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Original Article

Celecoxib reduces inflammation and angiogenesis in mice with adenomyosis

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Abstract: Objective: This study aimed to explore the effect of COX-2 selective inhibitor (celecoxib) on adenomyosis and its mechanism. Methods: By establishing a mouse model of adenomyosis and using celecoxib to treat adenomyosis, newly born female mice were randomly divided into a control group, adenomyosis model group, and celecoxib group. Hematoxylin-eosin (H&E) staining was used to observe the depth of endometrial infiltration of mouse adenomyosis. RT-PCR (reverse transcription PCR) and western blot were used to detect the expression of Cyclooxygenase-2 (COX-2), Vascular growth factor (VEGF), Nerve growth factor (NGF), and Corticotropin-releasing hormone (CRH) mRNA and protein in mice before and after celecoxib treatment. Results: After treatment with celecoxib, the depth of endometrial infiltration of mouse adenomyosis was reduced. COX-2 and VEGF decreased significantly after celecoxib inhibited expression of COX-2 ($P<0.001$), but there was no significant difference in the expression of NGF or CRH ($P>0.05$). Conclusion: This study indicated that COX-2 may be an important factor related to the pathogenesis of adenomyosis, and it may become an important molecular target for the treatment of adenomyosis.

Keywords: Adenomyosis, celecoxib, COX-2, VEGF, NGF, CRH, EMT

Introduction

Adenomyosis refers to a benign gynecological disease. The pathologic feature is diffuse or localized infiltration of the myometrium by endometrial glands and interstitial cells. The clinical symptoms include progressive dysmenorrhea, chronic pelvic pain, or subsequent symptoms (e.g., primary infertility), seriously affecting the patient's body and mind [1, 2]. Adenomyosis is closely related to endometriosis [3, 4], and they exhibit many common features for symptoms, histology and molecular changes [5-7]. Though they are benign diseases, they both exhibit several characteristics of malignant tumors (e.g., angiogenesis, local or distant invasion and metastasis) [8, 9]. However, some differences are revealed in their pathogenesis [10]. Over the past few years, studies have shown that angiogenesis, inflammatory mediators, and nerve growth factors (NGF) may be related to the occurrence, development, and pain symptoms of adenomyosis [11].

For the treatment strategy, surgical resection may be the recommendation for refractory adenomyosis, whereas many patients with fertility requirements and uterine preservation are reluctant to choose surgery [12, 13]. Accordingly, exploring new target drugs to treat adenomyosis is the aim of the present study. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used therapeutic drugs to treat inflammation and pain, and have extensive applications to treat rheumatoid joint disease and osteoarthritis. However, by its inhibiting effect on the expressions of COX-1 and COX-2 to varying degrees, it imposes damage on the gastrointestinal system [14]. COX-1 and COX-2 are isoenzymes performing prostaglandin synthesis. COX-1 is constitutively expressed in normal tissues, while COX-2 can be induced and up-regulated in pain and inflammatory pathways [15]. Celecoxib refers to a potent COX-2 selective inhibitor, inhibiting the prostaglandin cascade by preventing the binding of arachidic acid and the active site of cyclooxygenase-2. It is an anti-inflammatory, analgesic, and anti-

pyretic drug [16]. In recent years, celecoxib has also been investigated for tumor therapy by inhibiting angiogenesis, epithelial-mesenchymal transition (EMT), cell proliferation, and invasion [17-19].

COX-2 and VEGF facilitate the formation of new blood vessels, a necessary condition for the endometrium to invade the myometrium [20, 21]. Besides angiogenesis factors, inflammatory factors, and intimal and interstitial invasion are also pathogenic factors in adenomyosis [22]. Among inflammatory mediators, adrenal cortex hormone releasing hormone (CRH) is of critical significance. It is highly expressed in endometriotic lesions and related to stress and pain mechanisms [23]. Moreover, nerve growth factor (NGF) and its receptor are involved in the development of nociceptors, and facilitate the release of histamine from mast cells, accelerating the production of hyperalgesia. However, the correlation between COX-2, VEGF, CRH, and NGF with adenomyosis have rarely been explored. This study aims to explore the relationship between those cytokines with adenomyosis and attempts to explore their likely pathogenesis for adenomyosis.

