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Original Article

Effects of DMBG on Akt/HIF-1a signaling in the ovaries of polycystic ovary syndrome rats

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Abstract: Dimethylbiguanide (DMBG) is widely used to improve polycystic ovary syndrome (PCOS), but the precise mechanism remains unclear. The present study was conducted to examine the expression and contribution of protein kinase B (PKB/Akt)/hypoxia-inducible factor (HIF)-1a signaling during the development and treatment of PCOS. The results showed that HIF-1a mainly expressed in the granulosa cells of ovarian follicles and DMBG reversed PCOS-induced decrease of HIF-1a expression in the ovary. Further analysis found that endothelin (ET)-2, a HIF-1a target gene, expressed in same pattern with HIF-1a, indicating HIF-1a/ET-2 may play an important role in PCOS. Furthermore, the expression of Akt, a HIF-1a upstream gene, was also examined and then found that Akt expression decreased in PCOS ovaries and then increased after DMBG treatment. Interestingly, the expression of phosphorylated Akt (pAkt^{Thr308} and pAkt^{Ser473}) was similar with the change of Akt expression. Together, Akt/HIF-1a signaling pathway in the granulosa cells of ovaries was inhibited or damaged in some degree during the PCOS development, which could be reversed by DMBG drug intervention, suggesting this Akt/HIF-1a-mediated ET-2 signaling pathway may be an important mechanism regulating PCOS formation and treatment in mammalian ovaries *in vivo*, which should be a new clinical target for PCOS prevention and treatment in the future.

Keywords: Dimethylbiguanide, protein kinase B, hypoxia-inducible factor-1a, endothelin-2, polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is a major health problem in reproductive-aged women worldwide, which was first reported by Stein and Leventhal in 1935. In clinical practice, 75% of women with PCOS suffer from anovulation infertility [1-3], and 50% of them experience recurrent pregnancy loss [4-7], but the precise mechanism remains unclear. Our previous studies have indicated that hypoxia inducible factor (HIF)-1a-mediated endothelin (ET)-2 signaling plays a key role in the mammalian ovarian ovulation [8-13], which may also be an important mechanism regulating this disease. However, no any report was found at present about the expression changes of protein kinase B (Akt/PKB)/HIF-1a in the ovary of PCOS rats after dimethylbiguanide (DMBG) treatment. Clinically, DMBG is widely used to improve PCOS symptoms [11, 14-19], break the vicious cycle of the PCOS endocrine environment, and correct the high LH levels and hyperandrogenism [2, 3, 5-7, 16-18], but the mechanism of its action remains unknown.

Considering the action of HIF-1a/ET-2 signaling in mammalian ovulation, the present study used a PCOS rat model induced by letrozol to examine the expression changes and the possible role of Akt/HIF-1a signaling during the development and treatment of PCOS, based on our previous reports [8-13, 20-22]. In addition, the present study will shed light on the PCOS pathophysiological mechanism, and provide a new target for future clinical PCOS prevention and treatment.

Materials and methods

Animals

Sprague-Dawley rats were purchased from Wushi Experimental Animal Supply Co. Ltd.

