Culture of vitrified bovine ovarian tissue on agarose gel inserts maintains follicle integrity

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Short title: Bovine ovarian tissue vitrification-culture

Keywords: interleukins, follicle activation, caspase-3, cryopreservation, ovary

Word count: 5107

In brief

Ovarian tissue cryopreservation and culture provide an option for fertility preservation without tissue grafting, but need optimization. This study reveals that vitrified bovine ovarian tissue can be cultured on agarose gel and maintain follicle morphology, low activation and low apoptosis.

Abstract

Ovarian tissue preservation is hitherto a promising fertility insurance option for precious animals. Ovarian tissue vitrification and culture combined approach would eliminate the need of transplanting ovarian tissue to obtain mature oocytes. We aimed at optimizing vitrification and *in vitro* culture conditions for improved bovine ovarian tissue viability. Ovaries obtained from the slaughterhouse were punched into fragments and divided into three groups. Group 1 (fresh) was divided into two and immediately placed in two culture systems (culture inserts and agarose inserts). Group 2 was vitrified, warmed, and placed in the two culture systems while group 3 was only equilibrated then placed in the two culture systems. All cultures were maintained for six days and spent media were collected on alternate days for cytokine (interleukin 1β and interleukin 6) evaluation. Fragments were fixed for morphology assessment and immunohistochemistry. Higher percentages ($P<0.05$) of grade one (morphologically intact) follicles were observed in fragments on agarose compared to those on culture inserts at days two and four of culture. Conversely, we found higher ($P<0.05$) shifts of primordial follicles to transitional follicles in fragments on culture inserts vis-à-vis agarose inserts which was consistent with higher proportion of Ki-67 and MCM-7 and activated caspase-3 positive follicles. In conclusion, *in vitro* culture of bovine ovarian tissue on agarose inserts maintained follicle morphology, low follicle activation and low apoptosis compared to culture inserts.

Introduction

Cryostorage of the germplasm is a possible approach to preserve the fertility of a given individual for later application of assisted reproductive technology (ART) (Picton et al. 2000). It is especially important for fertility preservation of cancer patients destined to undergo gonadotoxic therapies particularly those that are unable to produce gametes (Rives et al. 2022). Similarly, gonadal tissue preservation is one of the first line approaches to be considered when precious animals die suddenly or when gonadotoxic therapies are indicated (Meirow et al. 1999, Valli-Pulaski et al. 2018). Ovarian tissue cryopreservation is the only acceptable option for restoring both reproductive and endocrine functions of the ovary (Macklon 2020, Picton et al. 2000), especially for the purpose of biodiversity conservation in young endangered animals (Comizzoli 2015). The conventional cryostorage technique of ovarian tissue is the slow freezing method (Faheem et al. 2011). Recently, the focus has been

gradually turning towards vitrification, which is relatively simple to carry out in field conditions, since the technique does not require sophisticated laboratory equipment. Studies indicating the superiority of vitrification over slow freezing have been reported ranging from better tissue integrity to resumption of folliculogenesis and steroidogenesis both post grafting and in *in vitro* culture (Amorim et al. 2012, Gastal et al. 2018, Herraiz et al. 2020, Marques et al. 2019, Xiao et al. 2013). However, a number of factors could affect the efficiency of ovarian tissue vitrification, which may include but not be limited to the choice of cryoprotectant (CPA), rate of tissue permeation of CPA (Lotz et al. 2020), equilibration temperature (Mouttham and Comizzoli 2016), speed of cooling, size of fragments (Amorim et al. 2011b), decortication technique and thawing protocol (Herraiz et al. 2020), and presence or absence of non-permeable CPAs (Elliott et al. 2017). Vitrification requires a high amount of CPA, enough to create a glassy solid state when rapid cooling is applied in liquid nitrogen (Amorim et al. 2011a, Kometas et al. 2021, Shi et al. 2017). Therefore, CPA permeation of ovarian tissue is a critical factor that determines the success of vitrification, especially looking at the complexity of cell types and presence of extracellular space (Lotz et al. 2020). Although the technology of ovarian tissue cryopreservation is no longer considered experimental in humans according to the American Society for Reproductive Medicine (ASRM 2019) and despite the fact that babies have been born from this technology (Donnez and Dolmans 2017), many challenges are yet to be resolved. The bovine species is an excellent clinical model used to properly optimize and validate protocols for ultimate translation to the human patient and to other mammals (Anderson and Baird 2019, Callejo et al. 2013).

The ultimate functional evaluation of thawed ovarian tissue may be after transplantation or *in vitro* culture. Warmed ovarian tissues can be xenografted in an immunocompromised animal or auto-transplanted (Kong et al. 2017). One of the limiting factors of this technique, apart from being invasive, is damage of the graft due to anoxia prior to establishment of a vascular

network, which may take several days in certain species (Anderson and Baird 2019, Kong et al. 2017, 2016, Liu et al. 2002, Van Eyck et al. 2009). Moreover, in some cases of cancer where individuals undergo gonadotoxic chemotherapies, the malignant cells may be present in the ovarian tissue thus risking the possibility of re-transplanting malignancy (Rosendahl et al. 2010). Furthermore, acute depletion of the follicular pool arising from over activation post grafting has been reported (Gavish et al. 2014, Masciangelo et al. 2019). A promising alternative to the transplantation of ovarian tissue is *in vitro* culture, which can be done for whole tissue (*in situ*) or isolated follicles (*ex situ*) (Gastal et al. 2019, Lunardi et al. 2016, O'Brien et al. 2003, Paynter et al. 1999, Shoorei et al. 2019, Sutton et al. 2021, Telfer et al. 2019). In addition, tissue damage from negative effects of vitrification were found to be ameliorated during *in vitro* culture of warmed ovarian tissue (Meng et al. 2022, Mouttham et al. 2015). However, *in vitro* culture of ovarian tissue is still experimental and more studies are required before it can be applied clinically (Telfer et al. 2019). The use of physical support such as hydrogel biomaterials is a well-known approach for supporting cellular integrity in tissue engineering (Awad et al. 2004). Regarding ovarian tissue, both culture inserts and hydrogels (agarose and alginate) have been reported for both *in situ* and *ex situ* culture of follicles (Laronda et al. 2014, Lunardi et al. 2016, West et al. 2007, Yang et al. 2017) (Fujihara et al. 2012). The main aim of this study is to optimize vitrification and subsequent *in vitro* culture of vitrified warmed bovine ovarian tissue. Therefore, the objectives were to evaluate the suitability of a vitrification protocol originally developed for larger ovarian tissue fragments intended for grafting, on smaller fragments suitable for *in vitro* tissue culture, and to test two *in vitro* culture conditions for sustaining bovine ovarian tissue viability after warming.

Materials and method

Chemicals and reagents

All chemicals were purchased from Sigma–Aldrich (Taufkirchen, Germany) unless stated otherwise.

Ovary transport and preparation of ovarian fragments

A total of 18 ovaries from 9 Belgian blue heifers were collected from slaughterhouse in a solution of normal saline and antibiotic (AB: Gentamicin 0.05 mg/mL (Gibco, Bleiswijk, Netherlands) in Dulbecco Phosphate Buffered Saline (DPBS), and immediately transported to the laboratory. No approval for use of animals for research was sought from ethics committee because ovaries were collected post mortem.

