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Effect of Pre-Treatment of Ovine Sperm on Male Pronuclear Formation and Subsequent Embryo Development Following Intracytoplasmic Sperm Injection

A Shirazi[1,2], M Derakhshan-Horeh[3], AA Pilvarian[3], E Ahmadi[2], H Nazari[2] and B Heidari[1,2]

[1]*Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran;* [2] *Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, SK, Canada;* [3] *Department of Physiology, Faculty of Basic Sciences, Payame-Nor University, Isfahan, Iran*

Contents

The objective of this study was to determine the effects of various methods of sperm pre-treatment on male pronuclear (MPN) formation and subsequent development of ovine embryos derived from *in vitro*-matured oocytes and intracytoplasmic sperm injection (ICSI). The effect of treatment of injected oocytes with dithiothreitol (DTT) on embryo development was also assessed. In Exp. 1, the injected oocytes with non-treated sperm were activated with three different procedures. The cleavage and blastocyst rates in those activated with DTT was lower ($p < 0.05$) than those activated with either ionomycin (Io) + 6-dimethylaminopurine (6-DMAP) or DTT + I + 6-DMAP. In Exp. 2, the effects of sperm pre-incubated with DTT, sodium dodecyl sulphate (SDS) or DTT + SDS as well as two-time frozen/thawed sperm (without cryoprotectant) on MPN formation and oocyte activation were examined. The non-treated sperm served as controls. The MPN formation in DTT + SDS group was higher ($p < 0.05$) than other groups except for freeze–thaw group. No difference in the rate of activated ICSI oocytes was observed among groups. In Exp. 3, the effect of pre-treatment of sperm on subsequent development of ICSI embryos and blastocyst cell numbers were examined. The rates of cleavage and blastocyst formation as well as the blastocyst cell numbers were similar among the pre-treated and control groups. In conclusion, pre-treatment of sperm with DTT + SDS positively affected MPN formation, although the subsequent development capacity of the resulting embryos remained limited. Moreover, DTT was not effective on oocyte activation compared with Io + 6-DMAP after ICSI.

Introduction

During the final stages of mammalian sperm maturation, their nuclear structure becomes progressively condensed and stabilized by the formation of disulphide bonds (Calvin and Bedford 1971; Marushige and Marushige 1975). It has been also proposed that decondensation of the sperm nucleus and formation of the male pronucleus (MPN) are affected by the structural stability of the sperm nucleus (Rodriguez et al. 1985; Perreault et al. 1988) which depends upon the association of sperm DNA with protamines.

In the process of normal fertilization, the sperm triggers a series of intracellular Ca^{2+} releases that activate the egg and results in MPF (M-phase promoting factor) inactivation, sperm head decondensation and pronuclei formation with the initiation of embryo development (Cuthbertson et al. 1981; Sun et al. 1994). In the ooplasm, factors supporting chromatin decondensation, such as reduced glutathione and nucleoplasmin, interact with sperm chromatin and induce remodelling of sperm chromatin to form a MPN (Perreault et al. 1984; Yanagimachi 1994; Collas and Poccia 1998; McLay and Clark 2003).

In several species such as mice (Kimura and Yanagimachi 1995), rabbits (Keefer 1989), humans (Van Steirteghem et al. 1993) and horses (Galli et al. 2002), intracytoplasmic sperm injection (ICSI) by itself is sufficient to activate the oocyte inducing sperm head decondensation, female and male pronuclear formation and initiating subsequent embryo development. In porcine, bovine and ovine, however, the conventional ICSI is not adequate for such events and a higher percentage of zygotes with male and female pronuclei and embryo development were obtained when ICSI has been followed by chemical stimulation (Rho et al. 1998; Tian et al. 2006; Shirazi et al. 2009). Additionally, in some species, in ICSI procedure, the retention of acrosome or subacrosomal perinuclear theca seems to prevent decondensation of sperm chromatin in ooplasm (Sutovsky et al. 1997; Hewitson et al. 1999; Katayama et al. 2002).

