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Basic Investigation

Experimental research into using bone-strengthening pill to treat ischemic necrosis of femoral head

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Abstract

OBJECTIVE: To study the use of a bone-strengthening pill to treat ischemic necrosis of the femoral head.

METHODS: A model of castration in rats with osteoporosis and a model of ischemic necrosis in the bilateral femoral heads of young rats were used to detect bone density, bone formation measurements, lipid deposition in the femoral head, the distribution of capillaries in the femoral head and other relevant biochemical indices.

RESULTS: In model rats, bone-strengthening pills were able to increase the bone density, the unit volume of bony trabeculae, the width of bony trabeculae, and the unit volume of the bony cortex. They could also enhance the mineral apposition rate in the femoral head and the seam width of osteoid deposition. Furthermore, there was a reduction in lipid deposition in the femoral head, and enriched distribution of arteries in the femoral head. Bone-related biochemical detections showed an increase in alkaline phosphatase staining and a decrease in tartaric acidic phosphatase staining.

CONCLUSION: The results show that bone-strengthening pills can obviously promote bony growth, cause an increase in bone density, restore blood circulation, inhibit the occurrence and development of bony necrosis, and accelerate the repair of necrotic bones, with no toxic side-effects.

Key words: Bone-strengthening pill; Necrosis of femoral head; Osteoporosis

INTRODUCTION

Avascular necrosis of the femoral head (ANFH) is a common clinical disease characterized by damage to bony trabeculae and marrow that is induced by the death of bony cells, hematopoietic cells in the marrow and the formation of lipid cells. It also usually stems from insufficient blood supply to the femoral head or the degeneration of bony cells, and predominantly affects middle-aged and young people. In advanced stages, collapse of the femoral head often causes dysfunction of the hip joint. Consequently, it is difficult to treat such a severe disease[1]. Factors that induce necrosis of the femoral head can be divided into the two types: trauma and non-trauma. The former mainly refers to femoral fracture, traumatic dislocation of the hip joint, acetabular fracture and intertrochanteric fracture, while the latter refers to various metabolic diseas-

es, such as mucopolysaccharidosis, blood disease, diver's bone disease, hormone supplements, and long-term alcoholic intemperance[2]. Here, we investigate and evaluate the curative effect and pharmacological action of a bone-strengthening pill for the treatment of ANFH.

MATERIALS AND METHODS

Drugs

Bone-strengthening pills were supplied by Beijing Kuangda Pharmaceutical Factory, with approval number: Z10970030. Compound danshen tablets were produced by Zhunhua Pharmaceutical Factory in Hebei Province, with batch number 960401. Prednisone acetate was produced by Beijing Chaoyang Pharmaceutical Factory, with batch number 960502.

Animals

Female wistar rats, weighing 200±20 g, were 3-4 w old. Baby rats were purchased from the animal center of China Institute of Drugs and Bio-products.

Main instruments

A LEICA RM2235 microtome (Leica Microsystems Inc. Bannockburn, IL, USA), an OLYMPUS BX40 microscope (Olympus medical system corp., Tokyo, Japan) and an Aeroset biochemical instrument (Abbott Laboratories, IL, USA) were supplied by the pharmacological laboratory under The China Academy of Chinese Medical Sciences.

Animal experiment

Model establishment: Model for induction of ischemic necrosis of the femoral head: The two front limbs of 10- to 15-day-old rats were excised at the upper side of the elbow, and the tail was snipped at its root. After cauterization, the baby rats were put back in their nest to be fed by their mothers. At the same time, baby rats with intact limbs were maintained as normal controls. At day 45, after the body weight of the model rats had increased to over 150 g, prednisone acetate was given intragastrically at a concentration of 10 mg/kg·d, three times per week for 12 w.

Model for castration: Under anesthesia, both ovaries of the female rats weighing 200±20g were removed. After cauterization, the wound was sutured and the rats allowed food and water ad libitum.

Group allocation and drug administration: Rats were split into 5 groups: control (untreated), model control (amputated but untreated), compound danshen tablet group, low-dose bone-strengthening pill group and high-dose bone strengthening pill group. In the normal control group, untreated rats were given intragastrically with 2 mL of distilled water, once per day. In the model control group, castrated rats were poured with 2 mL of distilled water once per day; and amputated rats were poured with 2 mL prednisone acetate. In the compound danshen tablet group, both castrated and amputated rats were poured with compound of danshen tablet at 0.2 g/kg in a final volume of 2 mL, once per day for a period of 12 w. In the bone-strengthening pill low-dose group, both castrated and amputated rats were poured with 2 mL suspension of bone-strengthening pill powder at a concentration of 1.14 g/kg, once per day for 12 w. In the bone-strengthening pill high-dose group, both castrated and amputated rats were poured with 2 mL suspension of bone-strengthening pill powder at a concentration of 2.28 g/kg, once per day for 12 w. Sixteen and five days prior to euthanasia, the rats were intraperitoneally injected with 30 mg/kg tetracycline.

