Artykuł przeglądowy *Review*

Cryopreservation of equine embryos obtained *in vivo*

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Summary

Cryopreservation of equine embryos eliminates the need to have a herd of recipient mares and to synchronize the donor and recipient mares. It also makes it possible to store embryos for long periods of time and transport them across international borders. The extracorporeal fertilization of equine oocytes by the intracytoplasmic sperm injection (ICSI) method, together with the procedure of live oocyte collection (Ovum Pick Up, OPU), has revolutionized the possibilities of transplanting frozen embryos. However, the high costs of OPU + ICSI and the large range of expected results in the form of ultimate, cultured and frozen embryos suitable for transplantation stimulate the current intensive search for increasingly effective methods of cryopreservation of embryos obtained not in the laboratory, but washed out after mare mating/insemination. The aim of this paper is to describe the methods of cryopreservation of equine embryos collected *in vivo*. There are two main methods of cryopreservation of equine embryos collected *in vivo*: slow freezing and vitrification (ultrafast freezing). Compared to the slow freezing of embryos, vitrification is a cheaper, simpler and faster procedure that does not require specialist freezing equipment, and, moreover, can be performed in the field, which is why it has found wider application in clinical practice. The main obstacle to cryopreservation of embryos in the field and obtaining an acceptable pregnancy rate after their transplantation (> 55%) is the need to obtain an embryo whose size does not exceed 300 µm, i.e. in the early stage of development (from morula to early blastocyst) between 144 and 168 hours after ovulation. Another factor that impacts the effectiveness of embryo cryopreservation is the presence of the embryonic capsule – an acellular glycoprotein envelope that is produced after the embryo descends from the oviduct into the mare's uterus along with the formation of a blastocyst. It has been shown that vitrification of punctured mare blastocysts smaller than 550 µm allows pregnancy to be achieved after thawing and transfer rate > 75%, regardless of whether the blastocyst fluid has been aspirated. In the case of embryos with a diameter of more than 550 µm, aspiration of the blastocyst fluid is necessary for effective vitrification.

Keywords: cryopreservation, embryos, vitrification, mare

Embryotransfer has long been one of the basic biotechniques used in assisted reproduction of horses. The ultimate result of embryotransfer depends on the quality of semen used for insemination, the effectiveness of the insemination procedure, the effectiveness of embryo recovery, the fertility of the donor mare and the embryo recipient and the synchronization of their estrous cycles, as well as the practical skills of the person who performs the procedure. The donor mare must be fertile and able to sustain the development of the embryo for at least about 7 days after ovulation (4).

Currently, embryos obtained directly from the donor are transferred to the recipient mare or subjected to procedures that allow them to remain outside the mare's body for a shorter or longer period of time during which they can be stored and transported. Embryos cooled to a temperature of 5°C in an appropriate medium can be effectively transplanted within 24 hours of collection (10, 11), whereas embryos frozen (cryopreserved) by appropriate techniques at –196°C can be stored practically without any time limits. This significantly simplifies the logistics of transplantation, reducing the

number of surrogates needed and eliminates the need for synchronization of the estrous cycle between the donor and the recipient mare. Frozen embryos can also be transported at any distance, subjected to genetic testing and used only after obtaining the results of these tests, e.g. tests that determine the sex of the embryo or exclude selected genetic disorders (12).

Initially, embryos obtained from the donor mares at the age of 6–8 days were frozen. The first pregnancy after transplantation of a previously frozen equine embryo was described in 1981, but unfortunately it was miscarried around day 60 (20). A year later, the first case of a foal born after transplantation of an embryo previously frozen was described (47). In both cases, the embryo was frozen by the slow freezing method. In 1994, the first pregnancy was obtained after transfer of an embryo subjected to vitrification (ultrafast freezing) (22).

