Journal of Animal and Feed Sciences, 18, 2009, 371–387

Oocyte and embryo cryopreservation – state of art and recent developments in domestic animals*

B. Gajda[1] and Z. Smorąg

National Research Institute of Animal Production,
Department of Biotechnology of Animal Reproduction
32-083 Balice, Poland

(Received 13 January 2009; revised version 19 March 2009; accepted 24 June 2009)

ABSTRACT

During the past few decades, significant progress in cryopreservation of mammalian oocytes and embryos has been observed. Transfer of cryopreserved embryos or oocytes resulted in live offspring in at least 25 species. So far, two major methods have been used for oocyte and embryo cryopreservation: conventional slow-rate freezing and vitrification. This review summarizes the progress in cryopreservation of mammalian oocytes and embryos that has been achieved by modifying oocyte/embryo susceptibility to cryopreservation or vitrification technology through such techniques as solid-surface vitrification, cryoloop, microdrop, cryotop, electron microscopy grids, nylon mesh, open pulled straw method or removal of lipids, addition of antifreeze protein or cytoskeleton-stabilizing agents, cholesterol or liposomes, centrifugation prior to cryopreservation or application of high hydrostatic pressure. In conclusion, the vitrification method opened new perspectives in cryopreservation of embryos and oocytes, both for *in vitro* fertilized and somatic nuclear transfer. Authors believe that new cryopreservation procedures which modify the susceptibility of oocytes and embryos to cryopreservation will gain importance as a major tool in mammalian gamete and embryo cryopreservation.

KEY WORDS: domestic animal, oocyte, embryo, cryopreservation, vitrification

CRYOPRESERVATION OF OOCYTES AND EMBRYOS: THE STATE OF THE ART

During the past few decades, significant progress in cryopreservation of mammalian oocytes and embryos has been achieved. Live offspring of at least

* A part of the paper was presented at the Central European Congress of Life Sciences EUROBIOTECH in Krakow, Poland (October 17-19, 2008)

[1] Corresponding author: e-mail: bgajda@izoo.krakow.pl

25 species resulted from transfer of cryopreserved embryos or oocytes. So far, two major methods have been used for oocyte and embryo cryopreservation: conventional slow-rate freezing and vitrification.

Conventional slow freezing was the first system to be used for embryo cryopreservation. In this system, controlled cooling rates allow extracellular and intracellular water exchange without serious osmotic effects or changes in cell shape. This technology has been used successfully to cryopreserve embryos of various species (Dobrinsky, 2002; Fuller and Paynter, 2007). However, unsatisfactory results have been reported for cells more sensitive to chilling, such as oocytes of different species or pig embryos. This can be explained by the decrease in permeability of the cytoplasmic membranes of oocytes during chilling procedure (Ruffing et al., 1993) and relatively high lipid content and/or lipid composition in porcine oocytes and embryos (Nagashima et al., 1994; McEvoy et al., 2000).

An alternative method of cryopreservation is vitrification, which uses a high concentration of cryoprotectant and rapid cooling rates to solidify solutions. Vitrification is a simple technology and it is potentially faster and less expensive than slow freezing. Moreover, it was shown to be more effective than slow freezing for material more sensitive to chilling. It is possible to obtain satisfactory survival rate (79%) of *in vitro* produced, vitrified cattle blastocysts, which showed an increased sensitivity to chilling and freezing (Vajta et al., 1996). Although some problems with vitrification remain to be fully addressed before it can become a routine cryopreservation technique, we believe that it shows much promise as a viable alternative to conventional freezing technology.

Cryopreservation of mammalian embryos is now a routine procedure, but considerable differences in efficiency exist depending on the origin of embryos, i.e. whether they are produced *in vivo* or *in vitro*, and on the stage of development and species involved.

