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Influence of storage at 5°C and vitrification on apoptotic changes in equine blastocysts

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Summary

The aim of the study was to determine the extent of nuclear DNA fragmentation in equine embryos at the blastocyst stage stored at 5°C for 6 h and 24 h or vitrified in straws or in the Rapid-i system. An additional aim was to determine the relationship between the age of fresh and stored embryos and the intensity of apoptosis. In this study, 42 embryos were analyzed by the TUNEL method and allocated to the following groups: fresh (control – 14 embryos), stored at 5°C for 6 h (7 embryos), stored at 5°C for 24 h (7 embryos), vitrified in straws (7 embryos), and vitrified in the ultra-fast Rapid-i system (7 embryos). It was found that 36.7% of fresh equine embryos had apoptotic nuclei, and the mean dead cell index (DCI) was 0.3%. This was evident as an increase in DNA fragmentation in equine embryos after vitrification in straws (2.3%) compared to fresh embryos (0.3%) and those vitrified in the Rapid-i system (0.3%). Embryo storage at 5°C did not affect the apoptosis frequency in nuclei regardless of the time of storage. DCI for embryos stored for 6 h was 0.44%, and after 24 h of incubation it was 0.64%. The intensity of apoptotic changes observed in equine embryos depended on their age and size. The apoptosis frequency was negatively correlated with the age and diameter of fresh embryos, embryos stored at 5°C, and embryos vitrified in Rapid-i, whereas for embryos vitrified in straws the correlation was positive.

Keywords: equine embryos, vitrification, embryo transfer, embryo apoptosis

Cryopreservation of embryos with liquid nitrogen at –196°C has been successfully used in the biotechnology of animal reproduction to preserve valuable genetic material and for the purpose of future embryo transfer (7, 23).

The sensitivity of embryos to cryopreservation differs depending on the species. Equine embryos are characterized by a decreased developmental potential after thawing or are incapable of surviving the freeze-thaw cycle. Therefore, in the case of horses, most procedures, such as embryo transfer and bisection, are carried out immediately after obtaining the embryos or after only a few hours of storage at approximately 4°C to 5°C (7). The possibility of cryopreserving equine embryos was first confirmed in 1982 by Yamamoto et al. (27), who obtained foals after the transfer of embryos that had been stored for a few hours at 0°C. The first practical applications of equine embryo preservation were reported as late as 1987 (5). Currently, storing equine embryos at 5°C for up to 24 h is widely used as part of assisted reproduction techniques prior to embryo transfer (14).

Methods for the vitrification of equine embryos are still in the experimental phase and thus are not commonly used in husbandry practice. This is mainly due to the presence of a capsule that reduces the penetration of the embryo by cryoprotectants as well as to the large amount of fluid in the blastocoel and the high intracellular lipid concentration (6, 26). The first pregnancies after equine embryo vitrification were obtained in 1994 (13). The improvement of equine blastocyst vitrification involves adaptation of procedures that were previously used to cryopreserve sheep and bovine embryos (4, 8).

All negative ultrastructural and biochemical changes in the embryo during cryopreservation are due to the decreasing temperature or the embryo's sensitivity to cryoprotectants present in the vitrification medium. Under unfavorable conditions, such as vitro culture and low temperature, the apoptotic process in embryos intensifies (2). Apoptosis occurs in embryos naturally, and there are many reports showing that it plays a major role in eliminating damaged cells during pre-implantation development and promoting proper

embryo development at post-implantation stages (9, 10). It is very likely that these changes are related to the embryo's sensitivity to external stimuli (25). It has been demonstrated that the frequency of apoptosis can be used as a marker of the embryo's developmental potential (9, 24). The storage of equine embryos at 5°C for 24 h does not significantly increase apoptotic changes (17).

The effects of slow freezing and vitrification of equine embryos on increasing DNA fragmentation have not yet been studied. It was reported that the vitrification procedure induces apoptotic changes in 78% of bovine embryos (16). Vitrified porcine embryos also demonstrate intensified apoptosis, as detected by the TUNEL assay. It has been shown that the dead cell index (DCI) and DNA fragmentation in the inner cell mass of blastocysts increase in embryos subjected to vitrification compared with control embryos (9).

