REPRODUCTIVE BIOLOGY

REVIEW

Factors and methods of pig oocyte and embryo quality improvement and their application in reproductive biotechnology

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SUMMARY

Compared to other mammalian species, pig oocytes and embryos are characterized by high lipid contents stored mainly as lipid droplets in the cytoplasm. This fact has a negative influence on manipulations on oocytes and embryos and, in general, biotechnological procedures are much less advanced in pigs than in cows. This paper discusses current methods for modifying porcine oocytes and embryos using *in vitro* culture or microsurgical manipulation, chemical agents such as cytochalasin B or D, physical means such as centrifugation or increased pressure and the biotechnological implications of these procedures. The presented methods make it possible to modify the characteristics of oocytes and embryos and thus increase their susceptibility to cryopreservation and cloning.
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INTRODUCTION

Mammalian oocytes and embryos are specific to each species. Porcine oocytes and embryos, compared with other mammalian species, are characterized by a high level of lipid content stored mainly as lipid droplets in the cytoplasm. Lipid droplets contain a mixture of different types of lipids surrounded by a single layer of phospholipids of varying composition [37]. The lipids are a structural component of a cell membrane and cytoplasm as well as a major source of energy necessary for normal development of an egg.

The success rate of cryopreservation of pig embryos appears to be highly correlated with cytoplasmic lipid content. This content depends primarily on the particular stage of embryo development [62]. It has been suggested that the lipid content found in early stage (from zygote to morula) porcine embryos is mostly responsible for the cooling intolerance of these embryos, whereas blastocysts are more resistant [19]. Moreover, it was demonstrated that the pig embryonic cytoskeleton is susceptible to damage during cryopreservation [14].

A high lipid content is responsible for the susceptibility of pig oocytes and embryos to various biotechnological manipulations such as cryopreservation, microsurgical bisection, sex determination, gene transfer or cloning decreases. Thus, methods that minimize negative influence associated with high lipid content in pig embryos should be developed. In this paper I will describe current methods for modifying porcine oocytes and embryos using *in vitro* culture, microsurgical manipulation, chemical agents such as cytochalasin B or D, and physical means such as centrifugation or increased pressure.

EFFICIENCY OF *IN VITRO* FERTILIZATION AND QUALITY OF CULTURED PIG EMBRYOS

The development of new techniques in pig reproduction, such as transgenesis and cloning, creates a large demand for oocytes and embryos. Efficient methods for *in vitro* production of embryos would largely solve this problem. Despite research conducted in many laboratories, pig embryo production technology has not progressed far enough. In

particular, the quality of embryos obtained after *in vitro* fertilization and culture continues to be much lower compared to *in vivo*-developing embryos [63]. The total number of cells in blastocysts produced *in vitro* ranges from 58 to 139 [20] compared to 150-250 cells in blastocysts obtained *in vivo*. In addition, blastocysts produced *in vitro* are characterized by a higher number of nuclei exhibiting DNA fragmentation, as demonstrated by Bryła et al. [4]. These authors established an average of 32 apoptotic nuclei in cultured blastocysts compared to seven found in blastocysts obtained *in vivo*. In *in vitro*-produced embryos, chromosomal abnormalities such as aneuploidy were found more frequently and probably resulted from polyspermic fertilization in pigs [56, 70]. Cytogenetic analysis of pig blastocysts produced *in vitro* [32, 72] showed that chromosomal aberrations occurred in approximately 45% of these blastocysts compared to 7% in embryos obtained *in vivo* [74]. In general, the transfer of mammalian embryos produced *in vitro* results in considerable developmental abnormalities such as increased embryo mortality, extended gestation and considerably greater body weight of progeny.

Selection of the best quality *in vitro* produced embryos requires evaluation of embryo competence to develop from early stages to parturition. Ulloa Ulloa et al. [72] claimed that to produce a high quantity of good quality pig embryos, the embryos must be subjected to rigorous selection two days after *in vitro* fertilization when they reach 3-4 to 5-8-cell stages, and then six days after fertilization when they reach the blastocyst stage. However, suboptimal culture conditions remain the major cause of lower quality of embryos obtained *in vitro*.