Materials and methods

Chemicals

Tamoxifen (Shanghai Fudan Fuhua Pharmaceutical Co., Ltd); Celecoxib (Pfizer Pharmaceuticals LLC), The H&E staining kit was purchased from Beijing Solarbio Technology Co., Ltd.

Mice adenomyosis model and animal treatment

6 ICR pregnant mice aged 17-19 days of gestation, weighing 38~40 g, were purchased from Qinglongshan Animal Breeding Farm in Jiangning District, Nanjing (Nanjing, China). The pregnant mice were kept in separate cages during the whole pregnancy period. Newborn female mice were selected for follow-up research. All mice could take in food and drinking water freely with 12 hours of light every day. According to the research of Parrott et al. [24], newborn female mice were selected and divided into control group (n=6), adenomyosis model group (n=6), celecoxib group (n=6) by random number method. The control group only took the solvent (the mixture of peanut oil/lecithin/condensed milk, the volume ratio was 2:0.5:3) all the time. The mice in the adenomyosis model group and celecoxib group received oral tamoxifen to induce adenomyosis. Mice in the adenomyosis model group and celecoxib group received 2.7 μmol/L tamoxifen suspended in a peanut oil/lecithin/condensed milk mixture (volume ratio 2:0.5:3) on the 2nd to 5th day after birth (birthday is the first day) at a dose volume of 5 μl/g, furthermore, from the 6th day, the mice in the celecoxib group were fed with celecoxib 30 mg/(Kg·d), and the adenomyosis model group received the same amount of solvent every day.

Specimen collection

Mice were breast-fed by mothers at the age of 1 to 21 days. From 22 d onwards, mice and mothers are kept in separate cages. Mice could eat and drink freely. The three groups (the celecoxib group, the adenomyosis group, and the control group) of mice were all raised for 3 months and then euthanized. Uterine tissues were taken, embedded in paraffin, and paraffin-embedded sections were subjected to H&E staining to observe the degree of myometrial gland infiltration.

In this study, all experiments were conducted under the guidance of the “Guidelines for the Care and Use of Laboratory Animals” of the National Research Council and approved by the Laboratory Animal Review Committee.

RT-PCR

The total RNA was extracted according to the instructions of the Trizol kit, and the expression of COX-2, VEGF, NGF, and CRH mRNA in adenomyosis were detected by two-step RT-PCR. The amplification program was as follows: 94°C for 5 min, followed by 28 cycles of 94°C for 30 s, annealing temperature 56°C for 30 s, and 72°C for 10 s, and finally 5 min at 72°C for a final extension. The product was subjected to 2% agarose gel electrophoresis, with the glyceraldehyde phosphate dehydrogenase (GAPDH) gene as an internal reference, and the gel electrophoresis image was photographed and saved using Image J software to analyze the expression of the target gene. Refer to **Table 1** for primer design.

Table 1. RT-PCR primer sequences

	Forward sequences	Reverse sequences
COX-2	5'-GGAATGTATGAGCACAGGAT-3'	5'-ATGATTAAACTTCGCAGGAA-3'
VEGF	5'-ATGAACTTTCTGCTCTCTTG-3'	5'-GTCGGGGTACTCCTGGAAGA-3'
NGF	5'-ATGTCCATGTTGTTCTACAC-3'	5'-CAGCTATTGGTGCAGTAGGG-3'
CRH	5'-ATGCGGCTGCGGCTGCTGGT-3'	5'-GACTTCTGTTGAGATTCCCC-3'
GAPDH	5'-GGTGAAGGTCGGTGTGAACG-3'	5'-CTCGCTCCTGGAAGATGGTG-3'

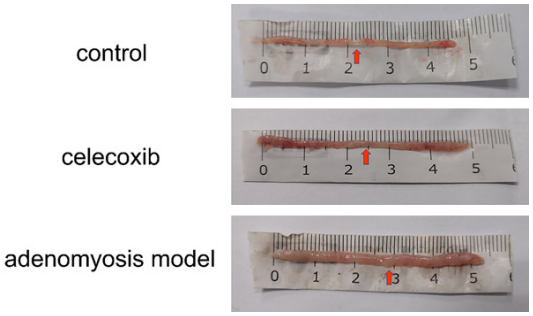

Figure 1. Uterus of 90-day-old in control group, celecoxib group, and adenomyosis model group. V-shaped mouse uterus was stretched into a straight line. The red arrow points to the cervix.

