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Reduced Red Blood Cell Membrane Damage in Streptozotocin-induced Diabetic Rats Supplemented With Roselle (UKMR-2) Calyx Extract

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Abstract: The aim of this study was to evaluate the effects of aqueous extract of roselle on the membrane composition of red blood cells. A total of 32 male Sprague-Dawley rats (230-250 g) were divided randomly into four groups. Diabetic rats were induced streptozotocin (45 mg kg^{-1}, i.v). The normal and diabetes groups were administrated with distilled water. The other normal and diabetes group were administrated with roselle aqueous extracts (100 mg kg^{-1}). After 28 days, the blood was drawn by sinus orbital for biochemical tests including membrane total protein, cholesterol and phosphatidylcholine and morphology of red blood cells was carried out through light microscope. In diabetic rats, the result showed the weight of rat, membrane total protein and Phosphatidylcholine (PCh) were significantly lower ($p<0.05$), while blood glucose and membrane cholesterol showed significantly higher ($p<0.05$) than control rats. In diabetic rats demonstrated with roselle, the result showed no significant difference ($p>0.05$) in weight and blood glucose compared to diabetic rats. The membrane total protein and PCh were significantly higher ($p<0.05$) than diabetic rats, whereas membrane cholesterol was significantly lower ($p<0.05$) compared to diabetic rats. The observation red blood cells morphology that showed echinocytes, schistocytes and Heinz body in diabetic rats was caused by oxidative stress damage. The morphology of red blood cells in diabetic rats supplemented with roselle is normal. Aqueous extract of roselle showed potential protective effects on membrane composition of damaged red blood cells.

Key words: Roselle, diabetes, red blood cells, membrane composition

INTRODUCTION

Diabetes Mellitus (DM) is a chronic disease where the pancreatic cells fail to produce sufficient insulin or unable to react to the insulin produced in the body. This condition inhibits the blood glucose from being absorbed into the cells, resulting in oxidative and hyperglycemia. Oxidative stress occurs due to the imbalance between radical-generating and radical-scavenging system. It is caused by the formation of Reactive Oxygen Species (ROS), non-enzymatic protein glycation, glucose autooxidation (Mullarkey *et al*., 1990), impaired glutathione metabolism (McLennan *et al*., 1991), alteration in antioxidant enzymes (Strain, 1991), formation of lipid peroxides (Baynes, 1991) and decreased ascorbic acid levels (Young *et al*., 1992). The imbalance in free radicals and lipid peroxidation causes detrimental damage to the red blood cells (RBCs) membrane (Acworth *et al*., 1997).

Roselle or *Hibiscus sabdariffa* Linn. is a tropical plant in the Malvaceae family which is well-known in Asia and Africa. It is commonly as an edible colorant in foods and drinks preparation. The calyx of roselle contains antioxidant such as anthocyanin, ascorbic acid, flavonoids and polyphenols (Tzu-Li *et al*., 2007). Previous studies have shown that the aqueous extract of roselle could inhibit low-density oxidation and decrease serum triglyceride and cholesterol in animal model (Chen *et al*., 2003).

RBCs are cells that are most exposed to peroxidative damage by ROS (Constantinescu *et al*., 1993). These cells are reliable model because they have simple internal structure, no nucleus and organelles in the cells (Battistelli *et al*., 2005). Although previous studies have shown the antioxidant effect of aqueous extract of roselle, there has been no study to evaluate its effect to RBCs membrane composition in diabetic rats. Thus, the aim of this study was to investigate the effects of

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aqueous extract of roselle in RBCs membrane composition in diabetic rats by measuring the concentration of membrane total protein, membrane cholesterol, membrane phosphatidylcholine (PCh) and observing the RBCs morphology.

MATERIALS AND METHODS

Animal and experimental design: A total of 32 male Sprague-Dawley rats (230-250 g) was provided by Animal Laboratory of research unit of Universiti Kebangsaan Malaysia. Diabetes was induced in 16 rats by intravenous injection of 45 mg kg^{-1} of STZ. Three days later, the glucose level of STZ-treated rats were measured. Rats with blood glucose levels higher than 15 mol L^{-1} were considered as diabetic.

