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Reproduction, Fertility and Development

Seven days *ex vivo* perfusion of whole ewe ovaries with follicular maturation and oocyte retrieval: towards the development of an alternative fertility preservation method

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ABSTRACT

Fertility preservation methods for prepubertal women about to undergo gonadotoxic chemo and/or radiation therapy are limited. Therefore, the aim of this study was to investigate the feasibility to develop an alternative fertility preservation method based on an *ex vivo* perfusion platform for whole ewe ovaries. Thirteen ewe ovaries were divided into two groups (group 1 and 2) that were perfused in a bioreactor for up to 7 days. Group 1 ($n = 3$) were stimulated with human menopausal gonadotropin (hMG) administered in single daily dose, while group 2 ($n = 10$) were stimulated continuously for 24 h. The perfused ovaries in group 1 showed no significant differences in follicular density, sub-follicular morphology and oocyte quality after ischaemia and after *ex vivo* perfusion compared with non-perfused control ovaries. The perfused ovaries in group 2 showed a significant decrease in the follicular reserve and oocyte quality compared with the control group. In total, 16 GV–MI oocytes were retrieved from both groups. This study describes for the first time the *ex vivo* maintenance of viable follicles of ewe ovaries with oocyte integrity and the retrieval of oocytes after *ex vivo* hormonal perfusion with two different protocols for up to 7 days.

Keywords: bioreactor, endocrinology, ewes, *ex vivo*, fertility preservation, follicular development, ovary, perfusion.

Introduction

Recent advances in chemo/radiotherapy protocols are providing a cure for many types of cancers. Cancer survivors need to be offered fertility preservation options (Letourneau *et al.* 2012; Benedict *et al.* 2018) since several cancer treatments can induce ovarian failure with consequent infertility (Wallace *et al.* 2005; Donnez *et al.* 2006, 2010; Bromer and Patrizio 2008). There are many strategies available today for fertility preservation in women requiring gonadotoxic treatments, including ovarian transposition, oocyte and/or embryo vitrification, and ovarian cortex transplantation (OCT) (Donnez 2013; Diaz-Garcia *et al.* 2018; Moravek *et al.* 2018). OCT is the only fertility preservation option available for prepubertal girls and for women who need prompt cancer treatment (Bromer and Patrizio 2008; Donnez and Dolmans 2013). Although live births after OCT have been reported, including one in a leukaemia survivor (Shapira *et al.* 2018), there are serious concerns that systemic cancers may metastasise to the ovaries posing the risk of re-introducing neoplastic cells at the time of OCT (Wallace *et al.* 2005; Dolmans *et al.* 2010, 2013; Donnez and Dolmans 2017). Folliculogenesis and retrieval of mature oocytes performed *in vitro* and *ex vivo* could be alternative methods for fertility preservation in these patients (Donnez and Dolmans 2017). Some progress has been made in terms of complete follicular growth and isolation of mature oocytes *in vitro* (Picton *et al.* 2008; Telfer and Zelinski 2013; Maffei *et al.* 2016; Chiti *et al.* 2017; McLaughlin *et al.* 2018). However, full development, from early follicular stages to a viable offspring, has only been described in rodent models (Eppig and Schroeder 1989; O'Brien *et al.* 2003; Vanacker *et al.* 2014;

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Laronda et al. 2017). The complex molecular events controlling follicular expansion and the long time required for folliculogenesis and oocyte maturity in large mammalian species are still poorly understood, creating challenges and limitations for *in vitro* studies (Edson et al. 2009; Figueiredo et al. 2011; Songsasen et al. 2012; Shea et al. 2014). A novel idea to overcome these hurdles, might be to explant an ovary from the patient prior to the cancer treatment, perfuse it *ex vivo* in a bioreactor, and perform ovarian stimulation in this artificial environment, which would have the advantage of exploiting the intact ovarian architecture to support folliculogenesis and oocyte maturation. The aim of this study was to evaluate follicular growth and oocyte survival and integrity after *ex vivo* whole ovarian perfusion using sheep ovaries. Potential ovarian tissue damage caused by any cold ischaemia events between the times from ovarian explantation to reperfusion, where the ovary is stored in a cold preservation solution, was also considered. We also investigated the possibility of retrieving mature oocytes using two different gonadotropin stimulation protocols during the normothermic perfusion in a bioreactor. Such perfusion bioreactor system could become a useful research platform for continued studies on how to maintain and stimulate fresh and cryopreserved ovaries *ex vivo* to develop alternative fertility preservation methods for young prepubertal female cancer patients or for patients in high risk of ovarian metastasis.

Materials and methods

Organ collection

Twenty-six ovaries from 13 sexually mature ewes (10–14 months old) of mixed breeds were retrieved at the local slaughterhouse between January 2020 and April 2020. All ovaries (n = 26) with intact vascular pedicles were directly dipped once in a 0.001% chlorhexidine solution dissolved in normal saline (Fresenius Kabi, Uppsala, Sweden) and then dipped three times in a sterile saline solution (0.9 mg/mL, Braun, Melsungen AB, Germany) to minimise contamination risk and tissue damage from the chlorhexidine solution. The collected ovaries were divided into an experimental group (n = 13) and a control group (n = 13) (Table 1). For the ovaries included in the experimental group, the ovarian artery was separated from the ovarian vein using a dissection microscope (Nikon, Tokyo, Japan). The ovarian artery was then cannulated with a 24 G cannula (Becton Dickinson infusion Therapy AB, Helsingborg, Sweden) and the venous outflow was left open. Sterile silk sutures 4–0 were used to fix the cannula to the ovarian artery as previously described (Wallin et al. 2009). The ovaries were then gently perfused with 2 mL ice-cold sterile phosphate-buffered saline (PBS, Gibco, London, UK) supplemented with heparin (50 IU/mL; Apoteket AB, Stockholm, Sweden), xylocaine (0.04 mg/mL; Astra Zeneca, Gothenburg, Sweden), piperacillin/tazobactam (10 μg/mL and 1.25 μg/mL; Stragen Nordic, Hillerød, Denmark) and Gibco's antibiotic–antimycotic (anti–anti; 1%; penicillin 10 000 U/mL, streptomycin 10 000 μg/mL and fungizone 25 μg/mL; Thermo Fisher Scientific, Stockholm, Sweden) until the organ blanched and clear liquid was seen flowing through the ovarian vein. All experimental ovaries were then submerged in cold (4°C) sterile PBS solution and placed on ice during transport to the laboratory. Each control ovary was dissected along the long axis and divided in two equal halves. One half was placed directly in 4% formaldehyde and served as the fresh control tissue for follicular assessment before *ex vivo* ovarian perfusion, and the other half was kept in the same cold preservation solution used for the transportation of the intact ovaries used for perfusion to the laboratory thus serving as control tissue to assess potential follicular damage from the cold ischaemia during transport. The average time between ovarian collection, cannulation and vascular perfusion was between 30 and 60 min, and the average cold ischaemia time was between 180 and 240 min (from ovarian collection to perfusion start).

