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Decreased expression of c-kit and telomerase in a rat model of chronic endometrial ischemia

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Summary

Background: It was unclear whether chronic endometrial ischemia contributed to the pathogenesis of thin endometrium and was associated with decreased endometrial stem/progenitor cell. Thus, we explored the role of chronic endometrial ischemia in the pathogenesis of thin endometrium and its effect on endometrial stem/progenitor cells apoptosis.

Material/Methods: *In vitro*, endometrial side population (ESP) cell apoptosis models were built, and apoptosis was quantified by fluorescence-activated cell sorter (FACS) analysis, pou5f1, and c-kit mRNA was detected by qPCR. *In vivo*, a rat model of chronic endometrial ischemia was induced by performing bilateral uterine artery ligation. TERT and caspase3 were detected by immunohistochemistry. Pou5f1and c-kit mRNA was examined by qPCR. C-kit, caspase3 and telomerase were detected by Western blot.

Results: In the *in vitro* endometrial SP (ESP) cells apoptosis model, we found that the apoptotic rate was gradually increased with time, prolonging the expression of TERT, and c-kit mRNA was gradually decreased. In the *in vivo* endometrial SP (ESP) cells apoptosis model, we found that endometrial thickness, luminal epithelium thickness, gland epithelium thickness and the number of glands in the experiment group were significantly decreased compared with those in the control group (P<0.05). The expression levels of c-kit, pou5f1 and telomerase was significantly lower in the experimental group than those in the control group (P<0.05). The expression level of caspase3 was significantly higher in the experimental group compared with that in the control group (P<0.05).

Conclusions: The present work shows that chronic ischemia and chronic endometrial ischemia-associated stem/progenitor cells apoptosis may be responsible for the pathogenesis of thin endometrium.

key words: thin endometrium • endometrial stem/progenitor cells • chronic endometrial ischemia • telomerase • c-kit

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BACKGROUND

An adequate thickness of the endometrium is indispensable for a successful pregnancy in assisted reproductive technology (ART) cycles [1,2]. Some studies have reported low pregnancy rates in the presence of thin endometrium [3]. Angiogenesis plays a crucial role in various female reproductive processes, including the development of a dominant follicle, formation of a corpus luteum and growth of the endometrium [4,5]. The process of angiogenesis is essential for the repair of endometrium after menstruation [6]. Miwa et al. [7] have shown that thin endometria are characterized by poor growth of glandular epithelium, high uterine blood flow impedance, decreased vascular endothelial growth factor (VEGF) expression, and poor vascular development. High blood flow impedance of radial arteries, which could be a trigger, impairs the growth of the glandular epithelium and results in a decrease in VEGF levels in the endometrium. Low VEGF causes poor vascular development, which in turn further decreases blood flow in the endometrium. This vicious cycle leads to a "thin" endometrium, which is related to impaired endometrial receptivity. The present results also suggest that high blood flow impedance of radial arteries at the start of the menstrual cycle can be a useful predictor of a thin endometrium, although the cause of high blood flow impedance of radial arteries in patients with a thin endometrium is unclear [7]. This hypothesis suggests that chronic endometrial ischemia is associated with the pathogenesis of thin endometrium. There is, however, no conclusive evidence regarding which pathways or molecular targets may be involved. Many of the current strategies for improving endometrial thickness focus on the use of aspirin and estrogen. However, there are many controversial studies regarding the effect of low-dose aspirin and estrogen on improving endometrial thickness and pregnancy rates [8].

The concept that endometrial stem/progenitor cells may be responsible for the high regenerative capacity of human endometrium was proposed many years ago [9–12]. Endometrial stem/progenitor cells have critical roles in the physiological remodeling and regeneration of the human uterus and the pathogenesis of gynecological diseases, including endometriosis and endometrial cancer [13,14]. Lorch et al. [15] determined that the average percentage of normal glands stained with telomerase was 94.5% on day 15 and 60% on day 24. With the diffuse pattern being present in 5.5% of nuclei on day 15 and no nuclei on day 24, the thin endometria had fewer mitotic figures on day 15, less telomerase staining on day 15(85%) and day 24(56%), and only 1.4% of the nuclei had diffuse staining on day 15 compared with normal endometria. Telomerase is a reverse transcriptase whose activity is associated with the immortality of iPS cells (induced pluripotent stem cells), embryonic stem cells, germ cells and cancer cells [16–20]. Decreased expression of telomerase in thin endometrium, which is unresponsive to estrogen replacement therapy compared with that in normal endometrium, indicates that thin endometrium may have a decreased amount of endometrial stem/progenitor cells [15].

