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ARTICLE

Assessing the effect of adipose-tissue-derived stem cell conditioned medium on follicles and stromal cells in bovine ovarian tissue culture

BIOGRAPHY

Francisco Vitale earned his medical degree in 2016, and completed his residency in gynaecology and obstetrics in 2021 at Hospital Universitario Austral (Argentina). He is currently a PhD researcher at the Université Catholique de Louvain (Belgium). His work focuses on developing strategies to improve the outcomes of ovarian follicles cultured *in vitro*.

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KEY MESSAGE

Follicular outcomes following in-vitro culture of human ovarian tissue remain suboptimal, mainly showing poor development and viability rates. This study showed that the addition of adipose-tissue-derived stem cell conditioned medium to culture enhances follicle survival, development and oestradiol production, and the viability of stromal cells.

ABSTRACT

Research question: Does adipose-tissue-derived stem cell conditioned medium (ASC-CM) supplementation enhance follicle and stromal cell outcomes *in vitro*?

Design: Bovine ovaries ($n = 8$) were sectioned and cultured *in vitro* for 8 days in two different groups: (i) standard culture (OT Ctrl D8); and (ii) culture with ASC-CM supplementation (OT + CM D8). Half of the culture medium was replaced every other day, and stored to measure the production of oestradiol. Follicle classification was established using haematoxylin and eosin staining. Follicle and stromal cell DNA fragmentation was assessed by TUNEL assays, while growth differentiation factor-9 (GDF-9) staining served as a marker of follicle quality. Additionally, three factors, namely vascular endothelial growth factor (VEGF), interleukin 6 (IL-6) and transforming growth factor beta 1 (TGF-β1), were evaluated in ASC-CM in order to appraise the potential underlying mechanisms of action of ASC.

Results: The OT + CM D8 group showed a significantly higher proportion of secondary follicles ($P = 0.02$) compared with the OT Ctrl D8 group. The OT + CM D8 group also demonstrated significantly lower percentages of TUNEL-positive follicles ($P = 0.014$) and stromal cells ($P = 0.001$) compared with the OT Ctrl D8 group. Furthermore, follicles in the OT + CM D8 group exhibited a significant increase ($P = 0.002$) in expression of GDF-9 compared with those in the OT Ctrl D8 group, and oestradiol production was significantly higher ($P = 0.04$) in the OT + CM D8 group. All studied factors were found to be present in ASC-CM. VEGF and IL-6 were the most widely expressed factors, while TGF-β1 showed the lowest expression.

Conclusions: Addition of ASC-CM to culture medium enhances follicle survival, development and oestradiol production, and promotes the viability of stromal cells. VEGF, IL-6 and TGF-β1 could be paracrine mediators underlying the beneficial effects.

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KEY WORDS

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INTRODUCTION

In-vitro culture (IVC) of ovarian tissue has emerged as a promising technique to develop mature metaphase II oocytes from the pool of primordial follicles in the ovarian cortex (*Telfer and Andersen, 2021*; *Telfer et al., 2023*). This approach avoids reimplantation of ovarian tissue, which is of great benefit to patients at high risk of ovarian metastases, such as blood-borne malignancies or ovarian cancers (*Dolmans et al., 2010*, *2013*). However, the outcomes of follicles developed in-vitro remain suboptimal, with poor follicle survival and development (*McLaughlin et al., 2018*; *Yang et al., 2020a*). Despite extensive efforts to optimize IVC conditions, studies have shown that only a limited proportion of primordial follicles are capable of progressing to the next growth stage without any significant damage, and the majority face death or growth arrest (*McLaughlin et al., 2018*; *Telfer et al., 2008*).

Intraovarian folliculogenesis encompasses a complex network of cell-to-cell communication and signalling pathways (*Cecconi et al., 2012*; *Craig et al., 2007*), so IVC conditions that best resemble in-vivo milieu are likely to generate less cellular distress and yield better outcomes. In recent years, numerous researchers have attempted to enhance IVC results through the use of different culture additives. It has been documented that cell-based co-culture systems may replicate the intraovarian microenvironment by releasing various paracrine factors (*Tagler et al., 2012*; *Tingen et al., 2011*; *Yang et al., 2020b*). The ability of adipose-tissue-derived stem cells (ASC), a type of mesenchymal stem cell, to promote cell proliferation, survival and tissue regeneration (*Al-Ghadban et al., 2021*; *Tsuji et al., 2014*) via secretion of a wide range of growth factors and cytokines encapsulated within exosomes (i.e. small extracellular vesicles that serve as carriers for cell-to-cell communication) is well known in the field of regenerative medicine (*Cacciottola et al., 2021*; *Kapur and Katz, 2013*; *Manavella et al., 2018*; *Trzyna and Banaś-Ząbczyk, 2021*). Supernatant from ASC culture, known as conditioned medium (CM), contains secreted exosomes and hence exerts the same beneficial effects as the original cells (*Bhang et al., 2014*). Indeed, the use of CM as opposed to cellular components has several advantages (*Gunawardena et al., 2019*; *Pawitan, 2014*). First, it avoids the possibility of two different types of cells competing for nutrients in the culture medium. Moreover, CM can be easily collected, stored and standardized, ensuring consistent and reproducible outcomes. Finally, CM circumvents any concerns associated with unwanted cell differentiation, which may arise when using stem cells in co-culture systems (*Sagaradze et al., 2019*).

In the field of regenerative medicine, it has been widely reported that cytokines and growth factors encapsulated within ASC-derived exosomes, including vascular endothelial growth factor (VEGF), interleukin 6 (IL-6) and transforming growth factor 1 (TGF-β1), promote a proliferative and anti-apoptotic effect on cultured cells (*Al-Ghadban et al., 2021*; *Trzyna and Banaś-Ząbczyk, 2021*). Besides its proangiogenic effects, VEGF has been described as a key factor involved in early follicle activation and development *in vitro*, mainly via the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway (*Irusta et al., 2010*; *Yang and Fortune, 2007*). Moreover, IL-6 has been associated with numerous biological follicle functions including promotion of in-vitro proliferation of granulosa cells and inhibition of cell apoptosis (*Tingen et al., 2011*). The role of TGF-β1 has been widely reported in the regulation of folliculogenesis (*Knight and Glister, 2006*). Indeed, TGF-β1 is known to stimulate proliferation and differentiation of granulosa cells in IVC systems (*Fazzini et al., 2006*).

