Sperm chromatin stability and its relationship with fertilization rate after Intracytoplasmic Sperm Injection (ICSI) in an assisted reproduction program

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Received: 1 April 2007 / Accepted: 19 October 2007 / Published online: 22 November 2007
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Abstract

Objective Evaluate sperm chromatin stability and its relationship with the rate of fertilization after procedures of intracytoplasmic sperm injection (ICSI) in a program of assisted reproduction.

Design Prospective study.

Setting Institute of Gynecology and Reproduction.

Patients Thirty-three women with their respective partners (12 couples in the study group and 21 couples in the control group) participating in a program of assisted reproduction. The study group was defined as men with >30% of non-decondensed spermatozoa (high sperm chromatin stability).

Interventions A part of each seminal sample was used to evaluate sperm chromatin stability under SDS and EDTA treatment and the second aliquot was used for the ICSI procedure. Fertilization was evaluated 16–18 h post sperm injection at a pronuclear stage. The fertilized oocytes were further cultured for 24–48 h before transfer to the patient.

Main outcome measures Fertilization rate.

Results Thirty-five oocytes (35.7%) in the group of study and 109 oocytes (78.9%) in the control group showed two pronuclei ($P<0.001$). The coefficient of determination between the SDS+EDTA (Grade 2) and rate of fertilization was $r^2=0.85$ ($P<0.001$) and the coefficient of regression was 1.72 ± 0.19 ($\beta\pm ES$) ($P<0.001$).

Conclusions High sperm chromatin stability is a factor which reduces the rate of fertilization after ICSI procedure.

Keywords Sperm chromatin stability · ICSI · Assisted reproduction · Fertilization rate · SDS+EDTA

Introduction

Intracytoplasmic sperm injection (ICSI) procedure has revolutionized the management of male-factor infertility allowing fecundity and pregnancy rates comparable to those obtained from in vitro fecundation conventional procedures [1, 2]. Although this method allows a closer approach between the sperm and the oocyte during the injection, this not always resulted in the development of a zygote. In human, lack of oocyte activation is responsible in most of the cases of fertilization failure (complete fertilization failure or low fertilization rates) after ICSI [3]. Although, there are still cases of fertilization failure after ICSI, this method has been proved to be useful including in cases of teratozoospermia [4].

Abnormalities in the male genome are a clear potential reason for post fertilization failure [5, 6]. Male infertility may arise due to high levels of loosely packaged chromatin and damaged DNA [6]. The achievement of a correct chromatin packaging level is essential for successful fertilization [5]. Then, sperm DNA integrity has been shown

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to be necessary for achieving and sustaining embryo development [6].

Chemical activation of oocytes may result in successful pregnancy in some cases [3] but in other cases with previous history of failed or low fertilization rate and 100% sperm abnormality, chemical activation of oocytes after ICSI may increase fertilization rate in some sperm morphological abnormalities (amorphous head and tapered head) but not in others (bent neck). However, there was no significant effect on early cleavage or number of good embryos in all subgroups of sperm morphological abnormalities [7] suggesting that in several cases another factor besides activation of oocytes is responsible for the success of the ICSI procedure. In pigs, the failure of male pronucleus formation was the major cause for the failure of fertilization in activated ICSI oocytes [8].

Aneuploidy and sperm premature chromatin condensation (PCC) have been described as prevalent causes of fertilization failure after ICSI [9]. Studies in unfertilized oocytes have demonstrated that 82% were still at metaphase II and 18% were activated [10]. However, many of the uncleaved human ICSI oocytes did not form the male pronucleus even after the oocyte had been activated [11] suggesting that activation of oocytes is not always associated with male pronucleus formation. In activated, unfertilized oocytes, there was a higher incidence of intact spermatozoa in these oocytes compared with metaphase II, unfertilized oocytes [10].

After sperm injection to oocyte, an appropriate decondensation of the nuclear chromatin and the subsequent formation of the male pronucleus is essential for fertilization and normal embryonic development. Higher incidence of intact spermatozoa in unfertilized oocytes suggests that sperm has high chromatin stability [12–14].

