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MODULATORY ROLE OF ANTIOXIDANT VITAMINS C AND E ON ERYTHROCYTE OSMOTIC FRAGILITY INDUCED BY CHRONIC SODIUM NITRATE ADMINISTRATION IN RATS

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ABSTRACT
Vitamin C and E supplements were administered to sodium nitrate-treated rats in order to examine the possible ameliorative effects of these antioxidants on erythrocyte osmotic fragility. Seventy adult Wistar rats were randomly divided into seven groups (n=10) and administered drugs or distilled water orally using a metallic canular for 60 days. Group I (control) received distilled water; Group II - 30 mg/kg $NaNO_3$; Group III – 30 mg/kg $NaNO_3$ + 500 mg/kg vitamin C; Group IV – 30 mg/kg $NaNO_3$ + 750 mg/kg vitamin C; Group V – 30 mg/kg $NaNO_3$ + 300 mg/kg vitamin E; Group VI – 30 mg/kg $NaNO_3$ + 400 mg/kg vitamin E; Group VII - 30 mg/kg $NaNO_3$ + 500 mg/kg vitamin C + 300 mg/kg vitamin E. Blood was collected from each animal at the end of the experiment to determine erythrocyte osmotic fragility. The results showed that, sodium nitrate caused significant decrease in erythrocyte osmotic fragility. Each of the vitamin administered separately increased erythrocyte osmotic fragility back to normal values. However, co-administration of the two vitamins very significantly increased erythrocyte osmotic fragility to above normal values. It was concluded that, vitamin C and E administered separately ameliorated sodium nitrate toxicity in a dose dependant manner. Co-administration of the two vitamins showed synergistic effect which was detrimental to the rats due to the risk of anaemia.

Keywords: antioxidants, vitamin C, vitamin E, sodium nitrate, erythrocytes, osmotic fragility, rats.

INTRODUCTION

Nitrogenous fertilizers used in agriculture contain different nitrate salts which are passed from the soil to different fruits and vegetables as well as ground and surface water which are eventually consumed by man (Manassaram *et al.*, 2006). Nitrates are also used in the industry for preservation (Wogan *et al.*, 1995) and coloring of tinned meat and sausages (Manassaram *et al.*, 2006) manufacture of matches and special cement (ATSDR, 2001), etc, which makes exposure to nitrates inevitable. The global challenge of food shortage and the impoverishment of soil in many parts of the world make the use of fertilizers and other modern farming techniques ever more imperative. In the human body, nitrates induce oxidative damage through the release of nitrite and NO (Oladele *et al.*, 1997). nitrates are also known to cause carcinogenic, mutagenic, teratogenic and embryotoxic effects (Kasyanenko *et al.,*1992). Vitamin C and E are known to be potent antioxidants (Ayo *et al.*, 2006; Suteu *et al.* 2007), hence, it is conceivable that, their administration (in the form of vitamins C and E supplements) into the body may augment the function of endogenous free radical scavengers such as superoxide dismutase, catalase and glutathione peroxidase thereby decreasing the deleterious effects of nitrates and nitrites. The aim of the present study therefore was to investigate the effect of chronic exposure to nitrates on osmotic fragility and the possible ameliorative effect of antioxidant vitamins C and E.

MATERIALS AND METHODS

Seventy (70) adult Wistar rats were used for the study. The animals were kept in large cages in the animal house for two weeks to acclimatise before commencement of the experiment. They were allowed free access to distilled water and fed pelletised growers feed (Vital Feed, Jos, Nigeria) before and during the experiment. Pelletised growers feed contains crude protein (14.5 %), fat (7.0 %), crude fibre (7.2 %), calcium (0.8 %), available phosphorus (0.4 %) (manufacturer's information leaflet). Drinking water was changed daily, and alternate day clearing and replacement of sawdust and droppings were carried out. Sodium nitrate salt (BDH Chemicals Limited, Poole, England) was dissolved in distilled water to make a stock solution containing 2.5 mg $NaNO_3$ in 0.1ml from which the animals were fed during the experiment. Tablets of Vitamin C (Em – vitamin C, 100mg tabs. Emzor Pharmaceutical Industries, Lagos, Nigeria) were crushed into powder to prepare a solution containing 25 mg of vitamin C in 0.1 ml. Similarly, capsules of vitamin E (Efishal 200™,Shalina Laboratories, Pvt, Mumbai, India) was cut open and emptied into a clean container. Vegetable oil was added to prepare a suspension containing 34 mg of the vitamin E in 0.1ml.

