

Full Length Article

Caffeine and oocyte vitrification: Sheep as an animal model

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ABSTRACT

Oocyte cryopreservation is a valuable way of preserving the female germ line. Vitrification of immature ovine oocytes decreased the levels of both maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) in metaphase II (MII) oocytes after IVM. Our aims were 1) to evaluate the effects of vitrification of ovine GV-oocytes on spindle assembly, MPF/MAP kinases activities, and preimplantation development following IVM and IVF, 2) to elucidate the impact of caffeine supplementation during IVM on the quality and development of vitrified/warmed ovine GV-oocytes. Cumulus-oocyte complexes (COCs) from mature ewes were divided into vitrified, toxicity and control groups. Oocytes from each group were matured *in vitro* for 18 h in caffeine free IVM medium and denuded oocytes were incubated in maturation medium supplemented with 10 mM (+) or without (–) caffeine for another 6 h. At 24 h.p.m., oocytes were evaluated for spindle configuration, MPF/MAP kinases activities or fertilized and cultured *in vitro* for 7 days. Caffeine supplementation did not significantly affect the percentages of oocytes with normal spindle assembly in all the groups. Caffeine supplementation during IVM did not increase the activities of both kinases in vitrified groups. Cleavage and blastocyst development were significantly lower in vitrified groups than in control. Caffeine supplementation during the last 6 h of IVM did not significantly improve the cleavage and blastocyst rates in vitrified group. In conclusion, caffeine treatment during *in vitro* maturation has no positive impact on the quality and development of vitrified/warmed ovine GV-oocytes after IVM/IVF and embryo culture.

1. Introduction

Cryopreservation of gametes and embryos has become an integral part of assisted reproduction technologies (ART). In particular, sperm cryopreservation has been the most widely and successfully used in humans as well as in variety of mammalian species [1]. Embryo cryopreservation has also become a routine practise in clinical ART and has resulted in delivery of many healthy babies [2]. However, embryo cryopreservation has some drawbacks; it requires the availability of male partner to produce those embryos, in addition, embryo cryopreservation is prohibited due to ethical, legal and religious implications in some countries [3]. Cryopreservation of unfertilized oocytes is an alternative option, giving flexibility in the time of *in vitro* fertilization (IVF) and a potential to establish oocyte banking with oocyte donation

[4–8]. There are two techniques applied to the cryopreservation of gametes and embryos: controlled slow freezing, which was favoured in early procedures, and ultrarapid cooling by vitrification, which is now widely used as it produces less damage to the oocytes and embryos than slow freezing [9].

Although extensive research have been conducted on the cryopreservation of metaphase II (MII) oocytes, vitrification of oocytes at this stage can disrupt the meiotic spindle [10], which could be avoided by freezing of oocytes at the germinal vesicle (GV) stages. Freezing of oocytes at this stage has many clinical advantages in human and animal reproduction. [11–15]. Though, numerous studies have been conducted to cryopreserve GV-oocytes in many mammalian species [12,13,15–27], and reportedly produced live births in humans and animals [17,28,29], the blastocyst development rate remains low

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[23,24,30]. One major obstacle is the requirement of the frozen GV-oocytes to be matured *in vitro* (IVM) prior to fertilization [19]. No standard IVM protocol for GV-oocytes has yet been established in humans and in some animal species [31–33].

It is well known that, oocyte meiotic maturation is controlled by the levels of two cytoplasmic protein kinases; maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK). High activities of both kinases are responsible for the onset of germinal vesicle breakdown and are also essential for the maintenance of the oocytes at MII-stage [34]. Previous studies showed that vitrification of immature ovine oocytes decreased the levels of both kinases after IVM [13].

Caffeine (1,3,7-trimethylxanthine), a phosphodiesterase inhibitor, has been reported to induce dephosphorylation of Y-15 and T14 of p34cdc2, which may have been occurred by inhibition of Myt1/Wee1 kinase resulting in an increase in the activity of MPF in cultured mammalian cells, *Xenopus*, and porcine oocytes [35]. In sheep, previous studies showed that treatment of *in vitro* matured oocytes with caffeine increased the activities of both MPF and MAPK kinases, improved frequencies of nuclear envelope breakdown and chromosome condensation of transferred nuclei, increased total cell numbers, and reduced the frequency of apoptotic nuclei in blastocyst embryos produced by somatic cell nuclear transfer (SCNT) [36–38]. Moreover, in aged denuded ovine oocytes, caffeine treatment increased the blastocyst development rates and decreased the frequency of polyspermy following IVF [39]. However, little is known about its role on the development of vitrified/warmed oocytes. Our aims were 1) to evaluate the effects of vitrification of ovine GV-oocytes on spindle assembly, MPF/MAP kinases activities, and preimplantation development following IVM and IVF, 2) to elucidate the impact of caffeine supplementation during IVM on the quality and development of vitrified/warmed ovine GV-oocytes.

2. Materials and methods

Unless stated otherwise, all chemicals and reagents were purchased from Sigma-Aldrich (Dorset, UK).