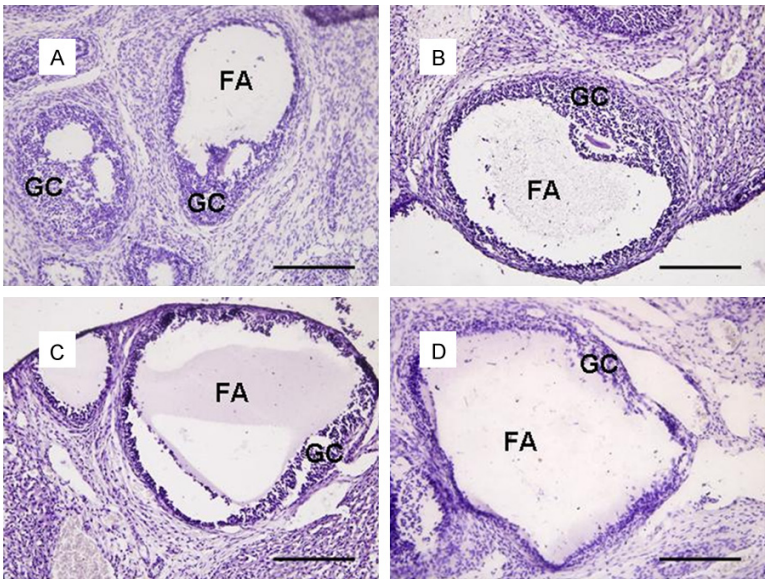


Figure 1. Ovarian histological examination. After fixation, the ovaries from each rat were embedded in paraffin, and 5-μm sections were cut and mounted on slides. The follicles develop normally in the control group (A and B), while PCOS ovaries contained many follicular cysts with degrading granulosa cells in the very thin layer of granulosa cells (C and D). (A) blank control group, (B) Vehicle control group, (C and D) PCOS group. GC: granulosa cell, AF: antral follicles, bar =100 μm.

(Fuzhou, China). The animals were maintained under a 14-h light/10-h dark schedule with continuous supply of chow and water. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee, Fujian Normal University.

Experimental design

Six-week-old female rats with two consecutive 4-day estrous cycles were randomly divided into three groups, including blank control group, vehicle control group and PCOS model group. PCOS was induced by i.g. 1 mg/kg/day letrozole dissolved in 1% caboxy methyl cellulose (CMC, 2 ml/kg) for 21 days, while the vehicle control was administered with the equal CMC. The rat model of PCOS was confirmed by vaginal smear and ovarian histology. Then, PCOS rats were subsequently received 300 mg/kg DMBG (Shanghai Sangon Biotech Ltd., Shanghai, China) for 4 weeks. The left ovary of the experimental animals was fixed and used for immunohistochemistry, the right ovary was frozen and used for detecting the expressions of functional proteins. The experiment was repeated two times.

Immunohistochemistry of HIF-1a, ET-2 and Akt

After fixation, the ovaries from each rat were embedded in paraffin, and 5-μm sections were cut and mounted on slides. The sections were then processed for immunohistochemical analysis with anti-HIF-1a antibody (1:500, Abcam, Cambridge, MA, USA), anti-ET-2 antibody (1:500, Abcam, Cambridge, MA, USA), anti-Akt antibody (1:500, Cell Signaling Technology, Beverly, MA, USA), anti-pAkt^{Thr308} antibody (1:500, Cell Signaling Technology, Beverly, MA, USA) and anti-pAkt^{Ser473} antibody (1:500, Cell Signaling Technology, Beverly, MA, USA). The sections were incubated at room temperature overnight with primary antibody. The immunoreactivity of the specific protein was visualized by the Elite ABC kit (BioGenex, San Ramon, CA, USA). Then the sections were counter-stained with hematoxylin and mounted with cover slips to identify the structure and types of cells in the rat ovary. The negative control used serum (Boster Biological Technology, Wuhan China) instead of primary antibody, and these slides were used for histological examination.

Western blot analysis of HIF-1a, ET-2 and Akt proteins

The protein expressions were examined by Western blot for for ET-2 with anti-ET-2 antibody (1:1000, Abcam, Cambridge, MA, USA), Akt with anti-Akt antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA), pAkt^{Thr308} with anti-pAkt^{Thr308} antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA), pAkt^{Ser473} with anti-pAkt^{Ser473} antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA) and -actin with anti-actin antibody (1:5000, Santa Cruz, Shanghai, China). The detailed process was described as our previous reports [8-13].

Statistics

Data are presented as the means ± SE. The significance of differences in mean values within

