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Extraction of breviscapine from *Erigeron breviscapus* and its effect on oxidative stress, inflammation, energy metabolism disorder and apoptosis in rats with uterine ischemia-reperfusion injury

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Abstract

This work aimed to prepare breviscapine from *Erigeron breviscapus* and investigate its protection mechanism on uterine ischemia-reperfusion injury (UIRI) in rats. Breviscapine was extracted and separated from *Erigeron breviscapus*. Sixty female SD rats were randomly divided into sham-operated, model and breviscapine groups. The breviscapine group was treated with 5 mg/kg breviscapine for three days. Then, the UIRI model was established in model and breviscapine groups via uterine artery ischemia for 30 min followed by reperfusion for 60 min. At the end of reperfusion, compared with model group, in breviscapine group the uterine tissue superoxide dismutase activity was increased, and the malondialdehyde level was decreased; the uterine tissue tumor necrosis factor α, interleukin (IL)-1β and IL-6 levels were decreased, and the IL-10 level was increased; the uterine tissue adenosine triphosphate, adenosine diphosphate, adenosine monophosphate and total adenine nucleotides levels were increased; the uterine cell apoptosis rate was decreased, the Bcl-2 protein expression level was increased, and the Bax protein expression level was decreased. In conclusion, breviscapine from *Erigeron breviscapus* can reduce the oxidative stress, inflammatory reaction, energy metabolism disorder and apoptosis in uterine tissue, thus exerting the protective effect on UIRI in rats.

Keywords: breviscapine; uterine; ischemia-reperfusion; oxidative stress; inflammation; apoptosis.

Practical Application: Breviscapine from *Erigeron breviscapus* is applied to preventing the uterine ischemia-reperfusion injury in rats.

1 Introduction

Ischemia reperfusion can cause the further aggravation of injury, which leads to the necrosis and dysfunction of involved tissues and organs, and eventually results in the infection, sepsis, multiple organ failure and other complications (Meyer et al., 1998). At present, the research of ischemia-reperfusion injury mainly focuses on the heart (Weerateerangkul et al., 2011), brain (Cao et al., 2007), liver (Montalvo-Jave et al., 2008), kidney (Li et al., 2010) and other organs, while the study of reproductive organ ischemia-reperfusion injury is less. However, any uterine surgery and invasive examination may cause the uterine ischemia-reperfusion injury (UIRI) (Kisu et al., 2017). In clinic, the most serious UIRI often occurs in placenta stripping, uterine rupture, cesarean section and abortion and induced labor.

Breviscapine, a kind of flavonoid, is the main active component of medicinal plant *Erigeron breviscapus* (Li et al., 2004). Modern pharmaceutical studies have found that breviscapine has anti-inflammatory (Wang et al., 2013), antioxidant (Wang et al., 2008), apoptosis inhibiting (Yiming et al., 2008) and other biological activities. Breviscapine has the protective effect on ischemia-reperfusion injury of heart (Wang et al., 2013), liver (Lin et al., 2016) and other organs. However, whether breviscapine has a protective effect on UIRI has not been reported. It is found that, the oxidative stress, inflammatory reaction, energy metabolism disorder and apoptosis are often involved in the ischemia-reperfusion injury (Kohli et al., 1999; Yassin et al., 2002; Wang et al., 2006; Zhao et al., 2016). In our study, we extracted and purified breviscapine from *Erigeron breviscapus*, and investigated its protection mechanism on oxidative stress, inflammation, energy metabolism disorder and apoptosis in rats with uterine ischemia-reperfusion injury.

2 Materials and methods

2.1 Extraction and purification of breviscapine from Erigeron breviscapus

One kilogram of *Erigeron breviscapus* was placed in the ultrasonic extractor, followed by adding 10 times (volume: mass) of 70% ethanol. The ultrasonic extraction was performed for 1 h. The extraction solution was filtered. After concentrating under reduced pressure, the crude breviscapine extract was obtained. HP-20 macroporous resin was placed in the chromatographic column. The crude breviscapine extract was loaded in the column using dry loading method. The elution was performed using 50% ethanol. The eluent was collected, and the solvent was removed under reduced pressure. The solid was dissolved

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in hot water. NaOH solution was added to the solution, and the pH was adjusted to 8.0. After filtering, the filtrate was collected, and was added with 10% H_2SO_4 solution to adjust the pH to 3.0. The temperature was kept at 50-55 °C. After standing for 15 min for precipitation, the precipitates were collected by centrifugation at 4000 r/min. The mother liquor was allowed to stand overnight for precipitation again. The other precipitates were collected by centrifugation. The two times of precipitates were combined, and washed with distilled water to pH 7.0. After vacuum drying at 50 °C, the final breviscapine product was obtained. The high performance liquid chromatography showed the content of breviscapine was 92.37%.

