Effects of Qigongwan on Wnt/β-catenin Signaling Pathway in Rats with Polycystic Ovary syndrome

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Abstract [**Objectives**] To explore the therapeutic effect and mechanism of Qigongwan on PCOS model rats by detecting the changes in sex hormone levels in rats with polycystic ovary syndrome (PCOS), and observing the effects of ovarian pathological morphological changes, apoptosis and expression of Wnt/β-β catenin signaling pathway protein. [**Methods**] Ten of 40 female SD rats were randomly selected as the normal group, and the other 30 rats were treated with letrozole combined with high-fat diet to establish the PCOS rat model. After successful modeling, the model group was randomly divided into Qigongwan group, positive Daying-35 (Ethinylestradiol and Cyproterone Acetate Tablets) group and model group, with 10 rats in each group. Qigongwan group was given 14.7 g/(kg · d) by gavage, Daying-35 group was given 0.21 mg/(kg · d) by oral gavage, and normal group and model group were given the same amount of distilled water, and the intervention lasted for 21 d. ELISA method was used to detect the levels of hormones such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), estradiol (E_2) and progesterone (P) in serum. HE staining was used to observe the pathological morphological changes of ovarian tissues; TUNEL staining was used to observe apoptosis of ovarian tissue granule cells; the expression of Wnt, β-catenin protein in rat ovarian tissue was detected by immunohistochemistry. [**Results**] (i) Compared with the model group, Qigongwan group and Daying-35 group could significantly increase serum E_2 and P levels, significantly reduce serum T levels ($P<0.01$), significantly reduce serum LH levels and LH/FSH ratio ($P<0.01$), and increase serum FSH levels ($P<0.05$) in different degrees. (ii) The results of HE staining showed that compared with the model group, Qigongwan and Daying-35 groups could improve follicular development and reduce atretic follicles in different degrees. Compared with Daying-35 group, the number of GC layers in Qigongwan group was significantly increased. (iii) The results of TUNEL staining showed that compared with the model group, the rate of TUNEL-positive cells in the Qigongwan group and Daying-35 group decreased significantly ($P<0.01$). (iv) The immunohistochemical results showed that compared with the model group, the expression levels of wnt and β-catenin in the Qigongwan group and the Daying-35 group increased in different degrees ($P<0.05$), and the expression range increased. [**Conclusions**] Qigongwan can regulate the secretion level of sex hormones such as FSH and LH, improve the pathological damage of ovarian tissue, and inhibit apoptosis of ovarian granule cells, and its mechanism may be related to the activation of Wnt/β-catenin signaling pathway.

Key words Qigongwan, Polycystic ovary syndrome (PCOS), Granulosa cells, Wnt/β-catenin signaling pathway, Apoptosis, Rat

1 Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder in women of childbearing age caused by the combined influence of genetic, environmental and other complex factors. Key clinical symptoms include: menstrual disturbance or amenorrhea, hyperandrogenemia, polycystic ovaries, insulin resistance, *etc.* Increasing evidence[1-2] indicate that increased apoptosis levels of granulosa cells (GC) are a key pathological mechanism for the development of PCOS. Among them, Wnt signaling pathway is a classical pathway that regulates cell proliferation and apoptosis, which is crucial in the development of ovarian follicles. At present, promoting the proliferation of PCOS ovarian GC and inhibiting its apoptosis is the main treatment strategy for PCOS[3]. According to the theory of Traditional Chinese medicine (TCM), the occurrence of PCOS is closely related to the dysfunction of the kidneys, spleen and liver, and the blockade of sputum and blood stasis. Many physicians believe that phlegm-dampness blocking the uterus is the main cause of PCOS[4-5]. Qigongwan has the functions of invigorating the spleen and eliminating dampness, promoting the circulation of qi and eliminating phlegm, opening the uterus and helping pregnancy, and has remarkable curative effect in the clinical treatment of PCOS. In this study, we observed the effect of Qigongwan on the expression of sex hormones, ovarian pathological histomorphology, apoptosis and the protein expression of Wnt/β-catenin signaling pathway in PCOS rats, to explore the therapeutic effects and possible mechanism of Qigongwan on PCOS rats.

