Inhibition of Fibroblast Activation by Components of Rhizoma Curcumae and Rhizoma Sparganii in A Rat Model of Uterine Leiomyoma

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Research

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Abstract

Background: Traditional Chinese medicine (TCM) often uses Rhizoma Curcumae and Rhizoma Sparganii (RCRS) as the natural herbs for the treatment of UL. RCRS has been shown to be effective in the treatment of UL in our previous study. This study was to investigate the molecular mechanism by which RCRS inhibits fibroblast activation protein (FAP) activation and prevents uterine leiomyoma in rats.

Methods: The SD rat model of uterine leiomyoma was established by estrogen and progesterone load combined with external stimulation. Subsequently, histological analyses, enzyme-Linked immunosorbent assays, western blotting were performed to evaluate the effect of the drug on uterine leiomyoma and its mechanism.

Results: Our data showed that treatment of rats with RCRS significantly reduced the expression of FAP, TGF- β (the FAP activating factor), and significantly decreased the phosphorylation of cell proliferation pathway-related signaling factors AKT/MEK/ERK, as well as the expression of the extracellular matrix component collagen.

Conclusions: Our results showed that RCRS is very effective in prevention and treatment of uterine leiomyoma in rats, and RCRS may exert its actions by inhibiting the activation of tumor-associated fibroblasts, inhibiting the cell proliferation, and improving tumor extracellular matrix.

Background

Uterine leiomyoma (UL) is the most common benign tumors of reproductive system in women of childbearing age with an incidence of about 70%, of which 25–50% of the patients show clinical symptoms (Stewart et al., 2016). Because the main stromal cells of UL are fibroblasts, it is also called uterine fibroids (Bhowmick et al., 2004). The main drugs used clinically to treat UL include mifepristone, letrozole, and vitamin D. Surgical treatment is currently the primary means of clinical treatment of UL, and hysterectomy is the only way to cure UL. However, these treatments have serious side effects, so it is urgent to use an effective and safe way to treat UL.

Rhizoma Curcumae and Rhizoma Sparganii (RCRS) is frequently used in patients with gynecological diseases such as uterine fibroids, dysmenorrhea and ovarian cysts improving blood circulation and remove stasis. Compatibility of Rhizoma Curcumae (*Curcuma phaeocaulis* Valetton) and Rhizoma Sparganii (*Sparganium stoloniferum* Buch.-Ham.) is a representative medicine for the treatment of UL in TCM (Yu et al., 2012). Chemical analyses show that Rhizoma Sparganii (RS) mainly contains volatile oil, flavonoids, saponins, organic acids, and other functional ingredients (Xu et al., 2015). Total flavonoids are one of the main active ingredients of RS and have pharmacological effects such as anticoagulation, antithrombotic and anticancer. Zedoary turmeric oil is a volatile oil extracted from Rhizoma Sparganii (RC). As an effective anti-tumor drug (Chang et al., 2019), the main active ingredients of zedoary turmeric oil are curcumin, curcumin diketone and β -elemene (Dosoky and Setzer, 2018).

RCRS, a combination of total flavonoids and zedoary turmeric oil, has a significant therapeutic effect on experimental uterine leiomyoma (Yu et al., 2014). Our previous studies have shown that the therapeutic effects of RCRS on UL include improving pathological status, reducing volume, and inhibiting myoma cell proliferation (Yu et al., 2014). Another study showed that the therapeutic effect of RCRS on UL may be related to the regulation of endocrine levels (Yu et al., 2019). The results of metabolomics prove that RCRS can reverse the abnormal metabolism in rats. But its further mechanism remains to be studied (Li et al., 2019). Due to the multi-channel and multi-target characteristics of TCM, the key targets for its efficacy are not clear and need to be studied. UL are composed of leiomyoma cells, fibroblasts and a large number of extracellular matrices (ECM) (Islam et al., 2018). Studies have shown that RCRS inhibits the proliferation of leiomyoma cells, but the mechanism is still unclear. Most studies of UL focused on leiomyoma cells, the mechanisms underlying tumor-associated fibroblasts (TAF) activation and UL development are not clear.

