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Artificial Activation of Mouse Oocytes with $SrCl_2$ with Minimal Detrimental Effect on Early Embryonic Development

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Authors' Contribution
AR and AS supervised and designed the experiments. AMA ans MAJ performed the experiments and compiled the data. AG and AJ executed statistical analysis and wrote the manuscript.

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Embryonic development, Mice, Parthenogenesis, Strontium chloride, Toxicity

ABSTRACT

Zygote, the first step in the life establishment, is sensitive to environmental impact. Any detrimental effect to this unit may lead to major abnormalities in future development. Artificial activation of oocyte is a one of key step in cloning. Strontium chloride ($SrCl_2$), a calcium oscillation inducing salt, is commonly used for the artificial activation mouse oocytes; however, limited reports are available to evaluate its negative impact. The objective of this study was to optimize the concentration of $SrCl_2$ for artificial activation of mouse oocytes and to evaluate its toxic effect on early embryonic development. Our results indicated that higher percentage of activation of mouse oocyte was achieved with 10mM and 15mM for period of 3 h and 6 h compared to the control group ($P<0.05$). To examine the toxic effect, 10mM $SrCl_2$ with 3 h exposure showed minimal detrimental effect on embryo development. Collectively, our findings revealed that 10mM concentration of $SrCl_2$ for 3 h exposure was appropriate for oocyte activation with the least toxic impact for embryonic development.

INTRODUCTION

Artificial activation is a developmental event that occurs without paternal contribution (Ramachandran *et al*., 2018). This is a key step for activation of reconstructed oocytes/embryos in the animal cloning program (Kishigami *et al*., 2006). Previously it was demonstrated that embryonic stem cell have been derived from the parthenotes (Varrault *et al*., 2018). These embryonic stem cells are equally competent to fertilized embryonic stem cell in terms of pluripotency and proliferation (Ju *et al*., 2008). Artificial activation provides useful information to understand the mechanism of fertilization and the contribution of paternal genome for the early embryonic development. It is complex process involving series of events that are essential for oocytes activation and early embryonic development. One of the most notable events is an increase of intracellular Ca^{2+} ions (Miyazaki *et al*., 1993; Lee *et al*., 2016) that causes activation of protein kinase C and reduction of the maturation promoting factor (Galis *et al*., 2020). Finally, meiotic resumption occurs resulting in activation of oocyte similar to the natural fertilization process (Jones *et al*., 1995; Sepulveda-Rincon *et al*., 2016).

The artificial activation of oocytes can be performed either by physical or chemical methods. The physical activation methods include electric stimulations (Mitalipov *et al*., 1999) or temperature variations (Stice *et al*., 1994) whereas in chemical method different chemical reagents are used for oocytes activation with different time frame. In the mouse model, strontium chloride ($SrCl_2$) has been used successfully for oocyte activation as it induces repetitive Ca^{2+} oscillations similar to those that occur during normal fertilization. Thus this method is assumed to resemble the natural fertilization even if it does not completely mimic the oocyte activation by sperm (Kishigami *et al*., 2007; Norozi-Hafshejani *et al*., 2018).

Although oocyte activation with $SrCl_2$ is achieved but very limited reports are available regarding the toxicological properties of $SrCl_2$ in living tissues. The objective of present study was to minimize the embryonic exposure to $SrCl_2$

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for an optimum activation process. Furthermore, during animal cloning, the addition of a cell permeable mycotoxin, cytochalasin B (CB), is also an integral part of activation protocol. CB prevents release of 2nd polar body by inhibiting the polymerization process and disrupting the actin filaments (Modlinski, 1980; Hou *et al*., 2016). This inhibition is important to maintain the diploid nature of oocyte during the parthenogenesis process. It is reported that, the diploid oocytes have more developmental competence as compared to haploid form (Liu *et al*., 2002; Ma *et al*., 2017). After optimizing the minimum exposure of $SrCl_2$ for activation process the minimum exposure time sufficient to maintain the diploid nature of chromosomes and effect of extra exposure timing with CB were also investigated. The objective was to minimize the embryonic exposure to $SrCl_2$ and CB for optimum activation.

