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FERTILITY PRESERVATION

Impact of human ovarian tissue manipulation on follicles: evidence of a potential first wave of follicle activation during fertility preservation procedures

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Abstract

Purpose The aim of this study was to investigate the impact of processing human ovarian tissue on follicle activation dynamics.

Methods Fresh ovarian tissue was retrieved from 9 women undergoing laparoscopic surgery for benign conditions. Biopsies from each patient were divided into 3 fragments, the first of which was immediately fixed in the operating room (T0) and the second and third just after processing at 25 (T25) and (T90) 90 min. To evaluate follicle activation, markers of the PI3K and Hippo signaling pathways were immunolabeled at each time point, targeting phospho-Akt (p-Akt) by immunohistochemistry and yes-associated protein (YAP) cellular localization in the granulosa cell layer by immunofluorescence.

Results Four hundred forty primordial follicles were evaluated for p-Akt and 420 for YAP. Significantly stronger p-Akt expression was observed at T25 (23.01 ± 13.45%; p=0.04) and T90 (38.99 ± 25.21%; p<0.001) than at T0 (2.72 ± 3.35%). A significant nucleus-to-cytoplasm shift in YAP was detected at T25 (1.21 ± 0.25; p=0.015 compared to T0 (0.95 ± 0.09), while T90 (1.10 ± 0.16) values were similar to T25.

Conclusion Our data prove that ovarian tissue manipulation significantly impacts follicle dynamics by stimulating the PI3K and Hippo signaling pathways involved in primordial follicle activation. Further experimental evidence must nevertheless be gathered to understand and gain control of follicle activation mechanisms in non-physiological conditions (like ovarian tissue manipulation), in order to optimize fertility preservation and restoration strategies.

Keywords Fertility preservation · Follicle activation · Ovarian tissue processing · PI3K/Akt pathway · Hippo pathway

Introduction

Primordial follicles (PFs) are fundamental developmental units of the mammalian ovary. They reside in the ovarian cortex and constitute the quiescent follicle reserve. Some are continuously recruited into the growing pool via a process called follicle activation. PFs are the most resistant to cryoinjury because of their small size, low metabolic rates, and absence of a zona pellucida [1]. This is why they are considered an essential resource for fertility preservation procedures, including ovarian tissue cryopreservation (OTC) and transplantation (OTT), as well as experimental techniques like in vitro culture (IVC) and in vitro activation (IVA). However, cultivating mature and competent oocytes from PFs is still challenging. Indeed, follicle development is a gradual process that requires orchestrated and sequential secretion of autocrine and paracrine factors from the oocyte, granulosa cells (GCs), and stromal cells, but also proper communication between all these compartments [2].

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Two main pathways play a crucial role in the follicle activation process, namely phosphoinositide 3-kinase (PI3K) and Hippo. The PI3K pathway is stimulated by various growth factors, and its activation triggers phosphorylation of its master regulator Akt, giving rise to a series of effectors involved in protein synthesis, cell growth, and oocyte-GC crosstalk [3–6]. The Hippo signaling pathway is disrupted in case of mechanical stress, such as ovarian fragmentation, inducing a cytoskeletal change that causes dephosphorylation of the yes-associated protein (YAP) and its shift to the nucleus, activating transcription factors involved in cell proliferation [7]. Synergic activation of these two pathways to promote GC growth was evidenced in a murine model by Devos et al., who demonstrated activation of kit ligand, a major stimulator of PI3K, after ovarian fragmentation for IVC [8]. During fertility restoration procedures, PFs enter the growing pool following extensive and accelerated awakening, much more so than in physiological conditions, raising questions about follicle survival and oocyte quality [9].