The nuclear chromatin stability is related to the amount of zinc present in the semen. Sperms exposed to sodium dodecyl sulphate (SDS) plus a zinc quelant, the ethylenediaminetetraacetic acid (EDTA) reduced chromatin stability and this chromatin decondensation is observed as the presence of swollen sperm heads. If in the presence of SDS+EDTA more than 30% sperms are intact (no decondensed), then the semen sample is diagnosed as having high sperm chromatin stability and this has been described as a cause of male infertility [12–14].

Men with high sperm nuclear chromatin stability showed low function of the seminal vesicles and this was associated with high seminal zinc concentration [15]. Furthermore, men with lower corrected seminal fructose levels, indicative of low seminal vesicles function, showed a lower response to the hamster test, evaluated through the low number of decondensed heads inside the oocyte [16]. Seminal zinc is responsible of the quaternary structure of the chromatin providing its stability [15, 16].

The aim of this study was to evaluate sperm chromatin stability and its relationship with the rate of fertilization after procedures of ICSI in a program of assisted reproduction.

Material and methods

Subjects

This is a prospective study in which 33 couples were examined, 12 of them corresponding to the study group (high sperm chromatin stability) and 21 couples corresponding to the control group (normal sperm chromatin stability), which entered to the Assisted Reproduction Program from the PRANOR group at the Assisted Reproduction Institute of Gynecology and Reproduction (Lima, Peru). We include those couples in which ICSI was considered as a unique procedure ($n=17$) or as an alternative procedure after a previous failure in an IVF cycle to accomplish pregnancy ($n=16$). The study was approved by the Institutional Review Board (IRB) at the Universidad Peruana Cayetano Heredia in Lima, Peru.

Semen samples

The semen samples were obtained from masturbation in aseptic conditions after 72 h of sexual abstinence. The samples were left in stand for 30 min to allow liquefaction; then each semen sample was separated in two equal aliquots and their characteristics were evaluated through WHO guidelines [17], except sperm morphology which was evaluated according to the strict criteria of Kruger [18].

One aliquot of the semen sample was only washed with Human Tubal Fluid (HTF) culture medium modified with Hepes (Irvine Scientific, USA) in cases with severe oligozoospermia, asthenozoospermia and necrozoospermia, or passed through a density gradient of Isolate® (colloidal suspension of silica particles) (95/70/50) (Irvine Scientific, USA) to recover sperms with better characteristics (lower cases of asthenozoospermia, oligozoospermia and teratozoospermia). The recovered sperms were used to proceed with ICSI.

Sperm chromatin stability assessment

The sperm nuclear chromatin stability (NCS) was evaluated in the second aliquot of semen through the Kvist method [19] modified by Gonzales and Sanchez [12]. For this purpose, the semen sample was washed with the culture medium twice ($n=5$) and in other cases through the density gradient ($n=28$).

The recovered sperms from each subject were incubated in three different media (Borate Buffer, SDS and SDS+

EDTA), just as previously described [12]. With this method, it is possible to obtain three grades as follows: no decondensation of head sperms (Grade 0), moderate decondensation of head sperms (Grade 1) and high decondensation of head sperms (Grade 2) as chromatin response. When the percentage of no-decondensed sperms in the presence of SDS+EDTA is >30% then sample is classified as high sperm nuclear stability [14].

Ovarian stimulation and oocyte retrieval

Women underwent controlled ovarian stimulation, in a short or long term protocol with recombinant FSH (Gonal-F®, Serono Laboratories) or human menopausal gonadotropin, HMG (Humegon®, Organon Laboratories) according to the stimulation protocols previously established [20]. The follicles were monitored by ultrasonography and programming oocyte aspiration when at least three follicles had a diameter greater than 18 mm. The follicular aspiration was done by vaginal ultrasound 36 h after the intramuscular application of human chorionic gonadotropin, hCG (10,000 IU) (Pregnyl®, Organon Laboratories). For the procedure, the patient was under general anesthesia with 200 mg of Propofol iv (Diprivan® 1% P/V; Astra Zeneca Laboratories, UK).

