Transplantation of intact rat gonads using vascular anastomosis: effects of cryopreservation, ischaemia and genotype

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BACKGROUND: A limited store of ovarian follicles is present at birth and its progressive decline during ageing is hastened by alkylating agents and ionizing radiation during treatment for cancer or autoimmune disease. Oligo- or azoospermia can arise for similar reasons in men. There is some experimental evidence showing that targeted gene deletion or drugs to produce hypogonadotrophism can protect germ cells from wastage. Another strategy for conserving fertility is to cryopreserve ovarian or testicular tissue for subsequent transplantation. To maximize gonadal function, it is desirable to preserve whole gonads for transplantation using vascular anastomosis. METHODS AND RESULTS: We investigated this strategy in the rat model. All freshly isotransplanted ovaries (n = 8) survived and resumed follicle growth and secretion and, although ischaemia for 24 h at 4°C did not disrupt ovarian function, the organs had fewer follicles. Four out of seven (57%) cryopreserved transplants survived for >60 days, were ovulatory and one pregnancy was established, but the ovarian reserve was compromised by fewer follicles. Ovarian allotransplants were vigorously rejected, even with moderate immunosuppression using cyclosporin A. On the other hand, only three out of seven (42%) fresh testicular isotransplants had active spermatogenesis, and none of the cryopreserved testes was functional. CONCLUSIONS: The effects of gonadectomy in rats can be reversed by isotransplants, but the results are more successful with ovaries than testes, and allotransplants were never successful. Intact cryopreserved ovaries can be restored to function after transplantation with vascular anastomoses.

Key words: cryopreservation/fertility/follicles/ovary/testis

Introduction

There is a limited endowment of follicles in the ovary at birth which declines during ageing (Faddy et al., 1992). Its disappearance is hastened by exposure to alkylating agents and ionizing radiation. Thus, as increasing numbers of young people become long-term survivors of cancer after successful treatment, more efforts are being made to alleviate the side-effects of drugs and radiation, notably infertility and premature menopause (Meirow, 2000). Iatrogenic destruction of germ cells is mediated by apoptosis, a process which can be inhibited by targeted mutagenesis of genes involved in the cell death pathway (Perez et al., 1999; Morita et al., 2000). At present, however, it is more practicable to safeguard human fertility using cryotechnology (Picton et al., 2000; Kim et al., 2001).

Storage of ovarian tissue at low temperatures is an alternative to using assisted conception technology (IVF) for banking embryos or oocytes, and is the only option available for prepubertal patients (Grundy et al., 2001). So far, however, only a few case reports have appeared in the clinical literature after implantation of frozen–thawed cortical strips (Oktay and Karlikaya, 2000; Oktay et al., 2001; Radford et al., 2001; Poiriot et al., 2002). A full span of menstrual life cannot be expected with this technique since the function of implants is limited by the small mass of ovarian tissue and the percentage of primordial follicles surviving ischaemia until revascularization occurs (Nugent et al., 1998; Baird et al., 1999). Primordial follicle-rich cortical ovarian tissue restored ovarian cyclicity and even pregnancy in several animal species, though, with few
exceptions, transplant activity was shorter than normal. In one study, for example, ≥50% of the follicles died during the period of ischaemia, although antioxidant treatment was able to reduce this wastage (Nugent et al., 1998).

Vascular transplants could, in theory, reinstate natural fertility after cryopreservation of ovaries and testes, but the diminutive calibre of blood vessels in small laboratory animals is demanding for microsurgery (Lee, 1974; Denjean et al., 1982; Comnier et al., 1985). We now report transplantation of the intact rat ovary and its proximal reproductive tract using vascular surgery. We have also investigated the possibility of allotransplantation, which might represent a radical alternative to existing oocyte donation technology.