Embryo source

Numerous studies indicate that embryos produced *in vitro* do not survive cryopreservation as well as those produced *in vivo* (Massip et al., 1995; O'Kearney-Flynn et al., 1998). Embryos produced *in vitro* are sensitive to chilling and freezing as a result of elevated lipid content, which can be modified by culture conditions. Removal of lipids by centrifugation increases their tolerance to chilling and *in vitro* survival of frozen-thawed embryos is improved after delipidation (Esaki et al., 2004). Reduction of the cytoplasmic lipid content in embryos using the metabolic regulator phenazine ethosulphate (PES) improves the cryotolerance of bovine embryos (Seidel, 2006). In our study on pig embryos produced *in vitro*

(Gajda et al., 2008a), we showed a significant decrease in lipid content after *in vitro* culture in PES supplemented medium.

Embryos cultured with PES also showed an increased ability to survive cryopreservation (Gajda et al., 2008b).

Several authors reported that embryos cultured in medium containing foetal calf serum (FCS) show retarded development and reduced quality (Abe and Hoshi, 2003), as well as reduced susceptibility to cryopreservation as a result of deviation in the relative abundance of developmentally important gene transcripts (Rizos et al., 2003). Studies on bovine embryos (Pugh et al., 1998, 2000) showed that FCS presence during embryo culture reduces their survival after cryopreservation. Those authors suggested that this negative effect is due to an excessive accumulation of lipids in embryos cultured with FCS. It therefore seems advisable to use protein-free media for embryo cryopreservation, or perhaps media with high-molecular-weight synthetic compounds such as polyvinylpyrrolidone (PVP) (Kuleshova et al., 2001), polyvinylalcohol (PVA) (Suzuki and Yoshioka, 2006), ficoll (Kasai et al., 1992; Gajda, 1996; Smorąg and Gajda, 1998) and hyaluronic acid (Joly et al., 1992; Palasz et al., 1993, 2008).

Vitrification appears to be preferable to conventional freezing for embryos produced *in vitro,* and most of the vitrification procedures designed for *in vivo* embryos have been adapted for *in vitro* embryos.

Stage of development

Embryos of different species at the same stage of development, as well as embryos of one species at various stages of development, display significant differences in cryotolerance. In cattle, for example, the survival rate of *in vivo* and *in vitro* produced oocytes and embryos is lower when they are cryopreserved at early stages of development than when they are cryopreserved as compacted morulae or blastocysts. Expanded and hatched porcine blastocysts survive better than other embryonic stages (Gajda and Smorąg, 2000). The most convenient stage for cryopreservation is the day-7 expanded blastocyst (Han et al., 1994; Hasler et al., 1997; Sommerfeld and Niemann, 1999).

Species

Cattle. The first calf originated from a frozen embryo was reported in 1973 (Wilmut and Rowson, 1973), and considerable progress has led to improved methods of cryopreservation of embryos produced *in vivo*. Embryo cryopreservation is an important tool for application of new biotechnologies such as cloning and transgenesis. Embryo freezing has been used in cryopreservation

and transfer of cloned embryos produced by somatic cell nuclear transfer (Nguyen et al., 2000) and blastomere nuclear transfer (Ushijima et al., 1999), and vitrified cytoplasts have been used in nuclear transfer (Booth et al., 1999). However, survival rates were lower (44%) for nuclear transferred than for *in vitro* fertilized embryos (78%) (Nguyen et al., 2000). In addition, cattle blastocysts injected with DNA have been cryopreserved and transferred, and resulted in pregnancies (Han et al., 2000). Survival rates of DNA-injected blastocysts were affected by freezing and by both the quality (78%-excellent, good-60%, fair-12%) and stage of development (early-48%, mid-52%, expanded-71%) of the embryos prior to freezing (Han et al., 2000). Pregnancies have been also produced using cryopreserved bovine blastocysts obtained by intracytoplasmic sperm injection (ICSI) (Keskintepe and Brackett, 2000).

Recently, major efforts have been made to improve the survival rates of *in vitro* produced embryos after cryopreservation. Compared to *in vivo* embryos, their IVF counterparts are more sensitive to cryopreservation and produce significantly lower pregnancy rates (less than 50%) (Agca et al., 1998). Developmental competence of IVF embryos strongly depends on the composition of culture media. However, after transfer, cryopreserved IVP cattle embryos can develop at rates similar to those of non-cryopreserved IVP embryos. Improvement of survival of cryopreserved cattle embryos can be achieved by optimizing culture conditions, selecting best quality embryos based on the kinetics of their development, or changing freezing procedures (Dobrinsky, 2002).