2.1. Oocyte collection

Ovine ovaries were collected from a local slaughterhouse (Nottingham, UK) and kept in a thermos flask filled with pre-warmed phosphate-buffered saline (PBS) at 25 °C during transportation to the laboratory. Cumulus–oocyte complexes (COCs) were aspirated using a 21-gauge needle attached to a 10-mL syringe from 2 to 3-mm follicles. The follicular fluid and COCs were placed into 50-mL conical tubes and kept in a warming box at 39 °C for 15 min so that COCs sank to the bottom of the tubes. The upper follicular fluid was removed and 5 mL of follicular fluid containing COCs was poured into a 90-mm Petri dish containing oocyte washing medium consisting of HEPES-buffered TCM 199 (H-TCM 199; Gibco BRL BRL/Life Technologies, Paisley, Renfrewshire, UK) supplemented with 10% v/v fetal bovine serum (FBS; Gibco). The COCs, with at least two to three compact layers of cumulus cells and a homogeneous cytoplasm, were selected under a light microscope (Leica, Wetzlar, Germany). The selected oocytes were randomly divided into three groups; vitrified, toxicity (exposed only to vitrification and warming solutions without freezing), and control (directly subjected to IVM).

2.2. Vitrification and warming of COCs

COCs were vitrified according to the method described by Moawad et al. [13]. Briefly, COCs were rinsed three times in base medium (BM; H-TCM 199 supplemented with 10% FBS), and then transferred into 500 µL equilibration solution (10% v/v ethylene glycol (EG) plus 0.25 M trehalose in BM) for 3 min on a warm stage at 39 °C. 3–5 oocytes were then transferred into a small droplet of 20 µL vitrification solution

(20% v/v EG and 20% v/v dimethylsulfoxide (DMSO)) before being immediately treated in warming solution as toxicity or vitrified. For vitrification, the cryoloop (Hampton Research, Aliso Viejo, CA, USA) was dipped in the vitrification solution so that a thin film was created by surface tension. Three to five oocytes were gently placed on the film using a glass-mouth pipette and the cryoloop device containing the oocytes was plunged directly into a cryovial that had been submerged and filled with liquid nitrogen (LN₂). The cap of the cryovial was then tightened and the vial was returned to LN₂. The whole process was performed in 1 min. For thawing, the cap of the cryovial submerged in LN₂ was carefully unscrewed and opened and the cryoloop containing the vitrified oocytes was transferred immediately into warming solution, which consisted of 500 mL BM plus 10% v/v EG and 1 M trehalose. The oocytes came off the cryoloop into the solution and were kept in this solution for 3 min before being transferred into 500 µL BM plus 0.5 M trehalose and then to BM (3 min in each solution). All solutions were kept at 39 °C. Finally, the COCs were examined morphologically to assess their viability under a stereomicroscope (MZ 12.5; Leica Microsystems, Wetzlar, Germany). Oocytes with a spherical and symmetrical shape and evenly granulated cytoplasm were regarded as viable, whereas oocytes exhibiting membrane damage, a swollen or ruptured zona pellucida and/or degenerated cytoplasm were considered as non-viable. Only morphologically viable COCs were selected for further experiments.

2.3. *In vitro* maturation

IVM was performed as described previously [15]. Briefly, COCs from vitrified, toxicity and control groups were washed twice in maturation medium (bicarbonate-buffered TCM 199 with Earle salts; Gibco) supplemented with 10% FBS, 5 µg/mL FSH (Vetropharm, Belleville, ON, Canada), 5 µg/mL LH (Vetropharm), 1 µg/mL 17β-oestradiol, 0.3 mM sodium pyruvate, 100 µM cysteamine and 50 µg/mL gentamicin. Groups of 40–45 COCs were then transferred into four-well dishes (Nunc, Roskilde, Denmark) containing 500 µL maturation medium covered with mineral oil. All dishes were pre-warmed in an incubator at 39 °C under 5% CO₂ in air before use. All COCs were cultured in the medium for 18 h under the same conditions.

2.4. Caffeine treatment

At 18 h post IVM, cumulus cells were removed from all the groups by repeated pipetting of COCs in H-TCM 199 supplemented with 4 mg/mL polyvinylpyrrolidone (PVP) and 300 IU/mL hyaluronidase. After washing in maturation medium, oocytes from each group were incubated in maturation medium supplemented either with 10 mM (+) or without (–) caffeine for another 6 h. IVM was then continued till 24 h.