Perfusion system

The ovaries (n = 13) were perfused in a modified closed circulation perfusion system assembled using equipment

Table 1. Study design and ovum pick-up results among the experimental groups 1 and 2 of *ex vivo* ewe ovarian perfusion.

	Perfused ovaries	Control ovaries	Ischaemia time	hMG protocol	Medium sampling timepoints	Perfusion time	Aspirated follicles and oocyte recovery rate (n, %)	Retrieved oocytes (n, grading)
Experimental group 1	n = 3	n = 3	3–4 h	Single daily dose 2 h: 0 IU/mL 2 h: 1.75 IU/mL 2 h: 0.87 IU/mL 18 h: 0 IU/mL	20 min, 1, 24 h, every 24 h	120–168 h (5–7 days)	14, 43%	6, MI
Experimental group 2	n = 10	n = 10	3–4 h	24-h continuous 2 h: 0 IU/mL 24 h: 1.75 IU/mL	20 min, 1, 24 h, every 24 h	72–144 h (3–6 days)	22, 45%	9 MI, 1 GV

GV, germinal vesicle; MI, metaphase I; hMG, human menopausal gonadotropin.

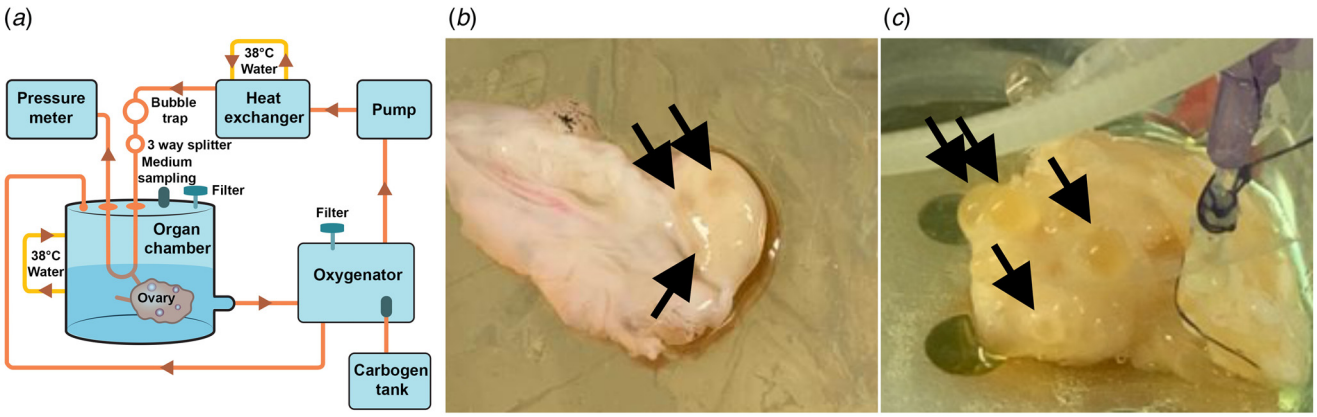

Fig. 1. (*a*) Bioreactor with peristaltic pump, oxygenator, heat exchanger, and organ perfusion chamber. The perfused ovary is completely submerged in the perfusion medium; (*b*) picture of ovary before perfusion. Small follicles are visible on the ovarian cortex (black arrows); (*c*) picture of ovary after perfusion (experimental group 2), immediately before ovum pick-up, showing ovarian cortex containing follicles with diameter between 3 and 5 mm (black arrows).

parts from a bioreactor set up previously assessed for *ex vivo* sheep uterus perfusion (Padma *et al.* 2019) (Fig. 1*a*). The ovaries were connected through the cannulated ovarian artery to the perfusion system. The bioreactor system recirculated the perfusate (closed system) which allowed the maintenance of a sterile environment and monitoring of temperature, oxygenation, perfusion pressure and flow throughout the duration of the experiment. The bioreactor consisted of three separate circuit loops: (1) the organ chamber that was combined with the media reservoir and a single ovary was completely submerged in the perfusion medium, (2) carbogen gas (95% O_2 and 5% CO_2) that oxygenated and buffered the perfusion media and (3) warm water (38°C) transported in a jacketed manner (physically separated from the two other circuits) ensuring a constant physiological temperature for the perfusate and the organ throughout the experiment. A peristaltic pump circulated the perfusate through the oxygenator (including a bubble trap) to the ovary in the organ chamber (Fig. 1*a*). The perfusion pressure was kept below 80 mmHg by regulating flow rate during the entire perfusion period in order to avoid ovarian vascular damage.

