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Recovery of fertility after grafting of cryopreserved germinative tissue in female rabbits following radiotherapy

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BACKGROUND: Many cancer survivors face infertility as a consequence of the aggressive treatment they must undergo. Cryopreservation of ovarian tissue before chemotherapy or radiotherapy may allow for tissue transplantation after the treatment, and restoration of fertility. We tested the potential of an orthotopic autografting of cryopreserved germinative tissue in female rabbits with ovarian failure following radiotherapy. METHODS: Ten adult multiparous female rabbits were randomly allocated into two groups, five in group I (control) and five in group II (transplant). All rabbits underwent right oophorectomy with cryopreservation of the germinative tissue, followed by sterilization of the remaining left ovary by radiotherapy. Later, group II rabbits received in the irradiated left ovary an implant of the frozen germinative tissue from the right ovary, whose small pieces were freely spread intracortically in a procedure we named 'intracortical sowing of germinative tissue' (ISGT). RESULTS: All group II rabbits conceived following spontaneous mating within 6 months of the transplant, whereas none of the remaining rabbits in group I had conceived up to 11 months after transplant. CONCLUSIONS: This study suggests that fertility can be restored in rabbits by sowing cortical tissue in a previously irradiated ovary. The clinical feasibility of this technique remains to be determined.

Key words: autograft/cryopreservation/fertility/ovary

Introduction

Modern treatment of malignant diseases by chemotherapy and radiotherapy has significantly extended many lives. Approximately 2% of all malignant diseases occur during childhood and adolescence (<14 years old), totalling 8600 new cases a year in the USA alone (Linet *et al*., 1999; Ries *et al*., 1999). However, an unfortunate side effect of this aggressive but life-saving therapy in children and in women of reproductive age is ovarian failure (Apperley and Reddy, 1995). It is estimated that half of the ovarian follicles (LD_{50}) are lost following a radiation dose of 400 centiGray (cGy) (Wallace *et al*., 1989). In women over 40 years old, 600 cGy is enough to decrease fertility. Although women under 40 years are less likely to lose ovarian function because of larger ovarian reserve, doses of 2000 cGy may produce ovarian failure (Lushbaugh and Casarett, 1976). Bath *et al*. (1999) studied the effect of radiation on ovarian function in children and adolescents. They observed that six out of eight young women given a total body irradiation of 1440 cGy developed ovarian failure (Bath *et al*., 1999). Furthermore, a significant proportion of irradiated young women who initially continue menstruating subsequently develop early menopause (Meirow and Nugent, 2001).

IVF with cryopreservation of the embryos is one option, prior to initiating cancer therapy, to prepare for ovarian failure. However, IVF inevitably would delay the critical therapy, and children and many young women who have no partner are not candidates. Thus, a second option might be the cryopreservation of unfertilized oocytes. Oocyte storage has faced technical difficulties compared with sperm or embryo cryopreservation because of the specific features of female germinal cells as documented by the low number of births achieved after oocyte cryopreservation (Fabbri *et al*., 2001). Despite the early disappointing results regarding survival, fertilization and cleavage rates, which led to only sporadic pregnancies in >10 years, the recent introduction of technical modifications such as ICSI and some changes in the freezing protocol have greatly improved the clinical efficiency of oocyte freezing, with the birth of several healthy children (Porcu, 2001).

On the other hand, cryopreservation of ovarian tissue before cancer therapy might allow for later transplantation of the germinative tissue, and restore not only fertility but also

ovarian endocrine function through an orthotopic grafting (Oktay and Karlikaya, 2000; Radford *et al*., 2001). Some authors have described different orthotopic grafting techniques in animals, with frozen–thawed ovarian cortical strips (Gosden *et al*., 1994), hemi-ovaries without vascular anastomosis (Salle *et al*., 2002) or the autotransplantation of the intact ovary with vascular anastomosis (Wang *et al*., 2002; Bedaiwy *et al*., 2003).

We report on a new experimental protocol that seems to have the potential to restore normal fertility in rabbits with ovarian failure caused by radiotherapy by the free transplantation of the cryopreserved germinative tissue of a healthy ovary removed prior to radiotherapy into the remaining irradiated ovary. The experiment described herein is an improved version based on the same technique used first and successfully with ewes (Almodin *et al*., 2002).

