Comparison between Zeta Potential and Hyalurenan Binding Methods to Improve Sperm Selection for Intracytoplasmic Sperm Injection (ICSI)

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ABSTRACT--- ***At present, sperm selection for ICSI only depends on morphology and motility, but these parameters may not be relevant the chromatin integrity. So sperm selection based on sperm functional characterized has been suggested. Thus, aim of this study was the comparison between two sperm selection method, HA binding and Zeta method, to select spermatozoa with normal morphology and intact chromatin.***

Methods:
Semen samples from 150 infertile couples referring to Nile Center For IVF were assessed during this study. Semen analysis was carried out according to WHO criteria. Semen divided in 3 groups. In 2 groups Zeta method and HA binding applied to select spermatozoa and 1 group as control. Sperm morphology, motility and DNA fragmentation were assessed by Papanicolaou staining, Chromomycin A3 (CMA3) staining, and SCD test, respectively.

Result:
Both HA binding assay and Zeta method are efficient to select sperm with normal morphology and motility. But in term of DNA fragmentation Zeta method appear to be more efficient to select sperm with low DNA fragmentation .

Conclusion:
The results of this study suggest that these sperm selection method can select spermatozoa with normal morphology and protamine deficiency and can use of selected sperm in ICSI. But Zeta method may be more efficient to select sperm with low DNA fragmentation. In patient with high DNA fragmentation, Zeta method can be useful to select spermatozoa for ICSI.

Keywords---- Hyaluronic acid (HA), Zeta potential, Intra-Cytoplasmic Sperm Injection (ICSI), Morphology, Motility, DNA fragmentation

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1. INTRODUCTION

The technological advance of intracytoplasmic sperm injection (ICSI) enables a single spermatozoon to be introduced into an oocyte (Palermo et al., 1992). During this procedure, the natural process of sperm selection is superseded by the embryologist and is based on sperm morphology and motility within the limits of microscopic magnification as well as the availability of motile spermatozoa. Recent studies show that spermatozoa selected with normal morphology and nuclear features using specialised microscopy may lead to higher fertilisation, implantation and live births (Bartoov et al., 2001, 2002, 2003; Berkovitz et al., 2005, 2006). Despite these improvements, concerns remain regarding insemination of spermatozoa with chromosomal aneuploidies and DNA fragmentation during ICSI. Indeed, it is shown that

