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Effects of Xuanhuang ointment on histomorphology and appearance of acute soft tissue injury in rats

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Abstract: Objective: To observe the effect of Xuanhuang ointment on the morphology and appearance of local soft tissue in rats with acute soft tissue injury. Methods: The acute soft tissue injury model of rats was established and randomly divided into Xuanhuang ointment group, safflower oil group and matrix ointment group. Another 5 rats were taken as the normal group. In addition to the normal group, apply ointment on the injured part of the right lower limb thigh, cover the injured tissue, and then wrap it with breathable film, medical tape and bandage. Change the dressing once every other day, three times in total. On the 3rd, 5th and 7th day of the experiment, the visual observation of the surface damage degree of local soft tissue in each group was compared; On the 1st, 3rd, 5th and 7th day after treatment, the local soft tissue was taken and the histomorphological changes were observed under light microscope. On the 7th day of the experiment, the histopathology of rats in each group was statistically compared. Results: The blood stasis at the injured part in Xuanhuang ointment group and safflower oil group disappeared significantly, and the repair of muscle fibers was faster than that in matrix ointment group. Conclusion Xuanhuang ointment has the effects of anti inflammation and detumescence, promoting blood circulation and removing blood stasis, and can accelerate the repair of muscle fibers.

1. Introduction

Acute soft tissue injury refers to the injury of muscles, ligaments, fascia, articular cartilage, joint capsule, synovial capsule and other parts below the skin and outside the bone. This kind of injury is on the rise today with the increasing number of high-energy sports. Traditional Chinese medicine has always occupied a unique and significant position in the field of treating soft tissue injury. Aiming at the pathogenesis of qi stagnation and blood stasis of soft tissue injury, on the basis of syndrome differentiation and treatment, many good attempts have been made to treat acute soft tissue injury with traditional Chinese medicine, and almost all treatment groups have reported satisfactory therapeutic effects. The external treatment of traditional Chinese medicine in the treatment of soft tissue injury is mainly to absorb the bioactive substances contained in the external traditional Chinese medicine through the skin at the injured part, and maintain the relatively stable blood drug concentration in the injured part, so as to achieve the effects of promoting blood circulation, removing

blood stasis, detumescence and analgesia [1]. On the basis of summarizing the research of other scholars, this experiment further studied the mechanism of Xuanhuang ointment in the treatment of acute soft tissue injury through randomized controlled animal experiments, and observed the effect of Xuanhuang ointment on the histomorphology and appearance of acute soft tissue injury in rats, so as to provide further scientific basis for the application and promotion of Xuanhuang ointment.

2. Materials and methods

2.1 Experimental animal selection

55 Whistar rats weighing 160~190g, half male and half female, provided by the experimental animal center of the Fourth Military Medical University, animal license No: SCXK (Shaanxi 2007-002). Adaptive feeding for 3 days before the experiment.

2.2 Experimental drugs and reagents

XuanHuang ointment is composed of rhubarb, gardenia, dandelion, papaya, Sichuan Mutong, earthworm, frankincense, myrrh, ground beetle. It is provided by the traditional Chinese medicine pharmacy of the Affiliated Hospital of Shaanxi University of traditional Chinese medicine, and the drug supervision is qualified. The pulverizer takes the above drugs as fine powder according to 100-120 mesh powder for standby. Preparation of sample drug: use Vaseline as matrix to make ointment with required concentration [2]. Safflower oil is provided by Changchun Jingkai Pharmaceutical Co., Ltd. (GYZZ z22024033, batch No. 20040103). Depilatory agent (sodium sulfide and water are prepared in the ratio of 8:2).

2.3 Establishment of acute soft tissue injury model in rats [3]

The rats were fixed on the self-made rat operating table, and a hollow tube with a diameter of 1.5cm and a height of 50cm was placed with a smooth wall. It was placed on the soft tissue of the right lower limb of rats (the hair of the thigh of the right lower limb was removed with 8% sodium sulfide hair remover one day before the experiment), and then a 100g weight was dropped freely from top to bottom to hit the soft tissue of the right lower limb of rats for three times, resulting in a non open soft tissue injury model with an area of about 4cm2 and obvious subcutaneous bleeding and swelling.

