ELSEVIER

Biochimica et Biophysica Acta 1523 (2000) 225–229

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Hemolysis of human red blood cells by riboflavin-Cu(II) system

Iyad Ali, Mansour K.M. Gatasheh, Imrana Naseem *

Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, India

Received 16 March 2000; received in revised form 13 June 2000; accepted 3 August 2000

Abstract

The photodynamic action of riboflavin is generally considered to involve the generation of reactive oxygen species, whose production is enhanced when Cu(II) is present in the reaction. In the present study we report that photoactivated riboflavin causes K^+ loss from fresh human red blood cells (RBC) in a time dependent manner. Addition of Cu(II) further enhances the K^+ loss and also leads to significant hemolysis. Riboflavin in a 2:1 stoichiometry with Cu(II) leads to maximum K^+ loss and up to 45% hemolysis. Bathocuproine, a specific Cu(I)-sequestering agent, when present in the reaction, inhibits the hemolysis completely. Free radical scavengers like superoxide dismutase, potassium iodide and mannitol inhibited the hemolysis up to 55% or more. However, thiourea was the most effective scavenger showing 90% inhibition. These results suggest that K^+ leakage and hemolysis of human RBC are basically free radical mediated reactions.

Keywords: Riboflavin; Copper; Oxygen radical; Red blood cell; Hemolysis

1. Introduction

In the presence of oxygen and visible light, riboflavin has been shown to be lethal to animal and human cells in culture and induce mutations in microorganisms [1,2]. We have previously shown that riboflavin in the presence of Cu(II) and light caused breakage of calf thymus DNA, supercoiled plasmid DNA [3] and degradation of bovine serum albumin [4]. Evidence from our laboratory and from other laboratories has shown that photoactivated riboflavin via its triplet excited state reacts with molecular oxygen and generates reactive oxygen species (ROS) [3,5,6]. These ROS are known to damage rat red blood cell (RBC) membrane, induce hemolysis [7], damage rat lens [8], inactivate enzymes [9,10], increase protein cross-linking [11,12], and destroy bilirubin [13], uric acid [14] and amino acids [15,16].

Oxidative modification of cellular constituents, including lipids, proteins and nucleic acids, has been implicated in the etiology of different pathological conditions and in ageing [17]. The damage caused by oxidative stress is expected to be exacerbated if the antioxidant enzymes themselves are damaged and inactivated by such events [18]. Several lines of evidence suggest that the generation of ROS is intimately associated with the photodynamic effect of many drugs involved in cancer therapy [19]. While singlet oxygen (O_2^1) is believed to be the major mediator of the reaction, oxygen species like the $O_2^{\bullet -}$ and $OH^{\bullet}$ are also known to induce deleterious effects including lipid peroxidation and membrane damage [20]. In fact, RBC have already been used as a target for $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2) [21]. However, the results obtained have been less than optimally clear because RBC contain the protective enzymes superoxide dismutase, catalase and glutathione peroxidase that are free radical scavengers. Furthermore, both methemoglobin [22] and oxyhemoglobin [23] can react with $O_2^{\bullet -}$ leading to precipitation of hemoglobin, thereby promoting hemolysis [24]. Several other hypotheses have been proposed to explain the mechanism of RBC hemolysis following oxidative stress in vivo and in vitro [25]. Hemoglobin appears to be the main site of damage when various oxidative drugs are used [26]. Under other oxidative conditions, the membrane appears to be the target of injury leading to hemolysis [27].

The administration of certain drugs, commonly designated as oxidants, stimulates the generation of ROS, which may overwhelm the cellular protective mechanism [28]. This is particularly likely when the defences of the RBC are already defective due to hereditary disorders [28].

The previous reports regarding the damaging effect of photoactivated riboflavin on RBC require the presence of serum [7]. In this paper we show that photoactivated ribo-

* Corresponding author. Fax: +91 (571) 401089;
E-mail: iyad74@mailcity.com

0304-4165/00/$ – see front matter
PII: S0304-4165(00)00126-4

flavin alone can cause K^+ loss from human RBC and for the first time report that it leads to hemolysis if Cu(II) is also present in the reaction.

