Methodology and characterization of an *in vitro* perfusion model for the mouse ovary

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Perfusion of intact ovaries *in vitro* has proved to be a suitable model for the study of ovarian events, such as ovulation and corpus luteum function, with advantages over cell culture systems of preserved three-dimensional structure with intact intercellular communication. This methodology has been described for several larger experimental animals. Development of genetically manipulated mouse strains and the larger availability of recombinant proteins and monoclonal antibodies in this species prompted the development of a mouse ovary perfusion system. In the present study, we describe the methodology for perfusion of mouse ovaries *in vitro* and characterize the model with respect to steroidogenesis, ovulatory efficiency and morphology. Swiss mice (30–45 g) were synchronized with a single injection of a GnRH agonist and laparotomy was performed on the morning of pro-oestrus. The right ovary and its vasculature, after cannulation on the arterial side, were surgically isolated and transferred to a recirculating perfusion system, and were perfused for 20 h. The ultrastructure of the ovaries after 20 h of perfusion was examined by transmission electron microscopy, which showed well-preserved cellular organelles and nuclei and distinct tight junctions between endothelial cells. Unstimulated ovaries did not ovulate and secreted small quantities of progesterone. After addition of hCG (50 iu) or ovine LH ($0.1 \mu\text{g ml}^{-1}$), ovulations were observed in all perfused ovaries (2.6 ± 0.5 and 2.0 ± 0.3 ovulations per treated ovary, respectively). There was a distinct rise in progesterone output after hormone addition. When a phosphodiesterase inhibitor, isobutylmethylxanthine ($0.2 \mu\text{mol l}^{-1}$), was added in combination with LH, the ovulation rate (7.4 ± 1.2) was similar to that *in vivo* and progesterone secretion was further increased compared with other treatments. Since the mouse has become a very important experimental animal in medical research and we have the ability to genetically manipulate this species, this methodology for perfusing mouse ovaries *in vitro* may be a useful tool in future studies of ovarian physiology.

Introduction

Perfusion of mammalian organs *in vitro* has been extensively used for studies of whole organ functions. A short-term *in vitro* perfusion methodology to study steroidogenesis in human ovaries was reported more than 30 years ago (Romanoff and Pincus, 1962). This technique was later adopted for longer term (> 10 h) *in vitro* perfusions of human (Stähler *et al.*, 1974), rabbit (Janson *et al.*, 1982) and rat (Brännström *et al.*, 1987) ovaries to study physiological processes, such as ovulation (Brännström and Janson, 1991) and the regulation of corpus luteum function (Sogn *et al.*, 1984). This method has also proved to be the only consistent method for inducing ovulation *in vitro* (Brännström and Janson, 1991). Furthermore, oocytes ovulated during *in vitro* perfusion of rabbit ovaries successfully underwent fertilization and normal pregnancies were established (Kobayashi *et al.*, 1983).

Growth factors and cytokines are known to play important roles in ovarian physiology (Adashi *et al.*, 1985; Brännström and Norman, 1993). With the development of recombinant proteins, monoclonal antibodies with neutralizing capacity and genetically manipulated animals, it has become increasingly possible to study the action of growth factors and cytokines. These experimental tools are commonly available for the mouse, whereas they are less readily available for rats and rabbits. Moreover, the availability of inbred mouse strains with special characteristics and the present knowledge of mouse genetics are much greater than those of any other animal (Paigen, 1995).

Possibly owing to the small size of the mouse and the extensive surgery involved in preparation of the isolated ovary, there are no published reports on perfusion of the mouse ovary to study metabolic and functional events. In this study we report, for the first time, the methodology involved in the successful *in vitro* perfusion of the mouse ovary with induction of ovulation *in vitro*.

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Materials and Methods

Hormones and chemicals

GnRH agonist (des-Gly¹⁰, D-Ala⁶), 3'-isobutyl-1-methylxanthine (IBMX), and gentamycin sulfate were purchased from Sigma (St Louis, MO). Ovine luteinizing hormone (NIH-LH-o25) was provided by NIDDK (Bethesda, MD) and the National Hormone and Pituitary Program (University of Maryland School of Medicine, Baltimore, MD). Bovine serum albumin (fraction V) was from Boehringer (Mannheim), heparin sulfate from Fisons (Thornleigh, NSW), insulin from NOVO (Copenhagen) and hCG from Organon (Oss, The Netherlands).