Ovaries were washed three times with washing solution (DPBS and AB) and two times in dissecting solution (Leibovitz medium (Gibco, Bleiswijk, Netherlands) and AB). At least 2 ovaries were used for each replicate experiment. Using a scalpel blade (Paramount Surgimed, New Delhi, India), the cortex of each ovary was sliced at an area with less visible follicles to make a 1 mm thick slice. A millimeter scale was placed beneath the dish containg the ovaries. A total of 76 equal ovarian tissue fragments were punched with 1.5 mm diameter biopsy punch (Kai medical, Oyana, Japan) from the sliced ovarian cortex for each experiment.

Experimental design

The detailed experimental design is presented in a flow chart (figure 1[I]) but briefly described as follows. Ovarian fragments were divided into 3 groups. Group 1 (fresh cultured [FC]) were immediately placed in culture for six days. Groups 2 and 3 were inserted on 30 G needles (four fragments per needle) to facilitate handling and maximize cooling rate. Three step equilibration and vitrification were performed. Group 2 (vitrified cultured [VC]) was vitrified and warmed, while group 3 was only equilibrated and warmed (equilibrated cultured [EC]). Each group was divided into two and placed in two culture systems (culture inserts and agarose inserts) for six days (see details below). Spent media were collected on alternate days from each well and sent for cytokine (interleukin 1β [IL-1β] and interleukin 6 [IL-6])

profiling. At the end of each culture period, fragments were weighed and fixed for 6 hours in Bouin's solution for morphology assessment and in neutral buffered formalin for immunohistochemistry. A total of six independent experiments were carried out.

Vitrification and warming

Vitrification procedure was based on the protocol described by Amorim et al. (2018, 2013). However, the tissue fragment size was reduced (1.5 mm diameter and 1 mm thickness) to conform with *in vitro* culture condition. Equilibration was performed in 5 ml tubes (Thermofisher, Roskilde, Denmark) at room temperature and based on a vitrification solution (VS) containing 10% (v/v) DMSO, 26% EG, 2.5% polyvinylpyrrolidone (PVP, MW 10 000) and 1 M sucrose in MEM (Gibco, Bleiswijk, Netherlands) + 20 mg/mL bovine serum albumin (BSA). Three step equilibrations involved 7 min in VS1 (25% VS in MEM + 20 mg/mL BSA), 4 min in VS2 (50% VS in MEM + 20 mg/mL BSA) and 3 min in VS3 (100% VS) at room temperature. Excess VS was soaked from the equilibrated fragments with sterile gauze then the needles were plunged directly into liquid nitrogen for vitrification (VC samples), while (EC) were directly passed into the different washes of warming solutions (WS).

Ovarian fragments were warmed in WS1 (1 M sucrose in MEM supplemented with 20 mg/mL BSA) at 37°C for 15 s and then moved through 3 washing steps with decreasing sucrose concentration at 37°C for 5 min each [WS2 (0.5 M sucrose in MEM supplemented with 20 mg/mL BSA), WS3 (0.25 M sucrose in MEM supplemented with 20 mg/mL BSA) and WS4 (MEM supplemented with 20 mg/mL BSA)].

Culture

The culture medium was composed of Waymouth's medium (Gibco, Bleiswijk, Netherlands) supplemented with insulin (10 µg/mL), transferrin (5.5 µg/mL), selenium (6.7 ng/mL), bovine serum albumin (1.25 µg/mL), sodium pyruvate (25 µg/mL) and gentamicin (0.05 mg/mL). Ovarian tissue fragments were cultured according to Yang et al. (2017) on culture inserts and

agarose inserts (Gohbara et al. 2010). Briefly, 1.5 g of agarose was heated and dissolved in distilled water (1.5% [w/v]) which corresponds to stiffness of 700 dynes/cm^2 (Balgude et al. 2001) and then poured and allowed to set in a 10-cm Petri dish. Hexahedrons (10 × 10 × 5 mm) were dissected and then soaked in the culture medium for at least 24 hours for media infiltration. Twenty-four well dishes containing untreated culture inserts 0.4 µm pore size were used (Thermofisher, Roskilde, Denmark). For each replicate, 9 culture inserts and 9 agarose inserts were used. Distilled water was placed in the 6 remaining empty wells to maintain adequate humidity in the dish. Culture medium was added to each well containing both inserts, so that the level of culture medium was near the upper surface of the agarose inserts thus, the agarose inserts were not completely submerged in the culture media. This creates a liquid gas interface that enhances gaseous exchange withing the tissue fragments (Gohbara et al. 2010). Four fragments were placed on each insert (agarose and culture) and kept separated from one another. Therefore, the groups were fresh cultured (FC); fresh cultured on agarose (FCA); vitrified cultured (VC); vitrified cultured on agarose (VCA); equilibrated cultured (EC) and equilibrated cultured on agarose (ECA). Culture was maintained for six days at 38°C and 5% CO_2, and 200 µL of spent media was replaced every other day and used for assay of cytokines.

Histology

Routine histology was carried out using an automatic tissue processor (Richard-Allan MICROM STP120 Thermo Scientific, Waltham, USA). Bouin fixed blocks were sectioned (5 µm thickness) and placed on glass slides and dried overnight. Hematoxylin and Eosin staining was conducted with Gemini AS automated slide Stainer (Epredia™ A81500001 Thermo Scientific, Waltham, USA) and cover slipped. Follicles were classified based on developmental stages as primordial when the oocyte was surrounded by flattened follicular cells; transitional when some the flattened follicular cells have been converted to cuboidal

189 cells or primary when all the follicular cells appeared cuboidal. Follicles were further graded
190 into four grades based on (Paynter et al. 1999) with modification as follows (figure 1[II]):
191 grade 1: spherical in shape, evenly distributed follicular cells, intact stroma, spherical oocyte
192 and intact nucleus and nucleolus; grade 2: spherical in shape, evenly distributed follicular
193 cells, intact stroma and spherical oocyte, misshapen nucleus and/or not homogenous
194 cytoplasm; grade 3: follicular cells pulled away from the stroma but oocyte spherical; grade 4:
195 follicular cells pulled away from the stroma and oocyte misshapen, vacuolated and pyknotic
196 nucleus of granulosa cells. Follicles were expressed as percentage of the total follicles
197 counted. Only follicles with visible nucleus were counted. Five sections were skipped
198 between counted sections to avoid double counting. Representative micrographs of the
199 different groups can be found in figure 2.

200 *Immunohistochemistry*

201 Immunohistochemical staining was performed automatically in a DAKO Autostainer
202 (California, USA) following the manufacturer's instructions for the three proteins assessed
203 namely: Ki-67, (a conventional intranuclear proliferation marker with high expression at the
204 G2 phase and mitosis), MCM-7 (less common but most reliable and sensitive proliferation
205 marker which essentially functions in the initiation and elongation of DNA replication)
206 (Juríková et al. 2016), and activated caspase-3 (commonly used as a reliable marker of
207 cellular apoptosis). Briefly, formalin fixed blocks were sectioned (5 µm thickness) and placed
208 on microscopic slides (Dako, California, USA). Slides were deparaffinized and rehydrated
209 prior to antigen retrieval which was performed at 97˚C for 20 min in antigen retrieval solution
210 [low pH (6.0) for Ki-67 and activated caspase-3; high Ph (9.0) for MCM-7 (Dako EnVision
211 Flex, Glostrup, Denmark)]. Subsequently, the sections were washed in wash buffer (Dako
212 EnVision Flex) and incubated with primary antibodies: anti-Ki-67 (clone MiB-1; Dako
213 EnVision Flex), anti-MCM-7 (1:100; Santa Cruz Biotechnology, Heidelberg, Germany) and

anti-activated caspase-3 (1:400; Cell signaling, Massachusetts USA) in a Dako Autostainer Link48 for 20 min at room temperature. EnVison FLEX (Dako EnVision Flex, Glostrup, Denmark) reagents were used for visualization of all the studied antigens and the slides were counterstained with hematoxylin (Dako EnVision Flex, Glostrup, Denmark), as recommended by the manufacturer. Bovine tonsil was used as a positive control and finally sections were cover slipped, digitally scanned, and evaluated with a case viewer software (3Dhistech version 2.3.2). Follicles counted as immunopositive for the protein targets assessed must have shown brown staining on at least one follicular cell (figure 3, 4 and 5).

