CEOG Clinical and Experimental Obstetrics & Gynecology

Effects of apelin on proliferation and apoptosis in rat ovarian granulosa cells

L. Shuang[1], W. Jidong[2], P. Hongjuan[3], Y. Zhenwei[4]

[1] Department of Gynecology and Obstetrics, Suining Central Hospital, Suining
[2] Department of Gynecology and Obsterics, Jinan Central Hospital Affiliated Shandong University, Jinan
[3] Deparment of Gynecology and Obsterics, Zhongshan Hospital, Wuhan
[4] Department of Gynecology and Obstetrics, the First Affiliated Hospital of Chongqing Medical University, Chongqing (China)

Summary

Objective: To investigate the influence of apelin on proliferation and apoptosis in rat ovarian granulosa cells (GCs) in vitro. *Materials and Methods:* Primary culture of SD rat ovarian GCs was cultured with different concentrations of apelin 10^{-8} mol/L, and APJ expression in GCs was inhibited by small RNA interfering (siRNA). Signaling inhibitor LY294002 of PI3K/Akt, HIMO intervention, MTT assay and flow cytometry were combined to observe the cell proliferation and apoptosis; Western blot was used to detect signaling protein expression related with cell apoptosis. *Results:* Compared with the control group, the differences on cell proliferation rate detected by MTT assay in APJ-siRNA group, LY294002 group, and HIMO group had statistical significance ($p < 0.05$). Protein expressions of Bad, Bax, and Foxo3a in GCs in APJ-siRNA group, LY294002 group, and HIMO group were apparently upregulated ($p < 0.05$), and the protein expression of Bcl-2 in APJ-siRNA group, LY294002 group, and HIMO group was obviously downregulated ($p < 0.05$). *Conclusion:* Apelin can promote proliferation of GCs and inhibit apoptosis via PI3/Akt signaling pathway.

Key words: Apelin; Granulosa cells; PI3K/Akt.

Introduction

Polycystic ovary syndrome (PCOS) is a common reproductive endocrine disease, with the clinical features of follicle atresia and chronic anovulatory. Although, there are numerous small follicles in bilateral ovaries of PCOS patients, they cannot produce mature follicles periodically, and the mechanism might be correlated with selection and development of follicles and imbalance of follicle atresia. Apoptosis of granulosa cells (GCs) has an important mechanism for follicles atresia, and extracellular signal, like hormone, growth factor, and cytokine, could result in cell proliferation or apoptosis. Previous research showed that the apelin levels in serum of PCOS patients increased, and might play a part in the pathogenesis of POCS [1-2]. Does the high-level apelin of PCOS patients affect the development of granulose cells? After the small interfering RNA (siRNA) is proposed to inhibit the expression of APJ, signaling inhibitor LY294002 and HIMO intervention were used in cultured granulose cells of apelin to observe the proliferation, Akt-mediated phosphorylation and protein expression of Bax, Bad, Foxo3a, and Bcl-2 in downstream of PI-3K signaling pathway, and to explore the specific mechanism of apelin to GCs in cellular and molecular level.

Materials and Methods

Materials

The sequence of APJ-siRNA was as follows: sense strand (5'-3'): 5'-GAGAACCGAUGCAUGAGAAdTdT-3', antisense strand (3'-5'): 3'-dTdTCUCUUGGCUACGUACUCUU-5'.

Experimental methods

The rat was fed adaptively for two days. Twenty-four hours after subcutaneous injection of pregnant mare serum gonadotropin (PMSG), the rat was killed by cervical dislocation, then the ovaries were removed, and the follicles were punctured by one-ml hollow-jet needle head in serum-free DMED/F12 medium to collect GCs, and they were prepared by blowing gently with Buckytube and passing through 200 mesh stainless steel cell sieve. After eight minutes of centrifuge with the velocity of 1,000 r/minute, the cells were collected by dropping supernatant. DMEM/F12 medium was added into the clusters of GCs in the bottom of centrifuge tube. After microscopic counting, the cells were inoculated into 25-ml culture flask according to 3.0×10^5/ml, and after cell adherence appeared when the cells were pre-cultured in incubator with DMEM/F12 medium (containing 15% fetal calf serum), the hatched temperature of 37°C and 5% CO_2 for 24 hours, the cells were continuously cultured by replacing culture solution. When the cell fusion reached to 80%, 0.1 pancreatin was used for cell dissociation for ten minutes, and the cells were collected by dropping supernatant after eight minutes of centrifuge with the velocity of 1,000 r/minute for passage. After making the slides of cells, HE staining and FSHR immunocytochemical staining were adopted for cell identification, and the operation methods were as the previous literature described [2].

