Zygote **25** (December), pp. 697–710. © Cambridge University Press 2017
doi:10.1017/S0967199417000569 First Published Online 28 November 2017

Sperm-derived factors enhance the *in vitro* developmental potential of haploid parthenotes

Ramya Nair[2], *Shahin Aboobacker*[2], *Srinivas Mutalik*[3], *Guruprasad Kalthur*[1] *and Satish Kumar Adiga*[2]
Clinical Embryology, Kasturba Medical College, Manipal University, Karnataka, India; and Department of Pharmaceutics, Manipal College of Pharmaceutical Sciences, Manipal University, Karnataka, India

Date submitted: 31.01.2017. Date revised: 01.08.2017. Date accepted: 17.08.2017

Summary

Parthenotes are characterized by poor *in vitro* developmental potential either due to the ploidy status or the absence of paternal factors. In the present study, we demonstrate the beneficial role of sperm-derived factors (SDF) on the *in vitro* development of mouse parthenotes. Mature (MII) oocytes collected from superovulated Swiss albino mice were activated using strontium chloride ($SrCl_2$) in the presence or absence of various concentrations of SDF in M16 medium. The presence of SDF in activation medium did not have any significant influence on the activation rate. However, a significant increase in the developmental potential of the embryos and increased blastocyst rate ($P < 0.01$) was observed at 50 µg/ml concentration. Furthermore, the activated oocytes from this group exhibited early cleavage and cortical distribution of cortical granules that was similar to that of normally fertilized zygotes. Culturing 2-cell stage parthenotes in the presence of SDF significantly improved the developmental potential ($P < 0.05$) indicating that they also play a significant role in embryo development. In conclusion, artificial activation of oocytes with SDF can improve the developmental potential of parthenotes *in vitro*.

Keywords: Cortical reaction, Developmental potential, Mitochondria, Parthenotes, Sperm-derived factor, Spindle assembly.

Introduction

In sexual reproduction, successful fertilization requires the union of maternal and paternal gametes, either of which seems to have a significant role to play in successful fertilization and embryonic development (McGrath & Solter, 1984). Maternal resources are directly available for the developing preimplantation stage embryos that support the development until embryonic genome activation takes place (Li *et al*., 2010). RNA profiling of murine zygote has confirmed the presence of around 380 sperm cytosolic RNAs (Park *et al*., 2013; Fang *et al*., 2014) indicating the transfer of cytosolic factors into the zygote. Therefore, it is probable that rather than acting as a mere vector for paternal genome, spermatozoa provide certain factors that regulate embryonic development (Parrington *et al*., 1996; Tang *et al*., 2000; Wu *et al*., 2001; Swann *et al*., 2004). Earlier studies have demonstrated that oocyte activation factors (Perry *et al*., 2000), centrosomes (Schatten & Sun, 2009), mRNAs (Sendler *et al*., 2013) and snRNAs (Krawetz *et al*., 2011; Kiani & Rassoulzadegan, 2013) from the spermatozoa have a significant role in early embryogenesis.

Microinjection of soluble cytosolic factors from spermatozoa (sperm-derived factors; SDF) can induce calcium oscillations similar to that occuring during normal fertilization (Swann, 1990; Kuretake *et al*., 1996; Parrington *et al*., 1996; Oko & Sutovsky, 2009). The candidate molecules identified as SDF responsible for oocyte activation are oscillin (Parrington *et al*., 1996), truncated c-kit receptor (Sette *et al*., 1997), phospholipase C zeta (PLCζ) (Saunders *et al*., 2002), glucosamine 6-phosphate isomerase and

[1]All correspondence to: Guruprasad Kalthur. Department of Clinical Embryology, Central Research Laboratory, Kasturba Medical College, Manipal University, Manipal 576104, Karnataka, India. E-mail: guru.kalthur@manipal.edu
[2]Clinical Embryology, Kasturba Medical College, Manipal University, Manipal 576 104, Karnataka, India.
[3]Department of Pharmaceutics, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal 576 104, Karnataka, India.

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

post-acrosomal WW-domain binding protein (PAWP) (Wu *et al.*, 2007; Aarabi *et al.*, 2014). These studies, reported in the literature, mainly focused on studying the increase in the intracellular calcium level by SDF.

Embryos developed from artificially activated oocytes in the absence of paternal contribution are known as parthenotes. They play an important role in the field of developmental biology and regenerative medicine. They serve as an excellent model to understand the role played by maternal and paternal genome during early embryogenesis. However, these embryos are usually characterized by poor and delayed *in vitro* development (Liu *et al.*, 2002; Hao *et al.*, 2006; Isom *et al.*, 2012), a high degree of cellular fragmentation, 2-cell stage arrest, decreased blastocyst rate and poor blastocyst quality (Liu *et al.*, 2002; Jeong *et al.*, 2005). Haploidy (Liu *et al.*, 2002), abnormal gene expression pattern and imprinting errors (Surani *et al.*, 1984; Solter, 1988) are considered to be the cause for such abnormal development in these embryos.

During normal fertilization, along with sperm nucleus, several cytosolic factors enter the ooplasm which helps in triggering the subsequent biochemical events in the oocytes initiating fertilization (Perry *et al.*, 2000; Krawetz *et al.*, 2011; Kiani & Rassoulzadegan, 2013). Therefore, arguably, the lack of paternal genome and/or the cytosolic factors might be one of the reasons for the poor development of artificially activated oocytes. However, there are no studies in the literature to ascertain their role in early embryo development. In this direction, the present study was undertaken to explore whether the presence of soluble sperm-derived factors (SDFs) in the culture medium during artificial activation of oocytes has any beneficial effects. Unlike in earlier studies, in which the sperm factors are microinjected to the oocytes to understand their role, we used a much simpler approach of supplementing SDF to embryo culture medium without using any microinjection methods.

Materials and Methods

Animal model

Inbred Swiss albino mice (8–10-week-old) maintained in the Central Animal Research Facility, Manipal University, were used for the study. The animals were maintained under standard conditions of temperature (25 ± 2°C), humidity (45–55%) and light (12:12 h of light and dark) and were fed with food and water *ad libitum*. Prior approval was obtained from the Institutional Animal Ethical Committee of Kasturba Medical College, Manipal University, to carry out the experiment (IAEC/KMC/64/2013). The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines on the care and use of laboratory animals.

Preparation of sperm-derived factors (SDF)

Caudae epididymis collected from adult Swiss albino male mice (8−10-week-old) was teased in 2 ml of M2 medium to release the spermatozoa. Motile spermatozoa were extracted by 'swim up' technique after centrifugation for 10 min at 1000 rpm. After determining the count and motility, spermatozoa were pelleted out by centrifugation and the count was adjusted to 8 million. To release the cytosolic factors, the spermatozoa were subjected to hypotonic treatment by resuspending the pellet in Milli-Q water for 1 h at 37°C as described by Lomeo & Giambersio (1991) followed by sonication at 90% power for 7 min at 37°C (Vibra-Cell, Sonics & Materials, Inc., CT, USA). To remove sperm tail and other debris, suspension was filtered using syringe filter of 0.22 µ pore size (Millipore, cat. no. SLGVO33RS). Pre-frozen sperm-derived factor was lyophilized (Daihan Labtech Co. Ltd, Korea) after the addition of sucrose (10%) and stored at −80°C until use. Lyophilized product was reconstituted with M16 medium (Stock 1 mg/ml) and diluted to obtain the required concentrations for experiments.

Superovulation and oocyte collection

Female Swiss albino mice (6–8-week-old) were superovulated by intraperitoneal (i.p.) injection of 5 IU pregnant mare serum gonadotropin (PMSG, cat. no. G4877, Sigma) followed by 10 IU human chorionic gonadotropin (hCG, Ovutrig, India) 48 h later. Mice were humanely sacrificed by cervical dislocation 13 h after hCG administration and oviducts were dissected out in M2 medium. Cumulus–oocyte complexes (COC) were released by carefully teasing the oviduct using a blunt needle and by observing under a stereomicroscope on a stage maintained at 37°C.

Parthenogenetic activation and embryo culture

The cumulus–oocyte complexes (COCs) retrieved from oviduct were washed in M16 medium and parthenogenetically activated with 10 mM strontium chloride ($SrCl_2$) for 3 h. COCs were incubated in hyaluronidase (1 mg/ml) prepared in M2 medium for 1 min at 37°C for denuding. Activation was confirmed by the presence of one pronucleus and two polar bodies (1PN/2PB) by observing at ×200 magnification under an inverted microscope fitted to a micromanipulator (IX70; Olympus, Tokyo, Japan). Activated oocytes were washed three times and cultured *in vitro* in M16 medium at 37°C and 5% CO_2 in air. Embryos were

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

scored regularly at every 24 h under an inverted microscope (×200 magnification) until they reached the blastocyst stage.

Collection of normally fertilized embryos

Superovulated female mice were kept for overnight mating with proven fertile males. Mating was confirmed by the presence of a vaginal plug and pronuclear stage (PN) embryos were collected in M2 medium at 16 h post hCG from the oviduct of mated females. For experiments with 2-cell stage embryos, embryos were collected from the oviduct of the mated females at 24 h post hCG.