In several species, permeabilization of the sperm membrane by physical means may have a role to play in facilitating decondensation and PN formation after ICSI. Sperm pre-treatment such as immobilizing sperm by crushing the sperm with the micropipette used for injection (Lacham-Kaplan and Trounson 1994), artificial removal of the acrosome and tail by sonication (Keefer 1989; Goto 1993), demembranation of spermatozoa by lysolecithin (Morozumi et al. 2006) and damaging the sperm membrane by freezing and thawing (Goto et al. 1990; Keefer et al. 1990; Goto 1993) have all been reported to improve results.

Treatment of spermatozoa with either the non-ionic detergent Triton X-100 (TX), or dithiothreitol (DTT) as well as sodium dodecyl sulphate (SDS); an anionic detergent has been also suggested to confer enhanced success on intracytoplasmic sperm injection in some species (Perreault et al. 1988; Chen and Seidel 1997; Rho et al. 1998).

One of the problems in sheep ICSI is the failure or rarely decondensation of sperm chromatins, resulting in a low rate of male pronuclear formation and normal fertilization in artificially activated ovine oocytes matured *in vitro* (Shirazi et al. 2009).

In view of the dependence of sperm nuclear decondensation on the reduction of disulphide bonds, the aim of this study is to determine how far the efficiency of ovine ICSI (judged by the rate of male and female PN formation and subsequent embryo development) can be improved by sperm pre-treatment with DTT. In

addition, considering the role of detergents in solubilizing proteins and disruption of sperm membrane, the probable synergistic effect between DTT and SDS on MPN formation is evaluated.

Material and methods

Unless indicated otherwise, all reagents in this study were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Experimental design

Experiment 1

Development of ICSI oocytes activated by DTT with and without ionomycin (Io) + 6-dimethylaminopurine (6-DMAP) (Table 1).

Table 1. Development of ICSI oocytes activated by Io + 6-DMAP with and without dithiothreitol (DTT)

Experimental Groups*	Activation	Manipulated oocytes No.	Cleavage n (%)	Blastocysts n (%)
IVF	–	551	447 (81.1)[a]	192 (34.8)[a]
Parthenogenetic	Io + 6-DMAP	152	123 (80.9)[a]	41 (27)[a]
ICSI	Io + 6-DMAP	277	217 (78.3)[a]	47 (17)[b]
ICSI	DTT	78	24 (30.8)[b]	0 (0)[c]
ICSI	DTT + Io + 6-DMAP	74	66 (89.2)[a]	7 (9.5)[c,b]
Sham	Io + 6-DMAP	88	67 (76.1)[a]	13 (14.8)[b]

[a,b,c]Number with different superscript in the same column differ significantly ($p < 0.05$).
*In ICSI groups, the sperm received no pre-treatment.
Io + 6-DMAP, Ionomycin + 6-dimethylaminopurine; ICSI, intracytoplasmic sperm injection.

To establish the better activation regimen, the various stimuli to induce oocyte activation were compared based on their effects on cleavage and subsequent embryo development *in vitro*. As a consequence of this experiment, the activation of oocytes in experiments 2 and 3 was accomplished with ionomycin followed 3 h later by 6-DMAP.

Experiment 2

The effect of pre-treatment of sperm with DTT, DTT + SDS, SDS and freeze–thaw on MPN formation (Table 2).

Fresh semen was subjected to swim-up procedure. After swim-up procedure, motile spermatozoa at the top were resuspended in the working drops and only fast-moving spermatozoa were allocated into control and pre-treated groups. Following ICSI, the activation of oocytes in all experimental groups was accomplished by ionomycin followed 3 h later by 6-DMAP. ICSI embryos produced with control and pre-treated spermatozoa were compared for the rates of MPN formation 16 h after ICSI. Replicates comprised of control and treated groups.

Experiment 3

The effect of pre-treatment of sperm with DTT, DTT + SDS, SDS and freeze–thaw on subsequent embryo development and blastocyst cell numbers (Table 3).