Materials and methods

Planning

Following the review of the literature (PubMed/Medline), the experimental protocol was established according to the International Norms for Animal Protection, and was sent to the Ethics Committees of Materbaby-Reprodução Humana e Genética, and the Medicine Department, Universidade Estadual de Maringá (UEM). The protocol was approved by both committees.

Subjects

The protocol was carried out with 10 New Zealand White (*Oryctologus cunicullus*) female rabbits, aged between 12 and 24 months, multiparous (one to six gestations), with weight varying between 3.2 and 4.7 kg, and two male rabbits of the same breed, with proven fertility and weighing ~4.0 kg, originally from the Cuniculture Department, Experimental Farm, UEM. The females were randomly allocated to two groups of five rabbits each: group I (control) and group II (implant). The animals were kept in individual cages, in the same environmental conditions until the beginning of the experiment.

Anaesthesia

Before being submitted to right oophorectomy, all the rabbits from both groups were put under fasting and water restriction for periods that ranged between 12 and 16 h. After that, they received 0.2 ml/kg of body weight of acepromazine at 0.2% (Univet, Cambuci, Brazil), i.m. as a tranquilizing and pre-anaesthetic medication. Later, animals were administered 0.1 ml/kg of atropine at 1% i.m. (Laboratorios Calbos, Curitiba, Brazil) and chlorihydrate of 2-(2,6-xylidino)-5,6-dihidro-4H-1,3-thiazine (Bayer, São Paulo, Brazil), 9.0 mg/kg of body weight i.m., as a sedative and a muscular tranquillizer associated with chlorihydrate of ketamine at 10% (União Química Farmacêutica Nacional, Embu-Guaçu, Brazil), 35.0 mg/kg of body weight i.m. as anaesthetic. They were assessed for the palpebral and mandibular reflexes, and for the response to stimuli, to pain and respiratory movements. Ventilation was controlled by the use of masks connected to an oxygen balloon (2 l/min).

Right oophorectomy

Once the anaesthetic level had been reached, the abdominal fur was sheared and antisepsis with iodine-polyvinylpyrrolidone at 1% was carried out. Then, medial laparotomy (10 cm) between the distal mammary glands was performed, sectioning the tissues layers using a cold blade scalpel until reaching the abdominal cavity. Wide access to the abdominal cavity was obtained by the use of pushers. Once the right ovary was isolated and identified, a suture at the insertion level of the meso-ovary ligament was done in the hilum using a mononylon thread 3-0, taking care to protect the uterine tube. The right ovary was then completely removed from all rabbits in both groups and sent to the laboratory for cryopreservation. After that, the left ovary was identified and isolated, and then it was fixed to the peritoneum of the anterior abdominal wall, 2 cm laterally and to the left of the abdominal incision with a mononylon thread 3-0. Close to the ovary, a tubular metal marker of 0.5 × 0.5 × 0.1 cm was placed on the meso-ovary ligament and on the peritoneum. A mark on the outside of the skin was made with Chinese ink, corresponding to the localization of the left ovary. The abdominal wall was sutured with mononylon thread 3-0. After recovery from anaesthesia, the rabbits were taken back to the bioterium at UEM where they were fed, watered and kept under daily care and observation until the beginning of the radiotherapy.

