ORIGINAL ARTICLE

# EFFECT OF ASCORBIC ACID AND ALPHA TOCOPHEROL ON IMMUNE STATUS OF MALE SPRAGUE DAWLEY RATS EXPOSED TO CHRONIC RESTRAINT STRESS

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**Background:** The immune system provides protection against infectious diseases or other insults. Psychological stress may alter antibody production through neurobiological pathways. Antioxidant supplementation is thought to improve immune status and thereby reduce infectious morbidity. The aim of this study was to determine the preventive effect of ascorbic acid and alpha tocopherol on immune status of rats exposed to chronic restraint stress. **Methods**: A total of 150 healthy male Sprague Dawley rats were included in the study. They were divided into 5 groups, each comprised of 30 rats. Group I was the control group on normal diet. Group II rats were exposed to chronic restraint stress for 6 hours daily for 15 days, without antioxidant supplementation, whereas rats of groups III, IV and V were given supplementation of ascorbic acid or alpha tocopherol or both respectively, for one month prior to exposure of rats to chronic restraint stress. Total leukocyte count (TLC) and lymphocyte counts was done, and serum immuno-globulins (IgG, IgA, IgM, and IgE) levels were estimated using ELISA. **Results**: Total leukocyte and lymphocyte counts and serum IgA, IgE, IgG, and IgM levels were found significantly ($p$<0.001) decreased in rats exposed to chronic restraint stress compared to the rats not exposed to the restraint stress. The combined supplementation of ascorbic acid and alpha tocopherol significantly ($p$<0.001) prevented the decline in total leukocyte and lymphocyte counts and serum immuno-globulins compared to the administration of either of the two antioxidants. **Conclusion**: Antioxidants (ascorbic acid and alpha tocopherol) given in combination produce greater beneficial effect in improving the immune status of rats exposed to chronic stress than individual supplementation of either ascorbic acid or alpha tocopherol.

**Keywords:** Stress, immune status, immuno-globulins, antioxidants, ascorbic acid, alpha tocopherol

## INTRODUCTION

The proliferation of T and B cells and lymphokine activated killer cells that are required to exert an effective defence against pathogens and tumour cells appear to be inhibited markedly upon exposure to environmental stresses.[1] The immune system protects the body from disease producing organisms and foreign bodies, (like antigens), by producing antibodies like IgG, IgM, IgA, and IgE with the help of antigen specific T helper cells. Psychological stress may alter antibody production through behavioural or neurobiological pathways. Stress influences the plasma and tissue concentration of many hormones that bind specific receptors on the membrane or in the cytoplasm of cells of the immune system, including the cells that participate in the production of antibodies.[2] Nutrition can have an important influence on immune function.[3] There are dietary components that are capable of boosting immune function. Antioxidant supplementation is thought to improve immunity and reduce infectious morbidity since these are essential micronutrients that are required for normal metabolic functioning of the body.[4] Studies of ascorbic acid at the molecular, cellular, and clinical levels conducted have revealed that it plays multiple biochemical roles. In addition to its participation in immunity, ascorbic acid also serves important enzymatic, antioxidant, and regulatory functions. Research shows that ascorbic acid raises the levels of IgG and IgM as well as the concentration of C3 in the bloodstream.[5] Increased levels of IgG, IgM levels were found in the women who supplemented their diet with vitamin C. Ascorbic acid destroyed those phagocytic derived highly reactive oxidant bio-chemicals that are toxic to the tissue cells. Ascorbic acid is essential for effective immune system.[6] Alpha tocopherol is an integral part of cellular membranes that has the role to defend the cell against oxidation. Within cells and organelles (e.g., mitochondria) vitamin E is the first line of defence against lipid peroxidation.[3]

The aim of this study was to determine the preventive effect of ascorbic acid and alpha tocopherol on immune status of rats exposed to chronic restraint stress.

## MATERIAL AND METHODS

The study was conducted at Centre of Research in Experimental and Applied Medicine (CREAM), Army Medical College, Rawalpindi, in collaboration with National Institute of Health (NIH) Islamabad for one

year from June 2008 to June 2009. A total of 150 healthy male Sprague Dawley rats were obtained (age 65±5 days; weight 250±50 gm) and were divided into 5 groups, each comprising of 30 rats.

