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Original Article

Exogenous estrogen promotes regeneration of rat endometrial stromal cells

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Abstract: Objective: To investigate the effect of exogenous estrogen therapy in improving regeneration of rat endometrial stromal cells in rats with thin endometrium. Methods: Rats with thin endometrium were modeled with 95% ethanol and were divided into 4 groups: acute injury treatment group (treated with estradiol benzoate, 20 μg/kg/d, after modeling), acute injury control group (treated with peanut oil, 20 μg/kg/d, after modeling), chronic injury treatment group (treated with estradiol benzoate, 20 μg/kg/d, after 3 estrus phase after modeling) and chronic injury control group (treated with peanut oil, 20 μg/kg/d, after 3 estrus phase after modeling). The endometrial thin degree was evaluated; endometrial morphology was observed by HE staining; the levels of cytokeratin and vimentin were detected by western-blot and immunohistochemistry. Results: The endometrium was significantly thicker and the expression of vimentin was stronger in acute injury treatment group and chronic injury treatment group, compared with the corresponding control groups (both $P<0.05$). The endometrial lining was evidently thicker and the vimentin expression level was obviously higher in acute injury treatment group than those of chronic injury treatment group (both $P<0.05$). Conclusion: Exogenous estrogen could promote endometrial cells regeneration *in vivo*, which might provide a new therapeutic method for the treatment of thin endometrium in women suffering infertility.

Keywords: Thin endometrium, cytokeratin, vimentin, rat model, estrogen

Introduction

Both high-quality embryo and receptive endometrium are essential to successful pregnancy [1, 2]. Endometrial thickness is the primary clinical metric for assessing uterine receptivity during assisted reproduction cycles [3]. Thin endometrium is defined as the endometrium is thinner than the minimum endometrial thickness that may maintain a successful implantation which is called threshold thickness. Approximately 0.6%-0.8% of patients suffering infertility due to thin endometrium [4]. The major clinical manifestation of thin endometrium is an unexplained low volume of menstrual blood (<30 mL). Miwa et al. first reported the pathological and physiological characteristics of thin endometrium and defined thin endometrium as that vascular endothelial growth factor was in low level and the grow of glandular epithelium was slow as well as the uterine artery blood flow and vascular dysplasia was with high resistance [5].

Various remedies have been proposed, such as given estradiol, vaginal sildenafil citrate, aspirin (low-dose), gonadotropin releasing hormone agonist, human chorionic gonadotropin and combined pentoxifylline and tocopherol [6-13]. Some researchers have tried endometrial biopsy, neuromuscular electrical stimulation on pelvic floor and intrauterine instillation of granulocyte colony-stimulating factor [14-17]. However, some patients still remain unresponsive even utilizing these remedies. Therefore, it is necessary to investigate some other innovative methods for the treatment of thin endometrium.

Estrogen is one of factors that affecting endometrium thickness [18]. Currently, few researches have reported the effect of estrogen on the grow promotion of endometrium, therefore, whether or not estrogen can improve thin endometrium regeneration need more evidences from *in vivo* experiments. Hence, our study aimed to go into the effect of estrogen on endometrial stromal cells regeneration in rats with thin endometrium.

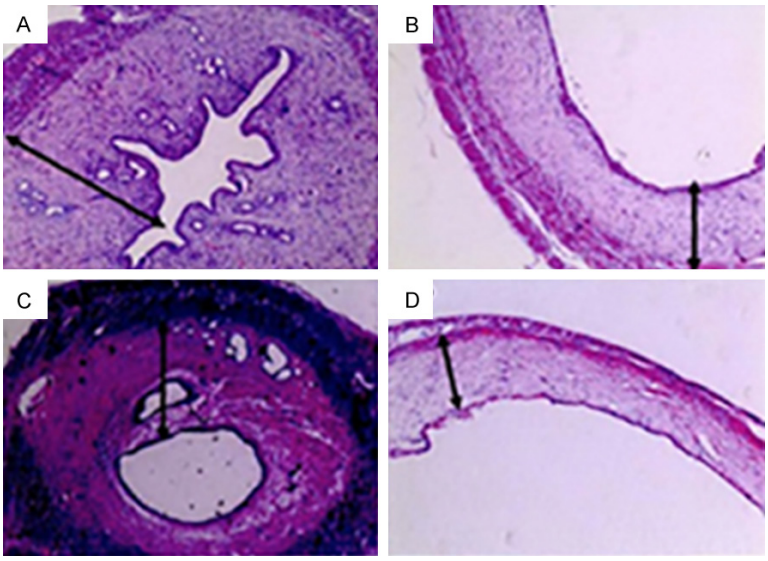

Figure 1. The morphology observation of the endometrium with HE staining of the four groups (80×). A: Acute injury treatment group, B: Acute injury control group, C: Chronic injury treatment group, D: Chronic injury control group.

