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Combination of Ascorbate and α-Tocopherol as a Preventive Therapy against Structural and Functional Defects of Erythrocytes in Visceral Leishmaniasis

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The redox unbalance in erythrocytes has been found to contribute significantly in the development of anemia in visceral leishmaniasis (VL). The present study revealed enhanced production of reactive oxygen species (ROS) and gradual depletion of α-tocopherol and ascorbate in the erythrocytes of infected animals. The response of erythrocytes to chronic treatment with antioxidants was studied in hamsters during leishmanial infection. Treatment with a combination of α-tocopherol and ascorbate proved to be the most effective preventive for the proteolytic degradation of erythrocyte membrane. Erythrocytes from infected animals were thermally more sensitive compared to the control ones. Combination of both antioxidants was most successful in resisting heat induced structural defects in the cells. Cross-linking of membrane proteins subsequent to oxidative damage in the red cells was accompanied by the formation of high molecular weight protein band at the top of the resolving gel in the presence of the cross-linking agent dimethyl-adepimidate (DMA). Marked inhibition of cross-linking was observed with combination of both antioxidants. Treatment with α-tocopherol and ascorbate together could withstand osmotic lysis of erythrocytes in the infected animals very efficiently. Decreased hemoglobin (Hb) level was successfully replenished and was coupled with significant increase in the life span of red cells after treating the animals with both antioxidants. Results indicate better efficacy of the combination therapy with α-tocopherol and ascorbate in protecting the erythrocytes from structural and functional damages during leishmanial infection.

Keywords: Visceral leishmaniasis; Anemia; Antioxidants; Free radicals

INTRODUCTION

Visceral leishmaniasis (VL) is predominantly a rural zooantroponose, which is transmitted to human beings by the bite of Cryptozoite dipterous insect (*Phlebotomous argentips*). Cardinal signs of this disease are prolonged fever, splenomegaly, pancytopenia and hypergammaglobulinemia.[1] Despite of the fact that anemia is one of the most striking clinical features of VL, the factors involved in its pathogenesis are not fully understood. Our earlier studies have shown enhancement in oxidative insult to red cells with the progress of anemia during leishmanial infection.[2] This perturbs the calcium homeostasis of erythrocytes inducing proteolytic degradation of cell membranes, which in turn precondition the red cells to premature lysis during this disease.[3] However, under normal condition, the red cells have the capacity to withstand the constant oxidative attack they face and can survive because of its in-built efficient antioxidant system.[4] Keeping this in mind, we investigated the antioxidant status of red cells during leishmanial infection. We further aimed at combating the damages resulting from oxidative assault to the red cells by subjecting the infected animals to chronic treatment with some non-enzymatic antioxidants.

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MATERIALS AND METHODS

Unless indicated, all chemicals and reagents were from Sigma–Aldrich Chemical Company, in the highest grade available. Radioactive labeled sodium chromate (sp act 94.2 Ci/gm) was purchased from Bhaba Atomic Research Centre, Mumbai, India.

Infection with Parasite

Leishmania donovani strain MHOM/IN/1983/AG83 was originally obtained from an Indian kala-azar patient. Every 6 weeks the strain was maintained in hamsters by intracardial passage. Amastigotes were isolated from the spleen of infected animal by the method of Hart *et al.*,[5] as modified by Looker *et al.*,[6] and were used for infection of hamsters (4–6 weeks) by injecting 10^7 amastigotes intracardially. Development and progress of anemia were monitored at different stages of infection by routine hematological methods.[7]

Antioxidant Treatment

Four to six weeks old Syrian golden hamsters (*Mesocricetus auretus*) weighing about 30–40 g were used in this study. Ascorbic acid was dissolved in 0.9% saline, whereas α-tocopherol was dissolved in minimum quantity of ethanol and diluted with 0.9% saline (0.1% v/v). At the 3 day after infection, both the drugs were administered orally at the doses of 2 mg/kg and 10 mg/kg of α-tocopherol and ascorbic acid, respectively. Untreated infected animals receiving ethanol-saline in place of drugs constituted the control group. Drugs were given biweekly and the animals were sacrificed after 3 months of chronic treatment.

