

Efficiency of Hyaluronic Acid Binding Ability to Improve Sperm Selection in Intracytoplasmic Sperm Injection (ICSI)

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ABSTRACT

Background: intra Cytoplasmic Sperm Injection (ICSI) is a reliable method for single sperm selection and injection to a large number of infertile patients. Human oocytes are naturally surrounded by cumulus cells embedded in the intracellular matrix made primarily of hyaluronic acid (HA), which plays a role as physiological selector for Intra-Cytoplasmic Sperm Injection (PICSI). Mature spermatozoa are able to bind to and digest HA for the best chance of reaching the oocyte and to maintain fertilization. The intact human spermatozoa are bound to immobilize HA surrounding oocyte *in vitro* and this leads to reduced risk of chromosomal imbalance or chromatin diseases.

Selection of spermatozoa by HA before ICSI may help to optimize the outcomes of the treatment; thus, our study aimed to compare ICSI results, based on the hyaluronic acid or traditional method for sperm selection. **Setting:** this study conducted at International Islamic Center for Population Studies and Research, Assisted Reproduction Unit, Al-Azhar University from January to October 2015.

Patients and Methods: 120 couples with male factor infertility and normal wives conducted at the Assisted Reproduction Unit in the International Islamic Center for Population Studies and Research (IICPSR), Al-Azhar University, Egypt, during the period from January to October 2015. All cases were clinically evaluated and eligible for analysis by using inclusion and exclusion criteria. All studied men subjected to ejaculated sperm prepared by traditional method and another prepared by PICSI method. ICSI performed simultaneously for different oocytes from the same woman using an ejaculated sperm by two methods from the same husband. ICSI outcomes compared to the two sequential attempts performed, respectively. Number and quality of oocytes, fertilization rates, rate of embryo cleavage and pregnancy outcomes were recorded. The collected data were tabulated and statistically analyzed.

Results: the present study showed a significant increase in the incidence of fertilization rate, the percentage of embryos with top grade in teratozoospermia and thawed semen groups with PICSI technique compared to the traditional method of ICSI groups. Finally, the percentage of pregnancy rate an increase in all groups manipulated with PICSI technique and this increase was statistically significant.

Conclusion: this study shed more light on the physiological sperm selection method (PICSI) which may improve fertilization and pregnancy rates compared to the traditional selected sperm in ICSI. It is concluded that PICSI technique in Assisted Reproductive Technology (ART) is one of the very important technique that improve fertilization in case of low fertilization rate after ICSI.

Keywords: ICSI, PICSI, Infertility, Physiological Selector, Fertilization rate, Pregnancy rate.

INTRODUCTION

ICSI has been successfully applied worldwide for several years, nevertheless we have no real knowledge regarding the hypothetical long-term side effects of ICSI; since the embryologist just select a single sperm blindly and inject it into the oocyte. In fact, some doubts about the safety of this technique can arise due to the fact that with ICSI some check points of natural fertilization are bypassed and some steps differ considerably from the physiological process; For instance, the introduction of the sperm tail into the ooplasm may cause sperm nuclear decompensation

problems ^[1]. It should be considered that ICSI may increase the risk of injecting spermatozoa with genetic or functional anomalies ^[2]. For these reasons and to minimize any risk related to ICSI, any new advance in this procedure that can help the operator to restore some of the basic physiological checkpoints and to stimulate the natural fertilization process should be welcome ^[3].

Hyaluronic acid (HA) is the main component of the extracellular matrix (ECM) of the cumulus oophorous. In the fertilization process, human

oocytes are naturally surrounded by HA, which is then involved in the mechanism of sperm selection. In fact, mature spermatozoa are able to bind to and digest HA and this is the best chance of reaching the oocyte and fertilizing it. The role of HA as a “physiologic selector” was also well recognized *in vitro*; it had been demonstrated that the spermatozoa that bind to immobilized HA *in vitro* were those having completed their plasma membrane remodeling, cytoplasmic extrusion and nuclear maturation^[4] and those with reduced risk of chromosomal imbalance or chromatin diseases. For these reasons, selection of spermatozoa before intracytoplasmic sperm injection (ICSI) might help to optimize the outcome of the treatment^[5].

