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Review article

Current and potential use of cryopreservation of farm animal embryos and oocytes

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The paper presents trends of development and current possibilities in cryopreservation of embryos and oocytes of cattle, pigs, sheep, goats and horses, as well as prospects for improving this technology. Two basic methods of cryopreservation, freezing and vitrification, are described. The paper discusses the main factors determining the suitability of embryos and oocytes for cryopreservation and possibilities for modifying it.

KEY WORDS: cryopreservation / embryo / oocyte / farm animals

The essence of cryopreservation of gametes and embryos is to stop their life processes for a practically unlimited time. This is as yet the only method by which this goal has been achieved, as attempts to lyophilize genetic material, which could have been an alternative to cryopreservation, have not produced the desired results.

The first experiments on cryopreservation of male gametes were undertaken as early as the 1870s, whereas methods of cryopreservation of female gametes and embryos do not have such a long history, dating back to the 1950s.

A breakthrough in the development of cryopreservation of genetic material was the discovery by Polge et al. of the protective role of glycerol in 1949. This enabled the development of a method of cryopreservation of sperm in the early 1950s, and twenty years later of embryos as well, owing to the theoretical work of the American cryobiologist Peter Mazur. The essence of this work was the assumption that for effective cryopreservation of ovum-sized biological objects the rate of cooling should be about 1°C/min. Based on these assumptions, Whittingam et al. [142] and Wilmut and Rowson [146] successfully froze mouse embryos in the early 1970s. This resulted in the application of these procedures to the cryopreservation of cattle and sheep embryos, and subsequently to those of a number of

other mammalian species, both laboratory animals and livestock, taking into account their species specificity in terms of capacity for cryopreservation.

Cryopreservation of embryos and oocytes can currently be carried out by freezing or vitrification. While there are fundamental differences between these two cryopreservation methods, both require control and optimization of conditions during each stage of the procedure.

Embryos and oocytes are frozen in freezers that enable a controlled decrease in temperature. In terms of cryobiology, freezing is the solidification of cooled liquid by crystallization. Intracellular crystallization is one of the main causes of cell damage during freezing. Cryopreservation by vitrification, in which the liquid is solidified in vitrified rather than crystal form, eliminates this cause of cell damage. The vitrification method is much simpler than conventional freezing methods. The main problems in vitrification are the toxicity of the protective compounds and osmotic damage. This is caused by the high concentration of protective compounds essential for vitrification. Nevertheless, this method now allows for highly effective cryopreservation of mouse, rat, rabbit, sheep, goat, horse and cattle embryos [41, 64, 115].

CATTLE

Freezing of embryos

Initially, bovine embryos were cryopreserved by slow freezing and slow thawing. This method was first used by Wilmut and Rowson in 1973 [146]. The procedure specific for this species involved the multistage addition and removal of the cryoprotectant at room temperature. The most commonly used cryoprotectants were dimethyl sulfoxide (DMSO), glycerol and ethylene glycol, and less often propanediol. No differences were noted in the survival rates of embryos frozen in ethylene glycol, DMSO and glycerol [13, 34], or in a mixture of glycerol and DMSO [72]. Ethylene glycol and glycerol are currently believed to be the most useful cryoprotectants for freezing bovine embryos. In subsequent studies, a shorter freezing programme was introduced, increasing the final temperature of the embryos to –30 or –35°C before their transfer to liquid nitrogen [123]. The survival rates of frozen embryos are similar for the slow and fast methods. The best survival rates for embryos frozen by the slow-freezing method (slow cooling to –60°C) can be achieved by slow thawing (about +20°C/min), whereas embryos transferred to liquid nitrogen at higher negative temperatures (–30 to –40°C) require rapid thawing (about +300°C/min). The results of transfer of frozen bovine embryos can be assumed to be about 10-20% lower than those obtained after transfer of fresh embryos.

When we freeze embryos obtained by in vitro fertilization and culture, we are dealing with organisms of reduced biological value. Studies have demonstrated that embryos obtained in vitro are less suitable for freezing than those obtained in vivo. More than 80% of cryopreserved bovine embryos obtained in vivo hatch in an in vitro culture, as compared to a hatching rate of only 20% for cryopreserved embryos produced in vitro [41]. The differen-

ces in the cryopreservation capacity of in vivo and in vitro embryos are presumed to be associated both with their water content [73] and with differences in the properties of the zona pellucida [103]. The zona pellucida of embryos produced in vitro has been shown to be more easily digested by enzymes, such as pronase, than that of embryos obtained in vivo [103]. Moreover, the survival of frozen embryos produced in vitro has been shown to depend on whether the culture was carried out in Menezo B2 or TCM 199 synthetic media or in a co-culture with oviductal epithelial cells or granulosa cells. This means that the efficacy of freezing embryos obtained in vitro will be more dependent on improvements in the technology of in vitro embryo production than on cryopreservation methods.