MATERIALS AND METHODS

BALB/c female mice (7-9 weeks old) were used as experimental animals. All mice were housed on 12 h dark/ 12 h light cycle and all the experimental protocols were performed accordance to the guidelines of Ethical Committee for use of laboratory animals at University of Veterinary and Animal Sciences.

Female BALB/c mice, were super stimulated as reported previously (Jamal *et al*., 2017). Briefly, equine chorionic gonadotropin (eCG 5.0 IU, Choronogest, Intervet) followed by 5 IU of human chorionic gonadotropin (hCG 5.0, IVF-C, LG Life Sciences) 48 h apart were injected intraperitoneally. Females were sacrificed by cervical dislocation and oocytes were collected by euthanizing females 13 h post hCG. Cumulus cells were released by treating with 300 µg/mL hyaluronidase (Sigma, H4272) in M2 media (Sigma, M7167) with gentle pipetting for 60s.

For zygote collection, super-ovulated females were exposed to breeding males and mating was confirmed by the formation of vaginal plug. Plug positive females were euthanized 18 h post hCG and zygotes were collected by puncturing ampulla in M2 medium (Sigma, M7167). The oocytes and zygotes were incubated at 37ºC for 30 min prior to further treatment.

Experiment 1 was conducted to optimize the treatment of $SrCl_2$ for oocyte activation. Oocytes were exposed to 5, 10 and 15 mM concentrations of $SrCl_2$ for 3, 6 and 9 h in Ca^{+2} free CZB medium. The medium was supplemented with cytochalasin B (CB; 5µg.mL) to maintain the diploid nature of developing embryos. All incubations were performed at 37 ºC and in 5% CO_2.

Experiment 2, was conducted to evaluate the toxic impact of $SrCl_2$ on normal embryonic development For this purpose, In-vivo fertilized zygotes were exposed with optimal treatments of experiment 1.

Experiment 3 was conducted to evaluate, whether the CB exposure time during experiment 1 (optimal treatment, 10 mM, 3 h) is sufficient for activating oocytes/embryos. Oocytes were exposed to CB (5µg. mL) for 0, 3 and 6 h intervals.

Activated oocytes were washed twice and incubated in CZB culture media at 37 ºC with 5% CO_2 until evaluated for developmental potential. The oocyte death rate and cleavage were observed at 1, 24 h after activation. Whilst, morula and blastocyst were observed 72 and 96 h post activation.

The statistical analyses were performed using the statistical software SPSS-17 version and statistical level was set at $P<0.05$. Binary logistic regression was used for analysis of the rate of cleavage, morula and blastocyst. Odds ratio was calculated for these parameters.

RESULTS

To determine the role of SrCl2 in artificial activation, oocytes were exposed with $SrCl_2$ (5, 10, 15 mM) for three different-time intervals (3, 6, 9 h). The data revealed that 10 and 15 mM concentrations of $SrCl_2$ for 3 and 6 h durations have more tendency for artificial activation of oocytes ($P<0.05$) (Fig. 1). The highest tendency (odd ratio; OD) for activation was calculated for 10 mM concentration for 3 h duration (OD = 2.94). To further determine the developmental fate of these artificially activated oocytes, the oocytes were cultured in M16 medium and early embryonic developmental potential was examined. The result indicated higher proportions of blastocyst formation at 10 mM SrCl2 for 3 h exposure relative to the other concentrations ($P>0.05$) (Fig. 1). When the negative impact of activation process in terms of dead oocytes was calculated, surprisingly it was observed that the lowest concentration 5 mM have a higher dead oocytes rate. However, 15 mM concentration of $SrCl_2$ for long duration exposure (9 h) also has the higher dead oocyte percentage ($P>0.05$) (Table I). So, 10 mM $SrCl_2$ concentration for 3 h durations was concluded as the optimum for mouse oocyte activation.

The data regarding the toxicological impact of $SrCl_2$ on fertilized zygotes showed that $SrCl_2$ did not affect the overall embryos developmental potential ($p>0.05$). However, the tendency at cleavage stage is more affected (1 vs 0.64) as compared to further developmental stages (morula blastocyst) (Table II).

The data regarding Cytochalasin-B (CB) treatment indicated that CB treatment significantly improved the activation potential of oocytes. The oocytes activated without CB has decreased cleavage rate and failed to develop to blastocyst as compared to other groups (Table III).

