentation.These men were effectively treated with IVF/ ICSI and fell into the group of men (<30%DFI) that had a 47% chance of initiating a term pregnancy (Saleh et al., 2002, Sakkas et al .,1996).

Female infertility factors also favor the selection of IVF/ ICSI. The fertility status of the female partner is related to the presence or absence of specific risk factors such as endometriosis or ovulatory dysfunction and to age. When the female partner has tubal disease or has undergone tubal ligation, sperm retrieval with IVF/ ICSI is clearly preferable, because it avoids subjecting both partners to reconstructive microsurgery. The female age is important because a female fertility progressively decreases after the age 35 - years and is limited after the age of 40-years (McDermott et al.,1996). Couples may consider sperm retrieval with ICSI when female partner is greater than 37-years of age. However, in couples in which the female partner approaches age 40, the success rate of treatment with or without ICSI decreases dramatically as well (Society for Assisted Reproductive Technology 1999).

Common methods of sperm retrieval are Microsurgical epididymal sperm aspiration (MESA), Percutaneous epididymal sperm aspiration (PESA),Testicular sperm extraction (TESE) and Percutaneous testicular sperm aspiration (TESA). Less frequent used sperm retrieval methods include vassal sperm aspiration and seminal

vesicle sperm aspiration aided by transrectal ultrasonography. There is not enough data to conclude that either the technique of sperm retrieval (open or percutaneous) or the source of sperm (testicular, epididymal, vasal or seminal vesicular) significantly effect pregnancy rates. Each technique and sperm source usually provides a sufficient number of sperm for ICSI and may provide enough viable sperm for cryopreservation (Jarow JP, 1996).

It has recently been demonstrated that 50% of the total group of NOA men will have a minute amount of ongoing spermatogenesis within their testicular parenchyma. Testicular sperm extraction (TESE) is surgically employed in NOA men in the hope of harvesting some of those individual sperm that might be sparsely scattered throughout the seminiferous epithelium, which is then used with ICSI to achieve biological fatherhood. Therefore, even the most severe forms of spermatogenic compromise may be treated with ICSI. In men with NOA, ≤60% were found to have some sperm in the testis (not quantatively sufficient to spill over into the ejaculate that could be retrieved in tiny amounts from the testes and used for successful ICSI (Silber et al., 2000).

Intracytoplasmic sperm injection (ICSI) has gained an increasing popularity due to its consistent fertilization rate and high pregnancy outcomes (Van Steirteghem et al., 1993, Palermo et al., 1995).

It appeared initially that in most severe cases, for instance those with apparently, 100% abnormal sperm morphology or cases where rare motile sperm were detected in the ejaculate could have pregnancy and delivery rates not apparently different from conventional IVF in men with normal sperm parameters (Nagy et al.,1995). By the use of ICSI procedure men with no sperm what so ever in their ejaculate could have children via sperm retrieval combined with ICSI. With obstructive azoospermia sperm could be retrieved from blocked epididymis or from the seminiferous tubules of the testes in virtually every case (Silber et al., 1995).

ICSI with surgical sperm retrieval procedures has been accepted as treatment of choice for azoospermia, which has been proven by many excellent fertilization and pregnancy outcomes of treatment cycles. A comparative study of conventional IVF versus ICSI for patients requiring microsurgical epididymal sperm aspiration (MESA)

gave overall fertilization rates and pregnancy rates of 45% and 47% respectively, for ICSI and 6.9% and 4.5% respectively, for IVF (Van Steirteghem et al.,1994). A lower blastulation rate has been demonstrated in ICSI as compared with IVF embryos, regardless of culture medium used or conditions. Embryos generated from spermatozoa of men with NOA have been shown a result of lower blastulation and implantation rate than embryos generated from ejaculated sperm or sperms from men with OA (Rosenlund et al., 1997). ICSI provided fertilization rates of 45 -75% per injected oocyte when surgically retrieved epididymal or testicular spermatozoa were used. Clinical pregnancy rates reported in the recent literature ranged from 26 -57% and delivery rates ranged from 18-54% (Balaban B et al., 1999).

There are slightly different clinical approaches employed to harvest testis tissue and sperm for ICSI. In Boston, TESE is typically carried out on a day remote from an ICSI cycle and if sperms are present the harvested testicular tissue is cryopreserved in multiple vials, each serving as a source of sperm for a later cycle of ICSI. In St.Louis all tissue extractions are microsurgical and co-ordinated with the day of oocyte retrieval during ICSI cycle. Most patients required little post-operative analgesia and no complications from TESE occurred. When ever possible, a 3mm portion of tissue was fixed for histological analysis in either Bouins or Zenkers solution and subsequently underwent standard Haematoxylin and eosin staining (Silber, 2000).

ICSI has been used largely to treat male infertility, with fertilization and pregnancy rates being comparable to those obtained in couples with good semen parameters undergoing standard in–vitro insemination. The evident ability of ICSI to achieve high fertilization and pregnancy rates has extended its application to azoospermic patients (Tournaye et al.,1994). Injected epididymal and testicular spermatozoa have been used to effect fertilization and pregnancy rates (Nagy et al., 1995). These fertilization rates were significantly lower than rates obtained with freshly ejaculated spermatozoa, but, increased fertilization and pregnancy rates were seen after a more aggressive permeabilization of sperm membrane (Palermo et al., 1996).

Palermo and Schlegel (1999) carried out a study on ICSI outcome in (308) cases according to the cause of azoospermia. The fertilization rate using fresh or cryopreserved epididymal spermatozoa was 72.4 % of (911) eggs for acquired obstruction, 73.1% of (1524) eggs for congenital cases, with clinical pregnancy rates

of 48.5% and 61.6% respectively. Spermatozoa from testicular biopsies fertilized 57.0% of (533) eggs in non-obstructive cases as compared to 80.5% of (118) eggs in obstructive azoospermia.

The clinical pregnancy rate was 49.1% for non-obstructive cases and 57.1% for testicular spermatozoa obtained in obstructive azoospermia. In cases of obstructive azoospermia fertilization and pregnancy rates with epididymal spermatozoa were higher than those achieved using spermatozoa obtained from testes of men with non-obstructive azoospermia.

Pregnancy loss in couples with surgically retrieved spermatozoa ranged between 4.0% and 15.4% comparable to that seen with ejaculated spermatozoa. The aetiology of azoospermia did not effect the live birth rate nor was there any relationship to neonatal malformation (Van Steirteghem et al., 1995). The only factor that clearly depresses the pregnancy rate with ICSI independently of the origin of the spermatozoon is maternal age (Silber et al., 1995).

Herman Tournaye (1995) carried out a study in which ICSI was done with spermatozoa recovered from a testicular biopsy specimen and few spermatozoa were recovered from wet prepararion of testicular biopsy not only in OA patients but also in NOA patients. In 32 patients out of 38 patients normal fertilization rate of 56.8% per successfully injected oocyte was obtained after ICSI of testicular spermatozoa. In 84% of the patients embryo were replaced with an overall pregnancy rate of 28.9% / testicular biopsy or 34.3% / embryo transfer, the results clearly indicate that at present an excisional testicular biopsy should be offered to all azoospermic patient irrespective of concentration of Follicular stimulating hormone (FSH), testicular size or medical history.

The first report of a pregnancy using testicular sperm for ICSI by (Schoysman et al., 1993) stimulated interest to others as minimum number of sperms were required for ICSI. Six pregnancies were achieved (one ended in miscarriage and 5 others were delivered) with ICSI and percutaneous fine needle aspiration (FNA) to obtain testicular sperms from 15 men. Although the method of using testicular FNA with ICSI is an exciting new development it should be reserved for men with irreparable

ductal obstruction because microsurgical vasovasostomy and vasoepididymostomy are more cost effective than testicular FNA with ICSI.

In a study of 197 ICSI treatment cycles (167 couples) were analyzed retrospectively, Fertilization rate after ICSI were relatively, high when non-motile testicular spermatozoa were used for microinjection, but, use of motile testicular spermatozoa were associated with still higher fertilization rate. Therefore, selection of motile spermatozoa is always preferable for ICSI (Joris et al., 1998).

The availability of ICSI has enabled the achievement of conception for men with NOA, it has also challenged scientist to consider whether there may be an increased incidence of congenital anomalies in children conceived with ICSI. It is reassuring that (Bonduelle et al.,2002) found no difference in the rate of congenital anomalies in 130 children born after ICSI compared with the 130 children born after IVF without ICSI.