The aim of the study was to investigate the impact of different methods of preservation on the intensification of apoptotic changes in equine embryos using the TUNEL assay.

Material and methods

Animals. The experiments were conducted according to guidelines approved by the Local Ethics Committee for Animal Experimentation in Cracow (no 37, 30 May 2016).

Embryos were obtained from 12 clinically healthy mares of the Konik Polski breed aged between 4 and 20 years. Ovulation in the mares was determined by rectal palpation and USG examination (MyLab30VET, Esaote, Italy). Immediately prior to ovulation, the mares were naturally mated or inseminated. If ovulation was not confirmed after 24 h, mating was repeated.

Reagents. The reagents used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Roche (Germany) if not stated otherwise. The equipment and reagents for collecting embryos were manufactured by Minitube (Germany), and those for vitrification by Vitrolife (Sweden).

Embryo flushing and evaluation. Embryos produced in vivo were obtained on days 6, 7 or 8 after ovulation. The uterus was flushed with PBS supplemented with 1% FBS (Sigma-Aldrich, USA) using the following equipment: Embryo Flushing Catheter, Flushing Y-junction Tubing with a high-flow connector, and Miniflush Filter (Minitube, Germany). The procedure was repeated four times using approximately 1.5 l of fluid for each flushing, depending on the size of the uterus.

Embryos were identified using a stereoscopic microscope (SMZ1000 Nikon, Japan) and evaluated based on their development stage, shape, turgor, and the number of degenerated blastomeres (McKinnon and Squires (1998). Embryos designated as class 1 and 2 with no distinct morphological alterations were used for further procedures. Blastocysts were randomly preserved at 5°C for 6 h or 24 h or vitrified using 0.5 ml straws (Minitube, Germany) or the Rapid-i device (Vitrolife, Sweden). The control group consisted of embryos that were evaluated directly after collection.

Embryo storage at 5°C. The embryos were transferred to 1.5 ml test tubes filled with EquiPRO Holding Medium (Minitube, Germany) that were placed in an Equitainer (Minitube, Germany) and stored at 5°C. After 6 h or 24 h of storage, the test tubes with embryos were removed from the Equitainer, the medium with embryos was transferred into sterile Petri dishes, and the embryos were fixed in 4% paraformaldehyde (PFA). Following identification and evaluation under a stereoscopic microscope (magnification: × 40), the embryos were transferred into PBS supplemented with 10% FBS and subjected to the TUNEL assay to detect apoptotic change.

Embryo vitrification in straws. The embryos were incubated in a vitrification medium using a commercial EQUIPRO Vit-Kit (Minitube, Germany). The embryo was placed in a drop of equilibration medium A and then in medium B at 26°C, for 5 min in each solution, followed by 30 sec exposure to a vitrification medium. Then, the embryo was transferred in a volume of approx. 50 µl of the vitrification medium to a previously prepared straw containing a column of a dilution medium and air. After closing the straw, it was held in liquid nitrogen vapor for 1 min and then plunged into a container of liquid nitrogen (MVE, Minitube, Germany).

Ultra-rapid vitrification (Rapid-i method). The embryos were vitrified using a closed vitrification system, Rapid-i (Vitrolife), and commercial cryoprotectants for vitrification, the EQUIPRO Vit-Kit (Minitube). The Rapid-i vitrification system consists of a plastic holder with a hole for inserting the embryo in a minimum quantity of the vitrification solution (approx. 2 µl) and a silicone storage straw (RapidStraw) providing additional protection for the embryo subjected to vitrification. Equilibration was carried out in two equilibration media, A and B, at room temperature (5 min in each solution). Then, the embryos were exposed for 30 sec to the vitrification medium, transferred individually into Rapid-i holes, and then placed in silicone storage straws (RapidStraw) that had previously been cooled in liquid nitrogen vapor. Each RapidStraw was cooled in a SmartBox (Vitrolife, Sweden) filled with liquid nitrogen. After closing the Rapid-i, the straws were closed with an ultrasonic sealer (Vitrolife, Sweden) and stored at –196°C in a container with liquid nitrogen (MVE, Minitube, Germany).