Experimental outline

To understand whether supplementation of SDF can improve the *in vitro* development of parthenogenetic embryos, oocyte activation was carried out in presence or absence of SDF. For this, COCs collected were parthenogenetically activated using 10 mM $SrCl_2$ in M16 without Ca^{2+} and Mg^{2+} supplemented with varying concentrations of SDF (5, 10, 20, 50, 75, or 100 µg/ml). Activated oocytes were washed (three times in M16 medium) post denuding and cultured in M16 medium until they develop to blastocyst stage. Blastocyst rate was calculated as the percentage of activated oocytes (pronuclear stage embryos) reaching the blastocyst stage.

To further understand whether the beneficial effect of SDF on early embryo development is stage specific, parthenotes at 2-cell stage were cultured in medium supplemented with varying concentrations of SDF (1, 2.5, 5, 10, 20, or 50 µg/ml) and the developmental potential was monitored until the blastocyst stage.

Detection of spindle assembly

Activated oocytes were fixed at 0, 3, 6 or 12 h (3 h after incubation in $SrCl_2$) for assessment of the spindle assembly using the method described by Nair *et al*. (2014). Briefly, activated oocytes were permeabilized by extraction buffer and fixed with ice cold methanol at −20°C for 12 min followed by blocking with 10% goat serum for 1 h. Primary antibody, monoclonal anti-α-tubulin antibody (cat. no. T9026; Sigma, USA) was diluted (1:100) in blocking solution and the embryos were incubated at 4°C overnight. These embryos were washed in PBS with 0.025% Triton X-100 (three times) followed by incubation in fluorescein isothiocyanate (FITC)-labelled anti-mouse antibody (cat. no. SC2010, Santa Cruz, 1:500 dilution) for 1 h at 37°C and washed three times before staining the nucleus with 4′,6-diamidino-2-phenylindole (DAPI). Embryos were mounted on a clean glass coverslip and images were acquired using a fluorescence microscope under ×400 magnification.

Cortical granules

Activated oocytes at 0 and 3 h were incubated in acid Tyrode medium for 1 min at 37°C and were later fixed using 4% PFA at 4°C for 24 h. These oocytes were blocked in Dulbecco's phosphate-buffered saline (DPBS) with 1% BSA, 0.01 M/l glycine and 0.05% Triton X-100 followed by staining with rhodamine labelled *Lens culinaris* agglutinin (LCA) (10 µg/ml) (cat. no. RL-1042, Vector Laboratories, Inc., CA, USA) for 30 min at room temperature in the dark. These oocytes were washed with PBS and images of cortical granules were grabbed using a micropublisher 5.0 RTV digital camera (Q Imaging, Surrey, Canada) fitted to a fluorescence microscope.

Sperm zona binding

Sperm zona binding assay was carried out as described by de Paola *et al*. (2015) with minor modifications. Briefly, caudae epididymis of 8–10-week-old Swiss albino mice were teased to release spermatozoa into 2 ml of M16 medium. Capacitation was carried out by incubating sperm suspension in 10% bovine serum albumin for 2 h at 37°C in 5% CO_2. Count and motility were determined post-capacitation and the sperm count was adjusted to 1×10^5 spermatozoa/ml, activated oocytes were incubated individually in 10 µl droplets of sperm suspension (1000 sperm/oocyte) for 1 h and washed gently in M16 medium to release the loosely bound spermatozoa. The oocytes were fixed using PFA and stained with propidium iodide (PI) and observed under a fluorescence microscope to count the number of bound sperm per oocyte.

Mitochondrial distribution pattern

The mitochondrial distribution pattern in oocytes activated in the presence or absence of SDF was assessed by rhodamine 123 staining as described previously (Nair *et al*., 2014). Briefly, the activated oocytes were incubated for 20 min at 37°C in M16 medium supplemented with 20 µg/ml of rhodamine 123 (cat. no. R8004, Sigma, USA). Stained oocytes were washed three times in M16 medium and mounted using antifade solution (cat. no. S3023; Dako, Carpinteria, CA, USA). Mitochondrial distribution pattern was assessed under a fluorescence microscope (×400 magnification; Imager-A1; Zeiss, Gottingen, Germany). According to the distribution pattern, oocytes were categorized as uniform, peripheral or semiperipheral and expressed in percentage (Nair *et al*., 2014).

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

Oct 4 expression

The expression of pluripotency regulator POU5F1 was assessed in embryos post 24 h of activation by immunofluorescence quantification as described by Kalthur *et al*. (2016) with minor modifications. Fixed embryos (4% PFA) were permeabilized (0.1% Triton X-100) and blocked [10% goat serum (Dako)] before incubating with primary antibody (cat. no. sc-5279; Santa Cruz Biotechnology, USA) overnight at 4°C. The embryos were washed three times (PBS with 0.025% Triton X-100) and incubated with FITC-conjugated goat anti-mouse IgG (cat. no. D0408; Santa Cruz, USA) for 1 h at 37°C. Images were grabbed using a micropublisher 5.0 RTV digital camera (Q Imaging, Surrey, Canada) and further analysed by Q-capture software. The fluorescence signal intensities were analysed from the grabbed images. The green channel was separated by Q-capture software (Media Cybernetics) to ensure that FITC signals and the background was subtracted before quantification of intensities.

Mass spectrophotometric analysis of SDF

Sperm-derived factors were subjected to MS/MS for mass spectrometric analysis of proteins present in the SDF. We used the lyophilized SDFs derived by the earlier method in this mass spectrometry analysis. The lyophilized SDF sample (about 2 mg each) was dissolved in 1 ml of water and methanol and passed through 0.22 µ membranes. The filtered solution was analysed by mass spectrometry (2000 QTRAP Nano spray™, MDS Sciex, a division of MDS Inc., Ontario, Canada) to identify possible protein components of SDF based on the m/z (Da) data obtained. Once the m/z charge ratio was obtained the raw data were used to obtain the deconvoluted mass with Dalton corrections.

Protein identification in sperm-derived factors using MS/MS-based peptide fingerprints:

Based on the corrected masses obtained, MS-fit was used to search the NCBI database for the peptide fingerprints (PF). The following settings were used, as shown in the Fig. 7. Masses above 531 to 1000 Daltons alone were selected for PFs. Masses below 531 Da were rejected as mass spectrometer background contaminants and solvents used in this study. As these proteins are not modified chemically we used the stable phosphate modifications available on proteins for the PFs. In total, 179 different proteins were identified through three sampling methods, with MOlecular Weight SEarch (MOWSE) scores between 50 to 3.49×10^7.

Statistical analysis

The data pertaining to developmental potential, cortical granule exocytosis, mitochondrial distribution pattern and spindle dynamics were represented as percentage; and sperm zona bindability and quantification of immunofluorescence were represented as mean ± standard error (SE) of the raw data. The validity of the data was analysed using chi-squared test and one-way analysis of variance (ANOVA) using the GraphPad InStat 3.0 statistical package (GraphPad Inc, USA). Differences were considered to be statistically significant if the *P*-value was <0.05.

Results

Role of sperm-derived factors during oocyte activation

Activation potential

To understand the role played by sperm-derived factors during oocyte activation, varying concentrations of SDF were supplemented in M16 medium with chemical activating agent $SrCl_2$. As shown in Fig. 1(*A*), the activation potential of oocytes in the control group ($SrCl_2$) was 93%. Incubating the oocytes with SDF alone did not result in oocyte activation. However, supplementation of SDF to the activation medium was found to result in approximately 98–99% activation at all concentrations used (5 to 100 µg/ml). The oocytes activated with $SrCl_2$ were haploid in nature and the presence of SDF during activation did not alter the ploidy status of activated oocytes.

Developmental potential

The embryos activated in the presence of varying concentrations of SDF were further cultured in M16 medium and scored at every 24 h intervals. The 2-cell rate in the control group was 84.42% (Table 1), which did not differ much in oocytes activated in the presence of 5, 10 or 20 µg/ml of SDF (87.14, 85.48 and 77.78% respectively). The highest 2-cell rate (94.74%) was observed in the 50 µg/ml group. At 96 h after culture, 39.6% of the control parthenotes underwent compaction (Fig. 1*B*), which was marginally lower at the 5 µg/ml (30.5%) and significantly lower ($P < 0.01$) at the 10 µg/ml concentrations (21.2%). Further increase in SDF concentration to 20 and 50 µg/ml increased the compaction rate to 35.4 and 47.05% respectively. The remaining embryos either arrested at the 2-cell stage or at the 6–8-cell stage. Activation in the presence of 100 µg/ml of SDF resulted in embryonic arrest at the 6–8-cell stage. However, the blastocyst rate was found to be increased in a dose-dependent manner up to 50 µg/ml. The highest blastocyst rate (32%) was obtained in the 50 µg/ml group, which was

