

Epigenetic Changes in Equine Embryos After Short-term Storage at Different Temperatures

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Abstract

Background: In embryos subjected to assisted reproductive techniques, epigenetic modifications may occur that can influence embryonic development and establishment of pregnancy. In horses, the storage temperature during transport of fresh embryos before transfer is a major concern. The aim of this study was to determine the effects of two storage temperatures (5°C and 20°C) on equine embryos, collected at day 7 after ovulation and stored for 24 hours, on the following characteristics: (i) morphological and histological development; (ii) expression of candidate genes associated with embryo growth and development, maternal recognition of pregnancy, methylation, and apoptosis; and (iii) gene-specific and global-DNA methylation. Embryos (n=80) were collected from Haflinger mares (n=15) on day 7 (n=60) or day 8 (n=20) after ovulation and assigned to 4 groups: day 7 control (E7F, fresh); day 7, stored for 24h at 5°C (E5C); day 7, stored for 24h at 20°C (E20C); and day 8 control (E8F, fresh 24h time control). The embryos and the storage medium from all groups were analyzed for: (i) medium temperature, pH, and lipid peroxidation (malondialdehyde; MDA), (ii), embryo morphology, mRNA expression, and DNA methylation (immunohistochemistry and gene-specific DNA methylation).

Results: Temperature during storage (E5C and E20C) did not affect embryo size (382 ± 47 and 553 ± 99 μm , respectively). There were no changes in pH and MDA accumulation irrespective of group. The relative mRNA abundance of specific genes related to growth and development (*POU5F1*, *SOX2*, *NANOG*), maternal recognition of pregnancy (*CYP19A1*, *PTGES2*), DNA methylation (*DNMT1*, *DNMT3A*, *DNMT3B*), and apoptosis (*BAX*) in the E5C and E20C were either up or downregulated ($P<0.05$) when compared to controls (E7F and E8F). The global methylation status, even as 5mC and 5hmC immune expression were similar among treatment groups. The specific genes *ESR1*, *NANOG*, and *DNMT1* were hypomethylated ($P<0.001$) in E20C embryos when compared to E8F (advanced stage).

Conclusions: Therefore, our study demonstrates for the first time the gene-specific and global-DNA methylation status of fresh equine embryos collected on days 7 and 8 after ovulation. In addition, short-term storage, regardless of temperature, modified gene expression and methylation of genes involved in embryo development and may therefore compromise embryo viability and development after transfer.

Introduction

Assisted reproduction techniques (ART) in horses have considerably advanced during the last decade. In this field, embryo transfer is still the most common ART applied to horses [1]. The production of embryos by ovum pick-up and subsequent intracytoplasmic sperm injection, however, is attracting increasing attention. Because of a limited availability of recipient mares at facilities that collect in vivo or produce in vitro embryos, the shipment of such embryos is required with increasing frequency. Similarly, techniques that require specific laboratory equipment as cryopreservation or preimplantation genetic diagnosis often demand overnight transport and sometimes transport back thereafter [2, 3]. Equine embryos are transferred at day 7 or 8 after ovulation [1, 4], with the majority developed to the blastocyst stage at the time of transfer [5]. For overnight transport, embryos are either cooled to 5 °C or kept at room temperature [6]. Therefore, transportation is a critical issue that can contribute to success or failure of subsequent embryo transfer.

During the development of preimplantation embryos, a wave of epigenetic reprogramming takes place to establish the totipotent state [7]. Epigenetics refer to certain hereditary DNA or chromosome alterations, such as methylation, histone modification, and genome imprinting [8, 9]. The reprogramming of DNA methylation is a necessary step for subsequent embryo development [10]. Processes involved in ART have been associated with disturbances in epigenetic reprogramming, increasing the incidence of imprinting disorders such as Beckwith-Wiedemann Syndrome (BWS), Angelman syndrome, and retinoblastoma in children conceived with the help of ART [11]. In cattle, the large offspring syndrome was described in calves derived from ART; this has been associated with loss of methylation and downregulation of certain maternal genes [12]. Exposure to environmental stimuli such as oxidative stress during ART procedures can contribute to abnormal DNA methylation and lead to impairment of embryonic development and failure in maternal recognition of pregnancy [13, 14].

During early embryonic development, an increased expression of growth factors leads to proliferation and prostaglandin production by trophoblast cells, likely involved in maternal recognition of pregnancy [15]. At the same time, DNA methyltransferases (DNMTs) play an important role in maintaining genome stability and integrity during development and epigenetic reprogramming [16] with *DNMT1*, *DNMT3A*, *DNMT3B*, and *DNMT3L* being the main genes involved in the establishment of methylation patterns required for cell lineage determination [17–19]. There is evidence that DNA methylation occurs mainly at the cytosines of cytosine-guanine dinucleotides (CpG) known as CpG sites [16]. Interactions between DNA and gene regulatory proteins can be critically influenced by the dynamics of DNA methylation. The genomic 5-methyl-2'-deoxycytidine (5mC) is recognized by methyl-DNA binding proteins that recruit histone deacetylases and could reduce via oxidation to 5-hydroxymethyl-2'-deoxycytidine (5hmC) [20]. Therefore, changes in 5mC and 5hmC can determine global DNA methylation and hydroxymethylation in tissues and cells.

Whereas extensive information is available on epigenetic changes in oocytes and conceptuses derived from ART in humans, mice and cattle [20–23], information with respect to horses is scarce [24, 25]. Our study therefore hypothesized that storage temperature contributes to epigenetic changes in equine embryos. To test our hypothesis, we evaluated if storage temperature of equine embryos alters gene expression leading to epigenetic effects involved in genetic reprogramming. Embryos collected on day 7 after ovulation were either stored at 5 °C or 20 °C for 24 hours. Fresh embryos collected on day 7 or 8 served as untreated controls. The following parameters were determined: (i) morphological and histological

development; (ii) gene expression of candidate genes associated with maternal recognition of pregnancy, embryo development, methylation, and apoptosis; and (iii) gene-specific and global DNA methylation.

Methods

Animals and reproductive management

All experimental procedures were performed according to Austrian animal welfare legislation and approved by the Austrian Federal Ministry for Science and Research (license number BMWFW-68.205/0135-WF/V/3b/2014). Fifteen healthy and fertile Haflinger mares (4–16 years old) were used as embryo donors. Animals were kept in a large paddock with access to a shed, fed with hay and mineral supplements twice daily, and water was available *ad libitum*. The ovaries and uterus of mares were scanned transrectally with an ultrasound machine (Mindray M9, Mindray, Shenzhen, China) equipped with 5–8 MHz linear-array transducer (6LE5Vs) to detect a 3.5 cm preovulatory follicle and uterine oedema characterizing estrous and determinate the time for artificial insemination [26]. Insemination was performed with either raw or extended (Equi Pro, Minitube, Tiefenbach, Germany) semen. One insemination dose contained at least 500 million progressively motile spermatozoa. Semen was collected from fertile stallions by artificial vagina using routine procedures [27]. The mares were inseminated at intervals of 48 h and checked every 24 h until ovulation was detected. No hormonal treatments were administered during the experimental period.

