DECREASED FERTILIZATION: HUMAN SPERM DNA FRAGMENTATION AND *IN VITRO* MATURATION OF OOCYTE IN STIMULATED ICSI CYCLES

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Abstract

Objective. The aim of this study was to evaluate the impact of sperm DNA fragmentation (SDF) on fertilization rate, and sperm nuclear decondensation after intracytoplasmic injection of sperm (ICSI) into cumulus-free germinal vesicle (GV) oocytes from stimulated cycles.

Methods and study design. After the retrieval of oocytes, the GV oocytes were cultured for 24 h. Oocytes that liberated polar bodies were injected with processed semen, and were used to evaluate SDF level. Based on SDF, the data were categorized into two groups. Group I in which $SDF \leq 30\%$ and group II in which $SDF > 30\%$. Fertilization (presence of two pronuclei) was checked 16-19 h after ICSI. Unfertilized oocytes were stained by Hoechst 33258 and examined to evaluate the undecondensed sperm head in oocyte. The rates of maturation, fertilization in fertilized IVM oocytes and the percentage of undecondensed sperm in the unfertilized oocytes were assessed according to SDF.

Results. Out of 146 GV oocytes that were subjected to IVM, 101 (69 %) developed to metaphase II. The fertilization rate of IVM oocytes in group II was significantly lower than that in group I ($P < 0.05$). Moreover, group I, had 25 % of their unfertilized oocytes containing condensed sperm, while group II had a significantly higher number (53 %) of unfertilized oocytes containing condensed sperm ($P < 0.05$).

Conclusion. SDF had a negative effect on the rate of fertilization in matured *in vitro* GV oocytes and could lead to an increase in the percentage of undecondensed sperm in IVM oocytes from stimulated cycles.

Key words: sperm DNA fragmentation, germinal vesicle oocyte, *in vitro* maturation, ICSI- fertilization, sperm nuclear condensation.

INTRODUCTION

After controlled ovarian stimulation, about 15-20% (1, 2) and in some cases, most of the follicles

(3) do not respond to the stimulation of the external gonadotropin and remain in immature germinal vesicle stage.

In vitro maturation of oocytes (IVM) provides an opportunity for them to be used as a valuable source of oocytes for intracytoplasmic injection of sperm (ICSI) during the second day, if some of these immature oocytes liberate their first polar body in laboratory conditions (4).

Despite several clinical studies that have been carried out, controversy continues to exist on the fertilization rate and developmental competence of these immature oocytes derived from stimulated ovaries (4-6). One possible explanation for the discrepancies reported in literature for the rates of fertilization in ICSI *in vitro* matured germinal vesicle (GV) oocytes could be that the studies did not take into account the contribution and significance of the sperm in fertilization.

Decondensation of the head of the sperm and formation of male pronucleus are necessary for successful fertilization. Defective chromatin decondensation has been linked to infertility (7-9).

There are two critical factors for proper sperm decondensation: sperm chromatin integrity and decondensation ability in the oocyte. Through bypassing the natural selection of sperm-zona penetration in ICSI method, the risk of oocyte fertilization by sperm containing damaged DNA will be increased. However, Sakkas *et al.* demonstrated that sperm chromatin damage may contribute to the failure of sperm decondensation after ICSI and thus result in failure of pronuclei formation and fertilization (7).

Meanwhile, the first step in sperm decondensation is the swelling of the sperm head,

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which is a required to ooplasmic factor. These factors depend on the maturational state of the cytoplasm.

Considering the fact that nuclear and cytoplasmic maturation do not synchronize on time in IVM oocytes (10) and defective sperm head decondensation factor in IVM oocytes, some studies reported that the fertilization rate in IVM oocytes is less than that in sibling oocytes that matured under physiological conditions (10).

In some of the infertile couples, the number of mature oocytes (metaphase II or MII) is not sufficient and only immature oocytes from stimulated cycles are available from the day of puncture. In addition, in processed semen, the level of sperm DNA fragmentation (SDF) is relatively high such that it cannot be recognized via conventional semen analysis. In such cases, routine intracytoplasmic injection of morphologically normal sperm with unknown DNA fragmentation level into *in vitro* matured oocytes is carried out after ovarian stimulation (11-17). In these cases, the two critical factors for successful fertilization may be impaired.