Sheep and goats. Since 1976, a number of studies reported a successful cryopreservation of sheep embryos, resulting in live offspring. First Willadsen et al. (1976), then Moor and Bilton (1977) and finally Smorąg et al. (1977) obtained live lambs following transfer of sheep morulae and blastocysts. The first successful vitrification of ovine embryos was carried out by Gajda et al. (1989). We demonstrated that the survival of transferred vitrified sheep morulae (60%) is comparable to that obtained after transfer of embryos frozen using the conventional slow method. In the latter study vitrification was also used to cryopreserve IVP sheep embryos (Ptak et al., 1999). The results of this study show that pregnancy rate for fresh and vitrified blastocysts did not differ significantly (47 vs 42%, respectively) but there were significant differences in lambing rates between the two groups (41 vs 23%, respectively). Although currently vitrification procedure still reduces developmental potential of ovine embryos, its efficiency remains too low for practical applications.

Less information is available in goat. Li et al. (1990) showed that after transfer of frozen-thawed goat early to hatched blastocysts, the pregnancy rate is similar to that observed for fresh embryos. Subsequent work showed that IVP goat embryos can survive cryopreservation (*in vitro*: 60%, *in vivo*: 45%) and produce live

offspring after embryo transfer (Tradi et al., 1999).

Surprisingly, small ruminant embryos produced *in vivo* or *in vitro* display similar survival rate and developmental competence following cryopreservation and embryo transfer (Dobrinsky, 2002). It should be possible to increase the efficiency of these procedures when the cryopreservation protocols, in majority developed for cattle embryos, are modified to take into account embryo culture and IVP embryo production systems for small ruminants.

Equine embryos. Horse embryos have special characteristics that pose problems for successful freezing. In early equine embryo development (between days 6 and 7), an acellular capsule is formed underneath the zona pellucida which may impair movement of the cryoprotectant into the embryo (Seidel, 1996). Among the factors determining embryo survival after freezing and thawing, the size of the embryo and the developmental stage appear to be more critical than the type of cryoprotectant used. The freezing protocol is similar to that used for bovine embryos, and glycerol is the usual cryoprotectant (Członkowska et al., 1985). Reasonable pregnancy rates of approximately 50% can be obtained for smaller (less than 250 μm in diameter) equine embryos. Pregnancies (40%) were reported after transfer of vitrified embryos (Hochi et al., 1994). Seidel (1996) used open pulled straw (OPS) vitrification for cryopreserving horse embryos at the morula and blastocyst stages. They hypothesized that a faster cooling rate would improve embryo survival after cryopreservation.

In order for cryopreservation of equine embryos to be as successful as in other domestic animal species, major cellular and molecular breakthroughs in equine embryology are needed in order to facilitate the development of reliable methodologies for equine embryo preservation.

Pig embryos. Intracellular lipids play a major role in the sensitivity of porcine embryos to chilling (Figure 1). This sensitivity seems to correlate with their sensitivity to cryopreservation. This is discussed in more detail in section "Modifying the susceptibility of mammalian oocytes and embryos to cryopreservation" (see pp. 379-381).

Successful cryopreservation of porcine embryos depends on several factors, among them the stage of embryo development, the type of cryoprotectants, and the source of embryos, as well as on the removal of cytoplasmic lipids before cryopreservation. Recently considerable progress has been made in the cryopreservation of pig embryos. Our studies showed that the developmental stage of expanded blastocyst in pig embryos is more suitable for vitrification than the hatched blastocyst stage, and we found that culture of pig embryos in North Carolina State University (NCSU)-23 medium for 6 to 7 days before cryopreservation improves their viability after vitrification in ethylene glycol and ficoll based solution (Gajda and Smorąg, 2000, 2002). Transfer of 147 vitrified

expanded blastocysts into 10 recipients, resulted in birth of 29 live piglets, with pregnancy rate of 50% (Figure 2; Gajda et al., 2004). Recent application of the OPS method to pig blastocysts has allowed excellent *in vitro* survival (Berthelot et al., 2000, 2001; Gajda and Smorąg, 2001), but pregnancy rates after transfer are quite variable, ranging from zero to 60% (Gajda et al., 2006).