The aim of the present study is to compare the ICSI outcome in various infertile couples in which the male subjects suffered from azoospermia, sperms for ICSI were retrieved through Percutaneous epididymal sperm aspirate and Testicular biopsy.

MATERIAL AND METHODS

This study included a retrospective analysis of Azoospermic patients who underwent Intracytoplasmic sperm injection (ICSI) at Islamabad Clinic Serving Infertile Couples, Islamabad, Pakistan.

Initially, the couple underwent a detail consultation in which their detailed history was taken and they were explained the basic physiology of infertility. Semen analysis of the husband was advised and hormonal profile of the female was taken on the third day of the menstrual cycle.

Semen analysis was done accordingly.

Sperm Preparation Method:

Sil Select (Fertipro) Gradient:

1. Semen analysis was performed as described in WHO manual (1992). Morphology was assessed on strict Krugers criteria. (kruger et al ., 1986, 1988).
2. 1- ml of Sil Select gradient lower layer (90%) was poured and overlaid with 1-ml of Sil Select gradient upper layer (45 %) in a 15-ml sterile polypropylene conical tube (Falcon ; Becton Dickinson , New Jersey , USA).
3. Liquefied semen sample was greatly layered on Sil Select gradient using sterile transfer pipette (Falcon; Becton Dickinson, New Jersey, USA).
4. The tube was centrifuged (Labofuge 300, Heraeus, Kendro Laboratory Products GmbH, Hanau,Germany) at 500 g for 10 minutes.
5. The pellet was gently aspirated using sterile glass Pasteur pipette (Borosilicate glass, Sigma Chemical Co., USA) and transferred into a clean, labeled 15-ml sterile polypropylene conical tube (Falcon; Becton Dickinson, New Jersey, USA).
6. 1-2 ml of culture medium (universal IVF medium, Mudi – Cult; Mollehaven, Jyllinge Denmark) was added to the tube and mixed gently.
7. The tube was again centrifuged at 250 g for 5 minutes.
8. The supernatant was discarded and the pellet was suspended again with 1 -2 ml of culture medium and step 7 was repeated.
9. Pellet was re- suspended in 0.5 - 1 ml of culture medium.

10. The final sperm concentration, % motility and sperm progression in the sperm preparation was determined using Markler chamber (Sefi-Medical instruments LTD, Israel).
11. The final sperm preparation, and a highly concentrated sperm preparation for ICSI procedure was incubated in the incubator at 37 C° and 5 % CO_2 for 30-60 minutes prior to insemination.

Subjects

The combination of ICSI and sperm retrieval procedures were offered to 105 couples in which the husbands had presented with azoospermia (obstructive and non-obstructive).

This study includes 105 female subjects and 105 male azoospermic patients, 50 patients with Non–Obstructive Azoospermia (NOA) and 55 patients with Obstructive azoospermia (OA),who underwent surgical sperm retrieval procedures like Percutaneous Epididymal Sperm Aspiration (PESA) or Fine Needle Biopsy (FNB) during a period of September 1999 to March 2005.

Diagnosis

The initial diagnosis of azoospermia is made when no spermatozoa can be detected on high powered microscope examination of centrifuged seminal fluid on at least two occasions(World Health Organization, 1999).

Percutaneous Epididymal Sperm Aspiration (PESA)

- Consent was signed by the patient before the procedure.
- Before an anesthetist anesthetized the patient, his name, wife's name and registration no. was confirmed by attending embryologist.
- Patient was anesthetized with injection Diprivan (Propofol 1%, 200mg/ 20ml) given according to dose of 1.5-2.5 mg/kg body weight. Along with the anesthesia continuous supply of oxygen was given to the patient. In this process several epididymal tubules were penetrated. Aspirate from the either left or right epididymis using disposable syringe with 0.2ml culture medium was taken by the surgeon , which was immediately passed on to the laboratory.

- Specimen was examined under the inverted microscope at x200 magnification for the presence of motile and non – motile sperm.
- When motile sperm were seen then, the specimen was used for ICSI procedure or was frozen for further use.
- When no motile sperm was seen then the other side was explored in the same manner.
- When no motile sperm were found in PESA from both left and right side then Testicular Biopsy was done.

Testicular Biopsy

When no motile sperm were seen on PESA then, Testicular biopsy was done using a syringe needle (18G). An aspirate from the testicles were taken and examined under the microscope at x 200 magnification.

Small pieces of testicular tissue were grounded in culture medium to release spermatogenic cells or mature spermatozoa from the tissue using the tissue grinder (Hunter scientific). The ground tissue (soup) was then placed in the center of culture dish, overlaid with liquid paraffin and examined under the inverted microscope at x200 magnification.

Tissue soup was then, explored for the presence of motile, non–motile sperm or spermatid.

If motile sperms were seen they were used for the ICSI procedure.

If only non–motile mature sperm were seen after exploring both sides then they were also used for ICSI procedure as there was probability of such sperm from tissue to be alive.

Before using sperm from tissue, they were fished out using ICSI injecting needle and placed in Polyvinylpyrrolidone (PVP) drop of ICSI culture dish prepared for insemination.

Treatment Protocol

Down – Regulation:

All female subjects were given long desensitizing protocol for down-regulation. Pituitary desensitization was done with subcutaneous administration of gonadotropin – releasing hormone agonist depot preparation (Decapeptyl 3.75mg; Ferring

Copenhagen NV, Denamrk) from the midluteal phase of the proceeding spontaneous menstrual cycle or after cyclic replacement of estrogens and progestins in amenorrhic patients for 21 days.

Desensitization was considered complete after four weeks of treatment if vaginal ultrasound (7.5 MHz probe, Aloka 500, Tokoyo, Japan) revealed the absence of follicle >10 mm in both ovaries.

Ovarian Stimulation

Follicular stimulation of the female subjects was carried out by using subcutaneous administration of recombinant FSH 50-IU preparation (Puregon ® ; NV organon ,Oss The Netherlands or Gonal – f ® ; Serono , Unterschleiβheim , Germany) .

The starting dose of gonadotrophins varied individually depending upon the age of the subject, basal serum follicular stimulating hormone (FSH) concentrations and response in previous treatment cycles.

The ovarian follicular response was monitored by transvaginal ultrasound three to four days after the commencement of the ovarian stimulation. The dose of medication was adjusted according to the response and monitoring was continued daily or on alternate days. When the size of the leading follicles on ultrasound was ≥20 mm in diameter, 10,000 IU of Human Chorionic Gonadotrophin (Profasi; Serono, Rome, Italy or Pregnyl; Organon , Oss, Holland) was administered intramuscularly.

Oocyte retrieval was done 35 ½ hours after Human Chorionic Gonadotropin (HCG) injection using the vaginal ultrasound technique under general anesthesia.

Oocyte/Egg Collection:

Egg collection was done 35½ hours after HCG-injection using vaginal ultrasound probe with 16G adapter and double lumen oocyte aspiration needle set (Cook Australia; Queens land, Australia). The follicular aspirate was immediately poured into 60 mm culture dish (Falcon Becton Dickinson and company, Franklin Lakes, U.S.A) and observed under phase contrast x10 magnification of stereomicroscope (Leica MZ12 ; Wetzlar,Germany)

Egg was identified by the presence of corona radiata and thick cumulus mass around it. As soon as the egg was found it was picked up using sterile glass Pasteur pipette (borosilicate, Sigma Chemical Co., USA) fixed on the tubing with 0.2 μm syringe

filter (Acrodisc; PALL Gelman Laboratoy) and a rubber teat, and transferred into the 35 mm culture dish (Falcon; Becton Dickinson, New Jersey, USA containing culture medium overlaid with liquid Paraffin oil (Medi-Cult; Mollehaven, Jyllinge, Denmark) preincubated at 37C°, 5 % CO_2 . All eggs were collected in the similar manner. When 4 – 5 eggs were collected, they were transferred to another culture dish, and incubated in the incubator (Nuaire 4500 E; Inc. Plymouth, Minnesota, USA) at 37 C°, 5% CO_2. Finally all, eggs were transferred to the incubator (Nuaire 4500 E ; USA) for about 1-2 hours prior to insemination by ICSI.