Freezing of oocytes

The first attempts at cryopreservation of mammalian oocytes date back to the 1970s, when methods developed for embryos were successfully used to freeze murine oocytes. Foetuses and offspring were then obtained from frozen murine oocytes that were thawed and then fertilized in vitro, but the efficacy of these attempts was low [143]. The procedures used proved ineffective for freezing bovine oocytes. Subsequent studies found that immature oocytes are less well-suited for cryopreservation than mature oocytes in metaphase II [77]. It was not until the early 1990s that the first calves were obtained from transplantation of embryos developing from cryopreserved oocytes that had matured in vitro [38].

A separate problem is the cryopreservation of immature oocytes (GV stage). The first research on the cryopreservation of immature cattle oocytes was carried out as a joint Polish-French undertaking [55]. It was then found that immature oocytes are not well-suited for cryopreservation, and freezing significantly impairs their ability to mature in vitro. The survival rates of oocytes obtained by these authors after thawing were low (6%). Although healthy offspring have been obtained from immature bovine oocytes cryopreserved by freezing [97, 129], the percentage of embryos reaching the blastocyst stage is still very low. In recent years, research on cryopreservation of bovine oocytes has primarily focused on increasing the efficacy of the individual steps of the method – cryopreservation by vitrification, and in vitro maturation and fertilization [90].

Vitrification of embryos

Bovine embryos were first preserved by vitrification [84, 85] using a procedure developed for mouse embryos. The vitrification mixture consisted of 25% glycerol and 25% 1,2-propanediol. After thawing the cryoprotectants were removed in a single step, using a 1M solution of sucrose. The transfer success rate following this procedure was 20% to 50% [80, 84]. This method was effective for embryos at the morula stage, whereas none of the vitrified blastocysts survived. Blastocysts are believed to be more sensitive than morulae to a high concentration of propanediol in the vitrification mixture. Propanediol is known to penetrate the cell more easily than glycerol, which may cause chemical or osmo-

tic damage to the cell. Attempts to modify the vitrification process by eliminating propanediol from the vitrification mixture have enabled effective vitrification of bovine blastocysts [138], although the in vitro survival rate of these blastocysts was not very high (57%).

In search of the optimal vitrification mixture for bovine embryos, Mahmoudzadeh et al. [81] conducted comparative tests of various vitrification media. The highest percentage, nearly 90% of embryos developing in vitro, was obtained following vitrification in a mixture of ethylene glycol, ficoll and sucrose (EFS) developed by Kasai et al. [63].

One of the important factors affecting the survival of vitrified embryos is their equilibration before vitrification. To avoid the adverse effect of high concentrations of cryoprotectants, the embryos were subjected to a multi-step equilibration procedure. In our own experiments [120] with bovine embryos equilibrated in three steps in a mixture of glycerol and propanediol, high embryo survival rates were observed both in vitro (90%) and in vivo (77%). These observations confirm the findings of other authors [66] who used a multi-step equilibration for vitrification of bovine blastocysts. A significantly higher in vitro survival rate was obtained after 4-, 8-, or 16-step equilibration than in the case of one or two steps [66].

An interesting possibility for increasing the suitability of embryos for vitrification is to subject them to subtle stress induced by high hydrostatic pressure [106] or an increased concentration of sodium chloride [78]. The method of pre-treatment of cells with increased hydrostatic pressure in combination with the Open Pulled Straw (OPS) technique has been successfully used for vitrification of bovine blastocysts obtained in vitro [106, 135].

The efficacy of bovine embryo vitrification is comparable to that of freezing [136, 137]. Similar percentages of seven-day-old bovine embryos developing in vitro have been obtained after cryopreservation by freezing (about 65%) and by vitrification (about 66%) [74, 109].

