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The cryopreservation of ovarian tissue: uses and indications in veterinary medicine

Banu Demirci[a,*], Jacqueline Lornage[b], Bruno Salle[b], Marie Thèrese Poirel[a], Jean François Guerin[b], Michel Franck[a]

[a]*Laboratoire de Zootechnie, Ecole Vétérinaire de Lyon, Marcy l'Etoile 69280, France*
[b]*Laboratoire de Biologie de la Reproduction, Hôpital Edouard Herriot/CECOS, Lyon, France*

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Abstract

Animal experiments have shown that cryopreservation of the ovarian cortex, containing primordial follicles, could be used to preserve gametes thereby restoring fertility in humans and animals. During the last 100 years, many hundreds of species have been lost, and a third of the breeding animals are threatened with extinction. To preserve genetic diversity, notably for the conservation of endangered species, it is essential to conserve female and male gametes. Today, biotechnologies such as artificial insemination and embryo transfer are used in breeding programs and are well developed. However, even using these advanced techniques, there are problems due to the limited number of individuals used as the source of gametes, so that the risk of inbreeding is high, even in large populations. To preserve genetic diversity, it is necessary to create gene banks of male and female gametes and embryos, using a very large number of individual donors. Cryopreservation of ovarian tissue could present a means for enlarging the gene pool. Cryopreserved ovarian tissue could be used in auto- or xenografts, or for in vitro maturation (IVM) of primordial follicles. In this review, we describe the processes for cryopreservation of ovarian tissue and the various possibilities for using it.

Keywords: Cryopreservation; Ovarian tissue; Indications; Veterinary medicine

1. Introduction

The cryopreservation of ovarian tissue containing primordial follicles is a new route in research on the conservation of female gametes. Several authors have already described the

* Corresponding author. Present address: International Clinical Trial Association, 21121 Fontaine-Les-Dijon, France. Tel.: +33-380-53-40-00; fax: +33-380-57-10-22.
E-mail address: bdemircifr@yahoo.fr (B. Demirci).

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use of cryopreservation of ovarian tissue in human medicine [1–3], primarily to restore fertility in women who have undergone chemotherapy or radiotherapy. This class of treatments is used not only for malignant diseases like cancer [2], but also in severe autoimmune pathologies such as rheumatoid arthritis [4].

Animals are used as experimental models to set up the protocols for freezing, grafting and culture systems. The best-studied animal model is the mouse, because it is easy to handle and the period of folliculogenesis is short [5–11]. Another animal model that has been well studied is the ewe, because ewe's ovary is similar to the human ovary in its architecture and physiology [12–20].

2. Why cryopreserved ovarian tissue?

The aim of ovarian tissue cryopreservation is to store primordial follicles located in the ovarian cortex, which represent the oocyte reserve. Limited successes with earlier methods have focused research on the freezing of immature oocytes inside primordial follicles. Yet, here also are differences. Cryopreservation of metaphase II oocytes gives disappointing results because of problems encountered during fertilization and embryonic development. Hardening of the zona pellucida, associated with premature cortical granule exocytosis stops the penetration of the spermatozoon. The rate of hatching blastocysts has been reported to diminish after freezing [21,22]. Moreover, even if chromosomes are situated on the meiotic spindle, there is a risk of an abnormal distribution, aneuploidy, during the first division of meiosis [23]. The freezing procedure can also damage the cytoskeleton by changes in the organization of the molecules and organelles [21]. The freezing of germinal vesicle oocytes could be an alternative because there is no risk of aneuploidy, however, hardening of the zona and damage to the cytoskeleton is still observed. In vitro maturation of cryopreserved oocytes has not yet been mastered and only one birth has been reported using frozen–thawed germinal vesicle oocytes [24].

The limited success with the methods described above has focused research on cryopreservation of the immature oocytes contained in primordial follicles, which are located in the ovarian cortex. This method is more likely to be successful, because the oocyte is less differentiated, it possesses fewer organelles, the zona pellucida and the cortical granules do not exist and the follicle is less prone to ischaemia [25].

