Selection of sperm based on combined density gradient and Zeta method may improve ICSI outcome


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BACKGROUND: Reducing the percentage of sperm anomalies in insemination samples remains a goal to be achieved in the intracytoplasmic sperm insemination (ICSI) procedure. The aim of this study was to evaluate the efficiency of density gradient centrifugation (DGC) and Zeta methods to recover sperm with intact chromatin, and to evaluate whether the combined DGC/Zeta procedure improved ICSI outcome.

METHODS: In Experiment 1, DGC and Zeta methods were carried out on 60 unprocessed semen samples. The samples were then assessed by chromomycin A3 staining, acridine orange test, terminal deoxynucleotidyl transferase dUTP nick end labeling assay and the sperm chromatin dispersion test for protamine deficiency and DNA fragmentation. In Experiment 2, sibling oocytes from 30 ICSI candidates were divided into two groups; one group was inseminated with sperm processed by DGC and the second with sperm processed by DGC/Zeta. The outcomes of 30 ICSI cycles were compared between the two groups and also with 34 ICSI candidates whose oocytes were inseminated by DGC-processed sperm.

RESULTS: Both procedures were efficient for the recovery of sperm with normal protamine content and low DNA fragmentation. However, the Zeta method yielded a greater number of sperm with less DNA fragmentation. Fertilization and pregnancy rates were improved following the combined DGC/Zeta procedure. Compared with DGC alone, the pregnancy rate appeared improved but this was not statistically significant ($P = 0.091$).

CONCLUSIONS: Combining DGC and Zeta procedures improves the quality of semen samples which may increase fertilization rates and possibly pregnancy rates.

Key words: Zeta method / density gradient centrifugation / DNA damage / fertilization rate / pregnancy rate

Introduction

Intra-cytoplasmic sperm insemination (ICSI) provides an ideal approach for the treatment of severe male factor infertility. Although this technology has revolutionized the treatment of infertile couples, apprehension about the incidence of birth defects in such children has not been fully allayed. Currently, selection of human sperm prior to assisted reproduction technique (ART) is based on criteria such as viability, motility and morphology which are far from ideal (Katz et al., 2002). Recently, much emphasis has been laid on DNA integrity, which is related to both the fragmentation status of DNA and proper chromatin packaging. It has been shown that sperm DNA anomalies reduce fertilization, embryo development, implantation and pregnancy rates. However, spermatozoa exhibiting large amounts of DNA damage may remain capable of fertilizing the oocyte and generating embryos with the potential to develop to term (Razavi et al., 2003; Gandini et al., 2004; Nasr-Esfahani et al., 2005). Damaged DNA that is carried into an oocyte by sperm may be repaired by the oocyte to a certain extent, although this potential depends on both oocyte quality and age (Twigg et al., 1998; Gandini et al., 2004). However, genetic defects can result when DNA damage is converted into a genetic or epigenetic mutation as a consequence of aberrant DNA repair before the S phase of the first mitotic division (Aitken and Krausz, 2001; Shimura et al., 2006). Thus, iatrogenic transmission of de novo genetic abnormalities may lead to increased numbers of miscarriage, childhood cancer and other diseases (Cox et al., 2002).
et al., 2002; Ørstavik et al., 2003; Hammadeh et al., 2006). Therefore, integrity of the paternal genome plays a key role in maintaining human reproductive potential, and the impact on conception of an altered paternal genome is certainly at least as important as that of the maternal genome.

Since the presence of a high percentage of spermatozoa with DNA damage may have a negative effect on the outcome of assisted reproductive technologies, the exclusion of such spermatozoa remains a goal to be achieved for optimal conception rates in assisted reproductive techniques (Benchaib et al., 2007; Bakos et al., 2008).

Semen processing is an integral part of all assisted conception procedures. Density gradient centrifugation (DGC) and swim-up procedures are routinely used in ART laboratories. Different kinds of procedures are employed in order to achieve for optimal conception rates in assisted reproductive technologies, the exclusion of such spermatozoa remains a goal to be achieved for optimal conception rates in assisted reproductive techniques (Benchaib et al., 2007; Bakos et al., 2008).