It has been estimated that 60–95% of the ovarian reserve is lost after OTT, also due to uncontrolled follicle activation [6, 10]. Experimental techniques like IVC and IVA, which could potentially take advantage of accelerated follicle activation to obtain as many mature oocytes as possible [11–13], are not yet considered safe and effective enough for use in humans. Retrieval of mature oocytes after IVC or IVA of follicles is indeed highly challenging in human beings, and one of the obstacles might well be this accelerated and dysregulated follicle activation. PF activation is the first step in their development, but there are safety concerns that early and non-physiological activation may trigger uncoordinated growth of oocytes and GCs [14]. Increasing evidence supports the notion that too early PF differentiation could impair genome integrity by altering DNA damage repair pathways [15], leading to inadequate and incompetent oocytes unable to support normal embryo development [16].

In light of these results, there is a growing need to elucidate the mechanisms behind PF dynamics, which are thought to act as a multiwave phenomenon [17], in order to find ways of optimizing procedures. Our study focuses on the mechanisms implicated in follicle activation during the initial phases of ovarian tissue processing. The aim is to understand whether the different steps involved in the processing of ovarian biopsies right after surgical retrieval might be responsible for the changes in follicle activation dynamics.

Materials and methods

Ethics approval

The use of human tissue was approved by the Institutional Review Board of the Catholic University of Louvain, and patients gave their written informed consent for the use of their ovarian tissue for research purposes.

Experimental design

Nine human ovarian biopsies were collected from patients aged 16–27 years (mean 25.25 ± 7.7 years) undergoing laparoscopic surgery at the Cliniques Universitaires Saint-Luc, Brussels. All nine women had undergone procedures for benign gynecological conditions. In patients with signs of ovarian disease, biopsies were taken from their contralateral healthy ovaries. After biopsy collection, one-third of the tissue per patient was cut in the operating room and immediately fixed in 4% formaldehyde, without any manipulation, to serve as a control sample (time zero (T0)). We selected specific time points based on the time required in our laboratory to process ovarian tissue for IVC, mimicking the phases of collection (T0), transportation (T25), and manipulation of the tissue (T90). Subsequently, half of the remaining fragments was fixed after 25 min (T25) and the other half after 90 min (T90) in order to mimic the phases of transportation and manipulation of tissue in IVC (Fig. 1).

The remaining tissue was transferred to the laboratory on ice in MEM-GlutaMax (Gibco, Life Technologies, Belgium) and dissected in a 10-ml pre-warmed dissection medium to remove any surplus medulla, as previously described [7]. It was cut into cortical fragments of 4×2×1 mm^3. We chose these dimensions as they are typically used for IVC, so our results can help other researchers in the field. Smaller dimensions are also known to have a greater impact on the Hippo pathway. Standardizing the size of all cortical fragments minimizes the potential differences in follicle activation associated with variability in Hippo signaling disruption during fragmentation.

Histology

All cortical fragments were fixed in 4% formaldehyde, embedded in paraffin, and serially cut into 5-µm sections. Every fifth section was stained with hematoxylin and eosin (H & E; Merck, Germany) for morphological evaluation and follicle count, while the remaining sections were assigned for immunohistological analyses. Slides were scanned with the Pannoramic P250 Flash III scanner (3DHISTECH, Hungary) at × 20 magnification and analyzed using CaseViewer (v2.2). Only follicles with a visible oocyte were taken into account. Follicles were counted in four non-consecutive sections per time point and then classified according to stage as primordial, intermediate, primary, and secondary follicles [18]. The percentage at each stage was established for comparison between groups ($\frac{\text{number of follicles per subgroup}}{\text{total number of follicles}}$). Follicle density was calculated by subtracting the total number of follicles from the surface area in mm^3 (n/mm^3). When calculating the area analyzed, we took into account the thickness of the sections by multiplying the area analyzed (in μm^2) by the thickness of the section (5 µm) to obtain the area in μm^3. Finally, we multiplied the above area by 10^{-9} to convert it to mm^3. Follicle density was therefore the

