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Protective effects of resveratrol against hypoxanthine-xanthine oxidase-induced toxicity on human erythrocytes

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ABSTRACT

An *in vitro* model of erythrocyte damage induced by hypoxanthine-xanthine oxidase (HX-XOD) was developed. The protective effects of resveratrol, a main component of red wine, were evaluated using this model. The results showed that resveratrol treatments maintained erythrocyte morphological and structural integrity, inhibited the formation of methaemoglobin, and suppressed the release of lactate dehydrogenase. Further investigation found that resveratrol attenuated the HX-XOD-induced reactive oxygen species generation, preserved erythrocyte antioxidant enzymes (SOD, CAT and GSH-Px) activities and increased GSH level. Also, resveratrol regulated the pyruvate kinase activity and pyruvate concentration, preserving the normal functioning of erythrocyte membrane ATPases (Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase). This study established an ideal cellular model for the research of erythrocyte antioxidant system, and revealed the protective mechanisms of resveratrol on human erythrocyte. The results of this study provided novel evidences for further researches on the utilization of resveratrol as a protector on human erythrocyte.

1. Introduction

Resveratrol (3,4′,5-*trans*-trihydroxystilbene) is a natural polyphenolic compound present in a wide variety of plants, such as grapes (Smoliga, Baur, & Hausenblas, 2011). The physiological function of resveratrol was first identified in an epidemiological study by the World Health Organization (WHO), which showed that though the French had relatively high fat intake, their incidence of cardiovascular diseases (CVD) was significantly lower than that of other nationalities. The effect was related to the relatively high intake of red wine by the French (Renaud & de Lorgeril, 1992). Further investigations showed that resveratrol was the main compound exerting a protective effect against cardiovascular disease (Siemann & Creasy, 1992). In recent years, research has demonstrated that resveratrol is of low-toxicity with biological activities that are beneficial to human health, such as antioxidation (Livingston et al., 2015), anti-cancer (Lin et al., 2013), anti-inflammatory (Mattison et al., 2014), and protective cardiovascular effects

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(Zordoky, Robertson, & Dyck, 2015). Within the cardiovascular system, erythrocyte is the most common type of blood cells. They are rich in haemoglobin and unsaturated fatty acids and with a large membrane surface area (Ahmad & Beg, 2013). Therefore, erythrocytes are vulnerable to oxidative stress, which can result in the destruction of the cellular membrane and even haemolysis. The oxidative damage of erythrocytes could also potentially cause certain CVDs (Rother, Bell, Hillmen, & Gladwin, 2005), indicating that the structural and functional destruction of erythrocytes may be related to cardiovascular disease risk. Research has shown that resveratrol can enter into human erythrocytes and accumulate in much higher concentrations compared to plasma levels (Rizvi & Pandey, 2010). The protective effect of resveratrol on erythrocytes is beneficial to cardiovascular health, but its protective mechanisms on human erythrocyte have been scarcely investigated.

To date, there are a large number of different assays to evaluate the antioxidant capacity of compounds *in vitro* and *in vivo*. Cellular models are one of the most effective methods to evaluate the antioxidant capacity and to elucidate the underlying mechanisms of oxidative stress (Niki, 2010). Owing to the fact that erythrocytes are vulnerable to oxidative stress, they can be used to evaluate the antioxidant capacity of compounds *in vitro*. However, many of the erythrocyte oxidative damage models are using 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) as a radical initiator (Mikstacka, Rimando, & Ignatowicz, 2010; Zhang et al., 2014), which did not uniformly mimic the *in vivo* conditions for the oxidative damage of erythrocyte. Therefore, it is urgently needed to develop an ideal erythrocyte oxidative damage model which can be used to study the protective effects of antioxidants on erythrocyte.

In this study, we established an *in vitro* model of erythrocyte oxidative damage induced by hypoxanthine–xanthine oxidase (HX–XOD) system, which simulated the principal pathway for the generation of free radicals in human body. Using this model, we aimed to investigate the protective effects and its mechanisms of resveratrol on human erythrocytes.