spermatozoa shape does not predict the presence or absence of chromosomal aneuploidies (Celik-Ozenci et al., 2003, 2004). In addition, diminished sperm maturity is associated with the possible presence of apoptosis and associated DNA fragmentation (Cayli et al., 2004; Huszar et al., 2007). Thus, sperm shape is an inadequate parameter for sperm selection. Other procedures should be used for sperm selection with normal DNA integrity; collectively known as chromatin integrity (Celik-Ozenci et al., 2003; Jakab et al., 2005). The literature studies reveal that many authors have implemented different procedures for selection of mature spermatozoa for the ICSI procedure. These procedures include: (i) sperm density gradients based on sperm mass to volume (Morrell et al., 2004), (ii) swim up based on sperm motility (Lopata et al., 1976), (iii) glass wool filtration based on self propelled movement of the spermatozoa and the filtration effect of glass wool (Henkel & Schill, 2003), (iv) zeta method and electrophoresis method based on sperm surface charges (Kaneko et al., 1984; Engelmann et al., 1988; Ainsworth et al., 2005; Chan et al., 2006), (v) hyaluronic acid (HA) binding method based on the presence of an HA receptor (Jakab et al., 2005) and (vi) sperm magnetic sorter based on apoptotic markers such as the presence of phosphatidyl- serine and FAS (Grunewald et al., 2001; Said et al., 2006) on the surface membrane of spermatozoa. Recently, more emphasis is given to the zeta and HA procedures. Mature spermatzoa possess an electric charge of)16 to)20 mV (Ishijima et al., 1991), which decreases with capacitation (Focarelli et al., 1990) or exposure to uterine neuraminidase and follicular fluid (Srivastava & Farooqui, 1980). This electric charge is termed the zeta potential and is reported to be due to sialoglyoproteins in the sperm membrane (Ishijima et al., 1991). In the epididymis, prostasomes link three forms of negatively charged gp20-CD52 glycopolypeptides to the sperm plasma membrane by glycosylphosphatidylinositol anchors (Kirchhoff & Hale, 1996; Rooney et al., 1996; Arienti et al., 1997; Yeung et al., 1997; Della Giovampaola et al., 2001; Ermini et al., 2005). This is the reason for sperm stickiness to surfaces in protein-free medium. Therefore, Chan et al. (2006), used this characteristic for sperm selection showing that this procedure selects spermatozoa with better quality; mainly in terms of morphology, DNA integrity and the absence of excessive histones (Chan et al., 2006).
During spermiogenesis, along with membrane remoulding and concomitant with formation of zona pellucida binding sites, the formation of an HA binding site occurs (Huszar et al., 1997). The assessment of HA binding is based on the proportions of bound spermatozoa with increased tail cross beat frequency versus unbound swimming spermatozoa that do not bind to HA (Huszar et al., 2004, 2007). Nonmotile spermatozoa without tail movement are not considered. It is shown that, as with zona binding, spermatozoa first bind to HA with an oriented head (Cherr et al., 1999; Vines et al., 2001). In addition, spermatozoa that bind to HA exhibit a uniform shape conforming to normal cells of the Kruger classification with strict criteria, which is based on the zona pellucida bound spermatozoa (Kruger et al., 1986; Gergely et al., 1999; Celik-Ozenci et al., 2004). It is also shown that an HA bound spermatozoon shows characteristics of a mature spermatozoon such as the absence of cytoplasmic residues (Jakab et al., 2005), excessive histones, apoptosis (Cayli et al., 2003, 2004; Celik-Ozenci et al., 2003; Seli & Sakkas, 2005) and high ceratin kinase activity (Huszar et al., 2004). These characteristics of HA binding spermatozoa became the basis for the prediction of fertility potential and the ICSI selection procedure (Cayli et al., 2003; Jakab et al., 2005; Nasr-Esfahani et al., 2008a). Thus, it is believed that this procedure may alleviate potential problems related to chromosomal aneuploidies and DNA fragmentation that presently cause concern regarding fertilisation following ICSI with just visually selected spermatozoa (Celik-Ozenci et al., 2004).

Following the first pregnancy and delivery by intracytoplas- mic sperm injection (ICSI), this procedure has been widely applied for treatment of infertility, particularly male factor infertility. ICSI has assisted many infertile couples to have children and will continue to do so (Palermo et al., 1992). In comparison to in vitro fertilization (IVF), this procedure may result in a higher fertilization rate and higher number of early cleaving embryos but lower blastulation, implantation and pregnancy rates, and possibly higher embryo anomalies and abortion rates (Lucas et al., 2010). One of the main reasons for these differences is the quality of sper- matozoa used for ICSI (Shoukir et al., 1998).

In natural fertilization following ejaculation, spermato- zoa migrate through several barriers and anatomical compartments including cervical mucosa, uterus, uterine tube, cumulus cells, zona pellucida (ZP) and finally, oolema before participating in fertilization (Suarez & Pacey, 2006). It has been shown that these barriers exclude immature and aneuploid spermatozoa from participating in fertilization (Suarez & Pacey, 2006). In ICSI, sperm selection is solely dependent on the embryologist's experience and is based mainly on sperm motility and morphology (Palermo et al., 1992). Recent studies reveal that these sperm characteristics do not exclude spermatozoa with DNA damage, especially in individuals with male factor infertility (Celik-Ozenci et al., 2004). Indeed, in these individuals, a higher percentage of spermatozoa with normal morphology has been shown to have damaged DNA compared to fertile controls (Avendan˜o et al., 2009). This suggests that ICSI may pro- vide an opportunity for damaged or aneuploid spermato- zoa to participate in the fertilization processes, which may have different consequences from failed fertilization and embryo development to increased rates of miscarriage and diseases in the offspring, including aneuploidy and possibly childhood cancer. Considering the widespread use of the ICSI procedure and the concerns about insemination of damaged spermatozoa into the oocyte, many researchers have focused on the development of novel sperm selection method for ICSI that is based on functional sperm proper- ties to reduce possible adverse effects of the ICSI procedure.