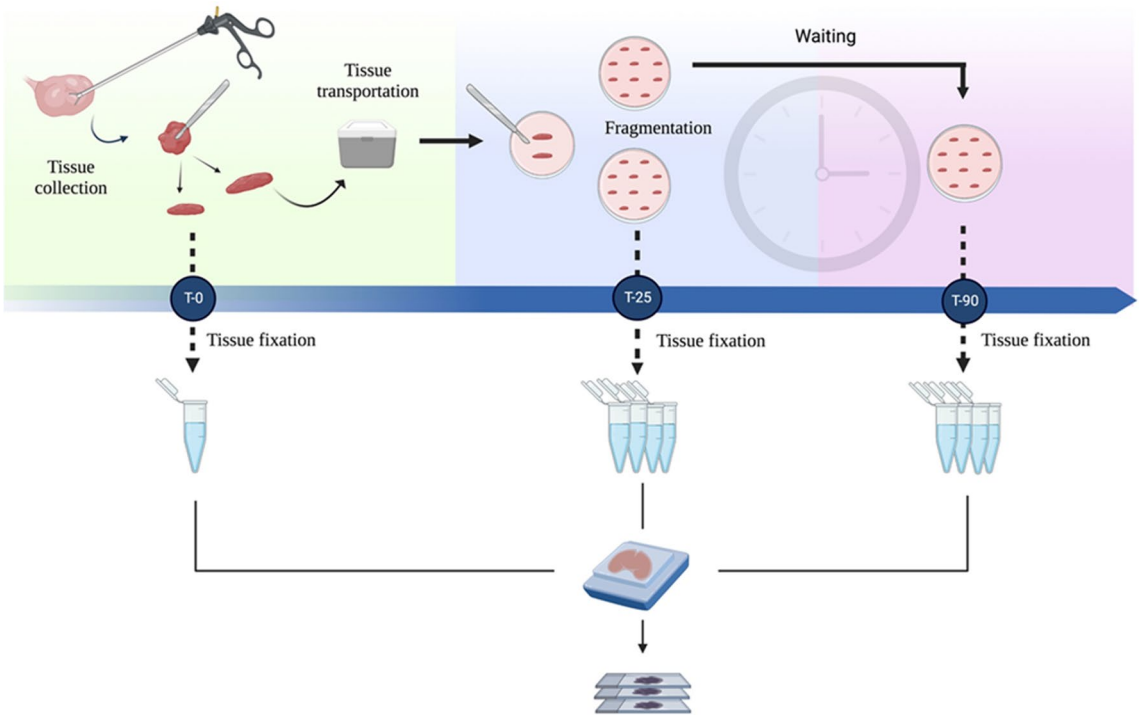

Fig. 1 Experimental design. Fresh ovarian tissue was collected from 9 women undergoing laparoscopic surgery and divided into 3 fragments apiece. The first was immediately fixed in the operating room (T0). The other two were processed by removing the medulla and fragmenting the cortex before fixation 25 (T25) and 90 (T90) min following biopsy retrieval. All cortical fragments were fixed in 4% formaldehyde and embedded in paraffin for histological analyses and immunolabeling

result of the total number of follicles divided by surface area in mm^3. We recruited mainly young patients hoping for high follicle density.

Immunolabeling

Three non-consecutive sections from each sample were earmarked for immunolabeling. Two main signaling pathways responsible for follicle activation, PI3K and Hippo, were investigated. The PI3K pathway was explored by analyzing its main activating factor, phospho-Akt (p-Akt), which initiates a phosphorylation cascade of downstream effectors involved in follicle activation. Disruption of the Hippo pathway was appraised by labeling YAP, a transcription factor whose cytoplasm-to-nucleus translocation leads to transcription of a number of factors that play a role in follicle growth and development. Since the specific aim was to assess follicle activation, only PFs were included in the statistical analysis.

Phospho-Akt analysis

Sections were deparaffinized with Histosafe (Yvsolab SA, Belgium) and rehydrated in 2-propanol (Merck, Germany). After blocking endogenous peroxidase activity with 3% hydrogen peroxide (H_2O_2) and demasking in Tris-EDTA for 20 min at 96 °C, non-specific binding sites were blocked by incubation in 10% fetal bovine serum for 60 min at room temperature (RT). Sections were first incubated overnight at 4 °C with rabbit anti-human p-Akt (SER 473, 1:100, Cell Signaling-Bioké), and then for 1 h with EnVision anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1, K001, Dako, USA). Negative controls were obtained using non-specific rabbit antibodies (10133249, Dako) and non-specific mouse antibodies (10132024, Dako), while human lung cancer specimens served as positive controls. Only follicles with a visible oocyte were considered for analysis.