IMPROVING EMBRYO QUALITY BY MODIFICATION OF EMBRYO CULTURE *IN VITRO*

The main factor affecting the yield and quality of pig blastocysts obtained in vitro are culture conditions. Many culture media have been tested to optimize these conditions [18, 26, 33, 43, 49, 51]. Currently, the most commonly

used media have a strictly defined chemical composition and, in addition to salts and proteins, they contain energy sources such as glucose, calcium lactate, pyruvate and amino acids. Several media such as modified Whitten medium [51], North Carolina State University (NCSU-23 or NCSU-37) medium [57] and porcine zygote medium (PZM; [77]) are available for the successful culture of embryos to the blastocyst stage.

Culture in the presence of serum or serum albumin

Embryo culture media contain many components of efficacy established experimentally. The most common component of culture media is protein in the form of bovine serum albumin (BSA) or fetal calf serum (FCS) which contain amino acids used by embryos as a source of energy while maintaining osmotic balance and stable pH [3]. The response of embryos to the addition of serum albumin or serum to culture medium, although not completely understood, depends on species and stage of embryo development. For example, bovine embryos during early development are most often grown in culture medium with serum albumin [3], although some authors prefer a serum supplementation [60, 75]. A beneficial effect of serum addition on the development of embryos (especially at more advanced stages of development) has also been reported in pigs [13, 41, 61]. The problem of serum albumin or serum supplementation has not been definitively resolved because some authors reported that protein has no effect on the *in vitro* development of pig embryos [6, 23, 36].

In our study [21], pig embryos were cultured in NCSU-23 medium most often supplemented with protein in the form of BSA or FCS. The BSA supplementation significantly increased the proportion of obtained blastocysts compared to both FCS-containing media and protein-free culture. Blastocysts cultured with BSA or FCS had a significantly lower number of nuclei displaying DNA fragmentation and showed a lower apoptotic index compared to blastocysts cultured without protein addition [21]. Studies of other researchers also demonstrated that BSA supplementation positively affected the first divisions of hamster embryos [34], the *in vitro* development of pig embryos [3, 29], the formation of pig blastocysts [59] and the number

of cells in bovine embryos [29]. FCS addition to the culture medium was also shown to have a beneficial effect on pig embryo development but only during the late stages of development [50, 61]. The unfavourable effect of FCS supplementation on pig embryo development was reported by Dobrinsky et al. [13]. It confirmed the findings of Bavister [3] who observed a similar effect for early bovine embryos. These observations have led Kim et al. [28] to propose a two-stage system for porcine embryo culture in which BSA is replaced with FCS as the morula develops into a blastocyst.

Culture with animal protein substitutes

The difficulty to standardize culture conditions with the use of animal protein in the form of serum or serum albumin as well as infection risk have caused a growing interest in the development of media with a strictly defined chemical composition. The use of these media might provide more reproducible culture conditions and eliminate the presence of non-specific or pathogenic factors. One attempt to replace estrous cow serum (ECS) was to use a strictly defined synthetic serum substitute (SSS) for oocyte maturation and *in vitro* culture of bovine embryos that were later cryopreserved [5, 64]. It was shown that embryos developed to the 8-cell stage in medium with SSS and then in medium with ECS were more susceptible to cryopreservation compared to embryos cultured in the presence of ECS alone. Similar findings were reported for SSS used with fresh and cryopreserved mouse and human embryos [9].

The first experiments in which bovine serum albumin was replaced with plant protein in bovine embryo freezing medium were carried out by George et al. [22]. These authors had previously used *in vitro* cultured embryos to test the toxicity of different wheat proteins and determined the threshold concentration of these proteins. In addition, they determined the protein content of culture medium below which a decrease in embryo quality was not observed after evaluation of the cell number and apoptosis level. The attempts at embryo cryopreservation showed that the replacement of BSA with plant protein did not negatively affect embryo survival and quality after thawing.

We attempted to replace BSA in pig embryo culture medium with plant protein (PP)[1]. No differences were found in the proportion of blastocysts obtained after culture with both BSA and PP. However, a higher mean number of cells was found in blastocysts cultured with PP (44.4) compared to blastocysts cultured with BSA (34.0). The results of experiments on *in vitro* development of pig embryos in medium with animal protein substitute are encouraging and prompt further research.

