Article

Artificial oocyte activation using calcium ionophore in ICSI cycles with spermatozoa from different sources

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Abstract

The present study evaluated the effect of artificial oocyte activation (AOA) with calcium ionophore A23187 on intracytoplasmic sperm injection (ICSI) cycles using spermatozoa from different sources. The 314 cycles evaluated were divided into three groups according to sperm origin; the ejaculated group ($n = 92$), the epididymal group ($n = 82$), and the testicular group ($n = 140$). Each group was further split into experimental subgroups, depending on whether or not AOA was performed. In addition, the cycles of women younger than 36 years were evaluated separately. For each experimental group, ICSI outcomes were compared between subgroups. No significant difference was observed between subgroups for all sperm origin groups. When evaluating only the cycles of women younger than 36 years of age, AOA increased the percentage of high-quality embryos (74.5 versus 53.0%, $P = 0.011$) and the implantation rate (19.3 versus 10.5%, $P = 0.0025$) when it was used with ejaculated spermatozoa, and the percentage of high-quality embryos (64.4 versus 50.3%, $P = 0.006$) when epididymal spermatozoa were used. These results may suggest that both sperm maturity and oocyte quality play a role in oocyte activation. However, this study is to be continued to confirm these findings.

Keywords: *calcium, epididymis, ICSI, spermatozoa, testicle*

Introduction

Male factor infertility is implicated in approximately 50% of couples treated with assisted reproduction techniques (Maduro and Lamb, 2002; Salgado Jacobo *et al.*, 2003) and, since its introduction in 1992, intracytoplasmic sperm injection (ICSI) has become the treatment of choice for severe male factor infertility (Palermo *et al.*, 1992). Azoospermia, defined as complete absence of spermatozoa from the ejaculate, is present in about 1% of all men (Willott, 1982) and in 10–15% of infertile men (Jarow *et al.*, 1989). The majority of cases are attributed to obstructive azoospermia, in which spermatogenesis is normal, but patients with nonobstructive azoospermia are characterized by impaired spermatogenesis (Tournaye *et al.*, 1997). Besides ejaculated spermatozoa, testicular and epididymal spermatozoa can be surgically retrieved from azoospermic patients for use in ICSI, which can result in successful fertilization and pregnancy (Craft *et al.*, 1993; Schoysman *et al.*, 1993).

Previous studies have indicated that ICSI outcomes are not related to sperm quality (De Vos *et al.*, 2003). Even though the ICSI fertilization rate is considered to be the highest among all

assisted reproduction treatments, fertilization failure is a recurrent phenomenon (Esterhuizen *et al.*, 2002), especially when non-ejaculated spermatozoa are used (Ghazzawi *et al.*, 1998; Ubaldi *et al.*, 1999).

Investigations of oocytes that remained unfertilized following ICSI revealed that missing or disturbed oocyte activation may be the most frequent cause of fertilization failure (Sousa and Tesarik, 1994), and premature chromosomal condensation in spermatozoa may be the next most common cause (Tejada *et al.*, 1992). Indeed, Nasr-Esfahani *et al.* (2007) demonstrated that after artificial activation, the fertilization rate associated with ICSI increased from 59.9% to 87.7%, and premature chromosomal condensation spermatozoa appeared to be present in over 50% of the remaining oocytes that failed to fertilize.

Oocyte activation is characterized by a dramatic rise in intracellular calcium concentration, which in mammals takes the form of calcium oscillations (Stricker, 1999; Hafez *et al.*, 2004) driven by an elevation in inositol triphosphate (IP3) concentrations (Miyazaki *et al.*, 1993; Rice *et al.*, 2000). The causative agent of these oscillations is proposed to be a recently described phosphoinositide-specific phospholipase C, PLC-ξ, which is a soluble sperm factor delivered to the egg following membrane fusion (Saunders *et al.*, 2007). Different methods have been proposed to overcome oocyte activation failure in ICSI cycles, including electrical (Zhang *et al.*, 1999; Manipalviratn *et al.*, 2006), mechanical (Tesarik and Sousa, 1995a; Tesarik *et al.*, 2002; Paffoni *et al.*, 2007), and chemical oocyte activation (Hoshi *et al.*, 1995; Rybouchkin *et al.*, 1997).

