ประสิทธิภาพของสารสกัดผักเม็กในการลดภาวะออกซิเดชันในหนูทดลองฮีโมลัยติคอะนีเมีย

Efficacy of Eugenia Grata Extract in Reduction of Oxidative Damage in Hemolytic Anemic Rats

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บทคัดย่อ

สาร Phenylhydrazine (PHZ) เป็นสารที่ทำให้เกิดฮีโมลัยติคอะนีเมียในสัตว์ทดลองโดยทำปฏิกิริยากับโมเลกุลของฮีโมโกลบินและชักนำให้เกิดภาวะเครียดจากออกซิเดชันส่งผลให้ระบบการไหลเวียนเลือดล้มเหลว นอกจากนั้นหนูทดลองที่ได้รับสาร PHZ จะแสดงภาวะความดันเลือดและความต้านทานหลอดเลือดต่ำ และลดการตอบสนองของหลอดเลือดต่อสารที่มีผลกับหลอดเลือดซึ่งอาจมีสาเหตุจากการลดลงของระบบต้านออกซิเดชันภายในร่างกาย ในปัจจุบันได้มีการทดสอบพบว่าผักพื้นบ้านของไทยมีฤทธิ์ต้านออกซิเดชันที่ดี ดังนั้นวัตถุประสงค์ของการวิจัยในครั้งนี้เพื่อศึกษาว่า สารสกัดผักเม็ก (*Eugenia grata*) สามารถลดภาวะเครียดจากออกซิเดชันที่เกิดขึ้นในหนูทดลองที่ถูกชักนำให้เกิดฮีโมลัยติคอะนีเมียด้วยสาร PHZ ได้ การศึกษาวิจัยทำได้โดยการป้อนสารสกัดผักเม็กขนาด 1 กรัม/กก/วัน เป็นเวลา 6 วันและในวันที่ 4 ของการทดลองหนูทดลองจะถูกชักนำให้เกิดฮีโมลัยติคอะนีเมียด้วยการฉีดสาร PHZ ขนาด 125 มก./กก. เข้าทางช่องท้อง เมื่อครบกำหนด 6 วันจะทำการเก็บตัวอย่างเลือดเพื่อวัดค่าฮีมาโตคริต และกลูทาไทออน ส่วนตัวอย่างพลาสมาจะตรวจวัดปริมาณ nitric oxide metabolites (NOx) จากนั้นหนูทดลองจะถูกทำให้สลบและทำการวัดค่าความดันเลือด อัตราการไหลเวียนเลือดของอวัยวะท่อนล่างและอัตราการเต้นของหัวใจ ผ่าน BIOPAC System ผลการทดลองพบว่าเมื่อให้สาร PHZ ความดันเลือด และระดับกลูทาไทออนในเลือดลดลงอย่างมีนัยสำคัญ ส่วนปริมาณ NOx เพิ่มขึ้นเกือบสองเท่าเมื่อเปรียบเทียบกับหนูปกติ ($p<0.05$) แม้ว่าการให้สารสกัดผักเม็กจะไม่สามารถป้องกันการเกิดฮีโมลัยติคอะนีเมียได้แต่สถานภาพการไหลเวียนเลือดและระบบต้านออกซิเดชันภายในร่างกายของหนูทดลองกลุ่มนี้ดีขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับหนูชีดกลุ่มควบคุมโดยสรุปแล้วสารสกัดผักเม็กให้ผลลดภาวะเครียดจากออกซิเดชัน ทำให้การใช้สารต้านออกซิเดชันภายในร่างกายลดลง และมีผลเพิ่มความดันเลือดแดงเพื่อปรับสภาพระบบการไหลเวียนเลือดของหนูทดลองฮีโมลัยติคอะนีเมียให้ดีขึ้น ซึ่งอาจเป็นแนวทางในการนำผักพื้นบ้านไปประยุกต์ใช้ในการลดภาวะเครียดจากออกซิเดชันในภาวะฮีโมลัยติคอะนีเมียในผู้ป่วยต่อไป

