Effect of injury-curing cataplasma on expression of AQP3 in skeletal muscles of rats by regulating cAMP-PKA signal pathway

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Abstract: Curing-injury Cataplasma to promote regulatory mechanism of acute closed soft tissue injury swelling of rats may be related to inhibit PGE2, regulating the cAMP - PKA signaling pathways, high expression of AQP - 3. SD rats. 48 rats randomly divided into three groups, each group of 16. Blank group: only hair removal treatment, marking the scope, not building; Control group: marking the depilation area shall be made within the scope of acute closed soft tissue injury model of rats, don't give medication; Experimental group: building local injury Papua gels, a fixed tape. Building after 1 d, 3 d, 7 d, 14 d four phase randomly put to death in the rat 4, only partial muscle tissue specimens after using Western Blot to detect AQP - 3 protein expression level, enzyme-linked immunoassay detection PGE2, cAMP, the expression of PKA, parallel correlation analysis. In 3 d, 7 d, 14 d three time points, AQP-3 and PKA of Experimental group on the expression is higher than the Control group ($P<0.01$), the expression of PGE2 and cAMP is lower than the Control group ($P<0.01$); And Experimental group, Control group and Blank group differences were statistically significant ($P<0.05$). Acute closed soft tissue Curing-injury Cataplasma to promote a swelling, may be related to inhibit PGE2, regulating the cAMP - PKA signaling pathways, high expression of AQP - 3.

Keywords: Curing-injury Cataplasma, AQP-3, PGE2, cAMP, PKA.

INTRODUCTION

The acute closed soft tissue injury refers to the injury on skin, muscle, ligament, surrounding nerves, vessels and other soft tissues resulting from acute traumas, and it is one of the common diseases in clinic (Yuan *et al*., 2008). In traditional Chinese medicine (TCM), it belongs to the "tendon injury", its pathogenesis is Qi-stagnation, blood stasis and collaterals disharmony (Chen, 2012). In TCM, promoting blood circulation, removing blood stasis and relieving swelling and pain are mainly adopted. Our hospital's empirical formula Zhishangsan (injury curing powder) has been applied in clinic for several decades, with obvious curative effect. Its modified cataplasm, with the characteristics of anti-inflammation, pain relief and improvement of microcirculation, can apparently eliminate the local swelling, alleviate pain after soft tissue injury and promote soft tissue repair (Li *et al*., 2013).

In this study, the acute closed soft tissue injury model was established on fat muscle at lateral rat's right posterior limb (Zhang *et al*., 2007) and then cataplasm was applied. Western blotting was performed to detect the AQP-3 expression level in skeletal muscle of the rat' s swelling model of acute closed soft tissue injury, enzyme-linked immunoadsorbent assay (ELISA) was adopted to detect the contents of PGE2, cAMP and PKA in skeletal muscle of the rat's swelling model of acute closed soft tissue injury. A study was performed on correlation between skeletal muscle swelling and signal pathways of AQP-3, PGE2 and cAMP-PKA after attacked by acute closed soft tissue injury, to explore the mechanism of injury-curing cataplasm in removing swelling after acute closed soft tissue injury.

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MATERIALS AND METHODS

Preparations of reagents and drugs

Drug composition: blood shell of 60g, 15g *Polygonum cuspidatum* and 10g *Lindera angustifolia* W.C. Cheng were specially prepared by our Pharmacy Department into the cataplasm, whereas the tissue lysis buffer (T-PER tissue protein extraction reagent) and ECL luminous fluid (Pierce ECL Western Blotting Substrate) were manufactured by Thermo, USA; AQP3 polyclonal antibody was manufactured by Santa Cruz, USA, prestained protein electrophoresis molecular weight (standard) was manufactured by Frementas, USA, SDS-PAGE gel preparation reagent system, Western blot electrophoresis buffer, membrane transfer buffer, primary antibody dilution buffer, blocking buffer, coating solution, secondary antibody dilution buffer, Tubulin antibody, HRP-labelled goat anti-rabbit IgG, cytokines ELISE kit, distilled water, labelled antigen and antibodies were all provided by Molecular Medicine Research Center of Xiangya Hospital.