2 Materials and methods

2.1 Animals Forth SPF-grade SD female rats, 5 - 6 weeks old, body weight of 180 - 220 g, were purchased from Beijing HFK Bioscience Co. Ltd. Animal production license No.: SCXK (Beijing) 2017-0001, room temperature (22 ± 2) ℃, relative humidity 55% - 65%.

2.2 Drugs and reagents Letrozole tablets, specification: 2.5 mg/tablet, Jiangsu Hengrui Pharmaceuticals Co., Ltd., batch No.: 22003153. Qigongwan is consisted of 20 g of Pinelliae Rhizoma (Banxia), 20 g of Cyperi Rhizoma (Xiangfu), 20 g of

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Chuanxiong Rhizoma, 20 g of Atractylodis Macrocephalae Rhizoma (Baizhu), 15 g of Massa Medicata Fermentata (Shenqu), 15 g of Poria (Fuling), 12 g of Atractylodis Rhizoma (Cangzhu), 12 g of Citri Exocarpium Rubrum (Juhong), 6 g of Glycyrrhizae Radix et Rhizoma (Gancao). All these drugs were provided and quality inspected by Xiyuan Hospital, China Academy of Chinese Medical Sciences, and were uniformly fried by Chinese medicine decoction machine to make concentrated liquid (containing crude drug amount 0.735 g/mL). Daying-35 (Ethinylestradiol and Cyproterone Acetate Tablets) was produced by Bayer HealthCare GmbH (Germany) with batch No.: KT09FJ52. Sodium carboxymethylcellulose (CMC-Na), Sigma Corporation, USA, batch No.: 1002375619.

2.3 Main instruments 3K18 centrifuge (Sigma, USA), Olympus BX53 microscope, TP1020 automatic dehydrator (Leica, Germany), RM2235 tissue microtome (Leica, Germany), Image-Pro Plus 6.0 (Media Cybernetics USA).

2.4 Methods

2.4.1 Molding. All experimental rats were subjected to adaptive feeding for 2 d, and then 10 of the 40 female mice were selected as normal groups by random number method. The normal group was fed with ordinary feed, and the remaining 30 rats were fed with letrozole combined with high-fat diet for modeling. The molding group was given letrozole solution 1 mg/(kg·d) dissolved in 0.5% (mass fraction) by gavage every day. At the same time, the rats were fed with high-fat diet (formula: 60% of common feed, 12% of egg yolk powder, 11% of lard, 5% of whole milk powder, 5% of sucrose, 5 percent of peanut, 1.5% of sesame oil and 0.5% of salt) for 21 consecutive days to carry out modeling treatment. The normal group was fed with the same volume of solvent. From the seventh day of modeling, a vaginal smear was performed daily and observed for two cycles, and the rats with persistent keratosis of vaginal epithelial cells was deemed as successful modeling[6-7].

2.4.2 Grouping and method of administration. After the model was successfully established, 30 modeled rats were randomly divided into model group, Chinese medicine Qigongwan group, positive drug Daying-35 group, 10 rats in each group. Qigongwan group and Daying-35 group calculated the dosage according to the body weight of 60 kg in women, and the dosage was calculated using the human and rat body surface area conversion method. Qigongwan group was given 14.7 g/(kg·d) by oral gavage once daily. Daying-35 group was given 0.21 mg/(kg·d) by oral gavage once daily. The normal group and the model group were given the same amount of distilled water by gavage, and the intervention period was 3 weeks. There were no animal deaths after the end of the experiment. After the last administration of rats in each group, rats were fasted after 20:00, measured the body weight of each group on the next day and recorded it, anesthetized with 10% chloral hydrate, collected about 5 mL of abdominal aortic blood, stood for 20 min and centrifuged at 3 000 r/min for 15 min, and absorbed the upper serum and placed it in a centrifuge tube. After blood collection, rats were sacrificed by cervical dislocation, and uterus and ovaries were uniformly removed, and fixed in 4% formaldehyde, so as to facilitate the subsequent HE staining and immunohistochemical detection.