In this study, we used a rat model of qi stagnation and blood stasis UL, in which UL was induced by estrogen and progesterone load combined with external stimulation (Zhao et al., 2018). The model is more suitable for TCM experiments according to the basic theory of TCM combined with western medical theory and experimental zoology. Since fibroblast activation leads to UL, the study assumes that RCRS inhibits UL through inhibiting TAF activation. Results of this study should provide experimental basis for using traditional Chinese medicine to treat UL.

Methods

Preparation of herbs

Preparation of herbal extract

The volatile oil of *Rhizoma Curcumae* was prepared and validated as described in our previous study (Zhao et al., 2018). A voucher specimen (NO.EZ 0704) was deposited in the Chengdu University of Traditional Chinese Medicine, Chengdu, China. *Rhizoma Sparganii* was identified as the *Rhizoma Sparganii* by Professor Teng Peng of Department of Pharmacy, Chengdu University of Traditional Chinese Medicine. When preparing the total flavonoids of *Rhizoma Sparganii*, weigh *Rhizoma Sparganii* powder 10g and add 8 volumes of 70% ethanol, heat and extract twice (1.5h each time), recover the solvent after filtration, and dry the extract in a 45 ° C drying oven to constant weight. A voucher specimen (NO.SL 0704) was deposited in the Chengdu University of Traditional Chinese Medicine, Chengdu, China.

Compound was used in four concentrations: original extract (1g crude extract/ml), 66.7% extract, 33.3% extract, and 16.7% extract. All extracts were stored at 4°C until use.

Preparation of herbal decoction

Herbs (*Rhizome Sparganii*: *Rhizome Curcumae* =1:1) were crush and soaked for 2h. Herb fragments were decocted in two steps, 1h for the first time and half an hour for the second time. After mixing and filtering,

the decoction was concentrated to 0.67 crude drug/mg and stored at 4°C.

Animal

Rats care and experimental procedures were performed under a protocol approved by the Animal Ethical Committee at the Chengdu University of Traditional Chinese Medicine (Animal Ethics Approval Number NO. 2017-08). Female Specific pathogen-free (SPF) SD rats (Animal License No. SYXK (Chuang) 2014-049) were purchased from Chengdu Dashuo Experimental Animal Tech (China). All rats were adaptively fed with ad libitum access to standard rodent diet and water for 4 days. All animals were sacrificed by cervical dislocation under 2% pentobarbital sodium(0.25ml/100g) after 5 weeks.

Study design

Seventy-two SD rats were randomly divided into 9 groups—control group, model group, RCRS-treated groups (66.7%, 33.3%, and 16.7%), RC-treated group, RS-treated group, decoction-treated group, and a positive control group. Model establishment began after adaptively feeding. The control group rats received no treatment. The remaining 8 groups were administered intragastrically with diethylstilbestrol (1.35 mg/kg) every day, and intramuscularly with 1 mg progesterone three times a week for five weeks.

At the beginning of the fourth week, the eight experimental groups of rats were injected subcutaneously with epinephrine hydrochloride (0.5 mg/kg/d), and one external stimulus was given 4 hours after the injection: (1) Upside down day and night for 24 hour; (2) 60db noise continuous stimulation for 3 hours; (3) continuous hanging tail upside down for 10min; (4) 5-10°C water bath for 4 minutes. It lasts for two weeks and guarantees that each stimulus is ≥ 2 times in two weeks.

At the beginning of modeling, drug (10ml/kg) was given to the intervention groups at the same time, and the corresponding groups were given separately (Fig.1). After 5 weeks, the rat serum was collected for ELISA after anesthesia; the rat uterus was divided into two parts, one for paraffin embedding and one for western blot analysis.