Stromal cell density

Nuclei of stromal cells were counted from an area of 5000 μm^2 selected on each section in a midway between the periphery and the center of the section. The number of caspase-3 positive stromal cells were also counted and presented as a ratio of the total stromal cells counted.

Assay of cytokines

Commercial ELISA kits for interleukin 1β (IL-1β) (Invitrogen, Vienna, Austria) and interleukin 6 (IL-6) (Invitrogen, Vienna, Austria) were used according to manufacturer's instructions to measure the concentration of IL-1β and IL-6 in the spent culture media. High concentrations of these cytokines were associated with an increased activation of primordial follicle pool in murine *ex vivo* ovarian cortex (Bromfield and Sheldon 2013). Standard curve was created for each plate using the absorbance of the standard and the concentrations provided by the manufacturer. Then, absorbance was measured at room temperature with Multiskan GO spectrophotometer (Thermofisher scientific, Vantaa, Finland) and data were normalized against the weight of the ovarian tissue fragments to 10 mg for IL-1β and 5 mg for IL-6 then concentrations were interpolated using the standard curve.

Statistical analysis

Discrete data obtained from counting follicles were analyzed using chi-square and presented in proportions as percentages. Concentrations of IL-1β were normally distributed (Shapiro-Wilk test), therefore analyzed using one way analysis of variance (ANOVA) while analysis of covariance (ANCOVA) was used to measure effect of the culture system. In the case of IL-6, wherein data were not normally distributed, Kruskal-Wallis test was used and 95% confidence interval was considered.

Results

Morphology

We investigated the effects of vitrification and culture system on the viability of primordial follicles within ovarian tissue fragments. When we consider the vitrified and equilibrated only groups, we found the lowest proportion of grade 1 follicles in the vitrified group ($P<0.05$) after six days of culture. Considering the two culture systems tested, the conventional culture inserts and agarose inserts, we observed higher proportion ($P<0.05$) of grade one follicles in fragments cultured on agarose (FCA and ECA) at day two of culture than those cultured on culture inserts (FC and EC). Similarly, at day four of culture, grade one follicles were significantly higher in VCA and ECA compared to those cultured on the conventional culture inserts (VC and EC, table 1).

To explore the initiation and maintenance of follicle growth, we classified the follicles based on stage of development (figure 1 [II]) and determined the proportions (table 2). significantly ($P<0.05$) higher proportions of transitional follicles were observed at culture day 2 and 4 in fragments cultured on the conventional culture inserts (FC, VC, and EC) with very low variability at day 6. The distribution of primordial and transitional follicles was similar but inversely associated. In other words, the proportion of primordial follicles reduces as the days in culture increases with simultaneous increase in the proportion of transitional follicles. In addition to the evaluation of follicles, the stroma where these follicles reside was also

evaluated. The stromal cell density which involved stromal cell counts per 5000 μm^2 did not show significant variations (P>0.05) among all the groups (table 3).

Immunohistochemistry

Immunolocalization of the two proliferation markers (Ki-67 and MCM-7) was carried out to validate the morphologically observed turnover of primordial to transitional follicles. In general, a higher proportion (P<0.05) of Ki-67 and MCM-7 positive follicles were recorded in fragments cultured on conventional culture inserts than in those cultured on agarose inserts which concurs with the distribution of the morphological classification (table 2). Interestingly, the two markers exhibited a very similar result although higher proportions were recorded in MCM-7. This is not surprising because MCM-7 is a protein that has been associated with initiation and elongation of DNA during replication, thus it can be detected earlier than Ki-67. Furthermore, apoptotic activity in the two culture systems was similar with the pattern of the proliferative activity. This means that, proportion of activated caspase-3 positive follicles were higher (P<0.05) in the culture system with conventional inserts than the culture system with agarose inserts. Similarly, activated caspase-3 positive stromal cells were significantly lower in fragments cultured on agarose inserts in most groups except at day 4 in EC (table 4).

Cytokines

One of the possible non-invasive ways of determining the integrity of cultured tissues is through spent culture media profiling. In this study, we evaluated two cytokines IL-1β and 6 in the spent culture media. The mean serial concentrations of IL-1β across the six-day culture did not vary significantly among the groups (table 5). Similarly, the concentrations of IL-6 were not different (P>0.05) across the six-day culture period. However, when we considered the effect of culture system, we found significant difference in IL-1β concentrations between culture on agarose and on culture inserts.

Discussion

The objectives of this study were to evaluate the suitability of a vitrification protocol, and two *in vitro* culture conditions for sustaining bovine ovarian tissue viability. The vitrification protocol was a modification of a technique that was successfully applied to non-human primate where, ovarian fragments (8x3x1 mm) were vitrified-warmed and autografted (Amorim et al. 2013). Amorim et al. (2018) confirmed that both reproductive and endocrine functions of the grafted ovarian tissue were restored 18 months post grafting. In these two studies from Amorim's lab, the same vitrification technique was applied although, the tissue size employed was designed to be suitable for tissue grafting as seen with previous studies involving transplantation post warming (Amorim et al. 2012, Kagawa et al. 2009). Therefore, we modified our tissue size to smaller fragments (1.5 mm diameter x 1 mm thickness) suitable for *in vitro* culture system (McLaughlin et al. 2018). In addition, it would simultaneously enhance tissue permeation rate of CPAs and increase cooling rate during vitrification. Furthermore, the vitrification protocol was composed of the best combination of different classes of CPAs including non-permeable CPAs (Elliott et al. 2017, Shahsavari et al. 2020) to ensure balanced osmotic changes during equilibration.

Morphologically, when we consider the vitrified and equilibrated groups, the lowest proportion of grade 1 follicles was recorded in the vitrified group on the sixth day of culture. This shows that exposure of tissue fragments to vitrification results in damaging effects to the tissue structural integrity. On the contrary, Mouttham et al. (2015) reported that exposure of bovine ovarian tissue to equilibrating solution (50% concentration of vitrification solution) resulted in the same morphological damage that is observed after vitrification. However, these negative effects are certainly dependent on the composition of the solution and the protocol employed (Amorim et al. 2011b).

