Fulvestrant, an estrogen receptor inhibitor, relieves postoperative hemorrhoid edema via up-regulation of miR-424-5p

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INTRODUCTION

Hemorrhoids are anal pad tissue and vascular proliferative disease with symptoms of bleeding, pain, prolapse, and perianal itching. The worldwide prevalence of hemorrhoids is as high as 39% [1,2]. The initial treatment for hemorrhoids includes dietary control, proper
hygiene, and use of stool softeners for easy defecation. Some people use external hemorrhoids bolts and ointment. Surgeries, including loop ligation, ligation and excision, have become the first choices for treatment of hemorrhoids. After surgery, the patient usually faces the risk of postoperative bleeding and tissue edema. The incidence of postoperative tissue edema is as high as 20 % [3].

In clinics, postoperative edema is more common in females than in males. Some studies have found that abnormal estrogen expressions in hemorrhoid tissues is associated with the development of the disease [4,5]. Elevated estrogen often further results in elevated estrogen receptor (ER) [1]. However, the relationship between ER and tissue edema after hemorrhoids surgery, and the specific mechanism through which ER affects tissue edema after hemorrhoids, remain unclear.

MicroRNAs (miRNAs) are non-coding small RNA molecules with total lengths of 19 - 23 bases [2]. The miRNAs regulate the expressions of multiple target genes in organisms, thereby affecting various physiological processes, including the proliferation, differentiation, and apoptosis of cells [3]. A previous investigation reported that the tissue miR-424 level was significantly reduced after hemorrhoid surgery. Research evidence shows that miR-424 regulated cell proliferation and suppressed estradiol-induced cell proliferation in endometrial cancer cells [6]. Fulvestrant is an estrogen receptor antagonist [7]. It also inhibits proliferation of carcinoma cells [8]. Nevertheless, there are no reports on the association between miR-424 expression and the effectiveness of Fulvestrant in reducing hemorrhoid edema.

The present research was carried out to determine ER expression in postoperative hemorrhoid edema tissues, and the possible mechanism of Fulvestrant-mediated reduction in postoperative hemorrhoids edema. In addition, the association of miR-424 expression with Fulvestrant-mediated mitigation of postoperative hemorrhoids edema was investigated, so as to generate scientific reference for management of the disease.

METHODS

Subjects

The study was approved by the Ethics Committee of Jinshan Hospital of Fudan University (approval no. (-2018-38-03). Written informed consents were obtained from the patients and/or guardians. Fourteen patients who had postoperative edema after mixed hemorrhoids (Miligan-Morgan) resection, and who underwent edema tissue resection in our hospital from December 2018 to June 2019 were included. The clinical diagnosis and treatment for hemorrhoids were conducted according to The American Society of Colon and Rectal Surgeons Clinical Practice Guidelines [9]. The operations were performed by an experienced physician.

Patients who satisfied the following inclusion conditions were subjects in the study: (1) Patients who had mixed hemorrhoids (Miligan-Morgan) and postoperative anal margin edema; (2) those aged over 18 years, and (3) patients who signed informed consent forms voluntarily. Exclusion criteria: (1) Patients with other anorectal diseases such as anal rectal tumors, ulcerative colitis, Crohn's disease, and anal skin diseases; (2) patients who had major medical conditions, including serious cardiovascular and cerebrovascular diseases, malignant tumor, paralysis, chronic liver and renal failure, immune system diseases, and HIV; (3) those with a long history of taking medication and others whose conditions were observed to be serious, and (4) women who were pregnant or breastfeeding.

Immunohistochemistry (IHC) analysis

Hemorrhoid edema tissues obtained from patients who developed postoperative anal edema were subjected to fixation in formalin, dried-out in ethanol gradient, and paraffinized. For IHC analysis, the paraffin-embedded tissues were sectioned. Then, the sections were dewaxed, hydrated, soaked in gradient of xylene-ethyl alcohol mixtures, and embathed 3 times with phosphate buffer saline (PBS), each for 3 min. Then, the sections were placed in 1 % bovine serum albumin (BSA; Beyotime) at room temperature for 20 min, followed by incubation for 12 h with primary anti-ERα immunoglobulin (1: 200) at 4 °C, and then with HRP-linked 2° immunoglobulin at room temperature for 20 min. Diaminobenzidine solution (Fuzhou Maxim Biotechnology Co. Ltd, China, DAB-1031) was used for staining. The sections were re-stained in hematoxylin (Nanjing Jiancheng Company, China, D005), and dehydrated in gradient ethanol and xylene for 20 min. Neutral gum sealing tablet (Sinopharm Chemical Reagent Co. Ltd, China, 10004160) was added to the sections. The ERα expression in tissue cells was examined under an optical
microscope (Olympus, Japan, BX43). All images were captured at x200 magnification.