Western blotting

We took the mouse uterus tissue from each group, extracted the total protein according to the kit instructions, determined the protein concentration by BCA method, separated the protein with 8% and 12% SDS-PAGE, then transfered to polyvinylidene fluoride (PVDF) membrane. Blocking was with 5% skim milk. Membrane was washed with TBST buffer 3 times Primary antibody was added (COX-2 rabbit.no.ab62331, 1:1,000; VEGF rabbit.AF5153, 1:1,000; NGF rabbit.no.ab52918, 1:1,000; CRH rabbit.no.DF6258, 1:1,000), and incubated at 4°C overnight. The membrane was washed with TBST solution 3 times. Horseradish Peroxidase (HRP)-labeled corresponding secondary antibody (diluted 1:4,000) was added and incubated at 37°C for 60 min. The membrane was washed again with TBST solution 3 times. Enhanced chemiluminescence (ECL) kit was used for substrate development. GAPDH was the internal reference. An image was taken after storage. Image J software was used for analysis, and the ratio of the target protein band to the gray value of GAPDH indicated the expression level of the target protein.

Statistical analysis

The SPSS statistical analysis software package 22.0 was used for statistical analysis. The comparison between multiple groups was performed by one-way analysis of variance (one-Way ANOVA), and the LSD method was used for multiple test verification. $P< 0.05$ indicated a significant difference.

Results

Detection of the modelling of adenomyosis in mice

According to the evaluation criteria of Bird et al. [25], one of the histologic diagnostic criteria of mouse adenomyosis is that endometrial glands infiltrate the myometrium but do not penetrate the serosa. The other diagnostic criteria are the destruction of concentric and longitudinal smooth muscle bands. In addition, the infiltration of the endometrium into the myometrium is graded as follows: Grade 0, the endometrium is not infiltrating into the myometrium; Grade I, subbasal adenomyosis (adenomyosis in a low-power field below the basal endometrium, without further penetration); Grade II, uterine adenomyosis penetrates into the middle of the myometrium; Grade III, adenomyosis penetrates beyond the middle of the myometrium. The uterus of mice of 90-day-old in control group, celecoxib group and adenomyosis model group are shown in **Figure 1**. H&E staining images of uterus of mice aged 90 days in the control group, celecoxib group and adenomyosis model group are shown in **Figure 2**. **Figure 2A** shows that the smooth muscle layer fibers of the mouse myometrium circulate centrally and regularly outside the endometrium, and there is no pathological change of adenomyosis. **Figure 2B** and **2C**, demonstrate that the endometrial glands are located in the muscular layer. At the same time, the smooth muscle structure of the muscular layer is obviously disordered, and the boundary with the endometrium is not clear. The depth of the endometrium infiltration into the myometrium of the celecoxib group was less than that of the adenomyosis model group. As demonstrated in **Figure 2D**, compared with the control group, the endometrial infiltration depth of mice in the adenomyo-