Animals

Female, unmated Swiss mice (University of Adelaide Central Animal Facility) of 4–6 months age (weighing 30–45 g) were kept under controlled conditions (14 h light:10 h dark) and fed water and pellets *ad libitum*. During the study, it became obvious that the leaner animals (< 35 g) were most suitable for surgery, owing to less intra-abdominal and retroperitoneal adipose tissue. The oestrous cycles of animals were synchronized using a modification of a procedure described by Vickery and McRae (1980); 20 µg of a GnRH agonist was injected i.p. at 12:00 h and mice were checked for pro-oestrus vaginal smears 3 days later. At this time, 80% of the animals exhibited pro-oestrus vaginal smears and this cycle was further confirmed by the presence of uterine fluid accumulation (Armstrong, 1968). Six animals were set aside and allowed to ovulate spontaneously *in vivo*. They were killed at oestrus and the number of oocytes in the ampullary region of the oviduct was counted under a microscope. The ovulation rate per ovary in these animals was 7.8 ± 2.3 .

Surgical technique

The surgical procedure used was a modification of the preparation of the rat ovary as described by Brännström *et al.* (1987) but with major differences due to the different vascular anatomy (Fig. 1) and the smaller size of the mouse.

On the morning of induced pro-oestrus, the mice were anaesthetized with an i.p. injection of ketamine:xylazine (60:10 mg⁻¹ body mass) followed by an i.p. injection of heparin (100 iu per animal). The right ovary with its vasculature was then isolated surgically. As the ovarian arteries of the mouse are too small to cannulate easily, the aorta was cannulated, with appropriate ligatures to direct the perfusion medium to the ovary. Only the right ovary from each mouse could be used, owing to the branching of the ovarian artery directly from the aorta on this side, as opposed to from the renal vessels on the left side (Fig. 1). The space in between the branching of the ovarian vessels and the kidney on the left side is too small to allow ligation without compromising ovarian flow.

The abdomen of the mouse was opened by a midline incision from the xiphoid process to the symphysis pubis. At this stage, the uterine horns were inspected for accumulation of fluid within the lumen of the uterus to ensure a pro-oestrous

Fig. 1. Illustration of the vascular anatomy of the mouse with ligatures indicated by dotted lines. ●, point of cannulation; ▲, position of catheter tip.

stage (Armstrong, 1968). The distal mesenteric artery was clamped and then cut between the clamp and the colon. The distal part of the colon was double ligated and resected in between ligatures followed by separation of the intestines from the retro-abdominal wall through dissection through the dorsal mesentery. Ligation of the left renal and suprarenal vessels was then performed, followed by excision of the left kidney. The left ilio-lumbar artery and vein were dorsal to the renal vessels and were subsequently double ligated and severed. The renal capsule on the right side was slit open by gentle pulling with forceps, followed by ligation of the vessels and excision of the kidney.

The right uterine branch of the ovarian artery and the uterine horn were ligated at the utero-tubal junction. By gentle dissection on the left side of the aorta and vena cava, three or four unpaired dorsal lumbar arteries were identified and then ligated. The accessible parts of these vessels were short and did not allow double ligation. Severance of the vessels was performed distal to the single ligatures at a later stage, towards the end of the operation. The vena cava and aorta were ligated just cranial to the iliac bifurcation, and cranial to this, a second suture was placed under the vena cava. The aorta was then tensioned by pulling the suture at the bifurcation and the aorta was cannulated in a retrograde direction with a 20G Teflon

catheter; the tip of the catheter was advanced to a position 2 mm from the branching of the right ovarian artery (Fig. 1). The aortic catheter was tied in place with the suture previously under the vena cava, which now included the vena cava, the aorta and the inserted catheter.

The right ovary was then freed from adherent tissue and the suspensory ligament was cut. The ovarian bursa was slit open and carefully peeled back. The ovary was gently lifted up and moved towards the centre to expose the left ilio-lumbar artery and vein, which were double ligated and severed. The lumbar dorsal arteries were then cut distal to the sutures which previously had been ligated. The aortic catheter was opened for a short time to allow flushing of the catheter. The aorta and vena cava were then ligated together cranial to the branching of the renal vessels, followed by severance of the vessels cranial to this ligature and caudal to the insertion sites of the cannulae. The vena cava was cut open to enable perfusate to escape after it had been through the ovary. The surgical procedure took approximately 30 min. The preparation was removed and placed in a small beaker with warm (37°C) 0.9% w/v NaCl.

