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ORIGINAL ARTICLE

In Vitro Perfusion of Whole Bovine Ovaries by Freezing Medium: Effect of Perfusion Rate and Elapsed Time after Extraction

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SUMMARY

Background: **Cryopreservation and transplantation of the whole ovary with vascular pedicle would be helpful to prevent posttransplantation ischemia. In fact, perfusion of the intact mammalian ovary through arteries and veins is the most technically difficult part of the whole cryopreservation process because of its complexity. It is important to develop the technology of long-time perfusion of intact ovaries by cryoprotectants at low temperatures because it was established earlier that 24-hour cooling to 5°C before cryopreservation is beneficial for the freezing of human ovarian tissue. The aim of this research was to study the effectiveness of perfusion of intact bovine ovaries with different rates of perfusion and elapsed time between extraction of these ovaries and beginning of perfusion.**

Methods: **Arteria ovarica was cannulated and ovaries were perfused with Leibovitz L-15 medium + 100 IU/mL heparin + 5% bovine calf serum + 6% dimethyl sulfoxide + 6% ethylene glycol + 0.15 M sucrose + Indian ink at room temperature (22°C). In the first cycle of experiments, ovaries (n = 145) were perfused for 60 minutes during 1 to 1.5 hours after extraction of ovaries in the slaughter house at perfusion rates of 150 mL/hour (2.5 mL/ minute), 100 mL/hour (1.67 mL/minute), 75 mL/hour (1.25 mL/minute), 50 mL/hour (0.83 mL/minute), 25 mL/ hour (0.42 mL/minute), and 12.5 mL/hour (0.21 mL/minute) for groups 1, 2, 3, 4, 5, and 6, respectively. In the second cycle of experiments, ovaries (n = 29) were perfused with a rate of 25 mL/hour (0.42 mL/minute) for 60 minutes during the following time-periods elapsed after extraction of ovaries in the slaughter house: 3 hours (n = 18), 4 hours (n = 5), 5 hours (n = 3), and 6 hours (n = 3) for groups 1, 2, 3, and 4, respectively. Ovaries in luteal and follicular phase of development were distributed randomly into groups. Successful perfusion of blood vessels was detected visibly by a blue coloration of the vascular pedicle and ovarian tissues. The percentage of Indian ink-perfused tissues was detected. The intensity of the vascular leakage and tissue damage was scored microscopically and noted as follows: lack of disruption (-), weak disruption (+), moderate disruption (++), and strong disruption (+++).**

Results: **The first cycle of experiments shows that an optimal perfusion rate was established for groups 4 and 5 (50 and 25 mL/hour, respectively). In the second cycle of experiments, good perfusion of ovaries with the perfusion rate of 25 mL/hour was established only for ovaries of group 1 (3 hours after extraction). The effectiveness of perfusion in group 2 (4 hours after extraction) was sharply decreased.**

Conclusions: **Effective perfusion of bovine intact ovaries with vascular pedicle with freezing medium (6% ethylene glycol + 6% dimethyl sulfoxide + 0.15 M sucrose) at room temperature includes a rate of perfusion 25 or 50 mL/ hour. Ovaries must be perfused no later than 3 hours after the death of animals.**

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KEY WORDS

cryopreservation, cryoprotectants, bovine, ovary, perfusion, vessels

INTRODUCTION

In medicine, because of the increasing effectiveness of cancer treatments and good long-term prognosis for young women, the problem of post-cancer infertility plays an important role. Because chemotherapy, depending on the treatment regime chosen, can be gonadotoxic and can lead to the functional death of ovaries. The cryopreservation of ovarian tissue before cancer therapy with retransplantation after convalescence is the key for solving this problem [1-3].

Experiments with cryopreservation of whole bovine ovaries with vascular pedicles are currently relevant for preservation of some endangered ruminant species as well as for medicine when these ovaries play the role of human model.

In contrast to investigations of different questions related to cryopreservation performed on small ruminant species (about 40 articles were published to date), data about cryopreservation conducted on large ruminant species are limited [4-8].

In a recent publication of a research group from France, which is one of the leaders of cryopreservation of whole mammalian ovaries [9], it is noted that cryoprotectant diffusion into perfused ovaries is a potentially limiting factor that has not been adequately investigated [9].

Cryopreservation and transplantation of the whole ovary with vascular pedicle would be helpful to prevent post-transplantation ischemia. However, data have provided evidence that after cryopreservation of whole ovaries, their viability is low [10,11].

