XENOTRANSPLANTATION OF OVARIAN TISSUE INTO MALE IMMUNODEFICIENT MICE

by

HUGO JOSE HERNANDEZ FONSECA

(Under the direction of BENJAMÍN G. BRACKETT)

ABSTRACT

A male immunodeficient mouse model for transplantation of ovarian tissue was investigated. Bovine and human ovarian tissues were surgically placed either under the kidney capsule or in the subcutaneous spaces of male non obese diabetic (NOD) severe combined immunodeficient (SCID) mice. Time intervals required for development of growing follicles were determined for neonatal and adult bovine ovarian tissue grafts. This interval was much shorter (P <0.01) in adult tissue than in one-week-old calf tissue, i.e. 55 vs 124 days. The increase in the proportion of growing follicles was coincidental with a decrease in the proportion of resting follicles. This increment in the growing follicle populations took place abruptly and was significant by 55 days and by 124 days after transplantation in the adult cow and calf ovarian grafts, respectively. Recovery of oocytes from bovine ovarian grafts was successful. Several immature oocytes were recovered and evidence of maturation in one oocyte was obtained after 24 hours of in vitro maturation. Treatment of host mice with an FSH:LH preparation increased follicular
development but did not enhance oocyte recovery rates. Human ovarian tissue grafted under the kidney capsule of intact male NOD SCID mice showed a greater proportion of growing follicles than similar grafts transplanted to the kidney of castrated hosts and to the subcutaneous space of intact hosts. However, no differences in follicular growth and development were detected between the intact/ kidney capsule and the castrated / subcutaneous groups. It is clear that the site of transplantation and presence or absence of the hosts’ gonads can potentially influence follicular development in xenotransplanted ovarian tissue. The male NOD SCID mouse model is a feasible alternative for xenotransplantation of mammalian ovarian tissue. This model should prove useful as a tool for further development of strategies for preservation of fertility of young female cancer patients and endangered species.

INDEX WORDS: Follicular Development, NOD SCID Mice, Oocyte Recovery, Ovarian Tissue, Xenotransplantation.
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CHAPTER 1

INTRODUCTION
INTRODUCTION

Purpose and Objectives of the Study

Xenotransplantation is viewed today as an alternative for fertility preservation for young female cancer patients and endangered species. The purpose of this study was to characterize and improve follicular development in a xenotransplantation model, using bovine and human ovarian tissue grafted into male immunodeficient mice. Follicular development to the antral stage has been successfully obtained after transplantation of ovarian tissue from many species into immunodeficient hosts. Nonetheless, optimal development conditions after transplantation of these tissues have not been well defined. The present research was conducted to better define some of the factors that will improve follicular development after xenotransplantation. Thus, a primary goal of this work was to provide researchers with important tools that can be used to further improve follicular growth under transplantation conditions. At the same time, the author hopes to provide clinicians and cancer patients with additional information that will help them obtain a realistic concept about the potential use of xenotransplantation for preservation of fertility.

Specific objectives of this study involving xenotransplantation of ovarian tissues in male non obese diabetic (NOD) severe combine immunodeficient (SCID) mouse hosts were: a) To determine an appropriate interval for xenografted newborn and adult bovine ovarian tissue to develop gonadotropin-responsive follicles; b) To determine an appropriate exogenous gonadotropin treatment that would promote follicular development and oocyte recovery; and c) To compare the effect of site of human ovarian
transplantation and of the presence or absence of the hosts’ testes on follicular survival and development.
CHAPTER 2

LITERATURE REVIEW
**Folliculogenesis and Oogenesis**

In vivo follicular and ovum development

In most mammals, including the human (Baker, 1963; Gosden, 1995; Motta et al., 1997), follicular formation starts and ends during fetal life (Hirshfield, 1991; Marion and Gier, 1971; Rüsse and Sinowatz, 1991). In a few species, such as rodents and rabbits, follicles appear later during the early neonatal period (Hirshfield, 1991; Marion and Gier, 1971; Rüsse and Sinowatz, 1991; Van den Hurk et al., 1995; Betteridge et al., 1989). In the cow, these first follicular structures called primordial follicles (30-50 µm in diameter) consist of a small oocyte (20-35 µm in diameter) surrounded by a single layer of flattened somatic (pregranulosa) cells (Figueiredo et al., 1993; Hulshof et al., 1994). At the time of birth in the human (Gougeon, 1993; Lunenfeld and Insler, 1993) and farm animals (Rüsse and Sinowatz, 1991; Erickson, 1966) there are hundreds of thousands of primordial follicles in the ovaries. Small groups of primordial follicles will gradually enter a growing phase (Scaramuzzi et al., 1980; for review see Van den Hurk et al., 1997) that most often ends in atresia (Baker, 1963). It is not well understood what factors or mechanisms induce a primordial follicle to abandon its resting state and begin to grow (activation). After activation, as pregranulosa cells acquire a cuboidal shape follicles will sequentially become intermediary and then primary follicles (in the cow: 40-60 µm in diameter, Figueiredo et al., 1993) when all granulosa cells forming the single layer are cuboidal. As the proliferative activity of granulosa cells increases the follicle becomes a multilayered structure. All follicles with more than one cuboidal granulosa cell layer are called secondary follicles (in the cow: max. of 150 µm in diameter, Lussier et al., 1987). In the human, pig and bovine, formation of the zona pellucida and of a thecal layer takes
place during the early growing stages of the secondary follicles (for review see Van den Hurk et al., 1997; Van den Hurk et al., 2000). Also during this stage the oocyte begins to grow reaching a diameter of 60 µm in the cow, 70 µm in the mouse, 80 µm in the human and 90 µm in the sow (for review see Van den Hurk et al., 2000). Progressive formation of a fluid filled cavity (called an antrum) and further increase in the diameter of the oocyte characterize the antral stage (for reviews see Van den Hurk et al., 1997; Van den Hurk et al., 2000). Growth and development of the oocyte is believed to be greatly determined by the surrounding granulosa cells. Projections from the granulosa cells penetrate the zona pellucida and form intercellular junctions with the plasma membrane of the oocyte. These connections allow exchange of regulatory factors that are essential for development of the oocyte and the follicle itself (Eppig, 2001; Senger, 1999).

In contrast to small laboratory rodents, where primary follicles only require 14-16 days to reach the preovulatory stage, in larger mammals complete folliculogenesis from activation of primordial follicles to the antral stage takes several months (Lunenfeld and Insler, 1993; Gougeon, 1996; Gougeon, 1986). In the cow, folliculogenesis starting at the primary stage requires around 12 weeks before an antral follicle is formed (Britt, 1991). Growth rate of a mammalian follicle changes according to the size of the follicle. Small preantral follicles have a slower growth curve than antral follicles (Gougeon, 1986; Lussier et al., 1987) and even among antral follicles growth rates vary greatly, with large follicles growing faster mainly due to development of the antrum rather than proliferation of granulosa cells as is the case for smaller follicles (Lussier et al., 1987).
Endocrine regulation

Initial migration of primordial germ cells to the gonadal ridge (undifferentiated gonad) occurs similarly in potential male and female embryos with completion at around 26 days of gestation in the human (Witschi, 1948; Gosden, 1995) and 30 days in the cow (Rüsse and Sinowatz, 1991). Female gonadal development occurs passively. In the case of a genetic female (XX) primordial germ cells will embed in the cortex of the undifferentiated gonad (now referred to as oogonia). Absence of the Y chromosome and hence of the testis determining factor results in development of the female gonad (for review see Senger, 1999).

Mitosis during the first part of fetal life increases the number of oogonia present in the ovary to around 7 million in the human and 2 million in the cow. Most of these oogonia degenerate by the time of birth reducing the number of follicles that are formed to 1 million and a few hundred thousand after birth, respectively (Van den Hurk et al., 1997). After mitosis, oogonia enter meiosis and are arrested in the diplotene stage of the prophase of the first meiotic division just before birth and are subsequently termed oocytes (Van den Hurk et al., 1995; for review see Gosden, 1995; Van den Hurk et al., 1997; Van den Hurk et al., 2000). Follicular formation begins during the fourth month of gestation in both human (Baker, 1963; Gosden, 1995; Mota et al., 1997) and cow (Betteridge et al., 1989; Van den Hurk et al., 1995). During the prepuberal period, although follicular growth occurs, the lack of adequate levels of gonadotropins impairs ovulation and all growing follicles undergo atresia. In the adult human and cow, there are successions of follicles that start growing during different times of the menstrual and estrual cycle, respectively (for review see Gougeon, 1986; 1993 and Van den Hurk et al.,
Most of these growing follicles never reach ovulation, instead the great majority degenerate (atresia) and with increasing age the number of follicles in the ovary decreases due mainly to this phenomenon. In the woman, only about 400 oocytes ovulate of the total number of primordial oocytes present at birth (approximately 1 million, see Gougeon, 1993).

There is some evidence that even though FSH is not essential, it does have a role in the development of small preantral (growing) follicles. Receptors for FSH have been detected in small follicles of cow, sheep and human (Wandji et al., 1992; Tisdall et al., 1995; Oktay et al., 1997). Nonetheless, it is clear that the major action of FSH is in the growth and development of antral follicles. Development of primordial follicles is variable among species (Shaw et al., 2000a). In mice, the gonadotropin independent phase is around 18 to 19 days and the subsequent gonadotropin dependent phase is 2 days. In large domestic species and primates, the growth phase is estimated to be more than 3 months and may take as long as a year, while the gonadotropin dependent stage is around 4 to 10 days.

After puberty, serum gonadotropin concentrations are elevated. These high FSH concentrations induce the recruitment of small antral follicles, which are gonadotropin sensitive. These follicles will grow and produce estradiol and inhibin, among many other steroids, growth factors, peptides and proteins. Of this cohort of recruited follicles some will be selected and continue to grow, as increasing levels of inhibin exert a negative feedback on the anterior pituitary and reduce the release of FSH. In monovulatory species (i.e. human and cow) only one of the selected follicles will become dominant. During dominance, the follicle will reduce its FSH requirements and LH receptors will appear on
granulosa cells. Estradiol production continues to increase until sufficient to trigger the preovulatory surge of LH that will induce ovulation of the dominant follicle (for review see Senger, 1999).

Effect of cancer treatments on ovarian follicles and oocytes

Cancer treatments designed to produce death of rapidly proliferating tumor cells in human or animal patients have irreversible damaging potential for developing germ cells, especially in females. Susceptibility of the oocyte to irradiation induced damages will vary according to its maturational stage. Primordial oocytes are more susceptible to irradiation than oocytes present in ovulatory size antral follicles (Ash, 1980). Also the doses of irradiation as well as the age of the patient at the time treatment is applied will affect the degree to which ovarian function is affected (Baker et al., 1972). Shortly after irradiation, oocytes become pyknotic, there is disruption of the nuclear membrane and fibrotic tissue (fibrosis) replaces the oocyte. The net effect of this scenario is an important reduction in the number of oocytes present in the ovaries (Himelstein-Braw et al., 1978). Gonadal failure (i.e. amenorrhea) has been reported after irradiation of the ovaries (Jacox, 1939).

As described for radiotherapy, ovarian lesions produced by chemotherapeutic drugs on the ovary include destruction of the follicles and fibrosis of the tissue. Similarly, the main effect is a reduction in the number of oocytes that can lead to ovarian dysfunction. The risk of suffering amenorrhea will depend on a series of factors that include: the type of chemotherapeutic agent (especially alkylating agents), the cumulative
dose, and the age of the patient at the time of treatment (Chapman et al., 1979; Wang et al., 1980; Uldall et al., 1972; Warne et al., 1973).