Preparation of Erythrocyte Membrane

Blood was collected from control and infected animals using heparin as anticoagulant. Cells were washed thrice with phosphate buffered saline (PBS, pH 7.4) and the buffy coat (mainly leukocytes) was separated from the erythrocytes by aspiration. Packed RBC's were then washed thrice with isotonic PBS (pH 7.4). Washed erythrocytes were hemolyzed in hypotonic lysing buffer and centrifuged at 27,000*g* for 30 min at 0–4°C. The process was repeated until the RBC membranes thus obtained were almost free of Hb.[8]

Analytical Determinations

Red Cell Survival

Red cell survival was estimated by enumerating the red cell half-life over time. Radioactive labeled sodium chromate (^{51}Cr) was injected intracardially to hamsters at a dose of 11 μCi/Kg body weight. The radioactive count of the red cells were taken at 7 days time interval till half of the radioactive ^{51}Cr injected had disappeared from circulation. The count on day "0" was taken as 100% radioactivity. The day at which 50% radioactivity disappeared was termed as $t_{1/2}$. Final results were expressed as $t_{1/2}$ in days.[9]

Measurement of Ascorbate and α-Tocopherol

Ascorbate concentration in cells was measured by HPLC using ion-pairing method of Pachla and Kissinger.[10] Cells were lysed by freeze thaw cycle in dry ice acetone and allowed to thaw on ice. The hemolysate was then ultra filtered according to the method of Iheanacho *et al.*[11] The clear ultra filtrate was used for the measurement of ascorbate. HPLC was carried out on a Waters Delta Pak C_{18} Column (300 μm, 5 μ) with a 4 mm guard column of same packing material. The mobile phase consisted of 80 mM ammonium acetate, 1 mM tridecylamine and 15% methanol, pH 5.2. Ascorbate was eluted at 5–6 min at a flow rate of 1 ml/min.

α-Tocopherol content was measured by HPLC according to the method of Lang *et al.*[12] 0.2 μl of packed cells or pellet of ghosts were mixed with 10 μl of methanol containing 0.5 mg of butylated hydroxy toluene, followed by 5–10 volumes of methanol containing 20 mM ascorbate. The lysate and ghost membrane extracts were chromatographed with 95% methanol as mobile phase on a Waters Delta Pak C_{18} Column (300 μm, 5 μ), with the 4 mm guard column of the same packing material. α-Tocopherol was eluted at 6.1–6.4 min at a flow rate of 1 ml/min.

Measurement of Reactive Oxygen Species (ROS)

Super oxide radical ($O_2^{\cdot-}$) in red blood cells was measured by using ferricytochrome C.[13] Optical density was read at 550 nm. $O_2^{\cdot-}$ concentration was calculated from the molar extinction coefficient of ferrocytochrome C, 28,000/M/cm.

H_2O_2 level in the erythrocytes was measured by using 2,6-DCPIP (2,6-dichlorophenol indophenol).[14] From the equimolar stoichiometry of the redox reaction involved, calculation of H_2O_2 concentration was done by using the molar extinction coefficient of oxidized 2,6-DCPIP (2.1×10^4/M/cm) at 610 nm.

Hydroxyl radical level ($^{\cdot}OH$) of erythrocytes was estimated by adding 100μM ascorbate, 10 μM 2-deoxy D-ribose and 20 mM Hepes buffer, pH 7.4 to 20% red cell suspension.[15] The reaction was initiated at 37°C by adding 100 μl H_2O_2 and stopped by adding 4% orthophosphoric acid. The deoxyribose damage which corresponds to $^{\cdot}OH$ was measured at

the absorbance of MDA at 532 nm using a molar extinction coefficient of 1.56×10^5/M/cm.