Looking at our two culture systems, this study recorded a higher percentage of grade 1 follicles in fragments cultured on agarose inserts within four days of culture. On the other

316 hand, a significant shift of primordial follicles to transitional follicles was observed from day
317 2 and 4 of culture. This trend was significantly higher in the system with conventional culture
318 inserts than in the system with agarose. This delineated a form of delayed activation in
319 fragments cultured on agarose inserts, perhaps associated with a hidden role of mechanical
320 signaling (Shah et al. 2018). Inert hydrogel biomaterials such as agarose or alginate are well
321 known in supporting cellular integrity in tissue engineering (Awad et al. 2004), but their
322 stiffness was inversely associated with the growth and development of secondary follicles
323 (West et al. 2007). In this regard it could be hypothesized that the stiffness of the agarose gel
324 inserts acting as extracellular matrix may have directly resulted in the delayed follicle
325 activation. Woodruff and Shea (2011) also hypothesized that follicle activation, health and
326 selection are dependent on physical environment where the follicle grows. Stromal cell
327 density did not vary significantly in the present study similar to an earlier finding where no
328 difference was reported in all treatment groups (Cavalcante et al. 2019).
329 The morphological values were reinforced with the more objective immunostaining (Hawes et
330 al. 2009). Immunosignals from both Ki-67 and MCM-7 showed similar pattern indicating a
331 significant increase in proportion of immunopositive follicles from day 2 of culture on
332 conventional culture inserts and on day 6 of culture on agarose. Generally, higher proportions
333 of both Ki-67 and MCM-7 were observed in fragments cultured on the conventional culture
334 inserts when compared with agarose inserts. Similarly, higher apoptosis (i.e., activated
335 caspase-3) was observed in both follicles and stromal cells in ovarian tissue fragments
336 cultured on the conventional culture inserts (table 3 and 4). This showed that despite having a
337 more robust and immediate primordial follicle activation in fragments cultured on culture
338 inserts, this condition was accompanied with lower viability. The high follicular cell
339 proliferation recorded may be attributed to the immediate nutrient availability for tissue
340 fragments since fragments were partially covered by a thin film of medium with a

simultaneous lower gas exchange. Morimoto et al. (2007) reported that higher oxygen tension is required to maintain human primordial follicle viability in *in vitro* culture up to 15 days.

In the current study we have found a high proportion of primordial follicle activation within the first 4 days of culture on inserts, whereas most follicle activation was seen at the sixth day of culture on agarose. Hyperactivation of primordial follicles has been postulated to be attributed to suppression of the Hippo pathway which could be associated to mechanical signals during tissue preparation (Grosbois and Demeestere 2018, Telfer et al. 2019). Ideally, global activation of primordial follicles may be desirable perhaps to produce high number of preantral follicles which can then be isolated for further development and maturation of oocytes in multistep culture condition (Telfer and Zelinski 2013). This is because, *in situ* culture of primordial follicles is an ideal condition to initiate follicle growth until preantral stage where they stagnate and mostly regress (Telfer et al. 2019). However, this trait of superhigh activation of primordial follicles is not devoid of consequences to ovarian tissue preservation technology, ranging from abnormalities to follicle development and atresia in *in vitro* culture to post graft follicle pool depletion referred to as follicle "burn out" (Bertoldo et al. 2018, Gavish et al. 2014). Although the burn out phenomenon that occurs after the ovarian fragment has been transplanted could be as a result of tissue ischemia that occurs prior to vascularization of the graft; there is evidence that it could be sequel to hyperactivation (Gavish et al. 2014). Thus, we hypothesize that the use of possible attenuating agents of follicle hyperactivation could serve as an alternative to follicle burn out. Although investigations would be necessary, since delayed follicle activation was obtained in fragments cultured on agarose, this could be an option. The use of *in vitro* culture on agarose before grafting should be tested, as well as extended culture period to achieve higher follicle activation *in vitro*.

In this study, we limited the culture period to six days to understand the activation pattern of the two culture systems. However, future perspective should include a longer duration

perhaps coupled with advanced molecular studies to understand the pathways involved which would likely lead to the ultimate goal of *in vitro* grown oocytes. Although producing a matured oocyte from primordial follicles have been demonstrated in the mice (Eppig and O'Brien 1996, O'Brien et al. 2003), the technology is at its infancy in the larger mammals (Telfer and Andersen 2021). Previously, studies have shown that, primordial follicle activation and development until the secondary stage is achievable *in situ* between 4 to 8 days in culture. However, further growth and development from the secondary stage requires follicle isolation and independent *ex situ* culture (Smitz et al. 2010, Telfer et al. 2019).

Furthermore, it is challenging to non-invasively evaluate the integrity of tissue explants in real time, and tissues are usually exposed to an invasive end point evaluation procedure such as histology. However, one of the alternatives is to analyse the spent media for cytokines, biopolymers, or nutrient utilization (Plekhanov et al. 2020). In the current study, we assayed cytokines (IL-1β and IL-6) which were found to be statistically the same in most treatments, notwithstanding, the significant variability between culture on agarose and on culture inserts with regards to IL-1β. This indicate that, a more robust analysis of the spent culture media may be useful to strengthen our understanding on ovarian tissue culture system and perhaps pave the way for optimisation of culture formulations. Moreover, this may further help in the development of procedures for non-invasive ovarian tissue evaluation. The more consistent pattern of IL-1β concentrations seen in fragments cultured on agarose could be due to their interaction with the agarose insert as an extra cellular matrix since IL-1β has multifaceted functions (Bent et al. 2018). In contrast, a study on murine *ex vivo* ovarian cortex reported an association between increased IL-1, IL-6 and IL-8 and increased activation of primordial follicle pool when the culture was challenged with lipopolysaccharides (Bromfield and Sheldon 2013). In an older *in vitro* study, supplementation with human recombinant IL-1β reversed an induced neurodegeneration (Strijbos and Rothwell 1995).

In conclusion, this study shows that vitrification of bovine ovarian tissue using a protocol originally developed for larger tissue dimension can be used for smaller dimension although deleterious effects of exposure to cryoprotectants still remain a big challenge. This study also shows that *in vitro* culture of bovine ovarian tissue on agarose inserts maintained good follicle morphology, low follicle activation, and low apoptosis of both follicles and stromal cells vis-a-vis culture inserts. This strongly indicates that agarose as a physical support could have played a role in the activation delay of follicles and could therefore be an attenuating option for fragments intended for grafting. It may equally serve as a model to further understand the mechanism of folliculogenesis *in vitro*.

Our findings provide baseline information that may be utilized for further studies to understand the basic physiology of initiation and maintenance of primordial follicle growth necessary for fine tuning *in vitro* culture conditions of ovarian tissue. More studies must be carried out to understand mechanisms responsible for the observed effect of culturing ovarian fragments on agarose inserts. There is no doubt that the current study is just a tip of the iceberg regarding vitrification and *in vitro* culture of ovarian tissue.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Regione Lombardia PSR INNOVA n. 201801061529, PSR R-INNOVA n. 202102146691, Polish National Agency for Academic Exchange under Grant No. PPI/APM/2019/1/00044/U/00001, Università degli Studi di Milano "Piano di Sostegno alla Ricerca 2021 (Linea 2 Azione A)" and Erasmus mobility program. This study was also carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA

(PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Author contribution statement

Conceptualisation and study design was performed by IMA, MC, AML, WN, GCL while experiments and data curation by IMA and HAH; resources and funding acquisition by WN, PD, GCL; data analysis by IMA, MC; data discussion and drafting of the manuscript by IMA and MC; critical review of the manuscript, AML, WN, PD, and GCL; supervision, GCL, and AML.

Acknowledgements

The authors deeply appreciate the technical support of Aleksandra Piotrowska, Olga Rodak, Lobke DeBels and Osvaldo Bogado Pascottini.