A further advantage, HA is containing products that had no known negative effects on post injection zygote development and it is metabolized by the oocyte^[6] HA represented also a more natural alternative material for handling spermatozoa before ICSI than the potentially toxic polyvinylpyrrolidone(PVP) used in conventional ICSI. This new approach to ICSI with HA-bound spermatozoa had been defined as “physiologic ICSI”; the positive effect of HA sperm selection was still to be confirmed in larger-scale studies^[7].

Sperm selection using HA expected to increase the implantation rate in ICSI cycles. A report had explored the characteristics of HA-binding spermatozoa in normospermic and teratozoospermia patients. However, limited data is available regarding the effects of injection of HA-selected sperm on the development of embryo in couples with normal semen parameters^[8]. The present study aimed to evaluate an alternative product for slowing sperm motility, which contains hyaluronic acid and measures its outcome (fertilization rate, embryos quality, implantation and pregnancy outcome).

SUBJECTS AND METHODS

this present study included 120 couples who referred to assist reproduction at the Fertility Clinic at the International Islamic Center for Population Studies and Research, Al Azhar University, Cairo, Egypt during the period from January to October 2015. The cases of the study were classified into 3 groups according to semen parameters:

Group I: normozoospermia which were divided into two subgroups 20cases ICSI and 20 cases PICSI.

Group II: teratozoospermia which were divided into two subgroups 20 cases ICSI and 20 cases PICSI .

Group III: thawed semen which were divided into two subgroups 20 cases ICSI and 20 cases PICSI.

All males were subjected to: complete semen analysis according to the method of WHO^[9].

a-Sample collection after three to five absent days.

b- Physical examination, which includes: semen volume - color - odour- liquefaction^[10, 11&12].

c- Microscopic examination to evaluate sperm concentration, motility and morphology by using ordinary light microscope^[13&14].

Categories of sperm movement^[9]:

1-progressive (PR) motility: spermatozoa moving actively.

2-non-progressive (NP): motility: all other patterns of motility with an absence of progression

3-immotility (IM): no movement.

Sperm preparation: by using sperm gradient medium and sperm washing medium to sperms to reach to a number of motile and morphologically normal sperm cells needed for assisted reproduction WHO^[9].

All female were subjected to Ovarian stimulation

Preliminary evaluations including general, local vaginal examination, ultrasound evaluation were done. Hormonal profile including estradiol (E₂), prolactin (PRL), luteinizing hormone (LH) and follicular stimulating hormone (FSH) were done by Vitek Immune Diagnostic Assay System VIDAS measurement, using the ELFA technique (Enzyme Linked Fluorescent Assay) on day three of the menstrual cycles^[15].

According to the ART protocols, women had ovarian gonadotropin stimulation drugs consisted of human menopausal gonadotropins (HMG) which contains equal concentrations of LH and FSH. All available human FSH pharmaceutical

preparations extracted from postmenopausal urine. While HMG might be used as a source of FSH, it has low specific activity and contains significant amounts of LH (as well as other proteins), which was thought to be associated with poor oocyte quality, reduced fertilization rates, lower embryonic viability and early pregnancy wastage. The development of other urinary FSH (u FSH) preparations (urofollitropin and highly purified urofollitropin), which contain significantly reduced or negligible quantities of LH, had resulted in higher pregnancy rates than with HMG [16].

The number of ampoules of initial gonadotropin dose used for ovarian stimulation was 75-300 IU/ml adjusted according to:

- 1- The patient's age, 2- Body mass index, 3- Baseline serum FSH concentrations on day 2 or 3 of menstruation and 4- Previous response to ovarian stimulation. However, it was costly and associated with risks including multiple pregnancy and ovarian hyper stimulation syndrome.

Cycle Monitoring

During treatment, the ovarian response monitored by

1. Vaginal ultrasound measurements of follicular growth starting on the sixth or seventh day of stimulation and repeated every two or three days according to follicular diameter. At each scan, the size and number of follicles were determined and recorded.
2. In normal responders, ovulation was triggered by administration of 10,000 IU human chorionic gonadotrophin (HCG) intramuscularly, when at least 4 follicles reached 18 mm. in diameter.