Culture with vitamin E

Vitamin E is a fat-soluble antioxidant that suppresses peroxidation of membrane lipids in animal cells [71]. Pig embryos have a relatively high content of lipids and therefore may be more susceptible to peroxidation. Peroxidation of fatty acids is known to inhibit the function of cells and might induce cell death [68]. One of the factors that can block lipid peroxidation is vitamin E. Vitamin E is considered as a major free radical scavenger in mammalian cell membranes [7]. Research conducted more than thirty years ago showed that vitamin E supplementation had a beneficial effect on the development of rat [69] and bovine embryos [42]. Recent studies have indicated that the vitamin E supplementation increases the proportion of bovine embryos reaching the blastocyst stage and reduces DNA fragmentation in cloned transgenic blastocysts of this species [76]. The addition of 100 μM of vitamin E to NCSU-23 medium increased the proportion of the obtained porcine blastocysts compared to the vitamin E-free group [21]. Similar observations were made by Olson and Seidel [42] who cultured bovine embryos for 5.5 days in a medium with 100 μM of vitamin E and transferred them to recipients. After flushing on day 7, vitamin E-treated embryos were approximately 63% larger than the control embryos.

We found that pig blastocysts cultured with vitamin E were of better quality than controls because they contained a smaller number of nuclei showing DNA fragmentation and had a lower apoptotic index [21]. Similar findings were reported earlier by Kitagawa et al. [30] who found that vitamin

[1]Gajda B, Grad I, Smorąg Z **2009** Effect of plant protein on development and quality of cultured *in vitro* porcine zygotes. *Reproduction, Fertility and Development* **21**(1) 157.

E at a concentration of 100 μM reduced H_2O_2 content, thus leading to limited DNA fragmentation. The positive effects of vitamin E on the proportion and quality of cultured bovine blastocysts and on the accumulation of lipids in these blastocysts were also reported by Reisa et al. [58].

Culture with phenazine ethosulfate

Recently, it has been found that the addition of phenazine ethosulfate (PES), a cell metabolic regulator, to the culture medium increases glucose metabolism and reduces the accumulation of lipids in cultured bovine embryos [8]. It was found that PES oxidizes NADPH to NADP as well as affects cell glucose metabolism and the amount of CO_2 produced from glucose [15]. In addition, PES elevates total CO_2 level in embryos, controls the pentose cycle and specifically stimulates the pentose phosphate cycle in mouse embryos [40]. We observed a slight increase in the proportion of cleaving pig embryos and the proportion of morulae and blastocysts obtained after culture in NCSU-23 medium with PES [21]. At the same time, blastocysts obtained after embryo culture in medium with lower concentrations of PES (0.025 and 0.05 μM) were characterized by a smaller number of nuclei showing DNA fragmentation and a lower apoptotic index compared to higher concentrations of PES (0.075 μM) or the control group. On the other hand, the number of cells in blastocysts after culture with PES was slightly lower compared to blastocysts from the control group. A positive effect of PES on the development of bovine embryos was reported by De La Torre-Sanchez et al. [8] and Barcelo-Fimbres and Seidel [2].

METHODS FOR IMPROVING CRYOPRESERVATION EFFICIENCY OF PORCINE OOCYTES AND EMBRYOS

Microsurgical removal of lipid compounds

A relatively high lipid content in pig embryos decreases as the embryo develops. The high lipid concentration makes pig embryos, especially at early

stages of development, exceptionally sensitive to cryopreservation. Freezing 2- to 8-cell stage pig embryos after microsurgical removal of lipids [38] increased susceptibility to cryopreservation. In this study *in vitro* development to the blastocyst stage was observed after thawing, and full *in vivo* development and normal progeny were obtained after transplantation into recipients. The delipated embryos survived cryopreservation regardless of being frozen immediately after lipid removal or after short culture while control embryos did not survive the freezing process. This study was the first to show directly that a high lipid content in pig embryos has a negative effect on the susceptibility of embryos to cryopreservation. It was also [12] found that pig embryos delipated at more advanced stages of development (morula/early blastocyst) demonstrated a much higher survival rate compared to embryos with intact lipids. A further study confirmed that both frozen and vitrified pig embryos with micro-surgically removed lipids can fully develop under *in vivo* conditions [39]. It was shown that after microsurgical removal of lipids, the vitrified pig oocytes can be efficiently fertilized *in vitro* and develop to the 8-cell stage and morula[1]. Although the microsurgical removal of lipids from embryos considerably improves cryopreservation efficiency, the method is not suitable for practical application because it is a time-consuming procedure.