2. MATERIALS AND METHODS

the study was approved by the hospital ethics committee. All patients gave their informed consent prior to inclusion in the study. Patients undergoing ICSI cycle were enrolled. The exclusion criterion regarding the wife are as follows: (1) age >38, (2) presence of any uterine anomalies like adenomyosis and fibroids larger than 3 cm in size, (3) any demonstrable hydrosalpinx, (4) moderate and sever endometriosis and (5) 3 or less oocytes at retrieval. 150 patients are prospectively randomized with the help of a computer generated randomization table after oocyte retrieval and were assigned to three groups: the ICSI group, were sperm selection for injection was based on visual assessment, the PICSI group, where sperm are selected based on their ability to bind to HA or the zeta potential group, where sperms are selected upon their surface charges. outcome measures studied are fertilization rate, number of top quality embryos and implantation rate. only fresh embryo transfers are included in the study.

Sperm analysis and sperm processing

Semen samples were collected from 150 infertile couples. A portion of semen was used for routine semen analysis and the remainder was washed twice with Ham's F10 (Sigma, St. Louis, MO, USA) + FCS10% (Gibco, Paisley, Scotland, UK), diluted to 5 million ml)1 and used for the assessment of sperm morphology according to strict criteria (Kruger et al., 1993). A Makler counting chamber was used for counting spermatozoa. After immobilising the cells with a fixing solution, the count was expressed as million per ml. Motility was evaluated using direct microscopic examination according to WHO criteria (World Health Organiza- tion, 1999) and morphology using Papanicolaou staining technique according to strict criteria (Kruger et al., 1993). All samples were assessed by one trained individual. As adequate amounts of spermatozoa were required for simultaneous analysis of both HA and zeta procedures, semen samples with concentrations lower than 5 million ml)1 and motility of less than 5% were excluded from this study.

Zeta potential sperm processing method

The zeta method was carried out according to Chan et al., (2006). Briefly, a diluted semen sample was centrifuged and the supernatant was discarded; making sure the minimum amount of medium containing serum remained in the tube. The pellet was subsequently mixed with 1 ml of serum free medium and exposed to a positive surface charge. To induce a positive charge, the tube was placed inside a latex glove to the level of the cap. While grasping the cap, the tube was rotated two or three turns and rapidly pulled out. Each tube was kept at room temperature for 1 min to allow adherence of the charged spermatozoa to the wall of the centrifuge tube. Tubes were held by the cap to avoid grounding of the tube. After 1 min, the tubes were centrifuged at 200 g for 5 min. The medium and pellet were discarded to remove non-adhering sperm and other cells. The tube's surface was washed with 0.2 ml of Ham's F10 + 10 FCS% to neutralise the charge on the tube wall and detach adhering spermatozoa. The collected medium at the bottom of each tube was repipetted and used to rinse the wall of the same tube several times to increase the number of recovered spermatozoa. The results were compared with washed semen samples. To minimise variation, a single trained individual carried out all the procedures. To verify that electrostatic charge was induced during zeta procedure, an electrostatic voltmeter was used (Alpha lab, Salt Lake City, USA).

Hyaluronic acid sperm selection

In the PICSI group, sterile PICSI dishes (Origio MidAtlantic Devices, USA) with three hyaluronan microdots attached to the interior bottom, were used. 10 µL droplets of culture medium (GMOPS, Vitrolife) were placed over the hyaluronan microdots and an elongated 10 µL drop of PVP was made below the drops, before covering the dish with oil. 1-2 µL of sperm suspension was then added to the hyaluronan microdot containing droplets. After 5 min of incubation at 37 ℃, HA bound sperm with normal morphology were removed with an injecting micropipette (TPC, Australia) to the adjacent PVP droplet

Susceptibility of spermatozoa to DNA fragmentation: Sperm chromatin dispersion test