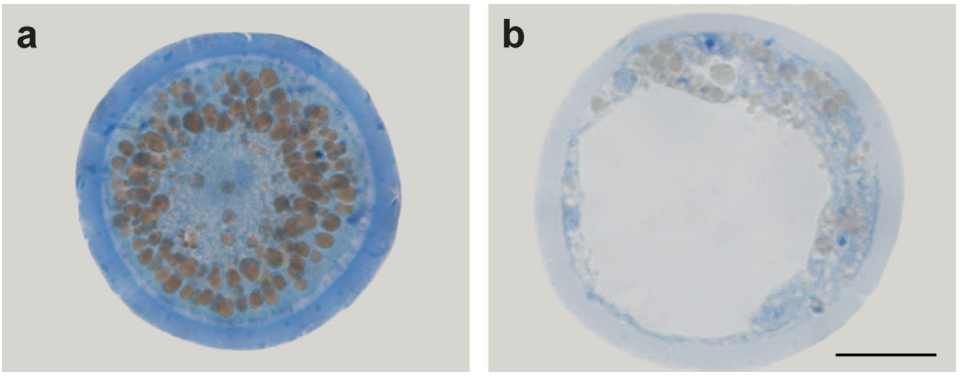

Figure 1. Porcine zygote (a) and blastocyst (b) stained with a mixture of Methylene blue and Azure II, observed under the light microscope. The lipid droplets arranged in the cytoplasm are seen as brown-stained. Scale bar = 35 μm

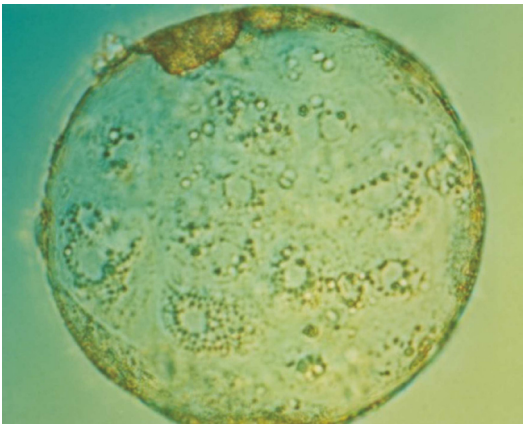

Figure 2. Vitrified-thawed porcine re-expanded blastocyst (original magnification x 100)

NEW TRENDS IN VITRIFICATION OF OOCYTES AND EMBRYOS

During the past few years, a significant progress has been made in the cryopreservation of oocytes and embryos of different species using new vitrification methods. Insufficiently rapid cooling rate is believed to be one of the challenges in vitrification process. In order to overcome this problem several methods have been developed like solid surface, cryoloop, microdrop, cryotop, electron microscopy grids or nylon mesh, and open-pulled straws. These methods allow to use of a minimal volume of freezing medium together with an extremely fast cooling rate, which shortens the time spent in the dangerous temperature zone, where sensitivity to chilling can affect the success rate.

Solid-surface vitrification (SSV). High rates of survival and development after solid-surface vitrification have been reported for *in vitro* matured oocytes from cows (Dinnyes et al., 2000) and goats (Begin et al., 2003). SSV uses a metal surface, precooled to -180^{o}C by partial immersion into liquid nitrogen, to cool microdrops of vitrification solution containing the embryos or oocytes. Recently SSV was used for vitrification of *in vitro* matured IVM porcine oocytes combined with a cytochalasin B pre-treatment (Somfai et al., 2006). The results indicate that SSV vitrification of IVM porcine oocytes allows the development of late-stage preimplantation embryos after parthenogenetic activation. This raises the possibility that SSV oocytes can be used for ICSI or nuclear transfer.

Cryoloop. One of the ultrarapid tools is the cryoloop. This consists of a tiny nylon loop mounted on a small stainless steel tube inserted into the lid of a cryovial. The cryoloop, was first applied to the flash freezing of protein solutions for analysis in crystallography (Parkin and Hope, 1998), and was later used to cryopreserve mammalian embryos and oocytes (Lane et al., 2001), resulting in births from vitrified blastocysts in the case of humans (Makkaida et al., 2001) and monkeys (Makkaida et al., 2003). Nevertheless, despite its successes, the cryoloop is a sensitive and fragile system that may increase the risk of accidental warming (Kuwayama, 2007).

