Article

Effects of failed oocyte activation and sperm protamine deficiency on fertilization post-ICSI

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Abstract

Sperm premature chromosomal condensation (PCC) has been associated with failed fertilization. Previous studies suggest that protamine deficiency or failed oocyte activation may make spermatozoa prone to PCC. However, it is not clear which of these two factors has a more profound effect on fertilization failure. In order to distinguish between these two phenomena, oocytes that failed to fertilize after intracytoplasmic sperm injection (ICSI) were artificially activated and the association between protamine deficiency and PCC was evaluated in the remaining oocytes that failed to fertilize. The results of this study reveal that after artificial activation, fertilization rate post-ICSI increased from 59.95 to 87.7% and PCC spermatozoa appeared to be present in over 50% of the remaining oocytes that failed to fertilize. The percentage of sperm PCC was significantly higher in protamine deficient samples, thus suggesting that after failed oocyte activation, sperm PCC induced by protamine deficiency may be considered as an alternative cause of failed fertilization post-ICSI. Furthermore, the results of this study did not show any correlation between pronuclei size asynchrony and protamine deficiency.

Keywords: *fertilization*, *ICSI*, *oocyte activation*, *protamine deficiency*, *sperm premature chromosomal condensation*

Introduction

Chromatin analysis of oocytes that failed to fertilize reveals that, after aneuploidy, sperm premature chromosomal condensation (PCC) is considered as the next most common cause of fertilization failure following intracytoplasmic sperm injection (ICSI) (Schmiady *et al*., 1986, 1996; Tejada *et al*., 1992; Mozdarani and Aghdaei, 2001). Rosenbusch suggested that the contribution of sperm chromatin anomalies to induction of sperm PCC should be studied (Rosenbusch, 2000).

A previous study showed that the number of human spermatozoa with PCC in unfertilized human oocytes was higher in protamine deficient patients compared with patients who had an adequate amount of protamine (Nasr-Esfahani *et al*., 2004a). One of the most important factors in determining the outcome of fertilization and embryo development is the quality of the oocyte (De Santis *et al*., 2005; Esfandiari *et al*., 2005; Ebner *et al*., 2006). Oocyte immaturity has been considered as one of the underlying factors in the induction of PCC. In order to exclude the effect of a cytoplasmic factor on PCC, mouse oocytes derived from an inbred strain were used to investigate the effect of protamine deficiency on the induction of PCC in human spermatozoa (Nasr-Esfahani *et al*., 2006). The results of this study also showed that sperm protamine deficiency, independently of oocyte maturity, can make spermatozoa prone to PCC. Thus, it was concluded that when spermatozoa are exposed to an environment with active meiosis promoting factor (MPF), such as the oocyte in metaphase II, protamine deficient spermatozoa are more likely to undergo PCC compared with spermatozoa with a normal amount of protamine, and therefore may result in failed fertilization (Nasr-Esfahani *et al*., 2006).

Protamine deficiency may directly affect the fertilization process or decrease the fertilization rate, due to co-occurrence of protamine deficiency with the late-stage spermiogenic

anomalies (Manicardi *et al*., 1995) such as defects in acrosome and perinuclear theca formation, membrane remodelling and other significant morphological and biochemical events that are necessary for normal sperm function and oocyte activation after sperm insemination (Courtot, 1991; Tesarik *et al*., 1994; Nasr-Esfahani *et al*., 2004a). Indeed, failed oocyte activation has been considered as one of the main causes of failed fertilization after ICSI (Sousa and Tesarik, 1994; Tesarik *et al*., 1994; Tesarik and Sousa, 1995a,b). Literature studies suggest that more than 80% of oocytes that failed to fertilize after ICSI contain spermatozoa (Flaherty *et al*., 1998). However, in these oocytes, it is not known whether failed fertilization is due to sperm factors, such as sperm protamine deficiency and inability of spermatozoa to activate the oocyte, or the oocyte factor (inability of oocyte to become activated by spermatozoa). Therefore, the aim of this study was to assess the chromatin status of oocytes that failed to fertilize and chemically activated oocytes that failed to fertilize after ICSI, in order to distinguish between the effects of sperm protamine deficiency and failed oocyte activation on failed fertilization.