In a study by Chapman et al., 1979, it was found that more than 80% of patients who had normal fertility pretreatment and received chemotherapy for treatment of Hodgkin’s Lymphoma, showed either primary ovarian failure or irregular ovarian activity. Even though women under 40 have a greater chance to be spared or to recover their normal fertility after cancer treatments, they are more likely to enter menopause earlier than normal women (Chapman et al., 1979; Fisher and Cheung, 1984). Thus, for any woman about to undergo cancer therapy (radiotherapy and/or chemotherapy) the risk of losing or compromising her subsequent fertility is very high.

Transplantation and xenotransplantation of ovarian tissue

Ovarian tissue transplantation was reported over a century ago (Morris, 1895). Initially, this technique was implemented in early endocrinological studies that provided many insights as to the physiological functions of the ovary in a variety of settings; e.g. Dunham et al. (1941) demonstrated viability of neonatal rat ovarian tissue in the anterior chamber of the eye; Pfeiffer (1934) reported mature function of rat neonatal ovary when placed in adult castrated host. Later studies on ovarian tissue grafts were successfully directed at restoration of fertility of various animal models: mice (Parrott, 1960; Jones and Krohn, 1960), hamsters (Parrott, 1959), and rabbits (Winston and McClure-Browne, 1974). Early cryopreservation experiments were also conducted on ovarian tissue prior to transplantation (Parkes and Smith, 1953; Parrott, 1960). Although, these early studies already reported great losses of oocytes due to ischemia (Jones and Krohn, 1960) it was
also clear that allogeneic ovarian grafts orthotopically transplanted (ovarian donor and recipient belong to the same species) were able to restore fertility (Parrott, 1960).

Research in cryopreservation of ovarian tissue decreased (Candy et al., 1995) as methods became available for the cryopreservation of oocytes (mice: Whittingham, 1977; cow: Schellander et al., 1988; rabbit: Al-Hasani et al., 1989) and development of in vitro fertilization technology (rabbit: Chang, 1959; cow: Brackett et al., 1982). However, current issues (as imminent cancer therapy) of relevance to human fertility (Bahadur and Steele, 1996; Moomjy and Rosenwaks, 1998) and the lack of large numbers of human oocytes (Candy et al., 1995) have renewed the interest in cryopreservation and transplantation of ovarian tissue.

Women about to undergo chemotherapy, radiotherapy, or surgery that may affect fertility are now being offered the opportunity to cryopreserve their ovarian tissue (Bahadur and Steele, 1996; Gosden et al., 1997) with the hope of possible future autotransplantation or xenogeneic transplantation followed by in vitro oocyte maturation and fertilization (Moomjy and Rosenwaks, 1998). Also, recent attention has been given to the preservation of endangered species with the utilization of these methods in conjunction with genome resource banks (Gunasena et al., 1997; Trounson et al., 1998; Wolvekamp et al., 2001). Thus, numerous studies have recently been conducted with the ultimate goal of developing protocols for cryopreservation and transplantation of ovarian tissue (Gosden et al., 1994a,b; Oktay et al., 1998; Gunasena et al., 1997; Candy et al., 1995; Weissman et al., 1999; Shaw et al., 2000a).

Ultimately, it is desired to be able to support oogenesis in vitro. However, there is only one report of term development (in the mouse) from an embryo derived from
primordial follicles, grown to yield oocytes matured and fertilized entirely in vitro (Eppig & O’Brien, 1996). During 3 weeks it was possible to follow development from ovarian tissue to a viable embryo in vitro (Eppig & O’Brien, 1996). Xenotransplantation of currently offers a more expedient and feasible means for obtaining development of follicles in frozen-thawed ovarian tissue. Xenotransplantation not only eliminates the risks (e.g. reintroduction of cancer cells in patients in remission) but the discomforts to the patients (e.g. surgery, hormonal stimulation and oocyte recovery) involved in autotransplantation (Aubard, 1999).

Gosden et al. (1994a) demonstrated that fertility could be restored to sheep after surgical removal of the ovaries, cryopreservation of the tissue, and subsequent autotransplantation. In another study, Gosden et al. (1994b) transplanted small pieces of sheep and cat ovarian cortex tissue under the kidney capsule of 8 week old bilaterally ovariectomized severe combined immunodeficient (SCID) mice. Due to a mutation on chromosome 16, SCID mice are characterized by agammaglobulinemia and an absence of mature B and T cells (Bosma et al., 1983, 1989; Sandhu et al., 1996). These mice are therefore able to accept foreign tissue grafts without eliciting an immune response that would normally destroy the transplanted tissue. The grafts became well vascularized and developed large follicles (some reaching the antral stage) with diameters up to 3 mm. The feline ovarian tissue produced more estrogen, beginning earlier, than ovine xenografts. Although the grafts survived (nine months post-surgery), no corpora lutea or corpora albicantia were found. This was not surprising for ovine follicles since, unlike feline follicles, they did not reach preovulatory sizes. Termination of development by atresia at the 3 mm stage, or earlier, might be due to insufficient gonadotrophic stimulation
or to physical restraint placed on the xenografts by the kidney capsule (Gosden et al., 1994b). Gosden et al. (1994 a,b) also noticed that primordial follicles have a significantly higher rate of survival than those in more advanced stages. Although ovarian tissue has an abundance of angiogenic factors (Rone et al., 1993), the early period of ischemia is detrimental to advanced follicle stages. The low metabolic rate of primordial follicles (Krohn, 1977) probably allows for survival and subsequent development following vascularization.

Candy et al. (1995) transferred previously frozen marmoset ovarian tissue under kidney capsules of 19 ovariectomized outbred nude (athymic) mice; 6 recipients similarly received fresh ovarian tissue. Ovarian tissue was recovered from 14/19 mice receiving frozen grafts and from all 6 mice receiving fresh tissue between 7 and 60 days after transplantation. Histologically, the number of follicles in the grafts did not appear to decrease with increasing time after grafting. Grafts of fresh tissue contained numerically more follicles compared to frozen tissue but there were no significant differences. Interestingly, presence of large antral follicles was reported for fresh grafts removed 16 to 50 days after transplantation and for frozen grafts removed at intervals between 21 and 32 days in the absence of exogenous gonadotropins. Apparently the endogenous gonadotropins of these ovariectomized nude mice were adequate to promote follicular growth and estrogen production in 83% of recipients as evidenced by vaginal cornification (Candy et al., 1995). These workers concluded that freezing and thawing did not substantially damage the tissue and that presence of antral follicles in ovarian grafts only 7 days after transplantation confirmed that follicles in the later stages of folliculogenesis can survive freezing. It was surmised that physical constraint of the
kidney capsule, inadequate vasculature or inadequate gonadotropic support may have accounted for apparent failure of further follicle growth (Candy et al., 1995).

Newton et al (1996) reported that frozen-thawed human ovarian tissue retained >50% of its primordial follicles after 18 days after grafting in SCID mice. Better results with cryopreservation followed use of DMSO, ethylene glycol or propanediol in contrast to glycerol as cryoprotectants. Human primordial follicles isolated from cryopreserved tissue retained >70% viability confirming that primordial follicles survive the steps of freezing and thawing relatively well (Oktay et al., 1997b). Hovatta et al. (1996) also reported high viability of human primordial follicles after the slow freeze-rapid thaw protocol.

Gunasena et al. (1997a) performed allogeneic transplantation of Institute of Cancer Research-strain (ICR) mouse ovarian tissue into the ovarian bursa of ovariectomized nude mice. The mice were monitored for the interval of appearance and duration of cornified epithelial cells in daily vaginal smears. No significant differences were found in the interval or the duration of appearance of cornified epithelial cells between mice receiving allografts of fresh or frozen-thawed ICR tissue after ovariectomy and the control group (sham-operated). Mated females produced litters after receiving the sham operation (3/4; 2, 7, and 9 pups), fresh ICR tissue (2/3; 4 and 5 pups), and frozen-thawed ICR tissue (1/4; 2 pups). The pups born from mothers receiving allogeneic transplantation all developed white haircoats documenting that they were from oocytes of the transplanted ICR tissue. Histological sections of transplanted tissue revealed well-developed antral follicles in controls and both allograft types (Gunasena et al., 1997a).
In a novel study, Oktay et al. (1998b) xenografted human ovarian tissue under the kidney capsule of SCID mice also bearing a mutation for hypogonadism (hpg) in order to study primordial follicle growth. This was the first report of survival of human ovarian tissue in the SCID mouse. Oktay et al. (1998b) were able to demonstrate that initial primordial follicle development was independent of exogenous FSH stimulation by examination of tissue sections 11 weeks after transplantation. At this time, no follicles were seen with more than two layers of granulosa cells, indicating that subsequent growth was dependant upon exogenous FSH. Also, during an additional period of 6 weeks, mice were treated with either FSH or saline. At 17 weeks after transplantation, no follicles beyond the two-cell layer stage were seen in saline treated mice. However, ovarian tissue in FSH-treated mice contained follicles in the antral stage up to 5 mm in diameter (Oktay et al., 1998b).

More recently, Oktay et al. (2000) revealed by morphological criteria and proliferating cell nuclear antigen (PCNA)-staining that human primordial follicles retain their capacity to initiate growth after being frozen-thawed and transplanted in SCID mice. Frozen-thawed ovarian grafts (originally from a 27 year-old woman) retained viability and still harbored an average of 75 primordial follicles/mm³ after 22 weeks of grafting. Great variability of follicle densities even in adjacent areas (Oktay et al., 1997b) makes it inaccurate to compare absolute numbers. Rather, ratios of “resting follicles (primordial stage)/total number of follicles” were proposed by Oktay et al. (2000) as a better means for assessing reproductive potential. Thus, after thawing and grafting the ratio of primordial follicles to total follicles had significantly dropped from an average of 0.94 ± 0.02 to 0.87 ± 0.01 (P = 0.008). Similar follicle densities were seen 18 weeks (126d) after
xenografting fresh ovarian tissue (Oktay et al., 1998b). Primordial follicle loss may occur during freezing, revascularization, or follicle growth initiation. Interestingly, the percentages of growing follicles were higher in xenografts than controls.

Weissman et al. (1999) transplanted human ovarian tissue subcutaneously into SCID mice carrying a nonobese diabetic mutation (NOD). Vaginal smears of female mice receiving subcutaneous xenografts after ovariectomy revealed the presence of cornified epithelial cells at intervals not different from sham-operated mice. Transplanted tissue was recovered in 92.6% of mice; others had been reabsorbed. Of the recovered tissue, 74% had developed follicles beyond the primordial stage. Subsequent to gonadotropin stimulation, 51% of grafts possessing follicles beyond the primordial stage contained follicles that were either pre-antral or larger. Graafian follicles with diameters up to 6 mm were present and could be visualized through the skin. Weissman et al. (1999) demonstrated that male mice served as better hosts of xenografts than did females, probably due to higher circulating androgens that could be used as substrate for conversion to estrogens. Other findings included a significantly better survival rate of xenograft follicles when the tissue transport medium was kept at 37°C (81.3%) instead of on ice (28.6%) and similar development of tissues in pituitary intact and down-regulated mice (Weissman et al., 1999).

Semple et al. (2000), collected bovine ovaries, that were transported to the lab in sterile PBS (37°C). After several washes, strips of ovarian cortex were made (approx. 1 x 2 x 2 mm) and held in either PBS with 8 IU human FSH/LH or in holding medium composed of Leibovitz’s L-15 with 10% steer serum and gentamicin. The strips were either transplanted immediately into immunosuppressed mice or cryopreserved. Three to
six fragments of fresh bovine ovarian tissue were inserted into dorsal subcutaneous space above the flank of SCID or NOD SCID mice. The grafts were allowed to develop for 2-3 weeks with injection (IP) of human menopausal gonadotropin (HMG; 4 IU/d for 14 d); then the tissue was removed, fixed and stained with hematoxylin and eosin. Of 31 recipients, donor tissue survival was confirmed histologically in 29 (94%) with primordial follicles, primary follicles, secondary follicles and antral follicles observed in 25 (81%), 22 (71%), 12 (39%), and 7 (22%), respectively (Semple et al., 2000). These results were similar to those utilizing human ovarian tissue (Weissman et al., 1999).