There has been no direct evidence to the relationship between thin endometrium and chronic endometrial ischemia. It was unclear whether chronic endometrial ischemia contributed to the pathogenesis of thin endometrium and whether it was associated with decreased endometrial stem/progenitor cell activity. We used Sprague-Dawley (SD) rats to create a model for chronic endometrial ischemia through ligation of the bilateral uterine arteries. By detecting expression of stem cell-related proteins, including telomerase (TERT), c-kit and pou5fl, the relationship between chronic endometrial ischemia and pathogenesis of thin endometrium was analyzed.

MATERIAL AND METHODS

In vitro, endometrial side population (ESP) cell apoptosis models were built

All animal experiments were approved by the Ethics Committees of Chongqing Medical University. Six-month-old virgin female Sprague-Dawley rats were maintained in collective cages in an appropriate room with controlled temperature and with a 12-h light cycle, and fed with standard rat chow and water. We isolated ESP cells from Sprague-Dawley rats as described by Masuda et al. [21]. All ESP cells were grown in IMDM containing 15% heat-inactivated fetal bovine serum and 100U/ml penicillin-streptomycin, and were incubated at 37° in a humidified atmosphere containing 5% CO_2 and 95% air. All cells in the testing were first passage. Select conditions that may be observed in ischemia were simulated by serum deprivation of the culture medium and by hypoxia. Cells were washed with serum-free IMDM and placed in serum-free medium in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 0, 2, 4 or 8 h.

Measurement of endometrial SP (ESP) cells apoptosis

We collected endometrial SP (ESP) cells by using trypsin without EDTA, and added 500 ul Binding Buffer into cell suspension, then added 2 ul Annexin V-FITC. After reacting for 20 min in a dark room at 25°C, the apoptotic cells were identified by flow cytometry. All experiments were repeated 3 times with similar results.

In vivo model of endometrial side population SP (ESP) cells apoptosis

Surgical methods were described by Simmons RA et al. [22]. In brief, all animals were acclimatized for 3 days before the experiment started. The experimental group rats were anesthetized with intraperitoneal xylazine (8 mg/kg) and ketamine (40 mg/kg), and both uterine arteries were ligated. Control group rats underwent the identical anesthetic and surgical procedure; in control group rats, neither of the uterine arteries was ligated. Vaginal smears were taken to determine estrous cycle phase [23]. Three months after the operation, the animals were killed under isoflurane anesthesia on the day of estrus. Then, random samples of uterus were frozen in liquid nitrogen. All samples were stored at –70°C.

Histological examination

For routine histology, 5 um sections of 10% formalin-fixed, paraffin-embedded tissue was prepared and stained with hematoxylin and eosin. All stained sections were assessed in a blinded fashion using a light microscope.

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Immunohistochemistry (IHC)

The tissue samples were fixed in formalin, embedded in paraffin, sectioned (5 μm thick), deparaffinized in xylene, dehydrated in a graded series of ethanol, subjected to antigen retrieval in citrate buffer (pH 6.0; Sigma) for 30 min in a steamer and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide and blocked with PBS containing 10% goat serum (sigma) for 1 h at 37°C. Next, slides were incubated with the primary antibody overnight at 4°C, which included polyclonal rabbit anti-telomerase (1 150; ZA-0239; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) and polyclonal rabbit anti-caspase3 (1:100; BS1518; Bioworld Technology, Inc.). Negative controls included omission of primary antibody and use of irrelevant primary antibodies. After several washes (3×3 min) to remove excess antibody, the slides were incubated with diluted (1:300) anti-rabbit biotinylated antibodies (Beijing Biosynthesis Biotechnology Co., Ltd, China) for 1 h. All the slides were washed in PBS and were incubated in avidin biotin peroxidase complex (ABC) (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) diluted 1:300 in PBS for 30min in humidified chambers at 37°C. DAB (Beijing Biosynthesis Biotechnology Co., Ltd, China) was used as a chromogen and hematoxylin was used as a nuclear counterstain.