Revised manuscript accepted for publication March 4, 2015

To assess the influence of apelin on proliferation and apoptosis of GCs and Akt protein expression, the GCs were inoculated into 96-well plates according to 2×10^3/hole; when the cell fusion reached to 50%, they were divided into two groups after culturing in serum-free culture for 24 hours: the control group was added with same volume of serum-free culture and the experiment group was added with same volume of apelin 10^{-8} mol/L; the culture solution was removed in both groups at 12, 24, and 48 hours of continuous culture, and 20 μL MTT solution was added in each hole; after another 48 hours of incubation, MTT solution was removed, and 200 μl dimethyl sulfoxide solution was added in each hole, oscillation for ten minutes, then optical density (OD) was determined at 490 nm of wavelength in ELISA to detect the cell proliferation; the cells were extended to culture dish with six-well plates, when the cells infusion reached to 80%, after serum-free culture for 24 hours, the control group was added with same volume of DMEM/F12 medium. While the experiment group, after culturing with apelin 10^{-8} mol/L for 12, 24, and 48 hours, 0.25% trypsin without EDTA was digested to suspension cells for five minutes of centrifuge with the velocity of 2.000 r/minutes and two times of cell rinsing with PBS, then resuspended, five μl Annexin V-FITC and 5 μl propidium iodide were added by well mixing, avoiding light reaction, and the apoptosis rate (each group set up five parallel holes) was detected within one hour by Annexin/PI. After the total cellular protein in both groups was extracted, the Western blot was adopted to determine the protein expression of Akt and p-Akt, and the operation methods were as the previous literature described [2].

Preparation of cell groups transfected by APJ-siRNA transfection reagent was diluted with serum-free DMEM/F12 medium, APJ-siRNA was diluted with RNase-free H_2O, and the above two reagents were mixed into 100 mol/L si-RNA-Lipo2000 for use. The cultured cells were inoculated into 96-well and six-well plates and cultured with DMEM/F12 (15% serum), and four groups were set up for transfection: (1) control group (DMEM/F12), (2) negative control group, (3) siGFP group, (4) APJ-siRNA group, and each group set up six parallel holes. An inverted fluorescence microscope was used to observe the transfection rate. The cell protein was extracted from six-well palates after 48 hours, and the APJ-siRNA could silence the APJ protein expression in GC successfully 48 h after transfection (Figure 1).

Influence of apelin on protein expression related to proliferation and apoptosis of GC treated with transfection and inhibitor the cells were inoculated into cell culture plate with 96-well and six-well plates, and divided into control group, APJ-siRNA group, inhibitor LY294002 group, and inhibitor HIMO group. When the cell infusion reached 40%, the control group was added with same volume of serum-free DMEM/F12 medium; the APJ-siRNA group was added with same volume of siRNA-Lipo2000 (100 nmol/L APJ-siRNA) mixed with serum-free DMEM/F12 medium; the inhibitor LY294002 group was added with LY294002 (ten μmol/L) dissolved by serum-free DMEM/F12 medium; the inhibitor HIMO group was added with same volume of HIMO (ten μmol/L) dissolved by serum-free DMEM/F12 medium. After treatment, the cells were cultured continuously for three hours in cell incubator, and then the four groups were all added with same volume of apelin 10^{-8} mol/L for culture. MTT assay was conducted 48 hours later. The cell proteins were extracted from six-well plates, and the protein levels of Akt, p-Akt, Bad, Foxo3a, Bcl-2, and Bax were detected by Western blot.