The main objective of this study was to determine whether the addition of CM from ASC to culture medium could improve the survival and development of ovarian follicles, and the viability of stromal cells in a bovine ovarian tissue IVC system. A secondary objective was to determine the presence of certain paracrine factors (VEGF, IL-6 and TGF-β1) in ASC-CM that may be involved in proliferation and anti-apoptosis cell mechanisms.

MATERIALS AND METHODS

Culture and passage of ASC

Previously characterized human ASC from female donors were acquired commercially (Stempro; Invitrogen, USA). They were subjected to IVC and passaged as described by *Manavella et al. (2018)*. Briefly, ASC were seeded at a density of 5×10^3 cells/cm^2, and cultured at 37°C in 5% CO_2 in MesemPRO RS medium (Gibco, USA) with 2% MesemPRO growth supplement (Gibco) and 1% L-glutamine (Gibco). After achieving 80–90% confluency, the cells were detached and passaged by enzymatic digestion using Accutase (Sigma-Aldrich, USA). All ASC used in the current study were between passages 3 and 4.

Preparation of ASC-CM

Upon reaching 80–90% confluency, the culture medium was replaced completely with serum-free alpha-modified Eagle's medium (α-MEM; Gibco) supplemented with 2 mg/ml glucose (Sigma, USA), and then cultured for a further 72 h at 37°C in 5% CO_2. This period of time was chosen to maximize the obtention of paracrine factors secreted by ASC. The collected supernatant was centrifuged at 1200 *g* for 5 min and filtered twice using a 0.22-μm filter, before being aliquoted and stored at -80°C.

ASC-CM analyses

Concentrations of VEGF (Cat: ab222510; Abcam, UK), IL-6 (Cat: ab178013; Abcam) and TGF-β1 (Cat: BMS249-4; Invitrogen) were measured in ASC-CM by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions. All experiments were carried out in duplicate. Sensitivity, intra-assay and interassay coefficients of variation are detailed in Supplementary Table 1.

Ovarian tissue processing and IVC

Fresh bovine ovaries (n = 8) were obtained from slaughtered animals (12–18 months) at the time of evisceration, and transported to the laboratory within 30 min in Leibovitz medium (Gibco) tubes covered in ice. Only normal-sized (4 × 2 × 2 cm), oval-shaped ovaries without visible cysts were used in the experiment. Upon arrival, the ovaries were sectioned into thin strips and any surplus medulla was removed using 10 ml pre-warmed dissection medium containing Leibovitz medium (Gibco) supplemented with 2 mM sodium pyruvate (Sigma), 2 mM glutamine (Sigma), 3 mg/ml bovine serum albumin (BSA; Sigma), 50 μg/ml penicillin G (Sigma) and 50 μg/ml streptomycin (Sigma). They were subsequently cut into fragments of 4 × 2 × 1 mm. One cortical fragment per sample served as a non-cultured control (D0) and was fixed immediately, while two fragments were placed in four-well culture dishes filled with 400 μl of pre-warmed culture medium

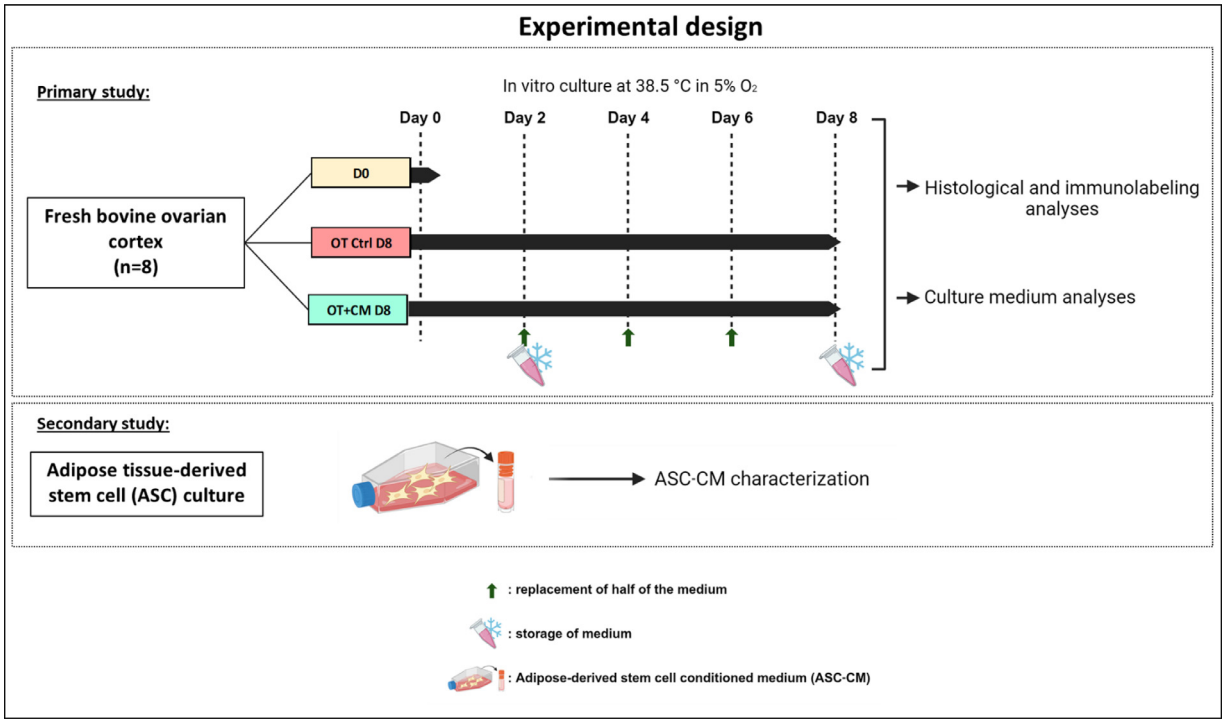

FIGURE 1 Experimental design. Primary study: Fresh bovine ovaries (n = 8) were fragmented into cortical fragments and cultured for 8 days in two different groups: ovarian tissue cultured alone (OT Ctrl D8); and ovarian tissue with conditioned medium (CM) derived from adipose-tissue-derived stem cells (ASC) (OT + CM D8). One cortical fragment per ovary served as the non-cultured control (D0). All cortical fragments were collected for histological and immunolabelling analyses. Half of the culture medium was replaced every other day, and medium from days 2 and 8 was stored at -80°C for future analysis. Secondary study: ASC-CM was analysed to identify and measure paracrine factors associated with proliferative and anti-apoptotic effects.