In order to forestall failed decondensation and fertilization of sperm containing damaged DNA into *in vivo* matured oocytes, there is need for evaluation of the influence of *in vitro* maturation of oocytes on decondensation rate with special attention to sperm chromatin integrity, which serves as donor of half of the embryo genome. In the current study, we cultured GV oocytes for 24 h and evaluated the rate of fertilization according to SDF level. Moreover, we examined the fate of sperm in unfertilized oocytes to evaluate the percentage of undecondensed sperm in these *in vitro* matured oocytes with respect to the SDF level in processed semen.

MATERIALS AND METHODS

Study subjects and design

The present project was approved by the ethics committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (1392-1-91-11402). A total of 50 infertile couples who had primary infertility were included in the study. All subjects were referred for microinjection following unsuccessful previous intrauterine insemination (IUI) cycles. Female participants aged between 25-35 years with at least two retrieved GV oocytes on the puncture day. Decision was made to include those participants who had no detectable fertility problems, including chronic anovulation, polycystic ovarian syndrome (PCOs),

endometriosis, and low ovarian reserve. Also, the male patients aged <45 years old and their routine semen analyses were evaluated to be normal according to World Health Organization criteria (WHO, 2010, Fifth edition); sperm concentration: $\geq 15 \times 10^6/\text{mL}$, total motility: >40% and normal morphology >4%. They also had no history of varicocele and urogenital surgery.

After providing comprehensive information about the study to infertile couple, a small part of the processed semen was used for evaluation of SDF level, and patients were classified into two groups with regard to the threshold value of 30% SDF: group I: $\text{SDF} \leq 30\%$ and group II: $\text{SDF} > 30\%$ (18, 19).

Sperm collection and preparation

After 2-3 days of sexual abstinence, semen samples were collected from the male participants by masturbation. After 30 minutes of liquefaction at 37°C and 5% CO_2 , discontinuous Allgrad (Life Global, Guilford, CT, USA) gradients (100:50) were implemented to process semen samples. In brief, semen samples (1 mL) were layered onto a discontinuous gradient of Allgrad consisting of a layer containing 50% Allgrad on top of 100% Allgrad, (1 mL of each solution). The tube was then centrifuged for 18 min at 300 g. Next, most of the supernatants were gently removed and the pellet was placed into a new, clean tube; the pellet was well washed twice at 350g for 5 min. After processing, the major portion of the semen sample was used for ICSI, and an aliquot of 200 μL of processed semen was used for SDF assessment. After preparation, conventional sperm parameters were determined according to the WHO criteria. Also, Kruger strict criteria were utilized in order to determine sperm morphology.

Evaluation of sperm DNA fragmentation

In the present study, sperm chromatin dispersion (SCD) test was used to assess sperm DNA fragmentation. In order to obtain sperm concentrations ranging between 15-20 million/mL, the processed sperm was diluted in phosphate buffered saline (PBS) buffer. An aliquot of low-melting-point agarose (Sigma-Aldrich chemical Company, St. Louis, Mo, USA) in Eppendorf tube (100 μL) was put in a water bath at 100°C for 5 minutes and was then incubated in desicator for temperature equilibration at 37°C. After incubation for 5 minutes, 50 μL of the sperm suspension was mixed with 100 μL of low-melting-point aqueous agarose (1%); the mixture was pipetted onto a glass slide already precoated with 0.65% standard agarose

and covered with a 22×22 mm coverslip. Afterwards, the slide was placed on a cold plate in the refrigerator (4°C) for 5 minutes and then the coverslip was removed carefully.