Semple et al. (2000) also worked with frozen tissue. Bovine ovarian cortex strips (~ 1 x 1 x 2 mm) were cryopreserved in holding medium containing 1.5 M ethylene glycol in 1 ml cryovials or 0.5 ml AI straws. The vials/straws were equilibrated in a programmable freezer at 20°C for 5 min, then cooled at 1°C/min to -7°C, seeded and held for 10 min, then cooled at 0.5°C/min to -60°C and plunged in LN. At the time of transplantation the ovarian tissue was thawed in air for 10 sec followed by immersion in a 37°C H2O bath. Tissue was washed twice in holding medium and once in saline, and then transplanted as above. At 2 weeks after transplantation 3 of 10 grafts exhibited good survival of ovarian tissue including presence of primary follicles in unstimulated recipients. In grafts maintained 4 weeks, 4/6 had good primary follicles (Semple et al., 2000). These results indicated that either fresh or frozen bovine ovarian tissue can survive several weeks after transplantation into a host mouse.

There is a cryopreservation protocol of ovarian tissue that has demonstrated effectiveness in all tested species ranging from mouse to elephant. Common morphological features across primordial follicles of different species probably explain
this broad application of a single cryopreservation protocol (Shaw et al., 2000b).

Primordial follicles retain viability in tissue after cryopreservation by a slow-freeze, rapid-thaw protocol (Oktay et al., 2000). Transplantation studies have demonstrated that such follicles can develop into antral stages and even result in pregnancies in laboratory and farm animals (Oktay et al., 1998a,b).

To minimize damage resulting from lipid peroxidation during ischemia (ischemia-reperfusion injury) in ovarian grafts Nugent et al. (1998) suggested precautions to include chilling the tissue to reduce production of reactive metabolites before reperfusion as well as adding free radical scavengers, e.g. superoxide dismutase and vitamin E. The latter treatment significantly reduced lipid peroxidation on day 3 and day 7 in mouse and human ovarian xenografts, respectively. A greater total follicle survival resulted in murine grafts in the vitamin E supplemented group, i.e 72% compared to 45% for non-treated controls (Nugent et al., 1998).

In vitro follicular development

Several culture systems in rodents have been able to sustain follicular development starting from different follicle stages. In fact, each follicle size at the beginning of culture requires a specific culture system and culture period for optimal development in vitro (for review see Smitz and Cortvrindt, 2002). Eppig and O’Brien, 1996, demonstrated complete in vitro development of mouse oocytes from the primordial follicle. Only one live pup was obtained after transfer to oviducts of pseudopregnant recipients of 190-2-cell embryos resulting from in vitro fertilization (IVF) of such oocytes. Even though in vitro culture of mouse follicles has resulted in offspring (Eppig
and O’Brien, 1996), the process has low efficiency. To date the mouse remains the only species where offspring have been reported after in vitro culture of early follicles (for review see Smitz and Cortvrindt, 2002).

Optimization of these culture systems requires the recognition of interrelationships between physical, biochemical and endocrine components of the system. As an example, it is known that in vitro follicle survival is FSH-dependent, however FSH concentrations in the medium will influence the metabolic needs and oxygen requirement of follicular cells. Similarly, the presence of serum in the medium can neutralize free radicals, the generation of which is influenced by the oxygen concentration (for review see Smitz and Cortvrindt, 2002).

As with any in vitro system, it is always a challenge to perfectly reproduce physiological conditions under which to study physiological events. This is also the case with the in vitro culture of follicles. This is especially true when we consider the supply of nutrients (e.g. growth factors, oxygen, energy substrates) to a follicle that is impossible to duplicate in vitro (for review see Smitz and Cortvrindt, 2002).

In vitro culture of follicles has also been reported in larger mammalian species, such as cats, pigs, sheep and cows. A common problem in working with these larger species is related to the difficulty in isolating small follicles from ovaries due to the dense fibrous tissue that surrounds these structures. This makes it necessary to employ enzymatic treatments, mechanical means or a combination of both to harvest large amounts of follicles. Enzymatic treatments can disrupt or damage thecal or granulosa cells; the latter are essential for follicle development (Eppig, 2001). On the other hand,
mechanical means are laborious, difficult and time consuming (for review see Smitz and Cortvrindt, 2002).

In larger species, including human and cow, in vitro culture of early follicles (primordial and primary stage) constantly results in massive activation of primordial follicles and a subsequent follicular degeneration (for review see Smitz and Cortvrindt, 2002). Relatively more success has been experienced when more advanced preantral follicles (secondary stage) of large mammals have been cultured in vitro. In sheep (Newton et al., 1999; Cecconi et al., 1999) and in pigs (Hirao et al., 1994) it has been possible to grow preantral follicles in vitro and to report occasional meiotic maturation of the oocytes. In cows (Gutierrez et al., 2000) and humans (Abir et al., 1997), in vitro culture systems were able to maintain viability of late preantral follicles for long term culture, although consistent data on oocyte meiotic competence is lacking.

Assessment of Oocyte Developmental Competence after Ovarian Transplantation

Pregnancies after ovarian transplantation

Successful cryopreservation of ovarian tissue has occurred in the mouse, cat, sheep, marsupials, elephant, monkey and the cow. In humans, Newton et al. (1996), reported a significantly important percentage of follicle survival after cryopreservation of ovarian tissue. The ability to cryopreserve ovarian tissue in liquid nitrogen for prolonged periods of time makes possible to consider this method of storing ovarian tissue from cancer patients. Such tissue could then be transplanted to the patient after they reach a cancer-free status.
Restoration of reproductive cycles and fertility has been achieved after transplantation of fresh and frozen-thawed ovarian tissue in mice (Carrol and Gosden, 1993; Gunasena et al., 1997a, 1997b; Sztein et al., 1998) and sheep (Gosden et al., 1994b). Incidence of pregnancy and the litter size of ovarian transplanted animals resulting after natural mating are decreased compared to sham operated controls (Gunasena et al., 1997b). Nonetheless, live births have resulted after ovarian transplantation.

Efforts to restore normal reproductive events in the human have met limited success. Transplantation of cryopreserved ovarian strips into the pelvic area has resulted in an increase in estradiol concentrations with an associated decrease in serum gonadotropins (Radford et al., 2001). These endocrine profiles indicated that the transplanted ovarian tissue was functional. Nonetheless, nine months after transplantation ovarian failure was reestablished (Radford et al., 2001). After administration of exogenous gonadotropins, evidence of follicular development and ovulation from ovarian grafts was documented in a woman autogenously grafted (Oktay and Karlikaya, 2000).

Fertilization (IVF) after ovarian transplantation

Sztein et al. (2000), demonstrated in the mouse, that oocytes recovered from frozen-thawed ovaries were capable of undergoing in vitro maturation, fertilization and development to the blastocyst stage. When these embryos were transferred to pseudopregnant recipients at the 2-cell stage, 19% of them developed to term. In a two-step approach that included allografting and in vitro culture of isolated follicles (mouse: Liu et al., 2001), it was demonstrated that oocytes recovered from frozen-thawed ovarian
tissue could mature, be fertilized, develop to the blastocyst stage and produce offspring (after transfer of late morula or early blastocysts). In sheep, oocytes were retrieved several months after orthotopic and heterotopic grafting, from both fresh and frozen-thawed ovarian tissue fragments (Aubard et al., 1999). These oocytes could be matured and fertilized. However, none reached the blastocyst stage.

In the human, from ultrastructural studies of cryopreserved and xenografted ovarian tissue (Nisolle et al., 2000), it was concluded that these procedures did not seem to damage the ultrastructure of primordial and primary oocytes and follicles contained in the grafts. Nonetheless, the investigators emphasized the need for assessing fertilization potential and developmental capacity in subsequent studies.

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CHAPTER 3

TIME COURSE OF FOLLICULAR DEVELOPMENT AFTER BOVINE OVARIAN TISSUE TRANSPLANTATION IN MALE NOD SCID MICE

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TIME COURSE OF FOLLICULAR DEVELOPMENT AFTER BOVINE OVARIAN TISSUE TRANSPLANTATION IN MALE NOD SCID MICE

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Abstract
To preserve potential fertility many young female cancer patients are electing to have ovarian tissue cryopreserved before being exposed to chemotherapy. Xenotransplantation of this tissue into immunodeficient mice has resulted in the development of antral follicles. However, the time course of follicular development in xenografted ovarian tissue has not been appropriately characterized. The aim of this study, using a bovine model, was to determine the appropriate interval for xenotransplanted ovarian tissue to develop gonadotropin-responsive follicles. A total of 25 male non obese diabetic (NOD) severe combined immunodeficient (SCID) mice were anesthetized and pieces of newborn calf and cow ovarian tissue were transplanted in dorsal subcutaneous (s.c.) spaces. At increasing intervals after surgery mice were euthanized and grafts were recovered, fixed in Bouins and embedded in paraffin. Six-micron sections were stained with hematoxylin and eosin. Five high magnification fields (HMF, 400 X) were examined in each section. In each HMF follicles were counted and classified as primordial, primary, secondary or
Percentages of growing follicles at different intervals were analyzed by ANOVA. Differences among means were determined by protected LSD. There was an increase (P<0.05) in the proportion of primary and secondary follicles on day 55 for the cow and, on day 124 after surgery for calf tissue compared to non-grafted, and xenografted ovarian tissues recovered at previous intervals. These observed increases were accompanied by a decrease in proportions of primordial follicles. Results suggest a sudden increase in the proportion of primary and secondary follicles due to progressive development of primordial follicles. In the NOD SCID mouse bovine follicles survived xenotransplantation and underwent development. A longer time interval was required for follicular development in the calf compared to adult cow ovarian tissues after xenotransplantation of ovarian tissue.

bovine, graft interval, SCID mouse, xenotransplantation

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Introduction

Xenotransplantation of ovarian tissue into immunodeficient mice is a model for studying follicular development [1,2] and effects of cryopreservation on follicle viability [3] as well as for potential utility in preservation of fertility in cancer patients [2, 4, 5], valuable farm animals, and endangered species [6,7]. In order to become a useful tool for preservation of fertility, more information is needed regarding follicular dynamics in xenografted ovarian tissue.

During folliculogenesis, primordial follicles gradually enter a growing phase of development [8, for review see 9]. After activation, the flat pregranulosa cells acquire a cuboidal shape, primordial follicles evolve into primary follicles with all granulosa cells forming the single layer becoming cuboidal [10, 11, for review see 9, 12]. As the proliferative activity of granulosa cells increases the follicle becomes a multilayered structure. All follicles with more than one layer of cuboidal granulosa cells are called secondary follicles [for review see 9, 13]. Subsequent follicular development involves formation of an antrum [antral follicle; for review see 9, 14] and an accelerated growth rate [cow: 15, human: 16]. In contrast to small laboratory rodents, where primary follicles only require 14-16 days to reach the preovulatory stage, in other mammals complete folliculogenesis from activation of primordial follicles to the antral stage takes several months [16, 17, 18]. In the cow, folliculogenesis from the primary stage to the antral follicle requires around 12 weeks [19].

Activation of primordial follicles with entry into the pool of growing follicles followed by growth up to the early antral stage (3-6 mm in diameter) has been achieved after xenotransplantation of ovarian tissue of several species into immunodeficient
rodents, e.g. SCID mice and nude mice and rats [cat: 1 sheep: 1, cow: 20, monkey: 21, elephant: 6, marsupials: 22, and human: 2].

Nugent et al., 1998 [23] suggested that ischemic conditions present during the first 48 h after ovarian transplantation [rats: 24] results in a major loss of follicles shortly after grafting [mouse: 25], especially of the growing follicular pool [guinea pig: 26, mouse: 27, cat and sheep: 1]. Approximately half of the primordial follicles can be anticipated to survive grafting [25], probably due to their lower metabolism [28]. Therefore, any growing follicles present in grafted ovarian tissue at an interval after the grafting procedure are assumed to have been derived from primordial follicles present at the time of transplantation [1].

