Improved Preservation Of Erythrocyte Antioxidant Capacity With Ascorbic Acid Reperfusion After Ischemia: A Comparative Study In A Rat Hindlimb Model

Y Coban, E Bulbuloglu, A Polat, F Inanc

Citation

Y Coban, E Bulbuloglu, A Polat, F Inanc. *Improved Preservation Of Erythrocyte Antioxidant Capacity With Ascorbic Acid Reperfusion After Ischemia: A Comparative Study In A Rat Hindlimb Model*. The Internet Journal of Alternative Medicine. 2004 Volume 2 Number 1.

Abstract

Aim of the study: We aimed to measure lipid peroxidation and antioxidant enzymes in erythrocytes before and after ischemia-reperfusion in a rat hindlimb model, and to compare the effects of reperfusion with Vitamin C and dexamethasone on the erythrocyte antioxidant capacity.

Material and method: Thiobarbituric acid -- reactive substances (TBARS), a measure of lipid peroxidation, Superoxide dismutase (SOD), Glutathion (GSH) and catalase (CAT) were determined in erythrocytes. Erythrocytes were prepared by classical washing method (0,9 %Nacl) from venous samples. A total of 42 rats were used in the study which were divided into 6 groups 7 rats in each as follows: control group or the sham-operated group, short ischemia-reperfusion, long ischemia-reperfusion, short ischemia and reperfusion with ascorbic acid (AA), short ischemia and reperfusion with dexamethasone, the double extremity I-R with short duration groups. A non-traumatic vascular clamp (Acland, V3 vascular clamp) was placed accross the right iliac artery for 45 minutes in short ischemia and for 3 hours in long ischemia groups. Venous blood samples were taken to evaluate the Cat, SOD, GSH and TBARS levels in erythrocytes at the completion of reperfusion.

Results: Elevated TBARS levels and decreased Cat, SOD and GSH levels in erythrocytes were seen after i-r of rat hindlimb ($P<0.001$). When compared with reperfusion with dexamethasone, AA had a more improved effect on antioxidant capacity of erythrocytes after ischemia of hindlimb in rats.

Conclusions: Reperfusion with AA after ischemia resulted an improved antioxidant capacity preservation in erythrocytes. In clinical setting, addition of vitamin C to reperfusion solution may be benefical.

INTRODUCTION

Erythrocytes are very susceptible to free radical-induced lipid peroxidation and are basic elements to be involved in the ischemia-reperfusion (I-R) injury. These cells are continuously exposed to high concentration of oxygen molecules and oxidative stress induced changes has been shown to occur in erythrocytes ([1,2]). They have been shown to be affected by several oxidative stress conditions such as intoxication, infections, hyperlipoproteinemia, diabetes mellitus, cigarette smoking ([3,4, 5,6]). I-R injury causes oxidative stress and impairs the erythrocyte antioxidant defense mechanisms. Excessive peroxidation of biomembranes is accepted as one of the processes by which tissues can be damaged during I-R ([7]). Reactive oxygen species can induce the oxidation lipids and proteins triggering disruptions in the erthrocyte membrane and eventually lead to hemolysis. Nutritional supplementation of multivitamin can beneficially modify defense system as indicated by the improved antioxidant vitamin status and elevated antioxidant enzymatic activities. Vigorous physical activity can also decrease the blood antioxidant defense capacity ([8]). Therefore, the antioxidant defense of the erythrocytes can be regarded as a dynamic system which is mainly affected by nutritional intake and systemic health status.

The antioxidant defense system consists of enzymes and antioxidant substances inluding tocopherols, ascorbic acid

and ceruloplasmin and albumin. The function of these systems is to keep free radicals under or at physiological levels. Ascorbic acid (AA), a plasma antioxidant and administration of AA prevented the immediate postnatal drop in AA and was not associated with evidence of hemolysis ([9]). Water soluble chain-breaking antioxidants such as AA can scavenge radicals residing aqueous phase. At time of reperfusion in skin flap salvage use of glucocorticoids, such as dexamethasone, has been shown to decrease I-R injury ([10]). The purpose of the present study were: 1) to determine the changes in the antioxidant levels of erythrocytes during I-R injury for assesment of the oxidant injury severity after ischemia-reperfusion in a rat hindlimb model, and 2) to determine the effects of AA and dexamethasone treatment on erythrocyte antioxidant capacity during reperfusion in a rat hindlimb model.