2. Materials and methods

Riboflavin, superoxide dismutase, β-carotene and bathocuproine were obtained from Sigma (St. Louis, MO, USA). Mannitol and cupric chloride were from British Drug Houses (BDH) (India). All other chemicals used were of the highest purity grade available commercially.

2.1. Preparation of RBC

RBC were prepared from fresh human blood by centrifugation at $1500 \times g$ for 10 min at room temperature. The cells were washed three times with 5 vols. of isotonic NaCl solution and suspended in the desired buffer.

2.2. Measurement of K^+ loss

Packed RBC were suspended in 3 ml of 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, varying amounts of riboflavin and Cu(II) to give 0.5% hematocrit. The reaction mixtures were incubated at room temperature in 800 lux of cool fluorescent light.

After centrifugation at $1500 \times g$ for 10 min the concentration of K^+ was measured in the supernatant using EEL flame photometry (Evans Electroselenium, Halsted, Essex, UK). For reference of 100% intracellular K^+, a sample of RBC was hemolyzed in distilled water and K^+ concentration determined in the supernatant after centrifugation as above.

2.3. Measurement of hemolysis

The percent hemolysis following incubation of RBC with riboflavin and Cu(II) was measured by reading the absorbance of the hemolysate at 415 nm as described by Yoshida et al. [29]. For reference, RBC were treated with distilled water and hemolysate read at 415 nm to obtain 100% hemolysis. All the experiments were carried out in triplicate and the mean values are reported.

Wherever specified, varying amounts of free radical scavengers or bathocuproine were included in the reaction mixture (see legends for details).

3. Results

It is well established that riboflavin in the presence of UV or visible light generates ROS [5,30]. Previous reports from this laboratory have shown that the presence of Cu(II) in the reaction results in the formation of more damaging species like $O_2^{\cdot}$ and $OH^{\cdot}$ via a modified Haber-Weiss reaction [6]. These ROS are known to damage macromolecules like DNA [3] and proteins [4] in vitro. As much work has already been reported on rat RBC [7] and other systems [8,14] it is, therefore, of interest to see the effect of these ROS on human RBC.

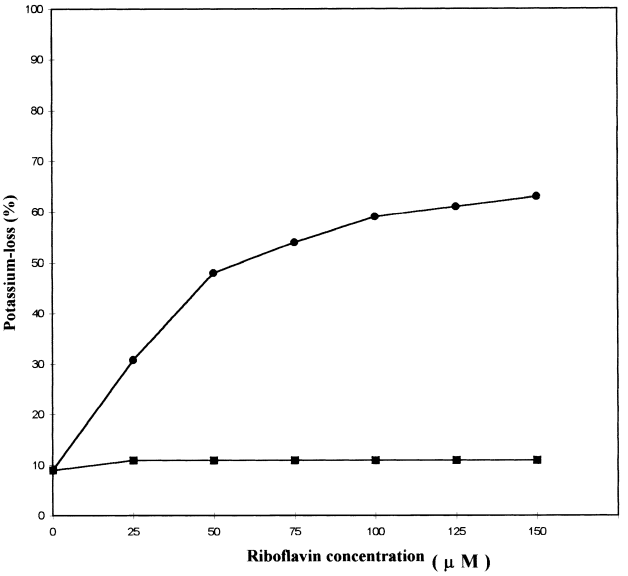

Fig. 1. Effect of different riboflavin concentrations on intracellular K^+ loss. Cells were incubated in 3 ml of buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 25–150 μM riboflavin to give 0.5% hematocrit. The percentage of intracellular K^+ loss was measured after 6 h of incubation in fluorescent light (●) and in the dark (■).

