

## CYTOCHROME B-5 REDUCTASE ACTIVITY AND METHEMOGLOBINEMIA IN HUMAN RED BLOOD CELLS OF THAI PATIENTS RECEIVING NITROGLYCERIN

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### ABSTRACT

Nitroglycerin and other organic nitrates have been used routinely in the treatment of angina pectoris and congestive heart failure. Clinical significant methemoglobinemia during administration of organic nitrates, including intravenous nitroglycerin, has been occasionally reported as a serious complication. In normal individuals, methemoglobin in red blood cell is maintained at a very low level (<1%). It is immediately reduced by cytochrome b<sub>5</sub> reductase to the normal hemoglobin moiety. Since acquired methemoglobinemia is not only resulted from exposure to a variety of oxidizing agents but also related to cytochrome b<sub>5</sub> reductase deficiency, the present study was aimed to investigate the relationship of cytochrome b<sub>5</sub> reductase activity and methemoglobinemia. 114 normal blood samples from male and female healthy volunteers of 20–65 years old were used as control group and 40 blood samples of patients receiving 24,000–104,000 µg (0.5–3.0 µg/kg/min) intravenous nitroglycerin were collected. The concentrations of methemoglobin in 57 men and 57 women healthy volunteers were  $0.05 \pm 0.02$  and  $0.08 \pm 0.02$  % of total hemoglobin (mean  $\pm$  SE) respectively. The cytochrome b<sub>5</sub> reductase activity in male was lower ( $p < 0.05$ ) than female. No severe methemoglobinemia was found in these patients. However, the methemoglobin concentration and cytochrome b<sub>5</sub> activity in the treatment group were significantly higher ( $P < 0.05$ ) than the normal group. Our results showed that methemoglobin content of erythrocyte was independent on the activity of cytochrome b<sub>5</sub> reductase. It was found that intravenous nitroglycerin administration in the total doses of 24,000–104,000 µg produced not only slight increase of methemoglobin content but also the significant increase of their cytochrome b<sub>5</sub> reductase activity. However, the increased methemoglobin levels of these patients were found to be within an acceptable level of normal condition. In conclusion, clinically significant methemoglobinemia was uncommon with low doses of intravenous nitroglycerin administration. In addition, the correlation between cytochrome b<sub>5</sub> reductase activity and nitroglycerin administration should be further investigated.