3. Principal uses of cryopreservation in veterinary medicine

3.1. Preservation of species and the fight to preserve genetic diversity

Once a year, the Commission for the Survival of Species of the International Union for the Conservation of Nature (IUCN), publishes a report entitled the Red Book, which evaluates the current survival status of animal species. According to the 1996 report [26], 25% of the existing species are threatened with extinction. Of the 26 orders of mammals, 24 include species close to extinction, and a thousand species have been lost during the last 100 years, and today a third of breeding animals are also threatened with extinction [27].

According to the Food and Agriculture Organization (FAO) of the United Nations, it is necessary to use the largest possible number of animal species for breeding in order to preserve genetic resources. In fact, the FAO recognizes that the conservation of endangered species is only possible by the conservation of the animals themselves, either by conserving these wild animals in their natural habitat or in another habitat such as a zoological park, or by the cryopreservation of spermatozoa and embryos.

The breeder's goal is to obtain genetic improvement in his herd in order to increase production, obtain better growth and fertility rates, and at the same time, increase resistance to infectious diseases [28]. However, the genetic progress obtained by selection from a population of a limited size inevitably increases relationships, within that population [29]. Thus, the preservation of genetic variability has been a major objective of breeders with small herds [30], but even with large herds the breeder is not free from the problem of inbreeding.

Van Tassell and Vanvleck [31] had estimated that 50% of genetic improvement in dairy cattle is accounted for solely by the selection of sires of sons for progeny testing. Furthermore, the dams of sons for progeny testing are usually the daughters of previous generations of sires of sons. Without any doubt, the efforts made to improve genetics have reached their goal; but as a consequence of this selection, relationships, and therefore, the average rate of inbreeding have increased regularly within most domestic animal populations [32].

3.2. Reduction of polymorphism, inbreeding and inbreeding depression

To have large genetic diversity in a breeding program, it is necessary that the number of founder animals (N_0) is as large as possible [33,34]. Although this rule is important, it is not always possible to achieve it. A breeder with a limited population maintains or increases the effective population (N_e) of males and females capable of procreating to produce the next generation, in order to reduce inbreeding. The actual population affects the inbreeding coefficient directly. This coefficient measures the percentage of identical alleles by line of descent on any locus of the individual. The variation in inbreeding (ΔF) of a population from one generation to another, initially defined by Wright [35], is calculated using the formula $\Delta F = 1/2N_e$. For effective population (the number of males (N_m) and females (N_f) which take part in the procreation of the next generation), the formula therefore is $\Delta F = 1/2\,(1/N_m + 1/N_f)$. From this equation, it is evident that if we increase the number of females as well as the number of males, the inbreeding coefficient will decrease. In fact, in small populations, the number of females is generally the limiting factor. Often, the breeder can use an increased number of males, in relation to the number of females, by the use of AI; in large populations, the number of females is high, so the number of males (N_m) is the limiting factor. To select a male for breeding, an index of genetic value is given to each male, and it is clear that the breeder will only take males with the highest index numbers (the first five for example). Whatever the size of the population, after some generations, the inbreeding variation (ΔF) will certainly increase, because there is not a large enough number of males and females at the same time.

Inbreeding causes an increase in homozygosity, which is accompanied by deleterious effects on the animal, such as the inability to survive or reproduce. This phenomenon is classically called inbreeding depression, which is expressed in reduced reproduction performance [36]. These deleterious effects are most often due to the recessive genes [37].

Franklin [38] and Soulé [39] found that populations with effective sizes of 50 or greater did not show inbreeding depression in the short term. For populations of smaller number (<50), the limited size of the population was responsible for inbreeding depression [40–43]. In the long term, inbreeding depression has been reported for populations with an effective size of 50 or even greater [44]. The results of Frankham et al. [45] have shown that it was difficult to define a threshold in order to differentiate these small and large populations.

4. Artificial insemination and embryo transfer: are these biotechnologies sufficient?

The population must be enriched directly from the founder animals to minimize the loss of genetic material: if a founder couple produce only one offspring, 50% of their alleles will be lost, but the production of two offsprings will produce a loss of 25%, and of three offsprings 12.5% [46]. Therefore, prolific births are essential to maintain genetic diversity. However, keeping a large population of animals is very costly, thus, the cryopreservation of gametes and embryos could be a solution for creating a real bank of genetic resources. The first generation of biotechnologies used artificial insemination (AI), which was initially employed at the beginning of the 20th century but was greatly used after the 1950s. The second generation was related to embryo transfer, from the beginning of the 1970s; in vitro fertilization, embryo sexing and cloning are the procedures being developed now, and they are considered as the third generation of the biotechnologies of reproduction. We will include cryopreservation of ovarian tissue in this last group.