Slides were evaluated independently by 3 pathologists for distribution and intensity of signal as described by De Falco et al. [24]. Intensity was scored from 0 to 3:0 (no immunopositivity); 1 (low immunopositivity); 2 (moderate immunopositivity); 3 (intense immunopositivity). An average of 22 fields was observed for each tissue.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from endometrial SP (ESP) cells, experimental and control group rats' endometrium using RNA prep pure Kit (Tiangen Biotech Co., Ltd). First-strand cDNA was synthesized using a PrimeScriptTM RT-PCR Kit (TaKaRa Biotechnology Co., Ltd). Pou5f1 and c-kit and mRNA expression levels were examined by quantitative real-time PCR with the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and All-in-One qPCR Mix (GeneCopoeia, Inc.). Â-actin values were used for normalization. Primers used for SYBR Green assay were purchased from GeneCopoeia, Inc. (MQP030962, MQP032441, and RQP048500). PCR amplifications were started with a 10min denaturation step at 95°C, followed by 40 amplification cycles (10 sec at 95°C, 20 sec at 60°C, 10sec at 72°C). Relative quantification of mRNA was performed using the comparative threshold cycles (CT) method. The value was used to plot the gene expression employing the formula $2^{-\Delta\Delta CT}$.

Western blot analysis

Expression of telomerase, oct4 and caspase3 protein was analyzed by Western blotting as described [25]. The primary antibodies used included polyclonal rabbit anti-telomerase (1:150; ZA-0239; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China); polyclonal rabbit anti-caspase3 (1:500;BS1518; Bioworld Technology, Inc.); polyclonal rabbit anti-c-kit (1: 500;BS3621; Bioworld Technology, Inc.) and polyclonal rabbit anti-β-actin (1:500; ZA-0239; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China). The bands were detected via enhanced chemiluminescence (ECL) reagent (Beyotime Institute of Biotechnology, Jiangsu, China) and quantified by ImageQuant 3.3 software (Molecular Dynamics).

Statistical evaluation

All values were expressed as mean ±SEM. Comparison of 2 means was made with Student's t-test. A p value of <0.05 was considered statistically significant.

RESULTS

Effects of Serum Deprivation and Hypoxia in endometrial SP (ESP) cells apoptosis

During ischemia many changes contribute to cellular death. To explore the effect of these stimuli, endometrial SP (ESP) cells were exposed to culture conditions including serum deprivation and hypoxia. Figures 1A and B show the percentage of endometrial SP (ESP) cells apoptotic cell number in response to serum deprivation and hypoxia treatment (2–6 hours). The flow cytometric detection indicated that along with increased time the apoptotic rate gradually increased. The real-time qPCR assay revealed that along with time prolonging, the expression of TERT and c-kit mRNA was gradually decreased (P<0.05; Figures 1C and D). These data suggest that serum deprivation and hypoxia, both components of ischemia *in vivo*, induced apoptosis in endometrial SP (ESP) cells. As a result of these findings, we adopted the following experiments, an *in vivo* model of endometrial SP (ESP) cells apoptosis to confirm the impact of ischemia on the endometrial stem cell population *in vivo*.

Histological findings

Endometrial thickness (H0), luminal epithelium thickness (H1), gland epithelium thickness (H2) and the number of glands (N) in the experimental group were significantly decreased compared with those in the control group (P<0.05; Figure 2, Table 1).

Immunohistochemistry (IHC)

In the samples examined we observed that caspase3 was intensively expressed in glandular epithelium and stromal cells in the experimental group; caspase3 was expressed at a low level in glandular epithelium in the control group. Telomerase expression level became moderate in the glandular epithelium and in stromal cells in the experimental group, and intense immunopositivity was found localized in the stroma of the control group (Figure 3).