To remove all blood from the preparation and to ensure a functional specimen, the preparation was perfused at this stage with 0.9% w/v NaCl administered by gentle use of a fine syringe or with an i.v. infusion container. During this procedure, the ovary became progressively paler in colour, as the blood was displaced from the specimen. The preparation was considered acceptable for perfusion if there was no visible arterial leakage, the blood flowed from the vena cava opening, and the ovary blanched after perfusion with saline.

Perfusion apparatus and procedures

The perfusion apparatus (Fig. 2) was a further development of the apparatus previously used for the rabbit and rat ovary (Janson *et al.*, 1982; Brännström *et al.*, 1987). The major modifications were that all glass parts were reduced in size (about $\frac{1}{3}$ of the original size) to allow for a smaller volume of perfusion medium and that Tygon tubing (with less adsorbance of steroids) replaced the previously used silicon rubber tubing in the parts through the pump. The medium used was M199 with Earle's salts (Gibco, Paisley) with additions of bovine serum albumin (4% w/v), gentamicin (50 $\mu\text{g ml}^{-1}$), NaHCO_3 (26 mmol l^{-1}) and insulin (0.02 iu ml^{-1}). The temperature of the medium was kept at 37°C by circulating heated water through jackets of the perfusion apparatus. The initial volume of perfusion medium was 25 ml, which was continuously oxygenated with 95% O_2 and 5% CO_2 .

During perfusion, the aortic catheter was connected to the outflow part of the bubble trap and the specimen was placed in the perfusion chamber, which was covered by parafilm. The pressure was adjusted to 80 mm Hg, which gave a flow rate of 0.5–0.6 ml min^{-1} . The ovaries were perfused for 1 h before any agents were added, to allow metabolic stabilization of the tissue. Human CG, LH and IBMX were dissolved in perfusion media and added in a volume of 0.5 ml. Samples of perfusion medium (0.5 ml) were taken at 1, 2, 3, 5, 7 and 20 h, using a sampling syringe during continuous recirculating perfusion.

Fig. 2. Photograph of perfusion apparatus. 1, bubble trap; 2, perfusion chamber; 3, medium reservoir/oxygenator; 4, gas humidifier; 5, peristaltic pump. Scale bar represents 15 cm.

Ovulation was assessed by counting the number of oocytes in the perfusion chamber. Ovulated oocytes were examined using Nomarski differential interference contrast optics, and ova with a germinal vesicle were considered to be immature, while those with germinal vesicle breakdown were regarded as undergoing maturation (Tsafirri and Kraicer, 1972). In some experiments, an excised ovary was placed in the perfusion chamber but only superfused with medium.

Assays

Progesterone concentrations in the perfusate were analysed with a radioimmunoassay kit purchased from Bioclone, Merrickville, VIC, and oestradiol concentrations were determined using a radioimmunoassay kit purchased from Serono, Rome. Intra-assay coefficients of variation were < 7% in both assays. The lowest standards of the assays were 1.0 pmol progesterone ml^{-1} and 0.05 pmol oestradiol ml^{-1} .

Fig. 3. Electronmicrographs of the ultrastructure of perfused mouse ovaries. (a) The granulosa (G) and thecal (T) cells contain extensive arrays of rough endoplasmic reticulum (RER). A Golgi complex (GC) is evident in one cell and the basement membrane (BM) of the granulosa cell layer is intact. Scale bar represents 1 μm . (b) Higher magnification of mitochondria in a thecal cell. Note the tubular cristae characteristic of steroid-producing cells. Scale bar represents 0.5 μm . (c) A tight junction (TJ) between two endothelial cell processes. Note the pinocytotic vesicles (arrows) and basement membrane (BM). Scale bar represents 0.25 μm .

Preparation for transmission electron microscopy

After a 20 h perfusion period, unstimulated and LH-treated ovaries were perfused with 5% (w/v) glutaraldehyde in 0.1 mol cacodylate buffer l^{-1} (pH 7.2), cut into 1 mm^3 pieces and then immersion-fixed at 4°C in the same fixative. After rinsing in cacodylate buffer, the pieces were post-fixed for 2 h with 1% (w/v) OsO_4 in cacodylate buffer and then stained en bloc with 1% (w/v) uranyl acetate in 70% (v/v) ethanol. After dehydration in a graded series of ethanol and equilibration in propylene oxide, they were embedded in Polarbed 812 resin (Fisons Scientific Equipment, Loughborough). Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Jeol 100s electron microscope operating at 80 kV.