When follicles are exposed to adverse conditions following xenotransplantation (e.g. ischemia, different body temperature and pH and immunologic rejection reactions), follicle development and oocyte quality could be affected [19].

Although not sufficiently studied, Weissman et al., 1999 [4] found that male NOD-SCID mice were better hosts compared to female NOD-SCID mice in terms of a higher number of large follicles present in human grafts. These authors hypothesized that the higher serum concentrations of androgens present in males served as substrates for estrogen production and consequently gonadotropins were more effective in stimulating follicle development [4]. Further support for this hypothesis is found in an in vivo. Using female monkeys it was found that only after 3-10 days of treatment with androgens there was a significant increase in the number of early growing [29] or primary follicles [30]. Levels of IGF-I receptor mRNA and its ligand in primordial follicles were also
significantly increased compared to those of control animals [30]. This suggests that androgens could induce follicle activation through the IGF system [30].

Although follicle stimulating hormone (FSH) plays a role in early follicular growth [31, 32], its major role is evident at the secondary and antral follicular stages [2, 33, 34, 14] probably due to an important increase in the number of FSH receptors [35, 36] and IGF-I receptors that can amplify the action of FSH at the follicular level [34]. This gonadotropin-dependent growth phase was demonstrated in humans [2]; when ovarian tissue was xenotransplanted into immunodeficient/ hypogonadotropic mice. Follicles were unable to advance beyond the secondary stage unless stimulated with FSH. Thus, antral development is dependent on gonadotropins and the presence of gonadotropin-responsive secondary follicles

However, the time course of follicular development in xenografted ovarian tissue, as well as the time required for surviving primordial follicles in xenografted ovarian tissue to develop to gonadotropin-responsive follicles has only been studied by Van den Broecke et al., 2001 [37]. They used human ovarian tissue xenografted into the subcutaneous space of NOD-SCID mice. In this study authors speculated that FSH-sensitive follicles present at 10 and 17 weeks after transplantation were stimulated to develop further to the antral stage after exogenous FSH administration [37]. Nonetheless, from their work it was not clear why 12 and 14 week transplantation intervals did not enable ovarian tissue grafts to respond similarly to the 10 and 17 week intervals. Several authors have pointed out that the outcome of ovarian tissue transplantation studies can be influenced by the source of the donor tissue [e.g. age: 22, 38 or hormonal pretreatments: 37], the administration of exogenous gonadotropins [2], the interval between grafting and
hormonal stimulation [37] as well as the time after grafting [39]. Such factors can further complicate complex interpretations of ovarian grafting studies. More research including studies with no interference from exogenous sources of gonadotropins are needed in order to understand follicular dynamics of ovarian xenografts.

The aim of this study, using a bovine model, was to characterize follicular dynamics in bovine ovarian tissue transplanted into immunodeficient mice, in order to determine the appropriate interval for xenotransplanted ovarian tissue to develop gonadotropin-responsive secondary follicles.

**Materials and Methods**

**Mice**

A total of 22 male NOD SCID mice (5 weeks of age; Jackson Laboratories, Bar Harbor, ME) were used. Upon arrival mice were held for 3 weeks before use to allow for acclimation. Mice were maintained in a positive pressure room isolated from other animals. Cages were filter-topped, and animals had free access to gamma-irradiated food pellets and sterile water. The daily light-cycle of the room was 12h L:12h D. Animals were inspected daily. All procedures were done aseptically under a laminar flow hood in a positive pressure room.

**Bovine Ovarian Tissue for Xenotransplantation**

Fresh bovine ovarian tissue was obtained from two dairy calves (1 week of age) and one adult beef cow (2 years of age). The calves were euthanized by captive bolt and exsanguination, all 4 ovaries were recovered. A unilateral transvaginal ovariectomy was performed on the cow. Ovaries were immediately placed in Leibovitz’s L-15 medium.
(Gibco BRL, Life Technologies, Rockville, Maryland, USA) supplemented with 50 µg/mL of gentamicin (SIGMA Chemical Co., St. Louis, MO, USA) and transported at approximately 25ºC to the laboratory within 5 min. Under laminar flow and aseptic conditions ovarian cortical tissues were isolated and cut into small pieces of approximately 3mm x 3 mm x 1 mm. The resulting tissue blocks were washed repeatedly in Leibovitz’s medium and transported in the same medium at 28 ºC to a positive pressure laminar flow room for grafting into NOD SCID mice.

Transplantation

Surgical procedures were carried out under a laminar flow hood and under aseptic conditions. Mice were anesthetized using Methoxyflurane (Metofane®, Schering-Plough Animal Health Corp., Union, NJ, USA) inhalation. Dorsal areas of each mouse were shaved and the skin aseptically cleaned immediately before surgery. A skin incision in the dorsal region allowed access to the subcutaneous space. Ovarian cortical tissue blocks were placed under the skin on both sides of the incision in mice receiving calf ovarian tissue; each host received 3-4 blocks of tissue. In mice receiving cow ovarian tissue, only one block of tissue was transplanted to each. Skin incisions were closed using metal clips. Post-surgical care was routine and the clips were removed once the skin had healed. One mouse died during surgery due to anesthesia and no other post-surgical complications were observed.

Four (two from calf and two from adult cow) non-transplanted ovarian tissue blocks were histologically fixed for day 0 (fresh non-grafted) controls. At increasing intervals after transplantation (days 20, 55, 84 and 124) 1 or 2 mice from each group (calf
or cow tissue) were euthanized by carbon dioxide intoxication. Grafts were recovered from the subcutaneous spaces, counted and fixed for further processing.

Histologic Processing and Examination

Recovered grafts were fixed in Bouin’s solution for approximately 6 h and then placed in 70% ethanol and stored at room temperature until they were embedded in paraffin. Embedded grafts were serially sectioned (6 micron thick sections) for a total of 50 sections per graft. Forty sections were stained with hematoxylin and eosin and every fifth section was left unstained for immunostaining. Stained sections were examined for the presence of follicles. Five high magnification fields (HMF, 400 X) were examined per section. In each HMF follicles were counted and classified as primordial, primary, secondary or antral [adapted from 2]. Only follicles containing an oocyte with a clearly visible nucleolus were counted. Primordial follicles were also counted even in the absence of a nucleolus but in the presence of an easily distinguishable nuclear membrane. Primordial follicles had one layer of flattened granulosa cells; the presence of at least one flattened cell was sufficient. Primary follicles were those with one layer of cuboidal granulosa cells. Secondary follicles had two or more layers of cuboidal granulosa cells and antral follicles had an antrum.

Assessment of Follicular Development

Growing follicles include primary, secondary and antral follicles. Total follicles include growing plus resting (primordial) follicles. Follicular development within each tissue type (adult vs neonatal) was assessed by examination of proportions of growing
follicles (growing follicles/total follicles) at different time intervals after transplantation. Follicular development within each interval (day of recovery after transplantation: days 0, 20, 55, 84 and 124) was assessed by examination of proportions of growing and resting follicles.

Proliferating Cell Nuclear Antigen

One ovarian tissue section/graft was randomly selected and immunostained for localization of proliferating cell nuclear antigen (PCNA) from a pool of paraffin embedded unstained sections (every fifth section of recovered grafts). PCNA is a 35 kD intranuclear polypeptide essential for DNA synthesis [41] that is maximally synthesized during the G1/ S-phase of the cell cycle [42]. Antibodies against PCNA allow detection of proliferating follicular cells, as an indication of tissue (e.g. follicle) viability and growth [43, 44]. Embedded sections were deparaffinized with xylene, rehydrated in ethanol and microwaved for 8 min in citrate buffer under full power (550 watts) to enhance antigen retrieval. Endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide in water. Sections were subsequently preincubated with nonimmune goat serum (Sigma, code G-9023, St. Louis, MO, USA) for 10 min at 25 ºC. The primary antibody, mouse monoclonal antibody to PCNA (Ready-to-Use DAKO® N-series, code N1529, Carpinteria, CA, USA) was added to each section and incubated for 10 min at 25 ºC. Then, the secondary antibody, a biotinylated goat anti-mouse antibody (Biotinylated link, Ready-to-Use DAKO®, Carpinteria, CA, USA) in PBS was applied for 10 min at 25 ºC. A 10 min incubation (25 ºC) with streptavidin conjugated to horseradish peroxidase (Peroxidase, Ready-to-Use DAKO®, Carpinteria,
preceeded a final 10 min incubation (25 °C) with 3,3’diaminobenzidine (DAB in chromogen solution, LSAB®2 System, Peroxidase, DAKO, Carpinteria, CA, USA). Sections were counterstained with hematoxylin (Gill II formula) and dehydrated and mounted on slides for microscopic examination. Control sections were treated similarly but with fetal calf serum (DAKO, Carpinteria, CA) substituted for the primary antibody.

Data and Statistical Analyses

Results are expressed as proportions of different follicle categories present in fresh (ungrafted control) or recovered grafts. For each category of follicles data is tabulated as a percentage of the total number of follicles counted at each time interval. Proportions of growing follicles at different intervals within tissue type (calf or cow) were analyzed by one-way ANOVA and Tukey’s pairwise comparisons were used to compare differences among means. MINITAB for Windows, version 13.31 (Preview Systems, Inc.) was used for statistical analysis. Proportions of growing follicles were analyzed by a two-sided Fisher’s exact test to determine differences among means between calf and cow tissues at different time intervals. Differences were considered statistically significant at P ≤ 0.05.

Results

Of a total of 21 recipients (calf tissue, n = 11; adult cow tissue, n = 10) grafts were recovered from 18 mice (85.7%), regardless of the grafting time or source of the ovarian tissue (calf tissue = 9/11; adult cow tissue = 9/10). On average, 56% (30/54) of grafts were reabsorbed, and 44% (24/54) were still present at the different times of recovery. In
several instances grafts were encapsulated. Macroscopic and histological examination revealed that the surviving grafts were vascularized.

The number of follicles present per HMF was almost four times greater in fresh calf tissue than in adult cow tissue (0.39 follicles/HMF vs 0.098 follicles/HMF, respectively). Although there was much variability in the number of follicles present in each graft, even within the same time interval and from the same source, normally calf ovarian tissue grafts had a greater number of follicles after recovery compared to adult cow tissue grafts (0.17 follicles/HMF vs 0.060 follicles/HMF, respectively). In both, calf and adult cow ovarian tissue there was a reduction in the number of follicles after recovery compared to fresh controls (calf: 0.17 vs 0.39 follicles/ HMF; adult cow: 0.060 vs 0.098 follicles/HMF, respectively).

The percentages of follicles in various stages at progressive intervals after xenotransplantation of calf ovarian tissue to NOD SCID mice are found in Table 1.1. There was a significant (P< 0.01) increase in the proportion of growing (primary + secondary + antral) follicles present at 124 days after transplantation (61%) compared to non-grafted tissue (day 0: 18%) and previous intervals (day 20: 24%; day 55: 10%; day 84: 15%, Fig. 1.1). This increase was accompanied by a decrease in the proportion of primordial follicles (Fig. 1.2 A, B and C). These results suggest a sudden increase in the proportion of primary and secondary follicles due to progressive development of primordial follicles.

The development of resting (primordial) follicles to growing (primary, secondary and antral) stages in cow implants was more abrupt compared to calf (Table 1.2). By only 55 days after transplantation there was a very significant increase (P< 0.001) in the
presence of growing follicles (100% by day 55; 96% by day 84; 95% by day 124, Fig. 1.1). Of these stages, the secondary follicle population increased with increasing time intervals to become the major growing follicle population (48% by day 55; 53% by day 84; 65% by day 124). Progression in development of follicles in cow grafts is shown in Fig. 1.2 D, E and F. Interestingly, the few well developed antral follicles that were present were observed at 124 days of transplantation in the adult cow graft and on days 0, 20 and 124 in calf ovarian tissue grafts.