composed of α-MEM supplemented with 25 mM HEPES (Gibco), 1 mg/ml BSA (Sigma), 3 mM glutamine (Sigma), 30 μg/ml penicillin G (Sigma), 50 μg/ml streptomycin (Sigma), 5 μg/ml transferrin (Fisher Scientific), 5 ng/ml selenium (Sigma), 50 μg/ml ascorbic acid (Sigma), 10 ng/ml insulin (Sigma) and 1 ng/ml human FSH (Gonal F; Merck Serono, UK). IVC was then carried out in two different groups: (i) ovarian tissue cultured alone (OT Ctrl D8); and (ii) ovarian tissue with ASC-CM (OT + CM D8). The OT + CM D8 group contained a combination of standard culture medium and ASC-CM at a 1:1 ratio from the beginning of culture. Every other day, half of the culture medium from both groups was changed. On days 2 and 8, retrieved culture medium was stored at -80°C for further analysis. A schematic design of the experiment is shown in FIGURE 1.

Both IVC groups remained in identical conditions for 8 days at 38.5°C in a low oxygen environment (5% O_2) using a hypoxic subchamber incubator (ProOx C21; BioSpherix, USA). In-vitro culture in physiological oxygen concentrations has previously shown an improvement in follicular viability (*Vitale et al., 2023*).

Sample processing

Ovarian fragments from all groups were fixed in 4% formaldehyde, embedded in paraffin and cut serially into 5-μm sections. Haematoxylin and eosin (H&E; Merck, USA) staining was performed on every eighth section to assess follicle density and classification. Sections in between underwent immunolabelling analyses. All slides were digitized using the Panoramic P250 Flash III scanner (3DHistech, Hungary) and analysed by CaseViewer (v2.3).

Histological evaluation

Follicle density and stage were investigated on five H&E-stained tissue sections per sample. To mitigate the possibility of counting twice, only follicles containing a visible oocyte were examined. Atretic follicles were separated from viable follicles if they showed ooplasm eosinophilia, pyknosis of granulosa cells and/or cytoplasmic contraction (*Gougeon and Chainy, 1987*). Follicle density was determined as the number of viable follicles per mm^3, taking section thickness into account. Follicles were only classified if they were viable according to developmental stage: (i) primordial (oocyte surrounded by a single layer of flattened granulosa cells; (ii) primary (oocyte surrounded by a single layer of cuboidal granulosa cells); and (iii) secondary (oocyte surrounded by two or multiple layers of cuboidal granulosa cells). The proportion of follicles at each stage was then established. Follicle and oocyte diameters of secondary follicles were assessed according to *Kitajima et al. (2014)*. For each measurement, two perpendicular diameters were recorded, and the average of these values was calculated (*Kitajima et al., 2014*).

Immunohistochemistry

Growth differentiation factor-9 (GDF-9) immunolabelling was used to evaluate follicle competence, as this factor has been reported to play a crucial role in promoting the early stages of folliculogenesis (*Hussein et al., 2006*). Four tissue sections per sample were analysed. Briefly, after blocking endogenous peroxidase activity with 3% H_2O_2 and demasking in citrate buffer (pH 6) for 75 min at 98°C, non-specific binding sites were blocked by incubation in 10% fetal bovine serum for

30 min. Sections were first incubated overnight with mouse anti-GDF-9 primary antibodies (1:1000, AB-325-AG012, AnshLab, RRID:AB_2756501), and then with EnVision anti-mouse HRP-conjugated secondary antibodies (1:2, K4001, Dako, RRID:AB_2827819). Human testicular cancer served as the positive control (Supplementary Figure 1), and mouse immunoglobulin G (Dako) served as the negative control. Slides were digitized using the Pannoramic P250 Flash III scanner (3DHistech), and growing follicles (primary and secondary) were examined for GDF-9 analysis. Using ImageJ software (http://rsb.info.nih.gov/ij/), growing follicles were digitally isolated from adjacent stromal cells with the 'clear outside' tool option. A colour threshold was then determined to separate GDF-9 signalling from the rest. After setting the scale of the image, the GDF-9-stained area and total area of each follicle were calculated in μm^2 and expressed as a percentage of GDF-9 staining.

TUNEL immunofluorescence

DNA fragmentation was analysed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assays using the In Situ Cell Death Detection Kit, TMR red (Roche, Switzerland), as described by *Vanacker et al. (2012)*. Four tissue sections per sample were analysed. Briefly, after pretreatment with 20 mg/ml proteinase K working solution (Roche) in 10 mM Tris-HCl for 30 min at 37°C, sections were incubated with a TUNEL reaction mixture: 50 ml enzyme solution (terminal deoxynucleotidyl transferase) and 450 ml label solution (nucleotide mixture in reaction buffer) for 60 min at 37°C. Finally, the slides were incubated in 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, USA) and covered with a fluorescent mounting medium (Enzo, USA). DNAse was used to damage DNA in positive control sections (Supplementary Figure 1), while negative controls were incubated with a label without enzyme solution. The slides were digitized using the Panoramic P250 Flash III scanner (3DHistech), and analysed with CaseViewer (v2.3). Follicles were classified as TUNEL-positive when the oocyte or ≥50% of granulosa cells were stained by TUNEL (*Amorim et al., 2012*). Results were presented as the percentage of TUNEL-positive follicles out of the total follicles. For stromal cell analysis, three areas showing TUNEL-positive stromal cells were examined at high magnification. The percentage of TUNEL-positive cells over TUNEL-negative (stained by DAPI alone) cells was calculated using ImageJ software (http://rsb.info.nih.gov/ij/).