2.2 Animal grouping and treatment

Sixty female SD rats (250 ± 20) g were randomly divided into three groups: sham-operated group, model group and breviscapine group, with 20 rats in each group. Three days before modeling, the rats in sham-operated group and model group were injected with 5 ml/kg normal saline through the caudal vein, once per day. The rats in breviscapine group were injected with 5 mg/kg breviscapine (Guangzhou Taiwei Biotechnology Co. Ltd., Guangzhou, China) through the caudal vein, once per day.

2.3 Modeling of UIRI

The rats in each group were given intraperitoneal injection of 3% pentobarbital Sodium (30 mg/kg) for anaesthesia, and then were fixed in supine position. A median incision was made into the abdomen. In model group and breviscapine group, the uterus was exposed and the uterine artery was isolated. The uterine artery was clamped with a non-invasive artery clamp for uterine ischemia for 30 min. Then, the artery clamp was released, and the reperfusion was performed for 60 min. In the sham-operated group, the uterus and uterine artery were exposed in the same way with other two groups, but the uterine artery was not clipped.

2.4 Determination of oxidative stress and inflammatory reaction indexes in uterine tissue

After 60 min of reperfusion, five rats of each group were taken and killed. The uterine tissue was taken and weighed. The homogenate of uterine tissue with normal saline was made. After centrifuging at 4 °C and 4000 r/min for 15 min, the supernatant was taken. The total protein content was detected by Coomassie brilliant blue method. The superoxide dismutase (SOD) activity was detected by xanthine oxidase method. The malondialdehyde (MDA) level was detected by thiobarbituric acid method. The levels of tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6 and IL-10 were detected by enzyme-linked immunosorbent assay. The detections were carried out in strict accordance with the instructions of kits.

2.5 Detection of high energy phosphates in uterine tissue

Five rats of each group were taken and killed. The uterine tissue was taken, and weighed. The uterine tissue was homogenized with 0.42 mol/L perchloric acid, and then was neutralized with 1.00 mol/L potassium hydroxide. The mitochondrial membrane of cells was smashed by ultrasonic cell pulverizer to release the energy substance into the solution. The mixture was centrifuged at 4 °C and 10000 r/min for 10 min. The supernatant was obtained, and was filtered with 0.2 μm microporous membrane. A 20 μL filtrate was taken for high performance liquid chromatography. The levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were analyzed, and the total adenine nucleotides (TAN) were calculated.

2.6 Determination of uterine cell apoptosis and expression of related proteins

Five rats of each group were taken and killed. The uterine tissue was taken and cut fully. After filtering with 300-mesh sieve, the cell suspension was obtained. The cell concentration was adjusted to 1×10^6 cells/L. The cell apoptosis was detected by flow cytometry, and the apoptosis rate was analyzed. In addition, the rest five rats of each group were taken and killed. The uterine tissue was taken, and homogenized. The protein was extracted. The expressions levels of B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma-2 associated X (Bax) protein were determined using western blot assays. All procedures were in accordance to the instructions of kits.

2.7 Statistical analysis

Data were presented as mean ± SD. The statistical analysis was performed using SPSS 20.0 software. The comparison of data was conducted using single-factor analysis of variance test with SNK-q test. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Comparison of SOD activity and MDA level in uterine tissue among three groups

After 60 min of reperfusion, the SOD activity in uterine tissue in sham-operated group, model group and breviscapine group was 144.45 ± 11.04, 83.05 ± 13.41 and 119.20 ± 15.79 U/mg, respectively, with MDA level of 1.72 ± 0.22, 3.69 ± 0.41 and 2.21 ± 0.38 nmol/mg, respectively. Compared with sham-operated group, in other two groups the SOD activity was decreased ($P < 0.05$), and the MDA level was increased ($P < 0.05$). Compared with model group, in breviscapine group the SOD activity was increased ($P < 0.05$), and the MDA level was decreased ($P < 0.05$) (Figure 1).