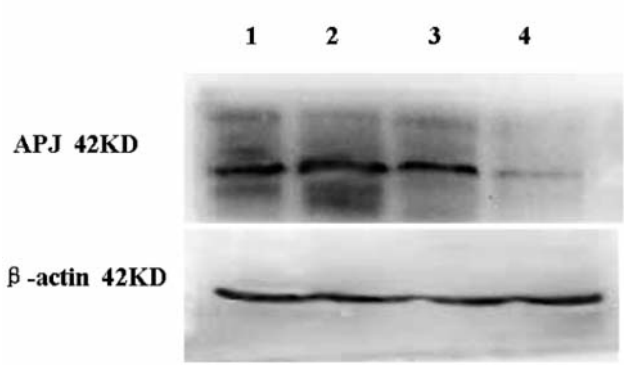

Figure 1. — APJ protein expression in GC 48 hour transfection with siRNA by Western blot. 1: the control group (DMEM/F12); 2: the negative control group; 3: the siGFP group; 4: the APJ-siRNA group.

Statistical analysis

The results were analyzed by SPSS13.0 software; all variables are presented as mean±SD; one-way analysis of variance was adopted in multi-group comparison; *t*-test was applied in the intergroup comparison.

Results

Identification of primary GCs in rat ovary HE staining showed that adherent cells were morphologically integral, with distinct edge, irregular polygons, oval nuclei, blue color, and reddish cytoplasm. FSHR was expressed in ovarian GC specifically and FSHR immunocytochemical staining showed

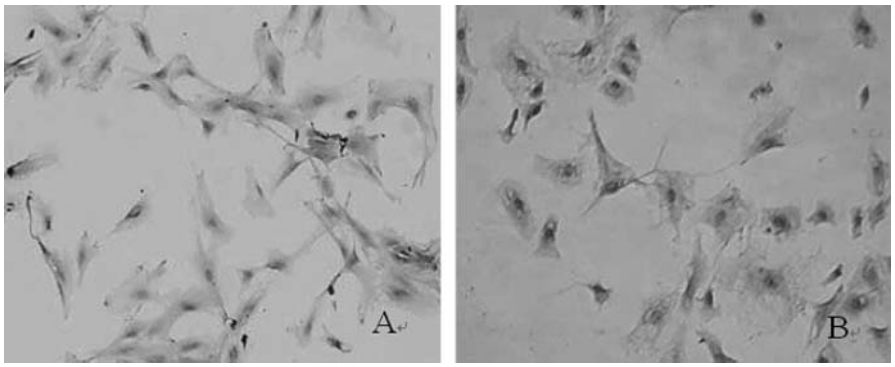

Figure 2. — Identification of primary GCs in rat ovary. A: observation of HE staining (×200); B: observation of immunocytochemical staining (S-P×400).

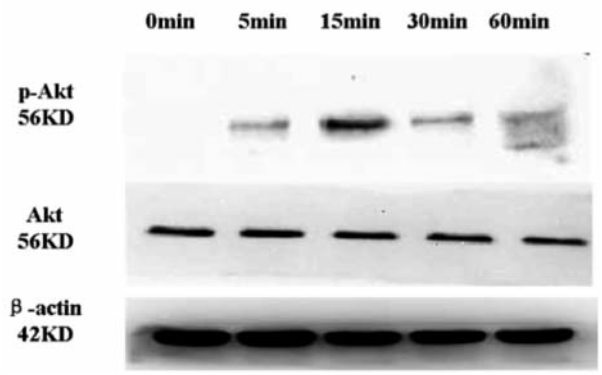

Figure 3. — Influence of apelin on Akt activation in GCs at difference times by Western blot.

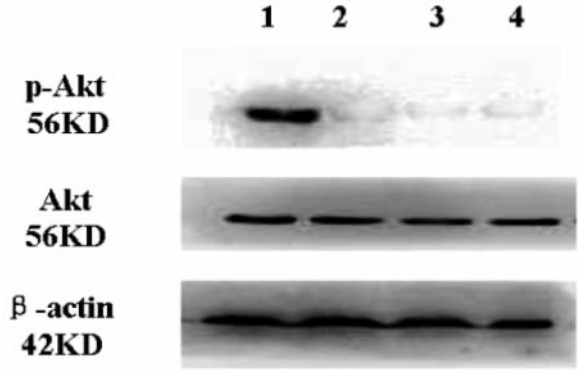

Figure 4. — Influence of apelin on Akt activation with signal inhibitor and siRNA by Western blot. 1: the control group; 2: APJ-siRNA group; 3: inhibitor LY294002 group; 4: inhibitor HIMO group.