Results

Ultrastructure of perfused ovaries

In general, the ultrastructure of the perfused ovaries was good and there was little evidence of injury or tissue degeneration after the 20 h perfusion. There were no marked differences between control and LH-stimulated ovaries. The basement membranes between the granulosa and thecal layers and around the endothelial cells were intact. The nuclei of granulosa and thecal cells had smooth borders, a double nuclear

envelope and nuclear pores. These cells contained active Golgi complexes, rough endoplasmic reticulum and mitochondria (Fig. 3a). Some of the mitochondria had tubular cristae, which are characteristic of steroid-producing cells (Fig. 3b), while others had transverse cristae. A few cells showed slightly swollen mitochondria, while others contained mitochondria with a very electron-dense matrix; these cells also contained large numbers of lipid droplets and were relatively devoid of rough endoplasmic reticulum (not shown). Endothelial cells around small blood vessels contained a wide array of organelles including Golgi complexes, and frequently showed pinocytotic vesicles on their adluminal and abluminal surfaces. Distinct tight junctions were present between endothelial cells (Fig. 3c).

Ovulations and oocyte maturation

Perfused unstimulated control ovaries ($n = 5$) did not ovulate. Stimulation *in vitro* with either hCG (50 iu), ovine LH (0.1 $\mu\text{g ml}^{-1}$) or LH + IBMX (0.2 mmol l^{-1}) resulted in ovulations from all five ovaries in each group (Table 1). The ovulation rate was higher in the LH + IBMX group than in the other groups. All ovulated oocytes in the LH- and hCG-stimulated groups exhibited germinal vesicle breakdown, while only 30% of ovulated oocytes in the LH + IBMX group were at the germinal vesicle breakdown stage.

Table 1. Ovulations in mouse ovaries perfused *in vitro*

Treatment	Number ovulating/ total number in group	Number of ovulations/ treated ovary (mean $\pm$ SEM)	Range
Control	0/5	—	—
hCG ^a	5/5	2.6 $\pm$ 0.5	1–4
LH ^b	5/5	2.0 $\pm$ 0.3	1–3
LH + IBMX ^c	5/5	7.4 $\pm$ 1.2	4–11

^ahCG (50 iu) was added at 1 h.^bOvine LH (100 ng ml⁻¹) was added at 1 h.^cOvine LH (100 ng ml⁻¹) and 3'-isobutyl-1-methyl xanthine (IBMX) (0.2 mmol l⁻¹) were added at 1 h.

Excised ovaries were superfused in an identical system to the one above to test whether perfusion of the vasculature was necessary to acquire ovulations. Four superfused hCG (50 iu)-stimulated and four unstimulated control ovaries did not ovulate.

Steroid release

The concentrations of oestradiol in the medium were below the detection limit in superfused ovaries (hCG-stimulated and control) and perfused control, LH- and hCG-stimulated ovaries at all time points. In the group of perfused LH + IBMX-stimulated ovaries, there were detectable concentrations of oestradiol after 2 h of perfusion (0.26 ± 0.09 pmol ml⁻¹), which increased gradually to 0.38 ± 0.07 pmol ml⁻¹ at 20 h. The concentration of progesterone measured in the perfusion medium represents a balance between progesterone secretion and adsorption, as progesterone in higher concentrations is

rapidly adsorbed to the surfaces of the system (Bruning *et al.*, 1981) with a $t_{1/2}$ of about 2.5 h (Brännström *et al.*, 1987). Progesterone was released in larger quantities than oestradiol in all treatment groups. In superfused control ovaries, there were low concentrations of progesterone in the medium throughout the perfusion time (3.3 ± 1.0 pmol ml⁻¹ at 20 h). When superfused ovaries were stimulated by hCG (50 iu), there was a slight, gradual increase in progesterone concentrations (11.8 ± 1.6 pmol ml⁻¹ at 20 h). Perfused ovaries released markedly larger amounts of progesterone. There was a small increase in the progesterone concentration in the control group, and the addition of either LH, hCG or LH + IBMX led to a marked rapid increase in progesterone release, reaching maximal concentrations at 3–7 h (Fig. 4).

Discussion

In vitro models may contribute to the identification of locally produced paracrine factors that regulate dynamic ovarian events such as ovulation. Experiments *in vivo* can be difficult to perform or to interpret owing to systemic influences. By perfusion of ovaries *in vitro*, the organ can be kept in a functioning state over extended periods, facilitating the study of these dynamic events.