It has been described that the calcium oscillation pattern during oocyte activation may influence not only fertilization but also embryo development and therefore the implantation (Rout *et al.*, 1997; Ozil *et al.*, 2006). The most commonly studied artificial oocyte activation (AOA) model uses the calcium ionophore A23187 (Dale *et al.*, 1999; Tesarik *et al.*, 2000; Kim *et al.*, 2001; Nakagawa *et al.*, 2001; Eldar-Geva *et al.*, 2003; Chi *et al.*, 2004; Murase *et al.*, 2004; Heindryckx *et al.*, 2005; Katsuki *et al.*, 2005) to increase the concentration of free Ca^{2+} in the cytosol, thereby mimicking the physiological cell-signalling mechanism (Wang E *et al.*, 1998).

There are few studies in the literature focused on AOA when spermatozoa with different types of abnormalities are used (Moaz *et al.*, 2006), or with spermatozoa from different origins (Ahmady and Michael, 2007; Check *et al.*, 2007) and the effects of AOA on embryo development and implantation are to be elucidated. The aim of the present study was to evaluate the effect of AOA, induced with the calcium ionophore A23187, on ICSI cycle outcomes using ejaculated, epididymal and testicular spermatozoa.

Materials and methods

Experimental design

The study was performed in 314 couples undergoing ICSI cycles for the first time between January 2006 and July 2007. Written informed consent was obtained, in which patients agreed to share the outcomes of their own cycles for research purposes, and the study was approved by the Brazilian National Committee of Ethics and Research (n.593/2007).

The cycles were divided into three experimental groups according to sperm origin: the ejaculated group (n = 92), the epididymal group (n = 82), and the testicular group (n = 140).

For each experimental group, cycles in which AOA was applied (AOA subgroup) were matched with cycles in which AOA was not applied (control subgroup). Matching was performed using the female's age and the number of oocytes recovered after follicular aspiration.

In order to exclude ovarian factor infertility, the cycles of woman younger than 36 years of age were also evaluated separately.

For surgical sperm retrieval in obstructive azoospermic patients, percutaneous epididymal sperm aspiration (PESA) was the first approach attempted, and testicular sperm aspiration (TESA) was performed when the former method failed. For retrieval from non-obstructive azoospermic patients, TESA was performed. The epididymal group was therefore composed exclusively of obstructive azoospermic patients. The testicular group was composed of both obstructive (n = 64) and non-obstructive patients (n = 76), and both types of azoospermia were equally distributed among the subgroups.

The following parameters were compared between the AOA and control subgroups of each experimental group: the normal fertilization rate, the percentage of high-quality embryos on the third day of development (day 3), the implantation rate, the clinical pregnancy rate, and the miscarriage rate.

The implantation rate was defined as the total number of gestational sacs divided by the total number of embryos transferred. Clinical pregnancy was defined as the presence of a gestational sac visualized by ultrasound 4–6 weeks after embryo transfer and miscarriage was defined as the spontaneous loss of a pregnancy before 24 weeks of gestation.

Sperm samples

Ejaculated spermatozoa were obtained by masturbation after 3–5 days of ejaculatory abstinence. After liquefaction at room temperature, sperm samples were prepared by either discontinuous density-gradient centrifugation (DDGC) or swim-up technique. For DDGC, the bottom fraction was aspirated and washed twice at 300 *g* for 8 min. For swim-up, sperm samples were diluted 1:1 with HEPES-buffered medium (Irvine Scientific, Santa Ana, USA), centrifuged at 300 *g* for 8 min, and incubated at 37°C for 1 h, allowing the spermatozoa to move from the resulting pellet to the over-layered culture medium.

After administration of cord block anaesthesia, testicular spermatozoa were obtained via TESA, which was performed by longitudinally inserting a 21-gauge butterfly needle into the superior testicle pole while avoiding the epididymis. Forward and backward movements were made and the needle direction was changed slightly to ensure parenchymal disruption for needle aspiration.