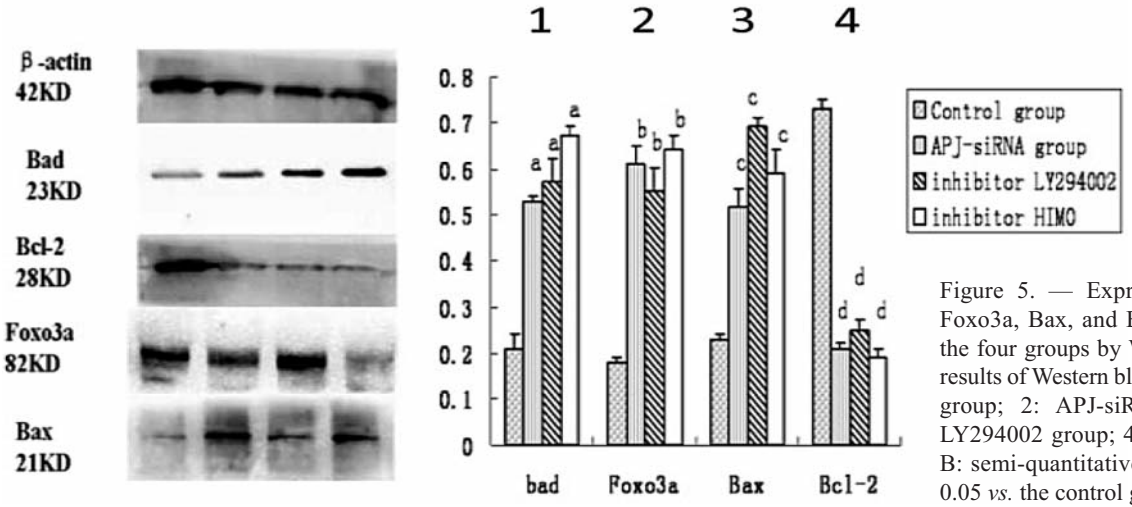

Figure 5. — Expression of Bad, Foxo3a, Bax, and Bcl-2 in GCs in the four groups by Western blot. A: results of Western blot; 1: the control group; 2: APJ-siRNA group; 3: LY294002 group; 4: HIMO group; B: semi-quantitative results; a: $p < 0.05$ *vs.* the control group.

that cytoplasm was medium brown with the positive rate of FSHR >90%, meeting the needs of the experiment (Figure 2).

Influence of apelin on proliferation of ovarian GCs by MTT assay Apelin 10^{-8} mol/L promoted the proliferation of GCs, with the proliferation rate was more than twice as high as the control group. OD values at 12, 24, and 48 hours in the experiment group were 0.30 ± 0.03, 0.58 ± 0.06, and 0.64 ± 0.02, respectively, compared with the control group at the same time (0.13 ± 0.02, 0.33 ± 0.07, and 0.35 ± 0.02 respectively), the difference was statistically significant ($p < 0.05$).

Influence of apelin on ovarian GCs by Annexin/PI detection Apelin 10^{-8} mol/L inhibited the apoptosis of GCs, the apoptosis rate of GCs at 12, 24, and 48 hours was 8.15 ± 0.43%, 7.16 ± 0.86%, and 6.07 ± 0.33%, respectively, in the experiment group, and 8.85 ± 0.57%, 10.24 ± 0.25%, and 15.43 ± 0.85%, respectively, in the control group. The difference between the two groups at the same time had statistical significance ($p < 0.05$).

