

# The Challenge of Ovarian Tissue Culture: 2D Versus 3D Culture

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## Research

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# Abstract

## Background

Cryopreservation of ovarian tissue is a powerful technique for preserving female fertility, as it can restore fertility and endocrine function. To increase the longevity of the transplant and decrease the risk of reimplantation of neoplastic cells, several studies have been carried out with the culture of ovarian tissue. The aim of this study was to compare a conventional (2D) culture with an alginate matrix three-dimensional (3D) model for ovarian tissue culture.

## Results

The ovarian tissue culture within the alginate matrix (3D) was similar to 2D culture, regarding follicular density, follicular cell proliferation and cell apoptosis in follicles and stroma. Stromal cells proliferation was kept stable in conventional culture but decreased in 3D culture ( $p = 0.001$ ). At 24 hours of culture, cytotoxicity was lower in the 3D model ( $p = 0.006$ ). As culture time increased, cytotoxicity seemed to be similar. Degradation of the tissue was suggested by the histological score analysis of tissue morphology during 72 hours of culture. Tissue injury was greater ( $p = 0.01$ ) in 3D culture due to higher interstitial oedema ( $p = 0.017$ ) and tissue necrosis ( $p = 0.035$ ).

## Conclusion

According to our results 3D culture of ovarian tissue has no advantage over 2D culture being more time consuming, difficult to perform and having worse reproducibility.

## 1. Background

Over the past decade, the cancer incidence was stable in women, with an overall decline in cancer mortality(1). This improvement in cancer survival is closely related to better oncological treatments that are potentially gonadotoxic, leading to premature ovarian failure in reproductive age patients and, consequently, infertility. Several approaches can be proposed to cancer patients, such as immature/mature oocyte cryopreservation, embryo cryopreservation and ovarian tissue cryopreservation (OTC). Fertility preservation through OTC is a powerful technique for preserving female reproductive potential; it can preserve thousands of ovarian follicles at one time and simultaneously restore endocrine function and fertility, allowing spontaneous conception(2, 3) and is the unique option for prepubertal girls and women who cannot delay the start of oncological treatments(4). According to American Society for Reproductive Medicine (ASRM), this approach is no longer considered experimental(3). However, there are some concerns about the graft's survival after transplantation and the potential risk of implantation of tumour cells(2, 3). Therefore, many studies are underway to overcome these limitations and new and experimental techniques have been developed, such as *in vitro* maturation.

The ovary morpho-functional unit is composed by an oocyte surrounded by somatic granulosa and theca cells. Basal folliculogenesis, as a complex process, involving a dialogue between the oocyte and its closely surrounding cells, through autocrine and paracrine bidirectional signalling(5, 6). Due to this cross talk, the maintenance of the follicle three-dimensionality during the whole growth process is crucial for the correct acquisition of the developmental competence(5). Though, recent research on *in vitro* follicle culture has shifted from two dimensional (2D) toward the use of three-dimensional (3D) structures(5). The use of a matrix maintains the follicle 3D architecture and mimics *in vivo* conditions, with a variable access to oxygen and nutrients(5, 7). This contributes to bridge the gap between conventional cell culture to animal models(8).

Historically, ovarian research is focused on folliculogenesis, but recently the ovarian stroma has become an exciting new field for research, holding critical keys to understanding complex ovarian dynamics(9). Activation of primordial follicles involve signalling pathways that reach the follicle through microvascularization present in ovarian cortex(6). Additionally, stromal tissue also plays an important role for the continued growth of follicles, a vascularized theca differentiates by recruitment of progenitor cells present in the ovarian cortex immediately adjacent to the follicle(6, 10). Therefore, the culture of fragments of ovarian tissue maintain the integrity and three-dimensional structure of the follicles supporting stromal tissue(10).

By culturing organs in pieces, several cultures from a single organ can be generated, increasing the number of experiments from a single animal(11). Culture of these tissue types in alginate hydrogels provides mechanical support to maintain the three dimensional architecture of the tissue, while the gel itself does not interact with the cells(11).

Alginate offers several advantages over other types of matrices. It is a biocompatible and bioactive natural matrix that floats in standard cell culture medium and provides mechanical support for growing tissues(11, 12).

The aim of this study was to compare a conventional 2D culture with an alginate matrix scaffold for ovarian tissue culture optimization.

## 2. Results

### 2.1. Tissue morphology and viability

The comparison of histopathologic scores is shown in Fig. 1. During the study period, degradation of tissue morphology was observed, with a significant increase in the score of all parameters.

According to Fig. 1.A., the increase of tissue necrosis score (TNS) is greater in the 3D culture ( $p < 0.001$ ,  $B = 0.033$ ,  $R^2_{adj} = 0.663$ ) than for 2D ( $p < 0.001$ ,  $B = 0.023$ ,  $R^2_{adj} = 0.381$ ). At 72 hours of culture, specimens of the ovary cultured within the alginate matrix (3D) showed higher scores for tissue necrosis (2D =  $1.700 \pm 0.291$  vs. 3D =  $2.550 \pm 0.138$ ,  $p = 0.017$ ).

The proportion of the degenerated follicles increased over time, identically in the two models (2D,  $p < 0.001$ ,  $B = 0.028$ ,  $R^2_{\text{adj}} = 0.617$ ; 3D,  $p < 0.001$ ,  $B = 0.031$ ,  $R^2_{\text{adj}} = 0.696$ ; Fig. 1.B.).

For the score of interstitial oedema (Fig. 1.C.), it was observed a greater increase in the 3D culture (2D,  $p = 0.001$ ,  $B = 0.018$ ,  $R^2_{\text{adj}} = 0.253$ ; 3D,  $p < 0.001$ ,  $B = 0.024$ ,  $R^2_{\text{adj}} = 0.428$ ). At 72 hours of culture, interstitial oedema was more evident in the 3D group compared to 2D (2D =  $2.000 \pm 0.224$  vs. 3D =  $2.650 \pm 0.130$ ,  $p = 0.035$ ).

The total histopathologic score (HS), which is the total score of three ovarian injury parameters, increased in both groups but more sharply in 3D culture (2D,  $p < 0.001$ ,  $B = 0.069$ ,  $R^2_{\text{adj}} = 0.477$ ; 3D,  $p < 0.001$ ,  $B = 0.088$ ,  $R^2_{\text{adj}} = 0.657$ ; Fig. 1.D.). Also, after 72 hours of culture, the total score was significantly higher in 3D culture than 2D culture (2D =  $6.200 \pm 0.569$  vs. 3D =  $8.050 \pm 0.302$ ,  $p = 0.01$ ).

During the study period, there was no changes in the amount of lactate dehydrogenase (LDH) released into the supernatant (Fig. 2.). The amount of LDH released was lower in culture with the alginate matrix than in conventional culture ( $1.16 \pm 0.10$  vs.  $1.66 \pm 0.11$ ,  $p = 0.006$ ), after 24 hours. However, no differences were found at 48 and 72 hours of culture.

## 2.2. Follicular analysis

From the histological evaluation, we observed that density of morphological normal follicles was maintained during the 72 hours of culture (Fig. 3.A.). However, the density of atretic follicles increased significantly in both groups (2D,  $p = 0.027$ ,  $B = 0.007$ ,  $R^2_{\text{adj}} = 0.102$ ; 3D,  $p < 0.001$ ,  $B = 0.009$ ,  $R^2_{\text{adj}} = 0.412$ ; Fig. 3.B.).