ICSI embryos derived from control and pre-treated groups were compared for the effects of sperm pre-treatment on cleavage, and subsequent development

Table 2. Effect of pre-treatment of sperm on male pronuclear formation after intracytoplasmic sperm injection

		Sperm head (%)				Oocytes (%)				
							Activated			
	Injected Oocytes No.	MPN	Swollen	Intact	Ejected	Not Activated*	1PN	2PN	3PN	Ana-Tel.
Control	165	61 (37)[a]	44 (27)[a]	38 (23)[a]	22 (13)[a,c]	47 (28)	25 (15)[a,b]	61 (37)[a]	3 (2)	29 (18)[a]
DTT	96	37 (38.5)[a]	13 (13)[b]	42 (44)[b]	4 (4)[b,c]	22 (23)	7 (7)[a]	58 (60)[b]	3 (3)	6 (6)[b]
DTT + SDS	53	31 (58.5)[b]	7 (13.2)[a,b]	9 (17)[a,c]	6 (11.3)[a,b,c]	12 (22.6)	4 (7.5)[a,b]	31 (58.5)[b]	0 (0)	6 (11.3)[a,b]
SDS	63	17 (27)[a]	28 (44.4)[c]	6 (9.5)[c]	12 (19)[a]	19 (30.1)	12 (19)[b]	24 (38.1)[a]	0 (0)	8 (12.7)[a,b]
Freeze-thaw	64	29 (45.3)[a,b]	27 (42)[c]	5 (8)[c]	3 (5)[c]	17 (26.6)	9 (14)[b]	29 (45.3)[a,b]	1 (2)	8 (12)[a,b]

[a,b,c]Number with different superscript in the same column differ significantly ($p < 0.05$)
*Oocytes with metaphase plate (MII oocytes).
MPN, Male pronucleus; 1PN, One pronucleus; 2PN, Two pronuclei, 3PN, Three pronuclei; Ana–Tel, Anaphase–Telophase; DTT, dithiothreitol.

Table 3. Development of intracytoplasmic sperm injection embryos injected with pre-treated sperm

Treatment groups	Injected oocytes No.	Cleavage n (%)	Blastocyst*			
			n (%)	ICM	TE	Total
Control	277	217 (78.3)	47 (17.0)	11.5 ± 2.3	43.2 ± 6.9	54.7 ± 8.9
DTT	168	144 (85.7)	28 (16.6)	20.2 ± 9.7	56.4 ± 11.2	76.6 ± 20.8
DTT + SDS	76	66 (87)	10 (13.2)	27.3 ± 8.3	86.8 ± 32.7	114 ± 40
SDS	97	80 (82.5)	14 (14.4)	13.6 ± 1.4	45.8 ± 3.6	59.4 ± 4.8
Freeze–thaw	94	70 (74.5)	17 (18.1)	20.0 ± 2.9	62.8 ± 9.6	82.8 ± 12.3

*The numbers of ICM, TE, and total cells expressed as mean ± SEM.
ICM, Inner cell mass; TE, Trophectoderm; DTT, dithiothreitol.

in vitro. Blastocysts produced with control and pre-treated sperm were subjected to double staining to establish whether the sperm pre-treatment could influence the quality of embryos in term of cell numbers and its allocation to inner cell mass (ICM) and trophectoderm (TE) cells.

Oocyte recovery and *in vitro* maturation

Ovine ovaries were collected at a local slaughterhouse and transported to the laboratory within 2–3 h in normal saline at temperature between 25 and 35°C. All visible follicles with a diameter of 2–6 mm were aspirated using gentle vacuum (30 mmHg) via a 20-gauge short-bevelled needle connected to a vacuum pump. The follicle content was released in pre-incubated HEPES-buffered TCM199 (H-TCM199), supplemented with 50 IU/ml heparin.

The method for *in vitro* maturation and production of sheep embryo was the same as described by Thompson et al. (1995) with minor modification. Briefly, the oocytes, with at least three layers of cumulus cells (COCs: cumulus–oocyte complexes), with a uniform granulated cytoplasm and homogenous distribution of lipid droplets in the cytoplasm were selected for the experiments. Before culturing, oocytes were washed in H-TCM199 supplemented with 5% FBS (Foetal bovine serum, Gibco 10270) and 2 mM glutamine. The oocyte culture medium (OCM) was bicarbonate-buffered TCM199 with 2 mM L-glutamine supplemented with cysteamine (0.02 mg/ml), hCG (1 IU/ml), FSH (0.05% IU/ml), E_2 (1 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), Na-Pyruvate (0.2 mM) and 10% FBS (Gibco 10270).