Stained slides were digitized using the Pannoramic P250 Flash III scanner (3DHISTECH, Hungary) at × 40 magnification and visualized with the Caseviewer software (v2.2). Images of ovarian tissue areas containing follicles were captured with Caseviewer and analyzed by Fiji v1.52 (NIH, USA), as previously described [19]. Briefly, PFs were digitally isolated from adjacent stroma by selecting a region of interest (ROI). A threshold was set to separate p-Akt staining from the rest. After determining the scale of the image, the p-Akt-immunolabeled area and total area of each follicle were calculated in square micrometers (μm^2) and expressed as a percentage of p-Akt-stained area out of total area per ROI.

YAP analysis

Sections were deparaffinized with Histosafe (Yvsolab SA, Belgium) and rehydrated in 2-propanol (Merck, Germany). 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 normal goat serum for 30 min at RT. Sections were first incubated overnight at 4 °C with mouse anti-human YAP (1:200, 12395S, Cell Signaling, USA) and then for 1 h with EnVision mouse HRP (1:2, K001, Dako, USA). Unmasking was achieved using a boric acid solution with H_2O_2 at 30% 1:1000 and tyramide chromogen for 15 min at RT (Alexa Fluor tyramide reagent, 1:200, B40953, Invitrogen), followed by a 20-min citrate buffer bath in the microwave. The cell nuclei were labeled by incubating them in Hoechst 333452 (1:1000, H3570, Invitrogen) for 10 min. Slides were also incubated in wheat germ agglutinin (WGA) (1:500, Biotium) for 10 min to stain the plasma membranes, before being mounted with HIGHDEF IHC fluoromount (Enzo, USA). Negative controls were obtained using non-specific rabbit antibodies (10133249, Dako) and non-specific mouse antibodies (10132024, Dako). Ovarian cancer tissue served as positive controls. Images of PFs were taken using the Axio Imager Z1 microscope with structured ApoTome illumination and a 63x/1.4 oil objective with laser power set at 405, 488, and 564 nm for Hoechst 33342, tyramide chromogen, and WGA, respectively. Native images were analyzed with Fiji v1.52 (NIH, USA) to calculate the mean signal intensity in the follicle nuclei and the cytoplasm, as previously described. Briefly, the GC layer of each follicle was defined as a ROI. A fixed threshold was then applied to the blue channel (DAPI) to delineate the nucleus within the considered ROI and to the red channel (WGA) to precisely delimit the cytoplasmic membrane of GCs. Hence, the mean intensity of the green signal was measured in the cytoplasmic and nuclear compartments within each considered ROI.

Statistical analysis

The GraphPad Prism software for Windows (v9.2, USA) was applied for statistical analyses. Since all data showed normal distribution, the paired *t*-test was used to evaluate follicle density and one-way ANOVA with Tukey's post hoc test to assess p-Akt and nuclear YAP. The results were expressed as mean ± SD. A p value <0.05 was considered statistically significant.

Results

Ovarian tissue morphology and follicle populations

Macroscopic examination did not reveal alterations in any analyzed samples. The tissue looked rose-colored with no necrotic areas. Histological evaluation at each time point showed follicles and stromal cells with normal morphology. Since we were not expecting any follicle growth in just 25 min, follicle density (no. follicles/mm^3 ± SD) and classification were evaluated on H&E-stained slides at T0 and T90. No statistically significant differences were found, either in follicle density (T0: 1668 ± 1676.6; T90: 1127 ± 1281.7) or in follicle classification, with the percentage of primordial follicles remaining unchanged after 90 min (T0: 76.7% ± 17.3; T90: 73.8% ± 13.4). Indeed, after 90 min, there was no modification to the GC layer, as we did not observe any statistically significant difference in the percentage of intermediate follicles between T0 and T90 (Table 1).