Immediately, the slide was immersed horizontally in a freshly produced acid solution (0.08N HCl) in the dark for 7 minutes at 22°C. Next, the slide was incubated in lysing solution I including 0.4 mol/L Tris, 0.8 mol/L dithiothreitol (DTT), 1% sodium dodecyl sulfate (SDS) and 50 mM ethylenediaminetetraacetic acid (EDTA) for 5 minutes, and in lysing solution II including 0.4 M Tris, 2 M Sodium chloride (NaCl), and 1% SDS for 10 minutes. The slide was then washed thoroughly in Tris-borate-EDTA (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5) for 2 minutes and dehydrated in increasing ethanol concentrations of 70%, 90%, 100%, respectively, each for 2 minutes, and were then left to dry out at room temperature.

The slide had to be stained to achieve bright field microscopy reading. This was carried out via Diff-Quick reagent (Medion Diagnostics, Düringen, Switzerland). A minimum of 300 sperm per sample were scored into four groups, according to our previous study (20) (Fig. 1):

I) Big-sized-halo nuclei: the width of the halo is equal to or higher than the core's minor diameter;

II) Medium-sized-halo nuclei: halo size is between the sizes of big and small halos;

III) Small-sized-halo nuclei: halo width is equal to or smaller than one third of the core's minor diameter;

IV) Without- halo nuclei.

Sperm cells with small halos and without halos

contain fragmented DNA. Processed semen samples were classified into two groups with respect to the threshold value of 30% SDF.

Ovarian stimulation

Similar controlled ovarian stimulation protocol was used in all participants (long protocol regime with gonadotrophin releasing hormone (GnRH) agonist. To suppress the ovarian activity, we used pituitary desensitization with 0.1 mg of Decapeptyl (triptorelin; Ferring Pharmaceutical, Germany) starting in the midluteal phase of the previous monthly cycle. Next, the GnRH analogue dose was reduced to 0.05 mg until the day of human chorionic gonadotrophin (HCG) administration. Ovarian stimulation of patients with recombinant FSH (Gonal-F) was obtained and continued by daily injections according to the patient's endocrine and ovarian ultrasonic responses to the extent that the observation of at least two or more follicles reached a mean diameter of 18–20 mm. Then, ovulation was induced with 10000-IU HCG, and transvaginal ultrasound-guided oocyte retrieval was performed 36 hours after HCG injection.

The oocytes were denuded of cumulus cells with hyaluronidase enzyme (Life Global, Guilford, CT, USA) and gentle mechanical pipetting after oocyte retrieval. The oocytes were divided into three groups according to their nuclear maturity profile, immediately after denudation: I) MII oocytes with first polar body, II) MI oocytes without first polar body, and III) Germinal vesicle oocytes comprising a prominent nucleus in cytoplasm.

Figure 1. A) Image of patient's processed semen with SDF≤30%. B) Image of patient's processed semen with SDF>30%. C) A group of different sperm halos; Large halo (head arrow ; ◀), medium halo (↔), small halo (small arrow; ◀) and without halo (*).

Oocyte in vitro maturation

In vitro maturation (IVM) was performed by culturing denuded GV oocytes for 24 h under mineral oil in 50 µl droplet of maturation medium containing bicarbonate-buffered TCM-199 with 2 mM L-glutamine supplemented with 10% FBS, 5.5 mg/mL sodium pyruvate, 25 µg/mL gentamycin sulphate, 5 µg/ml LH, 5 µg/mL FSH, and 1 µg/mL estradiol at 37°C, 5% CO₂ in a humidified atmosphere.

ICSI procedure

After 24 hours, only those oocytes that reached MII stage were used for ICSI. In brief, processed sperm suspension was added to the drop containing polyvinylpyrrolidone (PVP) and morphologically normal sperm were selected, and then immobilized and aspirated into the microinjection pipette. An MII oocyte was held on the holding pipette and an immobilized sperm cell was injected into the cytoplasm of the *in vitro* matured oocyte.

Assessment of fertilization

The injected oocytes were cultured into 30µl droplets of global total medium (Life Global, Guilford,CT, USA) covered with equilibrated mineral oil at 37°C and 100% humidity under a gas phase of 5% CO₂ in air. Sixteen-nineteen hours after ICSI, appearance of two pronuclei was taken as assessment criterion for normal fertilization. The percentage of the zygotes with two pronuclei over the sum of the injected *in vitro* matured MII oocytes was calculated as the fertilization rate.