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

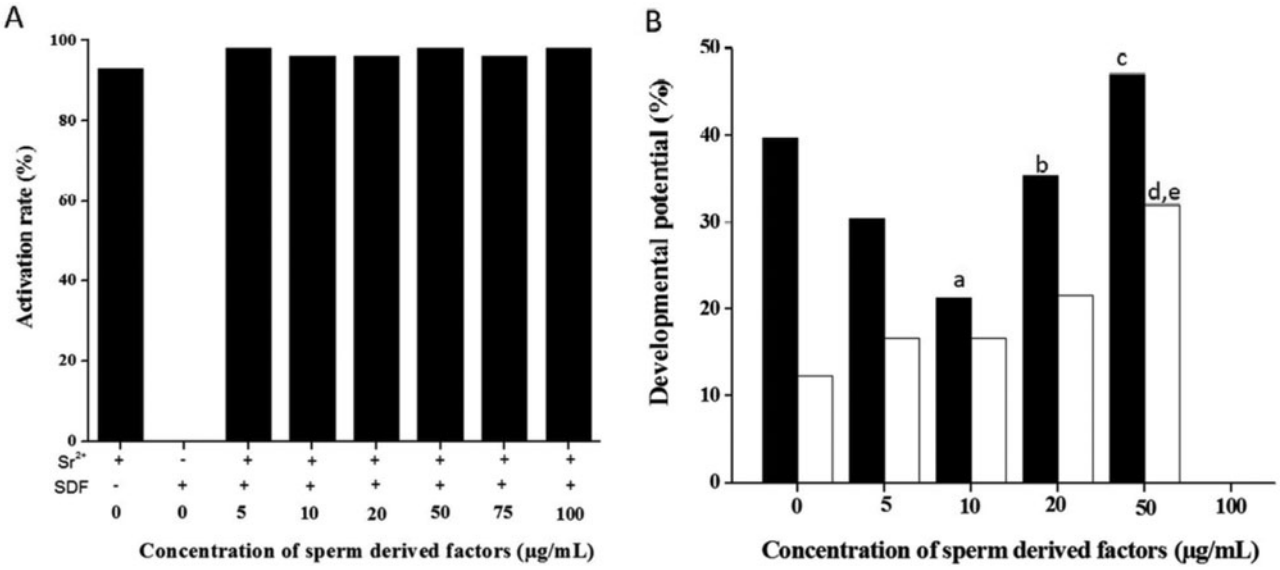


Figure 1 Activation potential and developmental potential of oocytes activated in the presence of sperm-derived factors. (*A*) Activation potential of mouse oocytes activated with Sr^{2+} in the presence of varying concentrations of sperm-derived factors ($n = 77$, 25, 70, 62, 63, 76, 57 and 45 in Sr^{2+} alone, SDF alone, 5, 10, 20, 50, 75, 100 µg/ml of SDF respectively in three trials). $\chi 2$ test was used for statistical analysis of the percentage of oocytes activated after supplementation with sperm-derived factors. (*B*) Developmental potential of parthenogenetic embryos activated in the presence of sperm-derived factors. 0 µg/ml of SDF corresponds to strontium alone (control). a $P < 0.01$ vs. C, b $P < 0.05$, c $P < 0.01$ vs. 10 µg/ml in morula rate; d $P < 0.01$ vs. C, e $P < 0.05$ vs. 5,10 µg/ml in blastocyst rate ($n = 77$, 25, 70, 62, 63, 57 and 45 in Sr^{2+} alone, SDF alone, 5, 10, 20, 50, 100 µg/ml of SDF in three trials). $\chi 2$ test was used for statistical analysis of the developmental potential of oocytes after activation in presence of sperm-derived factors ■ Morula rate; □ Blastocyst rate.

Table 1 2 cell rate of oocytes activated in presence and absence of SDF

Concentrations of SDF (µg/mL)	Total no. of MII oocytes	No. of oocytes developed to 2 cell embryos (%)	No. of fragmented and degenerated oocytes
0	77	65 (84.42)	12
5	70	61 (87.14)	9
10	62	53 (85.48)	9
20	63	49 (77.78)	14
50	57	54 (94.74)	3
100	45	39 (86.67)	6

significantly higher than that of the control (12.31%, $P < 0.01$).

Influence of SDF on formation of first cleavage axis

In oocytes activated with $SrCl_2$, at 18 h after activation, only 55.7% of the embryos had regular cleavage axis while the remaining embryos did not have clearly demarcated embryonic boundaries. However, these embryos developed to a 'proper' 2-cell stage by 24 to 36 h of culture suggesting delayed cleavage (Fig. 2). Interestingly, we observed that the 2-cell-stage embryos obtained after activation in the presence of SDF up to 20 µg/ml had a significantly increased number of embryos with a normal cleavage axis within 18 h of activation ($P < 0.0001$). Further increase in SDF concentration to 50 µg/ml decreased the percentage of embryos with a normal cleavage axis to 79.4%, although this percentage was still significantly higher than that of the control group ($P < 0.01$). At the highest SDF concentration, embryos with a normal cleavage axis at 18 h showed the lowest percentage compared with all other groups (14.2%, $P < 0.0001$).

Influence of SDF on cell cycle

To understand the influence of SDF on early embryonic development, further studies were performed with 50 µg/ml of SDF, which gave the maximum blastocyst rate. To understand whether the presence of SDF changed the cell cycle pattern, nuclear organization along with spindle dynamics was analysed at various

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

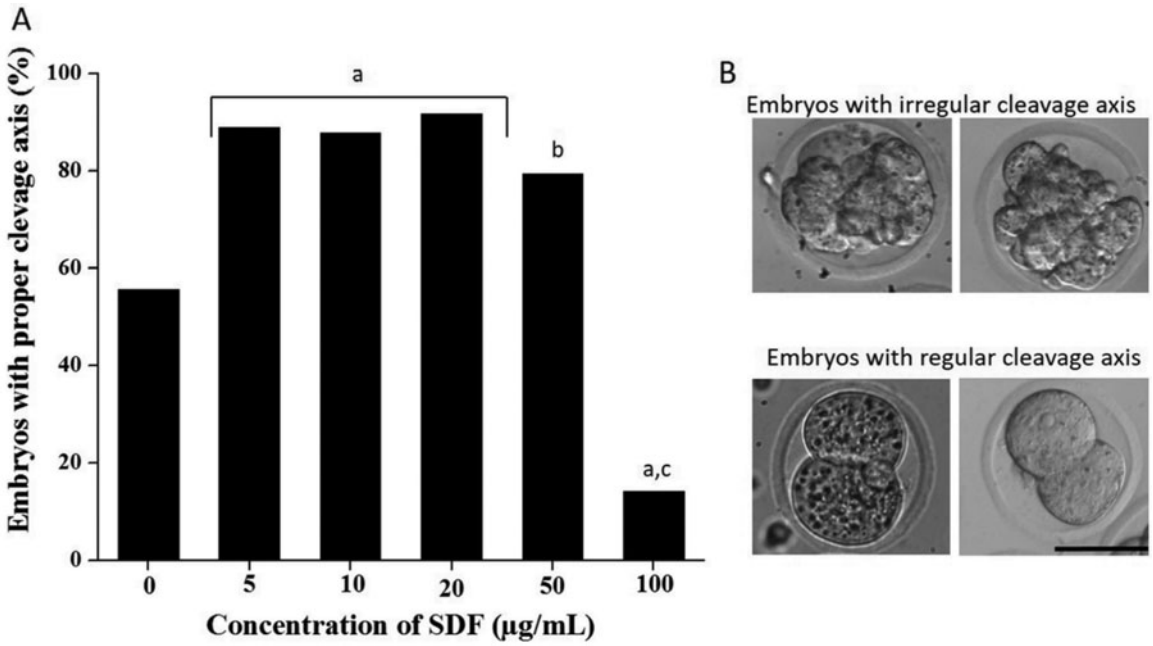


Figure 2 Embryos with proper cleavage axis after supplementation with sperm-derived factors. (*A*) Graph showing the percentage of embryos with proper embryonic axis. a $P < 0.0001$; b $P < 0.01$ vs. C, c $P < 0.0001$ vs. 5, 10, 20, 50 µg/ml ($n = 77$, 25, 70, 62, 63, 57 and 45 in Sr^{2+} alone, SDF alone, 5, 10, 20, 50, 100 µg/ml of SDF respectively in three trials). $\chi 2$ test was used for statistical analysis. (*B*) Images of embryos with irregular cleavage pattern at 18 h post activation (top panel), embryos with regular cleavage pattern at 18 h post activation (bottom panel). Bar represents 100 µm.

time intervals after activation. Oocytes activated in the presence or absence of SDF had all their activated oocytes at telophase at 0 h after activation with chromosomes moved to the spindle poles (Fig. 3). In the control group, at 3 h after oocyte activation, 64.30% of oocytes had their genome covered with a nuclear membrane and 21.40% of oocytes were in S phase. The remaining 14.30% were still in telophase. However, in oocytes activated in the presence of SDF, 50% of zygotes had crossed G1 phase and entered S phase at this interval. At 12 h, a shorter cell cycle was observed in oocytes activated with SDF group compared with the control group. In the SDF group, 68.75% were at early stages of cytokinesis marked by the appearance of a cleavage furrow, while in the control none of the activated oocytes had progressed to the 2-cell stage with 80% of these still in the G2M phase.

Cortical granule exocytosis

Cortical granule exocytosis was determined by an immunofluorescence method using LCA conjugated with rhodamine. Oocytes at the metaphase II (MII) stage served as the negative control, which had a uniform distribution of cortical granules (Fig. 4*Aa*), while the normally fertilized PN stage embryos which served as the positive control, had a cortical distribution pattern (94.12%) (Fig. 4*Ab*). MII oocytes incubated in the presence of SDF alone (without activating agent $SrCl_2$) also exhibited a uniform distribution of cortical granules indicating that SDF alone does not have any direct influence on cortical granule exocytosis (Fig. 4*Ac*). In artificially activated oocytes only 52% had cortical distribution immediately after activation (0 h) while 48% of oocytes had uniform distribution of cortical granules ($P < 0.0001$ vs. PN embryos). However, activation of oocytes in the presence of SDF increased the percentage of oocytes with cortical distribution (71.26%) at 0 h ($P < 0.0001$ vs. PN embryos), which did not change with increase in time (45.83 and 71.43% of oocytes with cortical distribution in oocyte activated in presence and absence of SDF) (Fig. 4*Ad*).