PI3K pathway activation

A total of 440 PFs were evaluated for p-Akt: 154 in the T0 group, 155 in the T25 group, and 131 in the T90 group. The percentage of p-Akt-stained area at T0 was 2.72 ± 3.35%. After manipulation, a gradual increase was observed after 25 min (23.01 ± 13.45%) and after 90 min (38.99 ± 25.21%), showing a statistically significant difference between both T0 and T25 (p=0.04) and T0 and T90 (p<0.001) (Fig. 2).

Hippo pathway disruption

We analyzed 420 PFs: 148 at T0, 146 at T25, and 126 at T90. YAP was evaluated in GCs by determining its subcellular localization. At T0, the ratio between nuclear and total concentrations was 0.95 ± 0.09, demonstrating a

Table 1 Follicle outcomes

Time points	Total follicle density (n/mm^3 ± SD)	Follicle classification			
		Primordial (% ± SD)	Intermediate (% ± SD)	Primary (% ± SD)	Secondary (% ± SD)
T0	1668 ± 1676.6	76.7% ± 17.3	15.7% ± 8.7	5.6% ± 8.3	2.0% ± 3.4
T90	1127 ± 1281.7	73.8% ± 13.4	20.6% ± 10.9	4.1% ± 4.5	1.4% ± 1.4

Percentages at each stage were established for comparison between groups: $(\frac{number\ of follicles\ per\ subgroup}{total\ number\ of follicles})$ ± SD. Follicle density was defined as the total number of follicles per mm^3 (n/mm^3)

predominantly cytoplasmic localization. After manipulation, greater nuclear localization was observed at both T25 (1.21 ± 0.25) and T90 (1.10 ± 0.16) compared to T0. Thus, we detected a significant cytoplasm-to-nucleus shift at T25 (p=0.015), after which it plateaued, with no further increase in the nuclear transition of YAP from T25 to T90 (Fig. 3).

We also evaluated the YAP fluorescent signal by considering the whole GC layer (nucleus and cytoplasm) and comparing its signal intensity at the three different time points. YAP protein levels showed no significant variation between T0 (24.582 ± 5.521), T25 (25.354 ± 6.242), and T90 (27.019 ± 8.766) (Fig. 3), demonstrating that over the course of 90 min, signal transmission is determined not so much by the amount of protein produced (which indeed remained stable for 90 min), but rather its cellular localization.

Discussion

The PF pool is the target population for fertility preservation strategies, as it is the source of oocytes potentially available for later fertility restoration. Extensive research has been conducted in attempts to elucidate factors that regulate follicle activation during fertility preservation procedures, in order to avoid massive follicle loss occurring in the event of fertility restoration [20]. Precocious follicle activation has been shown to occur as soon as 3 days after human ovarian tissue xenotransplantation [6, 21, 22], and its dynamics have also been largely investigated during the first 6 days of human ovarian tissue IVC [7, 17, 23]. However, fewer studies have addressed the follicle activation phenomenon during the very early steps of these procedures. It was recently demonstrated that follicle activation starts before IVC, immediately on day 0, and then appears to follow a multiwave pattern over the days, dropping to its lowest point on day 1 and peaking on day 6 [17].