The percentage of growing follicles was increased (P < 0.01) in cow tissue compared to calf beginning on day 55 after transplantation (see Fig. 1.1 and 1.2). This difference was still significant in ovarian grafts removed on day 124 after surgery.

Viability of growing follicles was evidenced by the positive PCNA reaction observed in immunostained sections of calf and cow tissue at the different time intervals.
Table 3.1 Percentages of follicles in various stages at intervals after xenotransplantation of calf ovarian tissue into 5-week-old male NOD SCID mice.

<table>
<thead>
<tr>
<th>Interval</th>
<th>No. Mice</th>
<th>No. Graft</th>
<th>Follicular Stages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resting</td>
</tr>
<tr>
<td>Day 0</td>
<td>-</td>
<td>2</td>
<td>83 ± 4.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 20</td>
<td>2</td>
<td>2</td>
<td>77 ± 1.88&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 55</td>
<td>2</td>
<td>3</td>
<td>90 ± 1.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 84</td>
<td>2</td>
<td>3</td>
<td>85 ± 6.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 124</td>
<td>3</td>
<td>5</td>
<td>39 ± 6.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b and c,d</sup> Significantly different, P< 0.01.

Values ± S.E.M.
Table 3.2. Percentages of follicles in various stages at intervals after xenotransplantation of adult cow ovarian tissue into 5-week-old male NOD SCID mice.

<table>
<thead>
<tr>
<th>Interval</th>
<th>No. Mice</th>
<th>No. Graft</th>
<th>Follicular Stages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resting</td>
</tr>
<tr>
<td>Day 0</td>
<td>-</td>
<td>2</td>
<td>94 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 55</td>
<td>2</td>
<td>2</td>
<td>0 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 84</td>
<td>2</td>
<td>2</td>
<td>4 ± 4.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 124</td>
<td>3</td>
<td>3</td>
<td>6 ± 6.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b and c,d</sup> Significantly different, P< 0.001.

Values ± S.E.M.
Fig. 3.1 Summary of data from Tables 1 and 2 presented as percentages of growing follicles in adult and neonatal bovine ovarian tissues at intervals after xenotransplantation.

Comparison between tissue type within each interval; values with different superscripts are significantly different, $P < 0.01$.

Comparison between different intervals within adult tissue; values with different superscripts are significantly different, $P < 0.001$.

Comparison between different intervals within neonatal tissue; values with different superscripts are significantly different, $P < 0.01$.

Insufficient data for statistical analysis.
Fig. 3.2. Histological sections of fresh (Day 0) non-grafted and grafted bovine ovarian tissues after increasing intervals, i.e. 55 and 124 days, in male NOD SCID mouse hosts. A, B, C Calf ovarian tissue and D, E, F Adult cow ovarian tissue on Day 0, Day 55 and Day 124, respectively. On Day 0 calf ovarian tissue had a greater density of primordial follicles: compare 2A with 2D. Xenografts removed on Day 55 revealed more resting than growing follicles in calf tissue but more growing follicles in adult cow tissue: 2B Primordial and primary follicles in calf graft (Day 55); 2E Early antral follicle in adult cow graft (Day 55). Growing follicles predominated in both calf and cow grafts removed on Day 124: 2C Primary and secondary follicles in calf graft (Day 124); 2F All stages of growing follicles in adult cow graft (Day 124). (Fig. 2 Stained with hematoxylin and eosin).
Discussion

There are many restrictions to obtaining healthy ovarian tissue (i.e. with abundant follicles) from young women or rare endangered species [7]. Adams and Pierson, 1995 [40] pointed out the similarities (e.g. similar ovarian size, similar diameter of mature follicle and corpus luteum, outermost cortex containing follicles, monovular and polycyclic, etc) and advantages that cow ovaries offered as a model for study of human ovarian follicular dynamics. Many of these similarities and others are also true for other farm and wild animals. For our study, bovine ovaries offered a major advantage of availability. The information generated by this model may contribute to preservation of fertility in the women, and in species under threat of extinction or farm animals of high genetic value.

Percentage of hosts in which at least one graft was recovered (85.7%) was acceptable as in other studies more than 90 % of xenografted hosts one or more grafts were recoverable [3, 4, 22, 45, 46]. Of the total number of transplanted grafts, here on average only 40 % were recovered (60 % of grafts were reabsorbed). This contrasts with the high survival rate observed when frozen-thawed human ovarian tissue was grafted under the kidney capsule [100/ 102, 98 %, 3] or in the subcutaneous space [31/32, 97 %;37] of immunodeficient mice. Even though our tissue was fresh at transplantation, this survival difference might be explained by species differences or the site of transplantation. A greater vascularization in the kidney is speculated to favor revascularization of the graft [1]. Nonetheless, both gross and histological examinations revealed that the surviving grafts were well vascularized and were easily distinguishable
from the surrounding host tissue. Grafts had a characteristic ovoid shape and were either white or “reddish” in color.

The reduction was not detected in human tissue by Van den Broecke et al., 2001 [47], before transplantation: 0.2 follicles / HPF vs after sc transplantation: 0.4 follicles/HPF). Although this might be explained by species differences, the fact that the earlier authors used tissue from infertile women with a mean age of 33 (range from 20-39) with a low number of follicles, could have severely hindered their ability to detect any differences [47].

Under our conditions a noticeable activation of primordial follicles and their subsequent follicular development required approximately 8-18 weeks (55-124 days). Similar intervals (8 –18 weeks) after transplantation into either female SCID mice [22] or nude rats [7] were required for preantral follicles to appear and constitute an important proportion of the follicles present in ovarian grafts from marsupials (Macropus eugenii and Lasiorhinus krefftii, respectively). Xenotransplanted elephant ovarian tissue had antral follicles at 10-11 weeks after transplantation [6], while evidence for cat ovarian tissue grafts indicates that 3-4 weeks are necessary for antral follicles to develop and 8-20 weeks was required when sheep ovarian tissue was transplanted [1]. In the case of human ovarian tissue xenotransplantation, follicle development to growing stages has been reported to require 12-16 weeks [37] and, antral follicle development (0.1 – 5.0 mm in diameter) reproducibly appeared after 20 weeks post-transplantation [5]. No difference in follicular development was reported between 20, 24, 30 or 36 weeks of transplantation [5]. This difference in the time of appearance of antral follicles is difficult to explain since ovarian donors in both studies were of similar ages (between 18- and 21-years-old)
and tissue recipients were female NOD SCID mice. Nonetheless, NOD SCID female
hosts were ovariectomized and grafts were placed under the kidney capsule in the Gook
et al., 2001 [5] study, while female NOD SCID hosts were intact and the grafts were
placed in the subcutaneous space in the Van den Broecke et al, 2001 [37] study. The
hormonal treatment received by the host in the former study was a lower dose (1 IU rFSH
every second day) given for a longer period of time (≥13 weeks) than in the latter study,
(in which 5 IU rFSH/day was given for 2 weeks). Another factor that could account for,
or contribute to, the observed difference is the fact that in the former work [37] ovarian
tissue was provided by a 21 year-old transsexual androgen pre-treated female and in the
later study [5] tissue for the fresh control tissue came from a 29 year old female with
breast cancer. As found in in vivo studies in nonhuman primates [29], androgens injected
in the transsexual patient could have stimulated early follicular development accounting
for the shorter time required for appearance of antral follicles. Taken together these data
and our results show that the time span necessary for follicular development to take place
after xenotransplantation is going to vary according to species and possibly even within
species due to hormonal environment and site of transplantation.

Britt [19] calculated 85 days (12 weeks) the time required by cattle follicles to
grow from primary to the antral stages. We estimate that the interval (55-124 days or 8-
18 weeks) required in this study by bovine ovarian grafts to show a significant shift in
follicle development is within an expected range of time necessary for primordial
follicles to reach the secondary and antral stages. Here, antral follicles only appeared
after 124 days (> 17 weeks) which although greatly exceeds the time span of 85 days [19]
might be accounted by the fact that it includes the time taken by primordial follicles to be
activated and reach the primary stage, which was not accounted by Britt’s estimations. It is well known that most of the time required for folliculogenesis is consumed by the early follicular stages [16].

Recovered calf ovarian grafts had different follicular dynamics compared to the adult cow ovarian grafts. In the calf tissue, a significant proportion of growing follicles appeared only at 124 days (P<0.01, compared to day 0), which greatly exceeded the time required by adult cow tissue (55 days) to reach a significant degree of growing follicle development (P<0.001, compared to day 0). Appearance of antral follicles at 20 and 124 days after transplantation, nonetheless it is most likely that a few very small antral or advanced secondary follicles survived transplantation and gave rise to these day 20 antral follicles [5, 21]. Data could be interpreted that the difference might be due to alterations in the time and magnitude of primordial follicle activation but not in the time required for progression to antral follicle formation. It is also possible that antral follicle formation is faster in the adult group but intervals chosen in this study do not allow such determination.

It was suggested using mice ovarian tissue that the response of a 50-day-old ovary to grafting may be different from that of a 200-day-old [48]. As soon as 5 to 25 days after transplantation of ovarian homografts, older ovaries lost a greater proportion of oocytes (74-77%) compared to younger ovaries (52%). Further evidence of the influence of age of the ovarian donor on the follicular development response after grafting was revealed by the work of Cox et al., 2000 [38]. Using mouse ovarian tissue, they reported that only 10-day-old ovaries had full follicular development (including late antral follicles and corpora lutea) 8 weeks after grafting. In contrast fetal and 3-day-old ovaries had follicular
development arrested at the early antral stage during the same time period. These data offer support to our finding that age of the donor will affect the follicular dynamics of growing follicles after transplantation into the host animal.

In conclusion, a significant proportion of growing follicles developed after transplantation of fresh bovine ovarian tissue into the subcutaneous space of an intact male NOD-SCID mouse. These growing follicles include, especially in the case of the adult tissue, an important proportion of secondary follicles that would be responsive to gonadotropins. Apparently, endogenous levels of gonadotropins present in the intact male NOD SCID mice are sufficient to stimulate antral follicle development in bovine ovarian grafts. Also, the age of the ovarian tissue donor influenced the time required for growing follicles to develop. Further research is indicated for assessment of oogenesis in conjunction with folliculogenesis in this model.