2.5 Detection of indicators

2.5.1 Detection of serum hormone levels by ELISA. Serum testosterone (T), estradiol (E_2), progesterone (P) and luteinizing hormone (LH) were detected in accordance with the instructions of the ELISA kit. LH, and then follicle-stimulating hormone (FSH) and the ratio of LH/FSH were calculated.

2.5.2 HE staining to observe ovarian histopathological changes. After 24 h of 10% neutral formalin incision and fixation of ovarian tissue, the material was taken, gradient alcohol dehydration, and the transparent tissue was immersed in paraffin wax at 60 ℃ to be embedded to make paraffin blocks. The paraffin block was continuously sectioned at 4 μm, baked, dewaxed by xylene, stained by HE, dehydrated, sealed and placed under a microscope to observe the histopathological changes of the ovary of rats.

2.5.3 TUNEL staining to observe apoptosis in ovarian tissue. Paraffin sectioned at 3 μm, conventional xylene dewaxing, gradient alcohol dehydration, 20 μg/mL protease K working solution to lyse protease. PBS rinsed, instilled with TUNEL mix 50 μL, cover glass added, placed in a dark box at 37 ℃ for 1 h, PBS rinsed, DAB color development, apoptosis nuclei showed pale yellow to dark brown. Took 3 consecutive non-repeating fields of view under a high magnification microscope, counted the total number of cells and the total number of positive apoptotic cells in each field of view, and calculated the apoptosis rate, namely, TUNEL positive rate (%) = Number of positive cells / Total number of cells × 100%.

2.5.4 Detection of the expression of Wnt, β-catenin proteins in rat ovarian tissues by immunohistochemistry. Paraffin sectioned at 3 μm, conventional xylene dewaxing, gradient alcohol dehydration, high-pressure antigen retrieval using citrate buffer, and 3% H_2O_2 blocking endogenous peroxidase for 10 min. PBS rinsed, added dropwise primary antibody Wnt, β-catenin (concentrations of 1/100, 1/200, respectively), incubated at 4 ℃ overnight, resumed the temperature at normal temperature for 0.5 h, added dropwise secondary antibody, incubated for 30 min, DAB color development, observed the degree of color development under a microscope, tap water rinsed after color development to terminate the reaction, hematoxylin counterstaining, dehydration, transparent treatment performed, and neutral gum sealed. Took 3 consecutive non-repeating fields of view under a high magnification microscope and used Image-pro Plus 6.0 image analysis software to determine the integral optical density (IOD) value of positive signals.

2.6 Statistical analysis SPSS 22.0 software was used for statistical data processing. The data obtained by the experiment such as T, E_2, P, LH, LSH, LH/LSH, TUNEL positive cell rate and other data belonged the measurement data, conformed to the normal distribution and the variance was uniform, and the data were

expressed in the mean ± standard deviation ($\bar{x} \pm s$). One-way ANOVA was used for the comparison of differences between groups, and *LSD* was used for analysis in pairwise comparisons, and $P < 0.05$ indicates the difference was statistically significant.

3 Results and analysis

3.1 Comparison of E_2, T, P expression in rat serum Compared with the normal group, the serum T level of the model group increased, and the levels of E_2 and P were significantly reduced ($P < 0.01$). Compared with the model group, the levels of E_2 and P increased and the level of T decreased in Qigongwan group and Daying-35 group ($P < 0.01$) as shown in Table 1.

Table 1 Comparison of serum T, E_2 and P expression levels in rats ($\bar{x} \pm s$, $n = 10$)

Group	T//nmol/L	E_2//Pmol/L	P//nmol/L
Normal	139.45 ± 9.32	37.21 ± 3.23	166.36 ± 5.21
Model	154.73 ± 5.41[a]	31.19 ± 6.93[a]	143.78 ± 4.52[a]
Qigongwan	140.97 ± 10.40[b]	35.09 ± 0.95[b]	161.47 ± 3.58[b]
Daying-35	141.24 ± 7.43[b]	35.89 ± 0.56[b]	163.50 ± 9.83[b]

Note: Compared with the normal group, [a]$P < 0.01$; compared with the model group, [b]$P < 0.01$.

