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ARTICLE

Human platelet lysate improves the growth and survival of cultured human pre-antral follicles

BIOGRAPHY

Cristina Subiran Adrados is a PhD student at the Laboratory of Reproductive Biology at the Copenhagen University Hospital. She has a background in stem cells science and reproductive medicine and her research focuses on female fertility preservation. She is currently working with human follicle culture and ovarian tissue cryopreservation.

Cristina Subiran Adrados[a,b,*], Jesús Cadenas[a], Mengxue Zheng[a,b], Sofie Lund[a], Elisabeth Clare Larsen[c], Mette Honnens Tanvig[d], Vinnie Hornshøj Greve[e], Paul Blanche[f], Claus Yding Andersen[a,b], Stine Gry Kristensen[a,c]

KEY MESSAGE

Follicle culture has the potential to become a clinical alternative for fertility preservation, especially for leukaemia and ovarian cancer patients. Here, we showed that human platelet lysate significantly increases growth and survival of pre-antral follicles cultured three-dimensionally for 8 days compared with other traditionally used protein sources.

ABSTRACT

Research question: How do platelet-rich plasma products like human platelet lysate (HPL) and umbilical cord plasma (UCP) affect the growth and survival of isolated human pre-antral follicles *in vitro*?

Design: Human pre-antral follicles ($n = 724$; mean diameter: 75 μm; range: 46–237 μm) were isolated from ovarian medulla donated by 14 patients undergoing unilateral oophorectomy for ovarian tissue cryopreservation. Follicles were encapsulated in 0.5% alginate and cultured for 8 days in media supplemented with 5% fetal bovine serum (FBS) ($n = 171$), 2.5% human serum albumin (HSA) ($n = 159$), 5% HPL ($n = 223$) or 5% UCP ($n = 171$).

Results: The survival probability was significantly higher in the group supplemented with HPL (80%) compared with the other three groups: FBS (54%, $P < 0.001$); HSA (63%, $P = 0.004$) and UCP (29%, $P < 0.001$). Surviving follicles in the UCP group had less defined follicular membranes and decompacted granulosa cell layers. The median growth of surviving follicles was significantly ($P < 0.001$) larger in the HPL group (73 μm) compared with any of the other three groups: HSA (43 μm); FBS (40 μm) UCP (54 μm). A descriptive analysis of follicular secretion of anti-Müllerian hormone and oestradiol did not reveal any difference between the groups. The detectability of follicular genes was high for *AR* (100%), *AMHR2* (100%) and *FSHR* (76%), whereas few follicles expressed *LHR* (20%).

[a] Laboratory of Reproductive Biology, The Juliane Marie Centre for Women, Children and Reproduction, University Hospital of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen, Denmark
[b] Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[c] The Fertility Clinic, University Hospital of Copenhagen, Rigshospitalet, Copenhagen, Denmark
[d] The Fertility Clinic, Department of Obstetrics and Gynecology, Odense University Hospital, Odense, Denmark
[e] Department of Obstetrics and Gynecology, Aarhus University Hospital, Palle Juul-Jensens Boulevard 82, DK-8200, Aarhus N, Denmark
[f] Department of Biostatistics, University of Copenhagen, Øster Farimagsgade 5, Entrance B, 2nd floor, 1014 Copenhagen, Denmark

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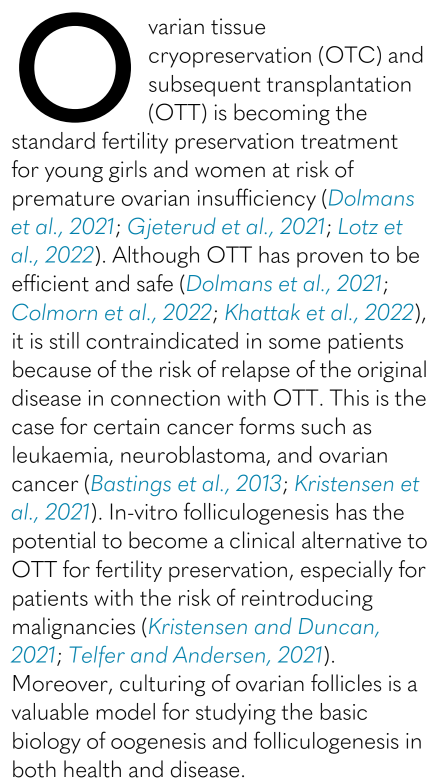
*Corresponding author: E-mail address: cristinasubiran@gmail.com (C. Subiran Adrados). https://doi.org/10.1016/j.rbmo.2023.06.011 1472-6483/© 2023 The Author(s). Published by Elsevier Ltd on behalf of Reproductive Healthcare Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)
Declaration: The authors report no financial or commercial conflicts of interest.

KEYWORDS
platelet rich plasma
human pre-antral follicles
fertility preservation
follicle culture

Conclusion: Human platelet lysate significantly improved survival and growth of cultured human pre-antral follicles compared with FBS, HSA and UCP. The use of HPL is a valuable improvement to culture human pre-antral follicles but further studies will have to prove whether the superiority of HPL translates into better quality oocytes.

INTRODUCTION



Ovarian tissue cryopreservation (OTC) and subsequent transplantation (OTT) is becoming the standard fertility preservation treatment for young girls and women at risk of premature ovarian insufficiency (*Dolmans et al., 2021*; *Gjeterud et al., 2021*; *Lotz et al., 2022*). Although OTT has proven to be efficient and safe (*Dolmans et al., 2021*; *Colmorn et al., 2022*; *Khattak et al., 2022*), it is still contraindicated in some patients because of the risk of relapse of the original disease in connection with OTT. This is the case for certain cancer forms such as leukaemia, neuroblastoma, and ovarian cancer (*Bastings et al., 2013*; *Kristensen et al., 2021*). In-vitro folliculogenesis has the potential to become a clinical alternative to OTT for fertility preservation, especially for patients with the risk of reintroducing malignancies (*Kristensen and Duncan, 2021*; *Telfer and Andersen, 2021*). Moreover, culturing of ovarian follicles is a valuable model for studying the basic biology of oogenesis and folliculogenesis in both health and disease.

In rodents, the first live birth resulting from complete in-vitro folliculogenesis was obtained in mice in 1996 (*Eppig and O'Brien, 1996*) and repeated in 2003 (*O'Brien et al., 2003*). In humans, the studies are limited owing to the scarcity of the material, but the results obtained during the last 10 years have been encouraging, with three scientific groups independently reporting derivation of mature human oocytes, i.e. metaphase II oocytes, from cultured pre-antral stage follicles. The first study in 2015 reported mature oocytes using secondary follicles as a starting point (*Xiao et al., 2015*) and two more recent studies reported development of mature oocytes from unilaminar follicles (*McLaughlin et al., 2018*; *Xu et al., 2021*). The oocyte maturation rates for cultured follicles remain low, however, and the multiple-step culture systems, culture media, materials and duration of the culture were substantially different between the three studies. Nonetheless, in-vitro folliculogenesis is a promising alternative for fertility preservation, but more research is needed to improve maturation rates and standardize the protocols for it to become a realistic option for fertility preservation.

