The clinical research for Ganoderan's effect on preventing and treating cerebral arteriosclerosis through inhibiting NADPH oxidizing enzyme expression

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Abstract: A lot of researches have verified that produced excessive reactive oxygen is one of the hazard factors causing atherosclerosis. NADPH oxidase is the main protease of vascular cell's producing reactive oxygen, the expression of its relevant subunits is closely correlated with the occurring and development process of atherosclerosis. Oxidizing reaction could damage organism tissue cells, ganoderan has very significant effect on the anti-oxidizing function of cell. The pharmaceutical research of ganoderan has significant meaning in curing diabetes mellitus, preventing and controlling arteriosclerosis. This paper is mainly to discuss the effect of anoderan's inhibiting NADPH oxidizing enzyme expression on preventing and treating cerebral arteriosclerosis and its action mechanism.

Keywords: Ganoderan, NADPH oxidizing enzyme ,arteriosclerosis.

INTRODUCTION

In recent years, the morbidity of cerebral arteriosclerosis has been increased gradually year by year. And it is a big killer nowadays. Coronary atherosclerosis does not develop from people's agedness, but emerges gradually with the increase of age. Most patients do not necessarily have clinical symptoms, which would be ignored easily. Serious patients could have symptoms of severe stroke; myocardial infarction which would be the threat to life. How to prevent and treat cerebral arteriosclerosis is a hard problem facing us (Fengling *et al*, 2012). Although the exact pathogenesis of AS (atherosclerosis) has not yet been clear so far, a lot of research have verified that NADHP oxidizing enzyme is closely correlated with the severity degree of AS disease. It is maybe due to that under some factor condition, the activity of NADPH oxidizing enzyme increases, ROS is excessively produced, then oxidative damage is caused and influences the diastolic function of blood vessel endothelium, thus arteriosclerosis is rendered. As a result, this paper is to find their correlation through aiming at studying the effect of ganoderan's inhibiting NADPH oxidizing enzyme expression on preventing and treating cerebral arteriosclerosis.

Ganoderma lucidum polysaccharide is one of the most effective ingredients in ganoderma lucidum. Thus it has drawn particular attention of medical science and technology workers, the related research and report are the most. It is already known that ganoderan has extensive pharmacological activity, which could enhance immunity of the organism, hypoxia ability of organism and eliminate free radicals (Zhijun *et al*, 2012), inhibit tumour, resist radiation, enhance the liver, bone marrow and blood's ability of compounding DNA, RNA and protein, as well as prolonging life-span. Ganoderan could also have the characteristics of stimulating the non-specific resistance, specific immune response of host, and inhibiting the biological activities of transplanted tumor. When molecular weight of polysaccharides is bigger than 1×104, it indicates strong acticity of inhibiting tumour, its active strength is also related with the bifurcation extent of polysaccharide chain and the number of hydroxide radical on branch. It not only has significant effect on CVD (cardiovascular disease), asthma, irritability, neurasthenia, stomach heat and so on, but also have function of reducing blood press and blood fat,solving blood stasis, improving blood circulation, beautifying skin (Xi *et al*, 2011) etc. Many kinds of pharmacological activities in lucid ganoderma are mainly related with ganoderan.

Oxidizing reaction could harm the tissues and cells of organism, and then chronic diseases like cancer, diabetes etc and aging effect would emerge. Thus searching effective antioxygenation is very significant. (Lijuan *et al*, 2010) have found from research that ganoderan could reduce the oxidative damage of tert-butyl hydroperoxide on human umbilical vein endothelial cell (HUVECs): CCK-8, Hoechst33258, colorimetry test and electron microscopic observations have represented that the survival rate of HUVECs in medication administration team of ganoderan is smaller than it in model set. (Xiao *et al*, 2009) have found from research that dose dependence of ganoderan could enhance the level of hydroxyproline and SOD. It indicates that ganoderan extraction could effectively relieve skin aging; there are other scholars studying the influence of ganoderan on serum antioxidant enzymes in rats with cervical cancer and immunoreaction.