Semen samples obtained from 60 individuals were divided into three equal portions. The control semen portion was washed with Ham’s F10 supplemented with 10% HSA and 10% FCS and centrifuged for 7 min at 300 g. The supernatant was discarded and the remaining pellet was washed in phosphate-buffered saline (PBS), pH 7.4. A droplet of this sperm suspension was smeared onto slides, air-dried and fixed by immersion in freshly prepared 4% paraformaldehyde in PBS for 25 min at 4°C. The slides were then washed in fresh PBS for 5 min at room temperature, treated with 0.2% Triton X-100 in PBS for 5 min and rinsed twice in PBS for another 5 min at room temperature. Excess liquid was removed by tapping the slides. The procedure was repeated for each batch of analyzed slides (Henkel et al., 2004).

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**Materials and Methods**

This study received the approval of the Institutional Review Board of Isfahan Fertility and Infertility Center and Royan Institute. Couples were informed that the use of the Zeta method did not expose sperm to chemicals or agents that were not routinely used in an ICSI procedure. Despite the absence of literature studies regarding the effect of the Zeta method on pregnancy and implantation rates, the ethical committee allowed couples to choose to receive embryos following the combined DGC/Zeta procedure or following the routine DGC procedure. Informed consent forms were signed by all patients. All chemicals were obtained from Merck (Darmstadt, Germany), unless otherwise stated.

**Sperm analysis and sperm processing**

Semen samples were obtained from patients who referred for both semen analysis and ICSI procedure to the Andrology Unit of Isfahan Fertility and Infertility Center. Routine semen analysis was carried out by light microscopy according to World Health Organization (WHO) criteria (WHO, 1999).

**Experimental design 1**

Semen samples obtained from 60 individuals were divided into three equal portions. The control semen portion was washed with Ham’s F10 + 10% albumin, the second portion was subjected to the Zeta method and the third portion was used for DGC. Sperm recovered from each group were evaluated for chromatin integrity using the sperm chromatin dispersion (SCD) test, acridine orange test (AOT), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and chromomycin A3 (CMA3) staining.

**Zeta method**

The Zeta method was carried out according to Chan et al. (2006). Immediately after semen dilution, 5 million/ml of diluted sperm were centrifuged in plastic tubes and the supernatant discarded, ensuring that only a minimum amount of serum-containing medium remained in the tube. The pellet was subsequently mixed with 1 ml of serum-free medium and exposed to a positive surface charge as follows: the tube was placed inside a latex glove up to the cap. The tube was then grasped by the cap, rotated two or three turns and rapidly removed from the glove. Each tube was kept at room temperature for 1 min to allow adherence of the charged sperm to the tube wall and then centrifuged at 200 g for 5 min. The medium and pellet were then discarded in order to eliminate any non-adhering sperm and other cells. Centrifugation does not alter the net charge on the tube (Chan et al., 2006). The tube surface was washed with 0.2 ml of Ham’s F10 + FCS 10% in order to neutralize the charge on the tube wall and to detach adhering sperm. The collected medium at the bottom of each tube was used to rinse the wall of the same tube several times in order to increase sperm recovery. To minimize variation, a single trained individual carried out all the procedures. An electrostatic voltmeter was used to verify that an electrostatic charge was induced during the Zeta method.

**Sperm preparation by DGC**

All procedures were conducted under sterile conditions. Two milliliters of a lower layer of 80% PureSperm gradient was transferred into a conical centrifuge tube. Two milliliters of an upper layer of 40% PureSperm gradient was then gently dispensed on the top of the lower layer. A liquefied semen sample was placed on the top of the upper layer, and the tube was centrifuged for 20 min at 300 g. The upper and lower layers were carefully aspirated without disturbing the pellet. Two to three milliliters of Ham’s F10 + 10% HSA was then added to the pellet which was resuspended and centrifuged for 7 min at 300 g. The supernatant was removed and the pellet suspended in a volume of 0.5 ml of Ham’s F10 + FCS 10% (Nasr-Esfahani et al., 2008a).

**TUNEL assay**

For the TUNEL assay, a detection kit (Apoptosis Detection System Fluoroscien; Promega, Mannheim, Germany) was used. Sperm suspensions were centrifuged for 10 min at 300 g and 4°C. The supernatant was discarded and the remaining pellet was washed in phosphate-buffered saline (PBS), pH 7.4. A droplet of this sperm suspension was smeared onto slides, air-dried and fixed by immersion in freshly prepared 4% methanol-free formaldehyde in PBS for 25 min at 4°C. The slides were then washed in fresh PBS for 5 min at room temperature, treated with 0.2% Triton X-100 in PBS for 5 min and rinsed twice in PBS for another 5 min at room temperature. Excess liquid was removed by tapping the slides. The procedure was repeated for each batch of analyzed slides (Henkel et al., 2004).