A key component of the follicle culture media is the nutrient source, with serum or plasma preparations typically being used. Platelet rich plasma (PRP) is produced by purifying and concentrating the plasma fraction of blood to concentrate the platelets about five-fold compared with peripheral blood (*Marx, 2001*). Upon activation, these platelets release a multitude of growth factors in high concentrations, including members of the transforming growth factor β superfamily (TGF-ß), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factors and other hormones, and cytokines related to tissue repair, angiogenesis and regeneration. The balance release of these biomolecules contained in platelets affects cell behaviour in terms of growth, recruitment and differentiation (*Everts et al., 2020*; *de Miguel–Gómez et al., 2021*). Despite a lack of consensus on the definition of PRP and standardization of purification protocols (*Tey, 2022*), most studies showed a positive effect of PRP during culture of many cell types, such as mesenchymal stem and stroma cells, bone tissue-derived cells and adipose cells (reviewed in *Anitua et al., 2022*). Adult peripheral blood is the most widely used source of PRP, but it can also be prepared using plasma from umbilical cord blood (*de Miguel–Gómez et al., 2020*; *Subiran et al., 2021*). A proteomic analysis comparing umbilical cord plasma (UCP) and peripherical blood PRP showed differences in the protein concentration and in protein compositions (*de Miguel–Gómez et al., 2021*). Collectively, PRP solutions are a promising cell supplement for clinical cell therapies, containing high concentrations of proteins and growth factors of human origin, reducing the risk of xeno-immunization and zoonotic transmission. The superiority of either peripheral blood or UCP as the source of PRP, however, remains to be determined.

Within reproductive medicine, PRP prepared from adult goat peripheral blood was able to support in-vitro maturation (IVM) of goat oocytes and had a protective effect on the mitochondrial activity and intracytoplasmic lipid content, which was not observed in the control group containing fetal bovine serum (FBS) (*Moulavi et al., 2020*). Interestingly, studies in mice have shown that PRP from murine adult blood used to culture ovarian pre-antral follicles improved oocyte maturation rates compared with FBS (*Taghizabet et al., 2022*). Murine follicle culture with murine umbilical cord-blood PRP significantly decreased follicular growth and blocked IVM completely (*Pazoki et al., 2015*). Moreover, human adult blood PRP supplementation increased growth and survival in three-dimensional group-cultured human primordial and small primary follicles co-cultured with ovarian stromal cells isolated from fresh and vitrified ovarian medulla tissue (*Hosseini et al., 2017*). Therefore, few studies have examined the effect of PRP on pre-antral follicle growth, and the results are variable possibly owing to different sources of PRP. The aim of the present study was to further investigate the effect of commercial PRP products, such as adult-peripheral and umbilical-cord blood, on the culture of isolated human pre-antral follicles.

MATERIALS AND METHODS

Ethical approval

The present study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Scientific Ethical Committee for the Capital Region of Denmark on 25 June 2020 (number H-2-2011-044). The storage and collection of patient data were approved by the Ministry of Health (number 30-1372) and by the Danish authorities to comply with European Union Tissue Directives.

Patients

Ovarian medulla tissue was donated by 14 patients (mean age 29.0 range 19.3–37.1) undergoing unilateral oophorectomy as part of OTC. The indications for fertility preservation were breast cancer (n = 8), chronic myeloid leukaemia (n = 1), Hodgkin's lymphoma (n = 1), sarcoma (n = 1), neurological cancer (n = 2) and rheumatoid arthritis (n = 1).

Experimental set-up

Human pre-antral follicles (n = 724) were isolated from surplus ovarian medulla tissue and divided into four experimental groups according to initial diameter (Supplementary Table). Each group was supplemented with either 5% FBS (Invitrogen, Gibco 10500-064) (ThermoFisher Scientific, Waltham, MA, USA), 2.5% HSA (CSL Behring 20%, Marburg, Germany), 5% HPL (catalogue number 06960) (Stem Cell Technologies, Kent, WA, USA) or 5% UCP (catalogue number 70020) (Stem cell Technologies, Kent, WA, USA). Follicles were cultured for 8 days, and growth and survival were assessed every second day during culture by microscopy. On day 8, spent medium was collected and anti-Müllerian hormone (AMH) and oestradiol concentrations were measured by enzyme-linked immunosorbent assay (ELISA). Surviving follicles were snap-frozen and the expression of *AR, AMHR2, FSHR* and *LHR* was analysed by quantitative reverse transcription polymerase chain reaction (FIGURE 1). The concentrations of the supplements were selected based on published research and our own experience with human follicle culture (*Pazoki et al., 2016*; *Yin et al., 2016*; *Hosseini et al., 2017*).

Follicle collection

Patients undergoing OTC for fertility preservation had one entire ovary surgically removed and delivered to the laboratory. The surgical procedure was carried out in the local hospital and two other collaborating hospitals in Denmark. Ovaries were delivered in McCoy's 5A (Invitrogen) (ThermoFisher Scientific, Waltham, MA, USA) at room temperature within 10 min (when the surgical procedure was carried out at the local hospital) or in Custodiol®-HTK (Koehler-Chemie, Germany) on ice within 4–6 h (when the surgical procedure was carried out at collaborating hospitals). The follicles were isolated as previously described (*Kristensen et al., 2011*) with minor modifications (FIGURE 1). First, medulla tissue pieces were subjected to mechanical digestion by continuously chopping for about 30 min in room temperature DMEM/F-12 media (Invitrogen, Gibco 21041-025) (ThermoFisher Scientific, Waltham, MA, USA) using the McIlwain Tissue Chopper (Cavey Laboratory Engineering Co. Ltd., UK). Subsequently, tissue was transferred to pre-warmed holding media supplemented with the digestion enzymes and incubated at 37°C for 45–60 min with gentle agitation. Holding media with the digestion enzymes consisted of McCoy's 5α culture medium containing 25 mM HEPES (Invitrogen, Gibco 22330-21) (ThermoFisher Scientific, Waltham, MA, USA), supplemented with 1 mg/ml human serum albumin (20%) (CSL Behring, Marburg, Germany), 2 mM Glutamax (Invitrogen, Gibco 35050-038), 0.05 mg/ml penicillin/streptomycin (Invitrogen, Gibco 15140122), 10 mg/ml insulin, 5.5 mg/ml transferrin and 6.7 ng/ml selenium (insulin-transferrin-selenium, Invitrogen, Gibco 41400-045) (ThermoFisher Scientific, Waltham, MA, USA), i.e. holding medium plus 0.2 mg/ml collagenase IV (Sigma-Aldrich, St Louis,