Experimental design

The perfused ovaries were allocated to two groups to assess follicular development and maturation using two different gonadotropin stimulation protocols (Table 1). Experimental group 1 (n = 3) consisted of ovaries that were perfused in the bioreactor (Fig. 1*a*) between 120 and 168 h with medium consisting of M199 (Thermo Fisher Scientific) supplemented with 2% bovine serum albumin (Roche Diagnostic GmbH, Mannheim, Germany), piperacillin/tazobactam (10 μg/mL and 1.25 μg/mL; Stragen Nordic), anti–anti (Thermo Fisher Scientific), 12 mL/L sodium bicarbonate 7.5% (Thermo Fisher Scientific), fetal bovine serum (FBS) 10% (Thermo Fisher Scientific), IGF-1 (50 ng/mL, Peprotech, Stockholm, Sweden) and HEPES (Sigma Aldrich, Stockholm, Sweden), with the addition of hMG (Menopur, Ferring Pharmaceuticals, Malmö, Sweden) administered in a single daily dose of 1.75 IU/mL for 2 h followed by 0.87 IU/mL for further 2 h. The hMG is a mixture (1:1) of follicle stimulating hormone (FSH) and luteinising hormone (LH) purified from the urine of postmenopausal women, both hormones are essential for follicle development and luteinisation (Campbell *et al.* 2007). Experimental group 2 (n = 10) consisted of ovaries that were perfused in the bioreactor between 72 and 144 h with the same medium used for group 1, but with hMG administered continuously at the same concentration of 1.75 IU/mL. Since gonadotropin secretion follows a pattern of daily peaks in cycling ewes (Baby and Bartlewski 2011), a single daily dose hMG media supplementation pattern was implemented in group 1 while a continuous hMG administration pattern was implemented in experimental group 2, as detailed in Table 1. The same cycle was repeated every 24 h. Human chorionic gonadotropin (hCG) (Pregnyl, MSD Sverige AB, Stockholm, Sweden) 50 IU/mL was added in the medium of 2 of the 3 ovaries in group 1 and of 6 of the 10 ovaries in group 2 during the last 24 h of perfusion before ovum pick-up (OPU) to trigger final oocyte maturation. OPU was performed at the end of the perfusion from visible follicles with a diameter between 3 and 5 mm (Fig. 1*b*, *c*), through gentle manual aspiration with an 18 G needle connected to a tube with an inner diameter of 2 mm (Rodriguez *et al.* 2006). The perfusion time was not constant among the experiments because the ovaries were retrieved from ewes that were not synchronised in their reproductive cycle. For this reason visible follicles amenable for OPU were achieved in different timepoints during the ovarian

perfusion. Additionally, 10 mL of the perfusion medium was sampled from the organ chamber at 20 min, 1 h, 24 h and at every 24 h during the perfusion (before any medium change). These perfusate samples were immediately frozen at −20°C and later biochemically analysed, as described below. At the end of the perfusion time, each ovary was fixed in 4% phosphate buffered formaldehyde for 24–48 h for later analysis.

Histomorphological examination and immunohistochemistry of ovarian tissue

Formaldehyde-fixed ovarian samples from both the perfused and non-perfused (control) ovaries were dehydrated and embedded in paraffin, sectioned at 4-μm thickness and stained with H&E according to standard protocols. Each specimen was examined in duplicate by two pathologists blinded to the sample groups. A minimum of two sections of each ovary were examined, taking at least ten sections (50 μm) apart from each other to avoid counting the same follicles twice. All ovarian-tissue slides were digitally scanned with a NanoZoomer S210 (Hamamatsu Photonics, Japan) and the surface area of the ovarian cortex on each section was measured using the NanoZoomer Digital Pathology.view 2 analysis software (Hamamatsu Photonics, Hamamatsu, Japan). The degree of injury was scored using digital pathology, according to previously established parameters (Paynter *et al.* 1999; Gandolfi *et al.* 2006). Non-damaged primordial follicles were identified as follicles with a spherical oocyte and a non-pyknotic nucleus, surrounded by one layer of flattened granulosa cells. Non-damaged primary follicles were identified as small spherical follicles that contained a GV stage oocyte surrounded by one to two layers of cuboidal granulosa cells. Secondary non-damaged follicles were identified as larger follicles surrounded by several layers of granulosa cells with or without small spaces between cells and containing a GV stage oocyte. Non-damaged antral follicles were identified by their characteristic fluid-filled antral cavity, where the oocyte lies at the edge in a mound made of granulosa epithelial cells (cumulus oophorus). Damaged follicles were classified as grade 1 follicles (G1) when theca and granulosa cells were pulled away from the edge, with disruptions and apparent loss of granulosa cells but with an oocyte maintaining its spherical shape. Grade 2 follicles (G2) were identified as follicles showing a greater disruption, the loss of granulosa cells and with theca cells pulled away from the follicle edge, with severe pyknotic nuclei in the granulosa cells and a misshapen oocyte with or without vacuolation or pyknotic nucleus (Fig. 2*a*–*f*). The overall ovarian histomorphological architecture was described with regards to abnormalities in the arteries and veins as endothelial detachment, internal elastic membrane rupture, or smooth muscle cell bloating (Koos *et al.* 1984; Milenkovic *et al.* 2011*b*). Follicular cell proliferation was studied by 3,3′-diaminobenzidine (DAB) immunohistochemistry for Ki-67, a nuclear protein associated with proliferation. Antigen retrieval was conducted in a pressure cooker with Diva Decloaker (Biocare Medical, HistoLab, Gothenburg, Sweden). Sections were exposed to hydrogen peroxide for 5 min, blocked with 5% bovine serum for 10 min and incubated with the Ki-67 antibody (antibody #15580; 1:500, Abcam, Cambridge, UK) overnight. Detection of the primary antibody was achieved by MACH 2 universal HPR (Biocare Medical) for 30 min, then Betazoid DAB for 5 min. Sections were then counterstained with hematoxylin. Apoptosis was confirmed by cleaved caspase 3 immunostaining (antibody Cat No. #9661; 1:300, Cell Signaling Technology, BioNordika, Stockholm, Sweden) followed by the highly sensitive MACH 3 polymer detection kit and by the Warp Red Chromogen kit (both from Biocare Medical) according to the manufacturers' instructions. This method clearly showed the different steps in the localisation of caspase 3 activation from cytoplasmic to nuclear translocation of the protein. Proliferation and apoptotic index were determined by calculating the percentage of Ki-67 and caspase 3 positive cells in hotspot areas of the ovarian cortex, examining all follicular subclasses separately (Fig. 2*g*, *h*).

Oocyte examination and maturation grading

Retrieved oocytes were examined using stereomicroscopy directly after OPU and were graded by an experienced embryologist who was blinded to the experimental groups. The number of retrieved oocytes, recovery rates and maturation grade were noted (Table 1).