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Abstract

Phenylhydrazine (PHZ) is the oxidative drug that induced hemolytic anemia in experimental animals by interaction with hemoglobin at which leads to the oxidative stress and consequently circulatory failure. Moreover, the PHZ-treated rats exhibited hypotension, low systemic vascular resistance and decreased vascular responses to endothelium-dependent vasodilator agents. The attenuation of vascular responses in these conditions may be due to a decrease in the antioxidant defense. Recently, our preliminary work has demonstrated that a local vegetable, *Eugenia grata* (EG), possessed strong antioxidant activities in many assay systems. Therefore, the aim of this study was to determine whether the EG extract would alleviate PHZ-induced oxidative damage in animal model of hemolytic anemia. Male Sprague-Dawley rats were orally administered with EG extract (1 g/kg/day) for six days while rats in the control group were fed with deionized water. On the fourth day of treatments, animals were induced hemolysis by intraperitoneal injection of 125 mg/kg PHZ. After 48 hours of PHZ administration, blood samples were collected from tail vein for reduced glutathione (GSH) and nitric oxide metabolites (NOx) assays, then animals were anesthetized and measured mean arterial pressure (MAP), and hindlimb blood flow (HBF). PHZ administration resulted in marked hypotension together with significant reduction in blood level of GSH and a twofold increase in plasma NOx. Although EG extract could not protect the animals from hemolytic anemia, a prompt recovery of blood pressure and increasing of antioxidant defense system was found in animals treated with EG extract. As a result, the EG-treated animals exhibited increased blood pressures, increased blood level of GSH and decreased plasma NOx when compared to the anemic control rats ($P<0.05$). In view of hemodynamic, MAP and hindlimb vascular resistance (HVR) of the anemic rats treated with EG were significantly increased whereas HBF was significantly decreased ($P < 0.05$). Our findings suggest that EG extract has an antioxidant effect to abrogate sustained hypotension and reverse the abnormal oxidative processes that may contribute to the development of hemolytic anemia, and offer a potential clinical means to alleviate the oxidative damage to the red cells of the hemolytic anemic patients.

คำสำคัญ : สารฟีนิลไฮดราซีน, ผักเม็ก, สารต้านอนุมูลอิสระ

Key words : Phenylhydrazine, Eugenia grata, antioxidant

Introduction

Hemolytic anemia is a disorder in which the red cells are prematurely destroyed (Turgeon, 1999). This abnormality of red cell feature found in patients with thalassemia (Schrier and Mohandas, 1992) that is the most common hereditary disease among Thai population. The disease is treated by chronic blood transfusion. However, this can cause severe iron overload resulting in progressive organ failure (Ciccoli *et al.*, 1999; Rodgers and Saunthararajah, 2001; Rund and Rachmilewitz, 2001). Phenylhydrazine (PHZ) is the derivative of the hydrazine which is toxic and can induce oxidative damage to hemoglobin and generates free radical as well as the increase in lipid peroxidation and hemolysis (Bates and Winterbourn, 1984;

Clemens *et al.*, 1984; Goldstein *et al.*, 1980). It is demonstrated that normal red cell treated with PHZ has a mimetic characteristic as those found in severe beta-thalassemia (Schrier and Mohandas, 1992). Moreover, the PHZ-induced anemic rats exhibited hypotension, low systemic vascular resistance and decreased vascular responses to endothelium-dependent vasodilator agents (Kukongviriyapan et al., 2000). The attenuation of vascular responses in these conditions may be due to a decrease in the antioxidant defense. Several studies have documented that vegetables and fruits are rich source of micronutrients with antioxidant properties. Recently, our preliminary works has demonstrated that some Thai vegetables possess strong antioxidant activities in many assay systems (Kwuangthip et al., 2002). Thus, it is noteworthy to investigate the antioxidant effect of a local vegetable extract in alleviation of oxidative damage in animal model of hemolytic anemia.