AI is one of most important procedure and the one most often used in breeding. From a selection of a very small number of males, it is possible to recover a sufficient quantity of spermatozoa to inseminate thousands of females per year. AI was first carried out on a dog, in 1780 by Spallanzani, an Italian physiologist [47]. Since then, this technique has been widely used and developed for cattle, pigs, sheep, goats and finally, horses. In 1949, the first trials for freezing sperm by Polge [48] revolutionized biotechnology: frozen sperm could be stored for long times and transported easily. A bank of male gametes was created from selected individuals for each breed within bovine, porcine, ovine, caprine, equine and canine species. Later, the knowledge of endocrinology of female domestic animals, notably the development of methods of estrus synchronization by the administration of prostaglandin and prostagens [49], enabled the improvement of the results of AI.

Nowadays, induction of ovulation by the synchronization of estrus, recovery of embryos and embryo transfer (ET) are well developed [49]. The nonsurgical collection of embryos from unstimulated [50] and stimulated [51] animals has been used since the end of the 1970s [52]. Offsprings were obtained from cows and sheep after in vitro maturation of oocyte followed by in vitro fertilization, in 1986 [53,54]. In sheep, oocytes in vitro maturation [55], in vitro fertilization and embryo culture [56] have been also well developed. From a report of the Association Europeenne de Transfert Embryonnaire (AETE) in 1999, 143,168 embryos, of which 9.13% were produced in vitro and 58.0% frozen, were transferred to cattle. The total number of embryos transferred in sheep was 6330, in pigs 534, in goats 176 and in horses 194 [57].

Embryo transfer would be more widely used, as would AI, if conservation procedures for gamete and embryo transport were improved. The limiting factor of these techniques remains in that they can only be used in countries where the techniques are already working well. Today, embryo cryopreservation is routinely performed for cattle and sheep, but for other species the procedure is still being developed [58].

Using these biotechnologies on a limited number of parents and grandparents allows an increase in the intensity of selection. In the USA, genetic diversity in cattle, sheep and goats increased between 1970 and 1996 due to the importation of animals. However, this diversity was originally due to the elevated number of breeders, to the low intensity of selection and the limited use of AI and other artificial procedures of reproduction. Conversely, today the Holstein breed is dominating for milk production in Europe, as well as in the USA. The large scale use of sons originating from fathers and grandfathers themselves originating from limited numbers produced by AI or by ET, results in a drastic reduction of genetic diversity [59].

The loss of genetic diversity has become a problem in dairy cattle. Wiggans et al. [36] showed that the coefficient of inbreeding in Holsteins born in 1990 varies between 0.38 and 0.01. They also showed that the estimation of milk production, lipid content and milk proteins are −29.6, −1.08 and −0.97 kg, respectively, for each 1% increase in inbreeding. Another study of the Holstein breed showed an increase in inbreeding from 0.5 to 3.2% between 1950 and 1990 [60]. The authors noted that the inclusion of unknown parents limited considerably the phenomenon of inbreeding.

AI and embryo transfer are not sufficient for fighting against inbreeding if the gamete reserve is limited. To avoid inbreeding the solution would be to create a gene bank of male and female gametes and embryos, formed the largest number of individuals.

The cryopreservation of ovarian cortex containing the primordial follicles would certainly be very useful in the conservation of female gametes. There are two possibilities: the extraction of slices of the ovarian cortex after puberty without disturbing the female reproductive system. Another way is to remove the whole ovaries after the animal's death, to be used later in techniques such as grafting or in vitro maturation.

5. Techniques for cryopreservation of the ovarian cortex

5.1. Freezing techniques

The important factors in freezing cells are the cooling rate, the nature and concentration of the cryoprotectant and the temperature at seeding.

