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Uterus Cryopreservation in the Sheep: One Step Closer to Uterus Transplantation

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Abstract. *Background: Uterus transplantation is the only way for women with no functional uterus to become pregnant. However, the technique is limited by the fact that an aggressive immunosuppression is necessary in order to avoid rejection of the graft. For better chances of finding a matching organ, which would minimize the immunosuppressive therapy, the establishment of cryobanks with a large number of uteri would be helpful. The aim of this study was to evaluate the feasibility to cryopreserve sheep uteri and to review the literature in this exciting new field of uterus cryopreservation and transplantation. Material and Methods: Ten sheep uteri were frozen either with slow cooling after perfusion with 10% dimethylsulfoxide, or without perfusion and the contractile ability was compared with that of fresh uteri. Results: All perfused uteri showed contractions after thawing, similar to those of the non-frozen uteri. Conclusion: This study shows that the perfusion of sheep uterus with a cryoprotectant prior to slow freezing allows the cryopreservation of the whole organ and maintains the functionality of the organ after thawing. The perfused sheep uterus provides an experimental model for further investigations with other cryoprotective agents and freezing protocols.*

Transplantation of the ovaries and uterus has gained new attention recently, since cancer treatment has become more successful at the cost of fertility loss (1, 2). In recent years, the field of cryopreservation of ovarian tissue has experienced a rejuvenation, after the first child birth following retransplantion of cryopreserved human ovarian tissue (1). More than 50 cases of transplantation of fresh and frozen ovarian tissue have been reported, mainly for premature ovarian failure (3-11). The practical utility of uterine transplants has not been established, but is now being investigated for possible applications for patients with congenital absence of the uterus (Mayer-Rokitansky-Kuster-Hauser syndrome), patients having had hysterectomy for benign and malignant uterine or cervical diseases, and patients with intrauterine adhesion (3, 6).

A feature of the uterus is that after transplantation, the patient needs to receive aggressive immunosuppression, which raises questions about the teratogenic effects of the treatment on an early pregnancy. Therefore it would be beneficial if a large number of uteri were available in order to facilitate a match with the most appropriate, human leucocyte-compatible recipient. This can only be achieved through long-term storage of uteri by cryopreservation.

Cryopreservation of whole organs has proved difficult for many reasons (12), nevertheless uterus cryopreservation may be possible since the uterus is a relatively simply organized organ. Our group has previously reported on the good functional activity of cryopreserved swine uteri after long-term storage (13-15). Here we report, for the first time, the successful cryopreservation of the sheep uterus which is even more similar to the human uterus and has already been successfully used in autotransplantation experiments (16).

Cryoprotective strategies, such as the introduction of a cryoprotectant agent and a controlled rate of freezing, have helped maintain the structural integrity of cells and tissues (17-26). The main problem is the perfusion of the cryoprotectant in the organ, which has to take place slowly in order to avoid cell membrane damage and to enable the cells to dehydrate before ice forms inside them. The aim of this study was to test the survival of frozen/thawed sheep uterus after cryopreservation with a slow freezing protocol.

Materials and Methods

Animals. Thirty ewe (*Ovis orientalis aries*) uteri were obtained from the local slaughterhouse. The female reproductive system in the sheep has a bicornate uterus with tortuous fallopian tubes. The mean

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Table I. *pH, HCO_3, and lactate in the perfused uteri before cryopreservation and 1 h after thawing and removal of the cryoprotectant.*

	pH (mean±SD)	HCO_3^- (mean±SD), mmol/l	Lactate (mean±SD), mmol/l
Fresh uteri (n=10)	7.3±0.17	13.5±1.00	1.1±0.30
Cryopreserved uteri with cryoprotectant (n=10)	7.2±0.11	12.5±1.06	1.2±0.12

weight of the ewe uteri was 40.4 g (30.5-50.0 g). They all came from healthy animals aged between 6 months to 2.0 years. The ewe uteri were separated from the rest of the body within approximately 2 min, shortly after the animal had been killed.

