The isolated perfused mouse uterus as a model for the study of implantation in vitro. Methodology and morphology

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INTRODUCTION

Mammalian implantation involves local hormonal signalling between the embryo and the uterus, and hormonal regulation between the ovary and the uterine-embryonal tissue (*Sherman & Salomon* 1975; *Heald* 1976; *Heap et al.* 1979; *Bazer et al.* 1983; *Hearn* 1986; *Miller* 1987). With the object of studying mammalian implantation mechanisms, several in-vivo and in-vitro systems have been employed (*Sherman & Wudl* 1976; *Chavez* 1984). In-vivo models incorporate all body systems and, therefore, provide general information about the resulting response to implantation, whereas in-vitro systems comprising cell cultures in contact with the embryo allow investigation of only initial embryo-uterine interactions. In previous studies, we have used the interaction of blastocysts with mouse and human uterine monolayer cultures and blastocysts to study the very early trophoblast-uterine attachment (*Lindenberg et al.* 1986; *Lindenberg et al.* 1988). However, this model is not suitable for investigating more than the initial stages of the implantation process as the epithelial-stromal relationship is not present. The presence of a uterine-stromal architecture is mandatory for implantation (*Finn* 1982; *Enders et al.* 1983; *Chavez* 1984; *Lundkvist & Nilsson* 1984; *Schlafke et al.* 1985). *Sengupta et al.* (1988) tried in an elaborated culture system to prepare uterine epithelial and stroma cells in culture preserving the epithelial-stromal relation seen in-vivo. Also this latter study failed to establish proper conditions for in vitro implantations.

With the object of studying implantation, while excluding extragenital influences on the process and while preserving the normal uterine epithelial-stromal relationship, we developed an in-vitro perfusion system of the mouse ovario-uterine tissue. The present paper describes the methodology of development of this system in which a mouse ovary, oviduct and uterus are surgically isolated en-bloc for perfusion in-vitro via the ovarian artery and an aortic segment.

MATERIALS AND METHODS

Animals

Randomly bred NMRI mice were maintained under a controlled ten-hour night, centered on midnight, and fed a standard diet ad libitum. Female mice weighing 25 to 35 grams were mated with fertile males. Mating was confirmed by the presence of a vaginal plug observed between 8 and 9 a.m. and the day of the plug was designated day 1 of pregnancy.

Surgical technique

On day 5 of pregnancy before 11 a.m. the mice were anaesthetized using sodium pentobarbitone (0.05 mg/g body weight) intraperitoneally and 50 IU heparin to avoid blood clotting. The abdomen was opened through a midline incision and the distal sigmoid colon was ligated and divided to give access to the retroperitoneum. Both kidneys were carefully removed to preserve the ovarian vessels. The left ovarian vessels usually pass over the lower pole of the left kidney and was always carefully dissected free before ligation of the renal vessels. All segmental vessels including the right ovarian vessels, were ligated and divided leaving the left ovarian vessels patent on an aortic segment. Thereafter, the left uterine horn was isolated, carefully preserving the supplying vessels. At the utero-vaginal junction the uterine horn with adjoining vessels was ligated and divided, leaving the ovario-uterine tissue attached to the aorta and vena cava on a pedicle. The aorta was then cannulated in a retrograde manner from the iliac bifurcation using a teflon catheter

(Viggo, Helsingborg, Sweden) with an outer diameter of 0.6 mm. Perfusion in situ was started immediately using HAM's F-10 medium supplemented with 100 IU/1 heparin and 21 mM HEPES buffer at a hydrostatic pressure of 40 mmHg and 37 C. As soon as the in situ perfusion was started the distal part of the uterine venous arcade was opened longitudinally without damaging the arterial supply, thereby providing sufficient venous drainage. The peritoneum covering the uterine horn and the superficial uterine myometrial layers were also divided longitudinally in the antimesometrial region in order to open smaller veins and lymph vessels. Finally, a ligature was firmly placed to ligate the aorta and vena cava cranial to the renal vessels (Fig. 1).