Histological analyses

For hematoxylin and eosin (H&E) staining, uterus was fixed overnight in 4% paraformaldehyde and then embedded in paraffin, sectioned, and stained.

For Immunohistochemistry studies, paraffin blocks were sectioned and blocked with blocking buffer. Immunostaining was performed using primary antibodies ER (anti-rabbit, ab3206, Abcam), PR (anti-rabbit, ab16661, Abcam), FAP (anti-rabbit, ab28244, Abcam) and visionized by goat anti-Rabbit antibody (SP-9001, Beijing Zhong Shan Golden Bridge Biotechnology, China) and 3, 3'-diaminobenzidine tetrahydrochloride.

Enzyme-Linked immunosorbent assays

Rat sera were collected, diluted, and divided in two aliquots. One aliquot was used to measure estradiol (E2) and the other aliquot used to measure progesterone (P). The assays were performed using ELISA kits

purchased from Nanjing Jiancheng Bioengineering Institute (#R20181226, and #R20181227 for E2 and P, respectively). and a Multiskan MK3 microplate reader (Thermo Lab systems).

Western blotting

The uterus tissue was cut, homogenized and centrifuged, and the supernatant was aspirated for use. Detection was performed using a BCA protein quantification kit (PICPI23223, Thermo). GAPDH used as loading control for error correction.

Statistical analysis

Data were analyzed using one-way analysis of variance. The results are expressed as the mean+SD. A *p*-value less than 0.05 was considered significant. If the data did not conform to a normal distribution, a nonparametric test was used.

Result

The total flavonoids identification

A total of 10 flavonoids compounds were isolated from Rhizome Sparganii, and their structures were identified. These compounds include 1,3-O-diferulyl glycerol, ferulic acid, vanillic acid, 5-hydroxymethyl furfural, β -sitosterol palmitate, β -sitosterol, rutin, Kaempferol, succinic acid and α -palmitic acid monoglyceride (Fig.2).

Development of rat model

Rats of the model group showed hair loss, decreased diet, and slow increase of body weight.

The uterus of the model group was dull, the texture was hard, and there were abnormalities, nodules and edema macroscopic (Fig.3A). At the same time, the transverse diameter and vertical diameter of the uterus in the model group increased, and the volume of the uterus also increased significantly.

Pathological examination showed that compared with the control group, the myometrial cells of the uterus of the model group were disorganized, the thickness of the muscle layer was different, the outline of the muscle fibers was unclear, and the muscle fiber cells showed various degrees of deformation or even necrosis (Fig.3B). This is similar to the histopathological changes in uterine fibroids in clinical people. Meanwhile, the levels of estrogen and progesterone in the serum and uterus of the model group were significantly higher (Fig.3C-F).

These results demonstrated that the rat model of UL is successful.

Effects of RCRS on the appearance of uterus in rats

Compared with the model group, the uterus of rat received medication was mostly symmetrical, the texture became soft and the thickness was uniform, and the uterine surface was smoother than the model group with no obvious swelling and ecchymoses. A small number of nodules can be seen on some uteri (Fig.3A).

Effects of RCRS on histological changes of uterus

There were 6 (6/8) cases in the positive group, 6 (6/8) cases in the water group, 6 (6/8) cases in the RC-treated group, 5 (5/8) cases in the RS-treated group, 3 (3/8) cases in the RS-treated (16.7%) group, and 5 (5/8) cases in the RS-treated (33.3%) group, and 5 (5/8) cases in the RS-treated (66.7%) group showed different degrees of degeneration (Fig.3B). Myometrium is disorderly arranged and changes in thickness. The muscle fibers are unclear and Light-stain. Muscle fibers show varying degrees of degeneration and necrosis. The nucleus volume increased and hyalinization can be seen in muscle fibers. These results show that RCRS treatment can significantly improve the histological conditions of UL.

