A BIOCHEMICAL STUDY ON HEMOGLOBIN

Enas, A. O.; Shehata, Y. M.; Eldawy, K. A.; Ghanem, A. A.*

Biochemistry Dept., Faculty of Vet. Medicine, Zagazig University;

* Forensic Science&Toxicology Dept., Faculty of Medicine, Mansoura University, Egypt

ABSTRACT

Organic nitrates are nitric oxide donors used as vasodilators in cardiovascular diseases. Hemoglobin binds to NO with great affinity and forms methemoglobin by oxidation in the erythrocyte, as a consequence an increase in reactive oxygen species (ROS) as superoxide and reactive nitrogen species (RNS) as peroxynitrite which may increase oxidative stress and lipid peroxidation and alter the erythrocyte antioxidant defense system. The aim of the present work is to study some biochemical effects of organic nitrates on hemoglobin and to evaluate the role of vitamin C (Ascorbic acid) as an antioxidant agent against the biochemical changes induced by different doses of organic nitrates (isosorbide mononitrate) given orally to male rats. The results revealed that treatment with organic nitrates caused marked elevation in the level of lipid peroxidation (estimated by the amount of MDA) and a decline in SOD, GSH and CAT activities accompanied with an increase in the rate of hemoglobin autoxidation and a decrease in iron and TIBC. The results also demonstrated a marked increase in hemoglobin derivatives as methemoglobin and carboxyhemoglobin. Treatment with vitamin C significantly reduced the changes caused by organic nitrates in all examined parameters. These results indicate that alterations caused by organic nitrates are connected with free radicals generation and used antioxidant effectively protect against intoxication.

Key words: *Organic nitrates, Vitamin C, Hemoglobin, Methemoglobin.*

INTRODUCTION

Organic nitrates are among the oldest and most commonly used drugs for the treatment of cardiovascular disease **(Tommaso and John, 2004)**. Organic nitrates are vasodilator drugs that cause the muscle in the walls of the blood vessels to relax, allowing the vessels to dilate. Organic nitrates are nitric oxide (NO) donors; in the vascular smooth muscle, nitric oxide induces relaxation, resulting in vasodilation of arteries and veins **(Jonathan, 1996)**. Xenobiotics like organic nitrates and nitrites are well-known medicinal agents, but they may also constitute endogenous, naturally occurring products of the reactions of NO, peroxynitrite and NO2 with lipids, and other biomolecules under oxidative and nitrosative stress **(Adrian et al., 2004)**. Therapy with different organic nitrates such as nitroglycerin, Isosorbide dinitrate and isosorbide mononitrate leads to increases in oxidative stress **(Thomas et al., 2007)**. Treatment with organ-

ic nitrates stimulates the production of reactive oxygen species (ROS) within the vascular tissue **(Munzel and Harrison, 1997)**. Superoxide dismutase (SOD), catalase and glutathione provide cellular protection against the damage caused by free radicals and reactive oxygen species (ROS). Measurement of these enzyme activities is an indirect and noninvasive method that could be used to assess oxidative stress **(Valko et al., 2006)**. Antioxidant enzymes in the blood are responsible to convert methemoglobin back to hemoglobin, and maintain methemoglobin levels normally do not exceed 1.0% **(Margret et al., 2007)**. Organic nitrates induced oxidation of hemoglobin (Hb) resulted in methemoglobin (MetHb) formation and O2.- generation, which caused lipid peroxidation **(Markovic et al., 2006)**. Vitamin C (Ascorbic acid) is a sugar acid with antioxidant properties, ascorbate is an antioxidant, since it protects the body against oxidative stress and is a cofactor in several vital enzymatic reactions **(Padayatty et al., 2003)**.