Cryopreservation

The right ovarian cortex of all animals (groups I and II) was excised, maintaining 1.0 mm thickness, washed 10 times in Dulbecco's phosphate-buffered saline (PBS) solution (Gibco, Grand Island, NY) at room temperature to remove excess blood, and cut into as many small pieces as possible (~1 mm^2 each), a procedure that took ~10 min to be completed. Two fragments of the ovarian tissue from each ovary were randomly selected and submitted to histological analysis. A modified slow freezing/rapid thawing protocol in a medium containing 1.5 mol/l dimethylsulfoxide (DMSO) was chosen (Newton *et al*., 1996). The pieces were placed in a freezing medium that consisted of PBS with 1000 mg/l D-glucose and 36 mg/l pyruvate (Gibco) containing 1.5 mol/l DMSO (Merck, Munich, Germany) and 10% fetal calf serum (Cultilab, Campinas, Brazil), and gently rolled (1 Hz) for 30 min at 4°C to promote rapid equilibration. The pieces were then loaded into 2.0 ml cryovials (Corning, Cambridge, Canada): two vials per ovary in 1.0 ml of the same solution. The cryovials were placed in a programmable Biocool freezer (Model BCIV40A, NY) at 0°C and cooled at a rate of 2°C/min to –9°C. Ice crystal nucleation was initiated by touching the side of the cryovials with forceps previously cooled in liquid nitrogen. After a further 5 min, the cryovials were cooled at rate of 0.3°C/min to –40° C. The vials were then plunged into liquid nitrogen at –196°C. The cryovials were kept frozen for 11 weeks until the thawing and implantation.

Radiotherapy

In the third week after oophorectomy, all rabbits were sedated with acepromazine at 0.2% (Univet, Campinas, Brazil) at a dose of 0.2 ml/kg of body weight, i.m. and submitted to a radiographic examination of the abdomen with antero-posterior and profile incidence. Using a dermal pencil, the localization of the left ovary was delimited with the help of the metal marker. In the fourth week after oophorectomy, having the localization marking of the metal tube radio opaque to the centre, the radiotherapy sessions started in a linear accelerator 4 MeV (Mega electrovolts). The sessions lasted for five consecutive days with a daily dosage of 200 cGy over an area of 5.0 cm^2 and 2.0 cm deep.

Thawing

Six weeks after radiotherapy was concluded, the cryovials with the germinative tissue from group II rabbits were warmed in air at room temperature (25°C) for 2 min and then immersed in a water bath at 37°C until the ice melted (2–3 min). The tissues were removed from the cryovials and then quickly washed seven times (~1 min each wash) in PBS with 1000 mg/l D-glucose and 36 mg/l pyruvate (Gibco) at room temperature for removal of the cryoprotectant. Two fragments of

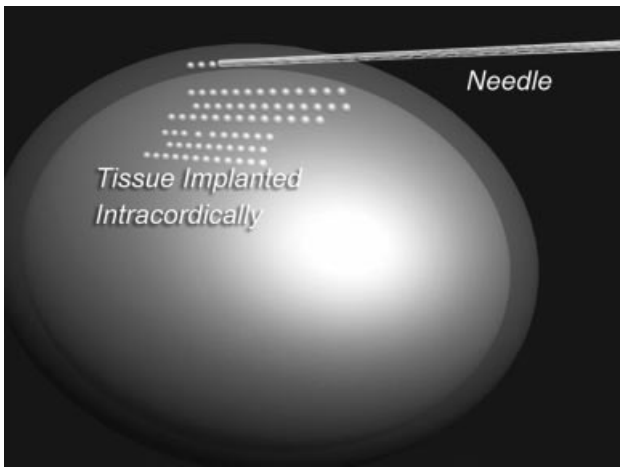

Figure 1. Graphic representation of the intracortical sowing of germinative tissue (ISGT) procedure. A hypodermic needle (18 gauge) is used to spread all the frozen–thawed germinative tissue from the right ovary into the cortex of the left ovary ('host ovary') in a motion analogous to the 'sowing' of a field.

the germinative tissue from each ovary were randomly selected and sent for histological analysis.

Implant

Simultaneously with the thawing, the animals in group II were submitted to surgery for the implantation under the anaesthetic procedure already described above. Following the laparotomy, an inspection of the abdominal cavity was carried out. The remaining irradiated left ovary was identified, isolated and released from its point of anchorage. The metal marker was removed and the gonad was returned to its original topography. The ovary was then biopsied for histological analysis. Hypodermic needles (18 gauge) were filled with fragments of the frozen–thawed ovarian tissue, which were then freely injected intracortically into the ovary, without any need for sutures, in a procedure we named 'intracortical sowing of germinative tissue' (ISGT) (Figure 1). Many gentle 'sowings' were performed all over the ovary until the entire thawed sample from the right ovary was implanted into the cortex. The time spent from the moment of thawing to the end of implantation was ~15 min. Post-operatively, the five grafted rabbits (group II) were housed with the non-grafted rabbits (group I) in individual cages under the same feeding, temperature and lighting conditions.