To validate the reproducibility of the experiment, the experiment was performed twice on the model rats with ischemic necrosis of the femoral head.

Indexing and detection

Three days prior to euthanasia, the bone density of the vertebrae was examined in all of the rats. On the day prior to euthanasia, rats were fasted. Under anesthesia, blood was taken from the abdominal aorta. The relevant biochemical indices in the serum were detected. After blood was extracted, the femoral artery was clamped at its root and about 3 mL of ink was injected into the contralateral femoral artery until the ink was seen in the rear limbs and at the root of the tail. The femoral heads, which remained unstained with ink, were removed and placed into neutral formaldehyde for fixation for subsequent pathological sectioning and staining using lipid dye and Hematoxylin and eosin (HE). The tibia on the same side was harvested, and a 3 mm fragment was cut below the platform and placed into neutral formaldehyde for fixation to prepare HE-stained pathological sections of decalcifying bone. The ink-dyed femur was sawn off at a point that was approximately 1 mm below the femoral head and placed into 95% alcohol for fixation for preparation of abrasive sections of non-decalcifying bone.

Toxicity tests and detection of the largest tolerance dose: The bone-strengthening pill (44g) was placed into 170 mL water, and prepared to the highest concentration of 0.2588 g/mL. After fasting overnight, each mouse was poured with 1 mL of the bone-strengthening pill mixture, 4 times per day, with the total weight 1.0352g of the bone-strengthening pill. The rats were given food and water ad libitum for 7 days and observed every day. On the 8th day, the rats were weighed and euthanized.

Statistical methods

All the data are expressed with ($\bar{x}$ ±s) , with significant differences set at P<0.01. All the statistical processes were treated with SPSS v10.0 statistical software (IBM Corporation, New York, United States).

RESULTS

Detecting bony density of vertebrae

Through serial sectioning, we compared the bone density of the cervical vertebrae. It can be seen from Table 1-1, 1-2, 1-3 that the two models can statistically reduce the bone density of rat spongy bone (P<0.01), as compared with the normal control group. The high-dose bone-strengthening pill also significantly enhanced bone density (P<0.05 or P<0.01), as compared with the model control group, whereas the low-dosage group only showed a mild increase. The compound danshen tablet could not enhance bone density in the rats.

Detecting measurement of bony state

Table 2-1, 2-2, 2-3 shows the result of detecting the unit volume of the bony trabeculae (TBV), the width of the bony trabeculae (MTPT), the unit volume of the bony cortex (CBV), and the osteocyte number within the hollow bone (NOcy). The results show that both rat models can inhibit bony growth. The volume and width of the bony trabeculae, and the volume of the bony cortex were significantly lower than those in the normal control group, and the NOcy also increased statistically (P<0.01). The high-dose bone-strengthening pill can clearly correct inhibition, but the effects of the low-dose bone-strengthening pill and compound danshen tablet were not obvious.

Observations on growth of local bony trabeculae of the femoral head, lipid deposition and vascular distribution

Lipid deposits in the femoral head: It has been discovered that rats poured with prednisone in the model control group have a higher proportion of lipid droplets in the cartilage cells of femoral head. Table 3-1, 3-2 shows that as compared with model control group, the high- and low-dose bone-strengthening pill can obviously reduce lipid deposition, with statistical significance (P<0.01).

Growth of bony trabeculae in the femoral head: As shown in Table 4-1, 4-2, 4-3, TBV, MTPT, MAR and MOSW obviously decline in femoral head of rats poured with prednisone, with statistical difference (P< 0.05 or P<0.01) as compared with normal control group. Judging from tissue section, break, damage and necrosis take place on the surface of femoral head, there is no column arrangement of cartilage cell layer, cells shrink, the nucleus concentrates and necrosis of marrow cells takes place. High dosage of bone-strengthening pill can obviously enhance several indexes (P< 0.05 or P<0.01), make cartilage cells arrange regularly, obviously decrease necrotic cells and make the surface of femoral head smooth without damage. Low dosage of bone-strengthening pill takes effect only on the second batch of rats. Compound danshen tablet has no effect.