Occyte Preparation For ICSI:

Half an hour after egg collection, the eggs with cumulus and corona radiate were treated with Hyaluronidase 80-iu /ml in HEPES buffered IVF culture medium (Medi – Cult; Mollehaven, Jyllinge, Denmark). The removal of the cumulus and corona cells was enhanced by aspiration of the complexes in and out of hand – drawn glass pipette (borosilicate, Sigma Chemical Co., USA) with different diameter opening 250-300 micrometer and 200 micrometer.

The eggs were subsequently rinsed several times in droplets of culture medium and then carefully observed under the inverted microscope at x200 magnification. This included an assessment of the egg, zona pellucida, the presence or absence of a germinal vesicle or the first polar body besides the assessment of nuclear maturity. The cytoplasm of the egg was examined for the presence of vacuoles or other abnormalities in the texture of the ooplasm. The eggs were then, incubated again in 25 µl microdrops of culture medium covered with Paraffin oil (Medi–Cult; Mollehaven, Jyllinge Denmark) at 37 C° and 5 % CO_2. ICSI was carried out on all morophological intact eggs that had extruded the first polar body (Metaphase II stage).

Intracytoplasmic Sperm Injection Procedure:

For microinjection procedure, 50-mm culture dishes with microdrops containing culture medium and Polyvinylpyrrolidone (PVP) overlaid with paraffin oil (Medi–Cult; Mollehaven, Jyllinge, Denmark) preincubated at 37 C°, 5 % CO_2 were used. Sperm from concentrated sperm preparation were added to a microdrop of culture medium and motile sperm from that microdrop were immobilized by adding them to (PVP) microdrop. The eggs were also placed in microdrops containing culture medium in the same culture dish.

ICSI procedure was carried out on the heated stage of an inverted phase contrast microscope under x200 magnification (Leica, DMIRB, Leica Microsystems, Wetzlar GmbH, Wetzlar,Germany) using micromanipulators (Research Instrument Inc. USA) and 30 degree bend microinjection and holding pipettes (Hunter Scientific , Essex , UK.). ICSI is the injection of a single spermatozoon into the ooplasm under the microscope with the help of micromanipulators microinjected eggs were then, rinsed in several microdrops of culture medium and then, incubated for 16 -18 hrs at 37 C and 5 % CO2.

Assessment Of Fertilization And Cleavage:

Fertilization was confirmed 18 hours after microinjection, the eggs were observed under the stereo microscope at x200 magnification for the presence of 2 – pronuclei and polar bodies.Fertilization was considered normal when two clearly distinct pronuclei (2PN) were present. Cleavage of embryos was confirmed after another 24 hours of in –vitro culture.

Assessment Of Embryo Quality:

All embryos were graded before embryo replacement. Following embryo grading system was used for the assessment of embryo quality (Veeck 1999).

Grade 1:

Preembryo with blastomeres of equal size and no cytoplasmic fragmentation.

Grade 2:

Preembryo with blastomeres of equal size and minor cytoplasmic fragmention covering ≥10 % but, less than 25 % of preembryo surface .

Grade 3:

Preembryo with blastomeres of equal or unequal size and fragmentation covering ≥25 % but not more than 50 % of preembryo surface.

Grade 4:

Preembryo with blastomeres of equal or distinctly unequal sizes and significant fragmentation covering more than 50 % of the preembryo surface.

Grade 5:

Preembryo with few blastomeres of any size and severe fragmentation covering ≥75% of the preembryo surface.

Embryo Transfer:

Embryo transfer was carried out using Sims-Wallace Embryo Replacement Catheter (SIMS –Portex Limited , Hythe Kent , UK) at 2-4 cell stage on Day -2, Day -3 or at blastocyst stage on Day -5 of egg collection under ultrasound guidance.

Luteal Support :

Progesterone (Cyclogest ® 400 vaginal pessaries ; Shire UK) was given from the day of egg retrieval and continued until pregnancy test was done after two weeks of embryo transfer. This luteal support was continued for another 12 - weeks in pregnant subjects.

Statistical Analysis

All the values were expressed as mean ± Standard error (S.E). Limit of significance was set at $P < 0.05$ .Mean values were compared using unpaired Students *t*-test.

The whole statistical analysis was done using the statistical Graph Pad Prism software verizon 4.03 (Graph Pad Prism Inc. USA).

Various rates were calculated in the following manner. Every value is for one patient per cycle.

1. Oocyte maturity rate (%) = $\frac{\text{Total No. of Metaphase II Oocyte}}{\text{Total No. of Oocyte retrieved}} \times 100$

2. Fertilization rate (%) = $\frac{\text{Total No. of 2- Pronuclei}}{\text{Total No.of Oocyte inseminated / microinjected}} \times 100$

3. Cleavage rate (%) = $\frac{\text{Total No. of Embryo cleaved}}{\text{Total No. of 2-Pronuclei}} \times 100$

4. Pregnancy rate per cycle (%) = $\frac{\text{Total No. of Pregnancies}}{\text{Total No. of cycles}} \times 100$

5. Clinical abortion rate (%) = $\frac{\text{No. of Miscarriage}}{\text{Total No. of Pregnancies}} \times 100$

6. Live birth rate per embryo transfer (%) = $\frac{\text{Total No. of live births}}{\text{Total No. of embryo transfers}} \times 100$

History Sheet			
Name of Female:		Age:	
Name of Male:		Age:	
Presenting Complaint:			
Hormonal Profile Of Female:			
USG (Ultrasonography of female):			
Semen Analysis Of Male:			

RESULTS

This study included a retrospective analysis of Azoospermic patients who underwent Intracytoplasmic sperm injection (ICSI) at Islamabad Clinic Serving Infertile Couples, Islamabad, Pakistan.

The infertile couples included in this study were categorized on the basis of motility of spermatozoa and the age groups of the female subjects.and were studied

The number and percentage of infertile subjects whose sperms were retrieved by Percutaneous Epididymal Sperm Aspirate (PESA) and Biopsy were categorized on the basis of motile and immotile sperm group which is given in table -1.

Table 1:

Number and Percentage of Subjects with Motile and Immotile Sperm Used in Percutaneous Epididymal Sperm Aspirate (PESA) and Biopsy in Intracytoplasmic Sperm Injection (ICSI) Cycles

Procedure	**Subjects with**				**Total No. of Subjects**
	Motile Sperm		**Immotile Sperm**		
	No.	Percentage	No.	Percentage	
PESA	47	85	8	14.5	55
BIOPSY	39	78	11	22	50

There were a total 105 infertile couples and out of 105 male partners 55 subjects underwent Pesa procedure. Out of 55 subjects 47 (85%) were with motile sperm and 8 (14.5%) were with immotile sperms. From a total of 50 subjects undergoing biopsy, 39 (78%) were with motile sperms and 11 (22%) were with immotile sperms. Three subjects out of the total 105 subjects underwent Pesa as well as Biopsy procedure.

Table 2:

Clinical Characteristics and Day Three Follicular Stimulating Hormone (FSH) Levels of Females Undergoing ICSI Cycles.

Characteristics	PESA		BIOPSY	
	Motile sperm	Immotile Sperm	Motile sperm	Immotile sperm
Age of female at presentation (yrs)	30.55 ± 0.82	31.25 ± 2.10	31.97±0.76	26.73 ± 1.84a*
No. of treatment Cycles	47	8	39	11
Day- 3 (FSH) levels of female miu/ml	6.362 ± 0.30	5.225 ± 0.37	6.774 ± 0.33	5.927 ± 0.70
Primary Infertility	47	8	39	11
No. of ampoules of Gonoadotropin	6.645±0.32	6.625±0.67	7.154±0.36	6.455±0.705

All values are expressed as mean ± standard error (S.E).
*b Highly significant ($P < 0.01$).
(a) motile sperm from biopsy vs immotile sperm from biopsy.

The age at presentation, treatment cycles, day-3 FSH levels and ampoules of gonadotropin used for ovarian stimulation are given in table -2. The age of the 47 female subjects, whose husbands underwent Pesa with motile sperm and 8 female subjects undergoing ICSI with immotile sperm retrieved through Pesa showed no significant difference ($P > 0.05$) in their age groups.

The follicular stimulating hormone were taken of day -3 of the menstrual cycle also showed no significant difference ($P>0.05$) when females who underwent ICSI with motile sperms and immotile sperms retrieved through Pesa were compared. The number of Puregon ampoules (6.645 ±0.32 miu/ml) used for the stimulation of ovaries of the female subjects whose oocyte were inseminated with motile sperm from Pesa in ICSI procedure were not significantly different from those who underwent ICSI with Pesa from immotile sperm (6.625 ± 0.67miu/ml).