MATERIAL AND METHODS

Female wistar rats weighing 250-350g were used in this study. All animals were housed in wire bottomed cages and allowed free access to standart rat chow and water. They were anesthetized with pentobarbital sodium 42 mg/kg intraperitoneally and with periodic supplementation (10mg/kg intraperitoneally) as needed. An ischemic model using the rat hindlimb was developed as previously described([11]). Bilateral groin incisions were made and iliac vessels were dissected. All surrounding tissues, except artery and vein were transected in order to prevent collateral circulation from the pelvis. The left iliac vein was canulated (24G, neoflon, Sweden) for drug administration and right iliac artery was separated for occlusion. Animals were divided into three groups, 7 rats in each as follows;

Group 1 was the control group or the sham-operated group Group 2 was the short ischemia-reperfusion group. Group 3 was the long ischemia-reperfusion group. Group 4 was the short ischemia and reperfusion with AA. Group 5 was the short ischemia and reperfusion with dexamethasone. Group 6 was the double extremity I-R with short duration group.

Table I summaries the details of groups. In all I-R groups except group 1, a non-traumatic vascular clamp (Acland, V3 vascular clamp) was placed accross the right iliac artery for 45 minutes in group 2 and for 3 hours in group 3. During this time, normal saline 1ml/kg/hr, were administered via left iliac cannula. In group 4 and 5 AA and dexamethasone at 15 mg/kg and 10 mg/kg doses within SF solution were administered at the same infusion rate, respectively. At the end of ischemic time, the clamp was removed from the right iliac artery and reperfusion started. In group 6, both iliac vessels were clamped and reperfusion was done via left jugular cannula. Reperfusion continued in group 2 for 45 minutes and in group 3 for 3 hours. In group 1, all the same procedure were done except vascular occlusion. Blood samples were taken from inferior vena cava for measurements of antioxidant enzymes activities and levels of reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS) in erythrocytes at the end of reperfusions in groups. In group 1, blood samples were taken just after the skeletalisation of the iliac artery and the iliac vein without clamping. After centrifugation of the blood, plasma and erythrocyte were separated. Erythrocyte was washed three times with 0.9% NaCl solution and packed. Erythrocyte and plasma were then stored at ?85 °C until study. The experiment was approved by our Institutional Committe of Animal Care.

Figure 1

Table I: Details of groups.

Group	Duration of ischemia and Reperfusion	Reperfusion With AA	Reperfusion with Dexamethasone	Reperfusion with SF	Number of extremity
1	None	None	None	None	None
2	45 minutes of I-R	None	None	+	1
3	3 hours of I-R	None	None	+	1
4	3 hours of I-R	+	None	None	1
5	3 hours of I-R	None	+	None	1
6	45 minutes of I-R	None	None	+	2

Figure 2

Table II: The mean Â± standart error (SE) values for erythrocyte catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities as well as thiobarbituric acid reactive substances (TBARS) levels in all experimental groups and piar-wise comparisions.