Sperm zona binding ability after oocyte activation

Sperm zona binding ability indicates the occurrence of zona reaction, which was assessed in oocytes activated in the presence or absence of SDF. The normally fertilized embryos a had significantly lower number of bound spermatozoa (04.79 ± 0.46) compared with MII oocytes (24.45 ± 01.56, $P < 0.001$, Fig. 4*Ba*). Artificially activated oocytes had significantly higher numbers of sperm bound to the zona (19.65 ± 01.26) when compared with PN stage embryos ($P < 0.001$, Fig. 4*Bb*)

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

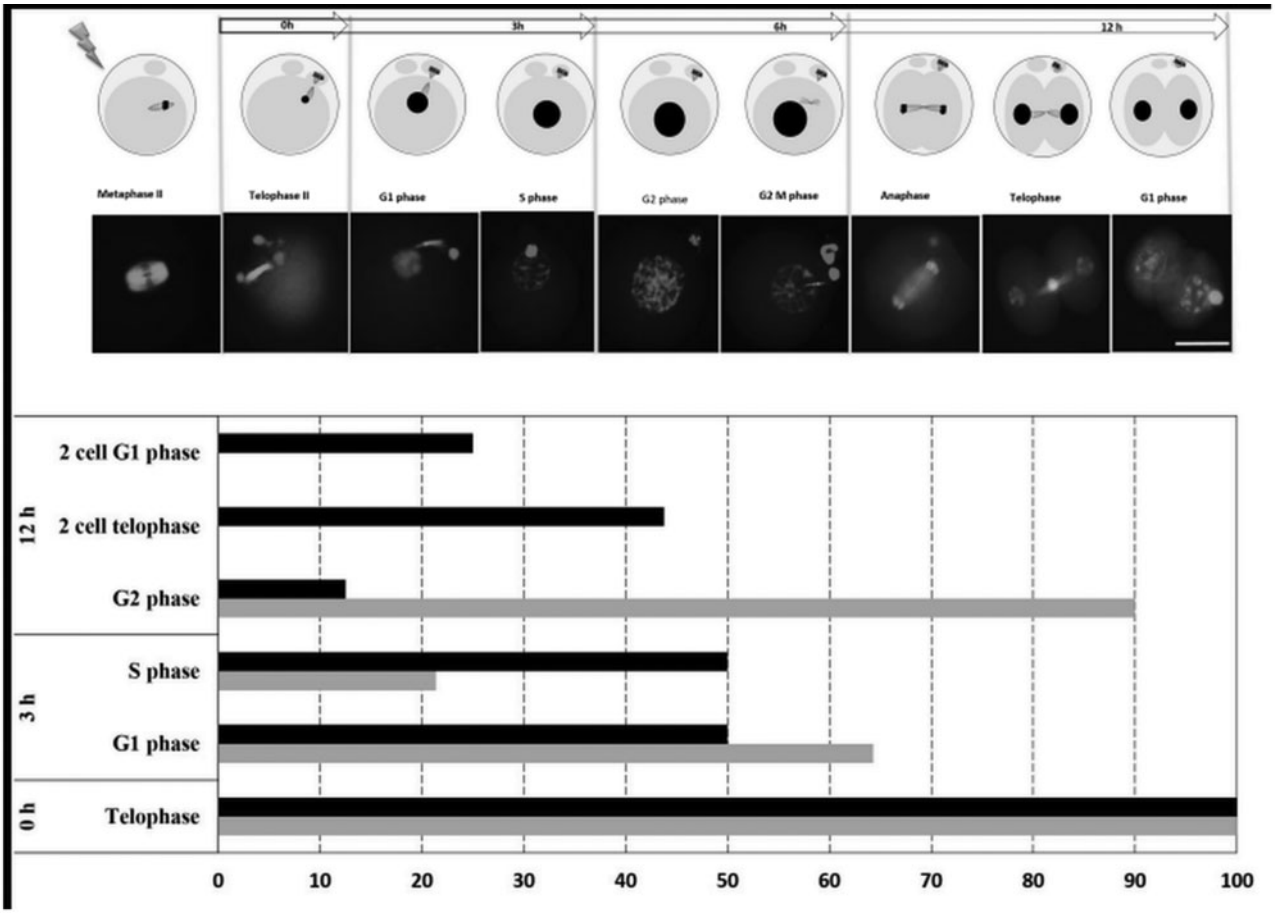


Figure 3 Cell cycle stages observed after each interval post artificial oocyte activation in oocytes. Representative images of spindle assembly and nuclear arrangement in oocytes at different cell cycle stages. The nucleus was stained with propidium iodide (red) and α-tubulin immunofluorescence detection was carried out for spindle assembly (green). Activated pronuclear embryos were fixed at specific intervals (0, 3, 6 and 12 h) and stained. The nuclear morphology and appearance of spindle is represented for each cell cycle point in the images. The images (12 h onwards) were pseudocolored with red. Bar represents 100 μm. Cell cycle stages observed in Sr^{2+} activated oocytes in the presence or absence of sperm-derived factors (50 μg/ml) at 0, 3 or 12 h post activation ($n = 15$ per group in all the intervals). Oocytes activated in the absence of SDF (control) and activated in the presence of SDF (50 μg/ml SDF) was assessed at different time intervals and the percentage of embryos in each stages of the cell cycle was plotted in the graph. Control; ▭ SDF; ■

and MII oocytes ($P < 0.05$). The presence of SDF during activation resulted in a decrease in the sperm zona binding ability (12.69 ± 0.71) when compared with the parthenogenetic control ($P < 0.01$, Fig. 4*Bc*), but was significantly higher than that of normally fertilized PN stage embryos ($P < 0.001$).

Mitochondrial distribution pattern in oocytes activated in presence of SDF

The mitochondrial distribution pattern was analysed at 0 and 3 h post activation. A uniform distribution of mitochondria was observed in approximately 65% of oocytes at 0 h (Fig. 5*Aa*). However, the presence of SDF during activation resulted in a significant decrease in the percentage of oocytes with centrally localized mitochondria ($P < 0.05$, Fig. 5*Ab*) while, in 16% of oocytes, aggregation of mitochondria was observed (Fig. 5*Ac*). Mitochondrial distribution pattern at 3 h post activation was unaffected with 85% oocytes having uniform distribution, while 10% had aggregated mitochondria. Oocytes activated with SDF had approximately 20% of oocytes with aggregated mitochondria.

Expression pattern of pluripotency gene oct4

The expression of pluripotency gene *Oct4* was analysed by quantification of immunofluorescence expression. *Oct4* expression was observed in both nucleus and cytoplasm and the intensity of fluorescence was found to be similar in normally fertilized and parthenogenetic