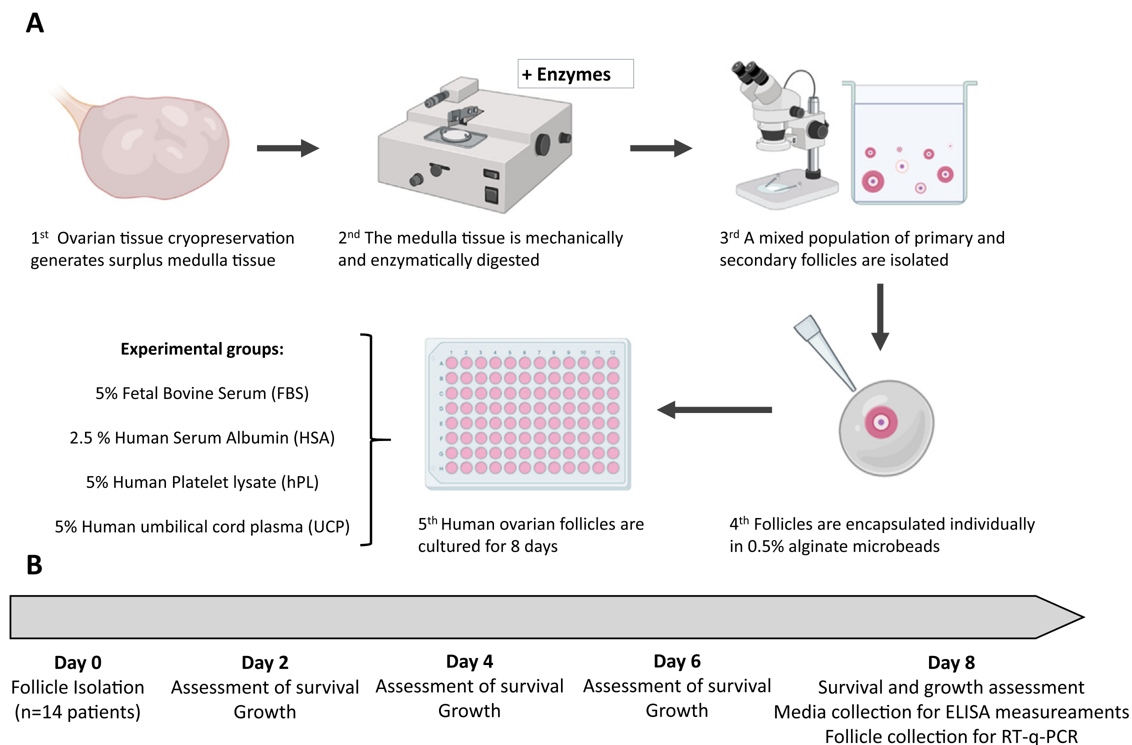


FIGURE 1 Experimental design. (A) Human pre-antral follicles (n = 724) were isolated from surplus ovarian medulla tissue donated by 14 patients undergoing unilateral oophorectomy as part of OTC for fertility preservation. Collected follicles were encapsulated in 0.5% (w/v) sodium alginate drops by pipetting 4 μl alginate drops into the cross-linking solution. The follicles were divided in four experimental groups: 5% fetal bovine serum (n = 171), HSA (n = 159), HPL (n = 223) and UCP (n = 171); (B) follicles were cultured for 8 days; growth and survival were assessed every second day during culture by microscopy. On day 8, anti-Müllerian hormone oestradiol concentrations were measured by enzyme-linked immunosorbent assay (ELISA) in media. Surviving follicles were snap-frozen and the expression of *AR*, *AMHR2*, *FSHR* and *LHR* was analysed by quantitative reverse transcription polymerase chain reaction (RT-q-PCR).

MO, USA) and 0.2 mg/ml DNase I (Sigma-Aldrich, St Louis, MO, USA). The enzymatic digestion was terminated by addition of an equal volume of 37°C digestion termination media consisting of holding medium supplemented with 10% FBS (Invitrogen, Gibco 10500-064) (ThermoFisher Scientific, Waltham, MA, USA) and 0.05 mg/ml penicillin/streptomycin (Invitrogen, Gibco 15140122) (ThermoFisher Scientific, Waltham, MA, USA). After enzymatic digestion, the cell suspension was aspirated up and down approximately 100 times with Pasteur pipette to release the follicles into the media. The cell suspension was screened in the flow bench using a Leica stereo MZ12.5 microscope to identify and collect isolated follicles. The follicles were washed in termination media and held in holding media until alginate encapsulation.

Follicle encapsulation and culture

Collected follicles were encapsulated in 0.5% (weight per volume) sodium alginate as previously described (*Kristensen et al., 2011*) with minor modifications. Liquid alginate (4 μl) containing one follicle was carefully dropped into a 35 mm culture dish filled with cross-linking solution (140 mM NaCl and 50 mM $CaCl_2$). To secure complete polymerization, the newly formed alginate bead was gently swirled around in the plate for 30 s. Immediately after, the alginate beads were removed and washed three times in culture media and each follicle-alginate bead was transferred to an individual well in a 96-well plate (Nuclon TM Delta 167008) (ThermoFisher Scientific, Waltham, MA, USA) containing 100 μl pre-equilibrated culture media: α-MEM (Invitrogen, Gibco 12571-063) (ThermoFisher Scientific, Waltham, MA, USA), 0.05 mg/ml penicillin/streptomycin (Invitrogen, Gibco, 15140122) (ThermoFisher Scientific, Waltham, MA, USA), 10 mg/ml insulin, 5.5 mg/ml transferrin, 6.7 ng/ml selenium (insulin-transferrin selenium, Gibco 41400-045) (ThermoFisher Scientific, Waltham, MA, USA), 50 μg/mlh ascorbic acid 9 (A4544-25G) (Sigma-Aldrich Life Sciences, St Louis, MO, USA), 2 IU/ml heparin (Amgros I/S, København, Denmark) and 100 mIU/ml recombinant FSH (Rekovelle, Ferring, Denmark). The follicles were divided in four experimental groups: 5% FBS (Invitrogen, Gibco 10500-064) (ThermoFisher Scientific, Waltham, MA, USA), 2.5% HSA (CSL Behring 20%, Marburg, Germany), 5% HPL (catalogue number 06960 (Stem Cell Technologies, Kent, WA USA) and 5% UCP (catalogue number 70020) (Stem cell Technologies, Kent, WA, USA).

The follicles were cultured at 37°C and 5% CO_2 for 8 days. Every second day, one-half of the culture media (50 μl) was exchanged with fresh media and survival and growth were assessed. The diameter was defined as the mean of two perpendicular lines drawn through the middle of the follicle, using the basal membrane as a measure. A non-blinded operator classified follicles as atretic if one of the following conditions was observed: the follicular membrane was disrupted; the oocyte became dark; and the granulosa cells structure was disrupted (see representative examples in Supplementary Figure). When a follicle was considered atretic, it was excluded from the analysis. After 8 days in culture, follicles and the spent media were collected and snap frozen for quantitative reverse transcription polymerase chain reaction or ELISA measurements.

Sodium alginate preparation

The stock of 10 mg/ml of sodium alginate (catalogue number W201502) (Sigma-Aldrich, St Louis, MO, USA) was prepared using deionized water. Organic impurities were removed by treatment with activated charcoal (0.5 g charcoal/g alginate). The active coal was removed via sterile filtration and the alginate solution was then lyophilized. The day before the follicle isolation and encapsulation, alginate aliquots were reconstituted with phosphate buffered saline (Invitrogen, Gibco, 14190136) (ThermoFisher Scientific, Waltham, MA, USA) (*Xu et al., 2009*).