Group I (Control) was fed on normal standard diet without any supplementation. They were supplied plain tap water for drinking. Group II (Stress) was fed on standard diet without any supplementations; however these rats were exposed to 6 hours restraint stress daily for 15 days. Group III (Ascorbic) were supplemented with ascorbic acid in the dose of 500 mg/l added in drinking water for one month before and during chronic stress. Group IV (Tocopherol) was given alpha tocopherol 300mg/l (supplement with soya bean oil) for one month before and during chronic stress. Group V (Combined) Ascorbic acid and alpha tocopherol was supplemented in the dose mentioned above and exposed to chronic stress in the similar manner.

Rats were exposed to chronic stress by placing each rat separately in mesh wire restrainer for 6 hours daily between 9 am and 4 pm for 15 days without food and water.

Intra cardiac sampling was done after two weeks exposure to restraint stress. Approximately 1.5 ml serum was obtained from each blood sample, transferred to serum tubes labelled and stored at -80 ºC in deep freezer till the assay for immunoglobulin levels by enzyme linked immunosorbant assay (ELISA) using Immunoperoxidase assay kits of Immunology Consultants Laboratory Inc. USA.[7] Estimation of total lymphocyte counts and absolute counts of other leukocytes were done by fully automated haematology analyser Sysmex KX-21[8] from total and differential leukocyte counts, by applying the formula:

$$\text{Absolute count of Lymphocytes} = \frac{\text{TLC} \times \%\ \text{Lymphocyte}}{100}$$

Similarly absolute counts of other leukocytes were estimated by applying the same formula.

Data were analysed using SPSS-15. ANOVA was used to compare the results followed by Post Hoc test to find the differences in pairs of group, and $p<0.05$ was taken as significant.

## RESULTS

Comparisons of total leukocyte and absolute counts of all leukocyte using one way ANOVA are presented in Table-1. There were significance differences ($p<0.001$) between the groups. Results of Post-Hock (Tukey) test, are presented in Table-2. Antioxidant supplementation of ascorbic acid and alpha tocopherol given separately or in combination significantly prevented the decline in total leukocyte and lymphocyte counts which were decreased as a result of chronic restraint stress. In the control group TLC was 8020±44 cells/µL, which decreased to 6785±78 cells/µL in the stress group. Combined supplementation group had the count at 7796±72 cells/µL, which was almost near to control. Similarly total lymphocyte count was found decreased to 6210±115 cells/µL in the stress group compared to control group (7194±107 cells/µL). But the count in combined supplementation group was near normal, i.e., 7206±60 cells/µL.

Immuno-globulin level of all 5 groups are presented in Table-3. Using one way ANOVA statistical significance of mean differences of immuno-globulins were assessed and variables with significant $p$ values were analysed by Post-Hock (Tukey) test and are presented in Table-4. Comparison of control and the stress groups revealed that levels of immuno-globulins were significantly decreased in stress group compared to the control group ($p<0.001$). Comparison amongst the groups given antioxidant supplementation, revealed that immunoglobulin levels were significantly raised in rats which were given combined supplementation ($p<0.001$).

**Table-1: Comparison of TLC and individual cells absolute counts between the groups by one way ANOVA**

| Variables (cells/µL) | Control (n=30) | Stress (n=30) | Ascorbic (n=30) | Tocopherol (n= 30) | Combined (n= 30) | *p* |
|---|---|---|---|---|---|---|
| **TLC** | 8020± 44 | 6785±78 | 7888±419 | 7971±77 | 7796±72 | <0.001 |
| **Lymphocyte** | 7194±107 | 6210±115 | 7326±73 | 7485±66 | 7206±60 | <0.001 |
| **Monocyte** | 50±1.7 | 58.96±10.7 | 55.13±1.08 | 52±1.12 | 53.4±9.2 | <0.001 |
| **Neutrophil** | 510±11.5 | 628±10 | 557±9.4 | 586±6 | 524±7.4 | <0.001 |
| **Basophil** | 10±0.94 | 3.93±0.69 | 10.33±0.92 | 5±0.61 | 6.86±0.86 | <0.001 |
| **Eosinophil** | 70.3±1.73 | 54±2.48 | 61.83±2.57 | 63.53±2.8 | 71.6±2 | <0.001 |