**Western blot assay**

The isolated hemorrhoid tissues were subjected to complete protein extraction using RIPA buffer, and the lysate protein content was measured with BCA procedure. Lysate protein was resolved electrophoretically with SDS-PAGE and transferred to PVDF membranes which were thereafter sealed using 5 % BSA. The PDVF membranes were then incubated with anti-ERα antibody (1:1000) and anti-VEGF antibody (1:1000; Proteintech Group) at 4 °C for 12 h, followed by incubation with HRP-linked 2° antibody (1:2000; Cell Signaling Technologies) at laboratory temperature for 40 min. Then, the membranes were stained with ECL (Millipore). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin (1:10000; Jiangsu KeyGEN BioTECH Corp. Ltd, Nanjing, China) was used as a standard protein. The G: BOX chemiXR5 system (Syngene, Cambridge, UK) and Gel-Pro32 software (Syngene) were used for the analysis of protein expression levels.

**Enzyme-linked immunosorbent assay (ELISA)**

The levels of total estrogens, including estrone (E1), 17beta-estradiol (E2), and estriol (E3) in hemorrhoid edema tissues were determined using Total Estrogen ELISA test kit. Each sample was determined in triplicate on a microplate reader (Molecular Devices, USA).

**Cell culture and treatments**

Human vascular endothelial cells were maintained in High Glucose DMEM containing 10 % FBS. The cells were incubated in medium at 37 °C to 60 - 70 % confluence. Then, the ER inhibitor Fulvestrant (MCE, Monmouth Junction, USA) was added to the medium in triplicate at a working level of 1 nM, followed by incubation for 24 h. For stimulation of ER, cell incubation was done with E2 (5 × 10⁻⁹, 10 × 10⁻⁹, and 15 × 10⁻⁹ mol/L) or DMSO (control) for 24 h., while miR-424-5p was over-expressed by exposing the cells to its mimic (GenePharma, Shanghai, China) in triplicate for 48 h. All cells were cultured at 37 °C and 5 % CO₂.

**Dual-luciferase reporter assay**

The MiRNA-target interaction was predicted and determined using dual-luciferase reporter assay system [10]. Luciferase vectors containing wild-type (WT) and mutant (Mut) reporters of ESR1 3'-UTR (psicHECKTM-2-ESR1-3'-UTR-WT/Mut; Promega, Mannheim, Germany) were used. The relative intensity of fluorescence was measured at 48 h post-cell transfection in vascular endothelial cells according to the manufacturer's instruction (E1910, Promega).

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Total RNA was extracted with RNAiso Plus General RNA Extraction kit (TaKaRa, Kyoto, Japan) according to the manufacturer’s instructions. The concentration and purity of total RNA was determined using ultrafine nucleic acid concentration tester, while RNA intactness was measured with electrophoretic analysis on agarose gel. The RNA was reverse-transcribed with Reverse transcription PrimeScriptTM RT Master Mix kit (Takara) according to the instructions of the manufacturers. The cDNA synthesis was done under the following conditions: 37 °C for 15 min, 85 °C for 5 sec, and 4 °C for 2 min. The product was directly employed for PCR amplification or stored at -20 °C. The PCR amplification was performed on an ABI StepOne Plus system fluorescence system fluorescence quantitative PCR instrument under the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 30 sec, and 60 °C for 40 sec. The PCR reaction curve was analyzed and Ct value was obtained. The comparative expression levels were calculated with 2⁻△△Ct procedure. The internal reference genes were GAPDH (for mRNA analysis) and U6 (for miRNA analysis).

**Statistics**

The SPSS 21.0 software was used for statistical analysis. Measured data are presented as mean ± standard deviation (SD), and were tested with paired-samples t-test. The comparison of counting variables was done using the chi-square test, while Pearson’s correlation analysis was used to determine relationships amongst variables. Significance was assumed at p < 0.05.