Observations on the vitrification of bovine embryos produced in vitro show that their potential for vitrification is lower than that of in vivo embryos [40, 47]. The survival rate of cryopreserved embryos obtained in vitro has also been shown to depend on whether the culture was carried out in synthetic media or in a co-culture with oviductal epithelial cells or granulosa cells. Hence it seems that the survival rates of cryopreserved embryos obtained by in vitro fertilization and culture are determined more by the culture method than by the cryopreservation method [84]. Attempts to modify media for culture of bovine embryos obtained after in vitro fertilization and then subjected to cryopreservation in a liquid with liposomes containing lecithin, sphingomyelin and cholesterol have not proved satisfactory [108]. It was only found that the addition of liposomes had no adverse effect on embryo development to the blastocyst stage or on survival after thawing. However, the presence of lecithin in liposomes reduced the survival rate of cryopreserved bovine embryos, which may suggest that it causes detrimental changes in cell membrane composition. Subsequent studies showed that culture in a protein-free medium in the form of serum prior to cryopreservation leads to a decrease in lipid levels in the embryos and improves the efficacy of cryopreservation [6].

The stage of embryonic development is another important factor affecting the survival of vitrified embryos obtained from in vitro fertilization and culture. These observations are confirmed by our own research on vitrification of bovine embryos obtained in vitro [40, 47], in which embryos in the morula stage showed greater sensitivity to vitrification than blastocysts.

In summary, vitrification may be an alternative to the traditional freezing method for cryopreservation of bovine embryos. This is confirmed by the comparable survival rates after non-surgical transplant of vitrified embryos (44.5%) and embryos frozen by the conventional method (45.1%) [26].

In 1994, work on bovine embryo vitrification carried out in the Department of Biotechnology of Animal Reproduction of the National Research Institute of Animal Production resulted in the birth of the first calf in Poland [120] following transfer of vitrified embryos.

Vitrification of oocytes

Experiments on vitrification of bovine oocytes have been performed on both immature oocytes (GV stage) and mature ones (metaphase II). Initial attempts at vitrification of oocytes in the GV stage failed to produce positive results [5, 138], but significant progress was achieved in vitrification of metaphase II oocytes. At that time the composition of the protectant mixture appeared to be the factor determining the survival of the vitrified oocytes [51, 141]. In experiments on the vitrification of mature oocytes in ethylene glycol or in a mixture of glycerol and propanediol, no survival was obtained in vitro [5], or only few oocytes survived [141]. In contrast, vitrification in a mixture of DMSO and ethylene glycol or DMSO, propylene glycol and acetamide resulted in a fairly high percentage of morphologically normal oocytes (about 90%) after thawing. They had a fairly good capacity to become fertilized, and the resulting blastocysts were able to fully develop in vivo. Another important factor for the survival of vitrified oocytes, according to the authors, was the multistage removal of the cryoprotectants after thawing.

In a study by Horvarth and Seidel [58] using cholesterol in the medium for cryopreservation of oocytes, there was a slight increase in the proportion of embryos dividing and developing into the 8-cell stage from oocytes exposed to cholesterol prior to vitrification and then fertilized in vitro in comparison to the control.

The use of a vacuum in a liquid nitrogen chamber (Vitmaster), in which vitrification is achieved by ultrafast cooling of the sample, and the open pulled straw technique (OPS) has enabled successful vitrification of bovine oocytes, both mature (metaphase II) and immature (GV stage) [4]. The authors cited achieved a high percentage of dividing embryos and blastocysts (72% and 38%, respectively) for vitrified oocytes in metaphase II, as well as fairly good results (27% dividing embryos and 14% blastocysts) for oocytes vitrified in the GV stage. Calves have also been obtained following transfer of embryos obtained from mature oocytes vitrified by OPS [135]. These results show that vitrification of both mature and immature bovine oocytes can be effective.

PIGS

Freezing of embryos

Pig embryos are less suitable for cooling and freezing than those of other mammalian species. This was already established in the first work on the preservation of embryos of this species, conducted in the mid-1970s [102, 145]. The low tolerance of pig embryos to cooling and freezing is due to the high lipid content in their cytoplasm [102]. The integrity of intracellular lipids was found to be disturbed during cooling, causing the destruction of the cytoplasm and resulting in irreversible degenerative changes in the embryo [89]. Furthermore, structural changes in lipids were observed in cells kept at +15°C [31]. Subsequent studies [92] have shown that the sensitivity of pig embryos to cooling is more dependent on the development stage of the embryo than in other species. For example, a significant difference has been noted in sensitivity to low temperatures between the hatched blastocyst and earlier stages of embryo development. Other studies [44, 93] have shown that sensitivity to low temperatures depends on whether the embryo developed in vitro or in vivo. These observations indicated that blastocysts hatched in an in vitro culture are less susceptible to damage at low above-zero temperatures than blastocysts hatched in vivo [93]. The specific sensitivity of pig embryos to low temperatures is also manifested in their reduced suitability for cryopreservation.

