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# Protective Effect of Erythropoietin on the Oxidative Damage of Erythrocyte Membrane by Hydroxyl Radical

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**ABSTRACT.** Treatment of red blood cells with the copper (II) ascorbate system causes increased lipid peroxidation, increased membrane microviscosity, and phospholipid translocation with a concurrent decrease in cytosolic catalase and glutathione peroxidase activities. All these changes are prevented if the cells are treated with erythropoietin prior to the exposure to copper (II) ascorbate. The present investigation further indicates that the oxidative damage brought about by copper (II) ascorbate is due to generation of hydroxyl radical and that erythropoietin plays a unique role in protecting the membrane from oxidative damage. BIOCHEM PHARMACOL **59**;4:419–425, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** erythropoietin; oxidative damage; hydroxyl radical, erythrocyte membrane

Ep† is a glycoprotein hormone secreted from the kidney in response to hypoxia in the renal tissues. The normal serum concentration of Ep varies from 8–20 mU/mL. In various anaemic states as well as in hypoxia, the concentration of Ep in plasma increases. The role of this hormone in erythropoiesis and haemoglobin biosynthesis is well known [1–4]. We reported earlier that starvation, which depletes the endogenous level of Ep, increases lipid peroxidation of red blood cell (RBC) membrane significantly, and that administration of Ep reverses the effect [5]. Recently, we observed that the copper (II) ascorbate system increases lipid peroxidation and decreases the spectrin content and $Mg^{2+}$-ATPase activity of the RBC membrane [6]. We further observed that administration of Ep completely protected the membrane from all these changes mentioned above [6]. The present paper addresses this issue of the protective role of Ep and further extends the study on the effects of copper (II) ascorbate and protection by Ep on the microviscosity and phospholipid translocation in RBC membrane. The modulation of cytosolic antioxidant enzymes of RBC was also studied under the same conditions. Our results suggest that the primary effect of copper (II) ascorbate is related to lipid peroxidation in RBC membrane, which subsequently produces other changes. The present investigation indicates that the oxidative damage brought about by the copper (II) ascorbate system is due to generation of $OH^{\cdot}$, and Ep, apart from its other functions, appears to protect the membrane from oxidative damage by $OH^{\cdot}$.

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†*Abbreviations:* DPH, 1,6-diphenyl-1,3,5-hexatriene; Ep, erythropoietin; $PA_2$, phospholipase $A_2$; PC, phospho-tidylcholine; RBC, red blood cells; $OH^{\cdot}$, hydroxyl radical; and $H_2O_2$, hydrogen peroxide.

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## MATERIALS AND METHODS

### *Chemicals*

Human recombinant erythropoietin, ascorbic acid, NADPH, BSA, bee venom $PA_2$, phosphatidylserine, phosphatidylethanolamine, PC, catalase, reduced glutathione, oxidised glutathione, glutathione reductase, and DPH were procured from Sigma. Thiobarbituric acid and DMSO were purchased from E. Merck. Benzenesulfonic acid and Fast Blue BB salt were obtained from Aldrich. Other reagents used were of analytical grade.

### *Isolation of Red Blood Cells*

Goat blood was collected in acid–citrate dextrose solution. The packed RBC were isolated by centrifugation at 3000 X *g* for 10 min at 4°. The plasma and the buffy coat were removed by aspiration and the red cells thus obtained were washed thrice with 0.9% NaCl solution.

### *Preparation of Erythrocyte Membranes*

Haemoglobin-free ghosts were prepared according to the method of Dodge *et al.* [7] with modifications [8]. Washed red cells were haemolysed with 40 vol. of 5 mM sodium phosphate buffer (pH 8.0) and centrifuged at 15,000 × *g* for 20 min at 4°. The pellet was washed several times until a colourless ghost was obtained.