Modeling and intervention

48 clean male SD rats 8 weeks old were provided by animal experiment center of Hunan University of Chinese Medicine, with weight of (300±20) g each. 1 hour before modeling, the lateral thigh of right posterior limb of each rat was depilated with 10% sodium sulfide, and the 48

rats were randomly divided into three groups: model group, drug treatment group, normal control group. Model group: with reference to literatures, weight dropping was performed to establish the swelling model of acute closed soft tissue injury on the depilated marked range, this group was only winded with bandage, but no drugs were given; drug treatment group: injury-curing cataplasm was scattered on the stricken area immediately after the establishment of swelling model of acute closed soft tissue injury and fixed with bandage. Normal control group: The lateral thigh of right posterior limb of each rat was depilated, the striking range was marked and winded with bandage, while modeling by striking was not performed.

Extraction and treatment of tissue specimens

On the 1st, 3rd, 7th and 14th day after modeling, the rats were randomly killed, 4 rats for each group at each time point. We take 1g muscle tissue from marked range, cleaned with normal saline and cut into pieces, then placed in a 2ml of freezing tube. The liquid nitrogen was added into the freezing tube for quick freezing, then the freezing tube was placed in a -80^{o}C refrigerator for later use.

Detection of expression of AQP-3 in skeletal muscle by Western-bolt

For extraction of total tissue protein, 50mg of muscle tissue was placed in a sterilized mortar, liquid nitrogen was continuously added, the tissue was grinded into the powder by charging, the powder was placed in a freezing tube, 1ml of tissue lysis buffer was added into the tube and shaken evenly, then cracked on the ice for 30min and centrifuged at 12000g in a centrifuge at 4^{o}C for 5min, the supernatant was extracted to detect the protein concentration.

Polyacrylamide gel electrophoresis

10% SDS-PAGE gel and 5% spacer gel were prepared, the corresponding protein sample was taken according to the measured protein concentration and added into the same-volume loading buffer, then boiled in the 100^{o}C boiling water for 5min. After loading, the gel was electrophoresed at 80V for 30min, when the dye leading edge was stained in the separation gel, the voltage was increased to 120V for continuous electrophoresis till Mark was at the bottom of separation gel (about 1.5h). PVDF was wetted for 60min (at constant current 300mA); 5% skimmed milk powder was used for blocking at room temperature for 2h, then set aside.

Western Bolt

TBS-T solution was used to rinse PVDF membranes for three times, AQP-3 primary antibody was added to TBS-T solution and reacted on shaking table at 4^{o}C for 12 hours, PVDF membranes were rinsed with TBS-T solution for three times, 10min for each time, then 1:2000 HRP-labeled goat anti-rabbit IgG secondary antibody was added to TBS-T solution and hatched at room temperature for 60min. PVDF membranes were rinsed with TBS-T solution for three times, 10min for each time, then developed with ECL luminous fluid, the grey level of each protein electrophoresis band indicated the expression of AQP-3 protein.

Enzyme-linked immunoadsorbent assay

After the standard was diluted on the ELISA Plate, then we add sample and enzyme, incubating and developing, and then we calculated PGE2, CAMP and KPA concentrations.

STATISTICAL ANALYSIS

SPSS 22.0 statistical software was used for analysis. The measuring data were expressed in mean ± standard deviation ($\vec{x}$ ±s). One-way analysis of variance and LSD method were used respectively to compare the data between groups at different time points. α=0.05 and P<0.05 showed the difference was statistically significant.

RESULTS

Expression of AQP-3 protein in skeletal muscle

After modeling, AQP3 expression in muscle for model group and drug treatment group changed in a curved manner, namely gradually increased in the first seven days, reaching the peak on the 7th day, then gradually decreased. After modeling, AQP3 expression in muscle for drug treatment group and model group at each time point was higher than that of the normal group (P<0.05), and that of drug treatment group was higher than that of the model group, but there was no apparent differences on the 1st day (P>0.05, fig. 1).