Sperm chromatin dispersion test was carried out according to Nasr-Esfahani et al., (2008a) on diluted semen samples. Slides were horizontally covered with a mix of Wright's staining solution and PBS (1 : 1) for 5–10 min with con- tinuous airflow. Slides were briefly washed in tap water and allowed to dry. A minimum of 500 spermatozoa per sample were scored under the 100· objective of the light micro- scope. Five SCD patterns were established. (i) Sperm cells with large halos (SCBH): whose halo width was similar to or higher than the minor diameter of the core. (ii) Sperm cells with medium size halos (SCMH): their halo size was between the large and small halos. (iii) Sperm cells with very small size halo (SCSH): the halo width was similar to or smaller than one-third of the minor diameter of the core. (iv) Sperm cells without a halo (SCWH). (v) Sperm cells without a halo or degraded (DC): similar to (iv) but weakly or irregularly stained. Sperm cells with very small halos, without halos and without halo or degraded contain fragmented DNA were considered as fragmented and finally percentage of DNA fragmentation was determined for each semen sample.

Evaluation of efficiency of HA and zeta procedures
For evaluation of efficiency of the two procedures, the difference between the mean values of sperm normal morphology, CMA3 positivity, DNA fragmentation in neat semen (control) and zeta or HA procedures were calculated and divided by the mean values of the neat semen times 100.

Ovarian stimulation
Each woman underwent the proper regulation and desensitization of controlled ovarian stimulation (COS) with various protocols of Gonadotropin releasing hormone (GnRH) agonist and Follicle stimulating hormone (FSH) during luteal phase. The follicular growth was monitored by using vaginal ultrasonography and detection of serum estradiol (E2) levels.

Oocyte retrieval, ICSI and embryo culture

Retrieval of oocytes is carried out by ultrasound-guided trans-vaginal aspiration, 36 hours after HCG administration. Follicular fluid is examined under microscope equipped with a heated stage for proper handling of oocytes at 37ºC. The oocytes retrieved are in the form of oocyte-corona-cumulus complexes. The cumulus cells are Graafian follicular cells that surround and nourish the oocyte during its development in the ovary. The innermost layer of cumulus cells, immediately adjacent to the zona pellucida, is called corona radiata. Corona radiata and cumulus cells maintain their contact with the oocyte at the time of ovulation, during a normal menstrual cycle, or after withdrawal by aspiration, in hormonally stimulated assisted reproduction cycles. The cumulus-corona mass has a fluffy appearance around the oocytes. The oocytes surrounded by a compacted mass of granulosa cells which holds the oocyte in the germinal vesicle (GV) stage (Rienzi, et al., 2008).

The identified oocyte-corona-complex are selected and transferred to 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES)-buffered culture medium in order to maintain the appropriate PH. Preparation of the retrieved mature oocytes should be carried out under conditions of constant PH of 7.3 and stable temperature of 37ºC. After collection, oocytes are denuded enzymatically by brief exposure and continuous pipetting on hyaluronidase enzyme. The corona cells are completely removed by pipetting through micropipettes. The mature metaphase II oocytes (MII) are then determined by the presence of the first polar body. ICSI is performed on mature oocytes using fresh sperms. Most of the procedures are performed with CO2-equilibrated culture media under paraffin/ mineral oil that prevents the evaporation of the media and minimize the fluctuations of both the PH and the temperature. After ICSI, the injected oocytes are cultured in 20µl drops of culture media under paraffin/ mineral oil in 6.0% CO2 incubator at 37ºC.

Fertilization Check

Fertilization is assessed on day one, approximately 17-19 hours after sperm micro-injection, and the normal fertilization is determined by the presence of two pronuclei and two polar bodies.

Normally fertilized oocytes should be spherical and have two polar bodies and two pronuclei (2PN). PNs should be juxtaposed, approximately the same size, centrally positioned in the cytoplasm with two distinctly clear, visible membranes (Tesarik and Greco, 1999; Tesarik et al., 2000; Scott, 2003).

The defined normal zygotes are grouped into two categories:

Group (1): Contains a number of normal zygotes that will be cryopreserved using the vitrification technique.