### *Treatment of Intact Erythrocytes with Copper (II) Ascorbic Acid and Preparation of the Haemolysate for Assay of Antioxidant Enzymes*

Two milliliters of 50% suspension of washed erythrocytes was incubated with different concentrations of Ep in a shaking water bath at 37° for 30 min. This incubation was continued for another hour with 0.2 mM $Cu^{2+}$ and 1 mM ascorbic acid. The incubation was terminated by addition of 10 μL of 10 mM EDTA and the copper ascorbate-treated erythrocytes were washed thrice with 0.9% NaCl solution. The packed cells were lysed with an equal volume of water, followed by centrifugation at 10,000 × *g* for 10 min at $0–4^0$. The supernatant (lysate) was used for the assay of catalase and glutathione peroxidase. Lysates from untreated washed packed cells and copper (II) ascorbate-treated but Ep-untreated packed cells were also prepared in a similar way and used for the assay of catalase [9] and GSH peroxidase [10], which served as the respective controls.

### *Assay of Catalase and Glutathione Peroxidase*

The activity of catalase in red cell lysate was measured by following the rate of decomposition of $H_2O_2$ by the method of Beers and Sizer [9]. The lysate was diluted 1:20 with normal saline for the assay of catalase activity. The incubation system contained 1.95 mL of 0.05 M phosphate buffer (pH 7.0) and 0.05 mL of the diluted lysate (enzyme). The reaction was initiated by the addition of 1 mL of $5 \times 10^3$ M solution of buffered $H_2O_2$ to the sample cuvette. An equal volume of 0.05 M phosphate buffer (pH 7.0) was added to the reference cuvette. Optical density readings were taken at 240 nm every 10 sec. The specific activity of the enzyme was expressed as ΔO.D./min/mg protein. GSH peroxidase activity was measured according to the method of Paglia and Valentine [10]. The assay system contained 0.043 M phosphate buffer with 2 mM EDTA (pH 7.0), 0.025 mM sodium azide, 0.15 mM glutathione, 1 U GSH, enzyme, and 0.25 mM NADPH. The reaction was started by the addition of 0.36 mM $H_2O_2$. The linear decrease of ΔA at 340 nm was recorded. The specific activity was expressed as μmol of NADPH produced/min/mg protein.

### *Incubation of Intact Erythrocytes with Copper (II) Ascorbic Acid Followed by $PA_2$ Treatment for the Study of Phospholipid Translocation*

Ten milliliters of 20% suspension of washed erythrocytes were treated with different concentrations of Ep prior to copper-ascorbate treatment and incubated in a shaking water bath at 37° for 30 min. It was further incubated with 0.2 mM $Cu^{2+}$ and 1 mM ascorbic acid at 37° for 1 hr. At the end of incubation, erythrocytes were washed twice with 0.9% NaCl and subjected to $PA_2$ treatment. Five milliliters of 50 mM Tris–HCl buffer pH 7.4 and 50 U of bee venom $PA_2$ were added to the washed copper (II) ascorbate-treated erythrocytes. The incubation was performed in a conical flask in a shaking water bath at 37° for 1 hr. Another batch of erythrocytes (without copper ascorbate and Ep) were treated in exactly the same way as stated above and designated as control. Bee venom $PA_2$ is non-permeable to the cells and specifically hydrolyses phospholipids present in the outer membrane bilayer [11]. Erythrocytes have been reported to stay intact during treatment of $PA_2$ from several sources [12]. To terminate the $PA_2$ action, 1 mL of 5 mM EDTA was added to the assay mixture and the cells were washed three times with cold 0.9% NaCl solution.

### *Lipid Extraction and Phospholipid Analysis by TLC*

Lipids were extracted from the washed packed red cells by the method of Rose and Oklander [13] and dried under nitrogen. The dried extracts were redissolved in a minimum volume of chloroform : methanol mixture (1:1, v/v), and 0.5 mg of lipid extract was applied on TLC plates (20 × 20, Silica Gel 60 from E. Merck). The plates were developed in an ascending fashion with chloroform : methanol : acetic acid : water (65:43:4:4, v/v), dried over hot air, and stained with iodine vapor. The different phospholipids were identified by using authentic phospholipids as markers. The spots corresponding to different phospholipids were scraped from the plates. After digestion of the phospholipids with perchloric acid on a sand bath at 180–200°, the liberated inorganic phosphate was measured according to the method of Ames and Dubin [14].