The mean age factor of 50 female subjects out of 105 infertile couples undergoing Biopsy, indicated that the age of female subjects undergoing ICSI with motile sperms (31.97 ± 0.76yrs) was significantly greater ($P<0.01$) than, the female subjects who

underwent ICSI with immotile sperms (26.73 ±1.84yrs).The 39 female subjects undergoing ICSI associated with biopsy with motile sperm underwent 39 treatment cycles and 11 female subjects who underwent ICSI with immotile sperms had 11 treatment cycles.

There was no significant difference (P>0.05) in the serum FSH levels of subjects whose husbands underwent ICSI with biopsy with motile sperms (6.774 ± 0.33 miu/ml) and subjects with immotile sperms (5.927 ± 0.70 miu/ml).The number of puregon ampoules (7.154 ± 0.36 miu/ml) used for the stimulation of ovaries of the female subjects whose oocyte were inseminated with motile sperms from biopsy in ICSI procedure was not significantly different (P> 0.05) from those who underwent ICSI with immotile sperms from biopsy (6.455± 0.70miu /ml).

There was no significant difference (P>0.05) in the age of female subjects undergoing ICSI with motile sperms retrieved from Pesa (30.55± 0.82 yrs) to the female subjects who underwent ICSI with motile sperms retrieved from biopsy (31.97±0.76 yrs). There were 47 infertile couples who underwent 47 treatment cycles of ICSI in which sperm were retrieved from Pesa, on the other hand there were 39 infertile couples who underwent 39 treatment cycles in which ICSI was done with sperms retrieved from biopsy. The mean ±SEM of FSH levels taken on day-3 of female subjects undergoing ICSI with motile sperms retrieved from Pesa indicated no significant difference (P<0.05),(6.363 ±0.30 miu/ml) to the female subjects who underwent ICSI with motile sperms retrieved through biopsy (6.774 ± 0.33 miu/ml). The number of puregon ampoules (6.645 ± 0.32 miu/ml) used for the stimulation of the female subjects whose oocyte were inseminated with motile sperms from Pesa in ICSI procedure were not significantly different (P>0.05) from those who underwent ICSI with motile sperm from biopsy (7.154 ±0.36 miu ml).

No significant difference (P>0.05) was obserevd between the age of the female subjects undergoing ICSI with immotile sperm (31.25±2.10 yrs) through Pesa to that of the female subjects who underwent ICSI with immotile sperm from biopsy (26.73±1.84 yrs).There were 8 infertile couples who underwent 8 treatment cycles of ICSI with Pesa and 11 infertile couples underwent 11 ICSI treatment cycles with biopsy.The FSH levels of day-3 of the female subjects undergoing ICSI with immotile

sperms (5.225± 0.37 miu/ml) were not significantly different (P>0.05) from the females undergoi'm ng ICSI with immotile sperms from biopsy (5.927±0.70 miu/ml).The number of Puregon ampoules (6.625±0.67 miu/ml) used for the stimulation of the ovaries of the female subjects whose oocyte were inseminated with immotile sperms in ICSI from Pesa were not significantly different (P>0.05) from those who underwent ICSI with immotile sperms from biopsy (6.455± 0.70 miu/ml).

Table 3:

Ovarian Response of the Female Subjects Undergoing ICSI – Cycles

Characteristics	PESA		BIOPSY	
	Motile sperm	Immotile sperm	Motile sperm	Immotile sperm
No. of oocyte retrieved	16.94±1.35 *b	8.875 ± 1.63	14.46 ± 1.12	13.91 ± 2.19
Oocyte maturity Rate %	81.03	88.7	78.7	93.4 d*
No. of metaphase II	13.72 ± 1.05c*	7.875 ± 1.60	11.38 ± 0.93	13.0 ± 2.16

All values are expressed as mean ± standard error (S.E).
*Significant (P < 0.05).
(b) motile sperm from Pesa vs immotile sperm from Pesa
(c) motile sperm from Pesa vs immotile sperm from Pesa
(d) motile sperm from biopsy vs immotile sperm from biopsy

Total number of oocyte retrieved , oocyte maturity rate and total number of metaphase –II retrieved are given in table -3.

There was significantly, higher (P< 0.05) number of oocyte retrieved from female subjects undergoing ICSI with motile sperm retrieved from Pesa (16.94 ±1.35) compared, to the oocyte retreievd from female subjects undergoing ICSI with immotile sperms retrieved from Pesa (8.875 ± 1.63).The oocyte obtained from the female undergoing ICSI with motile sperms retrieved from Pesa also showed a significantly higher (P<0.05) number of metaphase –II oocyte (13.72 ±1.05) than females undergoing ICSI with immotile sperm retrieved from Pesa (7.875 ± 1.60).
Oocyte retrieved in the female subjects undergoing ICSI with motile sperms retrieved from Biopsy (14.46 ± 1.12) were not significantly different (P>0.05) compared to the oocyte retrieved from female subjects undergoing ICSI with immotile sperms from biopsy (13.91 ± 2.19).The oocyte retrieved were analyzed no significant difference

(P> 0.05) was detected in the metaphase – II stage oocytes of the female undergoing ICSI with motile sperm retreived from biopsy (11.38 ± 0.93) to the female subjects undergoing ICSI with immotile sperms (13.0 ± 2.16) through biopsy.
There was no significant difference (P< 0.05) in the total no. of oocyte retreived from female subjects undergoing ICSI with motile sperms from Pesa (16.94 ± 1.355) to that of the female subjects undergoing ICSI with motile sperms from biopsy (14.46 ± 1.12) .Out of all these oocyte there was no significant difference (P> 0.05) in the metaphase –II stage of subjects undergoing ICSI with motile sperm (13.72 ± 1.053) from Pesa to the females undergoing ICSI with motile sperms retrieved from biopsy (11.38 ± 0.93).

There was no significant difference (P> 0.05) in the number of oocyte retrieved (8.875 ±1.63) and metaphase – II stage (7.875± 1.0) of female subjects undergoing ICSI with immotile sperms from Pesa to that of the female oocyte retrieved (13.91±2.19) and metaphase – II stage oocyte (13.0±2.16) of female subjects undergoing ICSI with immotile sperms from biopsy.

Oocyte maturity rate between subjects who underwent Pesa with motile sperm (81.03 %) had no significant difference (P = 0.289) to the oocyte maturity rate of subjects who underwent biopsy with motile sperm (78.7%) . Similarly, oocyte maturity rate between subjects who had ICSI with immotile sperms obtained from Pesa (88.7%) had no significant difference (P= 0.231) from the subjects who had ICSI with immotile sperms obtained from biopsy (93.4 %).

The oocyte maturity rate in the use of motile sperm obtained from Pesa (81.03 %) and use of immotile sperm obtained from Pesa (88.7%) indicated no significant difference (P = 0.145). The Oocyte maturity rate was highly significant (P = 0.001) in subjects in which ICSI was preformed with immotile sperms (93.4 %) retrieved from biopsy to that of subjects who underwent ICSI with motile sperm retrieved from biopsy (78.7 %).

Table 4:

Pronuclear Development, Fertilization And Cleavage Rate In Subjects Of PESA And Biopsy In ICSI Cycles

Characteristics	PESA		BIOPSY	
	Motile sperm	Immotile sperm	Motile sperm	Immotile sperm
No. of 2 - Pronuclei	9.255 ± 0.81e**	5.625 ± 1.36	6.462 ± 0.59	6.727 ± 1.15
Fertilization rate %	67 .4 f*	71. 4	56.7	71. 4
No. of cleaved embryos	7.191 ± 0.68	4 .125 ± 1.27	5.59 ± 0.52	5.273 ± 0.858
Cleavage rate %	77.7	73.3	86.5 g*	78. 3 h*

All values are expressed as mean ± standard error (S.E).
** Highly significant ($P < 0.01$).
(e) motile sperm from Pesa vs motile sperm from biopsy
(f) motile sperm from Pesa vs immotile sperm from Pesa
(g) motile sperm from Pesa vs motile sperm from biopsy
(h) motile sperm from biopsy vs immotile sperm from biopsy

Pronuclear development, fertilization and cleavage rates in Pesa and biopsy subjects undergoing ICSI are given in table -4.
Once the mature oocyte were micro-injected with sperms either motile or immotile, further fertilization was evaluated by the presence of 2- Pronuclei , there was no significant difference ($P > 0.05$) in the the 2- pronuclear stage of the couple who underwent ICSI with motile sperm (9.255 ± 0.81) from Pesa to the couple who underwent ICSI with immotile sperm from Pesa (5.625 ± 1.36).