**CMA3 staining**

Processed semen samples were fixed in Carnoy’s solution (methanol/glacial acetic acid 3:1) at 4°C for 5 min. Smears were prepared and each slide was treated for 20 min with 100 µl of CMA3 solution (Sigma, USA) [0.25 mg/ml in Mcllvaine buffer (7 ml citric acid (0.1 M) + 32.9 ml Na₂HPO₄ 7H₂O (0.2 M), pH 7.0, containing 10 mM MgCl₂)]. The slides were then rinsed in buffer and mounted with buffered glycerol (1:1). Microscopic analysis of the slides was performed on an Olympus
fluorescent microscope (BX51, Tokyo, Japan) with the appropriate filters (460–470 nm). On each slide, 200 sperm cells were evaluated. Evaluation of CMA3 positivity was carried out using Olyvia software. The pixel intensity of each sperm was recorded. Sperm with pixel intensity greater than 100 were considered to be CMA3 positive or protamine deficient, whereas those with pixel intensity lower than 100 were defined as either CMA3 negative or with a normal amount of protamine (Nasr-Esfahani et al., 2001).

Susceptibility of sperm to DNA fragmentation: SCD
Semen samples were washed with Ham’s F10 and then diluted to 5–10 million/ml. The SCD test was carried out according to Nasr-Esfahani et al. (2008b). Slides were covered with a mix of Wright’s staining solution and PBS (1:1) for 5–10 min with continuous airflow. Slides were briefly washed in tap water and allowed to dry. A minimum of 500 sperm per sample were evaluated under the ×100 objective of the light microscope. Five SCD patterns were established: (i) sperm cells with large halos whose halo width was equal to or greater than the minor diameter of the core; (ii) sperm cells defined as having medium size halos where halo size was interpolated between the sperm cells with high halos and those with very small halos; (iii) sperm cells with very small halo size: the halo width was one-third or less of the minor diameter of the core; (iv) Sperm cells without a halo: (v) sperm cells with very small or no halo and with degraded fragmented DNA. Finally, the percentage of sperm with small (iii), without halos (iv) and degraded (v) considered as percentage of DNA fragmentation for each semen sample.

Acridine orange staining
This is a method for determining single-stranded DNA. The metachromatic dye acridine orange intercalates with DNA and fluoresces green with double-stranded and red with single-stranded DNA.

Depending on sperm concentration, 10–100 μl of ejaculate was diluted 1:5 with Ham’s medium, washed and centrifuged twice for 10 min at 500g. The supernatant was discarded and the pellet was smeared onto a glass slide and air-dried. Slides were fixed in Carnoy solution (1:3 glacial acetic acid and methanol) for 2 h, washed with distilled water and stained for 5–10 min at room temperature with a freshly prepared acridine orange solution (10 ml 0.1% acridine orange in distilled water, 40 ml 0.1 mol/l citric acid, 2.5 ml 0.3 mol/l Na2HPO4). Finally, slides were washed carefully in distilled water, mounted with PBS and 200 randomly selected sperm were analyzed with an epifluorescence microscope. The percentage of red-fluorescing sperm (double-stranded DNA) was calculated (Nasr-Esfahani et al., 2001).

Experimental design 2
Patient selection
Couples that underwent ICSI due to male infertility were informed about both the routine sperm-selection procedure (DGC) and Zeta sperm-selection method. Out of 64 couples, 30 agreed to have half of their oocytes inseminated following the combined Zeta/DGC procedures and the second half of their oocytes to be inseminated following the DGC procedure alone. An additional 34 couples that were undergoing the ICSI procedure during the same period were used as a control group.

Sperm preparation for DGC and DGC/Zeta procedures
For the routine DGC procedure, PureSperm gradients 40 and 80% were used as described earlier. For the combined DGC/Zeta procedure, sperm were prepared by the DGC procedure and then immediately subjected to the Zeta method as described earlier. For both procedures, Sperm Rinse (Vitrolife, Gothenburg, Sweden) was used instead of Ham’s F10 + FCS.

