FSH Promotes Rat Follicle Development Through Inhibition of the Hippo Signalling Pathway

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Abstract

Purpose: Whether FSH promotes follicle growth by inhibiting the Hippo signalling pathway.

METHODS: Ovaries were cultured in vitro into a control group (no intervention), an FSH group (0.3 IU/mL FSH), and a VP group (10 μg/mL vetiporfin). HE staining and follicle counts were performed at each stage after 3 hours of in vitro culture. Immunohistochemistry was performed to study the expression levels of LATS2, YAP, PLATS2, and PYAP, and their expression levels in each group were also analysed by Western blot.

The number of secondary follicles was significantly increased in the FSH group, the arrangement of granulosa cells was neater, the nuclear fixation was reduced, and the number of atretic follicles was decreased in the VP group. The number of secondary follicles was significantly increased, the number of atretic follicles was reduced, and granulosa cell nuclear consolidation was reduced in the VP+FSH group. Immunohistochemistry showed that LATS2 and YAP expression levels were significantly increased and PLATS2 and PYAP expression levels were relatively decreased in the FSH group, PYAP and PLATS2 expression levels were significantly increased and YAP expression was significantly decreased in the VP group, and YAP and LATS2 expression levels were significantly increased and PYAP and PLATS2 expression levels were significantly decreased in the VP+FSH group. By Western blot, LATS2 and YAP were elevated and PYAP and PLAT2 were decreased in the FSH group, LATS2 and YAP were decreased and PYAP and PLATS were significantly elevated in the VP group, and LATS2 and YAP were elevated and PYAP and PLATS2 were decreased in the VP+FSH group.

CONCLUSION: FSH promotes follicle development by inhibiting the Hippo signalling pathway.

Introduction

Follicle-stimulating hormone (FSH) is a glycoprotein hormone secreted by gonadotropic cells of the pituitary gland and regulated by hypothalamic gonadotropin-releasing hormone (GnRH) pulses [1, 2]. In the ovary, FSH is required for follicle growth and oestrogen production [3, 4] and acts primarily through the FSHR receptor on the surface of follicular granulosa cells [5], which is present from the early preluminal follicle and regulates the growth of granulosa cells [3] [6], which, in turn, directly determines the fate of the follicle [7]. When FSH binds to FSHR, it induces cAMP and activates the protein kinase A pathway [8], and there is much evidence that other signalling pathways may also be activated by FSH; for example, FSH activates p38 mitogen-activated protein kinase (MAPK) in granulosa cells [9] and the growth signalling cascade via MAPK stimulation in ovarian tumour cells [10] and activates protein kinase B (PKB/AKT) and extracellular matrix signal-regulated kinases 1 and 2 (ERK1/2) [11]. FSH is the most important hormone regulating follicular growth and development and regulates follicular development through many signalling pathways after acting on FSHR receptors on the surface of the granulosa cell membrane, but many pathways remain unknown and need to be further explored.

The Hippo signalling pathway inhibits cell growth; it is named mainly after the protein kinase Hippo (Hpo) in Drosophila, which consists of a series of conserved kinases that control organ size mainly by regulating cell proliferation and apoptosis [12–15]. In the ovary, when the Hippo signalling pathway is inhibited, phosphorylation of its key factors, MST1/2 and LATS1/2, is also inhibited, ultimately leading to the entry of nonphosphorylated YAP into the nucleus, where it binds to the transcription factor TEMD and promotes transcription and granulosa cell proliferation. However, when the Hippo pathway is activated, it leads to MST1/2 activation, located upstream of YAP, and LATS1/2 turns on a series of phosphorylation reactions, which causes phosphorylation of YAP factor (PYAP), and the phosphorylated YAP stays in the cytoplasm, thus reducing YAP binding to TEAD and inhibiting transcriptional function and granulosa cell growth [16–24].