Histological analysis

All the samples for histological analysis were fixed in formalin (10%) and later the samples were embedded in paraffin, cut into 4 μm sections and stained with haematoxylin–eosin. The histological sections were observed under a light microscope and assessed qualitatively with regard to the presence of cells of germinative lineage in their different maturation phases, according to the classification by Turnbull *et al*. (1977), and with regard to their general morphological aspects.

Sexual behaviour

From the forth week after the implant, and then throughout the 11months of the experiment, male rabbits were alternately placed into each female's cage of both groups for periods of 10–15 min. After mating, the female rabbits were kept apart from the male. A week later, they were assessed for evidence of gestation. In the absence of gestation, they were again placed with a male rabbit for a new mating. For those female rabbits that did not consent to mating, the researchers carried out an induced mating by raising of the tail end to permit the males to act.

Before each mating, three observers jointly assessed the rabbit's vulval coloration, in an area with natural lighting, correlating the acceptance of mating with the vulval coloration. For this assessment, a colorimetric spectrum containing the shades white, pink, red and violet (Alvariño, 1998) was used.

Vaginal cytology

At 12–15 h after mating, a smear of the rabbit's vaginal mucosa was collected. The material was rolled over a glass slide and immediately stored in a flask containing ethyl alcohol at 96° for cellular fixing. They were then stained with methylene blue at 0.1%, and observed under the light microscope for the assessment of general morphological aspects and its correlation to their reproductive status.

Results

All the animals in groups I and II survived the various phases of the experiment, with no surgical complications being observed.

Sexual behaviour

The rabbits in group I showed themselves resistant to mating in all attempts up to the end of the experiment (11 months after the implant). They kept their tail end firmly against the cage's floor, producing noises and being aggressive towards the males, and they did not succeed in becoming pregnant even after the induced mating with the help of the researchers.

Initially, the rabbits from group II presented a similar behaviour to the rabbits in group I. Later, three of the rabbits in group II became receptive to mating from the 40th day after the implant, when a lordosis position that exposed the vagina and eased the penetration by the male was observed. These rabbits were named IIA, IIB and IIC. The other two rabbits in group II, IID and IIE, started to present the receptive behaviour from the fourth month after the implant.

The rabbits from group I presented a white vulval coloration in all observations up to the end of the experiment. The rabbits from group II clinically presented an alteration in the vulval coloration from white to red in an interval of 16 days from the end of the first month after the implant. It was observed that spontaneous mating took place when the vulval coloration had a red to violet aspect.

Pregnancy

In the third month after the implant, rabbits IIA, IIB and IIC became pregnant with a gestation that lasted for ~30 days. The other two rabbits in the group, IID and IIE, became pregnant in the sixth month after the implant. Also, in the sixth month after the implant, rabbits IIA and IIB becamet pregnant for the second time.

The pregnant rabbits built their dens in a wooden box placed inside the cage with the fur taken from their own bodies. When they gave birth, this den protected the litter until the time arrived for them to go out spontaneously. The rabbits were receptive to the litter, feeding them for ~2 months; no morphological alteration was observed in the offspring. The

Table I. Number of rabbits per litter

Animal	No. of rabbits (first gestation)	No. of rabbits (second gestation)
IIA	1	2
IIB	7	5
IIC	2	
IID	3	
IIE	2	

size of the litter in the seven pregnancies obtained during the study varied from one to seven (Table I).

Histological analysis

The macroscopic assessment of the ovarian tissue fragments obtained before cryopreservation revealed pink-white elastic tissues with fine granular surfaces. Under the light microscope at 100× magnification, the histological samples revealed preserved morphology in all the fields examined, with a well defined presence of germinative epithelium, albuginea and cortical tissue granule-albican bodies. Ovarian follicles in different phases of maturation were also seen, with a predominance of primordial follicles. The primordial and secondary follicles had appeared healthy in that it was possible to identify the zona pellucida, follicular cells, internal and external theca and the basal membrane (Figure 2).