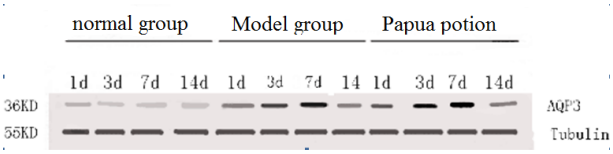

Fig. 1: Expression Changes of AQP-3 in Skeletal Muscle on the 1st, 3rd, 7th and 14th day

Expression of PGE2 in skeletal muscle

PGE2 expression in skeletal muscle for the model group and drug treatment group changed in a curved manner, namely gradually decreased on the first seven days, reaching the bottom on the 7th day, then gradually increased. On the 3rd, 7th and 14th day, PGE2 expression for drug treatment group and model group was respectively higher than that of the normal control group (P<0.01), and that of drug treatment group was higher than that of the model group (P<0.05), but there was no apparent differences on the 1st day (P>0.05, fig. 2).

Expression of cAMP in skeletal muscle

cAMP expression in skeletal muscle for model group and drug treatment group changed in a curved manner, namely gradually decreased on the first seven days, reaching the bottom on the 7th day, then gradually increased. On the 3rd, 7th and 14th day, cAMP expression for the drug treatment group and the model group was respectively lower than that of normal control group (P<0.01), and that of drug treatment group was higher than that of the model group (P<0.01), but there was no apparent differences on the 1st day (P>0.05, fig. 3).

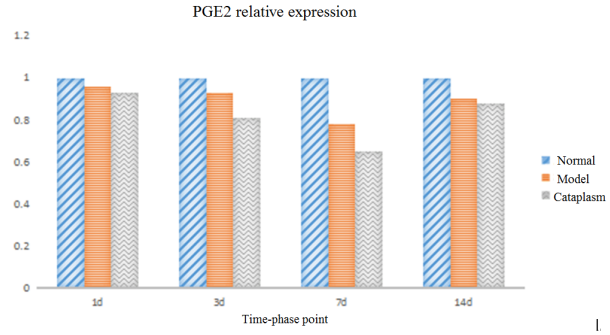

Fig. 2: Expression Changes of PGE2 in Skeletal Muscle on the 1st, 3rd, 7th and 14th day

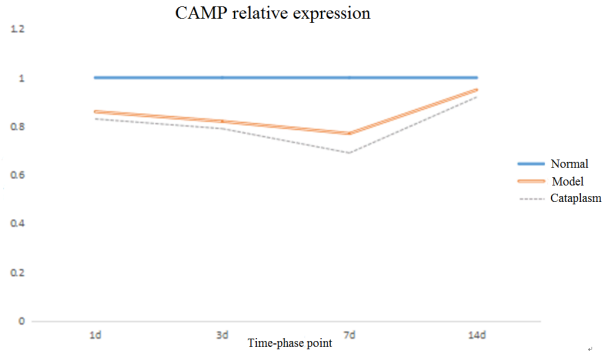

Fig. 3: Expression Changes of cAMP in Skeletal Muscle on the 1st, 3rd, 7th and 14th day

Expression of PKA in skeletal muscle

PKA expression in skeletal muscle for model group and drug treatment group changed in a curved manner, namely gradually increased on the first seven days, reaching the peak on the 7th day, then gradually decreased. On the 3rd, 7th and 14th day, PKA expression for drug treatment group and the model group was respectively higher than that of normal control group (P<0.01), and that of drug treatment group was higher than that of the model group (P<0.01), but there was no apparent differences on the 1st day (P>0.05, fig. 4).

DISCUSSION

In TCM, the acute closed soft tissue injury belongs to the "tendon injury", promoting blood circulation, removing blood stasis and relieving swelling and pain are mainly adopted, and external application of Chinese medicine is one of the features for treating this disease (Yu *et al*., 2016). The cataplasm modified by our hospital's empirical formula Zhishangsan (injury curing powder) is mainly composed of blood shell, *Polygonum cuspidatum, Lindera angustifolia* Cheng and other Chinese herbs (Zhang *et al*., 2007). It can remove swelling, relieve pain, promote blood circulation and remove the blood stasis, so, it can be used for treating the acute closed soft tissue injury; in recent years, most experimental studies about Chinese medicines in treating soft tissue injury made the general observations, pathological histology (Chen *et al*., 2002), biochemical factors and hemorheology as the observation indexes, to study its anti-inflammation, pain relief, swelling removal and other action mechanisms (Li *et al*., 2013). The preliminary experiments made by the research group have verified that cataplasm can remove swelling and relieve pain of acute closed soft tissue injury of rats, and it is related to expression of AQP-3. Aquaporin (AQP) is a kind of cell membrane channel protein with important physiological function, and it can quickly transfer water molecules in a specific manner (Antonova *et al*., 2015; Caziu *et al*., 2015).There are a total of 13 proteins, AQP3 is slightly expressed in skeletal muscle and myocardial tissue, but its action mechanism on physiological and pathological state of skeletal muscle is still unclear (Chen *et al*., 2012). Signal pathway of cyclic adenosine monophosphate (cAMP) is a significantly important signal transduction way in an organism, as a second messenger, cAMP is closely related to the cell regulation. cAMP signal pathway can regulate the cells by activating cAMP-dependent protein kinase (PKA)(Niu *et al*., 2007; Liu *et al*., 2012; Fang *et al*., 2016). PGE2 is an inflammatory reaction medium, and it can boost generation of cAMP mainly through EP receptor, to regulate the cells (Wang, 2010).

