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Recovery of natural fertility after grafting of cryopreserved germinative tissue in ewes subjected to radiotherapy

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Objective: To recover natural fertility of ewes that were subjected to ovarian failure induced by radiotherapy with an autologous orthotopic graft of cryopreserved germinative tissue.

Design: Experimental surgery study.

Setting: University hospital unit.

Animal(s): Adult ewes.

Intervention(s): Four ewes were submitted to right oophorectomy and posterior dissecting and freezing of the germinative tissue. Afterward, they were administered radiotherapy to induce infertility on the remaining left ovary. Later, two of the ewes had the thawed fragments of the right ovary injected inside the cortex of the irradiated left ovary in a "sowing" procedure that eliminated the need for sutures.

Main Outcome Measure(s): Recovery of fertility in ewes after transplantation of germinative tissue into the ovary destroyed by radiotherapy.

Result(s): The ewes were housed with fertile rams. Six months following the grafting, the rams impregnated the transplanted ewes. More than 2 years after radiotherapy, the nongrafted (control) ewes have not become pregnant.

Conclusion(s): Intracortical grafting of the germinative tissue circumvents the obstacle of vascular anastomosis with autologous ovarian implants. Patients could benefit from the subcortical grafting of germinative tissue in one of the ovaries, recovering fertility after radiotherapy treatment for malignancy. (Fertil Steril® 2004;81:160-4. ©2004 by American Society for Reproductive Medicine.)

Key Words: Autograft, ovary, cryopreservation, fertility

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The aggressive treatment of malignant diseases with chemotherapy and radiotherapy has extended many lives. An unfortunate side effect of this life-saving therapy in women of reproductive age is its effect on the germinative cells, which are susceptible to the toxicity of the treatment, causing early ovarian failure (1–4). It is a daunting prospect, especially for young patients who are still single and do not have any intention to become pregnant in the near future.

Women who receive a radiation dose of less than 500 cGy have a better chance of preserving ovarian function than those who receive more than 500 cGy (5). Some authors have suggested that a total dose of 600 cGy in women over 40 years old is enough to cause menopause, while a dose of up to 2,000 cGy would be necessary to affect younger women (3).

Besides radiation dose, patient age has great impact on the preservation of the ovarian function (6). Although some young patients have only temporary amenorrhea following radiotherapy, we know that a severe depletion of the follicular stocks may lead to early menopause (7). A mathematical model estimates that a reduction of 90% in the population of germinative cells in girls <14 years old may result in permanent ovarian failure at around 27 years of age (8).

For men, the freezing of semen has circumvented this problem (9). Unfortunately, eggs freeze poorly, and many authors have observed pregnancy rates that are unacceptably very low

(10–13). The first attempts at ovarian cryopreservation occurred in the 1950s when many authors tried to reestablish endocrine function in rodents after oophorectomy (14–16) or insert the cryopreserved tissue into an orthotopic site (17). In both cases, however, the results were unsatisfactory.

Cryopreservation of the ovarian tissue has recently been performed with very satisfactory results, maintaining the viability of germinative tissue (18–20). Restoration of fertility with the use of frozen-thawed tissue is still hypothetical without a consensus protocol (21). Three possible alternatives have been described: ovarian autograft (22, 23), ovarian xenograft (24) and in vitro maturation of primordial ovarian follicles (25, 26); however, no published reports have suggested that any procedure completely restores natural fertility.

We report a new and innovative protocol that is capable of restoring normal fertility in ewes with ovarian failure after radiotherapy by the reimplantation of cryopreserved germinative tissue into a "host ovary."

MATERIALS AND METHODS

After the approval by the Ethics Committee of the Materbaby — Reprodução Humana e Genética, four adult ewes with proven fertility each underwent a total right oophorectomy; the left ovary was also sutured to the anterior pelvic wall with a small metal marker to facilitate subsequent x-ray localization.

The right ovarian cortex was excised, maintaining 1.5-mm thickness, washed in several changes of room temperature Dulbecco's phosphate buffered saline (PBS) solution (GIBCO, cat. 14040-133; Gibco, Grand Island City, NB) to remove excess blood, and cut into as many small pieces as possible. A sample of the ovarian tissue was submitted for histological analysis to certify that it was normal at the outset.