MATERIAL AND METHODS

Fourty two male wistar rats weighing about 150-180g were used in these experiments. Isosorbide -5- mononitrate (ISMN) was used as organic nitrate. It was obtained in the form of tablets, dissolved in water and given orally at three different elevated doses (1.7 mg/ kg, 3.4 mg/ kg, and 6.8 mg/ kg body weight) for a period of 2 months. Vitamin C was dissolved in water, applied as a freshly prepared and given orally at a dose of (20 mg/ kg body weight). Animals were divided into seven main groups as follow: Group (1) was the untreated control group. Group (2): given ISMN1.7 mg/ kg. Group (3): given ISMN1.7 mg/kg followed by vitamin C. Group (4): given ISMN 3.4 mg/ kg. Group (5): given ISMN 3.4 mg/ kg followed by vitamin C. Group (6): given ISMN 6.8 mg/ kg. Group (7): given ISMN 6.8 mg/ kg followed by vitamin C. Blood was collected to investigate: Hemoglobin according to **(Moore et al., 1981)**. Methemoglobin **(Mitchell et al., 2001)**, carboxyhemoglobin, oxygen saturation, and oxygen content **(Zwart et al.,1984)**, hematocrit **(Brian et al., 2000)**, red blood cells **(Rodak, 1995)**, white blood cells **(Baker and Silverton, 1985)**, nitric oxide **(Montgomery and Dymock, 1961)**, glutathione (Beutler et al., 1963), catalase **(Cohen et al., 1970)**, superoxide dismutase (Dechatelet et al., 1974), malondialdehyde **(Draper and Hadley, 1990)**, iron and total iron binding capacity **(Perrotta, 1984)**, inorganic phosphorus **(Tietz, 2004)** and hemoglobin autoxidation rate **(Wallace, 1982)**. Data were analyzed using SPSS program (Statistical Package for the Social Sciences) version 13.

RESULTS

In groups given ISMN a significant decrease in hemoglobin, hematocrit, red and white blood cells, glutathione, catalase, superoxide dismutase, oxygen saturation, oxygen content, iron, total iron binding capacity, nitric oxide, and inorganic phosphorus comparing with control group. While a significant increase in methemoglobin, carboxyhemoglobin, malondialdehyde, and hemoglobin autoxidation rate. While in groups given vitamin C following ISMN a significant increase in hemoglobin, hematocrit, red and white blood cells, glutathione, catalase, superoxide dismutase, oxygen saturation, oxygen content, iron, total iron binding capacity, nitric oxide, and inorganic phosphorus. While a significant decrease in methemoglobin, carboxyhemoglobin,

malondialdehyde, and hemoglobin autoxidation rate comparing with groups given ISMN only of the same dose.

DISCUSSION

In the present study the decrease in hemoglobin concentration may be due to the effect of nitric oxide and nitrate ions liberated during isosorbide mononitrate metabolism which can oxidize hemoglobin to methemoglobin **(Bouchard et al. 1992)**. While the antioxidant effect of vitamin C that protects the blood from the oxidant effect of nitric oxide induces an improvement in hemoglobin **(Mary et al., 2005)**. Nitrate ions liberated during metabolism of isosorbide mononitrate can oxidize hemoglobin into methemoglobin **(Elizabeth, 2007). Lichstein et al., (2000)** reported an increase in methemoglobin concentration using ordinary doses of organic nitrates. Decreasing in methemoglobin concentration by using vitamin C may be due to the antioxidant effect of ascorbic acid (vitamin C). **Jacqueline et al., (2006)** recorded that the reduction of methemoglobin to hemoglobin by vitamin C is the nonenzymatic pathway that reducing methemoglobin and protect erythrocyte and concluded that erythrocyte alone had a negligible ability to reduce methemoglobin in the absence of exogenous ascorbate. Carboxyhemoglobin showed a significant increase in high doses only this may be due to the impairment in the antioxidant enzymes defense system of the erythrocyte which may make material changes in hemoglobin system by elevation of abnormal hemoglobin derivatives as carboxyhemoglobin **(Ersteniuk, 2002)**. Methemoglobinemia may also reduce the oxygen carrying capacity of the blood because methemoglobin cannot bind oxygen which reduce saturated oxygen and oxygen content. This decrease is significant in high doses **(Stepuro and Zinchuk, 2006)**. Reduction of hematocrit, RBC, and WBC may be a consequence of hemoglobin reduction and oxidative stress induced by nitric oxide. Highly significant decrease in the activity of SOD, and significant decrease in GSH level and CAT activity as well as very highly significant increase in MDA concentration may be due to increase of ROS and RNS these intermediates may participate in reactions giving rise to free radicals which increase by increasing ISMN dose **(Oxis, 2006)**. Vitamin C acts as a free radical scavenger may reduce the effect of free radicals **(Sally et al., 2003)**. Reduction of total phosphate at the expense of its organic part with a simultaneous increase of the role of inorganic phosphate during increase of methemoglobin in the erythrocytes after traditional therapy the methemoglobin level continued to rise while inorganic phosphate decreased in the erythrocyte. On the other hand, an improvement in inorganic phosphorus may be due to the protective role of vitamin C as an antioxidant which decrease lipid peroxidation and oxidative stress this protective role increase inorganic phosphorus concentration **(Annette, 2002)**.

