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Oocyte vitrification induces loss of DNA methylation and histone acetylation in the resulting embryos derived using ICSI in dromedary camel

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Research Article

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Summary

Oocyte cryopreservation has become an important component of assisted reproductive technology with increasing implication in female fertility preservation and animal reproduction. However, the possible adverse effects of oocyte cryopreservation on epigenetic status of the resulting embryos is still an open question. This study evaluated the effects of MII-oocyte vitrification on gene transcripts linked to epigenetic reprogramming in association with the developmental competence and epigenetic status of the resulting embryos at 2-cell and blastocyst stages in dromedary camel. The cleavage rate of vitrified oocytes following intracytoplasmic sperm injection was significantly increased compared with the control (98.2 ± 2 vs. 72.7 ± 4.1%, respectively), possibly due to the higher susceptibility of vitrified oocytes to spontaneous activation. Nonetheless, the competence of cleaved embryos derived from vitrified oocytes for development to the blastocyst and hatched blastocyst was significantly reduced compared with the control (7.7 ± 1.2 and 11.1 ± 11.1 compared with 28.1 ± 2.6 and 52.4 ± 9.9%, respectively). The relative transcript abundances of epigenetic reprogramming genes *DNMT1*, *DNMT3B*, *HDAC1*, and *SUV39H1* were all significantly reduced in vitrified oocytes relative to the control. Evaluation of the epigenetic marks showed significant reductions in the levels of DNA methylation (6.1 ± 0.3 vs. 9.9 ± 0.5, respectively) and H3K9 acetylation (7.8 ± 0.2 vs. 10.7 ± 0.3, respectively) in 2-cell embryos in the vitrification group relative to the control. Development to the blastocyst stage partially adjusted the effects that oocyte vitrification had on the epigenetic status of embryos (DNA methylation: 4.9 ± 0.4 vs. 6.2 ± 0.6; H3K9 acetylation: 5.8 ± 0.3 vs. 8 ± 0.9, respectively). To conclude, oocyte vitrification may interfere with the critical stages of epigenetic reprogramming during preimplantation embryo development.

Introduction

Oocyte cryopreservation is a rapidly developing assisted reproductive technology thanks to its indispensable importance for fertility preservation, oocyte donation programmes, and overcoming ethical issues related to embryo cryopreservation (Rienzi *et al*., 2010). In farm animals, the establishment of a cryobank of oocytes retrieved from high genetic merit animals would have practical importance for multiplication or conservation of elite genomes (Hosseini *et al*., 2015). The adverse effects of cryopreservation on oocyte competence, however, is still an open challenge in most mammalian species, as the live birth rate per vitrified mature oocyte is currently only 5.9% for mature (MII) human oocytes (Chamayou *et al*., 2011) and even less successful for *in vitro* maturation of immature oocytes (Schattman, 2015; Reader *et al*., 2017). Accordingly, investigations are underway to improve the current knowledge of oocyte cryobiology (Hosseini and Nasr-Esfahani, 2016).

The changes induced by exposition to cryoprotectants and subphysiological temperature in several aspects of oocyte physiology including biological behaviour (e.g. survival, fertilization, and early embryonic development) and structural cellular features (e.g. meiotic spindle assembly, chromosome alignment, mitochondrial potential and ultrastructure) have been extensively studied (Di Pietro *et al*., 2010; Hosseini and Nasr-Esfahani, 2016). More recent studies also investigated the effects of oocyte cryopreservation on mRNA transcripts related to cellular homeostasis including redox status, mitochondrial activity, oxidative stress, apoptosis, and cell cycle (Anchamparuthy *et al*., 2010; Di Pietro *et al*., 2010; Monzo *et al*., 2012; Chang *et al*., 2013; de Oliveira Leme *et al*., 2016; Hosseini and Nasr-Esfahani, 2016; Saadeldin *et al*., 2020).

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Importantly, by probing the transcriptome of mouse embryos developed from cryopreserved oocytes, Eroglu *et al.* (2020) showed that oocyte cryopreservation results in lasting injuries to oocytes that affected embryonic gene expression pattern characterized by distinctly upregulated and downregulated pathways. This may explain the poor development of embryos resulting from cryopreserved oocytes in the context of effects that cryopreservation has on embryonic genome activation and subsequent modulation of gene expression thereafter (Eroglu *et al.*, 2020).

Another area of concern that merits further study is the effect of cryopreservation on mRNA content of genes responsible for epigenetic reprogramming in newly developed zygotes (Shirazi *et al.*, 2016). Upon fertilization, the complementally parental genomes undergo significant epigenetic reprogramming changes including genomic transcription, genomic imprinting, X-chromosome inactivation, and induction of totipotency in the newly developed zygote (Hackett *et al.*, 2013). These epigenetic changes are governed by a myriad of known/unknown cytoplasmic factors including mRNAs, protein and other substrates. Therefore, any changes in the epigenetic machinery of oocyte could lead to subsequent changes in developmental competence of the developing embryos. Shirazi *et al.* (2016) demonstrated changes in transcript abundances of key epigenetic genes including DNA methyltransferases 1 (*DNMT1*) and 3b (*DNMT3B*), histone deacetylase (*HDAC1*), histone acetyl transferase (*HAT1*), *SMARG*, and *HMG3A* in vitrified ovine oocytes. However, Chen *et al.* (2016) did not observe any marked influence of oocyte vitrification on transcript abundances of vitrified bovine oocytes. In human, vitrification of immature oocytes resulted in reduced expression of *DNMT1* when assessed after *in vitro* maturation for 36 h (Shahedi *et al.*, 2017). This controversy may explain species-specific susceptibilities to vitrification, and perhaps other technical and biological differences that may preclude direct extrapolation of a final conclusion in other non-tried species. Another important question is to know if changes in epigenetic reprogramming transcripts could be translated to actual changes in the epigenetic stability of the resulting embryos. The answer to this question is reflected in a few recent studies in mouse (Cheng *et al.*, 2014; Cao *et al.*, 2019; Fu *et al.*, 2019) and bovine (Chen *et al.*, 2016). This would enable us to better discover the possible disturbing effects of oocyte vitrification on epigenetic stability of embryos during preimplantation and postimplantation development and the capacity of embryos to adjust the intimate and late effects of oocyte vitrification on epigenetics (Cheng *et al.*, 2014).