Following initial success with rat ovarian isotransplants, which was summarized in correspondence to Nature (Wang et al., 2002), we extended our study to investigate storage of ovaries at low temperatures above freezing and tolerance of allotransplantation with moderate immunosuppression. We also tested testicular transplants either using fresh or cryopreserved organs. The rationale for the male was the same as for females. Oligo- or azoospermia can arise from many causes, including cancer treatment (Damani et al., 2002; Thomson et al., 2002). Assisted reproduction involving frozen semen or microsurgical sperm aspiration for ICSI with cryopreservation if appropriate (Podsiadly et al., 1996; Patrizio, 2000) are widely practised, but cannot be used for pre-pubertal boys (Bahadur et al., 2000). Testicular banking offers a possibility of fertility conservation if the organ can be successfully transplanted, and this option too has now been tested in the rat model.

**Materials and methods**

**Transplantation surgery**

Adult female or male rats aged ~10 weeks old and weighing 200–250 g were obtained from Charles River (Canada). Inbred Lewis strain rats were used as donors and recipients for isotransplantation, whereas Brown–Norway strain rats were used as ovary donors for allotransplantation. The donor rats were anaesthetized with sodium pentobarbital (40 mg/kg). The right ovaries or testes, Fallopian tubes or vas deferens and the upper third of the uterus were dissected and branches of their vessels were divided. The ovaries were removed en bloc with their arteries and veins attached. In males, spermatic cord vessels were traced back to large vessels to create short cuffs of aorta and vena cava. The preparations were perfused with heparinized Ringer’s solution and subsequently with Wisconsin fluid in which they were immersed at 4°C overnight. The slides of ovaries were coded and the tissues were prepared as 6 μm sections. Vaginal smears were prepared daily for monitoring ovarian activity by perfusion. The organs were held in Wisconsin solution at 4°C for ±40°C, and then at 10°C/min to −85°C before plunging into liquid nitrogen for overnight storage. Thawing was carried out rapidly (>100°C/min) by swirling the organs in a water bath at 40°C. The cryoprotectant was washed out by reversing the concentration gradient by perfusion. The organs were held in Wisconsin solution at 4°C until transplanted (<1 h).

**Monitoring transplant function in vivo**

Vaginal smears were prepared daily for monitoring ovarian activity for ≥60 days after surgery. Four rats with fresh and four with cryopreserved isotransplants, whose cycles had been restored after 30 days, were tested for fertility with Lewis males for another 30 days. Two additional animals with fresh transplants were kept for an additional 50 days for testing ovulatory capacity by priming with i.p. injections of 50 IU equine CG and 50 IU hCG 48 h later so that ovulation could be verified the following day.

At autopsy, the ovaries and reproductive tracts were removed and examined for signs of ovulation and/or pregnancy. The Fallopian tubes and uteri were perfused with Trypan blue dye (0.025%, v/v; Sigma) to check if the tubes were patent. The completeness of oophorectomy on the contralateral side was confirmed at this time. Likewise the male gonad and tract were examined and organs of both sexes were weighed and then fixed in Bouin’s fluid overnight. The tissues were prepared as 6 μm serial sections stained with haematoxylin and eosin. The slides of ovaries were coded and the numbers of primordial and growing follicles were counted blindly in every tenth ovarian section and testes were studied morphologically.

**Hormone assays**

Rats were euthanized with carbon dioxide at a median time of 67.8 days (female) or 42 days (male) after transplantation. During terminal
anaesthesia, blood was withdrawn by cardiac puncture and stored as serum at -80°C. The concentrations of FSH and estradiol-17β in female rats and testosterone in male rats were measured using radioimmunoassay (sensitivity of 0.1 ng FSH per tube, Amersham Pharmacia Biotech, USA) and chemiluminescence (sensitivity of 37 pmol/l estradiol-17β; Bayer, USA) and ADVIA Centaur™ System (sensitivity of 0.35 nmol/l testosterone; Bayer, USA).

Statistics
The data were analysed by analysis of variance and Student’s t-test, P < 0.05 being considered significant.

Results
Ovary transplantation
From a total of 74 female transplant recipients, seven died before regaining consciousness and an additional rat died from infection. Most mortality occurred ~10 days after surgery and as a result of haemorrhage (10/74). All animals that survived longer were healthy and their body weight increased at a similar rate to intact controls.