Materials and Methods

1. Materials

Phenylhydrazine hydrochloride (PHZ), 5,5 dithio-bis-2-nitro-benzoic acid (DTNB), ethylene diamine tetra-acetic acid (EDTA), glutathione, glutathione reductase, G6P-disodium salt, sulfanilamide, naphthylene-diamine di-hydrochloride (NED), sodium nitrite and sodium nitrate were obtained from Sigma Chemical Co. Ltd., St. Louis, USA. NADPH, G6P-dehydrogenase and nitrate reductase were obtained from Boehringer Mannheim. Germany. Di-Potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Fluka Chemika Co. Ltd., Buchs, Switzerland.

2. Plant extraction

Eugenia grata (EG) is a selected vegetable for this study. 300 g. of whole plants were cleaned, chopped into small pieces and boiled in 600 ml of water for 45 min. After cooling down to the room temperature, the mixture was filtered through a coarse sieving cloth and the filtrate was centrifuged at 2500 rpm for 10 min. The supernatant was dried at reduced pressure by freeze-dryer vacuum evaporator. The dried extract was collected in a tight container and stored at -20°C until used.

3. Animals treatment

Male Sprague-Dawley rats (250g) were randomized into 3 groups (n=6/group). Rats were daily orally administered with deionized water (3ml/kg) and EG extract (1g/kg), respectively, for six days. On the fourth day of treatment, all studied animals were induced hemolytic anemia by peritoneal injection of 125 mg/kg PHZ. A group of normal rats administered with deionized water (DI) for six days were served as non-anemic controls.

4. Blood collection

After 48 hours of PHZ injection, blood samples were collected from the tail vein into capillary tube for the determination of hematocrit and into microcentrifuge tube containing 50 mM EDTA. 120 µl of whole blood was rapidly deproteinized with 120 µl of 10% sulfosalicylic acid (SSA) then mixed rapidly by vortex mixer and centrifuged at 4°C, 10000 rpm for 10 min. After that, 50 µl of the supernatant was collected for the analysis of GSH. The remaining whole blood was centrifuged at 4°C, 2500 rpm for 15 min and

collected plasma was stored at -80° C for determination of NOx level.

5. Analytical Procedures

5.1 Assay of reduced glutathione (GSH)

Reduced glutathione assay was performed essentially according to Tiezes method (Tietze, 1969) by added 50 (l of the supernatant to reacted with Ellmans reagent (0.2 M DTNB in 0.5 M phosphate buffer consisting of K_2HPO_4: KH_2PO_4= 4:1, pH 7.4 and 5 mM EDTA) and measured by a spectrophotometer (Jasco model 7800, Japan Spectroscope Co., Japan). Optical density reading was set at 412 nm. Standard curve was generated by using appropriate concentration of standards glutathione.

5.2 Assay of nitric oxide metabolites (NOx)

NOx assay was followed by Verdon and Dembny (Dembny et al., 1998;Verdon et al., 1995). Plasma samples were filled in centrifugal concentrators (NANOSEP™, Pal Filtron, USA) and centrifuged at 4°C, 6000 rpm for 90 min. After that, the supernatant was reacted with 80 uM NADPH, 4 mM G6P-disodium salt, 1.28 unit/ml G6P-dehydrogenase and 0.8 unit nitrate reductase, then incubated at 30°C for 30 min. and added in Griess solution (4% sulfanilamide in 0.3% NED). The sample was bring to measured with ELISA plate reader (Dynatech MR 5000, UK). Optical density reading was set at 540 nm. Standard curve was generated by using appropriate concentrations of standards nitrate and nitrite.