Effects of RCRS on the expression of FAP in uterus

Immunohistochemistry studies showed that the expression of FAP in the model group ($P<0.05$) increased significantly compared with the control group. The expression of FAP in RCRS-treated group (16.7%) was also increased. The effect of RCRS seemed to be dose-dependent as the low dose of the drug had a weaker inhibitory effect on FAP expression.

Compared with the model group, FAP expression was significantly decreased after RCRS (66.7%) or RC treatment ($P<0.05$). Positive control group showed a similar inhibitory effect on FAP expression ($p<0.01$) (Fig.4).

Effects of RCRS on the expression of TGF- β in uterus

Western blot analysis showed the expression of TGF- β was significantly increased in the model group compared with the control group ($p<0.01$).

Compared with the model group, the expression of TGF- β in the uterus in all drug intervention groups was significantly decreased ($p<0.05$). However, no statistical differences were detected in the control group. In the positive group, the expression of TGF- β was similar to that in each drug intervention group ($p<0.01$) (Fig.5A).

Effects of RCRS on the proliferation signaling pathway in uterus

To investigate the effects of RCRS on the expression of proteins regulating cell proliferation and apoptosis we analyzed the expression of AKT, ERK1/2 and MEK by western blot. Results showed that the drug treatment had no significant effect on the expression of these proteins. However, compared with the control group, the phosphorylation levels of AKT, ERK1/2 and MEK in the model group were significantly increased ($p<0.01$; $p<0.001$; $p<0.001$). The phosphorylation levels of AKT, ERK/2 and MEK were

significantly reduced in each drug intervention group compared to the model group. Positive group had similar effects on AKT and ERK1/2 phosphorylation ($p<0.01$; $p<0.001$; $p<0.05$) (Fig.6).

Effects of RCRS on the extracellular matrix in uterus

In order to study the effect of RCRS on the extracellular matrix of rat uterus, the expression of Collagen I and fibronectin, an important component of extracellular matrix, was analyzed by Western blot. Compared with the control group, the expression of collagen I and fibronectin protein in the model group was significantly increased ($p<0.001$; $p<0.05$).

Compared with the model group, except for the RS-treated group, the drug intervention groups reversed the expression of collagen I to varying degrees ($p<0.05$). This difference was particularly significant in the RCRS-treated (16.7%)/(66.7%) group ($p < 0.01$). Compared with the model group, the level of Collagen in the positive group was also decreased ($p<0.001$). there was no significant difference in the control group (Fig.5B).

The drug treatment showed no significant effect on fibronectin expression (Fig.5C).

Discussion

Uterine leiomyomas, also known as fibroids, affect up to 70% of women before menopause(Stewart et al., 2017) and cause various clinical problems, such as excessive uterine bleeding, algomenorrhea, infertility and miscarriage. Uterine fibroids are recognized as estrogen-dependent tumors, and their development is closely related to the coordination of estrogen and progesterone. However, no report was found in literature regarding the effects of RCRS component compatibility on TAF of UL. Therefore, in this study, we aimed to address this issue using a well-established rat model of UL. Morphological and pathologic data showed that RSRS treatment significantly improved. Based on that, we further examined whether RCRS improves UL by inhibiting TAF activation. As studies have shown that about 80% of fibroblasts are activated in tumor tissue, and FAP plays a key role in fibroblast activation(Räsänen et al., 2010)(Xouri et al., 2010). Angélica M. Santos (Santos et al., 2009) et al. showed that deletion of FAP gene in TAF significantly decreased the tumor cells proliferation activity, suggesting that FAP plays a key role in promoting tumor cells proliferation through TAF. Indeed, our western analysis results showed that the expression of FAP in uterus was significantly decreased in the RCRS component compatibility, indicating that FAP can be inhibited by RCRS component compatibility, which inhibits the activation of UL fibroblasts.