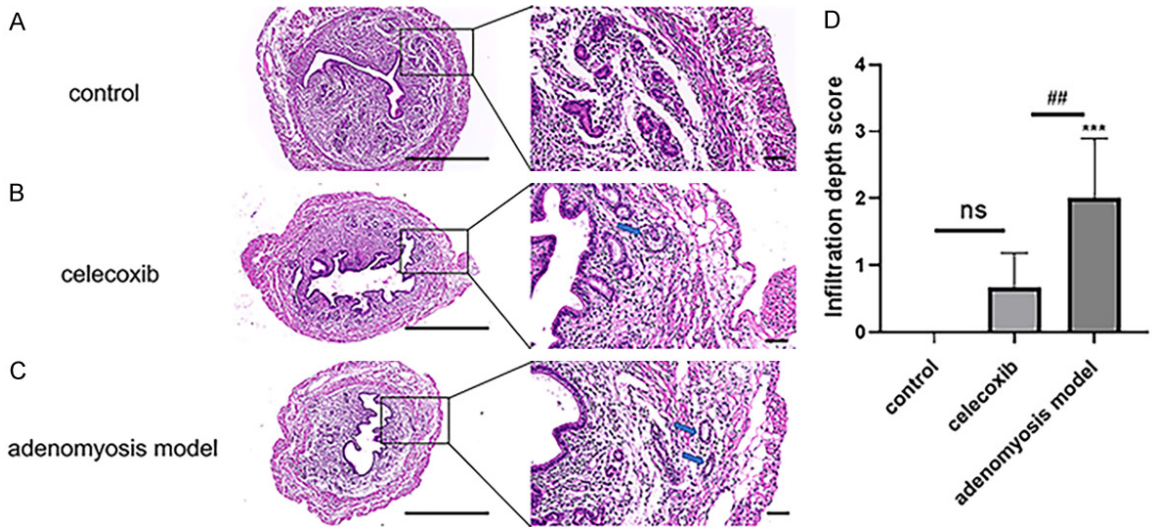

Figure 2. In a mouse model of tamoxifen-induced adenomyosis, Celecoxib reduces the depth of endometrial infiltration into the myometrium. (A-C) represent H&E stained images of mouse uterus in the control group, celecoxib group, and adenomyosis model group respectively. Ectopic endometrium gland (arrow) invades to the superficial and deep myometrium in (B and C) (scale bar: 100 μm and 20 μm). (D) represents quantified grades of myometrial infiltration by the endometrium in the control group, celecoxib group, and adenomyosis model group at 3 months after birth.

sis model group was significantly increased (P<0.0001), and the endometrial infiltration depth of the mice in the celecoxib group had no significant difference (P=0.1628). After celecoxib treatment, the depth of endometrial infiltration in mice was significantly reduced compared with the adenomyosis model group (P=0.004).

The effect of celecoxib treatment on COX-2, VEGF, NGF, CRH mRNA expression

In order to verify the expression of COX-2, VEGF, NGF, and CRH mRNA in mouse adenomyosis, RT-PCR was used to detect the expression of these transcripts. The expression of COX-2, VEGF, NGF, and CRH mRNA in the adenomyosis model group and celecoxib group were significantly higher than those in the control group (P<0.001) (**Figure 3**); similarly, the expression levels of COX-2 and VEGF mRNA in the celecoxib group were significantly lower than those in the adenomyosis model group (P<0.001), but there was no significant difference in NGF and CRH mRNA expression between the two groups (P>0.05).

The effect of celecoxib treatment on COX-2, VEGF, NGF, and CRH protein expression

The total protein extract of mouse uterine tissue was used to detect the expression of COX-2, VEGF, NGF, and CRH by western blot (**Figure 4**). The relative expression of COX-2, VEGF, NGF, and CRH protein in the celecoxib group and adenomyosis model group were significantly higher than those of the control group (P<0.001); Compared with the adenomyosis model group, the COX-2 and VEGF protein expression in the celecoxib group were significantly lower (P<0.001), but there was no significant difference in NGF and CRH protein expression between those two groups (P>0.05), which further confirmed the aforementioned findings.

Discussion

Tamoxifen refers to a selective estrogen regulator, while tamoxifen citrate exerts an anti-estrogen effect. The pathogenesis of tamoxifen-induced adenomyosis is not clear and it may display a relationship to the disorder of myometrial differentiation and structural remodeling [26]. In the present study, the method successfully employed tamoxifen to induce adenomyosis in newborn mice after adulthood, and no mice died. It was therefore suggested that this method can non-invasively and successfully induce the occurrence of adenomyosis in mice, and relevant pathophysiologic changes will be induced.