Epididymal spermatozoa were obtained by PESA, which was performed under local anaesthesia using a 27-gauge needle that was inserted into the epididymis. Gentle, negative pressure was applied as the epididymal fluid was aspirated.

For both PESA and TESA, the aspirated material was collected in a Falcon tube and washed with a minimum volume of culture medium at 37°C. The recovered material was checked for the presence of spermatozoon and centrifuged at 300 *g* for 8 min. When necessary, the fraction was diluted or concentrated.

Ovarian stimulation

Ovarian stimulation was achieved by long pituitary down-regulation using a gonadotrophin-releasing hormone agonist (GnRHa, Lupron Kit™, Abbott SA Societé Française des Laboratoires, Paris, France), followed by ovarian stimulation with recombinant-FSH (Gonal-F®, Serono, Geneva, Switzerland). Follicular development was followed by ultrasound starting on day 4 of gonadotrophin administration, and when adequate follicular growth and serum oestradiol concentrations were observed, recombinant human chorionic gonadotrophin (rHCG, Ovidrel™, Serono) was administered to trigger ovulation. Oocyte retrieval was performed 35 h after rHCG administration using transvaginal ultrasound guidance.

Preparation of oocytes

After retrieval, oocytes were incubated in culture medium (G-MOPS™-V3-Plus, VitroLife, Kungsbacka, Sweden) covered with mineral oil (Ovoil™, VitroLife) at 37°C and 6% CO_2 for 3 h. Cumulus cells were removed by a 30 s exposure to HEPES-buffered medium containing 80 IU/ml hyaluronidase (Irvine Scientific, Santa Ana, USA), after which coronal cells were manually removed using finely drawn glass Pasteur pipettes (Humagen Fertility Diagnostics, Charlottesville, Virginia, USA). The denuded oocytes were then assessed for nuclear status. Oocytes observed to have released the first polar body were considered mature and were used for ICSI.

ICSI and calcium ionophore treatment

For ICSI, oocytes were individually placed in 4 µl droplets of buffered medium (G-Mops™-V3-Plus, VitroLife). Spermatozoa was placed in a central 4 µl droplet of polyvinylpyrrolidone solution (PVP, Irvine Scientific) in a 50 × 40 mm glass culture dish (WillCo-dish®, New Jersey, USA) covered with warm mineral oil (Ovoil™). Sperm injection was carried out 38 h after recombinant HCG trigger on the heated stage (37°C) of an inverted microscope (Eclipse TE 300, Nikon®, Tokyo, Japan). After ICSI oocytes were incubated in culture medium containing 5 µmol/l of the calcium ionophore A23187 (4-bromo calcium ionophore A23187, Sigma B7272, EUA) at 37°C and 6% CO_2 for 30 min. The oocytes were then washed and incubated in culture medium (G-1™-V3-Plus, VitroLife) at 37°C and 6% CO_2.

Assessment of fertilization, embryo quality and embryo transfer

Fertilization was assessed 18 h after ICSI, and normal fertilization was declared when two clearly distinct pronuclei were present.

Embryo quality was evaluated under an inverted microscope (Eclipse TE 300), and the following parameters were recorded: (i) the number of blastomeres; (ii) the fragmentation percentage; (iii) variation in blastomere symmetry; (iv) the presence of multinucleation; and (v) defects in the zona pellucida and cytoplasm.

Embryo transfer was performed on the third day of development. High-quality (grade A) embryos were defined as those having all of the following characteristics: either 4–6 cells on the second day or 8–10 cells on the third day of development, less than 10% fragmentation, symmetric blastomeres, absence of multinucleation, colourless cytoplasm with moderate granulation and no inclusions, absence of perivitelline space granularity, and absence of zona pellucida dysmorphism. Grade B embryos were considered when showing the same characteristics as grade A embryos, expect for the fragmentation, which would be less than 25%.

For each couple, one to four embryos were transferred depending on the embryo quality and the female's age. Embryos of grades A and B were given priority in selection for transfer.