Macroscopically, the thawed fragments sampled were white, elastic and with fine granular surfaces. Submitted to assessment under the light microscope at 200× magnification, a preserved global architectonic structure, with a slight tumefaction without histological markers for ischaemia or tissue necrosis, was observed. The cortex was organized, and ovarian follicles in different phases of maturation could be seen, with a predominance of healthy looking primordial follicles, and preserved morphological aspects (Figure 3). There was no evidence of structural differences when compared with the fresh samples.

The samples of cortical tissue from the left ovaries of the rabbits from group II obtained after radiotherapy by a biopsy *in situ*, measuring ~1.5 × 1.0 × 1.0 mm, macroscopically were grey-white, elastic and with smooth and bright surfaces. Submitted to assessment under the light microscope at 200× magnification, they showed disorganization in the distribution of the cortical tissue form, absence of germinative epithelium, thickening of the albuginea and complete depletion of the follicular population (Figure 4).

Cytological assessment

The microscopic analysis of the vaginal smears obtained from the vaginal mucosa revealed the presence of preserved cells in all samples in group II, with morphologic characteristics of intermediary cells (Figure 5A), which shows a normal cyclical vaginal cytology (evidence of ovarian function). However, the sample analysis in group I showed evidence of nuclear alteration with the visualization of picnotic nuclei with morphological characteristics of deep cells in all the samples

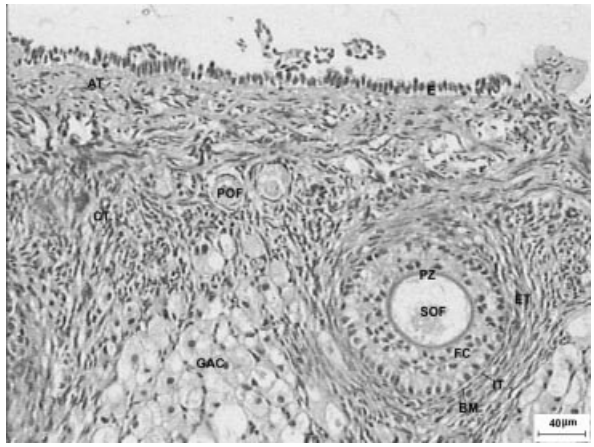

Figure 2. Light microscopy of rabbit ovarian tissue before freezing. The presence of healthy looking primordial and secondary ovarian follicles is evident. AT = albuginea, E = ovarian epithelium, CT = ovarian cortex, GAC = granulo-albican cells, POF = primordial ovarian follicle, SOF = secondary ovarian follicle, PZ = zona pellucida, FC = follicular cells, BM = basement membrane, IT = internal theca, ET = external theca. 100× HE.

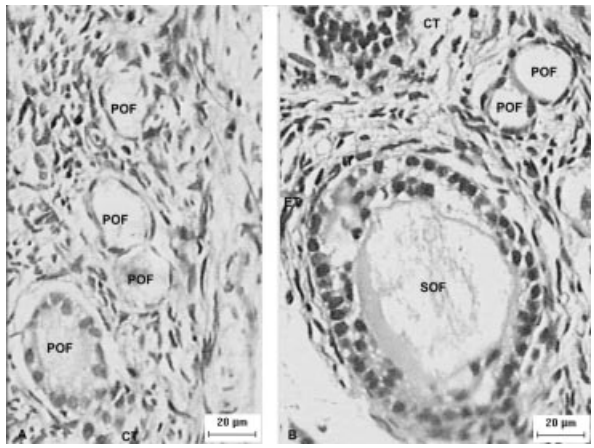

Figure 3. Light microscopy of rabbit ovarian tissue after freezing and thawing showing healthy looking primordial follicles (**A**) and primordial and secondary ovarian follicles (**B**). POF = primordial ovarian follicle, SOF = secondary ovarian follicle, CT = ovarian cortex, IT = internal theca, ET = external theca. 200× HE.