Adenomyosis is a highly estrogen-dependent disease. High estrogen induces EMT in epithe-

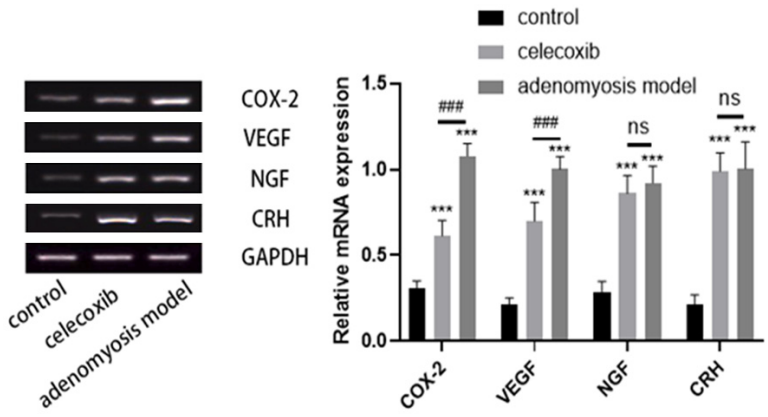

Figure 3. RT-PCR analysis of COX-2, VEGF, NGF, and CRH mRNA expression in each group of mice. Expression of COX-2, and VEGF mRNA was significantly inhibited by celecoxib, but the expression of NGF and CRH mRNA had no significant difference. $^{***}P<0.001$, $^{###}P<0.001$, $^{ns}P>0.05$.

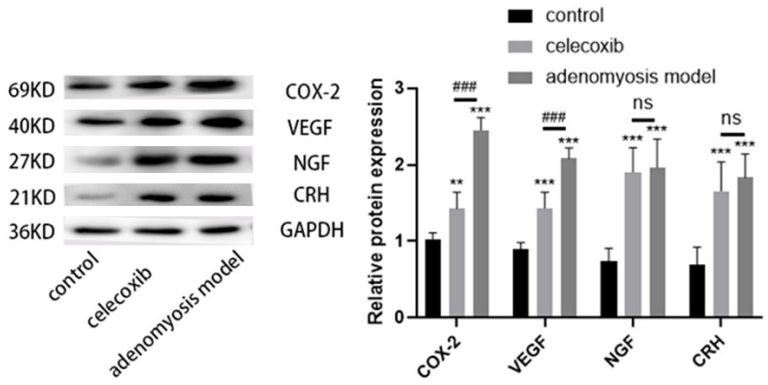

Figure 4. Western blot analysis of the COX-2, VEGF, NGF, and CRH protein in each group of mice. Celecoxib significantly inhibits the expression of COX-2 and VEGF, but has no effect on the expression of NGF and CRH protein. $^{**}P<0.01$, $^{***}P<0.001$, $^{###}P<0.001$, $^{ns}P>0.05$.

lial cells, and endometrial cells are significantly invasive after EMT, which promotes disease progression [27]. Thus, invasion and migration are considered two key factors in the pathogenesis of adenomyosis. As revealed by existing studies, COX-2 is overexpressed in the endometrium of patients with endometriosis and adenomyosis [28, 29]. COX-2 acts as a vital molecule in the inflammatory microenvironment, accounting for the synthesis of prostaglandins (PGs) by using arachidonic acid. The overproduction of PGS up-regulates the expression of VEGF. In return, VEGF is capable of stimulating the expression of COX-2 in the endometrium. A positive feedback loop was established between COX-2 and VEGF, conducive to the high concentration of COX-2, PGE2, and VEGF in the endometrium in adenomyosis [15]. COX-2 was demonstrated extensively to be closely related to the occurrence, development, and metastasis of tumors, which promotes the production of PGs and induces EMT by up-regulating the expression of COX-2 [30, 31]. Epithelial-mesenchymal transition (EMT) indicates the process by which epithelial cells lose their epithelial phenotype and acquire a mesenchymal phenotype. Estrogen-related epithelial-mesenchymal transition (EMT) may impact the invasiveness of endometrial cells in adenomyosis [32]. Moreover, EMT is considered the main mechanism of tumor progression, thereby promoting tumor cell migration, invasion, and metastasis [33, 34]. Furthermore, angiogenesis, i.e., the formation of new blood vessels from a pre-existing blood vessel network, is of essential significance for tumor migration, invasion, and metastasis. Given the infiltration and metastasis of VEGF and COX-2 in tumors, the expressions of the mentioned two factors may be related to the aggressiveness of the endometrium of adenomyosis. Celecoxib inhibits the expression of COX-2 and reduces EMT to prevent the invasion and metastasis of osteosarcoma cells in vivo and in vitro [30]. Moreover, celecoxib is capable of inhibiting the self-renewal of breast cancer stem cells and EMT, while attenuating metastasis and tumorigenesis [35]. As revealed from this study, the expressions of COX-2 and VEGF in the adenomyosis model group and the celecoxib group were higher than those in the controls. After the treatment with celecoxib, the results suggested ectopic glands in the uterine muscle, whereas the depth of the layer was reduced, and the expressions of COX-2 and VEGF were also significantly lower than those of the adenomyosis model group. It can be specu-