Thirty two rats were randomly divided into four groups: Normal (N), Normal+Roselle (NR), Diabetes (D) and Diabetes+Roselle (DR), with n = 8 in each group. N and D group were fed with distilled water while NR and DR group were administrated with 100 mg kg^{-1} aqueous extracts of roselle. All the administrations were done by force feeding for 28 days. The weight of rats was taken daily. After 28 days, the rats were sacrificed and their blood was drawn into EDTA tube by orbital sinus.

Extraction of roselle: The dried calyx of roselle species UKMR-2 was provided by Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Dried calyx was put into the blender containing distilled water and blended. The mixture was then poured into a pot and allowed to boil, after which they were allowed to cool at room temperature. The mixture was later filtered through gauze. The solution was freeze-dried to obtain the powder form of roselle which then was kept in 4°C freezer.

Packed red cells separation: The packed red cells were separated from plasma by centrifugation at 4000 rpm for 10 min (Eppendorf 5702R). The packed red cells (lower layer) were transferred to centrifuge tube and washed with 0.9% cold normal saline (with the ratio of 1:5) for three times at 4000 rpm for 15 min. After washing, the packed red cells were transferred into eppendorf tube and added with 0.9% normal saline for the use of storage in -40°C freezer.

Ghost membrane extraction: Extraction of ghost membrane was carried out based on Dodge *et al.* (1963) method. Packed cells were lysed by adding distilled water. The mixture was centrifuged at 16000 rpm for 20 min at 4°C (Mikro200R, Hettich Zentrifugen, Germany). After centrifugation, supernatant was discarded. The pellet (lower layer) was added with 5 mM PBS (pH 7.4) with stirring. The mixture was then centrifuged at 16000 rpm for 20 min at 4°C. The supernatant was discarded and this procedure was repeated for six times to form the ghost membrane. The ghost membrane was suspended into 5 mM PBS (pH 7.4) and kept in -40°C freezer.

Determination of RBC membrane total protein: RBC membrane total protein was determined based on Bradford (1976) method. Bradford solution was added into sample. The mixture was then vortexed and allowed to stand for 5 min at room temperature. The value of OD was measured with the wave length of 560 nm by using spectrophotometer (SECOMAM, AHS Laboratory supplies).

Extraction of RBC membrane lipids: RBC membrane lipids were extracted by method described by Folch *et al.* (1957). The purpose of extraction was to determine the concentration of membrane cholesterol and phospholipids. Sample was added with chloroform-methanol (2:1, v/v), then vortexed for 10 min and allowed to spin at 4000 rpm for 15 min at 4°C. After centrifugation, two layers were formed. The upper layer was discarded while the lower layer was dried by nitrogen gas.

Determination of RBC membrane cholesterol: RBC membrane cholesterol was determined by the method of Franey and Amador (1968). Extraction of membrane lipids were suspended with chloroform. The suspension was added with ethanol and then vortexed for 15 sec. The mixture was centrifuged at 3500 rpm for 3 min. After centrifugation, supernatant was pipeted into new test tube. Colour reagent was then added and stirred. Sulphuric acid was added slowly and allowed to stand for 10 min at room temperature. The value of OD was measured at 560 nm wave length.

Determination of RBC membrane PCh: RBC membrane PCh was determined based on Cynamon *et al.* (1984) method. Extraction of membrane lipids were suspended with chloroform. The suspension was added with ammonium ferrothiocyanate and then vortexed for 1 min. The mixture was then centrifuged at 2700 rpm for 5 min. After centrifugation, two layers were formed. The

upper layer was discarded while the lower layer was used for determination of OD value with the wave length of 485 nm.

Statistical analysis: Statistical comparison of data was done using Analysis of Variance (ANOVA) test. Values were expressed as Mean±SEM. Difference was considered to be statistically significant if the probability value was less than 0.05 ($p<0.05$).

RESULTS

Body weight: The study revealed significantly lower ($p<0.05$) of weight in D (246.85±4.11 g) and DR group (238.88±7.26 g) compared to N (364.43±3.79 g) and NR group (336.00±5.82 g). There was no significant difference between D and DR group. Results of weight are shown in Fig. 1.