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted individually from a subset of surviving follicles using Trizol reagent (Ambion) (Life Technologies, Carlsbad, CA, USA) and 1-bromo-3-chloropropane (Sigma-Aldrich, St Louis, MO, USA) and subsequently, with RNeasy Minikit 250 (Qiagen, Aarhus Denmark) following the manufacturer's instructions. The quantity and quality of the isolated RNA were evaluated using Agilent RNA 6000 Pico kit and Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). For each sample, cDNA was synthetized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, San Francisco, CA, USA) according to manufacturer's instructions. The following TaqMan probes were used: FSH receptor (*FSHR*; #Hs01019695_m1), Androgen receptor (*AR;* #Hs00171172_m1), AMH receptor 2 (*AMHR2*; #Hs00174915_m1) and LH receptor (*LHR*, #Hs00174885) beta-2-microglobulin (*B2M*, Hs00187842_m1) was used as the reference gene according to published data (*Cadenas et al., 2022*).

Enzyme-linked immunosorbent assay measurements

Quantification of human VEGF, PDGF-BB and TGF-ß was carried out three independent times in the same batch of FBS, HSA, HPL and UCP using the Human VEGF DuoSet Elisa (DY293B-05) (R&D Systems, Minneapolis, MN, USA), Human PDGF-BB DuoSet Elisa (DY220-15) (R&D Systems, Minneapolis, MN, USA) and Human TGF-ß DuoSet Elisa (DY240-05) R&D Systems, Minneapolis, MN, USA), respectively following the manufacturers' instructions. The FBS and HSA samples were undiluted; HPL samples were diluted 1:4 for human VEGF, 1:40 for human PDGF-BB and 1:2 for TGF-ß; UCP samples were diluted 1:8 for human PDGF-BB and undiluted for human VEGF and TGF-ß.

Quantification of AMH and oestradiol was carried out in the spent media for 349 of the surviving follicles: FBS (n = 72), HSA (n = 94), HPL (n = 144) and UCP (n = 39), collected on day 8 in the culture. Anti-Müllerian hormone was measured by picoAMH ELISA kits (AL-124-I) (Ansh Labs, Webster, TX, USA) and oestradiol was measured by ELISA (17-ESTR-3853B) (NovaTec, Hessen, Germany) following the manufacturer's instruction. Culture media were diluted 1:5 for AMH and 1:2 for oestradiol. A control of the concentration of AMH and oestradiol in the original media with the four supplements (FBS/HSA/HPL/UCP) separately was considered and subtracted from the results.

Statistical analyses

R version 4.2.2 (Team, R Core) (*R Foundation for Statistical Computing, Vienna, 2022*) was used for statistical analysis using and the packages 'lme4' (*Bates et al., 2015*) and 'GLMMadaptive' (*Rizopoulos, 2022*). Follicular survival was analysed by fitting a logistic mixed model with follicle survival as the outcome, patient as a random effect and experimental group as a fixed effect. This enabled comparison of the survival probability of a random follicle from a random woman between groups. These marginal probabilities were computed by marginalization of the subject-specific survival estimates (*Hedeker et al., 2018*).

Separate models were fitted to estimate and compare survival probabilities after 2, 4, 6 and 8 days. Median follicular growth for follicles surviving at day 8 was modelled by a linear mixed model with the logarithm of diameter growth as a continuous outcome, patient as random effect and experimental group and the logarithm of diameter at baseline (day 0) as fixed effects. We chose to log-transform the outcome (diameter growth) because a ratio scale was thought to be most relevant. That is, ratios of median diameter growths were modelled and estimated between groups via a linear mixed model of the log-transformed outcome (*Keene, 1995*). From the fit of both the linear and the logistic mixed model, appropriate 95% confidence intervals and *P*-values were extracted in addition to pointwise estimates. $P < 0.05$ was considered statistically significant. To evaluate the potential effect of transporting the tissues from local hospitals, transportation was added as a fixed effect in the linear and logistic mixed models. The mean gene expression level difference between follicles of any two groups were compared using a usual Welch' t-test.

RESULTS

Follicular survival and growth

A total of 724 pre-antral follicles (mean diameter: 75 μm, range: 46–237 μm) were collected from the surplus medulla tissue of 14 patients undergoing OTC. The follicles were divided into four experimental groups: FBS (n = 171), HSA (n = 159), HPL (n = 223) and UCP (n = 171). After 8 days of culture, the survival probability in the HPL group was significantly higher (80%, 95% CI 72 to 86) than in the other three groups: FBS (54% 95% CI 44 to 64; $P < 0.001$), HSA (63%, 95% CI 53 to 72; $P = 0.004$) and UCP (29%, 95% CI 21 to 38; $P < 0.001$). On the contrary, the probability of survival in the UCP group was significantly reduced compared with any of the other groups ($P < 0.001$ for all three groups) (FIGURE 2). Furthermore, the morphology of the surviving follicles in the UCP group showed a less defined follicular membrane and decompacted granulosa cell layers. The follicles in groups FBS, HSA and HPL presented with a similar morphology with a defined follicular membrane, smooth granulosa cell layers and clear oocyte in the middle (FIGURE 3A). Finally, the survival probability in the HSA group was not significantly different from the FBS group ($P = 0.474$) (FIGURE 2B).

The median follicular growth for surviving follicles was statistically significantly greater in the HPL group (73 μm [68–79]) compared with any of the other three groups: HSA (43 μm [40–47]; $P < 0.001$); FBS (40 μm [37–44]; $P < 0.001$) and UCP (54 μm [49–60]; $P < 0.001$). The median growth of the follicles from the UCP groups was also significantly larger compared with HSA and FBS ($P < 0.001$ for both) (FIGURE 3B and FIGURE 3C). Furthermore, the median growth of the groups HSA and FBS was not found significantly different ($P = 0.117$).

Concentration of anti-Müllerian hormone and oestradiol in the spent media

The concentration of AMH and oestradiol was measured in the spent media at day 8 individually for each follicle. The concentrations of AMH and oestradiol were below the lower detection threshold (<35 pg/ml for AMH and <30 pg/ml oestradiol) for 56% and 58% of the samples, respectively. Therefore, a detailed statistical analysis of the correlation between the diameter on day 8 and the concentrations could not be performed. A descriptive analysis of the data showed no difference between the experimental groups. It was noted, however, that for follicles with a diameter of 150 μm or less on day 8, the percentage of samples with values below the lower threshold for AMH per experimental groups were FBS (91%), HSA (48%), HPL (67%) and UCP (71%) but for follicles with diameter wider than 150 μm the percentages were lower for each experimental group FBS (10%), HSA (23%), HPL (18%) and UCP (54%) (FIGURE 4A). In a similar way, the percentage of spent media having below threshold values of oestradiol for follicles measuring 150 μm or less were FBS (86%), HSA (69%), HPL (67%) and UCP (46%), and for follicles wider than 150 μm on day 8 were FBS (70%), HSA (50%), HPL (20%) and UCP (45%) (FIGURE 4B).