All fresh ovarian transplants were still present at autopsy and were showing signs of normal function up to ~60 days post-operation. Vaginal smears indicated that estrous cycles had not been interrupted apart from brief irregularity after surgery. Ovarian and uterine weights were normal, nor were serum FSH or estradiol-17β significantly different to intact controls (Table I). These findings were consistent with the ovarian morphology, which was indistinguishable from controls [Figure 1(i), (ii)]. In addition, the numbers of primordial and growing follicles were not significantly different between controls and fresh transplants (Figure 2). These results indicated that the whole organ had survived with its intact vascular supply, because a non-vascularized implant would have lost follicles. Two additional animals injected with gonadotrophins after ~120 days after transplantation responded.

Although cold ischaemia for 24 h had not disrupted ovarian function, and ovarian morphology was normal [Figure 1(iii), (iv)], the organs showed signs of compromised function. The numbers of follicles were significantly reduced (P < 0.05) (Figure 2), as was uterine weight (P < 0.05) (Table I). Four out of seven cryopreserved transplants (57%) were surviving at the end of the study, and the animals had presented normal ovarian histology with follicles and corpora lutea at autopsy [Figure 1(v), (vi)]. These transplants too, however, had signs of compromised function. There was a longer delay until estrus smears resumed, serum FSH was higher than controls (P < 0.05) and serum estradiol was lower (P < 0.05).

Nevertheless, neither hormone was in the castrate range (Table I). Contralateral uterine weight in non-pregnant cryopreserved transplants was significantly lower than in fresh transplants (P < 0.01), consistent with the reduced estrogen levels (Table I). On the other hand, ovarian weight was higher, though these measurements were less reliable because adhesions occasionally made dissection more difficult. Where adhesions were present, it was difficult to determine the extent of collateral vascularization of the gonad and reproductive tract. The numbers of follicles in these cryopreserved ovaries were lower on average than controls (P < 0.01) (Figure 2), but results were variable. In one animal, follicles were virtually absent (although there was evidence of recent ovulation) and, in another, the numbers were in the lower range of normal.

The morphology of the Fallopian tube was not affected by 24 h of cold ischaemia [Figure 3(i)], and uterine tissues on both sizes of the utero-uterine Anastomosis were histologically indistinguishable and normal [Figure 3(ii), (iii)]. Only one animal was pregnant at autopsy, and it was in the cryopreserved group. It was not clear why other animals with transplants failed either to mate or to become pregnant because their uterus were patent according to the Trypan blue dye test. Fallopian tubes were sometimes shrunken, suggesting that there may have been some necrosis, and tubo-ovarian and other adhesions might have physically obstructed gamete transport and/or caused ovulatory dysfunction. The animal which became pregnant had the second largest number of follicles in its group, and its ovary contained 13 hyperaemic corpora lutea, which is typical of a young rat with a single ovary. The fetuses and their