3.2 Comparison of FSH, LH and LH/FSH values in rat serum Compared with the normal group, the LH and LH/FSH levels in the model group increased significantly, and the FSH levels decreased, and the differences were statistically significant ($P < 0.01$). Compared with the model group, the LH level and LH/FSH ratio were significantly reduced in Qigongwan group and Daying-35 group ($P < 0.01$), and the FSH level was increased in different degrees, and the difference was statistically significant ($P < 0.05$), as shown in Table 2.

Table 2 Comparison of serum FSH, LH and LH/FSH expression levels in rats ($\bar{x} \pm s$, $n = 10$)

Group	LH (U/L)	FSH (U/L)	LH/FSH
Normal	24.36 ± 0.78	7.87 ± 0.21	3.10 ± 0.09
Model	31.25 ± 1.12[a]	7.50 ± 0.26[a]	4.17 ± 0.20[a]
Qigongwan	25.72 ± 0.78[c]	7.90 ± 0.31[c]	3.26 ± 0.19[c]
Daying-35	25.54 ± 1.19[c]	7.81 ± 0.33[b]	3.27 ± 0.20[c]

Note: Compared with the normal group, [a]$P < 0.01$; compared with the model group, [b]$P < 0.05$, [c]$P < 0.01$.

3.3 Histopathological changes in rat ovaries Under the microscope, we observed that the normal group showed that follicles at different stages of development could be seen everywhere in rat ovarian tissues. Among them, the dominant follicle can be seen in the morphologically complete multilayer GC (mostly 6 – 8 layers), and the normal corpus luteum can be seen. The model group showed a decrease in the overall number of follicles, an increase in atresia follicles and cystic follicles, cystic changes in most follicles, a decrease in the number of GC layers (mostly 3 – 4 layers), a disordered arrangement, and a decrease in the corpus luteum. Qigongwan group showed that the overall number of follicles did not decrease significantly, it was visible that there were no significant reductions in the overall number of follicles, follicles at all levels could be seen, relatively few atresia follicles, a small number of follicles showed cystic changes, the number of GC layers increased significantly (mostly 4 – 6 layers), the arrangement was regular, a small number of corpus luteum and white bodies could be seen, and the number of GC increased. The effect of follicle development and atresia follicle improvement in Daying-35 group was the same as that of Qigongwan group, but the GC layer was less sparse (mostly 3 – 4 layers), as shown in Fig. 1.

3.4 Apoptosis of ovarian tissue in rats TUNEL results indicate positive expression within the GC nucleus of ovarian tissue, taking on a yellowish to dark brown staining. Only a small number of TUNEL-positive cells were scattered in the ovarian tissues of rats in the normal group. Compared with the normal group, the TUNEL-positive cell rate (58.72% ± 6.56%) in the model group was significantly higher than that in the normal group ($P < 0.01$). Compared with the model group, the TUNEL-positive cell rate ($P < 0.01$) decreased significantly in Qigongwan group (34.62% ± 2.47%) and Daying-35 group (36.25% ± 3.19%), among which there was no significant difference in the TUNEL-positive cell rate between Qigongwan group and Daying 35 group ($P > 0.05$).

3.5 Wnt and β-catenin protein expression in rat ovarian tissues The indicators Wnt and β-catenin expressions in the GC cell membrane and cytoplasm of ovarian tissues were light yellow to brownish yellow, mainly in the cell membrane, and occasionally expressed by follicular membrane cells. In this experiment, only the GC cell membrane expression part was counted, and Wnt and β-catenin proteins in the normal group were strongly positively expressed and the expression range was wide. Compared with the normal group, the expression levels of Wnt and β-catenin proteins in the model group decreased significantly ($P < 0.01$), and the expression rate decreased. Compared with the model group, the expression levels of Wnt and β-catenin in Qigongwan group and the Daying-35 group increased in different degrees ($P < 0.05$), and the expression range increased, as shown in Fig. 2-3 and Table 3.