Group (2): Zygotes of this group are allowed to resume their divisions in the same culture conditions 44hours micro-injection (day-2) forming 4-cell embryo with equal blastomeres and no fragmentations [small portions of cytoplasm enclosed by a cell memberane but usually not containing DNA are often formed during cell division. Fragmentation is therefore is defined as the presence of anucleate structures of blastomeric origin (Keltz et al., 2006)] before the embryos are cryopreserved using the same vitrification technique.

3. STATISTICAL ANALYSIS

Data was statistically analysed using IBM-SPSS version 20. One way analysis of variance (ANOVA) was applied to estimate the effect of the applied techniques on the studied parameters. The results were expressed as mean ± standard error of mean.

4. RESULTS

Table 1. One way-ANOVA to test the effect of the applied technique on the DFI, number of oocytes retrieved, FR and top quality embryos.

Parameter	Source	Sum of square	Degree of freedom	Mean square	$F_{calculated}$	P-Value
DFI	Between Groups	0.17	2	0.09	0.00	>0.05
	Within Groups	19758.63	102	193.71		
	Total	19758.80	104			
Number of Oocytes	Between Groups	55.94	2	27.97	1.234	>0.05
	Within Groups	2312.69	102	22.67		
	Total	2368.63	104			
FR (%)	Between Groups	46.42	2	23.21	1.846	>0.05
	Within Groups	1282.34	102	12.57		
	Total	1328.76	104			
Top quality embryos	Between Groups	101.80	2	50.90	7.229	<0.01
	Within Groups	718.16	102	7.04		
	Total	819.96	104			
Still birth rate (%)	Between Groups	0.501	2	0.251	1.008	>0.05
	Within Groups	25.346	102	0.248		
	Total	25.848	104			

$P>0.05$: insignificant effect; $P<0.01$: significant effect at $\alpha= 0.01$.

According to one way ANOVA, all the studied parameters were insignificantly differed among the applied techniques except in the top quality embryo rate was markedly differed (Table1).

Table 2. The female age, DFI, number of oocytes retrieved, FR and top quality embryos of control, zeta-processed and hyalurenase groups.

Parameter	Control (n=35)	Zeta-processed (n=40)	Hyalurenase (n=30)
Female age (years)	29.09 ± 1.06^a	27.83 ± 0.99^a	28.03 ± 1.12^a
DFI	42.74 ± 2.43^a	42.83 ± 2.08^a	42.83 ± 2.62^a
Number of oocytes retrieved	12.49 ± 0.92^a	11.78 ± 0.72^a	10.63 ± 0.76^a
FR (%)	7.43 ± 0.63^a	8.80 ± 0.60^a	7.43 ± 0.52^a
Top quality embryos (%)	3.74 ± 0.39^a	6.08 ± 0.48^b	5.10 ± 0.45^b
Still birth rate (%)	$13/35$ $(37.1\%)^a$	$21/40$ $(52.5\%)^a$	$12/30$ $(40\%)^a$

In each row, the mean values marked with the same superscript letter are similar (insignificant, P>0.05) whereas those with different ones are significantly differed (P<0.05).

In (Table2), Female age, DFI, Number of oocytes retrieved, FR(%) and, Still birth rate(%) showed homogenity among all the studied techniques.

The top quality embryo(%) in the Zeta processed group was in significantly higher than in the Hyalurenase group,but significantly higher than the Control group (Table2)