Groups	CAT (kg^{-1} Hb)	SOD (U g^{-1} Hb)	GSH (□mol g^{-1} Hb)	TBARS (µmol g^{-1} Hb)
Group 1	4.77 ± 0.13	0.174 ± 0.014	3.750 ± 1.461	17.18 ± 0.68
Group 2	2.60 ± 0,29	0.122 ± 0.015	2.36 ± 0.38	21.83±1.27
Group 3	2.59 ± 0.118	0.109 ± 0.010	1.25 ± 0.13	21.41±1.40
Group 4	3.68 ± 0.204	0.119 ± 0.007	2.43 ± 0.19	18.81±0.71
Group 5	2.79 ± 0.21	0.110 ± 0.014	2.02 ± 0.30	21.34±1.40
Group 6	2.24 ± 0.192	0.070 ± 0.020	1.39 ± 0.68	22.73±1.00
P				
1-2	0.0001	0.001	0.001	0.001
1-3	0.0001	0.001	0.0001	0.001
1-4	0.01	0.001	0.001	n.s.
1-5	0.001	0.001	0.001	0.001
1-6	0.0001	0.001	0.0001	0.001
2-3	n.s.	n.s.	0.01	n.s.
2-4	0.001	0.07	n.s.	0.001
2-5	n.s.	0.001	n.s.	n.s.
2-6	0.05	0.001	0.01	n.s.
3-4	0.001	n.s.	0.001	0.01
3-5	n.s.	n.s.	0.01	n.s.
3-6	0.05	0.001	n.s.	n.s.
4-5	0.001	n.s.	n.s.	0.01
4-6	0.0001	0.001	0.001	0.001
5-6	0.01	0.001	0.001	n.s.

N.S. :Non Signifigant

DETERMINATION OF CATALASE ACTIVITY IN ERYTHROCYTE

Catalase (CAT, EC 1.11.1.6) activity was determined according to Aebi's method ($_{12}$). The principle of the assay is based on the determination of the rate constant (s^{-1}, k) or the H_2O_2 decomposition rate at 240 nm.

DETERMINATION OF SUPEROXIDE DISMUTASE ACTIVITY IN ERYTHROCYTE

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al. ($_{13}$). The principle of the method is based on the inhibition of NBT reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the sample after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate.

DETERMINATION OF REDUCED GLUTATHIONE LEVEL IN ERYTHROCYTE

The level of reduced glutathione (GSH) in erythrocytes was determined by a modification of the method of Ellman ($_{14}$). Erythrocyte were deproteinated by addition of trichloroacetic acid (TCA). DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] was added to supernatants cleared by centrifugation (10 min, 3000 g/min). The formation of 5-thio-2-nitrobenzoic acid, which is proportional to total glutathione concentration, was monitored at 412 nm at 25°C against reagent controls.

DETERMINATION OF THIOBARBITURIC ACID REACTIVE SUBSTANCES LEVEL IN ERYTHROCYTE

The erythrocyte thiobarbituric acid reactive substances (TBARS) level was determined by a method ($_{15}$) based on the reaction with thiobarbituric acid (TBA) at 90–100 °C. In the TBA test reaction, malondialdehyde (MDA) or MDA-like substances and TBA react with the production of a pink pigment having an absorption maximum at 532 nm. The reaction was performed at pH 2–3 at 90 °C for 15 min. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. The results were expressed according to a standard graphic which was prepared from a standard solution (1,1,3,3-tetramethoxypropane).

STATISTICAL ANALYSIS

The results were expressed as means +/- SE (standart error) and data was analysed using two-way analysis of variance. SOD, CAT, GSH-Px, MDA were used as dependent variables. Time, number of ischemic extremity, vit C and dexamethasone administration were used as factors in the ANOVA. Bunferoni Posttest was used for pair-wise comparisions after ANOVA. P-values less than 0.05 were regarded as statistically significant.

RESULTS

The results are summarized in Table II. Control group erythrocytes showed significantly higher TBARS levels than other groups ($p<0.001$). GSH levels in control group erythrocytes were also significantly higher than the levels of other ischemia-reperfused groups ($p<0.001$ and $p<0.0001$). Intraerythrocyte SOD levels of ischemia-reperfused groups had significantly lower than controls ($p<0.001$).

Intraerythrocyte catalase levels showed a marked decrease in all groups except the group of i-r with AA ($p<0.001$). In this group the level of significance was at $p<0.01$ level. Interesting results were obtained, when multiple comparisions were done. One of these, significant decreases in Cat, SOD, and GSH levels occurred in one i-r extremity group, when compared with double extremity i-r group ($p<0.05$, $p<0.001$, and $p<0.01$, respectively). When comparing reperfusion with AA and dexamethasone in i-r groups, reperfusion with AA yielded a more improved levels in some antioxidants, i.e. catalase with a p value of 0,001 and GSH with a p value of 0.01. TBARS levels were also significantly lower in i-r group reperfused with AA ($p<0.01$). Short and long duration of i-r groups showed only difference in respect of GSH content of erythrocytes, the result was in short i-r group's favour ($P<0.01$).