### Table I. Restoration of ovarian function by transplantation of fresh organs or after cryopreserving or chilling

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Day when vaginal estrogencity reinitiated (% days post-operated)</th>
<th>Ovarian weight (ng)</th>
<th>Uterine weight (ng)</th>
<th>Serum FSH (ng/ml)</th>
<th>Serum estradiol (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact control</td>
<td>7</td>
<td>85.9</td>
<td>26.4 ± 3.4</td>
<td>289.9 ± 4.8</td>
<td>9.1 ± 0.4</td>
<td>416.9 ± 3.6</td>
</tr>
<tr>
<td>Fresh transplant</td>
<td>8</td>
<td>3.3 ± 1.6 (73.3)</td>
<td>33.1 ± 5.4</td>
<td>258.3 ± 23.9</td>
<td>9.3 ± 0.5</td>
<td>474.3 ± 80.6</td>
</tr>
<tr>
<td>Cryopreserved transplant</td>
<td>7</td>
<td>12.0 ± 2.5 (71.8)</td>
<td>51.8 ± 18.9</td>
<td>171.2 ± 34.5**</td>
<td>21.3 ± 6.2*</td>
<td>256.6 ± 61.8*</td>
</tr>
<tr>
<td>Cold ischaemia</td>
<td>4</td>
<td>3.0 ± 1.1 (78.5)</td>
<td>26.9 ± 4.1</td>
<td>196.2 ± 36.2*</td>
<td>11.5 ± 2.7</td>
<td>325.9 ± 66.9</td>
</tr>
<tr>
<td>Oophorectomy</td>
<td>4</td>
<td>-</td>
<td>95.2 ± 14.4**</td>
<td>58.4 ± 1.6**</td>
<td>4.3 ± 33.1**</td>
<td></td>
</tr>
<tr>
<td>Fresh allotransplant (CSA30)</td>
<td>6</td>
<td>-</td>
<td>80.8 ± 15.2</td>
<td>63.3 ± 24.5**</td>
<td>75.9 ± 11.0**</td>
<td></td>
</tr>
<tr>
<td>Fresh allotransplant (CSA60)</td>
<td>5</td>
<td>-</td>
<td>533.5 ± 104.6</td>
<td>93.1 ± 34.3**</td>
<td>69.2 ± 6.1**</td>
<td></td>
</tr>
<tr>
<td>Fresh allotransplant (CSA60)</td>
<td>6</td>
<td>-</td>
<td>339.7 ± 81.9</td>
<td>87.5 ± 25.0**</td>
<td>65.0 ± 13.4**</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM.
Allotransplants treated with immunosuppressant for 30 (CSA30) or 60 (CSA60) days.
Differences compared with controls were significant at *P < 0.05 and **P < 0.01.
*Below limits of detection.
placentas were normal in appearance and estimated to be ~16 days post-conception. One had implanted on each side of the uterine anastomosis, identified by a remnant of suture material. In contrast to isotransplants, allotransplants were aggressively rejected despite treatment with cyclosporin. Cycles were not restored, ovaries were grossly enlarged and uterine weights were in the castrate range (Table I). No follicles had survived and stromal tissue was fibrotic and heavily infiltrated with inflammatory cells [Figure 4(i), (ii)]. Serum FSH levels were higher than in other groups and estradiol levels were undetectable (Table I). The results with allotransplants in animals treated for either 30 or 60 days with cyclosporin A were almost identical, except for the degree of hypertrophy.

**Testis transplantation**

Out of a total 14 testicular transplants, two animals in the cryopreserved group died from thrombosis ~7 days after surgery. The surviving rats were healthy and gained weight after surgery. Seven surviving rats had received a fresh transplant and five received a cryopreserved testis, but all of the latter had atrophied by 6 weeks after surgery. Testicular weight and serum testosterone levels in both transplant groups were lower than in intact controls (Table II). In the fresh transplants, 60–80% of the seminiferous tubules were totally destroyed and lacking signs of spermatogenesis [Figure 5(ii) arrows]. The surviving tubules were found to be morphologically normal and spermatogenesis had generated germ cells at all stages of development [Figure 5(ii)] with abundant sperm in the epididymis [Figure 5(iii)]. However, the seminiferous tubules were significantly narrower compared with control rats [0.149 versus 0.198 mm, Figure 5(i), (ii)], indicating reduced spermatogenic activity and perhaps damage to the Sertoli cells. In contrast to these partially successful results, the testes after cryopreservation and transplantation were almost obliterated.

![Figure 1. Isotransplanted rat ovaries ~60 days after transplantation: (i) control showing a Graafian follicle (GF); (ii) ovaries transplanted freshly showing growing follicles (arrows); (iii) and (iv) ovaries transplanted after 24 h cold ischaemia at 4°C showing a Graafian follicle (GF) and a corpus luteum (CL); (v) ovaries transplanted after cryopreservation showing growing follicles (thick arrows) and primordial follicles (thin arrow), and (vi) two corpora lutea (CL). Scale bars = 50 μm. Haematoxylin and eosin.](https://academic.oup.com/humrep/article-abstract/18/6/1165/2913553)
by fibrosis. No trace of sperm production was found in these organs or adjacent epididymis [Figure 5(iv)]. In view of these results, it was irrelevant to test testicular allotransplantation.