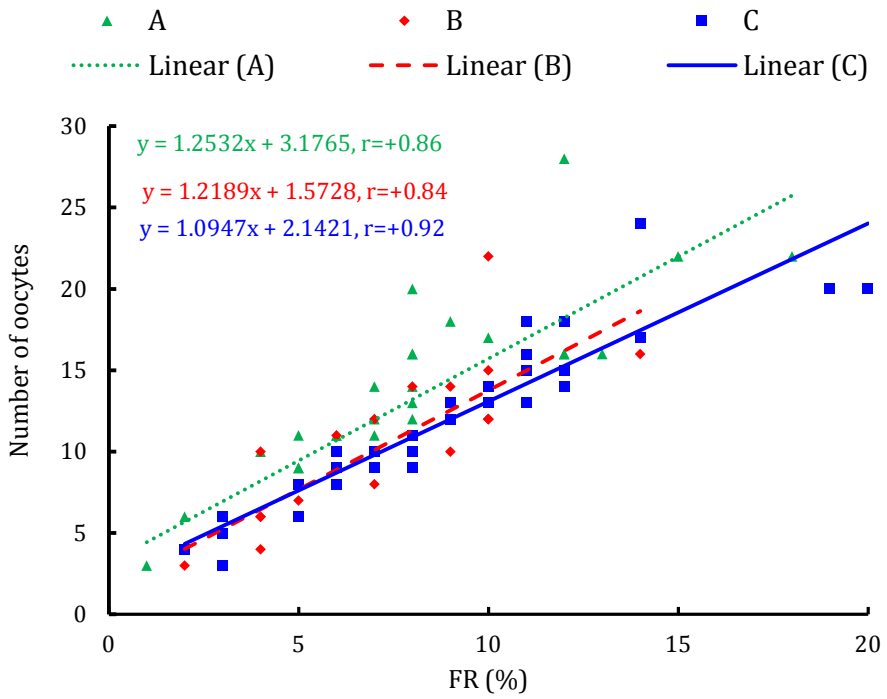

Figure 1. Relationship between the number of retrieved oocytes and the FR in normal (A), hyalurenase (B) and zeta processed (C) groups. r: correlation coefficient.

The Number of oocytes showed positive correlation within the FR(%) in all the standard group (Figure 1)

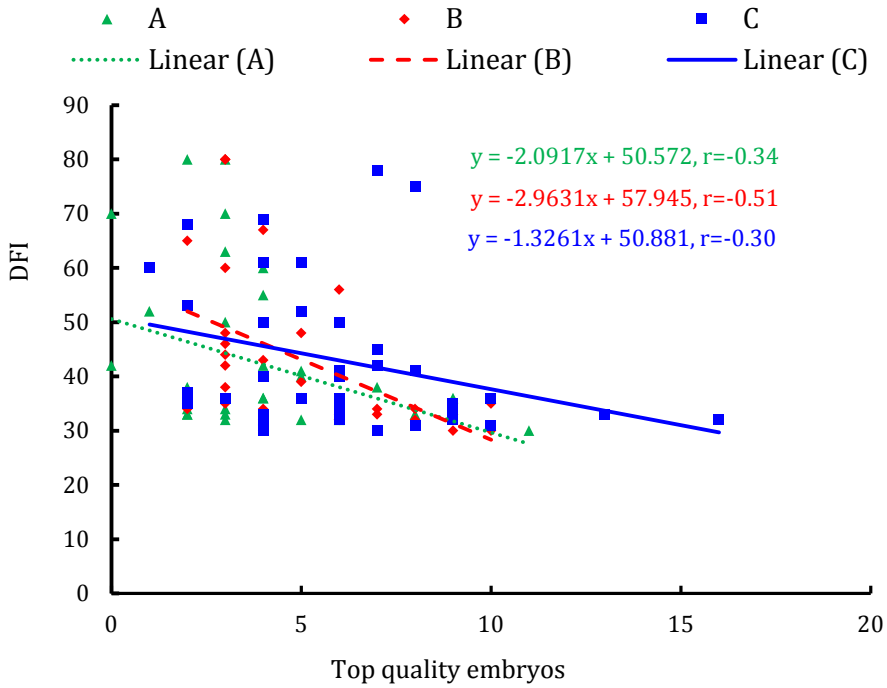

Figure 2. Relationship between the DFI and the top quality embryos (%)in normal (A), hyalurenase (B) and zeta processed (C) groups.