Procedure for ICSI

The aspirated oocytes of each patient were cultured in a HTF culture medium with 10% serum substitute supplement (SSS) (Irving Scientific, USA) under mineral oil at 37°C and in an atmosphere containing 5% CO₂. The oocytes of each patient in metaphase II were injected 40 h post-hCG injection according to the method described previously [21].

Fertilization was evaluated 16–18 h post sperm injection through the presence of two pronuclei and two polar bodies. The fertilized oocytes were transferred to HTF with 20% SSS (Irvine Scientific, USA) and cultured for another 24–48 h before being transferred to the mother uterus. A failure in the fertilization process was considered when the number of fertilized oocytes in relation to the number of injected oocytes was less than 50% [22]. Oocytes that failed to fertilize were evaluated microscopically to determine whether the failure of fertilization was due to problems in the chromatin decompaction process. No lysis of the oocytes was observed in the present study. All unfertilized oocytes were in metaphase II (no activated).

Statistical analysis

Rate of fertilization between study group and control group was assessed by chi square test. Data expressed as mean±

standard error of the mean (SEM) were assessed by Student's *t* test. The Spearman test was applied to determine the correlation between the fertility rate and the percentage of decondensed sperm in the presence of SDS+EDTA (Grade 2). A multivariate analysis was performed to determine the risk for success on the ICSI procedure. It was considered a statistical significant difference when $P<0.05$.

Results

Characteristics of the study (High sperm chromatin stability) and control (normal sperm chromatin stability) groups were similar. In fact, age of females (34.00 ± 4.55 years, mean±standard error of the mean for study group and 34.24 ± 3.66 years for control group, $P>0.05$), age of males (36.67 ± 6.29 and 37.81 ± 4.11 years for study and control groups, respectively, $P>0.05$) and time of infertility (4.17 ± 1.75 years and 4.29 ± 1.65 years, for study and control groups, respectively, $P>0.05$) were similar between both groups.

In Table 1 are values of the evaluation of sperm chromatin stability (SCS) in the study and control group. Data obtained with Borate Buffer alone or in combination with SDS, were similar in both groups; however, the study group had higher percentage of non decondensed sperm in the presence of SDS and EDTA (SDS+EDTA, Grade 0) and lower values of sperm highly decondensed (SDS+EDTA, Grade 2) in comparison with the control group ($P<0.001$).

Table 2 shows the number of aspirated follicles, number of injected oocytes, fertilized oocytes and the fertility rate of patients undergoing an ICSI procedure. The total number and the average of aspirated follicles, injected oocytes were similar in both groups; however, the fertilization rate was

Table 1 Values of the sperm chromatin stability test under Borate buffer (control), SDS and SDS+EDTA in semen samples classified as high sperm chromatin stability or normal sperm chromatin stability (control)

Sperms in	High sperm chromatin stability group [12]	Control group [21]
Borate Buffer (%)	100	100
SDS, Grade 0 (%)	61.92 ± 9.50	62.57 ± 10.69
SDS, Grade 1 (%)	27.42 ± 6.33	25.90 ± 5.03
SDS, Grade 2 (%)	9.42 ± 6.63	10.57 ± 6.99
SDS+EDTA, Grade 0 (%)	$50.92\pm8.03^*$	23.48 ± 11.80
SDS+EDTA, Grade 1 (%)	32.25 ± 5.72	33.62 ± 3.65
SDS+EDTA, Grade 2 (%)	$16.83\pm4.17^*$	45.29 ± 4.91

Data are mean±standard error of the mean. Number of subjects are between parentheses $P<0.001$ in relation to the control group with normal sperm chromatin stability.

Grade 0 no decondensed sperm heads, Grade 1 moderate decondensed sperm heads, Grade 2 highly decondensed sperm heads.