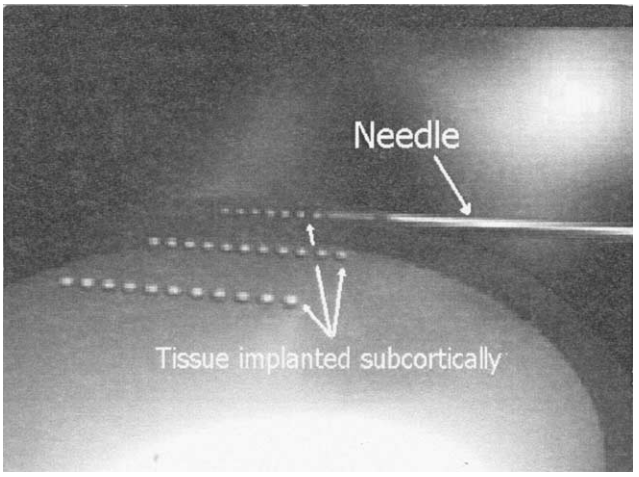
The pieces were placed in a freezing medium, which consisted of PBS with glucose and pyruvate (GIBCO, cat. 14287-080) containing 1.5 M dimethyl sulfoxide (Merck, München, Germany) and 10% fetal calf serum (Cultilab, Campinas, Brazil), and gently rolled (1 Hz) for 30 minutes at 4°C to promote rapid equilibrium. The pieces were then loaded into 2.0 mL cryovials (Corning, cat. 25704-2; Cambridge, Ontario, Canada; two vials per ovary) in 1.0 mL of the same solution.

The cryovials were placed in a programmable freezer (Biocool) 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 an additional 5 minutes, the cryovials were cooled at rate of 0.3°C per minute to −40°C. The vials were then plunged into liquid nitrogen at −196°C.

X-rays were taken 3 weeks later to locate the left ovary. All four ewes were then treated as far as the middle of the anteroposterior wall with 200 cGy for 10 days for a total of 2,000 cGy.

FIGURE 1

A graphic representation of the "Sowing Way Grafting" 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 ("host") ovary in a motion analogous to the sowing of a field.



Almodin. Recovery of fertility in ewes. Fertil Steril 2004.

Six weeks after radiotherapy was concluded, the cryovials were warmed in room temperature air for 2 minutes and then immersed in water at 37°C until the ice melted (2–3 minutes). The tissues were removed from the cryovials, and the cryoprotectant was quickly removed by repeated washing in PBS with glucose and pyruvate (GIBCO, cat. 14287-080). A sample of the tissue was examined histologically to confirm viability.

Two ewes were anesthetized, and the remaining irradiated left ovary was biopsied to determine whether viable germinative cells remained. Hypodermic needles (18 gauge) were filled with the fragments of the thawed ovarian tissue and were immediately injected inside the cortex of the irradiated left ovary in a procedure we called "Sowing Way Grafting," thus eliminating the need for sutures (Fig. 1). Many gentle spread "sowings" were performed until the entire thawed sample was implanted.

Postoperatively, the two grafted ewes were returned to pasture together with the two nongrafted (control) ewes. Four weeks later, the ewes were kept in a herd with rams and clinically evaluated each month.

All the samples for histological analysis were fixed for 2 days in 0.1:1,000 formalin (10%). The fixed ovarian samples were embedded in paraffin, cut into serial 5 μm sections, and stained with hematoxylin and eosin. All sections were examined microscopically at 100× magnification.

FIGURE 2

Ovarian tissue before freezing. It shows the cortex with primordial (PF), albuginea (A), primordial follicles in atresya (PFA), granulo-albicans cells (GAC), and theca-luteinic cells (TLC) (original magnification, 240×); haematoxylin and eosin stain.