The main role of FSH is to promote follicular granulosa cell growth. When the Hippo signalling pathway is inhibited, it can also promote granulosa cell growth; therefore, we hypothesize that FSH promotes follicular growth by inhibiting the Hippo signalling pathway. However, there is not much evidence of this phenomenon, and further proof is needed.

Materials And Methods

Experimental animals

Thirty-six 3-week-old SD rats (SPF class) were provided by the Laboratory Animal Center of Ningxia Medical University (Animal Certificate No. SCXK (Ning) 2020-0001). The experiments were performed in strict accordance with the Guidelines for Ethical Review of Laboratory Animal Welfare issued by the General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China and the National Standardization Administration of China.

Main reagents

FSH (follicle-stimulating hormone, Solarbio), VP (verteporfin, Solarbio); primary antibodies against YAP, PYAP (rabbit anti-mouse monoclonal antibody, produced by Abcam), LATS2, and PLATS2 (rabbit anti-mouse polyclonal antibody, produced by AffeBiotech); and secondary antibodies (HRP goat anti-rabbit IgG, from Abclonal) were purchased. A rabbit two-step immunohistochemistry kit was provided by ZhongShan Jinqiao; other reagents, such as ethanol and xylene, were analytically pure reagents from China.

In vitro culture and intervention of ovaries

The 3-week-old female rats were sacrificed by cervical dislocation, and the ovaries were removed by dissecting the skin and subcutaneous tissue with alcohol in the dorsal renal area. After the ovaries were stripped of their outer membranes, they were placed in culture dishes containing F12:DMEM (1:1) and divided into a CON group (no intervention), an FSH group (with the addition of 0.3 IU/mL FSH intervention; the FSH dose was chosen based on previous experiments verifying that FSH can promote

oocyte development after exceeding a threshold of 0.07 IU/mL [25], and previous experiments demonstrated that the use of 0.3 IU/mL FSH is the optimal dose for in vitro cultured ovaries [26]), a VP group (using 10 μg/mL Hippo signalling pathway YAP factor-specific inhibitor vetiporfin (VP) [27, 28] intervention, with the VP intervention dose referring to previous studies [29]), and a VP + FSH group (using a combined 10 μg/mL VP + 0.3 UI/mL FSH intervention). Ovaries in each group were incubated in a CO2 incubator at a constant temperature of 37°C for 3 hours and removed and rinsed three times with PBS to terminate the intervention, and the incubation time was based on previous experiments on in vitro culture and intervention of follicles and ovaries [30–32]. Some ovaries were fixed in 4% paraformaldehyde solution for 12 hours for morphological studies, and some ovaries were stored at -80 ℃ for molecular biology studies.

Morphological observations and follicle counts

After fixation, the ovaries of each group were routinely dehydrated, paraffin-embedded, and serially sectioned at 5-μm intervals, with an average of 10 sections selected for HE staining in each group, and six clear fields of view were randomly selected at equal intervals in each section under a 10× microscope and distinguished according to a previous study [33]. Granulosa cells were also observed for nuclear fixation and judged as normal or atretic follicles according to the condition of the granulosa cells, and the criteria were based on previous studies [34–36].

Immunohistochemical experiments

Ovarian paraffin sections were routinely dewaxed and hydrated, and the antigens were repaired by the high-pressure thermal repair method. The sections were placed in an autoclave, and citrate buffer was added, heated and boiled for 12 minutes and then cooled naturally. Endogenous peroxidase blocker was added for 10 minutes at room temperature, and primary antibodies against YAP, PYAP, LATS2, and PLATS2 (1:200) were added dropwise after PBS rinsing and incubated for 60 minutes at 37°C. The reaction enhancer was added for 20 minutes at room temperature, rinsed three times with PBS, added dropwise to enhance enzyme-labelled goat anti-rabbit IgG polymer and incubated for 20 minutes at room temperature. DAB colour development solution was added, and the colour was developed for five minutes at room temperature. Haematoxylin was restained for one minute; and alcohol fractionation in hydrochloric acid was performed for three seconds followed by rinsing to return the staining to blue for eight minutes, gradient alcohol dehydration, xylene transparency for 15 minutes, neutral resin sealing, and reading.