The DFI showed negative correlation within top quality embryos in all the standard group (Figure 2)

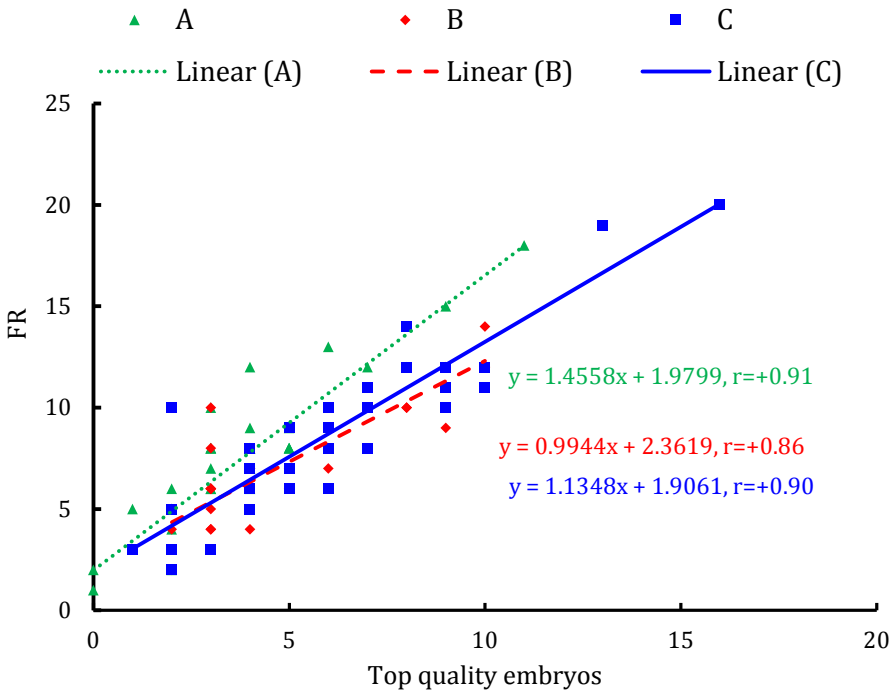

Figure 3. Relationship between the FR and the top quality embryos (%) in normal (A), hyalurenase (B) and zeta processed (C) groups.

The FR showed positive correlation within the top quality embryo in all the standard group (Figure 3).

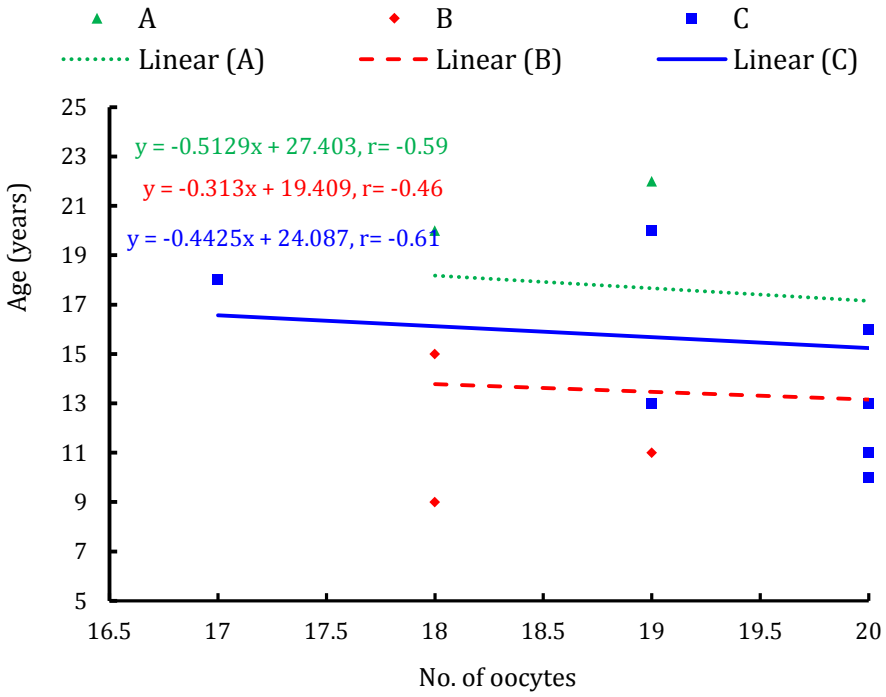

Figure 4. Relationship between the number of retrieved oocytes and the age of females in normal (A), hyalurenase (B) and zeta processed (C) groups.