Relative gene expression of *FSHR, AMHR2, AR* and *LHR* in cultured pre-antral follicles

The gene expression of *FSHR, AMHR2, AR* and *LHR* normalized to *B2M* was evaluated individually in a subset of 100 follicles, 25 per experimental group: mean diameter FBS (123 μm), HSA (132 μm), HPL (131 μm), UCP (132 μm). From those, only follicles in which RNA integrity number values exceeded 5 were included: FBS (n = 20), HSA (n = 25), HPL (n =23) and UCP (n = 17). The relative gene expression of *AR* was significantly lower in the HPL group compared with the HSA group ($P = 0.019$) and the UCP group ($P = 0.043$) but not compared with the FBS group ($P = 0.509$) (FIGURE 5A). Moreover, the relative gene expression of *AMHR2* in the HPL group was lower than the other three groups (HPL–FBS $P < 0.001$, HPL–HSA $P < 0.001$, HPL–UCP $P = 0.001$), but higher in the HSA compared with FBS (HSA–FBS $P < 0.001$) (FIGURE 5B). In a similar way, the relative gene expression of *FSHR* was lower in the HPL group compared with HSA and FBS (HPL–FBS $P < 0.001$, HPL–HSA $P < 0.001$) but not significantly different to UCP ($P = 0.379$) (FIGURE 5C). *LHR* was excluded from the further analysis due to the low number of follicles with a detectable level of expression (20% of the samples) (FIGURE 5D). Finally, although 100% of the follicles had detectable expression of *AR* and *AMHR2*, 76% expressed *FSHR* and 20% expressed *LHR* (FIGURE 5E).

Transportation of the ovary before ovarian tissue cryopreservation and follicle isolation

Of the 724 follicles, 355 follicles were collected from ovaries obtained at the local hospital with no transportation time (n = 8), and 369 follicles were collected from ovaries obtained from collaborating hospitals and transported to our facilities at a temperature of 0 to 4 °C (n = 6) within a period of 4–6 h. No effect of transportation was observed on the survival probability irrespective of experimental group (FBS, HSA, HPL and UCP) ($P = 0.352$). Moreover, no effect of transportation was observed on the median follicular growth for surviving follicles irrespective of experimental group ($P = 0.551$).

Quantification VEGF, PDGF-BB and TGF-ß in platelet rich plasma preparations

To determine how concentrated the PRP solutions were, the concentrations of VEGF, PDGF-BB and TGF-ß were measured in HPL and UCP. The other two serum sources, HSA and FBS, were also measured. Human serum albumin is a purified serum product; therefore, the concentration of VEGF, PDGF-BB and TGF-ß was zero. The concentration of TGF-ß in HPL (88933 ± 5360 pg/ml) (mean ± SD) was 11 times higher than the concentration in UCP (7928 ± 1336 pg/ml) and 20 times higher than the

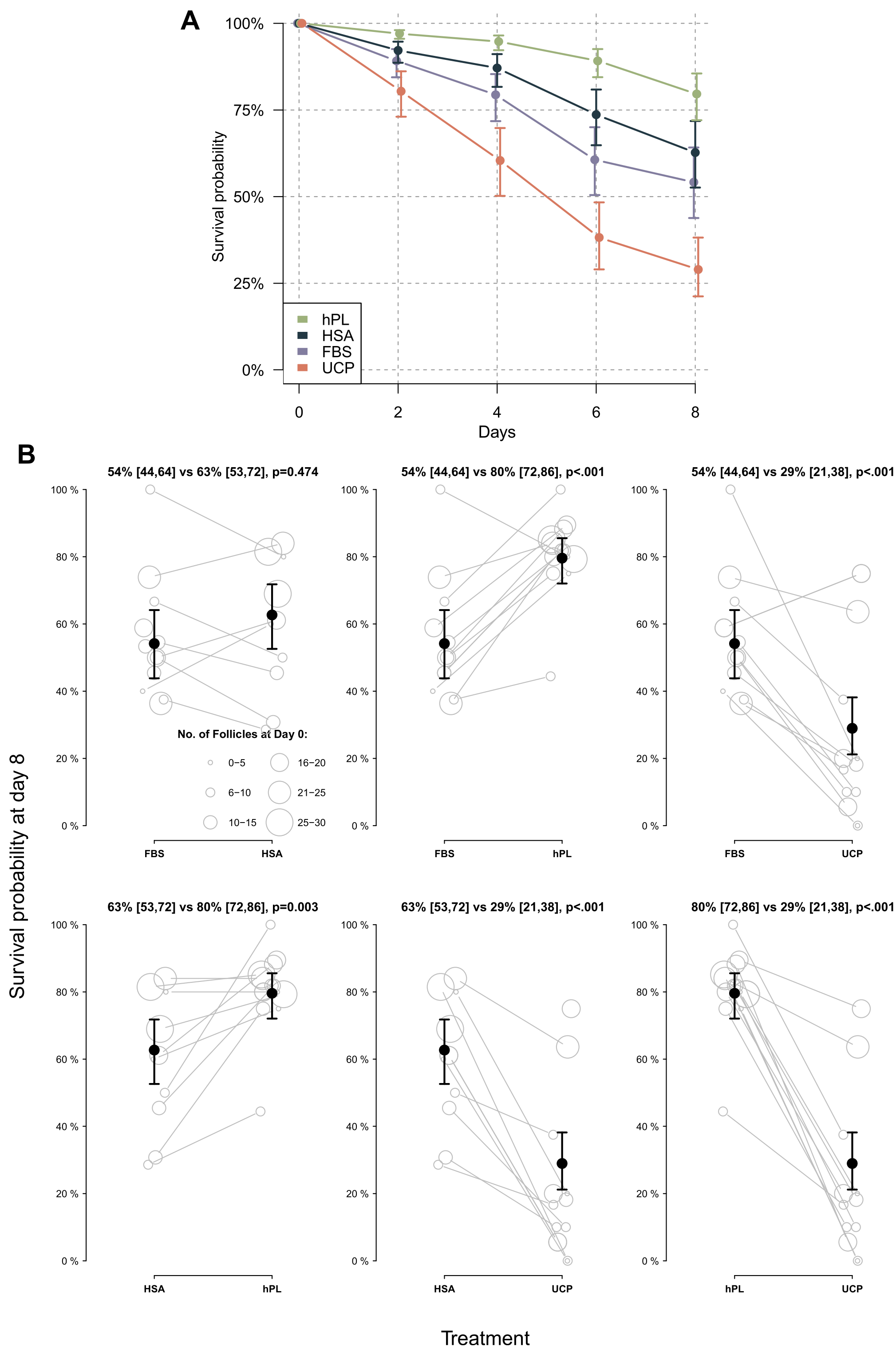


FIGURE 2 Survival analysis of follicles. A total of 724 follicles were cultured for 8 days in 0.5% alginate, divided in the four experimental groups: 5% fetal bovine serum (FBS) (n = 171), human serum albumin (HSA) (n = 159), human platelet lysate (HPL) (n = 223) and umbilical-cord blood (UCP) (n = 171). (A) Marginal survival probabilities curve in the four experimental groups, values represented as probability (95% confidence interval). Separate models were fitted to estimate and compare survival probabilities after 2-, 4-, 6- and 8-days; (B) comparison one to one of marginal survival probabilities at day 8 between the four experimental groups. Every circle represents one patient, the size of the circle represents the number of follicles cultured for that patient in that group. The allocation of follicles from 6 of the 14 patients is not evenly distributed across the experimental groups. The circles that are not connected to another, for each comparison, correspond to patients for which no follicles were allocated to the other treatment group. The bold dot and interval represent the mean probability and the (95% confidence interval). Significant differences between the groups are marked with P-values.