Oocyte collection, identification, grading and denudation

- 1- **Collection:** the follicular fluid was aspirated into sterile tubes (14 ml falcon) and the oocyte was identified in the horizontal laminar flow hood. The Oocyte –cumulus cells (OCC) were isolated under a dissecting microscope (Zeiss Stemi 2000-C Stereo Microscope) washed in global total w/HEPES Buffer (Life Global, Europe), then washed and placed into four well (nunc) dishes containing the same medium (**Fig .1**).

2- Denudation: the oocyte was placed in a 100 μ hyaluronidase 80 IU/ml (Life Global, Europe) 30-45 seconds, and then the oocyte was removed and placed in 100 μ l drop of global total w/HEPES Buffer (Life Global, Europe) to remove the corona cells (**Fig .2**) and assessment of oocyte grading [17] (**Table 1**). After denudation was completed, the oocyte washed in global total w/HEPES Buffer (Life Global, Europe) and put in injection dishes of ICSI & PICS, which was covered with 3ml of sterile equilibrated mineral oil.

ICSI procedure

ICSI procedure: ICSI procedure involves the injection of a single motile spermatozoon into the oocyte. The procedure carried out in a plastic microinjection dish containing micro droplets covered with mineral oil. A fraction (1 μ l) of the sperm suspension added to the periphery of the central polyvinylpyrrolidone (PVP) droplet.

PICSI procedure

PICSI dishes were conventional plastic culture dishes pre-prepared with 3 microdots of powdered HA (**Fig. 3**). The powdered HA was re-hydrated by adding 5 μ L droplets of fresh culture medium to each of the three microdots. A 2- μ L droplet with suspension of treated spermatozoa was then connected with a pipette tip to these culture medium droplets. The PICSI dish was incubated under oil; within 5 minutes; the bound spermatozoa were attached by their head to the surface of the HA-microdots and were spinning around their head.

An ICSI injecting pipette used to pick the best motile HA-bound sperm up and inject them one by one into the oocyte. The ICSI injecting pipette was previously loaded with a viscous medium (PVP or Sperm Slow) to facilitate sperm micromanipulation.

In PICSI, HA-sperm bound by the head to the bottom of the dish and have vigorous motility with the tail spinning around their head. HA-unbound spermatozoa, in contrast, swim free all around the droplet of culture medium with varied motility.

Follow up done considering the following:

- | | |
|-----------------------|-------------------|
| 1- Fertilization rate | 2- Cleavage rate |
| 3- Embryo grading | 4- Pregnancy rate |

Assessment of fertilization and embryo's quality:

Fertilization was assessed 16–18 h after microinjection. The injected oocytes were observed for any sign of damage and for the presence of pronuclei. Oocytes were classed as fertilized if two pronuclei (2PN) were present and the second polar body had been extruded. Approximately 72 h after microinjection, adequate number of embryos were transferred to recipient subjects^[18] (Figs. 4&5).

Embryo grading and transfer

Approximately 72 h after injection and according to the cell number and morphology of each embryo, there were four embryo-grading systems according to the method of Hill^[17]. **Grade A:** equal sized blastomeres without fragmentation, **Grade B:** slightly unequal blastomeres, up to 10% cytoplasmic fragments. **Grade C:** unequal sized blastomeres up to 50% fragments and large granules. **Grade D:** unequal blastomeres with significant fragmentation and large black granules. Day 3 embryos were transferred to recipient subjects according to the guidelines of the American Society of Reproduction. Excess good quality embryos were preserved. Fourteen days after embryo transfer, serum β -HCG was determined as a chemical pregnancy test (considered positive if >20 IU/L) and a transvaginal ultrasound scan of the uterus was done after 6-7 weeks of amenorrhea to determine whether a clinical pregnancy had been established (Intrauterine gestational sac visible).

RESULTS

1-Incidence of fertilization rate:

The incidence of fertilization rate showed in **table 2** revealed that, in normozoospermia PICS group was higher in comparison with ICSI ones. However, these differences were statistically insignificant ($P > 0.05$). While in teratozoospermia and thawed semen groups the fertilization rate was significantly higher ($P < 0.05$) in PICS was detected in comparison with ICSI.