Quantitative real-time polymerase chain reaction

Pou5f1 and c-kit mRNA were detected in all tissues studied. The expression of c-kit and pou5f1 mRNA was decreased significantly in the experimental group compared with the control group (p<0.05, Figure 4).
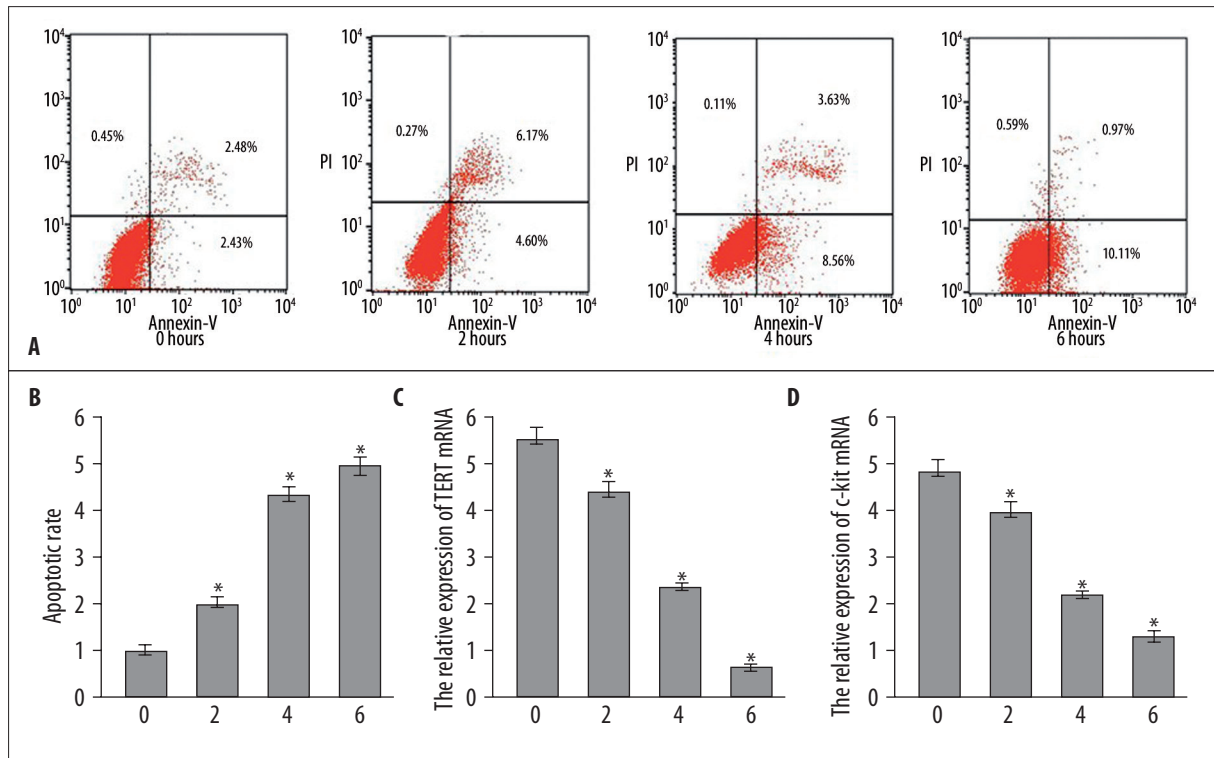
Figure 1. Serum deprivation and hypoxia trigger apoptosis in endometrial SP (ESP) cells. Endometrial SP (ESP) cells were incubated for 2, 4, and 6 hours in serum deprivation and hypoxia. Apoptosis was detected by flow cytometry. (**A, B**): Apoptosis was quantified by fluorescence-activated cell sorter (FACS) analysis, as confirmed by Annexin V and propidine iodide (PI). (**C, D**) The relative expression of TERT and c-kit mRNA was detected by qPCR. Data given as mean ±SEM. *p<0.05, *vs.* control group; Error bars, SEM.
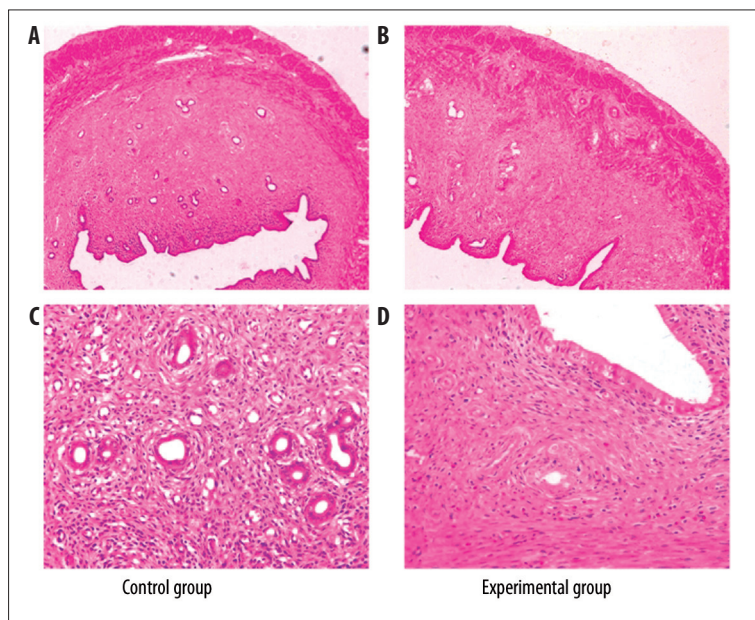
Figure 2. Histological examination of the rat endometrium. Hematoxylin and eosin (H-E) staining. (**A**) Representative endometrium sections of control rats; ×50 magnification. (**B**) Representative endometrium sections of experimental rats; ×50 magnification. (**C**) Representative endometrium sections of control rats; ×200 magnification. (**D**) Representative endometrium sections of experimental rats; ×200 magnification.