The solutions were kept at room temperature, and vitamin E was protected from direct contact with air and sunlight to avoid degradation, by stocking in a dark, air-tight jar. Appropriate amounts of $NaNO_3$

solution and the vitamins were collected using 1ml syringe for administration based on body weight of the animals.

The animals were randomly divided into seven groups of 10 rats each (n=10). They were administered drugs or distilled water orally using a metallic canular between the 8.00 am – 10.00 am daily for 60 days as follows: Group I (control) – received distilled water; Group II – received 30 mg/Kg $NaNO_3$; Group III – received 30 mg/Kg $NaNO_3$ + 500 mg/Kg vitamin C; Group IV – received 30 mg/Kg $NaNO_3$ + 750 mg/Kg vitamin C; Group V – received 30 mg/Kg $NaNO_3$ + 300 mg/Kg vitamin E; Group VI – received 30 mg/Kg $NaNO_3$ + 400 mg/Kg vitamin E; Group VII - received 30 mg/Kg $NaNO_3$ + 500 mg/Kg vitamin C + 300 mg/Kg vitamin E. The animals were weighed using a triple beam balance (Model OHAUS, 700 Series, Floram Park, N. J, U. S. A.) and values obtained were recorded at the beginning of the experiment and every two weeks. The doses of the drugs were adjusted according to the change in weights. At the termination of the experiment, the animals were anaesthetised by chloroform inhalation in a closed chamber and subsequently sacrificed. The thorax of the anaesthesized animal was cut open and with the aid of 5ml syringe with 21 Gauge needle, the pulsating heart of the rat was pierced and blood was aspirated. 3 ml of blood from each rat was transferred into each EDTA bottle for the determination of osmotic fragility.

Sodium chloride stock solution was prepared and maintained at pH of 7.4 (Faulkner and King, 1970). Ten test tubes, each containing 5ml of the NaCl solution of concentrations ranging from 0.00 to 0.90% were arranged serially in a test tube rack and labelled according to the concentrations of the NaCl. One set of the 10 test tubes was used to analyse each blood sample. 1ml syringe with 21 Gauge needle was used to transfer 0.02ml of blood (2 drops of 0.01 ml) from the EDTA bottle into each test tube of a set. Mixing was done immediately by gently inverting the test tube five times. The test tubes were allowed to stand for 30 minutes at room temperature (26 – 28^0C), after which the suspension was centrifuged at 2000 rpm for 10 minutes using model IEC HN – SII centrifuge. The supernatant was transferred into a glass cuvette and the concentration of haemoglobin was measured at wavelength of 540nm using a spectrophotometer (Spectronic–20, Bausch and Lomb, U. S. A.) by reading the absorbance. The same procedure was repeated for each of the blood samples. The % haemolysis was calculated using the formula of Faulkner and King (1970):

$$\% \text{ Haemolysis} = \frac{\textit{Optical Density of Test Solution}}{\textit{Optical Density of Standard Solution}} \; x \; 100$$

The data obtained are expressed as mean ± S. E. M. Differences in means of discrete parameters of any two groups were analysed using Student's *t*-test. Values of $p < 0.05$ was considered significant.

RESULTS

Effect of sodium nitrate: Percent haemolysis in the blood of rats treated with $NaNO_3$ was generally less than that of the control and the decrease was statistically significant at 0.60% and 0.50% NaCl concentration. There was significant negative correlation between percent haemolysis and percent NaCl concentration in both the control ($r = -0.87$, $p < 0.001$) and the nitrate-treated rats ($r = -0.94$, $p < 0.001$); that is, as the percent NaCl concentration decreased, percent haemolysis increased in both groups (Figure 1).

