Effects of *Bushenyiqihexue* Formula on the Endometrial Gland Apoptosis in Mice with Blastocyst Implantation Dysfunction

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Objective: To observe the effects of *Bushenyiqihexue* Formula (补肾益气和血方 Formula for Tonifying the Kidney, Replenishing *qi* and Harmonizing Blood, FTKRQHB) on the endometrial gland apoptosis in the mice with blastocyst implantation dysfunction. **Methods:** The mice with the first-day pregnancy were divided into the control, model and treatment groups, with 30 in each group, and blastocyst implantation dysfunction was induced by subcutaneous injection of mifepristone in the mice of the model and treatment groups. The pregnancy rate and implantation number of blastocysts were measured and the expressions of proliferating cell nuclear antigen (PCNA), Bax, Bcl-2, and activated caspase-3 were detected in all the three groups. **Results:** The model group had significantly depressed pregnancy rate, implantation number of blastocysts and apoptosis index, and elevated proliferation index of endometrial gland as compared with the control group ($P<0.05$ or $P<0.01$). Administration of FTKRQHB (the treatment group) resulted in significant increases in pregnancy rate, implantation number of blastocysts and apoptosis index of the endometrial gland, and a significant decrease in the proliferation index of the endometrial gland as compared with the model group ($P<0.05$ or $P<0.01$). The differences in the four indexes between the treatment group and control group were not significant statistically. The Bax and activated caspase-3 expressions in endometrial gland in the model group became significantly lower than that of the control group ($P<0.01$), whereas those in the treatment group were significant higher than that of the model group ($P<0.01$). However, the Bax and activated caspase-3 expressions in endometrial gland were similar in both treatment and control groups. **Conclusion:** Promoting the increases in Bax and activated caspase-3 expressions in the endometrial gland and bringing into balance between apoptosis and proliferation of the glandular cells at the implantation window phase by FTKRQHB may contribute to the effects of promoting the establishment of endometrial receptivity and improving blastocyst implantation dysfunction.

Key words: blastocyst implantation dysfunction; apoptosis; proliferating cell nuclear antigen (PCNA); Bax; Bcl-2; Caspase-3; *Bushenyiqihexue* Formula (Formula for Tonifying the Kidney, Replenishing *qi* and Harmonizing Blood, FTKRQHB)

Blastocyst implantation dysfunction has become the bottlenecking factor for a successful pregnancy. Improving embryonic implantation by promoting the establishment of endometrial receptivity is one breakthrough in treating barrenness in clinical practice. The establishment of endometrial receptivity is chiefly characterized by apoptosis of the endometrial gland at the implantation window phase. FTKRQHB has been shown in clinical practice to improve the embryonic implantation of the patients receiving multiple *in vitro* fertilization-embryo

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transfer unsuccessfully. In order to explore the mechanism of its action, the present study on the effects of FTKRQHB on the establishment of endometrial receptivity from apoptosis of the endometrial gland and on the relevant regulating and controlling factors were carried out.

MATERIALS AND MRTHODS

Laboratory Animals

The 100 male and 30 female Clean KM mice aged 8 to 12 weeks, weighing 28 to 20 g (Conformity Certificate No. 19–082), were supplied by Hubei Provincial Anti-epidemic Station**.**

Experimental Drugs

FTKRQHB consists of Sang Ji sheng (桑寄生 Ramulus Taxilli) 18g, Dan Shen (丹参 Radix Salviae Miltiorrhizae) 14g, Huang Qi (黄芪 Radix Astragali) 9g, Dang Gui (当归 Radix Angelicae Sinensis) 9g, and Chuan Xiong (川芎 Rhizome Chuaxiong) 9g. These Chinese drugs were made into the extractum (3.75 mg of crude drugs/1 ml of extractum) via decoction and alcohol sedimentation by Institute of the Integration of Traditional Chinese and Western Medicines, Tongji Hospital. Mifepriston tablets (25mg/1tablet, Batch Number: 010413) made by Beijing Third Pharmaceutical Factory were powdered and dissolved in propylene glycol at a concentration of 0.8 mg/ml.