Western blot

The expression level of c-kit and telomerase were significantly lower in the experimental group than in the control group. The expression level of caspase3 was significantly higher in the experimental group compared with the control group (P<0.05; Figure 5, Table 2).

DISCUSSION

We explored the impact of ischemia on endometrial SP (ESP) cells *in vitro* and *in vivo*. Firstly, we studied the effect of endometrial SP (ESP) cells that were exposed to culture conditions including serum deprivation and hypoxia. We found that along with increased time, the apoptotic rate

Table 1. The pathological changes of endometrium in experimental group and control group.

|	H0	H1	H2	N
	(mm)	(mm)	(mm)	
Control group	800±130	29.46±9.27	15.68±2.50	30±5.5
Experimental group	502±148*	17.20±4.98*	9.17±1.96*	18.9±4.8*

(The endometrial thickness (H0), luminal epithelium thickness (H1), gland epithelium thickness (H2) and the number of glands (N) in experiment group and control group rats. Data given as mean ±SEM. *p<0.05, *vs.* control group; Error bars, SEM).
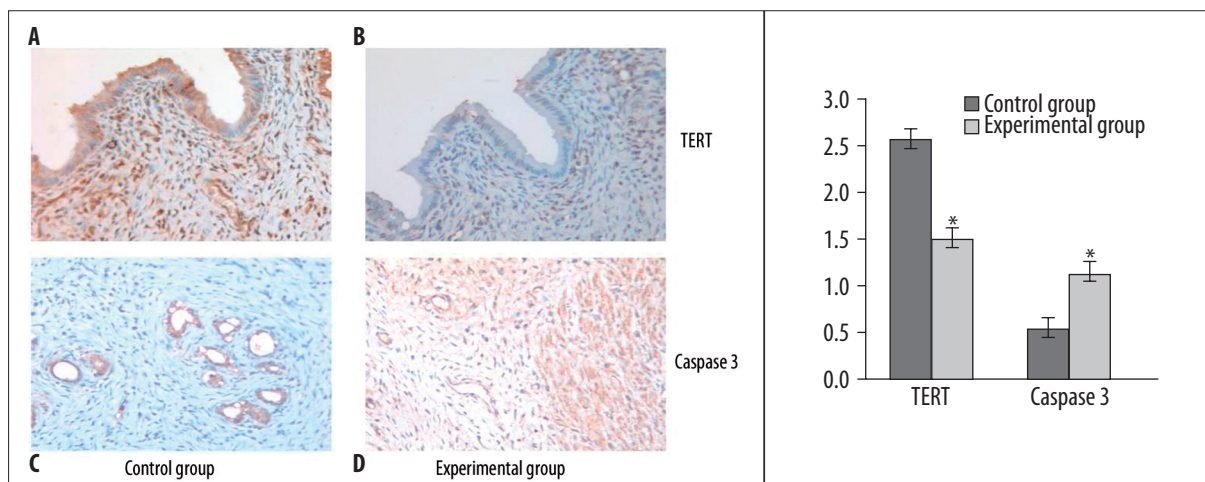
Figure 3. Representative immunohistochemical staining patterns of telomerase and caspase3 in rat endometrium. (**A**) Telomerase expressing cells in control rat endometrium; ×200 magnification. (**B**) Telomerase-expressing cells in experimental rat endometrium; ×200 magnification. (**C**) Caspase3-expressing cells in control rat endometrium; ×200 magnification. (**D**) Caspase3-expressing cells in experimental rat endometrium; ×200 magnification. *p<0.05 (*vs.* control group); error bars, SEM.