The incubation of human RBC in fluorescent light with increasing concentrations of riboflavin for 6 h resulted in the progressive loss of intracellular K^+ (Fig. 1). About 50% K^+ loss was achieved at 50 μM riboflavin. Further increase in the riboflavin concentrations did not result in any significant loss of intracellular K^+.

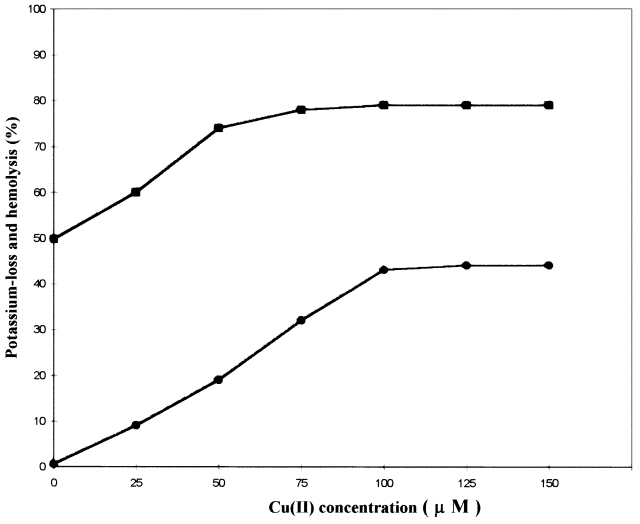

Fig. 2. Percent of K^+ loss and hemolysis of RBC induced by different concentrations of Cu(II) in the presence of riboflavin. Cells were incubated with 50 μM riboflavin and increasing concentrations of Cu(II). Hemolysis (●) and K^+ loss (■) were monitored after 6 h of incubation.

The percent K^+ loss and hemolysis of RBC by photoactivated riboflavin with increasing concentrations of Cu(II) are shown in Fig. 2. There was an initial increase in extracellular K^+ which stabilized at around 50 μM Cu(II). The maximum K^+ loss was 80% in the presence of Cu(II) which is 30% more than when RBC were incubated with riboflavin alone (Fig. 1). The presence of Cu(II) also induced significant hemolysis. It was appreciable at 25 μM concentration of Cu(II). A maximum of 45% hemolysis was, however, observed with 100 μM Cu(II). Further increase in Cu(II) concentrations did not lead to any significant increase in hemolysis. Metals like Zn(II), Fe(III), Mg(II) and Mn(II) when used with riboflavin instead of Cu(II) did not induce any significant hemolysis under the same standard reaction conditions (data not shown).

The percent K^+ loss and hemolysis in presence of 50 μM riboflavin and 100 μM Cu(II) were determined as a function of time (Fig. 3). After 1 h of incubation about 18% of the intracellular K^+ was lost into the medium without any significant hemolysis. Increasing the time of incubation to 2 h resulted in the release of 30% cellular K^+ and 10% hemolysis, and after 6 h 80% of the intracellular K^+ was lost with 45% hemolysis. In the absence of Cu(II), RBC did not exhibit any hemolysis even after prolonged incubation. In light-protected control samples, loss of cellular K^+ or hemolysis was not observed in the presence of both riboflavin and Cu(II) (data not shown), suggesting that the reaction is light mediated.

In order to investigate whether Cu(I) played a role in hemolysis, the Cu(I)-sequestering agent bathocuproine was included in the reaction containing riboflavin and Cu(II). Complete inhibition of hemolysis was achieved at 25 μM bathocuproine (Fig. 4) showing a Cu(I):bathocuproine stoichiometry of 4:1.