There is a clinical demand for oocyte cryopreservation of elite dromedary camels (*Camelus dromedarius*) in a cryobank setting. Our recent preliminary study showed particular sensitivity of MII oocytes in this species to the vitrification procedure, possibly due to their high lipid content (Moulavi and Hosseini, 2019). A study by Fathi *et al.* (2018) showed that vitrification of dromedary camel oocytes at the germinal vesicle stage using Cryotop supported superior results compared with solid surface vitrification following IVF. In a recent study, we showed that vitrification of camel MII oocytes adversely affects mitochondrial functions, although the effect was transient without compromising the developmental potential of the oocytes after parthenogenetic activation and intracytoplasmic sperm injection (ICSI) (Saadeldin *et al.*, 2020). However, the effect of vitrification on oocyte transcripts in association with subsequent effects on epigenetic stability of the resulting embryos remain unravelled. Moreover, while ICSI is routinely used for vitrified-warmed oocytes to overcome zona hardening and to ensure a reliable and timed insemination method (O'Neill *et al.*, 2018), the practical usage of ICSI for fertilization of vitrified oocytes has not been not investigated. Therefore, this study evaluates the effects of oocyte vitrification on maternal transcripts linked to epigenetic reprogramming and their subsequent effects on *in vitro* embryo development and epigenetic status of the resulting embryos during *in vitro* embryo development at the 2-cell and blastocyst stages.

Materials and methods

Unless specified otherwise, all chemicals and media were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA), respectively.

Oocyte preparation, in vitro *maturation (IVM), and vitrification*

The procedure for dromedary camel oocyte IVM has been described previously (Moulavi *et al.*, 2020). In brief, cumulus–oocyte complexes (COCs) were retrieved by aspiration of superficial follicles (2–8 mm) from abattoir-derived camel ovaries cold stored for 24–48 h at 10°C and using an 18-gauge needle into HEPES-buffered tissue culture medium 199 (HTCM199) supplemented with 3 mg/ml polyvinylpyrrolidone (PVP) and 2 IU/ml heparin. COCs with evenly granulated cytoplasm and at least three layers of compacted cumulus cells were selected for culture in groups of 30–35 in 500 µl of IVM medium prepared in Nunc 4-well dishes at 38.5°C, in 6% CO_2 in air and maximum humidity for 30–32 h. IVM medium was comprised of TCM199 supplemented with 10% fetal bovine serum (FBS), 2.5 mM Na-pyruvate, 50 µg/ml gentamycin, 1 mg/ml estradiol-17β, 10 µg/ml FSH, 10 µg/ml LH, 1 mM L-glutamine, 0.1 mM cysteamine, and 10 ng/ml EGF. Following IVM, cumulus cells attached to oocytes were removed by brief vortexing in 0.1% hyaluronidase in HTCM199 + 10% FBS. Only matured oocytes with the extruded/extruding first polar body were used for the experiments.

For vitrification, as described previously (Moulavi and Hosseini, 2019), denuded oocytes in each experimental group were incubated in 100 µl phosphate-buffered saline (PBS) supplemented with 20% FBS (basic solution: BS) for 1 min. In addition, three separate droplets of 100 µl equilibration solution [ES: 7.5% ethylene glycol (EG,) and 7.5% dimethyl sulfoxide (DMSO) in BS] were placed in the vicinity of each other and BS. Under a stereomicroscope, the BS droplet containing oocytes was gently connected to its nearest ES drop, and the BS-ES drop was connected to the next ES drop. Ultimately, the BS-ES-ES drop was connected to the third ES drop (BS-ES-ES-ES). The time taken between each step was 1 min. The equilibrated oocytes were then incubated in vitrification solution (VS: 15% EG, 15% DMSO and 0.5 M sucrose in BS) for 20 to 25 s. In the next step, five oocytes with the minimum VS medium were aspirated and placed on the tip of the Cryotop (Kitazato®, Japan) and quickly plunged into liquid nitrogen in less than 20 s. All incubations were performed at room temperature (RT). For warming, Cryotops were removed from liquid nitrogen and quickly plunged in warming solution 1 (WS1: 1 M sucrose in BS at 38.5°C). After 1 min, warmed oocytes were transferred into WS2 for 3 min (0.5 M sucrose in BS at RT) and then another 3 min into WS3 (0.25 M sucrose in BS at RT). Afterwards, the oocytes were incubated in the washing solution (BS) for 5 min. Vitrified-warmed oocytes were cultured for recovery in maturation medium for 0.5 h before being used for ICSI. The oocytes with morphologic evidence of shrinkage, cytoplasmic vacuolization, or leakage in plasma membrane were recorded as degenerated oocytes.

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Intracytoplasmic sperm injection and embryo development

Epididymal sperm were collected from cauda epididymis of testes derived from slaughtered mature bulls as described by Martinez-Pastor *et al.* (2006). The prepared semen samples were washed twice by centrifugation in HEPES-buffered Tyrode's albumin lactate pyruvate medium and the highly motile sperm was prepared by density gradient centrifugation and the prepared sperm pellet was diluted 1:10 in a Tris-based medium supplemented with 1% lecithin, 20% egg yolk, and 7% (v/v) glycerol, and then frozen and stored in liquid nitrogen until use. At 2 h before commencing ICSI, frozen semen thawed at 37°C and highly motile sperm was diluted and incubated in Tyrode's albumin lactate pyruvate medium containing 80 mM heparin + 15 mM glutathione (GSH) for 30 min at 38.5°C and 6% CO_2 in air for *in vitro* decondensation. Our previous study in bovine (Sekhavati *et al.*, 2012) and our preliminary study here (data not shown) showed that sperm *in vitro* decondensation before ICSI using heparin–GSH substantially increased the efficiency of male pronuclear formation and diploid *in vitro* development of embryos. Decondensed sperm cells were used for ICSI as routinely performed, except that the decondensed sperm is immotile and the inner diameter of the microinjection needle should be larger (about 10–15 µm) compared with usual ICSI. Vitrified-warmed and control oocytes at approximately 35 h post maturation were used for ICSI. At 30 min following ICSI, oocytes were activated using 5 µM ionomycin prepared in HTCM199 for 5 min, followed by washing in HTCM199. Activated oocytes were rested for 3 h in mSOF medium before being incubated in 2 mM 6-dimethylaminopurine for 4 h. The presumptive embryos were cultured (IVC) in groups of 6–8 in 20-µl droplets of mSOF under mineral oil at 38.5°C, 6% CO_2, and 5% O_2 in humidified air for 3 days before refreshing in fresh mSOF containing 10% charcoal stripped FBS for another 4 days. Cleavage was assessed on day 2 post ICSI and development of cleaved embryos to the morula, blastocyst and hatching/hatched blastocyst stages were assessed on days 4, 7, and 7–8 post ICSI, respectively (Moulavi and Hosseini, 2019).