2.4 Experimental scheme

Fifty rats, half male and half female, were taken. After successful modeling, all rats were fed with standard rat feed and drank freely under the same conditions (in the biochemical laboratory of Shaanxi University of traditional Chinese medicine, room temperature 19 ~ 24℃, relative humidity about 40%). They were randomly divided into 4 groups. 14 rats in the model group of acute soft tissue crush injury were recorded as a group (blank group), 12 rats in the matrix ointment control group as a group, 12 rats in the Xuanhuang ointment treatment group as a group and 12 rats in the safflower oil treatment group as a group. The patients were sacrificed 24 hours after the last administration. After modeling, the animals in each group were killed at three times: day 1, day 3, day 5 and day 7. Some rats were killed at each time. The skin of the hit part of the rats was cut with a scalpel, the subcutaneous tissue was separated, and the muscle tissue at the center of the injury was cut. Another 5 rats were taken as the normal group, and the tissues of the same part were taken.

2.5 Observe the histomorphology with naked eyes and observe it directly

Pathological and histomorphological examination, the steps are as follows:

1) Some rats were killed 24 hours after modeling and on the 3rd and 5th days of the experiment. All rats were killed on the 7th day of the experiment. The damaged central tissue was taken and cut with sharp scissors for $1\times1\times0.3cm^3$.The damaged tissue should be cut quickly to avoid squeezing and kneading the tissue and ensure the integrity of the research tissue.

2) Tissue fixation: Fix the damaged tissue block with 10% formalin solution.

3) Making paraffin sections ① Rinsing: rinse the fixed tissue with normal water repeatedly, and finally rinse it thoroughly with distilled water repeatedly; ② Dehydration:70% alcohol(60 minutes)→80% alcohol(60 minutes)→95% alcohol(60 minutes)→100% alcohol(60 minutes)→1/2 100% alcohol + 1/2 xylene(60 minutes);③ Transparent: permeating with 25%,50%,70%,90% xylene anhydrous alcohol; ④Wax dipping:put the muscle tissue and xylene into the 60℃refrigerator, take small pieces of pure paraffin with melting point of 52℃~56℃, gradually add them to the tissue, let the xylene dissipate gradually, and change the wax for 3~4 times; ⑤Embedding: pour the molten wax and muscle tissue into the prefabricated small carton while it is hot until it is completely solidified; ⑥ Sectioning: continuous tissue sectioning with a slicer; ⑦ Patch:apply a layer of adhesive on the quilt and add a drop of water; ⑧ Baking slices: dry the slices.

4) HE staining ①The slices were dewaxed in xylene for 5~10 minutes; ②Transfer into the mixture of xylene and pure alcohol (1:1) for about 5 minutes; ③Add 100% alcohol(3 minutes)→95% alcohol(3 minutes)→85% alcohol(3 minutes)→70% alcohol (3 minutes)→transfer into the dye solution; ④Hematoxylin staining for 5~10 minutes; ⑤Wash the excess dye solution on the glass slide with water, separate the color with 0.5%~1% hydrochloric acid alcohol(prepared with 70% alcohol) for a moment, and conduct microscopic inspection and control until the nucleus and chromatin in in the nucleus are clear, about 10 seconds; ⑥Rinse in running water for 15~30 minutes, or alkalize in lithium carbonate saturated solution for a short time to make the nucleus blue; ⑦Short wash with distilled water; ⑧Dye with 0.1%~0.5% eosin staining solution for 1~5 minutes. If it is difficult to dye, add 1~2 drops of glacial acetic acid to 100ml dyeing solution to make it easy to dye and not easy to decolorize; ⑨Dehydration: 70% alcohol (3 minutes)→80% alcohol(3 minutes)→95% alcohol(3 minutes)→100% alcohol(3 minutes); ⑩Xylene is transparent twice for about 10 minutes; ⑪Sealing:wipe off the excess xylene around the slice,do not dry up, quickly add an appropriate amount of neutral gum,and then seal it with cover glass.

3. Statistical Analysis

The statistical data were processed by Ridit analysis. There was significant difference between each group and the matrix ointment group ($P<0.05$).

4. Results and analysis

4.1 Visual observation results

After treatment, after the soft tissue of the rat's right rear thigh was hit, subcutaneous bleeding and swelling appeared rapidly at the injured part through naked eye observation, and the injury was continuously aggravated, which was most significant 24 hours after injury, and then decreased day by day. On the 5th day, the areas of local bleeding, blood stasis and edema in the blank group and matrix ointment group were still more than 1cm2; Most rats in Xuanhuang ointment and safflower

oil group were close to normal soft tissue, and there was significant difference compared with blank group and matrix ointment group ($P < 0.05$); There was significant difference between safflower oil group and Xuanhuang ointment group ($P < 0.05$).**(**See Table 1.**)**

(24 hours after modeling, the right lower limb of rats in each group was damaged, local swelling was obvious, and ecchymosis was visible, especially in the blank control group and matrix ointment control group)

Table 1: Effect of Xuanhuang ointment on acute soft tissue injury in rats.