Protamine content can be measured directly through protamine extraction and polyacrylamide gel electrophoresis, or indirectly by fluorochrome. *In situ*, fluorochromes such as chromomycin A3 (CMA3) compete with protamine. Bizzaro has reported that CMA3 staining is inversely related to the protamination state of spermatozoa (Bizzaro *et al*., 1998). Furthermore, CMA3 has been considered as a useful tool for the rapid screening of subfertility in humans, as it seems to allow indirect visualization of protamine deficient, nicked and partially denatured DNA (Bianchi *et al*., 1993, 1996; Manicardi *et al*., 1995) and it has been associated with ultrastructural anomalies of sperm chromatin (Iranpour *et al*., 2000). Interestingly, CMA3 staining has been shown to be increased in the sperm cells of infertile patients (Lolis *et al*., 1996; Razavi *et al*., 2003; Nasr-Esfahani *et al*., 2004a,b, 2005). Correlations between CMA3 staining in spermatozoa and assisted reproduction outcome have also been found. However, CMA3 staining cannot distinguish whether the potential protamine deficiency is due to lack of P1, P2 or a combination of both. Therefore, due to the large number of patients in this study, CMA3 staining was used as an indirect assessment of protamine content. In addition, in the previous study, it was postulated that protamine deficiency induces sperm PCC, and recovery from PCC may lead to pronuclei size asynchrony. Therefore, another aim of this study was to evaluate if there is any association between protamine deficiency and pronuclei size asynchrony.

Materials and methods

This study was approved by the ethical and scientific committee of Isfahan Fertility and Infertility Centre and Royan Institute. Semen samples were obtained from couples referred to Isfahan Fertility and Infertility Centre for ICSI treatment. The semen samples were collected by masturbation after 3–4 days of abstinence on the day of oocyte recovery. Routine semen analysis was carried out by light microscopy according to World Health Organization criteria (World Health Organization, 1999).

Experimental design

This study consisted of three groups of patients. In the first group of patients, unfertilized ICSI oocytes were subjected to chromatin analysis. In the second group of patients, unfertilized ICSI oocytes were artificially activated and then the remaining unfertilized oocytes were subjected to chromatin analysis. In the third group of patients, the size of pronuclei was assessed and related to protamine deficiency.

Group 1

A total of 765 oocytes were obtained from 56 patients undergoing ICSI. At 16–18 h post-insemination, oocytes were assessed for the presence of pronuclei. Unfertilized oocytes were selected and cultured in G1 medium (Vitrolife, Gothenburg, Sweden) for another 7–10 h, in order to check for signs of cleavage and to make sure that the 2 pronuclei (2PN) stage had not been missed. Unfertilized oocytes were treated and fixed as stated below for chromatin analysis.

Group 2

A total of 801 oocytes were obtained from 86 patients undergoing ICSI. At 16–18 h post-insemination, oocytes were assessed for the presence or absence of pronuclei. Unfertilized oocytes were selected and cultured in G1 medium for another 7–10 h, in order to check for signs of cleavage and to make sure the 2PN stage was not missed. Unfertilized oocytes were chemically activated by 10 µmol/l ionomycin for 10 min. These oocytes were washed with G1 medium and were cultured in this medium for a further 16 h, at which time they were assessed for the presence of pronuclei. Chemically activated oocytes that failed to fertilize were also treated and fixed for chromatin analysis.