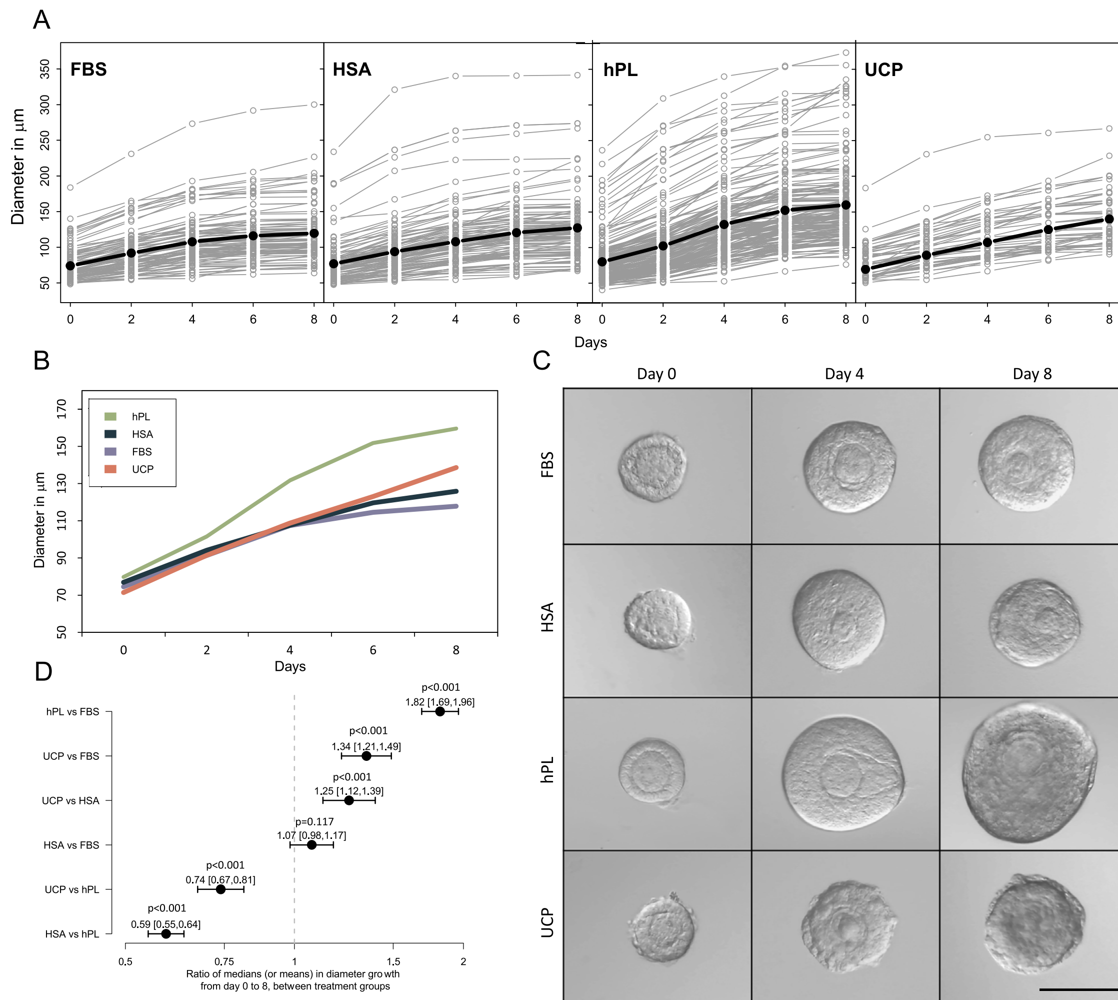


FIGURE 3 Follicular growth in surviving follicles. From the total of 724 follicles that were isolated on day 0, 401 follicles survived the culture period and were measured every second day; (A) Morphology of surviving human pre-antral follicles cultured for 8 days in in 0.5% alginate with fetal bovine serum (FBS), human serum albumin (HSA), human platelet lysate (HPL) and umbilical-cord blood (UCP). Brightfield images of follicles at day 0, day 4 and day 8 of the culture. Scale bar 200 μm; (B) comparison of ratio of medians of growth μm (95% confidence interval) between four experimental groups one to one. Estimated median differences in log-diameters between groups are presented exponentiated, to provide results that can be interpreted as ratios of median diameter growth between groups. $P < 0.05$ was considered statistically significant; (C) diameter of each surviving follicle every second day in the four experimental groups: FBS, HSA, HPL and UCP; bold line represents the mean follicular diameter in the group.

concentration in FBS (4480 ± 318 pg/ml). The concentration of VEGF in HPL (1794 ± 203 pg/ml) was 17 times higher than the concentration in UCP (105 ± 23 pg/ml). Finally, the concentration of PDGF-BB in HPL (17005 ± 3039 pg/ml) was 16 times higher than the concentration in UCP (1074 ± 533 pg/ml) (FIGURE 5F).

DISCUSSION

This study demonstrates that HPL from adult-peripherical blood increased growth and survival of isolated primary and secondary human follicles cultured in 0.5% alginate for 8 days compared with HSA, FBS and UCP. Interestingly, UCP also increased follicular growth compared with HSA and FBS but significantly reduced follicle survival and altered follicular morphology compared with the other three groups. In concordance with our study, adult blood PRP have previously been shown to increase the growth and survival of primordial follicles isolated from fresh and vitrified ovarian cortex tissue (*Hosseini et al., 2017*), confirming that PRP can consistently support the growth and survival of human pre-antral follicles at different developmental stages. Our study finds clear differences in the follicular outcomes with the use of HPL and UCP, which suggest that the diversity of growth factors in these blood products might have both positive and negative effects on follicle culture. The fact that the UCP group had the lowest percentage of surviving follicles underscores a potential negative effect of the product compared with the other groups. Additionally, these differences between UCP and HPL are in line with previous studies in a murine IVM model (*Moulavi et al., 2020*; *Taghizabet et al., 2022*). Murine adult blood PRP used to culture murine pre-antral follicles improved oocyte maturation rates (*Taghizabet et al., 2022*), whereas PRP from murine umbilical cord significantly decreased follicular growth and blocked IVM (*Pazoki et al., 2015*). These studies highlight that the use of undefined blood products like HPL and UCP most likely induce a fine balance of positive and negative influences which needs to be considered. The superiority of HPL in our study may reflect a higher concentration