2.5. Spindle and chromosome configuration

IVM oocytes were immunostained for tubulin and counterstained to assess chromosomes as previously described [13]. Briefly, oocytes (at least 44 oocytes/group) were fixed in 4% (w/v) paraformaldehyde (PFA) for 30 min at room temperature and then washed three times (10 min each) in PBS supplemented with 20% FBS (PBS-FBS). All oocytes were then transferred to PBS-FBS containing 0.5% Triton X-100 (permeabilization medium) and kept in this medium for 30 min at room temperature. Subsequently, the primary antibody (mouse monoclonal anti-α-tubulin antibody; 1:200 dilution) was added and oocytes were further cultured at 4 °C overnight. Then, oocytes were rinsed three times in PBS-FBS as described above before being incubated with a fluorescein isothiocyanate (FITC)-labelled goat secondary antibody (1:200 dilution) at room temperature for 60 min. Finally, oocytes were stained with 10 µg/mL Hoechst 33342 and then rinsed three times in PBS-FBS. Oocytes were then transferred to a small drop of Vectashield

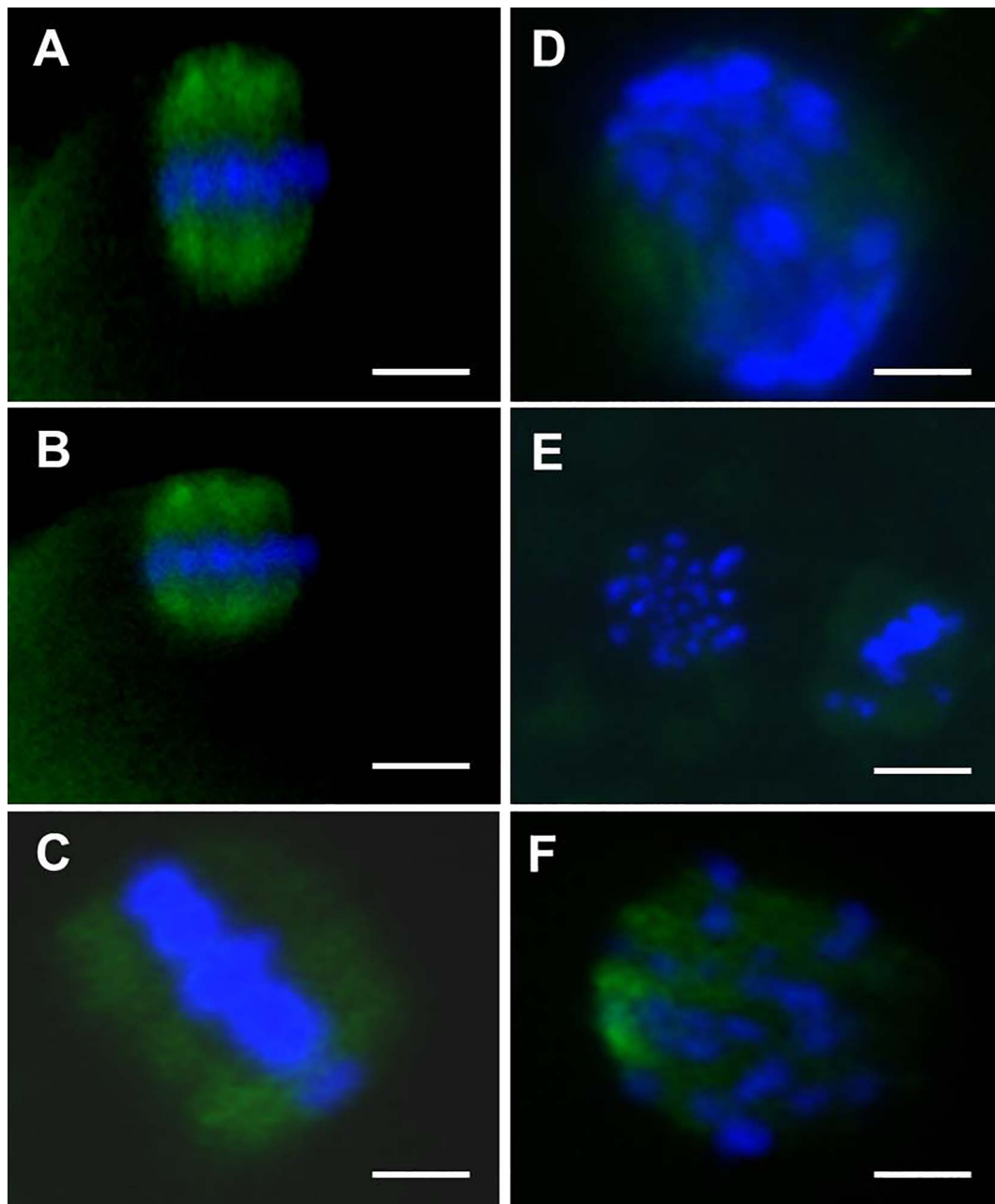


Fig. 1. Representative images of spindle (green) morphology and chromatin (blue) alignment in ovine oocytes following immunostaining at 24 h.p.m. (A–C) show normal morphology (symmetrical barrel shape meiotic spindle with chromatin aligned regularly along the equatorial plane of spindle), while (D–F) illustrates abnormal morphology in vitrified, toxicity and control oocytes, respectively. Scale bar = 50 μ m.

mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) on a diagnostic microscope slide (Erie Scientific, Portsmouth, UK) and then covered with a coverslip. The slides were examined by epifluorescence (Leica DMR, Wetzlar, Germany), and the images captured using a digital camera (Hamamatsu, Shizuoka, Japan) and analyzed by Simple PCI software (Compix, Cranberry Township, PA, USA). Based on the morphology of the meiotic spindles and chromosomes [13], oocytes with a classical symmetric barrel shaped spindle were considered normal, whereas oocytes with disorganized, clumped, dispersed or missing (completely or partially) spindles were considered abnormal. Similarly, normal chromosomes were defined as those in which two sets of chromosomes were regularly aligned along the equatorial plate of the meiotic spindle, whereas abnormal chromosomes appear clumped or dispersed (Fig. 1).