Evaluation of unfertilized oocytes using Hoechst dyes

All unfertilized *in vitro* matured oocytes were fixed in 4% (w/v) paraformaldehyde for 30 minutes. The oocytes were then stained with 2.5 mg/mL with 2.5 mg/mL Hoechst 33258 in 3:1 (v/v) glycerol/PBS for 30 minutes at room temperature in the dark. The stained oocytes were transferred to glycerol on glass slides, and were flattened *via* coverslips. They were instantly photographed to evaluate the sperm head status under an epifluorescence microscope equipped

with ultraviolet (UV) filters (Nikon, Tokyo, Japan).

The fate of the injected sperm cell was of high interest in this part of the study. After 16-19 hours since ICSI, sperm chromatin status in the cytoplasm of unfertilized IVM oocytes was categorized into two groups; I) condensed; II) decondensing or decondensed sperm head. The decondensation rate was calculated to be as the sum of moderate and grossly swollen sperm heads to the total number of stained unfertilized oocytes.

Statistical analysis

The calculations were performed using SPSS, v. 22. The rate of fertilization was compared making use of non-parametric analysis test (Mann-Whitney U-test) and to compare the rate of sperm head decondensation, independent samples t-test was used. The correlation between SDF and normal sperm morphology evaluated with Pearson correlation coefficient. Results were expressed as means ± SEM. P values <0.05 were considered statistically significant.

RESULTS

Demographic characteristics and reproductive history of infertile couples are summarized in Table 1. There were no differences in mean age, duration of infertility, and the number of previous IUI cycles. As shown in Table 2, the mean of SDF level and normal sperm morphology in two groups showed significant difference ($P < 0.05$). Also, Pearson's correlation coefficient revealed a negative correlation between normal sperm morphology and the SDF level.

A total of 146 immature oocytes from 50 stimulated cycles were collected from women with average ages of 31.2 ± 1.6 and 30.5 ± 1.3 in group I and II respectively. Among the GV oocytes subjected to IVM, 69 % progressed to MII stage 24 h after culture (Table 3).

The total number of injected oocytes after *in vitro* maturation and the fertilization rate are given in Table 4. The number of injected oocytes was 68 and 50 in group I and group II respectively. The rate of fertilization in group I was significantly higher than that in group II ($p < 0.05$).

Table 1. Demographic characteristics and reproductive history of infertile couples

Characteristic	Groups	
	SDF≤30%	SDF>30%
Female age (years) (Mean ±SEM)	31.2 ± 1.6	30.5 ± 1.30
Male age (years) (Mean ±SEM)	35.08 ± 3.60	34.52 ± 4.26
Infertility length (years) (Mean ±SEM)	1.75 ± 0.98	1.90 ± 0.89
Number of previous IUI cycle (Mean ±SEM)	2 ± 0.89	2.40 ± 1.34

Table 2. Mean sperm DNA fragmentation (SDF) and normal morphology in processed semen. Correlations between the percentage of SDF and normal morphology

Variables	Groups	
	SDF≤30%	SDF>30%
SDF in processed semen (Mean% ±SEM)	12.1 ± 4.24 ^a	63.92 ± 18.77 ^a
Normal sperm morphology (Mean% ±SEM)	18 ± 2.70 ^b	6.40 ± 0.81 ^b
Pearson's correlation coefficient		
Correlation between SDF and normal morphology	r	P value
	- 0.70	0.01

SDF: sperm DNA fragmentation; ^{a, b} p < 0.05, Similar letters indicate significantly difference between two groups.