Blood glucose: The concentrations of glucose in D (25.16±1.34 mol L^{-1}) and DR group (25.14±2.94 mol L^{-1}) were significantly higher than N (7.56±0.37 mol L^{-1}) and NR group (8.89±0.59 mol L^{-1}). There was no significant difference in D and DR groups (Fig. 2).

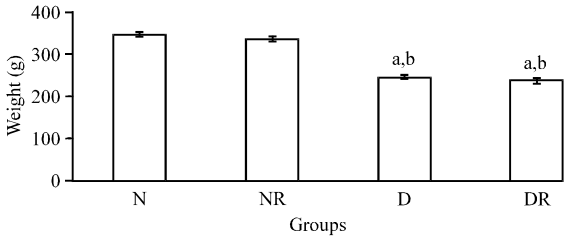

Fig. 1: Comparison of weights between groups. Values are expressed as Mean±SEM. [a]Significant difference compared to N group ($p<0.05$), [b]Significant difference compared to NR group ($p<0.05$)

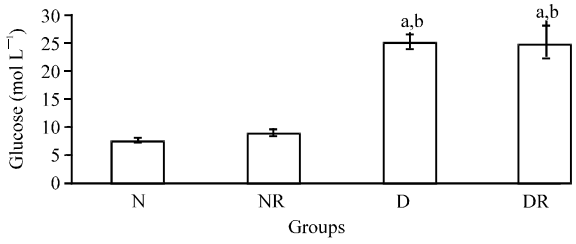

Fig. 2: Comparison of glucose levels between groups. Values are expressed as Mean±SEM, [a]Significant difference compared to N group ($p<0.05$), [b]Significant difference compared to NR group ($p<0.05$)

Membrane total protein: Figure 3 shows, the concentration of membrane total protein in D group (16.83±1.34 mg mL^{-1}) was significantly lower than N (91.45±2.31 mg mL^{-1}) and NR (86.60±2.16 mg mL^{-1}) group. Administration of aqueous extract of roselle in DR group (88.91±7.41 mg mL^{-1}) shown significantly higher than D group.

Membrane cholesterol: RBC membrane cholesterol was significantly higher in D group (0.31±0.02 mg mL^{-1}) as compared to N (0.16±0.02 mg mL^{-1}) and NR group (0.11±0.02 mg mL^{-1}). DR group (0.07±0.01 mg mL^{-1}) was shown significantly lower membrane cholesterol than D group (Fig. 4).

Membrane PCh: Figure 5 shows the level of membrane PCh for all experiments groups. The D (0.03±0.01 mg mL^{-1}) and DR group (0.06±0.01 mg mL^{-1}) were significantly lower in concentration of membrane Pch compared to N (0.28±0.03 mg mL^{-1}) and NR (0.35±0.04 mg mL^{-1}) group. Meanwhile, DR group was significantly higher than D group (0.06±0.01 mg mL^{-1} vs 0.03±0.01 mg mL^{-1}).

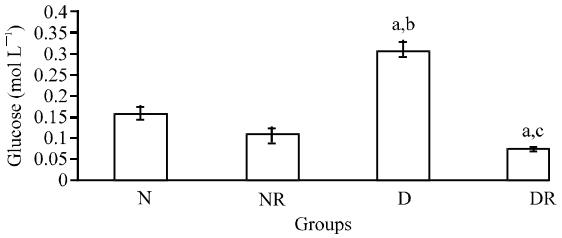

Fig. 3: Comparison of membrane total protein levels between groups. Values are expressed as Mean±SEM, [a]Significant difference compared to N group ($p<0.05$), [b]Significant difference compared to NR group ($p<0.05$), [c]Significant difference compared to D group ($p<0.05$)

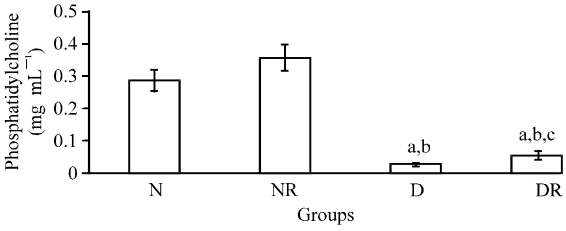

Fig. 4: Comparison of membrane cholesterol levels between groups. Values are expressed as Mean±SEM, [a]Significant difference compared to N group ($p<0.05$), [b]Significant difference compared to NR group ($p<0.05$), [c]Significant difference compared to D group ($p<0.05$)