Statistical analysis

Results are expressed as mean ± SD for numeric variables, while proportions (%) were used for categorical variables. Mean values were compared by Student's *t*-test, and proportions were compared using the chi-squared test or Fisher's exact test, depending on the sample size.

To study the influence of AOA on pregnancy and miscarriage rates, a binary logistic regression model was conducted. The results were presented as odds ratios (OR) and the corresponding 95% confidential interval (CI) calculated from the logistic regression model. Results were considered to be significant at the 5% critical level ($P < 0.05$). Data analysis was carried out using Minitab (version 14) statistical software.

Results

Overall experimental group

The general characteristics of the AOA and control subgroups when injected with ejaculated, epididymal or testicular spermatozoa were similar (**Table 1**). In addition, in the ejaculated group, no significant difference was observed between subgroups for sperm concentration (2.4×10^6/ml versus 2.7×10^6/ml for AOA and control respectively) and percentage of motile spermatozoa (67.8% versus 62.3% for AOA and control respectively).

No significant difference was observed between subgroups for all three sperm origin groups in terms of normal fertilization rate, percentage of high-quality embryos on the third day of development, implantation rate, pregnancy rate or miscarriage rate (**Table 2**).

Binary logistic regression analysis showed that calcium ionophore application was not a significant determinant of the likelihood of pregnancy or miscarriage when using ejaculated (OR = 0.86, 95% CI = 0.30–2.44; and OR = 1.6, 95% CI = 0.24–10.81; for pregnancy and miscarriage, respectively),

epididymal (OR = 1.46, 95% CI = 0.55–3.88; and OR = 0.64, 95% CI = 0.10–4.09; for pregnancy and miscarriage, respectively), or testicular spermatozoa (OR = 0.92, 95% CI = 0.41–2.06; and OR = 0.36, 95% CI = 0.08–1.64; for pregnancy and miscarriage, respectively).

Women younger than 36 years of age

In cycles of patients in which the woman's age was less than 36 years, no difference was observed in the fertilization rate (**Figure 1**), pregnancy rate (**Figure 2**), or miscarriage rate (**Figure 3**) between AOA and control subgroups from any sperm origin group. Nevertheless, AOA was able to increase the percentage of high-quality embryos (74.5% versus 53.0%, $P = 0.011$, for AOA and control respectively, **Figure 4**) and the implantation rate (19.3% versus 10.5%, $P = 0.0025$, for AOA and control respectively, **Figure 5**) when using ejaculated spermatozoa, and AOA also increased the percentage of high-quality embryos (64.4% versus 50.4%, $P = 0.006$, for AOA and control respectively, **Figure 4**) when using epididymal but not testicular spermatozoa.

Similar to the overall experimental group, when the woman's age was <36 years, binary logistic regression analysis also showed that AOA was not a significant determinant of the likelihood of pregnancy or miscarriage when using ejaculated (OR = 0.48, 95% CI = 0.11–2.06; and OR = 1.67, 95% CI = 0.07–37.73; for pregnancy and miscarriage, respectively), epididymal (OR = 1.85, 95% CI = 0.59–5.82; and OR = 0.56, 95% CI = 0.06–5.24, for pregnancy and miscarriage, respectively), or testicular spermatozoa (OR = 0.64, 95% CI = 0.22–1.85; and OR = 0.47, 95% CI = 0.07–3.34; for pregnancy and miscarriage, respectively).

Table 1. General characteristics of the artificial oocyte activation and control subgroups for each sperm origin group.