6. Hemodynamic study

The animal was anesthetized with sodium pentobarbital (65-75 mg/kg i.p.) and placed on a heating-pad, its body temperature was kept at 37°C. A tracheostomy was performed for spontaneously breathing. The right femoral artery was cannulated and connected to a pressure transducer for arterial blood pressure monitoring and recording with the data acquisition system (BIOPAC system, CA, USA). Hindlimb blood flow (HBF) was recorded by placing the electromagnetic flow probe around the abdominal aorta at which connected to the electromagnetic flowmeter (Carolina medical electronics, inc. U.S.A.). Hindlimb vascular resistance (HVR) was calculated from MAP and HBF.

7. Statistical analysis

Results were expressed as mean ± SE. Statistical analysis was performed by using the analysis of variance. P values less than 0.05 were considered significant.

Results

Results of hemodynamic studies were shown in Table 1. Hematocrit (Hct) of PHZ-treated rats were significantly decreased when compared with normal rats ($p<0.05$). In addition, the anemic rats showed markedly decreases in MAP and HVR whereas HBF was increased. While EG-treated rats remained in anemic state, MAP and HVR were significantly increased when compared with anemic control rats.

Treatment of rats with PHZ increased oxidative stress and reduced of GSH, an endogenous antioxidant, when compared with the normal rats. Although GSH level in EG-treated rats (569.30 ±17.81 uM/ml) was less than normal controls (825.19±26.20 uM/ml), however, it was significantly increased when compared with anemic control rats (465.95±11.89 uM/ml) (Fig.1). Moreover, it appeared that PHZ caused

a significantly increase in plasma NOx compared with normal rats (20.16±0.68 uM/ml). After EG-treatment, the level of plasma NOx was declined to 11.91±1.39 uM/ml.

Table1 Comparisons of hemodynamic data in all studied groups. Results showed that EG extract significantly improved circulatory status by preventing the reductions of MAP and HVR in anemic rats. * With respect to normal control ($p < 0.05$). + With respect to anemic control ($p < 0.05$).

Parameter measurements	Normal control	Anemic rats	
		DI 3 ml/kg	EG 1 g/kg
Hct (%)	46.89 ± 1.11	23.00 ± 0.62 *	22.88 ± 0.64 *
MAP (mmHg)	127.57 ± 1.95	63.04 ± 1.60 *	78.42 ± 3.06*+
HR (bpm)	354.48 ± 13.42	384.01 ± 5.27 *	357.82 ± 9.43
HBF(ml/min/100g tissue)	9.33 ± 0.61	17.62 ± 1.57 *	11.95 ± 1.15*+
HVR (PRU)	15.14 ± 1.17	3.86 ± 0.38 *	6.96 ± 0.84 *+