Another breakthrough was the mastery of extracorporeal fertilization of equine oocytes by the method of intracytoplasmic sperm injection (ICSI), which, together with the procedure of live oocyte collection (Ovum Pick Up, OPU), revolutionized the possibilities of transplanting frozen embryos. Currently, frozen equine embryos available commercially are most often obtained by ICSI performed on oocytes collected from mares by the TVA-OPU (ultrasound guided transvaginal aspiration of oocytes, TVA) method. However, the high costs of OPU + ICSI and the large range of expected results in the form of ultimately cultured and frozen embryos suitable for transplantation (34) stimulate the current intensive search for increasingly effective methods of cryopreservation of embryos obtained not in the laboratory, but washed out after the mare's mating/insemination. Research shows that in 2022, 24248 embryos were obtained *in vivo*, of which 22970 were transferred fresh and 49 were frozen (43). In the same year, 14242 embryos were produced *in vitro*, of which 2649 were transferred fresh and 3328 were frozen (43). An undeniable advantage of the latter method are significantly lower costs (e.g. the cost of embryo vitrification is 800 PLN in Poland and 300 USD in the U.S.) and the ability to perform all procedures in the field, without the need for a specialist laboratory capable of performing ICSI and further culturing the embryo thus obtained.

The aim of this paper is to present contemporary methods of cryopreservation of equine embryos obtained *in vivo* together with their limitations as an alternative to freezing embryos obtained by OPU-ICSI.

There is an ongoing research on effective methods of embryo manipulation and cryopreservation that could be applied in mobile practice using cheap and easily available equipment.

The conception rate after transplantation of fresh embryos is higher than 80% (30, 31, 44), and it is similar for embryos cooled to 5°C for a period not exceeding 24 hours (10, 11). The results of transplantation of frozen embryos vary depending on the origin of the embryo (obtained *in vivo* or produced *in vitro*).

Cryopreservation techniques for equine embryos obtained *in vivo*

The pregnancy rate for frozen and transferred embryos of small sizes (< 300 μm) ranges from 50% to 70% (24, 25, 29, 35). There are two main methods of cryopreservation of equine embryos: slow freezing and vitrification (36).

Slow freezing involves gradual exposure of the embryo to a cryoprotective agent (CPA) and controlled, stepwise cooling. The classic cryoprotective agent used in the slow embryo freezing procedure is glycerol (10%, 1.36 M), and the embryo is incubated in 2 or 4 glycerol solutions of increasing concentration. Other CPAs described in the literature are dimethyl sulfoxide (DMSO), 1,2-propanediol, ethylene glycol (1.5 M) (8), and methanol (2.5 M) (2), but their cryoprotective properties are weaker compared to those of glycerol. In a study by Hochi et al. (23) only ethylene glycol in combination with sucrose yielded a promising pregnancy rate after embryo thawing: 7/11 (63.6%) versus 2/8 (25%) for ethylene glycol and 3/8 (37.5%) for glycerine (23).

The basic condition for the effectiveness of the slow embryo freezing procedure is to establish the optimal cooling rate. Cooling the embryo too quickly leads to the formation of ice crystals and dehydration causing its destruction, which is why this procedure requires a specialized, programmed device characterized by high precision in lowering the temperature. Moreover, the straws should be incubated for a specified time at a temperature of 6–7°C to lead to the simultaneous, synchronized formation of ice crystals in the extracellular fluid as well. Therefore, slow freezing is a time-consuming process that usually takes from 1.5 to 2 hours (36).

An alternative to the slow freezing described above is vitrification, i.e. ultrafast freezing, which involves the immediate transition of intra- and extracellular fluids from the liquid phase to the solid phase, without the formation of ice crystals (36). Vitrification is possible only with the use of very high CPA concentrations (on average 4-5 times as high as those used in the slow freezing procedure) and a very fast lowering of the temperature by direct immersion in liquid nitrogen (27). Compared to the slow freezing of embryos, vitrification is a cheaper, simpler and faster procedure that does not require specialist freezing equipment and, moreover, it can be performed in the stable. The duration of the vitrification process is about 15 minutes (9, 15).