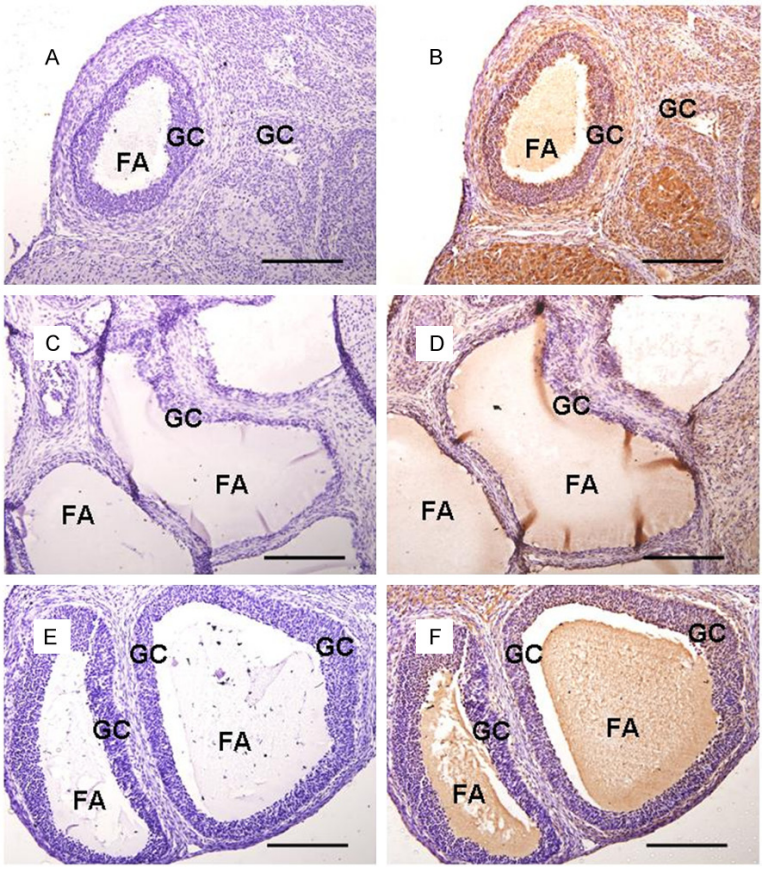


Figure 2. HIF-1a Immunohistochemistry in the ovaries of PCOS rats. Ovarian sections were immunostained for HIF-1a and counterstained with hematoxylin. The follicles develop normally in the control group (A and B), while PCOS ovaries contained many follicular cysts with degrading granulosa cells in the very thin layer of granulosa cells (C and D). DMBG rescued granulosa cells and follicular development (E and F). The HIF-1a immunohistochemical signals appear brown and the background counterstaining appears blue (B, D and F). Negative controls remained unstained, lacking primary antibody instead of serum (A, C and E). (A and B) control group, (C and D) PCOS group, (E and F) DMBG-treated PCOS group. GC: granulosa cell, AF: antral follicles, bar =100 μm.

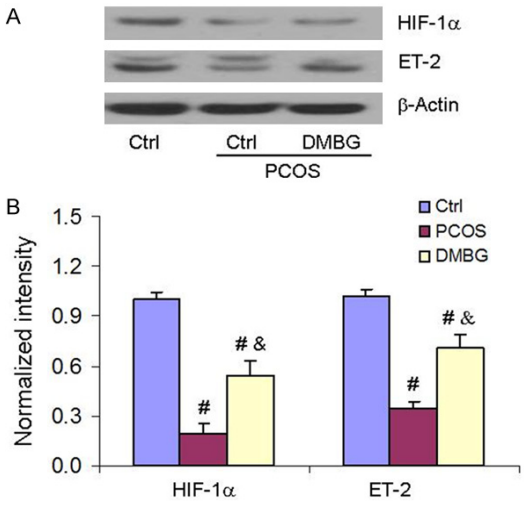


Figure 3. HIF-1a and ET-2 expressions in the ovaries of PCOS rats. Panel A: Representative ECL gel images of western blot analyses depicting the HIF-1a and ET-2 protein levels. Panel B: Summarized intensities of HIF-1a and ET-2 blots normalized to the control. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. #: $P<0.05$, v.s. control group, &: $P<0.05$, v.s. PCOS group.

and between multiple groups was evaluated using one-way ANOVA, followed by a Tukey's multiple range test. $P<0.05$ was considered statistically significant. # and & denote significant values ($P<0.05$).

Results

Ovarian histological examination

The present study first confirmed the PCOS rat model through histological examination of each ovary. Follicular development was normal in the control group (**Figure 1A** and **1B**), while there were many follicular cysts with degrading granulosa cells in the very thin level of granulosa cells (**Figure 1C** and **1D**). These results demonstrated that the PCOS rat model was developed successfully via letrozole induction.