Table 1. Comparison of the endometrial thickness between 4 groups ($\bar{x} \pm s$)

Groups	Rats (n)	Death rats (n)	Uteri (n)	Endometrial thickness (μm)
Acute injury treatment group	6	0	12	649.15±36.55*
Acute injury control group	6	1	10	221.24±38.19
Chronic injury treatment group	6	1	10	637.78±22.93#
Chronic injury control group	6	2	8	233.52±19.13

Note: *P<0.001, compared with acute injury control group; #P<0.001, compared with chronic injury control group.

Methods

Experiment design

All the animal procedures in this study were approved by the Institutional Animal Care and Use Committee.

The thin endometrium rat models were established by perfusion with 95% ethanol in uterus [19]. The Endometrial thickness was observed to judge if the rat model was established successfully. Accordingly, estrogen (20 μg/kg/d) was subcutaneous administrated on rat models. After twelve days, the effect of estrogen on endometrium was evaluated and the possible mechanism was investigated.

Experimental animals

Forty female adult Sprague-Dawley (SD) rats (weight 230~260 g) purchased from Shanghai Super--B&K laboratory animal Corp. Ltd., were fed in 25°C surroundings with 12-12 hour light-dark cycle and the food and water were free to them.

Animal model and application of estrogen

The thin endometrium rat model was performed with 95% ethanol and all models were established successfully [19]. Briefly, rats were anesthetized with 10% Chloral Hydrate (0.4 g/kg, i.p.) then, expose the uterus, clip each uterine horn with vascular clip, and inject 95% alcohol (0.5 mL) into the uterine horn with a 1 ml syringe and a 16-gauge needle for 5 min. After modeling, rats were placed in temperature and humidity-controlled incubation chambers until they awoke.

In clinic, acute or chronic thin endometrium was defined as the status has been lasting less than one menstrual cycle or more than 3 menstrual cycles, respectively. Considering that rats have an estrous cycle every 4-5 days, in this study, if exogenous estrogen plays a role on thin endometrium about 4-6 hours after modeling and three estrous cycles after modeling was evaluated.

Therefore, forty rats were divided into acute injury treatment group (n=6), acute injury control group (n=6), chronic injury treatment group (n=6), chronic injury control group (n=6): the acute injury control group was treated with estradiol benzoate diluted by peanut oil (20 μg/kg/d) per day for 5 days (starting from the model was established) by subcutaneous administration; the acute injury control group was treated with peanut oil (20 μg/kg/d) per day 5 days (starting from the model was established) by subcutaneous administration; the chronic injury treatment group was treated with estradiol benzoate diluted by peanut oil (20 μg/kg/d) per day for 5 days (starting from the third estrus phase after the model was established)