Table 3 Comparison of Wnt and β-catenin protein expression in rat ovarian tissues (IOD, $\bar{x} \pm s$, $n = 10$)

Group	Wnt	β-catenin
Normal	675.74 ± 55.23	783.16 ± 66.27
Model	448.45 ± 67.08[a]	562.19 ± 45.03[a]
Qigongwan	580.97 ± 50.20[b]	664.23 ± 45.51[b]
Daying-35	549.20 ± 61.43[b]	583.39 ± 58.46[c]

Note: Compared with the normal group, [a]$P < 0.01$; compared with the model group, [b]$P < 0.05$, [c]$P < 0.01$.

4 Discussion

PCOS is a common heterogeneous reproductive and metabolic disorder in women and a leading cause of infertility in women of reproductive age, accounting for 75% of anovulatory infertility[8]. In the long term, it may cause tumors, cardiovascular and cere-

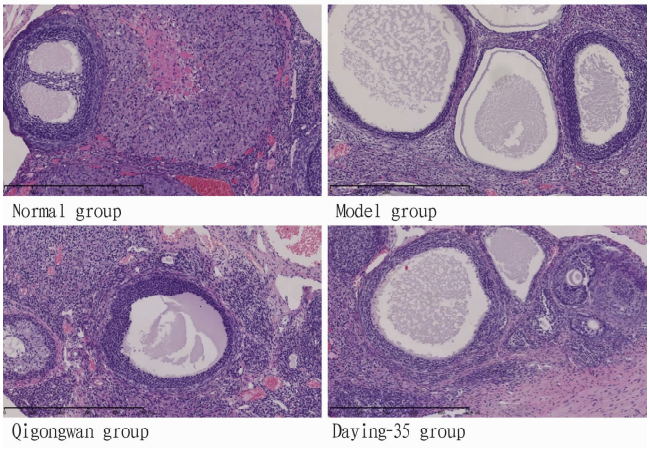

Fig. 1 Observation of ovarian tissue morphology in each group of rats (HE, ×400)

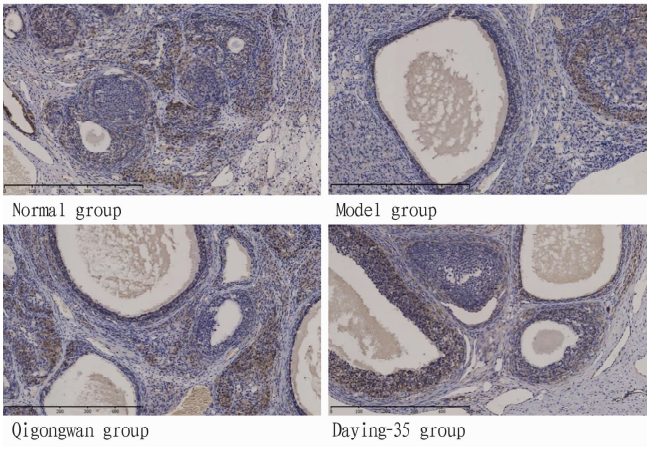

Fig. 2 Wnt protein expression in rat ovarian tissues in each group (IHC, ×400)