Experimental design

Equine embryos ($n = 80$) were collected on day 7 ($n = 60$) or day 8 ($n = 20$) after ovulation and assigned to 4 groups with 20 embryos per group: (i) day 7 control (E7F, fresh); (ii) day 7, 24 h at 5 °C (E5C); (iii) day 7, 24 h at 20 °C (E20C); (iv) day 8 control (E8F). For short-term storage, embryos were kept in holding medium (Minitube) within an Equitainer (Hamilton Biovet, Ipswich, MA, USA). The embryos and medium from all treatments were submitted to the following assessments: temperature, pH, lipid peroxidation, embryo morphology, mRNA expression, and DNA methylation (immunohistochemistry and gene-specific DNA methylation).

Collection and evaluation of embryos

Embryos were recovered on days 7 and 8 after ovulation (day 0, ovulation detection) using an intrauterine silicone 2-way Foley catheter CH 28 for mares (Minitube). The uterus was washed four times with 1 L of Ringer's lactate solution (Fresenius Kabi, Graz, Austria) prewarmed at 38 °C. The fluid recovered from the uterus was filtered through an embryo filter system (75 µm, EmCon embryo filter; Immunosystems, Spring Valley, WI, USA). The solution remaining in the filter cup was placed in a petri dish and analyzed under a stereomicroscope at 40 × magnification. Embryos were washed 10 times in holding medium (Minitube) to remove cellular debris. Embryos were measured with a microscopic scale and morphologically classified according to their quality on a scale from 1 (excellent) to 4 (degenerated) as described [28].

Holding medium temperature and pH

The temperature of the holding medium was recorded with a data logger (testo 175, Testo, West Chester, PA, USA) every 10 min for 24 h. Briefly, a second tube with the same volume of holding medium as for the embryo was placed inside the Equitainer and the fine sensor of the data logger was placed inside this holding medium for temperature monitoring.

The pH was assessed in a sample of the holding medium immediately before and after embryo storage for 24 h. The pH was assessed by a pH meter (SevenCompact S220-micro-kit, Mettler Toledo, Columbus, OH, USA) using the microelectrode (InLab Ultra-Micro-ISM, Mettler Toledo) for small sample volumes.

Lipid peroxidation

Lipid peroxidation was determined by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a fluorometric ($\lambda_{ex} = 532/\lambda_{em} = 553$ nm) product, proportional to the MDA present, using a commercial kit (Cat#MAK085, Sigma-Aldrich Co., St. Louis, MO, USA) and following the principles and methods previously described [29]. Briefly, the MDA standards were prepared by dilution of 10 µL of the 4.17 M MDA standard solution with 407 µL of water to prepare a 0.1 M MDA standard solution. Further, 20 µL of the 0.1 M MDA standard solution was diluted with 980 µL of water to prepare a 2 mM MDA standard to generate 0 (blank), 0.4, 0.8, 1.2, 1.6, and 2.0 nanomole standards. Later, a sample (20 µL) of the spent holding medium was gently mixed with 500 µL of 42 mM sulfuric acid in a microcentrifuge tube. Phosphotungstic acid solution (125 µL) was added to the samples, mixed by vortex, incubated at room temperature for 5 min, and then centrifuged at 13,000 × g for 3 minutes. The pellet was resuspended on ice with the 100 µL water/BHT solution (2%) and adjusted to a volume of 200 µL with water. The assay reaction was performed following the manufacturer instructions using a fluorometer (Victor 2D, Perkin Elmer, Santa Clara, CA, USA) and the data were analyzed by the software SoftMax Pro 6.5.1. (Molecular Devices, LLC, Sunnyvale, CA, USA). All samples and standards were run in duplicate.

Quantitative real-time PCR

Embryos from all groups ($n = 24$, 6 per group) were placed in 350 µL RLT buffer (Qiagen, Hilden, Germany) and stored at -80 °C. For RNA extraction from single embryos, 3.5 µL 2-mercapoethanol (Sigma-Aldrich) were added to the solution and RNA extraction and DNase I digestion were performed with the RNeasy Micro Kit (Qiagen) according to the recommended protocol for animal and human tissues. For qPCR, 6 µL of total RNA were

transcribed into cDNA using the SuperScript III First-Strand Synthesis system with random hexamer primers (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Two replicates per sample were reverse transcribed and pooled for qPCR. Primer and hydrolysis probes for the equine target genes *ATP1A1*, *BAX*, *BCL2*, *CYP19A1*, *DNMT1*, *DNMT3A*, *DNMT3B*, *DNMT3L*, *ESR1*, *H19*, *IGF1*, *IGF2*, *NANOG*, *POU5F1*, *PTGES2*, and *SOX2*, were designed using the PrimerQuest assay tool (<https://eu.idtdna.com/PrimerQuest/Home/Index>; Integrated DNA Technologies, Coralville, IA, USA) or taken from the literature [30–33]. Two reference genes (RG), *PSMB4* and *SNRPD3*, were included for normalization [34]. Assay details and full names of the genes investigated are listed in Table 1. All hydrolysis probes were dual-labelled with 6-carboxyfluorescein (FAM) on the 5' end and Black Hole Quencher 1 (BHQ1) on the 3' end. The assays were validated by generation of standard curves to determine PCR reaction efficiencies using the formula $E = 10^{-1/\text{slope}} - 1$ [35]. Efficiency-corrected C_q values were used for analysis. Real-time PCR quantification of the target genes using hydrolysis probes was performed as described [33]. The RG were measured with the fluorescent DNA dye SYBR Green. Reaction conditions have been described [34]. Target gene expression levels were normalized to the geometric mean of *PSMB4* and *SNRPD3* and relative expression changes were calculated with the comparative $2^{-\Delta\Delta CT}$ method [36].

Table 1
Primer for quantitative PCR.