Influence of apelin on Akt phosphorylation of PI-3K/Akt signaling pathway in GCs Apelin 10^{-8} mol/L began to activate the signaling protein at five minutes on GCs, phosphorylated Akt expression reached peak at 15 minutes, which showing the activation of PI3K/Akt signaling pathway; after treatment of APJ-siRNA, inhibitor LY294002, and HIMO, phosphorylated Akt protein did not express in the experiment group, while the control group it had positive expression, which showing that apelin promoted the phosphorylation of PI3K/Akt, but it was cancelled by PI3K inhibitor LY294002 and Akt inhibitor HIMO and APJ-siRNA (Figures 3, 4).

Analysis of cell proliferation after intervention included five visions of inverted fluorescence microscope to observe GSs transfected by fluorescence labeled siRNA at 12, 24, and 48 hours; no morphological abnormality was observed in all groups, the green fluorescence could be observed in the cells with successful transfection and the transfection

rate reached the peak of 80% at 48 hours. The OD values in the APJ-siRNA group, the inhibitor LY294002 group, and the inhibitor HIMO group were 0.34 ± 0.03, 0.36 ± 0.01, and 0.38 ± 0.04, respectively, which compared with the control group 0.61 ± 0.03, the differences had statistical significance ($p < 0.05$), suggesting APJ-siRNA, inhibitor LY294002, and inhibitor HIMO all could inhibit the effect of apelin on promotion of cell proliferation, especially the APJ-siRNA had the most obvious effect, but there was no statistically significant difference ($p > 0.05$) compared with the other two groups.

Analysis of protein expression after intervention compared with the control group showed that the differences on relative expression quantity of Bad protein, Bax protein, Foxo3a protein, and Bcl-2 protein in the APJ-siRNA group, the inhibitor LY294002 group, and the inhibitor HIMO group all had statistical significance ($p < 0.05$) (Figure 5).

Discussion

Apelin is a new peptide that was isolated from bovine stomach in 1998 by Tatemoto, and its gene is located on the human X chromosome. APJ, as apelin receptor, is lonely G-protein coupled receptor, consisting of 377 amino acid residues and seven spiral transmembrane proteins, and it belongs to the proteins associated with angiotensin type l receptor; moreover apelin/APJ system is widely distributed in the central nervous system and peripheral tissues [3]. Apelin is not only a vasoactive polypeptide, but also an adipose- derived hormone and neurotransmitter, involved in the progress of many diseases, like hypertension, atherosclerosis, cardiovascular diseases, diabetes mellitus, obesity, and so on [3-5]. The study shows that apelin promotes the proliferation of osteoblasts and vascular smooth muscle cells,and inhibits cell apoptosis and retina angiogenesis [6-8]. Previous study found that apelin/APJ system was expressed in ovarian GCs and theca-interstitial cells of PCOS rats abnormally [2]. Abnormal proliferation and apoptosis of GCs in follicles might generate numerous follicles into recruitment, and many antral follicles develop into PCOS, which is the pathological basis for development of multiple follicles [9]. The study further reports that apelin 10^{-8} mol/L can promote the anti-apoptotic effect of ovarian GCs in vitro of rats, and its proliferative effect and pro-apoptotic effect are time-dependent. The proliferative effect of apelin on brain microvascular endothelial cells of rats is enhanced with increasing concentration, while the proliferative effect of cells has no further increase when the concentration is more than ten ng/ml, considering ten ng/ml as the acceptable highest physiological concentration of cells [10], therefore, the proliferative effect and anti-apoptosis effect of apelin 10^{-8} mol/L can be taken as follow-up study.

Apelin promotes the proliferation of rat GCs in vitro with unclear mechanism. Recent discovery showed that apelin could induce the phosphorylation of signaling protein PI3K/Akt in vascular smooth muscle cells, promote proliferation, and PI-3K signaling inhibitor LY294002 and Akt signaling inhibitor 1701-1 could inhibit the proliferative effect of apelin significantly; hence the study concluded that the proliferative effect of apelin on vascular smooth muscle cells might be related with PI3K/Akt signaling pathway [7]. Another study indicated that apelin promoted proliferation of osteoblasts and inhibited cell apoptosis via PI3K/Akt signaling pathway, and the signaling inhibitor LY294002 and HIMO could inhibit the proliferative effect of apelin on osteoblasts [6].