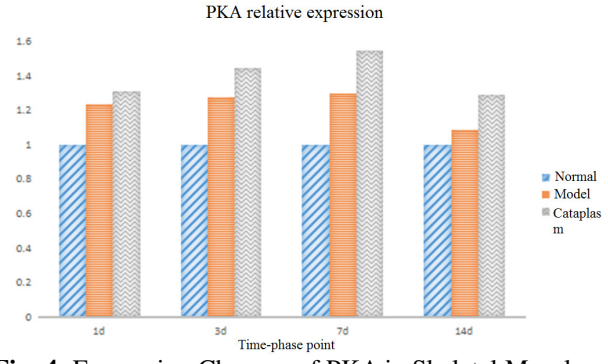

Fig. 4: Expression Changes of PKA in Skeletal Muscle on the 1st, 3rd, 7th and 14th day

The acute closed soft tissue injury can result in a series of pathological changes. PGE2 and other inflammatory mediums released, it could produce the swelling and pain-mediated signal transduction channels and regulate the protein expression level (Huang *et al*., 2011), so as to generate corresponding pathological and physiological effects. The results revealed that, after successful modeling, the rat's injured limb will rapidly have swelling, sub dermal ecchymosis and other symptoms; after rats are

attacked by the acute closed soft tissue injury (Divani *et al*., 2015), the differences of swelling removal effects between drug treatment group and normal group as well as between drug treatment group and model group are of statistical significance (P<0.05). In the normal control group, AQP-3, PGE2, cAMP and PKA are stably expressed (Wang, 2010). In the model group, AQP-3 and PKA expressions are gradually increased, reaching the peak on the 7th day, then gradually decreased, while still higher than the normal group on the 14th day. In the model group, PGE2 and cAMP expressions are both suppressed, reaching the peak on the 7th day, then AQP-3 and PKA expressions are gradually increased, on the 14th day, the AQP-3 and PKA expressions are still lower than the normal group (Yu *et al*., 2016). AQP-3 and PKA expressions for the drug treatment group are apparently higher than those of normal control group and model group, AQP-3 and PKA expression are gradually increased, reaching the peak on the 7th day, then gradually decreased, while still higher than those of model group on the 14th day; PGE2 and CAMP expressions for drugs treatment group are apparently lower than those of normal control group and model group, reaching the bottom on the 7th day, then the expressions are gradually increased, while still lower than those of model group on the 14th day (Cirak *et al*., 2015; Damyanov *et al*., 2015). The experimental results have shown that for the acute soft tissue injury, the external application of cataplasm self-prepared by our hospital can effectively remove the swelling of soft tissue and speed up the repair of injury (Antonova *et al*., 2015; Caziu *et al*., 2015). After the acute closed soft tissue injury, external application of cataplasm can effectively suppress the release of PGE2 and alleviate the release of inflammatory mediums around the injury, to suppress cAMP signal transduction, make PKA and AQP3 highly expressed, boost the removal of soft tissue swelling and speed up the repair of injury.

CONCLUSION

Based on the foregoing experimental results, the injury-curing cataplasm can remove the acute closed soft tissue swelling, which is possibly related to the high expression of AQP-3 by suppressing PGE2 and regulating cAMP-PKA signal pathway, but its exact action mechanism needs to be further studied.

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