Addition of protein to cryopreservation medium

Pig embryos produced *in vitro* in a medium with FCS demonstrated an increased susceptibility to cryopreservation [35]. However, there are reports indicating that embryos cultured under these conditions are characterized by retarded development and lower quality [1] as well as reduced susceptibility to cryopreservation associated with transcription factor gene damage [60]. Studies on bovine embryos [55] also confirmed the negative effect of FCS on embryo survival after cryopreservation. These authors suggest that excessive accumulation of lipids in embryos cultured in the presence

[1]Nagashima H, Kuwayama M, Grupen CG, Ashman RJ, Nottle MB **1996** Vitrification of porcine early cleavage stage embryos and oocyte after removal of cytoplasmic lipid droplets. *Theriogenology* **45** 180.

of FCS is the reason why this component has a negative effect on susceptibility to cryopreservation. Therefore, it would seem more advantageous for embryo cryopreservation to use protein-free media [36] or media with high-molecular-weight synthetic compounds such as polyvinylpyrrolidone (PVP; [31]), polyvinyl alcohol (PVA; [67, 73]), Ficoll [17, 19, 27, 65] or hyaluronic acid [25, 45-48].

Addition of cytoskeleton-stabilizing agents

One possible way to increase the efficiency of pig embryo cryopreservation is to add agents that stabilize cytoskeleton structure. Agents known to stabilize cytoskeleton structure include cytochalasin B, cytochalasin D and colchicine [10]. Dobrinsky et al. [11, 12, 14] found that cytochalasin B has a positive effect on *in vitro* and *in vivo* survival of vitrified expanding and hatching pig blastocysts. No improvements were found for morulae/early blastocysts. Confocal microscope analysis revealed considerable cytoskeleton damage in vitrified embryos not treated with cytochalasin B, whereas vitrified embryos treated with a cytoskeleton-stabilizing agent showed normal repolarization of microfilaments and other cytoskeleton components. These observations demonstrated that cryopreservation may affect cytoskeleton and that microfilament depolarization before vitrification considerably increases embryo survival after vitrification [14].

Addition of liposomes or cholesterol

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 failed to produce the expected results [55]. It was only found that the liposome supplementation had no negative effect on the development of embryos to the blastocyst stage and their survival after thawing. In contrast, the presence of lecithin in liposomes reduced the survival of cryopreserved bovine embryos which suggests that lecithin may have an adverse effect on changes in cell membrane composition. Using cholesterol in the oocyte cryopreservation medium, Horvarth and

Seidel [24] observed a slight increase in the proportion of cleaving embryos developing to the 8-cell stage compared to the control embryos.

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 early porcine embryos more susceptible to cooling and cryopreservation. On the other hand, however, centrifugation without lipid removal may affect the survival and developmental competence of frozen mature oocytes. Otoi et al. [44] found that although centrifugation has a negative effect on the bovine oocytes, its use is advantageous in the context of cryopreservation. A recent study with pig oocytes and embryos [66] showed a detrimental effect of centrifugation (10 000×g, 20 min) on the proportion of surviving vitrified oocytes, although the proportion of obtained parthenogenetic divisions was similar in the group of centrifuged and non-centrifuged oocytes (42 and 47%, respectively). Although centrifugation of zygotes had a slightly beneficial effect on the survival of zygotes after cryopreservation, it did not increase the developmental competence of surviving zygotes. Thus, centrifugation should be used before vitrification only to separate monospermic zygotes from polyspermic ones and, consequently, may increase the efficiency of their cryopreservation.

Application of high hydrostatic pressure

The possibility of using high hydrostatic pressure (300 to 800 bar) to increase cryopreservation efficiency for pig gametes and embryos was first demonstrated by Pribenszky et al. [52] and Du et al. [16]. These authors suggest that sublethal environmental stress induced e.g. by high pressure results in an increased concentration of specific chaperone proteins in male and female gametes and embryos. The synthesis of these proteins results in an increased tolerance of cells to stress induced by e.g. cryopreservation or culture *in vitro,* which in turn, improves the efficiency of the above biotechnological procedures. The method for treatment of cells with elevated hydrostatic

pressure was successfully used for cryopreservation of boar spermatozoa [53], vitrification of pig oocytes [16, 54], vitrification of bovine blastocysts obtained *in vitro*[1] and in pig oocytes intended for somatic cloning [16].

In summary, new biotechnological methods make it possible to modify the characteristics of oocytes and embryos and thus increase their susceptibility to cryopreservation and cloning, among others. It seems that these methods will gain importance to become major tools in mammalian gamete and embryo biotechnology in the future.

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