The present study showed a significant decrease in serum iron concentration and a significant decrease in total iron binding capacity (TIBC) in all ISMN treated groups. This result may be due to the nitric oxide effects on blood which increased with ISMN dose and also may be due to oxidative stress induced by nitric oxide which may decrease serum iron and decrease total iron binding capacity (TIBC) **(Joann, 2000)**. **Choi et al., (2002)**

recorded that NO concentrations correlated inversely with hemoglobin levels and that iron deficiency anemia increases with NO production, and elevated NO concentrations in iron deficiency anemia return to normal with iron supplementation. On the other hand, an improvement effect on serum iron concentration and total iron binding capacity (TIBC) was noticed by the effect of vitamin C. this improvement may be due to beneficial effect of vitamin C to enhance iron absorption (**Shersten et al., 2007**).

Table (1): Hemoglobin, methemoglobin, carboxyhemoglobin, oxygen saturation and oxygen content in different rat groups.

parameters	Animal Groups							ANOVA	
	control	ISMN 0.26mg	ISMN 0.26+VitC	ISMN 0.52mg	ISMN 0.52+VitC	ISMN 1.04mg	ISMN 1.04+VitC	F	P
Hb (g/dl)	15.43±0.05	13.79±0.11[a] -10.6*	14.69±0.1[a&b] -4.8*& +6.5**	12.71±0.21[a] -17.6*	14.52±0.08[a&b] -5.9*& +14.2**	11.79±0.11[a] -22.4*	14.02±0.13[a&b] -9.1*& +17.1**	94.5	<0.05
Methb (%)	0.15±0.03	1.02±0.01[a] +580*	0.43±0.3[a&b] +187*& -57.6**	1.72±0.04[a] +1047*	0.52±0.04[a&b] +246*& -69.8**	2.48±0.07[a] +1553*	0.6±0.04[a&b] +300*& -75.8**	369	<0.05
HbCO (%)	0.35±0.03	0.55±0.03 +57.1*	0.28±0.05 -20*& -49.1**	0.73±0.07[a] +109*	0.43±0.06[b] +22.9*& -41.1**	1.08±0.03[a] +208*	0.67±0.07[a&b] +91.4*& -38.0**	27.6	<0.05
$SATO_2$ (%)	67.4±0.3	64.3±0.4 -4.6*	66.9±0.6 -0.7*& +4.0**	62.2±0.5[a] -7.7*	65.8±1.04[b] -2.4*& +5.8**	60.7±0.5[a] -9.9*	65.6±1.5[b] -2.8*& +8.1**	7.5	<0.05
O_2ct (%)	16.7±0.06	15.9±0.1[a] -4.8*	16.3±0.04 -2.4*& +2.5**	15.8±0.07[a] -5.4*	16.1±0.07[a] -3.6*& +1.9**	15.4±0.1[a] -7.8*	15.8±0.08[a&b] -5.4*& +2.7**	24.7	<0.05

Results are presented as means ±SE and % of change.
a: Significant difference compared with control untreated groups at $p<0.05$.
b: Significant difference compared with ISMN treated groups of the same dose at $p<0.05$.
*: % of change compared with control untreated groups.
**: % of change compared with ISMN treated groups of the same dose.