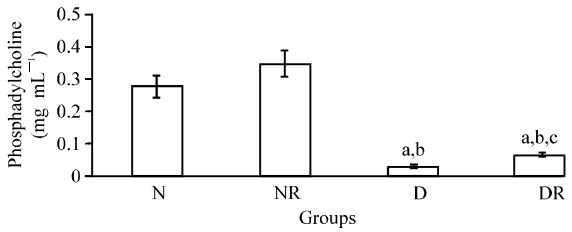

Fig. 5: Comparison of membrane PCh levels between groups. Values are expressed as Mean±SEM, [a]Significant difference compared to N group ($p<0.05$), [b]Significant difference compared to NR group ($p<0.05$), [c]Significant difference compared to D group ($p<0.05$)

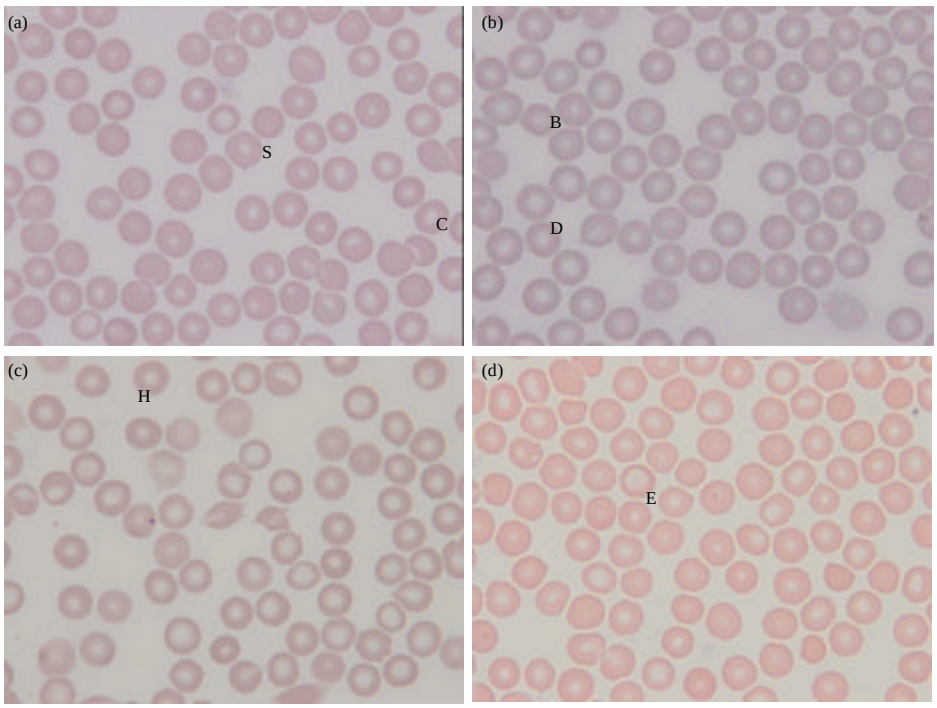

Fig. 6(a-d): Comparison of morphology from various experimental groups (100 x), The group of N (a) NR, (b) DR, (c) Showed normal morphology, D and (d) Showed abnormal morphology with the presence of echinocytes (E) Schistocytes (S) and Heinz body (H)

RBC morphology: Figure 6a-d show the blood smear of RBC from each of experimental group. RBCs of N, NR and DR group exhibited normal morphology. Echinocytes, schistocytes and Heinz body were present in the blood smear of D group.

DISCUSSION

Diabetes mellitus cause the production of ROS as a result of β-pancreatic cells damage (Aragno *et al*., 1999). The overproduction of ROS can occur in the RBCs membrane resulting in lipid peroxidation and eventually component structure damage (Acworth *et al*., 1997).