Table I. Effect of Strontium concentration and exposure timings on artificial activation and development of mouse oocytes.

Concentration	5mM			10 mM			15mM		
Timings (h)	**3**	**6**	**9**	**3**	**6**	**9**	**3**	**6**	**9**
Treated oocytes (n)	84	91	55	108	105	63	88	91	51
Dead oocytes	17%	16%	16%	9%	10%	13%	11%	12%	18%
Odd ratio	1	0.99	0.98	0.51	0.58	0.72	0.64	0.68	1.11
Cleavage	48%	50%	48%	*72%	*67%	56%	*69%	*69%	59%
Odd ratio	1	1.12	1.03	2.94	2.28	1.45	2.51	2.37	1.49
Morula	36%	42%	27%	50%	49%	25%	48%	47%	29%
Odd ratio	1	1.27	0.65	1.80	1.69	0.61	1.51	1.57	0.72
Blastocyst	21%	26%	13%	34%	32%	13%	31%	32%	17%
Odd ratio	1	1.33	1.39	1.89	1.73	1.29	1.62	1.81	0.74

*represents the significance value (P<0.05) with respect to reference category (5mM concentration for 3 h exposure) in binary logistic regression. Dead oocytes were observed 1 h after culturing of oocytes. Rate of cleavage, morula and blastocysts were observed 24, 72 and 96 h post activation. Morula and blastocyst (%) were calculated on basis of cleavage.

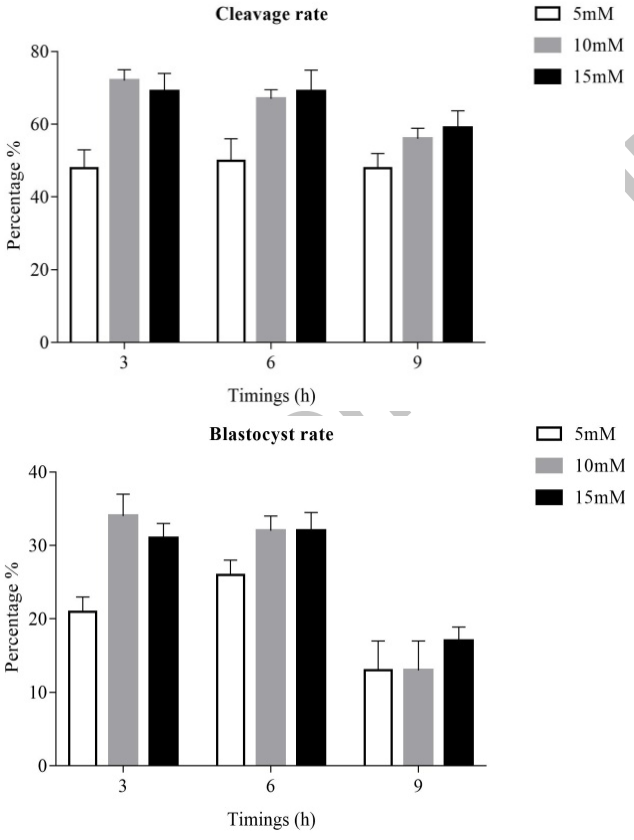

Fig. 1. The activation rate (cleavage rate and blastocyst rate) of BALB/c mice oocytes and their *in-vitro* developmental potential after treatment with 5, 10 and 15 mM $SrCl_2$ for 3, 6 and 9 h.

When the duration of CB treatment was evaluated then additional 3 h exposure did not significantly improve the cleavage rate and also embryonic development (Table III).

Table II. Effect of $SrCl_2$ (10 mM) treatment on mouse embryonic in-vitro development.

Timings (h)	0 h	3 h	6 h
Cleavage rate	93%	90%	90%
Odd ratio	1	0.64	0.64
Morulla	86%	85%	81%
Odd ratio	1	0.96	0.73
Blastocyst	75%	70%	68%
Odd ratio	1	0.76	0.71

Numbers of oocytes in each treatment were 30.

Table III. Effects of cytochalasin-B addition in activation media on mouse oocyte activation and development.