5. DISCUSSION

Due to concern about the quality of spermatozoa used for ICSI, much effort has been put into sperm selection procedures. Sperm selection procedures used in this study were the zeta and HA methods. Results of the current study showed that the zeta method was highly effective in terms of recovering spermatozoa with normal morphology, intact DNA and normal amounts of protamine. The efficiency of the zeta procedure relative to normal semen for

morphology, DNA integrity and protamine content was 67%, 44.6% and 13.1%, respectively. The recovery rate of spermatozoa after the Zeta method was similar to the recovery rate reported by Chan et al., (2006) (data not shown).

The results of the current study also revealed that sper- matozoa selected using HA procedure had significantly reduced amounts of protamine deficient spermatozoa. This, in turn, reflected the fact that selected spermatozoa had reduced amounts of excessive histone and possibly normally compacted chromatin (Cayli et al., 2003). In addition, spermatoza selected using HA procedure showed significantly lower levels of morphological anom- alies when compared with neat semen. However, DNA fragmentation was insignificantly reduced. The efficiency of the HA procedure relative to normal semen for mor- phology, DNA integrity and protamine content was 95%, 5.9% and 19.1%, respectively.

Comparison of the results between HA, and zeta methods revealed that both procedures significantly reduced morphological anomalies; but the HA procedure selected spermatozoa with significantly lower morphologi- cal anomalies than the zeta procedure. Protamine defi- ciency, an indicator of chromatin maturity, was also assessed in this study. The results revealed that both procedures were efficient in terms of spermatozoa selec- tion for protamine deficiency, but there was no significant difference between the two procedures. However, the results of SCD test revealed that zeta method was able to select higher percentage of spermatozoa with intact DNA, in comparison with HA procedure.

The difference observed between the zeta method and HA procedure can be due to differences in the mecha- nism of sperm selection. Zeta procedure selects spermato- zoa with respect to membrane surface charges or zeta potential while the HA procedure selects spermatozoa in a receptor-mediated manner. Zeta potential is likely to be induced by several surface glycoproteins, one of which is PH-20 – a receptor for HA. Therefore, a wider spectrum of functional glycoproteins may be involved in sperm selection in the zeta procedure. The fact that the HA procedure is a better selection procedure to recover spermatozoa with normal morphology could be explained by concomitant formation of HA receptors on a normal spermatozoon and membrane remoulding during sperm maturation. Although the efficiency of this procedure to recover spermatozoa with normal protamine content is not different between the two procedures, the zeta method appears to be more efficient for selecting sperma- tozoa with low DNA fragmentation when compared with HA procedure. This difference could again be due to the functional difference between zeta and HA procedures.

Analysis of correlation between semen parameters with DNA integrity and protamine deficiency, like the previous studies, reveals a significant positive correlation between sperm morphology and protamine deficiency (Nasr-Esfahani et al., 2001, 2008b; Razavi et al., 2003). This result emphasises that spermatozoa with normal morphology are more likely to have proper protamine content. Furthermore, DNA fragmentation is correlated to protamine deficiency, indicating that protamine defi- cient spermatozoa are prone to DNA damage (Tavalaee et al., 2008) (Table 2).

In this study, HA slides have been used. However, for ICSI, there are FDA approved dishes which can be used for sperm selection. Furthermore, due to the experimental design of this study, a substantial number of spermatozoa were required; therefore semen samples with density of higher than 5 million spermatozoa per ml were used. Considering the higher presence of sperm anomalies in semen samples with lower than 5 million, this could sug- gest that the two procedures might be more useful in ICSI patients with lower than 5 million spermatozoa per ml. Furthermore, each method has its own limitations. Both procedures are required to be carried out as soon as possible upon separation of spermatozoa from the semi- nal plasma, as surface marker changes take place with capacitation (Focarelli et al., 1990). HA procedure can only be performed on semen samples with some degree of motility, while zeta may be able to separate normal spermatozoa irrespective of sperm motility but may be limited to sperm count. In addition, we recommend that clinical efficiency of these methods in ICSI procedures should be investigated.

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