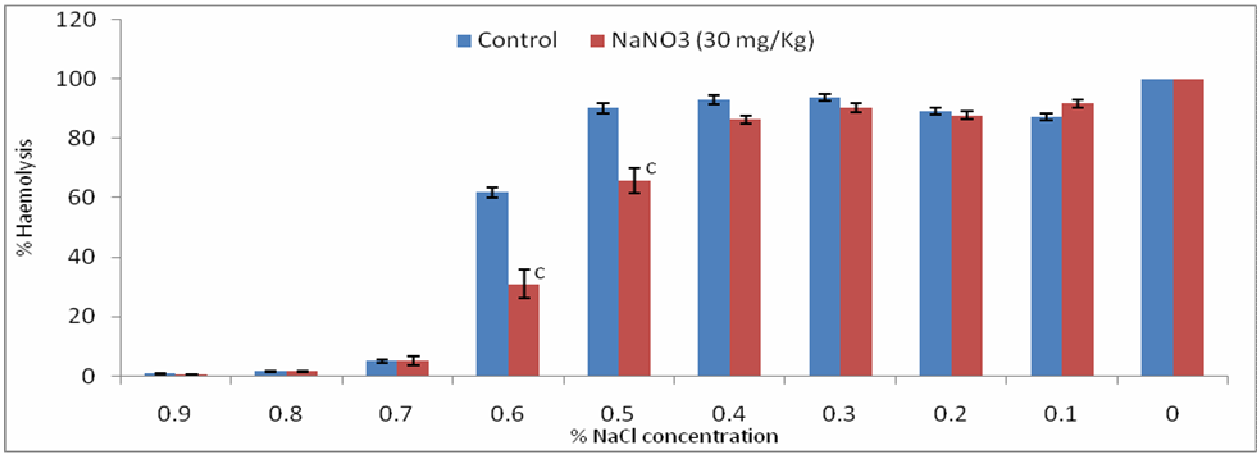

Figure 1: Percent haemolysis of control and nitrate-treated rats, ^{c} $p < 0.05$ compared to control.

Effect of vitamin C administration: Percent haemolysis in the blood samples of rats given 750 mg/kg vitamin C was significantly higher than in nitrate-treated rats at 0.70% and 0.60% NaCl concentration. Haemolysis was similar in rats treated with $NaNO_3$ + 750 mg/kg vitamin C and those treated with $NaNO_3$, at all other concentrations with maximum haemolysis occurring at 0.10% $NaNO_3$ concentration for both groups (Figure 2).

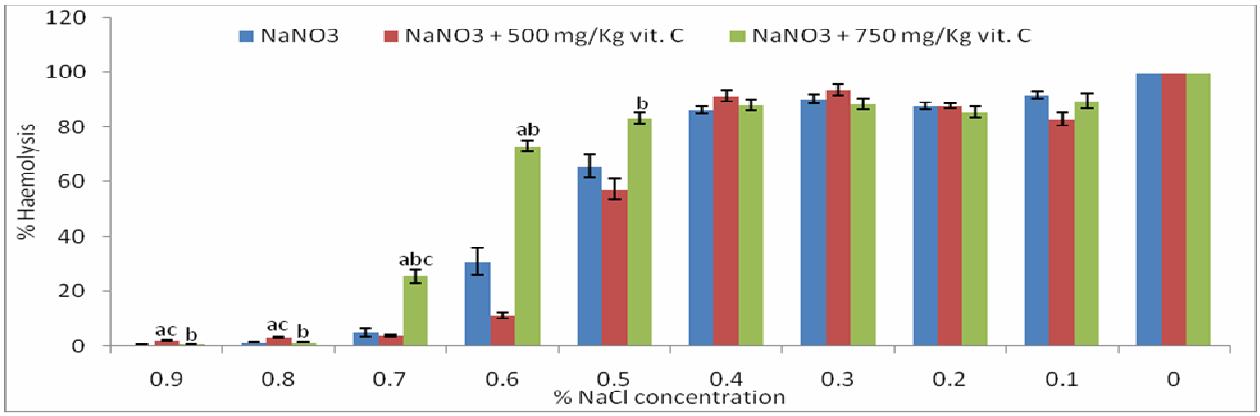

Figure 2: Percent haemolysis of nitrate- and vitamin C-treated rats.
[a] $p < 0.05$ compared to $NaNO_3$-treated rats, [b] $p < 0.05$ compared with $NaNO_3$ + 500 mg/kg vit. C-treated rats.
[c] $p < 0.05$ compared to control.

Effect of vitamin E administration: Percent haemolysis was higher at all NaCl concentrations in blood collected from rats treated with 300 mg/kg vitamin E when compared with the nitrate-treated rats, and the difference was statistically significant between 0.90% and 0.50% NaCl concentration. Maximum haemolysis was slightly higher in rats treated with 300 mg/kg vitamin E (92.37 ± 3.9) when compared with the nitrate-treated ones (91.76 ± 2.5), and it occurred at a higher NaCl concentration of 0.40% in the vitamin E-treated rats and 0.10% in the nitrate-treated. Percent haemolysis in rats treated with $NaNO_3$ + 300 mg/kg vitamin E was statistically similar to that of control between 0.60% - 0.10% NaCl concentration.