DISCUSSION

Erythrocytes are the first to react to increased activity of free radical oxidation and to exhaust their compensatory potential. Previous studies on erythrocyte antioxidant capacity and human disease relation showed that some changes in activities of the antioxidant enzymes in the cell may occur ([16]). GPX, reduced Glutathione (GSH), SOD, and CAT were measured in homogenous group of patients with untreated hairy cell leukemia and normal controls. GPX, CAT and SOD activities were significantly lower than in normal ([17]). The components of the blood antioxidant systems take a direct part in molecular mechanism of the body adaptation under conditions of viral hepatitis infections ([18]). It has been shown that cardiogenic shock patients had higher MDA and conjugated dienes and reduced activities of erythrocyte antioxidant enzymes and lower concentration of GSH, AA ([19]). In another study, patients with acute myocardial infarction has been shown to have low erythrocyte antioxidant enzyme activities and remains low during the post-infarction period of 7 days in patients without successful reperfusion ([20]). The post-transplantation period has been reported as characterized by progressive decrease in plasma total antioxidant status after successful transplantation ([21]). A markedly decreased antioxidant capacity seems to be a clinical picture in patients operated with cardiopulmonary bypass ([22]). Changed erthrocyte antioxidant activities were reported during I-R of the hearth ([23]). Decreased activities of SOD, Cat and low concentrations of GSH, AA and Vitamin E have been regarded as the result of increased utilization to scavenge peroxides ([24]). Plasma viscosity remains stable in serum oxidation of different degree, and therefore erythrocytes are responsible for changes in blood rheology during intensification of free radical oxidation ([25]).

It was proposed that a superoxide anion channel allows the transport of superoxide and other free radicals into the red cell, where they are deactivated by the erythrocyte antioxidant system which effectively prevents extensive oxidative damage to tissues ([26]). In addition to its function of gas exchange, the erythron provides a mechanism for the inactivation of reactive oxygen and oxide radicals in vivo. In carrying out this function, individual erythrocytes undergo changes in biochemical and structural properties, which are reflected by shape and functional alterations. This study primarily concerns with biochemical parameters of erythrocytes at the pre- and post- I-R injury. Although the exact mechanisms that lead to RBC deformability and shape changes after trauma/hemorrhagic shock remain unknown, these may be related with oxidative stress and its biochemical alterations in the erythron. Maturation of erythrocytes decreased GSH and all antioxidant enzymes. These maturational alterations are further intensified in diabetic 2 types ([27]). This study clearly shows that both short and long I-R injury decreases antioxidant enzymes of RBCs. Additionally this study compares the different amount of tissues, i.e. one extremity and double extremity, which rendered to I-R injury in the same experimental design. Hypothetically, the much tissue exposes to I-R injury, the more free radical damage occurs that eventually exhausts the antioxidant capacity of RBCs.

The erythrocyte has several membrane systems to protect itself against oxidation damage and hemolysis; these systems include superoxide dismutase, glutathione peroxidase and catalase. In clinical settings, it has been shown that multivitamin supplementation increase the antioxidant capacity ([28]). In this experimental setting adding AA to reperfusion solution had an improved effect only catalase level of ertyrocytes and this may be a reflection of the increased resistance of the erythrocytes to I-R injury due to AA supplementation. One of the most striking finding in our study was that TBARS in control and I-R with AA groups showed no significant difference ($p>0.005$). This may reflect the conservative effect of AA during ischemia-induced reperfusion in systemic blood.

CONCLUSION

We conclude that high-dose vitamin C administration diminishes early lipid peroxidation and preserves the

erythrocyte antioxidants after ischemia-reperfusion injury.This positive effect may help to maintain erythrocyte structural integrity after long lasted ischemia and following reperfusion. The findings of the study confirms the benefical effects of vitamine C seen previous studies ([29]). It is clear that some limitations present in this study.Although rats as an experimental model may not be a good model as they can synthesis AA, it was previously shown that high dose treatment with vitamin C could have scavenging effect on i-r injury and enhance the ischemia-reperfused skin flap survival in adult Sprague-Dawley rats ([30]). Future studies executed on rats mutant unable to synthesize AA will delineate the effects of vitamin C related to the subject ([31]).