RNA extraction, cDNA synthesis and relative quantitative PCR (RQ-PCR)

The transcript abundances of four well known epigenetic reprogramming genes (DNA methyltransferase 1 (*DNMT1*), DNA methyltransferase 3B (*DNMT3B*), Histone Deacetylase 1 (*HDAC1*) and suppressor of variegation 3–9 homolog 1 (*SUV39H1*)) and *ACTB* housekeeping gene were compared between vitrified and fresh oocytes, as described previously (Saadeldin *et al.*, 2019). In brief, the specific primers were designed using Primer-Blast 9 (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The expected products of primers in PCR were checked in Nucleotide-Blast 11 (https://blast.ncbi.nlm.nih.gov/Blast.cgi), which found no similarity with other dromedary camel genes. Table 1 represents the information regarding gene functions, primer sequences, annealing temperatures, and approximate product sizes of the amplified fragments of the candidate genes. The housekeeping gene *GAPDH* was used as the reference gene. Total RNA extraction from pooled vitrified and control oocytes was performed using the innuPREP RNA Mini Kit (Analytik Jena AG, Jena, Germany) according to the manufacturer's instruction. Total numbers of oocytes used for Rq-PCR were 33 and 30 for vitrification and control groups, respectively, in three replicates. RNA quantity and quality was checked using a NanoDrop™ spectrophotometer (Thermo Fisher, Waltham, MA, USA). Pulsed reverse transcription was conducted to generate complementary DNA (cDNA). Next, 20 ng RNA in a 20-µl reaction volume was used as the template for cDNA synthesis using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Reactions were subjected to 80 cycles of 2 min at 16°C, 1 min at 37°C, 0.1 s at 50°C, followed by final inactivation at 85°C for 5 min. The RT-qPCR was performed using a ViiA 7 thermal cycler (Applied Biosystems). The reactions comprised 100 ng cDNA, 1 µM forward primer, 1 µM reverse primer, and 1× Fast SYBR™ Green Master Mix (Applied Biosystems). The fold change and relative quantities of the target genes were calculated according to the $2^{-\Delta\Delta Ct}$ method. Reactions without reverse transcriptase or cDNA template resulted in no amplification. The thermal cycler was adjusted to 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s.

DNA methylation and H3K9 acetylation

As described previously (Hosseini *et al.*, 2016), embryos at the 2-cell and blastocyst stages were pooled for immunostaining. Pooled embryos in each stage were washed in PBS containing 1 mg/ml PVA (PBS/PVA) and then fixed in 4% paraformaldehyde in PBS for 30 min, permeabilized by 0.1% Triton X-100 in PBS/PVA for 15 min and blocked with 1% BSA for 1 h. For DNA methylation, embryos were first pre-treated with 4 N HCl for 60 min at RT, and then washed with PBS/PVA. Embryos were then incubated with the primary antibody: either mouse anti-5-methylcytosine (5-mC) antibody [5MC-CD] (ab73938, 1/100 dilution) (for DNA methylation) or mouse monoclonal anti-H3K9 (Invitrogen MA 523517, 1/500 dilution) (for H3K9 acetylation: H3K9ac), for 1 h at RT, followed by three washes in PBS/PVA. Embryos were then incubated with the secondary antibody goat anti-mouse IgG-TRITC (ab6786, Ex: 547 nm, Em: 572 nm, 1/2000 dilution in PBS containing 0.2% BSA) for 45 min at RT. DNA was counterstained with 1 µg/ml Hoechst 33342 stain. Preparations were washed in PBS before mounting and imaging. Negative controls were processed the same way with the exception that the primary antibodies were replaced with blocking buffer. Pixel intensity of randomly selected nuclei was quantified (ImageJ software; National Institute of Mental Health, Bethesda, MD, USA) and normalized to the background. The median pixel intensity of both nuclei in 2-cell stage embryos and 10–15 nuclei in each blastocyst and the average pixel intensity of fluorescence emission was detected.

Statistical analysis

All experimental groups were repeated at least three times. The data were analyzed by one-way analysis of variance methodology using the general linear models procedure from the SAS statistical package (SAS, 2003). Some dependents variables were subjected to logarithm transformation prior analysis. Ryan–Einot–Gabriel–Welsch–Quiot (REGWQ) test was used for comparisons of means, and significance was declared at $P \leq 0.05$.

Results

Effect of oocyte vitrification on oocyte survival and in vitro embryo development

In this study, the average rate of *in vitro* oocyte maturation was 83.6% (Table 2). The rate of oocytes that survived the vitrification-warming procedure was 91.8 ± 2.9%. No significant difference

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Table 1. Primers used for relative quantitative PCR