Oestradiol production

Oestradiol concentrations were measured in culture medium obtained from both IVC groups (OT Ctrl and OT + CM) on days 2 and 8 using an ELISA kit (Cat: ab108667; Abcam) in accordance with the manufacturer's instructions. All experiments were conducted in duplicate.

Statistics

GraphPad Prism software for Windows (v9.2) was used to conduct statistical analyses. One-way analysis of variance (ANOVA) was applied, followed by Fisher's least significant difference (LSD) post-hoc test. The paired *t*-test was used to analyse oestradiol production. Data expressed as a proportion were analysed after logit transformation, and results have been expressed as mean ± SD. A *P*-value <0.05 was considered to indicate significance.

RESULTS

Follicle outcomes

Follicle density, classification and morphology

In total, 1197, 807 and 1007 viable follicles were counted in the D0 group, the OT Ctrl D8 group, and the OT + CM D8 group, respectively. A significant decrease in follicle density was observed in both IVC groups (OT Ctrl D8: $P = 0.002$; OT + CM D8: $P = 0.010$) compared with the D0 group. However, the OT + CM D8 group exhibited significantly higher ($P = 0.025$) follicle density compared with the OT Ctrl D8 group (FIGURE 2A). Regarding follicle classification, there was a significant decrease in the proportion of primordial follicles in both IVC groups (OT Ctrl D8: 57.50% ± 5.37%, $P < 0.001$; OT + CM D8: 51.63% ± 4.80%, $P < 0.001$) compared with the D0 group (78.63% ± 3.29%), but a significantly higher proportion were detected in the OT Ctrl D8 group ($P = 0.033$) compared with the OT + CM D8 group. In terms of the proportion of primary follicles, significant increases were encountered in both IVC groups (OT Ctrl D8: 39.13% ± 4.91%, $P < 0.001$; OT + CM D8: 41.50% ± 5.18%, $P<0.001$) compared with the D0 group (18.88% ± 3.87%). The proportion of secondary follicles was significantly higher in the OT + CM D8 group (6.87% ± 3.27%) compared with the OT Ctrl D8 group (3.62% ± 3.20%, $P = 0.028$) and D0 group (2.37% ± 1.40%, $P = 0.003$) (FIGURE 2B). Concerning the proportion of atretic follicles between the two IVC groups, significantly lower values were obtained in the OT + CM D8 group (9.25% ± 3.41%, $P = 0.002$) compared with the OT Ctrl D8 group (23.75% ± 6.54%) (FIGURE 2C). Follicle and oocyte diameters were measured in morphologically normal secondary follicles. Twenty-four follicles were analysed in the D0 group, 32 in the OT Ctrl D8 group, and 60 in the OT + CM D8 group. No significant differences in follicle or oocyte diameters were observed between the groups (TABLE 1).

GDF-9 staining of follicles

In total, 177, 223 and 233 growing follicles were analysed in the D0 group, OT Ctrl D8 group and OT + CM D8 group, respectively. The OT + CM D8 group yielded the highest percentage of GDF-9 staining (22.80% ± 3.38%), and this differed significantly from the OT Ctrl D8 (16.38% ± 2.25%, $P = 0.001$) and the D0 (14.14% ± 2.16%, $P<0.001$) groups. The OT Ctrl D8 group displayed a significantly higher ($P = 0.033$) percentage of GDF-9 staining compared with the D0 group (FIGURE 3A,B).

DNA fragmentation

Follicles. In total, 448, 192 and 259 follicles were evaluated in the D0 group, the OT Ctrl D8 group and the OT + CM D8 group, respectively. The proportion of TUNEL-positive follicles was significantly higher in both IVC groups (OT Ctrl D8: 24.49% ± 6.36%, $P < 0.001$; OT + CM D8: 16.76% ± 7.53%, $P < 0.001$) compared with the D0 group (2.26% ± 2.11%). However, a significantly lower proportion was found in the OT + CM D8 group ($P = 0.014$) compared with the OT Ctrl D8 group (FIGURE 4A).

Stromal cells. The proportion of TUNEL-positive stromal cells was significantly higher in both IVC groups (OT Ctrl D8: 28.98% ± 4.88%, $P < 0.001$; OT + CM D8: 18.05% ± 3.48%, $P < 0.001$) compared with the D0 group (0.27% ± 0.11%). The OT + CM D8 group showed a significantly lower ($P = 0.001$) proportion compared with the OT Ctrl D8 group (FIGURE 4B).

Oestradiol production. In the group cultured with ASC-CM (OT + CM), there was a significant increase in oestradiol