Reagents

Anti-PCNA antibody was purchased from Neomarker Co., U.S.A., anti-Bax antibody and anti-bel-2 antibody from SantaCruz Co., U.S.A., anti-activated caspase-3 antibody from R&D Co., SP kit and DAB color kit from Beijing Zhongshan Bioengineering Company Limited, and the *in situ* cell apoptosis detection kits from Boehringer Mannhein Co., Germany.

Grouping and Treating

The male and female KM mice (1:2) were housed in a same cage for impregnation. Mating was evidenced by the appearance of a vaginal plug on the following morning, and the plugged females were designated as the first day of pregnancy. The mice with the first-day pregnancy were divided into the control, model and treatment groups, with 30 in each group. FTKRQHB was orally administered to the mice of the treatment group starting from the first day of pregnancy 0.2 ml/ a mouse, and equal volume of normal saline was orally administered to the mice of the control and model groups. Mifepristone solution was subcutaneously administered to the mice of the model and treatment groups 0.1 ml/a mouse, and equal volume of propylene glycol was subcutaneously administered to the mice of the control group from 7 a.m. to 8 a.m. on the fourth day of pregnancy. 10 mice of each group were sacrificed by cervical dislocating from 21:30 to 22:00 on the fourth day of pregnancy, and the uteri were excised. One uterine horn was fixed in 4% paraformal-dehyde and embedded with paraffin, and the other uterine horn was kept in liquid nitrogen. The remaining 20 mice of each group were sacrificed on the seventh day of pregnancy to observe the pregnancy rate and implantation number of blastocysts.

Detection of PCNA, Bax, Bcl-2, and Activated Caspase-3

Immunohistochemistry SP method was adopted for detection of PCNA, Bax, Bcl-2, and activated caspase-3. The PCNA positively stained cells (cells with pale brown stained nuclei) were counted in 10 random high-power fields of each sample under the light microscope. The proliferative index was the percentage of cells positive for PCNA. The cells positive for Bax, activated caspase-3 and Bcl-2 were characterized by pale brown stained cytoplasm. HPIAS-1000 pathology picture analysis system produced by Qianping Audiovisual Company of Tongji Medical College was used to measure their expressions, assessed by integrated luminosity of the positive signal.

Detection of Apoptotic Cells

The TUNEL method was used to assess the apoptotic cells. The positive nuclei with pale brown color were counted by 10 random high-power fields of each sample under the light microscope. The apoptotic index was the percentage of the positive cells.

Statistical Analysis

All the data were expressed as mean ± SD. Student's *t*-test and Chi-square test were performed to determine the significance of the difference between the covariates. The level of statistical significance was set as $P<0.05$. All the analyses were conducted with SPSS11.5 statistical software.

RESULTS

Pregnancy Rate and Mean Implantation Number of Blastocysts in All the Groups

As shown in table 1, the pregnancy rate and mean implantation number of blastocysts in the model group were significantly lower than those in the control group ($P<0.05$ or $P<0.01$), the pregnancy rate and mean implantation number of blastocysts in the treatment group were significantly higher than those in the model group ($P<0.05$ or $P<0.01$), and the mean implantation number of blastocysts in the treatment group was significantly lower than that in the control group ($P<0.05$).

Table 1. Pregnancy rate and mean implantation number of blastocysts in different groups ($\bar{x} \pm s$)

Group	Cases	Pregnancy rate (%)	Mean implantation number of blastocysts
Control	20	90	14.67±1.35
Model	20	25*	7.67±1.34**
Treatment	20	$55^{\triangle}$	$12.31\pm0.75^{*\triangle\triangle}$

Note: *P <0.05 and **P<0.01 as compared with the control group; and $^{\triangle}P$ <0.05 and $^{\triangle\triangle}P$ <0.01 as compared with the model group.