Gene	Primer (5′→3′)	Function	Product size (bp)*	Accession no.
DNMT1	F: TTGAGTCGGGAAGTGAACGG; R: TCTCCGAGGCATGCAAAGTT	The predominant form of the family of DNMT enzymes in mammals. Is involved in maintaining methylation patterns and also imprints during preimplantation and early postimplantation embryonic development (Trasler *et al*., 1996)	126	XM_010978348.1
DNMT3B	F: TTCCGTGAAACCAGGACTCG; R: GATTCGTCCACGTGATTGCG	Required for genome *de novo* methylation and it is essential for establishment of DNA methylation patterns during development (Yoder *et al*., 1997)	137	XM_010981933.1
HDAC1	F: GCTGTCTACTGGTGGCTCTG; R: CGGACTTCTTTGCATGGTGC	The major member of the family of HDAC. Actively involved during preimplantation embryo development. Allows histones to wrap the DNA more tightly thereby rendering the DNA transcriptionally silent (McGraw *et al*., 2003)	104	XM_010976074.1
SUV39H1	F: TGGCATCTCCAAGAGGAACC; R: GTCTGGGTATCCACGCCATT	A member of HMT family with essential roles for constitutive heterochromatin formation, gene repression, and maintenance of genome integrity. Mediates trimethylation of H3K9 and can target Dnmt3A/3B to establish a transcriptionally repressed state (de Oliveira Leme *et al*., 2016)	109	XM_010996871.1
ACTB	F: ACTTGCGCAGAAAACGAGAT; R: CACCTTCACCGTTCCAGTTT	Housekeeping	117	XM_010997926
GAPDH	F: TGCTGAGTACGTTGTGGAGT; R: TCACGCCCATCACAAACATG	Housekeeping	134	XM_010990867

*The melting curve for each primer was evaluated by ViiA^{TM}7 apparatus-associated software and the product size was confirmed by gel electrophoresis of PCR products on 1.5% agarose referring to a 1 kb DNA ladder (Invitrogen, Carlsbad, USA).

Table 2. The effect of vitrification-warming procedure on oocytes and subsequent embryonic development

Aspirated ovaries	89	
Retrieved oocytes	386	
Cultured COCs	255	
Maturation oocytes	213 (83.6 %)	
Matured oocytes used for each group	Vitrification	Control
	115	92
Degenerated after vitrification warming	10 (8.2 ± 3.3)*	–
Survived vitrification warming	105 (91.8 ± 2.9)	–
Degenerated after ICSI procedure	7 (6.1 ± 2.3)	4 (4.9 ± 3.5)
Survived ICSI procedure (cultured)	98 (93.6 ± 2.1)	88 (95.8 ± 2.8)
Cleavage	96 (98.2 ± 2)^{a}	64 (72.7 ± 4.1)^{b}
Morula formation	78 (80.1 ± 8.5)	47 (72.9 ± 7.6)
Blastocyst formation	7 (7.7 ± 1.1)^{b}	18 (28.1 ± 2.6)^{a}
Blastocyst hatching	1 (11.1 ± 11.1)^{b}	10 (52.4 ± 9.9)^{a}

*: Values are means ± standard error of the mean (SEM).
Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way analysis of variance (ANOVA) and the Tukey test.

was found between the survival rates of oocytes following ICSI procedure (93.6 ± 2.1 and 95.8 ± 2.8%, for vitrified and control oocytes, respectively). Following ICSI, the cleavage rates of vitrified and control oocytes showed a significant difference in favour of vitrified oocytes (98.2 ± 2 vs. 72.7 ± 4.1%, respectively). The rates of cleaved embryos that further developed to the morula stage were not significantly different between the two groups (80.1 ± 8.5 vs. 72.9 ± 7.6%, for vitrified and control oocytes, respectively). However, vitrification significantly reduced the rate of resulting embryos that could develop to the blastocyst stage compared with that of the control (7.7 ± 1.1 vs. 28.1 ± 2.6%, respectively). Moreover, the hatching rate of blastocysts derived from vitrified-warmed oocytes was significantly lower compared with that of control oocytes (11.1 ± 11.1 vs. 52.4 ± 9.9%). Morphological quality of developed blastocysts showed no apparent difference between the two experimental groups (Fig. 1*A*, *B*).

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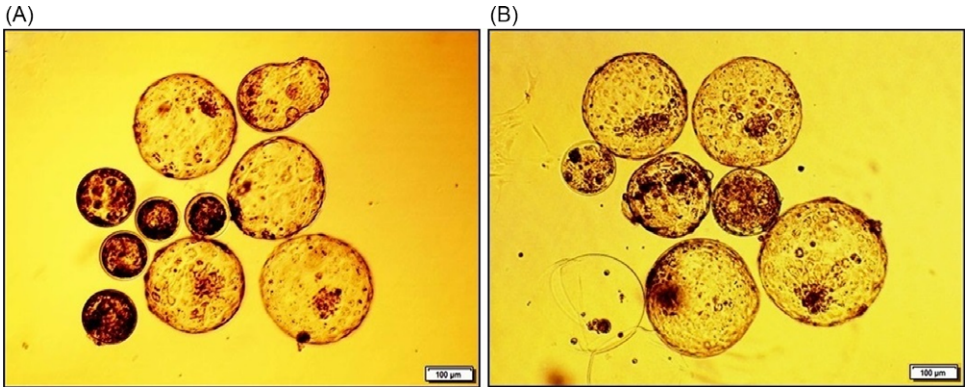

Figure 1. *In vitro* development of vitrified oocytes following ICSI in dromedary camel. Representative images of blastocysts developed in the vitrification (*A*) and control (*B*) groups. Scale bar: 100 µm.

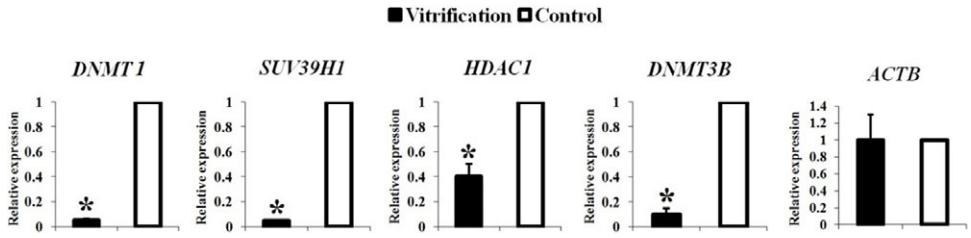

Figure 2. Effect of oocyte vitrification on transcript abundances of key epigenetic reprogramming genes in dromedary camel oocytes. Quantification of mRNA transcripts (*DNMT1*, *DNMT3B*, *HDAC1*, *SUV39H1* and *ACTB*) using RT-qPCR. Expression of mRNA in vitrified-warmed oocytes was normalized to that of fresh control oocytes. The primers used for amplification of these transcripts are listed in Table 1. Asterisk (*) indicates the significant difference between groups ($P < 0.05$, *t*-test). All experiments were independently repeated three times. Data were normalized against endogenous housekeeping gene *GAPDH*. For each replicate, 10–11 oocytes were pooled together. Total numbers of oocytes used for RT-qPCR were 33 and 30 for vitrification and control groups, respectively.

