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Attenuation of oxidative stress of erythrocytes by the plant-derived flavonoids vitexin and apigenin

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Vitexin belongs to the flavonoid carbon glycosides, apigenin is its aglycone. Studies have shown that both of them have antiviral, antioxidant and anticancer effects *in vivo* and *in vitro,* but the protective effects on human erythrocytes have not been reported. We studied and compared the protective effects of vitexin and apigenin against H_2O_2-induced oxidative damage in human erythrocytes and explored the structure-activity relationships. The experiment established the H_2O_2-induced oxidative damage model, with vitamin C (VC) as a positive comparison, to observe the changes of sulfhydryl content in the red cell membrane protein, erythrocyte morphology and membrane skeleton ultrastructure after oxidative damage. Research results showed that vitexin and apigenin could significantly reduce the degree of hemolysis, lower MDA content, and enhance the activities of erythrocyte antioxidant enzymes. In addition, vitexin and apigenin could restore cell surface morphology through protecting the sulfhydryl content in the red cell membrane protein. Among them, antioxidant capacities of apigenin at doses of 15 and 30 μg/ml were better than that of the same doses of vitexin, while effects of 60 μg/ml vitexin and 30 μg/ml apigenin were the same as 30 μg/ml VC. In conclusion, both vitexin and apigenin have protective effects against H_2O_2-induced oxidative damage of erythrocytes, which might be achieved through directing subdue oxygen free radical and protecting the antioxidant enzyme activity in cells and the sulfhydryl in the red cell membrane protein.

1. Introduction

Among studies on aging in modern medicine, the most influential theory on free radical damage was offered by Harman (1956). In 1968, McCord and Fridovich revealed the antioxidant effect of superoxide dismutases (SOD), created a new chapter of the free radical of biology (Fridovich 1922). In the process of research, the adverse effects and the cytotoxicity of free radicals on the body have attracted more and more attention. Oxygen free radicals in the human body are mainly free radicals, oxygen free radicals of high reactivity, many diseases, the aging of life (especially the diseases of aging) involve active oxygen free radical reaction (Wang Fuhai and Huang Chenghua 2013). A certain amount of oxygen free radicals is essential, but excessive accumulation will cause a series of biological responses, induces heart disease, cancer, atherosclerosis and other diseases (Beckman and Ames 1997; Kautmann et al. 2002; Li Xingtai and Ji Ying 2015; Goto and Radak 2013).

Erythrocytes could carry oxygen, scavenge free radicals, maintain the balance of blood flow and electrolyte, and other functions. Because erythrocytes often get contact with high pressure oxygen and free radicals, as their membranes are rich in unsaturated fats and iron molecules, which are powerful catalysts of free radical reactions, erythrocytes are considered to be one of the most vulnerable cells against oxygen free radicals. When erythrocytes are attacked by free radicals, polyunsaturated fatty acids of the cell membrane become peroxides, membrane proteins become broken and allosteric, intracellular enzyme activities are lost or reduced, the stability and stretch of the membrane skeleton network-like structure becomes lower, eventually leading to pathological changes of red blood cell morphology and function. Due to the particularity of erythrocytes, they became the best model for studying antioxidative pharmacology drug activity (Arbos et al. 2008).

Modern research has shown that flavonoid compound could repair endothelial cell damage induced by H_2O_2 through improving the activities of enzymes in endothelial cells and reducing the generation of free radicals (Zhang et al 2009; Cao Zhichao et al. 2013; Zhang Bo 2010; Zhejiang University 2010; Shi Tingting 2011).

Vitexin, belonging to the family of flavonoid glycosides, has been shown to confer resistance to viruses, oxidants and cancer (Zhang Xue and Xu Daohua 2013; Gu Chengbo et al. 2015; Yang Guodong 2011). Protective effects have also been demonstrated in ischemic and anoxic myocardial models, the generation of these effects may be implemented by inhibiting thrombosis, reducing ejection resistance and blood viscosity, improving blood flow and making erythrocyte morphology transformed (Zhu Lixia et al. 2014; Li et al. 2006; Qu Haiqi 2015; Prabhakar et al. 1981; Wang Jiannong et al. 2004). The experiments showed that vitexin could remove ·OH, O_2 and $^{-}$DPPH *in vitro* and has a protective effect on erythrocyte membranes (Yan Juan et al. 2010), but the mechanism has not been fully elucidated. Meanwhile, *in vivo* experiments showed that vitexin could slow

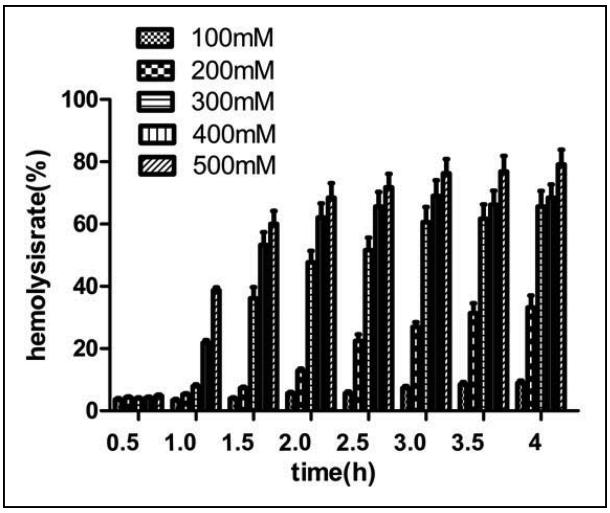


Fig. 1: Hemolysis of human erythrocytes. Figure 1 shows hemolysis rate of human erythrocytes induced by different concentrations of H_2O_2 at different time intervals. Data are presented as mean ± SD from six independent experiments.