### *Measurement of Membrane Microviscosity*

The microviscosity of RBC membrane was measured by the fluorescence depolarisation technique, using DPH as the fluorescence probe [15, 16]. A small volume of DPH solution (5 mM) in tetrahydrofuran was injected with rapid stirring into 1000 volumes of PBS at room temperature. The suspension was stirred for at least 2 hr till no odour of tetrahydrofuran was detected. In our experiments, both control and copper (II) ascorbic acid-treated membranes equivalent to 200 μg of protein were incubated in the presence and absence of Ep in PBS containing 1 μM DPH suspension for 2 hr at 37°. The measurements of fluorescence anisotropy were carried out in a fluorescence polarisation spectrometer (Perkin Elmer, IS-3) at room temperature using an excitation wavelength of 365 nm and an emission wavelength of 430 nm for DPH. The fluorescence anisotropy r was calculated using the equation:

$$r = (I_{II} - I_I)/(I_{II} + 2I_I)$$

where $I_{II}$ and $I_I$ are the fluorescence intensities oriented parallel and perpendicular to, respectively, the direction of polarisation of the excited light. The microviscosity parameter $[(r_0/r) - 1]^{-1}$ was calculated in each case knowing the maximal limiting fluorescence anisotropy $r_0$, which for DPH is 0.362 [17]. The microviscosity parameter used by us and various other workers [16, 18] is a convenient way of

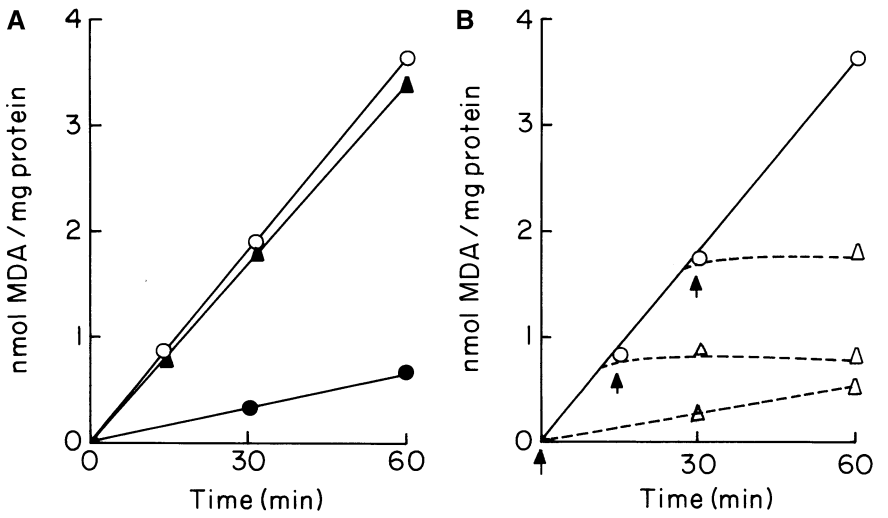


**FIG. 1. (A) Effect of erythropoietin on copper (II) ascorbic acid-induced lipid peroxidation in red blood cell membrane. (○) malondialdehyde (MDA) production in RBC membrane (Ep-untreated) in the presence of copper (II) ascorbic acid (●); MDA production in RBC membrane alone in the absence of copper (II) ascorbic acid; (▲) MDA production in RBC membrane treated with follicle-stimulating hormone (10 μg/mL) in the presence of copper (II) ascorbic acid. (B) Effect of the delayed addition of erythropoietin on copper (II) ascorbic acid-induced lipid peroxidation in red blood cell membrane. (○) MDA production in RBC membrane (Ep-untreated) in the presence of $Cu^{2+}$-ascorbic acid; (△) Addition of Ep (1 unit) at different time periods as indicated by arrow.**

quantifying the measurement and is strictly for the purpose of comparison and not an absolute measurement of microviscosity [19].

### *Determination of Lipid Peroxidation*

Lipid peroxidation was measured by the thiobarbituric acid assay method of Buege and Aust [20].

