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Oxidative hemolysis and osmotic fragility of erythrocytes in renovascular hypertensive and normotensive rats

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Oxidative hemolysis and osmotic fragility of erythrocytes in renovascular hypertensive and normotensive rats

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Introduction : *The imbalance of reactive oxygen species (ROS) in renovascular hypertension (RVH) that mediates oxidations of biological molecules and the membrane may cause oxidative cellular damage. The erythrocyte membrane is intrinsically prone to ROS-induced oxidative damage. Abnormal susceptibility of erythrocyte membrane to oxidative damage is known to reflect similar abnormalities in other organs and tissues.*

Objective : *To evaluate the membrane oxidative hemolysis and the osmotic fragility characteristics of erythrocyte between renovascular hypertensive and normotensive rats.*

Setting : *Burapha University*

Research design : *An experimental design*

Animals : *Male Sprague Dawley rats*

Methods : *The constriction of left renal artery was used to induce hypertension. The oxidative hemolysis of erythrocytes was performed by using the azo-compound 2,2'-Azobis (2-amidinoporpane) dihydrochloride (AAPH) as peroxyl radical initiator. The osmotic fragility of erythrocytes was performed in PBS containing various concentrations of NaCl.*

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Results : *The kinetic profile of the oxidative hemolysis curve was sigmoid where as RVH and normotensive erythrocytes profiles were not of significant difference. The osmotic fragility curve of RVH had the same pattern as that of normotensive ones.*

Conclusions : *There have been no definitive findings on the features of the erythrocyte membrane to oxidative hemolysis in RVH rats. This technique may not sensitive enough to measure peroxidative injury. There was no alteration in osmotic fragility of erythrocytes in RVH rats. The erythrocytes of RVH and normotensive rats were susceptible to hemolysis in the same pattern.*

Keywords : *Oxidative hemolysis, Osmotic fragility, Erythrocyte, Renovascular hypertension.*

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บทนำ : *โรคความดันโลหิตสูงที่เกิดจากการตีบหลอดเลือดแดงที่ไต มีผลทำให้มีการเพิ่มขึ้นของ reactive oxygen species (ROS) ซึ่งทำให้เกิดการทำลายโมเลกุลชีวภาพ เยื่อหุ้มเซลล์ และเป็นสาเหตุให้เซลล์บาดเจ็บ เยื่อหุ้มเซลล์เม็ดเลือดแดงมีองค์ประกอบของชีวโมเลกุลที่มีแนวโน้มถูกทำลายจาก ROS เหนี่ยวนำให้เกิดภาวะออกซิเดชั่น ความผิดปกติดังกล่าวสามารถสะท้อนถึงความผิดปกติในระดับอวัยวะและเนื้อเยื่อ*

วัตถุประสงค์ : *เพื่อประเมินการแตกของเม็ดเลือดแดงที่เหนี่ยวนำโดยภาวะออกซิเดชั่น และคุณลักษณะความเปราะบางออสโมติคของเม็ดเลือดแดงในหนูขาวที่เหนี่ยวนำให้เกิดความดันโลหิตสูงโดยการตีบหลอดเลือดแดงที่ไต และหนูขาวภาวะความดันโลหิตปกติ*

สถานที่ : *มหาวิทยาลัยบูรพา*

ลักษณะการวิจัย : *การทดลอง*

สัตว์ทดลอง : *หนูขาวเพศผู้ สายพันธุ์ Sprague Dawley*

วิธีการทดลอง : *ผ่าตัดผูกหลอดเลือดแดงที่ไตข้างซ้ายเพื่อเหนี่ยวนำให้หนูเป็นความดันโลหิตสูง ทำการเหนี่ยวนำให้เม็ดเลือดแดงแตกโดยภาวะออกซิเดชั่น จาก 2,2'-Azobis (2-amidinoporpane) dihydrochloride (AAPH) ซึ่งสร้าง peroxyl radical ทดสอบความเปราะบางออสโมติคของเม็ดเลือดแดงโดยการเปลี่ยนแปลงความเข้มข้น NaCl*