Similarly, there was no significant difference ($P>0.05$) in the 2- pronuclei stage of the subjects who underwent ICSI with motile sperm retrieved from biopsy (6.462 ± 0.59) to the subjects who underwent ICSI with immotile sperms retrieved from biopsy (6.727 ± 1.15). Highly significant difference ($P <0.001$) in the 2- pronuclei stage of subjects undergoing ICSI with motile sperms (9.255 ± 0.81) retrieved from Pesa was observed compared to the subjects undergoing ICSI with motile sperms retrieved from biopsy (6.462±0.59). No significant difference ($P> 0.05$) was detected between the 2- Pronuclei stage of subjects undergoing ICSI with immotile sperms (5.625 ± 1.36) and subjects undergoing ICSI with immotile sperms retrieved from biopsy.

Fertilization rate is significantly , higher (P =0.003) in subjects undergoing ICSI with motile sperm retrieved from Pesa (67.4%) to that of subjects undergoing ICSI with motile sperms retrieved (56.7%) from biopsy. Similarly, no significant difference (P> 0.05) between the fertilization rate of subjects undergoing ICSI with immotile sperms retrieved from Pesa (71.4 %) than the subjects undergoing ICSI with immotile sperm (71.4 %) retrieved from biopsy.

Once fertilization takes place then, cleavage of the embryo takes place which was analyzed and in subjects undergoing ICSI with motile sperm retrieved from Pesa (7.191 ± 0.68) to that of subjects undergoing ICSI with immotile sperms retrieved from Pesa (4.125±1.27) indicating no significant difference (P> 0.05).

Similarly, the number of embryo cleaved between the two groups of the subjects one who underwent ICSI with motile sperm retrieved through biopsy (5.590 ± 0.52) and the ones who underwent ICSI with immotile sperms retrieved through biopsy (5.273 ± 0.85) indicated no significant difference (P>0.05). No significant difference (P> 0.05) was noted in the number of cleaved embryos between subjects who underwent ICSI with motile sperm retrieved through Pesa (7.191 ± 0.68) and subjects who underwent ICSI with motile sperms through biopsy (5.590 ± 0.52).Similarly, there was no significant difference (P> 0.05) was observed in the number of cleaved embryo in subjects undergoing ICSI with immotile sperms retrieved through Pesa (4.125 ± 1.27) and in the subjects undergoing ICSI with immotile sperms retrieved through biopsy (5.273 ± 0.85).

Cleavage rate between subjects who underwent ICSI with motile sperm from biopsy (86.5 %) was highly significant (P= 0.001) to those subjects who underwent ICSI with motile Pesa (77.7%). But, no significant difference (P=0.534) between the subjects who underwent ICSI with immotile sperm retrieved through Pesa (73.3%) to the subjects who underwent ICSI with immotile sperms retrievd through biopsy (78.3 %).

Similarly, no significant difference (P= 0.546) was noted between the subjects who underwent ICSI with motile sperm through Pesa (77.7%) to subjects who underwent ICSI with immotile sperms through Pesa (73.3 %). Highly significant difference (P = 0.09) was observed between the subjects who underwent ICSI with motile sperms retrieved through biopsy (86.5%) to the subjects who underwent ICSI with immotile sperms retrieved through biopsy (78.3 %).

Table 5:

Pregnancy, Abortion and Live Birth Rate in PESA and Biopsy Subjects undergoing ICSI Cycles

Characteristics	PESA		BIOPSY	
	Motile sperm	Immotile sperm	Motile sperm	Immotile sperm
No. of embryo transferred	56.00 ±23.54	7.0 ± 7.0	44.50 ± 23.79	13.50 ± 5.72
Percentage of total pregnancies	36.1	12.5	48.7	–
Pregnancy rate per cycle	36.1	12.5	48.7	–
Abortion rate	64. 7	-	52.6	-
Live birth rate per embryo transfer	3.57	3.75	7.86i*	–

All values are expressed as mean ± standard error (S.E).
* Highly significant ($P < 0.05$).
(i) motile sperm from biopsy vs motile sperm from Pesa.

Pregnancy, abortion and live birth rate in Pesa and biopsy subjects undergoing ICSI are given in table -5.

The mean ± SEM of the total number of embryos transferred varied in all the four groups. In subjects who underwent ICSI with motile sperms retrieved through Pesa the number of the embryo transferred was (56.00 ± 23.54) whereas, in the subjects who underwent ICSI with immotile sperm retrieved from Pesa is (7.0 ± 7.0).
The mean ± SEM of the number of embryo transferred in subjects who underwent ICSI with motile sperm retrieved through biopsy is (44.50 ± 23.79) whereas the subjects who underwent ICSI with immotile sperms retrieved through biopsy (13.50 ± 5.72).

Pregnancy rate is (25.87 %) higher in subjects who underwent ICSI with motile sperms retrieved from biopsy (48.7%) to that of those subjects who underwent ICSI with motile sperms retrieved from Pesa (36.1 %).Abortion rate was also (18.70 %) higher in subjects who underwent ICSI with motile sperms retrieved from Pesa (64.7 %) to those subjects who underwent ICSI with motile sperms retrieved from biopsy (52.6 %).There was highly significantly (P=0.0001), live birth rate in subjects

who underwent ICSI with motile sperms retrieved from biopsy (7.86%) to that of those subjects who underwent ICSI with motile sperms retrieved from Pesa (3.57%). But, no significant difference (P=3128) was noted between the subjects who underwent ICSI with motile sperms through Pesa (3.57%) to that of those subjects who underwent ICSI with immotile sperms from Pesa (3.57%).

On the basis of age the present study includes the analysis of 102 infertile couples, in which after semen analysis the male subjects were detected to be azoospermic. They were offered ICSI treatment cycles with sperm retrieval processes like Pesa and Biopsy The couples were divided into 2 groups on the basis of age in both the categories of Pesa and Biopsy.

Group 1: 20 -29 yrs (Pesa)
Group 2: 30 39+ yrs (Pesa)

Group 3: 20 -29 yrs (Biopsy)
Group 4: 30 39 + yrs (Biopsy)

Table 6:

Various Age Groups of Subjects Undergoing Pesa and Biopsy

Total number of subjects	**Pesa**		**Biopsy**	
	Group-1	**Group-2**	**Group-3**	**Group-4**
102	20 -29 yrs	30 -39 +yrs	20 -29 yrs	30 -39 +yrs
Number	24	29	22	27
Percentage	23	28.4	21.5	26.4

The various age groups of subjects undergoing Pesa and biopsy are given in table -6.

There were total 24 (23%) patients of group-1 out of 102 infertile couples underwent ICSI treatment cycles with Pesa and 29 (28.4%) patients of group-2 underwent ICSI with Pesa. Similarly, 22 (21.5 %) patients of group-3 underwent ICSI with biopsy and 27 (26.4%) patients of group-4 underwent ICSI with biopsy.

Table 7:

Clinical Characteristics, Day three -Follicular Stimulating Hormone (FSH) Levels Of Female Subjects Undergoing Intracytoplasmic Sperm Injection

Characteristics	Pesa		Biopsy	
	20 -29 yrs	30 39 + yrs	20 -29 yrs	30 39 + yrs
	Group-1	Group-2	Group-3	Group-4
No. of treatment cycles	24	29	22	27
Female age at presentation (yrs)	25.53±0.35	33.17±0.76a**	27.36±0.28c**	34.15±0.68b**
Day- 3 FSH levels(miu/ml)	5.625±0.37	6.879±0.35	6.014±0.43	7.222±0.34
Primary Infertility	24	29	22	27

All values are expressed as mean ± standard error (S.E).
** Highly significant (P< 0.01).
(a) group-1 vs group-2
(b) group-3 vs group-4
(c) group-1 vs group-3

There were 102 infertile couples, out of which group-1 subjects underwent 24, group-2 underwent 29 ICSI cycles with sperms retrieved from Pesa, group-3 underwent 22 and group-4 underwent 27 ICSI treatment cycles with sperms retrieved through biopsy.