A disadvantage of vitrification is the use of high concentrations of CPA, which are toxic to the embryo. Therefore, it is important to adhere to the specified time of embryo immersion in individual media, especially in the final solution with the highest concentration. Some difficulty during the vitrification process is posed

by the manipulation of embryos in cryoprotectants because of their high density. Therefore, the operator carrying out this procedure needs to have appropriate experience in finding and transferring embryos (7). Currently, commercial kits for vitrification of equine embryos are available on the market.

Another study showed that embryos could be chilled for 16 hours prior to vitrification and that comparable pregnancy outcomes were achieved with them (15 out of 20 pregnancies with directly vitrified embryos and 13 out of 20 with recently chilled embryos) (24). This finding indicates that embryos can be collected in the stable, chilled and then shipped to an embryo transfer facility for actual vitrification.

The vitrification procedure developed by Eldrige Panusek et al. (15) seems to be widely used and consists in incubating the embryo in three vitrification solutions (VS): VS1 (1.4 M glycerol in phosphate buffered solution), VS2 (1.4 M glycerol and 3.6 M ethylene glycol), and VS3 (3.4 M glycerol and 4.6 M ethylene glycol).

From a practical point of view, the most common errors made during vitrification of equine embryos are as follows:

• exposure of the embryo to VS3 solution for more than 1 minute;

• transfer of the embryo from VS1 through VS2 to VS3 solution with too much fluid taken, which results in excessive dilution of the cryoprotectant level;

• VS3 solution is very dense, which causes the embryo to float on its surface, contributing to difficulties in finding it under a stereoscopic microscope and in further manipulation (38).

Limitations of cryopreservation of equine embryos

Eldridge Panuska et al. (15) published their results to assess the pregnancy rate obtained from embryos subjected to vitrification depending on the size of the embryo and the method of transplantation. The comparison concerned the use of embryos with a diameter of less than 300 µm and larger. Some embryos were transferred to the recipient mare by the direct method (i.e. with a straw directly after thawing), while others were removed from the straw after thawing, aspirated again into another straw and then transplanted to the recipient mare. No differences in pregnancy rates were found for these two methods. However, the size of the embryo was proved to be significant: in the case of embryos with a diameter of less than 300 µm, the pregnancy rate was 54% (26 out of 48), whereas in the case of embryos with a diameter exceeding 300 µm, no pregnancy was achieved (15). Thus, the main obstacle to the use of cryopreservation of embryos under field conditions and obtaining an acceptable pregnancy rate after their transplantation (> 55%) is the need to collect an equine embryo smaller than 300 µm, i.e. in the early stage of development (from morula to early blastocyst) between 144 and 168 hours after ovulation (3, 14, 35). Unfortunately, obtaining embryos of the appropriate size is difficult. Uterine flushing of the mare to obtain the embryo must be performed only a few hours after the embryo has descended from the fallopian tube into the uterus, where the time of descent into the uterus, as well as the rate of its development seem to vary depending on the season, type of semen used (fresh/frozen) and even the age of the mare (37). Flushing the uterus too early (e.g. on the 6th day after ovulation) may result in failure to obtain the embryo, which has not yet descended from the fallopian tube into the uterus, while performing the procedure too late may result in obtaining an embryo that is too large and therefore much more difficult to cryopreserve (6).

Eldridge-Panuska et al. (15) suggested the possibility of obtaining embryos smaller than 300 µm by flushing the mare's uterus 8 days after hCG induction of ovulation (assuming ovulation 36 hours after hCG administration).

An alternative method was proposed by Robinson et al. (32), which consists in the administration of PGE2 gel into the fallopian tube on the side where ovulation occurred 4 days after its occurrence, which contributed to premature descent of the embryo into the mare's uterus within 24 hours (32). This technique, however, is not used in clinical practice because of its invasive nature, i.e. the need for lateral laparotomy and the administration of PGE2 via a laparoscope into the mare's fallopian tube.

In summary, obtaining embryos of a size suitable for freezing is time-consuming and does not always produce the expected results. In case of emryos larger than 300 µm, the slow freezing procedure seems to be a protocol of choise, giving a chance for pregnancy but the success rateis low and doesn't exceed 20% (1, 35).