Group 3

A total of 1022 oocytes were obtained from 156 patients undergoing ICSI. At 16–18 h post-insemination, oocytes were assessed for the presence or absence of pronuclei. The size of each pronucleus was assessed by Zilos-tk laser software (Hamilton Thorne Biosciences, Beverly, MA, USA), developed for laser assisted hatching. Fertilized oocytes were considered to have asynchronous pronuclei if the difference between the sizes of two pronuclei was greater than 13% (Sadowy *et al*., 1998).

Sperm preparation

Semen samples were prepared for routine ICSI using discontinuous Pure Sperm gradients (80:40) (Nidacon, Gothenburg, Sweden). After insemination of oocytes, the remaining processed samples were used for evaluation of protamine deficiency and sperm morphology, using chromomycin A3 staining and modified Papanicolaou staining respectively.

ICSI

All the media were purchased from Vitrolife, G3 series plus, unless otherwise stated. After oocyte collection, the oocytes were treated in hyaluronidase (Hyase) in G-MOPS medium. Oocytes were then washed in fresh G-MOPS and transferred to G-oocyte under oil in a Falcon 1006 dish for microinjection.

The Pure Sperm processed semen sample was introduced into ICSI 100 (a viscous sperm handling solution) in the same dish. An Eppendorf micromanipulator mounted on a Nikon inverted microscope was used to perform ICSI. As far as possible, motile spermatozoa with the best morphology were selected for oocyte insemination. The injected oocytes were then washed and incubated in G1 medium. Fertilization was assessed by presence of pronuclei between 16–18 h after injection. In order to reduce female factors, any patients with fewer than four mature metaphase II (MII) oocytes that had survived the ICSI procedure were excluded from this study. Furthermore, immature, deformed and post-mature oocytes, or any oocytes with certain types of abnormality such as subzonal granulation or central granulation, were also excluded.

Evaluation of cleavage and embryo quality score

At 48–72 h after sperm injection, the embryos were scored for their cleavage and quality. The cleavage stages of embryos were recorded and the embryos were graded as A, B and C. Grade A embryos with even-sized blastomeres and less than 10% fragmentation were given score 3. Grade B embryos with even-sized blastomeres and between 10–50% fragmentation were given score 2. Grade C embryos with uneven-sized blastomeres and with >50% fragmentation were given score 1. The cumulative quality score of the embryos for each patient was calculated as follows: sum of scores of embryos/total number of embryos. Each embryo was also given a cleavage score that was calculated as follows: 2–3, 4–5, 6–8 and 9–16 cell embryos were given scores 1, 2, 3, and 4 respectively. The cleavage score of each patient was calculated as follows: sum of cleavage scores of embryos/total number of embryos.

Assessment of sperm morphology

Washed semen samples were stained using a modified Papanicolaou technique (Kruger *et al.*, 1986) and sperm morphology was assessed according to World Health Organization criteria. At least 200 cells were evaluated per slide (World Health Organization, 1999).

Assessment of protamine deficiency (chromomycin A3 staining)

Processed semen samples were fixed in Carnoy's solution [methanol: glacial acetic acid 3:1 (Merck, Germany)] 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 McIlvaine buffer (7 ml citric acid 0.1 mol/l + 32.9 ml $Na_2HPO_4.7H_2O$, 0.2 mol/l, pH 7.0, containing 10 mmol/l $MgCl_2$)]. The slides were then rinsed in buffer and mounted with buffered glycerol (1:1). Microscopic analysis of the slides was performed on an Olympus fluorescence microscope (BX51, Tokyo, Japan) with the appropriate filters (460–470 nm). On each slide, 100 sperm cells were evaluated. Evaluation of CMA3 positivity was carried out using Olysia software (Olympus). Pixel intensity of each sperm was recorded. Spermatozoa with pixel intensity greater than 100 were considered as CMA3 positive or protamine deficient, while those with pixel intensity lower than 100 were considered as CMA3 negative or with normal amount of protamine. Analysis of coefficient of variation using this software reveals that this method has lower intra-assay variation (data not shown) than the conventional method (Nasr-Esfahani *et al.*, 2001).