Analysis of Membrane Proteins

Protein profile of erythrocyte membrane was determined by SDS-PAGE using 4 and 10% polyacrylamide as the stacking and running gels, respectively according to the method of Laemmli.[16] The gels were stained with coomassie brilliant blue R-250 and scanned in a laser densitometer.

Determination of Crosslinking of Membrane Proteins

Homogenised red cell membranes were incubated with the crosslinking agent DMA for 60 min at room temperature. The reaction was terminated with 0.05 M ammonium acetate. The membranes were pelleted by centrifuging at 27,000*g* for 30 min and washed once in 10 M Tris-HCl (pH 7.4). SDS-PAGE of the crosslinked membranes was done according to the standard procedure.[17]

Analysis of Thermal Sensitivity

Erythrocytes were diluted to 10% hematocrit with 10 mM glycyl glycine containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 1 mM adenosine (pH 7.4) and subjected to heating in water bath at 46°C for 15 min. The cells were harvested by centrifugation at 1800*g*. Erythrocytes were then prepared for electron microscopy.[18] The cells were suspended in 10% hematocrit in PBS. Equal volumes of cell suspension and 2.5% glutaraldehyde were incubated at 15–20°C for 1 h. The cells were then washed to remove excess glutaraldehyde, and a very thin film of washed cells was spread on a cover slip. It was then dried and washed twice successively with increasing concentration of acetone in water.

Determination of Osmotic Fragility

Osmotic fragility of erythrocytes was determined by measuring their hemolysis in hypotonic buffer. Erythrocyte suspensions were added to a range of hypotonic (0.2–0.8%) PBS and the extent of hemolysis were determined spectrophotometrically at 620 nm.[19]

Statistical Analysis

Conventional methods were used for calculation of means and SD. Comparison between control and infected group was performed by unpaired student's *t*-test.

RESULTS

Antioxidant Level and the Status of ROS during Leishmanial Infection

Of the non-enzymatic defenses, important roles have been established for ascorbate in the cytoplasm while the foremost protective mechanism for red cell membrane can be attributed to α-tocopherol. There was gradual diminution in the levels of these two non-enzymatic antioxidants in the erythrocytes with the progress of infection as shown in Table I.

ROS like $O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$ etc. are potent oxidants, which contribute significantly to the pathogenesis of different types of disease. Table II shows increasing trends of both $O_2^{\cdot -}$ and H_2O_2 whereas $^{\cdot}OH$ radical was significantly elevated after 3 months of infection.

Effects of Antioxidant Treatment on Structural and Functional Properties of Red Blood Cells during VL

Ascorbate and α-tocopherol Level in Erythrocytes after Antioxidant Treatment

Chronic treatment with ascorbate was more effective in replenishing the decreased antioxidant levels in the erythrocyte of infected animals compared to the response obtained from similar treatment with α-tocopherol. However, simultaneous treatment with both the antioxidants helps to restore their levels almost to the normal status (Table III).

TABLE I Antioxidant level in erythrocytes from control and infected hamsters with the progress of infection. Ascorbate and α-tocopherol were measured by HPLC as described in "Methods" section

Post-infection period (in days)	Ascorbic acid (μM)		α-Tocopherol (μM)	
	Control (6)	Infected (6)	Control (6)	Infected (6)
30	26.7 ± 4.9	17.3 ± 3.2**	7.0 ± 1.1	5.2 ± 0.65**
60	25.6 ± 4.5	14.9 ± 2.9*	6.8 ± 0.9	3.9 ± 0.5*
90	24.5 ± 4.4	7.6 ± 0.98*	6.6 ± 0.85	2.2 ± 0.43*

Values are mean ± SD of four independent experiments. *$P < 0.001$, **$P < 0.01$, compared to their respective control groups. Figures in parenthesis represent number of animals in each group.