Distribution of capillaries in the femoral head:In the normal control group, rat femoral head has abundant capillaries interweaving into a net, the blood vessel has clear boundary, and dense capillaries in cartilage area form "bow-shaped ring". Sparse capillaries in the femoral head of rats poured with prednisone have a few branches, and the density of capillaries in cartilage area decreases. High dosage of bone-strengthening pill can obviously increase capillaries, and compound danshen tablet can also enrich capillaries.

Detection of biochemical indexes in blood

As shown in Table 5-1, 5-2, 5-3, rat TRAP staining in the model control group increases or has a tendency to increase, and ALP noticeably declines or shows a declining trend. These results indicate that the two models can strengthen bony absorption to inhibit bone formation. High-dose bone-strengthening pills can remarkably restore ALP levels and play an active role in promoting bone formation and inhibiting bone absorption. Compound danshen tablet played a certain role in reducing TRAP content, but had no effect on enhancing ALP levels. There were no obvious differences in blood calcium and phosphorus content in the various groups.

Toxicity and tolerance dosage tests

No abnormalities were observed with the largest tolerance dosage of the bone-strengthening pill given to the rats (corresponding to 184.04 times of the adult dosage). The acute toxicity test and chronic toxicity test were also normal. There was also no dysfunction in the blood, liver and kidney test on the relevant biochemical indices. No specific changes were found in the post-study pathological examination.

Table 1-1 Influence of bone-strengthening pill on bony density of cervical vertebrae of castration rats ($\bar{x}$ ±s)

Group	*N*	BMD
Normal control group	9	0.2977±0.02
Modal control group	10	0.2744±0.02*
Compound danshen group	10	0.2659±0.02
Bone-strengthening pill low dosage group	10	0.2780±0.02
Bone-strengthening pill high dosage group	10	0.2937±0.02##

Table 1-2 Influence of bone-strengthening pill on bony density of cervical vertebrae of the first batch rats poured with prednisone acetate ($\bar{x}$ ±s)

Group	*N*	BMD
Normal control group	14	0.2808±0.01
Modal control group	13	0.2625±0.02**
Compound danshen group	13	0.2608±0.02
Bone-strengthening pill low dosage group	12	0.2738±0.03
Bone-strengthening pill high dosage group	12	0.2801±0.01#

Table 1-3 Influence of bone-strengthening pill on bony density of cervical vertebrae of the second batch of rats poured with prednisone acetate ($\bar{x}$ ±s)

Group	*N*	BMD
Normal control group	10	0.2850±0.01
Modal control group	12	0.2637±0.02**
Compound danshen group	12	0.2673±0.02
Bone-strengthening pill low dosage group	12	0.2887±0.03
Bone-strengthening pill high dosage group	10	0.2879±0.02##

Notes: **P<0.01, *P<0.05 as compared with normal control group; ##P<0.01, #P<0.05 as compared with modal control group.

Table 2-1: Influence of bone-strengthening pill on measurement of bony state of castration rats (*n*=5, $\bar{x}$ ±s)

Group	TBV	MTPT	CBV	NOcy
Normal control group	42.87±6.94	113.60±7.83	97.57±0.90	7.23±2.16
Modal control group	21.31±4.11*	67.32±10.58*	93.03±2.44*	10.92±2.68*
Compound danshen group	29.70±10.02#	65.27±9.24	95.11±2.21	7.72±1.87#
Bone-strengthening pill low dosage group	24.89±4.60	64.30±7.50	94.22±2.04	10.07±2.98
Bone-strengthening pill high dosage group	29.02±3.50#	80.47±15.72#	98.31±0.68#	6.14±1.47#

Table 2-2 Influence of bone-strengthening pill on measurement of bony state of the first batch of rats poured with prednisone acetate (*n*=5, $\bar{x}$ ±s)

Group	TBV	MTPT	CBV	NOcy
Normal control group	37.43±0.08	85.41±10.39	96.16±0.02	4.58±1.01
Modal control group	23.49±0.08*	65.08±11.04*	93.76±0.01*	7.53±1.19*
Compound danshen group	23.99±0.09	70.29±14.47	94.22±0.03	6.49±1.14
Bone-strengthening pill low dosage group	23.31±0.03	74.31±6.52	93.55±0.04	4.81±0.76#
Bone-strengthening pill high dosage group	33.67±0.03#	83.74±17.21#	96.49±0.01#	3.68±0.44#

Table 2-3 Influence of bone-strengthening pill on measurement of bony state of the second batch of rats poured with prednisone acetate (*n*=5, $\bar{x}$ ±s)