Groups	Degree of lesion damage after modeling													
	Day 3				P Value	Day 5				P Value	Day 7				P Value
	-	+	++	+++		-	+	++	+++		-	+	++	+++	
Blank group	**0**	**0**	**0**	**12**	**>0.05**	**0**	**0**	**3**	**8**	**>0.05**	**0**	**3**	**5**	**2**	**>0.05**
Matrix paste group	**0**	**0**	**1**	**11**	**-**	**0**	**0**	**5**	**6**	**-**	**2**	**4**	**4**	**0**	**-**
Xuanhuang ointment group	**0**	**2**	**7**	**3**	**<0.01**	**0**	**7**	**4**	**0**	**<0.05**	**9**	**1**	**1**	**0**	**<0.01**
Safflower oil group	**0**	**13**	**5**	**6**	**>0.05**	**0**	**6**	**4**	**1**	**<0.05**	**7**	**3**	**0**	**0**	**<0.05**

Note: on the third day of modeling, n = 12; On the 5th day of modeling, n = 11; On the 7th day of modeling, n = 10; The degree of local soft tissue injury was observed and graded on the 3rd, 5th and 7th day of the experiment. The grading standard was: "-" no obvious swelling and ecchymosis; "+" no swelling, faintly visible ecchymosis. "+ +" swelling thickness is less than 1 time of the thickness of the same part of the left limb, and ecchymosis can be seen. "+ + +" the swelling thickness is more than twice the thickness of the same part of the left limb and / or there are obvious purple ecchymosis.

4.2 Pathological and histological observation results

The basic pathological changes of Xuanhuang ointment group, safflower oil group and model group were similar. 24 hours after trauma, subcutaneous edema, neutrophil infiltration, mild swelling, distortion or disappearance of damaged striated muscle fibers and other degenerative and necrotic changes were observed; Neutrophils and monocytes infiltrate in some necrotic muscle fibers, and a small amount of bleeding or hematoma formation and neutrophil infiltration can be seen between muscle fibers. On the first day after injury, the swelling of subcutaneous tissue was aggravated, the density of neutrophil infiltration in trauma lesions increased, and the degeneration and necrosis of striated muscle fibers occurred successively. On the 3rd day after injury, the edema of local subcutaneous tissue was reduced, and the density of neutrophils in trauma lesions continued to decrease, mainly monocyte infiltration; Fibroblasts proliferate actively, fibrous connective tissue proliferates, and many fibroblasts grow into hematoma; Some necrotic striated muscle fibers were replaced by new fibrous connective tissue. On the 5th day after injury, the neutrophils in the wound focus basically disappeared, the subcutaneous and muscle wound parts were basically filled with proliferative fibrous connective tissue, and the small blood vessels in the subcutaneous tissue increased, but the phenomenon of dilation and congestion was less. All rats were killed on the 7th day after injury, and some small blood vessels in subcutaneous tissue were dilated and congested; A large number of fibrous connective tissue proliferated in the muscle trauma, and some residual striated muscle fibers were divided by fibrous connective tissue; The hematoma was basically absorbed or organized. The formation of fibrous connective tissue in Xuanhuang ointment group was less than that in safflower oil group and matrix ointment group. The results showed that most rats in Xuanhuang ointment and safflower oil group were close to the normal microscopic soft tissue morphology, and there was significant difference compared with the blank group and matrix ointment group ($P < 0.05$); There was significant difference between safflower oil group and Xuanhuang ointment group ($P < 0.05$). The pathological sections of injured tissues of rats in each group on the 7th day are compared as shown in Table 2.

Table 2: Effect of Xuanhuang ointment on acute soft tissue injury in rats

Groups	Animals (n)	Lesion grading				P Value
		—	+	++	+++	
Blank group	10	0	1	6	3	>0.05
Matrix paste group	10	0	1	8	1	—
Xuanhuang ointment group	10	1	8	1	0	<0.01
Safflower oil group	10	0	7	3	0	<0.05

Note: the grading standard of pathological tissue injury: "-" has no obvious histopathological changes; "+" subcutaneous and muscle fibers were slightly edema and blood stasis, and only scattered neutrophils and other inflammatory cells were infiltrated; "+ +" moderate edema under the skin and between muscle fibers, a little degeneration and necrosis of muscle fibers, and obvious infiltration of inflammatory cells such as neutrophils; "+ + +" severe edema under the skin and between muscle fibers, degeneration and necrosis of a large number of muscle fibers, infiltration of inflammatory cells, or proliferation of inflammatory granulation tissue.