Effect of oocyte vitrification on maternal transcripts of epigenetic reprogramming genes

The relative abundances of four genes involved in epigenetic reprogramming were compared between control and vitrified-warmed oocytes. The genes include DNA methyltransferase 1 (*DNMT1*), DNA methyltransferase 3 beta (*DNMT3B*), histone deacetylase 1 (*HDAC1*), suppressor of variegation 3–9 homolog 1 (*SUV39H1*). The epigenetic roles of the selected genes are described in Table 1. The housekeeping genes *ACTB* were also included in the comparison to verify the normalization. As shown in Fig. 2, the oocyte vitrification-warming procedure resulted in significant reductions in the relative transcript abundances of all four epigenetic reprogramming genes ($P < 0.05$), but it did not affect the expression of the housekeeping gene *ACTB*.

Effect of oocyte vitrification on epigenetic status of 2-cell stage embryos

The effect of oocyte vitrification on global DNA methylation (Fig. 3*A*–*C*) and H3K9 acetylation (Fig. 4*A*–*C*) of the resulting ICSI embryos at the 2-cell stage was investigated. Accordingly, measurement of the 5mC fluorescence intensity showed that 2-cell embryos derived from vitrified oocytes were significantly hypomethylated compared with the control (relative intensity means: 6.1 ± 0.3 vs. 9.9 ± 0.5, respectively, $P < 0.05$) (Fig. 3*C*). Measurement of the H3K9ac fluorescence intensity showed a significant effect of oocyte vitrification on reduced histone acetylation level of the resulting embryos compared with that of the control (relative intensity means: 7.8 ± 0.2 vs. 10.7 ± 0.3, respectively, $P < 0.05$) (Fig. 4*C*).

Effect of oocyte vitrification on epigenetic status of the resulting embryos at the blastocyst stage

To understand if altered epigenetic status of early embryos derived from vitrified oocyte could persist at later stages of embryo development, blastocysts derived from vitrified-warmed and control oocytes were used to measure DNA methylation (Fig. 5*A*–*C*) and H3K9 acetylation (Fig. 6*A*–*C*). The global DNA methylation of blastocysts derived from vitrified oocytes showed a reduction compared with that in the control blastocyst, although this reduction was not statistically significant (relative intensity means: 4.9 ± 0.4 vs. 6.2 ± 0.6, respectively, $P > 0.05$) (Fig. 5*C*). Similarly, the H3K9 acetylation fluorescence intensity of blastocysts in the vitrification group showed a statistically non-significant reduction compared with the control blastocysts (relative intensity means: 5.8 ± 0.3 vs. 8 ± 0.9, respectively, $P > 0.05$) (Fig. 6*C*).

Discussion

Cryopreservation of oocytes retrieved from genetically superior animals has important practical importance for preserving of fertility in women and for multiplication and conservation of elite genomes in animals. Successful cryobanking of oocytes would also ensure a steady supply of oocytes for somatic cell nuclear transfer, stem cell production, and genetic engineering programmes (Hosseini *et al.*, 2015). While significant progress has been achieved in cryopreservation of farm animal oocytes with offspring born from frozen oocytes in cattle (Otoi *et al.*, 1996), horses (Maclellan *et al.*, 2002), and pig (Somfai *et al.*, 2014), this goal still remains in camels. In continuation with our previous study on vitrification and parthenogenetic activation of dromedary camel oocytes (Moulavi and Hosseini, 2019),

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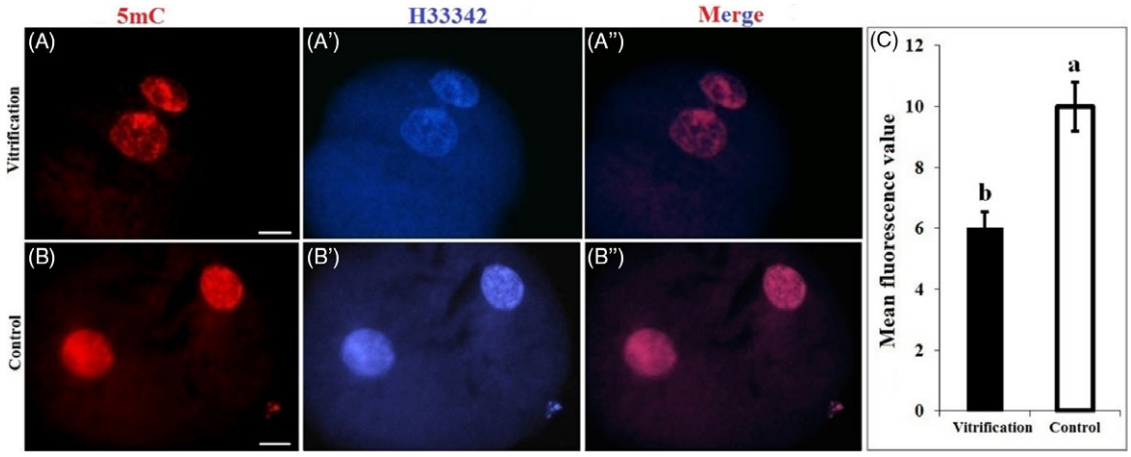

Figure 3. Effect of oocyte vitrification on global DNA methylation of the resulting 2-cell embryos in dromedary camel. *In vitro*-matured oocytes in the vitrification-warming and control groups used for ICSI, and the resulting 2-cell embryos were used for quantification of 5mC fluorescence intensity. (*A–B′′*) Representative images of 2-cell embryos immunostained for 5mC (red in *A* and *B*) followed by counterstaining with H33342 to visualize DNA (blue in *A′* and *B′*) and their merged views (*A′′*, *B′′*) in vitrification and control groups, respectively. (*C*) Quantification of 5mC signal intensities in 2-cell embryos derived from vitrified oocytes relative to that of the control oocytes. In each group, 11 embryos were used. Data are shown as mean ± SEM and different letters on the bars indicate a statistically significant difference between the two groups ($P < 0.05$). Scale bars: 25 µm.