Table 2 Rate of fertilization in the actual cycle of ICSI procedure

Variable	High sperm chromatin stability group [12]	Control [21]
Number of aspirated follicles	135	164
Number of retrieved oocytes	119	153
Number of injected oocytes	98	138
Number of fertilized oocytes	35	109
Rate of fertilization (%) (CI 95%)	35.7 (26.28–46.02)*	78.98 (71.23–85.45)

Cases include semen samples with high sperm chromatin stability. Control subjects are those whose semen samples have normal sperm chromatin stability. Data are mean±standard error of the mean. Number of subjects is between parentheses. CI 95% confidence interval at 95%. * $P<0.001$ in relation to the control group.

more than twice higher in the control group than in the group with high sperm chromatin stability (study group) ($P<0.001$). In those cases in which patients had a previous cycle of failed assisted reproduction, there were observed eight couples included in the control group and eight couples in the group with high sperm chromatin stability. The fertilization rate in the first cycle when they had an IVF previous was higher in the control group (77.88 ± 9.39) than in the group with high sperm chromatin stability (35.0 ± 12.24), $P<0.001$.

The group with sperm chromatin stability showed also lower fertility rates independent of the ovarian stimulation protocol with recombinant FSH or HMG. In any case, the group with high sperm chromatin stability showed lower rates of fertilization (HMG, $P<0.001$; FSHr, $P<0.001$) (Table 3).

Table 3 Fertility rate according to the ovarian stimulation protocol with recombinant FSH or Human Menopausal Gonadotropin (HMG) in control group and in those with high sperm chromatin stability (study group)

Variable	HMG		FSHr	
	Cases	Control	Cases	Control
Number of subjects	2	7	10	14
Number of follicles aspirated	10.00±5.66	7.43±2.44	11.50±8.73	8.00±4.87
Number of retrieved oocytes	9.00±5.66	6.00±2.31	10.10±7.49	7.43±3.32
Number of injected oocytes	8.00±4.24	6.43±2.23	8.20±5.88	6.64±3.08
Number of fertilized oocytes	2.00±1.41	5.00±1.63	3.13±2.17	5.29±2.58
Rate of fertilization (%)	23.50±4.95*	79.43±10.66	26.80±15.57**	79.36±15.49

Cases include semen samples with high sperm chromatin stability.

Controls are those whose semen samples have normal sperm chromatin stability. Data are mean±standard error of the mean.

* $P<0.01$ in relation to the control group stimulated with HMG.

** $P<0.001$ in relation to the control group stimulated with FSHr.

Table 4 Seminal parameters in the groups with high sperm chromatin stability and in those with normal sperm chromatin stability (control)

Variable	High sperm chromatin stability [12]	Control [21]
Seminal volume (mL)	1.70±0.84	1.65±0.53
Sperm count ($\times 10^6$ /mL)	31.75±30.79	38.29±24.82
Sperm motility Grade III (%)	22.17±12.54	22.24±8.89
Strict sperm morphology (%)	30.42±13.89	36.95±9.72
Sperm vitality (%)	70.92±18.03	70.09±13.15

Data are mean±Standard error of the mean. Number of subjects is between parentheses $P>0.05$.

Assessment of the semen parameter in men partner of the groups classified as controls and in those with high sperm chromatin stability (study group) showed that seminal volume, number of sperm per milliliter, motility grade III, strict Kruger morphology and sperm vitality were similar between the study group and the control group (Table 4).

Table 5 shows the values of fertilization rate according to the strict morphology classification ($<14\%$ normal morphology are teratozoospermics) and sperm chromatin stability ($<30\%$ SDS+EDTA, Grade 2; high sperm chromatin stability). The results showed that only samples of patients with high sperm chromatin stability showed a lower rate of fertilization after the ICSI procedure compared to the group with normal sperm chromatin stability ($p<0.001$).