The first report on the freezing of ovarian tissue was in the 1950s: grafts were obtained from mice after cryopreservation of ovarian tissue at −79 °C using glycerol as the cryoprotectant [5,61–63]. Authors reported 5% follicle survival after freezing: the cryoprotectant was glycerol but descent in temperature was not controlled precisely. Later, Candy et al. [7], showed that about 20% of the follicles survived in ovarian fragments frozen in glycerol, but using a more precise cooling rate.

At present, the long term conservation of all simple or complex cellular structures occurs at −196 °C, in liquid nitrogen. At this temperature, intracellular chemical reactions are

inhibited. During the drop in temperature, the physico-chemical properties of the conservation medium of the cells change. These modifications have a direct influence on cell biology. The control of the freezing and thawing rate, and the addition of cryoprotectant substances to the conservation medium, limits cell damage [64]. The freezing protocols of the last 20 years have improved cell survival compared to those of the 1960s, by using a slower rate of decrease in temperature. The standard protocol is based on an embryo freezing protocol [6] that corresponds to a very slow cooling rate (0.3 °C/min) with manual seeding.

Seeding is a necessary process to decrease the variations in temperature at the moment of ice nucleation. These variations are induced by the exothermic reaction arising from the formation of ice crystals. The seeding temperature varies according to the nature and the concentration of the cryoprotectant and the cooling rate. For example, for 1.5 M dimethylsulphoxide (DMSO), using the same freezing protocol (0.3 °C/min), different temperatures of seeding have been used: −7 °C [11,12,65], −8 °C [7,66] and −9 °C [65]. Before seeding, the crystallization temperature must be tested for a given sample, in the medium used, with a defined cryoprotectant, at a determined concentration.

The cryoprotectant most often used for sheep [12] and for mice ovarian tissue cryopreservation is 1.5 M DMSO [11,66–68]. DMSO is an excellent cryoprotectant, but because it causes destabilization of the cellular membrane and polymerization of microtubules leading to aneuploïdy (mutagen effect) [69], it is useful to compare DMSO with other cryoprotectants such as ethylene glycol, propylene glycol and glycerol, which are known to be less toxic. However, it is necessary to consider the toxicity of the cryoprotectant, its protecting effects and the cooling rate together, to evaluate the viability of the cell after cryopreservation. Comparisons of different cryoprotectants at a concentration of 1.5 M while using a standard cooling rate protocol (0.3 °C/min), show that the survival of primordial follicles is better with DMSO, propylene glycol and ethylene glycol in women [70] and in mice [7], and that there is no significant difference between DMSO and propylene glycol [65].

The protocol developed by our team, uses a cooling rate of 2 °C/min, with a semi-automatic seeding by release of negative calories within the freezing chamber. We have tested two cryoprotectants, DMSO and propylene glycol (PROH), at different concentrations: 1, 1.5 and 2 M after having previously tested the toxicity of these cryoprotectants at higher concentrations: 2, 4, 6.5, 9 and 10 M. The mortality rate of isolated small follicles was 4.6% for 2 M DMSO and 3.8% for 2 M PROH. We have reported that as the cryoprotectant concentration increased, the seeding temperature decreased. With a concentration 2 M DMSO we observed the temperature rise at −11 °C. The best results were obtained with 2 M DMSO, with a mortality rate of small isolated follicles of only 8.4%. These results were significantly better than with the standard protocol (17.6%) [17].

With the introduction of ovarian tissue banks for humans as well as animals it becomes possible to obtain fertilizable oocytes. The two feasible techniques are the grafting of ovarian tissue or in vitro maturation.

5.2. *Grafting*

i *Autograft*

The autograft can be performed either orthotopically on the ovarian pedicle or in the ovarian bursa, to obtain an ovulation followed by fertilization and a natural gestation,

or heterotopically, i.e. the ovarian tissue is grafted on another physiological site, for example subcutaneously, to achieve a folliculogenesis in in vivo conditions and recover the oocytes for an in vitro fertilization.

The first experiments on the autograft of cryopreserved tissues date back to the 1950s. The first pregnancy was reported in mice by Parrott in 1960 [5]. This work was taken up again in the 1990s by Gosden [12] who obtained the first pregnancy in a sheep after an orthotopic graft of frozen-thawed ovarian tissue.