ICSI procedure and embryo scoring
All media were purchased from Vitrolife G3 series plus, unless otherwise stated. A single-stimulation protocol was used for all patients. Briefly, ovarian stimulation was induced using buserelin (Aventis, Germany) from Day 21 of the previous cycle. Human menopausal gonadotrophin (Menogon, Ferring, Germany) in combination with recombinant FSH (Gonal-F, Serono, Switzerland) was administered daily from Day 2 of the cycle. Ovulation was induced by 10,000 IU hCG (Organon, Holland). Oocytes were retrieved by transvaginal needle-guided ultrasound at 34–36 h post-hCG. After collection, the oocytes were treated with hyaluronidase in G-MOPS medium.

Oocytes from the 30 couples undergoing DGC/Zeta were washed in fresh G-MOPS and then randomly divided into two groups and inseminated using: (i) sperm prepared by DGC (control) and (ii) sperm prepared by DGC/Zeta procedure. Oocytes from 34 control couples undergoing routine ICSI were inseminated using sperm prepared by the DGC method alone. ICSI was carried out in a Falcon 1006 dish that contained the G-oocyte and ICSI-100 (a viscous sperm-handling solution) using an Eppendorf micromanipulator mounted on a Nikon inverted microscope. The selection criteria for ICSI were sperm motility and morphology, irrespective of sperm-processing method. Around 16–18 h post-ICSI, fertilization was assessed by the presence of pronuclei. The fertilization rate was calculated from the ratio of fertilized oocytes to the total number of survived injected metaphase II oocytes, multiplied by 100 in both groups.

Cleavage rate was calculated from the ratio of cleaved embryos to the number of fertilized oocytes. Embryo quality was assessed at Days 2 and 3 post-oocyte retrieval, using a four-point score as described by Giorgetti et al. (1995). All cleaved embryos were assigned 1 point, and an additional point was added for each of the following features: absence of fragmentation (or fragmentation involving <25% of embryonic surface), absence of irregularities in blastomere size or shape, 4-cell stage on Day 2 and 8-cell stage on Day 3. Immature, deformed and post-mature oocytes or any oocyte with certain types of abnormality was excluded (Giorgetti et al., 1995).

Pregnancy rate was defined from ultrasonography findings showing at least one embryo with a fetal heart beat 5 weeks after transfer. Implantation rate was defined by the number of embryos with fetal heart beats per number of transferred embryos.

Statistical analysis
The mean, range of variables, coefficients of correlation and Student’s t-test were carried out using the Statistical Package for the Social Studies (SPSS 11.5; Chicago, IL, USA) software to analyze the correlation and compare results between different procedures. Levene’s test for equality of variances was carried out to assess normal distribution. P-values of <0.05 were considered significant.

The efficiency of each method was defined by determining the relative reduction in percent of DNA fragmentation with respect to control or raw semen sample. This was calculated by subtracting the mean percentage of DNA fragmentation in the processed group from the mean percentage of DNA fragmentation in the control group, divided by the mean percentage of DNA fragmentation in the control, multiplied by 100.

Results
Experiment 1
Table 1 gives an analysis of semen parameters (sperm density, motility, abnormal morphology) and the results of sperm chromatin integrity.
tests (percentage of CMA3, TUNEL, SCD and AOT) of unprocessed semen samples from the 60 patients who were evaluated in this study. Semen samples with sperm concentrations lower than 5 million/ml were excluded from this study as inadequate for further analysis.

Table II shows the correlations between semen parameters and the percentage of sperm with chromatin anomalies (assessed by CMA3, TUNEL, SCD and AOT) in unprocessed semen samples from 51 individuals. Only sperm with abnormal morphology showed a significant positive correlation with all of the sperm chromatin integrity tests. A significant negative correlation was also observed between sperm motility and CMA3 positivity.

Table III compares the mean values of CMA3, TUNEL, AOT and SCD in the control, Zeta and DGC groups. Comparison of the mean values of CMA3 positivity between the two procedures with the control shows that the percentage of CMA3-positive sperm was significantly reduced following Zeta and DGC procedures when compared with the control. However, the difference between the mean values of CMA3 positivity in the two procedures was not significant. The percentage of DNA-damaged sperm was also significantly lower following the Zeta method when compared with the DGC with respect to the three tests.