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They found that ganoderan could significantly reduce DPPH free radical, oxygen free radical and hydroxyl free radicals; it suggests that ganoderan could effectively enhance the scavenging activity of above free radical. The level of antioxidant enzymes' quantity and insulin in treatment group of patients with diabetes has increased significantly, the effect of lipid peroxidation and sugar content of blood has decreased significantly, all those demonstrate that ganoderan is effective antioxidant. (Haitao, 2012) have found from research that ganoderan is able to effectively reduce the oxidative damage of diabetic rats'pancreas and inhibit apoptosis through enhancing the activity of antioxidant enzyme, regulating bcl-2 expression and bax/bel-2 rate.

MATERIALS AND METHODS

Materials and reagent

CLP, cholesterol, sodium cholate, propylthiouracil, vitamin D_3, malondialdehyde (MDA), superoxide dismutase (SOD) test box, viable tissue's oxidative stress reactive oxygen species (ROS) primary fluorometric assay kit, immune antibody rats Nox4 CPV McAb, immune antibody rats p22phox polyclonal antibody.

40 healthy male rats are needed rat's weight is 180-200g. They are divided into five groups randomly: normal control group (CON), AS model group (AS), CLP prevention group (CLP_L, CLP_M, CLP_H) with low, medium and high CLP dose. In addition to normal group, the other four groups are given intraperitoneal injection of vitamin D_3 with 40mg·kg^{-1} dosage, high-fat diet (81.3% basal feed, 10% lard oil, 3%cholesterol, 0.5% sodium cholate, 5% white sugar, 0.2% propylthiouracil) will be fed to them. Prevention group are given gavage with CLP125, 250, 500mg·kg^{-1} every day, both normal group and model group are given the same amount of saline solution.

Method

12h abrosia is implemented on rats after 12 weeks, which will be of abdominal cavity anesthesia with ulla sugar in 20% concentration. Their blood is extracted through carotid artery intubation, rat's serum is segregated at 2000r·min-1. Test box is used to measure MDA, SOD content in serum of each group. Aorta is given HE dyeing, main artery's residual blood is washed with normal saline, and then it is fixed with 4% para formaldehyde solution. Ethanol dehydration is performed on them step-by-step, the embedding slice is applied with paraffin, the condition of aortic intima is observed with HE dyeing.

Blood vessel is taken out; its tissue is cut up by blade. Ganmed viable tissue's oxidative stressreactive oxygen species (ROS) primary fluorometric assay kit is used, fluorescence microplate is applied for detection at 490nm excitation wavelength and 520nm emitting wavelength.

Aorta is taken at the crotch of arcus aortae and aorta abdominalis, protein is extracted on tissue's cracking liquid ice which contains protease inhibitor. Quantitative protein is tested with BCA method. 8% SDS-PAGE gel electrophoresis is used to isolate protein, electric current 100·944· Chinese Pharmacological Bulletin 2012 Jul; 28 (7): membrane transferring is applied at 944 ~ 7V; 300 mA for 150 min. Then it is sealed with TBST containing 5% skim milk powder for 1 h. Nox4 and p22phox primary antibodies are added into it at 4□ overnight. d 2 is added with corresponding second antibody for 1h reaction at room temperature, then the membrane sweeping is given and gray level analysis is performed with image J. The data is analyzed and processed with statistical software, calculating data is denoted with x±s, interblock is analyzed through adopting single factor variance, pairwise comparison is performed through adopting SNK test (Feng *et al*, 2012).

RESULT

Compared with CON group, MDA, SOD in AS group increases significantly ($P<0.01$), MDA, SOD in medication administration team decreases significantly when compared with AS group ($P<0.01$), as it is shown in table 1.