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

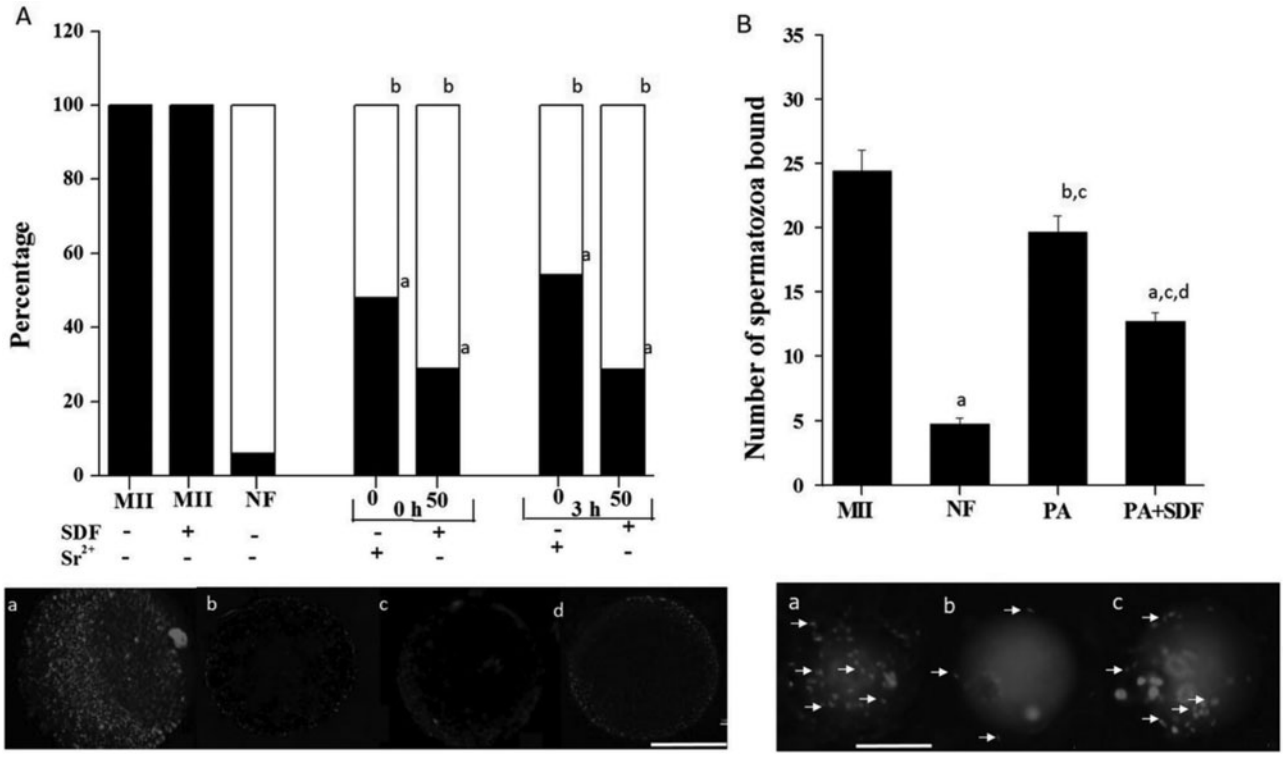


Figure 4 (*A*) Graph showing the percentage of oocytes with uniform and cortical distribution of cortical granules post activation with Sr^{2+} in the presence or absence of paternal factors. MII, MII oocytes; NF, normally fertilized pronuclear embryos; 0, oocytes activated with strontium in the absence of SDF; 50, oocytes activated in the presence of SDF (50 μg/ml). ■ Uniform; □ Cortical distribution. $N = 20$ per group in trials. χ2 test was used for statistical analysis. Representative images of cortical granule distribution in (a) MII oocyte; (b) PN stage embryo; (c) parthenotes; (d) parthenotes activated in the presence of SDF (50 μg/ml) by LCA immunofluorescence. Bar represents 100 μm. (*B*) Sperm bindability assay in oocytes activated in the presence or absence of SDF. MII, MII oocytes; NF, normally fertilized pronuclear embryos; PA, parthenogenetic embryos activated with strontium alone; PA+SDF, parthenogenetic embryos activated with strontium along with 50 μg/ml of SDF a $P < 0.001$, b $P < 0.05$ vs. MII, c $P < 0.001$ vs. PN, d vs. PA $N = 42$ for MII oocyte; PN for 62, parthenotes for 72, parthenotes with SDF for 26 in three trials. One-way ANOVA test was used for statistical analysis. Images represent the sperm zona binding pattern after staining with propidium iodide a MII oocytes, b PN embryos and c oocytes activated with strontium. The white arrow indicates the spermatozoa bound to zona. Bar represents 100 μm.

embryos after 24 h of activation (Fig. 5*B*). Parthenotes activated in the presence of sperm-derived factors also showed a similar expression pattern, indicating that SDF does not have any significant influence on *Oct4* expression.

Role of sperm-derived factors during embryonic development

To understand the role played by sperm-derived factors post activation, 2-cell stage parthenogenic embryos were cultured in M16 medium supplemented with varying concentrations of SDF. Embryos were scored at every 24 h and the blastocyst rate was calculated at 120 h of culture. Unlike during activation, the beneficial effect of SDF was observed at much lower concentrations (2.5 and 5.0 μg/ml), while 50 μg/ml concentration was found to be detrimental when supplemented in culture medium for the development of parthenotes. Parthenogenetic embryos cultured at lower SDF concentrations were found to have a marginally increased morula rate when compared with the control (39.60, 35.40, 49.18 and 52.20% in control, 1, 2.5 and 5 μg/ml groups respectively (Fig. 6). The control group (10 mM $SrCl_2$) showed a 12.31% blastocyst rate. While a dose-dependent increase was observed in parthenotes cultured at varying concentrations of SDF up to 5 μg/ml, parthenotes cultured at lower SDF concentrations (1 or 2.5 μg/ml) resulted in a blastocyst rate of 16.60 and 20.80%. A significantly increased ($P < 0.05$) blastocyst rate (26.80%) was obtained when parthenotes were cultured in M16 medium containing 5 μg/ml of SDF. However, at higher concentration (10 μg/ml), both morula rate

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

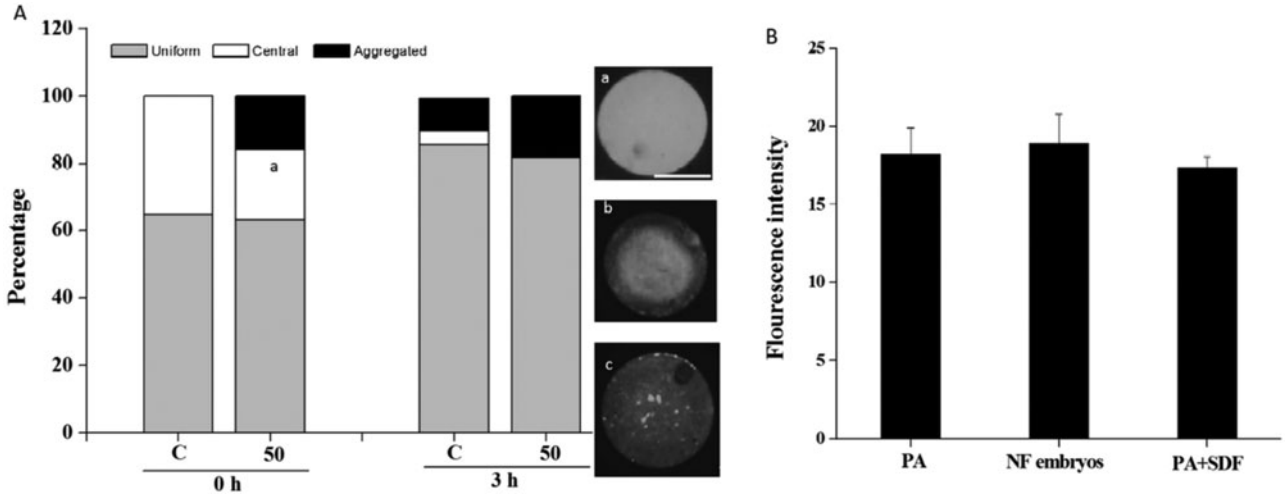


Figure 5 (*A*) Mitochondrial distribution pattern of oocytes activated in the presence or absence of SDF. C, oocytes activate with strontium alone; 50, oocytes activated with strontium along with 50 μg/ml of SDF. a $P < 0.05$ central distribution at 0 h. Uniform, Central, Aggregated distribution pattern of mitochondria. $N = 20$ per group in each of three trails. Images represent the distribution pattern of mitochondria post activation after staining with rhodamine 123. Representative images of a uniform, b central and c aggregated mitochondrial distribution patterns in oocytes are shown. Bar represents 100 μm. $\chi 2$ test was used for statistical analysis. (*B*) Oct 4 intensity in activated oocytes after 24 h of culture. PA, parthengenetic embryos activated with strontium alone; NF embryos, normally fertilized embryos; PA+SDF, parthenogenetic embryos activated with strontium along with 50 μg/ml of SDF. $N = 15$ per group in three biological replicates. One-way analysis of variance (ANOVA) was used for analysis of Oct 4 intensity.

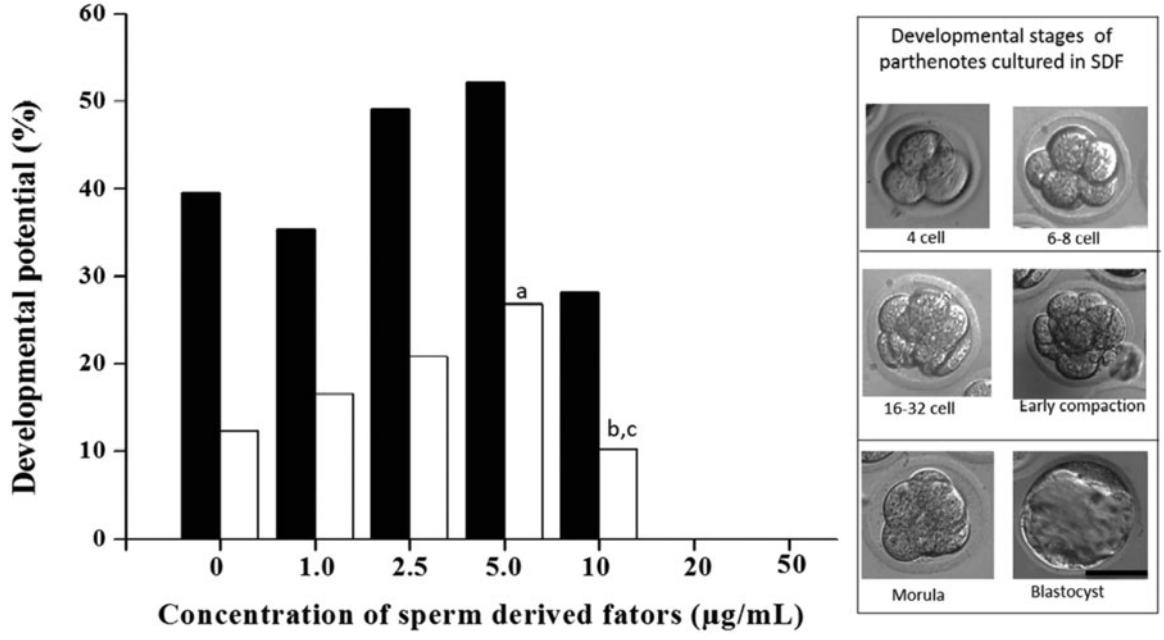


Figure 6 Developmental potential of embryos cultured in varying concentrations of SDF. a $P < 0.05$ vs. C, b $P < 0.05$ vs. 2.5, c $P < 0.01$ vs. 5 μg/ml SDF in blastocyst rate ($N = 45$ per group in three trials). $\chi 2$ test was used for statistical analysis. Images represent different cell stages of embryos during development. Bar represents 100 μm. Morula rate Blastocyst rate