**Table-2: Statistical differences of total and absolute counts of different cells between different pairs of groups using Post Hoc (Tukey's) test**

| Group comparison | TLC | Lymphocyte | Monocyte | Neutrophil | Basophil | Eosinophil |
|---|---|---|---|---|---|---|
| **Control vs Stress** | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Control vs Asorbic** | 0.063 | <0.001 | <0.001 | <0.001 | 0.934 | <0.001 |
| **Control vs Tocopherol** | 0.837 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Control vs Combined** | <0.001 | 0.988 | <0.001 | <0.001 | <0.001 | 0.042 |
| **Stress vs Ascorbic** | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Stress vs Tocopherol** | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Stress vs Combined** | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Ascorbic vs Tocopherol** | 0.479 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| **Tocopherol vs Combined** | 0.376 | <0.001 | 1.000 | <0.001 | <0.001 | <0.001 |
| **Combined vs Ascorbic** | 0.007 | <0.001 | <0.001 | <0.001 | <0.001 | 0.042 |

**Table-3: Comparison of serum IgA, IgE, IgG and IgM levels between different groups by One Way ANOVA all values are presented as Mean±SD**

| Immuno-globulins (ηg/ml) | Control (n=30) | Stress (n=30) | Ascorbic acid (n=30) | Tocopherol (n=30) | Combined (n=30) | *p* |
|---|---|---|---|---|---|---|
| **IgA** | 103.5±4.2 | 64.5±3.8 | 64.9±3.8 | 66.86±4.4 | 102±14.25 | <0.001 |
| **IgE** | 22.26±1.75 | 13.1±1.5 | 15.58±3.7 | 14.4±2 | 25±1.54 | <0.001 |
| **IgG** | 607±5.3 | 528±7.7 | 578±19.2 | 558±4.2 | 634.9±7.98 | <0.001 |
| **IgM** | 739±11.96 | 618.8±12.4 | 632±3.8 | 692±6.6 | 769.5±8.2 | <0.001 |

**Table-4: Statistical differences of serum IgA, IgG, IgM and IgE between different groups by using Post-Hock (Tukey) test**

| Group | IgA | IgG | IgM | IgE |
|---|---|---|---|---|
| **Control vs Stress** | <0.001 | <0.001 | <0.001 | <0.001 |
| **Control vs ascorbic** | <0.001 | <0.001 | <0.001 | <0.001 |
| **Control vs tocopherol** | <0.001 | <0.001 | <0.001 | <0.001 |
| **Control vs Combined** | 0.952 | <0.001 | <0.001 | <0.001 |
| **Stress vs Ascorbic** | 0.999 | <0.001 | <0.001 | <0.001 |
| **Stress vs Tocopherol** | 0.741 | <0.001 | <0.001 | 0.153 |
| **Stress vs Combined** | <0.001 | <0.001 | <0.001 | <0.001 |
| **Ascorbic vs Tocopherol** | 0.865 | <0.001 | <0.001 | 0.324 |
| **Tocopherol vs Combined** | <0.001 | <0.001 | <0.001 | <0.001 |
| **Combined vs Ascorbic** | <0.001 | <0.001 | <0.001 | <0.001 |

## DISCUSSION

The immune system, once thought to be autonomous is now known to respond to signals from many systems of the body especially the nervous system and endocrine system. Our study supports this view and has manifested a decline in immune status on exposure to chronic stress. Studies have revealed different methods for imparting chronic restraint stress to rats. Laifenfeld D *et al* proposed different means of chronic stress either by placing rats on hot plate, or keeping rats in cold.[9] Immobilization restraint chronic stress was more practical as compared to the exposure to extremes of temperatures because it might cause harm to the animal. Minimum recommended duration for chronic stress is 5 hours per day for 14 consecutive days.[10] In our study, we gave restraint immobilization chronic stress without giving food and water for 6 hours for 15 days. In the restrainer all limb movements of the rats were restricted.

In normal healthy human beings, neutrophil count range is 50–70%, and lymphocyte count is 20–40%. In rats, neutrophil and lymphocyte count is 72–94% while neutrophil count is 5–25%.[10] Differential analysis done by Doeing DC *et al* showed lymphocytes as the predominant cell type present in the peripheral blood of both male and female mice, comprising of 74% total leukocytes and 24% of neutrophil count.[11] Present study also revealed the same percentage distribution of white blood cells in healthy rats, i.e., lymphocytes as 89% whereas neutrophils as 7%.