ACKNOWLEDGEMENTS

The authors thanks to Dr.M.Hanefi EMRE, from Department of Phsiology, Medical Faculty of Inonu University for the support in biochemical measurements and preparation of the manuscript.

CORRESPONDENCE TO

Y.Kenan Coban MD, Dept. of Plastic Surgery, Sch. Of Medicine, Sütcüimam Univ.Kahramanmaras/Turkey
Email:kenancoban@ksu.edu.tr Phone:+903442212337
Fax:+903442212371

References

1. Suleyman H, Gumustekin K, Taysi S. Benefical effects of hippophae rhamnoides L. on nicotine induced oxidative stress in rat blood compared with vitamine E.Biol Pharm Bull 2002;25:1133-6.
2. Cutchom Mc, Kennedy DA, Terada LS, Emmett M, Waldion DL, Repire JF. Reperfusion of ischemic skeletal muscle cause erythrocyte hemolysis and decreases subsequent oxidant mediated lung injury.J Lab Clin Med 1991;118:352-8.
3. Gultekin F, Ozturk M, Akdogan M. The effect of organophosphate insecticide , chlorpyrifos-etyhl on lipid peroxidation and antioxidant enzymes (in vitro).Arch Toxicol 2000;74:533-8.
4. Kaminskaia GD, Abdullaev RIu. Red blood cell metabolic changes in patients with acutely progressive pulmonary tuberculosis. Probl Tuberkl 2002;2:33-6.
5. Efe H, Kirci D, Deger O, Yildirmis S, Uydu HA, Orem C. Erythrocyte Antioxidant Enzyme Activities and Lipid Peroxidation in Patients with Types IIb and IV Hyperlipoproteinemias. Tohoku Journal of Experimental Med 2004;202:163-172.
6. Yedgar S, Koshkaryev A, Barshtein G. The red blood cell in vascular occlusion. Pathophysiol Haemost Thromb.2002;32:263-8
7. Hersley K, Robinson KA, Gabbita SP, Selsman S , Floyd RA. Reactive oxygen species, cell signalling and cell injury.Free Rad Biol Med 2000;28:1456-62.
8. Machefer G, Groussard C, Rannou-Bekano F, Zouhal H, Faure H, Vincent S, Cillard J, Gratos-Delamarche A. Extreme running competition decreases blood antioxidant defense capacity. J Am Coll-Nutr 2004;23:358-64.
9. Bass WT, Malati N, Castle MC, White LE. Evidence for the safety of ascorbic acid administration to the premature infant. Am J Perinatol.1998;15:133-40.
10. Willemart G, Knight KR, Morrison WA. Dexamethasone treatment prior to reperfusion improves the survival of skin flaps subjected to secondary venous ischemia.Br J Plast Surg 1998;51:624-628.
11. Bozkurt AK. Alpha-tocopherol (Vitamin E) and iloprost attenuate reperfusion injury in skeletal muscle ischemia/reperfusion injury. J Cardiovasc Surg (Torino) 2002;43:693-6.
12. Aebi HE. Catalase of enzymatic analysis.In:Methods in enzymology.Enzymes 1:Oxidoreductases, Transferases, edited by Bergmeyer HU.Weinheim, Germany:VCH Verlagsgesell Schaft 1987;3:273-285.
13. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem 1988;34:497-500.
14. Ellman, GL. Tissue sulfhydryl groups. Arch. Biochem Biophys. 1959:8; 70-77.
15. Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. In: Packer L, Glazer AN, editors. Methods in enzymology: V 186, oxygen radicals in biological systems. California: Academic Press; 1990. p. 407-21.
16. Karatas F,Ozates I, Canatan H, Halifeoglu I, Karatepe M, Colak R. Antioxidant status status & lipid peroxidation in patients with rheumatoid arthritis.Indian J Med Res 2003;118:178-81.
17. Arrud VR, Salles TS, Costa FF. Glutathione peroxidase, reduced glutathione, superoxide dismutase and catalase in red cells of patients with hairy cell leukemia. Neoplasma 1996;43:99-102.
18. Shvalova EP, Antonova TV, Baranovskaia VB. The importance of the antioxidant protection systems of the blood in adaptation to the enfectious process in the enfectious process in viral hepatitis. Ter Arkh 1991;63:47-9.
19. Inal M, Alatas O, Kanbak G, Akyuz F, Sevin B. Changes of antioxidant enzyme activities during cardiopulmonary bypass. J Cardiovasc Surg (Torino). 1999;40:373-6.
20. Simic D, Mimic-Oka J, Pljesa M, Milanovic D, Radojevic S, Ivanovic B, Kolimanovska-Ostric D, Matic D, Simic T. Time course of erythrocyte antioxidant activity in patients treated by thrombolysis for acute myocardial infarction. Jpn Heart J 2003;44:823-32.
21. Pechan I, Danova K, Olejorova I, Halcok L, Rendekova V, Fabian J. Oxidative stress and antioxidant defense systems in patients after heart transplantation. Wien Klin Wochenschr 2003;113:648-51.
22. Pechan I, Olejarova I, Danova K, Fisher V, Minarova H, Dabisova A, Halcok L, Rendekova V, Gabaner I. Antioxidant status of patients after on pump and off-pump coronary artery bypass grafting. Bratisl Lek Listy 2004;105:45-50.
23. Inal M, Alt?nisik M, Bilgin MD. The effect of quercetin on renal ischemia and reperfusion injury in the rat.Cell Biochem Funct 2002;20:291-6.
24. Senthil S, Veerappen RM, Ramokriskna Rao M, Pugalendi KV. Oxidative stess and antioxidants in patients with cardiogenic shock complicating acute myocardial infarction. Clin Chim Acta 2004;348:131-7.
25. Roitman EV, Dement'eva II, Azizova OA, Nikitina NA, Gagaeva EV, Lopukhin IuM. Changes in blood rheological properties and erythrocyte osmotic resistance in activation of free radical processes Klin Lab Diagn 2001;3:42-3.
26. Richards RS, Roberts TK, McGregor NR, Dunstan RH, Butt HL. The role of erythrocytes in the inactivation of free