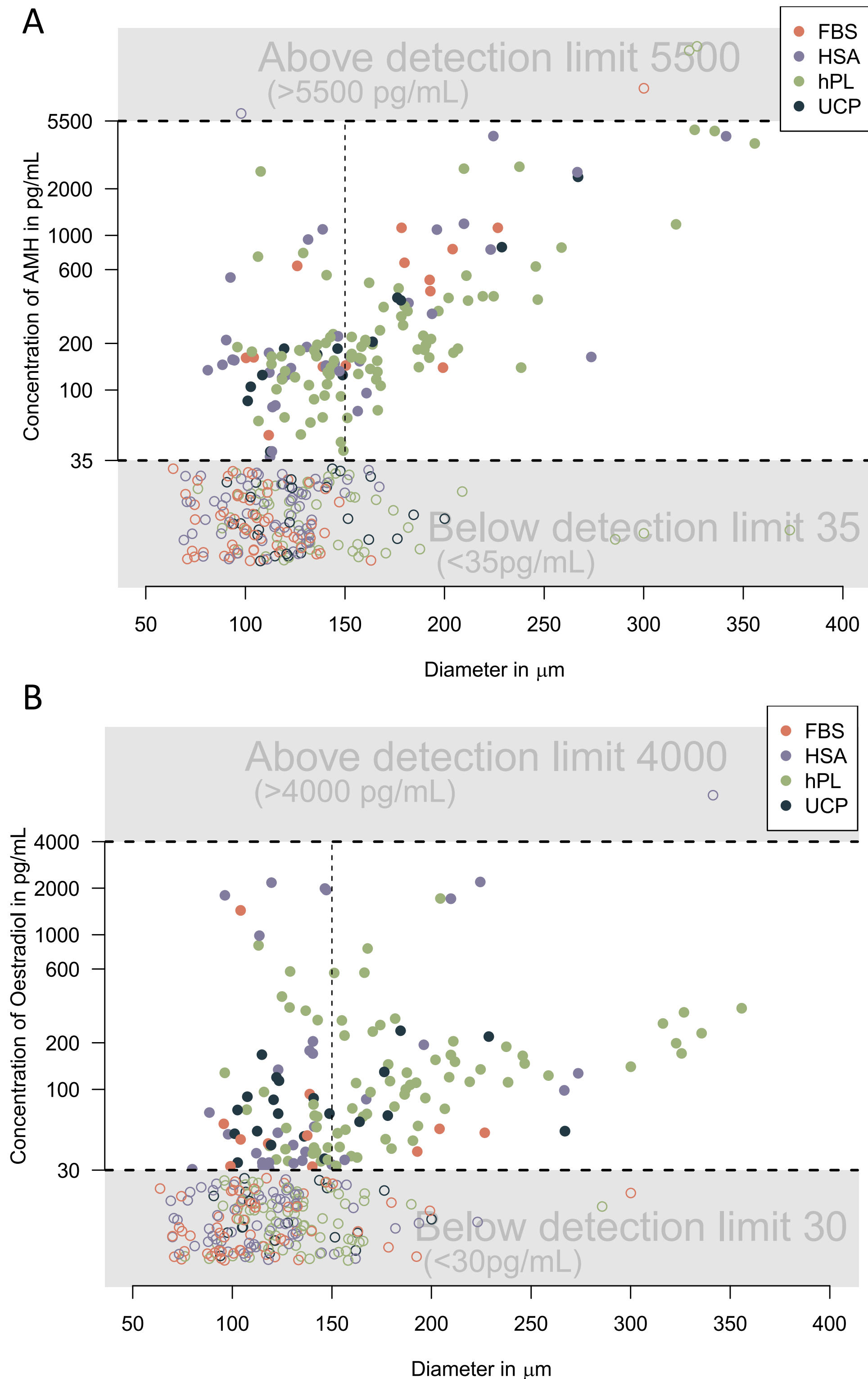


FIGURE 4 Anti-Müllerian hormone (AMH) and oestradiol secretion. Both AMH and oestradiol were measured individually for 349 of the surviving follicles (fetal bovine serum [FBS] $n = 72$), human serum albumin (HSA) ($n = 94$), human platelet lysate (HPL) ($n = 144$), umbilical-cord blood (UCP) ($n = 39$). Log transformed concentration of (A) anti-Müllerian hormone (AMH) (pg/ml) and (B) oestradiol (pg/ml) in the spent media at day 8 shown in the y axis and the follicular diameter in the x axis. Full dots represent the follicles which values were within the detectable limits, empty dots represent the follicles which values were out of the detection limits. The experimental group (FBS, HSA, HPL and UCP) is shown in colours.