Hormonal analysis

Oestradiol and progesterone concentrations in the undiluted sampled media were estimated to assess the follicular steroidogenic activity. The oestradiol concentration was estimated using sheep estrogen ELISA Kit (Reagent Genie, Dublin, Ireland). The coefficient of variation (CV) for oestradiol varied between 10 and 16%, the analytical sensitivity was 9.375 pg/mL and the detection range was 15.625–1000 pg/mL. The progesterone concentration was estimated using sheep progesterone ELISA Kit (Reagent Genie, Dublin, Ireland). The CV for progesterone varied between 6 and 11%, the analytical sensitivity was <0.188 ng/mL and the detection range was 0.0313–20 ng/mL.

Biochemical analysis

The concentrations of glucose, lactate, potassium, sodium, calcium and chloride in the perfusion medium were measured by a standard blood-gas analysis according to the manufacturer's protocols (RapidPoint 500 Blood Gas System,

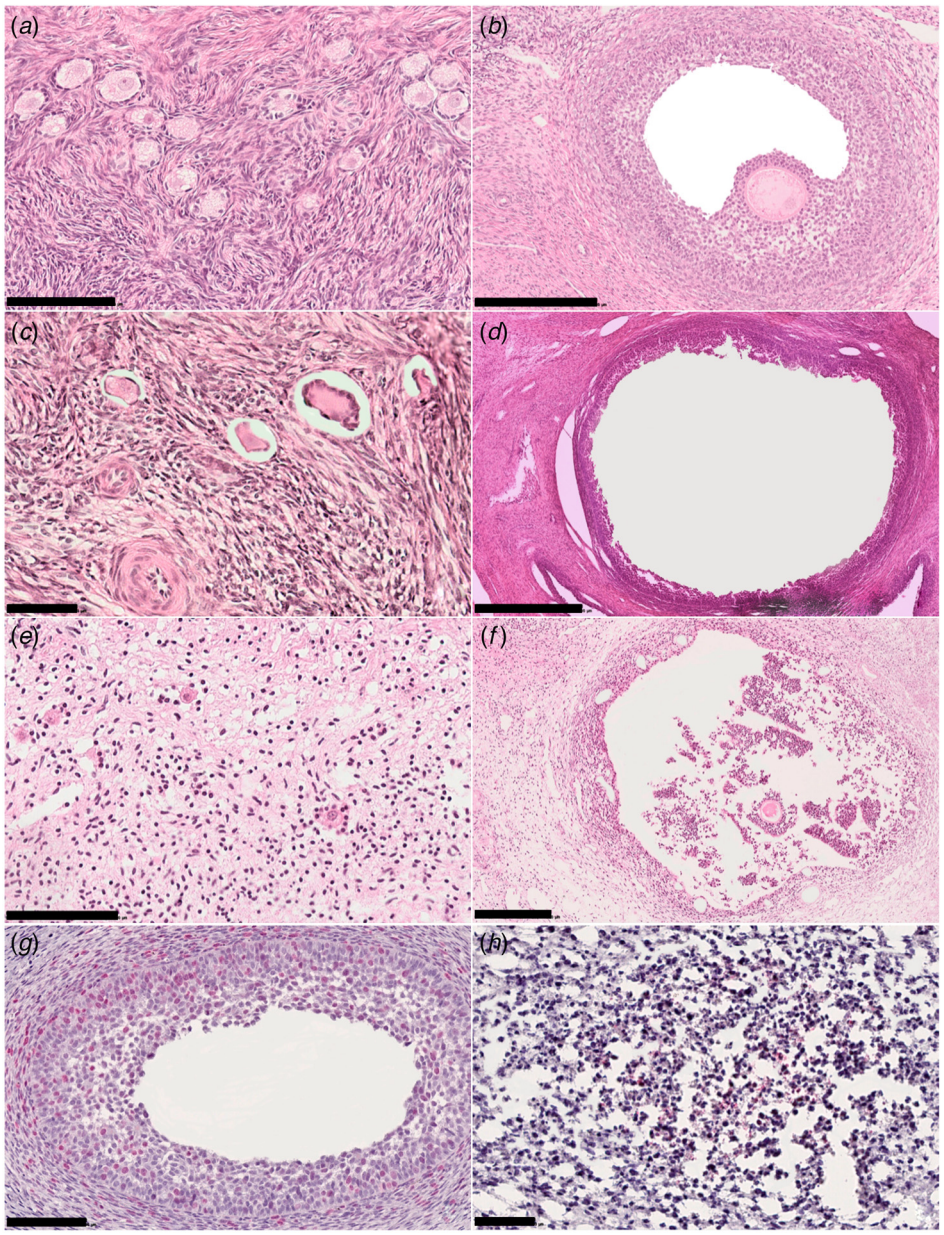

Fig. 2. Representative microscopic images of ischaemic and perfused ovaries showing follicles of different categories with H&E staining. (*a*) Non-damaged primordial follicles (scale bar = 100 μm); (*b*) non-damaged antral follicle (scale bar = 250 μm); (*c*) damaged G1 primordial follicles (scale bar = 50 μm); (*d*) damaged G1 antral follicle (scale bar = 500 μm); (e) damaged G2 primordial follicles (scale bar = 100 μm); (*f*) damaged G2 antral follicle (scale bar = 250 μm); (*g*) follicle with immunohistochemistry staining positive for Ki-67 (red) (scale bar = 100 μm); (*h*) follicle with immunohistochemistry staining positive for cleaved caspase 3 (red) (scale bar = 50 μm).

Siemens Healthcare, Erlangen, Germany) to assess the ovarian metabolic activity.