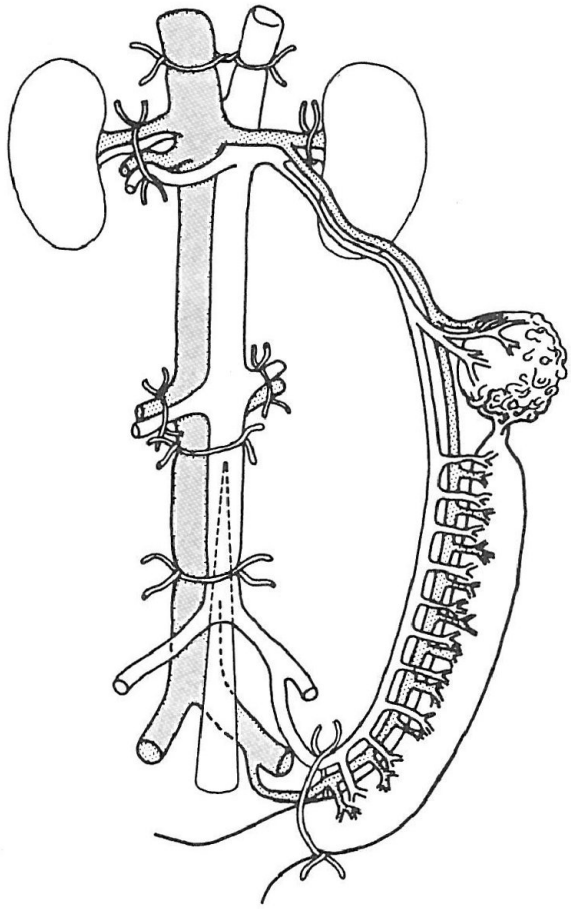

Figure 1. The figure illustrates the position of all the necessary ligatures and the perfusion cannula in the abdominal aorta prior to perfusion of the left murine ovario-uterine segment.

Immediately after this, the abdominal vena cava was opened between the teflon catheter and the cranial ligature without damaging the overriding right ovarian artery, thereby securing a sufficient venous outflow.

The left ovarian-tubal-uterine preparation, the ovarian vessels, the cannulated abdominal aorta and the vena cava were then dissected free en-bloc from the lumbar muscles (Fig. 1). Before the preparation was placed in the perfusion chamber, the ovarian bursa was also removed. To avoid drying of the tissues during the surgical procedure, the abdominal cavity was moistened regularly with warm saline. Most of the dissection work was done using an operating microscope (Zeiss, magnification 2.5 to 40 times). The time for the operative procedure did not exceed 30 minutes, including a maximum of 5 minutes for the in situ perfusion. At the end of the surgical procedure the right ovary and uterine horn were fixed in either 2% paraformaldehyde for light microscopy or 70% Karnovsky fixative (Lindenberg et al. 1986) for transmission electron microscopy.

The morphology of the right-side organs was used as a control assessment of the histology of the pre-perfusion tissue.

Perfusion procedure

The method is basically similar to the perfusion techniques previously described from this laboratory (*Janson et al.* 1982), although the entire system has been reduced in size to contain a total volume of only 20 ml of perfusion medium. The perfusion apparatus consists of a closed system in which the medium is recirculated (Fig. 2). All chambers are maintained at 37 C by warm-water jackets. A peristaltic pump circulates a major portion of the medium continuously through the oxygenator, while the portion reaching the tissue is regulated by adjusting the perfusion pressure according to a connected mercury manometer (Fig. 2). A perfusion pressure of 30 to 40 mmHg is applied. The ovarian-uterine preparation is placed in a perfusion chamber and the arterial cannula was connected to the perfusion line, the prepara-

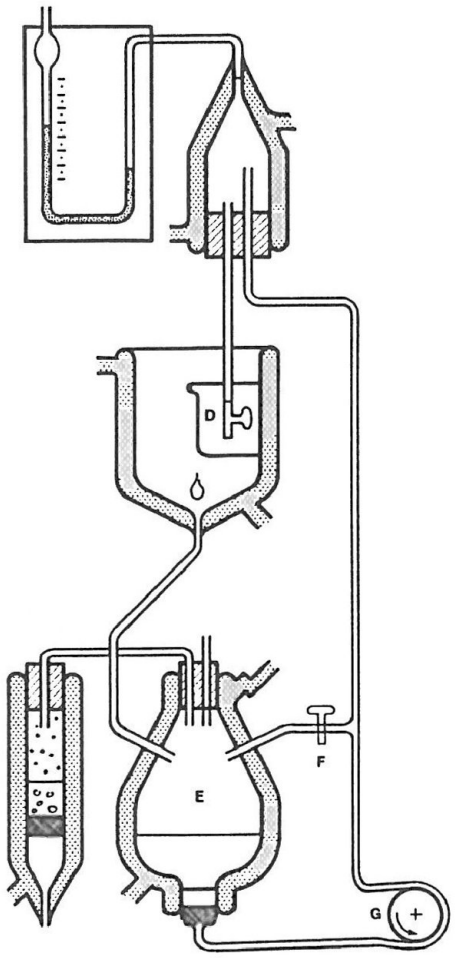

Figure 2. The perfusion apparatus consists of a closed system where the medium is recirculating. All chambers are maintained at 37 C by warm-water jackets (dotted areas). A peristaltic pump (G) circulates a major part of the medium continuously through the oxygenator (E), while the portion reaching the tissue is regulated by adjusting the perfusion pressure with a Hoffman clamp (F). The ovario-uterine preparation is placed in the organ chamber (D) and the arterial cannula connected to the perfusion line.