Microdrop vitrification method. This method involves dropping oocytes or embryos containing vitrification solution directly into liquid nitrogen. This method is successful because it eliminates the insulating effect of the container wall. Warming of oocytes is equally rapid when vitrified samples are dropped directly into a warm solution. This method was first proposed by Landa and Tepla (1990) for mouse embryos. It was then successfully used for bovine embryos (Riha et al., 1991), zygotes (Yang and Leibo, 1999), both mature oocytes (Papis et al., 2000) and immature oocytes (Kim et al., 2007), and pig embryos (Misumi et al., 2003). However, no further application of this technology has been published, probably because of the difficulties encountered when handling the embryos.

Cryotop (also called minimum-volume cooling). The cryotop has proven to be an easy and practical vitrification technique with extremely high cooling and warming rates. In this method, most of the vitrification solution can be removed after embryo loading, and therefore, the volume immersed directly into liquid nitrogen is extremely low. Embryos may pass rapidly through the dangerous zone (temperature range from +20 to -20°C), which decreases the risk of chilling injury. This method has been applied to various species for embryo cryopreservation, including the cow (Chian et al., 2004), rabbit (Hochi et al., 2004), buffalo (Muenthaisong et al., 2007), pig (Du et al., 2007) and human (Antinori et al., 2007).

Electron microscopy grids and nylon mesh. Alternative methods involving electron microscope (EM) grids, thin-walled open-pulled straws (OPS) or nylon mesh allow direct contact between embryos containing medium and liquid nitrogen that increases cooling and warming rates. These containers require less than 1-2 µl of an embryo suspension. However, the number of samples per container is restricted to 10-15 for EM grids and 4-6 for OPS (Martino et al., 1996; Matsumoto et al., 2001). Therefore, these methods are not suitable for vitrification of large numbers of oocytes or embryos. A membrane filter is useful for vitrifying large numbers of bovine oocytes. However, a bigger pore size is helpful for decreasing the volume of solution containing oocytes, which is the case with the EM grid (55 µm mesh size) and nylon mesh (60 µm mesh size). The EM grid has been used for vitrification of bovine cumulus-oocyte complexes (COCs) (Martino et al., 1996), bovine blastocysts (Park et al., 1999), and human zygotes (Park et al., 2000); nylon mesh may be applicable to other mammalian oocytes and embryos (Matsumoto et al., 2001; Abe et al., 2005). Given that a nylon mesh can easily handle a large number of oocytes or embryos, using this holder for the vitrification of GV bovine oocytes, for example, should facilitate gamete storage for further application in assisted reproductive technologies, such as *in vitro* fertilization, cloning, and stem cell biology.

Open Pulled Straw (OPS) method. Another method of vitrification is the open pulled straw method. In this method a standard plastic straw (0.25 ml) has previously been heat-pulled to half the diameter and thickness of the wall. The embryos in minimum volume of the vitrification solution (> 0.1 µl) are loaded by the capillary effect into the end of the straw. The straws are then immersed directly into liquid nitrogen allowing direct contact between the two solutions. Upon warming, the end of the straw is immersed into the medium, allowing the rapid dilution of cryoprotectant solution. The OPS method offers several advantages: extremely fast cooling and warming rates of more than 20 000°C/min, brief contact with concentrated cryoprotective additives (less than 30 sec over a range of 130°C), and the possibility of avoiding injury due to chilling and of decreasing

toxic and osmotic damage. With the introduction of the open pulled straw in 1997, the successful vitrification of embryos and oocytes from several different species have been reported: early stage bovine embryos produced *in vitro* (Vajta et al., 1997a), morula and blastocyst of pig (Berthelot et al., 2000; Gajda et al., 2006), horse (Oberstein et al., 2001) and cow (Hyttel et al., 2000), and porcine oocytes (Vajta et al., 1997b). Using this technique calves have been born following the transfer of embryos vitrified at both the oocyte and blastocyst stages (Vajta et al., 1999; Le Gal et al., 2000). In this way, OPS vitrification offers a new way to solve basic problems of reproductive cryobiology, and it may have a practical impact on animal biotechnology and assisted reproduction in humans.