Statistics

The distribution of the data was assessed using Kolmogorov–Smirnov and Shapiro–Wilk normality tests. The Wilcoxon Signed Rank test for related samples was used to compare the number of non-damaged and damaged follicles before perfusion, after cold ischaemia and after perfusion. Wilcoxon matched-pairs signed-rank test was used to compare the percentage of Ki-67 and cleaved caspase 3 positive follicle cells before and after perfusion. To assess any changes over time for hormonal-, metabolic- and electrolytic biomarker

concentrations in the perfusion medium, the coefficient from a univariable linear regression with perfusion time as covariate was calculated for each perfused ovary and the set of coefficients were tested with Wilcoxon signed-rank test. A *post hoc* analysis was also performed for these biomarkers with Wilcoxon signed-rank test for the changes from the start of perfusion to each measure after perfusion. Data herein is presented as mean (±s.d.) and a two-sided *P*-value of less than 0.05 was considered significant. All analyses were performed with SAS version 9.4 and Prism version 9.0.0.

Ethics approval and consent to participate

No ethical approval for the study was necessary since the ovaries were collected from animals that were bred for meat production with the possibility to use discarded organs for research (in line with the Swedish and European Union ethical regulations). Consent to participate was not applicable.

Results

Effect of perfusion on ovarian histomorphology, cell proliferation and apoptosis

Group 1

The comparison of the ovarian cortex follicular population between the non-perfused (control) ovaries, ovaries exposed to 3–4 h of cold ischaemia, and ovaries exposed to 120–168 h of normothermic perfusion, showed that the perfused ovaries had a good follicular preservation without a significant reduction in both the total and non-damaged number of primordial follicles per mm^2. Intact histomorphology was seen in 67% of the medullary arteries, in 0% of the ovarian arteries, in 100% of the medullary veins and in 50% of the ovarian veins of the perfused ovaries. Moreover, 33% and 100% of the ovaries after perfusion had intact endothelial layer and vascular wall morphology, respectively. However, in the case that one artery presented, for example, endothelial injury or vascular wall injury, it was counted as an injured vessel. All follicular subclasses were negative for Ki-67 (proliferation marker), which indicated no ongoing cell proliferation. Furthermore, cleaved caspase 3 (apoptosis marker) staining indicated that there was no ongoing apoptosis in any follicle type.

Group 2

The comparison between the ovarian cortex follicular population of the non-perfused control ovaries, after 3–4 h of cold ischaemia, and after 72–144 h of perfusion, showed that there was a significant reduction in the number of non-damaged primordial follicles per mm^2 after cold ischaemia (median values; 4.05 vs 2.32, $P = 0.039$), and after perfusion (median values; 4.05 vs 0, $P = 0.031$). This was also noticed for the total number of secondary follicles per mm^2 after ischaemia (median values; 0 vs 0.028, $P = 0.047$). Moreover, a significant increase was seen in the number of damaged, grade 2 (G2) primordial follicles per mm^2 after perfusion (median values; 0.021 vs 3.83, $P = 0.031$), indicating a low follicular and oocyte stability during the perfusion time. However, the perfused ovaries showed intact histomorphology in 33% of the medullary arteries, in 40% of the ovarian arteries, in 29% of the medullary veins and in 20% of the ovarian veins. Moreover, 14% and 43% of the ovaries after perfusion had intact endothelial layer and vascular wall morphology, respectively. After perfusion, follicular cells positive for cleaved caspase 3 were seen in non-damaged primordial follicles. However, there was no significant difference in follicular cell apoptosis comparing non-perfused (control) ovaries with perfused ovaries. Immunohistochemistry staining showed that primary, secondary and antral follicles were negative for Ki-67 and cleaved caspase 3, both indicating no ongoing apoptosis.

Effect of perfusion on steroid production

After assessment of the perfusion medium with ELISA, no significant changes were seen in oestradiol and progesterone concentrations in group 1 during the perfusion time (Fig. 3*a*, *b*). Oestradiol concentrations in the perfusion medium in group 2 significantly decreased between 20 min and all the later time points (Fig. 3*c*), indicating an insufficient gonadotropin follicle stimulation. In contrast, the significantly increased progesterone concentration in this group after 20 min (Fig. 3*d*) indicated an ongoing luteinising process of the growing follicles.

Effect of perfusion on metabolic and electrolytic biomarkers

After assessment of the perfusion medium with blood-gas analysis, no significant changes were seen in the concentration of metabolic and electrolytic biomarkers in group 1 (Figs 4*a*, *b* and 5*a*–*d*). Lactate, potassium, calcium and chloride concentrations in the perfusion medium in group 2 significantly increased between the perfusion start and the later time points (Figs 4*d* and 5*f*–*h*). In contrast, the glucose concentration significantly decreased in this group after the start of perfusion (Fig. 4*c*). Both metabolic and electrolytic changes in group 2 indicate active metabolic processes.

Effect of perfusion on oocyte recovery rate and quality

From fourteen aspirated follicles in group 1, six metaphase I stage (MI) oocytes were retrieved (oocyte recovery rate 43%) and from 22 follicles of group 2, nine MI and one germinal vesicle stage (GV) oocyte were retrieved (oocyte recovery