tion being suspended in its own venous effluent medium. At the end of a perfusion experiment, the tissue is fixed by continuing the perfusion and by replacing the perfusate with either 2% buffered paraformaldehyde or 70% Karnovsky fixative.

Medium

The perfusion medium consists of Medium 199 containing Earle's balanced salt solution and L-glutamine (Gibco. UK). This medium was supplemented with 0.026 M NaHCO, 75 µg/ml benzyl penicillin G potassium (USP), 50 µg/ml streptomycin, 0.2 U/ml heparin sulfate, 0.02 U/ml insulin, 3 nM estradiol-17b, 60 nM progesterone and 4% bovine serum albumin (Sigma Chemical Co., USA, Fraction V, fatty acid free).

Measurement of steroids in the medium

Samples of medium (1 ml) were collected during the perfusion and were replaced with an equal volume of fresh medium. The samples were kept frozen at -20 C for subsequent measurement of estradiol-17b and progesterone by radioimmunoassay (*Hillensjö et al.* 1976). The levels were expressed as nmol of steroid per liter of medium.

Light microscopic morphology

The ovarian uterine tissue was either immersed in 2% buffered formaldehyde or perfusion fixed with the same fixative. After primary fixation, the ovarian-uterine preparations were always divided into proximal, intermediate and distal uterine segments and an ovarian segment.

The tissues were dehydrated in graded alcohols from 70% to absolute, cleared in xylol and embedded in paraffin. The tissue segments were sectioned at 5 µm and stained with either hematoxylin or periodic-acid-Shiff.

Transmission electron microscopic morphology

The specimens were either immersed for 5 hours or perfusion fixed followed by immersion in 70% Karnovsky fixative for a total of 5 hours. Before further treatment of the tissues, the specimens were separated into the segments mentioned above. After repeated washing in 0.1 M cacodylate buffer pH at 7.3, the tissue was postfixed for 2 hours in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in graded alcohols and propylene oxide and embedded in Epon. Semithin sections were stained with toluidine blue, whereas ultrathin sections were stained with uranyl acetate and lead citrate. Representative sections from each of the segments of a perfusion preparation, were stained and observed.

A preparation was considered acceptable if in each segment there was no necrosis in any tissue and the uterine luminal epithelium was intact, and the ovary contained well developed corpora lutea. These criteria were based on initial light microscopic evaluation. Thereafter, transmission electron microscopy was done on selected sections.

Protocol for steroid adsorption in the perfusion system

Five sham perfusions, i.e. perfusions without any ovarian or uterine tissue in the apparatus, were carried out to determine the extent of steroid adsorption onto the surface of the perfusion equipment. Perfusate samples were taken at regular intervals and the estradiol and progesterone levels in these samples were estimated by radioimmunoassay.

Protocol for implantation time and steroid levels

With the object of confirming the time where implantation was initiated 8 mice were sacrificed at both 11 a.m. 3 p.m. on plug day 5. This interval included the initial 5 hours of the perfusion time used in this study. The mouse uteri were processed for light microscopy and serial sectioned to determine the progress of in-vivo implantations during this time.

In addition, twelve mice undergoing implantation were sacrificed on day-5 at 11 a.m. A 0.5 ml blood sample was collected from each mouse and the nine samples were analyzed for their 17b-estradiol and progesterone levels. An average level was determined for each steroid and, thereafter, both steroids were added to the perfusates to obtain these levels during uterine in-vitro perfusion.

Protocol for in-vitro perfusion of tissue preparations

Twenty-one ovario-uterine preparations were perfused for a period of 10 hours to observe uterine motility, analyze the levels of estradiol and progesterone in the medium and, study the morphology of the ovario-uterine specimens subsequent to the perfusion.

RESULTS

Hormone assays

The average steroid levels determined from 12 day 5, normally implanting mice, were 3.8 nM 17b-estradiol and 80 nM progesterone. These levels were added to perfusates of all subsequent perfusions.