Group	TBV	MTPT	CBV	NOcy
Normal control group	35.83±7.96	87.12±10.14	94.08±1.67	4.82±1.54
Modal control group	24.78±3.39*	71.56±9.20	93.22±1.92	9.60±1.76*
Compound danshen group	30.92±1.79	73.52±4.87	95.73±2.56	5.67±0.87#
Bone-strengthening pill low dosage group	30.60±4.96	76.17±12.68	96.17±1.04#	6.54±2.24#
Bone-strengthening pill high dosage group	32.96±6.48#	86.83±3.95#	96.07±1.36#	4.70±1.33#

Note: *P<0.01 as compared with normal control group; #P<0.01 as compared with modal control group.

Table 3-1 Influence of bone-strengthening pill on lipid deposit in cartilage cells of femoral head of the first batchof rats poured with prednisone acetate ($\bar{x}$ ±s)

Group	*N*	Rate of cells with lipid deposit (%)
Normal control group	9	4.74±5.11
Modal control group	7	16.64±7.42*
Compound danshen group	8	22.85±11.63
Bone-strengthening pill low dosage group	8	3.80±2.97#
Bone-strengthening pill high dosage group	12	4.63±2.22#

Table 3-2 Influence of bone-strengthening pill on lipid deposit in cartilage cells of femoral head of the second batch of rats poured with prednisone acetate ($\bar{x}$ ±s)

Group	*N*	Rate of cells with lipid deposit (%)
Normal control group	8	7.56±0.98
Modal control group	7	11.08±4.57*
Compound danshen group	10	10.80±4.28
Bone-strengthening pill low dosage group	10	4.84±3.26#
Bone-strengthening pill high dosage group	10	3.94±2.22#

Note: *P<0.01 as compared with normal control group; #P<0.01 as compared with modal control group.

Table 4-1 Influence of bone-strengthening pill on measurement of femoral head of castration rats (*n*=5, $\bar{x}$ ±s)

Group	TBV	MTPT	MAR	MOSW
Normal control group	47.93±0.06	87.76±23.26	—	—
Modal control group	29.47±0.05*	61.10±5.56*	—	—
Compound danshen group	45.44±0.05#	73.98±9.95#	—	—
Bone-strengthening pill low dosage group	38.15±0.08	71.64±14.34	—	—
Bone-strengthening pill high dosage group	45.17±0.05#	78.08±12.75#	—	—

Table 4-2 Influence of bone-strengthening pill on measurement of femoral head of the first batch of rats poured with prednisone acetate (*n*=5, $\bar{x}$ ±s)

Group	TBV	MTPT	MAR	MOSW
Normal control group	61.80±3.38	108.08±6.17	1.26±0.15	1.63±0.11
Modal control group	55.03±5.14*	3.53±12.28*	0.66±0.11*	0.83±0.05*
Compound danshen group	60.58±6.44	98.06±7.32	0.82±0.26	0.74±0.16
Bone-strengthening pill low dosage group	61.34±10.48	100.45±7.56	1.01±0.55	0.92±0.14
Bone-strengthening pill high dosage group	63.73±6.44#	108.20±6.56#	1.06±0.17#	1.44±0.29#

Table 4-3 Influence of bone-strengthening pill on measurement of femoral head of the second batch of rats poured with prednisone acetate (*n*=5, $\bar{x}$ ±s)

Group	TBV	MTPT	MAR	MOSW
Normal control group	60.71±0.04	106.31±6.37	1.08±0.22	1.40±0.06
Modal control group	50.06±0.04*	82.96±10.10*	0.68±0.06*	0.63±0.10*
Compound danshen group	50.21±0.06	96.51±14.19	0.67±0.11	0.66±0.10#
Bone-strengthening pill low dosage group	58.77±0.03#	90.99±3.19	0.98±0.22#	1.23±0.19#
Bone-strengthening pill high dosage group	60.76±0.06#	91.81±8.00	0.85±0.14#	1.61±0.75#

Note: *P<0.01 as compared with normal control group; #P<0.01 as compared with modal control group.