2.6. Analysis of MPF and MAPK activity

Analysis of MPF and MAPK activities was performed according to the method described by Ye *et al.* [40] with some modifications. Briefly, groups of 10 oocytes (3 replicates were repeated in each experimental group) were thoroughly washed in DPBS containing 0.1% polyvinyl alcohol (PVA) at 39 °C and then placed into 5 μ L of ice-cold lysis buffer containing 45 mM β -glycerophosphate (pH 7.3), 12 mM ρ -nitrophenylphosphate, 2 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), 12 mM $MgCl_2$, 12 mM ethyleneglycol bis (2-aminoethyl-ether) tetraacetic acid (EGTA), 0.1 mM EDTA, 20 mM Na_3VO_4 , 10 mM NaF, 2 mM dithiothreitol (DTT), 2 mM phenylmethylsulphonyl fluoride, 2 mM benzamidine, 20 μ g/mL leupeptin, 20 μ g/mL pepstatin A and 19.5 μ g/mL aprotinin. The samples were frozen in LN_2 and stored at -80 °C until analyzed. The activities of MPF and MAPK kinases were

measured simultaneously using histone H1 and bovine myelin basic protein (MBP) as their *in vitro* substrates, respectively. The oocyte lysate was thawed and then refrozen in liquid nitrogen (-196°C) once. The kinase reaction was started by adding the oocyte lysate to 5 μL kinase assay buffer containing 45 mM β -glycerophosphate (pH 7.3), 12 mM ρ -nitrophenylphosphate, 20 mM MOPS, 12 mM MgCl_2 , 12 mM EGTA, 0.1 mM EDTA, 2 mM Na_3VO_4 , 10 mM NaF, 4 $\mu\text{g}/\text{mL}$ H1, 6 mg/mL MBP, 40 μM protein kinase A (PKA) inhibiting peptide (Santa Cruz Biotechnology; Autogen Bioclear, Clane), 43 μM protein kinase C (PKC) inhibiting peptide (Promega, Southampton) and 10 Ci/mmol [γ - ^{32}P] ATP (PerkinElmer). The mixtures were incubated at 37°C for 30 min with gentle shaking. The reaction was stopped by adding 10 μL ice-cold $2 \times$ SDS sample buffer which was made from [125 mM Tris-Cl (pH 6.8) (Fisher Scientific), 200 mM DTT, 4% (w/v) SDS (Fisher Scientific), 0.01% (w/v) bromophenol blue and 20% (w/v) glycerol]. After boiling for 5 min, the substrates were separated by standard polyacrylamide gel electrophoresis (SDS-PAGE, 15% gels) using a Mini-Protean II dual slab cell (Bio-Rad, Hercules, CA) at 140 V for 1.5 h. Gels were dried on 3 mm filters and exposed to phosphor-screens (Fuji film). The phosphor images of gels (screens) were captured and the kinase activities were quantified using an FX phosphor image analysis system (Bio-Rad).

2.7. *In vitro* fertilization and embryo culture

IVF was performed using frozen-thawed semen pellets obtained from a Texel ram (Britbreed, Tranent, UK) as described previously [13]. Briefly, matured oocytes (24 h.p.m) were washed once in pre-warmed ovine fertilization medium (m-SOF supplemented with 2% sheep serum) at 39°C . Groups of 40–50 oocytes were then transferred into four-well dishes (Nunc) containing 500 μL fertilization medium and 2.0×10^6 spermatozoa/mL. After co-incubation of oocytes with spermatozoa for 18 h at 39°C in a humidified atmosphere of 5% CO_2 in air, presumptive zygotes were washed three times in 500 μL H-SOF containing non-essential amino acids and 4 mg/ml BSA at 39°C to remove adhered spermatozoa. After that, they were washed twice in embryo culture medium (C.SOF; m-SOFAaci containing BME-essential amino acid and MEM-non essential amino acids and FAF-BSA 4 mg/mL). Following washing, the embryos (15–20) were transferred into 50 μL drops of pre-warmed and equilibrated C.SOF-BSA culture medium covered with mineral oil and incubated at 39°C in a humidified atmosphere of 5% O_2 , 5% CO_2 and 90% N_2 until day 7 (Day 0 = Day of insemination). Cleavage was assessed at 24 and 48 h post insemination (h.p.i.). Development to morula and blastocyst was evaluated on day 5 and 7 p.i., respectively. Blastocysts were morphologically evaluated under a stereomicroscope and divided into early, expanded or hatched blastocysts. Mean cell numbers per blastocyst was evaluated by staining of blastocysts with 10 $\mu\text{g}/\text{mL}$ Hoechst 33342 [13]. The samples were examined under fluorescence microscope (Leica DMR, Germany) fitted with a digital camera (Hamamatsu, Japan) and image analysis software (Simple PCI, Compix Inc., USA).