References


CHAPTER 4

RECOVERY OF OOCYTES FROM BOVINE OVARIAN TISSUE TRANSPLANTED IN NOD SCID MICE

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TRANSPLANTED IN NOD SCID MICE

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Abstract

Hormonal stimulation following xenografting of ovarian tissue into
immunodeficient mice promoted antral follicle development. Efficient retrieval of
oocytes contained in these antral follicles is crucial in implementing a protocol involving
xenotransplantation for fertility preservation of cancer patients. Using a bovine model,
our objective was to recover oocytes from calf ovarian tissue transplanted into
immunodeficient mice. Fresh pieces of ovarian cortex, 1-2 mm³, from calves were placed
in subcutaneous spaces of male NOD SCID mice. Hormonal treatments were initiated 3
days after surgery. Control mice (n=3) received saline while treated mice (n=4/group)
received daily intraperitoneal injections of either 4 IU of FSH plus 4 IU of LH, 4 IU of
FSH or 1 IU of FSH for 14 days. Forty eight to 72 h after the last injection, mice were
euthanized and ovarian grafts were recovered. Serial sections of hematoxylin and eosin stained grafts were used for classifying and counting follicles. Randomly selected unstained ovarian sections were immunostained for nuclear proliferation antigen to ascertain viability. Other grafts were used for oocyte retrieval. Recovered oocytes were stained with orcein to assess maturation. Recovery of grafts (%) was no different across treatment groups. Treatment with FSH plus LH enhanced follicular development, but did not improve oocyte recovery. Evidence of maturation was only observed after incubation in vitro. Oocytes can be retrieved from xenotransplanted ovarian tissue.

follicular development, bovine, oocyte recovery, ovarian graft, Xenotransplantation

Introduction

The rescue of endangered species as well as the preservation of fertility in young human female cancer patients has renewed interest in cryopreservation and transplantation of ovarian tissue. Xenotransplantation of fresh or frozen-thawed ovarian tissue (Newton et al., 1996; Oktay et al., 1998; Weissman et al., 1999; Semple et al., 2000) into immunodeficient mice, immunotolerant of foreign tissue (Bosma and Carroll, 1991; for review see Sandhu et al., 1996), offers the advantage (Aubard, 1999; Shaw et al., 2000) for cancer patients in remission, of avoiding risk of reintroducing disease with autologous transplantation (i.e. replacement of their own ovarian tissues) after cryopreservation during active illness. Xenotransplantation also could release patients from discomforts (e.g. painful injections, hormonal screenings) and risks of hormonal overstimulation by restoring their ovarian tissue placed into tissue-hosts in which
follicular growth and development can be induced (Oktay et al., 1998; Weissman et al., 1999; Semple et al., 2000). This prospect for circumventing infertility inherent with cancer treatments holds promise providing oocytes can be obtained for assisted reproduction via in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

After xenotransplantation into an immunodeficient mouse host there is an ischemic period before revascularization of the graft (about 48 h in the rat, Dissen et al., 1994). Ischemia-reperfusion injury is most likely responsible for a drastic reduction in the follicular population of transplanted ovarian tissue (Liu et al., 2002). Most of the surviving follicles are primordial follicles and some primary follicles (Newton et al., 1996 Candy et al., 1997; Bland and Donovan, 1968; Felicio et al., 1984; Gosden et al., 1994). Some of these early stage follicles that survive are capable of developing up to the early antral stage (3-5 mm), presumably with viable oocytes, after several weeks or even months depending on the species (cat: Gosden et al., 1994, sheep: Gosden et al., 1994, cow: Semple et al., 2000, monkey: Candy et al., 1995, elephant: Gunasena et al., 1998, marsupials: Mattiske et al., 2002, and human: Oktay et al.; 1998).

Using a severe combined immunodeficient (SCID) and hypogonadotropic (hpg) mouse host, Oktay et al.(1998) clearly established that follicle development in human ovarian grafts will only develop past the early secondary stage following exogenous follicle stimulating hormone (FSH). Further work using ovariectomized immunodeficient mice also supported this concept as antral follicle development was not reached in ovarian grafts even after several months when no hormonal stimulation was given (sheep: Gunasena et al., 1997; human: Oktay et al., 2000). Nonetheless, the need for exogenous hormonal stimulation in host mice with an intact hypothalamo-pituitary axis has been
recently questioned (ovariectomized: Gook et al., 2001, Gosden et al., 1994, Candy et al., 1995, Candy et al., 1997; non-ovariectomized or intact: Cox et al., 2000), since endogenous levels of gonadotropins provided by the host mouse were apparently sufficient to stimulate antral follicle development (cow: Herrera et al., 2002). In addition to gonadotropins endogenous steroids may contribute to antral follicle development since male NOD SCID mice fostered better results in this regard than did NOD SCID female hosts (Weissman et al., 1999).

Development of antral follicles in ovarian grafts recovered from immunodeficient mice enabled the retrieval of oocytes by needle aspiration (Revel et al., 2001; Herrera et al., 2002). Oocytes thus obtained, can be matured (Herrera et al., 2002). However, the efficiency of this strategy is compromised by the prolonged time required for follicles to grow (human: Gougeon, 1996; Gougeon, 1986; cow: Britt, 1991), the loss of both grafts and follicles shortly after grafting (Liu et al., 2002) and the low number of antral follicles developed per graft, even with hormonal stimulation (Oktay et al., 1998). Previous experiences in the recovery of oocytes from human (Revel et al., 2001) and cow (Herrera et al., 2002) grafts have only resulted in 2 and 3 oocytes retrieved, respectively.

Our objectives were to employ bovine ovarian tissue in a model xenotransplantation system with male NOD SCID mouse hosts: 1) To evaluate the effect of exogenous gonadotropins on follicular development and survival; 2) To recover oocytes from bovine xenotransplanted ovarian tissue; 3) To initiate oocyte maturation in vitro after recovery from xenotransplanted grafts.
Materials and Methods

Male NOD SCID mice (n= 20, 6 weeks of age; Jackson Laboratories, Bar Harbor, ME) were hosts for transplanted bovine ovarian tissue. They were housed in a positive pressure room isolated from other animals. Cages were filter-topped, and animals had free access to gamma-irradiated food pellets and sterile water. The light: dark cycle of the room was 12 h:12 h. Fresh bovine ovarian tissue was obtained from six one-week-old dairy calves immediately after slaughter by captive bolt and exsanguination. Ovaries were placed in Leibovitz’s L-15 medium (Gibco BRL, Life Technologies, Rockville, Maryland) supplemented with 50 µg/mL of gentamicin (SIGMA Chemical Co., St. Louis, MO, USA) and transported to the laboratory within 5 min at ambient temperature (approx. 26°C). Ovarian medulla was separated from the cortex. Cortical tissue was cut into 1-2mm cubes. These blocks of ovarian tissue were washed repeatedly in Leibovitz’s medium supplemented with gentamycin (transport and wash medium) at room temperature (approx. 24 °C).

Fresh ovarian tissue blocks were transported in the above medium at 24 °C to a sterile positive pressure room for grafting into NOD SCID mice. All surgical procedures were performed under a laminar flow hood and under strict aseptic conditions. Mice were anesthetized by inhalation using Metofane® (Methoxyflurane, Schering-Plough Animal Health Corp., Union, NJ, USA). The dorsum of each mouse was shaved and the skin was cleaned with 70% ethyl alcohol and 0.05% chlorhexidine diacetate (Novalsan®, Fort Dodge Animal Health, Fort Dodge, IA, USA) for aseptic surgery. Two small skin incisions (one on each side) allowed access to subcutaneous spaces. Ovarian cortical blocks were placed under the skin through each incision. Each mouse received six blocks
of tissue (three on each side). Skin incisions were closed using metal clips; the clips were removed once the skin had healed. Post-surgical care was routine. One mouse in the control group died 3 days after surgery.

Six remaining ovarian tissue blocks not transplanted, were immediately fixed to provide day 0 controls (fresh).

Hormonal Treatments

Hormonal treatments were initiated three days after transplantation. Treatments were applied daily via intraperitoneally (0.1 ml). There were four treatment groups: Control mice (n=4) received saline while treated mice (n=5/group) received daily injections of either 4 IU of FSH (“Follistim®”, Organon) plus 4 IU of LH (“Humegon®”, Organon, West Orange, NJ, USA), 4 IU of FSH or 1 IU of FSH. Hormones were given for a period of 11-15 days.

Mice were euthanized by carbon dioxide intoxication 48 to 72 h after the last hormone injection. Grafts were recovered by dissection and representative pieces were fixed for histological study. At recovery, observations on the size, appearance, color and presence or absence of blood vessels associated with each graft were noted. At least four grafts from each treatment group was subjected to microscopic (40X) oocyte retrieval.

Oocyte Recovery

Randomly selected grafts for oocyte recovery were placed in Leibovitz’s L-15 medium supplemented with gentamycin at 24°C. Under a stereomicroscope, grafts were
carefully dissected. Whenever possible, follicles were isolated to improve oocyte recovery.

Upon microsurgical release from their follicles, the oocytes were held in Leibovitz’s L-15 medium supplemented with gentamycin at 24°C. Representative oocytes were stained with orcein (modified from Lorenzo et al., 1997) to assess maturation status. Briefly, oocytes were placed in a hyaluronidase solution (1 mg/mL) and vortexed for 5 min to remove cumulus cells. Denuded oocytes were placed on a slide under a cover slip. Oocytes were fixed in ethyl alcohol: acetic acid (3:1) for 16 h. After drying 5-10 min, staining was effected by passing orcein (1% solution) between slide and coverslip over an interval of 15 min.

Some oocytes were placed into oocyte maturation medium droplets (50 µL) for in vitro maturation (IVM) at 38.5°C under a humidified atmosphere mixture of 5% CO₂, 5% O₂ and 90% N₂ for 24 h. The maturation medium was Medium 199 (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 2.2 mg/mL NaHCO₃, 1 mg/mL polyvinyl alcohol (PVA), 50 µg/mL pyruvate, 10 mM HEPES, 0.25 mM L-glutamine, 0.1 mM cysteamine, 0.1 mM L-cystine, 50 µg/mL gentamicin, 0.1 IU/ml r-hFSH, 5.0 ng/ml r-hIGF-I. After IVM, one oocyte was stained with orcein (as above). Stained oocytes (before and after IVM) were microscopically examined at 400 X to assess the stage of nuclear maturation.

Histologic processing and examination

Grafts were fixed in Bouin’s solution for at least 6 h and then transferred to 70% ethanol and stored at 24°C until they were embedded in paraffin. Embedded grafts were
serially sectioned (6 micron). A total of 40 sections per graft were stained with hematoxylin and eosin. Follicles were counted and classified. To avoid redundancy in counting growing follicles only those primary, secondary and antral follicles with a clearly visible nucleolus were counted. Counted follicles were classified as primordial follicles (resting follicles) if they had one layer of flattened granulosa cells; the presence of at least one flattened cell was sufficient. Also, primordial follicles were included in the absence of a nucleolus if positive identification included a clearly discernable nuclear membrane. Primary follicles had one layer of cuboidal granulosa cells. Secondary (preantral) follicles had 2 or more layers of cuboidal granulosa cells and antral follicles had an antrum (Gougeon, 1986 as modified by Oktay et al., 1998) (Table 2.1). One randomly selected unstained serial section per treatment group was used for immunolocalization of proliferating cell nuclear antigen (PCNA).

Proliferating Cell Nuclear Antigen

Randomly selected ovarian tissue sections were immunostained for localization of proliferating cell nuclear antigen (PCNA). PCNA is a 36 kD intranuclear polypeptide essential for DNA synthesis (Xiong et al., 1991) that is maximally expressed during the G2-phase of the cell cycle (Liu et al., 1989). Follicle viability was indicated by a positive antibody response against PCNA, i.e. detection of proliferating follicular cells (following instructions of DAKO LSAB® System Instructions booklet, Carpinteria, CA, USA). Briefly, embedded sections were deparaffinized with xylene, rehydrated in ethanol and microwaved for 8 min in citrate buffer under full power (550 watts) to enhance antigen retrieval. Endogenous peroxidase activity was blocked by incubating the sections with
3% hydrogen peroxide. All incubations were at room temperature (24°C). Sections were preincubated with nonimmune goat serum for 10 minutes prior to addition of mouse monoclonal antibody (primary antibody) to PCNA (Ready-to-Use DAKO® N-series, code N1529, Carpinteria, CA) for 10 min. The secondary antibody, a biotinylated goat anti-mouse antibody (Biotinylated link, Ready-to-Use DAKO®, Carpinteria, CA) was applied for 10 min followed by 10 min incubation with streptavidin conjugated to horseradish peroxidase (Peroxidase, Ready-to-Use DAKO®, Carpinteria, CA) and a final 10 min incubation with 3,3′diaminobenzidine (DAB in chromogen solution, Ready-to-Use DAKO®, Carpinteria, CA). Sections were counterstained with hematoxylin (GILL II hematoxylin) and dehydrated and mounted on slides for microscopic examination. Control sections were similarly treated but with fetal calf serum instead of the primary antibody (DAKO, Carpinteria, CA). Presence of a red-color end product in the nucleus of granulosa cells and/or the nucleus of the oocytes indicated a positive reaction (DAKO LSAB® System Instructions booklet, Carpinteria, CA, USA).

**Assessment of Follicular Development and Survival**

Growing follicles included: primary, secondary and antral follicles (Oktay et al., 1998). Primordial follicles were considered to be resting follicles (Table 2.1).