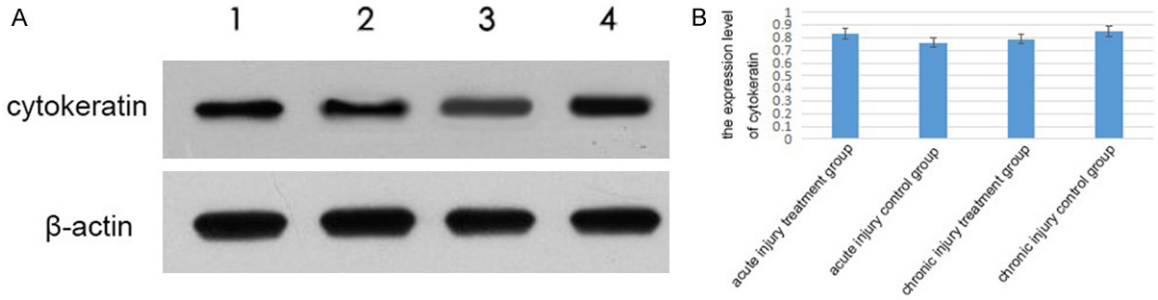

Figure 2. Expression of cytokeratin with western blot in Epithelial Cells. A: Group 1-4: acute injury treatment group, acute injury control group, chronic injury treatment group, chronic injury control group. B: The quantitative analysis of the relative expression level of cytokeratin in each group. The results showed that there was no difference between acute injury treatment group/acute injury control group and chronic injury treatment group/chronic injury control group of the expression of cytokeratin in epithelial cells.

Table 2. Comparison of the cytokeratin area per unit endometrium area between 4 groups ($\bar{x} \pm s$)

Groups	Uteri (n)	Cytokeratin area (%)
Acute injury treatment group	12	13.68±0.34
Acute injury control group	10	13.33±0.61
Chronic injury treatment group	10	12.89±0.39
Chronic injury control group	8	12.53±0.98

by subcutaneous administration; chronic injury control group was treated with peanut oil (20 μg/kg/d) per day for 5 days (starting from the third estrus phase after the model was established) by subcutaneous administration. After treatment, the health condition of the rats was monitored every 4-6 hours after surgery.

Specimen collection

The rats were anaesthetized with 10% Chloral Hydrate (0.2 ml/100 g, i.p.) at the forth estrus phase after administration of ES or peanut oil. The estrous cycle phase was confirmed by vaginal smear detection. The uteruses were separated and placed into 4% paraformaldehyde or stored at -80°C in reserve.

Immunohistochemistry

The uterine horns were embedded in paraffin and cut in to 4~6 mm slices, which were then deparaffinized in xylene, rehydrated (100%, 100%, 95%, 95%, 80%, 70%, 50%, 0%, 5 min each) and rinsed in water. Afterwards, the slices were incubated in 3% H_2O_2/methanol for 15 min at room temperature and blocked with 10% normal goat serum for 1 h. Then the slices were incubated with first antibody of cytokeratin (1:50) or vimentin (1:200) for 12-16 h at 4°C. Normal rabbit IgG was applied as negative control. After then, slices were incubated with HRP-conjugated goat anti-rabbit IgG in 10% goat serum (1:3000) for 1 h at room temperature. Next, the slices were counterstained with hematoxylin solution (Gill No.3, Sigma) for 5 min. The results were detected by Nikonmicroscope.

Hematoxylin-eosin staining

After fixing in 4% paraformaldehyde, the tissue samples were embedded in paraffin and cut in to 4~6 mm slices, which were then deparaffinized in xylene, rehydrated (100%, 100%, 95%, 95%, 80%, 70%, 50%, 0%, 5 min each) and rinsed in water. The slices were stained with hematoxylin for 5 min and eosin for 3~5 min. After staining, slices were dehydrated through an ethanol series and xylene and mounted with Permount TM mounting medium (Fisher Scientific, PA). The results were observed and measured by semi-automatic inverted biological microscope (with imaging system, DMI4000B, Zhengke, China).

Western blotting

After homogenate and centrifugation at 8000 rpm for 10 min at 4°C of the tissue, the concentration of protein was determined by BCA method. The proteins were separated by electrophoresis with 10% SDS-polyacrylamide gel and transferred to the polyvinylidene fluoride membrane, which were then blocked with 5% skimmed milk and incubated with the primary antibody overnight at 4°C, including anti-cyto-