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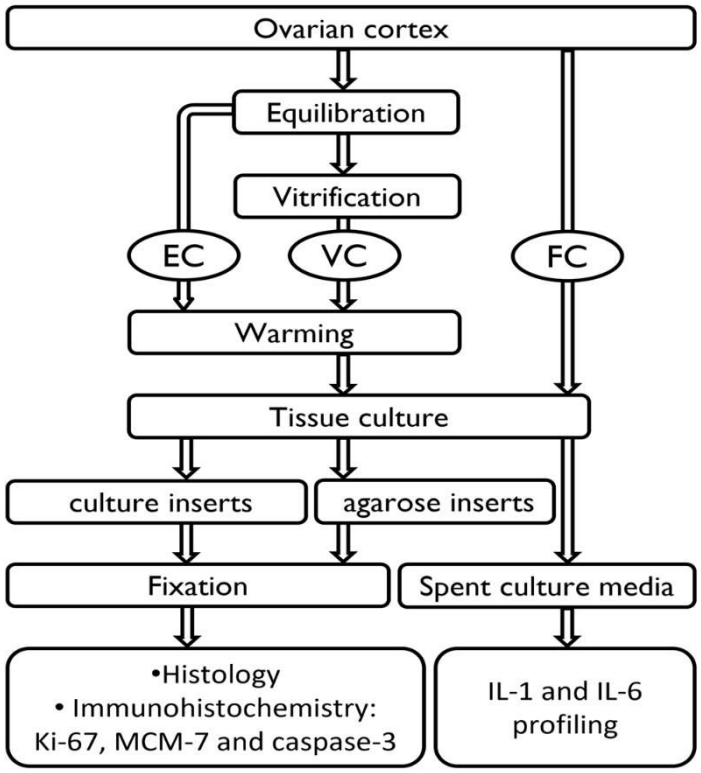
I



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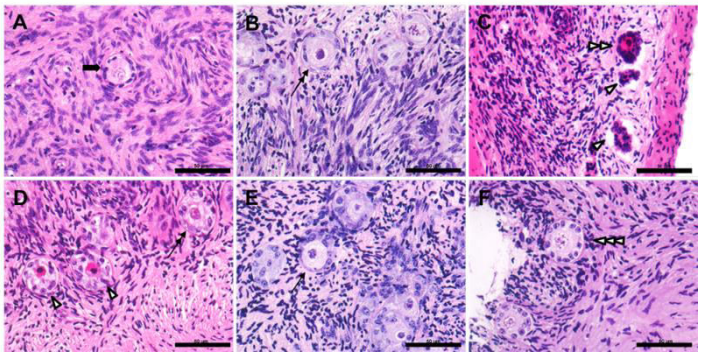

Figure 1. I; Experimental flow chart for bovine ovarian tissue vitrification, culture, and analysis; MCM-7: Minichromosome maintenance protein complex component-7; IL: Interleukin. II; Hematoxylin and eosin stained sections of bovine ovarian tissue fragments showing different follicle grading and classification. A, morphologically intact grade 1 primordial follicle showing spherical shape, evenly distributed follicular cells with intact stroma, spherical oocyte and intact nucleus and nucleolus (block arrow); B, grade 2 follicle showing spherical shape, evenly distributed follicular cells, intact stroma and spherical oocyte, misshapen nucleus (double head line arrow); C and D, grade 3 follicle (follicular cells pulled away from the stroma but oocyte spherical [double arrow heads]) and grade 4 follicle (follicular cells pulled away from the stroma and oocyte misshapen (arrowhead), vacuolated oocyte cytoplasm and/or pyknotic nucleus [arrowhead]) respectively; E, transitional follicle (line arrow) showing some the flattened follicular cells have been converted to cuboidal cells; F, primary follicle (triple arrowheads) showing all cuboidal follicular cells. Scale bar = 50 µm.

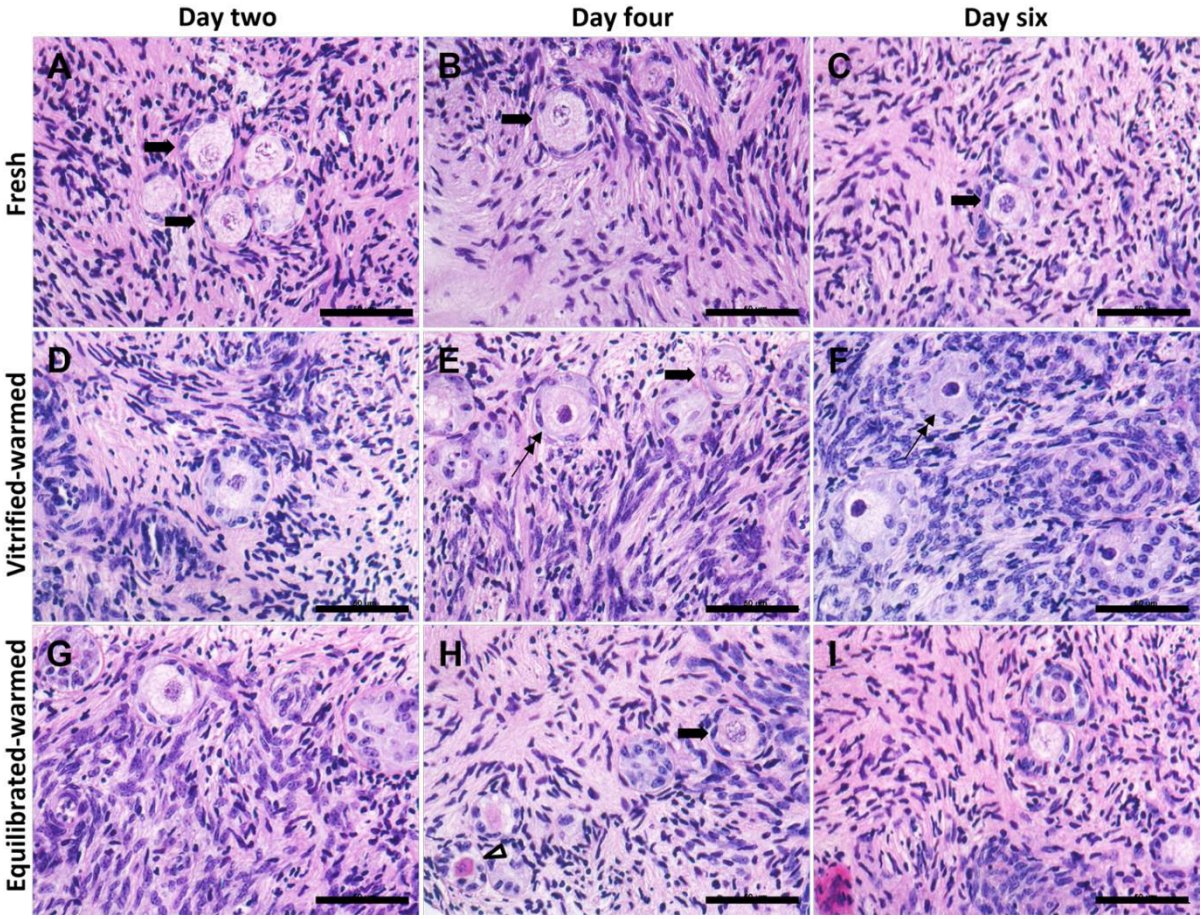

Figure 2. Bovine ovarian tissue fragments from all experimental groups and different culture periods sectioned (5 µm) and stained with hematoxylin and eosin. Fresh: A, B and C (day 2, 4 and 6 of culture respectively); Vitrified-warmed: D, E and F (day 2, 4 and 6 of culture respectively); Equilibrated-warmed: G, H, and I (day 2, 4 and 6 of culture respectively). Grade 1 follicles are indicated by the block arrows and double-head line arrows indicate grade 2, while arrowhead indicates grade 4 follicle. Scale bar = 50 µm.