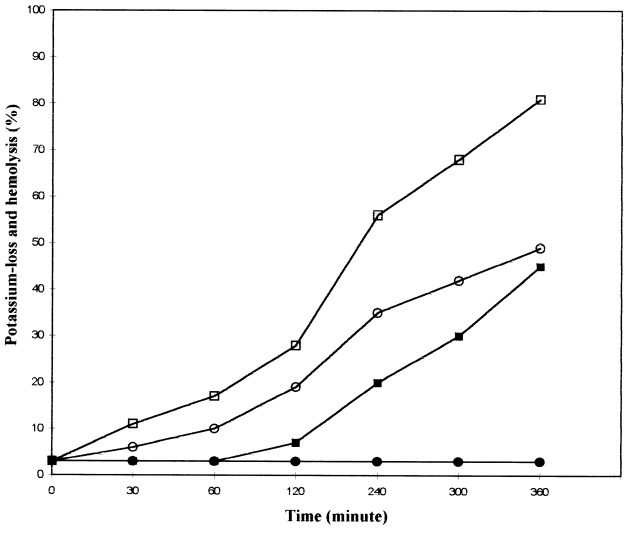

Fig. 3. Effect of increasing time of incubation on the hemolysis and intracellular K^+ loss from human RBC. Cells were incubated with 50 μM riboflavin and 100 μM Cu(II) for different time intervals. □, K loss; ○, K loss (without Cu(II)); ■, hemolysis; ●, hemolysis (without Cu(II)).

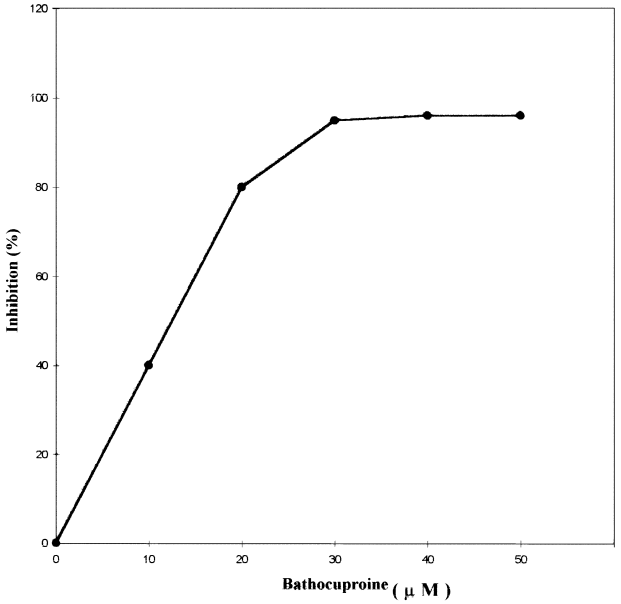

Fig. 4. Effect of bathocuproine on the hemolysis of RBC induced by riboflavin and Cu(II). Cells were incubated with 50 μM riboflavin, 100 μM Cu(II) and varying concentrations (10–50 μM) of bathocuproine. After 6 h incubation in fluorescent light, hemolysis was determined and compared with controls lacking bathocuproine.

Several free radical scavengers were included in the reaction to identify the major ROS participating in the RBC hemolysis. As shown in Fig. 5, potassium iodide, a scavenger of triplet oxygen, gave 60% inhibition of RBC hemolysis. Superoxide dismutase (SOD), the scavenger of $O_2^{\bullet-}$, showed 52% inhibition, while catalase, the scavenger of H_2O_2, and β-carotene, the scavenger of both O_2^1 and