lated based on the above data that inhibiting COX-2 cannot completely prevent the occurrence of adenomyosis, but it can reduce the degree of adenomyosis. Besides inflammation and angiogenesis, other factors or mechanisms may be also involved.

Nerve growth factor (NGF) is a multifunctional mediator. It can act as a pain mediator. Nerve growth factor is related to the pain of various diseases (e.g., osteoarthritis, synovitis and low back pain) [36]. NGF locally produced in patients with endometriosis helps produce and maintain endometriosis-related pain (e.g., dysmenorrhea and dyspareunia) [37]. Interestingly, as suggested from the research results of Barcena de Arellano [38] the overexpression of nerve growth factor in the peritoneal fluid of patients with endometriosis is not tightly associated with the production of endometriosis pain. It is therefore considered that the overexpression of NGF promotes the overexpression of proinflammatory factors and pain mediators, induces sensitization of nociceptors around the ectopic endometrium, and then produces pain sensation. The overexpression of factors and pain mediators leads to the sensitization of nociceptors around the ectopic endometrium, which in turn produces pain. As revealed from existing studies, in cultured endometriotic stromal cells, NGF is capable of stimulating COX-2/PGS signaling, which is considered a vital part of the pain attributed to inflammation in endometriosis [39]. Moreover, as revealed from existing studies, NGF in rat mast cells can induce COX-2 expression and its accompanying PGD2 production [40]. According to this study, the expression of NGF in the adenomyosis model group and the celecoxib group exceeded that in the controls, demonstrating that NGF may be involved in the occurrence of adenomyosis and pain-related symptoms. No significant difference was identified in the expression of NGF after celecoxib was employed, revealing that NGF impacts the formation of adenomyosis by other signal pathways, instead of the COX-2/PGS pathway, in the pathogenesis of adenomyosis. Accordingly, the expression of NGF did not significantly decrease after COX-2 was inhibited.

Corticotropin-releasing hormone (CRH) regulates the neurohormones of the hypothalamic-pituitary-adrenal axis, and is capable of participating in cell differentiation or acting as a pro-inflammatory factor [41]. In endometriosis, high expression of CRH induces an increase in the number of activated mast cells, probably causing inflammation and fibrosis of the endometrium [42]. In joint synovial tissue, CRH stimulates the production of PGE2 by COX-2 activity, and it acts in an autocrine manner in the synovium by facilitating the production of PGE2. Prostaglandin E2 was suggested to improve CRH synthesis in synovial cells of rheumatoid arthritis [43]. As revealed from the results of this study, the expression of CRH in the adenomyosis model group and celecoxib group was higher than that in the controls. After treatment with celecoxib, no significant difference was identified in CRH expression between the adenomyosis model group and celecoxib group, demonstrating that the production of CRH in adenomyosis is not completely dependent on PGS. It may be an inflammatory reaction attributable to adenomyosis, and it acts on the uterine tissue by an autocrine/paracrine method. The specific mechanism requires more specific studies.

In summary, the pathogenesis of adenomyosis displays a close relationship to COX-2. After the use of celecoxib to inhibit COX-2, the degree of infiltration of the muscularis in the endometrium of mice with adenomyosis was down-regulated. The mechanism by which celecoxib works may be multfactorial (e.g., inhibition of endometrial inflammation, EMT, and angiogenesis). Accordingly, this study deduces that inhibition of COX-2 may have promise for treating adenomyosis. This study performed only in vivo experiments and there was no in vitro detection of the expression of each factor after COX-2 inhibition. In addition, this experiment was an animal experiment, and the number of samples was small. The therapeutic mechanism of COX-2 selective inhibitors in adenomyosis requires in-depth studies.

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Disclosure of conflict of interest

None.

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