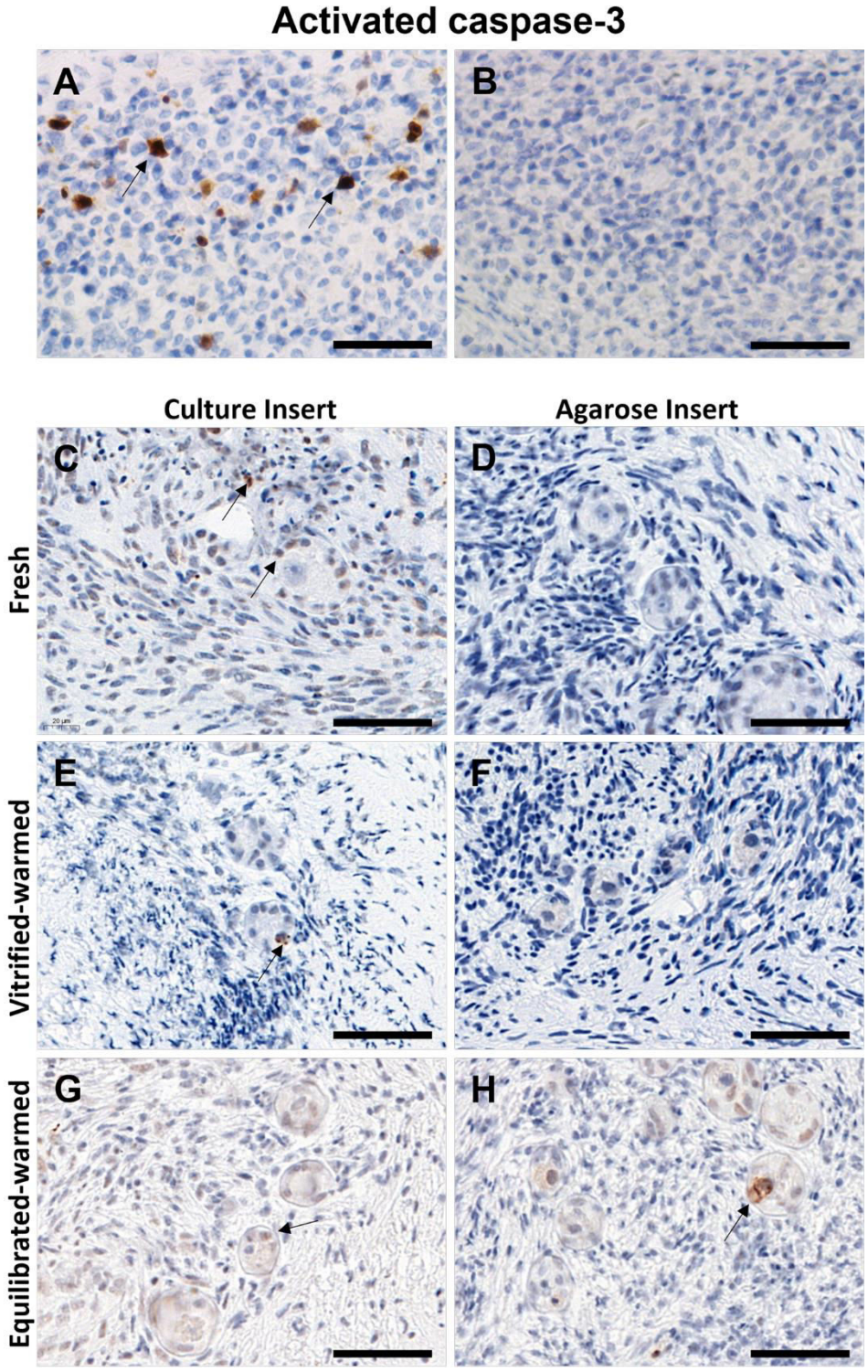

Figure 3. Activated caspase-3 immunohistochemical sections of bovine ovarian tissue fragments taken from the sixth day of culture. A and B represent the positive and negative controls for activated caspase-3 (bovine tonsil showing brown DAB (diaminobenzidine) positive germinal center cells (line arrows) in the positive control and DAB negative germinal center cells in the negative control); C, E and G represent the different treatments on agarose inserts while D, F and H represent the different treatments on culture inserts. Immunopositive nuclei are stained brown (line arrows). Scale bar = 50 µm.

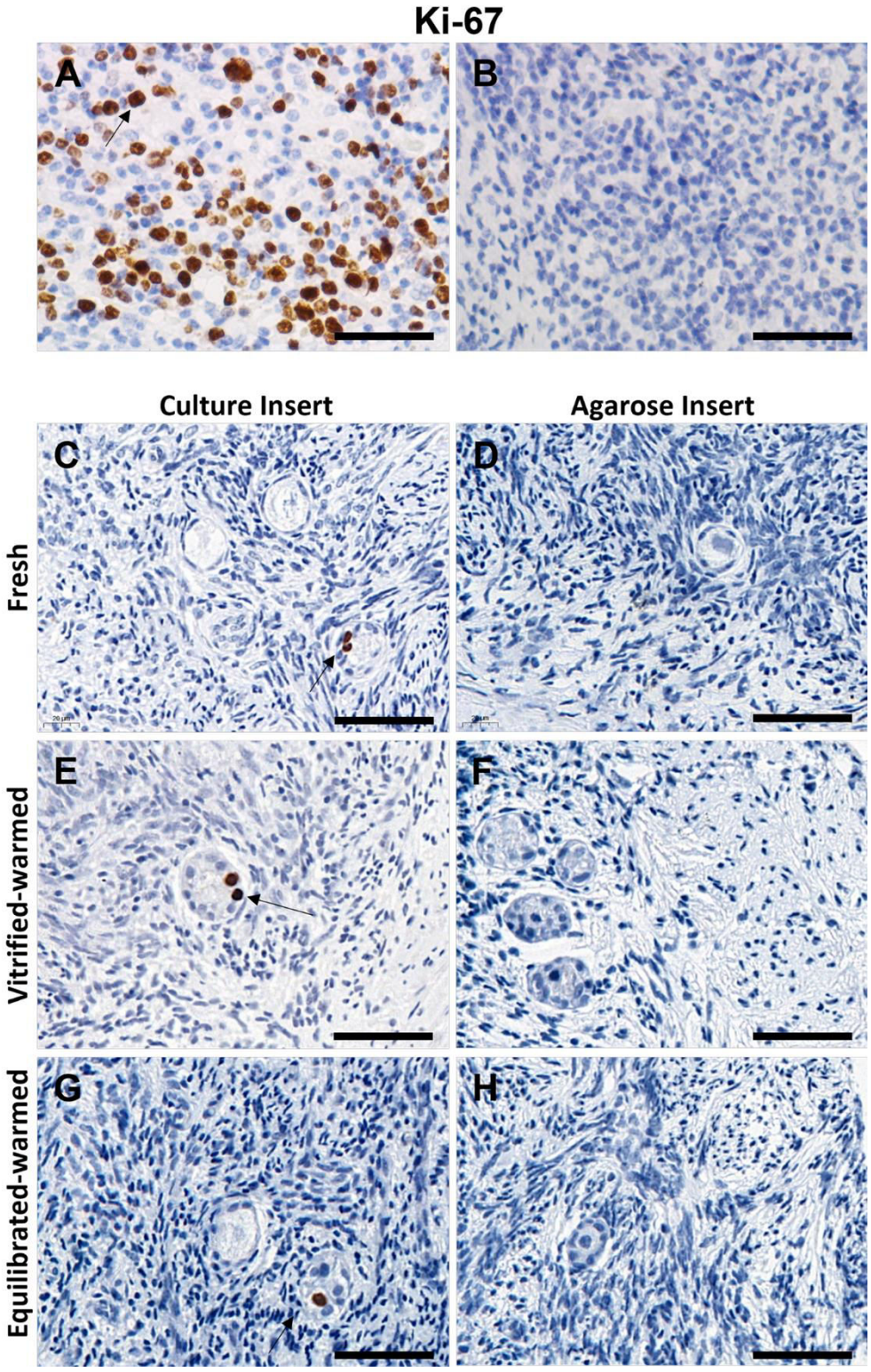

Figure 4. Ki-67 immunohistochemical sections of bovine ovarian tissue fragments taken from the sixth day of culture. A and B represent the positive and negative controls for Ki-67 (bovine tonsil showing brown DAB positive germinal center cells (line arrows) in the positive control and DAB negative germinal center cells in the negative control); C, E and G represent the different treatments on agarose inserts while D, F and H represent the different treatments on culture inserts. Immunopositive nuclei are stained brown (line arrows). Scale bar = 50 µm.