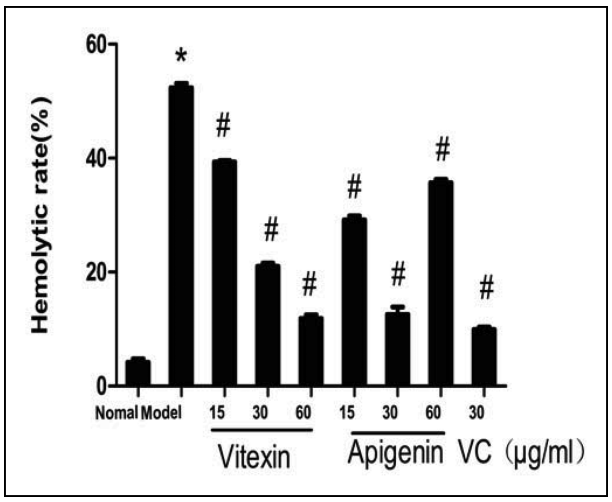


Fig. 2: Both vitexin and apigenin attenuated hemolysis rate of erythrocytes. Oxidative stress induced a significant increase in hemolysis rate of erythrocytes. Data are presented as mean ± SD from 10 individual experiments. * represents $p < 0.05$ compared to normal control and # indicates $p < 0.05$ compared to either oxidative stress erythrocytes.

D-galactose aging mice. The effects may be implemented by improving the abilities of antioxidant enzymes in the serum and organization, enhancing the ion transport activities of the cell membrane, reducing the contents of lipofuscin in brain tissue and improving morphology of cells abnormal to aging, etc. (Zhao Jun et al. 2013).

Apigenin, which has a chemical structure similar to vitexin, studies have shown that apigenin has antioxidant effects *in vivo* (Li Weilin 2014), effective in the prevention and treatment of a wide variety of tumors (Liu bin and Ba Yi 2013; Liu Jiaqi and Sun Yunyun 2012). For instance, Apigenin can significantly inhibit the growth of HeLa cervical cancer (Sui Haixia et al. 2011). In addition, apigenin plays a significant role in vasorelaxation, and suppresses the voltage dependent calcium channel, receptor maneuvering calcium channel, and inhibition of the inflow of extracellular calcium and potassium channel activation (An Fang et al. 2012).

At present, most of the studies of the protective effects of flavonoid compounds on erythrocytes were focused on the drug carrier (An Fang et al. 2012; XiYun et al. 2014) and protein components of the erythrocyte membrane (Cao Yuan 2008; Jiang Weihua 2005). Few research was focused on the integrity of the biological activity, and many studies used animal erythrocytes which are different from humans. Our research used the H_2O_2-induced oxidative damage model based on VC to compare the protective effects and mechanisms of vitexin and apigenin on erythrocytes, and provided a structure-activity basis for the selection and synthesis of anti-aging drugs.

2. Investigations and results

2.1. Establishment of the oxidative injury model

As shown in Fig. 1, H_2O_2 significantly induced oxidative stress in erythrocytes in a dose and time dependent manner. Over 4 h, there was no hemolysis in erythrocytes without H_2O_2 treatment. Erythrocytes treated with 100 and 200 mM H_2O_2 showed significant hemolysis after 3 h treatment. However, erythrocytes treated with 300, 400 and 500 mM H_2O_2 displayed significant hemolysis after 1 h exposure. Since there was a significant increase in hemolysis rate after 1.5 h treatment of erythrocytes with 400 and 500 mM H_2O_2 and no significant difference of hemolysis rate was observed between 400 and 500 mM H_2O_2 treatment from 1.5 h, the 400 mM concentration and 1.5 h exposure were selected for further investigations of the biological effects of vitexin and apigenin.

2.2. Effect of vitexin and apigenin on oxidative hemolysis of erythrocytes

The effects of vitexin and apigenin on the hemolysis rate of erythrocytes are shown in Fig. 2. Oxidative stress induced significant hemolysis of erythrocytes compared to normal erythrocytes ($p < 0.01$). Vitexin could significantly attenuate hemolysis of erythrocytes in both oxidative stress groups in a dose dependent manner. Vitexin low and medium dose groups had weaker anti-hemolytic effects than the VC (30 μg/ml) groups ($P < 0.01$, $P < 0.05$). Apigenin high and low doses groups have weaker anti-hemolysis effect than the VC group ($P < 0.01$, $P < 0.05$).