2. Materials and methods

2.1. Materials

Resveratrol (3,4′,5-*trans*-trihydroxystilbene; Res), quercetin (3,3′,4′,5,7-Pentahydroxyflavone; Que), hypoxanthine (HX), dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Co. (St. Louis, MO, USA). Lactate dehydrogenase (LDH), superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), catalase (CAT), pyruvate kinase (PK), pyruvate, Na^+/K^+-ATPase, and Ca^{2+}/Mg^{2+}-ATPase kits and xanthine oxidase (XOD) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Glutathione peroxidase (GSH-Px) and glutathione (GSH) kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Red wine (génesis) was purchased from Bodegas y Viñedos De Aguirre S.A. (Chile). Phosphate-buffered saline (PBS, pH 7.4) were purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Other chemicals were obtained from local sources and were of analytical grade.

2.2. Establishment of an erythrocyte oxidative damage model

2.2.1. Erythrocyte preparation

Blood samples were collected from healthy volunteers under 30 years of age. The blood samples were centrifuged at 1200 × g for 10 min at 4 °C, then the supernatant and white blood cells were discarded. Erythrocytes were washed with PBS (pH 7.4) three times and centrifuged at 1200 × g for 10 min. Erythrocytes with similar cell densities were obtained, and erythrocytes suspensions were prepared by re-suspending in 25 vol PBS containing 5 mM glucose.

2.2.2. Evaluation of methaemoglobin (MetHb)

MetHb saturation was measured with reference to the methods of Evelyn and Malloy (1938), and modified in our laboratory. Briefly, erythrocytes were collected and PBS was added. After complete freeze-thaw-induced haemolysis, the optical density (L1) was measured at 635 nm using a spectrophotometer. Neutral sodium cyanide (NaCN) solution was then added for 2 min to convert MetHb to MetHbCN. The optical density (L2) was then measured. The value of (L1-L2) was proportional to the MetHb saturation. The MetHb saturation was then calculated using the equation C_{MetHb} (g/L) = 1000 × (L1 – L2)/2.77. After that, potassium ferricyanide [$K_3Fe(CN)_6$] and NaCN were added to the sample to convert all haemoglobin (Hb) to MetHbCN, and optical density (L3) of total haemoglobin (tHb) was measured at 540 nm, tHb was calculated using the equation C_{tHb} (g/L) = L3 × 367.7, and the MetHb saturation was expressed as the percentage of MetHb in the Hb state, using the equation MetHb (%) = (C_{MetHb}/C_{tHb}) × 100%.

2.2.3. Optimization of HX–XOD treatment concentration and duration

Within HX–XOD radical generation system, while XOD is saturated, the generation of MetHb increased as the HX concentration increasing. At the XOD concentration of 0.2 U/ml, the following HX concentrations were tested: 0, 0.2, 0.4, 0.8, 1.0, 1.2, and 1.6 μmol/ml. After mixing HX and XOD evenly with the erythrocytes suspension, the samples were maintained at 37 °C in a dark environment and gently shaken for 60 min. At the same time, the erythrocytes were treated with HX and XOD separately in order to determine the compounds' individual effects on erythrocyte toxicity. After the reactions were completed, the method described above was used to determine the MetHb saturation.

In order to select an appropriate duration of erythrocyte damage, the optimum concentration of HX and XOD that described above were used. HX and XOD solutions were mixed evenly with the erythrocytes suspension, which were then maintained at 37 °C in a dark environment and incubated for 0, 20, 40, 60, 80, 100, and 120 min. Thereafter, the MetHb saturation was measured as described above.

2.3. Evaluation of the protective effects of red wine extract, quercetin, and resveratrol on erythrocytes against HX–XOD-induced oxidative damage

The raw red wine was evaporated to nearly dryness under vacuum at 40 °C to eliminate the alcohol, which was then