Seventeen perfusions were analyzed with respect to hormone levels. In figure 3, the changing concentration of progesterone during the sham perfusions and during experiments are shown. A significant ($P < 0.05$, t-test for non-paired data) decrease in the perfusate levels of progesterone was seen after 420 min (7 h) in the sham perfusions compared to the tissue perfusions. The sham perfusion media had a concentration of 20 nM progesterone compared to 70 nM progesterone in the perfused media. The estradiol did not show any variation with time in the sham or tissue perfusion, nor did the two exhibit any significant difference. The mean of these estradiol levels was 2 nM.

Implantation time

In the 8 uteri fixed at 11 a.m. and serially sectioned to determine the in-vivo implantation time, one was found not to be pregnant, while the 7 others contained a total of 14 blastocysts floating freely in the lumen and 22 blastocysts in the appositional and initial adhesive stages. In the 8 uteri fixed at 3 p.m. 10 blastocysts still

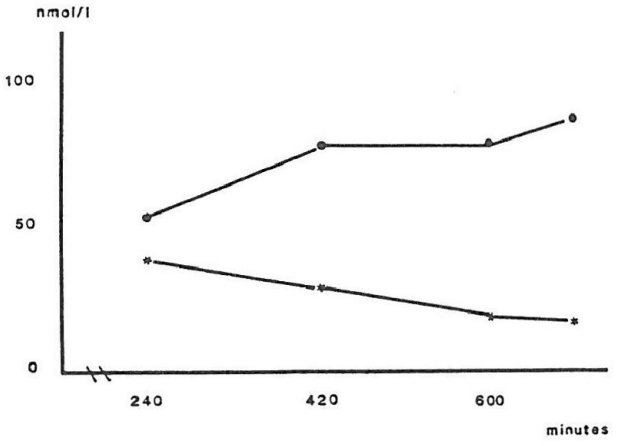

Figure 3. The mean levels of progesterone in the perfusate during sham perfusion (*) and during organ perfusion (O) for 10 hours.

remained free in the lumen, whereas 8 had reached the adhesive stage, and 6 were early implantation sites, with the blastocyst embedded in the stromal tissue. The beginning of the decidual reaction was also seen.

Light microscopic morphology of the perfused preparations

A total of 21 tissue preparations were perfused. Eighteen of these were found to exhibit spontaneous movement of the uterine segment during the whole period of in-vitro perfusion. Eleven of these 18 perfusions resulted in normal ovarian and uterine morphology. All of the uteri not showing spontaneous movement subsequently exhibited considerable necrosis.

The 10 perfused uterine horns, which were scored as morphologically abnormal, had remarkably distended lumina with degenerating and necrotic epithelium. The glandular tissue was sometimes better preserved than the luminal epithelium, while the stroma exhibited excessive edema and necrosis. In several of these 10 preparations, the ovaries were only mildly affected, having little edema and well preserved corpora lutea. All lymph vessels were distended.

The 11 perfused uterine horns, which were scored as being morphologically normal, exhibited intact columnar epithelial linings of the lumina and well preserved glands. There was no necrosis in the stroma although slight edema was present (Fig. 4). In the ovary, well developed corpora lutea were seen as well as follicles containing oocytes at different stages of development. Some degree of edema was always seen in the ovaries.

In the perfused preparations scored as morphologically normal, implantation sites were encountered ranging from very early blastocyst-attachment phases to conceptuses totally nidated into the endometrial stroma (Fig. 5). All implantation sites were found to be situated antimesometrially and decidual formation had occured.