**RESULTS**

**ER expression increased in perianal tissues after hemorrhoids surgery**

From December 2018 to June 2019, 185 patients were admitted with mean age of 45.29 ± 13.97 years, comprising 77 males and 108 females. Fourteen patients (7.57 %) developed postoperative anal edema (Figure 1 A). These consisted of 2 males (2.60 %) and 12 females (11.1 %) with mean age of 45.29 ± 13.97 years. Western blot assay indicated markedly higher ERα levels in postoperative edema samples than
in preoperative edema specimens ($p < 0.05$; Figure 1 B and C).

**ERα promoted tissue edema by inhibiting miR-424**

As shown in Figure 3 A, VEGF expression was significantly elevated after hemorrhoids surgery. The protein expression levels of ERα and VEGF were markedly higher in postoperative hemorrhoids edema samples than in preoperative hemorrhoid edema samples (Figure 3 B). To verify whether estradiol inhibited miR-424 via ER, different doses of E2 were added to primary cells extracted from preoperative tissues. The results showed that ERα and VEGF were increased linearly with concentration of E2 (Figure 3 C). However, miR-424 expression in primary cells was decreased by E2 (Figure 3 D). The overexpression of miR-424 significantly decreased VEGF expression (Figure 3 E and F), but did not change ERα protein expression. These results suggest that VEGF acted as the downstream target of ERα and miR-424.

**Fulvestrant promoted miR-424 and ERα and suppressed VEGF in preoperative primary cells**

Fulvestrant un-regulated miR-424 protein, but suppressed the protein expressions of ERα and VEGF in vascular endothelial cells (Figures 3 G and H). These results indicate the possibility of a fulvestrant-medicated mechanism for the treatment of postoperative hemorrhoid edema.

**Correlation between E2, ER, and miR-424**

Correlation analysis revealed marked correlations between postoperative levels of E2 and ERα, and miR-424. The level of E2 was positively associated with ERα (Figure 4 A), while the level of miR-424(-5p) had a significant negative correlation with E2 and ERα (Figures 4 B and C). The results of IHC examination also showed that ERα was markedly higher in postoperative edema samples than in preoperative samples (Figure 4 D).

**DISCUSSION**

Postoperative hemorrhoids edema is a common complication of hemorrhoids surgery. It is also an intractable complication. Once the postoperative edema occurs, it aggravates postoperative pain response, increases the risk of infection, and enhances the likelihood of re-operation. This study showed that the expression of the ESR1 gene and the VEGF and ERα proteins, and the
content of E2 were upregulated in postoperative anal edema tissues, when compared with preoperative hemorrhoid tissues. Moreover, the E2 treatment promoted protein expressions of ERα and VEGF in primary vascular endothelial cells, while the treatment with Fulvestrant reduced the expressions of ERα and VEGF. In addition, E2 promoted miR-424-5p expression, but this effect was suppressed by Fulvestrant. These results indicate that Fulvestrant might ameliorate postoperative anal edema by inhibiting the ERα/VEGF signaling axis.

Studies have shown that the levels of estrogens or ER are associated with the occurrence and development of hemorrhoids [4,11]. A study showed that ERs were present in the internal anal sphincter in < 30 % women undergoing hemorrhoidectomy [4]. It was also reported that estrogen receptors were present in the internal anal sphincter in 23.5 % of women of menstruating age, and in 11.8 % of post-menopausal women [4]. However, a newer investigation reported that there was no significant association between the changes in levels of hormones (estrogen, progesterone, and relaxin) and the development of hemorrhoidal disease [5]. In this study, there was higher incidence of hemorrhoid edema in females than that in males. In addition, the content of E2 and the level of ERα protein were higher in postoperative hemorrhoid edema tissues, when compared with preoperative hemorrhoid tissues. Thus, estrogen is linked to the incidence of postoperative hemorrhoids edema. Estrogen is an important steroid hormone which regulates the physiological function of the cardiovascular system, reproductive system, immune system, and nervous system [12]. Estrogen works by binding to the ERs. There is evidence showing that angiogenesis is related to the pathogenesis of hemorrhoids [13]. Estrogen and VEGF are both crucial actors in angiogenesis [14] and inflammation [15]. While ERα promotes angiogenesis [16], estrogen promotes vascular hyperplasia [17]. Indeed, VEGF is the main regulator that promotes angiogenesis [14]. The ERα protein participates in blood vessel generation by acting as a promoter of VEGF expression. Estradiol led to VEGF expression and cell multiplication in endometrial carcinoma [19]. However, inhibition of ER blocked the influence of estradiol on VEGF expression [19]. Moreover, miR-424 blocked E2-mediated cell multiplication in endometrial carcinoma [6]. Fulvestrant also inhibited the proliferation of cancer cells [8]. The present study indicated that the E2 treatment promoted the protein

