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Anti-Oxidative Stress and Anti-Apoptosis Effects of He Ying An Xin-Formula

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Abstract

Objective: To investigate anti-oxidative protective effects and potential anti-apoptosis mechanisms of He Ying An Xin-Formula (HYAX-F).

Method: VSMC were incubated by H_2O_2 (200μmol/L) for 2h as oxidative stress control group, same dosage of H_2O_2 was administered 2 hours after treatment with HYAX-F (100μg/mL, 50μg/mL, 25μg /mL) as therapeutic group and incubated for another 24 hours, cell supernant content of GSH and MDA were measured. Sub-cutaneous injection in back with D-galactose (125 mg/kg) to establish ageing rat model. Control group: D-galactose125 mg/kg, therapeutic groups: HYAX-F 500 mg/kg+D-galactose 125 mg/kg, HYAX-F 250 mg/kg+D-galactose 125 mg/kg, HYAX-F 125 mg/kg+D-galactose 125 mg/kg, normal group and young group. The RT-PCR technique was used to measure the expression of relevant genes, such as tumor necrosis factor- alpha (TNF-α) and B-cell lymphoma-2(Bcl-2) in the tissues of brain and liver.

Results: Compared with the control group, HYAX-F can significantly enhance the content of GSH ($P<0.05$) and decrease the content of MDA ($P<0.01$). Also HYAX-F can significantly decrease TNF-α expression and increase Bcl-2 expression in rats brain and liver tissues ($P<0.05$, $P<0.01$).

Conclusion: HYAX-F has greatly protective effects on anti-oxidative of rat VSMC and anti-apoptosis of ageing rats.

Keywords: He Ying An Xin-Formula (HYAX-F); Vascular smooth muscle cells; Anti-oxidative stress; Ageing rats; Anti-apoptosis

Introduction

He Ying An Xin-formula (HYAX-F) originated from "Nan Jing", which is a classical treatment principle to treat cardiovascular diseases. Clinically HYAX-F was used to treatment for chronic heart failure and ischemic heart disease, previous studies have demonstrated that it has the effect of improving cardiac function, slowing down the cardiac muscle remodelling, improving the neuroendocrine and hemodynamics [1], however the anti-oxidative stress and anti-apoptosis effects on ageing rats were not clear. In this study, we studied the effects of the HYAX-F on oxidative stress in rat vascular smooth muscle cells, and its mechanism of the anti-apoptosis effect of ageing rats.

Materials and Method

HYAX-formula extraction

HYAX-Formula was composed of the following herbs: *Cinnamomi ramulus, Paeoniae radix alba,* Poria*, Salviae miltiorrhizae radix et rhizoma, Panacis quinquefolii radix, Polygonati odorati rhizoma*. All herbs (500 g) were extracted with 70% EtOH (3000 ml) for 1.5 h, then collected extractions of three times, evaporating the solvent to get residue 93.5 g (yield of 18.7%). These extracts were stored at 4°C before use.

Cells culture and treatment

Rat vascular smooth muscle cells(VSMC) (Beijing dingguochangsheng Biotechnology Co., Ltd.) were maintained in high-glucose Dulbecco's modified Eagle's medium(Hyclone scientific, USA) supplemented with 10% calf serum(Difco International, Netherlands) at 37°C in a sterile 5% CO_2 incubator. When VSMC cultured to 80%-90% confluent monolayer cells, trysin-EDTA (Difco International, Netherlands) was used to dissociation cells and sub-cultured as a ratio of 1:3. Prior to treatment, VSMC were plated into 48-well plates (Costar, USA) at a density of 6×10^5 cells/ml. VSMC were induced by 200 μmol/L H_2O_2 for 2 hours as control group [2], while HYAX-Formula was treated as different dosages(100 μg/mL, 50 μg/mL, 25 μg/mL) for 24 hours as treatment groups before induced by 200 μmol/L H_2O_2 for 2 hours, then cultural supernatants were collected for GSH and MDA detections.

Measurement of GSH and MDA

VSMCs in 48-well plates were induced as previously described. The amount of supernatant GSH and MDA was determined with the GSH and MDA kits (Nanjing Jiancheng Bioengineering Institute, China).