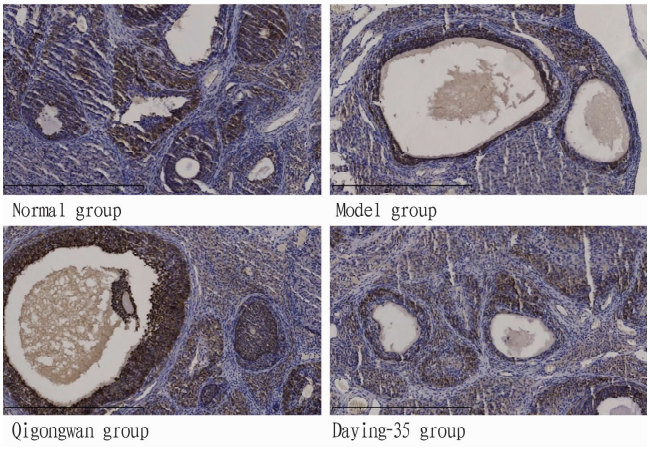

Fig. 3 Expression of β-catenin protein in rat ovarian tissues in each group (IHC, ×400)

brovascular diseases, abnormal blood lipids and blood glucose and other endocrine and metabolic system complications[9-10], seriously endangering the physical and mental health and family of patients. Modern epidemiological surveys show that the incidence of the disease in women of childbearing age is 6% -21%[11], so it is the main research direction in the field of reproductive medicine. The main clinical treatment methods include adjusting the menstrual cycle, ovulation induction, surgery, *etc.*, but there are many side effects and the treatment effect is not satisfactory. According to the TCM theory, this disease belongs to the categories of amenorrhea, oligomenorrhea, infertility, mass in the abdomen accumulation, uterine bleeding and so on. Successive generations of physicians believe that the main cause of PCOS is the internal arrest of water wetness, which accumulates into phlegm and blocks the uterus. Therefore, according to the principle of "treatment aiming at its root causes", clinical treatment should be based on drying and moisturizing phlegm. Qigongwan was first introduced in *Collection of Medical Prescriptions* written by the physician Wang Ang in the Qing Dynasty, the composition is mainly Pinelliae Rhizoma and Citri Reticulatae Pericarpium plus qi and blood revitalization medicine, which can cure women's human body fat phlegm, lipid membrane occlusion of the uterus, and infertility. A large number of clinical studies have proved that Qigongwan can reduce the body weight of PCOS patients, regulate HPO axis hormone secretion, promote the formation of dominant follicles and ovulation, and improve the clinical symptoms of PCOS patients[12-13]. However, the mechanism of treatment is still not clear.

The key pathological changes of PCOS are follicular development disorders, which are manifested as abnormal hormone secretion during follicular development, hyper-recruitment and atresia of follicles, abnormal increase in GC apoptosis, and nondominant follicle formation and ovulation. This experimental study showed that obvious polycystic dilated follicles, increased atresia follicles, no mature oocytes, decreased number of corpus luteum, disordered GC arrangement, thinning and degeneration in the model group rat ovary tissue. The ovary tissue of rats in the Qigongwan group showed that the morphology of follicles at all levels was normal, the number of GC layers increased compared with that in the model group, and a small amount of corpus luteum and white body were visible, indicating that ovulation may exist. The development of follicles is regulated by hormones and is closely related to ovarian GC[14]. GC contains FSH, E, and T-related hormone receptors that respond to FSH and LH stimulation, increase the transcription of the P450arom gene, promote androgen conversion to estrogen, and stimulate follicle growth and ovulation[15]. In addition, GC also has the ability to synthesize steroids such as E_2 and P, which enhances the positive feedback effect of LH ovulation peak, thereby inducing ovulation. Compared with the model group, the FSH of rats treated with Qigongwan was significantly increased, and LH, LH/FSH were significantly reduced. T levels were increased, while E_2 and P levels decreased, suggesting that Qigongwan intervention could improve the quality of PCOS rat follicles, increase the number of GC layers, and correct hormone imbalance. These show that Qigongwan has a good therapeutic effect on PCOS.

In recent years, studies have found that abnormal proliferative apoptosis of GC may be the pathological basis for the pathogenesis of PCOS[16-18]. After the follicles undergo recruitment, selection, and the formation of dominant follicles, the remaining

follicles undergo atresia, and the atresia of the follicles depends on the apoptosis mechanism of ovarian GC. The balance between GC proliferation and apoptosis signaling pathways determines the fate of follicles. By comparing the apoptosis of ovarian tissues in each group by TUNEL method, this experiment showed that the apoptosis rate of ovarian tissue in the model group increased significantly, while the apoptosis rate of PCOS rats decreased significantly after Qigongwan treatment, suggesting that Qigongwan could inhibit the apoptosis level of ovarian GC in rats, and Wnt/β-catenin signaling pathway is expressed in GC, which is involved in follicle development, ovulation, proliferation and apoptosis, and is closely related to GC dysfunction and PCOS.

