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Transplantation of Cryopreserved Mouse, Chinese Hamster, Rabbit, Japanese Monkey and Rat Ovaries into Rat Recipients

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Abstract: *Partial ovaries from mice, hamsters, rabbits, Japanese monkeys and rats have survived deep-freezing and returned to a normal morphological state after being thawed and transplanted into the rat uterine cavity. This report describes the ice-free cryopreservation of mouse and other ovaries at −196°C by vitrification. The vitrification solution was based on the solutions reported by Rall & Fahy [16]. After ovaries had been exposed to the vitrification solution, they were frozen, with their suspending medium, by liquid nitrogen. After freezing, the ovaries were thawed in 37°C water. The viability of the previously frozen ovarian tissue was tested by transplanting it into the uterine cavity of pseudopregnant rats. Seven days after transplantation, the ovaries were removed with the rat uterus, and stained with haematoxylin and eosin for histological examination. Survival of the frozen-thawed the ovaries in the rat uterine cavity demonstrates that these ovaries can tolerate exposure to osmotic dehydration and vitrification in a concentrated solution of cryoprotectant and are then immunologically acceptable to the uterine cavity.*

Key words: *ovary cryopreservation, ovary transplantation, rat uterine cavity, vitrification*

Introduction

Use of the ovary for cryobanking of female germ cells has many potential scientific applications, including the storage of DNA from rare, valuable or endangered animals. Several investigators reported that live births after autologous transplant of cryoperserved mouse ovaries [8, 11], cryopreservated ovaries transplantation of mouse ovaries [3, 9, 17] and antral follicles develop in transplanted cryopreserved African elephant ovarian tissue [10]. Spermatozoa are available for storage in their millions, and successful techniques have been available for many years [15], but the ease and success of artificial insemination techniques contrasts sharply with the problems associated with freezing mammalian oocytes. Transplantation of ovaries has been used as a method to enhance the reproductive performance of females. The prospects for graft survival were increased by transplanting ovarian tissue into various positions other than the normal anatomical site.

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Such positions have been chosen for their rich vascular supply or purported immunoprivilege, and include the anterior chamber of the eye, the neck and renal capsule. The majority of grafts have been to the kidney, since it has a high blood supply and high concentrations of angiogenic growth factors, but the uterus has a high blood supply and is also immunoprivileged. Transplantation procedures for ovaries would be enhanced if the ovarian tissue could be successfully cryopreserved and transplanted into the uterus. Beer *et al.* [1] found that intrauterine skin homografts survive in the uterus of the rat for the duration of pregnancy. That is, pregnancy may interfere with the afferent limb of the immunological reflex. The aim of this study was to develop a cryopreservation procedure for mammalian ovarian tissue and evaluate its survival in the uterine cavity.

Materials and Methods

Animals

Adult ICR mice, Chinese hamsters, Japanese white rabbits, Japanese monkeys, obtained from Clea Japan INC (Tokyo, Japan), and Wistar-Imamichi rats were obtained from Imamichi Institute of Animal Reproduction, and were laboratory-reared in an animal room which was air conditioned (23 ± 2°C) and illuminated from 07.00 to 21.00 hr. At ovary sampling, the donor animals were deeply anesthetized by the administration of a combination of sodium pentobarbital and diethylether. The animals' ages at the time of the experiments were mouse, 10 weeks; hamster, 10 weeks; rabbit, 5 months; monkey, 5 years and rats, 13 weeks of age.

Freezing

Ovaries were handled by the method of Rall & Fahy [16]. The ovaries were collected and subdivided into 4–8 pieces and, then placed in a freezing tube (Serum tube, Ms-4601, Sumitomo Bakelite Co. Ltd. Tokyo, Japan). The ovaries were washed in HB1 [16] solution, a modified Dulbecco's saline containing 1 mM phosphate buffer, 0.33 mM sodium pyruvate, 5.56 mM glucose, 3 ml^{-1} bovine serum albumin and 100 IU per m*l* of penicillin G, and then placed in a 1:4 dilution of the vitrification solution (VS1, 25% VS1=5.125% W/V DMSO, 3.875% acetamide, 2.5% propylene glycol and 1.5% polyethylene glycol [relative molecular mass 8000]) in HB1 for 15 min at 20°C to allow the tissue to be completely infiltrated by the cryoprotectants. The ovary suspensions were then placed in a cold room (at about + 4°C) where the ovaries were exposed to the vitrification solution in two steps. First the ovaries were transferred to a 1:2 dilution of VS1 for 15 min and then finally into VS1. After exposure to VS1 for 15 min, the ovaries were immediately transferred to liquid nitrogen. The frozen ovaries were preserved for about 30 days.