2-Number of transferred embryos:

The data in **table 3** showed that the number of transferred embryos was higher in normozoospermia in PICS group compared to ICSI. However, these differences were

statistically insignificant ($P > 0.05$). The embryo's number was significantly higher ($P < 0.05$) in teratozoospermia and thawed semen in PICS compared to that in ICSI.

3-Embryo grading:

Table 4 recorded an increase in the percentage of grade A embryo in PICS compared to ICSI in normozoospermia group, while grade B embryo was higher in ICSI compared to PICS; these differences were statistically insignificant ($P > 0.05$). In teratozoospermia and thawed semen groups, a significant increase was recorded in grade A embryos in PICS compared to those are ICSI. While in grade B, embryos were significantly higher in ICSI compared to PICS; these differences were statistically significant ($P < 0.05$).

4-Incidence of pregnancy:

The present data in **table 5** showed that the incidence of pregnancy test was insignificantly higher when the number of embryo transfer was > 3 embryos compared to the number of embryo transfer when it was 2-3 embryos in normozoospermia, teratozoospermia and thawed semen groups.

5- Pregnancy test:

The incidence of pregnancy test (**Table 6**) showed that: the +ve pregnancy test was significantly higher ($P < 0.05$) when the origin of embryo transfer was PICS compared to that of ICSI in both normozoospermia, teratozoospermia groups. While, in thawed semen, +ve pregnancy test it was insignificantly higher ($P > 0.05$) when the origin of embryo transfer was PICS compared to embryos originated from ICSI technique.

DISCUSSION

Primary indication for ICSI is male factor infertility constituting patients with subnormal semen parameters. However, ICSI used to treat couples with unexplained infertility, characterized by normal seminal sperm concentration and motility^[19]. Since sperm, quality plays an important role in determining ICSI outcome. Hyaluronic Acid (HA) has a natural sperm-selective function. It was demonstrated that, a method for *in vitro* selection of mature spermatozoa based on sperm-HA binding results in spermatozoa for ICSI with low incidence of aneuploidies and DNA damage.

Since the chromosomal status and the chromatin integrity are not predictable by the observation of sperm dimension and shape when performing conventional ICSI, the injection of aneuploid spermatozoa may generate chromosome aberrations in ICSI offspring; likewise, oocyte fertilization with spermatozoa with damaged DNA may lead to an increased risk of pregnancy loss^[20]. We tried to investigate if better sperm selection through PICSi would help improve outcome in patients with unexplained infertility. Our findings in 120 subjects undergoing ICSI monitored a statistically significant improvement in fertilization rate, embryo quality by favoring selection of spermatozoa with normal nucleus and intact DNA in PICSi group of teratozoospermia and thawed semen than ICSI in the same groups. Against this, in two studies with a small number of patients involved no differences in fertilization^[21], pregnancy and implantation rates^[22] observed when comparing HA-ICSI to PVP-ICSI.

Parmegiani *et al.*^[23], **Ménézo and Nicollet**^[24] and **Worriolow *et al.***^[25] observed when injecting HA-bound spermatozoa caused positive drift in fertilization and pregnancy rates, also a higher fertilization rate was observed when using PICSi technique, and a very low DNA fragmentation level by the Tunnel assay in both HA-bound sperm and sperm prepared by density gradient separation (1.17% and 1.59%, respectively)^[26&27]. In comparison with routine sperm preparation techniques, spermatozoa selected by HA binding resulted in significantly higher fertilization rates following ICSI^[28].

The contribution of sperm towards embryogenesis can be understood to be spread over two periods. First is the early period that extends from the point of fertilization to the period of early embryo development, generally considered to be up to the eight-cell stage. Second is the late period that extends from the eight-cell stage (point of embryonic genome activation) to birth and even beyond. In the early period, the sperm exerts its influence by affecting fertilization, syngamy and the first mitotic division. This achieved by oocyte activating factor, which brings about second meiotic division in the oocyte, and by proximal centriole, that is necessary for spindle assembly during