2.8. Statistical analysis

At least three replicates were repeated for each experimental group. Data were analyzed using Chi-squared test. MPF and MAPK activities were analyzed by un-paired student's *t*-test. All results are considered to be statistically significant at $P \leq 0.05$. The statistical analysis was performed using Graph Pad Software (<http://www.graphpad.com>).

3. Results

3.1. Effect of caffeine supplementation during IVM on spindle assembly and chromatin configuration of ovine oocytes vitrified at the GV-stage

As shown in Table 1, irrespective to caffeine supplementation, vitrification of ovine GV-oocytes reduced the proportions of oocytes with

normal spindle and chromosome configuration (43.8–47.8%) as compared with those in the control groups (67.6–77.3%). However, the difference between vitrified and control – oocytes (matured in caffeine free media) was not significant. Furthermore, the percentages of oocytes with missing spindle significantly increased in both toxicity and vitrified groups (22.7–28.3%) than in the control (6.8–9.1%). Caffeine supplementation during the last 6 h of IVM did not significantly improve ($P > 0.05$) the percentages of oocytes with normal spindle assembly and chromosome configuration in vitrified, toxicity and control groups.

3.2. Effect of caffeine supplementation during IVM on MPF and MAP kinases activities of ovine oocytes vitrified at the GV-stage

As presented in Fig. 2, the activities of both MPF and MAP kinases were reduced in vitrified groups as compared to the controls; however, the differences were not statistically significant. Exposure of oocytes to vitrification and warming solutions (toxicity group) did not affect the activities of both kinases in comparison with the control group. The levels of both MPF and MAP kinases were lower in vitrified oocytes matured *in vitro* in caffeine supplemented medium (vitrified+) than those matured in non-supplemented medium (vitrified–).

3.3. Effect of caffeine supplementation during IVM on cleavage rates and preimplantation development of ovine oocytes vitrified at the GV-stage following IVF and embryo culture

As summarized in Table 2, cleavage at 24 and 48 h.p.i. was significantly lower ($P \leq 0.05$) in vitrified groups either treated with (2.8% and 30.3%) or without (4.3% and 32.2%) caffeine than those in the other groups. Irrespective to caffeine supplementation, no significant differences were observed in the percentages of cleaved embryos at 48 h.p.i. between toxicity and control groups (values ranged from 58.1% to 78.6%). Development to morula (day 5 p.i.) was significantly lower ($P \leq 0.05$) in both vitrified groups (values ranged from 24.0% to 25.4%) than those in toxicity (45.1%) and control (41.6%) groups *in vitro* matured in caffeine supplemented media. However, no significant differences were observed in morula development between vitrified, toxicity, and control oocytes matured in caffeine free IVM media. Irrespective to caffeine supplementation, blastocyst rates were significantly lower ($P \leq 0.05$) in vitrified groups (1.7–4.2%) than those in toxicity and control ones (30.2–32.2%). The same trend was observed when blastocyst rates were calculated on the basis of the number of the cleaved embryos. Caffeine supplementation during the last 6 h of IVM did not improve blastocyst development in vitrified groups. Morphological evaluation of the blastocysts revealed no hatched blastocysts produced in both vitrified groups; however, from 8.3% to 16.1% were recorded in toxicity and control groups. Mean cell numbers per blastocysts were significantly lower ($P \leq 0.05$) in vitrified (+) blastocyst (54.0) as compared to other treatments (107.2, 117.3, 109.6, and 100.6, in toxicity and control + and –, respectively); however, this value did not differ significantly from those reported in the vitrified (caffeine –, 87.0) group (Fig. 3).

4. Discussion

In the present studies we hypothesized that incubation of vitrified/warmed ovine GV-oocytes with caffeine during IVM could restore spindle and chromosome integrities, the activities of MPF and MAP kinases, and embryo development following IVM/IVF. We demonstrated that vitrification of ovine oocytes at the GV-stage reduced the frequencies of oocytes with normal spindle configuration and MPF/MAP kinases activities and preimplantation development after IVM/IVF and embryo culture and caffeine supplementation during IVM failed to restore the quality and development potential of vitrified/thawed oocytes. In the present study the regimen of caffeine treatment (10 mM for

Table 1
Effect of caffeine treatment during IVM on spindle and chromatin configuration of ovine oocytes vitrified at GV-stage.