For a long time it was not possible to freeze pig embryos by methods that were successfully used to freeze the embryos of cattle, sheep or goats. The first successes in the cryopreservation of porcine embryos were mainly the result of the use of embryos at the appropriate stage of development, i.e. expanding or hatched blastocysts [54, 65]. The freezing procedure involved controlled slow cooling at a rate of 0.3°C/min and the use of glycerol as a cryoprotectant. However, the efficacy of these first trials was rather low, as only 9 piglets were born following transplantation of 77 cryopreserved embryos [54, 65].

In subsequent work on freezing pig embryos, the number of embryos used in the experiments was relatively low and the survival of the frozen embryos was assessed solely on the basis of their in vitro development [17, 95].

An important determinant of cryopreservation efficacy is the type of cryoprotectant used. The cryoprotectants most often used in experiments on freezing of porcine embryos have been glycerol [54, 61, 95], glycerol supplemented with lecithin [61] or glycerol with trehalose [17]. An unusual solution was the use of glycerol with the addition of chicken egg yolk [37], which resulted in the birth of one piglet after transplantation of 8 cryopreserved embryos.

Studies on the freezing of pig embryos, which have been carried out – as should be emphasized – on a very small amount of material, have shown that several basic conditions must be met for effective freezing. Blastocysts should be frozen at the stage near hatching. The media used for freezing should contain protein in the form of bovine serum

albumin. Glycerol (1.5 M) should be used as a cryoprotectant, although other cryoprotectants may be used. The freezing procedure should include prior cooling of the embryos in freezers, from room temperature to the seeding temperature at a rate of 1°C/min. Once crystallization is initiated, the embryos should be frozen at a rate of about 0.3°C/min to –35 or –38°C before being transferred to liquid nitrogen. The embryos should be thawed in a 35-37°C water bath using a 0.3-0.5 M sucrose solution to remove the cryoprotectants. The use of sucrose reduces the risk of damage caused by the cryoprotectant, including osmotic damage.

Freezing of oocytes

Research on cryopreservation of oocytes of livestock animals is least advanced in pigs, due to the specific sensitivity of the gametes and embryos of this species to cooling and cryopreservation. Studies on the sensitivity of pig oocytes to low temperatures indicate that immature oocytes surrounded by cumulus cells do not survive cooling even to +15°C [23]. The negative effect of low temperatures, manifested as depolymerization of the cytoskeleton, is probably linked to the penetration of cryoprotectants into the oocyte [101, 140] or to the cooling of lipids in the cytoplasm, which in turn have a destructive effect on cytoskeletal structure [76]. The latter hypothesis has been confirmed in studies on fresh oocytes subjected to centrifugation [76]. After a 48 h culture, the lipids were redistributed in the cytoplasm in the centrifuged oocytes, whereas in the oocytes frozen after centrifugation the lipid distribution was irreversible. According to the authors of the study, this is evidence that the cryopreservation process causes physicochemical changes in the lipids present in the cytoplasm of oocytes [76].

Subsequent studies confirmed that mature metaphase II oocytes were more suitable for freezing than immature oocytes [112]. Research on cryopreservation of porcine oocytes, apart from the search for optimal conditions of use of cryoprotectants [23, 79, 147], has also included attempts to use ‘antifreeze’ substances in the freezing process [26, 53, 114]. The use of these compounds in cryopreservation of oocytes and embryos is one of the directions of research [2, 39, 53].

Vitrification of embryos

As mentioned above, high fat content is a significant determinant of the suitability of porcine oocytes and embryos for cryopreservation. Content of fats is known to depend on the stage of development, the species and the physiological state. The highest content of fats is observed in embryos in the early stage of development. Among livestock species, their highest content is found in the oocytes and embryos of pigs [24]. In addition, porcine embryos produced in vitro have higher lipid content than those obtained in vivo [113]. This is unfavourable for cryopreservation. Fat content in oocytes and embryos can be reduced by microsurgical removal [91] or by polarization of lipids via centrifugation [33].

Porcine embryos produced in vitro have different sensitivity to cooling than the embryos of other mammalian species. Surprisingly, porcine blastocysts obtained from in vitro culture have been found to be less susceptible to damage at reduced temperatures than morulae or blastocysts that develop in vivo [40, 47].