Animals

The experiment was carried out in 40 ageing rats and 8 youth rats (male, weighing 220-240 g, Vital River Laboratory Animal Technology

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Co. Ltd., Beijing China) acclimated for 1 week before the experiments. All animals were fed with a standard diet and drink ad libitum, and adapted to the experimental conditions at 22 ± 2℃, humidity 60 ± 5% with a fixed 12-h artificial light period. The experimental mice were overseen and all protocols were approved by the Committee of the Animal Use and Care Committee of HeBei North University.

After 1week, 40 ageing rats were divided into 5 groups, except normal ageing rats group (group A), other ageing rats were subcutaneous injected 125 mg/kg D- galactose to induce aged rats model [3], such as control ageing rats group (group B), high dosage of HYAX-F group (500 mg/kg+125 mg/kg D- galactose, group D), medium dosage of HYAX-F group (250 mg/kg+125 mg/kg D-galactose, group E), and low dosage of HYAX-F group (125 mg/kg+125 mg/kg D-galactose, group F), 8 youth rats as normal group (group C). HYAX-F extractions suspended in 5% acacia solution (500 mg/kg, 250 mg/kg and 125 mg/kg) and vehicle (5% acacia solution) were administrated orally to ageing rats. After administration of 42 days, brain and liver were collected under anesthesia and immediately frozen in liquid N_2 and stored at -70℃ until use for Real-time Polymerase Chain Reaction (RT-PCR) analysis and tissue distribution analysis.

RT-PCR for relative genes expression in ageing rat brain and liver

Total RNA was extracted from tissue using TRIzol (Invitrogen life technologies, USA) according to the manufacturer's protocol. Samples (1 μg of RNA) were reverse-transcribed using a first-strand cDNA synthesis kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, USA) according to the manufacturer's instructions. Briefly, the total reaction volume was 20 μL with the reaction incubated as follows in an PE-480 HYBAID (Perkin Elmer, USA): 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, and hold at 4°C.

RT-PCR measurement of RNA expression

Real-time PCR was performed with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, USA) using Power SYBR® Green PCR master mix (Applied Biosystems, USA) according to the protocols provided by the manufacturer. Briefly, PCR was performed in a final volume of 20 μl including 10 ng sample cDNA, 5 μM specific forward and reverse primers, and 10 μl Power SYBR® green PCR Master Mix. PCR reactions consisted of an initial denaturating cycle at 95°C for 10 min, followed by 40 amplification cycles: 15 s at 95°C and 1 min at 60°C. The primers used were as Table 1. Results were presented as levels of expression relative to those of controls after normalization to GADPH using the $2^{-\Delta\Delta CT}$ methods. Analysis was carried out in triplicates.

Statistical analysis

Values are expressed as mean ± S.D. All the grouped data were statistically performed with SPSS 11.0. Significant differences between means were evaluated by one-way analysis of variance (ANOVA) and Tukey's Studentized range tests were used for post hoc evaluations. $P<0.05$ was considered to indicate statistical significance.

Results

Effects of HYAX-F on secretion of GSH and MDA of VSMCs in supernatant

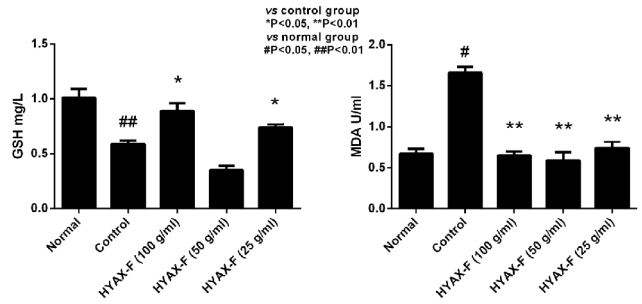
VSMCs were treated with the dosage of 100 mg/ml and 25 mg/ml of HYAX-F can promote GSH content in supernatant compared with control group ($P<0.05$). Moreover, each dosage of HYAX-F can significantly decrease MDA content in supernatant compared with control group ($P<0.01$). In control group, cells were merely induced by H_2O_2 to apoptosis, showed lower GSH content and higher MDA content in supernatant compared with normal group (Figure 1).