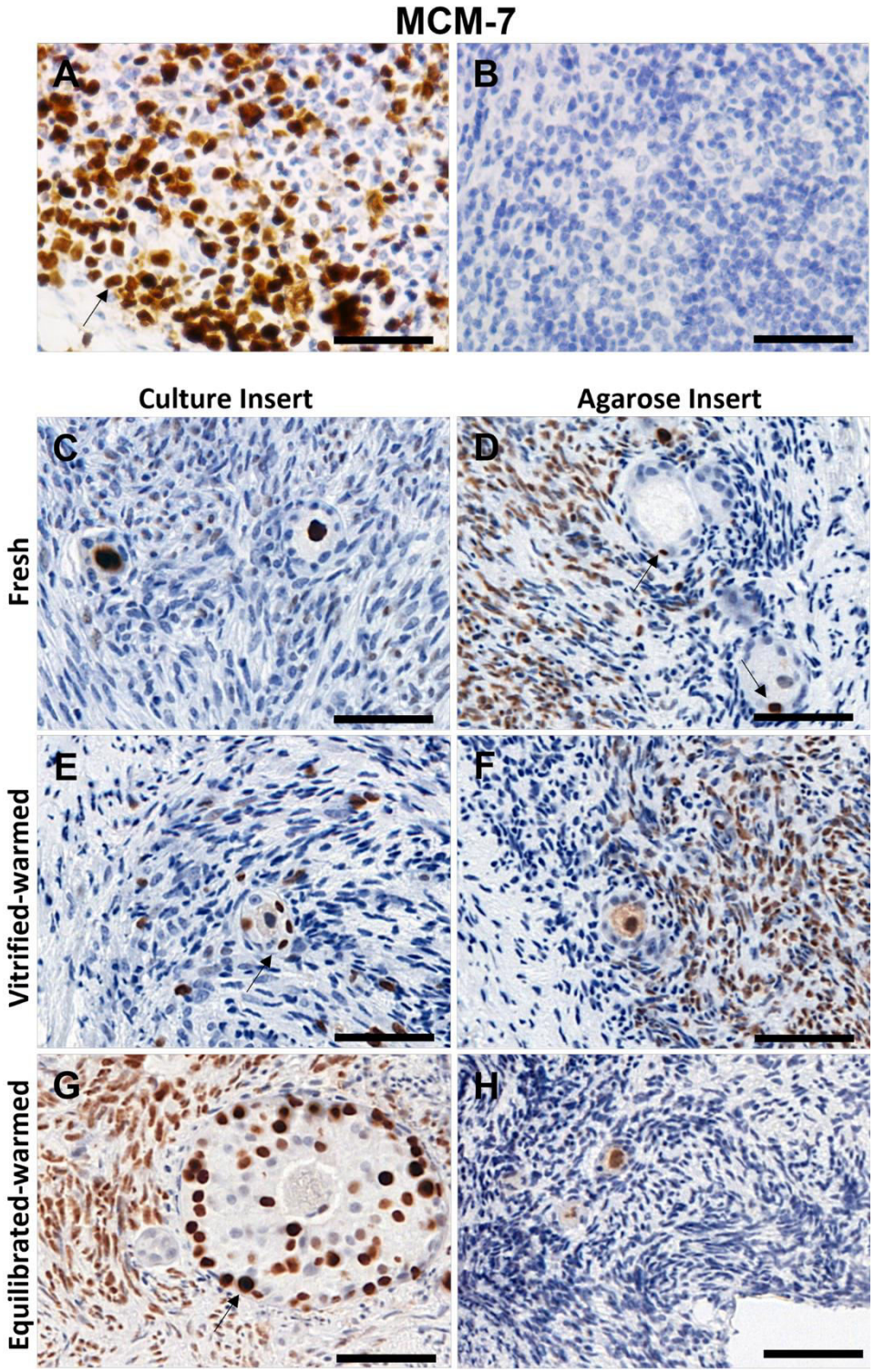

Figure 5. Minichromosome maintenance protein complex component-7 (MCM-7) immunohistochemical sections of bovine ovarian tissue fragments taken from the sixth day of culture. A and B represent the positive and negative controls for MCM-7 (bovine tonsil showing brown DAB positive germinal center cells (line arrows) in the positive control and DAB negative germinal center cells in the negative control); C, E and G represent different treatments on agarose inserts while D, F and H represent different treatments on culture inserts. Immunopositive nuclei are stained brown (line arrows). Scale bar = 50 µm.

Table Legends

Table 1: Follicle grading after different treatments of bovine ovarian tissue fragments.

Percentages (%) of the total number of follicles (n) counted in the six biological replicates. Values in the same column followed by a common superscript (a, b, c, d or e) are not significantly different (P>0.05). FC: Fresh cultured; FCA: Fresh cultured on agarose; VC: vitrified cultured; VCA: vitrified cultured on agarose; EC: equilibrated cultured; ECA: equilibrated cultured on agarose.

Table 2: Follicle classification after different treatments of bovine ovarian tissue fragments.

Percentages (%) of the total number of follicles (n) counted in the six biological replicates. Values in the same column followed by a common superscript (a, b, c, d or e) are not significantly different (P>0.05). FC: Fresh cultured; FCA: Fresh cultured on agarose; VC: vitrified cultured; VCA: vitrified cultured on agarose; EC: equilibrated cultured; ECA: equilibrated cultured on agarose.

Table 3: Stromal cells of bovine ovarian tissue fragments after different treatments.

The data in the column "Stromal cell density" are presented as mean ± standard deviation while data in the column "Caspase-3 positive" are percentages (%) of the stromal cells density counted in the five biological replicates. Values in the same column followed by a common superscript (a, b, c, or d) are not significantly different (P>0.05). FC: Fresh cultured; FCA: Fresh cultured on agarose; VC: vitrified cultured; VCA: vitrified cultured on agarose; EC: equilibrated cultured; ECA: equilibrated cultured on agarose.

Table 4: Immunohistochemistry of bovine ovarian tissue fragments after different treatments.

Percentages (%) of the total number of follicles (n) counted in the six biological replicates. Values in the same column followed by a common superscript (a, b, c, or d) are not significantly different (P>0.05). FC: Fresh cultured; FCA: Fresh cultured on agarose; VC: vitrified cultured; VCA: vitrified cultured on agarose; EC: equilibrated cultured; ECA: equilibrated cultured on agarose.

Table 5: Cytokine profile in spent media of bovine ovarian tissue fragments after culture.

Mean ± standard deviation obtained from the six biological replicates. Values in the same column followed by a common superscript (a or b) are not significantly different (P>0.05). FC: Fresh cultured; FCA: Fresh cultured on agarose; VC: vitrified cultured; VCA: vitrified cultured on agarose; EC: equilibrated cultured; ECA: equilibrated cultured on agarose.