**Key words :** methemoglobin, nitroglycerin, cytochrome b<sub>5</sub> reductase

## สมรรถนะของเอนไซม์ไซโตโครมบี 5 รีดักเตสและภาวะเมทฮีโมโกลบินสูงในเม็ดเลือดแดงของผู้ป่วยไทยที่ได้รับไนโตรกลีเซอริน

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### บทคัดย่อ

ไนโตรกลีเซอรินและสารประกอบอินทรีย์ในเตรทเป็นยาที่ใช้รักษาอาการปวดเค้นหน้าอกและสภาวะหัวใจล้มเหลว สภาวะเมทฮีโมโกลบินในเลือดสูงเป็นฤทธิ์ไม่พึงประสงค์อย่างรุนแรงที่เกิดขึ้นจากยาในกลุ่มนี้รวมทั้งการให้ไนโตรกลีเซอรินทางหลอดเลือดดำ ในคนปกติระดับเมทฮีโมโกลบินในเม็ดเลือดแดงมีอยู่ในระดับที่ต่ำมากคือน้อยกว่า 1 เปอร์เซ็นต์ โดยมีเอนไซม์ไซโตโครมบี 5 รีดักเตสทำหน้าที่รีดิวส์เมทฮีโมโกลบินในเลือดให้กลับไปเป็นฮีโมโกลบิน เนื่องจากสภาวะเมทฮีโมโกลบินในเลือดที่สูงขึ้นไม่ได้เกิดจากการสัมผัสกับสารที่เกิดออกซิเดชันได้ง่ายเท่านั้น แต่ยังสัมพันธ์กับการขาดเอนไซม์ไซโตโครมบี 5 รีดักเตสอีกด้วย การศึกษาครั้งนี้จึงต้องการตรวจหาความสัมพันธ์ของสมรรถนะเอนไซม์ไซโตโครมบี 5 รีดักเตสและสภาวะเมทฮีโมโกลบินสูงในเลือด กลุ่มควบคุมเป็นตัวอย่างเลือดจำนวน 114 ตัวอย่าง ที่ได้จากผู้บริจาคเลือดที่มีสุขภาพดีทั้งหญิงและชาย อายุ 20-65 ปี และตัวอย่างเลือดจากผู้ป่วยที่ได้รับการรักษาด้วยไนโตรกลีเซอรินทางหลอดเลือดดำในขนาด 24,000-104,000 ไมโครกรัม (0.5-3.0 ไมโครกรัม/กก./นาที) ค่าเฉลี่ย  $\pm$  ความคลาดเคลื่อนมาตรฐานของความเข้มข้นของเมทฮีโมโกลบินในชายและหญิงสุขภาพดีเป็น  $0.05 \pm 0.02$  และ  $0.08 \pm 0.02$  เปอร์เซ็นต์ของปริมาณฮีโมโกลบินทั้งหมดตามลำดับ สมรรถนะของไซโตโครมบี 5 รีดักเตสในเพศชายมีค่าต่ำกว่าเพศหญิงอย่างมีนัยสำคัญทางสถิติ ( $p < 0.05$ ) ไม่พบการเกิดภาวะเมทฮีโมโกลบินในเลือดสูงอย่างรุนแรงในผู้ป่วย อย่างไรก็ตามความเข้มข้นของเมทฮีโมโกลบินและสมรรถนะของไซโตโครมบี 5 รีดักเตสในกลุ่มที่ได้รับยาสูงกว่ากลุ่มคนปกติอย่างมีนัยสำคัญ ผลการศึกษาแสดงให้เห็นว่า ปริมาณเมทฮีโมโกลบินในเม็ดเลือดแดงไม่ได้ขึ้นกับสมรรถนะของเอนไซม์ไซโตโครมบี 5 รีดักเตส พบว่าการให้ยาไนโตรกลีเซอรินทางหลอดเลือดดำในขนาดรวม 24,000-104,000 ไมโครกรัม ทำให้เมทฮีโมโกลบินเพิ่มขึ้นเพียงเล็กน้อย แต่มีผลทำให้สมรรถนะของเอนไซม์บี 5 รีดักเตสเพิ่มขึ้นอย่างมีนัยสำคัญ และค่าเมทฮีโมโกลบินที่เพิ่มขึ้นในผู้ป่วยเหล่านี้ยังอยู่ในระดับที่เป็นปกติ โดยสรุปภาวะเมทฮีโมโกลบินในเลือดที่มีความสำคัญทางคลินิกไม่ค่อยเกิดขึ้นเมื่อได้รับไนโตรกลีเซอรินในขนาดต่ำๆ อย่างไรก็ตามก็ควรศึกษาต่อไปถึงความสัมพันธ์ระหว่างการเพิ่มขึ้นของสมรรถนะของเอนไซม์ไซโตโครมบี 5 รีดักเตสกับการได้รับไนโตรกลีเซอริน

คำสำคัญ : เมทฮีโมโกลบิน, ไนโตรกลีเซอริน, ไซโตโครมบี 5 รีดักเตส

## INTRODUCTION

Methemoglobinemia is a clinical condition characterized by cyanosis resulting from decreased oxygen transport. Methemoglobinemia may be inherited (e.g., abnormal hemoglobin or deficiency in methemoglobin reductase activity) or acquired. Acquired methemoglobinemia can result from exposure to certain drugs and chemicals such as nitrates and other direct oxidants, local anesthetics, aniline dyes and sulfonamides<sup>1</sup>. Organic nitrates such as nitroglycerin are often given intravenously at the onset of acute myocardial infarction. It is also very effective in the management of pain in patients with unstable angina. Nitrates caused venodilation and relief coronary vasoconstriction to ameliorate anginal attacks<sup>2</sup>. Besides common adverse effects of nitroglycerin, including headache and hypotension, clinical significant methemoglobinemia caused by denitration of nitroglycerin has been occasionally reported as a serious complication of intravenous nitroglycerin (NTG)<sup>3-5</sup>. In human erythrocytes, methemoglobin is reduced to oxyhemoglobin both by enzymatic and non-enzymatic systems. Under physiological conditions, methemoglobin reduction is accomplished mainly by red cell NADH-cytochrome b5 reductase. In hereditary cytochrome b5 deficiency and exposure to large amounts of oxidants, methemoglobinemia is postulated to be a potential adverse clinical consequence. This study was aimed to examine a possible correlation between the cytochrome b5 reductase in red blood cell activity and methemoglobin level in blood.

## MATERIALS AND METHODS

### Apparatus

UV-visible recording spectrophotometer (UV-1600A, Shimadzu) and high speed centrifuge (IE CB-22M) were used.