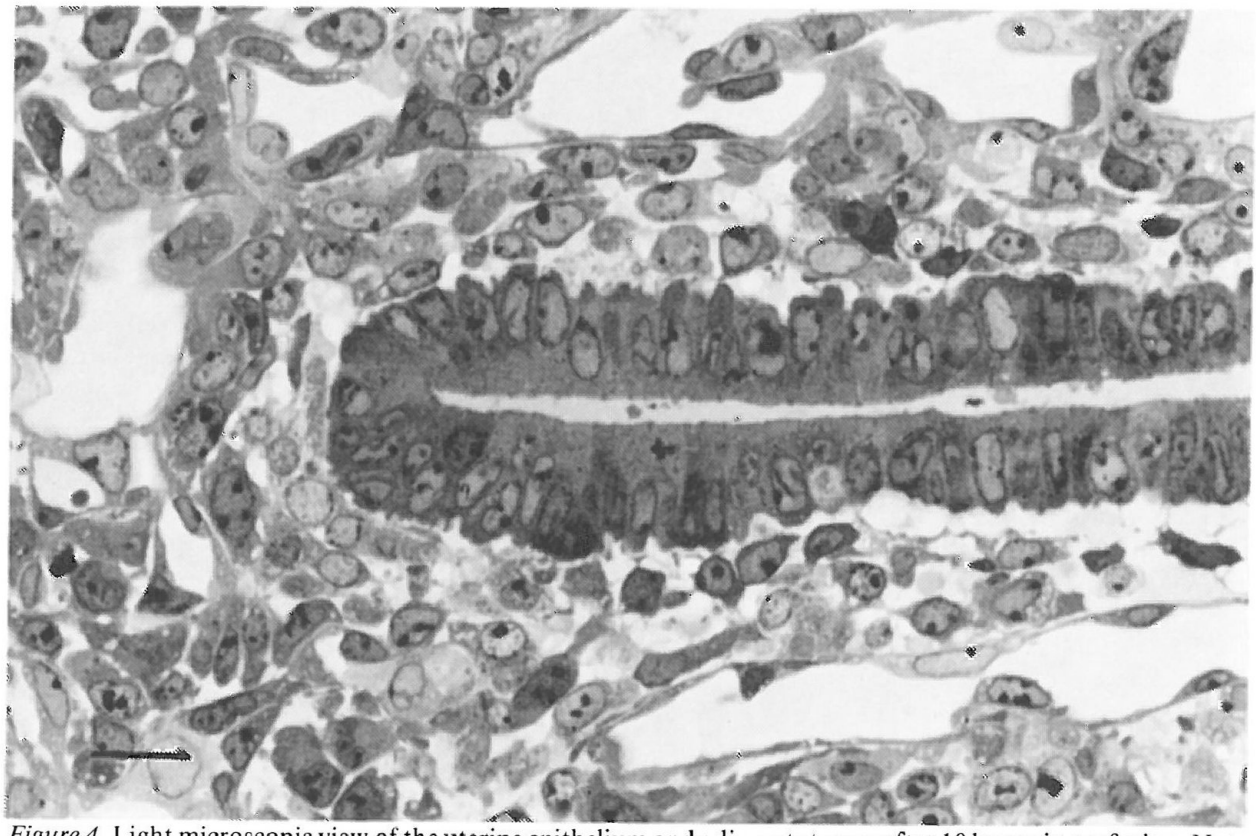

Figure 4. Light microscopic view of the uterine epithelium and adjacent stroma after 10 hours in perfusion. Note the intact epithelium and edema in the stroma. Several distended vessels are seen. Bar = 10 μm.