Gene symbol	Gene name	NCBI/Ensemble accession number	Oligo sequence (5' – 3')	Amplicon length (bp)	PCR efficiency (%)	R ² value	Reference
ATP1A1	<i>Equus caballus</i> ATPase Na ⁺ /K ⁺ transporting subunit alpha 1	NM_001114532.2, XM_023640223.1, XM_023640224.1	F: CTTGATGAACTTCAGCGCAAATA R: GGTGTAAGGGCATTGGGA P: TGAGCCGAGGCTTAACAACCTGCTC	104	94	0.990	This study
BAX	<i>Equus caballus</i> BCL2-associated X protein	XM_014729721.1 XM_014729717.1	F: AGGATGCGTCCACCAAGAAG R: CCTCTGCAGCTCCATGTTACTG P: CTCAAGCGCATCGGAGATGAGCTG	80	93.2	0.994	[33]
BCL2	<i>Equus caballus</i> B-cell lymphoma 2	XM_001490436.2	F: TTGGAAAGCCTACCACTAATTGC R: CCGTGTTTTATAGGCACAGGAGAT P: CCCACCTGAGCGGCTCCACC	74	92.6	0.998	[33]
CYP19A1	<i>Equus caballus</i> cytochrome P450 family 19 subfamily A member 1	NM_001081805.2 XM_005602588.2 XM_005602587.2	F: GGAGAGGAAACGCTCGTTATTA R: CCCATATACTGCAACCCAAATG P: ATCACTACTCCTCCCGATTTGGCA	107	99.2	0.999	This study
DNMT1	<i>Equus caballus</i> DNA methyltransferase 1	XM_014741825.1	F: GACCACCATCACGTCTCATTT R: CTCCTCATCCACAGAATTGTCC P: AAACGGAAACCCGAGGAAGAGCTG	97	100.5	1	This study
DNMT3A	<i>Equus caballus</i> DNA methyltransferase 3 alpha	XM_005600169.2 XM_005600168.2 XM_005600167.2 XM_005600170.2 XM_005600171.2	F: GATTATTGACGAACGCACAAGAG R: GTGTTCCAGGGTGACATTGA P: TGCAAATGTCTTCGATGTTCCGGC	112	100	0.998	This study
DNMT3B	<i>Equus caballus</i> DNA methyltransferase 3 beta	XM_001916514.4	F: CGAGTCTTGCCCTGTTTGAT R: GCGATAGACTCTTCACACACTT P: CGCCACAGGGTACTTGGTTCTCAA	110	100.6	0.999	This study
DNMT3L	<i>Equus caballus</i> DNA (cytosine-5)-methyltransferase 3-like	XM_014736476.1	F: GCCCTCACTTGGTTGGTTT R: CTTCACACAGGCACAGTTT P: CAAAGTGCCCATCTGCTCTGGAGA	98	100.5	0.999	This study
ESR1	<i>Equus caballus</i> estrogen receptor 1	NM_001081772.1	F: CACCCAGGAAAGCTCCTATTT R: CGAGATGACGTAGCCAACAA P: TCCACCATGCCCTCTACACATTTCC	110	101.8	0.999	This study
H19	<i>Equus caballus</i> H19, imprinted maternally expressed transcript	NR_027326.2	F: CCTCTAGCTCTGACTCAAGAATATG R: CAGGTCCATCTGGTTCCTTTAG P: ACTCAGGAATCAGCTCTGGAAGGT	103	94.3	0.992	This study
IGF1	<i>Equus caballus</i> insulin like growth factor 1	NM_001082498.2 XM_005606471.2 XM_005606472.2 XM_005606470.2 XM_005606469.2	F: TGCTTCCGGAGCTGTGATCT R: CCGACTTGGCAGGCTTGA P: AGGAGGCTGGAGATGTACTGCGCACC	67	102	1	[30]

R²: correlation coefficient of standard curve; F and R: forward and reverse primer; P: hydrolysis probe

Gene symbol	Gene name	NCBI/Ensemble accession number	Oligo sequence (5' – 3')	Amplicon length (bp)	PCR efficiency (%)	R ² value	Reference
IGF2	<i>Equus caballus</i> insulin like growth factor 2	NM_001114539.2	F: AAGTCCGAGAGGGACGTG R: ATTGCTTCCAGGCGTTGT P: CCCGTGGTCAAGCTCTTCCAGT	100	99.9	0.998	This study
NANOG	<i>Equus caballus</i> Nanog homeobox	XM_014740545.1 XM_001498808.1	F: ACAGCCCCGATTCATCCA R: TCTTTGCCTCGCTCGTCTCT P: CAGTCCCAGAGTAAAACCGCTGCCC	72	102.3	0.999	[31]
POU5F1	<i>Equus caballus</i> POU class 5 homeobox 1	XM_014734675.1 XM_001490108.5	F: CGGGCACTGCAGGAACAT R: CCGAAAGAGAAAGCGAACTAGTATTG P: TTCTCCAGGTTGCCTCTCACTCGGTTTC	73	100.8	0.999	[31]
PSMB4	<i>Equus caballus</i> proteasome subunit beta type IV	XM_001492317.4 XM_005610132.1 XM_008515015.1 XM_005613704.1	F: CTTGGTGTAGCCTATGAAGCCC R: CCAGAATTTCTCGCAGCAGAG	82	93.1	0.991	[34]
PTGES2	<i>Equus caballus</i> prostaglandin-endoperoxide synthase 2	NM_001081775.2	F: GAGGTGTATCCGCCACAGT R: AGCAAACCGCAGGTGCTC P: TCAGATGGAATGATCTACCCGCCTCA	81	92.3	0.996	[32]
SNRPD3	<i>Equus caballus</i> small nuclear ribonucleoprotein D3 polypeptide.	XM_001489060.4 XM_008511652.1	F: ACGCACCTATGTTAAAGAGCATG R: CACGTCCCATTCCACGTC	120	99.4	0.996	[34]
SOX2	<i>Equus caballus</i> SRY box 2	XM_003363345.3	F: TGCAGGCGCTGCACAT R: AGCGTGTACTTATCCTTCTTCATGAG P: ATAAATACCGTCCTCGGCGGAAAACCAA	91	99.3	0.998	[31]

R²: correlation coefficient of standard curve; F and R: forward and reverse primer; P: hydrolysis probe