In recent years, research on pig embryo cryopreservation has primarily focused on vitrification. Significant progress in the development of this method has been achieved by minimizing the volume of fluid containing the vitrified embryo (the OPS or SOPS method) [134]. This procedure, which allowed for a high rate of cooling during the vitrification process, resulted in a relatively high survival rate of pig embryos in vitro (morula: 14-70%, blastocyst: 67-73%) [9, 134] and in vivo (morula: 13%, blastocyst: 55%) [9, 10, 11]. Our own research has confirmed the positive effect of a minimized volume of vitrified sample in OPS straws on the survival of porcine embryos in the morula and blastocyst stages [43].

Modifications of the vitrification method using a high cooling rate include the droplet method for bovine oocytes [98] and embryos [111] and for porcine embryos [88]; cryoloop vitrification (CLV), used successfully for hamster embryos [67] and human embryos [68]; a method using EM grids [100]; the use of a metal cube covered with aluminium foil (solid surface vitrification – SSV); [8] and the use of liquid nitrogen cooled below –200°C in a Vitmaster [3]. Although these methods seem to be fairly attractive, their use for cryopreservation of porcine embryos is limited [4, 25].

In our own research on porcine embryo vitrification, the survival rate of blastocysts was about 30%, while no morulae survived at all [47]. These observations confirmed previous findings by Dobrinski and Johnson [27], who showed that 6- and 7-day pig embryos survived to a greater extent than 5-day embryos. The authors also found that one of their cryoprotectant mixtures (glycerol and serum albumin – VSa) was non-toxic irrespective of the age of the embryos. Similarly, in our own experiment [42], a 30-40% survival rate was obtained for morulae and blastocysts preserved in a mixture of ethylene glycol, ficoll and sucrose (EFS), while in our subsequent experiment [44] the ESF mixture proved to be more toxic to hatching blastocysts than to expanding blastocysts, which was reflected in the vitrification results. Experiments on vitrification of porcine blastocysts in subsequent years showed that, despite a high in vitro survival rate, the percentage of blastocysts surviving in vivo was much lower. By treating embryos with cytochalasin B prior to vitrification [28] or using the OPS method [11, 134], the possibility of effective cryopreservation of pig embryos at the morula stage has been demonstrated as well.

As a result of research on cryopreservation of pig embryos conducted in the Department of Biotechnology of Animal Reproduction of the National Research Institute of Animal Production, offspring has been obtained following transplantation of vitrified embryos [48]. These were the first piglets in Poland obtained after transplantation of cryopreserved porcine embryos.

Vitrification of oocytes

In studies on vitrification of immature porcine oocytes, cytochalasin B has been used as an inhibitor of actin microfilament polymerization [35, 59, 60], which specifically affects elements of the cytoskeleton, influencing its elasticity and making lipids less susceptible to cooling [18]. In these experiments, when oocytes were treated in this manner and vitrified in a mixture of glycerol and serum, 21% of thawed oocytes attained maturity in vitro [60]. A much higher percentage of mature oocytes (37%) was obtained after treatment with cytochalasin B and vitrification in ethylene glycol, and a slightly lower percentage (24%) after identical treatment and vitrification in a mixture of ethylene glycol and dimethyl sulfoxide [35]. It is also worth noting attempts to use the cytoskeleton stabilizer Taxol [36, 118, 128]. Taxol has been found to alter the microtubule dynamics in oocytes at the germinal vesicle breakdown (GVBD) stage, i.e. the dissolution of nuclear envelope [109]. The authors of two of the papers cited above [36, 128] are divided as to the beneficial effect of Taxol on the cryopreservation of pig oocytes.

Lipid compounds are a serious obstacle in cryopreservation of pig oocytes. To solve this problem, various strategies for reducing or removing some of the lipids or whole lipid droplets are used. The most commonly used methods have been microsurgical [52, 94] or mechanical, using centrifugation for lipid polarization [99]. It has been established that oocytes vitrified after microsurgical removal of lipid compounds can be effectively fertilized in vitro and develop into the 8-cell stage – morulae [94]. Hara et al. [52] used an interesting method of removing whole lipid droplets after prior centrifugation of oocytes in hypertonic glucose to obtain porcine oocytes in the GV pig stage that were better suited for cryopreservation than oocytes that were not treated in this manner.