Thawing

The ovary suspension was then warmed to 37°C and the remaining cryoprotectants washed away, first with 50% VS1, and then with 25% VS1. Finally the ovaries were washed in HB1 solution and 'cultured' in a rat uterine cavity.

Transplantation of cryopreserved ovarian tissue

The procedure for homoplastic grafting of rat ovary into the uterine cavity followed the method of Kagabu and Mamba [12]. The recipient rats, 13 weeks of age, copulated with vasectomized rats and 6 days later the pseudopregnant recipient rats were anaesthetized with 0.15 m*l*/rat Nembutal (Abbott, North Chicago, IL. USA). A frozen-thawed ovary was placed into the empty uterine cavity of the recipient and the uterus was sutured.

Assessment of cell viability after transplantation

The ovary and uterus complex were removed at 7 days after transplantation. The complex was fixed in Lavdovski's solution, dehydrated and embedded in paraffin wax, serially sectioned at 10 μm and stained with haematoxylin and eosin. Follicles were classified as healthy or atretic according to the criteria of Braw and Tsafriri [2].

Results

Grafts of frozen ovarian tissue removed 7 days after transfer contained healthy follicles (Fig. 1. A, B, C, D and E). Several grafts contained many large antral follicles (Fig. 1. A, E). Grafts that did not survive were necrotic (Fig. 1. F).

Discussion

We have shown that ovarian tissues from several species of mammals can be cryopreserved, with follicles