Western blotting

Ovarian tissue proteins were lysed and extracted using a protein extraction kit, and the protein sample concentrations were determined by the BAC method and paired. Electrophoresis was performed on a 10% SDS-PAGE gel with 8 μL (40 μg protein amount) of sample per well, and the proteins were transferred to PVDF membranes after electrophoresis. The membrane was blocked with 5% skim milk solution for 2 hours and incubated with primary antibodies against YAP, PYAP, LATS2, and PLATS2 (1:1000) overnight

at 4°C. The secondary antibodies (1:5000) were added the next day and incubated for one hour at room temperature with the ECL method to reveal the bands by luminescence and analyse the greyscale values of each group of proteins using ImageJ software, with β-actin as the internal reference.

Statistical analysis

SPSS 22.0 software was used for analysis, and statistical results are expressed as the mean and standard deviation (X ± S). Comparisons between groups were statistically calculated and subjected to chi-square analysis and one-way ANOVA, and full comparisons between each two groups were performed using the LSD method at $\alpha = 0.05$ as the test level, with $P < 0.05$ indicating a statistically significant difference.

Results

In the control group, the number of primordial follicles was the highest, the number of secondary follicles was slightly greater than the number of primary follicles but not obviously so, the morphology of follicles was generally good, and some atretic follicles with nuclei solidified by granulosa cells could be seen.

In the FSH group, there were relatively fewer primordial follicles and fewer primary follicles than in the control group, but there were significantly more secondary follicles, and the primary and secondary follicles were larger in diameter, with more neatly arranged granulosa cells within the follicles, fewer atretic follicles, and less nuclear fixation of granulosa cells.

In the VP group, compared with the control group, the number of primary follicles was increased, but the numbers of primary and secondary follicles were significantly reduced, the number of atretic follicles was significantly increased, the diameters of primary and secondary follicles were significantly smaller than those of the control and FSH groups, and the nuclear consolidation of granulosa cells was significantly increased. In the VP + FSH group, compared with the control group, there was a slight increase in the number of primordial follicles, a decrease in the number of primary follicles, a significant increase in the number of secondary follicles, a decrease in the number of atretic follicles, greater diameters of primary and secondary follicles, and a decrease in granulosa cell nuclear sequestration.

Table 1

HE staining 10X microscopy of the CON, FSH, VP, and VP+FSH groups for each stage of ovarian count

group	Primordial follicle (X±S)	Primary follicle (X±S)	Secondary follicle (X±S)	Atretic follicle (X±S)
CON	85.5±12.5	42.3±4.6	56.1±6.3	21.3±3.2
FSH	70.3±8.5*	34.4±3.3*	87.0±4.8*	15.2±1.6*
VP	105.0±13.9*	30.3±2.6*	37.3±4.6*	35.9±5.3*
VP+FSH	89.2±10.3*	37.8±3.0*	73.5±5.0*	18.5±3.3*

Compared to the control group, intervention with FSH resulted in a slightly lower number of primordial follicles, a slightly lower number of primary follicles, a significantly higher number of secondary follicles, and a significantly lower number of atretic follicles. After VP intervention, the number of primordial follicles was elevated, the numbers of primary and secondary follicles were significantly reduced, and the number of atretic follicles was significantly elevated. After intervention with VP + FSH, there was a slight increase in primordial follicles, a decrease in primary follicles, a significant increase in secondary follicles, and a slight decrease in atretic follicles. *=P < 0.05 vs. follicles at all stages in the CON group.