Further progress in the efficacy of oocyte cryopreservation methods has been achieved through the use of new techniques. The most important of these, as mentioned above, have been vitrification in a minimum-volume sample in open pulled straws (the OPS method) [133, 139] vitrification on the surface (cooled in liquid nitrogen) of a metal cube covered with aluminium foil (the SSV method) [50, 124] or vitrification on a thin plastic tip placed in a special tool called a Cryotop® [29, 35]. However, these methods have only slightly improved the survival rate of porcine oocytes.

A completely different approach to this problem is to apply high hydrostatic pressure, mentioned above, before the cryopreservation process. This causes the cells to 'adapt' to the next stress, i.e. cryopreservation, which may increase its effectiveness [105]. Attempts to use this technology for vitrification of porcine oocytes have resulted in 1.5-15.5% parthenogenetically developing blastocysts after thawing, as compared to 0.8-1.3% in the controls [29, 104]. It seems that the method using high hydrostatic pressure creates practical possibilities for increasing the effectiveness of cryopreservation of porcine oocytes.

In our own research, no pregnancy was obtained after 127 in vivo matured oocytes vitrified in a mixture of ethylene glycol and DMSO with the addition of foetal calf se-

rum were transplanted into four recipients. On the other hand, after transplanting 112 mature oocytes vitrified in a mixture of cryoprotectants without serum, pregnancy was obtained in two of four recipients [45]. Twelve live piglets were born and tested to verify their origin. Positive results were obtained for four piglets, which means that these were among the few piglets in the world to be born after transplantation of vitrified oocytes [46]. The study confirmed the results of previous experiments on bovine embryos [108, 131] showing a negative effect of the addition of serum on embryo survival after cryopreservation. These authors suggest that excessive lipid accumulation occurs in the presence of serum, which in turn has a negative effect on the suitability of the oocytes for cryopreservation.

In summary, on the one hand lipid compounds are a serious obstacle in cryopreservation of both embryos and oocytes, but on the other hand their role in the vital processes of cells as energy and building material is very important. More research is needed to improve the efficacy of methods to produce more stable and reproducible results while taking into account the role of lipids in cell function.

SHEEP

Freezing and vitrification of embryos

The first studies on the freezing and thawing of sheep embryos were based on cryopreservation methods for bovine embryos and were slightly modified. The first lamb obtained after transplantation of a frozen embryo was born in Cambridge in 1974 as a result of work by the Willadsen team [qtd. in 12], and the first in Poland was born in 1977 [122]. The most common cryoprotectants used were DMSO [16], ethylene glycol [86], glycerol [21] and methanol [22]. Research has shown that ethylene glycol is a better and far less toxic cryoprotectant for ovine embryos than DMSO [16]. High in vivo and in vitro survival rates for sheep embryos (73%) have been obtained by a controlled freezing method using 1.5 M ethylene glycol and 1 M sucrose solution for thawing. In addition, cryopreservation using ethylene glycol was found to be more effective in the case of embryos obtained in vivo [83]. However, Songsasen et al. [125] demonstrated that live offspring can be obtained following the transfer of sheep embryos frozen both in the morula and blastocyst stage.

In addition to the standard freezing method, vitrification of sheep embryos has also been attempted. Suitably modified procedures of this method of cryopreservation may be an alternative to current freezing methods and can be successfully used, for example, to conserve genetic reserves of vanishing breeds [62, 144], as in the case of the Polish Red cattle [144] or some breeds and lines of rabbits [121]. It seems that cryopreservation by vitrification may also be useful for embryos that are more sensitive to cooling, such as those produced in vitro, cloned, or subjected to manipulation, e.g. biopsy [19].

Important factors that may influence the survival of vitrified embryos include the composition of the vitrification mixture, the method of embryo transfer (direct or traditional thawing with morphological evaluation of embryos after thawing and/or culture), the physiological state of the embryo (obtained in vitro or in vivo) or culture methods.

The first successful attempts at vitrification of ovine embryos were made by Gajda et al. [49], Széll et al. [130], Martinez and Matkovic [82] and Ptak et al. [107]. The vitrification procedure used by Gajda et al. [49], based on the use of a mixture of glycerol and propanediol, resulted in vivo survival comparable to that of frozen embryos. Complete in vivo development of vitrified sheep embryos was made possible by transfer of embryos to the recipients directly (up to 2 min) after thawing and removal of the cryoprotectant. Subsequent studies investigated various types of vitrification mixtures, such as ethylene glycol and glycerol [83] or ethylene glycol and 1 M sucrose solution [1]. The use of the first of these vitrification mixtures resulted in a 52% pregnancy rate following vitrification of 6-day blastocysts [83]. In a study by Mermillod et al. [87], the percentage of births by ewes after transfer of fresh or vitrified blastocysts was similar (60% and 50%, respectively) when embryo culture was used after thawing. A higher percentage of pregnant females (81%) was observed in the case of direct transfer of cryopreserved embryos [83]. Subsequent research [qtd. in 19] confirmed observations by Gajda et al. [49] and Barila et al. [7] that the direct transfer of sheep embryos to the recipients increases the percentage of pregnant females. This procedure probably eliminates the negative impact of subjective selection of embryos after thawing or in vitro culture [19].