HIF-1a immunohistochemistry in the ovaries from PCOS rats

Our previous studies indicated that HIF-1a plays an important role in follicular development and ovulation [8-10, 20]. The present study therefore measured HIF-1a protein expression in ovaries from each group by immunohistochemistry (**Figure 2B**, **2D** and **2F**). HIF-1a was mainly expressed in the granulosa cells (**Figure 2A** and **2B**), and its expression level decreased in PCOS rats compared with the controls (**Figure 3C** and **3D**), while the big follicles existed and increased

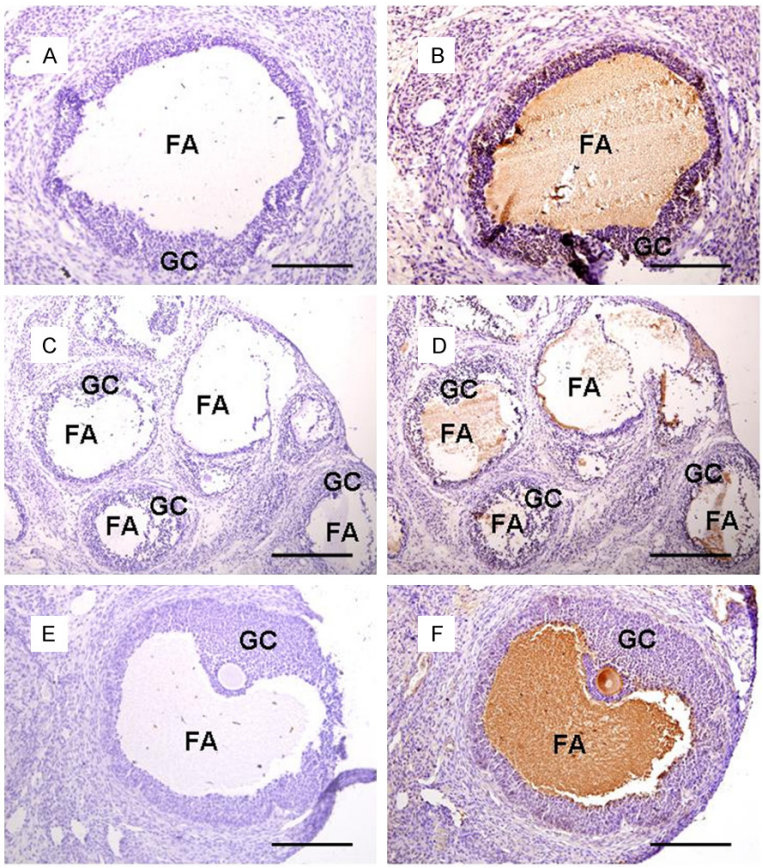


Figure 4. Akt Immunohistochemistry in the ovaries of PCOS rats. Akt immunohistochemical signals appear brown and the background counterstaining appears blue (B, D and F). Negative controls remained unstained, lacking primary antibody instead of serum (A, C and E). (A and B) control group, (C and D) PCOS group, (E and F) DMBG-treated PCOS group. GC: granulosa cell, AF: antral follicles, bar =100 μm.

(**Figure 2C** and **2D**). Interestingly, DMBG rescued HIF-1a expression and follicular development in the ovaries of PCOS rats (**Figure 2E** and **2F**), implying that HIF-1a is involved in PCOS development and DMBG treatment.

HIF-1a and ET-2 protein expression in the ovaries from PCOS rats

To confirm HIF-1a immunohistochemical results, the present study also examined HIF-1a protein expression by western blot (**Figure 3**). In PCOS rats, HIF-1a expression significantly decreased (**Figure 3**), while DMBG reversed this decrease (**Figure 3**). To further understand the possible role of HIF-1a in PCOS, the expression of the HIF-1a target gene ET-2 was also examined in the ovaries of each group (**Figure 3**). ET-2 protein expression decreased in PCOS rats (**Figure 3**), similar to HIF-1a expression (**Figure 3**). In DMBG-treated PCOS rats, ET-2 expression increased (**Figure 3**) compared to the PCOS group (**Figure 3**), indicating that ET-2 may also be involved in PCOS development and DMBG treatment. These results further indicated that the HIF-1a/ET-2 signaling pathway participated in PCOS development and DMBG treatment.