2.3. Effects of vitexin and apigenin on antioxidant enzymes, ATPase, ROS and MDA of human erythrocyte exposed to H_2O_2

When erythrocytes were treated with H_2O_2, there were significant increases in ROS and MDA content (Fig. 3). However, when erythrocytes were incubated with vitexin and H_2O_2, both ROS and MDA contents were gradually decreased in a dose dependent manner. Compared to the high and low doses of apigenin, the medium doses of apigenin has a better effect on antioxidant enzymes, ATPase, ROS and MDA ($p < 0.01$). But with the highest concentration of vitexin and the middle dose of apigenin, they could not reduce ROS and MDA content to normal erythrocyte levels. Moreover, H_2O_2 reduced anti-oxidative enzymes (SOD, CAT and GSH) and ATP in erythrocytes and both vitexin and apigenin could recover these enzymes activities to almost the levels of normal erythrocytes (Fig. 3).

2.4. Effect on methemoglobin in the erythrocyte

Effects on methemoglobin (MetHb) in the erythrocytes are shown in Fig. 4. After treatment with H_2O_2, the content of MetHb increased significantly in erythrocyte compared to normal erythrocytes ($p < 0.01$). Vitexin could significantly attenuate the content of MetHb in erythrocytes in both oxidative stress

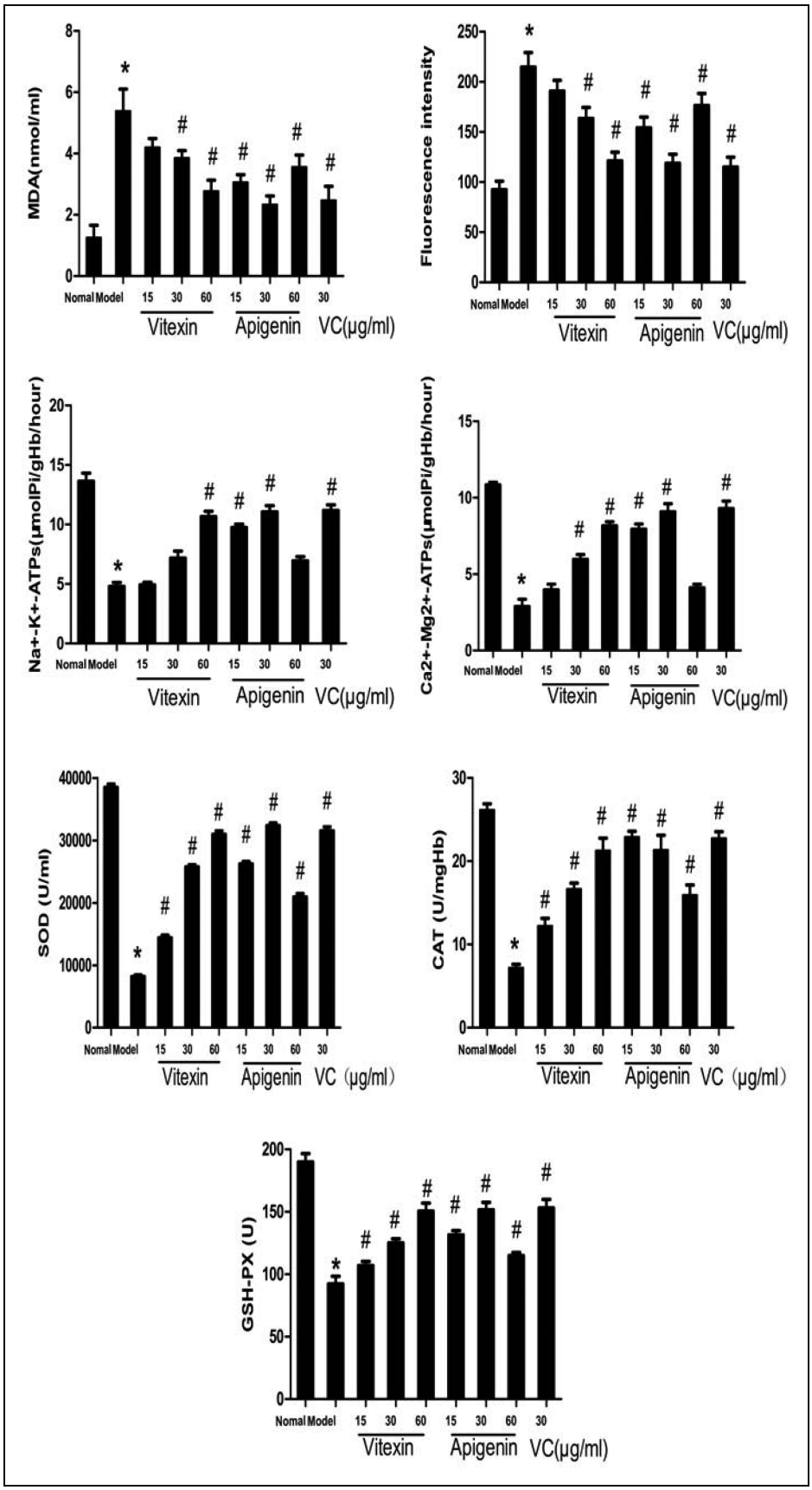


Fig. 3: Regulation of MDA, ROS, ATPase, total SOD, CAT, and GSH-PX in human erythrocytes under oxidative stress by Vitexin and Apigenin. The panels represent the results of MDA, ROS, ATPase, total SOD, CAT, and GSH-PX with treatment of Vitexin and Apigenin under oxidative stress respectively. Data are presented as mean ± SD from 10 individual experiments. * represents $p < 0.05$ compared to normal control and # indicates $p < 0.05$ compared to either oxidative stress.