CB exposure timings (addition in media)	0 h (no addition)	3 h (3h activation)	6 h (3h activation + 3h culture)
Cleavage rate	34%*	73%	73%
Odd ratio	0.20	1.03	1
Morula	0%*	87%	91%
Odd ratio	0.00	0.70	1
Blastocyst	0%*	46%	55%
Odd ratio	0.00	0.71	1

*represents the significance value (P<0.05) with respect to reference category (6 h) in binary logistic regression. Morula and blastocyst (%) were calculated on basis of cleavage. Numbers of oocytes in each treatment were 30.

DISCUSSION

Artificial activation of oocytes has been reported in the number of species including mouse (Ma *et al*., 2005), rat (Tomashov-Matar *et al*., 2005), rabbit (Ozil *et al*., 2001), bovines (Méo *et al*., 2004), porcine (Hao *et al*., 2006) and human (Versieren *et al*., 2010). Oocytes are usually arrested at the metaphase-II stage of meiosis until fertilized by sperm cell. The key factor for meiotic resumption is calcium oscillations (Lee *et al*., 2016). During artificial activation, calcium oscillations occur through stimulation of inositol triphosphate receptor (Zhang *et al*., 2005) which increases cytosolic calcium resulting in meiotic resumption (Murugesu *et al*., 2017). Different activation methods have been adopted in different species to improve the developmental potential of artificially activated oocytes; however, few studies evaluated the toxic effect of these reagents used in different activation protocol. Thus, it is important to evaluate the detrimental effect of these chemicals on early embryonic developmental competency and develop further strategies to reduce the toxic effects (Han *et al*., 2013). Therefore, in current study, we determined the mouse oocytes artificial activation with different concentrations of $SrCl_2$ (5, 10, 15 mM) for different time intervals (3, 6, 9 h) was observed. Moreover, detrimental effects of $SrCl_2$ and CB were also evaluated.

The activation process depends upon the frequency and amplitude of calcium oscillation. Comparatively high activation rates were obtained at 10 and 15 mM concentration for 3 and 6 h (Table I). These results were consistent with previous report (Loren *et al*., 2006) which demonstrated that 10 and 15 mM concentrations are equally competent for oocyte activation. 10 mM concentration has been declared more favourable for calcium oscillation (Cheek *et al*., 1993; Bos-Mikich *et al*., 1995). However, it has also been reported that 5mM concentration of $SrCl_2$ is suboptimal for calcium oscillation (Alberio *et al*., 2001). These results demonstrated that out of 10, 15 mM, the lower concentration 10 mM SrCl2 was found to be optimal for mouse oocyte activation.

In terms of exposure duration with $SrCl_2$, longer exposures resulted in higher number of morphologically abnormal oocytes (Table I). These findings was consistent with previous reports that longer exposure potentiate cleavage reulting in morphologically abnormal embryos (Han and Gao, 2013). Moreover, morphologically abnormal embryos were more at lower concentration (5 mM) as it falied to provoke sufficient signals for calcium oscillation.

Toxicological experiment demonstrated no significant difference at the different exposure time (Table II). It revealed that $SrCl_2$ is non-toxic for developing embryos. Although, it provokes calcium oscillations but it is reported that Ca^{2+} oscillations stop after meiotic resumption. Hence, strontium treatment may not intervene with the Ca^{2+}signalling process triggered during natural fertilization resulting in non-toxic for fertilized zygotes. Up to best of knowledge, the toxic effect of 10 mM $SrCl_2$ was reported the first time as no previous reports are available.

After evaluating the toxic effect of the best dose (10mM for 3 h), the next target was to elaborate the optimum exposure time of CB necessary for maintaining the diploid nature of activated oocytes. The results demonstrated a significant difference ($p<0.05$) of treatment groups with control (0 h). This was due to maintaining diploid nature of parthenotes by CB. The similar outcomes were reported previously that haploid parthenotes undergo fragmentation resulting in compromised development (Liu *et al*., 2002; Zhang *et al*., 2005). However, when CB exposure time (3, 6 h) was compared, no significant differences were observed (Table III). Additional CB exposure in culture media tends to increase in blastocyst development rate (Ma *et al*., 2005).

CONCLUSION

In conclusion, 10 mM concentration of $SrCl_2$ for 3 h exposure is an appropriate for oocyte activation and this treatment has least toxic impact on the future embryonic development. In addition, CB availability for this time duration is sufficient for further development but it needs to be investigated in details by conducting cytogenetic studies.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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