Akt immunohistochemistry in the ovaries from PCOS rats

Given HIF-1a is one target protein of Akt signaling, the present study further examine the expression of Akt and pAkt in each ovary. The results indicated Akt also expression in the granulosa cells of ovarian follicles (**Figure 4A** and **4B**), while a decreased expression of Akt was found in the ovaries of PCOS rats (**Figure 4C** and **4D**) and DMBG could rescue its expression in some degree (**Figure 4E** and **4F**). Akt expression pattern is similar with HIF-1a expression (**Figure 2**), suggesting Akt is also involved in PCOS development and DMBG treatment.

Activation of Akt signaling is mainly through $pAkt^{Thr308}$ and $pAkt^{Ser473}$, the present experiment therefore detected the expression of $pAkt^{Thr308}$ (**Figure 5**) and $pAkt^{Ser473}$ (**Figure 6**) via immunohistochemistry. Interestingly, the expressions of $pAkt^{Thr308}$ and $pAkt^{Ser473}$ were consistent with Akt (**Figure 4**) and HIF-1a (**Figure 2**) expression. The expression of $pAkt^{Thr308}$ was inhibited in PCOS group (**Figure 5C** and **5D**) compared with the control group (**Figure 5A** and **5B**) and DMBG partly rescued its expression (**Figure 5E** and **5F**), while a signifcant decrease of $pAkt^{Ser473}$ expression was found in PCOS group and an obvious increase of $pAkt^{Ser473}$ expression was found in DMBG-treated PCOS group (**Figure 6**), implying Akt may participate in the regulation of PCOS symptoms via HIF-1a signaling.

Akt protein expressions in the ovaries from PCOS rats

To confirm Akt immunohistochemical results, the present study also examined Akt, $pAkt^{Thr308}$

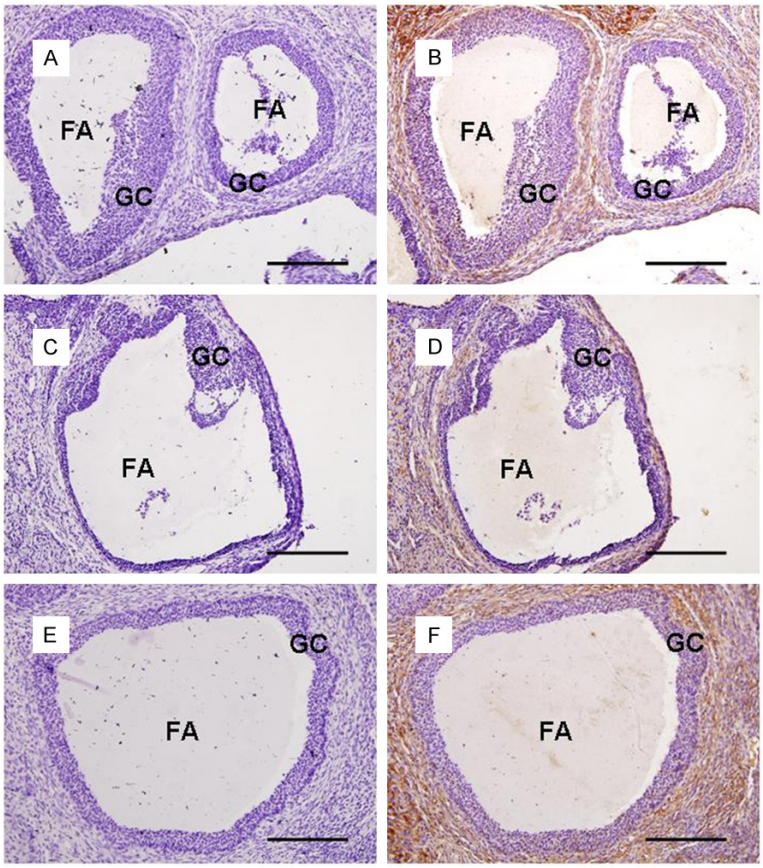


Figure 5. pAkt^{Thr308} Immunohistochemistry in the ovaries of PCOS rats. Akt-^{Thr308} immunohistochemical signals appear brown and the background counterstaining appears blue (B, D and F). Negative controls remained unstained, lacking primary antibody instead of serum (A, C and E). (A and B) control group, (C and D) PCOS group, (E and F) DMBG-treated PCOS group. GC: granulosa cell, AF: antral follicles, bar =100 μm.

and pAkt^{Ser473} protein expressions by western blot (**Figure 7**). In PCOS rats, all of Akt, pAkt-^{Thr308} and pAkt^{Ser473} expressions significantly decreased (**Figure 7**), while DMBG reversed these decreases (**Figure 7**). These results further indicated that the Akt/HIF-1a signaling pathway participated in PCOS development and DMBG treatment.