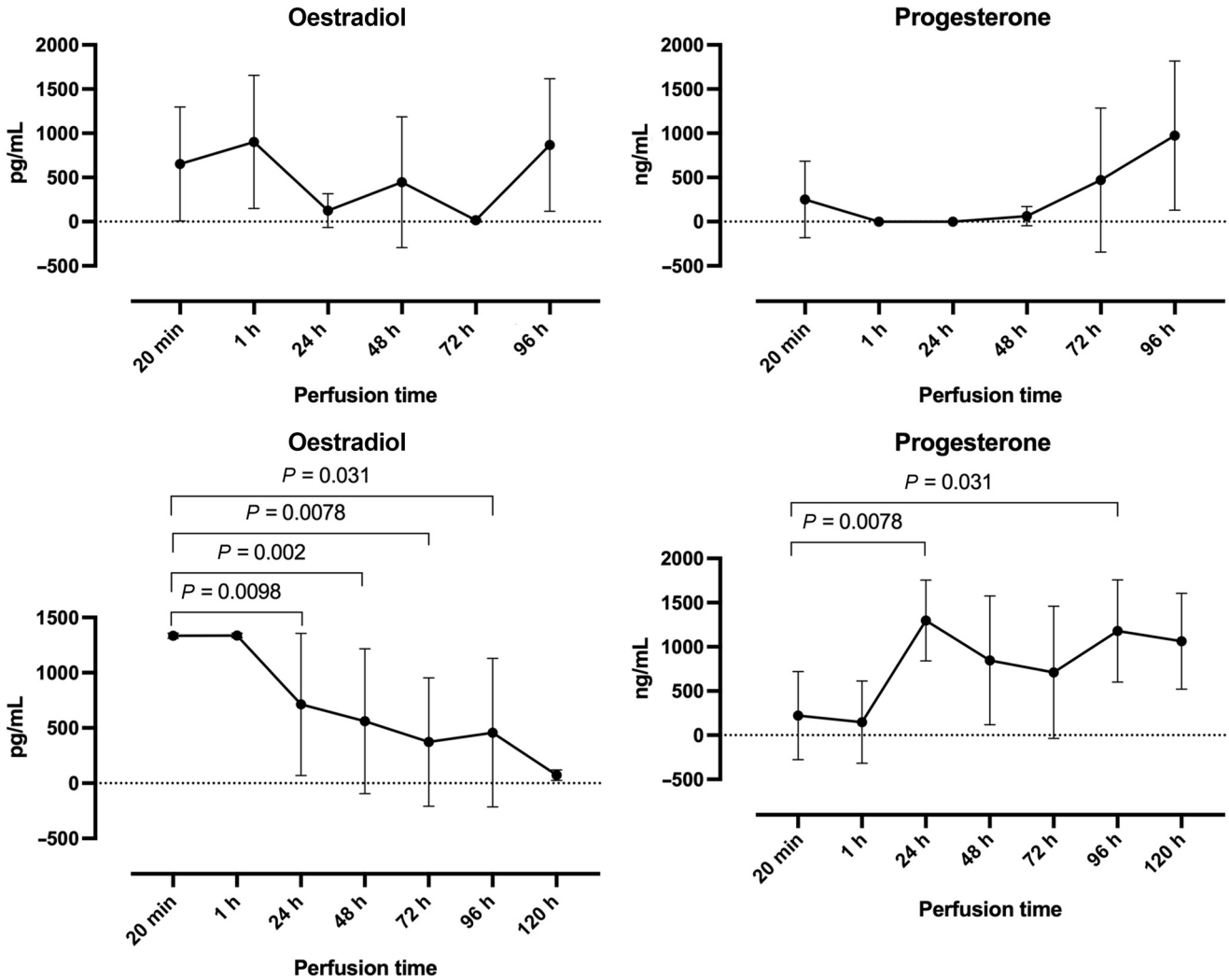

Fig. 3. Change in the concentrations of oestradiol and progesterone in the perfusion medium assessed at different time points during the ovarian perfusion (*a*, *b*) group 1; (*c*, *d*) group 2.

rate 45%) (Table 1). No metaphase II stage (MII) oocytes were retrieved from both groups. All retrieved oocytes had homogeneous cytoplasm and were partially covered by cumulus cells.

Discussion

In this study, we described an *ex vivo* perfusion device able to maintain whole sheep ovaries viable for up to 168 h (7 days). Two different gonadotropin stimulation protocols were assessed for *ex vivo* ovarian stimulation and the subsequent follicular development and histomorphology were evaluated. This study demonstrated successful harvesting of oocytes and to our knowledge, this is the longest *ex vivo* perfusion time (7 days) described to date for ewe ovaries. A number of studies have been performed on ovarian tissue perfusion from rodents (Koos *et al.* 1984; Sogn *et al.* 1984; Brännström *et al.* 1987; Brännström and Flaherty 1995), rabbit (Lambertsen *et al.* 1976; Hamada *et al.* 1977, 1979; Kobayashi *et al.* 1981*a*), sheep (Wallin *et al.* 2009; Milenkovic *et al.* 2011*b*; Maffei *et al.* 2016) and also from human tissue (Stähler *et al.* 1974; Abrahamsson *et al.* 1990; Milenkovic *et al.* 2011*a*; McLaughlin *et al.* 2018). However, a complete follicular development *in vitro* with subsequent oocyte maturation and live offspring has only been successful in rodent models (Eppig and Schroeder 1989; O'Brien *et al.* 2003). Progress in large animals, e.g. the sheep, has been limited due to the long time required for the *in vitro* follicular growth and oocyte maturation typical of large mammals (Shea *et al.* 2014). However, significant advances in organ reperfusion research using bioreactors and normothermic (37°C) conditions provide novel platform ideas on how to overcome these hurdles (Hosgood *et al.* 2018; Nasralla *et al.* 2018). In the study described herein, we used similar perfusion principles and found that a