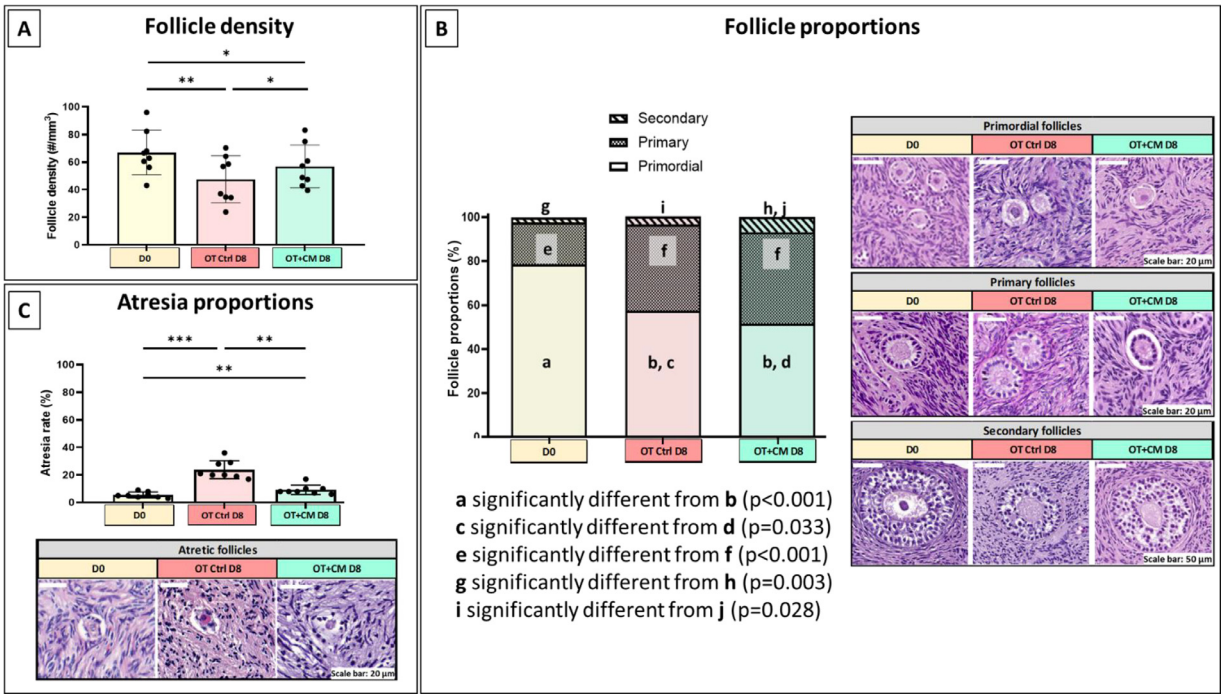

FIGURE 2 Density and proportion of follicles and proportion of atretic follicles. (A) Follicle density (no. of viable follicles/mm^3, mean ± SD) analysed by one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post-hoc test [**P = 0.002; *P = 0.010 (D0 versus OT + CM D8); P = 0.025 (OT + CM D8 versus OT Ctrl D8)]. (B) Proportion of follicles (percentage, mean ± SD) assessed by one-way ANOVA and Fisher's LSD post-hoc test, and representative photomicrographs of each stage of follicule development, namely primordial (oocyte surrounded by a single layer of flattened granulosa cells), primary (oocyte surrounded by a single layer of cuboidal granulosa cells) and secondary (oocyte surrounded by two or multiple layers of cuboidal granulosa cells). (C) Proportion of atretic follicles (percentage, mean ± SD) evaluated by one-way ANOVA and Fisher's LSD post-hoc test [***P < 0.001; **P = 0.008 (D0 versus OT + CM D8); P = 0.002 (OT + CM D8 versus OT Ctrl D8)], and representative photomicrographs of atretic follicles in each group. n = 8 ovaries per group. D0, non-cultured controls; OT Ctrl, ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

production by day 8 (84.11 ± 41.76 pg/ml, P = 0.022) compared with day 2 (57.02 ± 43.55 pg/ml). Furthermore, on day 8 of IVC, the OT + CM group yielded a significantly higher concentration of oestradiol compared with the OT Ctrl group (57.30 ± 27.41 pg/ml, P = 0.046) (FIGURE 5). The concentration of oestradiol was also measured in ASC-CM before being added to the OT + CM group, which displayed values below detectable levels (data not shown).

ASC-CM characterization and analyses. Concentrations of VEGF, IL-6 and TGF-β1 were measured in ASC-CM. Among studied factors, VEGF (531.41 ± 37 pg/ml) and IL-6 (545.43 ± 11.31 pg/ml) were the most highly expressed, while TGF-β1 showed values of 63.25 ± 2.10 pg/ml. In standard culture medium, the concentrations of these factors were below detectable levels (data not shown).

TABLE 1 SECONDARY FOLLICLE AND OOCYTE DIAMETERS

	D0[a] (24 follicles)	OT Ctrl D8[b] (32 follicles)	OT + CM D8[c] (60 follicles)	*P*-value
Follicle diameter (μm)	86.28 ± 27.84	88.92 ± 29.69	96.20 ± 34.04	a,b: 0.754 a,c: 0.261 b,c: 0.394
Oocyte diameter (μm)	40.17 ± 14.45	44.64 ± 16.54	47.14 ± 19.28	a,b: 0.252 a,c: 0.087 b,c: 0.635

Data are mean ± SD.

Assessed by one-way analysis of variance and Fisher's least significant difference post-hoc test.

D, day; D0, non-cultured controls; OT Ctrl: ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

DISCUSSION

This study demonstrates that the addition of ASC-CM to bovine ovarian tissue IVC medium enhances follicle survival, development and oestradiol secretion, and promotes the viability of stromal cells.

Supplementation of ASC-CM to culture medium had a positive influence on follicle stage progression, as shown by the lower proportion of primordial follicles and higher proportion of secondary follicles in the OT + CM D8 group compared with the OT Ctrl D8 group. Early-stage follicle development is a gonadotrophin-independent process, and requires local coordination of activating factors from somatic cell–oocyte bidirectional crosstalk (*Li and Albertini, 2013*). It was reported recently that CM from ASC contains various biomolecules, which could