Gene-specific DNA methylation analysis

The analysis of gene-specific DNA methylation was based on a protocol previously implemented for bovine oocytes [37]. Briefly, DNA from single equine embryos (n = 28; n = 7 per group) was isolated and bisulfite-treated with the EZ DNA Methylation Direct Kit (Zymo Research, Irvine, CA, USA) following the recommended protocol for samples containing up to 2×10^3 cells. Bisulfite-converted DNA was eluted with 10 μ l M-Elution Buffer (Zymo Research) into 1.5 ml DNA LoBind tubes (Eppendorf, Hamburg, Germany). Selection of target regions for bisulfite sequencing and primer design was done with the MethPrimer online tool [38]. The genomic sequences 2,000 bp upstream of the transcription start sites of the equine genes, *CYP19A1*, *DNMT1*, *DNMT3A*, *DNMT3B*, *ESR1*, *NANOG*, *PTGES2* and *SOX2*, were screened for CpG islands or CG-rich regions using the CpG island prediction function of the MethPrimer tool. For multiplex nested PCR, two sets of primers, an outer primer for a first-round multiplex PCR amplification of all 8 genes and an inner primer for a second gene-specific single nested PCR, were designed for each gene (Table 2). Primers were designed to bind outside the CG-rich areas and to amplify as many CpG dinucleotides as possible. Due to missing sequence data in the horse genome (EquCab2) upstream of the *PTGES2* gene, only one primer set could be designed for *PTGES2* which was used in both PCR reactions. The multiplex PCR was performed in 25 μ l reaction volumes including 200 μ M of each dNTP, 1 x buffer B2 (Solis BioDyne, Tartu, Estonia), 3 mM MgCl₂, 120 nM of each outer primer, 1.2 units HOT FIREPol DNA polymerase (Solis BioDyne) and 2 μ l bisulfite-treated DNA. The PCR reaction was carried out using the following temperatures: initial denaturation at 95 °C for 10 min, followed by 34 cycles of 95 °C for 30 sec, 54 °C for 30 sec, and 72 °C for 1 min, and a final elongation step at 72 °C for 7 min. One μ l of the multiplex PCR product was used as template for the subsequent nested PCR, performed in 25- μ l reaction volumes using the same mastermix components as described for the multiplex PCR except that the outer primer-mix was replaced with 500 nM of each gene-specific inner forward and reverse primer. The temperature protocol for multiplex PCR was also applied for the nested PCR, only the annealing temperature and cycle number were adjusted for each gene (*CYP19A1*: 56 °C, 35 cycles; *DNMT1* and *DNMT3A*: 59 °C, 35 cycles; *DNMT3B*, *ESR1* and *NANOG*: 60 °C, 35 cycles; *PTGES2*: 56 °C, 40 cycles; *SOX2*: 58 °C, 40 cycles). Aliquots of the PCR products were run in

a 2% agarose gel to confirm the correct size of the amplicons. The remaining aliquots were used for direct Sanger sequencing performed by Microsynth, Vienna, Austria. To improve the quality of the sequencing data, the amplicons of *ESR1* and *PTGES2* were isolated from the 2% agarose gel and purified using the Zymoclean Gel DNA Recovery kit (Zymo Research) prior to sequencing. Data analysis was performed with CLC Genomics Workbench 9 software (Qiagen). The sequence electropherograms were investigated for methylated "C" and unmethylated "T" peaks within a CpG context. CpGs with a "C" signal > 80% were categorized as methylated, whereas methylation values < 20% were categorized as unmethylated. Methylation values between 20 and 80% were designated as an unclear methylation status [37].

Table 2
Multiplex nested PCR primer for DNA methylation analysis.

Gene	Primer Sequence (5' – 3')	Amplicon length (bp)	Genomic localization (EquCab2)	Number of CpGs in inner amplicon	Number of sequenced CpGs
CYP19A1	Outer forward: TTTGTAGTTTTGATTGGTTGTTTTT	317	1:139092647– 139092963	-	-
	Outer reverse: CTAAACCCCATAAAACATCTCTTAC				
	Inner forward: TTTTTTTTGTAAGATTAGTGAGTATATTTA	212	1:139092716– 139092927	4	3
	Inner reverse: TTTCCAAAATTAAAAACATAACC				
DNMT1	Outer forward: AATTTTTTTTTAAGAGTTTGGTATGG	264	7:49740491– 49740754	-	-
	Outer reverse: ACCAATCCTCCTCTTTATACTAAAA				
	Inner forward: GAGTTTGGTATGGTATATAAGTGTGA	229	7:49740503– 49740731	9	8
	Inner reverse: AAAAAACTAACCTAAACTCACATC				
DNMT3A	Outer forward: GGGATTGATTAGATTTTTTAGAGAAG	316	15:70660175– 70660490	-	-
	Outer reverse: TAATAACACTAAATCCCTCCAAAAC				
	Inner forward: TAGGAGTTTAGTGGGGGAATAGT	200	15:70660225– 70660424	6	4
	Inner Reverse: ATAAAATAAATAAAACCCCTACACC				
DNMT3B	Outer forward: TTAAAGGGGAATAGTAGAAGTTTA	388	22:23584114– 23584353	-	-
	Outer reverse: CAACTCCAAAAATATTTAAATCAC				
	Inner forward: TATAGAGGATGGATTTGGGATTTTA	240	22:23584114– 23584353	10	8
	Inner reverse: ACTAAACACTCCCTACCCTAATACC				
ESR1	Outer forward: TTGTGGTAGGTATGAATTTTATGTG	334	31:15330033– 15330366	-	-
	Outer reverse: ATTACATATACAACCAACCACAAAC				
	Inner forward: AATTTTTAGTGGGAGGAAGTATAGTAT	226	31:15330075– 15330300	9	8
	Inner reverse: ACATAAACTAACAAAAACATCCC				
NANOG	Outer forward: TGGAAATATGGTGAATTTATAGGTAT	387	6:35487982– 35488368	-	-
	Outer reverse: AACTTAAATATCCAAACAAAAAACC				
	Inner forward: TTGGTAGATAGGATTAATTGAGAATT	237	6:35487994– 35488230	8	7
	Inner reverse: CAAACAAAAACCTTAAAAAATAC				
PTGES2	Forward: GATTTATTTAAGAGTGGGGGAGGT Reverse: CAATATAAAACCCCAACC	205	25:31473304– 31473508	17	11

Gene	Primer Sequence (5' – 3')	Amplicon length (bp)	Genomic localization (EquCab2)	Number of CpGs in inner amplicon	Number of sequenced CpGs
SOX2	Outer forward: ATTTTAAATATAGAATAAATTATGGAGAAG	302	19:20356881– 20357182	-	-
	Outer reverse: AAATAAAAATAAAACAAAACAAAATAAATA				
	Inner forward: ATAGAATAAATTATGGAGAAGTAAGGAG	253	19:20356889– 20357141	19	13
	Inner reverse: CTATCCTACTAAAATTTCAAAAACC				