Table (2): Hematocrit, red blood cells, white blood cells, nitric oxide, inorganic phosphorus, iron and total iron binding capacity.

parameters	Animal Groups							ANOVA	
	control	ISMN 0.26mg	ISMN 0.26+VitC	ISMN 0.52mg	ISMN 0.52+VitC	ISMN 1.04mg	ISMN 1.04+VitC	F	P
Hct (%)	56.7±0.5	50.7±0.6[a] -10.6*	53.2±0.4[a&b] -6.2*& +4.9**	47.9±0.5[a] -15.5*	51.2±0.4[a&b] -9.7*& +6.9**	45.6±0.5[a] -19.6*	53.7±0.5[a&b] -5.3*& +17.8**	54.4	<0.05
RBCsx10^6	5.5±0.06	4.4±0.09[a] -20.0*	5.2±0.07[b] -5.5*& +18.1**	4.1±0.04[a] -25.5*	5.0±0.04[b] -9.1*& +22.0**	3.8±0.05[a] -30.9*	4.6±0.09[a&b] -16.4*& +21.1**	59.6	<0.05
WBCsx10^3	12.0±0.07	10.8±0.1[a] -10.0*	11.8±0.07[b] -1.7*& +9.3**	9.5±0.1[a] -20.8*	11.7±0.04[b] -2.5*& +23.2**	8.0±0.07[a] -33.3*	10.4±0.06[a&b] -13.3*& +30.0**	311	<0.05
NO (µmol/L)	0.64±0.006	1.04±0.004[a] +62.5*	0.73±0.004[a&b] +14.1*& -29.8**	1.67±0.006[a] +160.9*	0.76±0.004[a&b] +18.8*& -54.5**	2.48±0.004[a] +287.5*	0.8±0.004[a&b] +25.0*& -67.7**	1843	<0.05
p. (mg/dl)	8.7±0.01	7.8±0.01[a] -10.3*	8.2±0.04[a&b] -5.7*& +5.1**	7.7±0.02[a] -11.5*	8.01±0.02[a&b] -7.9*& +4.0**	6.8±0.01[a] -21.8*	7.9±0.01[a&b] -9.2*& +16.2**	1017	<0.05
Fe (µg/dl)	339.5±0.9	300.4±1.0[a] -11.5*	328±1.4[a&b] -3.3*& +9.3**	272.8±2.6[a] -19.6*	316.6±1.0[a&b] -6.7*& +16.1**	226.5±1.1[a] -33.3*	293.6±2.0[a&b] -13.5*& +29.6**	622	<0.05
TIBC (mg/dl)	0.511±0.001	0.499±0.002[a] -2.3*	0.507±0.002[b] -0.8*& +1.6**	0.476±0.002[a] -6.8*	0.498±0.001[a&b] -2.5*& +4.6**	0.462±0.05[a] -9.6*	0.486±0.001[a&b] -4.9*& +5.2**	1096	<0.05

Results are presented as means ±SE and % of change.
a: Significant difference compared with control untreated groups at p<0.05.
b: Significant difference compared with ISMN treated groups of the same dose at p<0.05.
*: % of change compared with control untreated groups.
**: % of change compared with ISMN treated groups of the same dose.

Table (3): Glutathione, catalase, superoxide dismutase an malondialdehyde**.**

parameters	Animal Groups							ANOVA	
	control	ISMN 0.26mg	ISMN 0.26+VitC	ISMN 0.52mg	ISMN 0.52+VitC	ISMN 1.04mg	ISMN 1.04+VitC	F	P
GSH (mmol/Liter cells)	1.63±0.005	1.31±0.003[a] -19.6*	1.4±0.007[a&b] -14.1*& +6.9**	1.21±0.006[a] -25.8*	1.32±0.007[a&b] -19.0*& +7.4**	1.05±0.01[a] -35.0*	1.28±0.01[a&b] 21.5*& +21.9**	571	<0.05
CAT (mg/ml)	32.1±0.1	26.4±0.1[a] -17.8*	30.2±0.1[a&b] -5.9*& +14.4**	24.3±0.1[a] -24.3*	28.3±0.1[a&b] -11.8*& +16.5**	22.7±0.1[a] -29.3*	26.9±0.1[a&b] -16.2*& +18.5**	802	<0.05
SOD (mg/ml)	11.7±0.1	6.5±0.1[a] -44.4*	7.3±0.1[a] -37.6*& +12.3**	3.6±0.1[a] -69.2*	8.8±0.1[a&b] -24.8*& +144.0**	2.8±0.1[a] -76.1*	10.5±0.2[a&b] -10.3*& +275.0**	709	<0.05
MDA (nmol/ml/h)	187.3±1.3	220.7±1.1[a] +17.8*	193.8±1.7[a&b] +3.5*& -12.2**	238.4±1.5[a] +27.3*	213.7±1.0[a&b] +14.1*& -10.4**	339.9±1.9[a] +81.5*	219.4±1.42[a&b] +17.1*& -35.5**	1257	<0.05