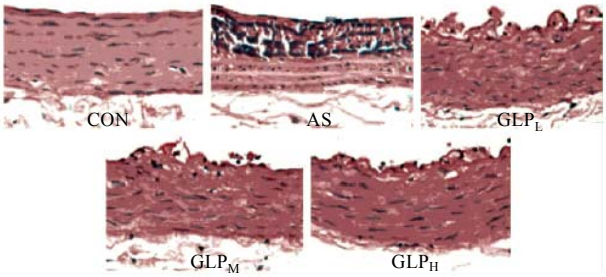

Fig. 1: HE staining of aorta (× 400)

The change of rat's atherosclerotic plaque is shown as fig 1. In normal control group, rat's thoracic and abdominal aorta wall has complete structure and distinctive gradation inner membrance is continuous and smooth, smooth muscle in middle layer is even without shrinking. In model group of atherosclerosis, endothelium of blood vessel wall falls off, inner membrance is thicker, the inner subcutaneous clearance is wider, the inner elastic plate is fractured, there are a lot of foam cells, smooth muscle cells and macrophages in plaque (Zhongjuan *et al*, 2012), fiber texture have hyperplasia with patchy calcification, and the smooth muscle of mesolamella has obvious atrophy. In GLP prevention group, endothelial cells fall off partially, its structure is complete, there is a little lipid deposition, rare fracture emerges on inner elastic plate.

The change of ROS content in aorta is shown as fig 2, ROS content of rat's aorta in AS group increases

obviously. When compared with AS group, ROS content in aorta of each CLP medication administration group decreases significantly (P<0.01).

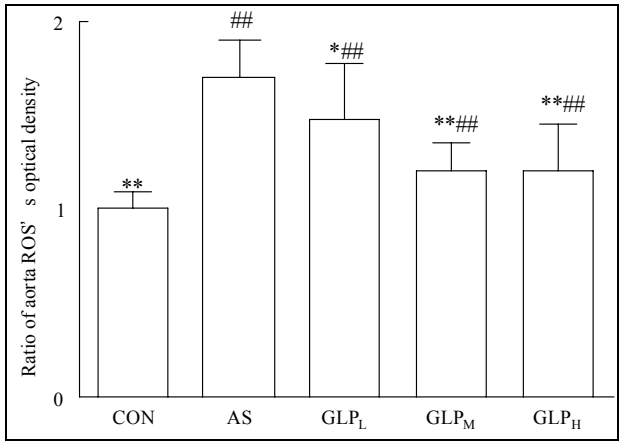

Fig. 2: ROS content in aorta (x±s,n=8)

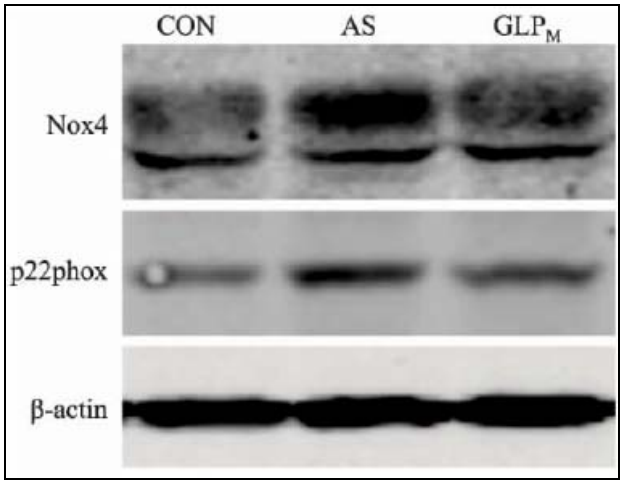

Fig. 3: western blot results

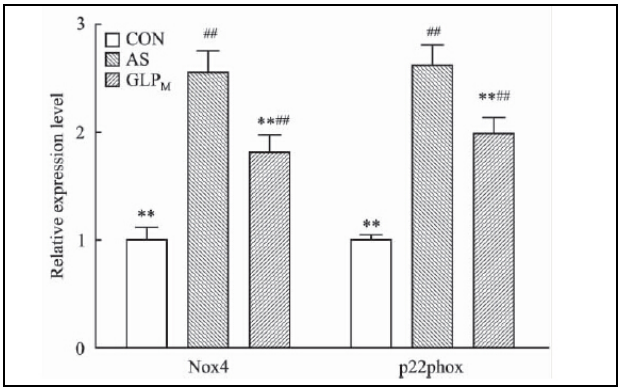

Fig. 4: the expression change of Nox4 and p22phox (x±s. n=3) * * P < 0. 01 vs AS; ##P < 0. 01 vs CON

The change of Nox4 and p22phox expression in aorta is shown as immunization print results in fig. 3, the amount of Nox4 and p22phox protein expression in AS group is higher than that in CON group (P<0.01), the amount of protein expression after GLP intervention decreased obviously (P<0.01).