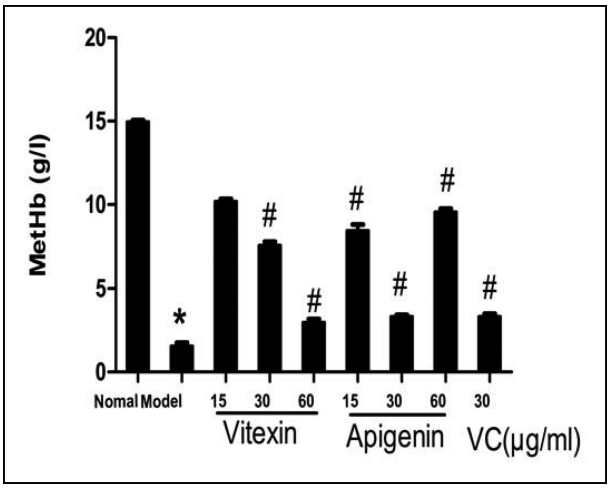


Fig. 4: Both vitexin and apigenin attenuated the content of MetHb in erythrocytes. Oxidative stress induced significant increase of MetHb in erythrocytes. Data are presented as mean ± SD from 10 individual experiments. * Represents $p < 0.05$ compared to normal control and # indicates $p < 0.05$ compared to either oxidative stress erythrocytes.

groups in a dose dependent manner. Compared to the high and low doses of apigenin, the medium doses of apigenin had a better effect on MetHb in the erythrocytes ($p < 0.01$). The high dose vitexin group showed no significant difference compared to the VC group, and the difference was also small between the medium dose apigenin group and the VC group, other medication groups had weaker activities than the VC group ($P < 0.01$).

2.5. Effects on amount of sulfhydryl content on the erythrocyte membrane

The effect on the amount of sulfhydryl content on the erythrocyte membrane is shown in Fig. 5. After treatment with H_2O_2, the content of sulfhydryl decreased significantly in treated erythrocytes compared to normal erythrocytes ($p < 0.01$). Medium doses of Apigenin has a better effect on sulfhydryl contented on the erythrocyte membrane ($p < 0.01$). Except the vitexin in high dose group and the apigenin medium dose group administration groups were weaker than the VC group ($P < 0.01$).

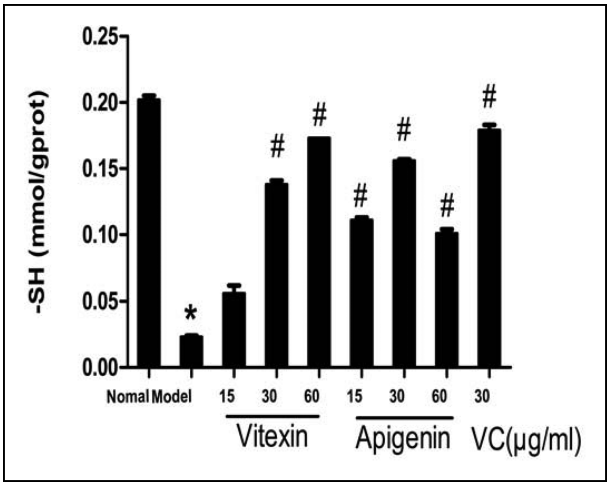


Fig. 5: Effects on amount of sulfhydryl contented on the erythrocyte membrane. Data are presented as mean ± SD from 10 individual experiments. * represents $p < 0.05$ compared to normal control and # indicates $p < 0.05$ compared to either oxidative stress erythrocytes.

2.6. Impact on the erythrocyte surface morphology

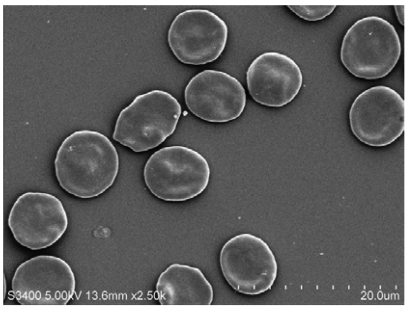
As shown in Fig. 6-1, normal erythrocytes are concentric type double concave discs, the surface is smooth, the margin neat and the dispersion uniform, cells with abnormal forms are rare. Figure 6-2 shows the picture of scouldning electron microscope after the cells were damaged by H_2O_2, cell morphology had significantly changed, the surface has spines, cell volume is reduced, cell morphology changed from double concave to circular, cells show shriveling, vesicle overflow and perforation. Figures 6-3 to 6-9 show cells proposed with different concentrations of vitexin, apigenin and VC before the damage. Spine was thinner or even disappearing, cell surface was smoother, cell adhesion conditions were improved, the number of normal erythrocytes had increased. These changes were positively correlated to drug concentration.