Regarding follicular classification, there was a significant decrease of primordial follicles in both types of culture (2D,  $p = 0.009$ ,  $B = -0.008$ ,  $R^2_{\text{adj}} = 0.169$ ; 3D,  $p = 0.036$ ,  $B = -0.006$ ,  $R^2_{\text{adj}} = 0.11$ ; Fig. 3.D.). The density of primary and secondary follicles remained during the 72 hours of culture. At 72 hours of culture, the density of primary follicles was significantly higher in the samples cultured with the alginate matrix (2D =  $0.138 \pm 0.064$  vs. 3D =  $0.318 \pm 0.063$  follicles/mm<sup>2</sup>,  $p = 0.035$ ; Fig. 3.E.).

## 2.3. Proliferation and apoptosis in ovarian tissue

The levels of apoptosis assessed through the percentage of follicles and stromal cells positive for Caspase 3 (AC-3), remained stable during the culture period; no significant differences were found (Fig. 4).

The percentage of proliferative follicles, assessed with Ki67 staining, remained stable during the culture period in both groups (Fig. 5.A.). However, the evaluation of proliferation in stromal cells revealed a significant decrease in the 3D culture ( $p = 0.001$ ,  $B = -0.003$ ,  $R^2_{\text{adj}} = 0.220$ ; Fig. 5.B.).

## 3. Discussion

Cryopreservation of ovarian tissue has been considered a reliable possibility for fertility preservation in young women at the risk of iatrogenic premature ovarian failure, due to oncological treatments. This is the only option for prepubertal girls and women who cannot delay the beginning of chemotherapy or cannot undergo ovarian stimulation protocol for oocyte or embryo cryopreservation.

Since 1996, when the first mouse originated from an oocyte entirely grown *in vitro*(13), follicle growth has been envisaged as a promising technique. The possibility to achieve complete oocyte development *in vitro*, in primates and most domestic species, has boosted research in this field. Different options were evaluated in order to overcome technical difficulties and an *in vitro* system was developed for culturing small pieces of ovarian cortex, enriched in primordial follicles(14).

Many culture approaches have been developed for different animal models; however, the optimal model has not been achieved. More efforts are needed in order to establish a reliable model for tissue culture.

Our work aimed to evaluate some drawbacks concerning the culture of ovarian tissue in a three-dimensional (3D) model. In this model OT was cultured in alginate matrix, which has been widely used for the *in vitro* culture of isolated follicles in mouse(15, 16) and rat(17). The biochemical properties of this matrix make it a good candidate for 3D models. It allows the bidirectional diffusion of hormones and other proteins that are essential for the tissue survival and also follicular development(18).

The histopathological evaluation of ovarian tissue (Fig. 1) elicited a global deterioration of tissue viability, and this decrease is time dependent. Tissue deterioration was observed in both 2D and 3D models, which is in accordance with other previously published work that report no difference in the cell viability, in the first days of culture in the 2D and 3D models(19). However, after 72h there is a significant decrease in the tissue viability in the 3D model. A decrease in tissue viability when the culture time is prolonged was previously described and a possible explanation for this is that the organ structure might retain the cellular waste produced, possibly inducing lack of oxygen and nutrients supply(20).

Retention in the matrix may explain the results obtained in the evaluation of cytotoxicity. Cell death and cytoplasmic membrane rupture releases LDH into the incubation medium. Alginate matrix has higher porosity, allowing the diffusion of small substrates (molecular weight (MW) < 20 kDa) at the same speed as water(21). However, bigger proteins, such albumin (MW = 69 kDa), have a lower diffusion. The high MW of LDH (140 kDa)(22) may retain it longer in the spheroid structure. Therefore, the lower cytotoxicity in 3D model at 24 hours of culture may occur due to LDH retention in the alginate matrix.

In order to establish a more detailed histopathological evaluation we developed a score, based in the evaluation of three histologic parameters: percentage of tissue in necrosis (TNS), interstitial oedema and follicular cell degeneration. TNS and interstitial oedema are close related parameters. Necrosis is characterized as an unprogrammed cell death process, which begins with cell swelling, resulting in cell membrane rupture and release of cell cytoplasmic content into the extracellular space (23), creating an extracellular movement of fluids. Our data show an increase in TNS and in interstitial oedema along experimental time, in both models. However, after 72h the increase in both parameters was greater in 3D

culture. In the interior of the 3D structure, the bioavailability of oxygen and nutrients may be limited, affecting cell survival over time and conditioning higher level of necrosis and release of intracellular content when compared to more superficial cells. In our characterization of tissue degeneration in *in vitro* culture models, we detected an abundant level of follicular degeneration (Fig. 1.B.), and no differences were observed in 2D versus 3D model. A more detailed analysis reveals an increase in follicular atresia (Fig. 3.B.) accompanied by a decreased in primordial follicles (Fig. 3.D.). Our results are in line with previous work, which had shown a time related increase of follicular atresia, in culture(24).

The biocompatibility of alginate matrix was intensively investigated *in vitro* and *in vivo* models, and it was described as not inducing an immune response(25), first by the absence of cell receptors to recognize alginate and second due the high purity commercialized alginate. This compound is extracted from brown algae, and may contain various impurities such as heavy metals, endotoxins, proteins, and polyphenolic compounds (26); however multi-step purification ensure the alginate high purity. Despite the safety of this compound, its widely use has been challenged by some drawback concerning the molecular size, charges and viscosity of alginate, different culture models, different implantation sites and different animal models employed(25).

A different proliferation rate is observed when 2D and 3D models are compared. As shown in the Fig. 5.B., the proliferation rate of stromal cells is decreased in the 3D model. The difference in the proliferation rates between 2D and 3D models was previously described; cell lines cultured in 3D system showed a reduced proliferation when compared with 2D model, and these effects is dependent of 3D model employed, more specifically is matrix dependent, and also dependent of specific cells properties(20). In the fields of fertility preservation, this effect was also reported in follicular *in vitro* culture, some authors have described that the physical properties of alginate matrices can limit the growth and development of earlier secondary follicles(16). However, this topic is very controversial, and it is continuously under review. Different reasons have been raised to explain these findings. In the context of our work, the rigidity of alginate matrix can be a possible explanation for the decrease in proliferation rate. Softer matrices with lower alginate concentrations(16) or the combination of different elements in the matrix to allow more space and the diffusion of macromolecules responsible for the tissue survival and growth were proposed.

Additionally, we evaluated the cellular death, using a caspase - 3 antibody, as a marker for programmed cell death. Caspase-3 is a well characterized protease, which plays an effective role in apoptosis(27, 28). The caspase-3 expression was already studied in granulosa(29) and teca cells(30) and also in oocytes(24). In our study, apoptosis was evaluated in stromal cells and follicles, the level of apoptosis in stromal cells and follicles was stable during the experimental time with no difference between conditions (2D vs. 3D model) (Fig. 4.). However, it is important to keep in mind that the apoptosis is a central process in the ovarian function and development, occurring since the foetal life, which reach mainly the oocyte, until adult life affecting the granulosa cells in growing follicles(27, 31).

In this study, we have performed a well-structured histopathological analyse and the major strengths of our study are the sample size and the duplicate and blind analysis of results. However, some points could be addressed in future research, to step up 3d model, namely the study of the tissue-matrix interactions and culture medium supplementation to decrease follicular atresia. Since the 3D model could be a reliable system for delivering drugs or molecules in a closed system.

## **4. Conclusions**

In conclusion, the ovarian tissue culture within an alginate matrix was similar to 2D culture, regarding follicular density, follicular cell proliferation and cell apoptosis in follicles and stroma. In 3D culture greater levels of tissue injury and oedema and lower stromal cell proliferation were seen. Therefore, there is no clear advantage in the 3D culture of ovarian tissue, as it is more time consuming, difficult to perform and less reproducible.