**Figure 3:** ER promoted VEGF by inhibiting miR-424. A: VEGF mRNA levels in preoperative and postoperative tissues, as measured using qRT-PCR analysis. B: Protein expressions of ERα and VEGF in preoperative and postoperative tissues. C: Protein expressions of ERα and VEGF in primary vascular endothelial cells preoperative hemorrhoid tissues. D: The influence of estradiol (E2) on miR-424 expression in primary vascular endothelial cells. E: The expression of miR-424(-5p) following transfection with miR-424-mimic. F: The effect of miR-424 overexpression on ERα and VEGF proteins. G: Fulvestrant promoted miR-424 expression. H: Fulvestrant inhibited both ERα and VEGF in primary vascular endothelial cells. ***P < 0.0001, compared with control.

**Figure 4:** Correlations of estradiol (E2) and ERα, with miR-424. A: Positive correlation between E2 and ERα. B: E2 was negatively correlated with miR-424. C: miR-424 was negatively correlated with ERα. D: ERα expressions in preoperative and postoperative hemorrhoid tissues, as measured via immunohistochemistry.
expressions of ERα and VEGF, but it decreased miR-424 expression in primary vascular endothelial cells. In contrast, the treatment with ER inhibitor Fulvestrant suppressed the expressions of VEGF and ERα but promoted that of miR-424. These results show that the miR-424/ERα/VEGF signaling might promote angiogenesis in hemorrhoids, and Fulvestrant might suppress angiogenesis in hemorrhoids tissues through the miR-424/ERα/VEGF signaling axis. Studies have shown that estrogen exerts anti-inflammatory effect [20]. Estrogen ameliorated inflammation and decreased the inflammatory biomarkers in a mouse airway inflammation model [15]. A study has shown that a 28-day E2 treatment markedly ameliorated airway inflammatory response by reducing expressions of NLRP3 inflammasome, caspase-1, and interleukin (IL)-1β in ovariectomized mice [15]. The fact that Fulvestrant treatment reduced ERα and VEGF expressions in primary vascular endothelial cells might suggest that Fulvestrant ameliorated inflammation. The miRNA regulatory member i.e., miR-424 is a widely studied factor, and it regulates the proliferation or growth of human cancer cells, including liver cancer, melanoma, and colorectal cancer, through the target genes [21]. It blocked E2-mediated cell multiplication in endometrial carcinoma by targeting G protein-coupled ER. Moreover, ESR1-induced upregulation of IncRNA LINC00511 promoted multiplication and invasiveness of ovarian carcinoma CAOV3 cells through suppression of miR-424-5p [22]. This research found that miR-424 was affected by ER and inhibited VEGF, and its level was promoted by E2 but suppressed by Fulvestrant. These results showed that the miR-424/ER/VEGF pathway plays a potent role in the pathogenesis and treatment of hemorrhoids edema.

CONCLUSION

This study shows that ERα and VEGF are upregulated in postoperative hemorrhoids edema tissues, when compared with preoperative hemorrhoid tissues. This indicates a specific mechanism for further studies on the pathogenesis of hemorrhoids edema after surgery. In vitro experiments in primary vascular endothelial cells show that reducing ER expression using Fulvestrant might be a therapeutic strategy for postoperative anal edema. However, the specific mechanism underlying ER-mediated development of hemorrhoids edema should be investigated in depth.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Fanyu Meng and Minning Xie conceived and designed the study, and drafted the manuscript. Fanyu Meng, Xinghua Chen, Huajiang Liu, Lei Zhang, and Ting Yu collected, analyzed and interpreted the experimental data. Xinghua Chen and Huajiang Liu revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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