Age of group-2 (33.17 ± 0.76) was significantly higher (P<0.01) than that of group-1 (25.53 ± 0.35yrs). Similarly, age of group- 4 (34.15 ±0.68 yrs) is significantly higher (P<0.01) than the age of group-3 (27.36 ± 0.28 yrs). The age of group-3 (27.36 ± 0.28 yrs) was significantly higher (P<0.01) than age of group -1 (25.53±0.35 yrs).But, no significant difference(P>0.05) in the age was detected in the group-2 (33.17 ±0.76 yrs) and group-4(34.15 ± 0.68yrs). No significant difference (P>0.05) in the FSH levels of group-1 (5.625±0.37 miu/ml), group-2 (6.879±0.35miu/ml), group-3 (6.014±0.43 miu/ml) and group -4 (7.222±0.34 miu/ml) was detected.

Table 8:

Ovarian Response Of Female Subjects Undergoing Intracytoplasmic Sperm Injection

Characteristics	Pesa		Biopsy	
	20 -29 yrs	30 39 + yrs	20 -29 yrs	30 39 + yrs
	Group-1	Group-2	Group-3	Group-4
No. of oocyte retrieved	18.9±2.0	13.62±1.49	14.32±1.31	14.15±1.39
Oocyte maturity rate	80.39	83.03d *	88.25e**	76.4
No. of metaphase- II	15.21±1.61	11.31±1.15	12.64±1.21	10.81±1.13

All values are expressed as mean ± standard error (S.E).

*Significant (P< 0.05).

** Highly significant (P<0.01)

(d) group-2 vs group-4

(e) group-3 vs group-4

Total number of oocyte retrieved, oocyte maturity rate and total number of metaphase – II retrieved are given in table -8. No significant difference (P>0.05) in the total number of oocyte retrieved between group-1(18.9±2.0), group-2(13.62±1.49), group-3 (14.32±1.31) and group-4 (14.15±1.39) was detected. Whereas the oocyte maturity rate detected no significant difference (P>0.05) between group-1 (80.39%) and group – 2 (83.03%) . However the oocyte maturity rate was significantly high (P<0.01) in group-3 (88.25%) to that of group-4(76.4%).But, no significant difference (P>0.05) were detected in comparsion of oocyte maturity rate amongst group-1 (80.39%) and group -3 (88.25%), and group-2 (83.03%) and group-4 (76.4%).Similarly, no significant difference (P>0.05) was detected in the metaphase –II stage between group-1 (15.21±1.61) and group-2 (11.31±1.15) and group-3 (12.64±1.21) and group-4 (10.81±1.13).However there was no significant difference (P>0.05) in the metaphase – II stage of group-1 (15.21±1.61) and group-3 (12.64±1.21).Similarly, no significant difference(P>0.05) was detected between group-2 (11.31±1.15) and group-4 (10.81±1.13).

Table 9:

Pronuclear Development, Fertilization And Cleavage Rate In Subjects Undergoing Pesa And Biopsy In (ICSI) Cycles

Characteristics	Pesa		Biopsy	
	20 -29 yrs	30 39+ yrs	20 -29 yrs	30 39+ yrs
	Group-1	Group-2	Group-3	Group-4
2- Pronuclei stage	11.21±1.36e**	6.96±0.63	5.5±0.83f**	6.37±0.66
Fertilization rate	73.69g**	61.58i *	43.52h**	58.90
No. of cleaved embryos	8.83±1.1j **	5.27±0.59	4.63±0.77	5.66±0.56
Cleavage rate	78.81	75.74	84.29	88.95

All values are expressed as mean ± standard error (S.E).

*Significant (P<0.05).

** Highly significant (P<0.01)

(e) group-1 vs group-2, group-1 vs group-3, group-1 vs group-2, group-1 vs group-3, group-2 vs group-4, group-1 vs group -2 ,group-1 vs group3 , group2 vs group4.

Pronuclear development, fertilization and cleavage rates in Pesa and biopsy subjects undergoing ICSI are given in table -9.The number of 2-PN retrieval was significantly higher (P<0.001) in group-1 (11.21±1.36) to that of group-2 (6.96±0.63).But, there was no significant difference (P>0.05) in the 2- PN stage of group-3 (5.5±0.83) and group-4 (6.37±0.66).Similarly, Group-1 (11.21±1.36) had significantly higher (P<0.01) number of 2-PN then , that of group-3 (5.5±0.83).But, there was no significant difference (P>0.05) in the 2-PN of group-2 (6.96±0.63) to that of group-4 (6.37±0.66).

Fertilization rate highly significantly raised in group-1(73.69%) to that of group-2 (61.58%) and group-3 (43.52%). The fertilization rate is also significantly higher (P<0.05) in group-4 (58.90 %) to that of group-3 (43.52%). Similarly, fertilization rate was also significantly higher in group-2 (61.58 %) to that of group-4 (58.90%).

The number of cleaved embryo are highly significantly raised in group 1 (8.833±1.10) to that of group-2 (5.27±0.59).but, no significant difference (P> 0.05) was detected in group-3 (4.63 ±0.77) and group-4 (5.66 ±0.56)The number of cleaved embryo in group-1 (8.833±1.10) were highly significant (P<0.01) to that of group-3

(4.63±0.77).but, no significant difference (P>0.05) was detected in comparison of group-2 (5.27± 0.59) and group 4 (5.66 ±0.569).There was no significant difference (P>0.05) in the cleavage rate of group-1(78.81 %) to that of group-2 (75.74%) and group-3 (84.29%).Cleavage rate also did not indicate significant difference between group-3 (84.29%) to that of group-4 (88.95%).But, there is significant difference (P<0.05) in the cleavage rate of group -2 (75.74%) to that of group-4(88.95%).

Table 10:

Pregnancy, Abortion and Live Birth Rate in Pesa and Biopsy Subjects in (ICSI) Cycles

Characteristics	Pesa		Biopsy	
	20 -29 yrs	30 -39 + yrs	20 -29 yrs	30 -39 + yrs
	Group-1	Group- 2	Group-3	Group-4
Pregnancy rate	37.5	24.1	18.1	55.5n*
No. of single pregnancy	0.37±0.10	0.24±0.08	0.18±0.08	0.55±0.09
Abortion rate	55.5	28.57	75	46.6
Live birth per embryo transfer	4.23o**	4.23	2.35	10.74m**

All values are expressed as mean ± standard error (S.E).

*Significant (P<0.05).

** Highly significant (P<0.01)

(m) group-4 vs group-3
(n) group-4 vs group-3
(o) group-1 vs group-3

Pregnancy abortion and live birth rate in Pesa and biopsy subjects in ICSI cycles are given in table 5 .There is no significant difference (P>0.05) in the pregnancy rate of subjects in group-1 (37.5 %) to that of group-2 (24.1%).But, the pregnancy rate was significantly higher (P<0.05) in group-4 ((55.5%) to that of group-3(18.1%) .No significant difference (P>0.05) was detected in the pregnancy rate of group-2(24.1 %) to that of group-4 (55.5%). No significant difference (P>0.05) in the abortion rate was detected between group-1 (55.5%), group-2 (28.57%), group-3 (75%) and group-4(46.6%).No significant difference (P>0.05) was detected in the live birth rate per

embryo transfer of group-1 (4.23%) to that of group-2 (4.23%). The live birth rate was significantly higher ($P<0.01$) in group-4 (10.74%) to that of group-3 (2.35%).Similarly highly significant difference ($P<0.01$) was detected in the live birth rate of group-1 (4.23%) to that of group-3 (2.35%), group-2(4.23%) and group-4 (10.74%). There one twin pregnancy in group-1 (Pesa) as compared to group-2 (Pesa) which had no multiple pregnancy.Similarly, group-3 (Biopsy) had one twin pregnancy as compared to group-4 (Biopsy) in which there were three twin pregnancies and one triple pregnancy.