Another factor determining the survival of cryopreserved ovine embryos is how they are obtained. There are significant differences in cell structure and biochemical processes between embryos obtained in vivo and in vitro, and this results in different degrees of suitability for cryopreservation processes. Furthermore, media of undefined composition and embryo culture methods used by various authors may contribute to increased sensitivity to cooling in embryos obtained in vitro and thus may affect the survival rate of cryopreserved ovine embryos [19].

Some modifications of the vitrification technique, including the use of open pulled straws, make it possible to achieve high ovine embryo survival rates both in vitro and in vivo [19, 26]. Some authors [132] have also drawn attention to the problem of the birth of lambs with structural defects or greater body weight following the transfer of embryos obtained in vitro and cryopreserved rather than after the transfer of fresh embryos.

Freezing and vitrification of oocytes

Cryopreservation of sheep oocytes, as in the case of other species, still faces many difficulties and the methods must be optimized. Numerous factors affect the survival of cryopreserved ovine oocytes. One of the main problems is their high sensitivity to freezing processes. Immature oocytes vitrified after prior removal of cumulus cells have

shown higher survival rates and better developmental competence than oocytes having these cells. Other studies [148], on the other hand, have found no effect of the presence of cumulus cells on the survival of mature ovine oocytes after vitrification by the SSV method.

Another factor that may affect the survival of frozen oocytes is preincubation with cytochalasin B (CB). Silvestre et al. [119], using CB a supplement for ovine oocyte vitrification, found that it did not affect oocyte maturation after thawing. In contrast, Bogliolio et al. [14] demonstrated that CB may have a negative effect on the survival of immature oocytes. Zhang et al. (148) showed that the presence of cytochalasin B has a positive effect on mature oocytes and reduces damage during the freezing process. The best results were obtained using CB at concentrations of 7.5 and 10 μg/ml of solution.

Another factor influencing the survival and developmental competence of oocytes is the method of cryopreservation. When conventional freezing methods were compared with vitrification by the SSV and Cryotop techniques, the best results were obtained using these two modifications of vitrification [30]. Oocytes vitrified by SSV had the highest survival rates, their mitochondria were unchanged, and their ooplasm structure was preserved [30].

GOATS

Freezing and vitrification of embryos

Much less research has been devoted to cryopreservation of goat embryos and oocytes. As in the case of sheep, the freezing method is based on techniques previously developed for cryopreservation of bovine embryos.

The literature indicates that cryopreserved goat embryos have higher survival rates than sheep embryos subjected to the same freezing and thawing procedures. Traldi et al. [132] compared the percentages of pregnant goats and sheep after transplantation of either fresh embryos or embryos vitrified in a mixture of glycerol and ethylene glycol. The percentages of pregnant females after transplantation of cryopreserved goat and sheep embryos produced in vitro were 60% and 41%, respectively, and 45% and 15% for embryos obtained in vivo [qtd. in 19, 132].

One of the decisive factors affecting the survival of cryopreserved goat embryos is their stage of development [75]. The highest survival rate of cryopreserved embryos has been demonstrated for expanding, hatching and hatched blastocysts, with DMSO or glycerol as a cryoprotectant [83]. A study by Li et al. [75] showed no significant differences in the percentage of pregnant females after transfer of fresh and frozen embryos (60% and 59%, respectively). Moreover, it has been demonstrated that live goat kids may be born following the transfer of embryos produced in vitro and cryopreserved [26].

Comparative studies of the survival of goat embryos cryopreserved by freezing and by OPS vitrification showed a higher survival rate for the vitrified embryos [32].

Freezing and vitrification of oocytes

The few studies [70] on the effect of cryopreservation on in vitro maturation of goat oocytes have shown that the vitrification process negatively affected their maturation and that the presence of propanediol as a cryoprotectant significantly reduced the percentage of fertilized oocytes, which may have been due to its harmful effect.