Table 3. Total number and maturational status of germinal vesicle oocytes after 24 hours culturing from stimulated cycles

Total number of germinal vesicle oocytes	Maturation status after 24h culture		
	MII	MI	GV arrest
146	101(69%)	20(14%)	25(17%)

As illustrated in Table 4, 55 unfertilized oocytes were stained by fluorochrome Hoechst 33258. About 3.5% of the stained oocytes did not show the presence of any sperm inside. Ejected sperm was 3.7% and 3.6% in group I and II respectively. In the rest of the oocytes examined in group I, ~25% contained sperm that retained their condensed nature, whereas 60% of the sperm were either decondensing or wholly decondensed. In group II, ~53% of the unfertilized oocytes were found to have undecondensed sperm head and ~38% contained decondensed or decondensing sperm. Sometimes, swollen sperm heads are closely

attached or trapped among the metaphase chromosomes of the oocyte (Fig. 2)

The rate of the sperm head that remained condensed in group I was significantly lower when compared with that in group II (p < 0.05). In addition, no significant percentage difference was found between group I and II of the decondensing or decondensed sperm (p > 0.05). Moreover, 15 and 9% of unfertilized oocytes were categorized as “others”. In group I, four unfertilized oocytes were not interpretable while two were not interpretable in group II.

Table 4. Mean sperm DNA fragmentation (SDF) and normal morphology in processed semen. Correlations between the percentage of SDF and normal morphology

Variable	Groups	
	SDF≤30%	SDF>30%
No. of oocytes injected	68	50
No. of 2PN oocytes (2PN/oocyte injected %)(Mean %±SEM)	41 (63.88± 6.27) ^a	22 (45.43±6.14) ^a
No. of oocytes stained with Hoechst 33	27	28
No. of oocytes with sperm inside	26	27
Unfertilized oocytes containing sperm		
Condensed sperm	7 (24.7±8.5) ^b	15 (52.61±8.7) ^b
Decondensed sperm	15 (60±11.26)	10 (38.38±3.6)
Others	4 (15.27±8.1)	2(9±5.5)

^{a, b} p < 0.05, Similar letters indicate significantly a difference between two groups. Figures in parentheses are Mean% ± SEM.

Figure 2. A) An in vitro-fertilized IVM human oocyte (Oocyte with two pronuclei (arrow) suggesting normal fertilization). B-D) Fluorescence micrographs of unfertilized oocytes stained with Hoechst 33258 and visualized under UV light after ICSI. Sperm nuclei in condensation form (arrow) (B). Various stages of sperm head decondensation are shown, from moderately swollen sperm head (C), to late stages; grossly decondensation form and one pronuclei (pn) (D). Fully decondensed sperm which is closely associated with the metaphase chromosomes of the oocyte. (D) A condensed sperm cell in the PVS. Sperm head (S), polar body (PB), metaphase plate (M).

DISCUSSION

The present study demonstrated that paternal chromatin damage had a negative effect on the rate of fertilization in cumulus-free germinal vesicle oocytes matured *in vitro* for 24 h from stimulated cycles. In addition, we found that in the unfertilized IVM oocytes derived from intracytoplasmic injection of sperm with high level of SDF in processed semen, the percentage of the remaining condensed sperm was higher than that of $SDF \leq 30\%$.

The rate of *in vitro* maturation of oocytes in stimulated cycles, as reported in the literature, varies between 35 and 75% (3, 21-24). The discrepancies in the reported maturation rates in these studies are probably the result of implementation of different study conditions including duration of the *in vitro* culture, media components and addition of different supplements; hormones, vitamins, antioxidants, growth factors and serum (22, 25, 26). Escrich *et al.* (23), reported the highest level of maturation rate (74.8%) in the stimulated cycle to date of cultured cumulus-free GV oocytes in human Tubal Fluid medium (hTF) for 48 h and demonstrated that most of the denuded GV oocytes collected from stimulated ovaries extruded the first polar body (PB) spontaneously after a minimum of 22 h.