PI-3K is a important member in super-family signal transduction pathway of growth factor, insulin, and insulin-like growth factors: both could change conformation of PI3K, generate kinase with the effect of second messenger in cytomembrane, and make protein signaling phosphorylated AKT (p-Akt) in downstream. a-Akt promotes cell proliferation and differentiation by inhibiting release of mitochondrial protein, regulating cell circle and so on. It was found that p-Akt could be detected five minutes after apelin was inoculated into cells, and reached to peak at 15 minutes. Further study showed that the proliferative effect of apelin was inhibited by APJ-siRNA, LY294002, and HIMO, as well as the phosphorylation of Akt was canceled. The above suggests after combining with receptor APJ, apelin promotes the proliferation of GCs via PI3K/Akt, and its proliferative effect can be inhibited by APJ-siRNA, LY294002, and HIMO.

Anti-apoptotic protein/ pro-apoptotic protein ratio determines the cell proliferation and apoptosis directly [11, 12]. A study suggested that Foxo3a was closely related to the early development and atresia of follicle [13]. It was found that the expressions of transcriptional regulatory factor Foxo3a released by GCs were higher in APJ-siRAN group, inhibitor LY294002 group, and HIMO group, which compared with the control group, the differences had statistical significance. Foxo3a is a number of the forkhead family transcription factors. Phosphorylated AKT can phosphorylate Foxo3a directly and be transferred to cytoplasm from cell nucleus with loss of transcriptional activity after combining with 14-3-3 protein, Foxo3a in cell nucleus can inhibit cell cycle protein to induce cell cycle arrest at G0/G1 period, and overexpression can also induce cell apoptosis [14, 15]. Pro-apoptotic effect of Bad in cells is mainly combined with Bacl-2 and Bacl-xL to form heterodimer; when activated, Akt phosphorylates the Ser136 in Bad, it generates the combination of 14-3-3 protein and Bad, inhibits Bad, and induces cell apoptosis [16, 17]. The study found that Bad protein expression in GCs in the experiment group was obviously higher than that in the control group, demonstrating APJ-siRNA 002, inhibitor LY294002, and HIMO all inhibit anti-apoptotic effect of apelin in GCs. In the process of programmed cell death, Bcl-2, as anti-apoptotic gene, and Bax, as pro-apoptotic genes play important role;

the former can inhibit various induction factors of cell apoptosis including free oxygen radicals, P53. and so on, while the latter can induce cell apoptosis mediated by interleukin-3. The study found that Bax protein was highly expressed in the experiment group, while the Bcl-2 was expressed significantly higher in the experiment group than that in the control group ($p < 0.05$), suggesting apelin can inhibit apoptosis in GCs in vitro, but APJ-siRNA, inhibitor LY294002 and HIMO all can cancel the anti-apoptotic effect of apelin on GCs.

Based on the above results, it is speculated that combined with APJ, high-expression of apelin in PCOS rats might promote the proliferation of GCs in follicles and inhibit apoptosis via PI3/Akt signaling pathway, and the apoptosis of GCs in small antral follicles is inhibited and follicles atresia cannot occur in time to create polycystic ovaries. Nevertheless, PCOS is an enigmatic reproductive endocrine disease. What is the influence of apelin on endocrine function of GCs? Is PI3K pathway is the only access for apelin to promote the proliferation of GCs? In-depth exploration has not yet been studied in the present study, therefore the apelin/APJ system and PCOS disease are worthy of further investigation.

References

[1] Cekmez F., Cekmez Y., Pirgon O., Canpolat F.E., Aydinöz S., Metin Ipcioglu O., *et al.*: "Evaluation of adipocytokines and insulin resistance inadolescents with polycystic ovary syndrome". *Eur. Cytokine Netw.*, 2011, *22*, 32.

[2] Luo S.,Wang J.D., Pan H.J., Hu X.J, Yao Z.W.: "Expression of Apelin/APJ in polycystic ovary of model rats". *Reproduction and Contraception*, 2011, *31*, 503.