3.2 Comparison of TNF-α, IL-1β, IL-6 and IL-10 levels in uterine tissue among three groups

Figure 2 showed that, after 60 min of reperfusion, in sham-operated group, model group and breviscapine group the TNF-α level in uterine tissue was 30.38 ± 5.30, 74.21 ± 11.21 and 46.09 ± 4.80 pg/mg, respectively, the IL-1β level was 18.16 ± 2.41, 48.83 ± 5.39 and 32.41 ± 7.05 pg/mg, respectively, the IL-6 level was 53.06 ± 7.24, 119.29 ± 13.68 and 84.72 ± 9.91 pg/mg, respectively, and the IL-10 level was 36.15 ± 5.73, 21.30 ± 3.42 and 28.24 ± 4.13 pg/mg, respectively. Compared with sham-operated group, in other two groups the TNF-α, IL-1β and IL-6 levels

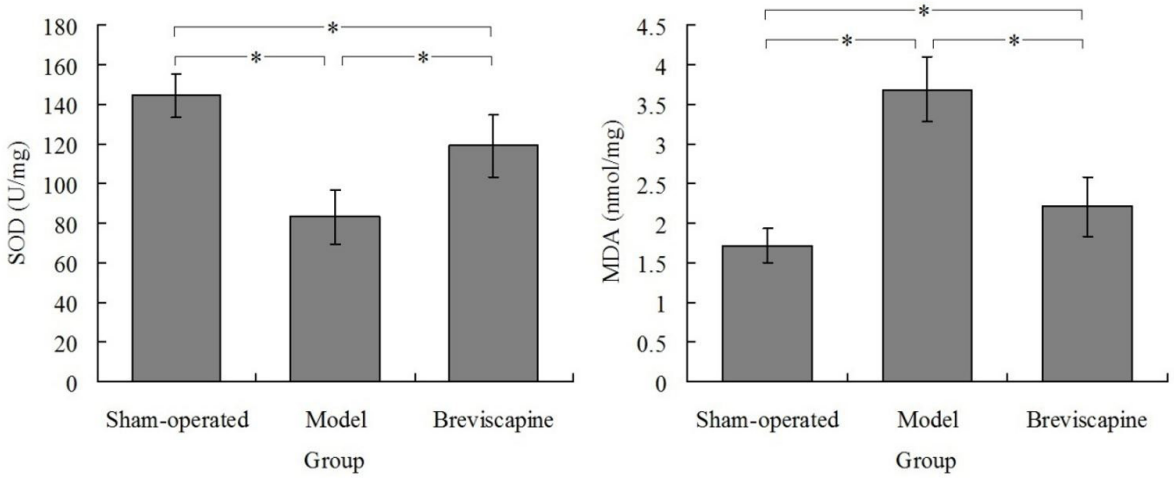

Figure 1. Comparison of SOD activity and MDA level in uterine tissue among three groups. *P < 0.05 for the comparison; SOD= superoxide dismutase; MDA= malondialdehyde.