Immunohistochemistry

In the con group, the expression levels of PYAP and PLATS2 were higher than those of YAP and LATS2, and were mainly expressed in granulosa cells and less in follicular membrane cells and follicular mesenchyme, and the expression levels of PYAP and PLATS2 were significantly higher in primordial and primary follicles than in secondary follicles, while YAP and LATS2, in contrast, were expressed in secondary follicles at higher levels than in primordial and primary follicles.

In contrast to CON, YAP and LATS2 expression levels were significantly increased in the FSH and VP + FSH groups, mainly in follicular granulosa cells, and were significantly higher in secondary follicles than in primordial and primary follicles.

Compared with CON, PYAP and PLATS2 expression levels were significantly increased in the VP group, mainly in follicular granulosa cells, and their expression levels were also significantly enhanced in follicular membrane cells and follicular mesenchyme, whereas YAP expression was significantly decreased in all stages of follicles, and there was a small decrease in LATS2 expression.

Figure 2 b (YAP), c (PYAP), d (LATS2), e (PLATS2) Immunohistochemical optical density value analysis of YAP, PYAP, LATS2, PLATS2 factor expression levels in the CON, FSH, VP, VP + FSH groups under 40X microscopy, compared with CON, YAP, LATS2 expression levels were significantly increased in the FSH and VP + FSH groups. There was a relative decrease in YAP and a small decrease in LATS2 in the VP group. There were relative decreases in PYAP and PLATS2 expression in the FSH vs VP + FSH group and significant increases in PYAP and PLATS2 expression in the VP group. *=P < 0.05 versus the CON group.

YAP expression was significantly higher in the FSH group than in the VP + FSH group and was relatively lower in the VP group than in the CON group. PYAP expression was relatively lower in the FSH group, significantly higher PYAP in the VP group, and relatively higher in the VP + fsh group compared with the CON group. LATS2 expression was relatively higher in the FSH and VP + FSH groups and relatively lower in the VP group than in the CON group. PLATS2 expression was relatively lower in the FSH group, significantly higher in the VP group, and relatively higher in the VP + FSH group than in the CON group.

Discussion

FSH is one of the most important factors in follicle development [4], and FSH acts on the FSHR receptor on the surface of granulosa cells [5] to regulate the growth of granulosa cells and follicle development through several pathways [9, 10].

Morphological observations and the follicle counting results of this study (Fig. 1, Table 1) showed that the number of secondary follicles was significantly increased in the ovaries cultured with FSH intervention compared to the control group, and the arrangement of granulosa cells within the follicles was also neater, with less nuclear fixation of granulosa cells and fewer atretic follicles. These experimental results indicate that FSH can promote follicle development, significantly pushing primary follicles to secondary follicles. Moreover, it plays a protective role in follicle development [37].

In the VP group, there were significant decreases in primary and secondary follicles, a significant increase in atretic follicles, and a significant increase in granulosa cell nuclear fixation relative to the control group. The results showed that VP exerted a significant inhibitory effect on follicle development, inhibiting the development of primordial follicles to primary follicles and inhibiting the development of primary follicles to secondary follicles, leading to more granulosa cells undergoing nuclear sequestration and more follicular atresia. Vp (vetiporfin) is a specific inhibitor of YAP, a factor downstream of the Hippo signalling pathway, which functions mainly by entering the nucleus to interact with the transcription factor TEAD to promote transcription and reproduction. TEADs contain a DNA-binding structural domain but lack an activation structural domain, whereas YAP lacks a DNA-binding domain but contains an activation domain. YAP binds to TEAD to form a YAP-TEAD heterodimeric transcription factor that promotes proliferation, and VP can selectively bind to YAP and inhibit the YAP-TEAD complex [27, 28, 38], thereby disrupting the nuclear transcriptional function of YAP-TEAD. In the VP + FSH group, the inhibitory effect of VP on follicle development was significantly reversed by FSH, with a significant increase in secondary follicles, a decrease in atretic follicles, and a decrease in granulosa cell nuclear sequestration. These results suggest that VP can significantly inhibit follicle development, and the inhibitory effect of VP on follicle development, as a specific inhibitor of YAP, a key factor of the Hippo signalling pathway, is most likely achieved by inhibiting the YAP factor [13]. FSH can significantly reverse the inhibitory effect of VP on follicle development, perhaps by inhibiting the Hippo signalling pathway to promote the expression of YAP.