TGF- β has pleiotropic effects and plays a key role in cell growth, differentiation, and immune response, and TGF- β 1 has important actions in pathological changes of fibrosis(Zhang et al., 2019). TGF- β can promote TAF activation and induce normal fibroblasts transformation(Lohr et al., 2014). Our data showed that the expression level of TGF- β 1 is increased in the uterus of the model group, suggesting that increased FAP expression is due to the increase of TGF- β . RSRC treatment reversed these abnormalities suggesting RSRC inhibits TGF- β expression and thus the growth of UL.

Abnormal cell proliferation is one of the characteristics of uterine fibroid formation (Moravek et al., 2015). PI3K-AKT signaling pathway and MAPK/ERK signaling pathway are classical pathways related to cell proliferation and apoptosis (Sun et al., 2016) (Hoxhaj et al., 2020). It has been reported (Sefton et al., 2015) that AKT inhibitors can activate the AKT pathway in uterine fibroids, which increase the phosphorylation level of AKT compared with normal uterine tissue, suggesting that AKT is a potential biomarker for UL. Our results showed that AKT, ERK, and MEK phosphorylation levels were elevated in the model group, whereas phosphorylation levels of AKT, ERK, and MEK were reduced after treatment with RCRS component compatibility. It has been shown in the literature that (Higashino et al., 2019) silencing of the TAF phenotype protein FAP can reduce the phosphorylation levels of AKT, ERK and MEK, indicating that AKT, ERK, and MEK phosphorylation levels are associated with FAP gene expression and TAF activation. This is consistent with our findings. These results indicate that the therapeutic effects of total flavonoids of *Rhizoma Sparganii*, volatile oil of *Rhizoma Curcumae* and their compatibility on UL may be related to the inhibition of FAP of TAF.

UL are mainly composed of fibroid cells, fibroblasts, and ECM. ECM mainly includes collagen I, fibronectin and laminin. A large amount of ECM deposition in uterine fibroid tissue is an important factor in promoting tumor development and metastasis (Bhowmick et al., 2004). Tumor stroma is an important factor in promoting tumor development and metastasis. It is mainly composed of ECM, fibroblasts, inflammatory cells and vascular smooth muscle cells. In addition, fibroblasts are in a static state in normal tissues, and are stimulated by factors such as trauma to secrete a large amount of ECM protein (Rodemann, 1991) (Brown et al., 2006) (Angeli et al., 2009). Therefore, the effects of RCRS component compatibility on collagen I and fibronectin in uterine tissue of UL model rats were investigated. The results showed that the expression level of collagen I in the uterus of UL model rats were reduced by the total flavonoids of *Rhizoma Sparganii* and volatile oil of *Rhizoma Curcumae* after treatment. However, the RCRS component compatibility ability to regulate the expression of collagen I in uterine tissue suggests that its mechanism of treatment of UL may be related to RCRS component compatibility inhibiting TAF activation by inhibiting FAP expression.

RCRS is a commonly used drug pair in TCM. They are often used in combination with 1:1 to treat various gynecological diseases. The compatibility of TCM can improve the performance of the drug and enhance the therapeutic effect of the drug. Some scholars have separated and identified the mixture of RCRS, and found that the active ingredients in the mixed liquid are several more than the simple RCRS liquid, and the active ingredients extracted by the mixed liquid have better anti-oxidation and anti-tumor effects. In the experiment, a group of RCRS component compatibility was set up, and a single component group of total flavonoids of *Rhizoma Sparganii* and a single component group of volatile oil of *Rhizoma Curcumae* were also set. The results showed that the concentration of P in the serum of UL rats in the RCRS component compatibility was significantly decreased, and the expression of progesterone receptor (PR), FAP, collagen I and TGF- β in UL rat uterus was significantly inhibited. Comprehensive analysis showed that the RCRS component compatibility was superior to the one-component of the total flavonoids of *Sparganium* and volatile oil in *Rhizoma Curcumae* in treating UL. Modern medicine uses a combination of drugs in the process of treating diseases, and the treatment effect received is often better than that of a single drug.

TCM has been practicing the concept of drug combination for thousands of years and has been validated in the clinical treatment of diseases. Our experimental results also illustrate the scientific and effective theory of the compatibility of TCM.