TABLE II Some ROS in erythrocytes during leishmanial infection. $O_2^{\cdot-}$ was measured by using ferricytochrome C. $^{\cdot}OH$ radical was estimated using 2-deoxy-D-ribose. H_2O_2 level was measured by using 2,6-DCPIP (oxidized blue dye). Experimental details are given under "Methods" section

Reactive oxygen species (ROS)	Control (6) (μM)	Infected (6) (μM)	% Increase from control
Super oxide radical ($O_2^{\cdot-}$)	0.024 ± 0.004	0.038 ± 0.007*	58.33
Hydrogen peroxide (H_2O_2)	0.18 ± 0.084	0.28 ± 0.05***	55.55
Hydroxyl radical ($^{\cdot}OH$)	5.4 ± 0.84	10.2 ± 2.03**	88.88

Values are the mean ± SD of four independent experiments. ***$P < 0.01$, **$P < 0.001$, *$P < 0.02$, compared to control animals. Figures in the parenthesis represent the number of animals used in each group. Results are from one time course (after 3 months of infection).

Protein Profile of Erythrocyte Membrane

Oxygen radicals have been found to influence fragmentation of membrane proteins in several red cell disorders. Our studies revealed accentuated degradation of protein bands 3 and 4.1 in the erythrocyte membrane of infected animals. Figure 1a shows the protein profile of red cell membranes after treating the infected animals with ascorbic acid and α-tocopherol. Ascorbic acid was slightly more effective (lane F) than α-tocopherol (lane E) in inhibiting the degradation of erythrocyte membrane proteins of infected hamsters. However, the most effective prevention of proteolytic degradation was observed after combination treatment with both α-tocopherol and ascorbic acid (lane D). The potency of the antioxidant treatment in protecting the degradation process can be assessed by determining the amount (in % from control) of band 3 and 4.1 proteins remaining after the degradation before and after antioxidant treatment (Fig. 1b).

TABLE III Effect of antioxidant treatment on the ascorbate and α-tocopherol level in erythrocytes of hamsters infected with *L. donovani*. Ascorbate and α-tocopherol were measured by HPLC as described in "Methods" section

Animals	Ascorbic acid (μM)	α-Tocopherol (μM)
Control	24.5 ± 4.4	6.6 ± 0.43
Infected	7.6 ± 0.98*	2.2 ± 0.43*
Infected + α-tocopherol	8.2 ± 1.83^{NS}	4.5 ± 0.86**
Infected + ascorbic acid	20.2 ± 1.65**	3.6 ± 0.62***
Infected + ascorbic acid + α-tocopherol	22.3 ± 3.52****	5.4 ± 0.97****

Values are the mean ± SD of four independent experiments. *$P < 0.001$ compared to control animals. NS, non significant, **$P < 0.01$, ***$P < 0.02$, ****$P < 0.001$ compared to infected animals. Results are from one time course after 3 months of infection.