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

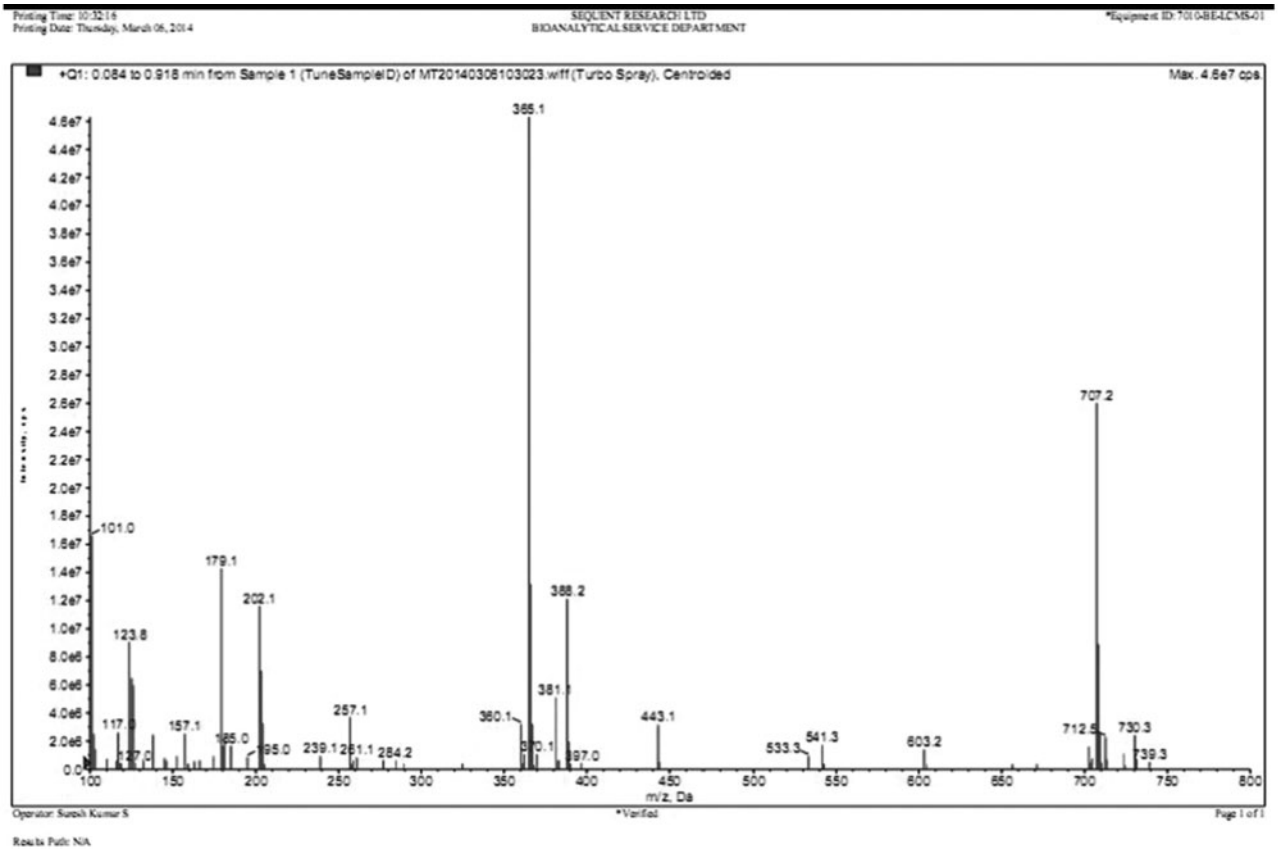


Figure 7 Mass spectra of sperm-derived factors.

and blastocyst rate were lower than that of the control (28.20 and 10.20% respectively). Furthermore, increasing the concentrations of SDF to 20 and 50 μg/ml resulted in failure of the embryos to undergo compaction.

Quantification and characterization of sperm-derived factors

Spectrophotometric analysis was carried out to estimate the amount of protein present in SDF. A standard graph was plotted with bovine serum albumin (BSA) (5 mg/ml) as the standard. The protein concentration of lyophilized product was 98 μg/ml. Further characterization of the obtained sperm-derived components was carried out by mass spectrophotometry. A representative mass spectrum of sperm-derived factor is shown in Fig. 7. The possible fragmentation products obtained indicated the presence of phosphatidylserine (385.30 k), phosphatidylethanolamine (744.03 k), phosphatidylcholine (768.00 k), sphingomyelin (740.867 k) and caveolin (25 k) in the lyophilized product. The analysis of mass spectrometric spectra revealed the presence of about 41 proteins, which were further classified based on their function (Table S1).

Discussion

In the present study, we demonstrated that the presence of SDF in the culture medium during artificial activation of oocytes can improve the developmental potential of haploid parthenotes without using any microinjection methods for the delivery of SDF into the oocyte. Previous studies have shown that haploid parthenotes are characterized by delayed embryonic development and decreased blastocyst rate (Kaufman & Sachs, 1975; Henery & Kaufman, 1992) which is also observed in the present study (Fig. 3). Beatty & Fischberg (1951) indicated that ploidy is one of the major factor that regulate the development of preimplantation embryos as both haploidy and polyploidy in mouse embryos can lead to a reduction in cell number. Similarly, Han *et al.* (1999), have reported the abnormal cleavage of polyploid embryos to three or more cells within 36 h of insemination in porcine embryos, while diploid embryos were still at the 2-cell stage.

The lack of paternal contribution in parthenotes is considered to cause poor *in vitro* development (Surani *et al.*, 1984; Solter, 1988). Along with the sperm nucleus, certain cytoplasmic factors are found to be released into the oocyte during fertilization.

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

Earlier studies have shown that the microinjection of SDF into oocyte results in calcium oscillations with a duration and amplitude similar to that observed during normal fertilization (Homa & Swann, 1994; Wu *et al.*, 1998) indicating that they have a significant role to play in fertilization. However, in these studies, SDF was microinjected into oocyte mainly focussing on understanding its effect on the intracellular calcium metabolism. None of these studies attempted to see whether the microinjection of SDF enhances the further development of activated oocytes. In this report, for the first time, we provide experimental evidence for the role of SDF in oocyte activation and early embryonic development of preimplantation embryos.

The presence of proteins that determines the fertility of spermatozoa has been observed in several species including human (Martınez-Heredia *et al.*, 2006; Domagala *et al.*, 2007; Secciani *et al.*, 2009), mouse (Stein *et al.*, 2006; Baker *et al.*, 2010; Guo *et al.*, 2010) and bovine species (Newton *et al.*, 2009; Saadi *et al.*, 2013). Mass spectrometric analysis in the present study revealed the presence of certain proteins that have been already reported to have role in fertility (Table S1).

Supplementation of SDF during oocyte activation was found to improve the development of haploid parthenotes. The presence of cytosolic sperm protein PLC zeta1 has been shown to activate the oocytes upon microinjection (Saunders *et al.*, 2002; Rogers *et al.*, 2004; Yoon *et al.*, 2012). In addition to this, microinjection of PLCζ mRNA was found to rescue oocytes with failed sperm oocyte activation in humans (Yoon *et al.*, 2008) and these embryos can progress up to the blastocyst stage (Saunders *et al.*, 2002; Yu *et al.*, 2008). However, we did not detect the presence of PLCζ in SDF in our study. But we observed the presence of proteins that are involved in the calcium signalling pathway such as tyrosine protein kinases SYK, solute carrier family 8 member 1 (Slc8a1), eukaryotic translation initiation factor 5A (Uorf1) and voltage gated calcium channels [calcium channel voltage dependent 1 type subunit (Cacnals), opioid receptor mu1] which might have helped in improving the developmental potential through their action on regulating the intracellular calcium level (Table S1). Further experiments are necessary to understand the exact mechanism of beneficial action by which these soluble factors from SDF induce the beneficial effects by entering into the oocyte or from outside.

To understand whether SDF involvement in fertilization and embryo development is stage specific, we cultured 2-cell-stage parthenotes in medium supplemented with SDF. The presence of SDF in culture medium during development was found to be beneficial, but was required at a much lower concentrations than that during oocyte activation. Following the first cleavage in mouse embryos, zygotic genome activation occurs, making it independent of parental genetic control. There are studies in the literature that indicate that paternal factors have a significant influence on the development of embryo up to blastocyst stage (Janny & Menezo, 1994; Liu *et al.*, 2012). SDF supplementation would have helped in regulation of the cell cycle of parthenogenetic embryos, as SDF contains few cell cycle regulators such as host cell factor C1 (HCFC1 protein) and tyrosine protein kinase Fe.

The paternal genome is reported to influence the first cell cycle in mouse (Renard & Babinet, 1986; Schabronath & Gärtner, 1988), in bovine (Eid *et al.*, 1994), as well as in humans (Tesarik *et al.*, 2002). We used nuclear and spindle dynamics to identify the stage of embryonic cell cycle. By 6 h post activation, parthenotes in the control group were found to enter M phase, which is evident from the formation of nascent spindle fibres. Normally fertilized embryos at the PN stage are reported to have a functional cell cycle check point at M phase (Fulka *et al.*, 1999). Oocytes activated in the presence of SDF were found to have decreased duration of G2M phase, as ~70% of embryos had already completed cytokinesis and progressed to the 2-cell stage by 12 h of activation, while oocytes activated in the absence of SDF were found to reach the 2-cell stage only by 24 h (data not given). Eukaryotic initiation factor 4E (Eif4Ebp1) present in SDF has been shown to play a significant role in regulation of G1S transition (Fingar *et al.*, 2004).