## **5. Methods**

### **5.1 Ethical statement**

The present study was approved by the Ethic Committee for Animal Experimentation (ORBEA Authorization number 11060495/23-11-2016) of the Faculty of Medicine of the University of Coimbra and performed according to the European Guidelines and Portuguese Law. The Guide for the Care and Use of Laboratory Animals of the National Institutes of Health was followed to take care of the animals and the ARRIVE guidelines to perform and report the experimental protocols.

### **5.2 Study design**

The experimental scheme is shown in Figure 6. Briefly ovarian tissue (OT) preparation, cryopreservation, 2D system or encapsulated model procedure and timescale for the experiment are shown.

### **5.3 Experimental animals**

The animal facility of the Faculty of Medicine of the University of Coimbra provided twelve female Rowet nude rats (RNU, homozygous) with 8-10 weeks age and an average weight of 200 g. Housing, under a 12 hours light/dark cycle, was carried in individually ventilated cages, with access to standard diet and filtered water ad libitum. Animals were distributed in a randomized manner through the study groups described below.

### **5.4 Ovaries collection and cryopreservation**

Surgical procedures were performed under inhalation anaesthesia with sevoflurane (5%) and with subcutaneous analgesia with carprofen (5mg/kg, making a total of 0.2 mL per animal).

For bilateral ovariectomy, animals were placed in a supine position and the abdominal wall was shaved, cleaned and sterilized with povidon-iodine solution. A longitudinal median laparotomy was performed with a 2 to 3 cm incision in the lower area of the abdomen. The ovaries were identified and removed with the ligation of the vascular pedicle(32,33). After ensuring adequate haemostasis, the abdominal cavity was closed in layers.

After ovariectomy, the ovaries were placed in cold Dulbecco's phosphatase-buffered solution (DPBS) (Biological Industries, Sartorius, Gottingen, Germany) supplemented with 10% foetal bovine serum (FBS) (Biological Industries, Sartorius, Gottingen, Germany) and each ovary was cut in 2 hemi-ovaries. The fragments were maintained in cold DPBS + 10% FBS before cryopreservation.

## **5.5 Cryopreservation Protocol**

The cryopreservation protocol consisted of a slow freezing and a rapid thawing method. The fragments were placed in a plastic cryovials (Nunc, Thermo Fisher) containing 1.5 mL of freezing media consisting in 1.5 M Ethyleneglycol (Sigma-Aldrich, St. Louis, Missouri, USA), 0.1% Sucrose (Merck, Darmstadt, Germany) and 10 mg/mL human albumin serum (HAS) (Grifols, Barcelona, Spain), and maintained in an ice bath. The cryovials were transferred to a rolling system for 30 min at 4°C to allow the cryoprotectant to enter the tissue. Then, cooling in a programmable freezer (Planner cryo 10 Series 2 Freezer) followed the subsequent protocol. The starting temperature was 0°C, and then it was slowly reduced to -9°C at a rate of -2°C/min. After a 5 min holding time at -9°C, manual seeding was performed, after then the cryovials were cooled to -40°C, at a rate -0,3°C/min, and the final step, a rapid decrease until -140 °C (-10°C/min). When the program was completed, cryovials were transferred into liquid nitrogen tank and stored until thawing.

In the experiment day, ovarian fragments were thawed. The vials were air-warmed for 30 sec and then immersed in a 37°C water bath for 5 min. The freezing media was removed at room temperature by stepwise dilution of freezing media in sequential thawing media stabilized at room temperature. Three culture dishes were filled with thawing medium I (0.75 M Ethyleneglycol + 0.25 M Sucrose in PBS + 10 mg/mL HAS), medium II (0.25 M Sucrose in PBS+ 10 mg/mL HAS), medium III (PBS + 10 mg/mL HAS). Ovarian fragments were transferred, using sterile forceps, into thawing medium I and stirred during 10 min at room temperature, afterwards, the same procedure was performed for thawing media II and III. Thawed tissue was transferred for PBS before culture.

## **5.6 Ovary culture**

Ovarian fragments were cultured in the growth media with and without encapsulation in an alginate matrix scaffold. The conventional culture was defined as 2D and the use of the scaffold as 3D. A 1.5% (w/v) solution of sodium alginate (Sigma-Aldrich, St. Louis, Missouri, USA) was prepared by mixing into sterile DPBS and heating to 37°C. To encapsulate the ovarian fragments, it was used the agarose (Invitrogen, California, USA) ring protocol adapted from Henry, 2015(34). Agarose rings were filled with a layer of alginate matrix, fragments were placed in the ring and covered with matrix. Cross-linking solution

(50 mM CaCl<sub>2</sub> + 140 mM NaCl) was added, allowing the solution to solidify into a gel around the ovarian organoid.

The gel-organoid was then placed in the growth media to be cultured for 24, 48 and 72 hours. The growth media consisted in  $\alpha$ -MEM (22561-021, ThermoFisher, Waltham, Massachusetts, USA), 10% FBS and 1/1000 penicillin/streptomycin (15140-122, Gibco, ThermoFisher, Waltham, Massachusetts, USA).

## 5.7 Histological Evaluation

Ovaries were fixed in 4% formaldehyde (Panreac Quimica Sau, Barcelona, Spain), embedded in paraffin, and sectioned serially at 5  $\mu$ m. Three sections per transplant were stained with haematoxylin and eosin (HE) for morphological analysis. The images were acquired on the Axio.Scan Z1 (Carl Zeiss), with a Plan-Apochromat 10x/0.8 lens, and photographed with the Zen 2 program blue edition (Carl Zeiss Microscopy GmbH, 2011). Histological analysis was performed blindly by a researcher, in two different times points, through the Image J software.

Follicles were quantified manually and, to avoid double counting, only follicles with a visible nucleus were taken into account. Only morphologically normal follicles were taken into consideration for further analysis. Follicles were then classified according to their maturity in primordial (constituted by a single layer of flattened granulosa cells), primary (they present a single layer of cuboid granulosa cells) and secondary (composed by two or more layers of granulosa cells around the oocyte)(35). The pre-antral and antral follicles were grouped with the secondary follicles. Follicular densities (number/mm<sup>2</sup>) were calculated after a manual surrounding of the cortical surface. Follicle atresia was assessed with morphologic criteria, such as, irregular shape, granulosa cell pyknosis, cytoplasmic contraction, presence of vacuoles and ooplasm eosinophilia(35).

## 5.8 Histological scoring system for *in vitro* experiences

The criteria for ovarian tissue viability was adapted from criteria previously described for ovarian tissue injury after *in vivo* experiences of ischemia/reperfusion(36–38). As shown in figure 7, histopathological examination of the tissue damage was performed in terms of three visual parameters, such as interstitial oedema, follicular cell degeneration and percentage of tissue in necrosis (TNS).

The follicles were histologically classified as degenerated, when they included cells with pyknotic nucleus, shrunken ooplasm and disorganized granulosa cells. Follicular degeneration score was calculated as a proportion of the degenerated follicles to the total number of follicles. The extent of overall necrosis within each ovary was quantified by a visual assessment of the percentage necrosis (TNS) for each specimen. Each parameter was scored using a scale ranging from 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe). Total scores were calculated according to these parameters. Ovary sections were double blinded analyzed.