Table 1

Groups	**Culture period**														
	Day two					Day four					Day six				
	%				n	%				n	%				n
Follicle grades (Gr)	Gr1	Gr2	Gr3	Gr4	Total	Gr1	Gr2	Gr3	Gr4	Total	Gr1	Gr2	Gr3	Gr4	Total
FC	6.6^{a,b}	72.9^{a}	2.2^{a}	18.2^{a}	362	12.0^{a}	65.7^{a}	3.9^{a,b,c}	18.3^{a}	382	12.5^{a}	62.5^{a}	1.0^{a}	24.0^{a}	208
FCA	33.6^{c}	34.4^{b}	8.1^{b}	23.9^{a}	259	15.5^{a}	36.0^{b,e}	1.9^{a,d}	46.7^{b}	317	8.4^{a,b}	37.4^{b}	2.8^{a,b}	50.9^{b}	214
VC	4.4^{a,b}	22.5^{c}	1.7^{a}	71.5^{b}	298	0.4^{b}	11.7^{c}	2.5^{a,b,d}	85.0^{c}	240	3.4^{b,c}	17.1^{c}	5.1^{b}	68.6^{c}	175
VCA	8.0^{b,d}	43.8^{d}	10.8^{b}	36.9^{c}	249	3.5^{c}	35.7^{b,e}	5.3^{b,c}	55.5^{d}	227	0.0^{d}	27.4^{d}	11.2^{c}	69.3^{c}	241
EC	3.7^{a}	29.0^{b,c}	3.0^{a,c}	64.3^{b}	297	2.5^{b,c}	30.2^{b,d}	1.3^{d}	66.0^{e}	318	6.4^{b,e}	23.6^{c,d}	2.5^{a,b}	67.5^{c}	203
ECA	13.2^{d}	35.5^{b,d}	6.8^{b,c}	44.5^{c}	220	9.4^{a}	43.4^{e}	6.9^{c}	40.3^{b}	159	2.9^{c,e}	25.6^{d}	6.5^{b,c}	64.9^{c}	308

Table 2

Groups	**Culture period**											
	Day two				Day four				Day six			
	%			n	%			n	%			n
	Primordial	Transitional	Primary	Total	Primordial	Transitional	Primary	Total	Primordial	Transitional	Primary	Total
FC	43.9[a]	37.3[a]	18.8[a]	362	36.4[a]	46.9[a,b]	16.8[a]	382	35.6[a]	46.2[a]	17.8[a,b,c]	208
FCA	90.0[b]	8.5[b]	1.9[b]	259	55.8[b]	27.8[c]	16.4[a]	317	37.9[b]	36.9[a,b,c]	25.2[c]	214
VC	53.4[c]	31.9[a]	14.8[a,c]	298	37.1[a]	40.8[a]	19.2[a]	240	37.7[a,b]	46.3[a]	16.0[a,b]	175
VCA	82.3[d]	13.7[b,c]	4.4[b]	249	60.4[b]	23.3[c]	16.3[a]	227	54.4[c]	29.5[c]	12.4[a]	241
EC	38.7[a]	30.6[a]	37.4[d]	297	21.7[c]	49.7[b]	28.6[b]	318	36.9[a,b]	41.9[a,b]	21.2[b,c]	203
ECA	74.1[e]	16.4[c]	9.5[c]	220	72.3[d]	22.6[c]	5.0[c]	159	45.8[b]	35.1[b,c]	19.2[a,b,c]	308

Table 3

Groups	**Culture period**					
	Day two		Day four		Day six	
	Cells/5000µm^2	%	Cells/5000µm^2	%	Cells/5000µm^2	%
Stroma	Stromal cell density	Caspase-3 positive	Stromal cell density	Caspase-3 positive	Stromal cell density	Caspase-3 positive
FC	71.2 ± 19.5	21.3[a]	97.6 ± 14.5	10.2[a]	79.4 ± 15.2	33.5[a]
FCA	95.8 ± 10.9	12.7[b]	105.4 ± 23.9	5.7[b]	89.0 ± 29.5	5.2[b]
VC	87.4 ± 21.0	8.2[c,d]	81.8 ± 20.0	9.8[a,c]	86.6 ± 27.0	9.2[c]
VCA	88.2 ± 27.5	6.3[c]	74.4 ± 15.7	4.3[b]	99.6 ± 19.4	3.6[b]
EC	96.4 ± 22.9	11.0[d]	94.2 ± 33.2	4.2[b]	98.0 ± 34.6	21.4[d]
ECA	106.6 ± 26.5	7.5[c,d]	110.0 ± 28.9	10.5[c]	90.4 ± 23.0	5.5[b]

Table 4

Groups	**Culture period**								
	Day two %(n)			Day four %(n)			Day six %(n)		
	Caspase-3	Ki-67	MCM-7	Caspase-3	Ki-67	MCM-7	Caspase-3	Ki-67	MCM-7
FC	63.4(123)^{a}	15.3(111)^{a,b}	32.4(108)^{a}	69.6(125)^{a}	62.9(124)^{a}	76.4(106)^{a}	76.6(167)^{a}	50.6(109)^{a}	74.5(98)^{a}
FCA	31.6(76)^{b,c}	2.8(71)^{c}	16.3(43)^{a}	50.4(133)^{b,c}	4.7(127)^{b}	35.0(58)^{b}	64.1(78)^{b,c,d}	29.0(131)^{b}	40.2(97)^{b}
VC	61.9(118)^{a}	33.5(179)^{d}	79.5(127)^{b}	64.5(93)^{a,c}	61.2(196)^{c}	87.3(79)^{a}	69.5(118)^{a,b}	57.7(78)^{a,b}	86.0(100)^{c}
VCA	34.8(112)^{b,c}	2.1(97)^{a,b}	10.8(37)^{a}	34.9(43)^{b,d}	0.0(51)^{b}	7.7(52)^{c}	26.8(97)^{e}	2.4(83)^{c}	34.6(78)^{a,b,c}
EC	52.0(152)^{a,b}	18.8(133)^{a}	60.7(107)^{c}	51.0(145)^{b}	48.6(109)^{d}	74.7(95)^{a}	88.6(123)^{c}	45.0(100)^{a,b}	65.0(60)^{a,b}
ECA	37.6(85)^{c}	6.4(78)^{b,c}	22.0 (50)^{a}	23.9(46)^{d}	4.5(44)^{b}	15.6(32)^{b,c}	54.3(140)^{d}	13.3(105)^{d}	50.8(65)^{a,b}

Table 5

Groups	**Culture period**					
	Day two		Day four		Day six	
	pg/mL	ng/mL	pg/mL	ng/mL	pg/mL	ng/mL
Cytokine	Interleukin 1β	Interleukin 6	Interleukin 1β	Interleukin 6	Interleukin 1β	Interleukin 6
FC	35.7 ± 12.8	171.4 ± 290.4	31.5 ± 12.5[a,b]	82.1 ± 138.3	32.2 ± 16.7	15.8 ± 25.1
FCA	48.2 ± 10.2	8.9 ± 15.2	57.2 ± 25.5[a,b]	116.8 ± 200.3	55.7 ± 17.3	33.5 ± 57.3
VC	31.7 ± 11.9	26.0 ± 44.1	30.8 ± 14.2[a,b]	4.2 ± 7.0	35.8 ± 12.1	7.6 ± 12.9
VCA	49.5 ± 15.7	2.8 ± 4.8	46.8 ± 20.9[a,b]	4.9 ± 8.3	42.1 ± 21.0	23.5 ± 40.4
EC	43.3 ± 18.0	84.5 ± 145.1	21.7 ± 14.3[a]	4.8 ± 8.1	50.2 ± 20.2	13.3 ± 22.4
ECA	46.3 ± 17.8	7.2 ± 12.2	58.9 ± 16.6[b]	7.9 ± 13.4	47.9 ± 29.9	37.1 ± 63.6