### Reagents

NADH was purchased from Sigma Chemical Company. Dry diethyl-aminoethyl-cellulose (DEAE, DE-52) was purchased from Whatman. Drabkin's solution was obtained from Life Science Dynamics Division of Arnaparn Co., Ltd. Other chemicals were analytical grade and obtained commercially.

### Specimens

Control group: Blood samples of 100 healthy blood donors, 50 males and 50

females obtained from the Thai Red Cross Society, Bangkok, were used as normal control subjects. The age range was 20 to 65 years.

**Sample group:** Blood was withdrawn from 30 patients admitted to the Coronary Critical Care Unit of Chulalongkorn Hospital who were treated with i.v. NTG. Inform consent was obtained from each patient. The age range was 50 to 65 years. 15 were males and 15 were females. NTG in concentration of either 100 or 400 µg/ml was infused continuously. The infusion rate was started at 5-10 µg/min and increased as tolerated to 25-50 µg/min. The total doses of NTG were between 24,000-104,000 µg.

Approximately 3 ml whole blood was freshly collected in a plastic tube with EDTA as anticoagulant and placed on ice for immediate transport to the laboratory, where it was analyzed at once for methemoglobin content and within 12 hours for cytochrome b5 reductase activity.

### Assay of methemoglobin in blood

Methemoglobin content was measured by micromethod described by Hegesh et al.<sup>6</sup>.

### Assay of NADH-cytochrome b5 reductase activity

NADH-cytochrome b5 reductase activity of erythrocytes was determined according to the method of Hegesh et al.<sup>7</sup>. The enzyme activity was measured in a reaction mixture which contained 20 µl of 0.25 M EDTA (pH 7), 100 µl of 0.05 M citrate buffer (pH 4.7), 3 ml of 0.5 mM potassium ferricyanide, 2 ml of 0.18 mM hemoglobin substrate (free from cytochrome b5 reductase), 3.48 ml distilled water and 10 µl of blood sample. Mixed and centrifuged the solution at 1500 x g for 5 minutes. 950 µl of hemolysate was pipetted in a cuvette having 1 cm light path and 50 µl of NADH was added and mixed. The reference cuvette contained the same constituents except NADH. The mixture was measured for increasing rate of absorbance at 575 nm.

### Statistical analysis

Results are expressed as mean ± standard error of the mean. Unpaired Student t-test was used to compare groups. Correlation between methemoglobin content and cytochrome b5 reductase activity in normal and treated groups were determined. P value < 0.05 was considered significant.

## RESULTS

There were age and sex variations of methemoglobin levels in normal erythrocytes. As shown in Table 1, there were significant difference between age groups and sexes. The average values  $\pm$  one standard error of mean of methemoglobin contents of male and female were found to be  $0.09 \pm 0.02\%$  and  $0.08 \pm 0.02\%$  respectively. Methemoglobin levels in both sexes ranged from 0 to 0.40%. The highest contents of methemoglobin in both sexes were in the age group of 41-50 years.

Results of cytochrome  $b_5$  reductase activities in male and female are shown in Table 2. The mean values  $\pm$  standard error of mean of cytochrome  $b_5$  reductase of male and

female were found to be  $3.12 \pm 0.05$  and  $3.32 \pm 0.05$  respectively.

Table 3 shows the levels of methemoglobin and cytochrome  $b_5$  reductase activity of normal subjects and treated patients. Significant increases of methemoglobin level and cytochrome  $b_5$  reductase activity in treated group were observed. When compared with the normal group with the same age of patients (> 50 years old), nitroglycerin produced significant increase in the amount of methemoglobin content and cytochrome  $b_5$  reductase activity.

No correlation between methemoglobin level and cytochrome  $b_5$  reductase activity was found in both normal group ( $r = 0.5$ ) and treated group ( $r = 0.25$ ).

Table 1. Methemoglobin content in normal volunteers.

Group	Sex Age (years)	N	Methemoglobin content (%)		Comparison	P value
			Range	Mean $\pm$ SE		
A	Male : 20-30	15	0	0	A vs B A vs C A vs D	<0.05 <0.05 <0.05
B	31-40	15	0-0.31	$0.04 \pm 0.03$	B vs C B vs D	<0.05 <0.05
C	41-50	15	0-0.33	$0.09 \pm 0.04$	-	-
D	50 <sup>+</sup>	12	0-0.38	$0.09 \pm 0.05$	-	-
E	TOTAL	57	0-0.38	$0.09 \pm 0.02$	E vs J	<0.05
F	Female : 20-30	15	0	0	F vs G F vs H F vs I	<0.05 <0.05 <0.05
G	31-40	15	0-0.39	$0.09 \pm 0.04$	G vs I	<0.05
H	41-50	15	0-0.39	$0.11 \pm 0.04$	H vs I	<0.05
I	50 <sup>+</sup>	12	0-0.40	$0.06 \pm 0.04$	-	-
J	TOTAL	57	0-0.40	$0.08 \pm 0.02$	E vs J	<0.05

Table 2. Cytochrome  $b_5$  reductase activities in normal volunteers.