Percent haemolysis was higher at all % NaCl concentrations in the blood collected from rats treated with 400 mg/kg vitamin E and the difference was statistically significant at 0.06% and 0.50% NaCl when compared with the nitrate-treated rats. Maximum haemolysis in the rats treated with 400 mg/kg vitamin E was 99.42 ± 7.1% at 0.40 % NaCl concentration, and 91.76 ± 2.5 % at 0.1 % NaCl concentration in the nitrate-treated rats. Percent haemolysis of rats treated with $NaNO_3$ + 400 mg/kg vitamin E was statistically the same as that of the control at all the NaCl concentrations. Generally, there was no statistically significant difference in percent haemolysis obtained in rats treated with the two different doses of vitamin E, except at 0.70 % NaCl concentration, when the percent haemolysis in rats treated with the lower dose was markedly higher than in those rats treated with the higher dose.

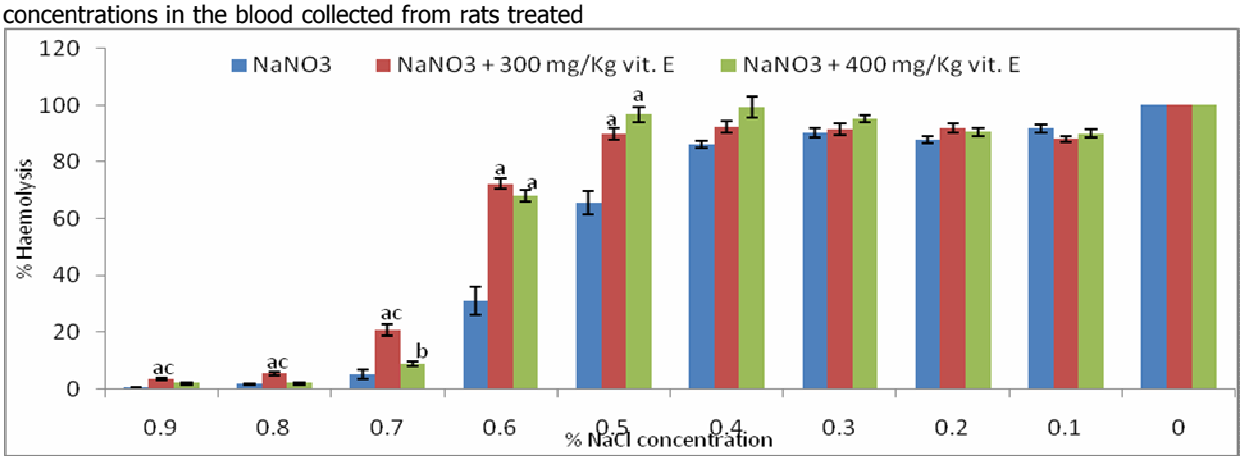

Figure 3: Percent haemolysis of nitrate- and vitamin E-treated rats.
[a] $p < 0.05$ compared to $NaNO_3$-treated, [b] $p < 0.05$ compared to $NaNO_3$ + 300 mg/kg vit. E-treated.
[c] $p < 0.05$ compared to control.

Effects of vitamin C and E co-administration: Percent haemolysis in the blood collected from rats co-administered vitamins C + E was higher in most of NaCl concentrations when compared with $NaNO_3$-treated, and the difference was statistically significant between 0.90 % and 0.40 % NaCl concentration. Maximum haemolysis was 95.41 ± 2,7 % at 0.40 % NaCl concentration for rats treated with vitamin C + E, and 91.76 ± 2.5 % at 0.10 % NaCl concentration for the nitrate-treated rats. Besides, co-administration of both vitamins also produced marked increase in percent haemolysis as compared to control at NaCl concentrations between 0.90 and 0.60 %.