Chromatin analysis of oocytes that failed to fertilize

Injected oocytes were processed individually. Each oocyte was placed in 0.5% sodium citrate (Merck) hypotonic solution for 9 min at room temperature. The oocyte was then transferred with a minimal amount of hypotonic solution on to a grease-free glass microscope slide and fixed with Carnoy's fixative (methanol:acetic acid 3:1).The slides were stained with 10% Giemsa (Merck) in phosphate-buffered saline, pH 6.8 for 10 min, and then analysed under a light microscope with ×1000 magnification. Chromatin of oocytes and spermatozoa were analysed. Spermatozoa with condensed chromatin were considered as intact. Spermatozoa with nuclei showing highly condensed chromatin mass with a small partly decondensed region at their periphery to slightly condensed structure with thin strands reaching out of the chromatin mass to long decondensed chromatin elements were scored as PCC. Spermatozoa with slightly or completely swollen heads were considered as partial nuclear chromatin decondensation (PNCD) or completely nuclear chromatin decondensation (NCD), respectively. Sperm heads that had not undergone any condensation changes were considered as intact.

Statistical analysis

Correlation coefficients and Student's *t*-test were calculated using the Statistical Package for the Social Studies (SPSS 11.5) software to compare results between different groups. A *P*-value of < 0.05 was considered to indicate statistical significance.

Results

The number of oocytes and their quality, number of injected oocytes and the chromatin status of oocytes that failed to fertilize, for groups 1 and 2, are summarized in **Table 1**. Comparison of results in the two groups shows: (i) percentage fertilization in group 1 and group 2 was 58.55 and 59.95%, respectively. In group 2, 178 out of 300 unfertilized oocytes that were chemically activated were subsequently fertilized, leading to a fertilization rate of 59.33%; (ii) chemical activation increased fertilization rate to 87.7% in group 2 (**Figure 1**); (iii) the percentage of intact, partial and complete nuclear chromatin decondensation and PCC in group 1 were 40.91 and 59.09%, respectively, while in group 2 they were 42.06 and 53.27%, respectively. Furthermore, in group 2, due to chemical activation, 4.67% of sperm proceeded and arrested in first mitosis.

The results in **Table 2** show a significant negative correlation between percentage CMA3 positivity and fertilization rate in both groups of patients (group 1: $r = -0.598$, $P \leq 0.001$ and group 2: $r = -0.334$, $P = 0.006$); however, the percentage fertilization in chemically activated oocytes that failed to fertilize showed no correlation with CMA3 positivity ($r = 0.04$, not significant). In group 1, a significant negative correlation was observed between percentage of PCC and fertilization rate

Table 1. Number and types of oocytes recovered and injected, and details of cytogenetic analysis in oocytes that failed to fertilize following intracytoplasmic sperm injection (ICSI) in two groups of patients.

	Group 1		*Group 2*	
	Number	*Number/ total (%)*	*Number*	*Number/ total (%)*
Oocytes retrieved	765	–	920	–
Germinal vesicle oocytes	46	46/765 (6.01)	59	59/920 (6.41)
Metaphase I oocytes	20	20/765 (2.61)	20	20/920 (2.17)
Metaphase II oocytes	690	690/765 (90.20)	801	801/920 (87.07)
Degenerate or deformed oocytes	9	9/765 (1.18)	40	40/920 (4.3)
Metaphase II oocytes injected	690	690/765 (90.20)	801	801/920 (87.07)
Oocytes survived post-ICSI	671	671/690 (97.25)	749	749/801 (93.51)
Fertilized oocytes	404	404/690 (58.55)	449	449/749 (59.95)
Failed to fertilize oocytes	267	267/690 (38.70)	300	300/749 (40.05)
Fertilized oocytes post-activation	–	–	178	178/300 (59.33)
Failed to fertilize oocytes analysed	179	–	122	122/300 (40.67)
Oocytes in metaphase II	120	120/179 (67.04)	57	57/122 (46.72)
Oocytes without spermatozoa	16	16/179 (8.94)	15	15/122 (12.30)
Fertilization failure[a]	154	–	122	107/122 (87.70)
Nuclear chromatin decondensation[b]	63	63/154 (40.91)	11	42.06
Premature chromosomal condensation[c]	91	91/154 (59.09)	57	57/107 (53.27)
Arrested[d]	–	–	5	5/107 (4.67)