Group	Sex Age (years)	N	Cytochrome $b_5$ reductase activity		Comparison	P value
			Range	Mean $\pm$ SE		
A	Male : 20-30	15	2.79-3.46	$3.05 \pm 0.06$	A vs B A vs C A vs D	<0.05 <0.05 <0.05
B	31-40	15	2.76-3.94	$3.11 \pm 0.09$	B vs S	<0.05
C	41-50	15	2.76-4.20	$3.09 \pm 0.11$	C vs D	<0.05
D	50 <sup>+</sup>	12	2.76-2.73	$3.26 \pm 0.18$	-	-
E	TOTAL	57	2.73-4.20	$3.12 \pm 0.05$	E vs J	<0.05
F	Female : 20-30	15	2.82-3.83	$3.35 \pm 0.09$	F vs G F vs H F vs I	<0.05 <0.05 <0.05
G	31-40	15	2.78-4.26	$3.29 \pm 0.09$	G vs H G vs I	<0.05 <0.05
H	41-50	15	2.89-3.87	$3.51 \pm 0.11$	H vs I	<0.05
I	50 <sup>+</sup>	12	2.78-3.64	$3.11 \pm 0.07$	-	-
J	TOTAL	57	2.78-4.26	$3.32 \pm 0.05$	E vs J	<0.05



**Table 3.** Comparison of methemoglobin level and cytochrome b<sub>5</sub> reductase in erythrocytes between normal subjects and patients receiving nitroglycerin.

Group	N	Methemoglobin content (%)	Cytochrome b <sub>5</sub> reductase activity (unit/min/g of hemoglobin)
		Mean ± SE	Mean ± SE
Normal	114	0.09 ± 0.01	3.22 ± 0.04
Normal (50-60)	25	0.09 ± 0.03	3.17 ± 0.06
Patients	40	0.10 ± 0.03*	3.34 ± 0.06

\* P&lt;0.05

## DISCUSSION

Methemoglobinemia is a clinical condition in which more than 1% of hemoglobin is oxidized to methemoglobin<sup>1</sup>. Methemoglobinemia may be inherited or acquired. The autosomal recessive condition results from an absence of cytochrome b<sub>5</sub> reductase that normally converts methemoglobin back to normal hemoglobin thereby leading to an increase in methemoglobin. Acquired methemoglobinemia is the most common form of methemoglobinemia and follows exposure to drugs or toxins. It is usually mild and poses no major clinical problems. Occasionally, however, it may be severe or even fatal<sup>1</sup>. The lethal level of methemoglobin in human is above 70%<sup>8</sup>.

Clinical significant elevation of methemoglobin level has been reported with the administration of nitroglycerin<sup>3,5,9-13</sup>. The results from our study showed that the usual dose of i.v. NTG caused no clinical significant methemoglobinemia. None of the patients had methemoglobin level more than 1%. Thus,

intravenous NTG administration in the total doses of 24,000-104,000 µg produced no clinical significant methemoglobinemia.

It has been previously reported that variation of cytochrome b<sub>5</sub> reductase activity is an age and sex dependent<sup>14-16</sup>. Our results was correlated well with the study of Moore et al.<sup>16</sup> which found a significant difference in erythrocyte enzyme activity between 270 Jewish males and 285 Jewish females ranging in age from 12 to 77 years. However, these methemoglobin levels were weakly correlated positively with the activity of cytochrome b<sub>5</sub> reductase in the treated group. In contrast to the study in patients with hereditary methemoglobinemia of Matsuki et al. (1981)<sup>17</sup>, cytochrome b<sub>5</sub> reductase activities in NTG-treated patients were significantly increased. The same incidence of increasing cytochrome b<sub>5</sub> reductase activity has also been reported recently in population exposed to high nitrate concentration in drinking water<sup>18</sup>. In view of these considerations, a possibility of adaptation of cytochrome b<sub>5</sub> reductase activity is suggested to be a regulatory mechanism.

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