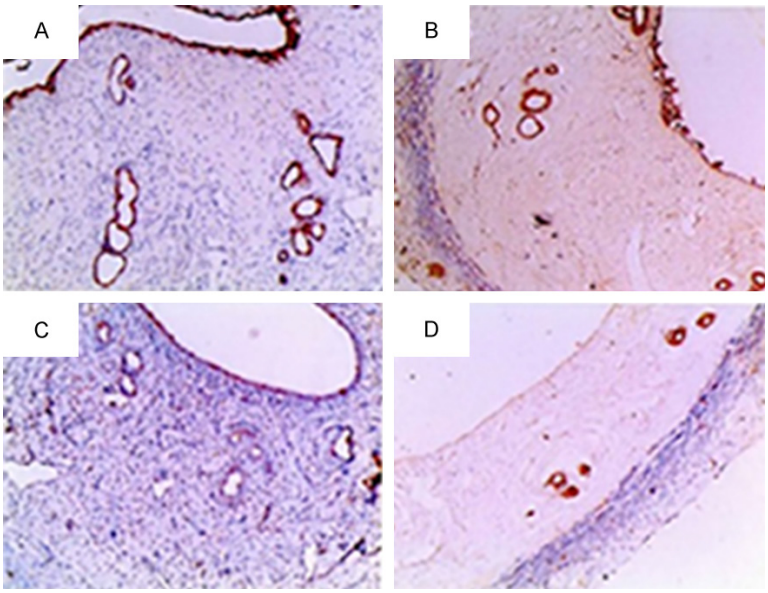

Figure 3. Regeneration of epithelial cells in the endometrium immunohistochemical staining with cytokeratin (200×). A: Acute injury treatment group, B: Acute injury control group, C: Chronic injury treatment group, D: Chronic injury control group. There was no difference between acute injury treatment group/acute injury control group and chronic injury treatment group/chronic injury control group. The yellowish-brown part indicated positive staining of cytokeratin.

keratin antibodies (1:50) and anti-vimentin antibodies (1:200). Thereafter, the membrane was washed and the second antibody anti-rabbit IgG (1:3,000) was added for the incubation for 2 h at room temperature. Finally, the membrane was developed by enhanced chemiluminescence (ECL, Amersham). The internal reference protein was β-actin. The expression level of cytokeratin and vimentin quantified as the relative gray value of β-actin.

Statistical analysis

The SPSS 19.0 software was used for statistical analyses. The measurements are expressed as means ± SD and the difference between groups were compared by post hoc Bonferroni tests. Values of $P<0.05$ were considered to be statistically significant.

Results

Histopathological observations

All rats recovered successfully after the surgery. Compared with the two control groups, the thickness of endometrium, endometrium superficial epithelia and the number of endometrial glands was evidently different in two treatment groups.

In the two treatment groups, the endometrial layer structure was intact as the thickness of endometrium was normal and there are more endometrial glands and capillaries, while the uterine horn in the two control groups was completely destroyed as the necrosis was extensive and there was even coagulative necrosis could be observed on endometrium layer and myometrium layer.

As shown in **Figure 1** and **Table 1**, apparently, there was evidently thicker endometrial lining in the two treatment groups compared with that in the two control groups (both $P<0.001$). However, the difference between the two treatment groups was insignificant ($P=0.404$).

Epithelial cells regeneration

The level of cytokeratin was detected by western blot and immunohistochemical staining to evaluate the regeneration of epithelial cells.

The western blot showed that there was only slightly difference in the cytokeratin level between treatment group and control group in both acute and chronic model (both $P>0.05$, **Figure 2**).

The result of immunohistochemical staining showed that the level of cytokeratin was centralized in the cytoplasm of the endometrial epithelium, of which the difference was insignificant between treatment group and control group in both acute and chronic model (both $P>0.05$, **Table 2**, **Figure 3**).

Stromal cells regeneration

The level of vimentin was detected by western blot and immunohistochemical staining to evaluate the regeneration of stroma cells.

The western blot showed that the vimentin level in the two treatment groups were significantly stronger compared with the two corresponding control groups (both $P<0.01$). The level of vimentin in the acute injury treatment