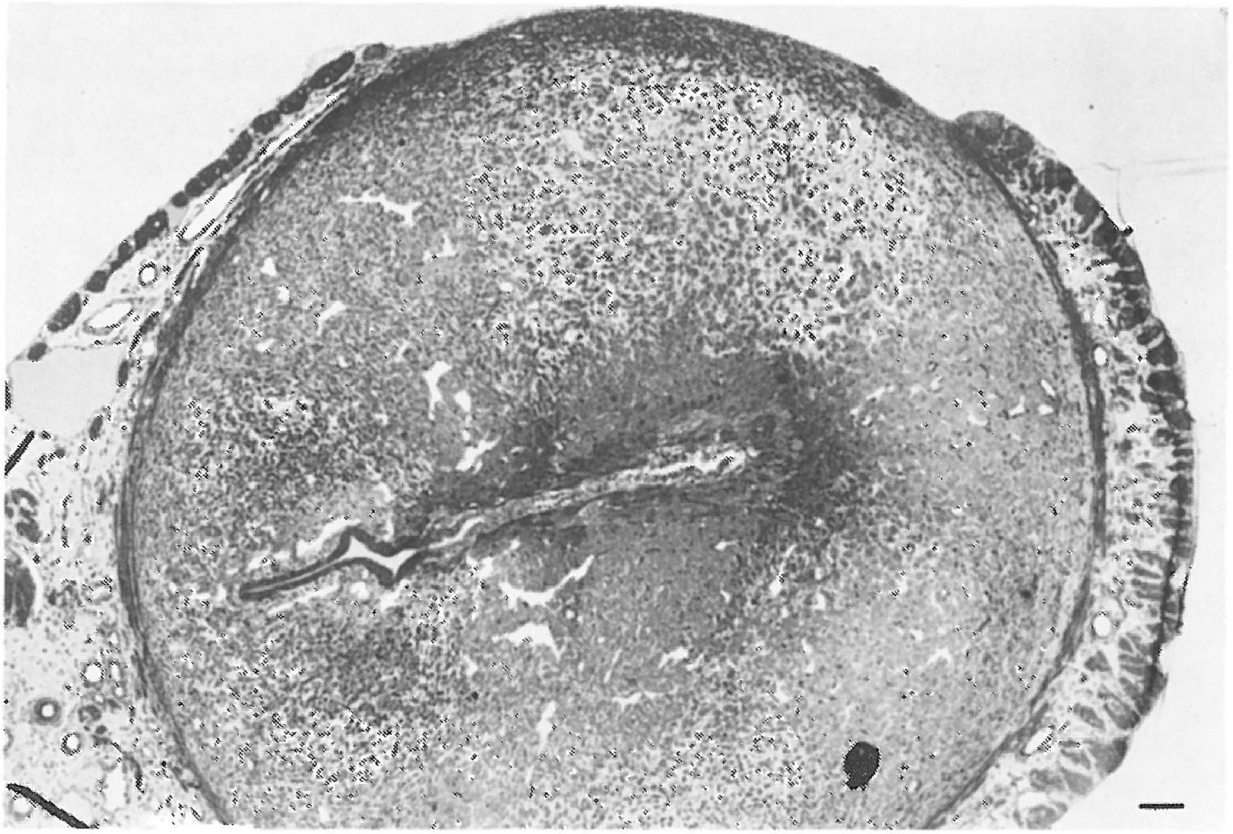

Figure 5. The light microscopic appearance of a uterine segment after 10 hours perfusion. An implanting blastocyst is seen at the antimetrial area as well as the beginning of decidua formation. The figure also illustrates the removed external muscular layer of the uterus (top). Bar = 100 μm.

Light microscopy of tissues prior to perfusion
Specimens taken from the contralateral, right uterine horn as controls did not show any signs of necrosis or edema.

Transmission electron microscopy of perfused uteri
Epithelium of the perfused uteri considered to be normal by light microscopy had microvilli (Fig. 6) and an ultrastructural morphology comparable with the non perfused uteri. In some cases necrotic epithelial cells appeared to be extruded from the epithelium in the perfused specimens. The stromal extracellular space was moderately distended due to edema (Fig. 6).

Transmission electron microscopy of perfused ovaries
The corpora lutea were composed of large luteinized cells filled with lipid droplets, an abundance of tubular mitochondria and smooth endoplasmic reticulum (Fig. 7).
Furthermore, oocytes within the follicles were well preserved in different stages of folliculogenesis. The stroma had signs of edema but no necrosis was found.

DISCUSSION
The preservation of tissue architecture following long term perfusion (10 h) and the continued release of steroids into the medium during perfusion indicates viability of the reproductive tissues. The crucial point for the present study is whether the implantation site occurring in-vitro appear normal. Implantation sites were found to be initiated or to progress in the antimesometrial region, and had a morphological appearance similar to that of implantation sites in intact animals. Many studies on mammalian implantation have exploided the advantages of in vitro culture. *Gleni-*

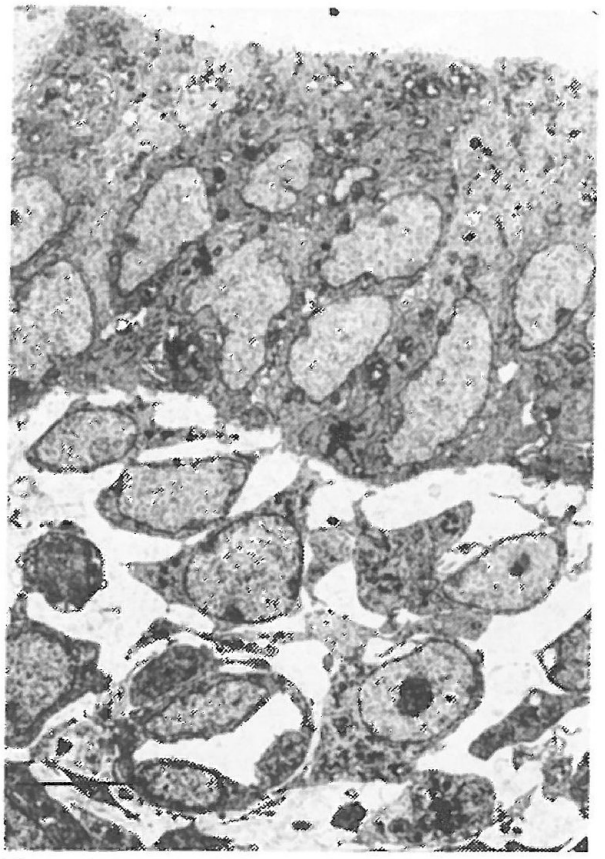

Figure 6. A transmission electron micrograph of the uterine epithelium after 10 hours of perfusion. Note the intact epithelium with microvilli and edema formation in the stroma. Bar = 10 μm.