2.7. Effects on erythrocytes membrane skeleton ultrastructure

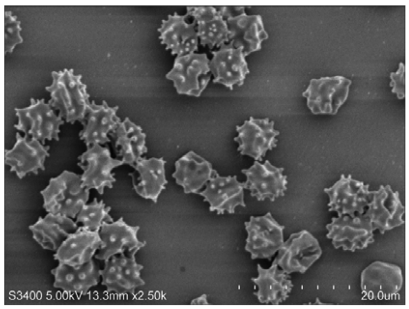
Usually the erythrocytes membrane skeleton is a polygon network structure, mostly a hexagon junctional complex connected with a spectrin tetramer, actin, band 4.1 protein, ankyrin and other membrane skeleton proteins. Spectrin is a link to connect all kinds of proteins into the reticular structure, as shown in Fig. 7-1, filament SP4 is a tetramer spectrin, and "J" dot in Fig. 7-1 represents the protein linked sites. From Fig. 7-1, the spectrin and complex on the normal erythrocyte membrane skeleton protein could be seen, it was complete, the structure was clear, and mesh was uniform, size was consistent. Figure 7-2 shows the erythrocytes membrane skeleton structure under 400 mmol L^{-1} H_2O_2 after damage. The network structure had largely disappeared, spectrin misses, fiber aggregates and hemoglobin settles in the skeleton. Figures 7-3 to 7-9 shows the erythrocyte membrane skeletons which were given different concentrations of unmarred vitexin, apigenin and VC before they were damaged. Medium doses of vitexin and a low dose of apigenin exerted a weak protection on membrane skeleton, spectrin split into pieces; with the increase of drug concentration, high doses of vitexin and medium doses of apigenin could recover the membrane skeleton network architecture partly, spectrin had become gradually complete, "J" dot was clearer. In Figure 7-9 it can be seen that VC Pre-protected could reduce the damage of H_2O_2 on membrane structure effectively, SP4 and "J" dot were clear. However, compared with the normal group, the membrane skeleton structure was loose, the mesh was larger and distribution of network fiber was uneven.

3. Discussion

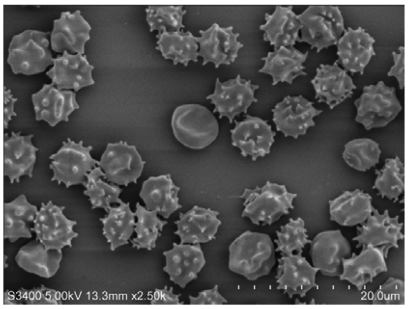
Cells exposed to large amounts of free radicals undergo oxidative damage and ultimately cause diseases (Wu Qixia 2003). Red blood cells are considered to be highly vulnerable to free radical damage, because they are susceptible to free radical attack and do not have nuclei and mitochondria. Treatment with H_2O_2 has become an important tool to investigate all kinds of oxidative cell damage (Brunauer 1994). Our results are consistent with the proposal that oxidative stress causes erythrocyte hemolysis. Suffering from oxidative damage will eventually lead to hemolysis. Thus the hemolytic test could be used to study antioxidant protection of erythrocytes. The antioxidant enzymes in erythrocytes mainly include SOD, GSH-Px and CAT, they could not only prevent damage from active oxygen, but also protect other antioxidant enzymes. SOD could disproportionate the super oxygen anion, so as to inhibit the super oxygen anion to inactivate GSH-Px and CAT, and the latter two kinds of enzyme could catalyze hydrogen peroxide into water and oxygen to pro-