The coefficient of regression between fertilization rate after ICSI and the highly decondensed spermatid chromatin values (SDS+EDTA, Grade 2) was statistically significant: 1.75 ± 0.19 ($\beta\pm SD$) ($P<0.001$). This suggest that at high percentage of sperm chromatin stability in the presence of SDS + EDTA (Low % of sperms Grade 2), the fertility rate decreases. The coefficient of determination (R^2) was 0.85

Table 5 Rate of fertilization in ICSI procedure according to sperm morphology or Sperm chromatin stability (Grade 2) under the exposure to SDS+EDTA

Variable	Rate of fertilization (%)
Normal sperm morphology	
<14% (Teratozoospermia)	63.33±32.15 (<i>n</i> =3)
≥14% (Normal)	59.73±29.64 (<i>n</i> =29)
Sperm chromatin stability Grade 2 under SDS+EDTA	
<30% (Normal)	26.25±14.22 (<i>n</i> =12)*
≥30% (High sperm chromatin stability)	79.38±13.78 (<i>n</i> =21)

Data are mean±standard error of the mean. *N*=number of subjects. Number of subjects is between parentheses. **P*<0.001 in relation to the control (normal) group.

(*P*<0.001), and the equation of regression: Fertilization Rate=11.72+1.72*(% sperms Grade 2 SDS + EDTA).

The multivariate analysis to assesses of the probability for success of the ICSI procedure controlling for SDS+EDTA (Grade 2) sperm chromatin stability, infertility type, infertility cause, years of infertility, number of aspirated follicles, injected oocytes, number of sperm, sperm with grade III motility, strict spermatid morphology, and sperm vitality as independent variables showed that only SDS+EDTA (Grade 2) was associated with the probability of success to the ICSI procedure with and coefficient of determination (*R*²) of 0.895 (Table 6).

Discussion

Intracytoplasmic sperm injection (ICSI) has resulted in the standard treatment for those cases of infertility caused by severe male infertility [23]. Despite of the evolution of

micromanipulation-assisted fertilization for the treatment of severe male infertility and refinement of diagnostic methods for the evaluation of sperm developmental potential, and development of new treatment regimens for the newly discovered abnormalities [23] it is not uncommon the fertilization failure (complete fertilization failure or low fertilization rates) after intracytoplasmic sperm injection (ICSI). In the majority of these cases, the unfertilized oocytes are inactivated [3]. This has been confirmed in the present study.

In addition, we provided direct evidence that failure to fertilize oocytes in ICSI procedures in our Assisted Reproduction Program were due to the existence of high sperm chromatin stability. For a successful fertilization and pronucleus formation is necessary a decondensation ability in the oocyte. The sperm abnormalities causing failure of sperm decondensation in the oocyte are unrecognizable by conventional semen analysis and different methods are used [12–14, 19].

Bjorndahl [24] demonstrated that the nuclear chromatin stability, which quaternary structure is zinc-dependent, is altered by a high molecular weight protein secreted by the seminal vesicles that selectively binds zinc ions in the ejaculate. Therefore, male that presented hypo-function of the seminal vesicles would have decreased zinc bindings levels, which would lead to spermatid nuclear chromatin hyper-stability and would affect the fertility capacity of the patient [15].

In the present study we have evaluated the degree of sperm nuclear chromatin condensation previous to the ICSI procedure and its relationship to the fertilization rate after ICSI, as a method to evaluate one of the two possible factors of failure in fecundation. In our results we found a lower fertilization rate in patients with high sperm nuclear

Table 6 Multivariate analysis to assesses the probability to fertilize an oocyte according to sperm chromatin stability, age of women, kina, cause and years of infertility and characteristics of the sperms in terms of number, motility, morphology and vitality