ster in 1961 cultured rabbit blastocyst on endometrial strips and *Grandt et al.* (1975) attempted to achieve implantation of mouse blastocysts onto the luminal epithelium of cultured mouse uteri. None of these studies demonstrated any decidua reaction which is thought to be important for normal implantation (*Lundkvist & Nilsson* 1984). In vitro culture systems comprising blastocyst attachment to plastic (*Gwatkin* 1966), monolayers of different type of cells (*Salomon & Sherman* 1975, *Herman* 1978, *VanBlerkom & Chavez* 1981, *Lindenberg et al.* 1986, *Camarate et al.* 1987, *Lindenberg et al.* 1988) or just components of the extracellular matrix (*Farac et al.* 1987) demonstrate aspects of the attachment phase but isolated from the ovarian-uterine-embryonal signalling present in vivo (*Heald* 1976, *Heap et al.* 1979, *Finn* 1982, *Bazer & Roberts* 1983, *Lundkvist & Nilsson* 1984, *Sherman & Salomon* 1975, *Hearn* 1986, *Miller et al.* 1987). To extend in vitro study of uterine-embryonal interactions a normal uterine epithelial- stroma relationship is mandatory as already seen in the in-vivo studies (*Nilsson* 1974, *Lundkvist & Nilsson* 1984). Moreover, an intact uterine ovarian axis is required to study the ovarian-uterine interaction (*Miller & McLean* 1987). The perfusion technique described here may provide a useful tool to elucidate the precisely coordinated inter-tissue signalling.

Factors such as the surgical approach, prevention of long periods of tissue ischemia, prevention of infection and edema, composition of perfusion media and maintenance of the perfusion equipment were found to be critical as was found previously in similar ovarian perfusion models for the rabbit (*Janson et al.* 1982) and the rat (*Sogn et al.* 1984). Two endpoints serving as criteria of viability were employed in both models, namely tissue morphology and the release of ovarian steroids into the perfusion medium. It was found that the morphology of the uterus was best preserved, especially the appearance of the epithelium and decidua, when bovine serum albumin was added (unpublished results) as was also found in the ovary model (*Bjersing et al.* 1981). A slight edema of the stroma, however, could not be prevented.

A significant release of ovarian steroid was recorded throughout the whole perfusion period. The progesterone concentrations in the medium were maintained at the levels found in plasma at the time of implantation by adding steroids in such a way as to balance the continuous ovarian production of progesterone throughout perfusion and the adsorption of this steroid to the glassware and tubing of the perfusion system (*Janson et al.* 1982). The results indicate that the perfusion model provides a tool for studying not only the initial attachment of trophoblast to uterine epithelial cells but also the later stages of the implantation process as the epithelium-stromal relation is preserved.