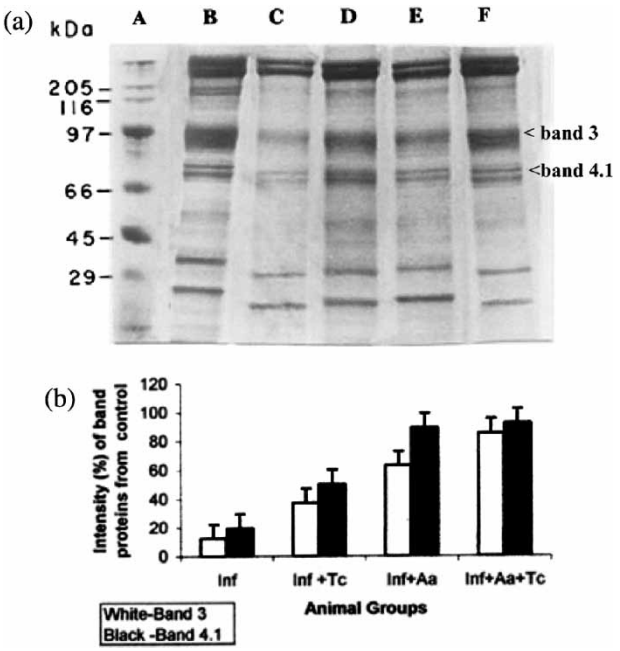

FIGURE 1 (a) Protein profile of erythrocyte membrane after antioxidant treatment. Standard molecular weight markers (lane A); Control membrane (lane B); 3 months infected membrane (lane C); infected (3 months) + ascorbic acid and α-tocopherol (lane D); infected (3 months) + α-tocopherol (lane E); infected (3 months) + ascorbic acid (lane F). Lane A was loaded with 10 μg of the markers whereas all other lanes were loaded with 50 μg of protein. The gel was stained with Coomassie Brilliant Blue R. The banding patterns seen here were identical to those seen in another three replicates. (b) Degradation of band 3 and 4.1 in the erythrocyte membrane of hamsters after antioxidant treatment. The intensities of band proteins on the SDS-PAGE (Fig. 1a) were determined by densitometry and the amount (%) remaining after degradation was calculated taking the control level as 100%. Values shown are mean ± SD of four determinations. Inf, Infected (3 months); Tc, α tocopherol; Aa, ascorbic acid.

Thermal Sensitivity of Erythrocytes

Thermal sensitivity of erythrocyte is highly dependent on the membrane skeletal proteins. Heating at 46°C transformed the shapes of the red cells from discocytes to spherocytes and poikilocytes in the infected animals (Fig. 2B). However, erythrocytes from control hamsters could retain their normal biconcave shape after similar treatment (Fig. 2A) Infected animals treated with antioxidants mostly maintained the normal shape of the erythrocytes after the heat treatment. Figure 2D) shows that ascorbic acid treatment was slightly more effective than α-tocopherol (Fig. 2C) in preventing the thermal shock. The infected animals treated simultaneously with both ascorbic acid and α-tocopherol was most successful in resisting the heat induced structural defects in erythrocytes (Fig. 2E).

Cross Linking of Membrane Proteins

The arrangement of skeletal proteins in red cell membranes from Leishmania infected hamsters was

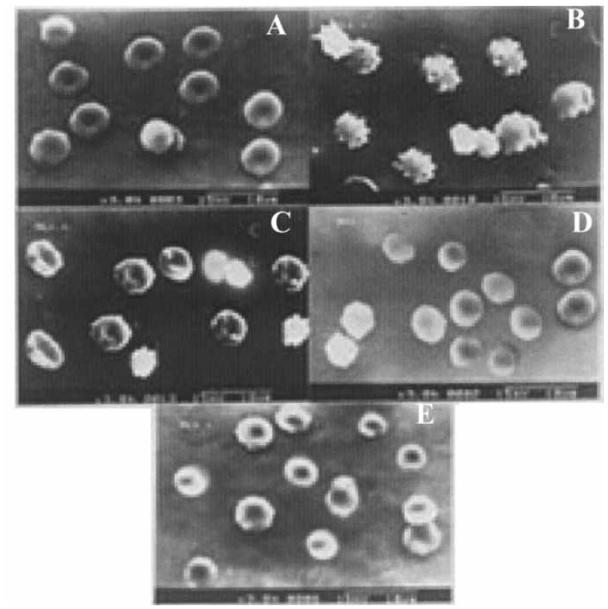

FIGURE 2 Scanning electron micrographs of erythrocytes after heat treatment. Cells were heated at 46°C. They were fixed in glutaraldehyde and coated with palladium-gold alloy. Cells were examined in Hitachi S-2360 N scanning electron microscope at a magnification of 3000× at 30° tilt angle. Control (panel A), 3 months infected (panel B), 3 months infected + α-tocopherol treatment (panel C), 3 months infected + ascorbic acid treatment (panel D), 3 months infected + ascorbic acid and α-tocopherol treatment (panel E). The figure represents three independent observations.