Global methylation - immunofluorescence staining for 5mC and 5hmC

Embryos (n = 28; n = 7 per group) from all treatment groups were prepared for immunofluorescence staining as previously described [39, 40] with minor modifications. Briefly, embryos were removed from the holding medium, washed in PBS, and fixed in ice-cold 4% paraformaldehyde (PFA; Sigma-Aldrich) for 25 min at room temperature (RT). Embryos were washed in PBS and kept for 25 min at RT in PBT (0.05% Tween-20 in PBS), permeabilized in 0.2% triton X-100 solution for 40 min at RT, washed 3 times and stored in 100 µL of PBT at 4 °C for the antibody staining. Embryos were depurinated in 4N HCl 0.1% Triton X-100 for 20 min at RT, washed in PBS and kept in PBT for 30 min at RT, and incubated in blocking solution (2% BSA in PBT) overnight at 4 °C. Later, embryos were incubated with the primary antibody (5mC mouse monoclonal antibody, EpiGentek, Farmingdale, NY, USA; or 5hmC mouse monoclonal antibody, Active Motif, Carlsbad, Ca, USA) at 1:200 in blocking solution for 1 h at RT, washed in PBT and incubated with Alexa Fluor 594 goat anti-mouse IgG (Thermo, Waltham, MA, USA) for 1 h in blocking solution at RT in the dark. Finally, embryos were washed in PBT, incubated in DAPI solution for 10 min at RT, then washed in PBT and prepared in a chamber for confocal microscopy (LSM 880, Carl Zeiss, Oberkochen, Germany). Scanning was conducted with Z stack of 25 optical series from the bottom to the top of the embryos with a step size of 65 µm to allow three-dimensional distribution analysis. Images were obtained at 20 × objective magnification and analysed using ImageJ software (version 1.50f).

Statistical analysis

The software SPSS version 24 (IBM-SPSS, Armonck, NY, USA) was used for statistical analyses. Data were tested for normal distribution by Kolmogorov-Smirnov test. Because embryo size was not normally distributed ($P < 0.05$), comparison of embryo size among groups was made with non-parametrical tests (Mann-Whitney test for fresh embryos collected on days 7 and 8, Wilcoxon test for the comparison of embryo size before and after storage in groups E5C and E20C, respectively). For the analysis of pH, lipid peroxidation, gene expression, and DNA methylation, one-way ANOVA with subsequent Tukey test were used to analyze differences among groups. Data are shown as mean ± standard error of the mean (SEM). A P value < 0.05 was considered statistically significant.

Results

Recovery rate and embryo morphology

A total of 80 embryos were obtained from 144 embryo flushing procedures; therefore, the overall recovery rate was 55% (80/144). Of the embryos collected, 92% were at the blastocyst and 8% at the morula stage. All embryos had a morphological classification of 1 or 2. Embryo size differed ($P < 0.05$) between fresh embryos collected on day 7 (E7F; n = 20) and day 8 (E8F; n = 16; Fig. 1). The size of embryos stored at 5 °C for 24 h (E5C; n = 20) increased ($P < 0.01$), whereas in embryos stored at 20 °C (E20C; n = 19) it decreased ($P < 0.05$). In 5 embryos (1 of E20C and 4 of E8F), data from size determination were not available. No significant difference in size among embryos collected on day 7 was detected. Fresh embryos collected on day 8 were larger ($P < 0.01$) than embryos stored for 24 h, irrespective of storage temperature. In 10 embryos (6 in E20C and 4 in E5C) of the storage groups, a shrinkage-like morphology was detected irrespective of temperature (Fig. 2). For the determination of gene expression and DNA methylation, only blastocysts of similar development stage and ≥ 300 µm in diameter at collection (in total n = 52) were used.

Medium temperature, pH, and lipid peroxidation

The temperature of the storage medium was constant for all embryos within the temperature groups during the storage period. The pH of the medium (7.22 ± 0.07 and 7.22 ± 0.09) was similar in groups E5C and E20C. In the spent holding medium, MDA accumulation could not be detected irrespective of treatment group.

Gene expression

The relative mRNA abundance of specific genes related to growth and development, embryo-maternal recognition, methylation, and apoptosis is depicted in Table 3. The gene *DNMTL3L* was not expressed in equine embryos irrespective of group. The expression of *IGF1*, *POU5F1*, *SOX2*, *NANOG*, *CYP19A1*, *PTGS2*, *DNMT1*, *DNMT3a*, *DNMT3b*, and *BAX* differed among groups ($P < 0.05$). Gene expression of *IGF1*, *CYP19A1* and *BAX* was similar in the two groups of stored embryos irrespective of temperature and control embryos collected on day 7, but higher in embryos collected on

day 8. A different pattern with regard to mRNA abundance was determined for *SOX2*, *NANOG*, *PTGES2*, *DNMT1* and *DNMT3b*; on the one hand the gene expression was similar in day 7 control embryos and embryos stored at 5 °C, and on the other hand day 8 controls and embryos stored at 20 °C. Gene expression of *POU5F1* and *DNMT3a* differed in E5C embryos in comparison to all other groups.

Table 3
Relative mRNA abundance (2 - delta CT) in fresh control (E7F and E8F) and stored embryos (E5C and E20C)[†].

Function	Gene	E7F	E5C	E20C	E8F	P-Value
Growth & Development	IGF1	3.50 ± 1.50 ^a	9.00 ± 6.02 ^{ab}	12.40 ± 4.46 ^{ab}	23.40 ± 5.50 ^b	0.037
	IGF2	2.20 ± 0.40	2.70 ± 0.77	3.30 ± 0.57	2.70 ± 1.02	NS
	H19	4.00 ± 0.58	4.54 ± 0.83	4.59 ± 1.00	6.60 ± 0.64	NS
	POU5F1	3.67 ± 0.30 ^{ab}	4.60 ± 0.38 ^a	3.27 ± 0.41 ^b	2.30 ± 0.41 ^b	0.004
	SOX2	30.90 ± 5.70 ^a	31.40 ± 8.28 ^a	8.80 ± 2.69 ^b	11.00 ± 3.72 ^b	0.01
	NANOG	38.50 ± 6.90 ^a	33.70 ± 8.16 ^a	9.60 ± 2.00 ^b	11.80 ± 3.10 ^b	0.002
	ATPA1A	8.4 ± 4.7	5.1 ± 2.5	6.1 ± 4.0	10.4 ± 7.1	NS
	Embryo-maternal communication	ESR1 [‡]	20.80 ± 8.91	n.d.	n.d.	8.30 ± 7.10
CYP19A1		8.45 ± 3.30 ^a	5.56 ± 1.76 ^a	7.36 ± 2.00 ^a	31.90 ± 10.80 ^b	0.013
PTGES2		24.00 ± 2.92 ^a	18.10 ± 2.79 ^a	8.30 ± 2.94 ^b	19.80 ± 2.60 ^a	0.006
Methylation	DNMT1	2.59 ± 0.17 ^a	2.60 ± 0.25 ^a	1.66 ± 0.18 ^b	2.80 ± 0.25 ^a	0.006
	DNMT3a	1.90 ± 0.10 ^a	2.37 ± 0.21 ^b	1.23 ± 0.12 ^a	1.93 ± 0.25 ^a	0.002
	DNMT3b	3.16 ± 0.18 ^a	3.56 ± 0.46 ^a	1.62 ± 0.15 ^b	1.70 ± 0.28 ^b	0.000
Apoptosis	BCL2	2.42 ± 1.03	1.50 ± 0.44	1.65 ± 0.30	1.92 ± 0.41	NS
	BAX	2.90 ± 0.32 ^{ab}	3.50 ± 0.45 ^a	2.50 ± 0.17 ^{ab}	1.70 ± 0.30 ^b	0.008