Embryo thawing. The thawing procedure consisted of taking a straw with a frozen embryo out of liquid nitrogen and holding it at room temperature (20 to 24°C) for 10 sec. Then, the straw was placed in a water bath for 10 sec at 20°C to 24°C. After removing the straw from the water bath, the vitrification medium and Vit-Kit Dilution Medium (Minitube, Germany) were mixed by gentle shaking. Then, the straw was opened, and its contents were placed in a drop of Vit-Kit Dilution Medium for 8 min, followed by 5 min in EquiPRO Holding Medium (Minitube, Germany). The next stage of the experiment consisted of evaluating the embryos under a stereoscopic microscope. After that, the embryos were fixed in 4% paraformaldehyde (PFA). Then they were sustained until DNA evaluation.

Tab. 1. Indicators of embryo apoptosis in all experimental groups

Experimental group	Embryos					
	n	Apoptosis detected n/%	Diameter (µm)	Total number of blastomeres	Number of apoptotic nuclei	DCI (%)
Fresh	14	5/36.7%	487.64 ± 196.66	1374.43 ± 1070.18	4.57 ± 8.23[b]	0.33
Stored at 5°C for 6 h	7	3/43%	530.00 ± 226.31	1561.57 ± 1113.21	7.00 ± 9.36[b]	0.44
Stored at 5°C for 24 h	7	3/43%	565.86 ± 235.96	1845.71 ± 1293.77	11.86 ± 17.01[b]	0.64
Vitrified in straws	7	5/71.4%	558.29 ± 257.10	1521.14 ± 1084.52	35.43 ± 34.72[a]	2.30
Vitrified in Rapid-i	7	3/43%	528.00 ± 188.97	1654.86 ± 1104.71	5.00 ± 9.20[b]	0.30

Explanations: a, b – different values within columns differ significantly ($p < 0.001$)

Embryos vitrified by the Rapid-i method were thawed by drawing the RapidStraw out of liquid nitrogen and placing it in a vertical position in the SmartBox. The holder with the embryo was immediately placed in 1 ml of Vit-Kit Dilution Medium (Minitube, Germany). After 8 min, the embryo was transferred for 5 min into EquiPRO Holding Medium (Minitube, Germany), heated up to room temperature, and evaluated under a stereoscopic microscope. Then, the embryos were fixed in 4% PFA. After that, they were sustained until DNA evaluation.

Evaluation of DNA fragmentation by the TUNEL assay. DNA fragmentation was analyzed by the TUNEL assay using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Germany).

The embryos were fixed in 4% PFA for 60 min at room temperature and permeabilized in PBS with 1.0% (v/v) Triton X-100 and 0.1% sodium citrate and washed in 50 µl of PBS-PVP. Embryos from the control group were fixed immediately after collection and evaluation. Embryos stored at 5°C were fixed directly after 6 or 24 h storage and microscopic evaluation. Vitrifited embryos were fixed after thawing and evaluation. Subsequently, they were incubated with reaction mixture consisting of nucleotides and terminal deoxynucleotidyl transferase (TdT) under mineral oil at 37°C in the dark for 60 min. Positive controls consisted of embryos incubated with RNA-free deoxyribonuclease (DNase) at 37°C in the dark for 60 min, while negative control samples consisted of embryos incubated in the reaction mixture without TdT. After incubation, the embryos were washed in PBS-PVP and transferred through a gradient (50-75-100% in PBS-PVP) of Vecta Shield with 4',6-diamidino-2-phenylindole (DAPI) (Victor Laboratories, CA). The embryos were placed in a drop of PBS-PVP and transferred onto a microscope slide. They were analyzed under a fluorescence microscope (Nikon Eclipse E600) with two types of filters: green fluorescence for apoptotic cells (wavelength 520 ± 20 nm) and blue fluorescence for all nuclei in the embryo stained with DAPI (wavelength 358-461 nm).

The assessment of nuclear DNA fragmentation consisted of comparing the total number of nuclei labeled with DAPI and the number of cells showing DNA fragmentation stained positively in the TUNEL assay. The following parameters were determined for all blastocysts in a given group: average total number of nuclei, average number of apoptotic nuclei, and dead cell index (DCI = No apoptotic cell/No of nuclei).