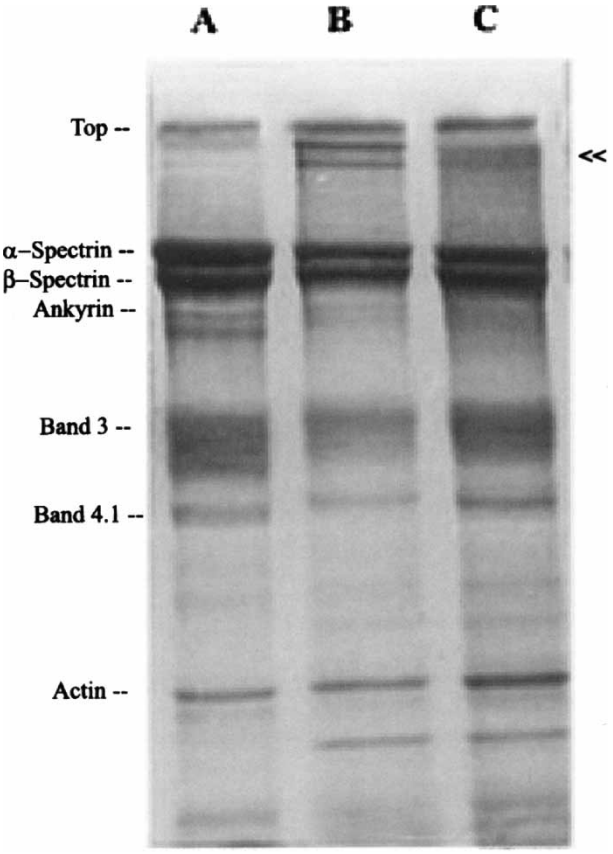

FIGURE 3 Effect of antioxidant treatment on the cross-linking of membrane proteins in the erythrocytes of hamsters infected with *L. donovani*. Crosslinking was done by incubating the membrane with cross-linking agent DMA. The gel was stained with coomassie brilliant blue R. Fifty microgram of protein was applied to each lane. A, control; B, 3 months infected; C, 3 months infected after treatment with ascorbic acid and α-tocopherol simultaneously. Crosslinked proteins are indicated in SDS—PAGE analysis with arrows. Band proteins are numbered according to the standard nomenclature for erythrocyte membrane proteins. The banding pattern seen here were identical to those seen in another three replicates.

studied using the bifunctional crosslinking agent DMA with a span of 8.6 Å. The membrane from infected source showed decrease in the intensities of spectrin, band 3 and 4.1, with the formation of high molecular weight protein band at the top of the resolving gel in the presence of cross linking agent (Fig. 3B). However, the ghosts from the control animals do not show any cross-linking with the same concentration of DMA (Fig. 3A). It is also clear from the figure that chronic treatment of infected hamsters with the combination of ascorbic acid and α-tocopherol could reduce the cross linking of membrane proteins to a considerable extent (Fig. 3C).

Osmotic Fragility of Red Cells

Figure 4 shows the hemolysis of erythrocytes in hypotonic saline compared to that produced by the hemolysis of the same volume of erythrocytes in water. The red cells of infected animals became highly fragile compared to the control ones. Treatment with ascorbic acid and α-tocopherol together could resist osmotic lysis efficiently as shown in the figure.

Effect of Antioxidant Treatment on the Hb Level and Survival of Erythrocytes

Table IV reveals decline in Hb level and reduction in the life span of erythrocytes in the infected animals as compared to the control ones. Treatment of the infected animals with the combination of antioxidants shows replenishment of decreased Hb level. This therapy was also successful in enhancing the life span of erythrocytes to a sizable range.