Follicular development was assess by examination of the proportion of growing follicles (No. of growing follicles/ total No. of follicles counted) present at the time of graft recovery. Follicular viability was confirmed by PCNA staining (described above).
Statistical Analyses

Results are expressed as percentages of resting and growing follicles present in grafts recovered from host mice that received different hormonal treatments. Percentages were analyzed by ANOVA. Protected LSD determined differences among means. Differences were considered statistically significant at P ≤ 0.05. SAS 8.2 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

Table 4.1 Classification of ovarian follicles

<table>
<thead>
<tr>
<th>Follicle type</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial*</td>
<td>1 layer of flat or a combination of flat and cuboidal pregranulosa cells</td>
</tr>
<tr>
<td>Primary+</td>
<td>1 layer of only cuboidal granulosa cells</td>
</tr>
<tr>
<td>Secondary+</td>
<td>2 or more layers of cuboidal granulosa cells</td>
</tr>
<tr>
<td>Antral+</td>
<td>Presence of fluid filled antral cavity</td>
</tr>
</tbody>
</table>

*a As modified by Oktay et al., 1998 from Gougeon, 1986.

* Resting follicles

+ Growing follicles
Results

Graft and oocyte recovery

Thirty-seven percent (42/114) of all grafts were recovered. At least one of the six grafts was recovered from each host. Recovery of grafts was similar across all groups (Table 2.2). All recovered grafts evidenced vascularization, macroscopically and histologically by vascular endothelium and presence of red blood cells.

On average 2.6 oocytes/host (50 oocytes recovered from 19 hosts; see Table 2.2) and 1.2 oocytes per graft were recovered (50 oocytes from 42 grafts; Table 2.2). Oocytes were more easily retrieved from grafts in FSH/LH treated mice. All seven oocytes stained with orcein immediately after recovery were immature, i.e. germinal vesicle stage. After 24 h of IVM one of the cultured oocytes (control group) showed signs of maturation (metaphase plate) after staining with orcein.

Hormonal treatment was not required for antral follicle development, as evidenced by the presence of a large percentage of antral stage follicles in the group that did not receive gonadotropin treatment. Fifty six percent of growing follicles in the control group were antral compared to hormone stimulated groups (FSH:LH= 11%; FSH 4IU= 0%; FSH 1IU= 4%). The FSH:LH treatment had a significant (P<0.05) effect on improving development of growing follicles (Table 2.3).
Table 4.2 Recovery of oocytes from calf ovarian tissue at 17 days after xenotransplantation into 6-week-old male NOD SCID mice.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. of hosts</th>
<th>No. grafts recovered/transplanted</th>
<th>No. oocytes recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>10/24</td>
<td>15</td>
</tr>
<tr>
<td>4 IU FSH/LH</td>
<td>5</td>
<td>11/30</td>
<td>17</td>
</tr>
<tr>
<td>4 IU FSH</td>
<td>5</td>
<td>11/30</td>
<td>6</td>
</tr>
<tr>
<td>1 IU FSH</td>
<td>5</td>
<td>10/30</td>
<td>12</td>
</tr>
<tr>
<td>Totals</td>
<td>19</td>
<td>42/114</td>
<td>50</td>
</tr>
</tbody>
</table>

*Hormone treatments started 3 days after xenotransplantation and were administered daily IP for 15 days prior to recovery of the grafts.
Table 4.3 Resting and growing follicles in calf ovarian tissue xenotransplants recovered from 6-week-old male NOD SCID mice after 15-day hormonal treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentages of total follicles (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting*</td>
</tr>
<tr>
<td>Control z</td>
<td>80 ± 15.27 (49)a</td>
</tr>
<tr>
<td>FSH:LH z 4 IU</td>
<td>53 ± 13.33 (8)b</td>
</tr>
<tr>
<td>FSH z 4 IU</td>
<td>84 ± 15.53 (31)</td>
</tr>
<tr>
<td>FSH z 1 IU</td>
<td>68 ± 14.29 (101)</td>
</tr>
</tbody>
</table>

* Primordial follicles
† Includes follicles in primary, secondary and antral stages.

a,b and c,d Different suscripts within columns denote differences, P< 0.05; Values ± S.E.M.

Data include one mouse per group following 11 days of treatment.
Discussion

We were able to retrieve at least one graft from each host. Nonetheless, in contrast with previous work, wherein more than 90% of recipients’ grafts were recovered (Semple et al., 2000; Mattiske et al., 2002; Newton et al., 1996; Weissman et al., 1999; Oktay et al., 2000), only 42 (36.8%) of 114 grafts originally placed in subcutaneous spaces survived xenotransplantation. On average, 2.2 of 6 grafts were recovered from each recipient. Better graft survival (100/102, 98%) was reported when human ovarian tissue was placed under the kidney capsule of immunodeficient mice (Newton et al., 1996). The kidney therefore may provide a better site. This is probably due to increased vascularization (Sherwood, 1989) and provision of cytokines to improve graft survival (Jakeman et al., 1992). However, comparison of the subcutaneous and renal sites did not reveal a difference in terms of egg survival within ovarian tissue transplanted between rats (Deanesly, 1957).

Oocyte harvest from the recovered grafts in the present study represented an improvement (50 recovered oocytes/ 19 hosts= 2.6 oocytes/ host, or 50 recovered oocytes/ 42 recovered grafts= 1.2 oocytes/graft; see Table II) compared with previous studies where no more than three oocytes were recovered (human ovarian tissue in nude mice, Revel et al., 2001: 2 oocytes recovered/ 9 grafts= 0.22 oocytes/graft ; cow ovarian tissue in Nude mice: Herrera et al., 2002: 3 recovered oocytes/8 hosts= 0.38 oocytes/host). This might be due to the fact that in those previous studies only aspiration with a needle was the recovery method. Our more aggressive recovery approach, in which careful dissection ensured isolation of even the smallest antral follicles, was apparently more appropriate.
As in previous work using human ovarian tissue (Revel et al., 2001), all seven oocytes recovered from xenografts and evaluated after staining were immature. Only Herrera et al., 2002, using cow ovarian tissue, has previously reported evidence of in vitro maturation of oocytes retrieved from xenografts. These authors found that extrusion of the polar body was evident in all three recovered oocytes 24 or 48 h after IVM at 38°C under 5%CO₂ in air. Under our IVM conditions, the only oocyte that was evaluated after a 24 h incubation period showed evidence of maturation. However, further research is indicated to assess fertilizability and developmental capacity of such oocytes.

To achieve large antral follicle development in ovarian grafts FSH is apparently essential as in human ovarian tissue xenotransplanted into immunodeficient/hypogonadotrophic mice, where follicles were unable to advance beyond the secondary stage unless stimulated with FSH (Oktay et al., 1998). In our bovine model, only the combined administration of FSH and LH induced enhanced follicular growth compared to controls. Earlier in SCID mice xenotransplanted with bovine (Semple et al., 2000) or human (Oktay et al., 1998, Weissman et al., 1999) ovarian tissue, treatments with FSH-like compounds with traces of LH activity were effective in stimulating follicular development. Even though follicular development can be induced solely by FSH administration after xenotransplantation (Gook et al., 2001, Van den Broecke et al., 2001), ovarian endocrine abnormalities have been reported when FSH is given alone to patients that lack endogenous gonadotropins (Couzinet et al., 1988; Shoham et al., 1991; Schoot et al., 1992, 1994; Balasch et al., 1995; Fox et al., 1997). Some LH is required to optimize follicular maturation and ovulation (Couzinet et al., 1988; Burgués et al., 2001;
Balasch et al., 1995). The latter consideration may help to explain our improved retrieval of oocytes from grafts recovered from FSH/LH treated mice.

References


CHAPTER 5

EFFECT OF SITE OF TRANSPLANTATION ON FOLLICULAR
DEVELOPMENT IN HUMAN OVARIAN TISSUE TRANSPLANTED INTO
INTACT AND CASTRATED IMMUNODEFICIENT MICE

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EFFECT OF SITE OF TRANSPLANTATION ON FOLLICULAR DEVELOPMENT OF HUMAN OVARIAN TISSUE TRANSPLANTED INTO INTACT AND CASTRATED IMMUNODEFICIENT MICE

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The University of Georgia, Athens, Georgia, U.S.A.

Abstract

Objective: To study the response of human ovarian xenografts to transplantation into different sites and in different host conditions.

Design: Controlled experiment.

Setting: Academic research laboratory, Department of Physiology and Pharmacology, The University of Georgia, Athens, Georgia, U.S.A.
Intervention(s): Human ovarian cortical pieces were transplanted either under the kidney capsule or in the subcutaneous space of intact or castrated male non-obese diabetic (NOD) severe combined immune-deficient (SCID) mice. Grafts were recovered following euthanasia.

Main Outcome Measure(s): Histological analysis and serum estradiol concentration measurements.

Result(s): After six months post transplantation, ovarian grafts transplanted under the kidney capsule of intact male mice had significantly (P<0.05) higher follicular development compared to the castrated/ kidney capsule and the intact/ subcutaneous groups. However, no difference was detected between the intact/ kidney capsule and the castrated/ subcutaneous groups. Serum estradiol concentrations, on average, were non-significantly increased in mice with ovarian grafts compared to mice without a graft.

Conclusions: Follicular development in xenotransplanted human ovarian tissue can be influenced by the condition of the host and the site of transplantation.

Introduction

Chemotherapeutic drugs for cancer treatment, especially alkylating agents, can produce infertility (e.g. primary ovarian failure or irregular ovarian activity) (1, 2). Frozen semen is effective to preserve future fertility in one third of male patients that returned to use their semen after chemotherapy (3). Freezing of human oocytes has encountered limited success (4, 5, for review see 6, 7). The ability to cryopreserve ovarian tissue as demonstrated in many species e.g. human, sheep and, rat (8-10), is an alternative for young female cancer patients faced with sterilizing treatments (6, 11).
Unlike collection of oocytes and production of embryos, in which hormonal stimulation and close monitoring of the patient is necessary, ovarian biopsy can be expediently done without delaying cancer treatment. Follicles in human ovarian tissue survive cryopreservation and are viable after thawing (10). Primordial follicles are more likely to survive cryopreservation (mouse: 9, 12) and grafting (cat and sheep: 13) while later stages disappear shortly after grafting (6). This higher survivability of primordial follicles is probably explained by their lower metabolic rate (14). Experiments where either fresh or frozen-thawed mammalian (human: 15, 16; cow: 17; cat and sheep: 13; elephant: 18; monkey: 19) ovarian tissue has been transplanted into immunodeficient (e.g. nude, severe combined immunodeficient) mice (xenotransplantation) have successfully developed antral follicles presumably derived from primordial follicles.

One of the preferred sites for grafting of ovarian tissue into tissue hosts has been under the kidney capsule. This transplantation site has been chosen due to its rich vascularization and for appropriately securing grafts in place, although, the pressure that the capsule exerts on the grafts to secure them might be responsible for limiting the expansion of growing follicles (13). Weissman et al., 1999 (16) examined the subcutaneous space as a transplantation site with the thought it would provide adequate room for follicle development to occur. Nonetheless, antral follicles did not surpass 6 mm in diameter (cat and sheep: 13; monkey: 19; human: 16, 20). Thus, the kidney capsule did not have any advantage as a transplantation site for ovarian tissue (8). It has been suggested that the site of transplantation is not critical, since even in the subcutaneous space re-vascularization quickly occurs (16).
Follicular development is enhanced in the absence of the host’s own ovaries (21). Wiessman et al., 1999 (16), compared immunodeficient female and male mice (i.e. NOD SCID mice) as recipients of human ovarian tissue transplanted to the subcutaneous space. After exogenous ovarian stimulation, males had significantly more large follicles than females. Nonetheless, given the nature of the endocrine environment of the male, the influence of the host’s gonads in terms of follicular development in ovarian grafts needs to be addressed.