syngamy and mitotic division. In the late period (8 cell stage and beyond), after the embryonic gene switches on, the sperm can influence embryogenesis by way of the genome that it contributes. If the genome altered by DNA breakages, it may manifest into a poor development to blastocyst stage, poor implantation or even a high risk of early pregnancy loss. DNA breakage is unlikely to affect embryogenesis in the period before the embryonic gene has been switched on^[29]. Hence a sperm with DNA fragmentation is more likely to affect implantation rates and early pregnancy loss rate and less likely to affect fertilization rates, cleavage speeds or day2/3 embryo quality.^[29 &30] No correlation between the HA-binding assays PICSi the degree of DNA damage^[31]. The HA-bound spermatozoa did not differ from HA-unbound ones as to DNA fragmentation (19.6% versus 21.4%, respectively). In spite of presence of difference between embryo quality in our study group PICSi of Normozoospermia and ICSI in the same groups, but this difference was not statistically significant. This finding might be due to the binding of the sperm to hyaluronic acid (HA) which correlates with the followings: maturity of cell, lower degree of chromosomal aneuploidy, decreased DNA fragmentation, increased chromatin integrity, normal head-morphology, consequently improved fertility potential and so improved the embryo quality. Our result agreed with previous studies of **Van Den Bergh *et al.***^[32] and **Parmegiani *et al.***^[33] which reported a significant improvement in the embryo quality in PICSi cases versus ICSI and revealed that injection of HA-bound spermatozoa (HA-ICSI) determined a statistically significant improvement in embryo quality and implantation when performing ICSI on a limited number of oocytes (between 1 and 3).

Injection of HA-bound spermatozoa improved embryo quality and development by favoring selection of spermatozoa with normal nucleus and intact DNA in fact; top-quality embryo rate is higher in HA-ICSI than in conventional PVP-ICSI and embryo development rate also had been found significantly increased^[34].

Against this, in a study with a small number of patients involved [18 patients and 44 patients^[35] no differences in fertilization was observed when

comparing HA-ICSI to PVP-ICSI. In addition, a retrospective comparison study of HA-ICSI versus an historical control group of PVP-ICSI, there was no statistical significant difference in fertilization^[36]. However, it was difficult to analyze the contribution of the spermatozoa in fertilization when ICSI performed on a limited number of oocytes. In spite of presence of difference between fertilization rates in our study group PICSU of Normozoospermia and ICSI in the same groups, but this difference not statistically significant.

However, an important difference regarded the quality of the embryos: 76% classified grade A in the first group (PICSU) and only 63.6% classified as grade A in the second group (ICSI), but this difference was not statistically significant^[37]. In our study, we found differences and increased pregnancy rate when number of embryo transfer (> 3 embryos) in comparison with embryo transfer (2-3 embryos) in all groups, but this difference was not statistically significant.

Our results is in harmony with a previous study of **Choel et al.**^[8] who investigated the efficacy of HA sperm selection in a specific patient group with previous poor fertilizations or repeated implantation failures despite having normal semen parameters. The authors reported no benefit in terms of fertilization or embryo quality in such patients.

The chance of pregnancy increased along with the number of embryos transferred^[38, 39&40]. This could be explained by the dialogue between the embryo and the endometrium before implantation^[41] or by the increased placental mass in early multiple implantations, which produces more hCG and progesterone than singleton placentas and might affect important factors in implantation^[42]. In our study, the pregnancy rate concerned either PICSU technique or mixed (ICSI & PICSU) techniques.

Therefore, in our study, we found differences in the pregnancy rate (statistically significant) in Normozoospermia, Teratozoospermia (PICSU) compared to ICSI. In spite of presence of difference between pregnancy rate in our study group (PICSU) of Thawed semen and ICSI in the same groups, but this difference was not statistically significant. This finding is in accordance with a previous work of **Menezo et al.**^[43] who found the same positive trend in

pregnancy rate (when injecting HA-bound spermatozoa) comparing 92 HA-ICSI versus 110 PVP-ICSI treatments.