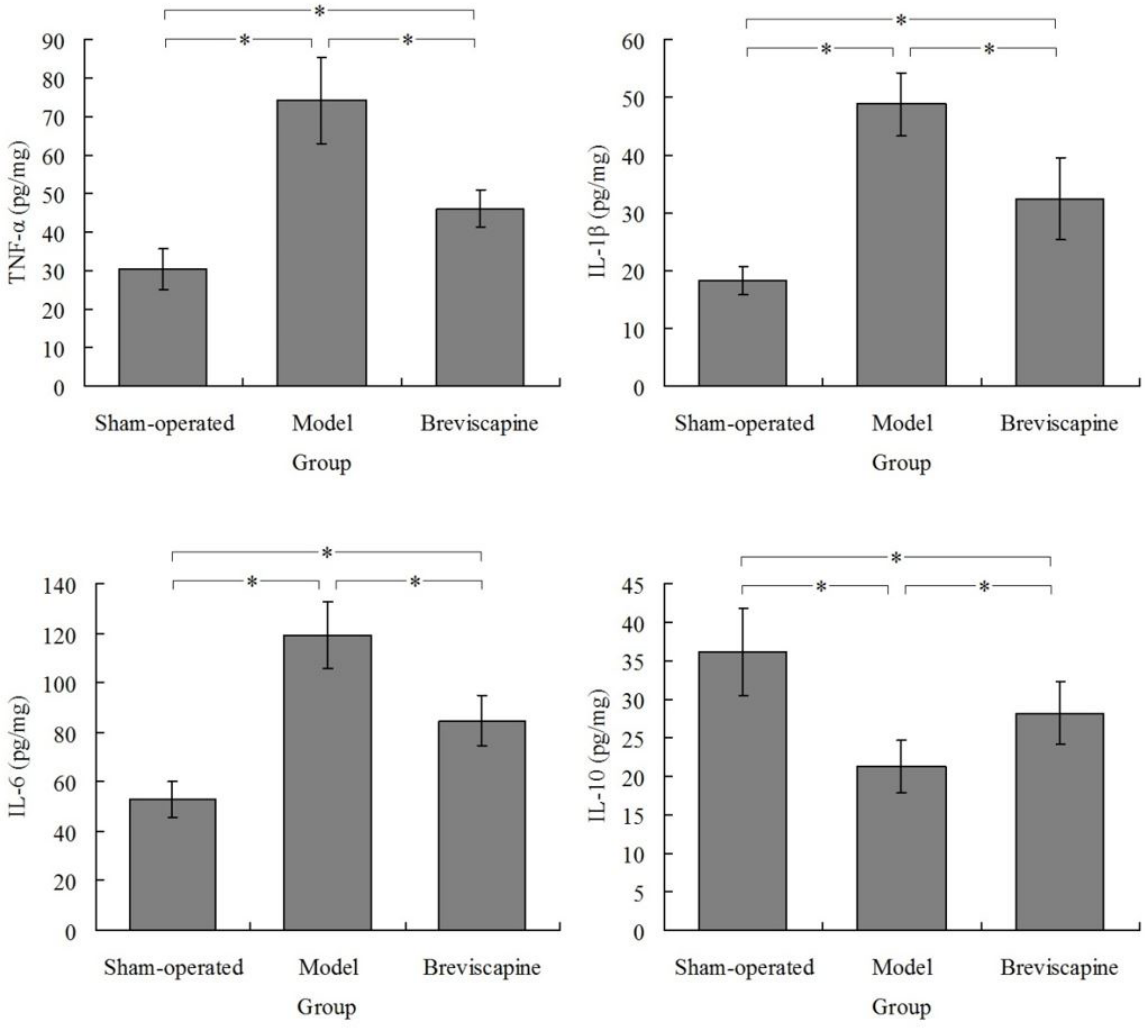

Figure 2. Comparison of TNF-α, IL-1β, IL-6 and IL-10 levels in uterine tissue among three groups. *P < 0.05 for the comparison; TNF-α= tumor necrosis factor α; IL= interleukin.

were increased (P < 0.05), and the IL-10 level was decreased (P < 0.05). Compared with model group, in breviscapine group the TNF-α, IL-1β and IL-6 levels were decreased (P < 0.05), and the IL-10 level was increased (P < 0.05).

3.3 Comparison of ATP, ADP, AMP and TAN levels in uterine tissue among three groups

After 60 min of reperfusion, in sham-operated group, model group and breviscapine group the ATP level in uterine tissue was 0.24 ± 0.03, 0.06 ± 0.01 and 0.15 ± 0.01 μg/mg, respectively, the ADP level was 10.68 ± 0.12, 0.09 ± 0.02 and 0.37 ± 0.03, respectively, the AMP level was 0.71 ± 0.13, 0.32 ± 0.04 and 0.59 ± 0.06 μg/mg, respectively, and the TAN level was 1.59 ± 0.21, 0.45 ± 0.07 and 1.12 ± 0.16 μg/mg, respectively. Each index in model group and breviscapine group was lower than that in sham-operated group (P < 0.05), but that in breviscapine group was higher than that in model group (P < 0.05) (Figure 3).

3.4 Comparison of uterine cell apoptosis rate among three groups

As shown in Figure 4, at the end of reperfusion, the uterine cell apoptosis rate in uterine tissue in sham-operated group, model group and breviscapine group was (0.04 ± 0.01)%, (0.36 ± 0.05)% and (0.25 ± 0.03)%, respectively. Compared with sham-operated group, the apoptosis rate in other two groups was increased (P < 0.05). Compared with model group, the apoptosis rate in breviscapine group was decreased (P < 0.05).