After grafting, the delay before resumption of steroidogenesis differs according to the species: between 15 and 23 days in mouse, independently of its age [8,11,71], and about 4–6 months in sheep [14,72]. The frozen-grafted ovary can remain hormonally active for 11 months in the case of the mouse [8] and for 22 months in sheep [72]. In sheep, reinitiating of follicular growth after grafting of frozen ovarian tissue was successful, preantral follicles of 80 μm diameter at 7 weeks, antral follicles of 250–350 μm at 10 weeks and ovulatory follicles at 13 weeks were observed [16]. Similar results were obtained by Baird et al. [72]: development of antral follicles from primary follicles occurred after 80 days of graft. Live births were obtained in mice [8,11,68] and more recently in a large mammal (sheep) [15].

ii *Xenograft*

The transplantation of ovarian tissue in immunodeficient animals, Nude (without T lymphocytes) or severe combined immunodeficiency (SCID), no B lymphocytes and no T lymphocytes), followed by in vitro maturation of the oocytes, is a good model for achieving in vivo folliculogenesis. Follicle development was observed in xenografts of frozen ovarian tissue originally from different species: marmoset [66], elephant [73] and wombat [74]. It has been impossible to reach preovulatory stage, probably because the endogenous gonadotrophins were not sufficient to support the follicular growth of the xenograft tissue [75]. Recently, Gook et al. [76] obtained antral follicles of 4–5 mm after a xenograft of frozen human ovarian tissue, with administration of follicle stimulating hormone (FSH).

The revascularisation of the graft is the key factor in ovarian grafting. The ovary produces angiogenic factors, which facilitate the migration of endothelial cells in the graft [77]. Baird et al. observed a decrease in the primordial follicles number after grafting: 65% in the fresh graft and 72% in the frozen grafts. They indicated that only 7% of the follicular loss was due to the freezing process itself, the main loss is due to ischaemia before revascularisation [72].

5.3. *In vitro maturation*

In vitro culture can be an alternative to ovarian tissue grafting. The use of frozen ovarian tissue requires that the cultures should start from primordial follicles. The initiation of in vitro folliculogenesis (transformation of the primordial follicles into primary follicles) was achieved for the first time in 1965 in mice [78]. The complete folliculogenesis process has been achieved in vitro, only in the mouse, by Eppig and O'Brien [19]: after a first culture step of the ovarian tissue to initiate growth, the secondary follicles were individually cultivated; following in vitro maturation and in vitro fertilization. A birth was obtained.

Although this experience has not been repeated, this work shows the possibility of a completely successful folliculogenesis with in vitro maturation. The time necessary to achieve complete folliculogenesis is longer for the sheep (more than 6 months) than for the mouse (22 days). A complete folliculogenesis has not been achieved in this species. The process can be divided into three main parts: growth initiation, formation of antral follicles from secondary preantral follicles or primary follicles, and the maturation of the oocyte to obtain a Metaphase II fertilizable oocyte.

Growth initiation of primordial follicles into primary follicles has been successfully achieved with cattle [79], baboons [80] and women [81,82]. In our work with sheep, on the fourth day of culture we observed a significant decrease in the amount of primordial follicles (from 72.9% (day 0) to 18.9% (day 4)) and an increase in the number of intermediate, primary and atretic follicles (from 13.7 to 30.3%, from 3.8 to 27.0% and from 9.4 to 22.9%, respectively) [18].

Preantral follicles have been cultivated after enzymatic [83–85] or mechanical isolation [9,86]. In rodents, starting from secondary follicles, Metaphase II oocytes were obtained after 14 days. The fertilisability of these follicles was similar to those of the control (in vivo maturation), and hatched blastocysts were obtained [9]. Metaphase II oocytes were obtained following the long-term maturation in vitro of small immature oocytes from cryopreserved mice ovarian tissues [87]. To date, only a few authors have validated their culture method with actual births [10,88,89]. It is important to note that these publications refer to works on the mouse, thanks to the short period of folliculogenesis. In sheep, antral follicles and few oocytes in Metaphase II have been obtained only from the culture of preantral follicles [20].

6. Conclusion

The intent of freezing the ovarian cortex is to create a bank of female genes, with the aim of protecting certain species in danger of extinction and conserving the genetic diversity of all species. However, difficulties remain in using this tissue, in which it is necessary to control ‘‘in vitro’’ all steps of follicular growth.

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