DISCUSSION

Our earlier studies revealed enhanced oxidative damage in the erythrocytes during leishmanial infection.[2] ROS have been implicated in various processes in health and disease.[20,21] The emphasis has been mainly on their detrimental effects causing tissue injury in several pathological conditions. Higher production of $^{\cdot}OH$ in comparison to ($O_2^{\cdot-}$) and H_2O_2 indicate hydroxyl radical to be the major harmful oxidant in erythrocytes during leishmanial infection.

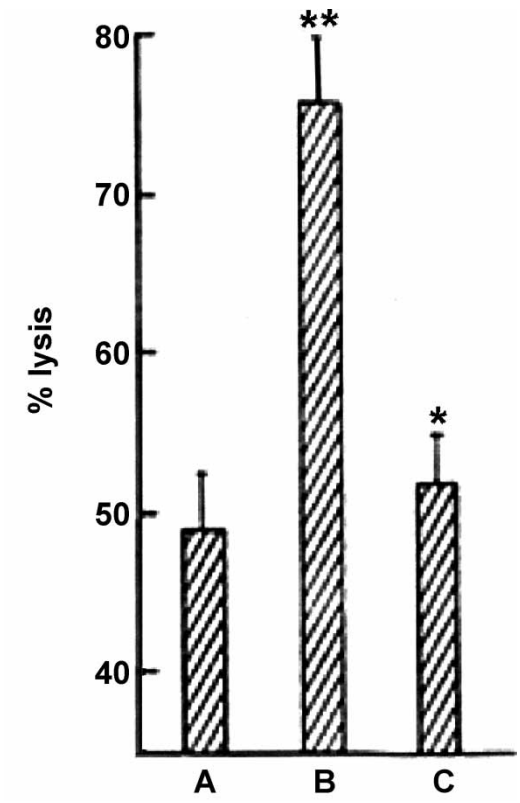

FIGURE 4 Osmotic fragility of erythrocytes after antioxidant treatment. Data are mean ± SD at four independent experiments. $^{**}P < 0.01$ compared to control; $^{*}P < 0.01$ compared to infected. A, control; B, infected (3 months); C, infected (3 months) + antioxidant (ascorbic acid and α-tocopherol) treatment.

Under normal condition, erythrocytes are equipped with efficient antioxidant system to combat the oxidative assault they face during their life span in circulation.[4] A positive correlation has been reported between the intracellular concentration of α-tocopherol and ascorbate in erythrocytes during leishmanial infection.[22] This corroborates with the concomitant decrease of these two non-enzymatic antioxidants observed with the progress of infection and suggest the deficiency of both to be responsible to some extent for the enhanced susceptibility of erythrocytes to oxidative injury in VL. To compensate the deficiency of these antioxidants in red blood cells, the infected animals were subjected to chronic treatment with α-tocopherol and ascorbic acid during post-infection period.

Replenishment of the decreased α-tocopherol level to some extent with the help of ascorbate supplementation may be correlated with the reduction of tocopheroxyl free radical and recycling of α-tocopherol by ascorbate. α-Tocopherol imparts its antioxidant potential solely on the red cell whereas ascorbate fails to stay within the membrane.[22] This explains the effect of α-tocopherol treatment,which was probably restricted only to membrane and could not affect the restoration of ascorbate level in the cytoplasm.

Erythrocyte membrane is composed of a cytoskeletal network, and a bilayer which are responsible for maintaining the membrane structural integrity and biconcave shape of the erythrocytes.[23] Oxidative attack on red cells can modify its proteins and enhance their degradation by intracellular proteolytic system.[24] Marked degradation of band 3 and 4.1 after 3 months of infection may lead to decreased binding of cytoskeleton to the membrane bilayer. This can finally result in the destabilization of cell membrane and early lysis of red cells in the infected animals.