Treatments	Caffeine	No. Oocytes	Spindle No. (%)			Chromosome No. (%)	
			Normal	Abnormal	Missing	Normal	Abnormal
Vitrified	+	46	22 (47.8) ^{ab}	11 (23.9) ^a	13 (28.3) ^a	22 (47.8) ^{ab}	24 (52.2) ^a
	-	57	25 (43.8) ^a	16 (28.1) ^a	16 (28.1) ^a	25 (43.8) ^a	32 (56.1) ^a
Toxicity	+	44	23 (52.3) ^{ab}	11 (25.0) ^a	10 (22.7) ^{ac}	23 (52.3) ^{ab}	21 (47.7) ^{ac}
	-	46	21 (45.7) ^a	13 (28.3) ^a	12 (26.1) ^a	21 (45.7) ^a	25 (54.3) ^a
Control	+	44	34 (77.3) ^b	6 (13.6) ^a	4 (9.1) ^{bc}	34 (77.3) ^b	10 (22.7) ^b
	-	102	69 (67.6) ^{ab}	26 (25.5) ^a	7 (6.8) ^b	69 (67.6) ^{ab}	33 (32.4) ^{bc}

Unless indicated otherwise, data show the number of oocytes at each category, with percentages given in parentheses. + means oocytes were incubated in maturation medium with 10 mM caffeine (18–24 h.p.m.), while; - means oocytes matured in medium without caffeine. Values with different superscripts in the same column differ significantly ($P \leq 0.05$).

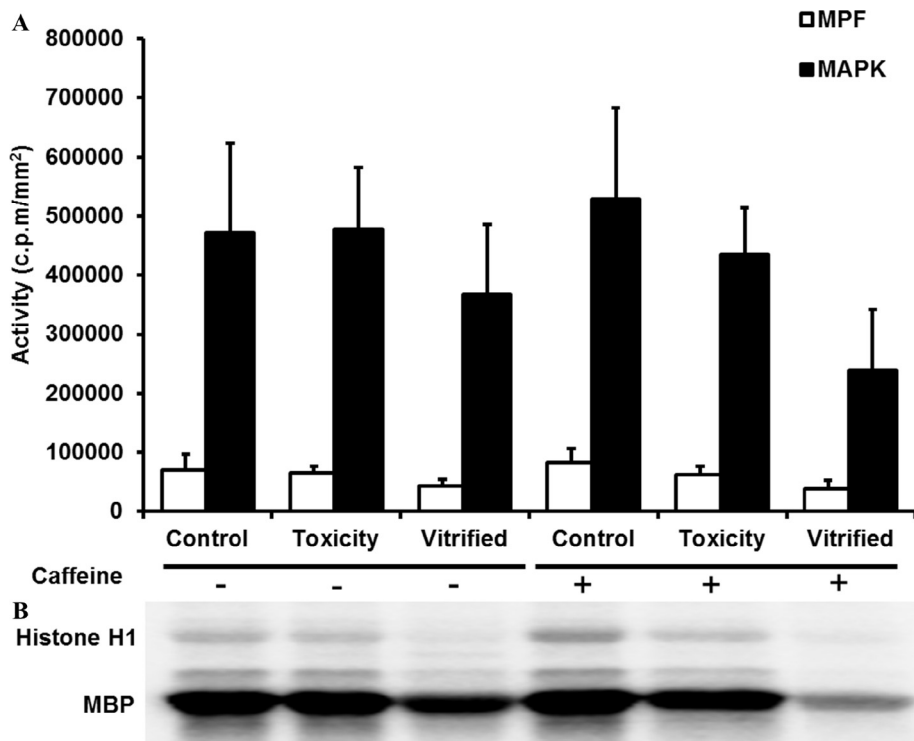


Fig. 2. Effects of caffeine supplementation during IVM on maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities of ovine oocytes vitrified at the germinal vesicle stage. Oocytes from control, toxicity and vitrified groups were incubated in IVM medium either supplemented with + or without - caffeine (10 mM) at 18–24 h.p.m., at 24 h.p.m., MPF and MAPK activities were detected in oocytes with an *in vitro* double-kinase by phosphorylation of histone H1 and myelin basic protein (MBP), respectively. A) Relative kinase activities (cpm/mm²). B) Phospho-image of PAGE, ³²P radioactivity representing kinase activities. Ten oocytes were analyzed in each group and three replicates were repeated for each experimental group. Data are presented as means ± SEM.

the last 6 h of IVM) was chosen based on the previous studies that have reported the positive impact of caffeine on *in vitro* matured ovine oocytes on the activities of MPF and MAP kinases and on the development of SCNT and IVF embryos [36–39,41]. In these studies the authors have reported the encouraging results based on exposure of denuded oocytes to caffeine; therefore, in our study we also used denuded oocytes to

study the effects of caffeine treatment. In mice, recent studies showed that supplementation of vitrification and warming solutions with caffeine maintained MPF levels and improved embryonic development after IVF of vitrified/warmed MII denuded oocytes [42]. The mechanism of action of caffeine on the levels of both kinases in oocytes is not well recognized and may differ between cumulus oocyte complexes

Table 2
Effect of caffeine treatment during IVM on developmental potential of ovine oocytes vitrified at the germinal vesicle stage.