In addition, the number of embryos with at least one apoptotic nucleus was established and compared with the number of embryos in a given group.

Statistical analysis. The results are presented as means ± standard deviation (SD). The differences between means were considered statistically significant at $p < 0.05$ and highly significant at $p < 0.01$. The calculations were performed using the Statistica software version 9.1 (StatSoft, Inc., 2010). A two-way ANOVA was used to analyze the effects of the type of storage and the day of obtaining the embryo. Tukey's test was used to detect statistically significant differences between means.

Results and discussion

The study analyzed 42 embryos at the blastocyst stage obtained from 12 mares after 96 uterine flushes. The average embryo recovery rate amounted to 43.7%. Indicators of embryo apoptosis for all experimental groups are presented in Table 1. The lowest DCI values and the fewest apoptotic nuclei were found in fresh embryos. In this group, apoptotic nuclei were observed in 36.7% of embryos. The largest percentage of embryos with fragmented DNA was observed after vitrification in straws (71.4%). DCI in that group amounted to 2.3%. Embryo storage was significantly correlated with the number of apoptotic nuclei ($p < 0.05$). Analysis of variance revealed the occurrence of a statistically significant effect of the type of preservation and the day of obtaining the embryo on DCI ($p < 0.05$). The highest DCI was found in embryos obtained on days 7 and 8 after ovulation and vitrified in straws (Tab. 2). With

Tab. 2. Values of the apoptotic index (DCI) in equine embryos, taking into account the day of collection post-ovulation (p.o.).

Preservation	Day of embryo collection (post ovulation)		
	6 days p.o. n = 12	7 days p.o. n = 17	8 days p.o. n = 13
Fresh	1.1	0.7	0.01
Stored at 5°C for 6 h	1.9	0.83	0
Stored at 5°C for 24 h	6.4[a]	1.2	0
Vitrified in straws	0.97[b]	2.2	2.6
Vitrified in Rapid-i	0.8[b]	0.6	0

Explanations: a, b – different DCI values within columns differ significantly ($p < 0.05$)

Tab. 3. Values of the DCI of equine embryos of different diameters

Experimental group	DCI, diameter < 400 µm (%)	DCI, diameter > 400 µm (%)
Fresh	0.70[a] (n = 5)	0.30 (n = 9)
Stored at 5°C for 6 h	1.90 (n = 2)	0.65 (n = 5)
Stored at 5°C for 24 h	6.40[b] (n = 2)	0.31 (n = 5)
Vitrified in straws	0.97[a] (n = 2)	2.44 (n = 5)
Vitrified inRapid-i	0.80[a] (n = 2)	0.50 (n = 5)

Explanations: a, b – different DCI values within columns differ significantly ($p < 0.05$)

respect to the other types of storage and fresh embryos, the greatest percentage of dead cells was observed in 6-day-old embryos, and the highest DCI was obtained for embryos stored at 5°C for 24 h (DCI = 6.4%). Statistically significant differences were found between embryos obtained on day 6 after ovulation that were stored at 5°C for 24 h and those vitrified in straws or by the Rapid-i method (Tab. 2). Analysis of DCI in all experimental groups taking into account diameters of the embryos revealed statistically significant differences between embryos with a diameter < 400 µm stored at 5°C for 24 h and fresh embryos or those vitrified in straws or by the Rapid-i method (Tab. 3).

The incidence of apoptosis has been suggested as an additional criterion in morphological embryo evaluation and a useful tool for improving the methods of preserving equine embryos. Apoptosis in equine embryos has not been extensively studied compared with other animal species. The present study, involving equine embryos at the blastocyst stage obtained on days 6, 7, or 8, revealed that apoptotic nuclei occurred in 36.7% of embryos, and the average dead cell index amounted to 0.3%. Moussa at el. (17) observed apoptosis in 81% of equine embryos obtained on day 7 after ovulation, while in our study apoptotic nuclei were detected in 33% of 7-day-old embryos. In bovine embryos, DNA fragmentation reached a level of 30% (3), while in pig embryos it amounted to 23% (18). Pomar et al. (2005), who analyzed equine embryos produced *in vivo* and considered to be grade I blastocysts, did not find any signs of apoptosis (21). Such discrepancies may arise from different criteria used in assessing apoptotic alterations. In the present study, apoptotic changes were detected on the basis of single-stranded and double-stranded DNA fragmentation stained in the TUNEL assay. This method was selected because it makes it possible to quickly detect and analyze apoptotic changes with the use of a fluorescence microscope. The TUNEL method is highly sensitive and capable of detecting six times as many apoptotic nuclei as other staining methods because, with this method, cells can be labeled in the early stages of apoptosis before morphological changes become identifiable.