Table 5-1 Influence of bone-strengthening pill on biochemical indices of castration rats blood ($\bar{x}$ ±s)

Group	*N*	TRAP(B-L)	ALP(IU)	Ca(mmol/L)	P(mmol/L)
Normal control group	9	1.84±0.51	3.24±0.73	—	—
Modal control group	9	2.41±0.48*	1.91±1.63*	—	—
Compound danshen group	9	1.44±0.50#	2.64±0.34	—	—
Bone-strengthening pill low dosage group	8	2.87±0.53	4.23±2.37#	—	—
Bone-strengthening pill high dosage group	10	1.94±0.47#	3.21±0.71#	—	—

Table 5-2 Influence of bone-strengthening pill on biochemical indices of blood of the first batch of rats poured with prednisone acetate ($\bar{x}$ ±s)

Group	*N*	TRAP(B-L)	ALP(IU)	Ca(mmol/L)	P(mmol/L)
Normal control group	14	2.25±0.67	4.16±2.96	2.30±0.29	2.16±0.54
Modal control group	12	2.87±1.06*	3.23±1.90*	2.15±0.22	2.04±0.23*
Compound danshen group	13	1.88±0.84#	3.77±1.99	2.11±0.27	2.41±0.46#
Bone-strengthening pill low dosage group	11	2.13±0.59	3.50±1.78	2.03±0.11	1.96±0.50
Bone-strengthening pill high dosage group	13	2.12±0.50#	4.07±1.25#	2.05±0.14	2.12±0.40

Table 5-3 Influence of bone-strengthening pill on biochemical indices of blood of the second batch of rats poured with prednisone acetate ($\bar{x}$ ±s)

Group	*N*	TRAP(B-L)	ALP(IU)	Ca(mmol/L)	P(mmol/L)
Normal control group	10	2.08±0.24	5.13±0.96	2.33±0.19	2.08±0.12
Modal control group	10	2.40±0.33*	4.57±1.44	2.27±0.15	2.38±0.26
Compound danshen group	10	2.48±0.97	3.20±3.32	2.34±0.24	2.29±0.17
Bone-strengthening pill low dosage group	10	2.31±0.35	2.07±2.28	2.21±0.20	2.15±0.38
Bone-strengthening pill high dosage group	8	1.47±0.32#	6.55±1.34#	2.22±0.16	2.04±0.23

Note: *P<0.01 as compared with normal control group; #P<0.01 as compared with modal control group.

DISCUSSION

Ischemic necrosis of the femoral head is caused by damage to the femoral head blood supply and mainly manifests in the death of bony cells, hematopoietic cells and lipid cells. Currently, it is believed that ischemic necrosis of the femoral head is caused by the comprehensive effects of biological factors and mechanical factors[3]. Solomon[4] posits that ischemia is caused by an interruption to or blockage of arterial blood flow, blockage of venous return, and/or blockage of sinusoids outside the blood vessels.

In recent years, there has been an increase in the number of patients with ischemic necrosis of the femoral head, which has led to increased research into its pathogenesis. Numerous reports suggest various causes of the pathogenesis of trauma- and hormone-induced ischemic necrosis of the femoral head. Phemster and others [5] put forward that lipid blockage in blood vessels may cause bony necrosis, which has since been clinically confirmed and accepted[6,7].

Some Chinese scholars[8-10] have used glucocorticoid hormone therapy or surgery to successfully create animal models with necrosis of the femoral head. They have discovered that bony cells in the femoral head and hematopoietic cells in the marrow cavity are damaged to different extents; deformation of bony trabeculae, which becomes sparse and breaks; a decrease or complete disappearance of tetracycline expression at the edge of the bony trabeculae; a decline in the rate of nuclein absorption; the presence of large lipid droplets in bony cells and other damage to cells; a decrease in the number of capillaries in the femoral head and a reduction in blood flow.

In this research, we have identified that the proportion of cells with lipid deposits markedly increases, with a large amount of lipid and lipid emboli along the vascular wall inside the marrow cavity. Bone-strengthening pills can reduce the deposition rate of lipids within the cells and noticeably decreases lipid attachment, thus contributing to the improvement of blood circulation in the femoral head. There was a significant increase in

TBV, MTPT, MAR, CBV and MOSW and a remarkable decrease in NOcy. At the same time, this study showed that a large distribution of capillaries in the femoral head, indicating that bone-strengthening pills can promote bony growth. This conclusion has been further confirmed by the increase in ALP and the decrease in TRAP staining.

Experiments have shown that bone-strengthening pills can 1) enrich distribution of capillaries in the femoral head; 2) decrease lipid deposition in bony cells; 3) improve the biochemical environment related to bony growth; 4) enhance the rate of mineral deposition in bones. Overall, these bone-strengthening pills act in a comprehensive manner to promote bony growth, increase bone density, restore blood circulation to the femoral head, inhibit the occurrence and development of necrosis, and accelerate bone repair in patients with AVNH.

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