This is the first report of perfusion of mouse ovaries *in vitro* combined with the induction of ovulation *in vitro*. In earlier studies on incubated mouse Graafian follicles, a very small number of follicles with signs of ovulation was observed (Neal and Baker, 1973; Boland *et al.*, 1995) and it is possible that some of these ruptured follicles were caused by mechanical trauma during preparation or incubation (Neal and Baker, 1973; Boland *et al.*, 1993). In the present study, the ovarian surface was protected by the ovarian bursa during surgery, so the

Fig. 4. Progesterone concentrations in the medium. Each symbol represents values obtained in five perfusions. Vertical bars indicate the SEM (a) control ovaries; (b) hCG-stimulated ovaries; (c) LH-stimulated ovaries; and (d) ovaries stimulated with LH and 3'-isobutyl-1-methylxanthine (IBMX).

bursa remained untouched until it was slit open before introduction of the ovary into the perfusion apparatus. Furthermore, during perfusion, the ovary was completely submerged in perfusion medium. This, in addition to the absence of ovulations from unstimulated control ovaries, rules out the possibility that ovulations from gonadotrophin-stimulated ovaries in the present study were due to mechanical injury to the follicle surface. The fact that superfused ovaries in our experimental model did not ovulate and secreted only small quantities of steroids suggests that superfusion is not a suitable method.

Perfused mouse ovaries exhibited a normal morphology after 20 h of perfusion. There were no signs of blood cells observed in any vessels, indicating a homogeneous perfusion. A limiting factor of perfusion *in vitro* is damage to endothelial cells with loss of intercellular tight junctions. In the present study, transmission electron microscopy revealed intact endothelial cells and basement membranes, indicating the absence of severe endothelial damage.

The reason for using adult cyclic mice in contrast to immature animals induced with eCG, which are commonly used in studies of reproductive functions in rats, was that at this stage the immature mice were considered too small for the extensive surgery to be carried out. However, a disadvantage of using adult cyclic animals in contrast to eCG-primed animals is that the ovary contains corpora lutea of different stages, the influence of which may complicate the interpretation of results.

The ovulation rate after treatment with only gonadotrophin was lower than that observed *in vivo*. However, the combined use of IBMX (to increase cAMP concentrations) and LH increased the ovulation rate to that which is normal *in vivo*, in accordance with observations in the rat ovary perfusion system (Brännström *et al.*, 1989). The observation that the ovulation rate can be increased in rat ovaries perfused *in vitro* by addition of several blood factors such as leukocytes (Hellberg *et al.*, 1991) and bradykinin (Brännström and Hellberg, 1989) suggests that several factors are lacking in the situation *in vitro* for maximal numbers of ovulations to take place. The reduction of ovulation rate *in vitro* can also partly be explained by a decreased flow to the ovary, owing to ligation of the ovarian branch of the uterine artery. It has been reported in rats that ligation of this artery causes a 40% reduction in ovulation rate (Peppler, 1976). In the perfusion methodology applied in the present study, the arterial side pressure is fixed and the flow will be the result of the pressure and the vascular resistance in the ovary. This technique enables the monitoring of the influence of vasoconstrictive agents on the ovarian vessels. In a slightly different perfusion system, which has been used for rabbit ovaries (Lambertson *et al.*, 1976) the arterial side pressure was not monitored and perfusion was performed with a set flow rate.

The concentrations of progesterone in the medium in the gonadotrophin-stimulated ovaries showed a marked increase in comparison to control ovaries. There was a continuous output of progesterone during the perfusion period, suggesting good viability of the ovaries. The concentration of progesterone in the medium corresponds well with concentrations in the tissue in a similar system for rat ovaries (Higuchi and Espey, 1989). The higher progesterone secretion from ovaries that had been exposed to IBMX may have been due to additional activation of preovulatory follicles or to activation of older corpora lutea.

Oestradiol concentrations were lower than progesterone concentrations and only detectable in ovaries stimulated by LH plus IBMX. This finding indicates that phosphodiesterase inhibition by IBMX increases the overall steroidogenic capacity of the perfused ovary. The steroid output from superfused ovaries was considerably lower than from perfused ovaries. This shows that the superfusion model is less responsive than the perfusion model in terms of steroidogenesis, and it is likely that only the surface part of the ovary is metabolically active.

In summary, we have described and characterized a model for perfusion of mouse ovaries *in vitro*, which enables induction of ovulation *in vitro*. This model could be useful in studies of ovarian function.

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