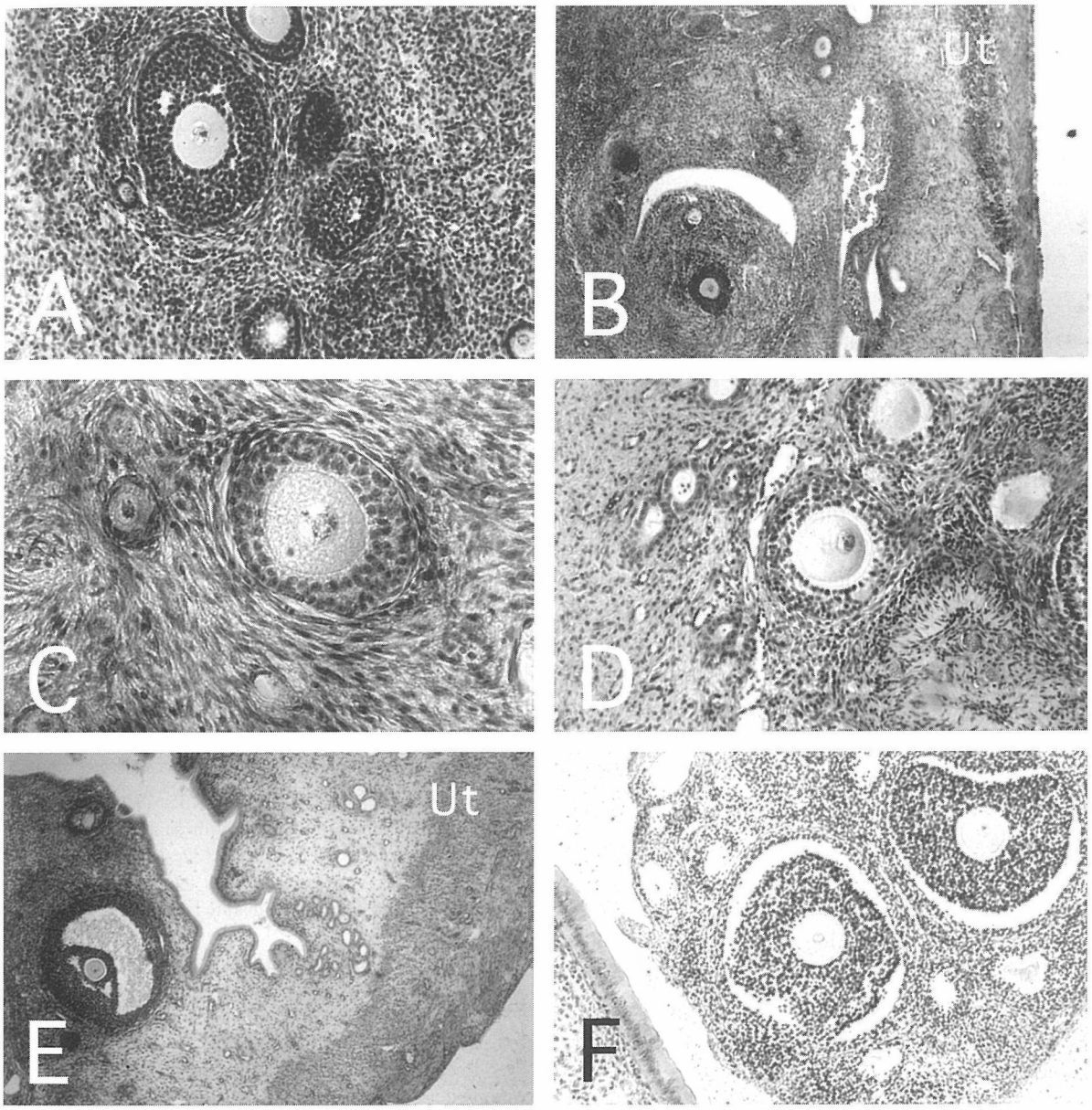

Fig. 1. Healthy antral follicles of frozen-thawed ovarian tissue transplanted into a rat uterine cavities and removed 7 days after transplantation. (A) Mouse follicles, (B) Chinese hamster follicles, (C) Rabbit follicles, (D) Japanese Monkey follicles, (E) Healthy large antral follicles of transplanted rat ovary (F) Necrotic mouse ovarian tissue. Ut; Uterus of recipient rat.

up to the small antral stage, and are capable of surviving freezing and thawing. Follicles develop to the large antral stage in the cryopreserved tissue after transfer to the uterine cavity of pseudopregnant rats.

Quantitative estimation of the effects of freezing and thawing on the ovarian tissues from the species studied were obtained by histological examination. These data suggest that freezing and thawing do not greatly damage the ovarian tissue. The presence of antral follicles in grafts as early as 7 days after transplantation confirms that follicles in the later stages of folliculogenesis can survive freezing but the number of healthy follicles per graft is low.

Cells are usually frozen by suspension in a cryoprotective solution. They are cooled at a rate that allows cellular dehydration, limits the extent of intracellular

Table 1. Morphologically healthy follicles in before and after freezing and grafted ovarian tissue

Animals	No. of samples	No. of healthy follicles larger than 250 μm per 4 pieces of divided ovaries			
		Follicles Surviving before freezing (A)	Follicles surviving after freezing	Follicles surviving after grafting (B)	Percentage of follicles surviving (B/A × 100)
Mice	4	59 ± 1	42 ± 3	10 ± 2	16.9
Chinese hamsters	4	59 ± 11	38 ± 6	8 ± 2	13.6
Rabbits	4	77 ± 20	51 ± 10	5 ± 2	6.5
Japanese monkeys	4	41 ± 11	29 ± 3	6 ± 2	14.6
Rats	4	98 ± 11	78 ± 9	11 ± 4	11.2

ice formation and avoids the adverse effects of osmotic stress during thawing. The preservation of tissue is more difficult because cells of different types have different requirements for optimal survival. In addition, cells in the middle part of the tissue may experience very different conditions during freezing and thawing. In spite of the fact that successful freezing relies upon the formation of extracellular ice to dehydrate the cells, this ice may disrupt the spatial integrity and functional connections between the cells of a tissue.

Several factors may have contributed to the poor rates of survival reported previously for ovarian tissue [4, 14]. Green *et al.* [7] reported that only 4% of previously frozen ovarian autografts survived and that this may reflect the inadequate penetration of cryoprotectant, particularly in cells at the center of the tissue. The relatively rapid rates of cooling (−2.8°C/min) may also have increased the risk of intracellular freezing. Leibo *et al.* [13] reported an increase in the occurrence of internal freezing in mouse oocytes when the cooling rate was increased to 3°C/min. Furthermore, where the whole surface area of the cells is not in contact with the cryoprotectant containing medium, dehydration during slow cooling will be limited and the risk of internal freezing further increased.