## 5.9 Immunohistochemistry Evaluation

Immunohistochemistry assay was performed with the cell proliferation biomarker rabbit polyclonal antibody Ki-67 (1:300; PA5-19462, Thermofisher, Waltham, Massachusetts, USA) and cell apoptosis rabbit polyclonal antibody caspase-3, AC3, (1:100; AHP2286, Bio-Rad Laboratories, Hercules, California, USA). Antigen retrieval was performed with CC1, pH 8 EDTA/Tris-based buffer (Ventana Medical Systems, Tucson, Arizona, USA), followed by primary antibody incubation according to manufacturer procedures. Detection of immunostaining was performed with an indirect multimer based revelation system conjugated with Horseradish Peroxidase (HRP) (OptiView DAB IHC Detection Kit, Ventana Medical Systems, Tucson, Arizona, USA), revealed with chromogenic precipitated by DAB. All the immunostained sections were then nuclear counterstained with haematoxylin, dehydrated in a graded series of ethanol, cleared in xylene and mounted using a synthetic mounting medium.

After the immunohistochemistry protocol, in which the incubation with the cell proliferation indicator (Ki-67) and cell apoptosis (AC3), the images were acquired on the Axio.Scan Z1 (Carl Zeiss), with a Plan-Apochromat 10x/0.8 lens and photographed with the aid of the Zen 2 blue edition program (Carl Zeiss Microscopy GmbH, 2011). The results analysis was double and blinded.

Regarding the stroma, the quantification of the area marked with each antibody was performed using the Image J program (Fiji version, 1.8.0, USA). Five zones of each sample were randomly selected through the application of a rectangular grid and the results subsequently presented as an average. In addition, follicles were classified as positive or negative for Ki67 and AC3. For this, positivity was considered when staining was observed in the oocyte and/or in at least one granulosa cell(39,40).

### **5.10 LDH assay (Cytotoxicity Evaluation)**

To evaluate the tissue viability, the lactate dehydrogenase (LDH) released in the culture medium from damage cells was measured using CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega G1780, Madison, Wisconsin, USA)(41–43). The assay was performed according to manufacturer's protocol. Briefly, after 24, 48 and 72h of culture the supernatant of each condition was collected and store at -20°C. In the day of LDH assay, the CytoTox96® Reagent was prepared mixing the buffer assay with the Substrate Mix. To test the effects of 2D *versus* 3D culture, 50 µL of culture medium was transferred for a 96 multi-well flat clear bottom plate and mixed with 50 µL CytoTox96® Reagent and incubated for 30 minutes, protected from light. After then, 50 µL of Stop solution was added to each well, and the optical density at 490 nm was measured using an EnSpire microplate reader. The absorbance values from the conditioned media supernatant was normalized to mean absorbance values calculated from control samples.

### **5.11 Statistical analyses**

The number of ovaries to be used was estimated using the G\*Power software version 3.1.9.4 (Kiel, Germany). A comparison of results between the 7 described groups was considered, with a type I error of 0.05 and a statistical power of 0.90, for an effect size of 0.60. In this way, a total sample size of 66

fragments (9.4 per group) was estimated, with an effective power of 0.901. Therefore, it was used 10 hemi-ovaries per group.

Statistical analyses were performed using the SPSS version 22.0 (IBM, Armonk, New York, USA). Simple linear regression over time was performed using bootstrapping strategy (1000 samples), and the regression coefficient (B) was reported. Normality distribution was evaluated to compare the two groups of study (2D vs 3D). Normally distributed variables were compared by means of t-student test and non-normally by means of Mann-Whitney test. A p-value of less than 0.05 was considered statistically significant. The results are expressed as mean  $\pm$  standard error of the mean (SEM).

## List Of Abbreviations

2D, two-dimensional; 3D, three-dimensional; AC3, caspase 3; ASRM, American Society for Reproductive Medicine; DPBS, Dulbecco's phosphatase-buffered solution; FBS, foetal bovine serum; HAS, human albumin serum; HE, haematoxylin and eosin; HS, histopathologic score; LDH, lactate dehydrogenase; OT, ovarian tissue; OTC, ovarian tissue cryopreservation; SEM, standard error of the mean; TNS, tissue necrosis score.

## Declarations

**Ethics approval and consent to participate:** The present study was approved by the Ethic Committee for Animal Experimentation (ORBEA Authorization number 11060495/23-11-2016) of the Faculty of Medicine of the University of Coimbra and performed according to the European Guidelines and Portuguese Law.