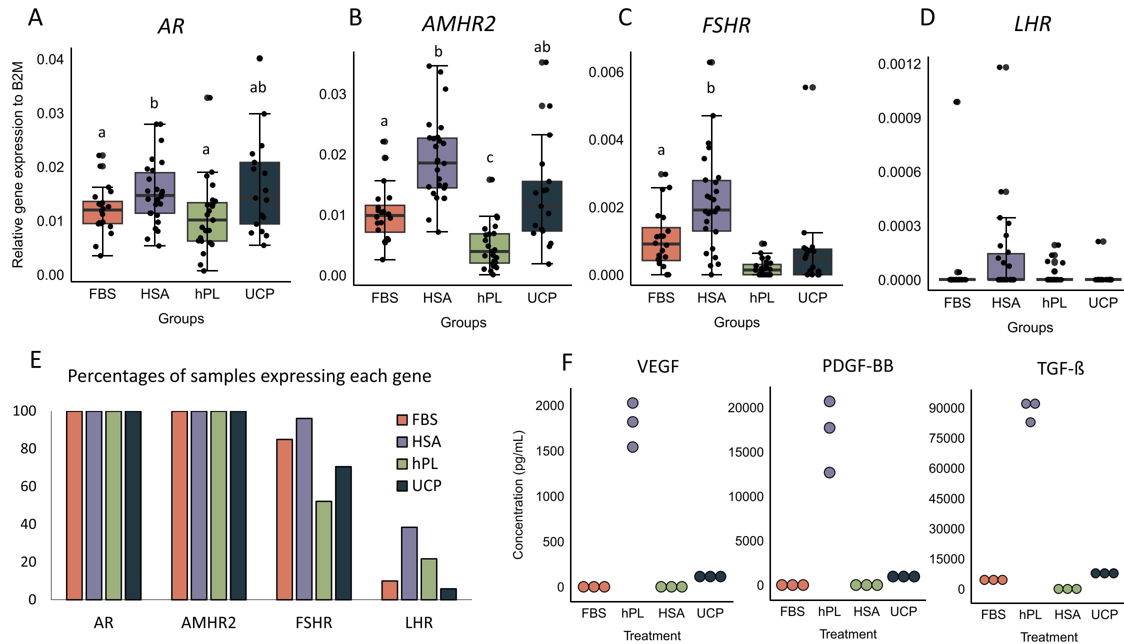


FIGURE 5 Gene expression was analysed in a subset of 85 surviving follicles (fetal bovine serum [FBS] (n = 20), human serum albumin (HAS) (n = 25), human platelet lysate (HPL) (n = 23), umbilical-cord blood (UCP) (n = 17); (A to D) relative gene expression of *AR, AMHR2, FSHR* and *LHR* in pre-antral follicles after 8 days culture with FBS, HSA, HPL and UCP. Box plots show median, interquartile range, maximum and minimum values. *P*-values for AR (FBS–HSA P = 0.036, FBS–HPL P = 0.509, FBS–UCP P = 0.083, HSA–HPL P = 0.019, HSA–UCP P = 0.0663 and HPL–UCP P = 0.043); *P*-values for *AMHR2* are (FBS–HSA $P < 0.001$, FBS–HPL $P < 0.001$, FBS–UCP P = 0.215, HSA–HPL $P < 0.001$, HSA–UCP P = 0.067 and HPL–UCP P = 0.001); *P*-values for FSHR are (FBS–HSA $P < 0.001$, FBS–HPL $P < 0.001$, FBS–UCP P = 0.379 HSA–HPL $P < 0.001$, HSA–UCP P = 0.001 and HPL–UCP P = 0.135). Groups with different letters indicate statistical differences ($P < 0.05$); (E) percentage of follicles expressing *AR, AMHR2, FSHR* and *LHR* per experimental group; (F) concentration of human VEGF, PDFG-BB and TGF-ß in FBS, HSA, PL and UCP in the same batch used for supplementation in three independent measurements.

and different composition of proteins, growth factors cytokines and chemokines (de Miguel–Gómez et al., 2021) compared with the other experimental groups. The concentration of three key human growth factors in the PRP products were analysed: PDFG-BB, TGF-ß and VEGF, and the concentration was 16, 11 and 17 times higher in HPL than in UCP, respectively. The difference could be due to the manufacturing method. Human platelet lysate is prepared by concentrating the plasma fraction from adult blood, lysing the platelets, and eliminating the platelet cellular bodies, whereas UCP is plasma from umbilical cord blood which has not been further concentrated. As expected, human PDFG-BB, TGF-ß and VEGF were low or not detected in HSA or FBS as HSA is a purified protein solution from serum and FBS is a bovine-derived product that only to a limited extend cross-reacts with human antibodies.

In human ovaries, AMH expression begins when primordial follicles are activated to grow and become primary follicles and increases in secondary follicles and peaks in small antral follicles (Weenen et al., 2004). Oestradiol is produced by granulosa cells of developing follicles (Shoham and Schachter, 1996). In the present study, the concentration of AMH in the spent media was lower than the detection limit (<35 pg/ml) for 69% of the follicles smaller than 150 μm and for 26% of the follicles bigger than 150μm (FIGURE 4A). Furthermore, the concentration of oestradiol was lower than the detection limit (<30 pg/ml) for 67% of follicles smaller than 150 μm and for 46% of the follicles bigger than 150 μm (FIGURE 4B), indicating that follicles are becoming steroidogenically active as they grow into larger stages. This suggests that the three-dimensional culture method supports the growth of follicles that can secrete AMH and oestradiol and, therefore, support folliculogenesis. Therefore, our results confirm and extend previous in-vitro studies reporting measurable concentrations in the spent media of AMH and oestradiol in the spent media from isolated follicles when reaching the secondary stage (Xiao et al., 2015; Xu et al., 2021).

To the best of our knowledge, this is the first study individually analysing gene expression in cultured human pre-antral follicles. *In vivo*, FSH is the essential driver of antral follicle growth, but the effect on pre-antral follicles is not well defined either *in vivo* or *in vitro*. Moreover, *FSHR* was shown to be expressed in human pre-antral follicles up to a certain extent in the primary and two-layered follicles (Oktay et al., 1997) and a constant low expression of *FSHR* was found in primary and secondary pooled follicles (Kristensen et al., 2015). First, the expression of typical granulosa cell markers: *AR*, *FSHR* and *AMHR* were analysed. Surprisingly, we found that the *FSHR* expression was lower than 0.00628 in all follicles within all treatment groups and even significantly downregulated in the group that presented the highest growth, HPL compared with the groups HSA and FBS. Although gene expression is not indicative of a functionally active protein, our results suggest that the increase in the growth of follicles treated with HPL might be gonadotrophin independent. During in-vitro maturation, downregulation of *FSHR* in the granulosa cells of the cumulus was accompanied by upregulation of the *LHR*

(Cadenas et al., 2021). The present study, however, found that few of the cultured preantral follicles expressed *LHR*, which is in line with our previous studies showing a lack of *LHR* in preantral human follicles less than 150 μm in diameter (Kristensen et al., 2015). Furthermore, gene expression of *AR* and *AMHR2* was detectable for all the follicles independently of the experimental group, whereas the expression of *FSHR* was observed in 76% and the LH in 20%. This suggested that *AR* and *AMHR2* are expressed in granulosa cells of secondary follicles but *FSHR* and to a higher extent *LHR* expression is generally low in cultured secondary follicles, suggesting that the preantral follicle growth is gonadotrophin independent to some extent.

We also found that transportation of the ovaries at 0–4°C before OTC did not affect follicular survival or follicular growth. In a similar way, several independent studies have been published to show no effect of transportation before OTC on the outcome of OTT (Jensen et al., 2017; Nikiforov et al., 2020; Lotz et al., 2022) (2, 38, 39). In contrast, transportation of the ovaries before OTC significantly reduced MII rates of cumulus oocyte complexes (COC) collected from the preparation media (Nikiforov et al., 2020). The detrimental effect of transportation on COC compared with follicles is probably because COC are more vulnerable to external changes than follicles.

A limitation of the study is the potential batch-to-batch variation in PRP products. which arises from the manufacturing process and the intrinsic differences between individual donors. In this case, although UCP is manufactured using single donor plasma, HPL is produced by pooling plasma from several donors attempting to reduce batch variability. Further studies should aim to address the potential batch-to-batch variations with the use of PRP products. Another limitation of the study is that only one concentration of each experimental group was tested. Ideally multiple concentrations of all four exogenous additives should be studied to fully assess their effect on follicle culture.

In conclusion, in-vitro folliculogenesis is an interesting fertility preservation option, especially for those patients with a risk of reintroducing malignancies. More research, however, is needed to standardize and improve the protocols. In the present study, we focused on optimizing a single aspect of the method: the nutrient–serum source in the culture media of pre-antral follicles. We demonstrated that a commercially available PRP from human peripheral blood, HPL, improved survival and growth of pre-antral follicles while UCP also increased growth but decreased survival rates dramatically and altered follicular morphology. Further studies, including extended culture, will have to prove the superiority of HPL compared with standard serum supplements like HSA and FBS and whether this translates into better quality oocytes collected from in-vitro produced antral follicles.

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AUTHORS' ROLES

ECL, MHT, VHG recruited the patients, CSA, JC, SGK and CYA designed the experiments; CSA, JC and MZ conducted the experiments; CSA, SL, PB, CYA and SGK analysed the data; CSA, JC, SL, PB, CYA and SGK evaluated the data; CYA and SGK supervised the project and CSA wrote the manuscript with contributions from all authors.

DATA AVAILABILITY

Raw data and description are available in supplementary materials

DATA AVAILABILITY

The data has been included as supplementary.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2023.06.011.

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