Immunohistochemical experiments revealed that LATS2 and YAP expression levels were significantly increased and PLATS2 and PYAP expression levels were relatively decreased in the FSH group compared to the CON group. LATS2 is a key molecule in the Hippo pathway cascade reaction, and its phosphorylation is a marker of Hippo pathway activation. Intervention of FSH in ovaries revealed elevated expression of LATS2 and decreased expression of PLAT2. These results suggest that FSH can inhibit the Hippo pathway by suppressing LATS2 phosphorylation and, in turn, the Hippo pathway. [13, 18, 39–42].

In the VP group, PYAP and PLATS2 were significantly increased, while YAP expression was significantly decreased, indicating that VP had a significant inhibitory effect on YAP and a small amount of inhibition

on LATS2. In contrast, in the VP + FSH group, YAP and LATS2 expression levels were significantly increased and PYAP and PLATS2 expression levels were significantly decreased, indicating that FSH can inhibit the Hippo signalling pathway and consequently promote YAP expression, reversing the specific inhibition of YAP by VP.

Past studies have shown that VP only specifically inhibits YAP factors and has little effect on LATS2, which is upstream of YAP in the Hippo signalling pathway [43], but we found that although there was only a small effect on LATS2 factors, VP not only acts on YAP factors in the Hippo signalling pathway only but also has significant effects on PYAP and PLATS. Perhaps the inhibitory effect of VP on YAP not only directly blocks the interaction of YAP with TEAD, thus blocking YAP-driven cell growth, but perhaps also indirectly inhibits YAP expression by causing the phosphorylation of factors upstream of the Hippo signalling pathway, thus blocking the growth-promoting effect of YAP. However, sufficient evidence is lacking, and further studies are needed.

In conclusion, the present study found that FSH, after acting on rat ovaries, promotes follicle development by inhibiting the Hippo signalling pathway and upregulating the expression of YAP, a key factor of the Hippo signalling pathway.

Declarations

Funding

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Conflicts of interest

The authors declare that they have no competing interests.

Ethics approval

This experiment strictly abides by the guidelines for ethical review of laboratory animal welfare issued by the General Administration of quality supervision, inspection and Quarantine of the people's Republic of China and the State Administration of standardization of the people's Republic of China.

Availability of data and material

The data sets supporting the results of this article are included within the article and its additional files.

Authors' contributions

Tairen Chen carried out most of the experiments, analyzed the data, and wrote a manuscript. Yuting Dong assisted in some experiments, editing and revising manuscripts. Mengjing Wu participated in some experiments. Qing Chang and Changchun Hei designed the experiment, supervised the research of all

aspects of the experiment, and guided the interpretation of the data and the revision of the manuscript. All authors read and approved the final manuscript

Consent for publication

Written informed consent for publication was obtained from all participants.