Proliferative and Apoptotic Status of Endometrium

As shown in table 2, the proliferative index of the endometrial gland in the model group was significantly higher than that in the control group ($P<0.01$), and the proliferative index of the endometrial gland in the treatment group was significantly lower than that in the model group. The difference in proliferative index of the endometrial gland between the treatment group and the control group was not significant. However，the apoptotic index of the endometrial gland in the model group was significantly lower than that in the control group ($P<0.05$), and the apoptotic index of the endometrial gland in the treatment group was significantly higher than that in the model group ($P<0.01$). The difference in apoptotic index of the endometrial gland between the treatment group and the control group was not significant. The differences in the proliferative index and apoptotic index of the endometrial stroma among the three groups were not statistically significant.

Table 2. Proliferative and apoptotic status of endometrium ($\bar{x} \pm s$)

Group	Cases	Proliferative index		Apoptotic index	
		Gland	Stroma	Gland	Stroma
Control	10	0.131±0.06	0.474±0.02	0.193±0.09	0.037±0.008
Model	10	0.368±0.05**	0.443±0.04	0.077±0.01*	0.040±0.008
Treatment	10	$0.185\pm0.02^{\triangle}$	0.450±0.02	$0.120\pm0.02^{\triangle}$	0.036±0.007

Note: *P<0.05 and **P<0.01 as compared with the control group; and $^{\triangle}P$<0.01 as compared with the model group.

Expressions of Bax, Bcl-2, and Activated Caspase-3 in Endometrium of the Three Groups

Expressions of Bax, Bcl-2 and activated caspase-3 were assessed by integrated luminosity of the positive signal. The mean integrated luminosity of Bax protein in the model group was significantly lower

than that in the control group (P<0.01), and the mean integrated luminosity of Bax protein in the treatment group was significantly higher than that in the model group (P<0.01). The difference in the mean integrated luminosity of Bax protein between the treatment group and control group was not statistically significant. The difference in the mean integrated luminosity of Bcl-2 among the three groups was not statistically significant. The mean integrated luminosity of activated caspase-3 was significantly lower than that in the control group (P<0.01), and the mean integrated luminosity of activated caspase-3 in the treatment group was significantly higher than that in the model group (P<0.05) but significantly lower than that in the control group (P<0.05).

Table 3. Mean integrated luminosity of Bax, Bcl-2 and activated caspase-3 in endometrium of the three groups ($\bar{X} \pm s$)

Group	Cases	Bax	Bcl-2	activated caspase-3
Control	10	36.73±7.63	17.20±22.9	30.73±1.30
Model	10	17.35±1.50**	10.63±6.00	22.14±2.58**
Treatment	10	28.99±7.42$^{\triangle\triangle}$	11.02±11.91	25.99±2.83$^{*\triangle}$

Note: *P <0.05 and **P <0.01 as compared with the control group; and $^{\triangle}P$ <0.05; $^{\triangle\triangle}P$ <0.01 as compared with the model group.