Group 1: oocytes that failed to fertilize after ICSI. In group 2, chromatin analysis was carried out on the remaining oocytes that failed to fertilize after artificial oocyte activation.
[a]Spermatozoa observed in oocytes that failed to fertilize.
[b]Intact spermatozoa, spermatozoa with partial or complete nuclear chromatin decondensation.
[c]Spermatozoa with premature chromosomal condensation in oocytes that failed to fertilize.
[d]Spermatozoa arrested in first mitosis.

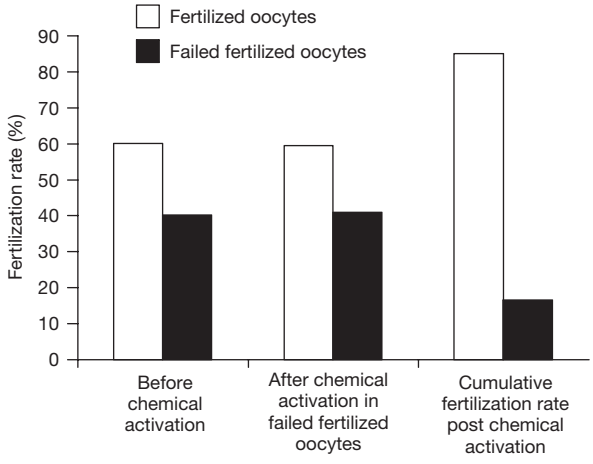

Figure 1. Percentage of fertilized and failed to fertilize oocytes and cumulative fertilization rate before and after chemical activation.

($r = –0.357$, $P = 0.009$); however, no significant correlation was observed between percentage of PCC and fertilization rate in the chemically activated oocytes that failed to fertilize. In group 1, no significant correlation was observed between CMA3 positivity and percentage PCC ($r = 0.145$, NS); however, in the chemically activated oocytes that failed to fertilize, a significant positive correlation was observed between percentage of PCC with percentage CMA3 positivity ($r = 0.344$, $P = 0.034$). In the chemically activated oocytes that failed to fertilize, a significant negative correlation was observed between spermatozoa with intact, partial and complete nuclear chromatin decondensation with both CMA3 positivity ($r = –0.333$, $P = 0.041$) and percentage of PCC ($r = –0.766$, $P < 0.001$), while such a significant observation was not observed in the first group.

In this study, patients were divided into two groups with low and high CMA3 positivity according to a previously defined cut-off value of 30% (Sakkas *et al.*, 1996; Nasr-Esfahani *et al.*, 2001). Analyses of data in these two groups of patients revealed that not only were fertilization rates different in the two groups, but also the percentage intact ($P = 0.001$), partial and complete nuclear chromatin decondensation ($P = 0.05$) and PCC spermatozoa ($P = 0.032$) were significantly different in group 2 (**Table 3**).

Table 4 shows the correlation between CMA3 positivity and sperm parameters, fertilization rate, cleavage and embryo quality scores in group 3. The results show a significant correlation between CMA3 positivity and sperm parameters (concentration $P = 0.009$; % motility $P = 0.001$; % abnormal morphology $P = 0.001$) and fertilization rate ($P = 0.001$); however, no significant correlation was observed with cleavage quality scores. In addition, a significant negative correlation was found between percentage sperm with abnormal head morphology and percentage oocyte with unequal pronuclei size ($r = –0.166$, $P < 0.01$).