Results are presented as means ±SE and % of change.
a: Significant difference compared with control untreated groups at p<0.05.
b: Significant difference compared with ISMN treated groups of the same dose at p<0.05.
*: % of change compared with control untreated groups.
**: % of change compared with ISMN treated groups of the same dose.

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الملخص العربى
دراســـة كيميائيـــة حيويـــة على الهيمـوجلـوبـين

إيناس أحمد عبدالفتاح يوسـف محمـد شـحاتـه
خليفـه الضـوى أحمـد عبدالعزيز أبوالفتوح غانم*

قسم الكيمياء الحيوية – كلية الطب البيطرى – جامعة الزقازيق

قسم الطب الشرعى والسموم – كلية الطب – جامعة المنصورة*

إن أدوية النيترات العضوية تسبب إتساع فى الشرايين التاجية للقلب وتستخدم فى الحالات المرضية التى بها ضيق فى الشرايين التاجية للقلب التى تؤدى إلى ذبحة صدرية وجلطة بالقلب. وقد تبين أن إستخدام أدوية النيترات العضوية قد يكون له بعض الآثار السلبية حيث أنه أثناء عملية أيض النيترات العضوية ينتج أكسيد النيتريك الذى بالرغم من أهميته فى إرتخاء الأوعية الدموية مما يزيد من تدفق الدم إلى القلب إلا أن أكسيد النيتريك أيضاً له قدرة عالية على أكسدة الهيموجلوبين إلى الميتاهيموجلوبين وينتج عن هذه الأكسدة عدد كبير من الشوارد الحرة التى قد تؤدى إلى أكسدة الدهون وحدوث بعض الأخطار الضارة الأخرى على الدم، ومن ناحية أخرى فإن إستخدام مانع تأكسد مثل فيتامين (ج) قد يحد من هذه الآثار الضارة لأكسيد النيتريك خاصة عند تناول جرعات عالية من النيترات العضوية، وقد لوحظ من هذه الدراسة حدوث إنخفاض ملحوظ فى تركيز الهيموجلوبين فى الدم ويزداد الانخفاض مع زيادة الجرعات، ارتفاع ملحوظ فى تركيز الميتاهيموجلوبين والكاربوكسى هيموجلوبين فى الدم ويزداد الارتفاع مع زيادة الجرعات، إنخفاض ملحوظ فى كمية الأكسجين المرتبطة بالهيموجلوبين ومحتوى الأكسجين بالدم خاصة فى الجرعات الثانية والثالثة وانخفاض ملحوظ فى الهيماتوكريت وعدد كرات الدم الحمراء والبيضاء فى الدم ويزداد الانخفاض بزيادة الجرعات، حدوث ارتفاع ملحوظ فى تركيز أكسيد النيتريك فى الدم ويتناسب طردياً مع ازدياد الجرعات، وجد ارتفاع ملحوظ فى مستوى نواتج التأكسد الفوقى للدهون المالون داى ألدهيد مع ازدياد الجرعات، وجد إنخفاض ملحوظ فى تركيز بعض مضادات الأكسدة بالدم CAT وSOD و GSH بزيادة الجرعات، وجد إنخفاض ملحوظ فى تركيز الفوسفور غير العضوى ومحتوى الحديد بالدم ويزداد الانخفاض بزيادة الجرعات، أما بالنسبة للجرعات التى تناولت النيترات العضوية بالإضافة لفيتامين (ج) فقد لوحظ تحسن واضح فى معظم القياسات السابقة، ومن هذه الدراسة تبين خطورة استخدام جرعات عالية من النيترات العضوية وأهمية تناول مضادات الأكسدة المتوفرة فى العديد من المواد الغذائية للحد من الآثار الضارة للأدوية المؤكسدة.