Using male NOD SCID mouse hosts for human ovarian xenografts, our objectives were: a) To compare the influence of the site of transplantation of ovarian tissue (kidney capsule vs subcutaneous space) on follicular survival and development; b) To compare the influence of the condition of the host (intact vs castrated) on follicular survival and development.

**Materials and Methods**

**Animals**

Male intact NOD SCID mice (n= 16, Jackson Laboratories, Bar Harbor, ME) were hosts of human ovarian xenotransplants. These animals were housed in an isolated positive pressure room. Cages were filter-topped, and animals had free access to gamma-irradiated food pellets and sterile water. The light:dark cycle of the room was 12L:12D. After arrival at the facility mice were allowed to acclimate for at least 6 weeks before their use.
Source of Human Ovarian Tissue

Following approval from the Institutional Review Board of The University of Georgia and Informed Consent prior to cancer chemotherapy two patients (ages 20 and 22) donated ovarian tissue.

Human ovarian tissue was obtained by biopsy. The ovarian cortical tissue (approx. 4 mm$^3$ pieces) was frozen following Newton et al., 1996 as modified by Oktay et al., 1998 and maintained in liquid nitrogen (-196 °C) until use. Thawing was carried out by placing the cryovial containing tissue in water at 25°C. Thawed pieces of tissue were washed twice in Leibovitz’s L-15 media supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, USA) and gentamicin (50 µg/mL; SIGMA CHEMICAL Co., St. Louis, MO, USA). They were then cut into smaller pieces i.e. approximately 2 mm with 1 mm of thickness.

Experimental Design

The effect of the condition of the host mice (intact vs castrated) as well as the site of transplantation (kidney capsule vs subcutaneous space) on follicular development of human ovarian grafts were evaluated.

All host mice (n=16) received 2 ovarian grafts each. The site of transplantation of the grafts as well as the gonadal condition of the male host varied: a) intact host/kidney capsule (n= 5); b) intact host/subcutaneous (n= 5); c) castrated host/ kidney capsule (n=3) and d) castrated/ subcutaneous (n= 3). Grafts were recovered after 190 days of xenotransplantation, following euthanasia by CO$_2$ toxicity.
Four mice (two intact and two castrated) were used as controls and did not receive ovarian grafts.

Surgery

Surgical procedures were carried out under a laminar flow hood under aseptic conditions. Mice were anesthetized using Methoxyflurane (Metofane®, Schering-Plough Animal Health Corp., Union, NJ, USA) by inhalation. Dorsal areas of mice were shaved and the skin aseptically cleaned immediately before surgery. A small skin incision allowed access to the subcutaneous space. Ovarian cortical tissue blocks (n= 2) were placed under the skin on both sides of the incision. To access the kidneys, a small incision on the abdominal musculature on each side behind the last rib was made, kidneys were exteriorized and a pocket was made by rupturing and separating the capsule from the surface of the kidney. A graft (n= 1) was placed under the kidney of each kidney. Occasionally rupture of the kidney capsule would limit the number of transplanted grafts to only one per host. Skin incisions were closed using metal clips. Post-surgical care included daily observation of mice for any signs of disease and removal of clips once the skin had healed. Castration was performed 10 days before transplantation of ovarian tissue.

Histologic Examination of Ovaries

In both experiments, recovered grafts were examined and fixed in Bouin’s solution for a minimum of 6 hours before being stored in 70% ethanol until they were embedded in paraffin (1-3 days later). Fifty serial sections (6 microns thick) were made
from each embedded ovarian graft. Most sections (n= 40) were stained with hematoxylin (Gill II hematoxylin) and eosin while every fifth section was left unstained and randomly selected for proliferating cell nuclear antigen (PCNA) detection. Five high magnification fields (HMF) of 400 X were examined in each stained section. Follicles were counted and classified as either resting (primordial) or growing (primary, secondary and antral) follicles. Primordial follicles had one layer of flattened, or a combination of flattened and cuboidal pregranulosa cells. Among the growing follicles, primary follicles had 1 layer of all cuboidal granulosa cells; secondary follicles had 2 or more layers of cuboidal cells and antral follicles presented a fluid filled antral cavity. To avoid counting follicles more than once, growing follicles were only counted in the section where the nucleolus was clearly visible. Resting follicles were counted even in absence of a nucleolus when a distinguishable nuclear membrane was present.

Both follicle development and survival were assessed by examination of the percentage of growing follicles (primary + secondary + antral) / total number of follicles (primordial + primary + secondary + antral) and PCNA immunostaining, respectively.

Proliferating Cell Nuclear Antigen

Randomly selected unstained ovarian tissue sections were immunostained for detection of PCNA. Paraffin embedded sections were deparaffinized by immersion in xylene and rehydrated in ethanol. In order to enhance antigen retrieval, sections were microwaved at 550 watts for 8 min in citrate buffer. To block endogenous peroxidase activity the sections were incubated with nonimmune goat serum (SIGMA, St. Louis, MO, USA) for 10 min at 25°C. Incubations with the primary (mouse monoclonal
antibody, Ready-to-Use DAKO® N-series, code N1529, Carpinteria, CA, USA) and secondary antibodies (Biotinylated link, Ready-to-Use DAKO®, Carpinteria, CA, USA), as well as with the peroxidase (horseradish peroxidase, Ready-to-Use DAKO®, Carpinteria, CA, USA) and the 3,3’-diaminobenzidine (DAB in chromogen solution, LSAB®2 System, Peroxidase, DAKO, Carpinteria, CA, USA) were carried out according to the DAKO LSAB® System Instructions booklet, Carpinteria, CA, USA. These sections were counterstained with hematoxylin (Gill II formula).

Estradiol Determination

On the day of graft recovery, before euthanasia, mice were anesthetized by inhalation of Methoxyflurane and 200-1000 µL of blood was obtained by cardiac puncture. Blood was allowed to clot at room temperature (24°C) for 30-60 min and then centrifuged to collect serum. Serum was stored at -20°C until assayed for estradiol. Estradiol was determined by a radioimmunoassay (RIA) procedure previously validated in blood samples of dogs, horses, cattle (Reimers et al., 1991) and rats (Lamb, 2000).

Statistical Analyses

Percentages of growing follicles (Table 3.2) in each treatment group were analyzed using a one-way ANOVA and comparisons among treatments were tested using Tukey’s pair-wise comparisons.
Results

When follicular development was represented as percentages of follicles that were growing, the intact/ kidney capsule group yielded a greater proportion of growing follicles (59%) than the castrated/ kidney capsule group (7.2%) (P< 0.05) (Table 3.1). Follicle survival across treatments was not different. Growing follicles in randomly stained sections showed a positive PCNA reaction.

Follicle diameters were as large as 15 mm in a graft recovered from a castrated/ subcutaneous mouse. Seven to 9 mm follicles were also found in two grafts recovered from two intact/ kidney capsule mice and one graft from an intact/ subcutaneous mouse.

Estradiol levels in serum of mice that had received ovarian grafts were not different across treatment groups (Table 3.1) hence data were pooled. On average estradiol levels in intact and castrated control mice (without ovarian grafts, 41.59 ± 9.40 pg/mL) were not different compared to levels found in grafted mice (342.46 ± 187.59 pg/mL).
Table 5.1 Resting and growing follicles present in human ovarian grafts recovered after transplantation into different sites of intact and castrated 12-week-old male NOD SCID mice.

<table>
<thead>
<tr>
<th>Testes/Graft Site</th>
<th>No. Resting follicles (%)</th>
<th>No. Growing follicles (%)</th>
<th>Totals (%)</th>
<th>Estradiol (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/SC</td>
<td>30 (61 ± 17.71)</td>
<td>19 (39 ± 17.71)</td>
<td>49 (100)</td>
<td>399.24 ± 339.24</td>
</tr>
<tr>
<td>I/KC</td>
<td>12 (41 ± 14.28)a</td>
<td>17 (59 ± 14.28)a</td>
<td>29 (100)</td>
<td>118.96 ± 18.63</td>
</tr>
<tr>
<td>C/SC</td>
<td>15 (68 ± 35.71)</td>
<td>7 (32 ± 35.71)</td>
<td>22 (100)</td>
<td>810.19 ± 711.81</td>
</tr>
<tr>
<td>C/KC</td>
<td>64 (92.8 ± 2.82)b</td>
<td>5 (7.2 ± 2.82)b</td>
<td>69 (100)</td>
<td>41.39 ± 2.73</td>
</tr>
</tbody>
</table>

Significant difference (P< 0.05) between groups is indicated by different superscripts a,b.

(Values ± S.E.M.)
**Fig 5.1** Human ovarian graft recovered from subcutaneous space of a castrated male NOD SCID mouse host. Large antral follicles are present; the most prominent follicle on the right was 15 mm in diameter.
Fig 5.2 Human oocyte recovered from ovarian graft. Oocyte is surrounded by cumulus cells.
Discussion

In contrast to previous reports (transplantation site: 8, 16) our data revealed a significant difference in follicular development after ovarian tissue xenografting to different transplantation sites (intact/kidney capsule, Table 3.1). It is clear from this study and other reports (primates: 19) that the hosts’ endogenous gonadotropin levels are sufficient to support antral follicle development in xenotransplanted grafts.

Results also suggest a difference between intact and castrated male hosts (intact/kidney capsule vs castrated/kidney capsule and castrated/subcutaneous vs intact/subcutaneous, Table 1 and intact/kidney capsule vs castrated/kidney capsule, Table 3.1).

Although estradiol concentrations were numerically greater in grafted mice compared to mice with no graft, there was no difference. Other studies have found that xenografts can greatly increase the serum estradiol concentrations of host mice (grafted) compared with ovariectomized non-grafted (13) or intact non-grafted (20) mice. An important finding is that, for the first time, follicles larger than 6 mm in diameter (15 mm) were found after xenotransplantation of ovarian mammalian tissue. This is especially noteworthy since grafting conditions in this model have allowed development to a size very close to the ovulatory size of human follicles (20mm, 19). In agreement with Weissman et al., 1999 (16), this report supports the use of males as hosts of human ovarian tissue. The biggest follicle measured was found in a graft recovered from the subcutaneous space, which, as suggested by Weissman et al., 1999 (16), provides more room for antral follicles to grow compared to the kidney capsule. Further research is
needed to better characterize optimal conditions for development of ovarian grafts in male NOD SCID mice.

References


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CHAPTER 6

CONCLUSIONS
CONCLUSIONS

This research has revealed that after transplantation of bovine and human ovarian tissue into NOD SCID mice, follicles will grow and develop into primary, secondary and antral follicular stages. It was established that the age of the ovarian donor animal could influence the interval necessary for the follicles to be activated and start growing. Xenografted adult bovine ovarian tissue required less time for follicle development to begin when compared with the calf tissue.

Recovery of oocytes from xenografted bovine ovarian tissue was shown to be feasible and oocyte maturation in vitro also was shown to be possible although a greater number of oocytes must be studied. The role of exogenous gonadotropins in follicular development, needs to be reexamined. Under our conditions, gonadotropins do not appear to be essential in terms of antral follicle development and oocyte recovery.

The site of ovarian tissue transplantation, as well as the presence or absence of the hosts’ testes are important factors that can influence the development of follicles in human ovarian grafts and should be considered when choosing xenotransplantation as a strategy for recovery of fertility in young female cancer patients.

Using this male mouse model for transplantation of human ovarian tissue we were able to obtain the largest follicle reported to date. A 15 mm antral follicle was obtained from the subcutaneous space of a castrated mouse host. Previous studies in many different species had not reported antral follicles larger than 6 mm in diameter. This argues for a better environment for follicular development offered by this model.

The male NOD SCID mouse model needs to be further studied and promises to be very useful not only in the development of strategies for preservation of fertility in young
female cancer patients and endangered species, but also for investigating the role of androgens in follicular development.