radicals. Med Hypotheses 1998;50(5):363-7.
27. Sailaja YR, Baskar R, Saralakumari D. The antioxidant status during maturation of reticulocytes to erythrocytes in type 2 diabetics. Free Radic Biol Med. 2003;35:133-9.
28. Cheng T, Zhu Z, Masuda S, Morcos NC. Effects of multinutritient supplementation on antioxidant defense system.J Nutritional Biochem 2001:12:388-95.
29. Matsuda T, Tanaka H, Yuasa H, Forrest R, Matsuda H, Hanumadass M, Reyes H.The effects of high-dose vitamin C therapy on postburn lipid peroxidation. J Burn Care Rehabil. 1993;14:624-9.
30. Zaccaria A, Weinzweig N, Yoshitake M, Matsuda T, Cohen M. Vitamin C reduces ischemia-reperfusion injury in a rat epigastric island skin flap model.
31. 1994;33:620-3.
32. Horio F, Takahashi N, Makino S, Hayashi Y, Yoshida A. Ascorbic acid deficiency elevates serum level of LDL-cholesterol in a rat mutant unable to synthesize ascorbic acid.J Nutr Sci Vitaminol 1991;37:63-71.

Author Information

Y. Kenan Coban, M.D.
Assistant of Professor, Dept. of Plastic Surgery, School Of Medicine, Sütcüimam University

Ertan Bulbuloglu, M.D.
Assistant of Professor, Dept. of General Surgery, Sütcüimam University

Alaadin Polat, M.D.
Dept. Of Physiology, Sch.of Medicine, Inonu University

Fatma Inanc, M.D.
Assistant of Professor, Dept of Biochemistry, Sch. Of Medicine, Sütcüimam University