Sperm origin	*Subgroup*	n	*Female age (years)*	*Male age (years)*	*No. of follicles*	*No. of oocytes*	*No. of transferred embryos*
Ejaculate	AOA	46	34.4 ± 5.5	39.8. ± 8.7	17.7 ± 10.4	11.2 ± 9.8	1.71 ± 0.96
	Control	46	34.0 ± 5.5	38.1 ± 7.8	18.1 ± 10.1	11.3 ± 10.9	2.03 ± 1.17
Epididymis	AOA	41	31.6 ± 4.8	43.8 ± 6.9	19.8 ± 12.1	12.6 ± 8.3	2.51 ± 1.3
	Control	41	34.2 ± 5.0	46.0 ± 7.0	20.4 ± 12.0	14.6 ± 8.7	2.51 ± 1.2
Testicle	AOA	70	34.3 ± 4.1	41.3 ± 7.9	17.6 ± 9.7	13.3 ± 7.5	2.23 ± 1.10
	Control	70	34.1 ± 4.8	44.3 ± 8.1	18.3 ± 9.9	13.6 ± 7.8	2.51 ± 1.30

Values are mean ± SD. AOA = artificial oocyte activation. There were no statistically significant differences between AOA and controls for any of the sperm categories.

Table 2. Effect of artificial oocyte activation using calcium ionophore in patients with spermatozoa retrieved from ejaculate, epididymis or testicle.

Sperm origin	*Subgroup*	n	*Fertilization rate (%)*	*High-quality embryos (%)*[a]	*Implantation rate (%)*	*Pregnancy rate (%)*	*Miscarriage rate (%)*
Ejaculate	AOA	46	(220/317) 69.4	(116/220) 52.7	15.3	(11/46) 23.9	(4/11) 36.3
	Control	46	(259/340) 76.2	(134/259) 51.7	12.0	(10/46) 21.7	(4/10) 40.0
Epididymis	AOA	41	(254/519) 48.9	(139/254) 54.7	16.7	(11/41) 26.8	(3/11) 27.3
	Control	41	(399/599) 66.6	(212/399) 53.1	19.7	(13/41) 31.7	(3/13) 23.1
Testicle	AOA	70	(473/935) 50.6	(189/473) 40.0	13.1	(17/70) 24.2	(8/17) 47.1
	Control	70	(534/952) 56.1	(236/534) 44.2	15.1	(16/70) 22.8	(4/16) 25.0

AOA = artificial oocyte activation.
There were no statistically significant differences between AOA and controls for any of the sperm categories.
[a]Grade A embryos.

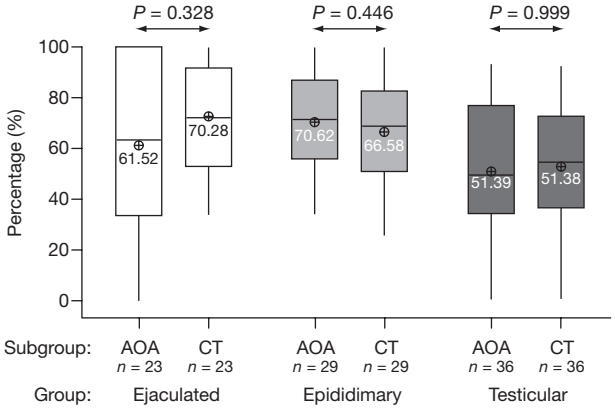

Figure 1. Fertilization rate in the artificial oocyte activation (AOA) and control (CT) subgroups after injecting ejaculated, epididymal or testicular spermatozoa for cycles in which woman's age was less than 36 years. There were no statistically significant differences between AOA and controls for any of the sperm categories. (The symbol + inside a circle indicates the median value; the horizontal line indicates the mean value.)

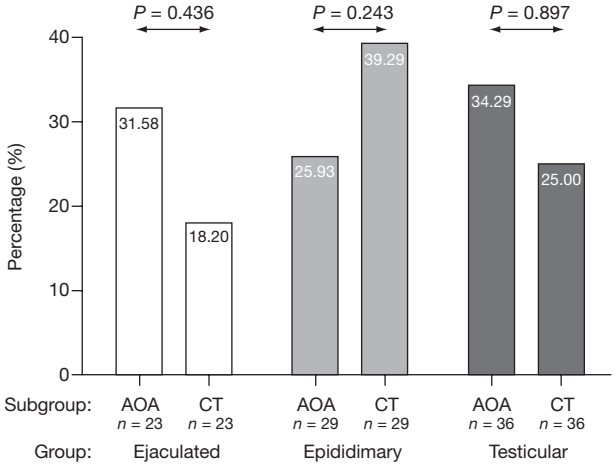

Figure 2. Pregnancy rate in artificial oocyte activation (AOA) and control (CT) subgroups after injecting ejaculated, epididymal or testicular spermatozoa for cycles in which woman's age was less than 36 years. There were no statistically significant differences between AOA and controls for any of the sperm categories.