Study design. Ten sheep uteri were cryopreserved according to a slow freezing protocol with 10% dimethylsulfoxide (DMSO) (group A) and 10 were frozen without perfusion simply in a freezer at –18˚C (group B). Ten non-frozen ewe uteri were perfused for control purposes (group C).

Perfusion system. After cannulation of both uterine arteries with 16–24-gauge needles (Abbocath-T, Abbott, Ireland), depending on the size of the uterus, the organ with adnexa was placed in a controlled-temperature perfusion chamber (Karl Lettenbauer, Erlangen, Germany) filled with the perfusion medium (Figure 1). The uterus was then connected bilaterally to two reservoirs containing the perfusion buffer (Krebs–Ringer bicarbonate-glucose buffer; Sigma, Deisenhofen, Germany) (Figure 2).

The perfusion medium was oxygenated with carbogen gas (95% oxygen and 5% carbon dioxide) and then pumped into the uterine arterial catheters with two roller pumps. The flow rates of the perfusion medium and oxygenation were constantly monitored and kept at 15 ml/min and 0.05 bars, respectively, with an ideal pressure rate of 100 mmHg being maintained throughout the duration of the experiments.

Cryopreservation protocol. The cryopreservation protocol has been described elsewhere (12). In brief, the uteri were perfused with the cryoprotectant solution (10% DMSO; Merck, Hohenbrunn, Germany) in PBS (PBS Dulbecco; Biochrom, Berlin Germany). Then they were left to soak in the solution for an additional 30 min at 4˚C. After pre-equilibration, the uterus with the cannula was placed in the cryocontainer (1000 ml; Rotilabo, Carl Roth, Karlsruhe, Germany) and cooled to subzero temperature in a freezer at –70˚C. After 24 h, the cryocontainer was transferred to a freezer at –150˚C. The uteri were kept there for 4 weeks. Thawing was carried out quickly in a 60˚C water bath, and removal of DMSO was achieved by perfusion at room temperature with a reversed sucrose concentration gradient (0.25 M, 0.1 M, and 0 M sucrose in Krebs–Ringer solution). The uterus was then transferred to the perfusion chamber and perfused with Krebs–Ringer perfusion medium at 37˚C for an additional 30 min before contractions were induced with oxytocin.

Intrauterine pressure measurement. Intrauterine pressure was recorded with an intrauterine pressure catheter (NEUROVENT-P 3F electronic semiconductor pressure sensor; Rehau, Erlangen, Germany). This pressure catheter has an outer diameter of 1 mm and one pressure sensor. The intrauterine catheter was connected to a data logger (MPR 1; Raumedic, Rehau) and pressure changes were recorded on a personal computer.

Induction of rhythmic uterine contractions. Oxytocin (Syntocinon, Novartis, Nuremberg, Germany) was used at a concentration of 0.5 IU as a bolus to induce rhythmical contractions.

Vitality parameters. Samples of the perfusate were taken before DMSO perfusion and cryopreservation and after thawing and removal of the cryoprotectant for measurements of pH, hydrogen carbonate (HCO_3^-) and lactate as previously described (27).

Statistical analyses. Data evaluation was performed using Microsoft Excel (Excel 2003 SP3; Microsoft, Redmont, USA) and statistical evaluations were performed using the *t*-test. A *p*-value of 0.05 was considered as statistically significant.

Results

Assessment of contraction ability. All ten non frozen and all ten uteri that had been cryopreserved with the slow cooling method showed rhythmical contractions comparable to those of fresh uteri. Figure 3 shows a typical intrauterine pressure profile of a uterus after cryopreservation with perfusion. After administration of oxytocin, the uterus started to contract (indicated with an increase of intrauterine pressure). The intrauterine pressure increase of the slowly cryopreserved uteri was 22.6±10.6 mmHG (mean±SD). None of the uteri frozen quickly and without perfusion showed any signs of contractions.

Vitality parameters. Table I shows the values (mean±SD, n=10) for pH, HCO_3^- and lactate in the perfused uteri before cryopreservation and one hour after thawing and removal of the cryoprotectant. No significant differences were observed.