ผลการทดลอง : *การแตกของเม็ดเลือดแดงจากภาวะออกซิเดชั่นตามฟังชันก์ของเวลา มีลักษณะกราฟเป็นรูปซิกมอยด์ ซึ่งไม่มีความแตกต่างอย่างมีนัยสำคัญ ระหว่างหนูขาวที่เหนี่ยวนำให้เกิดความดันโลหิตสูงโดยการตีบหลอดเลือดแดงที่ไต และหนูขาวภาวะความดันโลหิตปกติ เช่นเดียวกับกราฟการแตกของเม็ดเลือดแดงจากภาวะออสโมติคตามฟังชันก์ของความเข้มข้น NaCl ที่มีลักษณะเหมือนกันทั้งสองกลุ่ม*

สรุป : *ผลการศึกษาครั้งนี้ไม่สามารถสรุปถึงปรากฏการณ์ของเยื่อหุ้มเซลล์เม็ดเลือดแดงที่เหนี่ยวนำให้แตกโดยภาวะออกซิเดชั่น ในหนูกลุ่มที่เหนี่ยวนำให้เป็นความดันโลหิตสูงโดยการตีบหลอดเลือดแดงที่ไต เทคนิคที่ใช้อาจจะไม่มีความไวพอที่จะวัดระดับความผิดปกติดังกล่าว นอกจากนี้ยังไม่พบความแตกต่างของความเปราะบางออสโมติค เม็ดเลือดแดงที่ได้จากหนูทั้งสองกลุ่มมีลักษณะการแตกเหมือนกัน*

คำสำคัญ : *การแตกของเม็ดเลือดแดงโดยออกซิเดชั่น, ความเปราะบางออสโมติค, เม็ดเลือดแดง, ความดันโลหิตสูงโดยการตีบหลอดเลือดแดงที่ไต*

Renovascular hypertensive (RVH) rats are commonly used as experimental models for study of hypertension. As demonstrated by Goldblatt [1] renal artery occlusion creates ischemia, which causes the stimulation of rennin-angiotensin system, leading to an increase in oxidative stress. Reactive oxygen species (ROS) may play a dual role in hypertension. On the one hand, they may cause a loss of bioavailability of nitric oxide, thereby causing vasoconstriction and elevation of blood pressure; on the other hand, enhanced production of ROS may serve as trigger mechanism for oxidative damage of numerous macromolecules, in part through the activation of membrane-bound NADH and NADPH oxidases.[2,3] These oxidase enzymes are present in endothelial cells, vascular smooth muscle cells, phagocytic mononuclear cells and erythrocytes.

The imbalance of ROS in hypertension is also suggested by the observation of increased level of lipid peroxides and decreased concentrations of antioxidant in plasma of hypertensive patients.[4-6] ROS that mediated oxidations of biological molecules and membrane may cause oxidative cellular damage. Extensive lipid peroxidation in biological membranes cause loss of fluidity, decrease in membrane potential, increase permeability to ions and occurlysis. The erythrocyte membrane is intrinsically prone to ROS-induced lipid peroxidation due to its high content of polyunsaturated lipids and it has been extensively used to investigate the role of oxidative membrane damage in various pathological conditions.[7,8] Abnormal susceptibility of erythrocyte membrane to oxidative damage is known to reflect similar abnormalities in other organs and tissues.

Since the membrane and indeed the cellular intrinsic properties of erythrocyte-related hypertension are very different from those of normal subjects, the membrane structures themselves must be significantly perturbed.[9,10] According to the above-mentioned findings, we hypothesized that erythrocytes from RVH rats were more fragile than those from nomotensive ones. The ability of erythrocyte membrane to maintain its structure integrity in this study was evaluated by two parallel techniques in order to better establish the fragility characteristics of erythrocyte and to uncover clinically significant differences between RVH and normotensive rats. The oxidative hemolysis test addressed the evaluation of susceptibility of erythrocytes to peroxidation. The osmotic fragility test measured the stability of erythrocytes to withstand hemolysis in decreasing osmotic gradients which may vary considerably in different conditions.