Treatment	Caffeine	No. Oocytes	No. Cleaved (%)		No. Morula (%)	No. Blastocysts (% of oocytes)				Total blastocysts (% cleaved at 48 h.p.i.)
			24 h.p.i.	48 h.p.i.		Early	Expanded	Hatched	Total	
Vitrified	+	142	4 (2.8) ^a	43 (30.3) ^a	36 (25.4) ^a	5 (3.5) ^{ac}	1 (0.7) ^a	0 (0.0) ^a	6 (4.2) ^a	13.9 ^a
	-	115	5 (4.3) ^a	37 (32.2) ^a	28 (24.3) ^a	0 (0.0) ^c	2(1.7) ^{ac}	0 (0.0) ^a	2 (1.7) ^a	5.4 ^a
Toxicity	+	98	31 (31.6) ^b	77 (78.6) ^b	53 (45.1) ^{bc}	9 (9.2) ^a	10 (10.2) ^b	12 (12.2) ^b	31 (31.6) ^b	40.3 ^b
	-	93	21 (22.6) ^b	54 (58.1) ^b	33 (35.5) ^{ac}	3 (3.2) ^{ab}	12 (12.9) ^b	15 (16.1) ^b	30 (32.2) ^b	55.5 ^b
Control	+	149	47 (31.5) ^b	94 (63.1) ^b	62 (41.6) ^{bc}	11 (7.4) ^{ab}	10 (6.7) ^{bc}	24 (16.1) ^b	45 (30.2) ^b	47.9 ^b
	-	96	52 (45.2) ^c	67 (69.8) ^b	37 (38.5) ^{ac}	10 (10.4) ^b	11 (11.5) ^b	8 (8.3) ^b	29 (30.2) ^b	43.3 ^b

Cleavage rates were evaluated at 24 and 48 h post insemination (h.p.i.). Morula and blastocyst rates were evaluated on Days 5 and 7 post insemination, respectively. Unless indicated otherwise, data show the number of oocytes at each stage, with percentages given in parentheses. + means oocytes were incubated in maturation medium supplemented with 10 mM caffeine (18–24 h.p.m.), while; - means oocytes matured in medium without caffeine. Values with different superscripts in the same column differ significantly ($P \leq 0.05$).

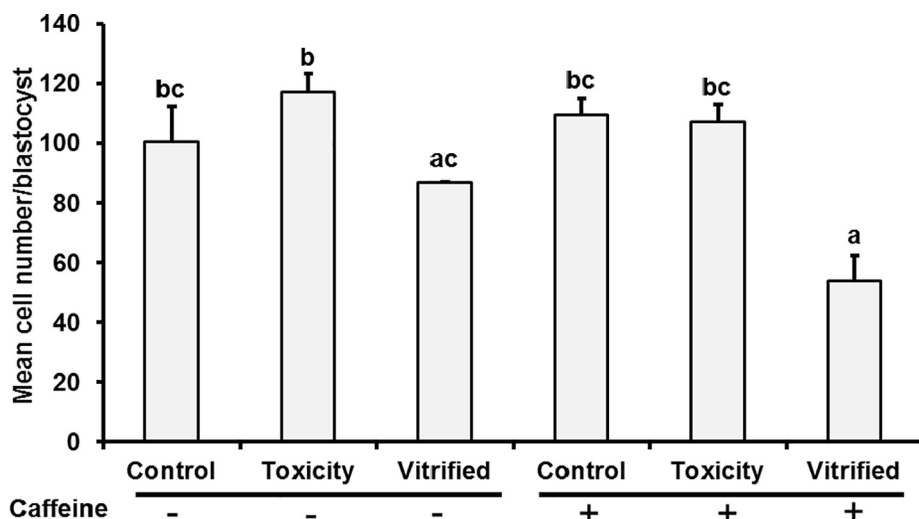


Fig. 3. Effects of caffeine supplementation during IVM of ovine oocytes vitrified at the germinal vesicle stage on mean cell numbers of blastocysts produced after IVF and embryo culture. Mean cell numbers per blastocyst were determined by staining of day 7 blastocysts with Hoechst 33342 (10 µg/mL). Data are presented as means ± SEM. Bars with different letters denote significant differences at ($P \leq 0.05$).

and denuded oocytes [39].