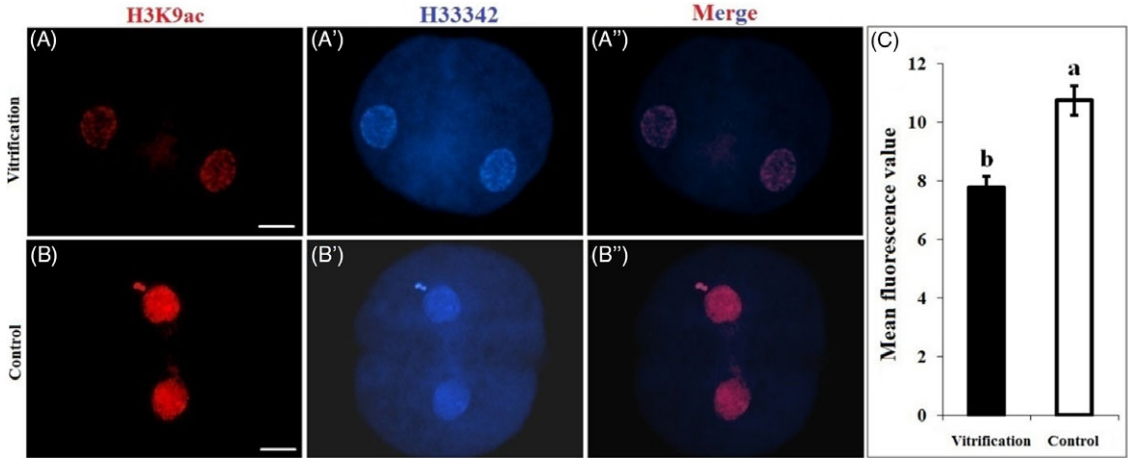

Figure 4. Effect of oocyte vitrification on histone acetylation of the resulting 2-cell embryos in dromedary camel. *In vitro*-matured oocytes in the vitrification-warming and control groups used for ICSI, and the resulting 2-cell embryos were used for quantification of H3K9 fluorescence intensity. *A–B′′*) Representative images of 2-cell embryos immunostained for H3K9 (red in *A* and *B*) followed by counterstaining with H33342 to visualize DNA (blue in *A′* and *B′*) and their merged views (*A′′*, *B′′*) in vitrification and control groups, respectively. (*C*) Quantification of H3K9 signal intensities in 2-cell embryos derived from vitrified oocytes relative to that of the control oocytes. In each group, 11 embryos were used. Data are shown as mean ± SEM and different letters on the bars indicate a statistically significant difference between two groups ($P < 0.05$). Scale bars: 25 µm.

this study investigated how oocyte vitrification may affect developmental competence and epigenetic status of the resulting ICSI embryos in this species. Obtained results indicate that *in vitro*-matured vitrified-warmed oocytes support cleavage and *in vitro* embryo development following ICSI. Although the cleavage rate was unexpectedly increased following vitrification warming, the decreased blastocyst rate indicated the impaired developmental potential of vitrified oocytes, a finding that is in agreement with our previous study in this species (Moulavi and Hosseini, 2019) and studies in other species [human (Rienzi *et al.*, 2010), bovine (Spricigo *et al.*, 2015), mouse (Cao *et al.*, 2019), goat (Srirattana *et al.*, 2013), and ovine (Hosseini and Nasr-Esfahani, 2016)]. Therefore, the observation of higher cleavage rate despite the subsequent lower blastocyst rate may explain an indirect effect of oocyte vitrification on the incidence of spontaneous activation, which results in the progression of oocyte meiosis and embryo mitosis in the absence of participation of the male genome. Spontaneous activation of vitrified oocytes has been demonstrated previously (Gardner *et al.*, 2007), and studies showed that vitrification of MII oocytes caused a decrease in MPF level due to the reduction in *CYCLIN-B* mRNA, which makes oocytes prone to spontaneous activation (Asgari *et al.*, 2012). Moreover, the blastocyst rate of vitrified oocytes that underwent ICSI in this study (7.7%) was lower, not only compared with fresh oocytes in the same study (28.1%), but also compared with the 22.9% blastocyst rate that was obtained in our previous study after parthenogenetic activation of vitrified-warmed oocytes in dromedary camel (Moulavi and Hosseini, 2019). The first part of the results in this study, therefore, indicated that in dromedary camels, as in other farm animal species, cryopreservation of MII oocytes is still not optimized and vitrification significantly reduces oocyte developmental competence. The development of an optimized oocyte vitrification protocol needs further studies focusing on the cryobiology of camel oocytes at cellular and molecular levels.

The newly developed embryos of mammals inherit a huge stockpile of maternal mRNAs, which are crucially important

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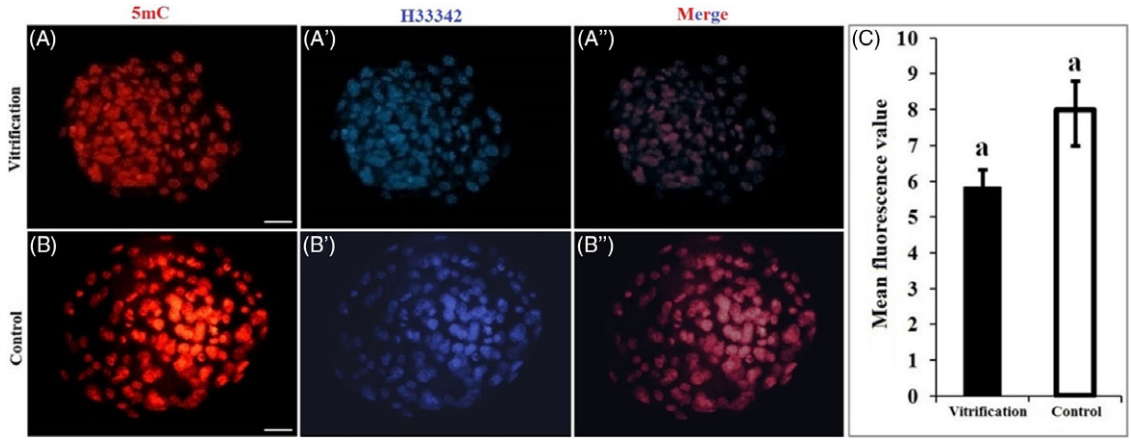