Figure 1 depicts the ability of Zeta and DGC procedure to recover sperm with intact DNA and normal protamine content. The efficiency of the Zeta method to separate sperm with normal protamine and intact DNA appears to be higher than that of the DGC procedure for all four sperm integrity tests.

**Experiment 2**

In this study, 30 couples agreed to have half of their oocytes inseminated by sperm processed by the combined DGC/Zeta procedure. Of the 30 couples, 28 received at least one embryo from oocytes inseminated by this means. Embryos from two individuals were vitrified. The control group consisted of a further 34 other couples who were undergoing the ICSI procedure during the same period.

Table IV shows that the percentage of fertilization was significantly different between sibling oocytes inseminated by sperm prepared by DGC and by sperm prepared by DGC/Zeta (52.39 versus 65.79%, \( P = 0.032 \)). However, the percentage of fertilization was not different

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**Table I** Descriptive analysis of semen parameters and sperm integrity tests in raw samples

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Number of cases</th>
<th>Mean ± SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (million/ml)</td>
<td>60</td>
<td>55.7 ± 20.4</td>
<td>5.00</td>
<td>90.00</td>
</tr>
<tr>
<td>%Sperm motility</td>
<td>60</td>
<td>49.6 ± 13.2</td>
<td>15.00</td>
<td>75.00</td>
</tr>
<tr>
<td>%Abnormal sperm morphology (WHO)</td>
<td>56</td>
<td>69.8 ± 11.2</td>
<td>30.00</td>
<td>94.00</td>
</tr>
<tr>
<td>%CMA3 positivity sperm</td>
<td>51</td>
<td>41.4 ± 11.0</td>
<td>15.00</td>
<td>78.00</td>
</tr>
<tr>
<td>%DNA fragmentation (TUNEL)</td>
<td>55</td>
<td>15.8 ± 8.1</td>
<td>6.00</td>
<td>52.00</td>
</tr>
<tr>
<td>%DNA fragmentation (SCD)</td>
<td>51</td>
<td>50.4 ± 15.6</td>
<td>18.00</td>
<td>88.00</td>
</tr>
<tr>
<td>%DNA fragmentation (AOT)</td>
<td>60</td>
<td>49.6 ± 15.1</td>
<td>20.00</td>
<td>78.00</td>
</tr>
</tbody>
</table>

WHO: World Health Organization.
CMA3: chromomycin A3.
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.
SCD: sperm chromatin dispersion.
AOT: acridine orange test.

**Table II** The correlation between semen parameters and protamine deficiency and DNA fragmentation assessed by CMA3, TUNEL, AOT and SCD in unprocessed semen samples from 51 individuals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>%CMA3 positivity sperm</th>
<th>%DNA fragmentation (TUNEL)</th>
<th>%DNA fragmentation (AOT)</th>
<th>%DNA fragmentation (SCD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (million/ml)</td>
<td>−0.122</td>
<td>0.130</td>
<td>0.135</td>
<td>0.281</td>
</tr>
<tr>
<td>%Sperm motility</td>
<td>−0.445</td>
<td>0.145</td>
<td>−0.098</td>
<td>0.081</td>
</tr>
<tr>
<td>%Abnormal sperm morphology</td>
<td>0.782</td>
<td>0.541</td>
<td>0.566</td>
<td>0.777</td>
</tr>
</tbody>
</table>

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.
SCD: sperm chromatin dispersion.
AOT: acridine orange test.
CMA3: chromomycin A3.
**Significant at P-value < 0.01.**

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between the oocytes inseminated with DGC and those in the control group of patients. Percentage cleavage and embryo score on Day 2 were not significantly different between the groups. Although the embryo score on Day 3 appeared to be higher when compared with the control group, this difference was not statistically significant ($P = 0.09$).

The pregnancy and implantation rates in couples receiving at least one embryo from the Zeta group were 53.57% and 26.18%, respectively, whereas in the control group, they were 33.33% and 15.80%, respectively. Pregnancy ($P = 0.091$) and implantation ($P = 0.152$) rates were not significantly improved in the Zeta group when compared with the control.