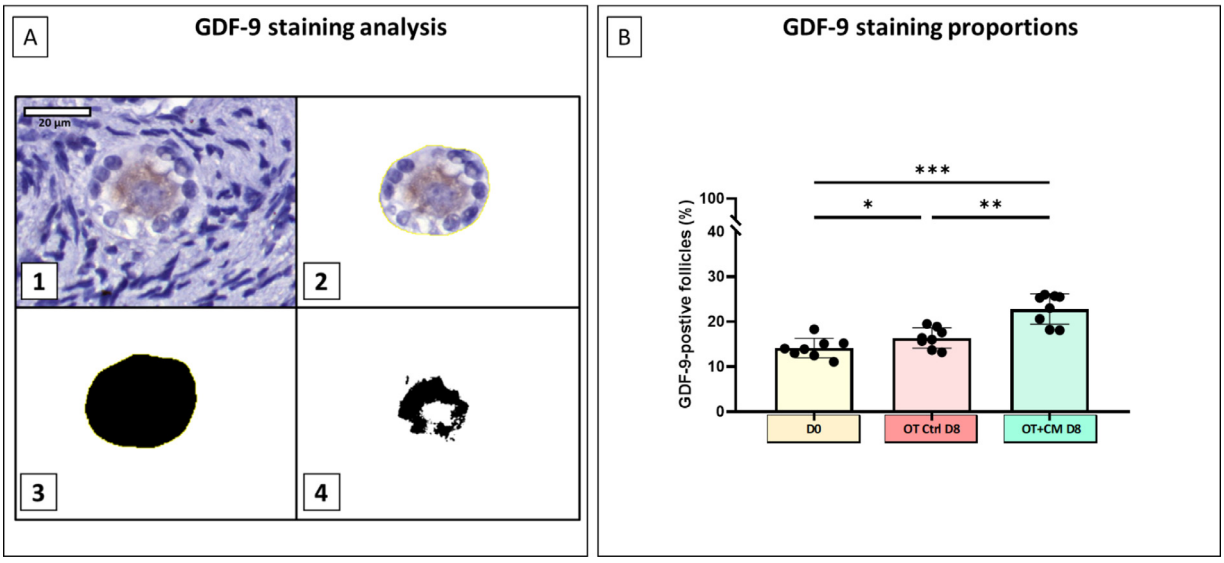

FIGURE 3 Analysis of growth differentiation factor-9 (GDF-9) staining and proportion of follicles stained. (A) GDF-9 staining analysis. (1) Growing (primary) follicle surrounded by stromal cells. Scale bar = 20 μm. (2) Digital follicle isolation from surrounding cells. (3) Measurement of total follicle area. (4) Colour deconvolution and measurement of GDF-9 area within the follicle. (B) Proportion of follicles stained with GDF-9 (percentage, mean ± SD) assessed by one-way analysis of variance and Fisher's least significant difference post-hoc test (***$P < 0.001$; **$P = 0.001$; *$P = 0.033$). $n = 8$ ovaries per group. D0, non-cultured controls; OT Ctrl, ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

modulate follicle activation signalling pathways (*Cacciottola et al., 2023*; *Jiao et al., 2022*; *Tomaszewski et al., 2019*). In line with the current findings, *Hosseini et al. (2019)* detected an increased rate of follicle development in human ovarian cortical fragments co-cultured with mesenchymal stem cells for 8 days compared with ovarian cortex cultured alone.

Despite the significant increase in the proportion of secondary follicles in the OT + CM D8 group in this study, there were no significant differences in the diameters of secondary follicles or oocytes between any of the groups (**TABLE 1**). Only secondary follicles were considered in this analysis, as they represent the main follicle

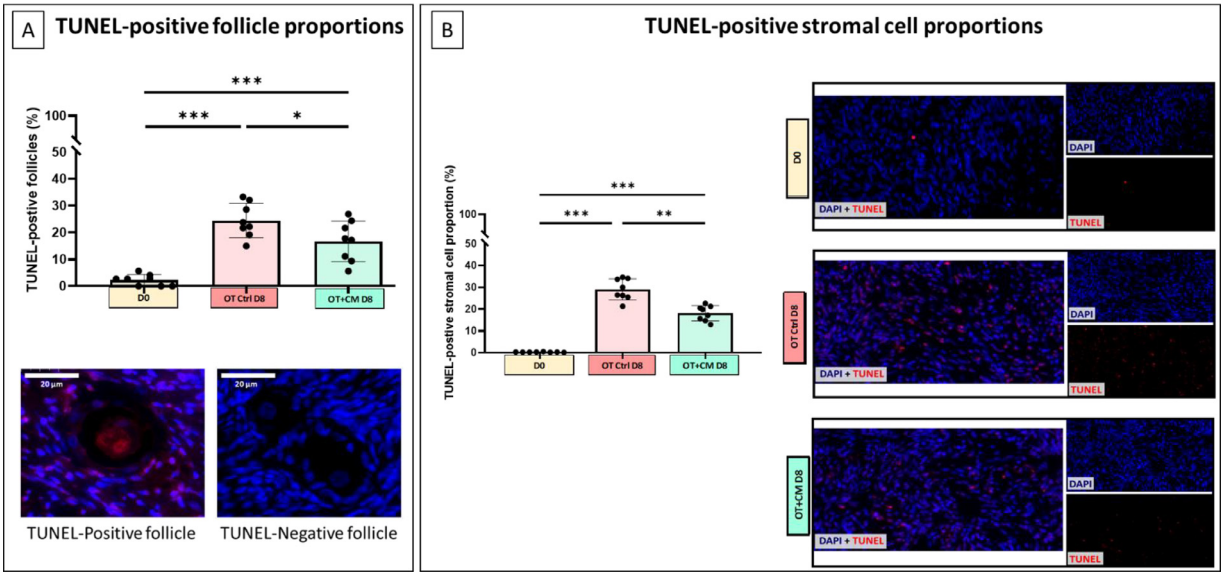

FIGURE 4 Proportions of TUNEL-positive follicles and stromal cells. (A) Proportion of TUNEL-positive follicles (percentage, mean ± SD) analysed by one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post-hoc test (***$P < 0.001$; *$P = 0.014$). Representative photomicrographs of TUNEL-positive and TUNEL-negative follicles. TUNEL (red), DAPI (blue). Scale bar = 20 μm. (B) Proportion of TUNEL-positive stromal cells (percentage, mean ± SD) assessed by one-way ANOVA and Fisher's LSD post-hoc test (***$P < 0.001$; **$P = 0.001$). Representative photomicrographs of TUNEL-positive and TUNEL-negative (stained by DAPI alone) stromal cells in each group. TUNEL (red), DAPI (blue). $n = 8$ ovaries per group. D0, non-cultured controls; OT Ctrl, ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