Figure 5. Effect of oocyte vitrification on global DNA methylation of the resulting blastocysts in dromedary camel. *In vitro*-matured oocytes in the vitrification-warming and control groups used for ICSI, and the resulting embryos were cultured up to the blastocyst stage to be used for quantification of 5mC fluorescence intensity. (*A*–*B*′′) Representative images of blastocysts immunostained for 5mC (red in *A* and *B*) followed by counterstaining with H33342 to visualize DNA (blue in *A*′ and *B*′) and their merged views (*A*′′, *B*′′) in vitrification and control groups, respectively. (*C*) Quantification of 5mC signal intensities in blastocysts derived from vitrified oocytes relative to that of the control oocytes. In each group, 7 blastocysts were used and in each blastocysts, 10–15 nuclei were analyzed. Data are shown as mean ± SEM and common letters on the bars indicate the lack of a statistically significant difference between two groups ($P < 0.05$). Scale bars: 25 µm.

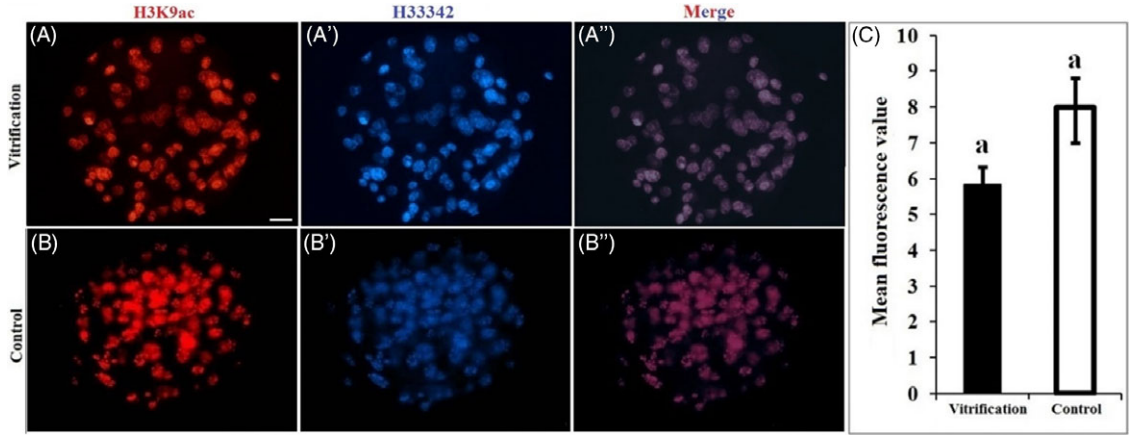

Figure 6. Effect of oocyte vitrification on histone acetylation of the resulting blastocysts in dromedary camel. *In vitro*-matured oocytes in the vitrification-warming and control groups used for ICSI, and the resulting embryos were cultured up to the blastocyst stage to be used for quantification of H3K9 fluorescence intensity. (*A*–*B*′′) Representative images of blastocysts immunostained for 5mC (red in *A* and *B*) followed by counterstaining with H33342 to visualize DNA (blue in *A*′ and *B*′) and their merged views (*A*′′, *B*′′) in vitrification and control groups, respectively. (*C*) Quantification of H3K9 signal intensities in blastocysts derived from vitrified oocytes relative to that of the control oocytes. In each group, 7 blastocysts were used and in each blastocysts, 10–15 nuclei were analyzed. Data are shown as mean ± SEM and common letters on the bars indicate the lack of a statistically significant difference between two groups ($P < 0.05$). Scale bar: 25 µm.

during early zygotic mitotic divisions until the activation of major portions of the embryonic genome occurs at a species-specific stage of development, i.e. the 8–16-cell stage in cow (Sirard, 2012). Available data on molecular profiling of vitrified oocytes and ovarian tissues are somewhat contradictory, from no or weak to significant effects of cryopreservation compared with their control counterparts (for review see Monzo *et al.*, 2012). The effects of vitrification on genes linked to epigenetic reprogramming have received less attention despite the large influence they have on cell specification and transcriptional regulation during embryo development (Shirazi *et al.*, 2016). Focusing on epigenetic-related genes, we observed that vitrification resulted in a significant reduction in the transcript abundances of all four genes assessed, *DNMT1*, *DNMT3B*, *HDAC1*, and *SUV39H1*. In ovine, Shirazi *et al.* (2016) observed a significant reduction in the expression profile of *DNMT1*, *DNMT3B*, *HDAC1*, and two other epigenetic genes (*SMARCAL1* and *HMGN3a*), while the relative abundance of *SUV39H1* remained unchanged between vitrified and fresh oocytes in their study. In mouse, the increased expression levels of all four types of *DNMT*s (*DNMT1*, *DNMT3A*, *DNMT3B*, and *DNMT3I*) were significantly reduced in vitrified oocytes (Cheng *et al.*, 2014). Assessment of the relative gene expression of the ten-eleven-translocation (*TET*) family of dioxygenases that oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) in DNA showed that *TET2/3* did not change in the first 2 h of vitrification warming in mice, but increased significantly after 2 h in mouse (Hajj and Haaf, 2013). Although our observation was in line with the aforementioned reports confirmed an adverse effect of vitrification on transcript abundances of epigenetic-related genes, it was still unclear if these changes in the transcript levels could lead to epigenetic instability of the resultant embryos. Based on the epigenetic roles played by

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the four genes that were assessed in this study (Table 1), and if the changes in transcripts could lead to parallel changes in abundances of the associated enzymes, one may argue that embryos derived from vitrified oocytes should be globally hypomethylated and hypoacetylated compared with the embryos derived from fresh oocytes. This hypothesis was the main question of the third experiment in this study.