The selected COCs were pooled and randomly distributed in maturation droplets (15 oocytes in 50 μl) and covered by sterile paraffin oil in a 60-mm Petri dish (353004; 60 mm Easy Grip Dish; BD Biosciences, Franklin Lakes, NJ, USA) and were then incubated for 24 h at 39°C and atmosphere of 5% CO_2 in air and maximum humidity.

Preparation of sperm and *in vitro* fertilization

Fresh semen was collected from a Lori-Bakhtiari breed ram of proven fertility. For swim up, 80–100 μl of semen was kept under 1 ml of HEPES-Synthetic Oviduct Fluid-BSA (HSOF-BSA) in a 15-ml conical Falcon tube at 39°C for up to 45 min.

After swim up, the 700–800 μl of the top fluid were then added to 3 ml of HSOF-BSA, centrifuged twice at 200 g for 3 min and the final pellet resuspended with HSOF-BSA.

Before insemination, the oocytes were washed four times in HSOF and once in fertilization medium (SOF enriched with 20% heat-inactivated oestrous sheep serum) and were then added to fertilization drops. The HSOF and fertilization media were the same as used by Tervit et al. (1972), with modification for preparing HSOF (20 mM of $NaHCO_3$ was substituted with 20 mM HEPES; 10 mM free acid and 10 mM Na salt). Both media were supplemented with antibiotics.

Insemination was carried out by adding 1×10^6 sperm/ml to the fertilization medium. A 5-μl aliquot of sperm suspension, containing 1×10^6 sperm/ml, was added to fertilization medium that included 10 oocytes per 45-μl fertilization drop. Fertilization was carried out by co-incubation of sperm and oocytes in an atmosphere of 5% CO_2 in humidified air at 39°C for 22 h.

Sperm pre-treatment

After swim up, the sperm were incubated in HSOF-BSA medium containing: I) 5 mM DTT for 20 min, II) 5 mM DTT plus 0.1% SDS for 20 min and III) 0.1% SDS for 20 min. The sperm were then washed twice in HSOF-BSA and prepared for injection in the same medium. In group IV) after swim up, the sperm without cryoprotectant was transferred to the −20°C for 5 min and then plunged directly into liquid nitrogen for 1 min and thawed immediately in 37°C. This procedure (freezing in liquid nitrogen and thawing at 37°C) was carried out two times. The non-treated sperm served as control. In pre-treated and control groups, the sperm were prepared in HSOF-BSA for injection.

Intracytoplasmic sperm injection (ICSI)

ICSI was performed using an IX71-Olympus inverted microscope with Nomarsky optics (IX71; Olympus, Tokyo, Japan). After IVM, the oocytes, in groups of 15, were denuded and then placed into a drop of 50 μl of injection medium (HSOF-BSA) covered with mineral oil. The prepared sperm was diluted 1 : 1 with 12% polyvinylpyrrolidone in PBS immediately, just before microinjection. One drop of injection medium (50 μl) with two drops (10 μl) of diluted sperm with PVP were arranged in two columns on the lid of a 60-mm tissue culture dish (353004; 60 mm Easy Grip Dish; BD Biosciences). The inner diameter of injection and holding pipettes were 5–6 and 20–30 μm, respectively. For injection, the sperm were aspirated tail-first and injected into the ooplasm through the zona pellucida. In case of DTT-treated sperm, altered morphology of the sperm head was used as an additional selection criterion (Fig. 1). In control and DTT groups, before sperm aspiration, the sperm were individually immobilized by touching the midpiece.

During injection, the first polar body was either in the 6 or 12 o'clock position, and the injection pipette was in the 3 o'clock position. Prior to injection, the ooplasm was first aspirated to approve that the oolema was broken. The spermatozoon was injected into the ooplasm with a minimum volume of medium (< 5 pl) at the 3 o'clock position. Sham injections were performed in a similar manner. After ICSI, oocytes were washed three times in HSOF containing 6 mg/ml BSA, and then activated.