CONCLUSION:

Our results suggested that even though PICSU may lead to selection of sperm of superior quality in terms of DNA integrity and a lower aneuploidy rate, no apparent clinical improvement was visible. We conclude that there was a trend towards better fertilization (statistically significant) in the study group PICSU of teratozoospermia and thawed semen than ICSI in the same groups. In our study, we found a positive drift in embryo quality (statistically significant) in the study group PICSU of teratozoospermia and thawed semen than ICSI in the same groups. In our study, we found differences and increased the pregnancy rate when number of embryo transfer (> 3 embryos) more than embryo transfer (2-3 embryos) in all groups, but this difference was not statistically significant. In our study, the pregnancy rate concerned either PICSU technique or ICSI. Therefore, we found differences in the pregnancy rate (statistically significant) in normozoospermia, teratozoospermia and thawed semen with PICSU compared to ICSI technique. We believe that PICSU technique in ART is one of very important technique that improved fertilization rate.

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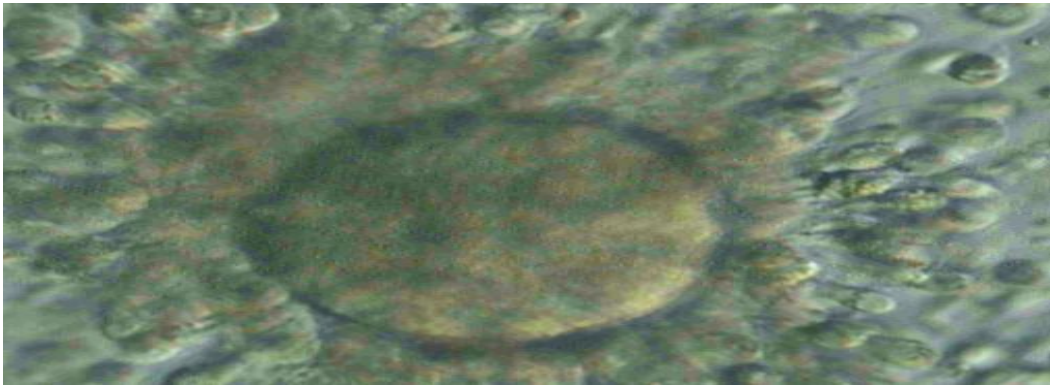


Fig.1: photomicrograph of the oocyte surrounded by the cumulus masse

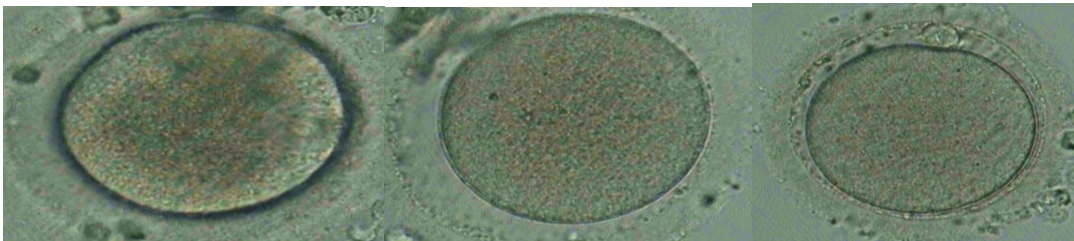


Fig.2 : photomicrographs of (A) oocyte at germinal vesicle stage (GV), (B) oocyte at metaphase I stage and (C) oocyte at metaphase II.

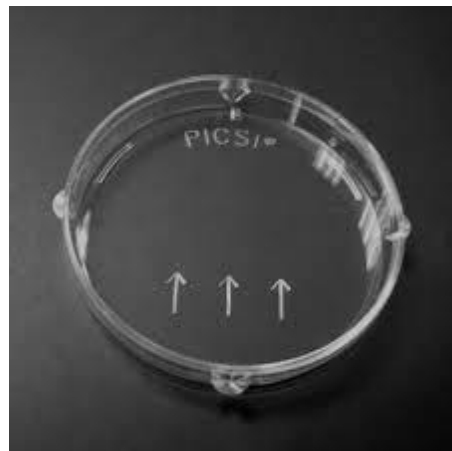


Fig. 3: photograph showing the PICSI dish.