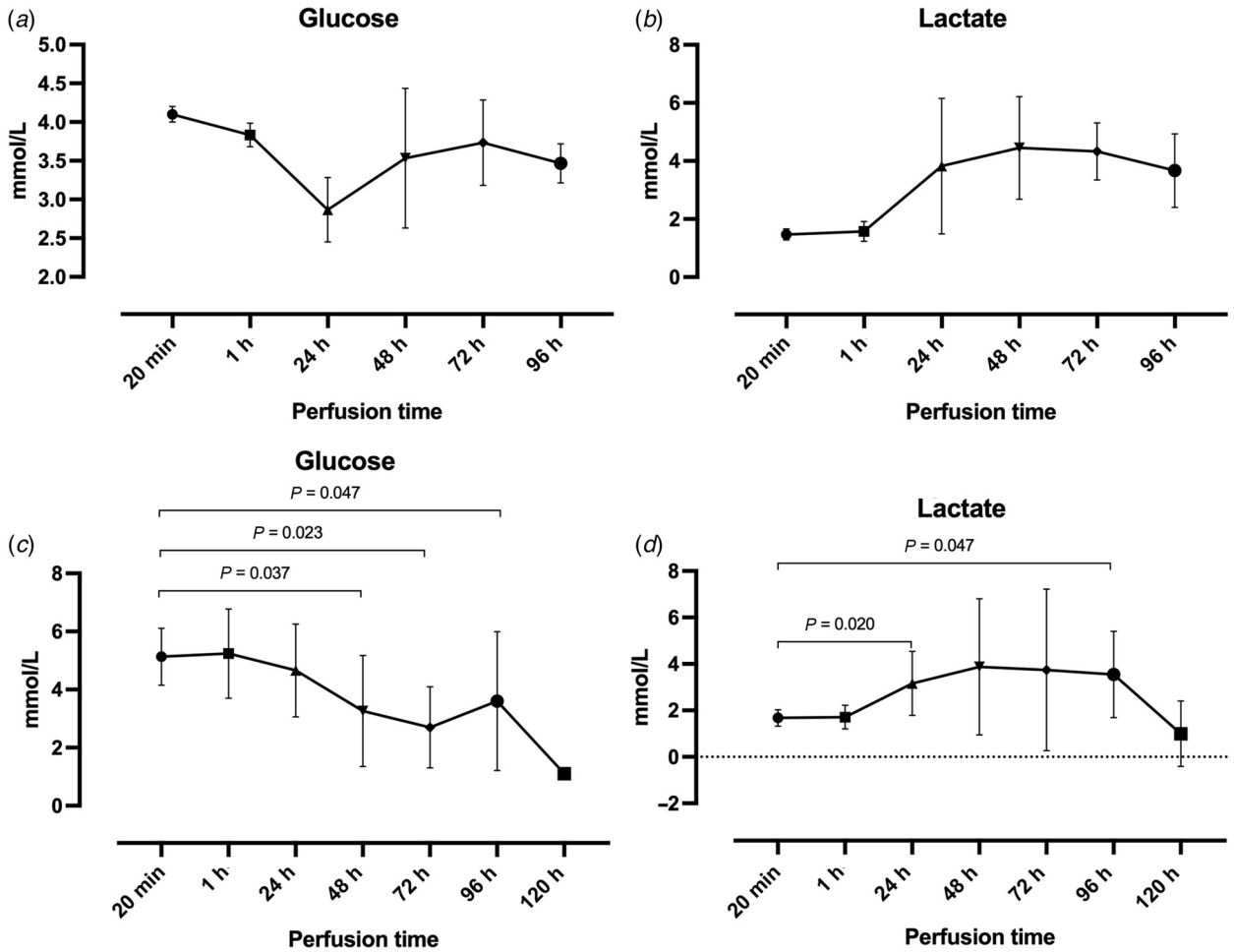

Fig. 4. Change of concentrations of glucose and lactate in the perfusion medium assessed at different time points during the ovarian perfusion (*a*, *b*) group 1; (*c*, *d*) group 2.

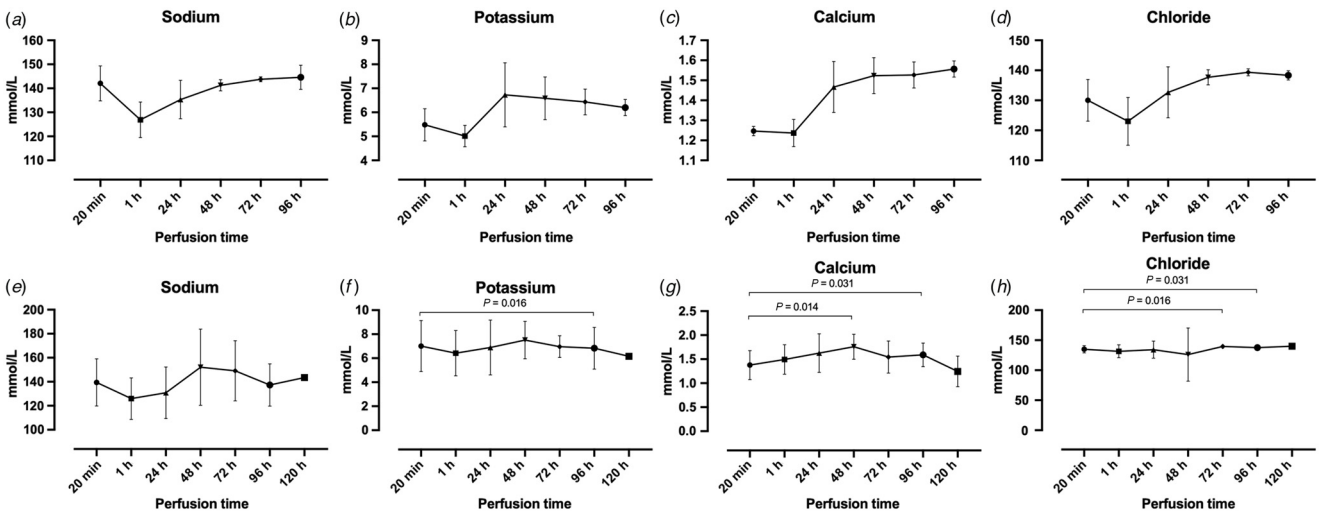

Fig. 5. Change of concentrations of sodium, potassium, calcium and chloride in the perfusion medium assessed at different time points during the ovarian perfusion (*a*–*d*) group 1; (e–*h*) group 2.