The Wnt/β-catenin pathway regulates the proliferation, apoptosis, differentiation and migration functions of cells by regulating the expression of the downstream intranuclear proto-oncogene c-Myc and cyclinD1[19]. β-catenin is the core molecule in the Wnt pathway, localized in the cytoplasm in the inactivated state, and under normal circumstances, the cytoplasmic glycogen synthase kinase-3β (GSK-3β) complex degrades β-catenin by phosphorylation and ubiquitination, making its concentration level low and unable to mediate downstream signals. When cells are stimulated by specific factors, Wnt binds to frizzled receptors located on the surface of the cell membrane, induces the dissociation of the GSK-3β complex, relieves the inhibitory effect on β-catenin, and activates downstream target genes. The effect of Wnt/β-catenin pathway on ovarian development is reflected in the interaction between Wnt/β-catenin and sex hormone receptor pathway, which is an important regulatory pathway for ovarian steroid hormone secretion. On the other hand, it determines the fate of cells, regulates cell survival, proliferation, differentiation and apoptosis activities, and is closely related to important ovarian development events such as GC proliferation, ovulation, luteinization and follicular atresia[20]. Previous studies have shown that the Wnt pathway can promote the secretion of follicle GC estrogen, regulate FSH and LH hormone secretion, and have a positive effect on steroid production and ovulation[21]. Wu Xueqing *et al.* [22] found that the expression of β-catenin and its downstream target proteins (Survivin, BMP4) in PCOS patients was inversely proportional to the expression of the apoptotic factors Bax and Bcl-2, and that the WNT/β-catenin pathway was involved in regulating apoptosis in ovarian cells and was an important mechanism for PCOS formation. Experts and scholars have gradually realized that Wnt/β-catenin may become a new therapeutic target for PCOS, but there have been no relevant studies to verify it. In this experiment, the expression levels of Wnt and β-catenin in the model group were significantly lower than those in the normal group, and Qigongwan group improved significantly compared with the model group, reflecting that Qigongwan could increase the expression of wnt/β-catenin signaling pathway and reduce GC apoptosis.

In summary, this study indicates that Qigongwan can improve PCOS symptoms, promote follicle development and ovulation, and its mechanism may be related to activating Wnt/β-catenin signaling pathway, reducing GC apoptosis response, and regulating ovarian hormone secretion, thereby promoting follicle development and ovulation. However, the sample size of this experiment is small, and a large number of experiments and clinical studies are needed for further verification in the future.

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(To page 83)

to in manipulation treatment. Wang Guojun[18] pointed out that the concept of "emphasizing both tendons and bones" has a very long history, and attention should be paid to the concept of "emphasizing both tendons and bones" in the diagnosis and treatment of common bone injury diseases. Throughout history, medical practitioners have emphasized the importance of tendons and bones, advocating for the principle of "treating both tendons and bones equally, emphasizing both tendons and bones, and balancing them". Especially for manipulation treatment, it should pay full attention to the roles of "tendons and bones". The mechanical imbalance of tendons and bones is the key to the pathogenesis of thumb stenotic tenosynovitis. The treatment results showed that the total effective rate, VAS score, and recurrence rate of the observation group were significantly better than those of the control group, indicating that the functional recovery of the observation group was more significant.

In summary, as an innovation in traditional Chinese medicine techniques, the four-step tendon regulating manipulation has significantly better therapeutic effects than traditional techniques, and has greater advantages in improving pain and function. It is worth promoting in clinical practice. However, there are few cases of observed patients this time, and the treatment and observation cycles are relatively short. Further research is needed to determine their long-term efficacy.

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(From page 15)

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