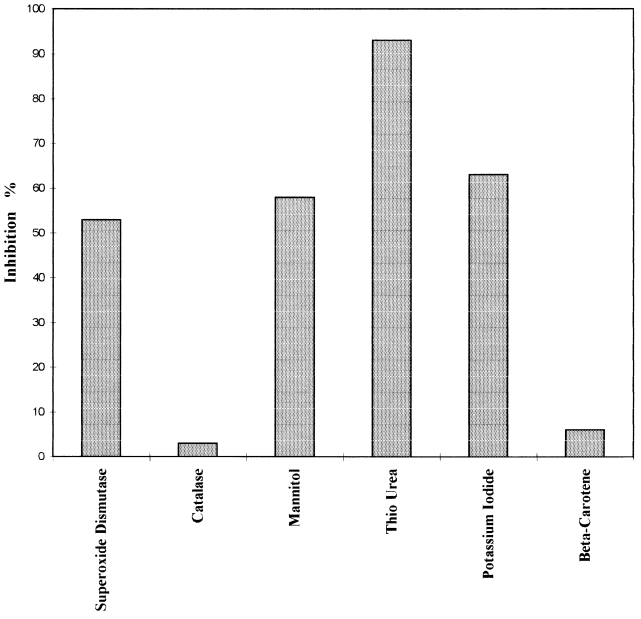

Fig. 5. Inhibition of RBC hemolysis by various free radical scavengers. Cells were incubated with 50 μM riboflavin and 100 μM Cu(II) and 0.1 mM of either mannitol, thiourea, potassium iodide, β-carotene or 20 μg/ml of SOD or catalase. The incubation was for 6 h in fluorescent light.

nitric oxide (NO•), did not show any inhibition. Mannitol and thiourea, both scavengers of OH•, showed 63% and 93% inhibition respectively, indicating OH• as the major ROS involved in the reaction.

4. Discussion

Riboflavin or vitamin B_2 is the prosthetic group of several proteins and enzymes which is reversibly reduced by hydrogen atoms. When exposed to light, riboflavin absorbs energy and reacts via its triplet excited state with other molecules such as protonated substrates or molecular oxygen generating ROS [31]. These ROS are well known for their damaging effect on several biological molecules [3,4]. The present observations illustrate the effect of photoactivated riboflavin on intracellular K^+ loss and hemolysis of human RBC. In the presence of riboflavin and visible light, significant loss of intracellular K^+ is observed (Fig. 1) which is probably due to an alteration in the permeability of the RBC membrane. In addition, this result also shows that the photosensitized riboflavin rendered the cell membrane permeable to K^+, but not to large molecules like hemoglobin as no hemolysis was observed under these conditions.

Riboflavin in the presence of Cu(II) and visible light has been shown to cause breakage of calf thymus DNA [3] and fragmentation of proteins [4]; therefore, the effect of riboflavin on human RBC was also studied in the presence of Cu(II) (Fig. 2). The hemolysis of RBC started after a lag of 60 min, when intracellular K^+ loss was already 18% and increased progressively up to 6 h with the maximum being 45%. However, after 6 h the maximum K^+ loss was 80%. No further hemolysis was observed up to 10 h of incubation in light (data not shown).

The effect of Cu(II) on RBC leading to hemolysis may arise from the fact that Cu(II) interacts with photoactivated riboflavin to provide a long-lived excited species. Direct charge transfer from excited riboflavin can then generate the singlet and triplet oxygen [6]. Other ROS such as OH• or hydroxide ion (OH^-) are also generated in the reaction via a modified Haber-Weiss reaction when Cu(II), water and molecular oxygen are present. In our previous study [3] we found that the addition of Cu(II) to riboflavin reaction inhibited the photodegradation of riboflavin and in this process Cu(II) is reduced to Cu(I). To see if Cu(I) is also involved in RBC hemolysis reaction, riboflavin-Cu(II) mediated hemolysis was carried out in the presence of bathocuproine, a known Cu(I)-sequestering agent. Complete inhibition was achieved at 25 μM bathocuproine (Fig. 4), confirming once again that Cu(II) is reduced to Cu(I) and the latter is an essential intermediate in the reaction.

Scavengers of various ROS were also included in the reaction (Fig. 5). The results of this experiment strongly suggest OH• as the major ROS involved in the RBC damaging reaction. This observation is based on the fact that thiourea substantially and D-mannitol to a lesser extent prevented hemolysis. On the other hand, potassium iodide gave 63% inhibition, suggesting that singlet and triplet excited states of oxygen also play a significant role in hemolysis. This is in agreement with our previous report on a bacterial system [6] where we have proposed a scheme for photodegradation of riboflavin in which the presence of Cu(II) generates O_2^1 and OH• from $O_2^{\bullet -}$ via a modified Haber-Weiss reaction.