Development of technology of long-time perfusion of intact ovaries by cryoprotectants at low temperatures is important because earlier it was established that 24 hour cooling to 5°C before cryopreservation is beneficial for freezing of human ovarian fragments [12]. It was established that the quality of follicles and the intensiveness of neovascularization observed in ovarian tissue precooled both before culture and cryopreservation was drastically increased [12].

The aim of this research was to study the effectiveness of perfusion of intact bovine ovaries with different rates of perfusion and different elapsed times between extraction of these ovaries and beginning of perfusion.

MATERIALS AND METHODS

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

The technology of the local slaughterhouse allows obtaining ovaries during the 10 to 15 minutes following the death of animals. Ovaries with vasculature were cut from the dorsal aorta, placed in a thermos without liquid, and transported to the laboratory within 30 to 40 minutes. In laboratory, all preparatory manipulations of ovaries for perfusion and the process of perfusion with perfusion medium were performed at room temperature (22°C). Ovaries were isolated one by one, but connective tissues *(mesoovarium)* were not removed (Figure 1a). The phase of the ovary (follicular or luteal) was determined after postperfusion dissection of the ovary by the absence or presence of a fresh (red or orange) *corpus luteum*. The vessels (*arteria ovarica* und *vena ovarica*) (Figure 1b) were transected about 20 - 25 cm under the ovary.

The *arteria ovarica* (inner diameter, 0.8 - 1.2 mm) (Figure 1b) was prepared, and a 22 G catheter (Introcan Safety; B. Braun Melsungen AG, Melsungen, Germany) was introduced to this vessel, fixed using nylon sutures (Figure 1 c, d, e), and perfused with Leibovitz L-15 medium + 100 IU/mL heparin + 5% bovine calf serum + 6% dimethyl sulfoxide + 6% ethylene glycol + 0.15 M sucrose + 25% Indian ink.

In the first cycle of experiments, ovaries (n = 145) were perfused for 60 minutes during 1 to 1.5 hours after extraction of ovaries in the slaughter house at perfusion rates: 150 mL/hour (2.5 mL/minute), 100 mL/hour (1.67), 75 mL/hour (1.25 mL/minute), 50 mL/hour (0.83 mL/minute), 25 mL/hour (0.42 mL/minute), and 12.5 mL/hour (0.21 mL/minute) for groups 1, 2, 3, 4, 5, and 6, respectively.

In the second cycle of experiments, ovaries (n = 29) were perfused using perfusor Secura FT (B. Braun Melsungen AG, Melsungen, Germany) at a rate of 25 mL/hour (0.42 mL/minute) for 60 minutes during the following time-periods alapsed after extraction of ovaries in the slaughter house: 3 hours (n = 18), 4 hours (n = 5), 5 hours (n = 3), and 6 hours (n = 3) for groups 1, 2, 3, and 4, respectively. Ovaries in luteal and follicular phase of development were distributed randomly into groups. Successful perfusion of blood vessels was detected visibly by a blue coloration of the vascular pedicle and ovarian tissues. The percentage of Indian ink–perfused vessels was noted. The intensity of the vascular leakage and tissue damage was scored microscopically and noted as follows: lack of disruption (-), weak disruption (+), moderate disruption (++), and strong disruption (+++).

Effectiveness of perfusion was evaluated using ANOVA. The level of statistical significance was set at $p < 0.05$.

RESULTS

After successful perfusion, approximately 95% of the ovarian tissue and vascular pedicle had a blue colour. The ovarian tissue has unstained areas, showing an incomplete perfusion of the freezing medium (Table 1, Figure 2).

Table 1. Effectiveness of perfusion of the whole bovine ovaries by freezing medium.

Group	Speed of perfusion		Ovaries in group n	Saturated tissues %	Intensity of vascular and tissue damage
	mL/hour	mL/minute			
1	150	2.5	6	95	+++
2	100	1.67	12	95	++
3	75	1.25	12	95	+
4	50	0.83	50	95	-
5	25	0.42	50	95	-
6	12.5	0.21	15	15	-

Freezing medium includes Leibovitz L-15 medium + 100 IU/mL heparin + 5% bovine calf serum + 6% dimethyl sulfoxide + 6% ethylene glycol + 0.15 M sucrose + Indian ink.
All rates are significantly different ($p < 0.05$).