The weight of STZ-induced diabetic rats showed significantly lower than both of the control groups, similarly found in previous study (Suthagar *et al*., 2009). According to Chatterjea and Shinde (2002), the reduction of weight could be due to the excessive catabolism of protein and adipose tissue in the body. The process of catabolism of protein and adipose tissue is important as a compensation process to produce energy since the metabolism of glucose fail in diabetic rats. Nevertheless,

the weight of diabetic rats supplemented with roselle was not increased. This could be due to the rats' compensation state to treat the symptoms of diabetes.

The STZ-induced diabetic rats showed higher blood glucose level than control group as shown similarly in previous study (Akbarzadeh *et al*., 2007). According to Holemans *et al*. (1997), STZ prevents DNA synthesis in β-pancreatic cells by inhibiting many enzymes involved in DNA synthesis. Besides that, it also results in the degeneration and destruction of DNA in β-pancreatic cells. As a consequence, the β-pancreatic cells are unable to produce insulin therefore caused the increase of blood glucose level. However, the supplementation of aqueous extract of roselle was unable to reduce down the blood glucose level in diabetic rats. It might be due to the dysfunction of adipose tissue and inhibition of proliferation of adipose tissue in diabetic rats (Hajer *et al*., 2008). Thus, it reduced the uptake of blood glucose in adipose tissue (Yosaph *et al*., 2009).

This study showed that the membrane total protein was lower in diabetic rats. The membrane total protein in DM patients was found significantly lower than healthy individuals due to the production of ROS (Adewoye *et al*., 2001). Hyperglycemia caused non-enzymatic protein glycation and production of polyol compounds which may cause damage of tissue in the body (Ahmed, 2005). On the other hand, diabetic rats with administration of aqueous extract of roselle results in significantly higher membrane total protein compared to diabetic rats without roselle. According to Rosalina Tan *et al*. (2011), antioxidants such as polyphenol and flavonoid increased total protein plasma. Hence, the anthocyanin in roselle may increase the total protein plasma in which influenced the membrane total protein by increasing its level.

The result of increased membrane cholesterol in diabetic rats in current study is supported by Shinde *et al*. (2010), whereby high level of glucose prolonged the time of LDL cholesterol in blood circulation. According to Andallu *et al*. (2001), membrane cholesterol exchange with plasma cholesterol can occur. Diabetic rats supplemented with roselle showed lower cholesterol membrane compared to normal rats and diabetic rats, similar to previous study (Olatunji *et al*., 2005). It was suggested that the cause could be due to the increase in low Density Lipoprotein (LDL) receptor in liver and decrease in conversion of Very Low Density Lipoprotein (VLDL) to LDL.

In this study, the result showed PCh level was lower in diabetic rats. This could be due to the damage of PCh induced by non-enzymatic glycation (Ellenberg and Rifkin, 1983). Administration of aqueous extract of roselle in diabetic rats showed higher PCh compared to diabetic rats. This is supported by a study that used vitamin E and vitamin C as antioxidant that increased the membrane phospholipid (Shinde *et al*., 2010). According to May (1999), vitamin C could protect components in the cytoplasm and membrane from damage by ROS. Thus, the presence of antioxidants (anthocyanin and ascorbic acid) in roselle showed the similar effect.

The RBC morphology in diabetic rats showed the presence of echinocytes, schistocytes and Heinz body, while normal RBC morphology was shown in diabetic rats supplemented with roselle. Decrease in membrane total protein caused alteration of the structure of RBC (Adewoye *et al*., 2001). According to Allen *et al*. (2006), the increase in cholesterol membrane in diabetic patient increased the rigidity of RBC membrane. The decrease in membrane phospholipid was a consequent of oxidant damage that caused changes in RBC morphology from normal to abnormal (Suwalsky *et al*., 2008). Changes of membrane composition may affect membrane fluidity, deformability, rigidity, and hence alteration of the morphology of RBC and thus affecting the cellular integrity.

CONCLUSION

In conclusion, a decrease in membrane total protein and PCh and an increase in membrane cholesterol were observed in diabetic rats. It is suggested that roselle could exhibit antioxidant effect that may reduce the damage of membrane composition of RBCs and minimize the abnormal morphology of RBCs.

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