[†]Embryos day 7 fresh, E7F; stored at 5°C, E5C, and 20°C, E20C; Fresh embryos day 8, E8F. [‡]E5C and E20C either did not express ESR1 or do not have enough samples for statistical comparison and, therefore, values were not considered (n.d.). ^{ab} Differences among groups within genes are indicated by superscript letters and are highlighted in gray (P < 0.05).

Gene-specific DNA methylation

The percentage of overall DNA methylation and the overview of DNA methylation results of single CpGs of the specific genes are depicted in Table 4 and Fig. 3, respectively. The predicted promoter region of *CYP19A1* was fully methylated whereas *PTGES2* and *SOX2* were unmethylated in all embryos irrespective of treatment group. For *DNMT1*, the methylation status was similar in the two groups of fresh embryos and embryos stored at 5 °C, whereas it was lower in embryos stored at 20 °C. The methylation status of *NANOG*, however, was higher in fresh day 8 embryos than in the other three groups.

Table 4
Percentage of methylation in the CpG islands of the specific genes[†].

Function	Gene	E7F	E5C	E20C	E8F	P-Value
Growth & Development	SOX2	2.2	1.1	0.0	0.0	0.62
	NANOG	46.9 ^a	63.3 ^{ab}	53.1 ^a	85.7 ^b	< 0.0001
Embryo-maternal communication	ESR1	64.3 ^{ab}	39.3 ^{bc}	28.6 ^c	78.6 ^a	< 0.0001
	CYP19A1	100.0	100.0	100.0	100.0	NS
	PTGES2	0.0	0.0	0.0	0.0	NS
Methylation	DNMT1	89.3 ^{ab}	85.7 ^{ab}	75.0 ^b	96.4 ^a	0.009
	DNMT3a	78.6 ^a	100.0 ^b	89.3 ^{ab}	100.0 ^b	0.006
	DNMT3b	53.6	30.4	44.6	33.9	0.052

[†] Specific genes related to growth & development, embryo-maternal recognition, and methylation functions in fresh control (E7F and E8F) and stored embryos (E5C and E20C); embryos day 7 fresh, E7F; stored at 5 °C, E5C, and 20 °C, E20C; Fresh embryos day 8, E8F. ^{ab} Differences among groups within genes are indicated by superscript letters and are highlighted in gray (P < 0.05).

Global methylation – 5hmC and 5mC

The fluorescence intensity (255 to 4.97×10^6) varied among embryos. The global methylation was, however, not affected by treatment irrespective of the antibody (5mC or 5hmC; Fig. 4). When immune expression was compared between antibodies within each treatment, E7F had a greater ($P < 0.05$) expression of 5hmC compared to 5mC. Among all other groups, expression did not differ between 5hmC compared to 5mC.

Discussion

To the best of our knowledge, the present study is the first to analyze the effects of storage temperature (5 and 20 °C) on development, relative mRNA abundance and DNA methylation in equine embryos processed for shipment using a commercial holding medium.

Untreated fresh embryos collected on day 7 and day 8 were included as controls to determine changes in relative mRNA abundance and DNA methylation associated with embryo age itself. Whereas difference in size of embryos collected on days 7 and 8 after ovulation as well as a considerable variance in size of embryos collected on the same day is not surprising and in agreement with previous studies [41–43], effects of storage temperature on equine embryo size have not yet been reported. Interestingly, embryonic size decreased slightly but significantly in embryos stored at 20 °C whereas it increased in embryos stored at 5 °C. The rapid increase in size of equine embryos starting on day 7 mainly depends on influx of fluid into the blastocoel allowed by the formation of an osmotic gradient due to activity of $\alpha 1/\beta 1$ Na⁺/K⁺-ATPase (reviewed by [43]). This enzyme has been detected in horse embryos not earlier than day 8 after ovulation [44]. The present results suggest that storage at 20 °C inhibits the activity of this enzyme, but in comparison to in vivo-produced embryos decreased enzyme activity is present in embryos stored at 5 °C. Interestingly, in all embryos analyzed for gene expression in the present study, *ATP1A1*, the gene encoding for the $\alpha 1$ subunit of the Na⁺/K⁺-ATPase, was detected, but there were no differences in relative mRNA abundance among groups. Gene expression is, however, not necessarily associated with enzyme activity. In day 8 embryos, immunohistochemistry of Na⁺/K⁺-ATPase revealed a pronounced protein expression of this enzyme in the whole trophoblast [45]. This suggests that the technique would not allow to detect differences among groups in the present investigation. We did not detect changes in pH and oxidative balance of the holding medium during storage irrespective of temperature; however, in approximately 25% of the embryos a partial separation of the trophoblast from the zona pellucida occurred. This finding is in accordance with a previous description of morphological abnormalities after 12 or 24 h of cooled storage in equine embryos [28] and may also be associated with delayed formation of an osmotic gradient due to impaired activity of $\alpha 1/\beta 1$ Na⁺/K⁺-ATPase and subsequent collapse of the blastocoel.

Among the genes we assessed in this study, *SOX2*, *NANOG* and *DNMT3B* are associated with pluripotency, and have been detected in equine pluripotent cells and in equine MSCs [46]. Interestingly, relative mRNA abundance of these genes was affected by storage temperature in a way that it was similar in day 7 control embryos and embryos stored at 5 °C on the one hand and day 8 controls and embryos stored at 20 °C on the other hand. Changes in *PTGES2* and *DNMT1* relative mRNA abundance followed the same pattern. This suggests that storage of equine day 7 embryos at 20 °C does not prevent changes in relative mRNA abundance that also occur between day 7 and day 8 in embryos in utero. Consequently, the storage temperature of 20 °C may be beneficial for embryo development when compared to a storage temperature of 5 °C where gene expression of the respective candidate genes stayed at the same level as in the control embryos collected on day 7. The relative mRNA abundance of other candidate genes, however, namely *IGF1*, a gene associated with embryonic development because it increases cell proliferation and decreases cell apoptosis [47, 48], *CYP19A1*, the gene encoding aromatase, and the proapoptotic gene *BAX*, was similar among stored embryos irrespective of temperature as well as in control embryos collected on day 7, but differed in embryos collected on day 8. This suggests that embryo storage at 20 °C

may have beneficial effects in comparison to 5 °C regarding the expression of some but not all genes. These findings are in agreement with higher pregnancy rates after transfer of shipped equine embryos with an arrival temperature between 10 and 16 °C in comparison to embryos transported in cooler conditions [49].