The dead cell index amounted to 1.1% for embryos in the early blastocyst stage obtained on day 6 after ovulation and decreased with the growth of embryos to 0.7% on day 7 after ovulation. Apoptotic changes in embryos flushed on day 8 after ovulation were incidental (0.01%) and were detected in only one of 7 embryos. It is highly suggestive that, after being initiated approximately 6 days after ovulation, apoptosis gradually decreases with embryo growth (3, 17). The apoptotic index in bovine blastocysts was also inversely proportional to the total number of nuclei. A similar trend was identified in murine and human embryos (12). Furthermore, the apoptotic index in expanding murine blastocysts was lower than it was at earlier developmental stages (11).

The purpose of this study was to establish the effect of different methods of embryo preservation on the intensification of apoptotic changes in equine embryos. Cryopreservation is a strong external stimulus that activates proapoptotic factors and aggravates signs of late-stage apoptosis. According to our own studies, apoptotic changes in 6-, 7-, and 8-day-old embryos are not increased after storage at 5°C for 6 h. The dead cell index in that group was 0.44%, similar to that for fresh embryos (0.3%). Prolonging cryopreservation for up to 24 h had an insignificant effect on DNA fragmentation, increasing the index to 0.64%. These results are consistent with those reported by Moussa et al. (2004), who found that storing equine embryos at 5°C for up to 24 h did not intensify apoptotic changes (17). It can be assumed that the storage of equine embryos for up to 24 h does not reduce their developmental potential and indicates the practical usefulness of such storage in other biotechnological procedures. The efficiency of low temperature storage, especially in liquid nitrogen, depends directly on the age and size of embryos. The pregnancy rate obtained by transferring small vitrified embryos with a diameter of < 400 µm ranged from 45% to 67%, whereas for embryos with a diameter of > 400 µm it was significantly lower and ranged from 0% to 38% (1, 22, 28). Studies aimed at improving the efficiency of large embryo vitrification are focused on increasing the speed of freezing the vitrification medium and the embryo. These conditions can be obtained by ultra-rapid vitrification methods, such as the cryotop or cryoloop, which are successfully applied in human fertility clinics. Our study involved the analysis of apoptosis in embryos vitrified in straws and by the Rapid-i system. The analysis of equine embryos after vitrification in straws showed a positive TUNEL reaction, confirming the occurrence of apoptosis in 71.4% of embryos. The number of cells with apoptotic nuclei almost doubled compared with embryos evaluated immediately after flushing (36.7%). There is no information in the available literature on vitrification of equine embryos by ultra-rapid methods, nor its evaluation by the TUNEL assay. So far, ultra-fast methods, such as Rapid-i have been used only for vitrification of mare oocytes (19, 20). In our study, DCI after vitrification in the Rapid-i system was lower compared with vitrifica-

tion in straws. Vitrification in Rapid-i did not intensify apoptosis irrespective of embryo size, and DCI after vitrification in Rapid-i was at the same level as the index obtained for fresh embryos (0.3%) (Tab. 3). Although positive results were obtained in morphological evaluation, in some embryos nuclear fragmentation was intensified. Analysis of apoptosis should involve concurrent application of several detection methods to provide a detailed insight into mechanisms underlying apoptotic changes and the impact of thermal stress on equine embryos.

The results obtained in this study suggest that the value of the death cell index (DCI) indicates the quality and developmental abilities of vitrified equine embryos. The TUNEL method can be used to evaluate the effectiveness of embryo preservation techniques.

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