Previous reports have suggested that normal follicular development [4], including ovulation [5], is possible in ovarian tissue grafted to heterotopic sites. Gosden *et al.* [6] reported that cat, but not sheep, follicles reached the pre-ovulatory stage in ovarian tissue transplanted under the kidney capsules of immuno-deficient mice. In the present study follicles did not appear to develop into healthy antral follicles in the uterine cavity but the likelihood of observing mature pre-ovulatory follicles just at the stage of ovulation was low because of the small number of grafts analyzed.

The present findings show that the uterus is an extremely hospitable implantation site for ovarian tissue. This is not surprising since the uterine cavity is the "natural incubator" for ovarian tissue. Transplantation of ovarian tissue to rat uterine cavity provides an assay of its developmental potential and will facilitate the development of cryopreservation procedures for many mammalian species.

References

1. Beer, A.E. and Billingham, R.E. 1970. Implantation, transplantation, and epithelial-mesenchymal relationships in the rat uterus. *J. Exp. Med.* 132: 721–736.
2. Braw, R.H. and Tsafriri, A. 1980. Effect of PMSG on follicular atresia in the immature rat ovary. *J. Reprod. Fertil.* 59: 267–272.
3. Cox, S.L., Shaw, J., and Jenkin, G. 1996. Transplantation of cryopreserved fetal ovarian tissue to adult recipients in mice. *J. Reprod. Fertil.* 107: 315–322.
4. Deanesly, R. 1954. Immature rat ovaries grafted after freezing and thawing. *J. Endocrinol.* 11: 197–200.
5. Felicio, L.S., Nelson, J.F., Gosden, R.G., and Finci, C.E. 1983. Restoration of ovulatory cycles by young ovarian grafts in aging mice: Potentiation by long-term ovariectomy decreases with age. *Proc. Nat. Acad. Sci. USA.* 80: 6076–6080.
6. Gosden, R.G., Baird, D.T., Wade, J.C., and Webb, R. 1994. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at −196°C. *Hum. Reprod.* 9: 597–603.
7. Green, S.H., Smith A.U., and Zuckerman, S. 1956. The number of oocytes in ovarian autografts after freezing and thawing. *J. Endocrinol.* 13: 330–334.
8. Gunasena, K.T., Villines, P.M., Critser, E.S., and Critser, J.K. 1997. Live births after autologous transplant of cryopreserved mouse ovaries. *Human Reprod.* 12: 101–106.
9. Gunasena, K.T., Villines, P.M., Critser, E.S., and Critser, J.K. 1997. Allogeneic and xenogeneic transplantation of

cryopreserved ovarian tissue to athymic mice. *Bio. Reprod.* 57: 226–231.

10. Gunasena, K.T., Lakey, J.R., Villines, P.M., Bush, M., Raath, C., Critser, E.S., McGann, L.E., and Critser, J.K. 1998. Antral follicles develop in xenografted cryopreserved African elephant (Loxodonta africana) ovarian tissue. *Anim. Reprod. Sci.* 53: 265–275.
11. Gunasena, K.T., Villines, P.M., Critser, E.S., and Critser, J.K. 1997. Live births after autologous transplant of cryopreserved mouse ovaries. *Hum. Reprod.* 12: 101–106.
12. Kagabu, S. and Mamba, K. 1992. Heteroplastic graft of Japanese monkey, golden hamster, rabbit and mouse ovary into rat uterine cavity. *Jpn. J. Fert. Steril.* 37: 658–660.
13. Leibo, S.P., McGrath, J.J., and Cravalho, E.G. 1978. Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. *Cryobiology* 15: 257–271.
14. Parkes, A.S. and Smith, A.U. 1953. Regeneration of rat ovarian tissue grafted after exposure to low temperatures. *Proc. R. Soc. Lond. Ser. B* 140: 455–470.
15. Polge, C., Smith, A.U., and Parkes, A.S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperature. *Nature* 164: 666–667.
16. Rall, W.F. and Fahy, G.M. 1985. Ice-free cryopreservation of mouse embryos at −196°C by vitrification. *Nature* 313: 573–575.
17. Sztein, J., Sweet, H., Farley, J., and Mobraaten, L. 1998. Cryopreservation and orthotopic transplantation of mouse ovaries: New approach in gamete banking. *Bio. Reprod.* 58: 1071–1074.