Review Article
Total flavonoids of Rhizoma Drynariae play a protective role on steroid-induced avascular necrosis of femoral head by mediating PI3K/Akt signaling pathway

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Abstract: This study aimed to explore the in vitro and in vivo protective mechanism of total flavonoids of Rhizoma Drynariae (TFRD) on steroid-induced avascular necrosis of the femoral head (SANFH) via PI3K/Akt signaling pathway. The isolated human bone marrow microvascular endothelial cells (HBMEC) of femoral head without internal lesion in patients undergoing hip replacement were collected and the SANFH in vitro model was established by hydrocortisone. The effect of HBMEC on serum VEGF and NO levels, serum calcium, blood phosphorus and ALP activities, bone mineral density (BMD) detection, bone trabecular area ratio and empty bone lacuna rate, and PI3K/Akt signaling pathway under TFRD intervention were evaluated. SD rats were purchased and the SANFH in vivo model was established by intramuscular injection of methylprednisolone into the hind legs to evaluate the effect of TFRD intervention on trabecular area ratio, bone lacuna rate and femoral head microvascular quantity. TFRD can significantly promote the cell angiogenesis ability of SANFH in vitro model and activate PI3K/Akt signaling pathway. It also can dramatically increase the trabecular area ratio and the number of femoral head microvessels, and reduce the rate of empty bone lacunae. TFRD plays an in vitro and in vivo protective role on SANFH by mediating PI3K/Akt signaling pathway.

Keywords: Steroid-induced avascular necrosis of the femoral head, total flavonoids of Rhizoma Drynariae, PI3K/Akt signaling pathway, protective effect

Introduction

Osteonecrosis of the femoral head (ONFH) is also called avascular necrosis of the femoral head. Its main pathogenesis is bone ischemia caused by blocked blood supply, which is divided into traumatic and non-traumatic ONFH clinically. A vital cause of non-traumatic ONFH is the widespread use of large doses of glucocorticoids [1, 2]. Steroid-induced avascular necrosis of the femoral head (SANFH) is the main clinical morbidity type, accounting for 57% of the total morbidity due to excessive use of glucocorticoid, which leads to femoral head ischemia and massive death of bone cells [3, 4]. ONFH will cause secondary collapse of femoral head and osteoarthritis as the disease progresses, resulting in unbearable pain and joint rigidity, which will bring great physiological and psychological burden to patients [5].

There are many theories about the pathogenesis of SANFH, among which the theory of abnormal levels of vasoactive factors is gradually accepted. Abnormal levels of vasoactive factors lead to reduced angiogenesis, decreased microcirculation, ischemia and hypoxia in femoral head, apoptosis of osteoblasts and necrosis
of femoral head [6-8]. PI3K/Akt signaling transduction pathway plays a key role in angiogenesis. PI3K/Akt is gradually phosphorylated by activating the kinase system, thus activating pathways to regulate cytokines, such as vascular endothelial growth factor (VEGF) and nitric oxide (NO), which are crucial vasoactive factors in the body and promote angiogenesis [9, 10]. The traditional Chinese medicine, total flavonoids of Rhizoma Drynariae (TFRD), has its advantages in prevention and treatment of osteoarthritis. The medicinal part of Rhizoma Drynariae is the rhizome of the polypodiaceae drynaria fortunei or drynaria baronii, while the TFRD is extracted from its dried rhizome and is the main effective components of Rhizoma Drynariae [11, 12].

Studies have shown that TFRD has pharmacological effects in orthopaedics, such as treatment of osteoporotic fractures [13]. In this study, SANFH rats are taken as research objects to explore TFRD’s effect on SANFH and to assess its in-vivo and in-vitro protection mechanism through PI3K/Akt signaling pathway.

Materials and methods

Experimental animals

Thirty-six healthy male SD rats with a body mass of 350±20 g were used. The rats were housed 2 in each cage, 12 h in day and night with a humidity of 37%-42%, and free to eat and drink water. One-week after adaptive feeding, they were randomized into 3 groups, 12 in each group.