Methylation of DNA occurs during the migration of proliferating primordial germ cells but demethylation in postmigratory germ cells. A second wave of DNA demethylation takes place in cleavage stage embryos with DNA methylation being minimal at the blastocyst stage ([50]; recently reviewed by [51]). In the present study, only minor differences in the methylation status of candidate genes were detected between fresh embryos collected on day 7 and day 8 and the two groups of stored embryos. An interesting finding was hypermethylation of *NANOG* in embryos collected on day 8 in comparison to the other groups. A hypomethylation of *DNMT3a* in fresh day-7 embryos in comparison to all the other groups where embryo age was 8 days is most probably only age-related. Changes in the methylation status of genes that regulate embryo development have been described in porcine blastocysts during in vitro culture and were suggested to contribute to early pregnancy loss [52]. In the present study, either demethylation or a delay in methylation of CpG islands of specific genes could be a consequence of maintaining the transcriptional activation [53] under the temperatures used during storage compared to physiological temperatures. The embryo environment associated with ART had long-lasting consequences in mouse, ruminant and human embryos [54–56]. A major setback associated with the introduction of in vitro production of ruminant embryos was the occurrence of the “large offspring syndrome” [54]. This condition has caused considerable loss after transfer of in vitro produced ruminant embryos and has been linked to the use of serum in the culture media [54] altering gene expression and DNA methylation [12]. Comparable problems, however, have not yet been described in foals derived by ART which is in agreement with the findings of the present study with only minor changes of methylation status of the candidate genes.

In conclusion, our study has investigated for the first time the relative mRNA abundance of some candidate genes as well as the global and gene-specific DNA methylation status of fresh equine embryos collected on days 7 and 8 after ovulation. Moreover, we demonstrate that short-term storage of embryos alters the expression of genes involved in embryo development and methylation. Results suggest some beneficial effects of storage at 20 °C in comparison to 5 °C.

Abbreviations

ART

Assisted reproduction techniques; BWS:Beckwith-Wiedemann Syndrome; CpG:cytosine-guanine dinucleotides; DNMTs:DNA methyltransferases; MDA:malondialdehyde; PBS:Phosphate-buffered saline; PCR:Polymerase chain reaction;

Declarations

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Authors' Contribution

Conceptualization: GDAG, DS, CA. Data curation: GDAG, RE, CA. Investigation and methodology: GDAG, MM, DS, RE, CA. Writing: GDAG, CA. Writing – review & editing: GDAG, DS, MM, RE, CA. All authors read and approved the final manuscript.

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Availability of data and materials

The data analyzed during the current study are available from the corresponding authors on reasonable request.

Ethics approval and consent to participate

All experimental procedures were performed according to Austrian animal welfare legislation and approved by the Austrian Federal Ministry for Science and Research (license number BMWFV-68.205/0135-WF/V/3b/2014).

Consent for publication

Not Applicable.

Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figures

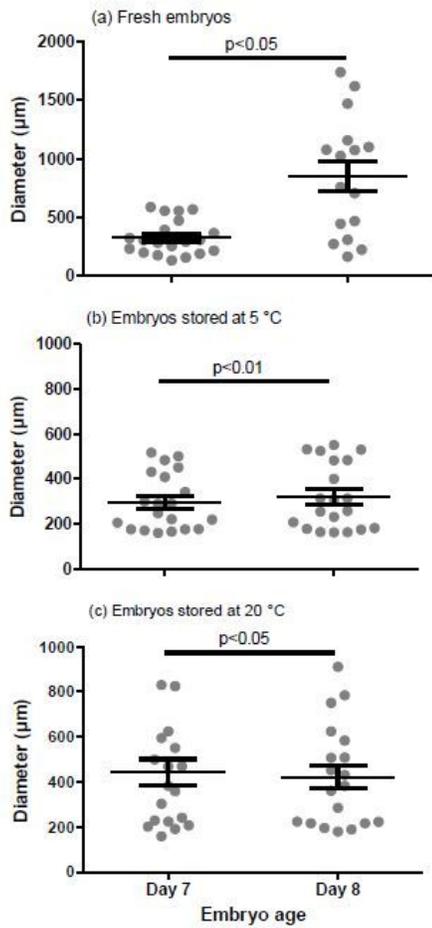


Figure 1

Diameter of fresh embryos. (A) collected on day 7 (E7F) and day 8 (E8F) after ovulation, and before (d7) and after 24 h (d8) short-term storage at (B) 5 °C (E5C) and (C) 20 °C (E20C). Sig-nificant differences are indicated in the figure.