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Figures

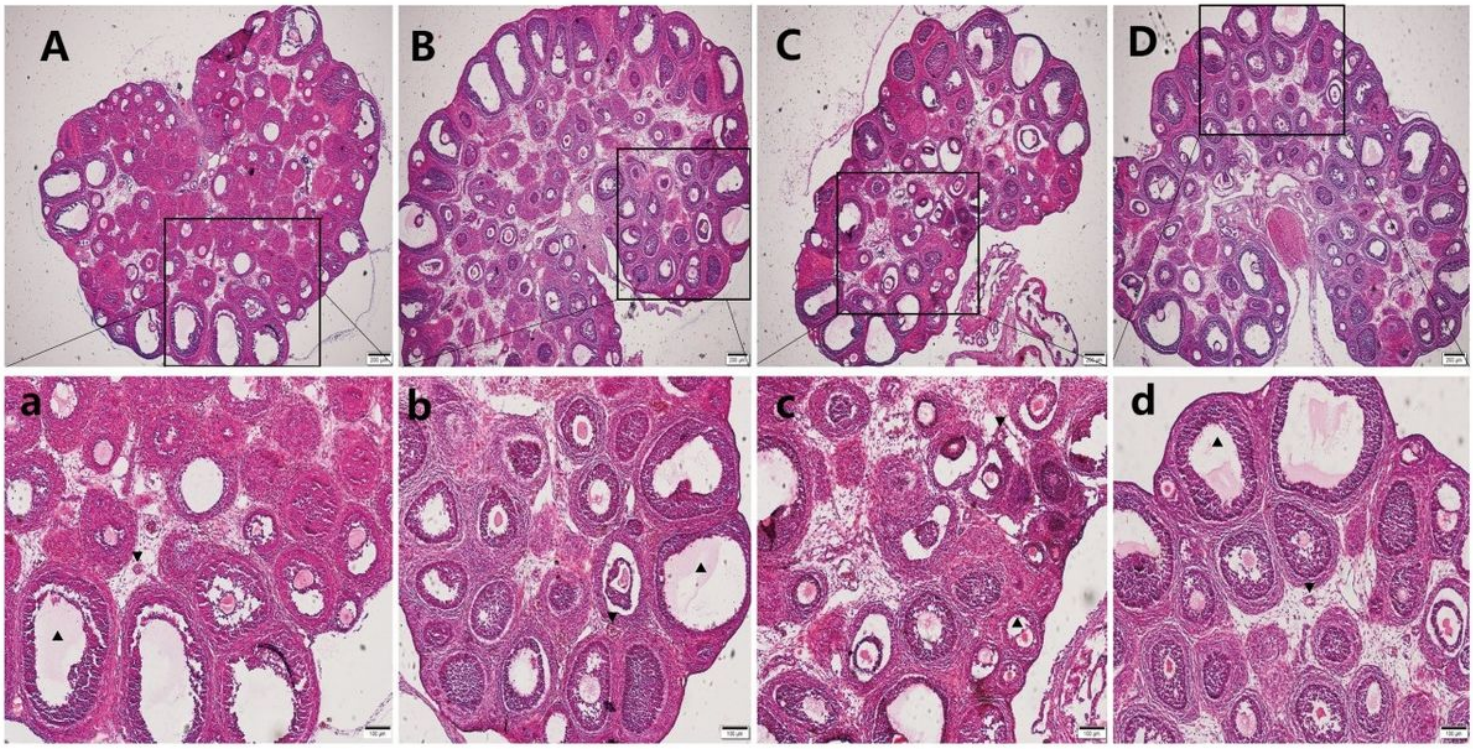

Figure 1

HE staining at 4X vs. 10X microscopic observation in the no intervention group (A,a), FSH (B,b) group, VP (C,c) group, and VP+FSH (D,d) group. ▲ are secondary follicles, ▼ are primary follicles. Bar=200 μm in the 4X plot, bar=100 μm in the 10X plot.