Independent variable	β	SE	<i>P</i>	Confidence interval at 95%	
SDS+EDTA (Grade 2) %	1.75	0.19	0.0001	1.31	2.12
Age of patient (years)	−0.74	0.99	0.466	−2.79	1.32
Kind of infertility	21.25	10.82	0.063	−1.26	43.75
Cause of infertility	2.66	2.65	0.326	−2.84	8.18
Years of infertility	0.69	2.27	0.762	−4.02	5.42
Number of aspirated oocytes	0.68	1.08	0.532	−1.56	2.92
Number of injected oocytes	−0.72	1.61	0.058	−4.08	2.63
Sperm count (×10 ⁶ /ml)	0.01	0.14	0.923	−0.28	0.31
Sperm motility grade III (%)	0.13	0.46	0.774	−0.82	0.31
Normal morphology (%)	−0.04	0.39	0.922	−0.86	0.78
Sperm vitality (%)	−0.32	0.27	0.258	−0.89	0.25

Dependent variable: Fertilization rate (FR). β = coefficient of regression, *SE* = standard error, *P* = probability, *N* = sample size. SDS+EDTA (GRADE 2) high decondensation of head sperms. Low values of sperms grade II means high sperm chromatin stability. *R*² = 0.895, *P* = 0.0001, *N* = 33.

chromatin stability in the presence of SDS+EDTA, which would be suggesting that the fecundity failure was related to the chromatin hyper-stability. This will avoid the formation of the male pronucleus.

Other authors with a different design to the present study did not find correlations between the chromatin decondensation or chromatin decondensation rate in vitro and the fertilization rates in ICSI procedure [25]. Our design grouped semen samples as having normal sperm chromatin stability or high sperm chromatin stability. After ICSI procedure, a sample with high sperm chromatin stability showed a fertilization rate of 26.25% compared to 79.38% in the group with normal sperm chromatin stability. When samples were separated according to normal or abnormal sperm morphology differences in fertilization rates were not observed.

Sakkas et al. [26] examined unfertilized oocytes, using the fluorochrome Hoechst 33342, to determine whether a relationship exists between failure of fertilization and sperm chromatin quality. They found that poor chromatin packaging and/or damaged DNA may contribute to failure of sperm decondensation after ICSI and result in failure of fertilization. Then, It is, suggested that a highly stable sperm chromatin is associated with DNA damage and this should be contributing to a failure in the decondensation process after the sperm deposition into the oocyte as it has been observed in the present study. Other authors studied decondensation ability of spermatozoa from normal samples and their fertilizing performance in an in vitro fertilization (IVF) program. Fertilization occurred when the decondensation percentage of sperm nuclear chromatin was more than 70% [27].

There are several reports that suggest that oocytic maturation may occur after the fecundation process, affecting the embryo development, pregnancy and implantation process [28]. In this study we have injected during the ICSI cycles, oocytes in metaphase II characterized by the presence of the first polar body in the perivitelline space which decreases the possibility of injection of immature oocytes.

On the other hand, there are several technical aspects related to the injection and deposition of the sperm inside the oocyte that could influence the result of the ICSI procedure. It is necessary an inverted microscope equipped with micromanipulators and injectors to make the injection procedures, and glass micro-needle correctly prepared, with appropriate characteristics and with an adequate position over the inverted microscope. However, during this study, there has not been any lysis of the oocytes as a result of a bad injection procedure, or cases in which the sperm have been deposited in the perivitelline space.

It is interesting that our findings indicate that high sperm chromatin stability is predictor of low fertilization rate than

strict sperm morphology. In fact, a similar rate of fertilization was observed when samples with normal sperm morphology or abnormal sperm morphology were used for the ICSI procedure. Efficacy of ICSI for teratozoospermic samples has been recently confirmed [29].

Therefore, due that patients in whom it has been demonstrated high spermatid nuclear chromatin stability, an important factor during fecundation process, which would be directly affecting the fertility rate after ICSI procedure, it would be important to consider the nuclear chromatin stability evaluation previously to this procedure, and to estimate the success rate and/or to search for new alternatives.

Subjects with high sperm chromatin stability should be treated previously to an ICSI procedure. One attempt to treat these cases have been described previously [14].

Although study suggest a role of high sperm chromatin stability as related to failure in fertilization rates in ICSI procedures, a greater number need to be assessed in future studies.

Acknowledgements Authors thanks to Cynthia Gonzales-Castañeda for her help in the English edition.

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