3.5 Comparison of Bcl-2 and Bax protein expressions in uterine tissue among three groups

Figure 5 showed that, after 60 min of reperfusion, in sham-operated group, model group and breviscapine group the Bcl-2/β-actin ratio was 0.73 ± 0.07, 0.19 ± 0.03 and 0.43 ± 0.04, respectively, and the Bax/β-actin ratio was 0.12 ± 0.01, 0.60 ± 0.12 and

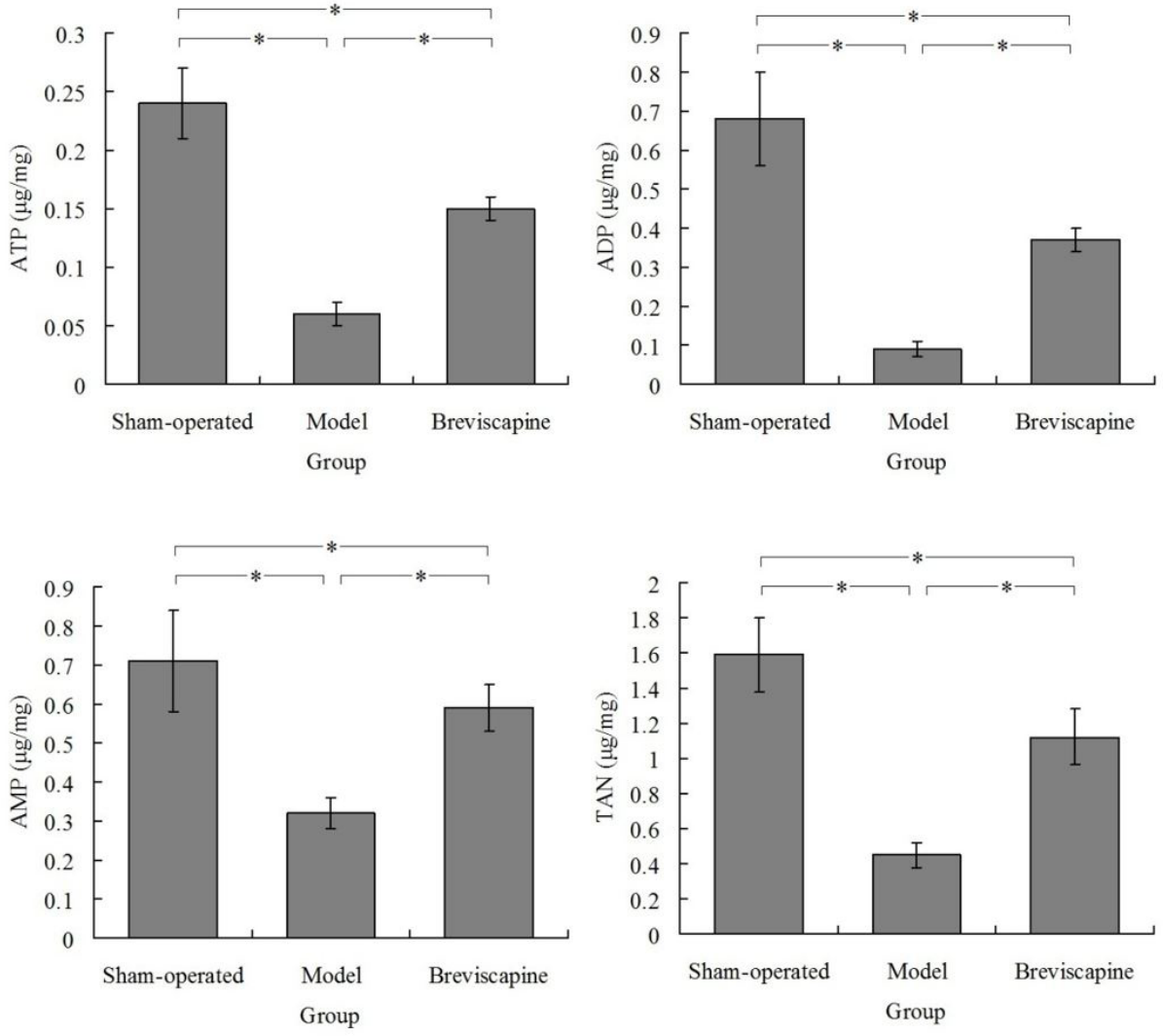

Figure 3. Comparison of ATP, ADP, AMP and TAN levels in uterine tissue among three groups; *P < 0.05 for the comparison. ATP= adenosine triphosphate; ADP= adenosine diphosphate; AMP= adenosine monophosphate; TAN= total adenine nucleotides.