DISCUSSION

The complex and fine changes usually take place in the endometrium at the implantation window phase, which form a favorable microenvironment for the blastocyst implantation. The apoptosis of the glandular epithelium occurring exactly at this phase is closely linked to establishment of the endometrial receptivity. Von RU et al.[1] found that the apoptosis occurring in the glandular epithelium of the endometrial stratum basale during on the 19–20th day of the menstrual cycle, subsequently the apoptosis took place in the glandular epithelium of the functional basale. At this time, the proliferative reaction and Bcl-2 expression occurred only in the stroma. The glandular apoptosis occurring at this phase are supposed to play an important role in establishment of the endometrial receptivity. Both the disappearance of Bcl-2 expression and the increase of Bax expression are the inducing factors for glandular apoptosis at this phase. Gao F et al. found that the endometrial gland revealed the apoptosis in the afternoon 4 days after gestation, that is, the prophase of blastocyst implantation in the mice (the blastocyst implantation in mice started at 22 p.m. to 24 p.m. on the 4th day after gestation).[2] After the mice were treated by mifepristone, the apoptosis of the glandular cells was markedly decreased, thus affecting establishment of the endometrial receptivity. In FTKKQHB, Sang Ji sheng (桑寄生 Ramulus Taxilli) can exert the effect of invigorating the kidney essence, which is used as the sovereign ingredient; Huang Qi (黄芪 Radix Astragali) is used as the minister ingredient for invigorating the spleen that provides the material basis for the acquired constitution to nourish the congenital essence for the fetus; and the drugs for nourishing blood, regulating blood and harmonizing blood, such as Dan Shen (丹参 Radix Salviae Miltiorrhizae), Dang Gui (当归 Radix Angelicae Sinensis), and Chuan Xiong (川芎 Rhizome Chuaxiong), are added to regulate the Chong and Ren channels. In clinical practice, the therapeutic results are satisfactory. 3 The increasing of pregnancy rate correlates closely with the glandular apoptosis and the establishment of the endometrial receptivity. Generally, the blastocyst implantation in the mice comes on during 22 p.m. to 24 p.m. in the 4th day of gestation. Sampling in this series was carried out during 21:30 p.m. to 22 p.m., namely, at the time exactly prior to the blastocyst implantation in the mice. Because whether the endometrial receptivity is established at this phase or not is very important to the blastocyst implantation. It is found in the present experiment that subcutaneous injection of mifepristone could produce a remarkable decrease in pregnancy rate, implantation number of blastocysts and apoptosis of the endometrial gland in

the mice, with a relative increase of the proliferation; while the mice previously treated by FTKRQHB showed a remarkable elevation in apoptosis of the endometrial gland and a reduction of the proliferation mainly occurring in the stroma, with no significant differences in the apoptosis and proliferation as compared with the normal mice, indicating that FTKRQHB could improve the imbalance between apoptosis and proliferation of the endometrial gland cells in the mice with blastocyst implantation dysfunction, and promote the establishment of the endometrial receptivity.

The Bcl-2 protein family is the prevailing regulator of apoptosis. Bcl-2 and Bax have been studied thoroughly and deliberately. Bax can form the homodimers itself, with the pro-apoptotic activity. However, Bax can also form the heterodimers with Bcl-2 when Bcl-2 is overexpressed, showing the anti-apoptotic activity. Activation of the caspases family is indispensable to their meditating effect on apoptosis. Caspase-3 is a key kinase that initiates apoptosis and also the main effect molecule for apoptosis. Apoptosis is irrevocably launched when caspase-3 is processed into active enzymes.

It was found in the present study that high expression of Bax at the implantation window phase occurred in the glandular epithelium and the surface epithelium of the endometrium, especially in the glandular epithelium; but the Bcl-2 expression occurred in the stroma. Mifepristone could produce a decrease in Bax expression in the glandular epithelium but showed no effect on Bcl-2 expression in the stroma. FTKRQHB was able to improve the low expression of Bax in the glandular cells of the endometrium in mice with blastocyst implantation dysfunction. The activated caspase-3 expression at the implantation window phase occurred in both the glandular epithelium and the surface epithelium of the endometrium, especially in the glandular epithelium, but it was hardly present in the stroma. The activated caspase-3 expression in the glandular epithelium was significantly inhibited after mifepristone was administered on the 4th day of gestation; however, FTKRQHB could significantly improve the activated caspase-3 expression in the glandular epithelium.

The results from the present study showed that the Bax and activated caspase-3 were largely expressed in the glandular epithelium, and the apoptosis occurred at the implantation window phase; the reducing of Bax and activated caspase-3 expressions by mifepristone might be one of the mechanisms leading to poor apoptosis and sthenic proliferation of the glandular cells; and FTKRQHB could exert the effect of regulating the imbalance between apoptosis and proliferation of the glandular cells to promote establishment of the endometrial receptivity and improve blastocyst implantation dysfunction by way of promoting the expressions of Bax and activated caspase-3 in the gland.

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