In the present study, 69% of cumulus-free GV oocytes collected after superovulation progressed to MII after 24 h *in vitro* culture. This is a finding that is close to the results achieved by Farsi *et al.* (24) who demonstrated that 70% of the retrieved GV oocytes matured to MII in GI media after 24-30 h of incubation. In stimulated ICSI cycles, denudation of cumulus cells is inevitably performed following oocyte retrieval in order to evaluate the oocytes' nuclear maturity status. Thus, we also used cumulus-free germinal vesicle oocytes for *in vitro* culture. However, Kim and coworkers (21) investigated the impact of the presence of cumulus cells on the maturation rate of GV oocytes in stimulated cycles and reported that the presence or absence of cumulus cells did not have any significant effect on the maturation rate of GV oocytes from follicles primed with gonadotropin (21).

However, immature human oocytes that are retrieved from stimulated cycles have the capacity for fertilization and can produce early embryos *in vitro* (27), although the report of previous studies on different fertilization rates varied between 44.2 and 79.8% (4-6). Here, the variation rates of fertilization in ICSI-IVM oocytes may be attributed to inadequate attention to the significance of the sperm in fertilization. As mentioned

above, paternal genome quality is an important factor determining a successful fertilization process in ICSI method, irrespective of the normal appearance of some sperms. The selected sperms for injection contained fragmented DNA (28) and could not be recognized in routine sperm analysis.

So far, many studies have assessed the impact of SDF on the rate of ICSI-fertilization from oocytes that were already mature at the time of oocytes denudation (14, 29); however the results were contradictory. Some clinical studies have found no significant correlation between sperm DNA damage and fertilization rates *in vitro* (11-14, 30), whereas others reported a negative correlation between sperm DNA damage and fertilization rates (15-17, 31, 32).

In the current study however, for the first time, we reported the negative effect of SDF on fertilization rate in cumulus-free immature human oocytes derived from stimulated and ICSI cycles. It was observed that the fertilization rate was significantly lower in group II as opposed to that in group I. The fertilization rates in patients with $SDF > 30\%$ (~64%) from GV-IVM oocytes that were obtained from stimulated ovaries were close to those obtained in the study reported by Farsi *et al.* (65%) (24) and Reichman *et al.* (62.1 %) (3). Similar to our study, stripped GV oocytes were cultured overnight, and sufficient motile sperm of gross normal morphology were implemented for ICSI in both studies. Sometimes, despite the successful injection of sperm into each oocyte, the rates of fertilization could be of dismal quality. In group II with $SDF > 30\%$, our fertilization rate was close to that reported in the study of Shu *et al.* (44.2%) (4), indicating that a significant proportion (56%) of the oocytes were unfertilized and thus potentially wasted.

Unlike our study, none of these studies specified the SDF level in ICSI cycles. Sakkas *et al.* (7) demonstrated that intracytoplasmic injection of sperm with a high level of abnormalities in the sperm chromatin into *in vivo* matured oocytes may lead to decondensation failure in sperm nucleus and thus an apparent failure of fertilization. They suggested that decondensation failure of damaged DNA may be due to its incapability for decondensation physically and mechanically or it may be unsuccessful in the initiation of regulatory mechanisms that ultimately leads to head decondensation and male pronucleus formation. However, the lacuna in our current knowledge was comprehension of the role of SDF in the sperm nucleus decondensation and fertilization in human unfertilized oocytes that matured *in vitro*. Accordingly, in the

present study, to examine the sperm fate in IVM oocytes based on SDF level, we stained unfertilized IVM oocytes using fluorochrome dye. Among the oocytes subjected to staining, approximately 3.5 % showed no sign of sperm inside the ooplasm. Flaherty *et al.* (33), Sakkas *et al.* (7), and Lopes *et al.* (34) reported that 19, 16, and 16.7% of the oocytes examined in that order, had ejected sperm after ICSI. They suggested that the ejection of the sperm was most probably because of a technical error during the ICSI procedure, although in the present study, contrary to their reports, ejection of the sperm from the oocyte into perivitelline space (PVS) occurred only in ~3.5% of all stained unfertilized oocytes.