Main reagents and instruments

TFRD (Beijing Qihuang Pharmaceutical Co., Ltd., Z20133051), methylprednisolone sodium succinate for injection (Fuan Pharmaceutical (Group) Co., Ltd., H20183039), VEGF enzyme-linked immunosorbent assay kit (Zhen Shanghai and Shanghai Industrial Co., Ltd., hz-R0020c), chloral hydrate (Qingdao Yulong Algae Co., Ltd., H3702673), β-actin, Akt, p-Akt rabbit anti-mouse polyclonal antibody (Shanghai Hengfei Biotechnology Co., Ltd., M1000170, K005124P, bs-12458R-2), BS-1101 microplate reader (Molecular Devices, SpectraMax iD5) NO detection kit (Shanghai Jingkang Bioengineering Co., Ltd., JK-(a)-2328), electrophoresis apparatus (Anatech (Beijing) Co., Ltd, Wealtec ELITE300), DXA dual-energy x-ray absorptiometry (InAlyzer, Beijing B&E Teksystems Co., Ltd.).

Modeling and grouping

Twenty-four healthy SD rats were selected as the research objects. Gluteal muscle was injected with methylprednisolone sodium succinate injection at a dose of 21 mg/kg, and then injected at 8 am in the morning every day for 3 days to duplicate the rat model of SANFH. Twenty-four model rats of SANFH were randomized into the model group (MG) and the experimental group (EG), 12 in each group. In addition, 12 healthy SD rats were taken as the control group (CG) and injected with 9% sodium chloride solution daily at 8 am, continuously for 3 days. On the first day after successful modeling, rats in the MG were given 6 ml/kg normal saline and those in the EG were given 6 g/L TFRD solution at 35 mg/kg per day, which was equivalent to the adult dose of 0. 75 g/d. Rats in each group were given gastric lavage for 8 weeks, and they were weighed once a week and fed normally.

Outcome measures

(1) Pathological sections of femoral head and bone mineral density (BMD) detection: Eight weeks after administration, pentobarbital sodium was injected intraperitoneally for anesthesia; femoral heads of rats in each group were fixed in paraformaldehyde solution, and then embedded in ethanol gradient dehydration paraffin, sectioned, dewaxed, stained by hematoxylin-eosin (HE), sealed and observed under optical microscope. The pathological morphology of femoral head tissue of rats in each group was observed. BMD of femoral head region of rats in each group was measured by BMD surveying instrument. Trabecular area ratio and empty bone lacuna rate were detected. (2) Detection of serum VEGF and NO levels, blood calcium, blood phosphorus and ALP activity: Eight weeks after administration, pentobarbital sodium was injected intraperitoneally for anesthesia, and abdominal aorta blood was collected and centrifuged. After serum was collected, serum VEGF level and ALP activity were detected by enzyme-linked immunosorbent assay, and serum NO level was tested via chemical method. (3) Detection of Akt and p-Akt protein expression levels in femoral head: Eight weeks after administration, pentobarbital
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sodium was injected intraperitoneally for anesthesia, femoral head was taken, total protein in femoral head was extracted, concentration of total protein was detected, and total protein was separated by electrophoresis. The membrane was transferred, sealed and washed, and β-actin rabbit anti-mouse polyclonal antibody, Akt rabbit anti-mouse polyclonal antibody and p-Akt rabbit anti-mouse polyclonal antibody were added. The membrane was incubated overnight at 4°C and washed, and then it was incubated 2 h with secondary anti-body under normal temperature and cleaned. Subsequently, it was coated with luminescent reagent, exposed, and scanned. Finally, gray value of each strip was measured by ImageJ and analyzed.

(4) Apoptosis detection: paraffin section: after cells were dewaxed and hydrated, the apoptosis of bone cells was observed under microscope based on the manual of TUNEL apoptosis kit. Apoptosis index (AI) was calculated. Calculation formula: AI = number of apoptotic cells/total number of cells × 100%.

Statistical methods

All the data were processed by SPSS22.0, and the measurement data were expressed as x ± sd, and the comparison among groups was analyzed via one-way analysis of variance (ANOVA). P<0.05 was considered statistically significant.

