Original Article

In vitro Effect of Ethanol on Erythrocyte Methemoglobin and Reduced Glutathione Concentrations

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Background: *Ethanol is well known as oxidative stress inducing factor. Many studies demonstrated that after alcohol intaking both in experimental animals and human, it has an impact on erythrocytes.* ***Objectives:*** *To study effect of ethanol (EtOH) on Methemoglobin (MetHb) and glutathione (GSH) levels in erythrocytes after in vitro exposure.* ***Materials & Methods***: *Erythrocytes from 20 adult human subjects were treated with 25, 37.5, 50 and 62.5 mg/dL of ethanol (EtOH) as oxidant agent. We compared Methemoglobin (MetHb) and glutathione (GSH) levels in erythrocytes before and after exposure to EtOH.* ***Results:*** *Increasing of EtOH concentration and incubation time caused increasing of MetHb levels ($p < 0.05$). We found that MetHb levels in post-incubated erythrocytes were higher than those in untreated erythrocytes from three to ten times. Moreover, our results were demonstrated that high EtOH concentrations and long incubation time resulted in reducing of GSH levels in erythrocytes ($p < 0.05$). GSH levels in post-incubated erythrocytes were lower than those in untreated erythrocytes ranging from 0.32 to 8.14 mg/dL.* ***Conclusions***: *EtOH as oxidative damage indicator induced MetHb formation and GSH depletion in vitro.*

Keywords: ● *Increased methemoglobin* ● *Reduced glutathione* ● *Ethanol* ● *Erythrocytes*

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บทคัดย่อ

การศึกษาเปรียบเทียบปริมาณเมธฮีโมโกลบินและกลูตาไธโอนในเม็ดเลือดแดงก่อนและหลังการกระตุ้นด้วยเอทานอลที่มีความเข้มข้นต่างกันในหลอดทดลอง

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บทคัดย่อ *เอทานอลเป็นปัจจัยที่กระตุ้นให้เกิดออกซิเดทีฟสเตรท จากการศึกษาจำนวนมากแสดงให้เห็นว่าหลังจากการดื่มแอลกอฮอล์ทั้งในสัตว์ทดลองและมนุษย์มีผลกระทบกับเม็ดเลือดแดง* ***วัตถุประสงค์*** *เพื่อศึกษาเปรียบเทียบระดับเมธฮีโมโกลบินและกลูตาไธโอนในเม็ดเลือดแดงก่อนและหลังการกระตุ้นด้วยเอทานอลที่มีความเข้มข้นต่างกันในหลอดทดลอง* ***วิธีการศึกษา*** *นำเม็ดเลือดแดงมากระตุ้นด้วยเอทานอลที่มีความเข้ม 25, 37.5, 50 และ 62.5 มิลลิกรัม/เดซิลิตร และวัดปริมาณเมธฮีโมโกลบินและและกลูตาไธโอนก่อนและหลังการกระตุ้นด้วยเอทานอลเมื่อครบ 1, 2 และ 3 ชั่วโมงตามลำดับ* ***ผลการศึกษา*** *เมื่อเพิ่มความเข้มข้นของเอทานอลและเวลาในการกระตุ้นทำให้ปริมาณเมธฮีโมโกลบินในเม็ดเลือดแดงเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) และพบว่าปริมาณเมธ-ฮีโมโกลบินหลังจากการกระตุ้นมีมากกว่าก่อนถึง 3-10 เท่า ส่วนปริมาณของกลูตาไธโอนในเม็ดเลือดแดงลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเพิ่มความเข้มข้นของเอทานอลและเวลาในการกระตุ้น เมื่อเปรียบเทียบปริมาณกลูตาไธโอนก่อนและหลังการกระตุ้นมีประมาณ 0.32-8.14 มิลลิกรัม/เดซิลิตร* ***สรุป*** *เอทานอลมีผลทำให้มีการเพิ่มขึ้นของระดับเมธฮีโมโกลบินแต่ทำให้ระดับกลูตาไธโอนลดลงในเม็ดเลือดแดงในหลอดทดลอง*

คำสำคัญ: ● *การเพิ่มขึ้นของเมธฮีโมโกลบิน* ● *การลดลงกลูตาไธโอน* ● *เอทานอล* ● *เม็ดเลือดแดง*