Discussion

Uterus transplantation is the only possibility for women with no functional uterus to conceive and deliver their own child. In recent years there have been many reports on uterus transplantation attempts (28) and it is predicted that uterus transplantation could reach the clinical stage within 2-3 years, if high activity of research within this field is continued (2). In uterus transplant, the organ would be removed from a living or recently deceased donor and transferred to a recipient. Embryos previously created by *in vitro* fertilization would be transferred to the uterus and, after a child was born, the uterus would be removed to avoid a lifetime of taking powerful immuno-suppressant drugs to prevent rejection. Uterus transplantation

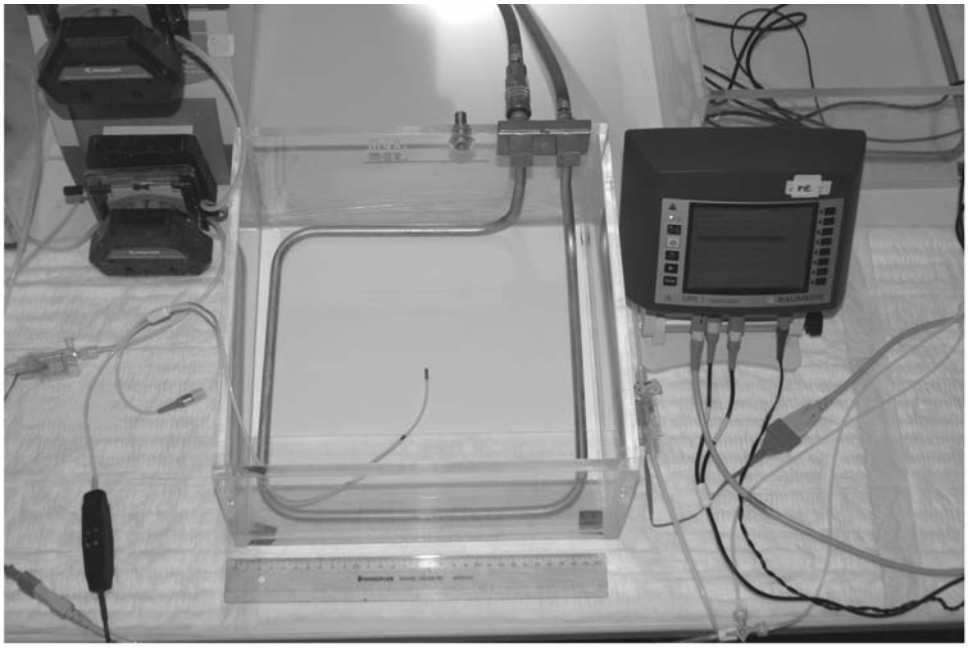

Figure 1. *Perfusion chamber with equipment*.