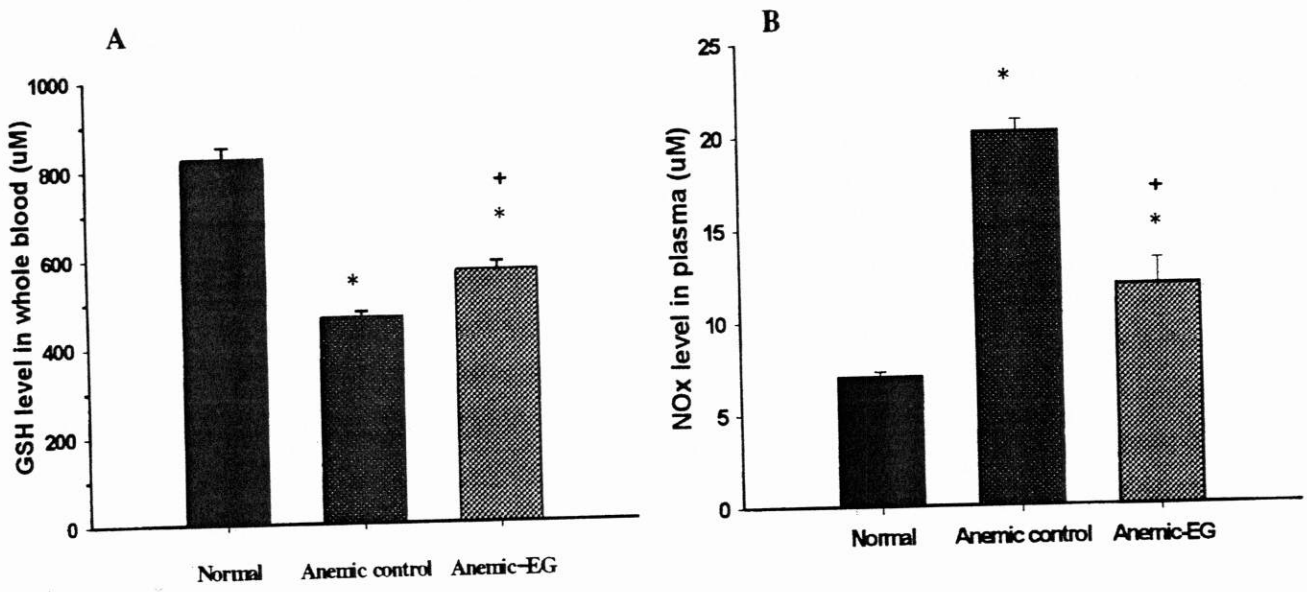

Fig. 1 Comparisons of GSH in the whole blood (A) and plasma NOx (B) of the animal studies. Under hemolytic anemic condition, it was found a depletion of the endogenous antioxidant and increase in nitric oxide production. A significant increase of blood GSH in EG-treated animals compared with the anemic controls indicating the antioxidant effect of EG extract. * With respect to normal control ($p < 0.05$). + With respect to anemic control ($p < 0.05$).

Discussion

Increased oxidative stress in PHZ-treated rats have been suggested to contribute to disturbances in hematological profiles and cellular membrane damage (Ferrali *et al.*, 1992; Ferrali *et al.*, 1997). Moreover, PHZ also induced circulatory failure as those found in this study including marked anemia, hypotension and low vascular resistance (Pakdeechote et al., 2001).

Recently, our preliminary works has demonstrated that some Thai local vegetables possess strong antioxidant activity in free radical scavenging assay. *Eugenia grata* is one of the local vegetables that favorites among Thai people in Northeast region. It is demonstrated that EG extract can directly scavenge free radicals, inhibit free radical production and has strong reducing power. Moreover, EG extract can inhibit free radical production in rat peritoneal macrophage (Kwuangthip *et al.*, 2002). The present study showed that EG extract was able to reduce the severity of PHZ-induced oxidative damage. However, the EG extract could not protect against the PHZ-induced decrease in the concentration of hemoglobin and the number of erythrocytes because the data of the present study indicate that a treatment with PHZ induced severe hemolytic anemia. Hemolysis and associated anemia are complex process, not exclusively relate to oxidative stress, but possibly involving mechanisms which are not influenced by antioxidant. For example, in another study sulfhydryl oxidation and protein aggregation in hemoglobin-free human erythrocyte membranes were not prevented by any of the antioxidants tested or by antioxidant enzymes (Hashmi et al., 1996).

The results of the present report indicate that the protection afforded by EG extract is due to an antioxidant activity. This possibility is supported by (i) the increasing of GSH level, which may due to the expansion of GSH production or diminution of GSH use; (ii) the ability to prevent lipid peroxidation of EG extract which scavenge free radical, as those found in the previous studies (Pakdeechote et al., 2001), (iii) the improvement of hemodynamics i.e. MAP, HBF and HVR, show the effect of EG on vascular function. In summary, EG extract can minimize oxidative stress in red blood cells by reducing both plasma MDA and NOx. It also abrogated hypotension and low systemic vascular resistance, thus supporting the role of antioxidant agent in this hemolytic anemic model. Innovative use of vegetables in hemolytic anemic condition is an important indication and should be further explored.

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