### *Measurement of Hydroxyl Radical*

($OH^{\cdot}$) was measured using DMSO as a $OH^{\cdot}$ scavenger according to Babbs and Steiner [21]. Chemically, DMSO yields a stable non-radical product, methanesulfonic acid (MSA), on reaction with $OH^{\cdot}$. Accumulation of MSA in the DMSO-pretreated $Cu^{2+}$ ascorbate system in the presence and absence of different concentrations of Ep was measured to estimate $OH^{\cdot}$ generated therein. The MSA so formed was allowed to react with Fast Blue BB salt to yield a yellow reaction product ($\lambda_{max}$ 425 nm) which was measured spectrophotometrically. Benzenesulfonic acid was used as the standard. $OH^{\cdot}$ generated was expressed as nmol/mL of the reaction mixture.

### *Statistical Significance*

All values were reported as means ± SD and Student's *t*-test was used for determining statistical significance.

## RESULTS

Copper (II) ascorbic acid has been found to increase lipid peroxidation of RBC membrane significantly, and administration of Ep prior to oxidative stress completely prevents lipid peroxidation (Fig. 1A). Follicle-stimulating hormone, another glycoprotein, exhibits no significant effect on copper (II) ascorbic acid-induced lipid peroxidation of RBC membrane (Fig. 1A). To understand and confirm whether the changes brought about by Ep were due to protection, the effect of the delayed addition of Ep on copper (II) ascorbate-induced lipid peroxidation in RBC membrane was studied (Fig. 1B). In this experiment, Ep (IU), added at zero time of incubation, showed almost complete protection of the RBC membrane from lipid peroxidation. Moreover, addition of Ep (IU) at different time periods after the initiation of peroxidation prevented further production of malondialdehyde. These results indicate that Ep protects the RBC membrane from lipid peroxidation. A significant decrease in the activity of the protective enzymes, i.e. catalase (42%) and glutathione peroxidase (44%) of the red cells following copper (II) ascorbate treatment, was observed (Table 1). Treatment of the cells with Ep prior to copper (II) ascorbate treatment prevented this inhibition of the activities of these enzymes.

In normal erythrocytes the phospholipid distribution across the membrane bilayer is asymmetric, sphingomyelin and PC being predominantly present in the outer layer and all phosphatidylserine (PS) and most phosphatidylethanolamine (PE) present on the inner side of the membrane. In our study, the free radicals generated by copper (II) ascorbate induced a significant translocation of PS and PE from the inner to the outer leaflet and of PC from the outer to the inner leaflet (Table 2) of the red cell membrane. PC exhibited a reduction in values from 19.5 to 12.11 nmol of phosphate/mg lipid, which indicates significant translocation of PC from outer to inner leaflet, while the value of 23.86 indicates that EP has protected this translocation. PE and PS values indicate the same phenomenon. Table 3 shows an increase in the microviscosity of the RBC membrane due to copper (II) ascorbic acid treatment. In this case as well, Ep prevented the increase in microviscosity of RBC membrane due to copper (II) ascorbic acid treatment, thus exerting a protective influence on the erythrocyte membrane.

In order to determine whether the combination of $Cu^{2+}$ and ascorbate in our system produced lipid peroxidation through the generation of $OH^{\cdot}$, the effect of superoxide dismutase (SOD) and catalase were determined. Table 4

**TABLE 1. Effect of copper (II) ascorbic acid on the catalase and glutathione peroxidase activity of red blood cell and its protection by erythropoietin**

| System | Catalase activity (ΔO.D./min/mg protein) | % Control | Glutathione peroxidase activity (μmol NADPH produced/min/mg protein) | % Control |
|---|---|---|---|---|
| RBC | 0.540 ± 0.02 | 100 | 0.387 ± 0.01 | 100 |
| RBC + Ep (0.2 U) | 0.491 ± 0.01 | 91 | 0.341 ± 0.01 | 88 |
| RBC + $Cu^{2+}$ + ascorbate | 0.312 ± 0.003* | 58 | 0.215 ± 0.01* | 56 |
| RBC + $Cu^{2+}$ + ascorbate + Ep (0.2 U) | 0.480 ± 0.03† | 89 | 0.410 ± 0.02‡ | 106 |