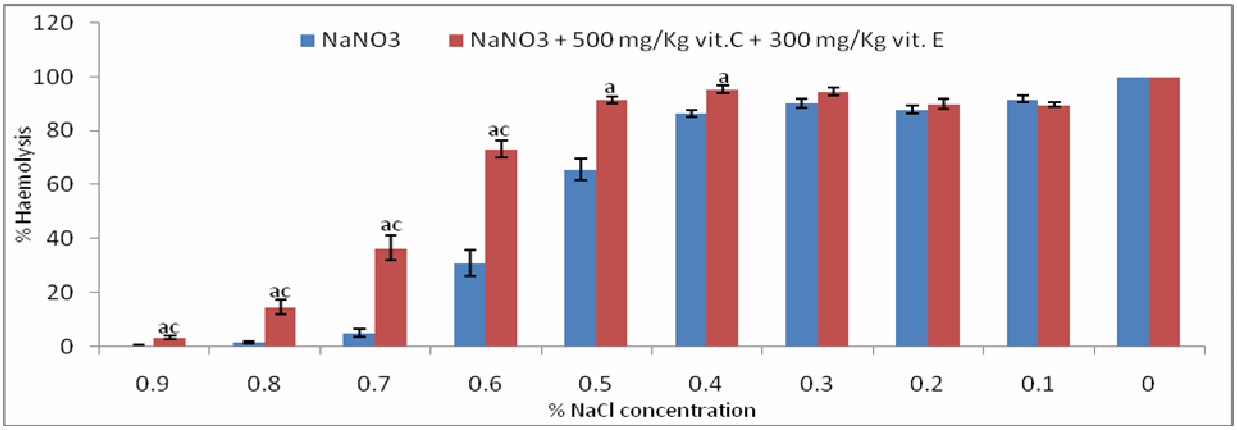

Figure 4: Percent haemolysis of nitrate- and vitamin C + E-treated rats.
[a] $p < 0.05$ compared to $NaNO_3$-treated, [c] $p < 0.05$ compared to control.

DISCUSSIONS

Chronic administration of sodium nitrate decreased the percent haemolysis, that is, it decreased osmotic fragility of erythrocytes of the exposed rats (figure 1). This result contradicted the previous findings that nitrates, through oxidative process change membrane structural state by decreasing membrane fluidity provoking haemolysis (Batina, 1998). Oxidative injury to erythrocyte membrane was reported to cause formation of pyknocytes (Fischer *et al.*, 1985; Bensoltane *et al.*, 2006) - shrunken and condensed cells - which result in increased ability to resist osmotic stress. A recent study by Bensoltane *et al.* (2006) reported that rats subjected to sub-chronic nitrate effect developed capacities of adaptation. A similar finding of adaptation was reported in sheep (Sinclair and Jones, 1964). Thus, the decrease in osmotic fragility of erythrocytes after chronic exposure to sodium nitrate poisoning in the present study may be due to the ability of the erythrocytes to develop adaptive compensatory changes via homeostatic mechanisms to reduce the toxicity.

Administration of 750 mg/kg vitamin C to $NaNO_3$-treated rats increased erythrocyte osmotic fragility of $NaNO_3$-treated rats to values statistically similar to that of control (reversed the $NaNO_3$ effect) (Figure 2). This shows the ameliorative effect of the vitamin at that dose. This effect was apparently due to the ability of the vitamin to strengthen the physical integrity of erythrocyte membrane (Awodi *et al.*, 2005) and through free radical quenching activity (Gecha and Fagan, 1992). Thus, vitamin C may be of benefit during chronic nitrate exposure.

There was significantly higher percent haemolysis at 0.90% and 0.80 % NaCl concentration in blood samples collected from rats treated with 500 mg/kg vitamin C when compared with nitrate-treated ones (Figure 2). Furthermore, maximum haemolysis was higher in the blood of rats given 500 mg/kg vitamin C (93.59 ± 3.8) when compared with the $NaNO_3$-treated rats (91.76 ± 2.5), and it occurred at a higher NaCl concentration of 0.30% and 0.10% successively. Percent haemolysis of erythrocytes of rats treated with 750 mg/kg vitamin C was generally higher than those of the $NaNO_3$-treated and the $NaNO_3$ + 500 mg/kg vitamin C-treated rats at concentrations between 0.90 and 0.5. % NaCl and was statistically similar to that of the control. This shows the dose-dependant effect of vitamin C in chronic nitrate toxicity and agrees with previous findings that the effect of vitamin C in oxidative stress-related toxicity increases with an increase in dose (Ambali *et al.*, 2007).

Vitamin E at both doses increased the osmotic fragility of the nitrate-treated rats back to normal especially the dose of 400 mg/kg (Figure 3), indicating that the vitamin counteracted the effect of $NaNO_3$ on osmotic fragility. This demonstrates the ameliorative effect of vitamin E, which is known to be a potent antioxidant (Ayo and Oladele, 1996; Whitehead and Keller, 2002). Erythrocyte osmotic fragility of rats treated with the two doses of the vitamin was statistically similar as shown in Figure 3, thus revealing no dose-dependant effect. However, a different dose margin may show a different result.