Figure 2 shows the percentage of pregnancy and implantation rates in individuals who received all embryos from DGC-inseminated oocytes, and in the control group that received 1 (Z1), 2 (Z2), 3 (Z3) or 4 (Z4) embryos from density gradient centrifugation (DGC)/Zeta-inseminated oocytes.
In order to exclude confounding effects, other parameters including mean male and female age, number of oocytes, number of embryos transferred, female factors (endometriosis, tubal adhesion, PCO), semen parameters and number of repeated cycles were analyzed between the two groups. No significant differences were observed (data not shown).

Discussion

Sperm selection for assisted reproduction should aim to minimize the risk of abnormal sperm participating in the process of fertilization. The ideal sperm-separation technique should eliminate non-viable spermatozoa, leukocytes, bacteria and other sources of contamination. It should be quick, easy, cost effective and able to isolate as many mature spermatozoa as possible without inducing damage to the sperm (Colleu et al., 1996; Henkel and Schill, 2003; Jakab et al., 2005; Varghese et al., 2007).

Recently, different researchers have aimed to reduce the number of sperm with chromatin abnormalities. Little is known about the relative influence of such chromatin abnormalities on embryonic development and the ensuing offspring. However, many studies have shown that paternal genomic alterations may compromise not only fertilization and embryo quality, but also embryo viability and progression of pregnancy, and may cause spontaneous miscarriage or biochemical pregnancy (Razavi et al., 2003; Moustafa et al., 2004; Nasr-Esfahani et al., 2004; Zini and Libman, 2006; Deemeh et al., 2007; Ozmen et al., 2007; Tavalaee et al., 2008a).

In the current study, we compared the efficiency of the Zeta and DGC methods, using different techniques to assess DNA fragmentation and protamine deficiency. This showed that Zeta is more efficient than DGC for recovering sperm with intact chromatin. We found a significant positive correlation between abnormal sperm morphology with protamine deficiency and damaged DNA. A negative significant correlation has been observed between sperm motility and protamine deficiency (Nasr-Esfahani et al., 2007a, b, 2008b; Tavalaee et al., 2007), suggesting that abnormal sperm are more likely to be protamine deficient. Previous studies suggested that DNA damage may be attributed to abnormal chromatin packaging (Sakkas et al., 1999; Nasr-Esfahani et al., 2006; Aitken and De Iulis, 2007; Tavalaee et al., 2008b). A positive correlation was observed between DNA fragmentation and protamine deficiency, suggesting that protamine-deficient sperm are prone to DNA damage.

Our results show that the mean percentage of CMA3 positivity and DNA-damaged sperm was significantly lower following Zeta and DGC procedures than that of the control. Similarly, previous studies have shown that sperm chromatin integrity improves after semen preparation by DGC or Zeta procedures (Angelopoulos et al., 1998; Morrell et al., 2004; Chan et al., 2006; Kam et al., 2007). The novelty of our study is the use of different techniques, including TUNEL, acridine orange staining and SCD test for the assessment of DNA damage.

In this study, the percentage of sperm with normal morphology was improved by using Zeta and DGC procedures in comparison to control. Both procedures were efficient in recovering sperm with normal protamine content, although the Zeta method was more effective in terms of recovering sperm with intact or undamaged DNA. Although the efficiency of sperm selection in terms of protamine deficiency was not different between the two procedures, the Zeta method appeared to be more able to select sperm with intact DNA. The Zeta method was more efficient in terms of selection of sperm with lower DNA fragmentation as measured by TUNEL, AOT and SCD.

The difference observed between the DGC and Zeta procedures may reflect different mechanisms of sperm selection. The Zeta method selects sperm with respect to membrane surface charges or Zeta potential, which is likely to be induced via several surface sialoglycoproteins (Srivastava and Farooqui, 1980; Focarelli et al., 1990; Ishijima et al., 1991; Iqbal and Hunter, 1995; Della Giovampaola et al., 2001). The mechanism involved in the Zeta method is based on the greater net negative charge of mature sperm due to membrane sialoglycoproteins, specifically, gp20-CD52 glycopeptides, which are acquired during transition through the epididymis (Kirchhoff and Hale, 1996). Thus disruptions in the sperm maturation process result in reduced negative charge.

Indeed, sperm given time to replace somatic histones with protamines for optimal chromatin packaging would, additionally, acquire epididymal proteins to yield a negative Zeta potential. Therefore, changes in protein composition in the membrane could be concomitant with histone/protamine exchange, which make sperm prone to DNA damage. In the DGC procedure, sperm isolation is based upon the ratio of sperm mass to volume. During this procedure, highly motile sperm with compact DNA move actively in the direction of the sedimentation gradients (Sakkas et al., 2000; Henkel and Schill, 2003).