Values are means ± SD (N = 3).
* $P < 0.001$ against RBC.
† $P < 0.01$ against RBC + copper (II) ascorbate.
‡ $P < 0.001$ against RBC + copper (II) ascorbate; assay procedure has been described in the text.

shows that both SOD and catalase and their combination decreased lipid peroxidation significantly, thereby providing protection to the RBC membrane from lipid peroxidation and suggesting that the oxidative damage is due to the generation of the $OH^{\cdot}$. In a chemically defined system, copper (II) ascorbate in the absence of Ep produced 415 nmol of $OH^{\cdot}$/mL reaction mixture. With increasing concentrations of Ep (1U) and (2U) and under the identical conditions, the amount of $OH^{\cdot}$ detected, gradually decreased from 415 nmol to 210 and 85 nmol/mL, respectively (Fig. 2).

## DISCUSSION

The use of copper (II) ascorbate as a free radical generating system is well established [22–24]. In the present paper, free radicals were generated *in vitro* using the copper (II) ascorbate system. According to recent concepts [24], the metal-catalyzed autoxidation of ascorbate is accompanied by the reduction of the transition metal ions and the formation of hydrogen peroxide. Subsequently, the reduced metal ions react with hydrogen peroxide via the Fenton reaction:

$$M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + OH^- + OH^{\cdot}$$

giving rise to $OH^{\cdot}$, which are responsible for the biological damage. The data presented herein showed that RBC membrane becomes oxidatively damaged in the presence of Cu/ascorbate. The significant inhibition of lipid peroxidation by superoxide dismutase (SOD) and catalase suggests that both $O_2^-$ (superoxide anion) and $H_2O_2$ were needed to produce the proximal oxidant, probably $OH^{\cdot}$. Thus, the protection provided to the RBC membrane from lipid peroxidation by SOD and catalase suggests that the copper (II) ascorbate system initiates lipid peroxidation through the generation of $OH^{\cdot}$. Here, we observed that the copper (II) ascorbate system generated nearly 400 nmol of $OH^{\cdot}$/hr/mL of reaction mixture as measured by the method of Babbs and Steiner [21]. In the presence of Ep, the levels of $OH^{\cdot}$ gradually decreased from 415 nmol/mL (without Ep) to 210 and 85 nmol/mL with 1 and 2 U of Ep, respectively, thereby indicating a scavenging action of Ep. Hydroxyl radical is highly reactive and can react at or near its site of formation. Hence, $OH^{\cdot}$ formation *in vivo* will be site-specific to the points at which metal promoters of the Fenton reaction are located. Thus, the biological damage done by $O_2^-$ and/or $H_2O_2$, which is mediated by $OH^{\cdot}$, depends on the location of the metal ion complexes catalytically active in the reaction [25]. Site specifically generated $OH^{\cdot}$ in the copper (II) ascorbate system could damage RBC membrane *in vitro*. There may be numerous sites on the RBC membrane protein where transition metals remain complexed and $OH^{\cdot}$ is eventually generated on these transition metals (e.g. $Cu^{2+}$) in the presence of a reductant (such as ascorbate), which ultimately brings about the damage. The externally added $Cu^{2+}$ may also bind to these sites and further potentiate the damage. However, the possibility of a global damage of RBC membrane proteins *in vitro* by the copper (II) ascorbate system may not be ruled out, although this hypothesis

**TABLE 2. Effect of copper (II) ascorbic acid on the phospholipids of RBC membrane and its protection by erythropoietin**

| Phospholipids | nmol of phosphate/mg lipid | | |
|---|---|---|---|
| | Control membrane | Copper (II) ascorbic acid-treated membrane | Copper (II) ascorbic acid + Ep (0.2 U)-treated membrane |
| Phosphatidylcholine (PC) | 19.5 ± 2.3 | 12.11 ± 0.38 | 23.86 ± 0.76* |
| Phosphatidylethanolamine (PE) | ND | 18.4 ± 0.13 | 12.24 ± 0.31* |
| Phosphatidylserine (PS) | ND | 20.81 ± 0.05 | 13.8 ± 0.47* |

Values are means ± SD (N = 3). ND, not detectable.
* $P < 0.001$ against copper (II) ascorbic acid-treated membrane; assay procedure has been described in the text.