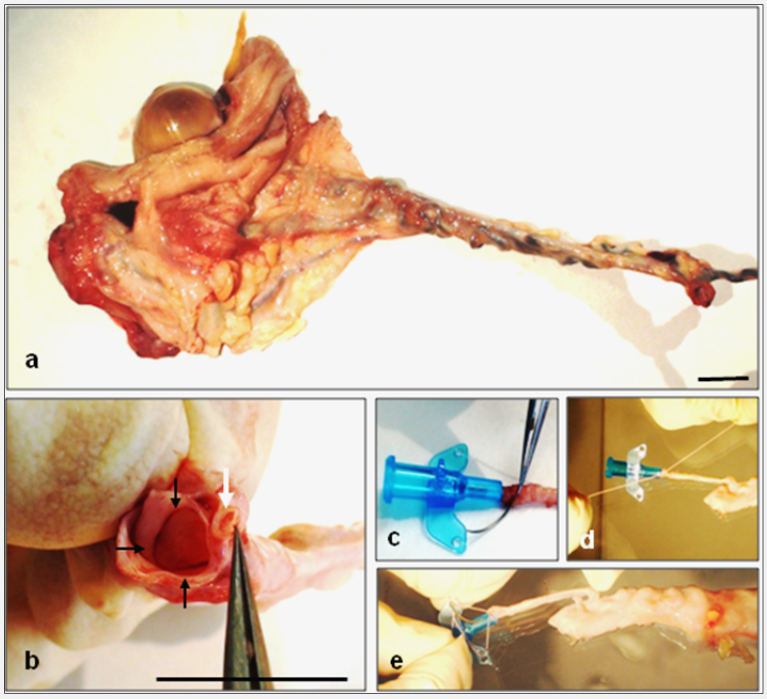

Figure 1. Preparation of intact bovine ovary with vascular pedicle for perfusion.
(a) beginning of ovary perfusion; (b) *arteria ovarica* [white arrow], *vena ovarica* [black arrows]; and (c, d, e) introduction and fixation of catheter in *arteria ovarica.*
Bar = 1 cm.

Vascular leakage and tissue damage as a result of high pressure of the perfusion (freezing) medium with varying intensities (strong (+++), lack (++), and weak (+)) were observed with different perfusion rates of 150, 100, and 75 mL/hour for groups 1, 2, and 3, respectively (Table 1).

An optimal perfusion rate was established for ovaries of groups 4 and 5 (50 and 25 mL/hour, respectively) (Table 1, Figure 2).

In the second cycle of experiments, good perfusion of ovaries with the perfusion rate of 25 mL/hour was established for ovaries of group 1 only (3 hours after ex-

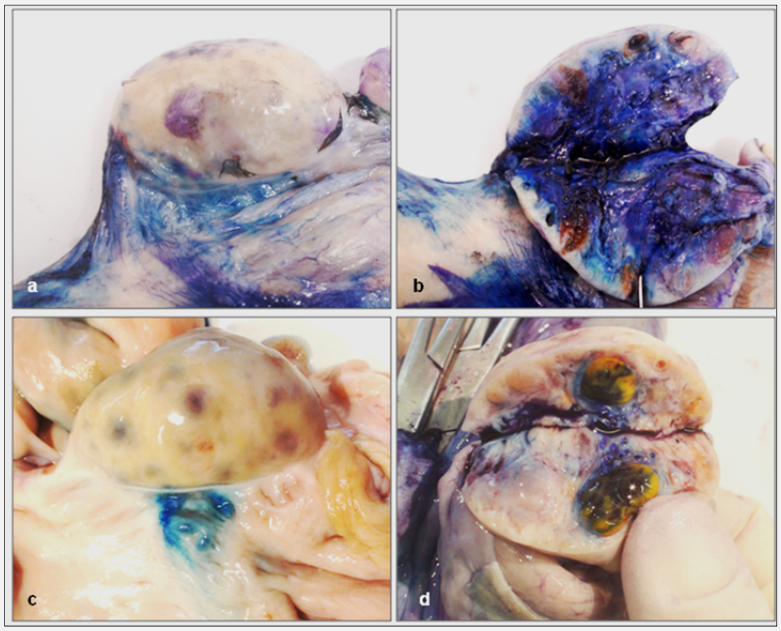

Figure 2. End of perfusion of intact bovine ovary with vascular pedicle with freezing medium.
(a, b) ovary after successful perfusion [~ 95% of tissues], and (c, d) ovary after unsuccessful perfusion [~ 15% of tissues].
Freezing medium includes Leibovitz L-15 medium + 100 IU/mL heparin + 5% bovine calf serum + 6% dimethyl sulfoxide + 6% ethylene glycol + 0.15 M sucrose + Indian ink.

traction). The effectiveness of perfusion in group 2 (4 hours after extraction) was sharply decreased.