Table 2. Correlation between a range of parameters and fertilization rate or protamine deficiency (CMA3 positivity) in two groups of patients. Values are correlation coefficients (r).

Parameter	*Group 1*		*Group 2*	
	Fertilization rate	*CMA3 positivity*	*Fertilization rate*	*CMA3 positivity*
Per cent fertilization	–	–0.598 (0.001)	–	–0.334 (0.006)
Per cent fertilized oocytes post-activation	–	–	0.044 (NS)	0.040 (NS)
Premature chromosomal condensation[a]	–0.357 (0.009)	0.145 (NS)	–0.119 (NS)	0.344 (0.034)
Nuclear chromatin decondensation[b]	–0.254 (NS)	0.154 (NS)	0.178 (NS)	–0.333 (0.041)
Per cent abnormal sperm morphology	0.123 (NS)	–0.145 (NS)	–0.079 (NS)	0.348 (0.004)

Values in parentheses are *P*-values. NS = not statistically significant.
aSpermatozoa with premature chromosomal condensation in oocytes that failed to fertilize.
bSpermatozoa with intact, partial or complete nuclear chromatin decondensation.

Table 3. Comparison of a range of parameters between subgroups with chromomycin A3 (CMA3) positivity lower or higher than 30%, in two groups of patients. Values are percentages, presented as mean ± SD, unless otherwise stated.

Parameter	*Group 1*			*Group 2*		
	CMA3 < 30%	*CMA 3 > 30%*	*P-value*	*CMA3 < 30%*	*CMA3 > 30%*	*P-value*
No. of metaphase II oocytes	8.35 ± 4.8	7.87 ± 3.3	NS	7.40 ± 4.06	8.91 ± 5.33	NS
No. oocytes survived post-ICSI	3.00 ± 1.87	3.70 ± 2.11	NS	7.40 ± 4.06	8.91 ± 5.33	NS
Fertilization	64.69 ± 10.16	36.87 ± 12.97	0.001	69.89 ± 17.38	55.99 ± 24.68	0.010
CMA3-positive spermatozoa	21.36 ± 1.43	53.74 ± 3.33	0.001	21.36 ± 6.34	54.64 ± 15.42	0.001
Intact spermatozoa[a]	70.25 ± 4.62	48.11 ± 4.26	0.002	65.00 ± 47.43	17.59 ± 26.93	0.001
Premature chromosomal condensation[b]	50.73 ± 33.98	48.87 ± 33.06	NS	25.00 ± 42.49	61.78 ± 42.25	0.032
Nuclear chromatin decondensation[c]	11.89 ± 4.21	7.09 ± 2.41	NS	0.00	10.26 ± 27.43	0.050
Arrested[d]	–	–	–	10.00 ± 31.62	6.69 ± 21.10	NS

NS = not statistically significant; ICSI = intracytoplasmic sperm injection.
[a]Intact spermatozoa in failed fertilized oocyte .
[b]Spermatozoa with premature chromosomal condensation in oocytes that failed to fertilize.
[c]Spermatozoa with partial or complete nuclear chromatin decondensation.
[d]Spermatozoa arrested in first mitosis.

Table 4. Correlation between a range of parameters and percentage of chromomycin A3 (CMA3) positivity spermatozoa.

Parameter	*CMA3 positivity*	
	r	*P-value*
Concentration (×10^6/ml)	–0.236	0.009
% Motility	–0.321	0.001
% Abnormal morphology	0.563	0.001
% Unequal pronuclei size	0.105	NS
% Fertilization	–0.526	0.001
Cleavage score on day 2	0.059	NS
Cleavage score on day 3	0.064	NS
Embryo quality score on day 2	0.037	NS
Embryo quality score on day 3	0.033	NS

r = correlation coefficient; *P*-values are given where $P < 0.05$ (taken as statistically significant); NS = not statistically significant.