**TABLE 3. Effect of copper (II) ascorbic acid on the microviscosity of RBC membrane and its protection by erythropoietin**

| System | $[r_0/r) - 1]^{-1}$ |
|---|---|
| RBC membrane | 2.26 ± 0.04 |
| RBC membrane + Ep (0.1 U) | 2.20 ± 0.02 |
| RBC membrane + copper (II) ascorbic acid | 2.44 ± 0.02 |
| RBC membrane + copper (II) ascorbic acid + Ep (0.1 U) | 2.07 ± 0.08 |

Values are means ± SD (N = 4).

appears remote due to very high reactivity of the $OH^{\cdot}$ in diffusion-controlled fashion.

The copper (II) ascorbate system has been found to increase lipid peroxidation and decrease the spectrin content and membrane-bound $Mg^{2+}$-ATPase activity of RBC membrane, and these effects are prevented on pretreatment with Ep [6]. Furthermore, our present work illustrates that this same system significantly inhibits the protective enzymes catalase and GSH peroxidase, translocates the phospholipids of the erythrocyte membrane, and increases membrane microviscosity. All these changes are prevented by prior incubation of the system with Ep. The inhibition of activity of the antioxidant enzymes catalase and GSH peroxidase probably aggravates the oxidative damage on red cell membrane. Either an increase in the concentration of oxidant species and/or a decrease in the antioxidant levels could lead to oxidative stress conditions [26]. Under such conditions, increased radical concentrations would lead to damage of most biomolecules, among them antioxidant enzymes. An increased rate of radical production frequently elicits, as a response, an increase in the levels of antioxidant enzymes. However, in the initial stages of the insult and/or under high rates of radical input, enzyme inactivation prevails and enzymatic activities are reduced, leading to autocatalysis of the oxidative damage process. Decreased activities of the antioxidant enzymes have been described in several *in vivo* situations, such as administration of doxorubicin and nitroxantrone [27], triiodothyronin [28], lindane [29], occlusion reperfusion cycles [30], septic syndrome [31], and UV exposure [32].

**TABLE 4. Effect of catalase and SOD on copper (II) ascorbate-induced lipid peroxidation in RBC membrane**

| System | nmol MDA formed/mg protein |
|---|---|
| Control membrane | 0.64 ± 0.00 |
| Membrane + 0.2 mM $Cu^{2+}$ + 1 mM ascorbic acid | 3.74 ± 0.24* |
| Membrane + 0.2 mM $Cu^{2+}$ + 1 mM ascorbic acid + catalase (200 U) | 0.995 ± 0.04† |
| Membrane + 0.2 mM $Cu^{2+}$ + 1 mM ascorbic acid + SOD (200 U) | 0.899 ± 0.02† |
| Membrane + 0.2 mM $Cu^{2+}$ + 1 mM ascorbic acid + catalase (200 U) + SOD (200 U) | 0.833 ± 0.04† |

Values are means ± SD (N = 3).
* $P < 0.001$ against control membrane.
† $P < 0.001$ against control and copper (II) ascorbate.

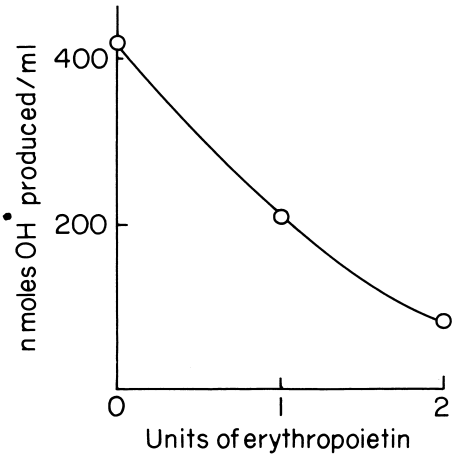


**FIG. 2. Effect of erythropoietin on hydroxyl radical generation by the $Cu^{2+}$-ascorbic acid system. The assay system contained 500 mM DMSO, 0.2 mM $Cu^{2+}$, 1 mM ascorbic acid, 10 mM phosphate buffer pH 7.4, and 0, 1, and 2 units of Ep, respectively.**