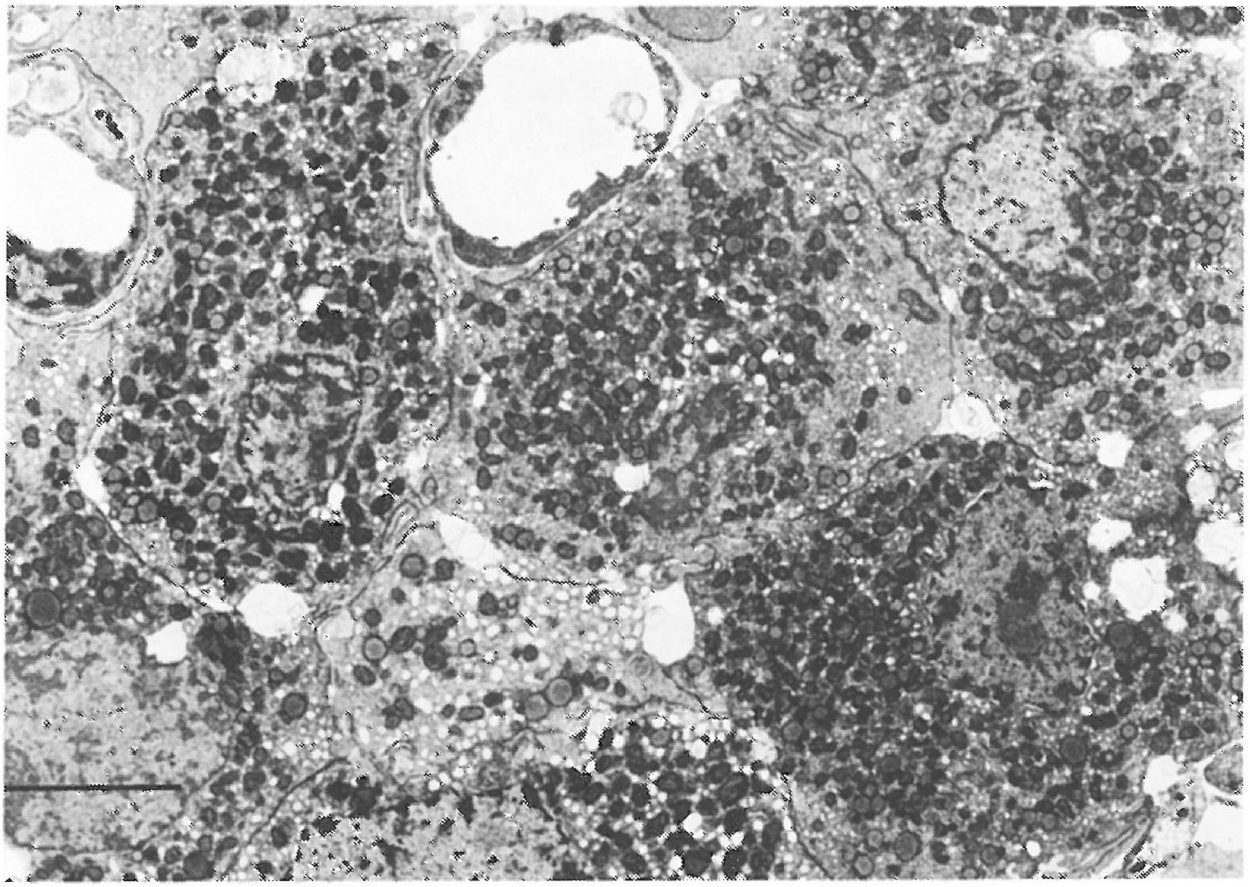

Figure 7. A transmission electron micrographic view of luteinized cells of the corpus luteum after perfusion. The cells contain an abundance of mitrochondria and smooth endoplasmic reticulum indicating steroid synthesis. Bar = 10 μm.

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Summary
In order to facilitate investigations of mammalian blastocyst implantation in the endometrium, an in-vitro organ perfusion technique was developed. This technique was designed to avoid the drawbacks of in-vivo and cell culture investigations, while retaining physiological resolution of the endo- and paracrinology and specifically a normal epithelium to stroma relationship. The ovary, oviduct and uterine horn from 21 mice were perfused in-vitro for 10 hours. The surgical techniques for isolation of the organs as well as the perfusion procedure are described. The resultant morphology of the perfused tissue, including implantations is described and illustrated by light- and transmission electron microscopy. The model seems to be useful for studying the mammalian implantation as implantation takes place and decidua is formed during perfusion.

Sammendrag
En in-vitro organ perfusions model er udviklet m.h.p. at studere blastocyst implantationen i endometriet. Denne teknik er anvendt for at kunne undgå nogle af de fejl, man ellers må acceptere ved in-vivo eller celle-kultur undersøgelser. I modellen bevares de normale endo- og paracrine funktioner for cellerne, samtidig med at den normale epithel og stroma arkitektur er intakt. Ovariet, oviducten og det ene uterine horn fra 21 mus blev i 10 timer perfunderet in-vitro. Den kirurgiske teknik og perfusionsproceduren er beskrevet. Morfologien efter perfusion er beskrevet v.h.a. lys- og transmissions elektronmikroskopi. På baggrund af de præsenterede resultater anser vi denne model for brugbar ved studiet af implantationen, idet blastocyster implanterer og decidua dannes under perfusionen.

Yhteenveto / K. Pelkonen
Artikkelissa kuvataan elinperfuusioon perustuva in

vitromenetelmä, joka helpottaa nisäkkään blastokystin endometriumiin tapahtuvan implantaation tutkimista. Menetelmä suunniteltiin siten, että sillä vältettäisiin in vivo- ja soluviljelmötekniikoiden heikkoudet. Menetelmässä perfusoittin 21 hiiren munasarjaa, munanjohdinta ja kohdun sarvea 10 tuntia in vitro. Artikkelissa kuvataan sekä elinten eristämiseen käytetty kirurginen tekniikka että perfuusionmenetelmä. Perfusoidun kudoksen ja implantaattien morfologia esitetään sekä valoettä transmissionelektronimikroskooppisin kuvin. Malli näyttää soveltuvan nisäkkään implantaation tutkimiseen, koska siinä sekä tapahtuu implantaatio että deciduan muodostuminen perfuusion aikana.

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