DISCUSSION

Ischemia is the serious problem accompanying the process of extraction, cryopreservation, and retransplantation of the ovarian tissue.

It is known that the presence of blood vessels is a very important factor for successful ovarian tissue transplantation including establishment of the blood supplies crucial for the survival of ovarian follicles [13]. It was shown that transplanted immature rat ovaries become profusely re-vascularised within 48 hours after autotransplantation [13]. In the cortex, development of primordial follicles is fully dependent on stromal vessels [14]. Prior to re-vascularisation, implants are vulnerable to ischemia, which is the main obstacle for the survival of tissue after transplantation. Such damage can lead to a 30 to 70% reduction in graft size accompanied with fibrotic changes [15]. The hypoxia observed during the first 5 days after grafting and ischemic damage occurring during this period could induce primordial follicle loss [16-18] and disorders of follicular activation [19, 20].

When talking about cryopreservation of the whole ovary with post-thawing retransplantation through vascular anastomosis, the problem of neovascularization will be solved "automatically."

We have performed our investigations on bovine ovaries, which are noted to be a good model for the cryopreservation of the whole human ovary with its vascular pedicle. We selected to use only the cow from three "popular" mammalian species for the following two reasons.

The first is "anatomical and physiological" factors. We fully share the points of view of Gerritse et al. [4] and Zhang et al. [6], which performed their experiments using bovine ovaries as a model system for human. The authors explained that they used bovine ovaries because of the following: (i) the ovaries are comparable in size with human ovaries; (ii) only one or two follicles are maturing per cycle as the maturation of multiple follicles greatly influences the ovarian volume and, therefore, the outcome of the freezing process; and (iii) cows have a monthly cycle. The authors [4,6] did consider bovine, porcine, and ovine ovaries for their investigations as possible candidates for a model system. Volume measurements indicated that, whereas porcine ovaries are comparable in size to human ovaries, bovine ovaries are considerably larger, and ovine ovaries are much smal-

ler. These authors underline that in pigs, multiple follicles mature per cycle, possibly resulting in a different architecture and/or vasculature of the ovaries compared with ovaries in which only one or two follicles mature each month. Therefore, these authors [4,6] selected bovine ovaries as a model system [4,6].

Second, we chose the cow as a model for our cryoinvestigations for "cryobiological" reasons.

As a rule, cryoinvestigations in human tissues are based on successfully used protocols emerging from similar experiments performed on laboratory and agricultural animals. Before it was possible to freeze human cells, animal tissues had been successfully cryopreserved. However, it is necessary to take into account that human cells have their own peculiarities. One such attribute is the presence of intracellular lipids. Intracellular lipids are a 'stumbling block' for cryopreservation.

Data demonstrating the role of these intracellular structures during cryopreservation have been published [21]. The method proposed involves polarization and removal of cytoplasmic lipids from oocytes or embryos before cryopreservation. Nagashima et al. [21] were the first to successfully grow embryos from GV-porcine oocytes that were cryopreserved following delipidization. Using this method the authors avoided negative effects caused by cooled intracellular lipids. According to the data provided by the authors, removal of intracellular lipids does not lead to a worsening of further development of oocytes and embryos. Successful oocyte vitrification after removal of cytoplasmic lipids leads to the question of possible changes in the physiochemical properties of cytoplasmic membrane lipids arising at low temperatures, which were discussed as a significant cause of cryobiological problems during experiments.

We believe that it is impossible to dismiss classic data about the role of intracellular lipids as energetic materials of oocytes and building materials for membranes of future embryos. The fact that the volume of mitochondria as well as lipid vesicles increases during oocyte development to metaphase II (MII) stage indirectly confirms this point. Moreover, Sathananthan et al. [22] have shown that in the cell complex called 'smooth endoplasmic reticulum-lipid globules-mitochondria' reticulum-globules-mitochondria connections do exist.

They have also shown that these connections may be damaged after oocytes cooling or freezing.

In the overwhelming majority of work studying the effect of cooling on mammalian oocytes and embryos, a negative cryo-influence is explained in terms of the effect on cytoskeletal elements.

For example, cooling of human oocytes causes deploymerization of cytoskeletal protein structures and most mouse oocytes cooled to 25°C for 10 minutes had an abnormal cytoskeleton [23].

We believe that the negative effect of cooling on tissues can be explained by the effect of cooling lipids on cytoskeletal structures. Whilst performing our investigations on porcine oocytes, we found that following centrifugation, redistribution of lipids occurs within 48 hours of *in vitro* culture in oocytes not exposed to vitrification/warming (data not published).