Conclusion

In this study, the UL model in rats was established to evaluate the effect of RCRS on UL. The results show that RCRS can significantly improve the macroscopic and pathological state of uterus in rats with UL, reduce the expression of TGF- β and FAP protein in the uterus, decrease the expression of signal factors (AKT, ERK and MEK) in the cell proliferation related pathway and regulate the secretion of collagen I in ECM. It is suggested that RCRS may achieve the effect of treating UL by inhibiting the activation of TAF, the expression of factors of cell proliferation pathway (AKT, ERK and MEK) and reducing collagen I in ECM. The data presented in this study may provide a new idea and therapeutic approach for UL and lays the foundation for further investigation in the treatment of UL.

Abbreviations

UL, uterine leiomyoma; TCM, traditional Chinese medicine; RCRS, Rhizoma Curmae and Rhizoma Sparganii; ECM, extracellular matrices; TAF, tumor-associated fibroblasts; ER, estrogen receptor; PR, progesterone receptor; E2, estradiol; P, progesterone; FAP, fibroblast-activating protein; TGF- β , transforming growth factor- β ;

Declarations

Ethics approval

Rats care and experimental procedures were performed under a protocol approved by the Animal Ethical Committee at the Chengdu University of Traditional Chinese Medicine (Animal Ethics Approval Number NO. 2017-08).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YWF: Validation, Investigation, Writing-Original Draft; YMZ: Validation, Data Curation, Writing-Original Draft; YL: Ideas, Methodology; TP: Resources; YK: Resources; XMS: Writing- Reviewing and Editing; GW: Writing- Reviewing and Editing; FP: Conceptualization, Writing-Reviewing and Editing; CHY: Conceptualization, Writing- Reviewing and Editing, Project administration. All authors read and approved the final manuscript.

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Figures

Figure 1

Schematic design of the experiment.

Figure 2

Compounds in the total flavonoids of *Rhizoma Sparganii*. 70% ethanol was used to extract the total flavonoids of *Rhizoma Sparganii*, and 10 compounds were identified, including 1,3-O-diferulyl glycerol, ferulic acid, vanillic acid, 5-hydroxymethyl furfural, β -sitosterol palmitate, β -sitosterol, rutin, Kaempferol, succinic acid and α -palmitic acid monoglyceride.

Figure 3

Preparation of uterine fibroid model by estrogen and progesterone load combining with external stimulation. (A) Representative uterus appearance of rats in each group. (B) Representative histological changes of uterus in each group. Immunohistochemistry shows levels of (C) PR and (D) ER in uterus. The levels of (E) P and (F) E₂ in serum were measured by ELISA. (n=8; mean+SD, **p<0.01, *p<0.05 compared with control group; magnification 400×).

Figure 4

Effects of RCRS on the expression of FAP in uterus. Representative immunohistochemistry graphics of FAP in uterus. (n=8; mean+SD, ##p<0.01, #p<0.5 compared with model group; **p<0.01, *p<0.05 compared with control group; magnification400×)

Figure 5

Effects of RCRS on the expression of TGF-β and extracellular matrix in uterus. Representative western blotting band of TGF-β, collagen, fibronectin. The black borders are added to the image to distinguish different bands. (n=8; mean+SD, ###p<0.001, ##p<0.01, #p<0.5 compared with model group; ***p<0.001, **p<0.01, *p<0.05 compared with control group).

Figure 6

Effects of RCRS on the proliferation signaling pathway in uterus. Representative western blotting band of p-AKT, AKT, p-ERK1/2, ERK1/2, p-MEK2 and MEK2. The black borders are added to the image to distinguish different bands. (n=8; mean+SD, ###p<0.001, ##p<0.01, #p<0.05 compared with model group; ***p<0.001, **p<0.01, *p<0.05 compared with control group).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.tif](#)
- [NC3RsARRIVEGuidelinesChecklist2014.docx](#)
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