5. Conclusions and Discussion

Acute soft tissue injury is a common and frequently occurring disease in clinical orthopedics. The injury causes local tissue redness, pain and capillary rupture, which limits the patient's ability to move and seriously affects the quality of life [4]. Soft tissue injury belongs to the category of "tendon injury" in China's traditional medicine. Acute soft tissue injury is mostly caused by blood stasis block, and its main pathogenesis is blood stasis and qi stagnation and disharmony [5].

Acute inflammation includes the anti-inflammatory effect of inflammation caused by soft tissue injury, generally including swelling inflammation model, granuloma model and immune inflammation model. The swelling and inflammation model was used in this experiment. The self-made injury frame was used to make the weight (100g) fall from the height of 50cm, resulting in a local traumatic hematoma at the mark (the right thigh of rats). The striking area was 4cm2. Subcutaneous ecchymosis was visible after striking, and the swelling was obvious after 1h. No fracture, no skin rupture. The histopathological changes of the animal model of acute soft tissue injury prepared by this method are similar to those of clinical soft tissue injury. Three days after the weight (100g) hit, the subcutaneous tissue adhered, the superficial muscles had bleeding, the surrounding muscles were pale and had poor elasticity. Under light microscope, there was extensive bleeding in the muscle, turbidity and swelling of muscle fibers, rupture of some muscle membranes, necrosis of some muscle fibers and edema around them. Seven days after the blow, there was still adhesion and a small amount of bleeding between the skin and subcutaneous tissue, and a small amount of blood stasis in the muscles. Under light microscope, hyaline degeneration of muscle fibers and edema of muscle stroma were observed, accompanied by a large number of granulocyte infiltration. Pathological examination showed different degrees of bleeding, thrombosis, tissue necrosis and inflammatory reaction in subcutaneous fat, capillaries, muscle tissues, ligaments and joints. The clinical manifestations were blood stasis, burning pain, subcutaneous ecchymosis and cyanosis.

This model is consistent with clinical acute soft tissue injury. The selection of weights used to prepare the model is determined by pre-test of 10, 50 and 250g weights. The falling of 250g weight causes local skin rupture and exudation of tissue fluid, which is easy to lead to fracture. The falling of 50g weight caused local swelling, but the subcutaneous ecchymosis and subcutaneous nodules were not obvious. Therefore, 100g heavy hammer is used to cause local soft tissue damage, no fracture, no skin rupture, and obvious subcutaneous ecchymosis and swelling. Traditional Chinese medicine mainly attributed the main pathological mechanism of acute soft tissue injury to blood stasis and qi stagnation and disharmony of collaterals. It is considered that the disease belongs to the

category of "tendon injury". "Su Wen · Yin Yang Ying Xiang Da Lun" said: "Qi injury pain, form injury swelling", which clearly pointed out that pain and swelling are the main clinical manifestations of acute traumatic hematoma [6].

Modern medicine believes that trauma is the main cause of acute soft tissue injury. Generally, external forces act on the human body indirectly or directly, resulting in local tissue swelling or wall rupture of microvessels [7]. On the one hand, external force can directly cause local tissue cell degeneration and necrosis; At the same time, due to the destruction of blood circulation, trauma, aseptic inflammatory reaction and other reasons, the local internal environment changes, hypoxia and metabolic disorder of tissue cells will continue to cause degeneration and necrosis of tissue cells and aggravate the damage of local tissues. After a few hours, there will be inflammatory reaction, which shows congestion, serous exudation and leukocyte migration, release of inflammatory mediators and accumulation of metabolites, so local redness, swelling and pain. Aseptic inflammation is the most important pathological change after acute soft tissue injury [8]. Neutrophils are one of the main inflammatory cells in the early stage of aseptic inflammation in trauma lesions. From the results of this experiment, xuanhuang ointment can significantly inhibit the local sterile inflammatory response of trauma, promote the absorption of inflammation and the dilution and operation of inflammatory mediators. The results of this experiment confirmed that xuanhuang ointment can significantly reduce the bleeding, congestion and expansion of blood vessels around the damaged local muscle fiber stroma, and reduce the phenomenon of red blood cell deposition. At the same time, it significantly reduced the infiltration of neutrophils in the local muscle stroma and around the blood vessels.

In conclusion, xuanhuang ointment can promote the dissipation of local congestion and accelerate the repair of injured muscle fibers, which shows that one of the mechanisms of xuanhuang ointment in the treatment of acute soft tissue injury may be related to the fact that xuanhuang ointment can significantly inhibit the inflammatory reaction of injured local soft tissue.

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