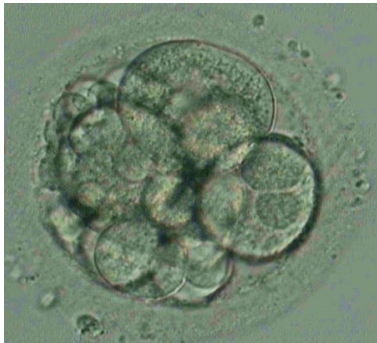
Fig .4: photomicrographs showing (a) fertilized oocyte with one polar body and there is a pronuclei, (b) Fertilized oocyte with two cells; (c) Fertilized oocyte with four cells.



Embryo Grade (A)



Embryo Grade (B)



Embryo Grade (C)



Embryo Grade (D)

Fig. 5: photomicrographs showing the cleaving embryos scored according to equality of blastomeric size and proportion of nucleated fragments observed by inverted microscope (Magnification x 400).

Table1: assessment of oocyte grading ^[17].

Grade	Characteristics
Grade 1 (immature oocyte, prophase I)	Shows a centrally located germinal vesicle. No polar body present.
Grade 2 (nearly mature, metaphase II)	No polar body, no germinal vesicle.
Grade 3 (mature/ preovulatory, metaphase II)	Sometimes appears loosely aggregated extruded polar body, no nucleus with clear ooplasm, homogeneously granulated.
Grade 4 (postmature)	Polar body is still intact or fragmented. Ooplasm may be slightly darkened, mainly granulated. Oocyte is still round.
Grade 5 (atretic nonviable)	Atresia occurs in all oocytes from early immature to postmature stages. Polar body and nucleus are degenerated, if present. Ooplasm is dark and vacuolated. Uneven surface and very irregular shape of the oocyte; a preivitelline space is obvious Clearly visible dark (brush-like) zona pellucida.

Table 2: relation between incidence of fertilization rate in normozoospermia, teratozoospermia and thawed semen by ICSI and PICS techniques.

groups	incidence of fertilization rate		significance
	ICSI	PICSI	PICSI & ICSI
normozoospermia	92.33±16.10	95.6±9.05	P > 0.05
teratozoospermia	85.2±19.1	90.5±14.4	P < 0.05*
thawed semen	81.12±21.81	88.4±14.4	P < 0.05*

Table 3: relation between numbers of transferred embryos in normozoospermia, teratozoospermia and thawed semen by ICSI and PICS techniques.

groups	No. of transferred embryos		significance
	ICSI	PICSI	PICSI & ICSI
normozoospermia	91.5±16.5	94.1±10.8	P > 0.05
teratozoospermia	80±21.9	88.1±17.4	P < 0.05*
thawed semen	74.9±22.8	85.3±17.4	P < 0.05*

Table 4: relation between embryo grading in normozoospermia, teratozoospermia and thawed semen by ICSI and PICS techniques.

groups	embryo grading	ICSI	PICSI	P value
normozoospermia	Grade A	67.8±35.3	96.7±12	P > 0.05
	Grade B	32.2±35.35	3.3±12.1	
teratozoospermia	Grade A	74.9±35.4	95.8±12.9	P < 0.05*
	Grade B	25.1±35.4	4.2±13.1	
thawed semen	Grade A	77.7±31.5	95.2±15.2	P < 0.05*
	Grade B	22.3±31.7	4.8±15.2	

Table 5: relation between number of embryo transfer and pregnancy test in normozoospermia, teratozoospermia and thawed semen

groups	number of embryo transfer			P value
	pregnancy test	2-3 embryos	> 3 embryos	
normozoospermia	Positive	60%	100%	P > 0.05
	Negative	40%	0%	
teratozoospermia	Positive	60%	65%	P > 0.05
	Negative	40%	35%	
thawed semen	Positive	64.71%	100%	P > 0.05
	Negative	35.29%	0%	

Table 6: relation between embryo transfer (ET) origin and pregnancy test in normozoospermia, teratozoospermia and thawed semen.

groups	pregnancy	Embryo transfer origin (ET)		P value
		ICSI	PICSI origin	
normozoospermia	positive	41.70%	75%	P < 0.05*
	negative	58.30%	25%	
teratozoospermia	positive	0%	78.79%	P < 0.05*
	negative	100%	21.21%	
thawed semen	positive	58.33%	75%	P > 0.05
	negative	41.67%	25%	