Results

TFRD’s effect on serum Ca and P contents

There were remarkable differences in blood calcium between groups compared by one-way ANOVA (P<0.05); the blood calcium content in the MG was dramatically lower than that in normal rats (i.e. blank group) (P<0.05). There was no marked difference between the blank group (BG) and the EG (P>0.05); the blood calcium in the EG was dramatically higher than that in the MG (P<0.05). There was marked difference of blood phosphorus between the three groups compared by one-way ANOVA (P<0.05); the blood phosphorus in the MG was markedly higher than that in normal group (P<0.05). There was no obvious difference in blood phosphorus between the BG and the EG (P>0.05); the blood phosphorus in the EG was dramatically lower than that in the MG (P<0.05) (Table 1).

TFRD’s effect on ALP activity

The ALP activity of each group was dramatically different from that of other groups compared by one-way ANOVA (P<0.05). Compared with the ALP activity in the BG and the MG, the activity in the MG decreased markedly (P<0.05), suggesting that osteoblast metabolism decreased and bone resorption increased; compared with the EG, the ALP activity in the BG had no marked change and obvious difference (P>0.05), while the activity in the EG was dramatically higher than that in the MG (P<0.05) (Table 2).

TFRD’s effect on area ratio of trabecular bone and empty bone sink rate

The trabecular area of each group was remarkably different from that of the other groups compared by one-way ANOVA (P<0.05). Compared with the BG and the EG, the trabecular area ratio in the MG was at a lower level, with statistically significant difference (P<0.05). There was no obvious difference between the EG and the BG (P>0.05). The rate of empty bone lacuna in each group was remarkably dif-

<table>
<thead>
<tr>
<th>Table 1. Effect of TFRD on serum Ca and P contents (x ± sd)</th>
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<tr>
<td>Group</td>
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<tr>
<td>Blank group</td>
</tr>
<tr>
<td>Model group</td>
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<tr>
<td>Experimental group</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>P</td>
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</table>

Note: through Tukey test, compared with the BG, *P<0.05; compared with MG, *P<0.05.

<table>
<thead>
<tr>
<th>Table 2. Effect of TFRD on ALP activity (x ± sd)</th>
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<tbody>
<tr>
<td>Group</td>
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<tr>
<td>Blank group</td>
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<tr>
<td>Model group</td>
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<tr>
<td>Experimental group</td>
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<td>F</td>
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<td>P</td>
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</table>

Note: through Tukey test, compared with the BG, *P<0.05; compared with MG, *P<0.05.
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Compared with the BG and the EG, the empty bone lacuna rate in the MG was higher, with statistically significant difference (P<0.05). There was no remarkable difference between the EG and the BG (P>0.05) (Figure 1).

Comparison of BMD of femoral head of rats in each group

BMD of rats in the MG was remarkably lower than that in the BG and the EG (P<0.05). There was no marked difference in BMD of femoral head between the EG and the BG (P>0.05), suggesting that it was dramatically lower in the MG than that in the normal rats (BG), and it was increased in the EG to nearly normal level after treatment (Table 3; Figure 2).

Table 3. Comparison of BMD of femoral head of rats in each group (x ± sd)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BMD (g/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank group</td>
<td>12</td>
<td>0.236±0.010</td>
</tr>
<tr>
<td>Model group</td>
<td>12</td>
<td>0.181±0.037</td>
</tr>
<tr>
<td>Experimental group</td>
<td>12</td>
<td>0.228±0.041</td>
</tr>
</tbody>
</table>

F = 10.090, P<0.001

Note: through Tukey test, compared with the BG,
*P<0.05; compared with MG, **P<0.05.

Comparison of VEGF and NO levels in serum of rats in each group

The comparison between serum VEGF and NO in each group was tested by one-way ANOVA,

Figure 1. Comparison of ratio of trabecular area and empty bone lacuna in each group. (A) The trabecular area ratio in each group is significantly different from that in other groups compared by one-way ANOVA (P<0.05). Compared with the blank group and the experimental group, the trabecular area ratio in the model group is at a lower level, and the difference is statistically significant (P<0.05). There is no marked difference between the experimental group and the blank group (P>0.05). The empty bone lacuna rate of each group is compared (B), and the empty bone lacuna rate of each group is dramatically different compared by one-way ANOVA (P<0.05). Compared with the blank group and the experimental group, the empty bone lacuna rate in the model group is higher, and the difference is statistically significant (P<0.05). There is no remarkable difference between the experimental group and the blank group (P>0.05).