Effects of HYAX-F on expression of TNF-α and Bcl-2 genes in brain and liver of ageing rats

HYAX-F can significantly down-regulate TNF- **α** expression in brain and liver of ageing rats compared with control group($P<0.05$, $P<0.01$). High-dosage and low-dosage of HYAX-F can significantly up-regulate Bcl-2 expression in brain and liver of ageing rats compared with control group as well ($P<0.05$, $P<0.01$) (Figures 2 and 3).

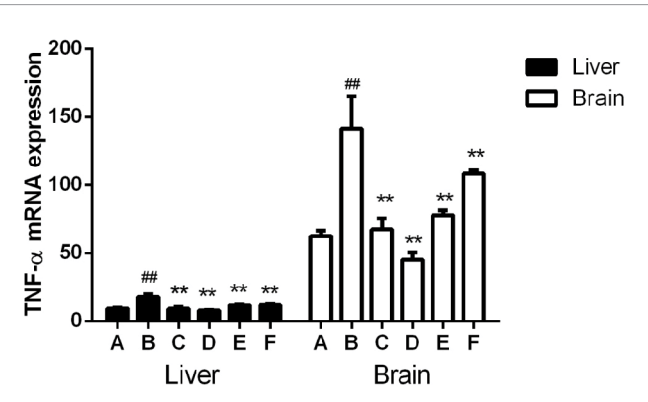
Discussion

Vascular ageing has been implicated in the progression of age-related cardiovascular disorders. Epidemiological discover ageing is associated with an increased prevalence of cardiovascular disease, vascular smooth muscle cells (VSMC) comprise the major arterial cell population, and changes in VSMC contribute to alterations in vascular remodelling and cell signalling. Cellular senescence is a permanent



Compare with control group*<0.05, **<0.01
Compare with normal group #<0.05, ##<0.01

Figure 1: Effects of HYAX-F on secretion of GSH and MDA of VSMCs in supernatant ($\bar{x} \pm s$, n=6)

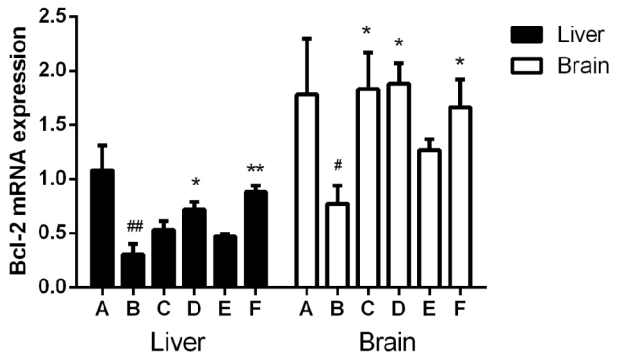


Compare with control group*<0.05, **<0.01
Compare with normal group #<0.05, ##<0.01
Group A=normal ageing rats group
Group B=control ageing rats group
Group C=youth rats group
Group D=high dosage of HYAX-F group (500 mg/kg+125 mg/kg D- galactose)
Group E=medium dosage of HYAX-F group (250 mg/kg+125 mg/kg D-galactose)
Group F=low dosage of HYAX-F group (125 mg/kg+125 mg/kg D- galactose)

Figure 2: Effects of HYAX-F on expression of TNF-α genes in liver and brain of ageing rats($\bar{x} \pm s$, n=6).

Gene	Forward	Reverse
TNF-α	5'-TGGCGTGTTCATCCGTTCT-3'	5'-CTGAGCATCGTAGTTGTTGGAA-3
Bcl-2	5'-ATCGCTCTGTGGATGACTGA-3	5'-GTCTGCTGACCTCACTTGTG-3
GAPDH	5'-ACAGCAACAGGGTGGTGGAC-3	5'-TTTGAGGGTGCAGCGAACTT-3

Table 1: Gene-specific primers used for RT-PCR.