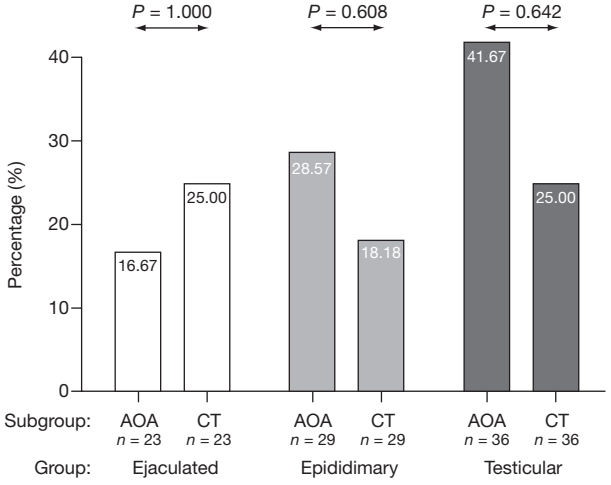

Figure 3. Miscarriage rate in artificial oocyte activation (AOA) and control (CT) subgroups after injecting ejaculated, epididymal or testicular spermatozoa for cycles in which woman's age was less than 36 years. There were no statistically significant differences between AOA and controls for any of the sperm categories.

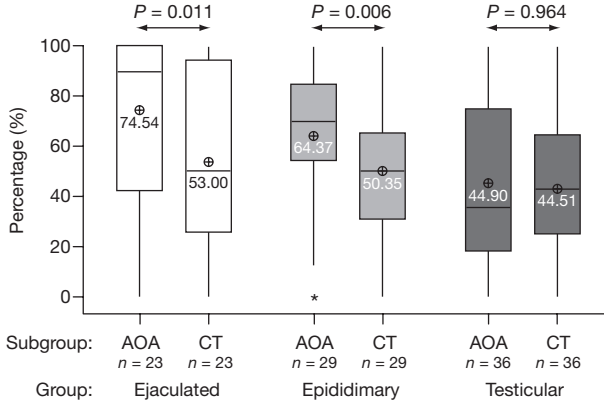

Figure 4. Percentage of high-quality embryos (grade A) in artificial oocyte activation (AOA) and control (CT) subgroups after injecting ejaculated, epididymal or testicular spermatozoa for cycles in which woman's age was less than 36 years. (The asterisk denotes an outlier value; the symbol + inside a circle indicates the median value; the horizontal line indicates the mean value.)

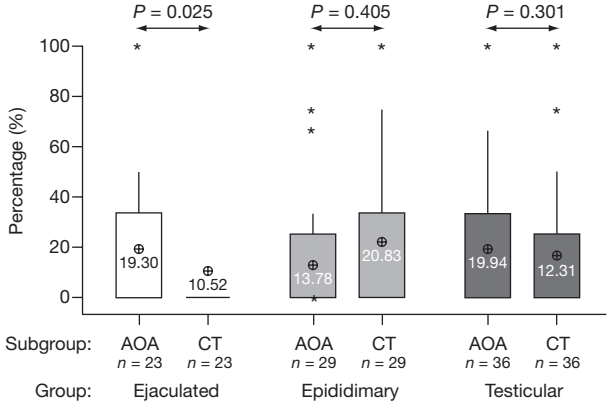

Figure 5. Implantation rate in artificial oocyte activation (AOA) and control (CT) subgroups after injecting ejaculated, epididymal or testicular spermatozoa for cycles in which woman's age was less than 36 years. (Asterisks denote outlier values; the symbol + inside a circle indicates the median value.)