Discussion

In recent decades there have been striking advances in the treatment of cancer and some non-malignant diseases by treatment with potentially sterilizing chemotherapy and/or radiotherapy. As survival and cure rates rise, the focus is turning to the late effects of treatment, among which loss of fertility and gonadal failure are of high importance. Various options are available for protecting fertile potential. The first is sperm cryopreservation for adult males (Witherington et al., 1977) and embryo banking for females and now even a possibility with mature oocytes (Trounson and Mohr, 1983; Fabbri et al., 2001). Other strategies that are currently under consideration are the use of drugs to protect gonadal function in vivo (Tilly and Kolesnick, 1999). Success rates with cryopreservation are variable, but some of these strategies are still more theoretical than practicable. Importantly, none of these options apply to the pre-pubertal child, yet there is a markedly rising number of girls and boys who have survived potentially sterilizing treatment for malignancy (Aziz, 2002). For children, as well as for young adults, there is a possibility of banking immature germ cells in gonadal tissue or as whole organs for subsequent transplantation.

Most experience to date has been based upon cryopreservation of gonadal tissue slices. Provided the tissue is thin enough, immersion in cryoprotectants may be adequate for avoiding serious damage from intracellular ice crystals during cooling or rewarming. Successful transplantation of ovarian tissue from a number of species has now been recorded, including laboratory rodents (Carroll and Gosden, 1993; Harp et al., 1994; Candy et al., 2000), farm animals (Gosden et al., 1994; Baird et al., 1999) and monkeys (Schnorr et al., 2002). Moreover, a few case reports indicate there are prospects for clinical application (Oktay and Karlikaya 2000; Radford et al., 2001). The attraction of this strategy is that both the endocrinology and natural fertility of the individuals would be restored by transplantation. The disadvantage is that implantation of tissue without vascular reanastomosis leads to ischaemic damage to the tissue and depletion of germ cells and their supporting somatic cells. Fortunately for the ovary, primordial follicles appear to be relatively resistant to ischaemia, although losses
always occur (Nugent et al., 1998). Likewise, there is damage to the seminiferous tubules when slices of testicular tissue are transplanted (Schlatt et al., 2002; Shinohara et al., 2002). Whilst orthotopic transplantation provides a possibility of restoring natural fertility, heterotopic transplants require assisted reproductive technology to realize the fertile potential of the tissue. A more radical procedure is to recover spermatogonial stem cells from the testis for cryopreservation. Experiments in rodents have demonstrated that these cells restore fertility to the sterilized testis after transfer to the seminiferous tubules (Avarbock et al., 1996). It is questionable if germ cell transfer or tissue implantation can achieve sufficiently high efficiency for restoring a full lifespan of fertility.

Gonadal transplants have been tested throughout the 20th century, and some extraordinary claims, which were often not verifiable, were made about their efficiency in humans (Nugent et al., 1997). Attempts were also made to cryopreserve whole organs, such as the kidney, but they were either unsuccessful or unrepeatable (Guttman et al., 1976). One of the most serious problems was vascular injury, probably resulting from osmotic damage or chemical toxicity from cryoprotective agents. Our study shows that, even after storage at liquid nitrogen temperature, a whole rat ovary can be transplanted successfully and restore follicular activity and secretion. Moreover, there is a possibility of pregnancy after these procedures, indicating potential new strategies for fertility conservation in animals and humans. However, ovarian allotransplants were vigorously rejected in this species, which appears to rule out ovarian donation except when the donor and host have a close tissue match. Unlike the ovaries, testicular isografts did not survive if the organs had been cryopreserved, although fresh organs were more successful.