However, when polarized oocytes are vitrified/warmed, the lipid polarization is irreversible. This, in our opinion, suggests that the vitrification/warming process induces an alteration to the physiochemical properties of intracellular lipids.

It is known that MII oocytes are more resistant to freeze damage than GV-stage oocytes. We consider that this may be due to differences in the properties of cytoskeletal elements. One important difference is that the configuration of microtubules and microfilaments is different at these two stages of oocyte maturation. Cytoskeletal elements in GV-oocytes appear straight and rigid, while the appearance of microfilaments and microtubules in MII stage oocytes is undulating and flexible. Based on the hypothesis of a complex interaction between the lipid phase of cells and the elements of the cytoskeleton, hardening of these lipids might cause deformation and disruption of the cytoskeleton. In the case of the rigid GV-oocyte cytoskeleton this apparently results in permanent damage while in the more flexible MII-oocyte cytoskeleton, permanent damage is absent. Cytochalasins have a specific, reversible effect on cytoskeletal elements making them more flexible and less susceptible to the effect from cooled lipids.

An optimal protocol of cryopreservation must prevent the alterations of the physiochemical properties of cooled lipids, avoid irreversible damage to the lipid globule membranes, and protect the cooling reticulum-lipid connections from destruction.

It is known that bovine oocytes are to a considerable extent more cryostable than porcine oocytes. There is also information suggesting that the diameters of bovine and porcine intracellular lipid vesicles are different. The characteristics of the intracellular lipid granule membranes are also a topic of discussion.

We compared the ultrastructure of lipid droplets, and the effect of cooling on intracellular lipid vesicles of bovine and porcine GV-oocytes [24]. It was shown that lipid droplets of bovine cells have a homogenous structure. The utilization of lipids takes place directly from these vesicles without formation of interim lipid compounds. In contrast, there are two kinds of lipid droplets in porcine GV-oocytes: 'dark' and homogenous vesicles next to 'gray' vesicles with electronlucent streaks. Vesicles of each specific group are connected to each other. After 12-hour culture, the formation of the cisternal smooth endoplasmic reticulum layer is always associated with 'gray' lipid vesicles. This is evidence that during oogenesis lipolysis takes place only in 'gray' vesicles. It is supposed that cytoplasmic lipolysis has two stages: 'dark' vesicles change into a 'gray' form followed by a utilization of these 'gray' lipids. Furthermore, both types of lipid droplets in porcine oocytes changed morphologically during cooling: they turned from a round into a spherical form with lucent streaks. Lipid droplets in bovine GV-oocytes revealed no visible morphological changes after cooling [24].

In order to compare intracellular lipids and the cryostability of ovine and human pronuclear embryos we vitrified embryos of both species and evaluated the ultrastructure of intracellular lipids before and after vitrification. Cryopreservation of embryos was performed according to the method previously described for ovine GV-oocytes with two different methods of removal of cryoprotectant: step-wise and direct rehydration. We noted, that in contrast to human pronuclear embryos, where direct rehydration has a mortal effect after thawing, ovine pronuclear embryos show high developmental rates (31 - 34%) after *in vitro* culture [25].

Fresh lipid droplets in both species, human and bovine, are homogenous in structure. It was noted that after vitrification the intracellular lipids in cryopreserved human embryos underwent no visible morphological changes while distinct changes were observed in the lipid droplets of the ovine embryos. These alterations, attributable to the vitrification process, reflect changes in the physical and chemical properties of the lipids such as hardening.

We suggest that the specificity of human cells, reflected by their high sensitivity to osmotic processes, is related to the specificity of both intracellular lipids and cytoplasmic and organelle membranes. Lipids are the most cryo-labile intracellular compounds of oocytes and embryos. Indeed, the specific nature of intracellular lipids in pig oocytes makes them practically unsuitable for cryopreservation, particularly vitrification. It has been observed that the appearance of intracellular lipids of fresh human cells before and after vitrification shows no changes. We compared these lipids with those of ovine cells subjected to the same protocol which was used for human oocytes with direct rehydration in our previous investigations. These lipids of viable embryos showed ultrastructural changes after vitrification not noted in the human oocytes [26].

Bearing in mind the resistance of ovine cells to direct post-thaw rehydration, we were able to observe hardening, increased density, and morphological alterations in the intracellular lipids of all cooled ovine oocytes. These alterations were absent in all human oocytes, which were clearly unable to tolerate direct rehydration. It may be assumed that within the same cell, the structure of intracellular and membrane lipids are the same.