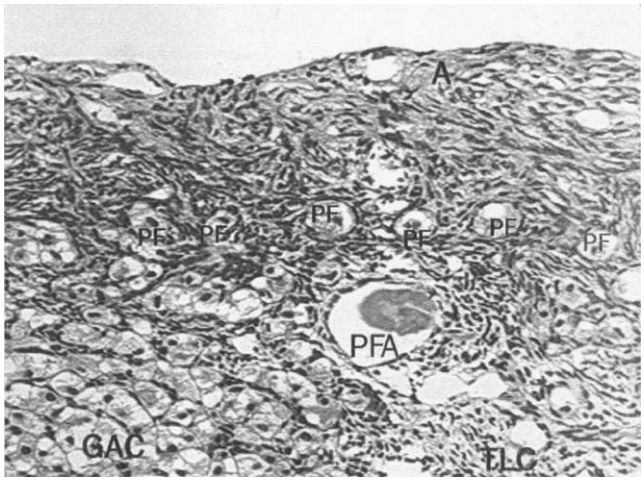


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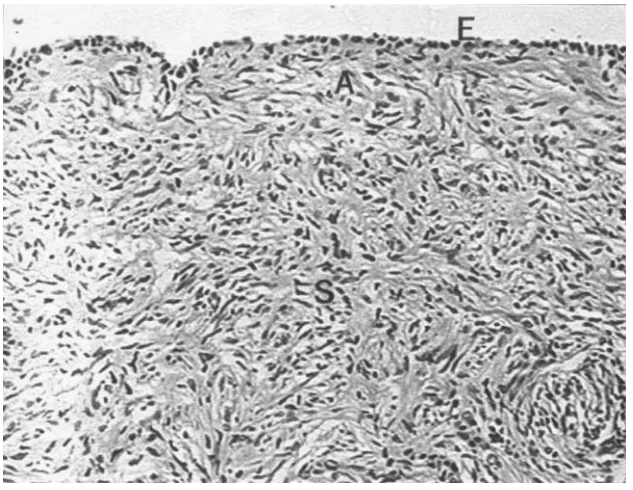
FIGURE 4

Ovarian tissue after radiotherapy. It shows a cortex and medullar with atrophy and without the germinative tissue. It shows only epithelium (E), albuginea (A), and ovarian cortex stroma (S) (original magnification, 200×); haematoxylin and eosin stain.



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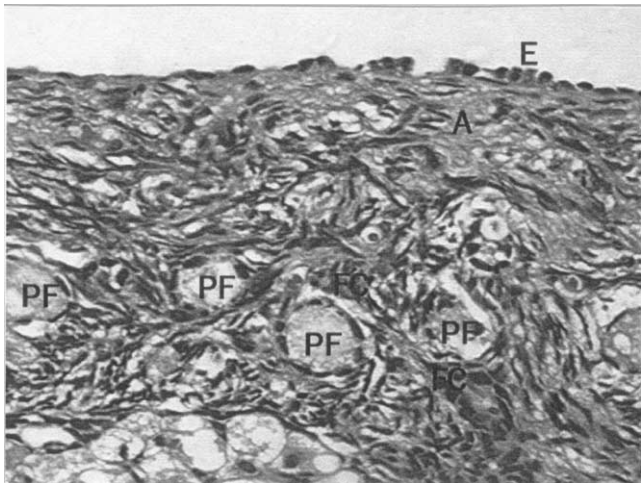
RESULTS

The tissue samples obtained before (Fig. 2) and after cryopreservation (Fig. 3) demonstrated healthy looking germinative tissue with primordial, secondary and cavitary follicles, as described by Turnbull et al. (27). The samples collected from all irradiated ovaries revealed fibrosis in the germinative tissue without any type of follicles, which confirmed the impossibility of this ovary to recover natural fertility without intervention (Fig. 4).

FIGURE 3

Ovarian tissue after thawing. It shows a cortex with the primordial follicles (PF), follicles cells (FC), albuginea (A) and epithelium preserved (original magnification, 400×); haematoxylin and eosin stain.



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Six months after the grafting, the two grafted ewes became pregnant — one of them with twins. The lambs were born healthy and are still alive today. More than 2 years after radiotherapy, the two, nongrafted (control) sheep have not become pregnant. One of the grafted sheep got pregnant for a second time, giving birth to another healthy lamb.

DISCUSSION

Numerous animal and human studies document that primordial follicles are relatively resistant to the freezing-thawing procedure (28, 29). With the exception of glycerol, cryoprotectants (propanediol [PROH], ethylene glycol, and dimethyl sulfoxide [DMSO]) make little difference in the success rate (7). The choice of a cryoprotectant depends on the time and temperature of incubation.

Higher survival rates are observed when primordial follicles from mice are exposed to 1.5 M of DMSO and PROH for just 5 minutes at room temperature (28). The incubation time must be increased with ovarian tissue from sheep and humans because it is more fibrous and the cryoprotectant penetration is reduced (19, 23, 30). We chose to incubate the ovarian tissue for 30 minutes and to work at 4°C with DMSO

because it penetrates the tissue more rapidly than propanediol at 4°C (19).

