

*Full Length Research Paper*

# The effects of ascorbic acid and $\alpha$ -tocopherole on leukocyte count of sodium nitrate-treated Wistar rats

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The experiments were performed with the aim of investigating the possible ameliorative effects of ascorbic acid and  $\alpha$ -tocopherole supplements administered to sodium nitrate-treated rats on total and differential leukocyte counts. Seventy (70) adult Wistar rats, divided into seven groups of 10 rats each were used for the study. They were administered drugs or distilled water orally using a metallic canula between 8.00 to 10.00 am daily for 60 days as follows: Group I (control) received distilled water; Group II received 30 mg/kg NaNO<sub>3</sub>; Group III received 30 mg/kg NaNO<sub>3</sub> + 500 mg/kg AA; Group IV received 30 mg/kg NaNO<sub>3</sub> + 750 mg/kg AA; Group V received 30 mg/kg NaNO<sub>3</sub> + 300 mg/kg vitamin E; Group VI received 400 mg/kg NaNO<sub>3</sub> + 300 mg/kg vitamin E and Group VII received 30 mg/kg NaNO<sub>3</sub> + 500 mg/kg AA + 300 mg/kg vitamin E. At the end of the experiment, the rats were sacrificed and blood was collected for the determination of total and differential leukocyte count. The results showed that sodium nitrate administration induced leukocytosis, which was enhanced by AA administration at both the higher and lower doses as evidenced by increased neutrophil and lymphocyte counts as well as increased monocyte count at the lower dose. Similarly,  $\alpha$ -tocopherole at both doses enhanced NaNO<sub>3</sub> toxicity by increasing lymphocyte and total leukocyte counts. In conclusion, co-administration of the two antioxidant vitamins showed negative synergistic effect.

**Key words:** Ascorbic acid,  $\alpha$ -tocopherole, sodium nitrate, leukocytes, rats.

## INTRODUCTION

Sodium nitrate is one of the constituent salts of nitrogenous fertilizers. Recently, in many African countries, there has been a sharp increase in the use of nitrogenous fertilizers, pesticides, insecticides, herbicides, fungicides and other chemical agents in agriculture (Oladele et al., 1997). Nitrates from fertilizers eventually end up as residues in different quantities in the foodstuffs of plant origin which are consumed by man and their excess quantity adversely affects man and animals (Antipina et al., 1990). Foodstuffs of animal origin are also source of nitrate intake because nitrates are used in the preservation (Buglak et al., 1989; Prugar and Prugarova, 1990)

and colouring (IPCS, 1999; Manassaram et al., 2006) of tinned meat and sausages. Nitrogenous fertilizers, especially nitrates and nitrites, are of great importance and concern to man because they possess mutagenic, carcinogenic, teratogenic and embryotoxic properties (Antipina et al., 1990; Kasyanenko et al., 1992). The major source of nitrate in the human body is through intake of food and water (IPCS, 1999). Vegetables may account for more than 70% of the nitrates in a typical human diet (ATSDR, 2001). Drinking water, which accounts for up to 21% of total nitrates intake in a typical human diet (Wogan et al., 1995; ATSDR, 2001; Manassaram et al., 2006), may contain variable amounts of nitrates. While acute poisoning usually occurs following intake of large doses of nitrates and nitrites, chronic poisoning occurs as a result of intake of small toxic doses over a long period.

It has been established that in the human body, nitrates

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induce oxidative damage through the release of free radicals (Azhipa et al., 1990; Rubenchik et al., 1983; Babsky and Shostakovskaya, 1992; McAllister et al., 1995; Oladele et al., 1997; Singhal et al., 2001; Manassaram et al., 2006). Vitamin C also known as ascorbic acid (AA) and  $\alpha$ -tocopherole (vitamin E) are potent antioxidants capable of reducing oxidative damage by augmenting the function of endogenous free radical scavengers such as superoxide dismutase, catalase and glutathione peroxidase (Gecha and Fagan, 1992; Williams, 1997; Whitehead and Keller, 2002; Son et al., 2004; Ayo et al., 2006; Suteu et al., 2007), thereby decreasing the deleterious effects of nitrates and nitrites. The aim of the study was to evaluate the activity of ascorbic acid and  $\alpha$ -tocopherol during chronic nitrate toxicity on total and differential leukocyte counts.