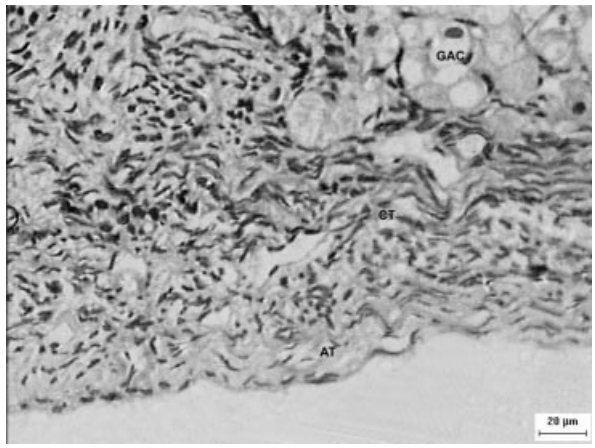

Figure 4. Light microscopy of rabbit ovarian tissue after radiotherapy. GAC = granulo-albican cells, CT = ovarian cortex, AT = albuginea. 200× HE.

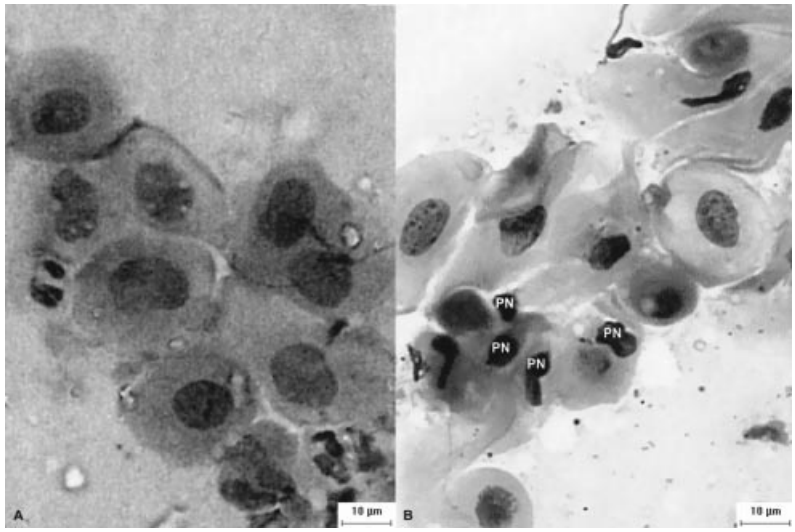

Figure 5. Light microscopy of the vaginal mucosa smear of rabbits submitted to radiotherapy and implant (group II), showing intermediary cells (**A**), and without implant (group I), showing deep cells with picnotic nuclei (PN) (**B**). 400×.

(Figure 5B), which shows vaginal cytology in permanent diestrus (ovary not active).

Discussion

Modern techniques for the treatment of malignant diseases have greatly improved the chances of cure in children and young women. One of the consequences of this success, however, is a life in menopause, and having no chance of conceiving on their own. In the last decade, there has been intense investigation in order to find ways that might give women treated for malignancy at reproductive age the opportunity to have children.

At present, cryopreservation of ovarian tissue pieces, rather than the whole human ovary, or oocytes from antral follicles, provides the most practical and useful means of preserving a patient's fertility (Oktay, 2001). Numerous animal (Candy *et al*., 1997) as well as human (Gook *et al*., 1999) studies have demonstrated that primordial follicles are relatively resistant to the freezing–thawing procedure.

With the exception of glycerol, cryoprotectants (propanediol, ethylene glycol and DMSO) make little difference to the success rate (Newton *et al*., 1996). The histological analysis results in the present study show that samples of tissue obtained after cryopreservation (Figure 3) presented healthy looking germinative architecture very much in the same way as the samples collected fresh (Figure 2). Although histological analysis of samples of tissue is not conclusive for tissue viability, the results strongly suggest that the freeze–thawing protocol used did not damage the integrity of the germinative tissue.

In theory, frozen-banked ovarian tissue can be used in a number of ways to restore fertility (Newton, 1998). Nevertheless, insertion of cryopreserved ovarian tissue at an orthotopic site is the only method by which 'natural' fertility can be restored (Nugent *et al*., 1997). With this kind of procedure, it would be possible not only to restore endocrine cycles, doing away with the necessity for hormone replacement therapy in patients with ovarian failure, but also to recover natural fertility.