**Consent for publication:** Not applicable

**Availability of data and materials:** Not applicable

**Competing interests:** none

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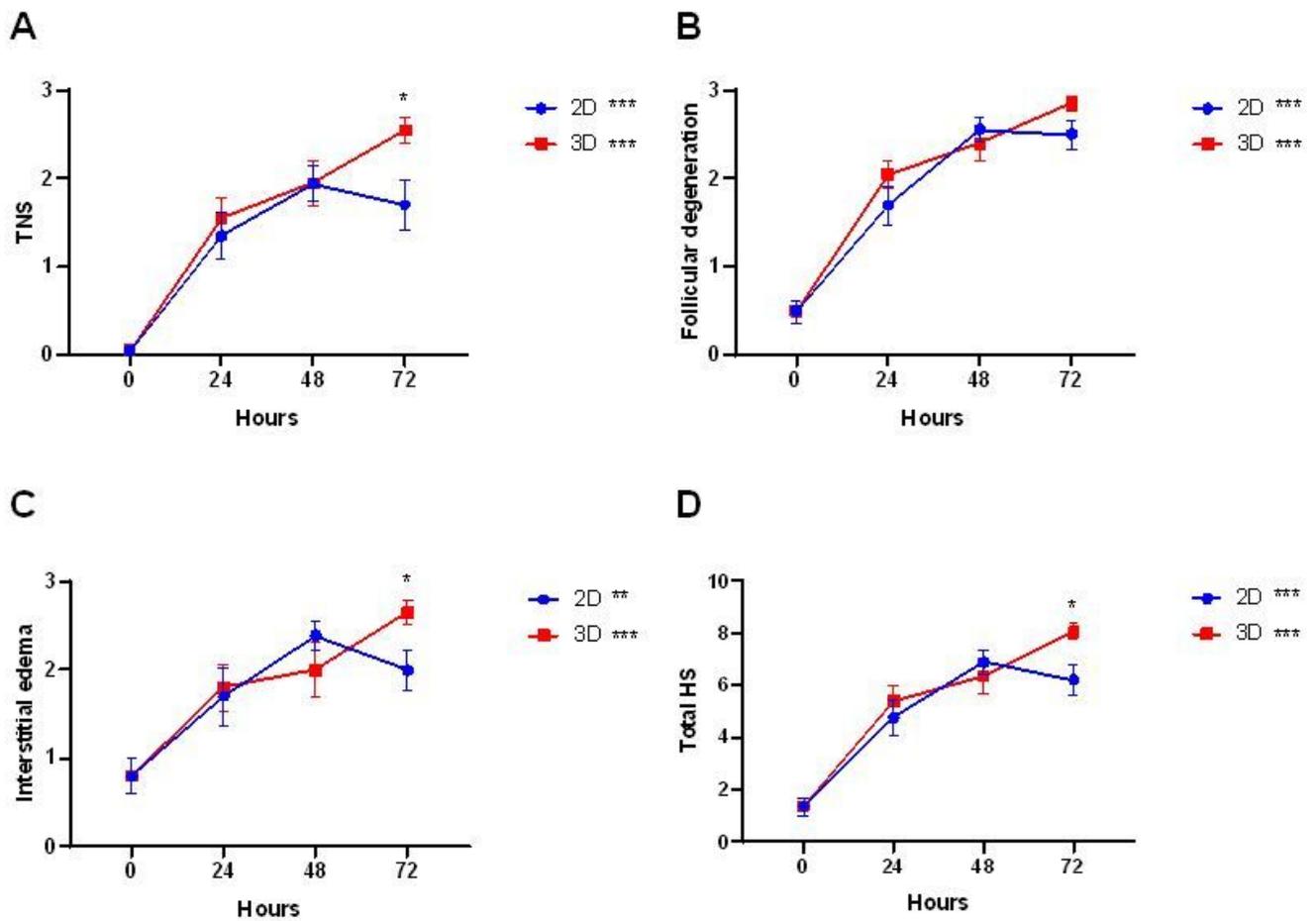
## References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin.* 2020;70(1):7–30.
2. Duncan FE, Feinberg E, Brannigan RE, Edmonds M, Ataman L, Woodruff TK. Fertility Preservation [Internet]. Eighth Edi. Yen & Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management: Eighth Edition. Elsevier Inc.; 2019. 857-886.e6 p. Available from: <https://doi.org/10.1016/B978-0-323-47912-7.00033-0>
3. Practice Committee of the American Society for Reproductive Medicine. Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion. *Fertil Steril* [Internet]. 2019;112(6):1022–33. Available from: <https://doi.org/10.1016/j.fertnstert.2019.09.013>
4. Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Obstet Gynecol.* 2005;105(1):214.
5. Belli M, Vigone G, Merico V, Redi CA, Zuccotti M, Garagna S. Towards a 3D culture of mouse ovarian follicles. *Int J Dev Biol.* 2012;56(10–12):931–7.
6. Monniaux D, Cadoret V, Clément F, Dalbies-Tran R, Elis S, Fabre S, et al. Folliculogenesis. *Encycl Endocr Dis.* 2018;377–98.
7. Kapałczyńska M, Kolenda T, Przybyła W, Zajączkowska M, Teresiak A, Filas V, et al. State of the art paper 2D and 3D cell cultures – a comparison of different types of cancer cell cultures. *Arch Med Sci.* 2018;14(4):910–9.
8. Jensen C, Teng Y. Is It Time to Start Transitioning From 2D to 3D Cell Culture? *Front Mol Biosci.* 2020;7(March):1–15.
9. Kinneer HM, Tomaszewski CE, Chang FL, Moravek MB, Xu M, Padmanabhan V, et al. The ovarian stroma as a new frontier. *Reproduction.* 2020;160(3):R25–39.
10. Scott J, Carlsson IB, Bavister BD, Hovatta O. Human ovarian tissue cultures : extracellular matrix composition , coating density and tissue dimensions. *Reprod Biomed Online* [Internet]. 2004;9(3):287–93. Available from: [http://dx.doi.org/10.1016/S1472-6483\(10\)62143-8](http://dx.doi.org/10.1016/S1472-6483(10)62143-8)
11. King SM, Quartuccio S, Hilliard TS, Inoue K, Burdette JE. Alginate Hydrogels for Three-Dimensional Organ Culture of Ovaries and Oviducts. *J Vis Exp.* 2011;52(June):4–9.
12. Jackson KS, Inoue K, Davis DA, Hilliard TS, Burdette JE. Three-dimensional ovarian organ culture as a tool to study normal ovarian surface epithelial wound repair. *Endocrinology.* 2009;150(8):3921–6.
13. Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod.* 1996;54(1):197–207.
14. Smits J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K, et al. Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation. *Hum Reprod Update.* 2010;16(4):395–414.
15. Pangas SA, Saudye H, Shea LD, Woodruff TK. Novel Approach for the Three-Dimensional Culture of Granulosa Cell-Oocyte Complexes. *Tissue Eng.* 2003;9(5).
16. Xu M, West E, Shea LD, Woodruff TK. Identification of a stage-specific permissive in vitro culture environment for follicle growth and oocyte development. *Biol Reprod.* 2006;75(6):916–23.

17. Heise M, Koepsel R, Russell AJ, McGee EA. Calcium alginate microencapsulation of ovarian follicles impacts FSH delivery and follicle morphology. *Reprod Biol Endocrinol*. 2005;3:1–8.
18. Amorim CA, Van Langendonck A, David A, Dolmans MM, Donnez J. Survival of human pre-antral follicles after cryopreservation of ovarian tissue, follicular isolation and in vitro culture in a calcium alginate matrix. *Hum Reprod*. 2009;24(1):92–9.
19. Gurski L, Jha A, Zhang C, Jia X, Farach-Carson M. Hyaluronic acid-based hydrogels as 3D matrices for in vitro evaluation of chemotherapeutic drugs using poorly adherent prostate cancer cells. *Biomaterials* [Internet]. 2009;30(30):6076–85. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf>
20. Edmondson R, Broglie JJ, Adcock AF, Yang L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay Drug Dev Technol*. 2014;12(4):207–18.
21. Brito IR, Lima IMT, Xu M, Shea LD. Three-dimensional systems for in vitro follicular culture: overview of alginate-based matrices. *Reprod Fertil Dev*. 2014;26:915–30.
22. Javed MH, Azimuddin SMI, Hussain AN, Ahmed A, Ishaq M. Purification and characterization of lactate dehydrogenase from Varanus liver. *Exp Mol Med*. 1997;29(1):25–30.
23. Lee SY, Ju MK, Jeon HM, Jeong EK, Lee YJ, Kim CH, et al. Regulation of Tumor Progression by Programmed Necrosis. *Oxid Med Cell Longev*. 2018;2018.
24. Ojala M, Erkkilä K, Tuuri T, Sjöberg J, Suomalainen L, Suikkari AM, et al. Cell death and its suppression in human ovarian tissue culture. *Mol Hum Reprod*. 2002;8(3):228–36.
25. Orive G, Ponce S, Hernández RM, Gascón AR, Igartua M, Pedraz JL. Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginates. *Biomaterials*. 2002;23(18):3825–31.
26. Lee KY, Mooney DJ. Alginate: Properties and biomedical applications. *Prog Polym Sci*. 2012;37(1):106–26.
27. Hussein MR. Apoptosis in the ovary: Molecular mechanisms. *Hum Reprod Update*. 2005;11(2):161–77.
28. Cohen GM. Caspases: Executioners of Apoptosis. *Biochem J*. 1997;326:1–16.
29. Izawa M, Nguyen PH, Kim HH, Yeh J. Expression of the apoptosis-related genes, caspase-1, caspase-3, DNA fragmentation factor, and apoptotic protease activating factor-1, in human granulosa cells. *Fertil Steril*. 1998;70(3):549–52.
30. Krajewska M, Wang HG, Krajewski S, Zapata JM, Shabaik A, Gascoyne R, et al. Immunohistochemical analysis of in vivo patterns of expression of CPP32 (Caspase-3), a cell death protease. *Cancer Res*. 1997;57(8):1605–13.
31. Milenkovic M, Diaz-Garcia C, Wallin A, Brännström M. Viability and function of the cryopreserved whole rat ovary: Comparison between slow-freezing and vitrification. *Fertil Steril*. 2012;97(5):1176–82.