## MATERIALS AND METHODS

Seventy (70) adult Wistar rats were used for this study. Ethical guidelines on handling of experimental animals of the Ahmadu Bello University, Zaria, Nigeria, were strictly followed. The animals were kept in large cages in the animal house for two weeks to acclimatise before commencement of the experiment. They were given free access to distilled water and pelletized growers feed (Vital Feed, Jos, Nigeria) before and during the experiment. The pelletized growers feed contained crude protein (14.5%), fat (7.0%), crude fibre (7.2%), calcium (0.8%) and 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  $\text{NaNO}_3$  in 0.1 ml from which the animals were fed during the experiment. Tablets of AA (vitamin C, 100 mg tablets Emzor Pharmaceutical Industries, Lagos, Nigeria) were crushed into powder and dissolved in distilled water to form a solution containing 25 mg of AA in 0.1 ml of the solution. Similarly, capsules of vitamin E (EFISHAL 200™, Shalina Laboratories, Pvt., Mumbai, India) were cut open and emptied into a clean container. Vegetable oil was added to prepare a suspension containing 34 mg of the vitamin E in 0.1 ml of the suspension. 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 and air-tight jar. Appropriate amounts of  $\text{NaNO}_3$  solution and the vitamins were collected using 1 ml syringe for administration based on body weights of the animals.

### Experimental design

The animals were divided into seven groups of 10 rats each. They were administered drugs or distilled water orally using a metallic canular between 8.00 and 10.00 am daily for 60 days as follows: Group I (control) received distilled water; Group II received 30 mg/kg  $\text{NaNO}_3$ ; Group III received 30 mg/kg  $\text{NaNO}_3$  + 500 mg/kg AA; Group IV received 30 mg/kg  $\text{NaNO}_3$  + 750 mg/kg AA; Group V received 30 mg/kg  $\text{NaNO}_3$  + 300 mg/kg vitamin E; Group VI received 400 mg/kg  $\text{NaNO}_3$  + 300 mg/kg vitamin E and Group VII received 30 mg/kg  $\text{NaNO}_3$  + 500 mg/kg AA + 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 anaesthetized animal was cut open and with the aid of 5 ml syringe with 21 gauge needle, the pulsating heart of the rat was pierced and 1 ml of blood was aspirated and emptied into an EDTA bottle.

### Laboratory analysis

Total leukocyte count was carried out using standard laboratory method as described by Dacie and Lewis (1991). 0.5 ml of blood was drawn into WBC pipette and diluted with WBC diluting fluid (1:20), mixed and allowed to stay for 5 to 10 min. The diluted blood was introduced into the improved Neubauer Counting Chamber. The cells were counted using HM – Lux microscope (Germany).

The differential leukocyte count was also carried out using the standard laboratory method of Dacie and Lewis (1991). The cells were counted under the microscope using oil emersion at the magnification of 1000x.

### Statistical analysis

The data obtained in the study were expressed as mean  $\pm$  S.E.M. Differences in means of discrete parameters of any two groups were analysed using student's t-test. Values of  $p < 0.05$  were considered significant.

## RESULTS

### Effects of sodium nitrate administration

Total leukocyte count of the nitrate-treated rats was significantly higher than that of the control (Table 1). Lymphocyte and monocyte counts were slightly higher in the nitrate-treated rats when compared with the controls, but the difference was not statistically significant.

### Effect of ascorbic acid administration

Neutrophil and monocyte counts of the rats treated with  $\text{NaNO}_3$  + 500 mg/kg AA were significantly higher than those of the nitrate-treated as seen in Table 1. In addition, lymphocyte, neutrophil and monocyte counts of the rats were significantly higher than those of the control. Neutrophil and lymphocyte counts of rats administered 750 mg/kg AA were significantly higher than those of the control. Furthermore, monocyte count of rats treated with 750 mg/kg AA was significantly lower than that of the rats treated with 500 mg/kg AA.

### Effect of $\alpha$ -tocopherol administration

Total white blood cell (WBC) counts of the  $\text{NaNO}_3$ -treated, 300 mg/kg vitamin E treated and 400 mg/kg vitamin E treated rats were statistically the same and significantly higher than those of the control. Similarly, lymphocyte counts of the vitamin E treated groups were statistically

**Table 1.** Leucocyte counts of control, NaNO<sub>3</sub>-treated and NaNO<sub>3</sub> + vitamin C-treated rats (Mean ± S.E.M.).

Group Leucocyte	Control (distilled water) (n = 10)	NaNO <sub>3</sub> treated (30 mg/kg) (n = 10)	NaNO <sub>3</sub> + AA treated	
			500 mg/kg (n = 8)	750 mg/kg (n = 10)
Total WBC (x 10 <sup>9</sup> /L)	173.9 ± 4.1	193.7 ± 5.9 <sup>c</sup>	208.1 ± 8.0	204.3 ± 2.1
Neutrophils (x 10 <sup>9</sup> /L)	37.1 ± 0.9	48.0 ± 1.5	54.3 ± 2.1 <sup>ac</sup>	47.6 ± 0.5 <sup>c</sup>
Lymphocytes (x 10 <sup>9</sup> /L)	125.4 ± 3.0	134.1 ± 4.2	139.7 ± 2.1 <sup>c</sup>	146.3 ± 1.5 <sup>ac</sup>
Monocytes (x 10 <sup>9</sup> /L)	11.4 ± 0.3	12.0 ± 0.5	14.2 ± 0.5 <sup>ac</sup>	8.6 ± 0.1 <sup>b</sup>
Eosinophils (x 10 <sup>9</sup> /L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.1 ± 0.0
Basophils (x 10 <sup>9</sup> /L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