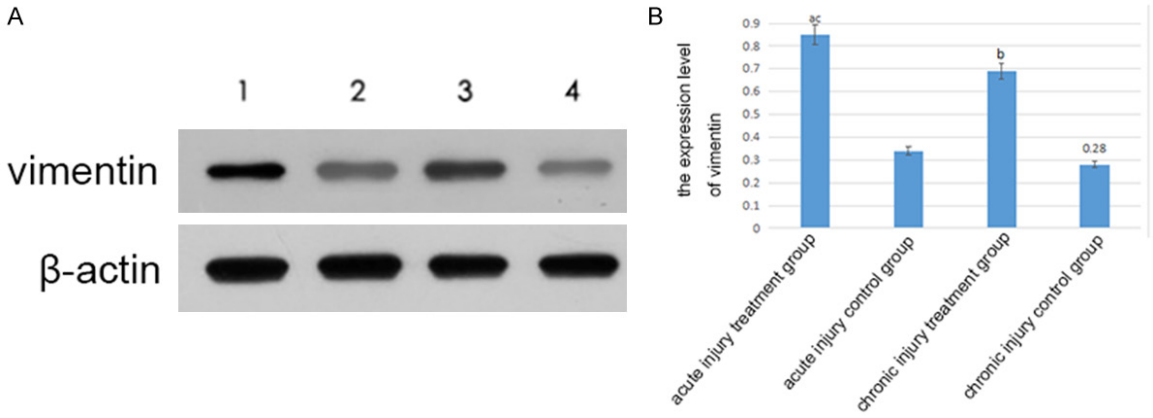

Figure 4. Expression of vimentin with western blot. Group 1-4: acute injury treatment group, acute injury control group, chronic injury treatment group,chronic injury control group. Compared with the corresponding control group, [a]P<0.01, [c]P<0.01; compared with the acute injury treatment group [b]P<0.05.

Table 3. Comparison of the positive vimentin area in per unit endometrium area between 4 groups ($\bar{x} \pm s$)

Groups	Uteri (n)	Vimentin area (%)
Acute injury treatment group	12	12.97±0.55[a]
Acute injury control group	10	5.03±0.56
Chronic injury treatment group	10	10.15±0.64[b,c]
Chronic injury control group	8	5.44±0.51

Note: Compared with the corresponding control group, [a]P<0.001, [b]P<0.001; compared with the acute injury treatment group, [c]P<0.001.

group was significantly stronger than that of the chronic injury treatment group (P<0.05). However, there was no significant difference between the two control groups (P>0.05, **Figure 4**).

The results of immunohistochemical staining showed that the expression of vimentin was mainly centralized in cytoplasm of endometrial stroma cells. The level of vimentin in the two treatment groups were significantly stronger than that in the two corresponding control groups (both P<0.001). The vimentin expression in the acute injury treatment group was obviously higher than that in the chronic injury treatment group (P<0.001), while the difference between the two control groups was insignificant (P>0.05, **Table 3**, **Figure 5**).

Discussion

In this study, the therapeutic effect of exogenous estrogen on rats with thin endometrium was evaluated and its effects on acute or chronic injury werewas compared. Histologic evaluation of the uterine indicated that the model was established successfully, moreover, subcutaneous administration of exogenous estrogen played an positive role on the thin endometrium regeneration; conversely, the two control groups showed that the necrosis was extensive and there was even coagulative necrosis could be observed on endometrium layer and myometrium layer, which also proved that the exogenous estrogen had therapeutic effects on thin endometrium. Guo el al. reported that the cellular response to estrogen is regulated both by the binding between ER and estrogen response element and the signal pathways [20]; Qiu el al. found that estrogen could regulate the expression of angiogenic factors [21]; the result of report from Jiang el al. showed that estrogen could increase the expression level of TGF-β and VEGF to promote endometrium repair in intrauterine adhesion patients after transcervical resection of adhesion [22]. Therefore, we speculated that estrogen may exert its therapeutic effects via binding to the estrogen receptor (ER), thus to promote endometrial repair and cyclical endometrial vascular reconstruction [23, 24]. Additionally, studies have proved that exogenous estrogen could improve women pregnancy rates [25], indicating that exogenous estrogen may also promote the regeneration of endometrium via improving ovarian response.

Cytokeratin, a kind of structural specificity protein, specifically expresses in epithelial cells