32. Di Loreto VE, Pera LI, Rigalli A. Experimental Surgical Models in the Laboratory Rat. 1st Editio. Rigalli A, Di Loreto V, editors. 2009. 149–151 p.
33. Khajuria DK, Razdan R, Mahapatra DR. Descrição de um novo método de ooforectomia em ratas. Rev Bras Reum [Internet]. 2012;52(3):462–70. Available from: <http://www.scielo.br/pdf/rbr/v52n3/v52n3a16.pdf>
34. Henry L, Labied S, Fransolet M, Kirschvink N, Blacher S, Noel A, et al. Isoform 165 of vascular endothelial growth factor in collagen matrix improves ovine cryopreserved ovarian tissue revascularisation after xenotransplantation in mice. Reprod Biol Endocrinol. 2015;13:1–10.
35. Gougeon A. Regulation of ovarian follicular development in primates: Facts and hypotheses. Endocr Rev. 1996;17(2):121–55.
36. Asfour V, Polson A, Varma R, Menon P. Evaluating ovarian preservation after ovarian torsion using the ovarian preservation score and tissue necrosis score. Clin Obstet Gynecol Reprod Med. 2015;1(2):34–9.
37. Guven S, Muci E, Unsal MA, Yulug E, Alver A, Duman MK, et al. The effects of carbon dioxide pneumoperitoneum on ovarian blood flow, oxidative stress markers, and morphology during laparoscopy: a rabbit model. Fertil Steril [Internet]. 2010;93(4):1327–32. Available from: <http://dx.doi.org/10.1016/j.fertnstert.2008.10.053>
38. Eser A, Hizli D, Haltas H, Namuslu M, Kosus A, Kosus N, et al. Effects of curcumin on ovarian ischemia-reperfusion injury in a rat model. Biomed Reports. 2015;3(6):807–13.
39. Luyckx V, Scalercio S, Jadoul P, Amorim CA, Soares M, Donnez J, et al. Evaluation of cryopreserved ovarian tissue from prepubertal patients after long-term xenografting and exogenous stimulation. Fertil Steril. 2013;100(5).
40. Dolmans MM, Binda MM, Jacobs S, Dehoux JP, Squifflet JL, Ambroise J, et al. Impact of the cryopreservation technique and vascular bed on ovarian tissue transplantation in cynomolgus monkeys. J Assist Reprod Genet. 2015;32(8):1251–62.
41. Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods. 1983;64(3):313–20.
42. De Michele F, Poels J, Weerens L, Petit C, Evrard Z, Ambroise J, et al. Preserved seminiferous tubule integrity with spermatogonial survival and induction of Sertoli and Leydig cell maturation after long-term organotypic culture of prepubertal human testicular tissue. Hum Reprod. 2017;32(1):32–45.
43. Keshaw H, Forbes A, Day RM. Release of angiogenic growth factors from cells encapsulated in alginate beads with bioactive glass. Biomaterials. 2005;26(19):4171–9.

## Figures



**Figure 1**

HS results regarding TNS (A), follicular degeneration (B), interstitial oedema (C) and total HS. Data presented as mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

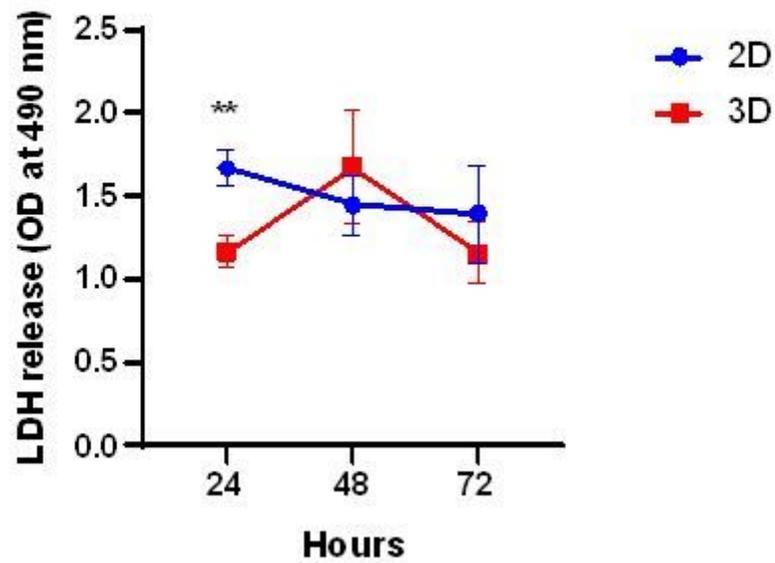
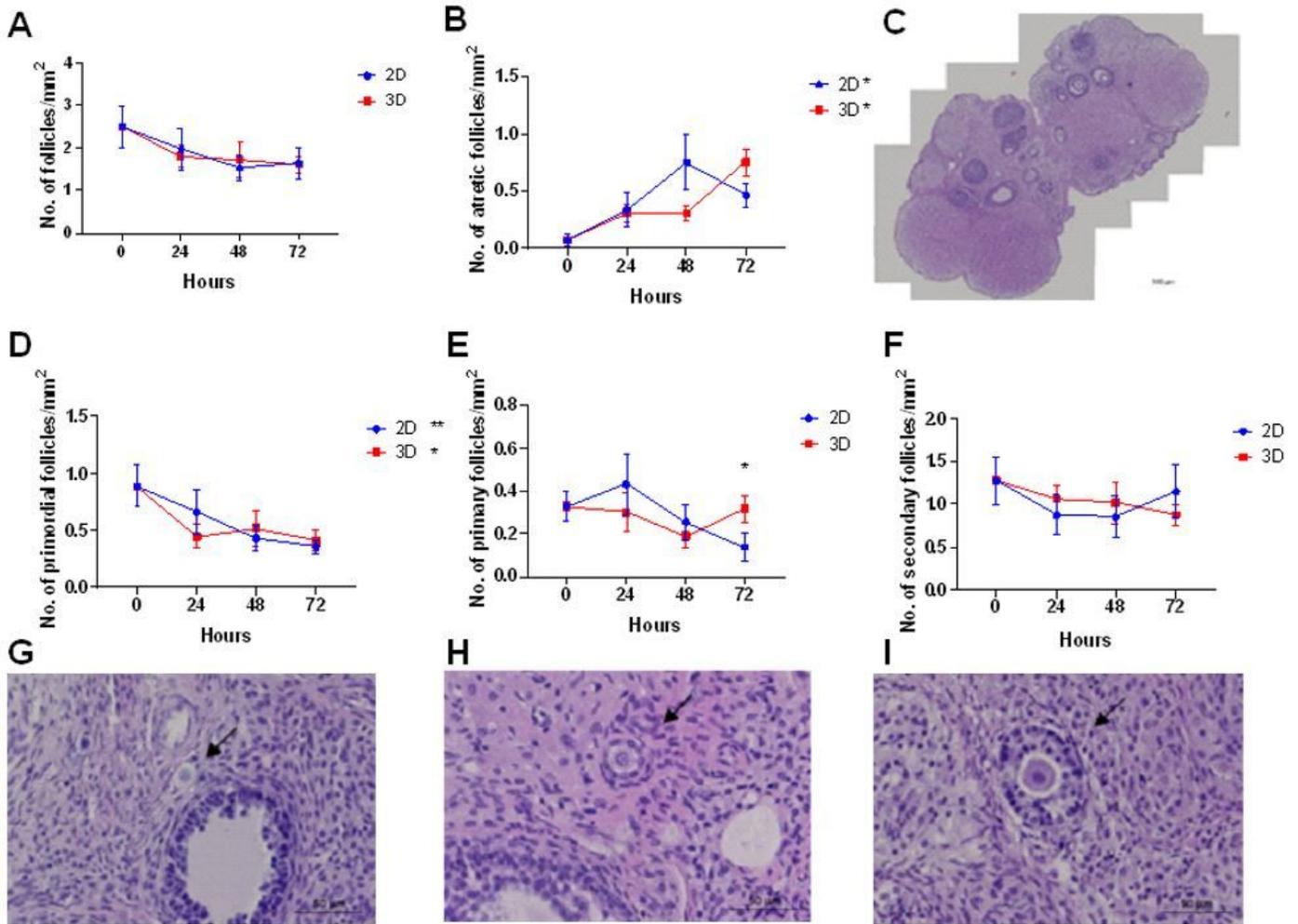