Materials and Methods

Materials

The following reagents were obtained from either Sigma-Aldrich or Merk: sodium chloride (NaCl), sodium phosphate dibasic (anhydrous) (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), 2,2'-Azobis (2-amidinoporpane) dihydrochloride (AAPH).

Experimental animals and renal artery constriction procedure

Male Sprague Dawley rats were purchased from the National Laboratory Animal Care, Mahidol University and were approximately 14 to16 weeks at the time of experimentation. The animals were allowed to acclimate for at least one week before experimentation. All animals were housed in the animal facilities at the Department of Medical Science with a light cycle of 12 hours light/dark and allowed free choice access to standard rat chow and water. Animal

procedures in this study were approved by the Ethics Committee on Experimental Animal, Burapha University.

Renovascular hypertension was induced by left renal artery constriction as demonstrated by Goldblatt. [1] All the rats were randomly divided into 2 groups (n = 8 per group): normotensive and hypertensive group. The rats were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneal), from which an incision was made in the mid-abdomen, and the left renal artery was carefully dissected from the renal vein under aseptic conditions. A stainless wire (0.3-mm diameter) was parallel-placed on the left renal artery of 8-week-old rats and constricted the left renal artery with a rope. After retrieving the stainless wire, the abdomen was closed. The rats were placed in a heating cage until they awoke. After operation, all rats were allowed an ordinary rat chow diet and tap water as desired and kept on a 12-hours light/dark. The systolic blood pressure (SBP) was measured by an indirect tail-cuff sphygmomanometer in preheated (37 °C, 15 min) conscious rats before and at weekly intervals for 6 - 8 weeks after renal artery constriction. Rats were considered to be hypertensive when SBP was increased significantly compared to the control.

Preparation of erythrocyte suspensions

Whole blood was obtained from male Sprague Dawley rats via heart puncture, and collected in a heparinized tube. Erythrocytes were centrifuged and washed three times with 10 volumes of 10 mmol/L phosphate buffered saline (PBS; 125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4). The plasma and buffy coat were carefully removed by aspiration after each wash. The washed erythrocytes were finally resuspended in PBS and adjusted to a hematocrit (HCT) of 4 %.

Eight animals of each group were used, and erythrocytes from one animal of each group were analyzed on the same day.

Hemolysis Assay

Oxidative Hemolysis Test

Erythrocyte hemolysis mediated by AAPH, a peroxyl radical initiator, was measured according to the method of Niki *et al.* with minor modification.[11] The rate of peroxyl radical generation can be controlled by adjusting the concentration of AAPH. Preliminary experiments were performed to choose the dose of AAPH to be used by incubating erythrocytes in the presence of various concentration of AAPH range from 10 to 100 mM as a function of time incubation. In every experiment, the concentration of AAPH was kept at 50 mM while 3 hours was appropriated to be the incubation period in this study.

Erythrocyte suspension at 4 % hematocrit was incubated with 50 mM AAPH (in PBS at pH 7.4). This reaction mixture was shaken gently while being incubated for the indicated time at 37 °C. At the indicated time, the reaction mixture (0.5 ml) was diluted with PBS (1.5 ml) and control was diluted with distilled water (1.5 ml) to induce 100 % hemolysis. The hemoglobin contents of the supernatant after centrifugation were determined by measuring the absorbance (Abs) at 540 nm. The percent hemolysis was calculated by using the following equation:

$$\% \text{ Hemolysis} = \frac{\text{Absorbance at 540 nm of sample}}{\text{Absorbance at 540 nm of control}} \times 100$$

Osmotic Fragility Test

The osmotic fragility of erythrocytes was performed as described by Parpart *et al.* [12] Briefly, erythrocyte suspension at 4 % hematocrit was added into PBS tubes with increasing concentration of NaCl (%) 0, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.7 and 0.9. The tubes were gently mixed and incubated at 37 ^{o}C for 20 min. Then, the samples were centrifuged at 1500 g for 10 min and the supernatants were measured at 540 nm. The percentage of hemolysis in each concentration of NaCl was calculated and compared with that of complete hemolysis. The results were expressed as % hemolysis.