MODIFYING THE SUSCEPTIBILITY OF MAMMALIAN OOCYTES AND EMBRYOS TO CRYOPRESERVATION

Important advances have been made in cryopreserving oocytes and embryos by modifying their susceptibility to the procedure. The techniques applied include removal of lipids, addition of antifreeze protein or cytoskeleton-stabilizing agents, addition of cholesterol or liposomes, centrifugation prior to cryopreservation, or application of high hydrostatic pressure.

Microsurgical removal of lipid compounds. As already mentioned, the lipid content of pig embryos is relatively high and decreases as the embryo develops. This high lipid concentration makes pig embryos, especially those at early stages of development, exceptionally sensitive to cryopreservation. Freezing pig embryos that are at the 2- to 8-cell stage and from which lipids have been microsurgically removed (Nagashima et al., 1994) makes them more susceptible to cryopreservation. In that study, *in vitro* development to the blastocyst stage was observed after thawing, and complete *in vivo* development and normal offspring after transplantation were obtained. The study also found that the delipidated embryos survived cryopreservation regardless of whether they were frozen immediately after lipid removal or after a brief culturing period. In contrast, no control embryo survived the freezing process. This study was the first to show directly that the high lipid content of pig embryos has a negative effect on the susceptibility of embryos to cryopreservation. Another study (Dobrinsky, 1999) found that delipidated pig embryos at more advanced stages of development (morula/early blastocyst) show a much higher survival rate than do embryos with intact lipids. Other work confirmed that both frozen and vitrified pig embryos from which lipids have been micro-surgically removed can develop fully *in vivo* (Nagashima et al., 1995). A study with vitrified pig oocytes showed that after microsurgical removal of lipids, the oocytes can be efficiently fertilized *in vitro*

and can develop to the stage of the 8-cell and morula (Nagashima et al., 1996). Although the microsurgical removal of lipids from embryos considerably improves cryopreservation efficiency, the method is not suitable for practical application because it is time-consuming.

Non-invasive removal of lipids. Recently, an alternative non-invasive delipidation method has been reported. This method does not require micromanipulation and it depends on partial digestion of the zona pellucida, which allows full separation of lipids during centrifugation. The non-invasive delipidation method has been successfully used for cryopreservation of *in vitro* pig morula (Esaki et al., 2004), oocytes (Du et al., 2006) and cloned blastocysts (Nakayama et al., 2008).

Addition of antifreeze protein. Injection of antifreeze proteins (AFPs) into embryos can improve the success of cryopreservation, since they inhibit the growth of ice crystals as well as the recrystallization of water during thawing. These antifreeze proteins are naturally expressed in sub-arctic fish species whose embryos show greater tolerance to freezing. Moreover, they can protect cell membranes from cold-induced damage (Rubinsky et al., 1991) and inhibit ice recrystallization (Knight et al., 1984). The protective effect of antifreeze protein depends on the concentration and type of AFP used. Recent studies have shown that AFP can protect embryos from chilling, and that this effect may be due to stabilization of the cellular membranes during chilling rather than to inhibition of ice crystal formation (Robles et al., 2007). Further experiments are needed to determine the effect of the addition of antifreeze protein on embryo cryosurvival and chilling resistance.

Addition of cytoskeleton-stabilizing agents. One possibility to increase the efficiency of pig embryo cryopreservation is to add agents that stabilize cytoskeleton structure in embryos, because it is known that it may be damaged by the cryopreservation process. Agents known to stabilize cytoskeleton structure include cytochalasin B, cytochalasin D and colchicine (Dobrinsky, 1996). Dobrinsky et al. (1997, 2000), who investigated the effect of cytochalasin B on depolarization of microfilaments and the efficiency of pig embryo vitrification, found that this factor increases on *in vitro* and *in vivo* survival of vitrified expanding and hatching blastocysts. In contrast, no improvement in survival was observed for morulae/early blastocysts. Cell analysis under a confocal microscope revealed considerable cytoskeleton damage in vitrified embryos not treated with cytochalasin B, whereas vitrified embryos treated with a stabilizing agent showed mostly normal repolarization of microfilaments and other cytoskeleton components. These observations are evidence that cryopreservation may affect the cytoskeleton and that microfilament depolarization before vitrification considerably increases embryo survival after vitrification (Dobrinsky, 1999).