Activation of ICSI ova

Within 1 h after injection, the injected oocytes (ICSI groups) were activated as the following groups: (i) Exposure to 5 μM ionomycin in HSOF with 1% FBS for

5 min, culture in IVF-SOF for 3 h to allow extrusion of the second polar body, and then exposure to 1.9 mM 6-DMAP prepared in HSOF at 39°C, 5% CO2 in air for 3 h; (ii) Exposure to 2 mM DTT in HSOF at 38.5°C for 20 min and washed twice (10 min each) in HSOF; (iii) Exposure to 2 mM DTT in HSOF at 38.5°C for 20 min and washed twice (10 min each) in HSOF accomplished with ionomycin followed 3 h later by 6-DMAP.

After activation, oocytes were washed in HSOF and placed into 20-μl drops of IVC medium. Denuded IVM oocytes and sham-injected oocytes after activation with Io + 6-DMAP were considered as controls of the chemical (parthenogenetic group) and mechanical activations. In parthenogenetic group, the oocytes were treated with 6-DMAP directly after activation with ionomycin.

In vitro culture

Presumptive zygotes in IVF, ICSI, Sham, and parthenogenetic groups were allocated to 20 μl culture drops (five to six embryos/drop) containing SOF supplemented with 2% (v/v) BME-essential amino acids, 1% (v/v) MEM-non-essential amino acids, 1 mM glutamine and 8 mg/ml fatty acid free BSA. Embryos were cultured for 8 days at 39°C under mineral oil in a humidified atmosphere of 5% CO_2, 7% O_2 and 88% N_2. On the third and fifth day of culture (Day 0 defined as the day of fertilization), the medium was replaced with fresh culture medium supplemented with 10% charcoal stripped FBS. The rates of cleaved embryos (day 3) and blastocysts (day 7) were expressed on the basis of the number of oocytes at the onset of culture.

Nuclear staining

Sixteen hours after ICSI, the injected oocytes were transferred in ice-cold ethanol containing 10 μg/ml Hoechst 33342 for 15 min. The oocytes were directly mounted into the small droplet of glycerol on glass slide and examined under an epifluorescent microscope (IX71; Olympus, Tokyo, Japan). The nuclei appeared blue, caused by DNA labelling with the membrane permeable Hoechst 33342. The criteria used for defining fertilization vs parthenogenetic activation was the presence of both female and male pronuclei and one or two polar bodies in the absence of sperm head within the oocyte or perivitelline space. The lack of a second polar body in some oocytes was, probably, because of the loss of one polar body during culture and/or fixation. The oocytes with one or two PN (± a second polar body) in the presence of sperm head within the oocyte or perivitelline space were considered to have been activated parthenogenetically.

Differential staining

Differential staining of ICM and TE compartments was carried out on Day 7 blastocysts. Briefly, blastocysts were incubated in TX prepared in the base medium (HSOF containing 5 mg/ml BSA) for 20 s. The blastocysts were then stained in the base medium containing 30 μg/ml propidium iodide (PI) for 1 min. After two washes in the base medium, the blastocysts were transferred in ice-cold ethanol containing 10 μg/ml Hoechst 33342 for 15 min. The blastocysts were directly mounted into the small droplet of glycerol on glass slide and examined under an epifluorescent microscope (IX71; Olympus, Tokyo, Japan). ICM nuclei appeared blue, caused by DNA labelling with the membrane permeable Hoechst 33342, and TE cells appeared red because of staining of nuclear DNA with the membrane impermeable PI.

Statistical analysis

Data were collected over at least five replicates. Chi-square and Fisher Exact Test was applied when qualitative evaluation was considered.

The difference in blastocyst cell numbers between experimental groups was analysed using one-way ANOVA. When ANOVA revealed a significant effect, comparison of means among groups was performed using Tukey test. When normality test was failed, the Kruskal-Wallis One Way Analysis of Variance on Ranks was applied. Data were expressed as mean ± SEM. All analyses were conducted with SPSS Version 11.5 (SPPS Inc., Chicago, IL, USA) and $p < 0.05$ was considered significant.

Results

Experiment 1

Effect of oocyte activation after ICSI, parthenogensis and sham injection on subsequent embryo development.