Statistics and data processing

All results are expressed as means ± standard error of mean (S.E.M) of 6 -10 different trials and analyzed with the software Microcal™ Origin 6. Different groups were assessed by analysis of variance (ANOVA). Significance was defined as $P < 0.05$. All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS).

Results

Blood Pressure

The mean SBP in male Sprague-Dawley rats was 155 ± 10 mmHg before renal artery constriction, and it rose progressively to a peak mean value of 180 ± 8 mmHg at 8th weeks postoperation in hypertensive groups (Fig.1). In this regard, the SBP was increased up to 16 %. The SBP in normotensive groups was found to be 158 ± 11 and 168 ± 6 at 1st and 8th weeks, respectively, which increased only 6 %. The percent of increased blood pressure in hypertensive groups was significantly higher than that of normotensive ones ($P < 0.05$).

Time-dependent rate of AAPH-induced erythrocyte hemolysis

When erythrocytes were incubated in PBS (without AAPH), they were stable and no significant hemolysis within 4 hours, thus to exclude any membrane-perturbing effect of the experimental conditions (data not shown). When a water-soluble radical initiator, AAPH (final concentration 50 mM), was

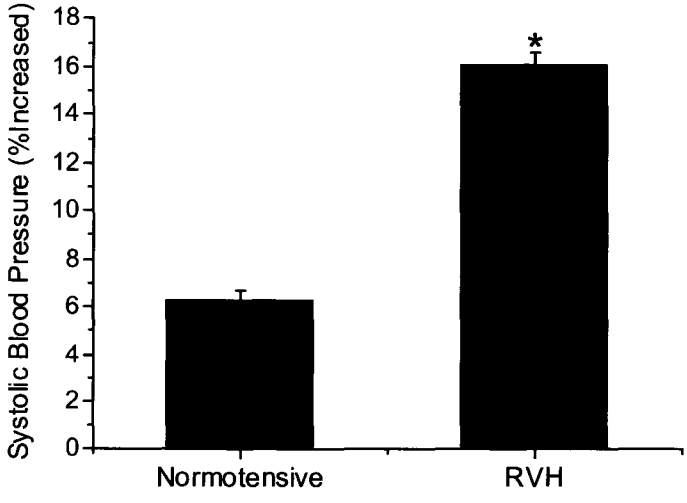

Figure 1. The percent increased in systolic blood pressure from RVH and normotensive rats.

* = $P < 0.05$ versus normotensive rats. Data are expressed as mean ± S.E.M of n = 8 samples.

added to the erythrocyte suspension, it induced hemolysis in a time-dependent manner. The kinetic profile of the oxidative hemolysis curve was sigmoid from which one can calculate several quantitative parameters. The lag time, defined as the absence of hemolysis, reflects the endogenous antioxidant of the cell to inhibit peroxyl radicals. After depletion of all endogenous antioxidants, hemolysis occurred rapidly.[13] The other parameters were the time required to achieve 50 % hemolysis and maximum hemolysis.

A typical example of the time-dependent hemolysis curves of erythrocytes from RVH and normotensive rats is shown in Fig. 2. In erythrocyte exposed to AAPH, hemolysis started after 100 min incubation and plateau (98 -100 % hemolysis) at approximately 170 min incubation. The lag time of hemolysis in RVH (98 ± 2.34) is similar to that of normotensive one (101 ± 1.47). The 50 % hemolysis of RVH (128 ± 3.4) is not statistically significant than that of normotensive ones (123 ± 3.66). The maximum hemolysis of RVH (167 ± 1.77) is not significantly different than that of normotensive ones (168 ± 2.55). In this experimental condition, the time-dependent hemolysis curves of erythrocytes from RVH and normotensive rats were not of significant difference in all 3 parameters as showed in Table 1.