Co-administration of vitamins C and E significantly increased the osmotic fragility of $NaNO_3$-treated rats to a level higher than that of control (Figure 1 and 4). This effect is detrimental to the health of the animals as it predisposes them to anaemia. Further studies on the effect of co-administration of these vitamins to normal rats on osmotic fragility may require further clarification. The above result also demonstrated the synergistic effect of the two vitamins as reported by Khmelevsky and Poberezkina (1990). However, the synergistic effect seen in the present study is negative in nature. This is contrary to the findings of of Appenroth *et al.* (1997); Altuntas *et al.* (2002); Gokalp (2003); Khadkhodaee *et al.* (2005) and Suteu *et al.* (2007), who showed the protective effects of vitamins C and E in chronic toxicity associated with oxidative stress induced by different chemicals. Worthy of note is the fact that different mechanisms of action have been reported for both nitrates (Dutsheyko, 1989; Antipina *et al.,*1990; Donavan, 1990; Sidoryak and Minyaylenko, 1991; Zadorozhnaya, 1991; Kasyanenko *et al.,*1992; Karpovsky *et al.,*1994; IPCS, 1999; ATSDR, 2001) as well as vitamins C (Gecha and Fagan, 1992; Williams, 1997; Proteggente *et al.,* 2001; Singhal *et al.,* 2001;

Whitehead and Keller, 2002; Balz, 2003; Guan *et al.,* 2004; Son *et al.,* 2004; Awodi *et al.,* 2005) and E (Boadi; 1991; Packer, 1991; Javouhey-Donzel *et al.,* 1993; Gulthrie and Picciano, 1995; Karlson, 1997; Azzi and Stocker, 2000; Jain *et al.,* 2000; Sushil *et al.,* 2000; Droge *et al.,* 2006). This suggests that the interaction of these substances *in vivo* will be complex and is likely to result into a wide range of effects. The nature of the administered chemical, the dose, duration, as well as the route of administration in the present study, which may allow interaction between $NaNO_3$ and the vitamins in the rats' stomach before absorption, may explain the observed effects.

The doses of 500 mg/kg vitamin C and 300 mg/kg vitamin E showed similar effects on osmotic fragility when administered to $NaNO_3$-treated rats except for concentrations of 0.70, 0.60 and 0.50 % NaCl where vitamin E markedly increased osmotic fragility more than vitamin C (Figure 2 and 3). This indicates that vitamin E, which is lipid-soluble, has more potent antioxidant activity than vitamin C, thus agreeing with previous reports (Tsuchihashi *et al*., 1995; Ocak *et al*., 2007). Both vitamins at higher doses improved erythrocyte osmotic fragility to normal values where they produced similar effects except for 0.70% NaCl concentration where vitamin E showed similar effect to control while vitamin C significantly increased osmotic fragility more than control and vitamin E-treated groups (Figure 2 and 3). This indicates the ameliorative effect in chronic nitrate poisoning, with vitamin E (400 mg/kg) having a better effect. This is in agreement with the previous findings (Ayo and Oladele, 1996; Awodi *et. al*., 2005).

The increase in osmotic fragility observed in rats treated with the lower doses of both vitamins C and E was ameliorative more than the combination of both vitamins which showed higher erythrocyte osmotic fragility than the control group (Figures 2 and 3). However, the increase in erythrocyte osmotic fragility observed in the vitamins C + E- co-administered rats (Figure 4) is detrimental due to the risk of anaemia. This finding, again, indicates synergism between the vitamins in agreement with other findings (Hanafy and Sultan, 2004; Ocak *et al*, 2007; Suteu *et al*., 2007). Hence, concurrent oral intake of these vitamins should be avoided by individuals with long-term exposure to nitrates.

Conclusions

Based on the results obtained in this study, it was concluded that, sodium nitrate caused significant toxicity by decreasing erythrocyte osmotic fragility of the treated rats. Separate administration of both vitamin C and E ameliorated sodium nitrate toxicity by increasing erythrocyte osmotic fragility back to normal in a dose dependant manner, with vitamin E producing the better effect. However, co-administration of the two vitamins increased osmotic fragility to values significantly above the normal which is detrimental to the rats due to the risk of anaemia.

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