Discussion

A variety of stimuli can induce artificial oocyte activation, and combining calcium ionophore treatment with ICSI has been shown to be effective in achieving satisfactory results in fertilization, implantation, and pregnancy (Hoshi *et al.*, 1995; Tesarik and Sousa, 1995b; Rybouchkin *et al.*, 1997; Kim *et al.*, 2001; Nakagawa *et al.*, 2001; Eldar-Geva *et al.*, 2003; Chi *et al.*, 2004; Murase *et al.*, 2004; Katsuki *et al.*, 2005). Nevertheless, the effect of AOA in oocytes injected with spermatozoa of different origins is still unclear. A recent case report related a successful pregnancy and delivery following the ICSI of a frozen–thawed nonviable testicular spermatozoa and the induction of AOA with a calcium ionophore. It was suggested that the key point in fertilization of nonviable spermatozoa is oocyte activation (Ahmady and Michael, 2007).

In another study, oocytes injected with testicular spermatozoa, which were retrieved from a globozoospermic patient, and subsequently exposed to calcium ionophore failed to fertilize, which suggested an association between the acrosome and the activity of the oocyte-activation sperm factor, PLC-ξ (Check *et al.*, 2007).

The aforementioned studies were case reports, in which only one cycle was described. The present report is the first large study comparing the effect of AOA induced with the calcium ionophore A23187 on ICSI cycles using ejaculated, epidydimal and testicular spermatozoa. These clinical findings demonstrate that treatment with calcium ionophore is able to improve the embryo quality when ejaculated or epididymal spermatozoa are used and the implantation rate when ejaculated spermatozoa are injected as long as the oocytes are derived from women less than 36 years of age.

It has been proposed that human oocyte activation during fertilization is mediated by a two-step pattern of increases in intracellular Ca^{2+} concentrations (Tesarik and Mendoza, 1999). The first component, activated after the beginning of gamete fusion, has been termed the trigger and is required for the first sperm-induced increase in the free ooplasmic calcium concentration (Tesarik *et al.*, 2000). The second, subsequent component, the termed oscillator, is characterized by a series of shorter Ca^{2+} transients. The oscillator alters the functional parameters of the oocyte's internal calcium stores so as to make them capable of supporting the ongoing series of calcium oscillations (Tesarik and Mendoza, 1999).

Even though the free intracellular calcium trigger can be induced by exogenous physiological stimuli (Zhang *et al.*, 1999; Yanagida *et al.*, 2001; Tesarik *et al.*, 2002; Manipalviratn *et al.*, 2006; Paffoni *et al.*, 2007), repetitive calcium oscillation is critical for successful oocyte activation in mammals (Williams, 2002), and nearly all parthenogenetic activation agents that work via calcium increase fail to cause oscillations (Swann *et al.*, 2004).

With respect to the present study, the reason why the oocytes injected with ejaculated and epididymal spermatozoa were able to positively respond to AOA, while those injected with testicular spermatozoa could not, may be explained by a relationship between sperm maturity and the function of oocyte activation. One such hypothesis would be that PLC-ξ is at a different concentration or is inactivated in immature spermatozoa relative to mature or partially mature spermatozoa. Therefore, it could also be postulated that a testicular spermatozoon is unable to maintain the calcium oscillations in the ooplasm after artificial stimulation of oocyte activation.

Previous findings comparing pregnancies after injection of round or elongated spermatids (Fishel *et al.*, 1995; Tesarik *et al.*, 1995; Tesarik, 1996) raise questions as to the stage of spermatogenesis at which the soluble sperm factor first appears. Analysis using polymerase chain reaction revealed that PLC-ξ expression occurs in spermatids but not during the earlier stages of spermatogenesis (Saunders *et al.*, 2002). Nevertheless, fertilization and pregnancy rates after injection of spermatid cells have tended to be lower than those after injection of mature or testicular spermatozoa (Vanderzwalmen *et al.*, 1997). Furthermore, spermatid-injected mouse oocytes required an extra activation stimulus to start normal embryonic development (Kimura and Yanagimachi, 1995), suggesting that immature spermatozoa may have impaired fertilization function and that the sperm factor may be incompletely developed or inactive, at least in immature mouse spermatozoa.