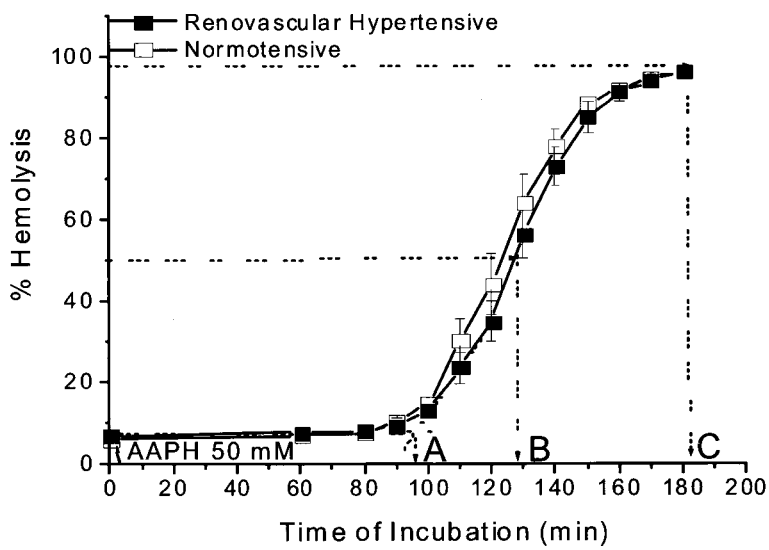

Figure 2. Time-dependent hemolysis curve of erythrocytes incubated at 37°C in the presence of 50 mM AAPH. Arrows indicate the parameters which can be calculated: (A) lag time (B) time at which 50 % hemolysis and (C) maximal hemolysis are reached. Data are expressed as mean ± S.E.M of n = 8 samples.

Table 1. Parameters measured from the hemolysis curves of erythrocytes. NS = non statistic significance. Data are expressed as mean ± S.E.M of n = 8 samples.

	Renovascular Hypertensive Rats	Normotensive Rats
Lag time (min)	98 ± 2.34^{NS}	101 ± 1.47
50 % Hemolysis (min)	128 ± 3.4^{NS}	123 ± 3.66
Maximum Hemolysis (min)	167 ± 1.77^{NS}	168 ± 2.55

Osmotic fragility of erythrocyte hemolysis

In RVH erythrocytes, the minimum hemolysis was noted at NaCl concentration gradient of 0.47 % and the completed hemolysis was at NaCl concentration of 0.15 %. In normotensive erythrocytes, hemolysis commenced at NaCl concentration gradient of 0.47 %, a salt gradient was not significantly different from that of RVH. The maximum hemolysis in this group was the same NaCl concentration gradient as that of RVH rats, 0.15 %.

The effects of hypertension on osmotic fragility, evaluated from the curves of hemolysis as a function of NaCl concentration, were shown in Fig. 3. The osmotic fragility curve of RVH was the same pattern as that of normotensive ones. The mean corpuscular fragility (MCF) was extrapolated from the osmotic fragility curves. MCF represents the NaCl concentration which has resulted in 50 % hemolysis. The MCF of erythrocytes from RVH (0.28 ± 1.55) was similar to that of normotensive ones (0.29 ± 1.4).

Discussion

Since RVH has been reported to cause oxidative stress, [2,3] one can expect increase of ROS in response to suspected abnormal oxidative damage of biomembrane and erythrocyte hemolysis. In RVH rats, the erythrocyte hemolysis curve mediated by AAPH showed the same fragility as compared to normotensive rats. Similarly, erythrocyte osmotic fragility of both groups failed to differ from each other. The above observation reflects the same intrinsic membrane properties and surface area to volume ratio in erythrocytes of both groups.

Studies with isolated erythrocytes, which have no cell organelles, may provide a simple and reliable measure for oxidative damage because the interaction of AAPH with plasma membranes must be the initial step for cellular damage. The peroxyl radicals generated from azo compound from AAPH induce the chain oxidations of lipids and proteins in the erythrocytes membrane components and eventually