<sup>a</sup> p < 0.05 compared to NaNO<sub>3</sub>-treated; <sup>b</sup> p < 0.05 compared to NaNO<sub>3</sub> + 500 mg/kg AA treated; <sup>c</sup> p < 0.05 compared to control; n = number of animals.

**Table 2.** Leukocyte counts of control, NaNO<sub>3</sub>-treated and NaNO<sub>3</sub> + vitamin E-treated rats (Mean ± S.E.M.).

Group Leucocyte	Control (distilled water) (n = 10)	NaNO <sub>3</sub> treated (30 mg/100g bw) (n = 10)	NaNO <sub>3</sub> + Vitamin E treated	
			300 mg/kg (n = 10)	400 mg/kg (n = 10)
Total WBC (x 10 <sup>9</sup> /L)	173.9 ± 4.1	193.7 ± 5.9 <sup>c</sup>	193.7 ± 5.7 <sup>c</sup>	199.7 ± 4.4 <sup>c</sup>
Neutrophils (x 10 <sup>9</sup> /L)	37.1 ± 0.9	48.0 ± 1.5	45.7 ± 1.4	45.9 ± 1.0
Lymphocytes (x 10 <sup>9</sup> /L)	125.4 ± 3.0	134.1 ± 4.2	137.1 ± 4.1 <sup>c</sup>	144.0 ± 3.1 <sup>c</sup>
Monocytes (x 10 <sup>9</sup> /L)	11.4 ± 0.3	12.0 ± 0.5	9.1 ± 2.3	9.0 ± 3.1
Eosinophils (x 10 <sup>9</sup> /L)	0.0 ± 0.0	0.0 ± 0.0	4.1 ± 0.3	4.1 ± 0.1
Basophils (x 10 <sup>9</sup> /L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

<sup>c</sup> p < 0.05 compared to control; n = number of animals.

**Table 3.** Leukocyte counts of control, NaNO<sub>3</sub>-treated and nitrate plus AA + E co-administered rats (mean ± S.E.M.).

Group Leucocyte	Control (distilled water) (n = 10)	NaNO <sub>3</sub> treated (30 mg/100 kg) (n = 10)	NaNO <sub>3</sub> + Vitamin E treated (500 mg/kg AA + 300 mg/kg vitamin E) (n = 10)
Total WBC (x 10 <sup>9</sup> /L)	173.9 ± 4.1	193.7 ± 5.9 <sup>c</sup>	198.3 ± 2.5 <sup>c</sup>
Neutrophils (x 10 <sup>9</sup> /L)	37.1 ± 0.9	48.0 ± 1.5	39.3 ± 0.5 <sup>a</sup>
Lymphocytes (x 10 <sup>9</sup> /L)	125.4 ± 3.0	134.1 ± 4.2	140.8 ± 1.8 <sup>c</sup>
Monocytes (x 10 <sup>9</sup> /L)	11.4 ± 0.3	12.0 ± 0.5	9.3 ± 0.1
Eosinophils (x 10 <sup>9</sup> /L)	0.0 ± 0.0	0.0 ± 0.0	3.4 ± 0.0
Basophils (x 10 <sup>9</sup> /L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

<sup>a</sup> p < 0.05 compared to NaNO<sub>3</sub> treated; <sup>c</sup> p < 0.05 compared to control; n = number of animals.

the same but significantly higher than that of the control (Table 2).

#### Effects of ascorbic acid and α-tocopherol co-administration

Neutrophil counts of the rats co-administered with NaNO<sub>3</sub> + 500 mg/kg AA and 300 mg/kg vitamin E were significantly lower than those of sodium nitrate-treated rats. In addition, total WBC and lymphocyte counts of the AA + vitamin E-co-administered rats were significantly higher

than that of the control (Table 3).

#### Comparison between the effects of ascorbic acid (50 mg/kg) and α-tocopherol (300 mg/kg) administration

The changes caused by the two vitamins at the lower doses were similar; AA increased the neutrophil and monocyte counts, while α-tocopherol increased the total WBC and lymphocyte counts when compared with the control (Tables 1 and 2). There was no statistically significant difference between the values of the observed

parameters.