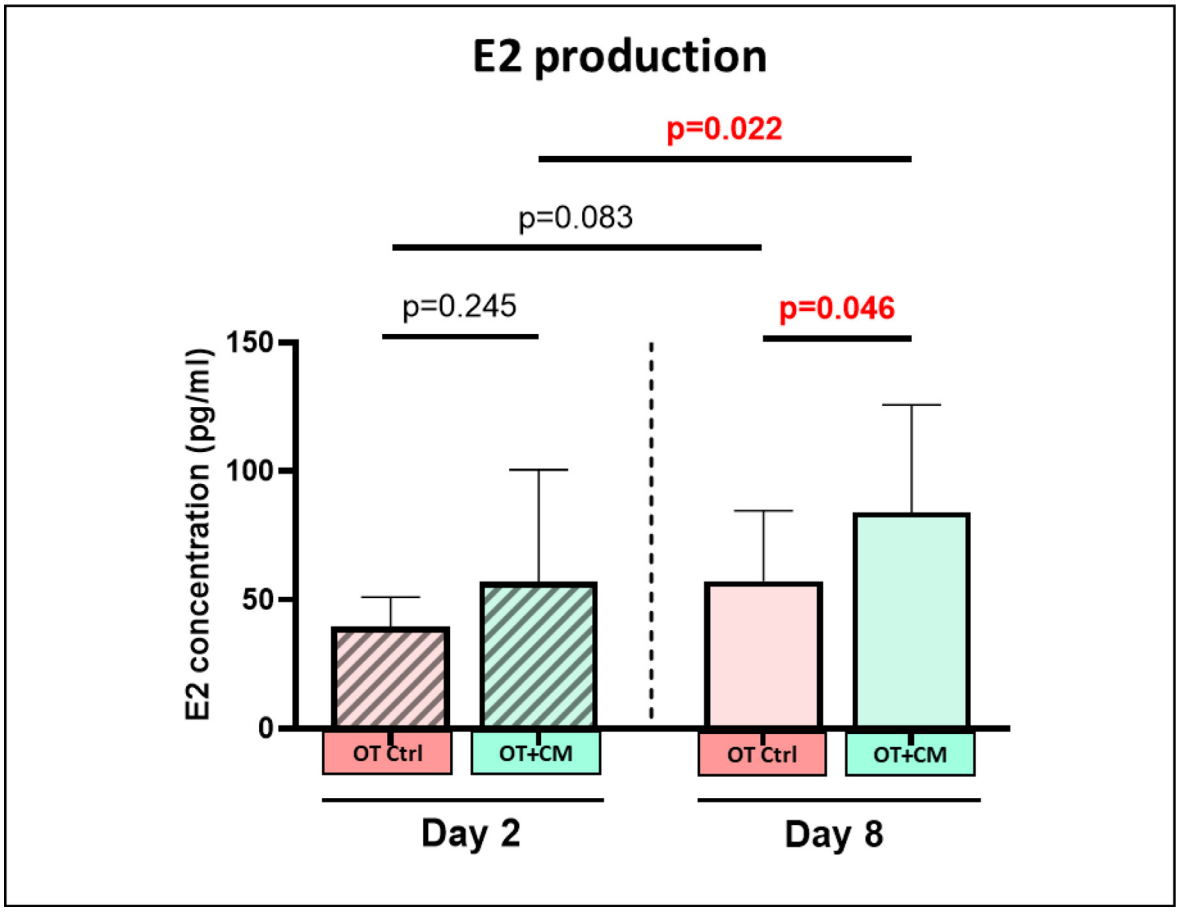

FIGURE 5 Production of oestradiol (E2; pg/ml, mean ± SD) analysed by paired *t*-test. $n = 8$ ovaries per group. OT Ctrl, ovarian tissue cultured alone; OT + CM, ovarian tissue with conditioned medium derived from adipose-tissue-derived stem cells.

outcome following the follicle activation step (after 8 days of IVC). However, it is important to emphasize that follicle growth is a slow process *in vitro*, requiring several cycles of replication of granulosa cells (*Braw-Tal and Yossefi, 1997*). Indeed, previous research from *Itoh and Hoshi (2000)* identified a substantial increase in the diameter of bovine follicles when co-cultured with mesenchymal stem cells alone after 30 days of culture. It is therefore possible that the culture period used in the present study (8 days) may not have been long enough to elucidate discernible differences. Something else to consider is that once follicles reach the secondary stage in the multistep IVC system (*McLaughlin et al., 2018*), they should be isolated from the surrounding ovarian cortex and cultured further to continue their development. It is likely that secondary follicles in the present study had reached their growth limit due to the influence of surrounding ovarian cells.

In recent years, it has been acknowledged that oocyte-derived factors from the TGF-β superfamily, particularly GDF-9, regulate follicle stage transition through distinct functions, including growth and expansion of adjacent granulosa cells (*Hreinsson et al., 2002*; *Orisaka et al., 2006*). GDF-9 is currently identified as a key indicator of follicle quality and competence (*Hussein et al., 2006*). Upon activation, the oocyte of the recruited follicle starts secreting GDF-9, which directly triggers the proliferation of granulosa cells, causing follicle stage transition through the developing phases (*Gilchrist et al., 2008*; *Li and Albertini, 2013*). In the present study, the percentage of GDF-9 staining was significantly higher in growing follicles (primary and secondary) in the OT + CM D8 group compared with the OT Ctrl D8 group. Based on these findings, paracrine factors contained within ASC-CM were able to promote follicle progression via stimulation of oocyte-derived factor GDF-9. In line with these results, *Xia et al. (2015)* reported increased expression of GDF-9 in isolated human preantral follicles co-cultured with mesenchymal stem cells.

ASC-CM supplementation boosted the proportion of morphologically viable follicles (non-atretic) compared with IVC without ASC-CM. As stated above, only viable follicles were taken into account to calculate follicle density. It is well known that follicle density decreases during ovarian tissue IVC, which was evidenced in the present study. However, the data demonstrated that follicle density was significantly higher in the OT + CM D8 group compared with the OT Ctrl D8 group. Similar results were obtained by *Sousa et al. (2021)* when investigating the impact of goat ovarian tissue co-cultured with mesenchymal stem cells over 7 days. These differences may be explained by the pro-survival properties of ASC-derived paracrine factors (*Ai et al., 2023*; *Cacciottola et al., 2023*; *Song et al., 2017*). In order to further explore the mechanisms behind cell death, DNA fragmentation was evaluated by TUNEL assays. The OT + CM D8 group yielded a lower proportion of TUNEL-positive follicles compared with the OT Ctrl D8 group. Consistent with the present results,

Green et al. (2019) reported higher viability of mouse follicles co-encapsulated with mouse ASC compared with follicles cultured alone. Similarly, another study demonstrated a significant decrease in the rate of follicle apoptosis when CM from mesenchymal stem cells was added to standard IVC medium (Jia et al., 2017).