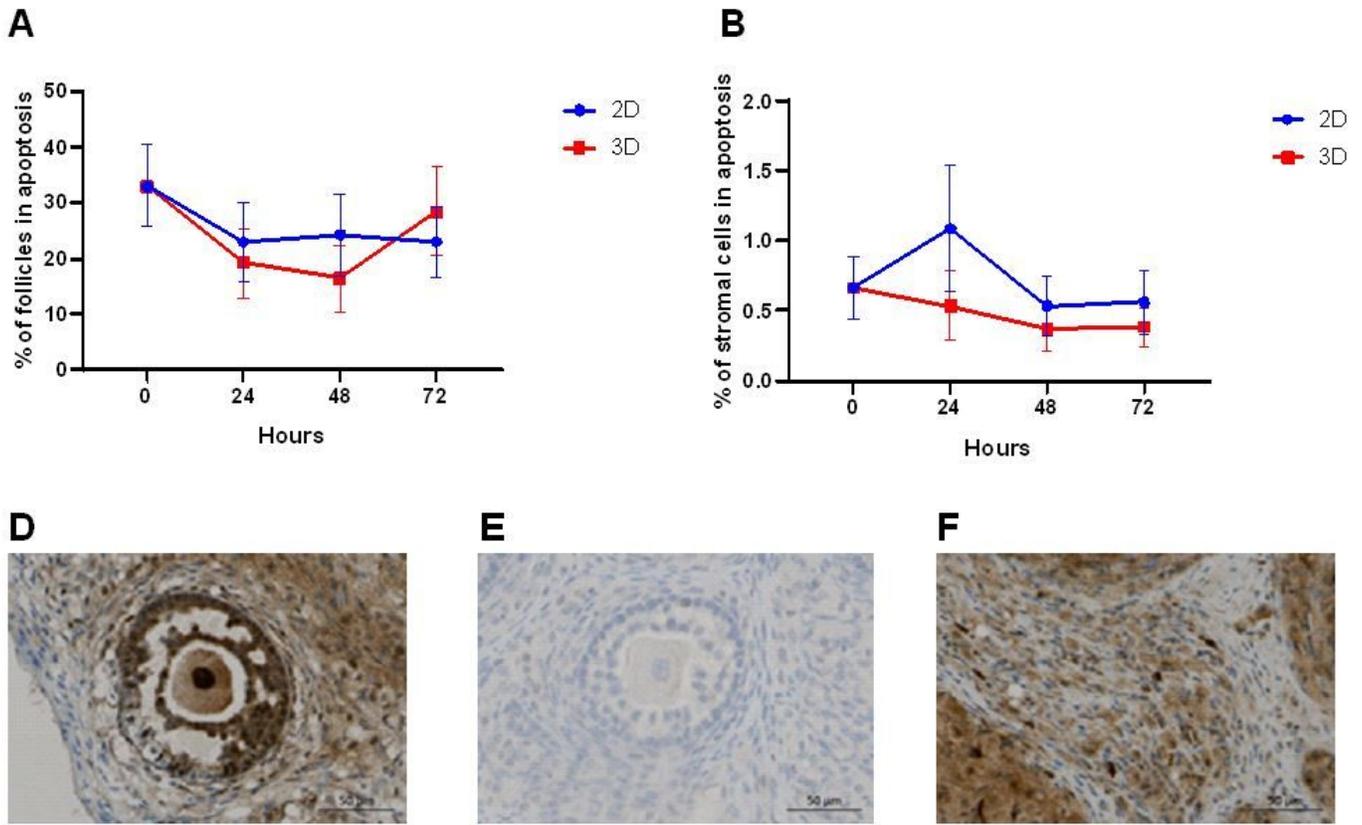
Figure 2

Cytotoxicity analysed through the release of LDH into culture supernatants. Data presented as mean of optical density (OD)  $\pm$  SEM. \*\*p<0.01.



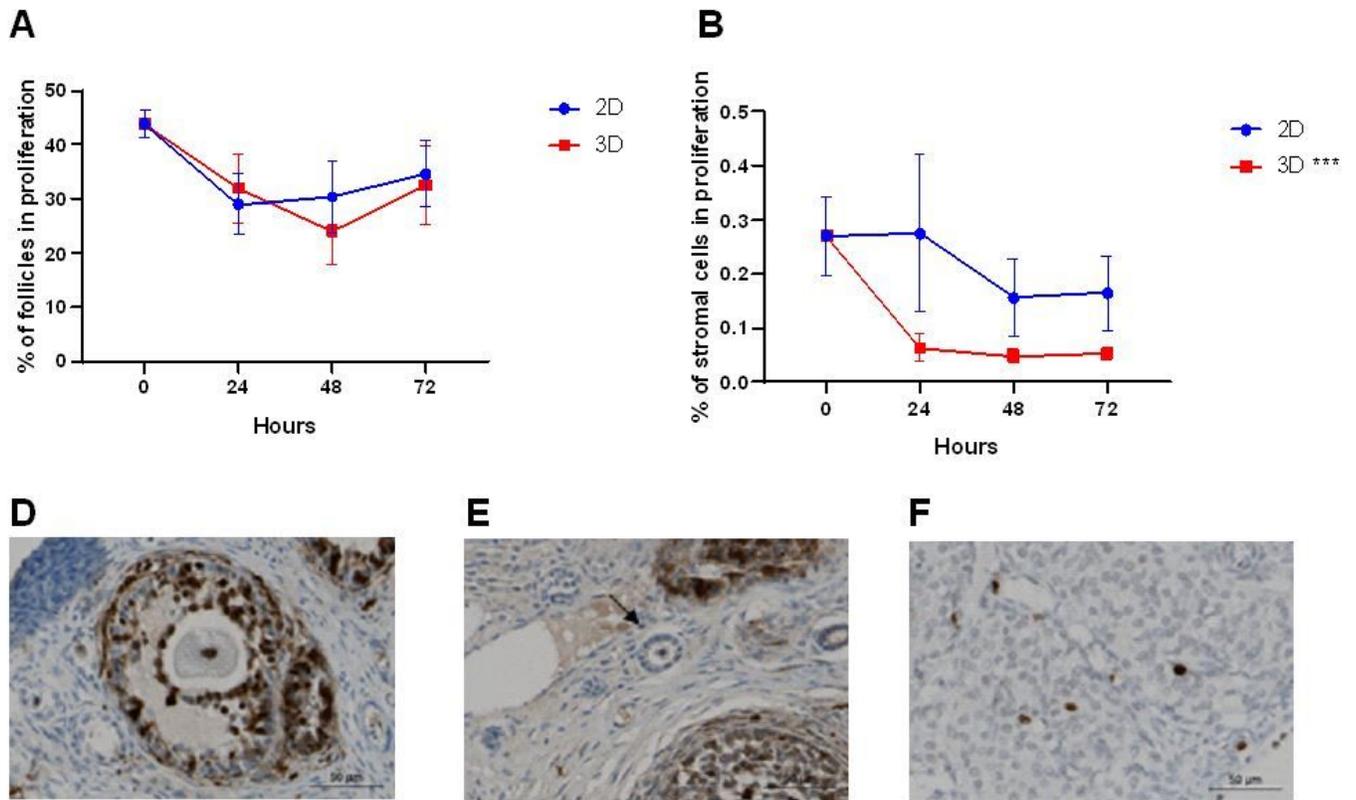
**Figure 3**

Follicular analysis. Representative images of ovarian tissue (C) and morphological normal primordial (G), primary (H) and secondary (I) follicles (arrow). Density of follicles classified as normal (A) and degenerated (B) according to oocyte and granulosa cells morphology. Density of primordial (D), primary (E) and secondary (F) follicles. Original magnification x200. Data presented as mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 4**