### **Comparison between the effects of ascorbic acid (750 mg/kg) with that of $\alpha$ -tocopherole (400 mg/kg) administration**

Similarly, the changes caused by the two vitamins at the higher doses were similar; AA increased neutrophil and lymphocyte counts, while  $\alpha$ -tocopherol increased total WBC and lymphocyte counts when compared with the control (Tables 1 and 2). There was no statistically significant difference between the values of the observed parameters.

### **Comparison between the effect of ascorbic acid administration and ascorbic acid + $\alpha$ -tocopherol co-administration**

Neutrophil count of rats co-administered 500 mg/kg AA + 300 mg/kg vitamin E was significantly lower than that of the nitrate-treated and 500 mg/kg AA-treated groups (Tables 1 and 3).

### **Comparison between the effects of $\alpha$ -tocopherol administration and ascorbic acid + $\alpha$ -tocopherol co-administration**

Neutrophil count of rats co-administered with AA + vitamin E was markedly lower than that of nitrate-treated and 300 mg/kg vitamin E-treated groups (Tables 2 and 3).

## **DISCUSSION**

This study showed that sodium nitrate significantly increased the total leukocyte count in the treated rats. This result is in agreement with previous findings of Bruning-Fan and Kaneene (1993) who reported compensatory erythrocytosis, neutrophilia and eosinophilia following chronic exposure to high nitrate levels. Similarly, erythrocytosis and leukocytosis were observed following acute exposure to nitrates (Nikolov, 2001). In addition, the result was consistent with the reports of Manassaram et al., (2006) that nitrate administration induced hypoxia, resulting in compensatory increase in the production of cellular components of blood by the bone marrow.

Neutrophil, lymphocyte and monocyte counts were significantly higher in rats treated with sodium nitrate + 500 mg/kg AA, when compared with the same parameters in rats treated with only sodium nitrate (Table 1). This indicates that AA at this dose enhanced the toxic effects of nitrates. This finding was, apparently, due to the prooxidant effect of AA reported by Seyfulla and

Borisova (1990). The result supports the findings of Jeffrey (1998) that AA acts as a prooxidant through the formation of ascorbyl radical, which increases the antioxidant burden of the body.

Furthermore, neutrophil and lymphocyte (but not monocyte) counts of rats treated with 750 mg/kg AA were significantly higher than those of control (Table 1), suggesting that AA at this dose also enhanced the toxic effects induced by NaNO<sub>3</sub> on leukocyte counts in the rats. This finding also showed that the vitamin at different doses exerted different effects on sodium nitrate-induced toxicity on leukocyte counts. This is in agreement with previous findings that demonstrated the multiple mechanisms of the action of AA in the body (Gecha and Fagan, 1992; Podmore et al., 1998; Singhal et al., 2001; Whitehead and Keller, 2002; Guan et al., 2004; Awodi et al., 2005).

The co-administration of AA and  $\alpha$ -tocopherol to NaNO<sub>3</sub>-treated rats showed that neutrophil count of rats co-administered AA and  $\alpha$ -tocopherol was significantly lower than those of the control and nitrate-treated groups (Table 3). In addition, neutrophil count was also lower when compared with that of the rats administered the vitamins separately (Tables 1 and 2). This indicates the synergistic effect of the two antioxidant vitamins. Such synergistic effect of AA and  $\alpha$ -tocopherol was shown by Khmelevsky and Poberezkina (1990) in humans. Although the mechanism of synergistic action of AA and  $\alpha$ -tocopherol was not elucidated in this study, it has been shown that AA enhances the antioxidant effects of  $\alpha$ -tocopherol through the conversion of oxidised forms of  $\alpha$ -tocopherol back to  $\alpha$ -tocopherol (Whitehead and Keller, 2002). The results obtained in the study disagree with the findings of Appenroth et al. (1997), Altuntas et al. (2002), Gokalp (2003), Khadkhodae et al. (2007) and Suteu et al. (2007), who showed the protective effects of AA and  $\alpha$ -tocopherol in chronic toxicity associated with oxidative stress induced by different chemicals. The nature of the administered chemical and the route of administration in the study, apparently, allowed interaction between NaNO<sub>3</sub> and the vitamins in the stomach of the rats before absorption. This may explain the disparity.

In conclusion, sodium nitrate administration adversely affected the rats resulting to leukocytosis. This effect was enhanced by AA administration at both the higher and lower doses as evidenced by increased neutrophil and lymphocyte counts, as well as increased monocyte count at the lower dose. Similarly,  $\alpha$ -tocopherol at both doses enhanced NaNO<sub>3</sub> toxicity by increasing lymphocyte and total WBC counts. Co-administration of the two vitamins showed negative synergistic effect. Therefore, AA and  $\alpha$ -tocopherole intake, especially in combination, should be done with great caution during long-term nitrate exposure due to their potential adverse effects.

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