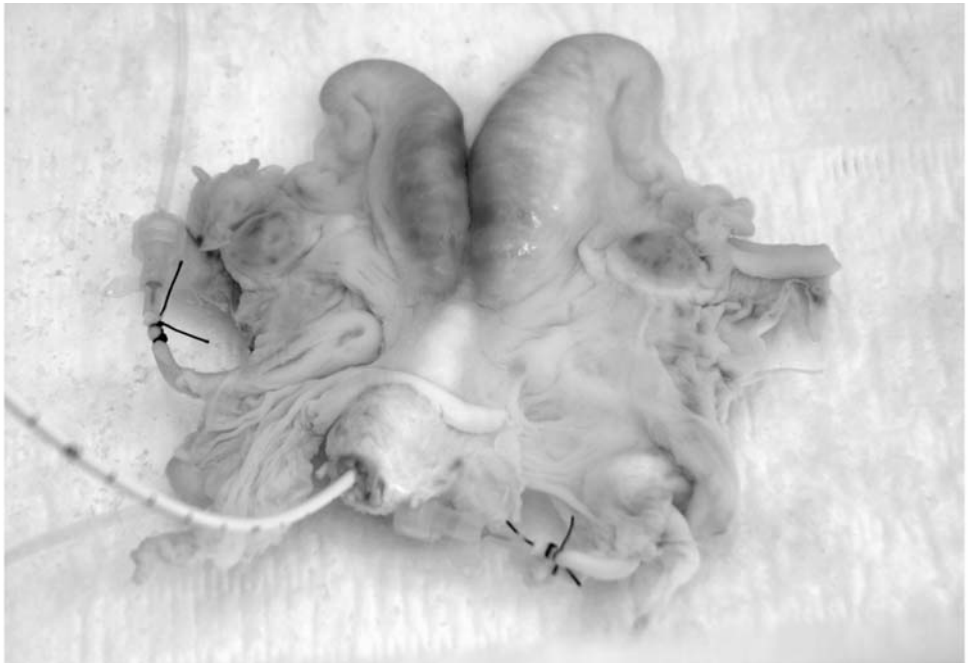

Figure 2. *Sheep uterus with arterial catheters*.

is not an emergency practice and therefore if enough uteri were available there would be enough time to locate a matching recipient. This could only be achieved through the establishment of cryobanks where uteri could be preserved at low temperatures for long periods of time.

Successful organ preservation is an important aspect of transplantation and ensures the maintenance of organ viability until implantation into the recipient. In cryobiology, the aim since the very beginning of research in this field has been to prolong the viability of fresh donor organs and the most

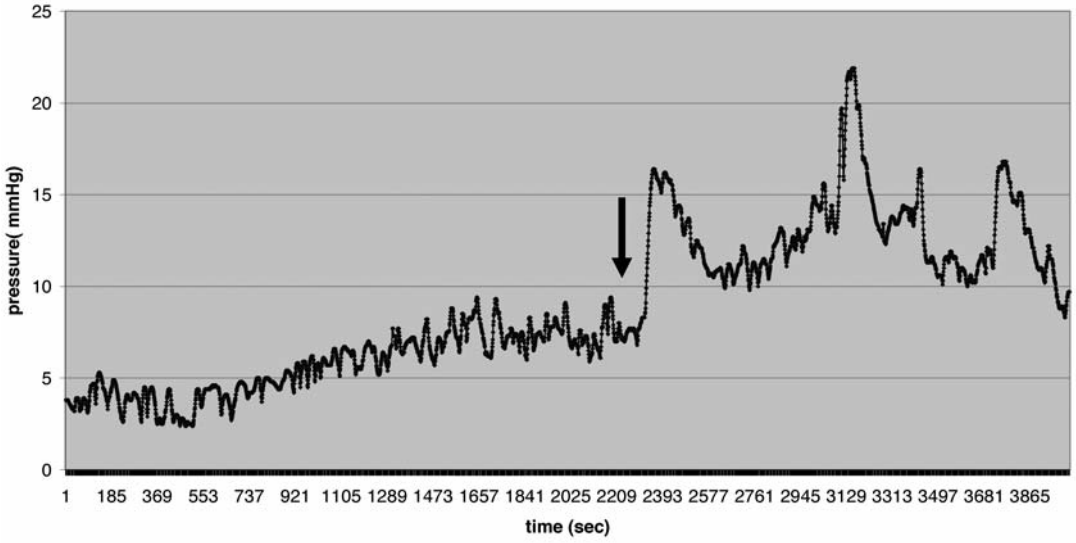

Figure 3. *Typical intrauterine pressure profile of an uterus after cryopreservation with perfusion and thawing (group A). (Arrow indicates adminstration of 0.5 IU oxytocin).*

efficient method of organ preservation is still that of cryostorage below –130˚C (12). Cell suspensions, isolated cells, sperms, oocytes and even embryos and ovarian tissue have been successfully cryopreserved for longer periods of time (29, 30). Tissue and whole-organ cryopreservation has always proved difficult due to the many different cell types present, limitations of heat and mass transfer, ischemia reperfusion injury, and problems with intravascular ice formation (31).

Regarding uterus transplantation, it has been recently reported that cold ischaemia allows the perfusion of the organ for up to 12 hours (32). And a recent report from Brannstrom *et al*. showed that the tolerance for cold ischaemia in mice from the time the uterus is taken out until it can be placed in the recipient is around 24 hours (2). The uterus has an additional advantage in that although it is a big hollow muscular organ, it has not the dense and highly organized structure of a heart or kidney, which are difficult to cryopreserve (33). As early as 1960, Farrant reported on the recovery of contractile responses in guinea-pig uteri *in vitro* after freezing to dry ice temperature and thawing, showing that even with the older methods of cryopreservation, the uterus is an organ suitable for cryopreservation (34). The uterus of the guinea pig is much smaller than a human or sheep uterus.