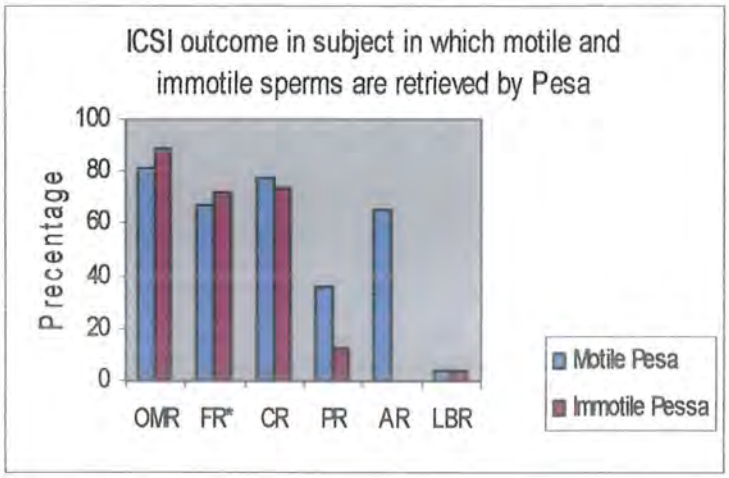
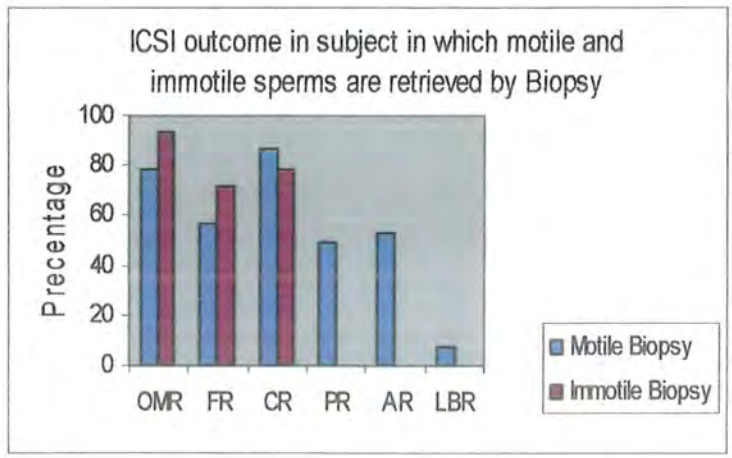


Fig 1. ISCI outcome in subject in which motile and immotile sperms and retrieved by Pesa

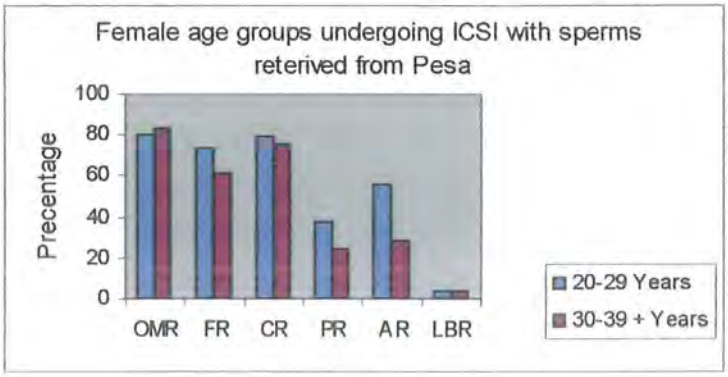
* Significant ($P<0.05$)

OMR = Oocyte maturity rate
FR = Fertilization rate
CR = Cleavage rate
PR = Pregnancy rate per cycle
AR = Abortion rate
LBR = Life Birth rate per embryo transfer



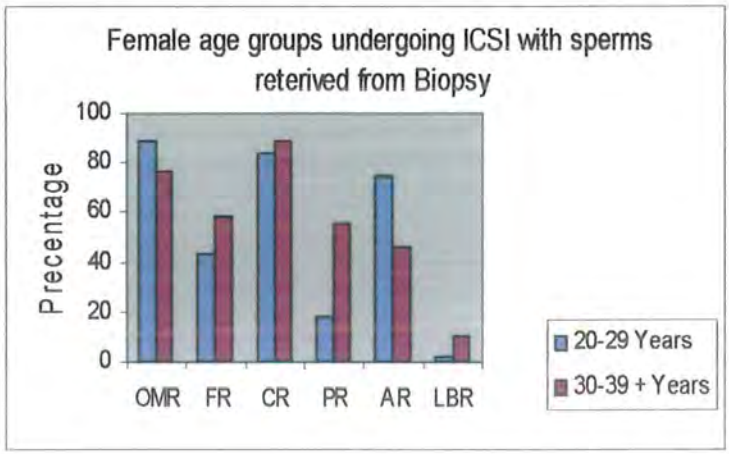
* Significant (P<0.05)
** Highly significant (P<0.001)

Fig 2. ISCI outcome in subject in which motile and immotile sperms and retrieved by Biopsy



* Significant (P<0.05)
** Highly significant (P<0.001)

Fig 3. Age group of female subject undergoing ICSI with sperms retrieved by Pesa



* Significant (P<0.05)
** Highly significant (P<0.001)

Fig 4. ISCI outcome in various female age groups

DISCUSSION

In this retrospective study data was examined which was collected from ICSI cycles that were preformed with the use of epididymal and testicular retrieved sperm, with the incentive to develop the best infertility management strategy for the azoospermic patients. A total of 105 Primary infertile couple with azoospermic male subjects were treated at Islamabad clinic serving infertile couple Islamabad.

The couples were offered ICSI treatment cycles with sperms retrieved from Pesa and Biopsy. Motility of the sperms has proven to play an important role in ICSI outcome as the sperms with improved forward progression, facilitate and help to expedite the identification and collection of vital spermatozoa. In the present study it was observed that in most testicular biopsies where spermatozoa were present, at least a few sperms cells usually displayed a sluggish, twitching type of motility (Tournaye et al. 1996) observed in patients with 100% dead spermatozoa in their ejaculate, motile spermatozoa could still be recovered from a testicular biopsy and can be used for ICSI procedure.

The major concern when confronted with an azoospermic patient in assisted reproductive program is that the patients correct etiology should be determined and corresponding probability of obtaining spermatozoa from various sperm retrieval procedures should be successful. In the present study while selecting spermatozoa for microinjection motile spermatozoa were used that is those with some signs of motility, but it was not always that motile spermatozoa were used because at times immotile spermatozoa were also use to microinject the oocyte retrieved from the female subjects.

Once the mature oocyte were microinjected and further fertilization was observed by the presence of 2-pronuclear development. The number of 2- PN retrieved in the subjects where ICSI was preformed with motile sperms through Pesa was significantly higher ($P<0.001$) than those where motile sperms were retrieved through Biopsy. In the present study the spermatozoa were divided into two groups, motile and immotile group but, various studies have divided the spermatozoa according to

obstructive azoospermia (OA) and non obstructive azoospermia (NOA) (Schlegel2004). The above results are different from the observation done by Friedler et al (2002) in their observation the 2-PN number was similar in both subjects of OA and NOA.Those subjects which underwent ICSI with motile sperms through Pesa had a significantly higher (P=0.003) fertilization rate as compared to those subjects who underwent ICSI with motile sperms through biopsy. Buffat et al. (2006) also in his studies of outcome of ICSI from sperms of different origin i.e epididymal and testicular sperms also confirmed that epididymal spermatozoa should be preferred to testicular spermatozoa irrespective of the aetiology of OA. This observation is similar to the observation made by Mansour et al. (1997) who compared the outcome of ICSI using epididymal sperm from patients with (OA) and the use of testicular sperm in (NOA) and found a significantly, lower fertilization rate in the latter group (59.5 vs 39 %).Similar, observation was mentioned by Palermo et. al (1999) who reported a significantly higher fertilization rate of 73% in 241 cycles of (OA) using epididymal sperm compared with 57 % in 53 cycles of NOA.

Further cleavage rate of the embryo was significantly higher (P<0.001) in the subjects undergoing ICSI with motile sperms retrieved by Biopsy compared to those subjects who underwent ICSI with motile sperms through Pesa. Similarly, cleavage rate of subjects undergoing ICSI with Biopsy with motile sperms was significantly higher (P=0.09) to those subjects who underwent ICSI with immotile sperms through biopsy (Schwarzer et al .2003). In the subjects who underwent ICSI with motile sperms through Biopsy had pregnancy rate which was (25.87%) higher to the subjects who underwent ICSI with motile sperms through Pesa. There was significantly higher live birth rate (P<0.001) in the subjects who underwent ICSI with motile sperms through biopsy as compared to the subjects who underwent ICSI with motile sperms through Pesa (Matyas et al.2005). There was significantly higher abortion rate in subjects undergoing ICSI with motile sperms through Pesa to that of subjects undergoing ICSI with motile sperms through biopsy. A retrospective study was carried out in a hospital based infertility center. In this there were subjects diagnosed with azoospermia and severe oligospermia who had undergone a sperm retrieval procedure inconjunction with ICSI cycles. The impact of sperm motility on fertilization and clinical pregnancy rates were determined. The motile and non motile sperm groups differed in the number of mature oocyte retrieved (10.7 ± 5.8 vs 13.4 ±

6.0), fertilization (56.7 % ± 59.1 %) and embryo conservation rates were (35.9 % vs 39.3 %) were statistically, similar, Clinical pregnancy rates did not differ between the motile and (38.5 %) and non motile (31.2%) groups nor did they differ between obstructive and non obstructive patients 35.3 % vs 26.7 %) (Matyas et al.2005).