Table 1: comparison of MDA, SOD levels of serum in rats of each group after 12 weeks'administration (x ± s, n =8)

Group	MDA/umol·L^{-1}	SOD/NU·ml^{-1}
CON	6.2±1.0$^{\#\#}$	160.23±32.58$^{\#\#}$
AS	10.3±2.1*	206.01±39.34**
CLP_L	8.7±0.9* $^{\#\#}$	178.04±15.23* $^{\#\#}$
CLP_M	7.1±1.2* $^{\#\#}$	170.85±23.07* $^{\#\#}$
CLP_H	7.3±1.6* $^{\#\#}$	174.57±33.78* $^{\#\#}$

*P<0.05, * *P<0.01vsCON; $^{\#\#}$P<0.01vsAS

DISCUSSION

We all always think that oxygen is good for human beings, but this kind of thought has been overturned after the SOD (superoxide dismutase) of superoxide anion radical is clearly discovered, some generation product of oxygen and its derivative metabolites are all discovered to be harmful to organism. In the metabolic process of vital movement, all kinds of free radicals will be generated constantly, superoxide anion free radical and hydroxyl radical are two kinds of most representative free radical, as well as its active derivatives, such as H2O2, RO·, RO2 and ROOH (Nie *et al*, 2013). Superoxide anion free radical not only has important biological function, but also has close relationship with many diseases, and it is the first free radical of all oxygen radical, then generates other free radicals after a series of reaction, thus it has especially important significance.

Ganoderan could clean up a variety of reactive oxygen species (ROS) generated from physical, chemical and biology source, reduce the production of lipid peroxide malondialdehyde (MDA), increase (superoxide dismutase) (SOD), glutathione peroxidase activity and so on, as well as playing an important role (Jianguo, 2010) in pharmacology of Chinese medicine. The mechanism of its action has following several possible explanation: (1) ROS is directly removed. Polysaccharide could capture the ROS generated from the reaction of lipid peroxidation chain, reduce the length of lipid peroxidation chain, thus could block or slow down the proceeding of lipid peroxidation.In view of OH·, it could rapidly merge with captured hydrogen atom on polysaccharide hydrocarbon chain into water, while the carbon atom of polysaccharide would leave behind a single electron and become carbon free radical, then form peroxy radical after further oxidation, finally resolve into the product with no harm to organism; for O_2, polysaccharide could make oxidation reaction with it for the purpose of elimination; for singlet oxygen, it could transmit excitation energy to polysaccharide, which would be in excited state, while itself returns to ground state.(2) Metal ion needed by ROS is generated through complexation. OH on polysaccharide

ring could generate the metal ion, which is necessary to OH· etc. OH on polysaccharide ring could perform complexing with the required metal ion (such as Fe^{2+}, Cu^{2+} etc) which could generate OH· etc, and make it could not generate the hydroxyl radical which starts the lipid peroxidation or decompose the lipid peroxide generated from lipid peroxidation, thus inhibit the generation of ROS. (3) SOD is promoted to be released from the surface of cell. Polysaccharide could play its role of clearing up ROS through promoting SOD to be released from the surface of cell, and then prevent the chain reaction caused by free radical, as well as the precaution of senility. (4) The activity of antioxidant enzymes is improved. Polysaccharide could play its role of antioxygenation (Feng, 2010) through enhancing the activity of enzyme such as SOD, CAT, CSH-P_x and so on.