Evaluation of the epigenetic marks in this study showed that oocyte vitrification could lead to the loss of DNA methylation and histone acetylation of the resulting embryos at the 2-cell stage. These epigenetic changes in 2-cell embryos could be considered relevant to the reductions observed in the transcripts of *DNMT1*, *DNMT3B*, *HDAC1*, and *SUV39H1* in vitrified oocytes, provided that the roles played by these genes were in epigenetic modification of DNA (Table 1). In agreement, Chen *et al.* (2016) detected reduced levels of DNA methylation and H3K9me3 in oocytes and early ICSI embryos derived from vitrified bovine oocytes. Suo *et al.* (2010) demonstrated a significant increase in AcH4K12 levels in vitrified oocytes and abnormal chromatin distribution or acH4K12 patterns in respective zygotes before and after pronuclear formation in mice. A reduction in global genomic methylation of vitrified mouse MII oocytes and the resulting 2-cell to 8-cell stages embryos was reported by Liang *et al.* (2014). Moreover, Fu *et al.* (2019) showed that, in mice, the levels of 5mC and 5hmC were significantly decreased 2 h after vitrification. Mechanistically, these epigenetic changes may be caused by either an indirect effect of vitrification on transcripts of genes linked to epigenetic reprogramming, as supposed in this study, or to direct effects of vitrification on reduction of DNA methyltransferases or excessive relaxation of chromosomes in vitrified-warmed oocytes (Cheng *et al.*, 2014; Chen *et al.*, 2016). As the maternal genome undergoes a gradual pattern of DNA demethylation followed by *de novo* DNA methylation during the normal course of early embryo development in mammals (Suo *et al.*, 2010; Cheng *et al.*, 2014; Hosseini *et al.*, 2015; Chen *et al.*, 2016), an immediate question was whether initial epigenetic changes of maternal genome induced by vitrification can lead to subsequent changes in the resulting embryos. Therefore, the last part of this study was to understand whether epigenetic changes that were detected following oocyte vitrification in the resulting 2-cell embryos persisted during later stages of *in vitro* embryo development.

In this study, blastocysts derived from vitrified oocytes had reduced levels of DNA methylation and H3K9 acetylation. Although this may indicate a possible risk for long-term inheritance of epigenetic instabilities caused by oocyte vitrification, the levels of epigenetic differences between vitrified and fresh blastocysts seem to be diluted during mitotic divisions. Available data in mouse indicate that epigenetic changes occur following oocyte vitrification may be partially adjusted by the blastocyst stage, whereas the others could persist and lead to loss of DNA methylation of *H19*, *PEG3*, and *SNRPN* imprinted genes (Cheng *et al.*, 2014). In bovine, oocyte vitrification reduced DNA methylation and H3K9me3, but increased H3K9 acetylation in early cleavage embryos. When developed to the blastocyst, inner cell mass showed no difference in DNA methylation, H3K9me3, and acH3K9 in the inner cell mass, whereas decreased levels of DNA methylation and acH3K9 observed in trophectoderm cells (Chen *et al.*, 2016). By tracing the reprogramming of 5mC and its derivatives in resulting mouse embryos, Cao *et al.* (2019) observed that oocyte vitrification did not alter the dynamic changes of 5mC, 5hmC and 5fC before the 4-cell stage, whereas the levels of 5hmC and 5fC significantly reduced from the 8-cell stage onwards. Although these results suggest that oocyte vitrification could interfere with critical stages of epigenetic reprogramming during preimplantation embryo development, when the epigenome appears to be most plastic, the epigenetic plasticity steadily decreases during later stages of development (Hajj and Haaf, 2013). In the extent of present knowledge, the phenotypic consequences originating from epigenetic changes caused by oocyte vitrification remain largely unknown. Moreover, differential contributions of chemical, thermal and osmotic shocks of oocyte vitrification procedure to the gene expression profile and epigenetic status of the resulting embryos are not well understood. Specifically, some cryoprotectants (CPAs) used during cryopreservation due to their special chemical formula have affinity for the reaction with the epigenetic composition of cells (Hu *et al.*, 2012). For example, Hu *et al.* (2012) showed that dimethyl sulfoxide (DMSO), a commonly used CPA, significantly reduced DNA methylation of oocytes, whereas in contrast, propylene glycol, another commonly used CPA, significantly increased DNA methylation in cryopreserved oocytes. In this regard, novel approaches of oocyte cryopreservation for intra-oocyte injection of safer and natural CPAs, such as trehalose, may subvert any detrimental effects of CPAs on epigenetic stability of the oocyte epigenome (Eroglu *et al.*, 2009). Changes observed in the epigenetic status of vitrified oocytes, corrected using current chemical epigenetic modifiers, remain to be understood, because during any intervention to reverse these epigenetic changes, the effects on the oocytes and the resulting embryos will be applied to the level of all genes.

In conclusion, the results of this study indicated that *in vitro*-matured vitrified-warmed oocytes support cleavage and *in vitro* embryo development following ICSI in dromedary camels. Vitrification significantly reduced the transcript abundances of four key genes involved in epigenetic modification and led to corresponding changes in epigenetic stability of the resulting embryos. In contrast, hypoacetylation of H3K9 may render the chromatin of the embryos closed, thereby less transcriptionally active. Although differences in global DNA methylation and histone acetylation did not reach a statistically significant level for the number of the analyzed blastocysts, this does not exclude the possibility that the epigenetic status of different genes might have been altered significantly as a result of vitrification (Saenz-de-Juano *et al.*, 2014; Li *et al.*, 2016). The long-lasting effects that assisted reproductive technologies may have on the integrity of developing embryos have received much attention recently. Although human studies are fewer compared with animal model studies due to legal issues, growing numbers of studies have shown evidence of transcriptional, proteomics, metabolic and even phenotypic changes in neonates born following assisted reproductive technologies, including oocyte/embryo cryopreservation (Dupont and Sifer, 2012; Saenz-de-Juano *et al.*, 2014; Li *et al.*, 2016; Wu *et al.*, 2018; Garcia-Dominguez *et al.*, 2020). To best of our knowledge, this is the first study on the effect of MII-oocyte vitrification on *in vitro* development of ICSI embryos in dromedary camels.

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Conflicting interests. Authors declare that there is no competing interest in this study.

Ethical standards. None. The authors of this study did not work with any living animals in the context of this study.

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