The cellular penetration is similar for the two cryoprotectants at 37°C, but they are more cytotoxic at this temperature (31). In Figure 3, we can see healthy looking germinative tissue with primordial, secondary, and cavitary follicles, demonstrating that the freezing-thawing protocol adopted was successful.

Despite the excellent results obtained with the preservation of follicles after freezing, prior investigators had great difficulty reestablishing ovarian function after transplanting cryopreserved ovarian tissue. Subcutaneous auto-transplant without vascular anastomosis has been performed in mice, allowing the reestablishment of hormonal production and dispensing with artificial hormones, but natural fertility could not be restored. Grafting the thawed tissue at heterotopic site with a rich vascular bed may be an alternative option. Endocrine cycles could still be restored but ovarian stimulation, follicle aspiration, and in vitro fertilization (IVF) is needed for conception (32, 33).

Reproductive performance (i.e., mean number of pups per litter and number of litters per female) was similar in recipients with grafts of fresh and frozen ovaries, and in nontreated control female mice from the same strain as the ovary donor (34).

The viability of the ovarian cortex when transplanted fresh (32) or frozen (21, 35) has also been verified in animals as well as human beings. The recovery of fertility through orthotopic transplantation in rodents has been performed with relative success (34, 36). In these procedures, however, it has generally been necessary to perform vascular anastomosis to obtain a better blood supply to decrease the germinative tissue loss.

Success has also been reported when the transplant of hemi-ovaries or cortical strips was carried out with suture but without vascular anastomosis (21, 22, 33). In this case, it was expected that the transplanted tissue would be supplied and revitalized by blood contribution through contact; however, a loss of follicles occurs due to ischemia during the establishment of grafts of ovarian tissue (37). In excess of 50% of the follicles are lost within 15 days of grafting of fresh or frozen ovaries in DMSO (28). This strongly suggests ischemia is the cause of the poor follicle survival, and not, as argued by others, damage to the germinative cells during freezing and thawing (23, 28, 33, 38). In addition, whereas only 7% of the follicles are lost during freezing-thawing procedure, a striking 65% are lost during revascularization (39).

A mathematical model suggests that follicle loss accelerates as the population size falls to $<25{,}000$, and that ovarian function is lost when the number is depleted to $<1{,}000$ (8). Even if a substantial proportion of follicles survive after frozen-thawed tissue is grafted into patients with ovarian failure, the total follicle population will still be low in such a way that ovarian function may only be regained for a short time (7).

We froze the germinative tissue together with the ovarian cortex in very small pieces so that the cryoprotectant could act with greater efficiency. We only removed one of the ovaries and left the other to act as a "host" site for the frozen tissue. We think that the preservation of just one ovary is enough to recover natural fertility.

The ovary submitted to radiotherapy had all its germinative tissue destroyed (Fig. 4). Its size was reduced, but its vascularization was kept intact, thus becoming the natural "host" site for the frozen tissue. The thawed tissue was injected with a needle inside the cortex along all the extension of the "host ovary" in a procedure that is analogous to the "sowing" of a field (Fig. 1), assuring contact between the implanted tissue fragments and the blood supply, without the need for sutures. All the tissue from the frozen ovary was implanted. This experiment was performed 2 years ago and the grafted ewes are still fertile today.

CONCLUSIONS

It is possible to recover the fertility of ewes submitted to radiotherapy for sterilization with the grafting of cryopreserved germinative tissue using the "Sowing Way Grafting" procedure. The ovary that undergoes radiotherapy becomes the "host ovary," offering the possibility for the recovery of natural fertility and pregnancies without the need for treatment such as IVF.

We are proposing an innovative technique for the grafting of germinative tissue without sutures, which holds the potential of restoring normal fertility to thousands of women rendered sterile each year because of the aggressive treatment of malignancies. As promising as it may appear to be, we think that this is just the first step in a series of animal investigations needed to assess the long-term effects of this type of implantation. We do not know, for instance, for how long the host ovary will be able to maintain its fertility and if any future hazards will occur due to freezing. A more detailed study with a greater, and more representative, number of subjects is required to confirm the hypothesis.

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