Cytoplasmic streaming along with microtubule polymerization is also found to play an important role in exocytosis of cortical granules immediately after sperm entry into oocytes (Kim *et al.*, 1996). There are contradictory reports on the stimulus of cortical granule exocytosis. Horvath *et al.* (1993) reported that sperm act as the stimuli, while other studies have indicated that repetitive calcium oscillation triggers cortical granule exocytosis (Kline & Kline, 1992; Abbott & Ducibella, 2001). The increase in the percentage of oocytes with cortical granule exocytosis after activation in the presence of SDF observed in this study could be due to the ability of SDF to increase amplitude and duration of calcium oscillations during artificial activation. However, this aspect needs to be studied further.

The number of sperm bound to parthenotes in the presence of SDF was also found to be significantly lower, indicating the occurrence of a zona reaction. However, it was not efficient as the sperm-induced zona reaction as observed in pronuclear embryos (Fig. 5*B*). Tatone *et al.* (1994) have reported the occurrence of a transient phenomenon of sperm block within 30 min of artificial oocyte activation, unlike a permanent sperm-induced block in normally fertilized zygotes. The interval assessed in the present study is 3 h after the addition of strontium, by which time the

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

block to further binding of the spermatozoa must have been reduced.

Apart from the effect of SDF on oocyte activation and early events of fertilization, we were interested to understand the cytoplasmic quality of the embryos developed. Mitochondria, which is the major cytoplasmic organelle and an indicator of developmental potential of embryos (Acton *et al.*, 2004; Schatten *et al.*, 2014) were analysed in oocytes activated in the presence or absence of SDF. SDF supplementation during oocyte activation however did not alter the distribution of mitochondria until after 3 h of activation. The uniform distribution of mitochondria was observed in artificially activated oocytes both in the presence or absence of SDF and was similar to that of earlier reported studies (Katayama *et al.*, 2006). The aggregation that was observed post activation could be due to reorganisation of the oocyte cortex during cortical granule exocytosis after fertilization, as indicated by Katayama *et al.* (2006).

The expression of the pluripotency gene (*POUF51*), which regulates early embryogenesis was also analysed to understand whether the presence of SDF during activation had any influence on the expression of pluripotency gene. Quantification of immunofluorescent signals indicated that, at early intervals of fertilization, embryo quality is unaffected even after SDF supplementation in parthenotes. Oct 4 is a maternal transcript protein and is usually found to be downregulated after the 2-cell stage in the mouse (Palmieri *et al.*, 1994; Wu & Schöler, 2014) for proper embryonic development (Zuccotti *et al.*, 2011).

In conclusion, this is the first study that gives experimental evidence for the role of SDF during oocyte activation and early embryonic development. As the supplementation of these factors can improve the blastocyst rate in haploid parthenotes it can have a beneficial role in derivation of haploid stem cells for experimental and therapeutic purposes. We used haploid parthenotes to understand the beneficial role of sperm-derived factors on development. Further studies are necessary to understand whether diploid parthenotes will show a similar response, whether the components present in soluble SDF are able to enter the ooplasm without any microinjection method and how they improve the cleavage pattern in parthenotes.

Acknowledgement

Authors thankfully acknowledge the financial support from the Department of Health Research (DHR), Indian Council of Medical Research (ICMR), Government of India (DHR/HRD/Women scientist/Type-V/8/2014–15) and Department of Biotechnology (DBT), Government of India (BT/PR15130/GBD/27/328/2011).

Author contributions

RN and SA performed the experiments; GK designed the study and wrote the manuscript; SKA and SM helped in statistical analysis and writing the manuscript.

Supporting Information

Additional Supporting Information is available online at the publisher's website.

Table S1

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199417000569

References

Abbott, A.L. & Ducibella, T. (2001). Calcium and the control of mammalian cortical granule exocytosis. *Front. Biosci.* **6**, 792–806.

Acton, B.M., Jurisicova, A., Jurisica, I. & Casper, R.F. (2004). Alterations in mitochondrial membrane potential during preimplantation stages of mouse and human embryo development. *Mol. Hum. Reprod.* **10**, 23–32.

Aarabi, M., Balakier, H., Bashar, S., Moskovtsev, S.I., Sutovsky, P., Librach, C.L. & Oko, R. (2014). Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *FASEB J.* **28**, 4434–40.

Baker, M.A., Reeves, G., Hetherington, L. & Aitken, R.J. (2010). Analysis of proteomic changes associated with sperm capacitation through the combined use of IPG-strip pre-fractionation followed by RP chromatography LC-MS/MS analysis. *Proteomics* **10**, 482–95.

Beatty, R.A. & Fischberg, M. (1951). Cell number in haploid, diploid and polyploid mouse embryos. *J. Exp. Biol.* **28**, 541–52.

de Paola, M., Bello, O.D. & Michaut, M.A. (2015). Cortical granule exocytosis is mediated by alpha-SNAP and N-ethylmaleimide sensitive factor in mouse oocytes. *PLoS One* **10**, p.e0135679.

Domagała, A., Pulido, S., Kurpisz, M. & Herr, J.C. (2007). Application of proteomic methods for identification of sperm immunogenic antigens. *Mol. Hum. Reprod.* **13**, 437–44.

Eid, L.N., Lorton, S.P. & Parrish, J.J. (1994). Paternal influence on S-phase in the first cell cycle of the bovine embryo. *Biol. Reprod.* **56**, 1232–7.

Fang, P., Zeng, P., Wang, Z., Liu, M., Xu, W., Dai, J., Zhao, X., Zhang, D., Liang, D., Chen, X. & Shi, S. (2014). Estimated diversity of messenger RNAs in each murine spermatozoa and their potential function during early zygotic development. *Biol. Reprod.* **90**, 94.

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

Fingar, D.C., Richardson, C.J., Tee, A.R., Cheatham, L., Tsou, C. & Blenis, J. (2004). mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. *Mol. Cell. Biol.* **24**, 200–16.

Fulka, J., First, N.L., Fulka, J. & Moor, R.M. (1999). Checkpoint control of the G2/M phase transition during the first mitotic cycle in mammalian eggs. *Hum. Reprod.* **14**, 1582–7.

Guo, X., Shen, J., Xia, Z., Zhang, R., Zhang, P., Zhao, C., Xing, J., Chen, L., Chen, W., Lin, M. & Huo, R. (2010). Proteomic analysis of proteins involved in spermiogenesis in mouse. *J. Proteome Res.* **9**, 1246–56.

Han, Y.M., Abeydeera, L.R., Kim, J.H., Moon, H.B., Cabot, R.A., Day, B.N. & Prather, R.S. (1999). Growth retardation of inner cell mass cells in polyspermic porcine embryos produced *in vitro*. *Biol. Reprod.* **60**, 1110–3.

Hao, Y.H., Lai, L.X., Liu, Z.H., Im, G.S., Wax, D., Samuel, M., Murphy, C.N., Sutovsky, P. & Prather, R.S. (2006). Developmental competence of porcine parthenogenetic embryos relative to embryonic chromosomal abnormalities. *Mol. Reprod. Dev.* **73**, 77–82.

Henery, C.C. & Kaufman, M.H. (1992). Cleavage rate of haploid and diploid parthenogenetic mouse embryos during the preimplantation period. *Mol. Reprod. Dev.* **31**, 258–63.

Homa, S.T. & Swann, K. (1994). Fertilization and early embryology: A cytosolic sperm factor triggers calcium oscillations and membrane hyperpolarizations in human oocytes. *Hum. Reprod.* **9**, 2356–61.

Horvath, P.M., Kellom, T., Caulfield, J. & Boldt, J. (1993). Mechanistic studies of the plasma membrane block to polyspermy in mouse eggs. *Mol. Reprod. Dev.* **34**, 65–72.

Isom, S.C., Whitworth, K.M. & Prather, R.S. (2012). Timing of first embryonic cleavage is a positive indicator of the in vitro developmental potential of porcine embryos-derived from *in vitro* fertilization, somatic cell nuclear transfer and parthenogenesis. *Mol. Reprod. Dev.* **79**,197–207.

Janny, L. & Menezo, Y.J. (1994). Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. *Mol. Reprod. Dev.* **38**, 36–42.

Jeong, Y.J., Cui, X.S., Kim, B.K., Kim, I.H., Kim, T., Chung, Y.B. & Kim, N.H. (2005). Haploidy influences Bak and Bcl-xL mRNA expression and increases incidence of apoptosis in porcine embryos. *Zygote* **13**, 17–21.

Kalthur, G., Salian, S.R., Nair, R., Mathew, J., Adiga, S.K., Kalthur, S.G., Zeegers, D. & Hande, M.P. (2016). Distribution pattern of cytoplasmic organelles, spindle integrity, oxidative stress, octamer-binding transcription factor 4 (Oct4) expression and developmental potential of oocytes following multiple superovulation. *Reprod. Fertil. Dev.* **28**, 2027–38.

Katayama, M., Zhong, Z., Lai, L., Sutovsky, P., Prather, R.S. & Schatten, H. (2006). Mitochondrial distribution and microtubule organization in fertilized and cloned porcine embryos: implications for developmental potential. *Dev. Biol.* **299**, 206–20.

Kaufman, M.H. & Sachs, L. (1975). The early development of haploid and aneuploid parthenogenetic embryos. *Development* **34**, 645–55.

Kiani, J. & Rassoulzadegan, M. (2013). A load of small RNAs in the sperm—how many bits of hereditary information? *Cell. Res.* **23**, 18–9.