The relationship between increasing age and the progressive decline in fertility has been widely discussed. It was reported that human fertility starts to decline markedly around the age of 35 years (van Zonneveld *et al.*, 2001; te Velde and Pearson, 2002), and age-related infertility is associated not only with a quantitative but also with a qualitative decline in the ovarian reserve (Westergaard *et al.*, 2007).

In the present study, calcium ionophore treatment improved ICSI outcomes only in oocytes derived from women aged less than 36 years, which suggests that not only the spermatozoa but also the oocyte plays a role in oocyte activation. It has been postulated that PLC-ξ is inactive inside spermatozoa, and that it is activated upon introduction into the oocyte (Swann *et al.*, 2004). In addition, it has been postulated that the action of the sperm factor is not a passive process, but instead depends on oocyte agents, such as sperm nucleus decondensation factor (SNDF) (Dozortsev *et al.*, 1997).

Even though it has been demonstrated that AOA can promote a rise in intracellular calcium concentration, which can result in higher fertilization rates, in the present study, no improvement in fertilization was found when a calcium ionophore was applied. Nevertheless, further embryonic development was found to be positively affected by AOA. The hypothesis that Ca^{2+} oscillations may provide more than merely a stimulus for meiotic resumption and that they may play a role in long-term embryonic events was previously discussed (Bos-Mikich *et al.*, 1997; Ozil and Huneau, 2001). It has been shown that differences in Ca^{2+} signalling patterns can have effects not only on implantation and post-implantation development but also on long-term fetal morphology (Ozil and Huneau, 2001) and weight variation in offspring (Ozil *et al.*, 2006).

The exact mechanism by which intracellular Ca^{2+} influences embryonic development is not completely understood; however, it has been postulated that the calcium oscillation pattern may partly act through gene expression regulation (Rout *et al.*, 1997; Ozil and Huneau, 2001).

Treatment with calcium ionophore has been extensively studied in different species. The use of calcium ionophore in pigs induced

62% of oocytes to form a pronucleus (Wang WH *et al*., 1998) and at an appropriate concentration it also inhibited the polyspermia in-vitro fertilization (Asano and Niwa, 2004). In a bovine system, the rate of oocyte activation was dependent on the calcium ionophore dose concentration, used during incubation (Chung *et al*., 2000) and equine oocytes were effectively activated with 50 mmol/l calcium ionophore, without sperm penetration, suggesting that this would be a useful protocol for activation after cloning (Choi *et al*., 2001).

Besides calcium ionophore treatment, strontium-induced AOA has been reported in humans. Yanagida *et al*. (2006) achieved fertilization, pregnancy and childbirth after combining strontium treatment with ICSI. The health risks associated with exposing gametes to either strontium or calcium ionophores remain to be definitively proven. Although possible risks associated with exposing human oocytes to chemical agents may still be uncovered, calcium ionophore treatment has been widely applied in human oocytes with no evidence of negative health effects.

In 1997, calcium ionophore treatment was tested in a clinical trial, and a healthy infant was delivered (Rybouchkin *et al*., 1997). In 2001, pregnancy and delivery resulting from frozen–thawed embryos after AOA treatment with a calcium ionophore were achieved (Kim *et al*., 2001). In addition, ICSI and calcium-ionophore-mediated oocyte activation generated successful pregnancies and deliveries for normozoospermic patients with repeated failed fertilization attempts (Eldar-Geva *et al*., 2003) and extremely low fertilization rates (Chi *et al*., 2004).

Although the findings of this study are interesting, the present trial has some limitations, especially when the sub-groups were formed with a small number of patients in each arm. Therefore, a well-conducted, prospective randomized study with a larger number of patients is needed to give the definitive answers.

In summary, these results suggest that AOA may be a useful tool to improve ICSI outcomes in younger patients when ejaculated or epididymal spermatozoa are used, which supports the theory that sperm maturity and oocyte quality both play roles in oocyte activation. However, confirmation of these findings awaits a larger prospective trial with an increased number of patients.

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