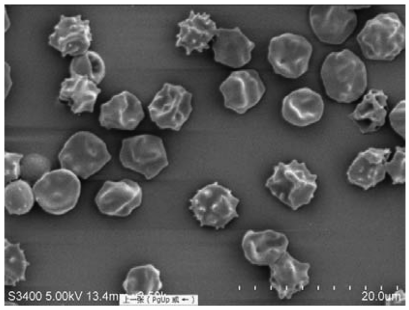
6-1. Normal group



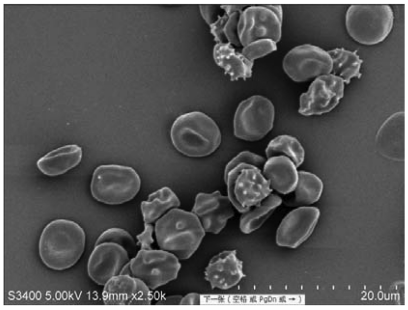
6-2. Model group



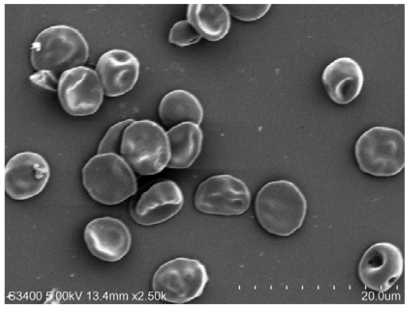
6-3. 15μg/ml Vitexin



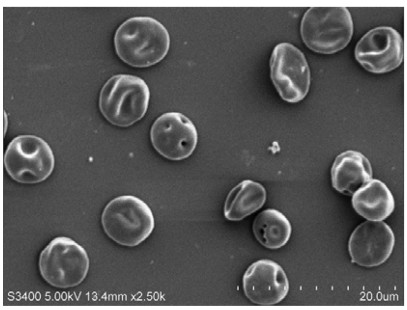
6-4. 15μg/ml Apigenin



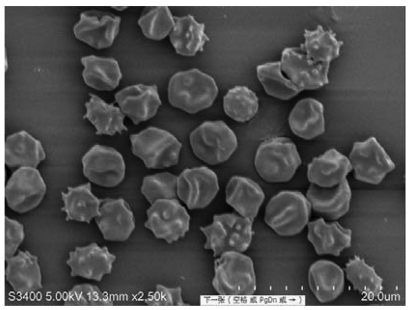
6-5. 30μg/ml Vitexin



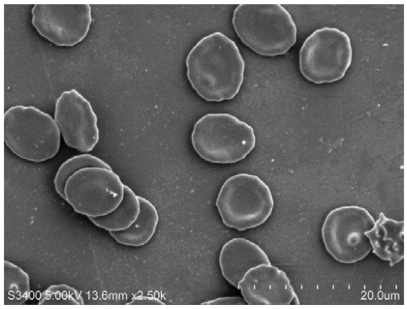
6-6. 30μg/ml Apigenin



6-7. 60μg/ml Vitexin

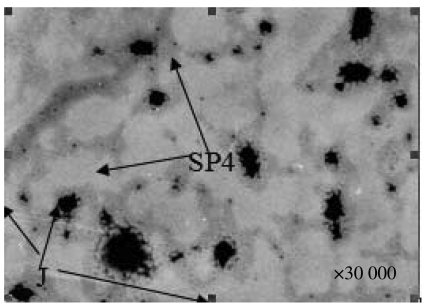


6-8. 60μg/ml Apigenin

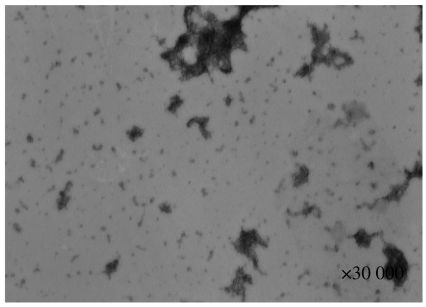


6-9. 30μg/ml VC

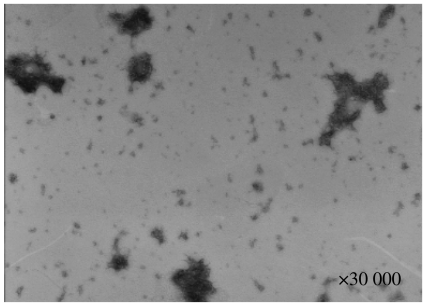
Fig. 6: Shape changes of human erythrocytes induced by H_2O_2.



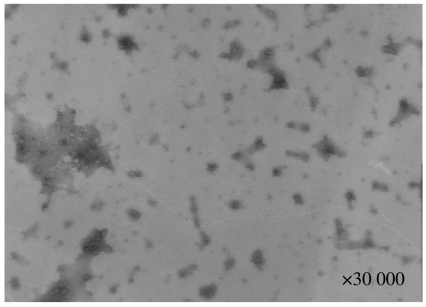
7-1. Normal group



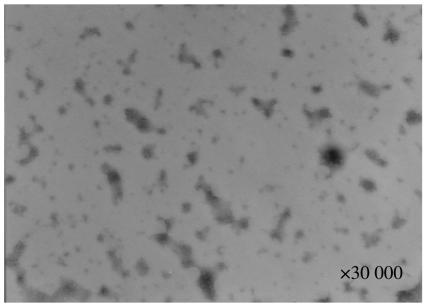
7-2. Model group



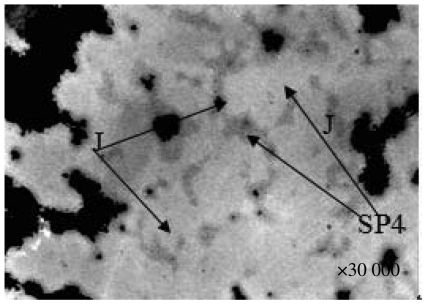
7-3. 15 μg/ml Vitexin



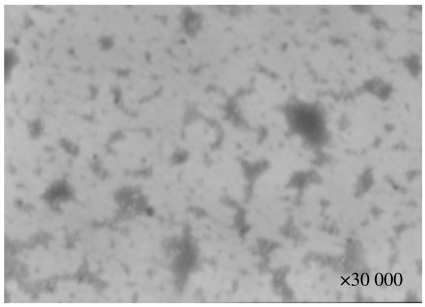
7-4. 15μg/ml Apigenin



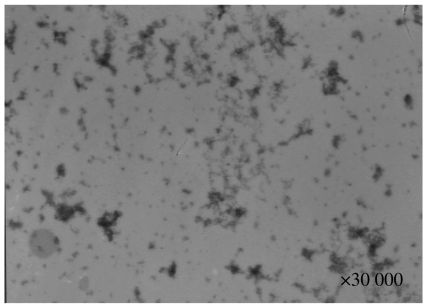
7-5. 30μg/ml Vitexin



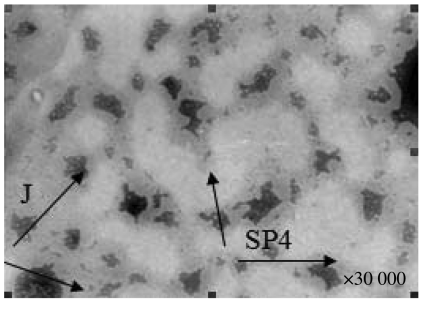
7-6. 30μg/ml Apigenin



7-7. 60μg/ml Vitexin



7-8. 60μg/ml Apigenin



7-9. 30μg/ml VC

Fig. 7: TEM results of human erythrocyte induced by H_2O_2.