Addition of cholesterol or liposomes. Attempts to modify culture media for bovine embryos obtained after *in vitro* fertilization and then cryopreserved in a medium supplemented with liposomes containing lecithin, sphingomyelin and cholesterol were carried out by Pugh et al. (1998). It was found that the liposome supplement had no negative effect on the development of embryos to the blastocyst stage or on their survival after thawing, whereas the presence of lecithin in liposomes reduced the survival of cryopreserved bovine embryos, which may suggest that lecithin has an adverse effect on changes in cell membrane composition.

Horvarth and Seidel (2006) increased the proportion of cleaving embryos that developed to the 8-cell stage, by adding cholesterol to the oocyte cryopreservation medium before vitrification, followed by *in vitro* fertilization. However, this study shows that, although significant improvement in post-thaw oocyte viability and early cleavage was observed after addition of cholesterol, blastocysts rates remained comparable to those derived from non-treated cryopreserved oocytes.

Centrifugation prior to cryopreservation. Polarization of lipid droplets as a result of centrifugation is a good method for visualizing pronuclei in pig zygotes. Moreover, lipids polarized by centrifugation can be mechanically removed, which makes pig embryos at early stages of development more susceptible to cooling and cryopreservation. On the other hand, centrifugation without lipid removal may affect the survival and developmental competence of frozen mature oocytes. Otoi et al. (1997) found that although centrifugation has a negative effect on bovine oocytes, its use is advantageous in the context of cryopreservation. A recent study with pig oocytes and embryos (Somfai et al., 2008) showed that centrifugation (10 000 *g* for 20 min) reduced the rate of surviving vitrified oocytes, although the proportion of parthenogenetic divisions was similar in the group of centrifuged and non-centrifuged oocytes (42 and 47%, respectively). Meanwhile, although zygote centrifugation slightly improves their survival after cryopreservation, it does not increase the developmental competence of surviving zygotes. Thus, those authors suggested that centrifugation should be used before vitrification only to separate monospermic zygotes from polyspermic ones, which may increase the efficiency of their cryopreservation.

Application of high hydrostatic pressure. The possibility of using high hydrostatic pressure (300-800 bar) to increase cryopreservation efficiency for pig gametes and embryos was first demonstrated by Pribenszky et al. (2005a,c) and Du et al. (2008). Those authors suggested that, sublethal environmental stress, induced, for example, by high pressure, increases concentration of specific chaperone proteins in gametes and embryos. Synthesis of these proteins results in an increased tolerance of cells to stress induced by treatments such as cryopreservation or culture *in vitro*, which, in turn, increases the efficiency of the above biotechnological procedure. Cell treatment with elevated hydrostatic

pressure was successfully used for cryopreservation of boar spermatozoa (Pribenszky et al., 2005a; Kuo et al., 2007), vitrification of pig oocytes (Du et al., 2008; Pribenszky et al., 2008), and vitrification of bovine blastocysts obtained *in vitro* (Pribenszky et al., 2005b, 2007).

CONCLUSIONS

In the near future, use of cryopreserved embryos will be a routine alternative for breeding programmes in most domestic animal species, but the technology should be improved. The vitrification method opened new perspectives in cryopreservation of embryos and oocytes, both for *in vitro* fertilization and somatic cell nuclear transfer procedures. By its technical potential, this method creates a substantial improvement of the efficiency of cryopreservation. We believe that new cryopreservation procedures which modify the susceptibility of oocytes and embryos to low temperature will gain importance as a major tool in mammalian gamete and embryo cryopreservation.

ACKNOWLEDGEMENTS

The Figure 1 presented in the article was donated by my colleague Dr. Marek Romek from the Institute of Zoology, Jagiellonian University in Krakow (Poland).

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