To elucidate the impact of ovarian tissue manipulation on fertility preservation outcomes, we decided to investigate follicle activation by analyzing PI3K activation and Hippo pathway disruption in non-physiological conditions, like ovarian biopsy retrieval and ovarian cortex fragmentation. Although no difference was found in the proportion of PFs over the course of 90 min (by follicle classification analysis on histological sections), which was expected, our study showed that rapid and

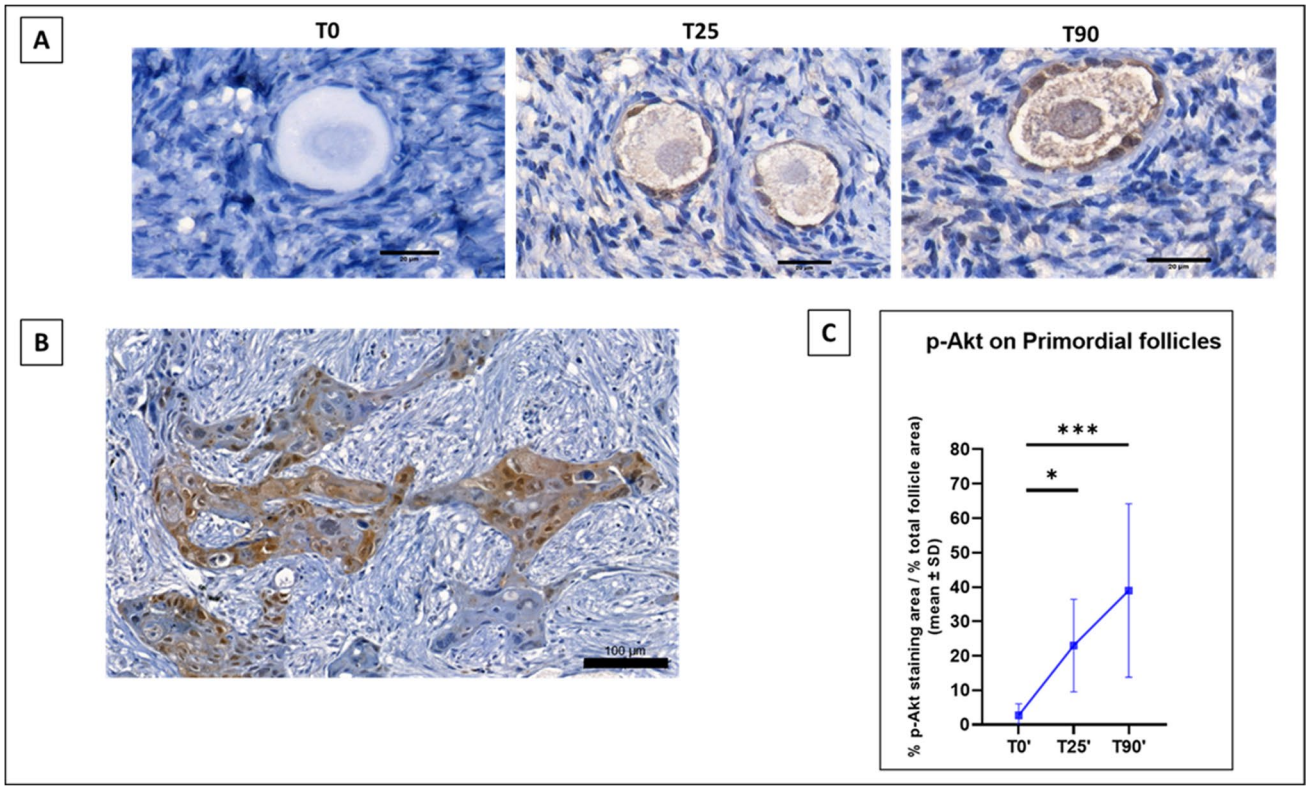

Fig. 2 PI3K pathway activation. **A** P-Akt immunostaining on primordial follicles at different time points. (1) T0 (no staining), (2) T25 (weak staining of the GC layer and oocyte cytoplasm), (3) and T90 (intense staining of the GC layer and oocyte nucleus and cytoplasm). Scale bar = 20 µm. **B** P-Akt immunostaining in human lung cancer served as a positive control. Scale bar = 100 µm. **C** P-Akt staining analysis on primordial follicles (as a percentage, mean ± SD) analyzed using one-way ANOVA and Tukey's post hoc test. Significant differences between time points are indicated by *p <0.05 and ***p<0.001. T0, time zero; T25, time 25 min; T90, time 90 min