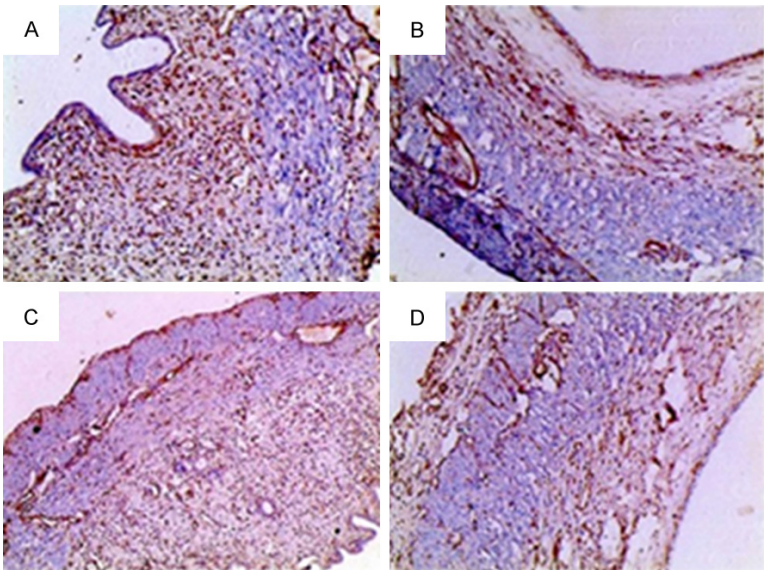

Figure 5. Regeneration of stroma cells in the endometriumimmunohistochemical staining with vimentin (200×). A: Acute injury treatment group, B: Acute injury control group, C: Chronic injury treatment group, D: Chronic injury control group. The yellowish-brown part indicated positive staining of cytokeratin.

and net-like distributed throughout the cytoplasm, so the change of distribution of cytokeratin can indicate the change of the area of the epithelial cells, thus the development of epithelial cells can be observed. Similarly, vimentin mainly distributes in stromal cells, so its distribution can indicate the grows condition of stroma cells and some endothelial cells. In our study, the level of vimentin in the two treatment groups were obviously higher than that of the two control groups, which in the acute injury treatment group was even higher, suggesting that exogenous estrogen could promote the regeneration of stromal cells and endothelial cells.

However, the therapeutic effect of exogenous estrogen on thin endometrium was weakened if the administration of the estrogen was delayed. We have found that compared with acute injury treatment group, the endometrial lining was thinner and the level of vimentin was lower in the chronic injury treatment group. The reason might be that the delayed administration of exogenous estrogen could not induce stem cells to regenerate endometrial cells because the inflammation induced by the modeling was diminished progressively.

There are some limitations in this study, for example we did not further investigate the deeper mechanism for the effects of estrogen on the regeneration of endometrial cells and the indicators were relative less. Therefore, in the future, further study is needed to explore the exact mechanism of the effects of exogenous estrogen on thin endometrium.

In conclusion, exogenous estrogen could promote endometrial cells regeneration *in vivo*, which might provide a new therapeutic method for the treatment of thin endometrium in women suffering infertility.

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Disclosure of conflict of interest

None.

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References

[1] Barash A, Granot I, Fieldust S and Or Y. Successful pregnancy and delivery of a healthy baby after endometrial biopsy treatment in an in vitro fertilization patient with severe Asherman syndrome. Fertil Steril 2009; 91: 1956, e1951-1953.

[2] Wang X, Gu BX, Luo TM, Chen YH, Zhang CL and Li M. Pregnancy outcome after transplantation of frozen-thawed high-quality embryo in PCOS patients. Reproduction and Contraception 2016; 36: 270-275.

[3] Granberg S, Wikland M, Karlsson B, Norstrom A and Friberg LG. Endometrial thickness as measured by endovaginal ultrasonography for identifying endometrial abnormality. Am J Obstet Gynecol 1991; 164: 47-52.