Figure 2. BMD of femoral head of rats in each group is significantly lower than that of blank group and experimental group (P<0.05). There is no marked difference in BMD of femoral head between the experimental group and the blank group (P>0.05).
SANFH is a pathological process of bone trabecula and marrow necrosis in the local area of the femoral head caused by the death of active ingredients of the femoral head due to the destruction of the local blood supply of the femoral head as a result of a large amount or long-term application of hormones. It belongs to non-traumatic osteonecrosis of the femoral head and has occupied the first place [14, 15]. At present, along with the widespread or even abuse of hormone and the phenomenon that some diseases must be treated with hormone for a long time, the morbidity of this disease has shown an increasing trend year by year [16]. However, the early detection of this disease is difficult, the late disability rate is high, the course of the disease is long, and the prognosis is poor. Once the disease occurs and is not controlled in time, the quality of life of patients will be seriously affected, so it has attracted more and more attention from the medical community [17, 18].

The animal model of SANFH is mainly rabbit, but the gene similarity between rabbit and human is lower than the gene similarity between rat and human, and the femoral head of rat has similar anatomical structure as human, and its

**Table 4.** Comparison of VEGF and NO levels in serum of rats in each group (x ± sd)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>VEGF (pg/ml)</th>
<th>NO (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank group</td>
<td>12</td>
<td>32.713±4.568</td>
<td>15.791±2.803</td>
</tr>
<tr>
<td>Model group</td>
<td>12</td>
<td>20.834±4.926*</td>
<td>10.591±1.392*</td>
</tr>
<tr>
<td>Experimental group</td>
<td>12</td>
<td>30.237±5.183*</td>
<td>14.363±2.953*</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>19.640</td>
<td>14.030</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: through Tukey test, compared with the BG, *P<0.05; compared with MG, *P<0.05.

**Table 5.** Comparison of Akt and p-Akt protein expression levels in femoral head of rats in each group (x ± sd)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Akt</th>
<th>p-Akt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank group</td>
<td>12</td>
<td>0.472±0.062</td>
<td>0.522±0.033</td>
</tr>
<tr>
<td>Model group</td>
<td>12</td>
<td>0.494±0.095</td>
<td>0.773±0.076*</td>
</tr>
<tr>
<td>Experimental group</td>
<td>12</td>
<td>0.481±0.075</td>
<td>0.831±0.042*</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>0.238</td>
<td>112.500</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.789</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: through Tukey test, compared with the BG, *P<0.05; compared with MG, *P<0.05.

**Figure 3.** There was no obvious difference in Akt protein expression levels between the three groups (P>0.05). The p-Akt protein expression levels in femoral head of rats in the MG and the EG were remarkably higher than those in the BG (P<0.05), and the expression levels in femoral head of rats in the EG were markedly higher than those in the MG (P<0.05) (Table 5; Figure 3).

TFRD's effect on apoptosis of femoral head cells in rats

The results of apoptosis showed that the apoptosis indexes in the MG were markedly higher than those in the BG (P<0.05), and the drug effect in the EG could reduce those indexes dramatically (P<0.05) (Table 6).

Discussion

SANFH is a pathological process of bone trabecula and marrow necrosis in the local area of the femoral head caused by the death of active ingredients of the femoral head due to the destruction of the local blood supply of the femoral head as a result of a large amount or long-term application of hormones. It belongs to non-traumatic osteonecrosis of the femoral head and has occupied the first place [14, 15]. At present, along with the widespread or even abuse of hormone and the phenomenon that some diseases must be treated with hormone for a long time, the morbidity of this disease has shown an increasing trend year by year [16]. However, the early detection of this disease is difficult, the late disability rate is high, the course of the disease is long, and the prognosis is poor. Once the disease occurs and is not controlled in time, the quality of life of patients will be seriously affected, so it has attracted more and more attention from the medical community [17, 18].