Discussion

The present results clearly demonstrated that Akt and HIF-1a expressed in the granulosa cells of PCOS ovaries and DMBG rescued the decreased expressions of Akt and HIF-1a in PCOS ovaries in some degree, suggesting that Akt/HIF-a signaling may play an important role in ovarian dysfunction in PCOS rats, especially ovulatory failure.

It is well-known that PCOS is characterized by hyperandrogenism, ovulatory process dysfunction and polycystic ovaries [22, 23], but the precise pathogenesis of PCOS still remains unclear, although it is usually diagnosed during the early reproductive years, but still occurs in approximately 4% to 18% of reproductive-aged women [24, 25]. Therefore, a PCOS rat model was used in the present experiment to investigate the expression and contribution of Akt and HIF-1a during PCOS development and treatment. The results of ovarian histology confirmed PCOS model developed successfully and further found many follicular cysts in PCOS ovaries. Our previous studies have indicated HIF-1a participated in the process of follicular growth, development and ovulation [8-13, 20, 26-28]. The specific expression of HIF-1a in the ovarian granulosa cells may be involved in the change of oxygen supply [10, 29] and take part in the regulation of ovarian functions [8-13, 20], which is related with decreased oxygen pressure of the intrafollicle microenvironment during the follicular development [9, 29-31]. But about the mechanism of HIF-1a regulating PCOS development is still unknown, since current researches have shown that HIF-1a/ET-2 signaling regulates ovulation [8-13, 20, 29], which may play an important role in PCOS development and regulation.

Recently, our research has already showed the expression and clinical significance of the HIF-1a/ET-2 signaling pathway during the development and treatment of PCOS, but the regulatory mechanism needed further investigation, such as the role of Akt in PCOS. Given HIF-1a is a target protein of Akt signaling pathway [11-13, 20, 28], which may also be involved in PCOS development and treatment. The present study therefore examined the expression of Akt in the ovaries of PCOS rats, and then found the

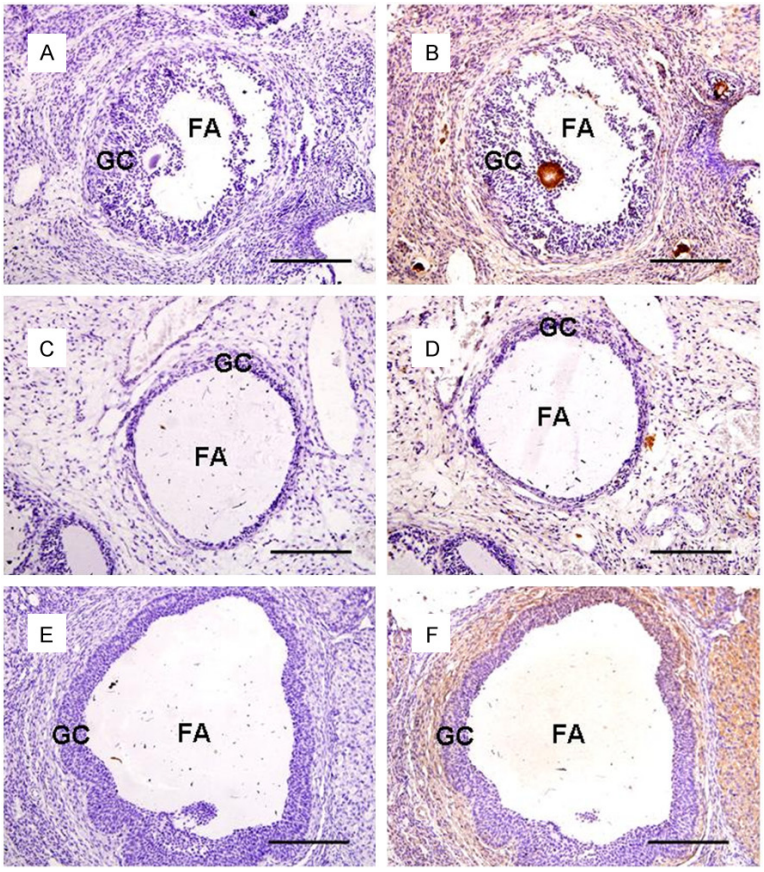
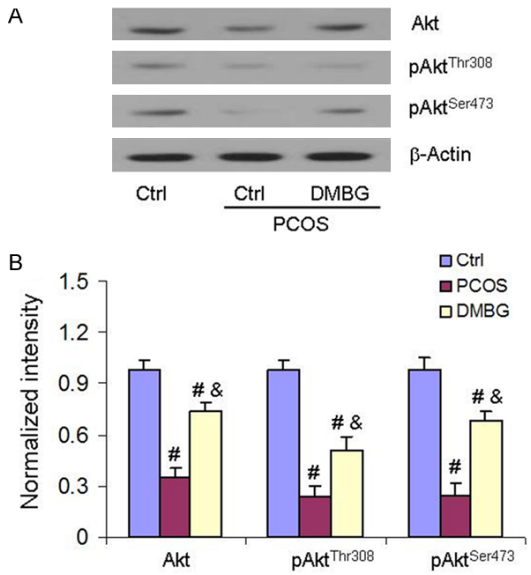