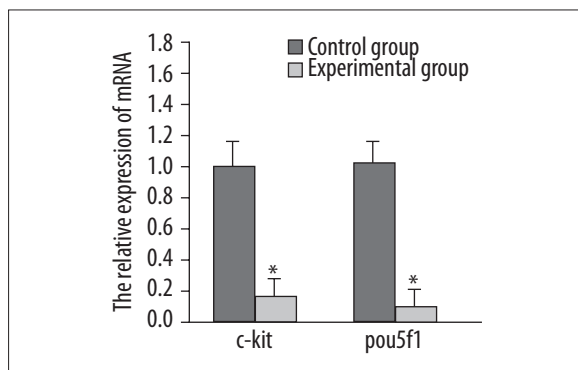
Figure 4. Quantitative real-time PCR analysis of c-kit and pou5f1 mRNA expression in control and experimental rat endometrium. Relative decreased expression of c-kit and pou5f1 in experimental rat endometrium versus control rat endometrium, as calculated by the $2^{-\Delta\Delta Ct}$ method. *p<0.05 (*vs.* control group); error bars, SEM.
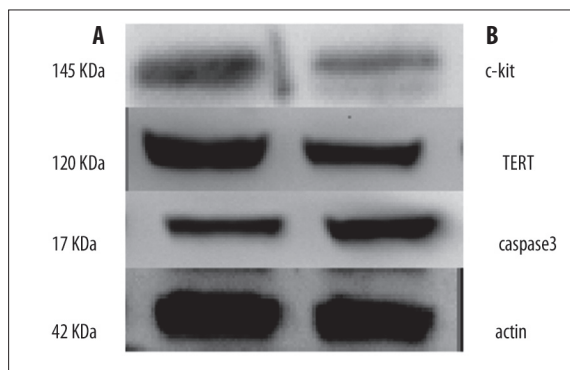
Figure 5. Western blot analysis of telomerase, c-kit and caspase3 in rat endometrium. (**A**) Control rat endometrium (**B**) experimental rat endometrium.

was gradually increased, the expression of TERT and c-kit mRNA was gradually decreased. These data suggest that serum deprivation and hypoxia, both components of ischemia *in vivo*, induced apoptosis in endometrial SP (ESP) cells.

Uterine blood flow is very important for endometrial growth [26]. High blood flow impedance of uterine radial arteries

leading to chronic endometrial ischemia may be involved in the pathogenesis of poor endometrial growth in patients with thin endometrium [7,27]; however, it is difficult to study the mechanism of thin endometrium in clinical samples. Therefore, a nice model is necessary. In the present study, we have, for the first time, developed a model of chronic endometrial ischemia induced by performing bilateral uterine artery ligation in the rat. The reduction of endometrial thickness, luminal epithelium thickness and the number of glands has been identified in our experiment.