Better efficacy of the combination treatment with α-tocopherol and ascorbate in comparison to the treatment with each of these antioxidants alone in resisting the proteolytic degradation of erythrocyte membrane, support the possibility of a mediating role of α-tocopherol in the transmembrane transfer of ascorbate derived electrons during leishmanial infection.[22]

Thermal sensitivity of erythrocytes is highly dependent on the membrane skeletal proteins. Any change in their structural association, alignment with each other will affect this property of cell membrane.[25] Defects in the skeletal components have been described in several hemolytic anemias', which may give rise to mechanically and thermally unstable erythrocytes.[23] Erythrocytes from infected animals were found to be thermally more sensitive than the red cells from the control animals. Heat treatment affected the biconcave shape of erythrocytes during leishmanial infection and protection offered by the antioxidant treatment against this thermal shock points to an interacting role of α-tocopherol and ascorbate in enhancing the antioxidant activity of the treated cells. As the combination therapy of α-tocopherol and ascorbic acid was more effective than each of these antioxidants alone in rectifying oxidative hazards in erythrocytes during leishmanial infection, combination of these

TABLE IV Hb level and life span of erythrocytes of infected hamsters after antioxidant treatment. Life span of erythrocytes was measured by labeling the red cells with ^{51}Cr. $t_{1/2}$ represents the day at which 50% of the injected radioactivity disappeared from circulation. Experimental details are given under the "Methods"

Animals	Hemoglobin content (gm/dl)	Red cell life span ^{51}Cr $t_{1/2}$ in days
Control	16.2 ± 1.20	22
Infected	10.0 ± 0.89*	8
Infected + ascorbic acid and α-tocopherol treated	13.5 ± 1.4**	16

Values are mean ± SD of four independent experiments. $^{*}P < 0.01$ compared to control animals, $^{**}P < 0.05$ compared to infected animals. Results are from one time course (after 3 months of infection).

two was used for treatment in subsequent experiments. As the oxidative assault to red cells progressed, it was associated with enhanced formation of MDA during the post-infection period.[2] MDA is known to be capable of cross-linking membrane proteins through their carbonyl groups.[26] Oxidative damage of membrane lipids and subsequent cross-linking of membrane proteins indicate derangement of skeletal proteins[27] in the erythrocytes of the infected animals. Favourable arrangements of the skeletal and integral membrane proteins are required to build up the protein meshwork which is responsible for the shape and stability of the red cells. Distance between the functional groups of DMA is 8.6 Å. Subtle change in the membrane protein organization may be sufficient for the formation of DMA cross-links. The result suggests that the membrane proteins of the red cells in the infected animals undergo spatial redistribution, so that they become cross-linkable by the DMA concentration, which fails to cross-link the membrane proteins from the control animals.

Prevention of this cross linking process by chronic treatment with the combination of antioxidants may be considered as a protective measure against the hemolysis by free radicals during leishmanial infection.

Accentuated degradation of both cytoskeletal and integral membrane proteins can affect the permeability of red cell membrane[28] and can finally result in the destabilization of cell membrane leading to the early lysis of erythrocytes in the infected animals. Treatment with ascorbic acid and α-tocopherol together could resist the osmotic lysis of erythrocytes in the infected animals very efficiently. Combination drug treatment showed potency towards the replenishment of decreased Hb level and life span of erythrocytes in the infected animals. The results indicate the efficacy of chronic antioxidant therapy in the rectification of anemia along with the oxidative damages in erythrocytes during leishmanial infection.

Since ascorbate cannot stay within the membrane, its antioxidant activity does not directly interfere with the peroxidation of membrane lipids, but may do so indirectly either by reducing tocopheroxyl free radical in the lipid bilayer or through transmembrane electron transfer in the erythrocytes.[22] This probably explains the cooperative behaviour of α-tocopherol and ascorbate in maintaining the antioxidant reserve of erythrocytes and thereby protecting the affected cells from premature destruction during VL.

Acknowledgements

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