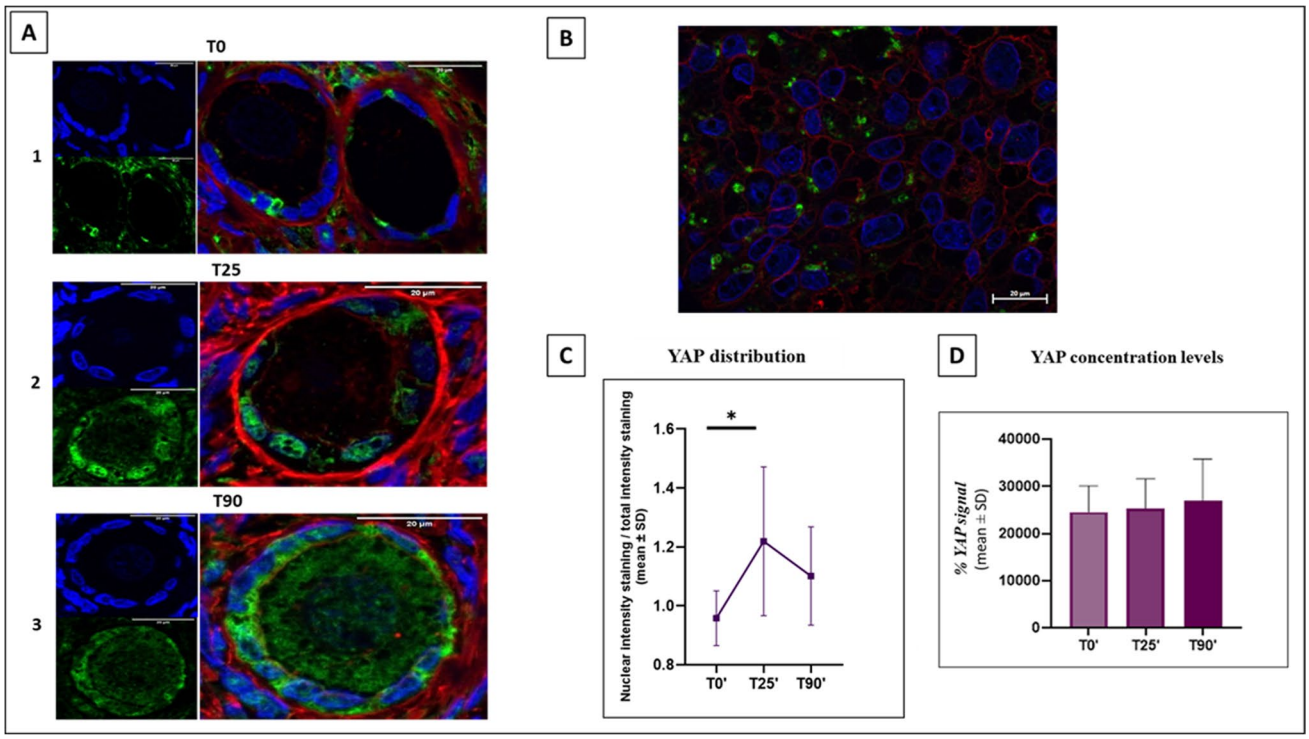

Fig. 3 Hippo pathway disruption. **A** Immunofluorescence targeting YAP (green) and subcellular localization analysis in GC nucleus (DAPI, blue) and cytoplasm, delimited by plasmatic membrane counterstaining (red). (1) T0: predominantly cytoplasmic YAP localization; (2) T25: predominantly nuclear YAP localization; (3) T90: Both cytoplasmic and nuclear YAP localization, with nuclear preponderance. Scale bar = 20 µm. **B** Immunofluorescence targeting YAP (green), nucleus (DAPI, blue), and cytoplasm, delimited by plasmatic membrane counterstaining (red) in ovarian cancer tissue served as positive control. Scale bar = 20 µm. **C** YAP distribution trend at different time points (nuclear mean intensity staining/total mean intensity staining ± SD) in primordial follicles evaluated by one-way ANOVA and Tukey's post hoc test. A significant difference between time points is indicated by * ($p< 0.05$). **D** Total YAP concentration levels (nucleus and cytoplasm of the GC layer). No significant differences were found between time points. T0, time zero; T25, time 25 min; T90, time 90 min