0.36 ± 0.07, respectively. Compared with sham-operated group, in other two groups the Bcl-2/β-actin ratio was decreased ($P < 0.05$), and the Bax/β-actin ratio was increased ($P < 0.05$). Compared with model group, in breviscapine group the Bcl-2/β-actin ratio was increased ($P < 0.05$), and the Bax/β-actin ratio was decreased ($P < 0.05$).

4 Discussion

When the ischemia-reperfusion occurs, the xanthine oxidase is activated, and the neutrophil respiration bursts. This increases the production of oxygen free radicals and decreases the activity of endogenous free radical scavenging enzymes. The oxygen free radicals begin to attack the unsaturated fatty acids in cell membrane, causing a series of free radical chain reactions, which further leads to the damage of cell structure and function (Granger et al., 1986). SOD is the main scavenger of endogenous oxygen free radicals, and its level can directly reflect the ability of body in scavenging free radicals (Pigeolet et al., 1990). MDA is one of the main lipid peroxidation products. Its level can reflect the degree of lipid peroxidation and indirectly reflect the degree of body damage (Inder et al., 1996). In the present study, the breviscapine was extracted and purified from *Erigeron breviscapus* and its protection mechanism on UIRI in rats was investigated. Results showed that, after 60 min of reperfusion, compared with model group, in breviscapine group the uterine tissue SOD activity was increased, and the MDA level was decreased. This indicates that, breviscapine has the anti-oxidative stress function in protecting against UIRI in rats.

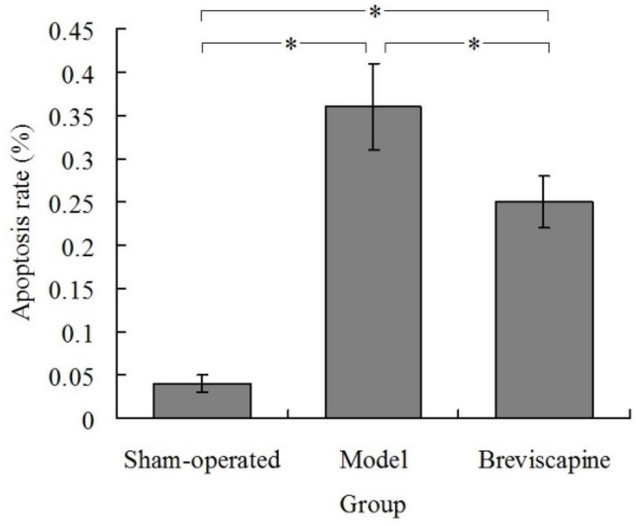

Figure 4. Comparison of uterine cell apoptosis rate among three groups; *$P < 0.05$ for the comparison.

Inflammatory reaction is a common pathophysiological phenomenon, which is caused by many factors. Inflammatory reaction is widely involved in the progression of aging, tumorigenesis, myocardial infarction, cerebral infarction and other diseases. There are also inflammatory reactions in the process of ischemia-reperfusion injury (Cao et al., 2007). The inflammatory cytokines are mainly secreted by immune cells. Under normal circumstances, the content of cytokines is very low, but their expressions increase rapidly after stimulation by various factors such as infection, trauma and ischemia. The over-expression of inflammatory cytokines can lead to or aggravate the tissue damage (Hommes & van Deventer, 2000). TNF-α, IL-1β and IL-6 are pro-inflammatory cytokine, and IL-10 is anti-inflammatory cytokine. The level change of their levels indirectly reflects the degree of inflammatory reaction (Meador et al., 2008). Results of this study showed that, compared with model group, in breviscapine group the uterine tissue TNF-α, IL-1β and IL-6 levels were decreased, and the IL-10 level was increased. This suggests that, the breviscapine has the anti-inflammatory effect in rats with UIRI.

It is found that the energy metabolism disorder is the initiating factor of ischemia-reperfusion injury (Zhao et al., 2016).