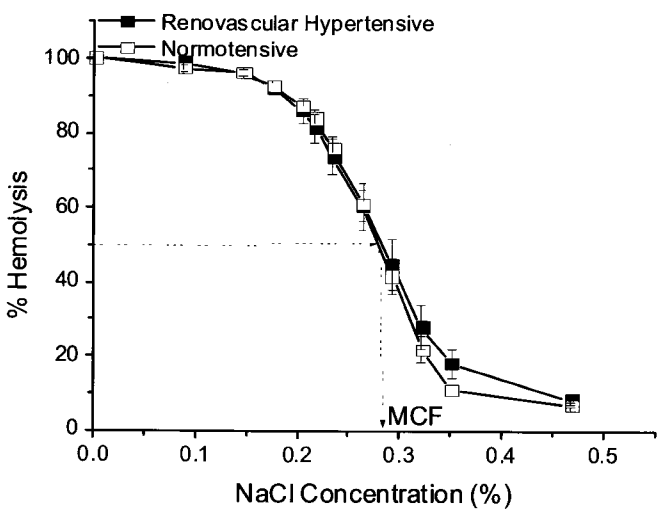

Figure 3. Osmotic fragility curves of RVH and normotensive rats. The degree of hemolysis was calculated by comparing with 0 % NaCl in PBS which represented 100 % lyses. Mean corpuscular fragility (MCF) was NaCl concentration for 50 % hemolysis. Data are expressed as mean ± S.E.M of n = 8 samples.

cause cellular lyses.[14] The oxidation of erythrocytes membranes serves as a model for the oxidative damage of biomembranes. We therefore studied erythrocytes to determine the effect of oxidative stress induce membrane oxidative hemolysis in RVH and normotensive rats.

From the kinetic profile of the time-dependent hemolysis curves, they demonstrated that the susceptibility of rat erythrocyte membrane to oxidative damage had the same degree in both groups. The endogenous antioxidant activity (lag time) in hypertensive groups may not be different than those of normotensive ones. Although the RVH was associated with increase ROS production, there have been no definitive findings on the features of the membrane oxidative damage in RVH erythrocytes. This technique may not sensitive enough to evaluate membrane oxidative injury. It may be implied that NADH/NADHoxidases in RVH are considered as the major sources of ROS in vascular smooth muscle cells more than in erythrocyte membrane.[15] We acknowledge that the present study did not assess all parameters of oxidative stress and antioxidant system. These include malondialdehyde, conjugated dienes, superoxide radical, superoxide dismutase, catalase and glutathione peroxidase. Adding these parameters would increase the validity of our study.

We further investigated the integrity of erythrocytes by measuring the changes in erythrocyte osmotic fragility. The osmotic fragility of erythrocytes reflects their ability to withstand hypotonic gradient, which allows cells to increase their volume by 70 % before the surface membrane is stretched and lyses occur.[16] The cell lyses due to hypotonic solution is related to their shape, deformability, surface area/ volume ratio and intrinsic membrane properties. Osmotic fragility has been applied to the study of membrane permeability and found to be altered in various pathological conditions including cancer, anemia and thalassemia .[17,18] The preliminary results of our study showed that the hematocrits were 48.5 ± 0.5 and 48.5 ± 0.87 in RVH and normotensine rats respectively. In this study, the only one constriction of renal artery was performed and SBP reached peak at 6-8 weeks postoperatively therefore lack of chronic renal failure that leaded to anemia. We found that there was no alteration in osmotic fragility of erythrocytes in RVH rats. This is the first study that has evaluated osmotic fragility in RVH rats, and erythrocyte membrane integrity is negatively affected by RVH.

Garay *et al.*[19] reported that the abnormality in erythrocyte membrane transport have been recognized in patients with essential hypertension but not in those with renovascular hypertension. The intrinsic abnormality of erythrocyte membranes such as lipid composition and fluidity underlines the defects that may be genetically linked to the pathogenesis of hypertension process. Essential hypertension is a polygenic disorder that results from the inheritance of a number of susceptibility genes.[20] Hypertension is a complex phenomenon, and essential hypertension may be different from renovascular hypertension in details. There is a lack of agreement about the membrane alteration that is linked to RVH process. We have approached this question by using 2 techniques to evaluate erythrocyte fragility. In the present data, it can be concluded that erythrocytes of normotensive and RVH rats are susceptible to hemolysis in the same pattern.

Acknowledgments

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