Such kind of experimental AS animal model is usually applied for modeling early lesion of AS, which is first represented in aorta. Oxidative stress indicators' MDA level in serum of AS group is significantly higher than that in CON group, MDA is the lipid peroxide generated by ROS' attacking polyunsaturated fatty acids of cytomembrane. It will indirectly reflect the production of ROS and its degree of damage to tissue.The test is performed through combing with aorta ROS kit, and shows that there maybe exists the decrease of antioxidant defense mechanism and increase of generating ROS *in vivo* under AS condition in collective. The pharmacological study of ganoderan verifies that it has a variety of biological activity and pharmacologic action, except for main regulation of cotton-padded clothes and anti-tumor effect. CLP could reduce hypoxic injury in primary cortical neurons through inhibiting oxidant stress Ganoderma lucidum polysaccharide peptide has the effect of anti-oxidative damage on human umbilical vein endothelial cells. The above experiments have confirmed that CLP has the function of slowing down oxidative stress in rats and could repress the development process of aortic sclerosis (Zhang *et al*, 2013).

At present, SOD is thought to be the natural antioxidant enzymes, which could effectively clear up ROS *in vivo*. The experiment demonstrates that the content of SOD serum in AS model group has increased significantly when compared with normal group. It could be explained as that the synthesis secretion of antioxidant enzyme after increasing ROS during the course of disease would get compensatory increase within certain schedule, while the experiment suggests that this compensatory mechanism is unable to eliminate too much ROS accumulated *in vivo*. CLP is able to effectively reduce the ROS level in aorta and MDA value of serum, it represents that CLP maybe prevent atherosclerosis progression through reducing the generating process of ROS.

At present, NADPH oxidizing enzyme is considered to be the main peroxisomes which generates ROS in blood vessel. NADPH oxidizing enzyme is involved in the whole process of AS occurring and development. NADPH oxidizing enzyme contains 5 subfractions: Cytochrome of cell membrane components b558 (gp091phox and p22phox) and cytoplasmic components p47phox, p67phox as well as tiny CTP binding protein rac etc, where p22phox is of high expression in various cell. In recent years, as least two kinds of new gp91phox isozyme family, namely Nox3, Nox4, Nox5 and other member are discovered. In vascular cell, endothelial cells and smooth muscle cells have two main expression, namely Nox1 and Nox4.Yang *et al* (Yang *et al*, 2010) etc have discovered in atherosclerotic body that the shoulder part of coronary plaque has a lot of peroxide sedimentary, meanwhile the combination body of elevated p22phox and Nox2 is mainly located in the macrophage of plaque.Nox4 is positioned on the main source of ROS in blood vessel, and NADPH oxidase activity in vascular smooth muscle mainly depends on Nox4 expression. The differentiation phenotype of vascular smooth muscle plays a critical role in the pathological process of atherosclerosis. In Nox family like Clempus *et al*, only Nox4 is thought to play a decisive role in the atomization process of vascular smooth muscle. Our experiments have represented that CLP pharmacological intervention could effectively lower the p22phox and Nox4 expression of protein in aorta. This maybe mainly due to that CLP is able to lower the ROS level of AS rats' aorta and control atherosclerosis. The detailed mechanism needs to be further studied.

CONCLUSION

Atherosclerosis has already become the main problem which poses threat to human health. How to prevent and control atherosclerosis is one of the challenges facing human beings. Ganoderan could effectively inhibit the pathological process of atherosclerosis, and lower the oxidative stress indicators of MDA, SOD content in serum and ROS content in aorta etc, then reduce the Nox4, p22phox protein expression of artery. Through lowering the expression level of NADPH oxidase protein such as Nox4, p22phox etc, aortic oxidative stress reaction will be inhibited and atherosclerosis will also be improved. Ganoderan has precious medicinal value; further study about its antioxidant effect etc is very beneficial for curing and preventing diseases. At present, the research for antioxidant polysaccharide is still in initial stage, and there exists many problem to be solved. Firstly, the function mechanism of polysaccharide antioxidant is still not clear; secondly the best dosage and access to antioxidant polysaccharide need to be researched.

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