However, data obtained in the current study revealed that patients with SDF >30 % in processed semen had a significantly higher number of unfertilized oocytes (53 %) that contained condensed sperm. Our data is in consonance with that of Sakkas *et al.* (7), who examined the presence of damaged DNA in the sperm of patients treated with ICSI making use of in-situ nick translation, and evaluated unfertilized mature MII oocytes obtained from stimulated ovaries using the flurochrome Hoechst 33342 to determine whether a relationship exists between failure of fertilization and sperm chromatin damage. Separation of patients into two groups was based on their sperm chromatin abnormality (Chromomycin A3 (CMA3) >30% and endogenous nicks > 10%); they showed that there was no difference between the fertilization rates after ICSI. They also found that patients with a high degree of chromatin damage (nicks >10% of their sperm) had a significantly higher number of unfertilized oocytes (~50%) that contained condensed sperm. As a conclusion, they claimed that a significant factor that results in failure of sperm nucleus decondensation and fertilization could stem from paternal chromatin.

In addition, Lopes *et al.* (34) investigated the percentage of maternal and/or paternal fragmented DNA in unfertilized oocytes after ICSI using a modified version of the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin end-labeling (TUNEL) method. They found that 50 % of oocytes that showed no pronuclear formation contained chromatin with damaged DNA and hypothesized that intracytoplasmic injection of sperm containing abnormal chromatin into oocyte would probably result in failure of sperm decondensation and fertilization.

Moreover, Flaherty *et al.* (33) showed that ~30 and 10% of the unfertilized activated and MII oocytes respectively, contained undecondensed sperm head,

irrespective of the sperm DNA integrity. Nevertheless, some studies found no correlation between chromatin decondensation test and fertilization rate (35-37).

In consonance with Sakkas *et al.* (7), our data showed that high values of sperm DNA fragmentation lead to unfertilized IVM- oocytes that contain higher percentages of condensed sperm. Consequently, a high level of SDF in processed semen for ICSI may impede the completion or initiation of decondensation and therefore lead to a failure in fertilization regardless of the oocyte possessing the necessary mechanism to initiate decondensation.

We cannot claim that the fertilization failure was totally because of a sperm defect; it also seems possible that damaged DNA sperm will have a role in the failure of the decondensation process in IVM oocytes and not in *in vivo* matured oocytes. Nevertheless, this is the first study that has demonstrated that damaged DNA may contribute to the failure of sperm decondensation after intracytoplasmic sperm injection into *in vitro* matured cumulus-free germinal vesicle oocytes.

De los Reyes *et al.* (10), evaluated the sperm head decondensation capacity of dog oocytes that matured *in vitro* (during 0, 48, 72 and 96 h in TCM-199) or *in vivo* (50-72 h after ovulation). After IVF, the oocytes were fixed and stained by DAPI (4',6-diamidino-2-phenylindole). Their data showed that nearly 97% of ovulated oocytes contained decondensed sperm chromatin while sperm decondensation rate in *in vitro* matured oocytes during 48, 72 and 96 h were 65.7, 80.5 and 85% respectively. They concluded that the decondensation ability of IVM oocytes increased up to 72 h of culture and suggested that cytoplasmic maturational state of the oocytes affected the sperm decondensation factors, which is directly associated to culture time rather than nuclear maturation. Our report is in consonance with their data.

Recently, Meseguer *et al.* (38) reported that the effect of SDF on pregnancy outcome depends on oocyte quality. Decreased normal fertilization in IVM oocytes that were injected with sperm containing damaged DNA may be due to nuclear development in IVM oocytes that did not completely synchronize with cytoplasmic maturation. Defective important factors in IVM oocytes that regulate sperm decondensation are not obviously defined and needs further attention.

In conclusion, in the present study, we found that SDF>30% can result in a decrease in fertilization rate and an increase in the percentage of undecondensed sperm head in denuded germinal vesicle. We therefore suggest that the analysis of DNA

integrity of processed sperm can enhance ability to predict the rate of fertilization after *in vitro* maturation of oocytes, especially when the oocytes have failed to mature adequately in stimulated cycles and we have to use *in vitro* matured oocytes to increase the number of embryos available for transfer or cryopreservation.

Conflict of interest

We declare that there is no conflict of interest.

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