Subsequent research found goat oocytes to be suitable for vitrification by both the SSV and CLV techniques [110]. Moreover, the cryoprotectants used in both vitrification techniques were found not to be toxic to the maturing and developing oocytes and goat embryos. However, none of the vitrified oocytes developed to the blastocyst stage after fertilization.

Another factor that may affect the survival rate and percentage of fertilized oocytes is the presence of cumulus cells during cryopreservation. Vitrified immature oocytes from which the cumulus cells were removed have been shown to have lower survival rates, and a lower percentage of them retain their normal morphology as compared to oocytes with these cells. In addition, immature goat oocytes have been shown to tolerate the cryopreservation process better than mature oocytes [110].

HORSE

Freezing and vitrification of embryos

Cryopreservation of horse embryos is still an unsolved problem due to the high cost of research. Few equine embryos are obtained worldwide, and there have been few reports of embryo transfer. An additional factor limiting the acquisition of more embryos is that superovulation cannot be induced in mares. Moreover, the presence of a characteristic capsule, which forms in the early embryonic stage [26, 83, 127], may impair the penetration of the cryoprotectant substance into the embryo and thereby reduce its potential for freezing [83, 127]. In contrast to other mammalian species, equine embryos exhibit individual, variable tolerance for cryopreservation methods [26]. Legal questions are another important factor limiting the development of methods of cryopreservation of equine oocytes and embryos; for most breeds, foals obtained from cryopreserved embryos cannot be registered in the herd books [127].

It was not until the early 1980s that the first foals were obtained after transplantation of embryos frozen in liquid nitrogen. Another successful experiment in which horse embryos were preserved in liquid nitrogen and a foal was born was conducted during an international exchange between Poland and England in 1985 [15].

A review of previous work on the cryopreservation of horse embryos indicates that controlled freezing methods based on cryopreservation techniques for other mammalian species were initially used [116]. However, these methods did not produce satisfactory results in terms of embryo survival and thus were not implemented in commercial embryo transfer programmes. In later research [117], the method of vitrification in OPS straws was used for cryopreservation of horse embryos.

Equine embryos are designated for cryopreservation on the 6th day after ovulation [83, 127]. The first foal from cryopreservation of a 6-day embryo was obtained in 1982 by Yamamoto et al. [qtd. in 127]. In this species, the survival rate of cryopreserved embryos mainly depends on their size and stage of development. Evidence of this is found in results reported by Lascombes and Pashen [69], who froze embryos of less than 220 μm and obtained a 56% pregnancy rate. Attempts at cryopreservation of embryos in the morula or blastocyst stage have shown that the higher the freezing rate (0.3 to 0.5°C/min), the better the survival rate of the embryos [26]. Glycerol has proved to be the best cryoprotectant in this species [20, qtd. in 83 and 127]. However, the procedure for freezing and thawing embryos is identical to that used for bovine embryos [qtd. in 83]. Modifications of these techniques (e.g. adding 100 mM L-glutamine to the glycerol solution) resulted in a pregnancy rate of 50% [qtd. in 83 and 127].

Investigation of the effect of blastocyst size on their survival after vitrification [57] using an EFS vitrification mixture revealed that the embryo survival rate decreased as the size of the embryo increased. Other studies [71] have attempted to determine the effect of the presence of the capsule on the speed at which the cryoprotectant penetrates the embryo, and found that after digestion of the capsule with enzymatic compounds the cryoprotectant better penetrates the embryo. This technique has been used for cryopreservation of embryos larger than 220 μm, which had low survival rates without enzyme treatment [127].

Freezing and vitrification of oocytes

In the case of equine oocytes, the first attempts at cryopreservation using ethylene glycol as a cryoprotectant did not produce satisfactory results [126]. The survival rate of oocytes was 16% and 17% for controlled freezing and vitrification, respectively. Attempts to assess the effect of the degree of oocyte maturation after cryopreservation [56] showed lower survival rates in both immature and mature equine oocytes as compared to bovine oocytes vitrified by the OPS technique [126]. Furthermore, mature equine oocytes have been shown to better tolerate the freezing process than immature ones. Ethylene glycol has proven to be the best cryoprotective agent for equine oocytes [126]. The use of modifications of the vitrification method, such as CLV and OPS, has significantly improved the survival rates of equine oocytes [126].

The unsatisfactory pregnancy rates following transfer of embryos obtained from cryopreserved oocytes indicate that procedures for preservation of equine oocytes at low temperatures require further investigation [96, 126].

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