tect SOD, so that all those three enzymes form a protection system (Sun Xuemin 2007). MDA is the main product of lipid peroxidation reaction, and its amount reflects the degree of cell damage indirectly. Erythrocytes are rich in ATPase, mainly the Na^+-K^+-ATPs and Ca^{2+}-Mg^{2+}-ATPs, which participate in material transportation, information transmission and energy supply (Zhang Wei et al. 2007; Wang Xinling et al. 1999; Shi Wenjing et al. 2012; Yan Xiaoyiing et al. 2013; Kumar and Maurya 2014; Kumar et al. 2014). Our experiments showed that vitexin and apigenin could play positive roles in inhibiting erythrocyte hemolysis, recover these enzyme activities to almost the levels of normal erythrocytes, and reduce ROS and MDA content.

Hemoglobin function is closely related to its structure. Hemoglobin forms cross-linked complexes with band 3 protein and contractile proteins, the cell density could increase, the deformation will be reduced (Salhany 1998). Our results show that vitexin and apigenin could reduce the formation of methemoglobin, playing a role in stabilizing membrane proteins and maintaining membrane structure. Under physiological conditions, erythrocyte surface morphology is an important parameter to evaluate other structure functions of the parameters. The change of cell surface morphology affects not only the aggregation, adhesion, immune function and metabolism of the cells, but also erythrocyte deformation ability and rheological properties (Wang Hongru 1997; Zhao Chunting 1997; Meiselman et al. 1999; Zhang Min et al. 2012). Experimental results have shown that vitexin and apigenin could restore membrane protein aggregation caused by oxidative damage to some extent. Qualitative determination of sulfhydryl has shown that, this protective function was completed probably by inhibiting the auto-oxidation of membrane proteins and preventing protein crosslinking. Ren Jing (2009) found that EGCG has protective effects on erythrocyte membrane protein damage caused by lysozyme fiber, consistent with our experimental results. We also found that vitexin and apigenin could restore the integrity of the membrane skeleton connection compounds and spectrins and help to reshape the network structure of the fiber. This explains why vitexin and apigenin could reduce the skeleton protein damage, reduce the protein aggregation, and inhibit membrane skeleton damage.

Although vitexin and apigenin have similar structures, apigenin showed better biological activity than vitexin. One possible reason is the glycosylation of vitexin on ring A causing a loss of the planar structure bending into a certain angle, causing steric hindrance to affect the antioxidant activity. This is consistent with resuts reported by Du Chunfang (2011) who studied the influence of 6-,8-C glycosylation on antioxidant activity. Consistent with Jin Yue (2006), quercetin and its antioxidant activity of monosaccharides and disaccharides successively weakened when the molecular glycosyl increased.

4. Experimental

4.1. Drugs and chemicals

Anticoagulant fresh blood (without white blood cells), (Zhangjiakou center blood stations); vitexin, (self-control, purity of 98% or more); apigenin, (Tianjin YiFang Science and Technology Co., Ltd); ascorbic acid (vitamin C, Tianjin chemical reagent factory); MDA, SOD (total), ATP, CAT and GSH-Px, MetHb, Sulfhydryl assay kit (Nanjing JianCheng Biotechnology Research Institute, Batch Number: 20120417, 20120417, 20120420, 20120419, 20120417, 20120801, 20120801, respectively). Wide range of protein molecular weight standards (Thermo companies in the United States) 2 × the sample buffer, active oxygen detection kit, PMSF, Bradford protein concentration determination kit (the BiYunTian Biotechnology Research Institute), Tris, acrylamide, TEMED (Promega Company), SDS (Biomol Company), glycine, Coomassie brilliant blue R - 250 (Amresco company), AP (Sigma Company), methylene double acrylamide (Fluka Company), methanol (Thermo Fisher); DMSO (Tianjin DaMao chemical reagent factory); 30% H_2O_2 (Tianjin Kemiou Chemical Reagent Co., Ltd.); twelve water disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, ice acetic acid (The above reagents are pure home-bred analysis).

4.2. Preparation of main solutions

Vitexin (10,0 mg) was precisely weighed and dissolved, mixed with 100 μL dimethyl sulfoxide (DMSO) to make it fully dissolved, then joined with CH_3OH to get a 5 mg/ml stock solution of vitexin, which was diluted with CH_3OH to different concentrations for adminsitration.

Apigenin solution were prepared the same way.

Vitamin C (10,0 mg) was precisely weighed and dissolved, mixed with 100 100 μL DMSO to make it fully dissolved, then join with CH_3OH to get a 5 mg/ml stock solution of vitamin C, which was diluted with physiological saline to different concentrations.