Regarding the pregnancy rate and live birth rate it was significantly higher in the subjects who underwent ICSI with motile sperm through biopsy to that of those subjects who underwent ICSI with motile sperm through Pesa. But, the abortion rate was raised in subjects undergoing ICSI with motile sperms through Pesa than to those subjects where motile sperms from biopsy were used (Balogh et al.2005). This is different from the study made by (Vicari et al. 2001) who reported higher fertilization, pregnancy rates which ultimately lead to higher ongoing / delivery rates for subjects with OA significantly higher abortion rate were reported in NOA subjects.

The conclusion drawn from the present study is that no matter what site of sperm retrieval is used that is either Epididymis or Testes, the sperm motility has great importance as the results in present study have shown that pregnancy rate and live birth rate was significantly raised in subjects where motile sperms retrieved through either biopsy or Pesa when used for ICSI. But, the overall success rate was more in the subjects who underwent ICSI through motile sperms retrieved through Biopsy.

REFERENCES

American Society For Reproductive Medicine, Fertil Steril September 2004. Vol 82, Suppl. 1.

American Urological Association, Fertil Steril September 2004; Vol 82, Suppl.1.

Balaban B, Urman B, Sertac A, Alatas C, Aksoy S, Mercan R and Nuhoglu A: In – vitro culture of spermatozoa induces motility and increases implantation and pregnancy rates after testicular sperm extraction and intracytoplasmic sperm injection. Hum Reprod 1999, 28082811.

Bonduelle , Liebaers, Devroey P and Van Steirteghem A. Neonatal data on the cohort of 2889 infants born after ICSI 2002 Hum Reprod ;17 : 671 – 694.

Carson SA, Pisarki MD, Casson PR ,Cisneros PL, Lamb DJ, Lipshultz LI, Buster JE. Fertilization after standard in vitro fertilization versus intracytoplasmic in subfertile males using sibling oocyte. Fertil Steril 1999 ; 71 : 627 - 32

De Croo I, Van Der Elst J, Everaert K, De Sutter P, Dhont M. Fertilization, pregnancy, and embryo implantation rates after ICSI in cases of obstructive and non – obstructive azoospermia. Hum Reprod 2000 ; 15 : 1381 – 8 .

Friedler S, Raziel A, Schachter M, Strassburger D, Bern O, Ron-ELR. Outcome of first and repeated testicular sperm extraction and ICSI in patients with non-obstructive azoosperima hum. Reprod 2002; 17: 2356-2361.

Jarow JP. Seminal vesicle aspiration of fertile men . J Urol 1996 ; 56 : 1005 –7.

Jarrow JP, Espeland MA and Lipshultz li. Evaluation of the azoospermic patient. J Urol 1989 ;142: 162 -5.

Joris H, Bonduelle M,Hofmans K and Van Steirteghem A. Mental development of 201 chidern at 2 years of age 1998 Lancet 351; 1535.

Mansour RT, Aboulghar MA, Serour GI, Fahmi I, Ramazy AM and Amin Y; intracytoplasmic sperm injection using microsurgically retrieved epididymal and testicular sperm. Fertil. Steril 1997; 65: 566-572.

Nagy Z, Devroey P, Silber S, Van Steirteghem A and Liu J : Using ejaculated, fresh and frozen – thawed epididymal and testicular spermatozoa gives rise to comparable results after sperm injection. Fertil. Steril 1995 ;63 : 808 - 815.

Palermo GD, Schlegel PN, Colombero LT, Moy- F and Rosewaks Z.. Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy. Hum Reprod 1996 ; 11 : 1023 – 9.

Palermo GD, Schlegel PN, Hariprashad JJ, Ergun B, Mielnik A, Zaninovic N, Veeck

LL and Rozenwaks Z. (1999). Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men. Hum. Reprod; 14: 741-748.

Rosenlynd B, Sjoblom P, Dimitrakopoulos A, Hillensjo T. Epididymal & testicular sperm for Introcytoplasmic sperm injection in the treatment of obstructive ozoospermia Acta Obstet Gynecol Scand 1997; 75: 135-9.

Sakkas D, Urner F, Bianachi PG, Bizzaro D, Wagner I. Sperm chromatin abnormalities can influence decondensation after intracytoplasmic sperm injection. Hum Reproduction 1996; 11:837 – 43.

Saleh RA, Agarwal A, Nelson DR, Nada EA, El Tonsy MH, Alvarez JG. Increased sperm nuclear damage in normozoospermic infertile men:A prospective study. Fertil Steril 2002 ; 78 :313- 8

Schoysman R , Vanderzwalmen P, Nijs M, Segal L, Segal-Bertin G, Geerts L. Pregnancy after fertilization with human testicular spermatozoa 1993 Lancet ; 342: 1237.

Silber SJ, Nagy Z, Devroey P, Tournay H. and Van Steirteghem AC. (1997). Distribution of spermatogenesis in the testicles of azoospermic men: the presentce or absence of spermatids in the tests of men with germinal failure Hum, Reprod, 12, 2422-2428.

Silber SJ, Van Steirteghem AC, Liu J, Nagy Z, Tournaye H and Devroey P. High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicular biopsy. Hum Reprod 1995; 10 :148-52.

Silber SJ. Microsurgical TESE and the disturbution of spermatogenesis in non-obstructive azoospermia. Hum Reprod 2000; 15 : 2278-84.

Society for Associate Reproductive Technology, The American Society for Reproductive Medicine. Assistant Reproductive technology in the U.S. 1996. Results generated from the American Society for Reproductive Medicine/Society for Assistant Reproductive Technology REgistery. Fertil Steril 1999; 71: 798-807

Sousa M, Cremades N, Silva J, Oliveira C, Ferraz L, Teixeira da Silva J. Predictive value of testicular histology in secretory azoospermic subgroups and clinical outcome after microinjection of fresh and frozen – thawed sperm and spermatid. Hum Reprod 2002 ; 17 : 1800 – 10 .

Thonneau P, Marchand S, Tallec A, Ferial ML, Ducot B, Landsec J. Incidence and main cause of infertility in a resident population (1, 850, 000) of three French regions (1988 – 1989). Hum Reprod 1991. 6: 811-6.

Tounaye H, Verhayen G, Nagy P, Ubaldi F, Goosens A , Silber S. Are there any predictive factors for successful testicular sperm recovery in azoospermic patients.

Hum Reprod 1997 ; 12 : 80 – 6 .

Tournaye H, Devroey Silber SJ. Van Steirleghen A, Nagy-Z, Liv J. Normal pregnancies resulting from testicular extraction and intraytoplasmic sperm injection for azoospermia due to maturation arrest Fertil-Steril 1996; 66:110-7.

Tournaye H, Silber SJ, Nagy ZP, Liu J, Devroey P and Van Steirteghem A. Microsurgical epididymal sperm aspiration and intracytoplasmic sperm injection: a new effective approach to infertility as a result of congenital bilateral absence of vas deferens. Fertil Steril 1994 ; 61 : 1045 - 51.

Uehara S, Hashiyada M , Sato K , Sato y , Fujimori K and Okamura K. Preferential X – chromosome inactivation in women with idiopathic recurrent pregnancy loss. Fertil Steril 2001 ; 76 : 908 – 914.

Van Steirteghem A.C , Liu J , H . Higher success rate by intracytoplasmic sperm injection than by subzonal insemination . Report of a second series of 300 consecutive treatment cycles. Hum Reprod 1993 ; 8:1055 -1060.

Van Steirteghem AC, Silber SJ, Nagy ZP, Liu J and Devroey P.Conventional in-vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. Hum Reprod 1994 ; 9 : 1705- 9

Vicari E, Grazioso C, Burrello N, Cannizzaro M, D Agata, and Calogero AE. Epididymal and testicular sperm retrieval in azoospermic patients and the outcome of interacytoplasmic sperm injection inrelation to the etiology of azoospermia Fertil steril 2001; 75: 215-216.

World Health Organization. WHO laboratory manual for the examination of human semen and semen – cervical mucus interaction. New York. Cambridge University Press, 1999.