One of the main issues with an orthotopic procedure is related to the site that will host the transplant. The pioneering studies of Gosden and his group demonstrated that sheep could get pregnant again after the orthotopic autotransplantation of cryopreserved ovarian cortical pieces on the infundibulopelvic ligament. One out of six sheep became pregnant from a frozen–thawed graft with no abnormality in the newborn animal (Gosden *et al*., 1994). Frozen–thawed fragments of ovarian cortex were also autografted on the uterine horns of six ewes. Although ovulation resumed in most ewes, none of the ewes grafted orthotopically became pregnant at a synchronized time (Aubard *et al*., 1999). Despite their relative success, the poor results indicate that a more adequate site to receive a long-term transplant should be considered.

In an ideal situation, an ovary should be in its original place, if 'natural' fertility is actually to be achieved. Thus, the use of intact frozen-banked ovaries would probably be the best option since both ovaries could be preserved for future transplant. Recently, the function of cryopreserved ovaries could be restored after transplantation in rats using vascular anastomosis, but pregnancy rates were quite low (Wang *et al*., 2002; Yin *et al*., 2003). It has also been shown that the transplantation of a frozen–thawed sheep ovary by perfusion, with micro-vascular anastomosis, could partially restore hormonal function (Bedaiwy *et al*., 2003). Perfusion could improve the cryopreservation of intact human ovaries, but this has been complicated by the technical difficulty of obtaining ovaries with intact vascular pedicles as well as optimally cryopreserving vascular, germinal and stromal components with larger and fibrous human ovaries. This technique needs to be perfected in animal studies first. Because of this, currently the most feasible method is cryopreservation of thin ovarian cortical pieces, which allows easier penetration of cryoprotectants into the tissue.

Since ovarian transplantation is out of reach at the present moment, and because initial attempts at orthotopic ovarian transplantation met with limited success, a modified approach needed to be developed to enhance the prospects of pregnancy and longevity of the grafts. It was, therefore, decided to keep the rabbits' left ovaries in their original site, while the right ovaries were removed to supply the transplant tissue. At the moment of the implant, it was observed that, after radiotherapy, the left ovaries had reduced in size but their vascularization had been preserved and, as a consequence, they became a 'natural' recipient for the germinative tissue transplant. That is why it was decided they should be referred to as 'host ovary'. Theoretically, the preservation of germinative tissue from just one ovary should be enough to recover natural fertility.

The idea of transplanting frozen–thawed germinative tissue to a host ovary is not totally new. Radford *et al*. (2001) grafted a cortical strip on the left ovary and another at the site of the right ovary of a woman who had been treated for Hodgkin's lymphoma. Seven months after reimplantation, the patient menstruated, but subsequently hormone concentrations went back to levels seen with ovarian failure. An important difference in this experiment is the fact that the patient's ovary had been exposed to chemotherapy and radiation prior to cryopreservation and transplantation, perhaps explaining their lack of success and short-term function in the opposite side where strips were grafted on the peritoneum.

Despite the excellent results obtained with the preservation of follicles after freezing, follicle survival rate after the implant is a serious concern. While 7% of the follicles were lost during freezing and thawing, 65% were lost during the revascularization period (Gosden *et al*., 1994; Baird *et al*., 1999). Aubard *et al*. (1999) using both orthotopic and heterotopic grafting procedures obtained similar results using fresh and frozen–thawed grafts, which strongly suggests that the key factor responsible for poor follicle survival is the post-grafting ischaemia and not damage of the germ cells during freezing and thawing (Aubard *et al*., 1999). Many other studies have also concluded that ischaemia may be responsible for follicle loss (Nugent *et al*., 1997; Gosden, 2000; Liu *et al*., 2002).