The results of these experiments suggest that the Zeta method is more efficient for the separation of mature sperm, especially with minimal DNA damage. Therefore, in the second experiment, immediately following semen processing with DGC, the Zeta method was carried out on the processed sample, and the recovered sperm were used for the insemination of half of the sibling oocytes. A combined DGC/Zeta procedure was used instead of a direct Zeta procedure to produce a cleaner fraction, to reduce the possibility of infection and to use the advantages of the two procedures simultaneously. Table IV shows a significantly greater fertilization rate in the sibling oocytes inseminated by DGC/Zeta-processed samples compared with those inseminated by samples processed by DGC alone.

No statistically significant differences were observed between cleavage rate and embryo score on Day 2. The embryo score on Day 3 appeared higher in the DGC/Zeta group when compared with the DGC group, but this was not statistically significant. Thus, it appears that semen samples undergoing DGC/Zeta processing have a higher potential to induce fertilization when compared with DGC processing alone. This difference may be due to the selection of mature sperm with a normal protamine content and possibly a higher ability to induce oocyte activation. Furthermore, the advantage of applying DGC followed by the Zeta method may enhance sperm quality. Previous studies have suggested that DNA fragmentation may not exclude sperm from participating in the fertilization process, so that the increased recovery using the Zeta method of sperm with low DNA fragmentation may not be relevant to fertilization rates. However, the effect of DNA damage on fertilization outcome is controversial (Henkel et al., 2003; Razavi et al., 2003; Nasr-Esfahani et al., 2005; Zini and Libman, 2006). The higher quality of embryos on Day 3
may be due to lower DNA damage observed in the Zeta-processed samples and to the fact that embryonic genomic activation, which relies on parental DNA integrity, is implemented on Day 3 post-fertilization. The results of this study suggest that pregnancy (P = 0.091) and implantation rates (P = 0.152) in the couples receiving at least one embryo from the DGC/Zeta group may be higher than the group receiving embryos following DGC alone, but the results were not statistically significant. This difference might have achieved statistical significance if all embryos in the treatment group were from DGC/Zeta or if higher numbers of patients were included. Therefore, in order to further elucidate the effect of Zeta method on ICSI outcome, the couples were grouped according to the number of embryos derived from the DGC/Zeta group. As shown in Fig. 2, implantation and pregnancy rates appeared to increase as the number of the embryos derived transferred following DGC/Zeta increase. In addition, out of eight couples receiving embryos solely following DGC/Zeta, six became pregnant, thus resulting in pregnancy and implantation rates of 75 and 46.9%, respectively.

In a similar study by Dirican et al. (2008), sperm were selected according to an early apoptotic marker, annexin V, using a magnetic cell sorter. This procedure reduced the number of sperm with DNA fragmentation and appeared to increase the pregnancy rate. Ainsworth et al. (2007) used an electrophoretic method, based on sperm surface electric charge, which suggested that sperm selected according to negative surface charge have less DNA fragmentation. This procedure was implemented by Fleming et al. (2008) in 28 ICSI candidates on sibling oocytes. Embryos were transferred according to best morphology, and patients who received embryos following this procedure appeared to have higher pregnancy rates. However, there was no statistical analysis of these results. The conclusion of this study was that the method is faster and simpler than DGC and reduces reactive oxygen production (Fleming et al., 2008). Therefore, selection of sperm with efficient methods such as electrophoretic, magnetic cell sorter or Zeta may open a new chapter on sperm-selection procedures for ICSI.

The Zeta method has a number of limitations. It must be carried out immediately after the separation of sperm from the seminal plasma, since changes in surface markers take place with capacitation (Focarelli et al., 1990). Furthermore, as reported by Chan et al. (2006), this procedure has a low recovery rate (8.8%).

In conclusion, Zeta sperm selection appears to be a simple and inexpensive procedure, without requiring additional instruments, for selection of intact sperm with low DNA damage. The study suggests that sperm selected by the Zeta method may have less DNA fragmentation compared with those selected by conventional DGC. Therefore, combining these two procedures may further improve the quality of sperm selected for ICSI and lead to higher pregnancy rates. The current preliminary study needs to be further validated in a more extensive randomized trial.

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