Another factor influencing the effectiveness of embryo cryopreservation is the presence of the embryonic capsule, i.e. an acellular glycoprotein envelope that is produced after the embryo descends from the oviduct into the mare's uterus along with the formation of the blastocyst (5, 17). The thickness of the capsule is negatively correlated with the effectiveness of cryopreservation (26). The lower effectiveness of cryopreservation may result from the slow penetration of glycerol and ethylene glycol through the embryonic capsule (19). The good results of cryopreservation of embryos produced *in vitro*, in which the embryonic capsule is absent, seem to additionally confirm this theory (18). Attempts have been made to increase the penetration of CPA into the embryo by partial digestion of the embryonic capsule with trypsin before cryopreservation, but the results were contradictory (28, 39), which is why this method has not been used in clinical practice.

Cryopreservation of embryos larger than 300 µm

A breakthrough in cryopreservation of embryos larger than 300 µm was a study by Choi et al. (12), who, while developing an embryo biopsy technique for genetic diagnosis before transfer, noticed that puncturing

and collapsing the embryo did not impair its viability. This led to further studies to verify the usefulness of this method of „reducing" embryos. In the next study, a piezoelectric drill was used to puncture the embryonic capsule and trophoblast, and then blastocyst fluid was aspirated before embryo vitrification. The study resulted in a promising pregnancy rate of 71% (5/7) with embryos smaller than 650 µm (13).

One of the limitations of cryopreservation of embryos collected *in vivo* is the difficulty of recovering an embryo smaller than 300 µm, i.e. around 6 days after ovulation (6, 37). Effective cryopreservation of embryos larger than 300 µm allows the embryo recovery procedure to be performed around 7-8 days after ovulation, which is much easier in practice (6).

The embryo manipulation method was further analyzed, which resulted in the conclusion that a higher pregnancy rate occurs in embryos manipulated with a micromanipulator compared to those manipulated manually (16, 21, 46). It should be noted that a serious disadvantage of manual embryo puncture is the need for high operator skill, despite which irreversible damage to embryos occurs more often than it does in the case of manipulation with a micromanipulator (46).

Vitrification of punctured blastocysts smaller than 550 µm made it possible to achieve pregnancy rate > 75%, regardless of whether blastocyst fluid was aspirated. In the case of embryos of more than 550 µm in diameter, aspiration of blastocyst fluid is necessary for successful vitrification (45).

Sanchez et al. (33) modified a vitrification method designed for cows, applied it to equine embryos larger than 300 µm using a hemi-straw, and achieved a high pregnancy rate of around 70% (41, 42). Furthermore, Sanchez's studies confirmed that the diameter of the equine embryo determines the choice of the cryopreservation protocol (33).

In the case of slow freezing of embryos larger than 550 µm after their prior puncture, a significant increase in the number of dead cells and cytoskeletal damage was observed, which was not observed when similar embryos were vitrified (40). Possibly, the small hole made in the capsule and/or the time used for the equilibration are insufficient for adequate influx of cryoprotectant and efflux of intracellular water. Another possible cause of the higher level of damage during slow freezing is the poor freezing protocol used in the research: embryos were frozen at 0.5°C/min, and 85% of blastocoel fluid was aspirated before cooling (40). It is recommended that the cooling rate be reduced to –0.3°C/min from –6°C to –30°C and to –0.1°C/min from –30°C to –33°C and that the blastocoel fluid volume be reduced by about 50% (28). Reduction of the cooling rate prolongs the total cooling period and allows larger embryos to dehydrate more completely as the freezing process progresses, and the cryoprotectants also have more time to equilibrate inside and outside the cells (1).

Cryopreservation of equine embryos obtained *in vivo* is an alternative to the Ovum Pick Up (OPU)/ Intracytoplasmic Sperm Injection (ICSI) procedure. It seems crucial to develop vitrification protocols for embryos larger than 300 µm that can be used with cheap, easily accessible equipment under field conditions. This will result in rapid breeding progress and make it available for smaller horse farms as well. Currently, the authors' team is working towards this objective.

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