The current study also demonstrated a significant improvement in the viability of ovarian stromal cells in the OT + CM D8 group compared with the OT Ctrl D8 group. These specialized cells are known for their role in orchestrating essential functions within the ovarian microenvironment. They provide signalling cues that facilitate proper folliculogenesis (Kinnear et al., 2020; Telfer et al., 2023), offer structural and biochemical support (Ouni et al., 2019), and regulate immune responses and inflammation through release of cytokines (Zhang et al., 2020). Compromised viability of stromal cells could disrupt these intricate signalling networks, potentially leading to impaired follicle development and reduced oocyte quality.

Steroid hormones such as oestradiol have proved vital to the in-vitro development of bovine preantral follicles, enabling oocytes to acquire the necessary competence as they transition through the growth stages (Chauvin et al., 2022; Drummond, 2006). Granulosa cells and theca cells of growing follicles secrete oestradiol, and this production increases proportionally with follicle stage progression and proliferation of granulosa cells (Tajima et al., 2006). As expected, medium collected at the end of IVC from the OT + CM D8 group had a significantly higher concentration of oestradiol compared with the OT Ctrl D8 group, reflecting the higher proportion of secondary follicles in the OT + CM D8 group. These results confirm the findings of Park et al. (2021), who reported that the expression of two key steroidogenic genes, namely aromatase and steroidogenic acute regulatory protein, was significantly higher in isolated mouse granulosa cells after being cultured with CM from mesenchymal stem cells.

In the OT + CM group, tissue was cultured in a medium comprising 50% standard culture medium and 50% ASC-CM (1:1 ratio). This strongly implies that the paracrine factors present in ASC-CM provide a greater benefit than the compounds found in the standard culture medium for cultured ovarian tissue.

Subsequent studies could assess whether varying the proportions of ASC-CM and standard culture medium leads to additional modifications in follicle outcomes. Furthermore, a broader proteomics analysis could contribute to better insight into the factors contained in ASC-CM.

As described in earlier studies (Borgese et al., 2020; de Souza et al., 2020; Tomaszewski et al., 2019), the present study found notable concentrations of VEGF, IL-6 and TGF-β1 in ASC-CM. Besides its proangiogenic effects, VEGF has been shown to be a strong cell survival factor (Byrne et al., 2005; Kosaka et al., 2007). By activating the PI3K/Akt pathway, VEGF has been described as a key factor involved in early follicle activation and development (Irusta et al., 2010; Yang and Fortune, 2007). VEGF receptors have been found to be expressed in bovine follicles (Greenaway et al., 2004), and the concentration of VEGF mRNA increased gradually through the growth stages (Yang and Fortune, 2007). Yang and Fortune (2007) also demonstrated that addition of VEGF to culture medium increased the proportion of secondary follicles in a dose-dependent manner in bovine cortical fragments cultured for 10 days. Studies have also shown that various interleukins, such as IL-6, are involved in ovarian folliculogenesis (Tomaszewski et al., 2019). IL-6 receptors were found mainly in granulosa cells and theca cells of developing bovine follicles (Samir et al., 2017). This multifunctional interleukin has been associated with numerous biological activities, including proliferation of granulosa cells and inhibition of cell apoptosis (Tingen et al., 2011). Previously, it has been proposed that IL-6 induces anti-apoptotic gene expression (B-cell lymphoma 2 gene family) (Karaoz et al., 2010). In addition, the role of TGF-β1 has been widely reported in the regulation of folliculogenesis (Knight and Glister, 2006). Oocytes produce TGF-β1, which is critical for oocyte–granulosa cell communication, formation of transzonal projections and intercellular metabolic cooperation (Chen et al., 2015). Indeed, TGF-β1 is known to stimulate proliferation and differentiation of granulosa cells in IVC systems in cows (Fazzini et al., 2006), and several TGF-β receptors (TGFBR1, TGFBR2 and TGFBR3) have been documented in granulosa cells of bovine follicles (Matiller et al., 2019). In addition, TGF-β1 plays an important role in the formation of extracellular matrix and secretion of its components. TGF-β1 supplementation has been found to promote collagen production and boost proliferation of chicken granulosa cells *in vitro* (Zhou et al., 2021).

The current study has both strengths and limitations. One of its strengths lies in the assessment of follicle development *in vitro* from different aspects, such as the proportion of secondary follicles, GDF-9 expression and oestradiol production, which adds robustness to the results. Additionally, the findings successfully demonstrate the beneficial impact of ASC-CM on both cell compartments, namely ovarian follicles and stromal cells. In recent years, an increasing body of evidence has highlighted the essential role of stromal cells in folliculogenesis. On the other hand, the main limitation of the study is that only three paracrine factors were assessed in ASC-CM. Further proteomics studies on ASC-CM are currently being studied by the authors' team to encompass novel target factors, and improve understanding of the protective effects it exerts on ovarian tissue cultured *in vitro*.

In conclusion, this study demonstrates that the addition of ASC-CM to bovine ovarian tissue IVC medium enhances follicle survival, development and oestradiol secretion, and promotes the viability of stromal cells. ASC-derived paracrine factors (VEGF, IL-6 and TGF-β1) associated with pro-survival and anti-apoptotic effects were identified in CM. Overall, these findings on the beneficial role of ASC-CM on IVC outcomes for ovarian tissue offer a firm foundation for further investigations into optimal culture conditions.

DATA AVAILABILITY

Data will be made available on request.

AUTHOR CONTRIBUTIONS

F.V.: experimental design, experimental procedures, analyses, statistical analysis, interpretation of results and article preparation. L.C.: experimental design, statistical analysis and interpretation of results. A.C: experimental design and interpretation of results. L.H: analyses and interpretation of results. J.D.: interpretation of results, article preparation and revision. M.M.D.: experimental design, interpretation of

results, and article preparation and revision.

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SUPPLEMENTARY MATERIALS

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