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Introduction

Oxidative stress in erythrocytes can occur in many pathways and affect chemical change. It can cause hemoglobin (HB) oxidizing to methemoglobin (MetHb) that contains oxidized ferric iron Fe^{+3} rather than the reduced ferrous form Fe^{+2} found in Hb. Ferric iron has slightly greater affinity for oxygen which shifts the oxygen dissociation curve to the left resulting in decreased release of oxygen in tissues.[1-5] Ethanol is well known as oxidative stress inducing factor. Several studies demonstrated that after alcohol intaking both in experimental animals and human, it has an impact on many organs such as liver, heart and cells especially erythrocytes.[5-8] From the experiment in rats, they showed that a 2 mL/kg dose of EtOH inhibited G6PD activity significantly, decreased inhibition rate after EtOH admistration and I_{50} from *in vitro* study was 17 mM.[5] Turkeys were given ethanol in drinking water for 15 weeks had *in vivo* indications of heart dysfunction but levels of the antioxidant enzymes SOD, catalase and glutathione peroxidase were elevated and 10% decreasing in GSH.[6] Studying in rat model demonstrated that they were significantly decreased in erythrocyte superoxide dismutase and Na+, K(+)-ATPase activities, but catalase levels were sharply increased in one hour after ethanol intoxication.[7] Bulle S and his group carried out the study in human male volunteers aged between 35-45 years with a drinking history of 8-10 years. They found that plasma marker enzymes AST, ALT, ALP and γGT, plasma and erythrocyte membrane lipid peroxidation, erythrocyte lysate nitric oxide (NOx) levels were increased in alcoholic subjects.[8] GSH plays an important role in the detoxification of ethanol. Many studies demonstrated that Ethanol induced a decrease in GSH (reduced glutathione) concentration in hepatocytes.[9-13] Therefore, ethanol administration may lead to GSH depletion in the red blood cells (RBCs) because EtOH in the bloodstream can penetrate into erythrocytes.

The purpose of this study was to evaluate *in vitro* exposure of erythrocytes samples to ethanol which induced increasing of erythrocyte MetHb levels and decreasing erythrocyte GSH levels. MetHb concentration was measured in ethanol-treated erythrocytes having a maximum absorbance at a wavelength of 630 nm. GSH concentration was determined by evaluating the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) by sulfhydryl compounds from the formation of a yellow colored anionic product whose absorbance was measured at 412 nm. Data were analyzed using Student's *t-test* and were expressed as the mean ± standard deviation. A *p-value* of < 0.05 was considered significant.

Materials and Methods

Preparation these reagents: Ethanol, 1.67% metaphosphoric acid, 0.3 M disodium hydrogenphosphate (Na_2HPO_4), 0.9% sodium chloride, 5% triton X-100, 1% potassium cyanide (KCN), 10% potassium ferricyanide [$K_3(Fe(CN_6))$] and 0.2% 5,5'-dithiobis-2-nitrobenzoic acid (DTNB).

Blood samples and preparation of ethanol-treated erythrocytes

Fresh blood samples were obtained from 20 non-smoking healthy adults. All volunteers wrote consent form before blood taking. 5 mL venous blood sample was collected from each subject in K3-EDTA coated tubes after overnight fast and processed immediately. The whole blood samples were determined hemoglobin concentration in g/dL. The samples were centrifuged at 3,000 ×*g* for 10 minutes. The plasma and the buffy coat were removed by aspiration, and the erythrocytes were washed three times with 0.9% NaCl. Red blood

cells were exposed to EtOH as oxidant agent by suspending one volume of erythrocytes in nine volumes of different concentrations (25%, 37.5%, 50%, and 62.5%) of EtOH. This procedure resulted in the conversion of Hb to MetHb. The EtOH-treated samples were incubated at 37°C with continuous shaking for 1, 2 and 3 hours. These samples served for MetHb and GSH measurements at 1, 2 and 3 hours after EtOH adding. MetHb formation and GSH inhibition were determined as oxidative damage indicators.

Measurement of methemoglobin

Place 2.0 mL of 0.3 M phosphate buffer into each of two test tubes (A and B). Add 100 μL of the ethanol-treated red blood cell and 3 ml of 5% triton X-100 into each tube and mixed. The diluted specimen in tube A was read in a spectrophotometer at 630 nm and the absorbance reading noted (D1), and then 50 μL of 1% potassium cyanide was added to the mixture to convert MetHb to cyanmethemoglobin and the absorbance was read (D2). MetHb concentration was calculated as OD1-OD2. 50 μL of 10% potassium ferricyanide was added into tube B and recorded the absorbance (D3), then 50 μL of 1% potassium cyanide was added to the mixture and recorded the absorbance (D4). Hb concentration was calculated as OD3–OD4. Calculation of MetHb concentration expressed as percentage of Hb concentration as following fomular.