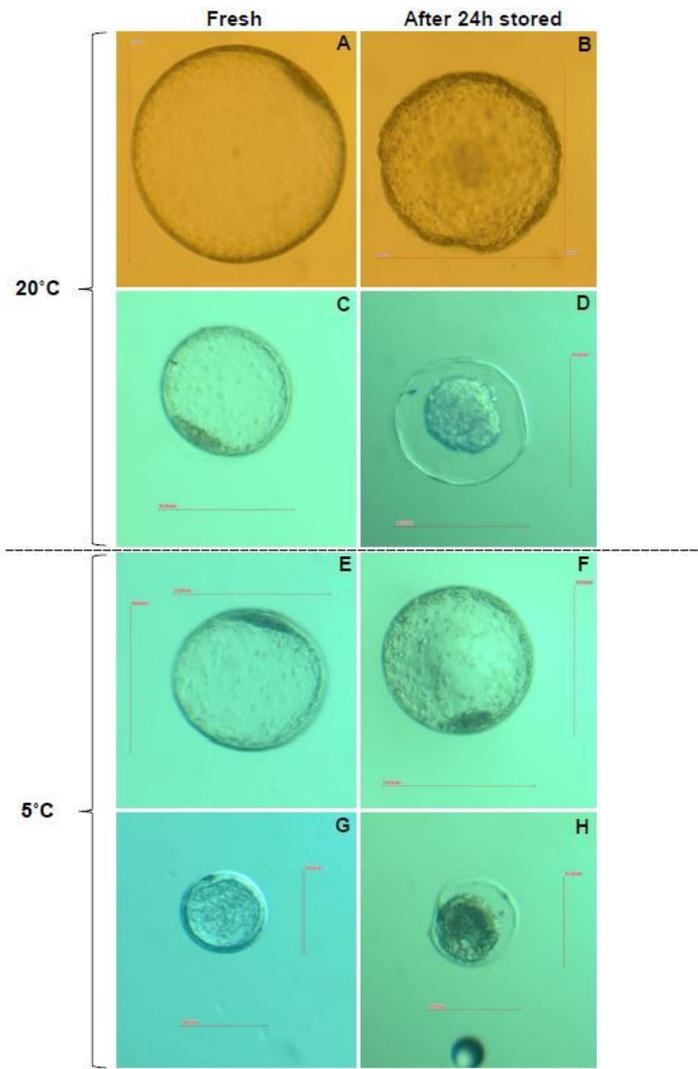


Figure 2

Illustration of fresh equine embryos (E7F) and stored in either 20°C or 5°C for 24h. (A, C, E, G) Embryos at blastocyst stage with normal morphology before submitted to storage; (B) embryo with a slight shrinkage of the embryonic cells and few areas of detachment between the trophoblast cells, zona pellucida and embryonic capsule; (F) embryo with normal morphology after storage time; (D, H) embryos with strong shrinkage morphology of the embryonic cells.

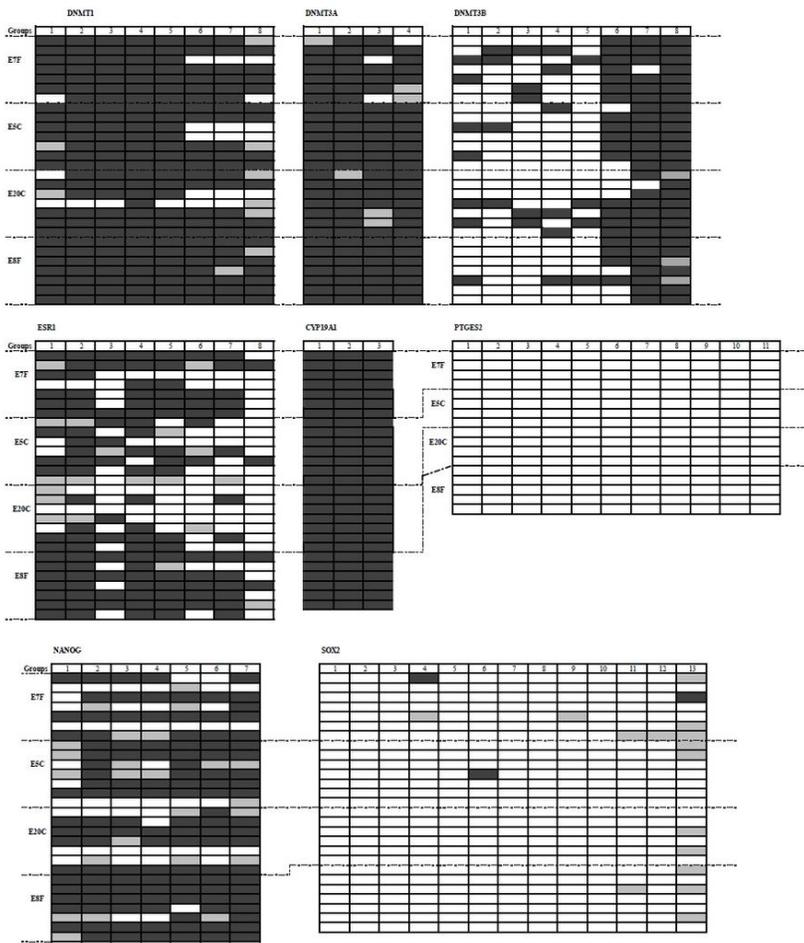


Figure 3

Overview of DNA methylation results of single CpGs for the selected genes. DNA methylation (DNMT1, DNMT3A, DNMT3B), embryo-maternal recognition (ESR1, CYP19A1, PTGES2), and growth and development (NANOG, SOX2) of the equine embryo. White box, unmethylated CpG; black box, methylated CpG; gray box, unclear methylation state. One row refers to one embryo; and the number of columns refers to the number of CpGs sequenced in the CG-rich region (methprimer CpG island finder) upstream of the gene (Table 2).

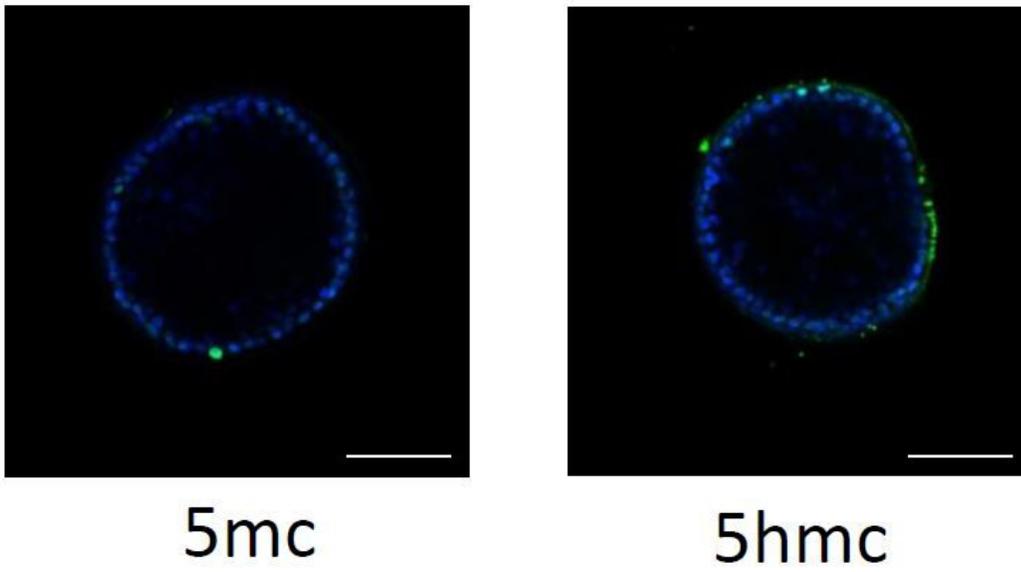


Figure 4

Illustrative image of 5mC and 5hmC immunolabeling in equine stored embryo at 20°C after 24h. Equine embryos had similar 5mC and 5hmC immunolabeling among treatments. Green label-ing represents methylated stained cells and the blue labeled cells are stained with DAPI to allow the identification of the embryonic cells and permit the quantification of global methylation. Scale bar, 100µm.