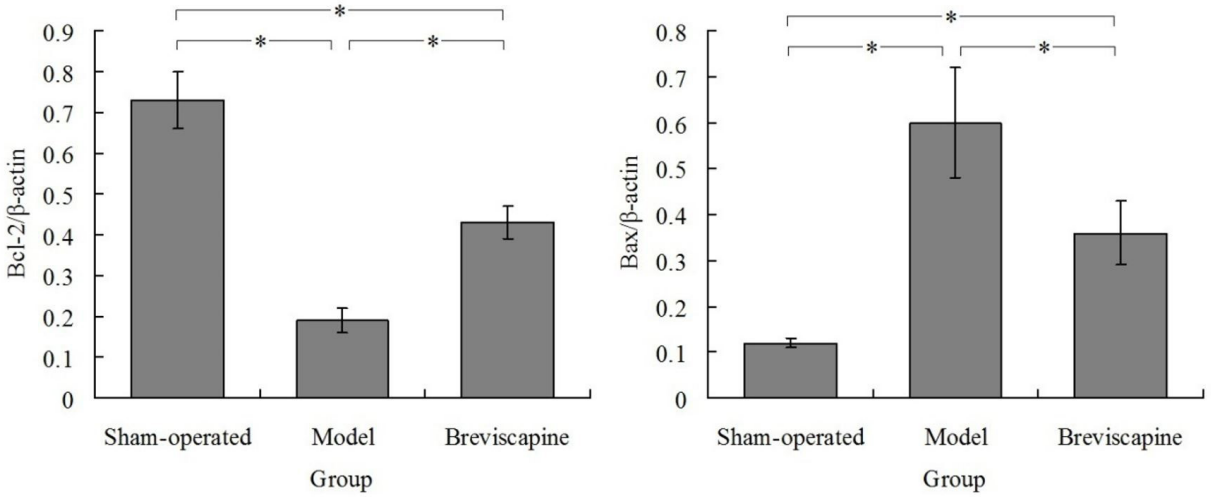

Figure 5. Comparison of Bcl-2 and Bax protein expressions in uterine tissue among three groups. *$P < 0.05$ for the comparison; Bcl-2= B-cell lymphoma-2; Bax= B-cell lymphoma-2 associated X.

The generation and preservation of ATP is an important prerequisite to protect the integrity of tissue structure and function. However, when the tissue ischemia or ischemia-reperfusion occurs, the aerobic oxidation of fatty acids and glucose is blocked, and the energy supply for this pathway is reduced or even interrupted. The ATP is provided only by anaerobic glycolysis, but the amount of ATP provided by this pathway is small, which will lead to the cell metabolic disorder, dysfunction and tissue structure damage. As the decreased ATP level cannot provide enough energy, the body degrades ADP, AMP, adenosine and hypoxanthine to provide the energy. This will further reduce the levels of ATP, ADP and AMP in body (Griffiths & Halestrap, 1993). Results of our study showed that, compared with model group, the uterine tissue ATP, ADP, AMP and TAN levels in breviscapine were obviously increased. This indicates that, breviscapine can reduce the energy metabolism disorder in rats with UIRI.

Apoptosis, also known as programmed cell death, is characterized by formation of apoptotic corpuscle, and is regulated by its internal genes. There is apoptosis in uterus tissue after ischemia-reperfusion (Okazaki et al., 2005). Bcl-2 protein is a transmembrane protein, which plays an important role in anti-apoptosis. It can block the apoptosis caused by oxygen free radical, ischemia and hypoxia (Shimizu et al., 1995; Satoh et al., 1996). Bax protein is a pro-apoptotic protein, which plays a pro-apoptotic effect by inhibiting the activity of Bcl-2. The expressions of these two proteins are in a dynamic balance of antagonism, and regulate the apoptosis together (Korsmeyer et al., 1993). In our study, at the end of reperfusion, compared with model group, in breviscapine group the uterine cell apoptosis rate was decreased, the uterine tissue Bcl-2 protein expression level was increased, and the Bax protein expression level was decreased. Form this we can deduce that, breviscapine can reduce the uterine cell apoptosis in UIRI rats by regulating the Bcl-2 and Bax expressions.

5 Conclusion

In conclusion, breviscapine from *Erigeron breviscapus* can reduce the oxidative stress, inflammatory reaction, energy metabolism disorder and apoptosis in uterine tissue, thus exerting the protective effect on UIRI in rats. This study has provided a clue for further clarifying the protection mechanism of breviscapine on UIRI.

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