As shown, the rate of cleavage was significantly lower in ICSI embryos activated by DTT compared to those activated by Io + 6-DMAP or DTT + Io + 6-DMAP ($p < 0.05$). The total blastocyst rate on Day 8 in the ICSI groups ranged between 0% and 17%. No blastocyst was achieved in DTT activated ICSI embryos. The rate of blastocyst in IVF and parthenogenetic groups was significantly higher than ICSI and sham groups ($p < 0.05$; Table 1).

Experiment 2

Effect of sperm pre-treatment with DTT with and without SDS as well as the effect of two-time frozen–thawed sperm (without cryoprotectant) on male pronuclear formation.

The percentage of MPN formation in ooplasm of those oocytes injected with sperm pre-treated with DTT + SDS was significantly higher than pre-treated and control groups except for the oocytes injected with frozen–thawed sperm.

The percentage of ICSI oocytes with swollen sperm head was lowest in DTT groups (DTT and DTT + SDS) compared with other groups. Conversely, the proportion of oocytes with intact sperm head in those injected with DTT-pre-treated sperm was significantly higher than other groups ($p < 0.05$). More than 50% of DTT-treated sperm displayed altered morphology; under phase contrast, the heads of those sperm exhibited prominent bending near equatorial segment

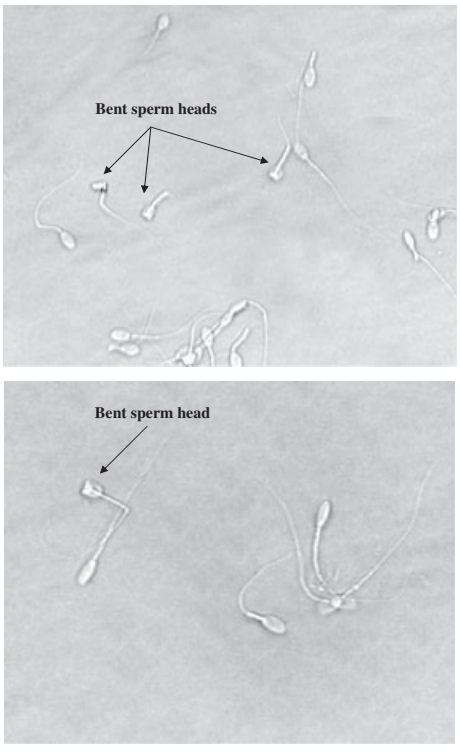

Fig. 1. Dithiothreitol treated ram sperm

(Fig. 1). The percentage of ejected sperm from injected oocytes ranged from 4% to 19% among treatment groups (Table 2).

The difference in proportion of ICSI oocytes which had not been activated after artificial activation (I + 6-DMAP) was insignificant among groups. In activated oocytes, the percentage of ICSI oocytes with one pronucleus (1PN) in SDS group was higher than DTT group while the percentages of activated oocytes with two pronuclei (2PN) in DTT and DTT + SDS were significantly higher than SDS and control groups. There was no significant difference in the percentages of oocytes with three pronuclei (3PN) among groups. The percentage of ICSI oocytes with anaphase–telophase (Ana–Tel) nucleus was lowest in DTT group and ranged from 6% to 18% among groups (Table 2).

Experiment 3

In vitro development of ICSI oocytes injected with pre-treated sperm.

The difference in cleavage and blastocyst rates among oocytes injected with pre-treated and non-treated sperm was insignificant. Likewise, despite the big difference in mean numbers of ICM, TE and total cells between SDS + DTT and control groups, the difference attributable to big variation in cell numbers was not significant Table 3.

Discussion

Treatment of spermatozoa with either the non-ionic detergents such as TX or DTT has been suggested to confer enhanced success on intracytoplasmic sperm injection in several species (Suttner et al. 2000; Asada et al. 2001; Galli et al. 2003; Morozumi et al. 2006; Tian et al. 2006). In this study, pre-treatment of sperm with DTT + SDS led to the highest rate of MPN formation among ICSI oocytes. It seems DTT [an agent that specifically reduces disulphide bonds (S = S)] and SDS (an anionic detergent which disrupts non-covalent bonds) could exert their synergistic effect on MPN formation through sperm nuclear decondensation and sperm membrane damage, respectively (Perreault et al. 1988; Chen and Seidel 1997; Rho et al. 1998).