Our research group has extensive experience in the use of the extracorporal perfused swine uterus as an animal model for the study of uterine physiology (35-40). We recently reported on the successful cryopreservation of a whole swine uterus after perfusion with a cryoprotectant and a slow freezing protocol, with complete recovery of contractile responses after thawing (13, 14). Findings from these studies prompted us to examine a further animal model for cryopreservation studies. The ewe is one of the most commonly used animals in reproductive studies because the uterus is of a similar size to that of humans. Differences from the swine uterus include the fact that the horns are not so long and the uterus wall of the nonpregnant ewe is thicker. Furthermore, the sheep uterus has already been successfully orthotopic autotransplanted (16). The same group was able to show that in the sheep model, the vascular connections could be established in a manner similar to that used for clinical renal transplantation and that would be required in a human uterus transplantation situation. The first successful uterus autotransplantation in the ewe has recently been reported (41).

The principle of organ perfusion with a cryoprotectant agent is today widely used in order to slow the development of ischaemic injuries and prolong the tolerable ischaemic time (42). In our experiments, perfusion with DMSO was carried out prior to cryopreserving the sheep uterus in accordance with freezing protocols similar to those previously described (17, 43-45). DMSO is widely used in cryobiology as a cryoprotective agent due to its good tissue permeability properties (46). Our experiments showed that a 10% DMSO concentration provides adequate cryoprotection. We were also able to show that the perfusion of the organ before freezing initiation is the main, if not the only factor, that allows the organ to survive the slow cooling procedure, since the uteri frozen without perfusion did not survive the freezing.

Validation of survival of the whole organ after freezing and thawing is the main problem, since organ functions depend not only on isolated cell integrity, but also on the maintenance of its whole architectural and structural integrity. Our group has reported that in the swine uterus, the

ability to respond to oxytocin stimulation correlates with other histomorphological evaluation possibilities such as histological examination and LIVE/DEAD assay showing vitality of the cryopreserved organ (11). In this experimental model, survival of the uteri was assessed with the induction of spontaneous or induced rhythmical contractions of the uterus after cryopreservation, which provides the most convincing evidence that the ewe uterus is functional after the freezing/thawing procedure. The next step in the evaluation procedure would be to retransplant a cryopreserved ewe uterus and produce offspring. The success of the Gosden group, who transplanted cryopreserved uteri and ovaries in the rat and achieved subsequent pregnancies, allows us to believe that it can also be successful in larger animals (47). Similar results have been achieved with fresh uterine transplants in mice (42), sheep (16) and in goats (43).

To the best of our knowledge, this is the first report of a successful cryopreservation of a sheep uterus. Long-term preservation of uteri may prove to be important for the purposes of restoring fertility in selected patients in whom uterine transplantation may be indicated (Rokitansky syndrome, hysterectomy after an obstetric rupture, *etc*.). In 2000, a human uterine transplantation was carried out to treat infertility in a woman who had lost her uterus due to postpartum haemorrhage. However, the first uterine transplant had to be removed after 99 days due to thrombosis and necrosis from torsion of the uterine vessels (48). Although human uterine transplantation is technically feasible, it remains a risky procedure that is not life-saving and it involves a huge number of medical and bioethical issues which are still unanswered (26). Finally, successful cryopreservation of swine uteri and ewe uteri may also serve as an example showing that whole-organ cryopreservation is possible.

Conclusion

This study shows that the perfusion of sheep uterus with a cryoprotectant prior to slow freezing, allows the cryopreservation of the whole organ and maintains the functionality of the organ after thawing. The perfused sheep uterus provides an experimental model for further investigations with other cryoprotective agents and freezing protocols. Our studies with swine and sheep uteri, which are of a similar size to the human uterus, suggest that cryopreservation of the human uterus is also feasible.

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