The above findings assume significance in view of the fact that photodynamic therapy is increasingly used with great success for the treatment of a variety of tumors and cancer and attempts are being made to extend its use in the treatment of other clinical conditions [1,2]. Riboflavin is an important constituent of our daily diet. It is found in free and conjugated form in almost all biological tissues and fluids. The normal serum contains up to 8 μM loosely bound copper. Other biological fluids may also contain loosely bound copper in the low micromolar range [32,33]. Gutteridge defines loosely bound copper as that copper which is available for binding to a chelating agent like 1,10-phenanthroline. It is possible that this loosely bound copper can be mobilized by riboflavin. Therefore, the risk of free radical mediated damage to tissue increases during phototherapy of various clinical disorders. However, further studies are necessary to evaluate the role of free radicals in cell damage, to understand their mechanism of action and conditions of their formation in vivo.

Acknowledgements

The authors are grateful to Prof. S.M. Hadi, chairman Department of Biochemistry, Prof. M. Saleemuddin and Dr. M. Mirza for their help and fruitful discussions. The help of Dr. R. Mahmood for the preparation of the manuscript is thankfully acknowledged.

References

[1] R.B. Webb, M.M. Malina, D.F. Benson, Action spectrum for mutagenesis by visible light in *E. coli*, Genetics 56 (1967) 594–595.
[2] M.O. Bradly, N.A. Sharkey, Mutagenicity and toxicity of visible fluorescent light to cultured mammalian cells, Nature 266 (1977) 714–726.
[3] I. Naseem, M. Ahmad, R. Bhat, S.M. Hadi, Cu(II) dependent degradation of DNA by riboflavin, Food Chem. Toxicol. 31 (1993) 589–597.
[4] M. Jazzar, I. Naseem, Enhanced protein degradation by photoilluminated riboflavin in the presence of Cu(II), Biochem. Mol. Biol. Int. 34 (1994) 883–895.
[5] I. Naseem, M. Ahmad, S.M. Hadi, Effect of alkylated and intercalated DNA on the generation of superoxide anion by riboflavin, Biosci. Rep. 8 (1988) 485–492.
[6] M.M. Jazzar, I. Naseem, Genotoxicity of photoilluminated riboflavin in the presence of Cu(II), Free Radic. Biol. Med. 21 (1996) 7–14.
[7] Y. Suzuki, T. Miura, T. Ogiso, Riboflavin photosensitized hemolysis