The proportion of swollen sperm head in DTT group was lower than other groups except for DTT + SDS group. Accordingly, the proportion of intact sperm head in DTT group was highest among groups ($p < 0.05$). From the previous text, it could be inferred that DTT by itself was incapable to improve sperm nucleus decondensation and MPN formation. This finding was in agreement with previous experiments indicating that DTT in the absence of any ionic detergent did not destabilize the nuclear matrix (Ward 1994; Nadel et al. 1995; Yong et al. 2005). Although the difference in proportions of activated oocytes among groups was insignificant, the proportion of injected oocytes with 2PN was significantly higher in DTT and DTT + SDS groups compared to the other groups except for the freeze–thaw group. The higher proportion of injected oocytes with 2PN respect to the rates of MPN formation in DTT (60% vs 38.5%) and SDS (38.1% vs 27%) groups could be interpreted as a parthenogenetic activation in these groups. As the artificial activation protocol for all injected oocytes was identical (Io + 6-DMAP), whether the sperm pre-treated with DTT or SDS could exert any further stimuli on oocyte activation via more release of sperm-borne oocyte-activating factor (SOAF; Saunders et al. 2002) and hence more exposure of oocyte cytoplasm to SOAF (sperm-specific phospholipase C, PLCζ) need to be further investigated. This hypothesis, however, could not be applicable for the DTT + SDS group because the proportions of MPN and 2PN were the same in this group (Table 2). In the other hand, if sperm pre-treated with SDS or DTT could exert some sort of parthenogenetic activation, why in combination of both reagents this effect was not evident. It could be explained by this fact that in the presence of both DTT and SDS, the sperm cells became more prone to MPN formation rather than the presence of DTT or SDS alone. On the other hand, the higher proportion of oocytes with MPN in DTT + SDS group predisposed the conditions for normal fertilization and as a consequence decreased the occurrence of parthenogenesis.

Additionally, considering the activation procedure in injected oocytes, 3-h interval between treatments with Io and 6-DMAP, the majority of oocytes should extrude the second polar body. The presence of swollen or intact sperm head besides 2PN in ooplasm, however, was indicated that in some oocytes the second polar body has not been expelled from the cytoplasm. Therefore, it could be inferred that in DTT and SDS groups at least a proportion of the produced blastocysts were diploid parthenotes. In another experiment (unpublished data), we found that in sham injected and parthenogenetically

activated oocytes, considering 3-h interval between Io and 6-DMAP treatments, almost 37% of produced parthenotes were diploid.

Based on our previous finding, neither permeabilization of the sperm membrane by physical means, crushing the sperm tail with the micropipette used for injection, nor the injection procedure by itself were sufficient to activate the ovine oocyte and MPN formation after ICSI. Therefore, in this study, the artificial activation of injected oocytes was considered as an essential step of ICSI procedure. As shown, artificial activation with DTT was inefficient compared to the activation with Io + 6-DMAP, in term of cleavage and blastocyst rates (Table 1). This finding was in contrary to the report indicating that the treatment of bovine ICSI oocytes with DTT resulted in increased cleavage and blastocyst rates in the group of non-activated embryos and in acceleration of blastocyst development in the group of activated embryos (Galli et al. 2003). It seems treatment of injected ovine oocyte with DTT was ineffective on oocyte activation and as a consequence the normal events such as sperm nuclear decondensation and subsequent embryo development were disturbed. The difference between ovine and bovine oocytes in response to DTT might be related to the difference in oocyte susceptibility to DTT in these species.

In this study, the rate of blastocyst formation in parthenogenetic group was significantly higher than corresponding value in ICSI and sham-injected oocytes, it seems the subsequent embryo development has been negatively influenced by ICSI procedure.

Moreover, the higher blastocyst rate in parthenogenetic group compared with sham-injected oocytes might be related to the difference in activation procedure in those groups. Indeed, 3-h interval between treatments with Io and 6-DMAP in sham group and the absence of the time interval in parthenogenetic group, predispose the activated oocytes to haploid and diploid parthenogenesis in sham and parthenogenetic groups, respectively. It has been shown that the haploid parthenogenetic embryos are developmentally delayed and that the majority of them because of suboptimal genomic imprinting fail to reach the blastocyst stage compared with diploid counterparts (Henery and Kaufman 1992; Kim et al. 1997).