Hypoxic conditions may be the reason why more follicles are lost initially after transplantation rather than the freeze–thaw procedure (Aubard *et al*., 1999). Nevertheless, the limited oxygen supply to the tissue may be responsible for inducing angiogenesis by the upregulation of angiogenic vascular endothelial growth factor (VEGF) (Laschke *et al*., 2003). Ovarian tissue is a rich source of angiogenic factors that undoubtedly encourage rapid migration of endothelial cells into the grafts and an early restoration of the blood circulation (Rone *et al*., 1993). The problem is with the centre of the tissue that would be ischaemic for a few days after grafting (Gosden *et al*., 1994). As ovarian tissue revascularization has been the subject of intense investigation, in a recent primate study, some authors have even attempted to enhance angiogenesis by administering VEGF (Schnorr *et al*., 2002), without any obvious success.

In an attempt to minimize ischaemia, the frozen–thawed graft tissue was freely implanted in the 'host ovary' by a procedure we called ISGT (Figure 1). This is a very simple procedure in which all cryopreserved pieces of tissue were injected into the cortex of the host ovary using a wide-bored needle, ensuring the contact of the tissue fragments with the blood supply without the need for sutures. As the protocol did not require any sutures, cortical tissue could be cut into very small fragments. This seems not only to facilitate the action of the cryoprotectants, but it is also expected to decrease the ischaemia time. Frozen–thawed follicles freely transplanted into the striated muscle of the dorsal skinfold chambers of hamsters have already been shown to induce adequate angiogenesis and revascularization (Laschke *et al*., 2003).

As a result of ISGT, all transplanted rabbits in group II recovered their natural fertility by littering at least once. Two of the rabbits had litters for a second time during the experiment. The rabbits that received the implant seem to have recovered their normal hormonal activity, documented by the vaginal mucosa pattern (Figure 5A), which is consistent with a normal estrogenic state. The variation of the vulval coloration is also an indirect parameter of the hormonal levels in the reproductive cycle of the rabbits (Alvariño, 1998). Moreover, they periodically showed acceptance of mating associated with the change in the vulval coloration to red, which indirectly demonstrated the presence of cyclical hormonal activity.

A factor to be considered with the free transplantation, however, is that it must be carried out as superficially as possible to minimize the possibility of architectural disturbance by injecting the cortical tissue into the stroma. There is also the possibility of inclusion epithelial cyst formation due to the morphological alterations produced by possible reacting fibrosis nidus. This, however, should not be a hindrance to new pregnancies when we consider the large numbers of follicles that theoretically are grafted.

The very high fertility rate observed with ISGT indicates that the procedure was successful in restoring natural fertility to the rabbits. It may be speculated, however, that fertility might have been recovered not due to the transplantation of the frozen–thawed tissue but due to pre-existing primordial follicles that had survived radiotherapy. In theory, the 'sowing' procedure itself might have increased the revascularization and enabled these animals to conceive with the remaining primordial follicles in the 'host ovary'. Nevertheless, we find this possibility highly improbable for several reasons.

First of all, the rabbits were submitted to a very high dose of radiotherapy. In women, a dose of 5000–10 000 cGy is necessary to cause infertility (Herrmann, 1997). In the case of rabbits, we believe that a dose of 1000 cGy should be more than enough to cause infertility. In fact, the biopsy samples from the irradiated ovaries showed an unhealthy looking tissue without follicle population (Figure 4), indicating that the radiotherapy dose to which the rabbits were submitted might have been enough to cause infertility. This really seems to be the case since the rabbits in the control group never became pregnant during the experiment. The non-grafted rabbits also presented a cytological picture consistent with a hypo-estrogen state (Figure 5B). Their refusal to mate, associated with a white vulval coloration, demonstrated the absence of adequate hormonal activity. Even if some follicles might have survived,

a mathematical model for women has shown that ovarian function is lost when the number of follicles is depleted to <1000 (Faddy *et al*., 1992).

In conclusion, the high fertility rate results obtained in this experiment encourage us to believe that the ISGT procedure is a feasible theoretical animal model for future transplantation. It also suggests that fertility can be restored to rabbits by sowing of cortical tissue inside the cortex of a previously irradiated ovary. The clinical feasibility of this technique, however, remains to be determined. As promising as it might seem, more investigations are needed to assess the long-term effects of this type of implantation.

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