Discussion

Upon entry of the spermatozoon into the ooplasm, sperm nuclear decondensation factors (SNDF) enter the spermatozoon and oocyte activation begins. Following entry of SNDF into the spermatozoon, the head swells and sperm-associated oocyte activating factors (SAOAF) are released, resulting in MPF inactivation (Dozortsev *et al.*, 1995, 1997). Therefore, 4–5 h after sperm entry, resumption of meiosis and sperm head decondensation, histone–protamine replacement, takes place, followed by pronucleus formation (Dozortsev *et al.*, 1995). Failure to fertilize has been attributed to inability of the oocyte to induce SNDF, partial or complete lack of SAOAF in spermatozoa, failure of MPF inactivation and presence of excessive histone, which may lead to premature chromosome condensation (PCC).

The results of this study show that fertilization rates in two groups of patients were 58.55 and 59.95%, respectively.

Analyses of oocytes that failed to fertilize showed that over 80% of analysed oocytes post-ICSI contained spermatozoa. In the second group of patients, due to chemical activation, fertilization increased from 59.95 to 83.74%, indicating that failed oocyte activation may account for around 60% of failed fertilization post-ICSI. A review of published studies shows that different chemicals have been used for oocyte activation, resulting in activation rates of 66–90% for fresh oocytes (Yamano *et al.*, 2000; Murase *et al.*, 2004; Yanagida *et al.* 2006). The result of this study using ionomycin and aged oocytes post-ICSI led to 59.33% fertilization, which is close to the lower end of the reported rates.

Sperm chromatin analysis of chemically activated oocytes that failed to fertilize revealed that 53.28% of these spermatozoa showed PCC, while the status of spermatozoa in the rest of the oocytes was intact, partial or complete nuclear chromatin decondensation, arrested in the first mitosis, or the oocyte did not contain any spermatozoa. Therefore, it can be concluded that after failed oocyte activation, sperm PCC may be considered as the second cause of failed fertilization.

In both groups of patients, a significant negative correlation was observed between CMA3 positivity with fertilization (**Table 2**), as in previous studies, showing that protamine deficiency is involved in the mechanism of failed fertilization (Nasr-Esfahani *et al.*, 2001; Razavi *et al.*, 2006). However, absence of correlation between PCC with CMA3 positivity in the first group of patients suggests that other factors may be involved in induction of PCC. The results of the second group of patients demonstrate that this factor may be failed oocyte activation. However, when this defect was overcome by chemical activation, a significant correlation was observed between percentage of PCC with CMA3 positivity ($r = 0.344$, $P = 0.034$), suggesting that after failed oocyte activation, PCC may be considered as the second cause of failed fertilization and may be related to protamine deficiency. Analysis of PCC in patients with CMA3 positivity of lower and higher than 30% further verified the above hypothesis. In addition, in these two subgroups of patients, fertilization rate and percentage of spermatozoa with intact head in the failed activated oocytes and unfertilized oocytes were significantly different (**Table 3**). In patients with low CMA3, the mean number of intact heads was higher, while the mean percentage of spermatozoa with PCC was lower. In contrast, in patients with high CMA3, the mean number of intact heads was lower, while the mean percentage of spermatozoa with PCC was higher. These results may indicate that in the protamine deficient samples, the main reason for failed fertilization appears to be PCC, possibly induced by protamine-deficiency. An explanation for protamine deficiency, leading to PCC and thereby resulting in failed fertilization, has been provided below.