Stress has been associated with transient immuno-suppression. In the present study TLC, total lymphocyte count and immuno-globulins were the immune markers. Total leukocyte count was compared amongst different groups, and it was found that there was significant reduction in TLC from 8,020±44 to 6,785±78 cells/µl following exposure to chronic stress when compared to the rats without stress. This stress related reduction in TLC was prevented in rats who were given ascorbic acid, alpha tocopherol or combined supplementation. Total lymphocyte count decreased from 7,197 to 6,210 cells/µl. In addition eosinophil and basophil counts were reduced, while neutrophil and monocyte counts got increased. Rats administered with ascorbic acid or alpha tocopherol or combined supplementation, manifested improved cell count. Antioxidant supplementation prevented the decline in immune cell count and kept the count near normal. Yin D *et al*[12] conducted a study on mice to evaluate the effect of restraint stress on lymphocyte count by placing mice in a centrifuge machine for 12 hours daily for 2 days. Mice showed 35–49% reduction in lymphocyte count in the spleen compared with the unstressed control. In our study there was 15% reduction in lymphocyte count. Studies conducted by Zager *et al*[13] on rats also manifested the decrease in lymphocyte count due to stress. In their study 24 and 96 hours sleep restriction (SR) for 21 days by the modified multiple-platform method, and their respective 24-h recovery periods, affect immune activation in rats and found a significant decrease in total leukocyte and lymphocyte counts along with immunoglobulin levels.

Total serum immuno-globulins A, E, G and M levels were found significantly decrease in rats exposed to chronic restraint stress as compared to the healthy control group. Multiple studies have supported that chronic stress led to decreased levels of IgA, IgM and IgGs. Animal study conducted by Tournier JN *et al* on mice showed 30% reduction in immunoglobulin levels and 20% decrease in total leukocyte count after chronic restraint stress.[14] In our study we found 35% decline in serum IgA and IgE , 11% in serum IgG and 16% in serum IgM levels. The human based study of Hucklebridge *et al*[15] had documented the decreased levels of IgA on exposure to chronic stress. In their study only one immunoglobulin (IgA) was evaluated.

However, Herbert *et al*[16], evaluated all the immuno-globulins. IgA and IgM were found to be decreased by 13% and 24% respectively on exposure to stress. In their study IgA was masured in saliva. A study conducted by Chen and Edwin, documented the effect of socioeconomic stress on immune status.[17] In their study, immune marker like IgE and neuro-endocrine marker like cortisol were measured in asthmatic individuals belonging to low socioeconomic status and compared with individuals belonging to high socioeconomic status. The results revealed the decreased cortisol and increased IgE levels in individuals of low socioeconomic status.

Immunoglobulin levels were found significantly raised in group of rats which were given either ascorbic acid or alpha tocopherol supplementation separately or in combination ($p<0.001$), but combined supplementation proved more beneficial as regards the levels of all the immuno-globulins. Studies conducted by Hung Pham on Sprague Dawley rats observed the effect of alpha tocopherol on IgE levels and found their increased level.[18] However alpha tocopherol when administered through injection along with selenium showed more increased levels of immuno-globulins G and M, which may be due to synergistic effect of the two antioxidants.[19] The combined effect of ascorbic acid and alpha tocopherol on immune status has been studied sparsely. Eichenger *et al* documented that ascorbic acid supplementation led to the enhanced levels of alpha tocopherol.[20] Another documented evidence could be that, alpha tocopherol enhanced the tissue ascorbic acid concentration. Vitamins C and E function as water-soluble and lipid-soluble chain-breaking antioxidants, respectively, and protect lipids, proteins, and membranes from oxidative damage. Vitamin C scavenges oxygen radicals in the aqueous phase, whereas vitamin E scavenges oxygen radicals within the membranes. Vitamin C regenerates vitamin E by reducing vitamin E radicals formed when vitamin E scavenges the oxygen radicals. This interaction between vitamin C and vitamin E radicals can take place not only in homogeneous solutions but also in liposomal membrane systems where vitamins C and E reside separately outside and within the membranes respectively, and vitamin C can act as a synergist.[21] This is supported by a study conducted by Wambi *et al*[22] who evaluated the role of combined supplementation of ascorbic acid and alpha tocopherol in protecting haematopoietic cells and improving animal (mice) survival after total body irradiation. Total leukocyte count was found significantly higher in mice given combined supplementation as compared to those given separately.

The use of antioxidant supplements can be one of the means by which we can prophylactically protect our body from the harmful effects of stress. Intake of antioxidant vitamins may result in a significant increase in lymphoproliferative capacity and in phagocytic functions of polymorphonuclear neutrophils.

## CONCLUSION

The combined supplementation of ascorbic acid and alpha tocopherol renders beneficial effect in improving the immune status (lymphocyte count and immunoglobulin levels) of Sprague Dawley rats.

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