Figure 6. pAkt^{Ser473} Immunohistochemistry in the ovaries of PCOS rats. Akt^{Ser473} immunohistochemical signals appear brown and the background counterstaining appears blue (B, D and F). Negative controls remained unstained, lacking primary antibody instead of serum (A, C and E). (A and B) control group, (C and D) PCOS group, (E and F) DMBG-treated PCOS group. GC: granulosa cell, AF: antral follicles, bar =100 μm.

Figure 7. Akt, pAkt^{Thr308} and pAkt^{Ser473} protein expressions in the ovaries of PCOS rats. Panel A: Representative ECL gel images of western blot analyses depicting the Akt, pAkt^{Thr308} and pAkt^{Ser473} protein levels. Panel B: Summarized intensities of the Akt, pAkt^{Thr308} and pAkt^{Ser473} blots normalized to the control. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. #: $P<0.05$, v.s. control group, &: $P<0.05$, v.s. PCOS group.



expression pattern of Akt protein is very similar with HIF-1a protein, suggesting Akt may regulate PCOS syndrome through HIF-1a signaling. For further identifying the role of Akt during this process, the present study also detected the expression changes of its active forms, pAkt^{Thr308} and pAkt^{Ser473}. Similar results were found in PCOS rats and DMBG-treated PCOS rats, implying pAkt^{Thr308} and pAkt^{Ser473} regulated PCOS development and DMBG treatment. These findings indicated Akt/HIF-1a signaling was involved in the development and treatment of PCOS.

Clinically, DMBG is used to improve PCOS symptoms [14-16, 25], our present results found DMBG increased the expression of HIF-1a and ET-2 in granulosa cells of PCOS rat ovaries after treatment, indicating HIF-1a/ET-2 signaling may play an important role in PCOS treatment as our previous investigations [8, 9, 32]. Further analysis of Akt, pAkt^{Thr308} and pAkt^{Ser473} expression changes demonstrated that DMBG rescued PCOS-induced decrease of these three proteins in PCOS ovaries, which providing a new research direction for PCOS development and treatment.

At present, there is much progress in the pathophysiology of PCOS, but the detailed mechanism of PCOS is still not completely understood. To our knowledge, the present study is the first

time to provide direct evidence that Akt/HIF-1a signaling expression was inhibited in PCOS ovaries and DMBG rescued the damage of this pathway during the treatment of PCOS. Furthermore, Akt/HIF-1a agonists afford opportunities for the development of novel treatments for the formation and development of PCOS, and provide a new target for clinical PCOS prevention and treatment.

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Disclosure of conflict of interest

None.

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