It is well known that normal spindle configuration and chromosome alignment in MII-oocytes is crucial for successful fertilization and embryo development after IVF [43]. The effects of cryopreservation on an oocyte's spindle assembly and chromosome arrangement have been reported in different species, including the mouse [10,25], human [44,45], bovine [46,47], porcine [48], equine [26], and ovine [13,49]. In sheep, previous studies have shown that spindle configuration in vitrified-warmed MII oocytes is influenced by the cryodevice used, with higher percentages of oocytes with abnormal spindle morphology noted following the use of open pulled straw (OPS) and cryoloop compared with cryotop devices [49]. In the present study we found that vitrification of ovine GV-oocytes negatively impacted spindle assembly and chromosome arrangement after IVM. We also noticed that caffeine supplementation during the last 6 h of IVM did not improve spindle alignment in vitrified group (Table 1). In contrast to our observations, previous studies showed that caffeine treatment at 20 mM during the last three hours of IVM before vitrification was able to keep the normality of chromatin organization in vitrified/warmed ovine MII-oocytes in similar values as in the control group. Discrepancies between the results could be due to the stage at which oocytes were vitrified, oocyte quality, method of vitrification, and the regimen of caffeine treatment. In mice, previous studies showed that caffeine treatment during IVM impacts the spindle morphology in dose dependant manner; a high percentage of oocytes with normal spindle morphology were noticed in 5 or 10 mM caffeine treated groups as compared to those treated with 0 or 1 mM [50]. Perturbations in the meiotic spindle configuration following IVM of vitrified GV-oocytes could be associated with cryodamage to key regulatory proteins, such as MPF and MAPK, which are involved in spindle formation and microtubule organization [13]. Herein, there was a tendency for the activity of both kinases to be lower in vitrified oocytes compared with control oocytes, although the differences failed to reach statistical significance. However, the activities of both kinases were comparable in the toxicity group and the control one. These observations indicate that cryopreservation may induce the degradation of molecules involved in the signalling pathways that are involved in the regulation of kinase activity. Previous studies have reported that the activity of MPF decreased significantly in OPS, cryoloop, and cryotop vitrified-warmed MII ovine oocytes compared with fresh controls; however, this activity was restored after 2 h *in vitro* culture in OPS- and cryoloop-vitrified oocytes, but not in cryotop-vitrified oocytes [49]. Our results also showed that caffeine supplementation during IVM did not improve the levels of MPF and MAPK in vitrified oocytes suggesting that the damaging effects on both kinases during vitrification could not be restored by caffeine treatment.

Further studies are needed to elucidate the effects of other caffeine concentrations or methods of treatment on the quality of vitrified oocytes. In sheep, it has been shown that the treatment of COCs with 20 mM caffeine for the last 3 h of IVM and before vitrification stabilizes the activity of MPF at similar levels to that of control oocytes [51]. In mice, recent studies have illustrated that caffeine supplementation at 10 mM during vitrification and warming of MII-oocytes maintained MPF activity after cryopreservation [42].

The deleterious consequences arising from cryodamage may also appear during cleavage or pre-implantation development [52]. In fact, it has been documented that, vitrified-warmed oocytes cleave and reach blastocyst stage in significantly lower proportions as compared to control [12,15,53,24]. The reduction in developmental rates obtained after IVM and IVF of vitrified/warmed immature oocytes can be ascribed to structural, biochemical, and molecular changes that could occur as a consequence of vitrification process [12–15,25,24] or as a result of exposure to cryoprotectants [54]. Our results showed that frequencies of cleaved embryos (24 and 48 h.p.i.) and blastocyst rates were significantly lower in vitrified oocytes than in toxicity and control groups. We also demonstrated that caffeine supplementation during the last 6 h of IVM did not improve these percentages in vitrified oocytes (Table 2). Again, under our experimental conditions, these results confirm inability of caffeine to restore the quality and development of vitrified/warmed oocytes. The positive impact of caffeine on developmental potential of vitrified/warmed oocytes has been reported in different species. For example, in sheep, previous studies showed that treatment of oocytes before vitrification with 20 mM caffeine for the last three hours of IVM reduced spontaneous activation than non-treated groups [51]. The authors attributed their findings to the prolonged period of meiotic arrest of vitrified MII oocytes in caffeine treated group [51]. It has been documented that caffeine could maintain the quality of aged oocytes safely in mouse as aged oocytes treated by caffeine has a potential to be activated [55]. In mice, supplementation of vitrification and warming media with 10 mM caffeine significantly improved blastocyst rates after IVF of MII-oocytes [42]. In cattle, pre-treatment of oocytes with caffeine before IVM has shown to delay meiotic resumption and improved blastocyst quality and their tolerance to cryopreservation [56]. Regarding the quality of *in vitro* produced blastocysts, our results revealed that vitrification and warming of oocytes reduced the mean cell numbers/blastocyst as compared with the control; however, the difference was not statistically significant. We also demonstrated that caffeine treatment failed to recover or even it dramatically reduced the quality of blastocysts (Fig. 3). In mice, it has been shown that vitrification of MII-oocytes in the presence of 10 mM caffeine did not significantly impact the total cell

numbers per blastocyst [42].

The results presented herein indicate that exposure of oocytes to vitrification solutions (toxicity group) has no detrimental impact on the development of oocytes. This improvement in the toxicity trials may be related to the strict short exposure time to the cryoprotectants during the vitrification and warming procedures. This regimen of exposure is beneficial to surpass the toxic effects of high concentrations of chemicals.

5. Conclusions

In conclusion, our results suggest that vitrification of ovine GV-oocytes negatively impact oocyte quality and development after IVM/IVF and embryo culture. Supplementation of *in vitro* maturation media with 10 mM caffeine during the last 6 h of IVM failed to restore the spindle integrities, MPF/MAPK activities and subsequent development of vitrified/warmed oocytes at the germinal vesicle stage. Further studies are needed to test different regimen (i.e. different concentrations, various time and methods of treatment) of caffeine on developmental potential of cryopreserved oocytes.

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Competing interests

All authors declare no competing interests.

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