$$\%\text{MetHb} = \frac{(\text{OD1-OD2}) \times 100\%}{(\text{OD3-OD4})}$$

Reduced glutathione determination

Aliquot of 200 μL of EtOH-treated red blood cells was hemolyzed with 1.8 mL of distilled water and 3.0 ml of 1.67% metaphosphoric acid and filtered. 100 μL of the hemolysate was added to 400 μL of 0.3 M Na_2HPO_4 buffer, and followed by 50 μL of 0.2% 5,5'-dithiobis(2-nitrobenzoic acid), the absorbance was then recorded at 412 nm. GSH concentration was calculated as followed.

$$\text{GSH mg/mL erythrocyte} = \frac{\text{OD} \times 12.5 \times 307.3}{\% \text{ hematocrit}}$$

Results

The effect of EtOH on erythrocyte MetHb concentration

In order to investigate the effect of EtOH on MetHb concentration in RBCs, we designed to expose RBCs with EtOH in various time and measure MetHb at the different time line (*see Materials and Methods*). As shown in Table 1 and Figure 1, there was a statistically significant increase in MetHb concentration in erythrocytes exposed to increasing concentrations of EtOH and vary incubation time compared with the control untreated erythrocytes. Untreated erythrocytes MetHb levels measured at 1, 2 and 3 hours were 1.69, 2.10 and 2.92%, respectively. Erythrocyte MetHb measured at 1, 2 and 3 hours post-incubation with 25 mg/dL EtOH was 3.16, 4.17 and 5.31%, respectively, with 37.5 mg/dL EtOH was 3.99, 5.07 and 6.01%, respectively, with 50 mg/dL EtOH was 4.75, 6.74 and 9.28%, respectively,

Table 1 Erythrocytes methemoglobin measured at 1, 2 and 3 hours incubation with 25, 37.5%, 50%, and 62.5% of ethanol.

Time	Methemoglobin (mean ± SD) treated with Ethanol				
	Untreated control	25 mg/dL	37.5 mg/dL	50 mg/dL	62.5 mg/dL
1 h	1.69 ± 0.86	3.16 ± 0.94	3.99 ± 1.15	4.75 ± 1.15	6.07 ± 1.24
2 h	2.10 ± 0.84	4.17 ± 1.49	5.07 ± 1.47	6.74 ± 2.47	8.89 ± 3.47
3 h	2.92 ± 1.44	5.31 ± 2.29	6.00 ± 2.39	9.28 ± 4.29	20.80 ± 14.58

Table 2 Erythrocytes reduced glutathione levels measured at 1, 2 and 3 hours incubation with 25, 37.5%, 50%, and 62.5% of ethanol.

Time	Reduced glutathione (mean ± SD) treated with Ethanol				
	Untreated control	25 mg/dL	37.5 mg/dL	50 mg/dL	62.5 mg/dL
1 h	24.40 ± 3.75	24.08 ± 5.12	21.35 ± 4.55	19.27 ± 3.49	15.59 ± 2.75
2 h	22.61 ± 4.30	19.25 ± 3.01	17.56 ± 2.89	16.69 ± 3.04	14.47 ± 3.79
3 h	21.76 ± 5.05	19.15 ± 5.96	17.47 ± 6.22	17.73 ± 9.07	18.67 ± 8.39

and with 62.5 mg/dL EtOH was 6.07, 8.89 and 20.80%, respectively. The mean MetHb concentration in the control group was 2.92 by 3 hours whereas with the highest EtOH concentration (62.5 mg/dL) the erythrocytes MetHb was 20.80, there was a statistically significant increase in MetHb concentration.

The effect of EtOH in erythrocyte GSH concentration

As shown in Table 2, there was a statistically significant decrease in GSH concentration in erythrocytes exposed to increasing concentrations of EtOH and incubation time compared with the control untreated erythrocytes. The mean GSH concentration in the control group was 21.76 whereas with the highest EtOH concentration (62.5 mg/dL) the erythrocytes GSH was 18.67 by 3 hours, there was a statistically significant decrease in GSH concentration.

Discussion

Incubation of erythrocytes with oxidant increases MetOH levels accompanied by decrease in GSH levels and induction of oxidative stress in erythrocytes. GSH plays an important role in the detoxification of EtOH and leads to GSH depletion in the red blood cells. In this study, EtOH was able to both induce MetHb formation and a decrease in GSH concentration in erythrocytes *in vitro*. MetHb and GSH levels were measured by 1, 2, and 3 hours after incubation the erythrocytes with EtOH (25%, 37.5%, 50%, and 62.5%). These results indicated that with higher concentration of EtOH and longer incubation time induced an increase in MetHb concentration and a decrease in GSH.

Conclusion

In conclusion, the results observed in this study demonstrated that human erythrocytes incubated with EtOH *in vitro*, there was an increase in MetHb concentration and a decrease in GSH. Further studies are required to determine the amount of EtOH diffuse into the erythrocytes having direct effect to oxidative stress.

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