Compare with control group*<0.05, **<0.01
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Compare with normal group #<0.05, ##<0.01
Group A=normal ageing rats group
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Group D=high dosage of HYAX-F group (500 mg/kg+125 mg/kg D- galactose)
Group E=medium dosage of HYAX-F group (250 mg/kg+125 mg/kg D-galactose)
Group F=low dosage of HYAX-F group (125 mg/kg+125 mg/kg D- galactose)
Figure 3: Effects of HYAX-F on expression of Bcl-2 genes in liver and brain of ageing rats($\bar{x} \pm s$, n=6).

non-replicating state characterized by growth arrest, increased oxidative stress, telomere and mitochondrial dysfunctions. In cultured cells, critical DNA damage triggered by a variety of chemical agents and stresses including oxidative stress and activation of oncogenes induces rapid "stress-induced premature senescence". VSMC senescence in atherosclerotic plaques is a characteristic feature of atherosclerosis and is associated with increased levels of reactive oxygen species (ROS). ROS increases intracellular (DNA) damage and ultimately can elicit the onset of apoptosis or the induction of cellular senescence. H_2O_2 can stimulates NAD(P)H oxidase to generate O_2^-, which is the main source of ROS generated in VSMCs. Enhanced production of ROS and insufficient removal by scavenging systems are hallmarks of vascular ageing [2,4,5]. The excessive ROS lead to the dysfunction of cell and body, even induce apoptosis.

In this study the changes in oxidative stress biomarkers were detected, such as GSH and MDA. GSH is an important endogenous antioxidant enzyme in organism, which plays an important role in cellular protection against oxidative damage. The reduction of its vitality will result in a certain degree of decline in antioxidant capacity of the body, which will lead to the accumulation of free radicals, and eventually lead to a certain degree of oxidative damage. MDA is the organic compound with the formula $CH_2(CHO)_2$, its reactive species occurs naturally and is a marker for oxidative stress, which is used as a biomarker to measure the level of oxidative stress in an organism. In our experiment, the oxidative stress injury model of VSMC was simulated by H_2O_2 (concentration 200 μmol/L). Results showed that compared with control group, HYAX-F (100 μg/ml and 25 μg/ml) could promote GSH synthesis of VSMC for nearly 1.5-fold and 1.3-fold respectively, indicated HYAX-F anti-oxidative damage partly depend on increase the level of GSH. Whereas compared with VSMCs treated with H_2O_2, the levels of MDA were significantly lower in VSMCs which was incubated with HYAX-F at each concentration (2.2-fold to 2.8-fold). HYAX-F showed magnificently oxidation resistance in H_2O_2 caused oxidative stress vascular smooth muscle cells.

Along with oxidative stress, apoptosis is believed to be intimately involved in the progression of ageing. Ageing is a prominent risk factor for cardiovascular disease, with age the blood vessel wall broadens and develops a thickened intima consisting of infiltrating vascular smooth muscle cells(VSMCs) and resulting in local inflammation [6,7]. Ageing increases oxidative stress and inflammation [8]. Apoptosis is regulated by apoptosis modulating proteins, which are divided into two major categories: pro-apoptotic proteins and anti-apoptotic proteins. Apoptosis is the result of the loss of balance between these two kinds of proteins. TNF-α is known to be one of the cytokines that can induce apoptosis by its receptor pathway, which can initiate the cascade reaction related to apoptosis and nerve injury. TNF-α transfer into intracellular by combine with its receptor, uptake by target cell lysosomal resulted in reduced lysosomal stability and leakage of enzyme, causing cell lysis, it can also change metabolism of glucose in target cells, causes a decrease in intracellular pH, leading to cell death [9,10]. Bcl-2 plays an important role in the regulation of apoptosis as well. Except grow factors caused apoptosis, ROS is the main cause of cell death, while Bcl-2 acts to inhibit apoptosis by suppressing ROS production [11]. In this study, the gene expression of TNF-α and Bcl-2 were used to evaluate the anti-apoptosis effect of HYAX-F. Results showed that compared with D-galactose induced ageing senescent rat, gene expression of TNF-α both in liver and brain magnificently down-regulated. Whereas HYAX-F (500 mg/kg and 125 mg/kg) could significantly up-regulate Bcl-2 gene expression both in liver and brain.

Conclusion

HYAX-F can resist H_2O_2 caused VSMC from oxidative stress damage, and regulate gene expression of TNF-α and Bcl-2 in ageing senescent rat liver and brain to anti-apoptosis. We also partly confirmed the mechanism of HYAX-F regulated cardiovascular disease by anti-oxidative stress and anti-apoptosis.

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