The phospholipids are a suitable target in the course of the oxidative process due to the presence of esterified polyunsaturated fatty acids in their molecular structure. In normal erythrocytes, phospholipid distribution across the membrane bilayer is asymmetric, sphingomyelin and (PC) being predominantly present in the outer layer and all phosphatidylserine (PS) and most phosphatidylethanolamine (PE) present on the inner side of the membrane [11, 12]. In our study, the free radicals generated by copper (II) ascorbate attack the fatty acid side chains of the membrane phospholipids and induce a significant translocation of PS and PE from the inner to the outer leaflet and PC from the outer to the inner leaflet of the red cell membrane. Prior incubation of the cells with Ep tends to protect the cells from such translocation of phospholipids. If unsaturated fatty acids were released from the membrane phospholipids during lipid peroxidation, a change in membrane fluidity might be expected [18]. In our study, an increase in membrane microviscosity of RBC membrane was observed due to free radicals produced by copper (II) ascorbate. In this case as well, Ep prevented the increase in microviscosity of RBC membrane, thus exerting a protective influence on the erythrocyte membrane.

Ep is a glycoprotein hormone (MW 34,000 Da) containing approximately 30% carbohydrate, 11% sialic acid, 11% total hexose, and 8% *N*-acetylglucosamine. Cross *et al.* have shown that small glycopolypeptides are effective scavengers of $OH^{\cdot}$ [33]. Such scavenging action, as they have pointed out, is to be expected from the high sugar content of the glycopolypeptides. Thus, protection by Ep may be mediated through the scavenging action of its sugar moiety. Moreover, the protein moiety of Ep contains three more basic than acidic amino acids, and charged residues constitute 27% of the total which are irregularly distributed

[34]. These basic amino acids and the charged residues may act as the 'sink' for the generated hydroxyl radicals. In the early 1980s, researchers made the pertinent observation that free histidine, or serum albumin, lowered the bulk concentration of $OH^{\cdot}$ in a system in which the radical was generated by a $Cu^{+}/Cu^{2+}$ couple; it was suggested that the radical was still produced, but was not liberated because it attacked the histidine, free or in peptide linkage [35]. Besides histidine, glycoproteins containing cysteine and tryptophan are also susceptible to peroxide attack [35]. These observations substantiate the view that Ep protects the red cell membrane from lipid peroxidation possibly by scavenging the hydroxyl radicals generated in the system, and as a result subsequent effects on phospholipid translocation and membrane microviscosity change.

The antioxidant function of Ep had not been investigated until recently when a few workers observed that when recombinant human erythropoietin (rhEp) was administered to haemodialysis patients [36–39] and to patients suffering from anaemia of chronic renal failure [40, 41] for a considerable period of time, there was a significant reduction in lipid peroxidation and an enhancement of SOD, catalase, and other antioxidant activities. In premature rabbits, rhEp treatment has been shown to improve antioxidant status possibly by mobilizing the non-heme iron in the extracellular fluid to new reticulocytes and young red blood cells [42]. This could make non-heme iron unavailable for production of oxygen radicals via the Fenton reaction. However, these findings are only indicative of an antioxidant function and are far from establishing Ep's antioxidant role, which remains a virgin area.

The results of our and earlier studies as well as facts located in its structure now seem to indicate that Ep may act both as a direct as well as indirect antioxidant [43]. By scavenging oxyradicals, it may serve the role of a direct antioxidant, and by stimulating the other antioxidant-defensive mechanism(s), it may act as an indirect antioxidant. The information available thus far, though meager, indicates that the Ep has the potential to act both as a direct as well as indirect antioxidant, an issue that remains open to debate. Further studies are in progress to understand the exact mechanism of protection by Ep.

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