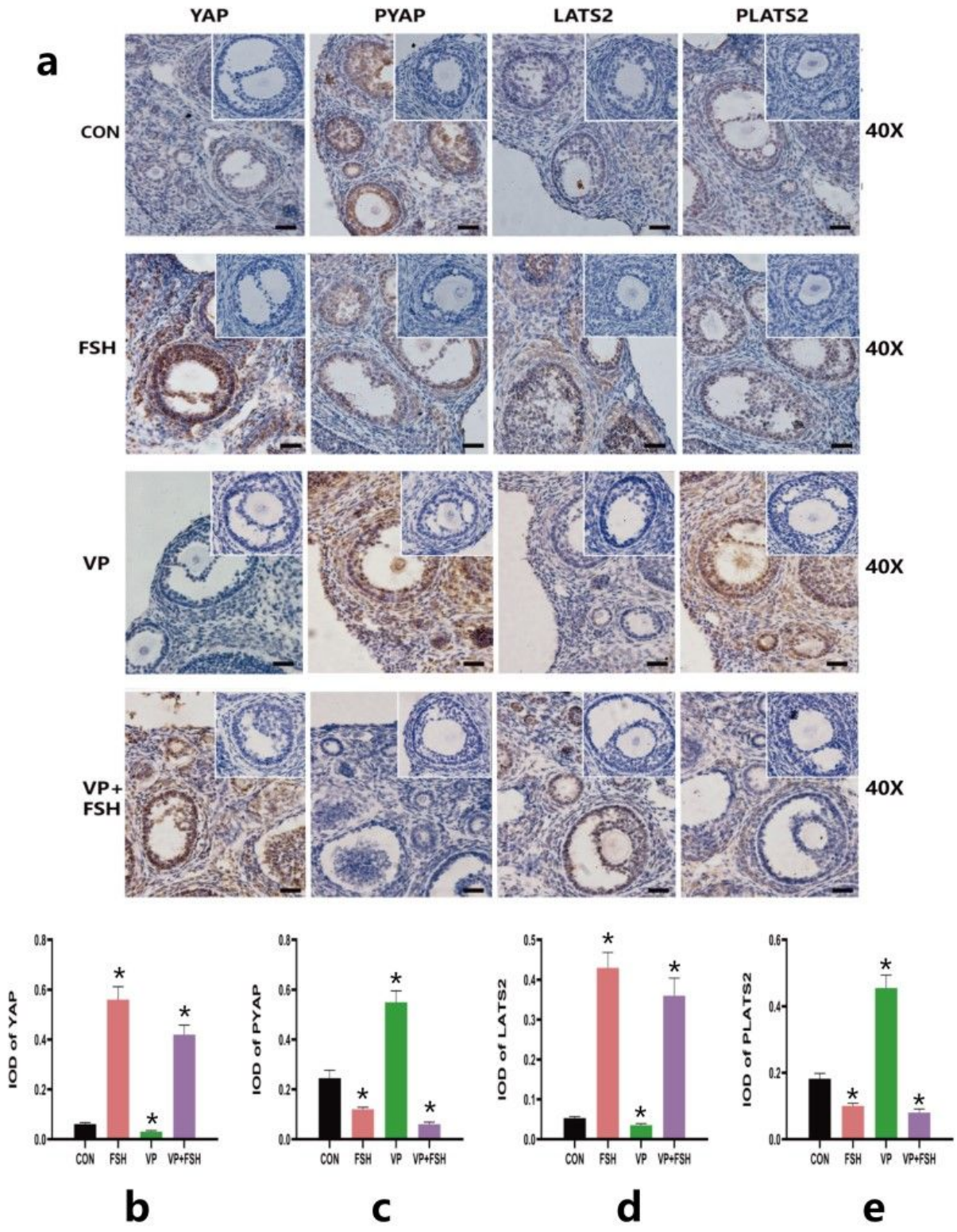

Figure 2

2a, Immunohistochemical staining of ovaries in the CON, FSH, VP, and VP+FSH groups was observed at 40X to evaluate the expression levels of YAP, PYAP, LATS2, and PLATS2 in each group, with positive expression in brown and nuclear haematoxylin staining in blue. The small image in the upper right corner of each large panel is the negative control group.