30% H_2O_2 was diluted to the required concentration with normal saline.

4.3. Preparation of human erythrocyte suspension

Anticoagulant fresh blood was received from healthy donors (Zhangjiakou Center Blood Stations). Plasm, white film, platelets, lymphocytes, monocytes and neutrophils were carefully removed after centrifugation (4 ° C, 2500 rpm, 10 min). Rred blood cells at the bottom were harvested after repeatedly washing in PBS (pH 7.4) cooled in advance. The packed cells with PBS were made in a volume ratio of 2% of the blood cell suspension after centrifugation (4 °C, 2500 rpm, 10 min) (Porat et al. 2006).

4.4. Establishment of the oxidative damage model

Erythrocyte suspension (2 %, 2.0 mL) was added to H_2O_2 (2.0 mL) at different concentrations and the final concentrations of H_2O_2 were 0. 100, 200, 300, 400 and 500 mM. The group without H_2O_2 was the control group. Erythrocytes were taken from each tube every 30 min after incubation at 37 °C, diluted 10-fold with 0.9% saline or distilled water (ddH_2O) and then centrifuged at 2500 rpm for 10 min at 4 °C. The supernatants were collected to measure the absorbance value at 412 nm. The final hemolysis rate was determined by the final concentration of H_2O_2 and incubation time with the following formula: hemolysis rate (%) = $A/A_0 \times 100\%$ (A, absorbance value of sample diluted with saline; A_0, absorbance value of sample diluted with ddH_2O.

4.5. Experimental sectionalization and drug delivery

The experiment includes a normal group, a model group, vitexin (15, 30, 60 μg/ml) different dose groups, apigenin (15, 30, 60 μg/ml) different dosage groups and a vitamin C (30 μg/ml) positive control group. According to the group, 100 μL vitexin, apigenin or VC were added to the red blood cells as protective agents, to the normal control group and the model group the same amounts of solvents (DMSO and CH_3OH) were added. The centrifugal tubes were gently shaken to make them fully contacted and evenly mixed, then incubated in a boiling water bath for 30 min at 37 °C. Finally, to these tubes 400 mM H_2O_2 normal saline solution were added and the tubes were incubated for 1.5 h at 37 °C.

4.6. Effect on oxidative stress in erythrocytes

Oxidative stress in erythrocytes was determined by measuring enzymatic levels of CAT, glutathione peroxidase (GSH-Px), and ATPase. Moreover, ROS and MDA were also measured. After establishment of erythrocyte oxidative damage, cytoplasmic proteins were employed for determination of activities of SOD, CAT, GSH-Px, and ATPase. ROS and MDA in erythrocyte cytoplasm were extracted by their methods respectively. All these parameters were measured according to the instructions of the available kits.

4.7. Effects on methemoglobin and sulfhydryl content of erythrocyte proteins

Methemoglobin has a characteristic absorption peak at 630 nm, and there is an equal optical density value of methemoglobin (MetHb) and reduced hemoglobin (Hb) at 602 nm. This relationship could be used to calculate the content of methemoglobin.

According to the sample kit instructions, detection was completed within one hour, and calculation followed the formula:

Amount of methemoglobin/liter = $(A_{630nm}\text{-}r \times A_{602nm})/A_{602nm} \times (R\text{-}r) \times 100\% \times$ hemoglobin/liter

where A_{630nm} is the absorbance value of the sample at 630 nm; A_{602nm} is the absorbance value of sample at 602 nm; R and r are constant, R = 1.81; r = 0.14.

According to Ellman's method, different concentrations of vitexin, apigenin and VC were added to 100 mL membrane proteins and incubated in a boiling water bath for 1 h at 37 °C. To the the model group and the normal group the same doses of normal saline were added. Hydrogen peroxide solution was

added, and then incubated for 1.5 h; finally, sulfhydryl content was measured according to the kit instructions.

4.8. Electron microscopic observation of erythrocyte morphological changes

For examination of erythrocyte membrane surface morphology, erythrocytes from oxidative stress and natural aging samples were mixed with 2% glutaraldehyde at a ratio of 1:100 (v/v) and then fixed overnight. After washing three times with cold PBS, the samples were placed in an ion sputtering device to be sprayed with metal after drying and then photographed using a S-3400N scanning electron microscope. For examination of erythrocyte membrane skeleton structure, erythrocytes from oxidative stress and natural aging samples were mixed with NaPi solution (5 mmoL/L, pH 7.0) containing 2.5% (W/V) TritonX-100 at 1:4 ratio (v/v) and then incubated at 0 °C for 1 h. The mixture was dropped onto a copper grid (200 mesh), washed three times with ddH_2O and exposed to 1% uranyl acetate for 5-10Ýs for negative staining. The H-7500 TEM was used to observe the erythrocyte membrane skeletal structure after drying with filter paper.

4.9. Statistical processing

Statistical evaluations were carried out using Statistical Package for Social Sciences (SPSS for Windows, version 17.0). All values were expressed as the mean ± standard deviation (SD). Differences between groups were analyzed by Student's *t*-test. For all tests, *P* values of less than 0.05 or 0.01 were considered significant.

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