Table 2. The relative levels of expression of c-kit, telomerase and caspase3 protein in control and experimental rat endometrium.

	c-kit	Telomerase	Caspase3
Control group	0.75±0.09	0.87±0.12	0.40±0.08
Experimental group	0.31±0.13*	0.64±0.19*	0.67±0.23*

(Data given as mean ±SEM. *p<0.05, *vs.* control group; Error bars, SEM).

These changes are consistent with pathology of patients with thin endometrium, which is characterized by poor growth of glandular epithelium and high uterine blood flow impedance. Our SD rats treated with bilateral uterine artery ligation can serve as a good model for chronic endometrial ischemia. In addition, the pathological changes in the rats support the hypothesis that chronic endometrial ischemia is associated with the pathogenesis of thin endometrium.

C-kit, telomerase and Pou5f1 are been proven to be associated with the features of stem cells of endometrium, including undifferentiation and proliferative potential [16–20,28,29]. C-kit is a stem cell factor receptor that has been described as an undifferentiated marker in the human endometrium [28]. Telomerase is a reverse transcriptase whose activity is associated with the immortality of iPS cells (induced pluripotent stem cells), embryonic stem cells, germ cells and cancer cells [16–20,30]. Changes in telomerase activity during the menstrual cycle reflect the proliferative potential of the endometrium [31]. Decreased expression of telomerase in thin endometrium that is unresponsive to estrogen replacement therapy compared with normal endometrium indicates that thin endometrium may have a decreased activity of endometrial stem/progenitor cells [15]. Pou5f1, as a nuclear transcription factor, encodes a transcription factor that is involved in mediating the maintenance of the undifferentiated state, and is widely regarded as a hallmark of pluripotent stem cells [29]. In a numbers of stem cell studies, apoptosis was present in various stem cells, such as progenitor cells and mesenchymal stem cells [32–34]. The death-related cysteine proteases of the caspases family play a central role in the execution phase of apoptosis. Caspase3 is a member of this family, which is present in a wide variety of cells and has been found to be activated in nearly every model of apoptosis, including neuronal progenitor cell apoptosis and mesenchymal stem cell apoptosis [32–34]. In the *in vitro* model of ESP cells apoptosis, we found that, together with ESP cells apoptosis, the expression of c-kit and TERT was decreased. The reduced c-kit, pou5f1 and telomerase expression and the increased expression of caspase3 in the experimental group indicate that chronic endometrial ischemia maybe contribute to ESP cell apoptosis, which is involved in the pathogenesis of thin endometrium. Moreover, abnormal alteration between caspase3 and telomerase/pou5f1/c-kit suggest that there is a deregulation of endometrial stem cells in the endometrium of experimental group rats.

Stem cell therapy is crucial in treatment of many diseases [35,36]. It has been reported that chronic ischemia may lead to stem/progenitor cell apoptosis. Telomerase expression is decreased in chronic heart failure [37]. Poor blood supply and low oxygen tension caused mesenchymal stem cell (MSC) apoptosis in the ischemic myocardium [38]. MSCs undergo caspase-dependent apoptosis in response to hypoxia and serum deprivation, which are both components of ischemia *in vivo* [34]. Bone marrow-derived MSCs contribute to endometrial regeneration and are progenitors of endometrial stromal fibroblasts [39]. Our study indicates that chronic endometrial ischemia induced an increased apoptosis of endometrial stem/progenitor cells.

Decreased expression of telomerase in thin endometrium that is unresponsive to estrogen replacement therapy compared to normal endometrium indicates that thin endometrium may have a decrease of endometrial stem/progenitor cells [15]. Combined with the findings above, we hypothesize that chronic endometrial ischemia-associated stem/progenitor cell apoptosis may be involved in the pathogenesis of thin endometrium.

Conclusions

We developed an ideal model of thin endometrium induced by performing bilateral uterine artery ligation in the rat. We also demonstrated that chronic endometrial ischemia contributes to the pathogenesis of thin endometrium. Chronic endometrial ischemia-associated stem/progenitor cells apoptosis may play a crucial role in this disease.

Conflict of interest statement

The authors have no conflict of interest regarding this manuscript.

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