Considering the presence of both female and male pronuclei as a prerequisite for the normal fertilization, it could be expected that in ICSI oocytes the higher proportion of 2PN (female and male pronuclei), the higher subsequent embryo development (cleavage and blastocyst rates) would be. While, in the current study, there was no such a relation between the proportion of injected oocytes with 2PN and the subsequent rates of cleavage and blastocyst formation among groups. Therefore, the question is why despite the higher proportions of 2PN oocytes in DTT + SDS and DTT groups, the rates of blastocyst formation in these groups were not higher than other groups (Table 3).

The previously arisen question could be answered by the findings of Szczygiel and Warda (2002) indicating that simultaneous treatment of spermatozoa with detergent and DTT induces extensive chromosomal breakage. It has been shown that mouse spermatozoa treated with both TX and DTT simultaneously and then used for ICSI resulted in broken paternal chromosomes in the zygote (Szczygiel and Warda 2002). Whether SDS as an anionic detergent in combination with DTT could similarly exert such a chromosomal defect should be further investigated. It has been, however, demonstrated that treating human spermatozoa with TX before ICSI, is more beneficial rather than detrimental on pre-implantation embryo development and has been resulted in the fastest and most efficient oocyte activation and sperm head decondensation (Kasai et al. 1999). As for DTT, there are several reports indicating the positive effect of this reagent on the efficiency of ICSI in some species (Rho et al. 1998; Suttner et al. 2000; Asada et al. 2001). Furthermore, based on report, incubation of zygotes with DTT was found to prevent chromosomal aberrations that would have otherwise occurred (Tateno and Kamiguchi 1999). In this study, however, it is possible that sperm pre-treatment with combination of SDS and DTT can adversely affect sperm chromatin structure that is necessary for normal embryonic development.

In mouse, oocytes injected with sperm nuclei that were treated with alkyltrimethylammonium bromide (ATAB; an ionic detergent) combined with or without DTT both developed to the pronuclear stage but only those nuclei that were treated with ATAB, which contained stable nuclear matrices, had the ability to participate fully in embryogenesis after ICSI (Ward et al. 1999).

Therefore, from the previous textit could be inferred that despite MPN formation in DTT + SDS group, combination of these agents could destabilize the sperm nuclear matrix in a manner that led to the improper incorporation of male and female pronuclei. Moreover, it has been suggested that sperm nuclei have mechanisms for chromosomal degradation (endonucleases) that can be activated by detergent and DTT (Szczygiel and Warda 2002).

It has been demonstrated that when sperm heads had no traces of the acrosome, acrosomal matrix, plasma or nuclear membranes, and had only a partial perinuclear theca or even in the completely absence of perinuclear theca, were fully capable of normal embryogenesis when used in ICSI (Wyrobek et al. 1990; Kuretake et al. 1996). Thus, it seems that in some species sperm nucleus is the only component of the spermatozoa that is required for embryonic development when used with ICSI (Ward et al. 1999).

The insignificant difference in MPN and blastocyst formation rates after ICSI between pre-treated sperm and those became immotile by two-time freezing and thawing was partially in agreement with the results in porcine species (Tian et al. 2006).

Concerning the quality of produced blastocysts assessed by blastocyst cell numbers, there was no significant difference among oocytes injected with treated or non-treated sperm which was in accordance with the report in swine species (Tian et al. 2006).

In conclusion, pre-treatment of ovine sperm with DTT in combination with SDS is effective for facilitating sperm decondensation and MPN formation. However, subsequent embryo development is not positively

influenced by that treatment. Moreover, DTT is not effective as much as Io + 6-DMAP on ovine oocyte activation after ICSI.

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Conflicts of interest

None of the authors have any conflict of interest to declare.

Author contributions

A Shirazi: Study design and supervision; M Derakhshan-Horeh: Doing experiment; Pilvarian AA: Study design; E Ahmadi: Doing experiment; H Nazari: Doing experiment; B Heidari: Statistical analysis.

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Author's address (for correspondence): A Shirazi, Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran. E-mail: abs890@mail.usask.ca; shiraziabbas@yahoo.com