[3] Tatemoto K., Hosoya M., Habata Y., Fujii R., Kakegawa T., Zou M.X., *et al.*: "Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor". *Biochem. Biophys. Res. Commun.*, 1998, *251*, 471.

[4] Kleinz M.J., Davenport A.P.: "Emerging roles of apelin in biology and medicine". *Pharmacol. Ther.*, 2005, *107*, 198.

[5] Krist J., Wieder K., Klöting N., Oberbach A., Kralisch S., Wiesner T., *et al.*: "Effects of weight loss and exercise on apelin serum concentrations and adipose tissue expression in human obesity". *Obes. Facts*, 2013, *6*, 57.

[6] Xie H., Tang S.Y., Cui R.R., Huang J., Ren X.H., Yuan L.Q., *et al.*: "Apelin and its receptor are expressed in human osteoblasts". *Regul. Pept.*, 2006, *134*, 118.

[7] Liu C., Su T., Li F., Li L., Qin X., Pan W., et al.: "PI3K/Akt signaling transduction pathway is involved in rat vascular smooth muscle cell proliferation induced by apelin-13". *Acta Biochim. Biophys. Sin. (Shanghai)*, 2010, *42*, 396.

[8] Kasai A., Ishimaru Y., Kinjo T., Satooka T., Matsumoto N., Yoshioka Y., *et al.*: "Apelin is a crucial factor for hypoxia-induced retinal angiogenesis". *Arterioscler. Thromb. Vasc. Biol.*, 2010, *30*, 2182.

[9] Matsuda F., Inoue N., Manabe N., Ohkura S.: "Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells". *J. Reprod. Dev.*, 2012, *58*, 44.

[10] Olszanecka-Glinianowicz M., Madej P., Nylec M., Owczarek A., Szanecki W., Skałba P., *et al.*: "Circulating apelin level in relation to nutritional status in polycystic ovary syndrome and its association with metabolic and hormonal disturbances". *Clin. Endocrinol. (Oxf.)*, 2013, *79*, 238.

[11] Shimojo N., Jesmin S., Zaedi S., Soma M., Maeda S., Yamaguchi I., *et al.*: "Changes in important apoptosis-related molecules in the endothelin-1-induced hypertrophied cardiomyocytes: effect of the pretreatment with eicosapentaenoic acid". *Exp. Biol. Med. (Maywood)*, 2006, *231*, 932.

[12] Dorn G.W. 2nd.: "Apoptotic and non-apoptotic programmed cardiomyocyte death in ventricular remodelling". *Cardiovasc. Res.*, 2009, *81*, 465.

[13] Matsuda F., Inoue N., Maeda A., Cheng Y.A.; Sai T., Gonda H., *et al.*: "Expression and function of apoptosis initiator FOXO3 in granulosa cells during follicular atresia in pig ovaries". *J. Reprod. Dev.*, 2011, *57*, 151.

[14] Shen M., Lin F., Zhang J., Tang Y., Chen W.K., Liu H.: "Involvement of the up-regulated FoxO1 expression in follicular granulosa cell apoptosis induced by oxidative stress". *J. Biol. Chem.*, 2012, *287*, 25727.

[15] Karman B.N., Basavarajappa M.S., Hannon P., Flaws J.A.: "Dioxin exposure reduces the steroidogenic capacity of mouse antral follicles mainly at the level of HSD17B1 without altering atresia". *Toxicol. Appl. Pharmacol.*, 2012, *264*, 1.

[16] Parborell F., Abramovich D., Irusta G., Tesone M.: "Angiopoietin 1 reduces rat follicular atresia mediated by apoptosis through the PI3K/Akt pathway". *Mol. Cell Endocrinol.*, 2011, *343*, 79.

[17] Yoo N.J., Kim M.S., Lee S.H.: "Expression and mutation analyses of Fas, FLIP and Bcl-2 in granulosa cell tumor of ovary". *Tumori*, 2012, *98*, 118e.

Address reprint requests to:
W. JIDONG, M.D.
Department of Gynecology and Obstetrics
Jinan Central Hospital
Affiliated Shandong University
105 Jiefang Road
Jinan 250013 (China)
e-mail: obster_jd@163.com