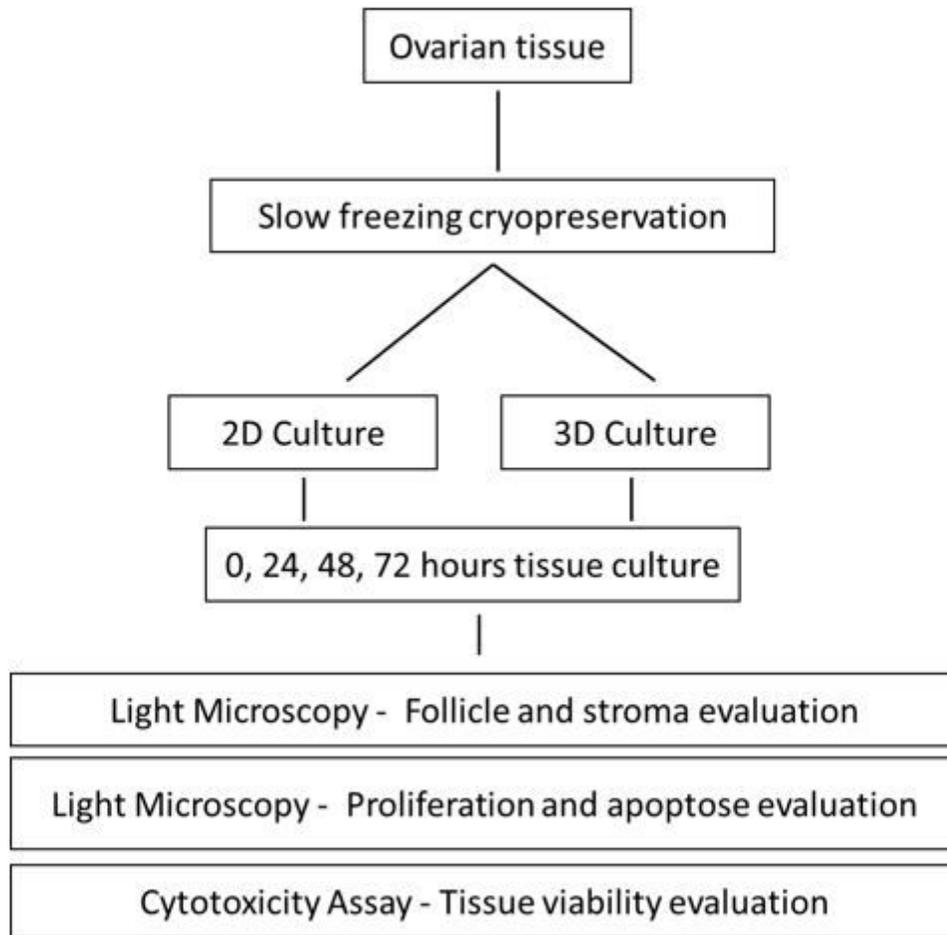
Tissue apoptosis evaluated through AC3 staining in follicles (A) and stromal cells (B). Representative images of AC-3 staining in a secondary follicle (D) and stromal cells (F). A negative secondary follicle is represented in figure E. Original magnification x200. Data presented as mean  $\pm$  SEM.



**Figure 5**

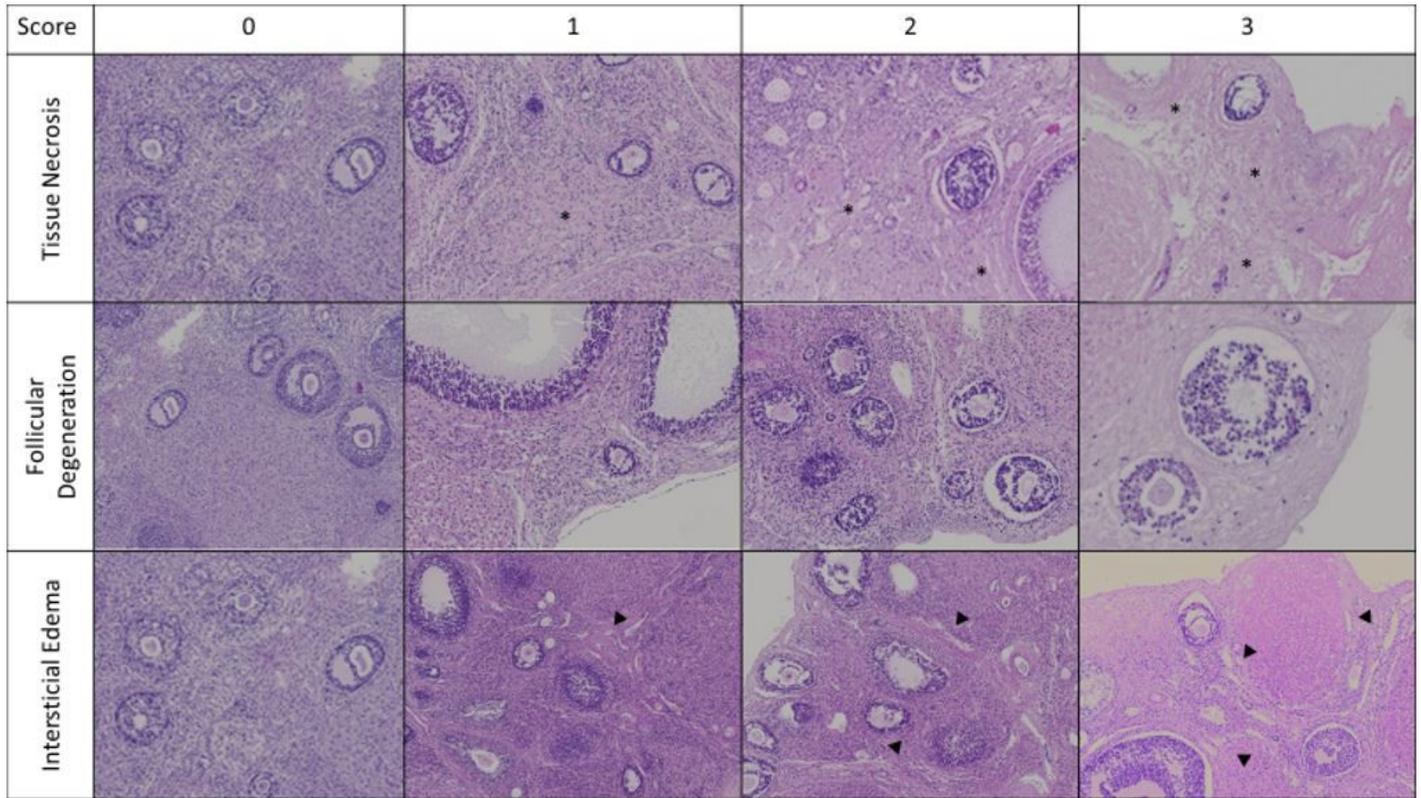
Tissue proliferation evaluated through Ki67 staining in follicles (A) and stromal cells (B). Representative images of Ki67 staining in a secondary follicle (D) and stromal cells (F). A negative primordial follicle (arrow) is represented in figure E. Original magnification x200. Data presented as mean  $\pm$  SEM.

\*\*\* $p < 0.001$ .



**Figure 6**

Schematic representation of the study. The figure shows the experimental design of the comparative study of in vitro culture system for ovarian tissue – 2D versus 3D.



**Figure 7**

Histopathologic score (HS) represented in haematoxylin and eosin-stained ovarian sections. The images show areas of tissue necrosis (\*), follicular degeneration and interstitial oedema (▼). A scoring system where none=0, mild=+, moderate=++ and severe=+++ was applied.