Synchronization of cell cycles between spermatozoa and oocyte is related to sperm chromatin status, as nucleoprotamine is inert to active MPF and upon oocyte activation, meiosis promoting factor (MPF) becomes inactive. During the early hours of oocyte activation, spermatozoa undergo nuclear decondensation by protamine–histone exchange (Perreault *et al.*, 1988; Perreault, 1990; Dozortsev *et al.*, 1995), and once MPF has been inactivated, both spermatozoon and oocyte have a nucleohistone structure and they are in the G1 stage of the cell cycle. However, if a spermatozoon with partial or complete absence of protamine, for example a round spermatid, is injected into an oocyte, sperm PCC takes place, resulting in asynchronization of the cell cycle between spermatozoon and oocyte (Tesarik *et al.*, 1996; Aslam and Fishel, 1999), and this phenomenon may partially account for the underlying mechanisms of failed fertilization after ICSI.

In addition, chromatin remodelling, involved in histone protamine exchange, is a concomitant event with formation of perinuclear theca containing SAOAF involved in oocyte activation (Dozortsev *et al.*, 1995, 1997). It has been shown that round-headed spermatozoa from infertile patients contain less protamine, more histones and intermediate proteins than normal spermatozoa (Blanchard *et al.*, 1990).

A study by Ohtsuki *et al.* (1996) suggests that protamine, with oligo-arginine cluster, may be involved in oocyte activation through activation of casein kinase II (CKII); therefore, spermatozoa with chromatin anomalies may affect oocyte activation. On the other hand, one must consider the fact that mouse spermatozoa lacking P2 protamine have been shown to activate oocytes after ICSI (Cho *et al.*, 2003), which may suggest that the aforementioned phenomena may function independently of each other in failed fertilization.

Based on the results of a previous study, and in agreement with the results of the present study, it could be speculated that when a protamine-deficient spermatozoon enters an oocyte, PCC takes place depending on the degree of protamine deficiency (Nasr-Esfahani *et al.*, 2006). However, in such a circumstance if the oocyte becomes activated, spermatozoa might recover from PCC and undergo male pronucleus formation. The recovery of spermatozoa from PCC may lead to a lag or delay between male and female pronucleus formation, which may result in asynchronization of male and female pronucleus size, which in turn may affect embryo development and implantation rate (Scott *et al.*, 2000). Indeed, asynchrony of pronucleus development has been suggested to correspond to prolongation interval between the appearance of the male and female pronucleus (Tesarik and Greco, 1999). Therefore, in this study in the third group of ICSI candidates, CMA3 positivity and sperm morphology were assessed and the differences in size of pronucleus were recorded. The result of this study showed no correlation between CMA3 positivity and pronucleus asynchrony. The failure to observe any correlation between CMA3 positivity and pronucleus asynchrony may indicate that protamine-deficient spermatozoa are unable to recover from failed fertilization. Another explanation for the latter statement might be that the semen sample diagnosed as protamine deficient via global assessment such as CMA3 staining (Aoki *et al.*, 2006) may possess a small population of cells with normal protamine content, and these spermatozoa may be chosen for insemination by the sperm selection procedure during ICSI. It is important to note that direct assessment of the selected spermatozoa for insemination was not applicable in this study. A weak positive correlation between sperm head morphology and pronucleus asynchrony, suggesting a certain type of anomaly, may be involved in this phenomenon, which requires further investigation. Recent studies postulated that pronucleus asynchrony may be related to sperm DNA fragmentation, and male pronucleus involved in DNA repair may lag from female pronucleus and lead to pronucleus asynchrony (Muriel *et al.*, 2006). Indeed, previous studies reveal that protamine-deficient

spermatozoa are prone to DNA fragmentation compared with normal spermatozoa (Nasr-Esfahani *et al.*, 2004a, 2006; Aoki *et al.*, 2005).

Furthermore, a recent study by Aoki *et al.* (2006) simultaneously assessing protamine content, DNA integrity and sperm cell viability, shows that individual sperm cells displaying the lowest protamine concentration display diminished viability and increased sperm cell susceptibility to DNA damage.

In conclusion, the results of this study suggest that after oocyte activation, sperm protamine deficiency may account for failed fertilization after ICSI and protamine deficiency may make spermatozoa prone to PCC following ICSI.

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