In the rat model, vascular surgery is technically demanding, but the small size of the organs is favourable for cryopreservation. The vascular architecture of the reproductive system in this species requires the ovary to be transplanted with its adjacent Fallopian tube and a segment of the uterus. In larger species and in humans, an en-bloc transplant would not be required, for which anastomosis of the ovarian pedicle would be technically straightforward. Indeed, this procedure has already been used in monkeys (Scott et al., 1981) and sheep (Jeremias et al., 2002). In clinical practice, we envisage that one of the pair of ovaries would be left in situ so that an intact pedicle is available for exchanging the sterilized organ with the frozen and thawed ones once the patient is ready for autotransplantation. In the present study the equilibrium cooling method was used with a standard cryoprotectant (DMSO) because this has already proved successful with ovarian tissue (Baird et al., 1999). The cryoprotectant was introduced and washed out by slow perfusion, and a sugar was added as a further precaution to reduce osmotic damage. This model enabled function of the transplants to be monitored non-invasively using vaginal smears to indicate estrogen secretion. Confirmatory endocrine data were obtained by serum hormone measurement and by gross and microscopical examination of the gonads. There was concordance between gonadal morphology and size and the circulating levels of gonadotrophins and sex steroids, which were correspondingly elevated and decreased, respectively, in hypogonadal animals. However,
the ultimate test of function in successful transplants is fertility, and this was demonstrated by pregnancy in one rat which, surprisingly, was in the cryopreserved ovary group. Clearly it is desirable to confirm and extend this study, although the labour and surgical skill required will likely delay progress towards optimizing protocols, and avoiding thrombi and necrosis. Evidently, a period of about 1 h ischaemia was not detrimental to function because the fresh transplants did not suffer significant follicle losses. However, 24 h of ischaemia had detrimental effects, although the mean number of follicles was higher than in the cryopreserved group. This result implies that there may be significant impairment in potential fertility if, for example, organs are shipped on ice between centres without cryopreserving. Should these techniques ever be applied for the conservation of fertility in animals or patients, cryopreservation protocols and alternative strategies need to be refined and tested. Vitrification, which can prevent ice formation in tissue, should be considered although ultra-rapid freezing of large organs of primates and farm animals is problematic and equilibration with high concentrations of cryoprotective agents will be lengthy (Al Aghbari and Menino, 2002). These problems will also have to be addressed for conventional cryopreservation methods.

The results of testicular transplantation were much less successful than with the ovary, probably because of the long spermatic vessels and vulnerability of this organ to ischaemia. With fresh transplants, three out of seven organs maintained spermatogenesis and secreted testosterone. Nevertheless, some damage was observed, and tubules were narrower, implying that Sertoli cells and/or germ cells were less abundant and the daily production of sperm was reduced. None of the cryopreserved testes were functional after transplantation, and testosterone levels were in the castrate range. These disappointing results should not discourage attempts using modified protocols because testicular banking could benefit some patients, especially pre-pubertal children, seeking fertility conservation.

Lastly, studies were carried out to investigate the survival of gonadal allotransplants. The rationale for these studies was that organ donation could theoretically be used for treating patients with premature gonadal failure or gonadal agenesis, and might therefore serve as an alternative to conventional oocyte donation. Combinations of inbred rat strains were chosen on the basis of extensive studies of other organ transplants, and the dose of cyclosporin was selected because it permitted rat cardiac allotransplants to survive when the Lewis and Brown–Norway combination was used (Harrison and Madwed, 1999). However, these doses were unsuccessful in preventing rejection of allotransplants of ovaries. Estrous cycles were never reinitiated, follicles disappeared completely and serum FSH and estradiol levels were in the castrate range. It therefore appears that ovarian allotransplantation is not an option, unless there is a closer match of MHC haplotypes or more aggressive immunosuppression is used.

Figure 5. Isotransplanted rat testes 42 days after surgery. Normal spermatogenesis in seminiferous tubules of control organs (i) and fresh transplanted testes (ii), but destroyed tubes were also apparent (ii, arrows). Cryopreserved organs were sterile after transplantation (iv, right part). Sperm were present in the lumen of epididymides of fresh transplanted organs (iii) but not cryopreserved testes (iv, left part). Scale bars = 50 μm. Haematoxylin and eosin.
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References

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