[4] Zhao J, Tian T, Zhang Q, Wang Y and Li Y. Use of granulocyte colony-stimulating factor for the treatment of thin endometrium in experimental rats. PLoS One 2013; 8: e82375.
[5] Miwa I, Tamura H, Takasaki A, Yamagata Y, Shimamura K and Sugino N. Pathophysiologic features of "thin" endometrium. Fertil Steril 2009; 91: 998-1004.
[6] Chen MJ, Yang JH, Peng FH, Chen SU, Ho HN and Yang YS. Extended estrogen administration for women with thin endometrium in frozen-thawed in-vitro fertilization programs. J Assist Reprod Genet 2006; 23: 337-342.
[7] Dumitru DM, Onofriescu M, Carauleanu A and Radulescu D. The endovaginal ultrasound finding of a thin and regular endometrium is uncommon in endometrial cancer. Rev Med Chir Soc Med Nat Iasi 2009; 113: 132-135.
[8] Hsieh YY, Tsai HD, Chang CC, Lo HY and Chen CL. Low-dose aspirin for infertile women with thin endometrium receiving intrauterine insemination: a prospective, randomized study. J Assist Reprod Genet 2000; 17: 174-177.
[9] Demir B, Dilbaz S, Cinar O, Ozdegirmenci O, Dede S, Dundar B and Goktolga U. Estradiol supplementation in intracytoplasmic sperm injection cycles with thin endometrium. Gynecol Endocrinol 2013; 29: 42-45.
[10] Liu SM, Zhou YZ, Wang HB, Sun ZY, Zhen JR, Shen K, Deng CY and Lang JH. Factors associated with effectiveness of treatment and reproductive outcomes in patients with thin endometrium undergoing estrogen treatment. Chin Med J (Engl) 2015; 128: 3173-3177.
[11] Papanikolaou EG, Kyrou D, Zervakakou G, Paggou E and Humaidan P. "Follicular HCG endometrium priming for IVF patients experiencing resisting thin endometrium. A proof of concept study". J Assist Reprod Genet 2013; 30: 1341-1345.
[12] Reynolds K, Khoury J, Sosnowski J, Thie J and Hofmann G. Comparison of the effect of tamoxifen on endometrial thickness in women with thin endometrium (<7 mm) undergoing ovulation induction with clomiphene citrate. Fertil Steril 2010; 93: 2091-2093.
[13] Gleicher N, Vidali A and Barad DH. Successful treatment of unresponsive thin endometrium. Fertil Steril 2011; 95: 2123, e2113-2127.
[14] Li Y, Pan P, Chen X, Li L, Li Y and Yang D. Granulocyte colony-stimulating factor administration for infertile women with thin endometrium in frozen embryo transfer program. Reprod Sci 2014; 21: 381-385.
[15] Eftekhar M, Sayadi M and Arabjahvani F. Transvaginal perfusion of G-CSF for infertile women with thin endometrium in frozen ET program: a non-randomized clinical trial. Iran J Reprod Med 2014; 12: 661-666.
[16] Lebovitz O and Orvieto R. Treating patients with "thin" endometrium-an ongoing challenge. Gynecol Endocrinol 2014; 30: 409-414.
[17] Jing Z, Qiong Z, Yonggang W and Yanping L. Rat bone marrow mesenchymal stem cells improve regeneration of thin endometrium in rat. Fertil Steril 2014; 101: 587-594.
[18] Zhang GR, Yu X, Xu YL, Wang TT and Lang JH. Endometrial thickening in 98 patients received selective estrogen receptor modulators therapy. Journal of Reproductive Medicine 2016; 25: 937-942.
[19] Zhang YH, Wang CF, Chen ZY, Zhang RH, Li RJ and Li HX. Establishment and preliminary identification of mouse thin endometrium model. J Shanxi Med Univ 2017; 48: 432-435.
[20] Guo RX, Wei LH, Tu Z, Sun PM, Wang JL, Zhao D, Li XP and Tang JM. 17 beta-estradiol activates PI3K/Akt signaling pathway by estrogen receptor (ER)-dependent and ER-independent mechanisms in endometrial cancer cells. J Steroid Biochem Mol Biol 2006; 99: 9-18.
[21] Qiu Y, Lu YY and Zhang JQ. Effect of estrogen on growth of endometrial cancer cells and expression of angiogenic factors. Chin J Oncol Prev Treat 2012; 4: 29-33.
[22] Jiang YN. Effect of different doses of estrogen on endometrial repair and prognosis in patients with moderate to severe IUA after hysteroscopic adhesion excision. Journal of Taishan Medical College 2018; 2: 185-187.
[23] Senturk LM and Erel CT. Thin endometrium in assisted reproductive technology. Curr Opin Obstet Gynecol 2008; 20: 221-228.
[24] Shen MS, Wang CW, Chen CH and Tzeng CR. New horizon on successful management for a woman with repeated implantation failure due to unresponsive thin endometrium: use of extended estrogen supplementation. J Obstet Gynaecol Res 2013; 39: 1092-1094.
[25] Le AW, Shan L, Wang ZH, Dai XY, Xiao TH and Zuo R. Effects of icariin on the expression of ER, VEGF, and KDR in the endometrial cells of thin endometrium. Genet Mol Res 2015; 14: 11250-11258.