significant follicle activation had already occurred after 25 min. In fact, for the PI3K pathway, it only took the time needed to harvest, transport, and process the tissue to activate PFs, with rates almost 20% higher than in controls. The same was observed of the Hippo pathway, where a significant nucleus-to-cytoplasm shift in YAP was also encountered after 25 min. However, no difference was noted between the three time points in total YAP concentration levels. This finding is interesting, as it confirms that these signaling pathways take advantage of the properties of tyrosine kinase receptors, which, in order to act quickly, use phosphorylation cascades by exploiting pre-synthesized proteins [24, 25]. Only around 24 h later does new protein synthesis commence, upon specific transcription factor activity in DNA [24, 25].

As already suggested by other authors [7, 8], our data evidenced simultaneous trigger of the two pathways, since they were both activated after tissue manipulation at T25, but appear to have followed a different trend thereafter. Expression of p-Akt continued to increase (by T90 the percentage of stained area had climbed to 38.99%), but YAP reached its intranuclear concentration peak at T25. One hypothesis is that after 25 min, mechanical stress due to fragmentation ends, thereby curtailing nuclear translocation of YAP, whose intranuclear levels nevertheless remain higher than at T0. Thus, while the Hippo pathway plateaus at the end of tissue manipulation, p-Akt levels continue to rise, proving that other factors may be involved in follicle activation during these early stages of ovarian tissue processing.

First, disconnection of ovarian tissue from the bloodstream, resulting in reduced oxygen supply, could trigger hypoxic stress that has been shown to be related to follicle activation. In this regard, Shiratsuki et al. [26] demonstrated the impact of hypoxia-induced factor 1 (HIF1) on Akt-mTOR signaling in a bovine model by illustrating increased vascular endothelial growth factor levels, causing enhanced GC proliferation.

Second, separation of the cortex from the medulla after retrieval could result in loss of inhibitory stimuli like anti-Müllerian hormone and activin A, as well as estradiol and progesterone. Indeed, these hormones are all produced by growing follicles that, because they reside in the medulla, provide important negative paracrine feedback on PFs [27–29].

Our results demonstrate that ovarian fragmentation plays a key role in the dynamics of PF activation. Several studies have investigated how ovarian fragmentation could potentially boost follicle growth in patients with a diminished ovarian reserve [30–32]. Díaz-García C et al. [32] observed Hippo pathway disruption 1 h after fragmentation by detecting a decrease in the p-YAP/YAP ratio and an increase in the expression of CCN proteins and BIRC. However, the role of the Hippo pathway is still contentious, as other studies have failed to confirm its contribution to follicle activation and growth [16, 30, 33]. One possible explanation may be linked to different patient characteristics in terms of ovarian reserve and fertility potential [16, 27, 30]. Our study was conducted using ovarian tissue from young and potentially fertile patients, proving that at least in this subpopulation, ovarian fragmentation appears to be able to initiate follicle activation by triggering both PI3K and Hippo pathways.

Conclusion

Our study provides strong evidence that manipulating ovarian tissue during the initial phases of human ovarian tissue processing has a significant impact on the dynamics of early follicle development. We have successfully demonstrated the involvement of two key pathways in follicle activation, namely PI3K and Hippo. Indeed, our findings contribute to the overall understanding of the early phases of PF activation, allowing us to adapt and improve current strategies. We believe that using a pharmacological approach that acts on the PI3K and Hippo pathways immediately after tissue harvesting may actually prevent overly early follicle activation and drive follicle growth, yielding competent and fertilizable oocytes. However, further investigations and more comprehensive studies into follicle activation during these early phases of ovarian tissue processing must be conducted to validate our results.

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Declarations

Conflict of interest The authors declare no competing interests.

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