Kim, N.H., Day, B.N., Lee, H.T. & Chung, K.S. (1996). Microfilament assembly and cortical granule distribution during maturation, parthenogenetic activation and fertilisation in the porcine oocyte. *Zygote* **4**, 145–9.

Kline, D. & Kline, J.T. (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**, 80–9.

Krawetz, S.A., Kruger, A., Lalancette, C., Tagett, R., Anton, E., Draghici, S. & Diamond, M.P. (2011). A survey of small RNAs in human sperm. *Hum. Reprod.* **26**, 3401–12.

Kuretake, S., Kimura, Y., Hoshi, K. & Yanagimachi, R. (1996). Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biol. Reprod.* **55**, 789–95.

Li, L., Zheng, P. & Dean, J. (2010). Maternal control of early mouse development. *Development* **137**, 859–70.

Liu, L., Trimarchi, J.R. & Keefe, D.L. (2002). Haploidy but not parthenogenetic activation leads to increased incidence of apoptosis in mouse embryos. *Biol. Reprod.* **66**, 204–10.

Liu, W.M., Pang, R.T., Chiu, P.C., Wong, B.P., Lao, K., Lee, K.F. & Yeung, W.S. (2012). Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proc. Natl. Acad. Sci. USA* **109**, 490–4.

Lomeo, A.M. & Giambersio, A.M. (1991). 'Water-test': a simple method to assess sperm-membrane integrity. *Int. J. Androl.* **14**, 278–82.

Martínez-Heredia, J., Estanyol, J.M., Ballescà, J.L. & Oliva, R. (2006). Proteomic identification of human sperm proteins. *Proteomics* **6**, 4356–69.

McGrath, J. & Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179–83.

Nair, R., Singh, V.J., Salian, S.R., Kalthur, S.G., D'Souza, A.S., Shetty, P.K., Mutalik, S., Kalthur, G. & Adiga, S.K. (2014). Methyl parathion inhibits the nuclear maturation, decreases the cytoplasmic quality in oocytes and alters the developmental potential of embryos of Swiss albino mice. *Toxicol. Appl. Pharmacol.* **279**, 338–50.

Newton, L.D., Kastelic, J.P., Wong, B., Van der Hoorn, F. & Thundathil, J. (2009). Elevated testicular temperature modulates expression patterns of sperm proteins in Holstein bulls. *Mol. Reprod. Dev.* **76**, 109–18.

Oko, R. & Sutovsky, P. (2009). Biogenesis of sperm perinuclear theca and its role in sperm functional competence and fertilization. *J. Reprod. Immunol.* **83**, 2–7.

Palmieri, S.L., Peter, W., Hess, H. & Schöler, H.R. (1994). Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev. Biol.* **166**, 259–67.

Park, S.J., Komata, M., Inoue, F., Yamada, K., Nakai, K., Ohsugi, M. & Shirahige, K. (2013). Inferring the choreography of parental genomes during fertilization from ultralarge-scale whole-transcriptome analysis. *Genes. Dev.* **27**, 2736–48.

Parrington, J., Swann, K., Shevchenko, V.I., Sesay, A.K. & Lai, F.A. (1996). Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. *Nature* **379**, 364–8.

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press

Perry, A.C., Wakayama, T., Cooke, I.M. & Yanagimachi, R. (2000). Mammalian oocyte activation by the synergistic action of discrete sperm head components: induction of calcium transients and involvement of proteolysis. *Dev. Biol.* **217**, 386–93.

Renard, J.P. & Babinet, C.H. (1986). Identification of a paternal developmental effect on the cytoplasm of one-cell-stage mouse embryos. *Proc. Natl. Acad. Sci. USA* **83**, 6883–6.

Rogers, N.T., Hobson, E., Pickering, S., Lai, F.A., Braude, P. & Swann, K. (2004). Phospholipase Cζ causes Ca2+ oscillations and parthenogenetic activation of human oocytes. *Reproduction* **128**, 697–702.

Saadi, H.A.S., van Riemsdijk, E., Dance, A.L., Rajamanickam, G.D., Kastelic, J.P. & Thundathil, J.C. (2013). Proteins associated with critical sperm functions and sperm head shape are differentially expressed in morphologically abnormal bovine sperm induced by scrotal insulation. *J. Proteomics* **82**, 64–80.

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K. & Lai, F.A. (2002). PLCζ: a sperm-specific trigger of Ca2+ oscillations in eggs and embryo development. *Development* **129**, 3533–44.

Schabronath, J. & Gärtner, K. (1988). Paternal influence on timing of pronuclear DNA synthesis in naturally ovulated and fertilized mouse eggs. *Biol. Reprod.* **38**, 744–9.

Schatten, H. & Sun, Q.Y. (2009). The role of centrosomes in mammalian fertilization and its significance for ICSI. *Mol. Hum. Reprod.* **15**, 531–8.

Schatten, H., Sun, Q.Y. & Prather, R. (2014). The impact of mitochondrial function/dysfunction on IVF and new treatment possibilities for infertility. *Reprod. Biol. Endocrinol.* **12**, 1.

Secciani, F., Bianchi, L., Ermini, L., Cianti, R., Armini, A., La Sala, G.B., Focarelli, R., Bini, L. & Rosati, F. (2009). Protein profile of capacitated versus ejaculated human sperm. *J. Proteome Res.* **8**, 3377–89.

Sendler, E., Johnson, G.D., Mao, S., Goodrich, R.J., Diamond, M.P., Hauser, R. & Krawetz, S.A. (2013). Stability, delivery and functions of human sperm RNAs at fertilization. *Nucleic Acids Res.* **41**, 4104–17.

Sette, C., Bevilacqua, A., Bianchini, A., Mangia, F., Geremia, R. & Rossi, P. (1997). Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa. *Development* **124**, 2267–74.

Solter, D. (1988). Differential imprinting and expression of maternal and paternal genomes. *Annu. Rev. Genet.* **22**, 127–46.

Stein, K.K., Go, J.C., Lane, W.S., Primakoff, P. & Myles, D.G. (2006). Proteomic analysis of sperm regions that mediate sperm-egg interactions. *Proteomics* **6**, 3533–43.

Surani, M.A.H., Barton, S.C. & Norris, M.L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* **308**, 548–50.

Swann, K.A.R.L. (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development* **110**, 1295–302.

Swann, K., Larman, M.G., Saunders, C.M. & Lai, F.A. (2004). The cytosolic sperm factor that triggers Ca^{2+} oscillations and egg activation in mammals is a novel phospholipase C: PLCζ. *Reproduction* **127**, 431–9.

Tang, T.S., Dong, J.B., Huang, X.Y. & Sun, F.Z. (2000). Ca (2+) oscillations induced by a cytosolic sperm protein factor are mediated by a maternal machinery that functions only once in mammalian eggs. *Development* **127**, 1141–50.

Tatone, C., Van Eekelen, C.G. & Colonna, R. (1994). Plasma membrane block to sperm entry occurs in mouse eggs upon parthenogenetic activation. *Mol. Reprod. Dev.* **38**, 200–8.

Tesarik, J., Mendoza, C. & Greco, E. (2002). Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. *Hum. Reprod.* **17**, 184–9.

Wu, H., He, C.L. & Fissore, R.A. (1998). Injection of a porcine sperm factor induces activation of mouse eggs. *Mol. Reprod. Dev.* **49**, 37–47.

Wu, H., Smyth, J., Luzzi, V., Fukami, K., Takenawa, T., Black, S.L., Allbritton, N.L. & Fissore, R.A. (2001). Sperm factor induces intracellular free calcium oscillations by stimulating the phosphoinositide pathway. *Biol. Reprod.* **64**, 1338–49.

Wu, A.T., Sutovsky, P., Manandhar, G., Xu, W., Katayama, M., Day, B.N., Park, K.W., Yi, Y.J., Xi, Y.W., Prather, R.S. & Oko, R. (2007). PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J. Biol. Chem.* **282**, 12164–75.

Wu, G. & Schöler, H.R. 2014. Role of Oct4 in the early embryo development. *Cell Regen.* **3**, 1.

Yoon, S.Y., Jellerette, T., Salicioni, A.M., Lee, H.C., Yoo, M.S., Coward, K., Parrington, J., Grow, D., Cibelli, J.B., Visconti, P.E., Mager, J. & Fissore, R.A. (2008). Human sperm devoid of PLC, zeta 1 fail to induce Ca^{2+} release and are unable to initiate the first step of embryo development. *J Clin. Invest.* **118**, 3671–81.

Yoon, S.Y., Eum, J.H., Lee, J.E., Lee, H.C., Kim, Y.S., Han, J.E., Won, H.J., Park, S.H., Shim, S.H., Lee, W.S. & Fissore, R.A. (2012). Recombinant human phospholipase C zeta 1 induces intracellular calcium oscillations and oocyte activation in mouse and human oocytes. *Hum. Reprod.* **27**, 1768–80.

Yu, Y., Saunders, C.M., Lai, F.A. & Swann, K. (2008). Preimplantation development of mouse oocytes activated by different levels of human phospholipase C zeta. *Hum. Reprod.* **23**, 365–73.

Zuccotti, M., Merico, V., Bellone, M., Mulas, F., Sacchi, L., Rebuzzini, P., Prigione, A., Redi, C.A., Bellazzi, R., Adjaye, J. & Garagna, S. (2011). Gatekeeper of pluripotency: a common Oct4 transcriptional network operates in mouse eggs and embryonic stem cells. *BMC Genomics* **12**, 345.

https://doi.org/10.1017/S0967199417000569 Published online by Cambridge University Press