Given the detrimental role of lipids during cryopreservation, the lipid cryostability in contrast to the osmotic instability of human cells is still far from being fully understood. Direct post-thaw rehydration induces lethal osmotic effects in human pronuclear oocytes but is successful for ovine oocytes. A correlation between the cryostability of mammalian oocytes and the ultrastructure of intracellular lipids is proposed for further investigations. Taking into account that the ultrastructure of intracellular lipids of laboratory and agricultural animals are different from human intracellular lipids, we believe that using animal oocytes as a model for human oocytes in cryo-investigations is questionable [26].

However, taking into account all the above, of the three species of agricultural animals, pig, cow, and sheep, bovine ovarian tissue is a more suitable model for human because intracellular and membrane lipids are most osmotic-sensitive which is similar to human cells. Just this bovine model was used in our experiments.

To date, there are no data about successful transplantation of human intact ovary with its vascular pedicle after cryopreservation. However, cryopreservation of whole ovaries with vascular anastomosis with post thawing retransplantation can be considered a promising strategy for cancer patients. Some reports have described fresh (nonfrozen) whole ovaries with vascular anastomosis in human, and one pregnancy was noted [27-31].

The problem of cryopreservation of intact ovary with its vascular pedicle in animals is far from being solved. Despite numerous attempts and evidence of restoration of long-time follicular development [10,11,32-41], only three births after retransplantation of frozen ovine ovaries were noted.

The important role of perfusion of whole ovine ovaries by cryoprotective medium was recently shown [9]. An observational study of 360 ovine ovaries stained by *in vitro* perfusion with a qualitative marker of tissue blood supply was performed. A logistic regression model was built to identify factors associated with incomplete ovary staining. Whole ovine ovaries with their vascular pedicles were perfused at 0.35 mL/minute for 2 hours at 39°C under 19 experimental conditions. The pedicles were removed, and the ovaries were cut and photographed. The unstained area of the sliced surface was measured. 64.4% of the ovaries were observed to have unstained areas. Multivariate analysis found that incomplete ovary staining was independently associated with lower experimenter experience, smaller ovary slice surface area, and presence of a corpus luteum. The presence of unstained areas was independent from experimental conditions. The rate of incomplete ovary staining decreased from 83% to 60% [9]. The formulated conclusion of these experiments is that blood supply impairments that result in incomplete perfusion might adversely affect outcomes after whole ovary cryopreservation. Improved perfusion techniques should enhance success [9].

The Indian ink perfusion studies by Gerritse et al. [4] were performed on intact bovine ovaries. The authors observed that the larger vessels were well perfused, whereas the smaller ones and the capillaries were less well perfused. The authors assumed that the relatively smaller molecular weights of cryoprotective agents, such as dimethyl sulfoxide and sucrose, imply that they would be subject to fewer molecular filtering mechanisms of endothelial cells and basal membranes [4].

In our experiments, the role of perfusion solution, which includes 5% bovine calf serum + 6% dimethyl sulfoxide + 6% ethylene glycol + 0.15 M sucrose, takes the place of the freezing medium. In fact, the presence of these components will not increase the permeability of the perfusion medium into small capillaries. The

presence of cryoprotectants (especially the nonpermeable cryoprotectant sucrose) and bovine calf serum in the perfusion solution can explain that for successful perfusion of small blood vessels and especially capillaries, we need a perfusion medium speed that is slower than those in the experiments of Gerritse et al. [4] (2.5 mL/minute). Increasing of the perfusion rate in ovaries of group 3 (1.25 mL/minute) leads to vascular leakage and tissue damage.

The elapsed time between the death of the animals in the slaughter house including removal of the ovary and the start of the perfusion process is an important parameter of the cryopreservation technology because of ovarian ischemic damage. We have shown that this period at room temperature must be no longer than 3 hours because beyond this time, the sealing of both smaller and bigger vessels by coagulated erythrocytes begins. Certainly, this period can be increased by using lower temperatures for the transportation of ovaries.

In conclusion, effective perfusion of bovine intact ovaries with vascular pedicle by freezing medium (6% ethylene glycol + 6% dimethyl sulfoxide + 0.15 M sucrose) at room temperature includes a perfusion rate of 25 or 50 mL/hour. Ovaries must be perfused within 3 hours after the death of animals.

Declaration of Interest:

The authors of the manuscript declare no conflict of interests.

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