The animal model of SANFH is mainly rabbit, but the gene similarity between rabbit and human is lower than the gene similarity between rat and human, and the femoral head of rat has similar anatomical structure as human, and its
biomechanics of femoral head is closer to human [19, 20]. Therefore, rats are used as the model replication objects in this study, and Escherichia coli endotoxin and methylprednisolone are commonly used at home and abroad to replicate SANFH. From the results of BMD measurement of pathological sections of femoral head observed in the general state, the model replication is successful. Pathological sections can accurately observe the pathological state of femoral head and the efficacy of drugs. Pathological examination results are the golden standard for diagnosing various diseases clinically. The morphological structure of femoral head in SANFH patients will be seriously changed, and BMD will also be reduced markedly [21]. BMD of femoral head is a commonly used method for clinical evaluation of femoral head necrosis and efficacy. In this study, BMD of the femoral head of the SANFH rats treated with TFRD has also been effectively improved, which shows that through pathological examination and BMD evaluation, TFRD can improve the anatomical morphology of the necrotic femoral head and has remarkable efficacy on the SANFH model rats.

PI3K/Akt signaling transduction pathway plays a vital role in angiogenesis [22]. PI3K can regulate cell metabolism, proliferation and apoptosis. It is a crucial signaling messenger, and it can participate in activation of Akt-dependent signaling pathway [23]. Akt is the central downstream effect factor of PIP3. PIP3 can gradually phosphorylate AKT protein. Activated Akt can phosphorylate a variety of proteins, and phosphorylated Akt can regulate a variety of active factors such as VEGF and NO, thus exerting its biological effect of mediating cell growth and development and vascular regulation [24]. In this study, the VEGF and NO levels in serum of SANFH rats treated with TFRD increased, which indicated that the therapeutic mechanism of TFRD on SANFH model rats might be to promote angiogenesis, rebuild blood supply and circulation, and advance new bone formation in necrotic parts by increasing the levels of angiogenic active factors (such as VEGF and NO) in serum.

Hip joint is an important joint for human body to bear weight. Dense bone trabeculae is arranged inside femoral head, which can support bone. Changes in the arrangement of bone trabeculae will cause changes in the mechanical properties of cancellous bone. When avascular necrosis of bone cells is caused by poor local blood supply of femoral head, bone trabeculae will be sparse or even fractured, resulting in fracture or local collapse [25, 26]. This experiment revealed that: Compared with the BG and the EG, the area ratio of bone trabecula in the MG was at a lower level, and the differences were statistically significant (P<0.05), suggesting that local bone trabecula decreased after femoral head necrosis; compared with the BG, the area ratio of bone trabecula in the EG and the BG were both at a higher level, and there was no statistical difference between both groups (P>0.05), which indicated that TFRD could improve the area ratio of bone trabecula and prevent and treat SANFH. Bone lacuna is the cell body of bone cells, and those cells are the main cells that maintain the metabolism of mature bone and play a vital role in bone absorption and formation. When the bone cells are irreversibly damaged, it can lead to death, and then there will be empty bone lacunae, making them lose their own functions. The ratio of empty bone lacunae depends on the number of femoral head lacunae and empty bone lacunae. When the activity of osteoblasts decreases, the formation of new bone decreases, while the activity of osteoclasts increases, the necrosis of osteoclasts increases, which leads to an increase in the number and ratio of empty bone lacunae. This experiment displayed that: Compared with the BG and the EG, the empty bone lacuna rate in the MG was at a higher level, and the difference was statistically significant (P<0.05), which indicated that the empty bone lacuna increased obviously after femoral head necrosis; compared with the BG, the empty bone lacuna rate of both groups was at a lower level, and the difference was not statisti-

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Apoptosis indexes</th>
</tr>
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<tbody>
<tr>
<td>Blank group</td>
<td>12</td>
<td>3.621±0.410</td>
</tr>
<tr>
<td>Model group</td>
<td>12</td>
<td>22.464±3.301*</td>
</tr>
<tr>
<td>Experimental group</td>
<td>12</td>
<td>7.276±1.468*#</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>271.900</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 6. Comparison of apoptosis test results of femoral head cells of rats in each group (x±sd)

Note: through Tukey test, compared with the BG, *P<0.05; compared with MG, *P<0.05.
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cally significant (P>0.05), which indicated that TFRD could prevent and treat SANFH by reducing the empty bone lacuna rate.

To sum up, TFRD plays an in vitro and in vivo protective role on SANFH by mediating PI3K/Akt signaling pathway.

Acknowledgements

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Disclosure of conflict of interest

None.

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References