of rat erythrocytes in the presence of serum, J. Pharm. Dyn. 5 (1982) 568–575.
[8] U.P. Andley, B.A. Clark, Conformational changes of beta H-crystalline in riboflavin sensitized photooxidation, Exp. Eye Res. 47 (1988) 1–15.
[9] J. Jedziniak, M. Arredondo, U.P. Andley, Oxidative damage to human lens enzymes, Curr. Eye Res. 6 (1987) 345–350.
[10] E. Silvia, Rate constant studies of the dye-sensitized photoinactivation of lysozyme, Radiat. Environ. Biophys. 16 (1979) 71–79.
[11] U.P. Andley, B.A. Clark, Spectroscopic studies on the photooxidation of calf lens gamma-crystalline, Curr. Eye Res. 7 (1988) 571–579.
[12] J.D. Goosey, J.S. Zigler, J.H. Kinoshita, Cross-linking of lens crystalline in a photodynamic system: a process mediated by singlet oxygen, Science 208 (1980) 1278–1280.
[13] E. Knoblock, F. Mandys, R. Hodr, Study of the mechanism of the photochemical oxidation of bilirubin by high performance liquid chromatography, J. Chromatogr. 428 (1988) 255–263.
[14] J. Newburger, A.b. Combs, T.F. Hsu, In vitro photodecomposition of uric acid in presence of riboflavin, J. Pharm. Sci. 66 (1977) 1561–1564.
[15] J. Bhatia, D.K. Rassin, Photosensitized oxidation of tryptophan and hepatic dysfunction in neonatal gerbils, J. Parenter. Nutr. 9 (1985) 491–495.
[16] W.R. Frissell, C.W. Chung, C.G. Mackenzie, Catalysis of oxidation of nitrogen compounds by flavin coenzyme in the presence of light, J. Biol. Chem. 234 (1956) 1297–1302.
[17] B. Halliwell, Oxidants and human disease: some new concepts, FASEB J. 1 (1987) 358–364.
[18] T. Tabatabaie, R.A. Floyd, Susceptibility of glutathione peroxidase and glutathione reductase to oxidative damage and the protective effect of spin trapping agents, Arch. Biochem. Biophys. 314 (1994) 112–119.
[19] M. Hoebeke, H.J. Schiuntmaker, L.E. Jannink, T.M.A.R. Dubbelman, A. Jakobs, A.V.D. Vorst, Electron spin resonance evidence of the generation of superoxide anion, hydroxyl radical and singlet oxygen during the photohemolysis of human erythrocytes with bacteriochlorin a, Photochem. Photobiol. 66 (1997) 502–508.
[20] D. Dolphin, Photomedicine and photodynamic therapy, Can. J. Chem. 72 (1994) 1005–1013.
[21] E.W. Kellogg III, I. Fridovich, Liposome oxidation and erythrocytes lysis by enzymically generated superoxide and hydrogen peroxide, J. Biol. Chem. 252 (1977) 6721–6728.
[22] H.C. Sutton, P.B. Roberts, C.C. Winterbourn, The rate of reaction of superoxide radical ion with oxyhaemoglobin and methaemoglobin, Biochem. J. 155 (1976) 503–510.
[23] R.E. Lynch, G.R. Lee, G.E. Cartwright, Inhibition by superoxide dismutase of methaemoglobin formation from oxyhaemoglobin, J. Biol. Chem. 251 (1976) 1015–1019.
[24] R.E. Lynch, I. Fridovich, Effect of superoxide on erythrocytes membrane, J. Biol. Chem. 253 (1978) 1838–1845.
[25] M. D'Aguino, S. Gaetani, M.A. Spadoni, Effect of factors of favism on the protein and lipid components of rat erythrocyte membrane, Biochim. Biophys. Acta 731 (1983) 161–167.
[26] B. Goldberg, A. Stern, The role of the superoxide anion as a toxic species in the erythrocytes, Arch. Biochem. Biophys. 178 (1977) 218–225.
[27] R.J. Trotta, S.G. Sullivan, A. Stern, Lipid peroxidation and haemoglobin degradation in red blood cells exposed to t-butyl hydroperoxide. Dependence of glucose metabolism and haemoglobin status, Biochim. Biophys. Acta 679 (1981) 230–237.
[28] M.R. Clemens, H.D. Waller, Lipid peroxidation in erythrocytes, Chem. Phys. Lipids 45 (1987) 251–268.
[29] Y. Yoshida, K. Kashiba, E. Niki, Free radical-mediated oxidation of lipids induced by haemoglobin in aqueous dispersions, Biochim. Biophys. Acta 1201 (1994) 165–172.
[30] K. Kuratomi, Y. Kobayashi, Studies on the interactions between DNA and flavins, Biochim. Biophys. Acta 476 (1977) 207–217.
[31] E. Frati, A.M. Khatib, P. Front, A. Pauasyn, F. Aprile, D.R. Mitrovic, Degradation of hyaluronic acid by photosensitized riboflavin in vitro modulation of the effect by transition metals, radical scavengers, and metal chelators, Free Radic. Biol. Med. 22 (1997) 1139–1144.
[32] J.M.C. Gutteridge, Copper-phenanthroline induced site specific oxygen radical damage to DNA, Biochem. J. 218 (1984) 983–985.
[33] D.A. Rowley, B. Halliwell, Superoxide dependent and ascorbate dependent formation of hydroxyl radicals in the presence of copper salts: a physiologically significant reaction, Biochim. Biophys. Acta 225 (1983) 279–284.