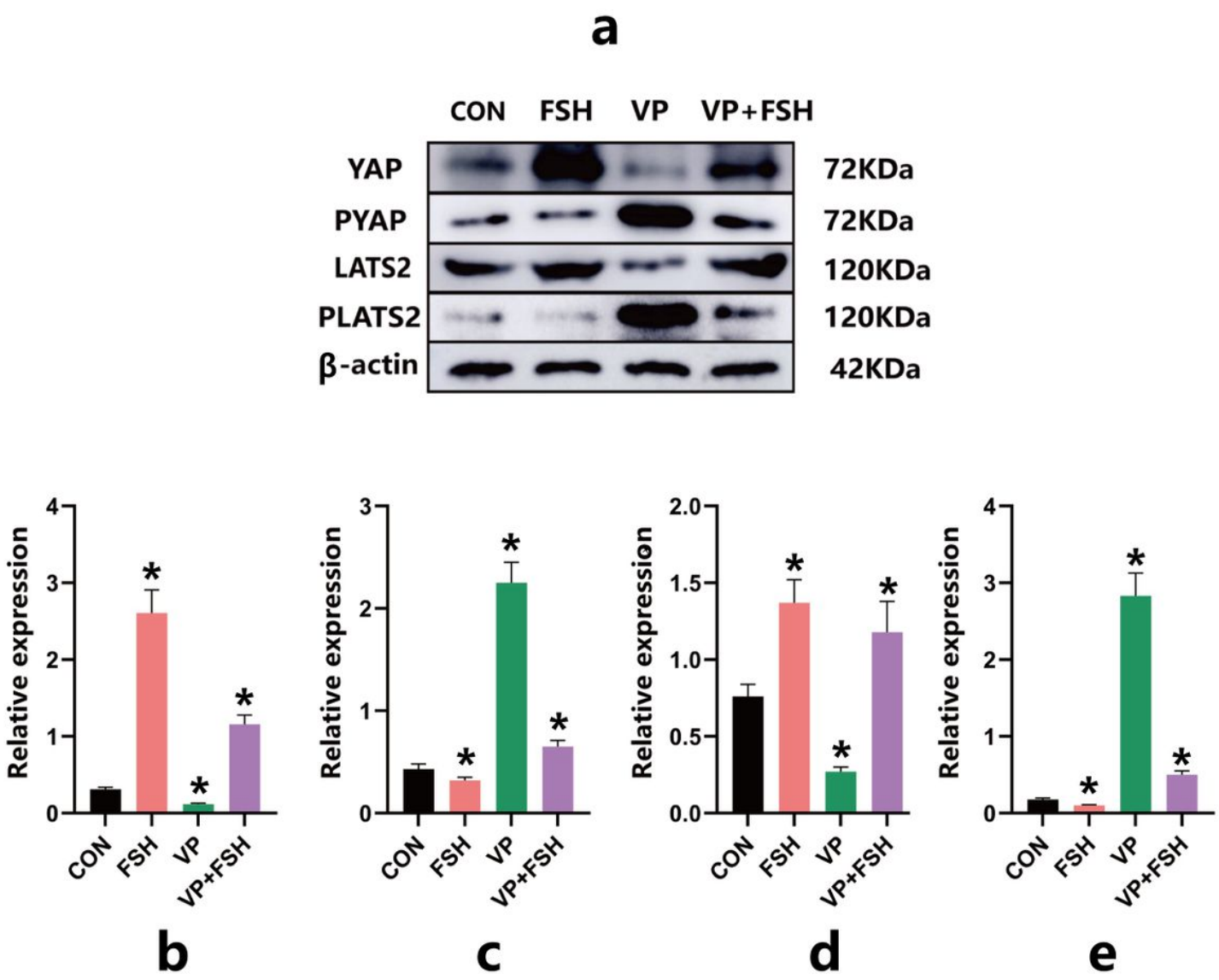

Figure 3

3a, Western blot analysis of YAP, PYAP, LATS2, and PLATS2 expression levels in the CON and FSH, VP, and VP+FSH groups. Figure 3b (YAP), c (PYAP), d (LATS2), e (PLATS2). Relative expression levels of YAP, PYAP, LATS2, and PLATS2 factors in CON and FSH, VP, VP+FSH groups compared with β-actin. β-actin was the internal reference. *=P<0.05 versus the CON group.