hMG-enriched medium used in a closed bioreactor system can maintain the general health of the ovary after explantation, and that the follicular sub-populations could be protected from apoptosis. Moreover, no significant damage was observed in the sub-follicular morphology (the theca and granulosa cells), or in oocyte integrity compared with control ovaries. Interestingly, the primordial follicles were particularly stable in group 1 where ovaries were stimulated with hMG administered in a single daily dose, while a significant depletion of the non-damaged primordial and total secondary follicular density, with concomitant increase in G2 damaged primordial follicular density was seen in group 2. Perhaps, this difference was due to the modifications made in the pulsatile hormone dosage, since a more gradual hormone administration (as used in group 1) may ensure a better protection of the immature follicles pool. Furthermore, no differences in follicular apoptosis were seen after perfusion in any of the follicular subclasses in group 1. However, the relatively intact histomorphology of both the arterial and venous ovarian pedicles in both groups after the extended ovarian perfusion, together with the good cellular survival and minimal tissue damage is a significant achievement considering the long perfusion times explored. Our results are consistent with, and expand on, the previous short-time ovarian perfusion studies: 2 h of perfusion in ewes (Wallin *et al.* 2009), 5 h in human (Abrahamsson *et al.* 1990; Milenkovic *et al.* 2011*a*), 12 h in rabbit (Lambertsen *et al.* 1976; Hamada *et al.* 1977, 1979; Kobayashi *et al.* 1981*a*) and 20 h in rat and mouse (Koos *et al.* 1984; Sogn *et al.* 1984; Brännström and Flaherty 1995). Our results support the findings of another study that used a complex perfusion medium supplemented with insulin, IGF-I and FSH that resulted in a successful 96-h long perfusion time (Maffei *et al.* 2016). Perhaps it could be beneficial to supplement ovarian perfusion protocols with growth factors and hMG. An unexpected finding of our study was the observation that, despite the long perfusion time, there was no cell damage and apoptosis of follicular subclasses as shown by the absence of cell proliferation and apoptosis markers (Ki-67 and cleaved caspase 3, respectively). These results may be explained by the low density of higher follicular classes found in the perfused ovaries of both groups, and/or that the gonadotropin insensitivity of the primordial follicles prevented the development of early-stage follicles. This normal hormone insensitivity in small follicles is caused by a low expression of hormone receptors and, in future studies, alternative growth stimulants should be included in the perfusate to better promote primordial follicle development. Correspondingly, oestradiol concentrations in group 1 did not significantly increase during the perfusion, suggesting the need for protocol optimisation (higher gonadotropin levels and addition of growth stimulants) in the perfusate to initiate the primordial follicle growth and to support granulosa and theca cells. Interestingly, the oestradiol concentrations significantly decreased during the perfusion in group 2, suggesting that a continuous (24 h daily) gonadotropin administration is suboptimal. However, another plausible explanation for the low oestradiol concentrations could be related to the repeated perfusate changes, every 24 h, that could have diluted the oestradiol concentrations measured in both groups. These perfusate changes were necessary to prevent accumulation of toxic levels of lactate and other metabolic byproducts associated with a high ovarian metabolic rate and the absence of a systemic clearance of these byproducts *in vitro/ex vivo*. The progesterone levels significantly increased during perfusion in group 2, but to a lesser extent in group 1. The elevation of progesterone levels in group 2 were likely associated with the high and non-pulsatile hCG supplementation in the perfusate that probably supported early luteinisation of the higher-class follicles. Previous ovarian perfusion studies performed on different models have also shown steroid hormonal secretion in the perfusion medium. For example, the addition of forskolin stimulated an increase of oestradiol and progesterone levels in the perfusate after 2 h in ewe ovary perfusion (Wallin *et al.* 2009). Therefore, the addition of forskolin to the perfusate in future experiments could be beneficial using our *ex vivo* ovarian perfusion platform. Steroid hormones were also produced from perfused human postmenopausal ovaries in response to hCG during a 5-h perfusion period (Abrahamsson *et al.* 1990; Milenkovic *et al.* 2011*a*). Previous ovarian perfusion studies with rabbit, rodents and human models used shorter perfusion times than what is biologically required to retrieve fully mature oocytes (Stähler *et al.* 1974; Lambertsen *et al.* 1976; Hamada *et al.* 1977, 1979; Wallach *et al.* 1978; Kobayashi *et al.* 1981*b*; Koos *et al.* 1984; Sogn *et al.* 1984; Brännström *et al.* 1987; Sogn *et al.* 1987; Brännström and Flaherty 1995). In a recent study, an extended *ex vivo* whole ewe ovary perfusion was achieved, although no ovum pick-up was performed (Maffei *et al.* 2016). To our knowledge, our study is the first to report oocyte retrieval after extended ewe ovarian perfusion. A total of 16 oocytes (1 GV and 15 MI) were retrieved from groups 1 and 2 after hMG ovarian stimulation. The oocyte recovery rates (43–45%) were comparable with a previous study where oocyte retrieval was performed post-mortem, using similar ovum pick-up parameters as presented herein (Rodriguez *et al.* 2006). Despite the absence of MII oocytes, the retrieval of 15 MI oocytes that could be maturated *in vitro* (IVM) is an encouraging result. It is known that oocyte immaturity (GV/MI), in sheep is expected because the process of *in vitro* embryo production is still inefficient (approximately 70–90% of immature oocytes undergo maturation). Thus, the use of IVM of retrieved immature oocytes is considered necessary before any *in vitro* fertilisation (IVF) should be performed (Zhu *et al.* 2018).

Conclusion

Our study sought to find a novel method for enhancing the options of fertility preservation for prepubertal girls prior to chemo/radiotherapy or for women with cancer at high risk of ovarian metastasis. The strengths of this study are the extended perfusion times achieved herein (up to 7 days) and that a total of 16 GV–MI oocytes were retrieved from both groups. Long perfusion times will be necessary to give sufficient time for *ex vivo* follicle development and oocyte harvesting. The addition of piperacillin/tazobactam in the perfusate presented herein resulted in extended perfusion times without contamination. Additionally, we assessed all follicular sub-classes separately using cell proliferation and apoptosis indexes to evaluate potential negative influences caused by the perfusion. A limitation to our study includes the collection of ovaries from ewes of unknown cycle and reproductive history. However, organs collected from the slaughterhouse provided a large number of ovaries without the need for costly large animal experiments and this approach does not require any ethical permission within the European Union since the animals are bred and culled for food production. Another limitation is that IVM was not performed to assess the viability of the retrieved oocytes. However, the experimental platform presented herein will serve as an excellent whole ovary perfusion model to conduct future experiments that could elucidate what additional factors may be beneficial to stimulate *ex vivo* follicular maturation. In clinical settings, such a platform might be used for fertility preservation by promoting *in vitro* follicular growth and harvesting oocytes. Once perfected, this option could be very beneficial for prepubertal girls of for patients with systemic cancer that have high risk of ovarian metastasis thus precluding ovarian tissue freezing for future regrafting. Future studies should assess the effect on follicular growth, viability and oocyte maturation of higher doses of gonadotropins, and other growth stimulants, administered in the perfusate, in combination with more refined ovum pick up parameters.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

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