**Pharmacological and Biochemical modulation of stress markers by L-NAME and L-Ascorbic Acid in chronic restraint model in Wistar rats**

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Running Title: Pharmacological and Biochemical Modulation of Stress Markers in Rats

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**Abstract**

**Background**

Stress is the psycho-physiologic reaction of the body to diverse stimuli including emotional or physical stimuli that imbalance the homeostasis and is also known to trigger various stress markers. Despite the stressors of different types, chronic stress in particular, is known to influence the physiological milieu and breakdown of adaptive mechanisms consequently aggravating the morbid states.

**Objectives**

The present study was designed to evaluate the modulatory role of stress marker by N-nitro-L-arginine-methyl ester (L-NAME) and L-Ascorbic acid (L-AA) in experimental model of chronic restraint stress (RSx21) in Wistar rats.

**Methods**

MDA and GSH levels were determined by the method of Okhawa et al 1979 and Ellman 1959 respectively, the SOD and catalase levels were estimated by the method of Nandi and Chatterjee 1988 and Aebi 1984 respectively.

**Results**

Results from our study reveal the significant enhancement of malondialdehyde (MDA) level while significant attenuation of superoxide dismutase (SOD), reduced glutathione (GSH) and catalase levels in chronic stress group compared with vehicle (non-stress) group. The MDA level was found to be increased by L-NAME (10 and 50 mg/kg) in chronic restraint (RSx21) induced rats as compared to vehicle treated RS group. Antioxidant L-AA (100 and 200 mg/kg) significantly reduced MDA level in chronic stress situation. However, L-NAME and L-Ascorbic Acid were found to cause an increase in level of plasma SOD, GSH and catalase when compared with vehicle treated RS group. On the other hand, L-AA (100 and 200 mg/kg) reversed these RS induced changes in these oxidative parameters.

**Conclusions**

Hence, results from the study underlined the intricate role of antioxidants as evidenced by reversal of oxidative stress markers that command a vital role in the development of morbid condition.

**Key words**: Restraint Stress, Chronic Stress, Antioxidant, N-nitro-L-arginine-methyl ester, L-Ascorbic acid

**1. Introduction**

The term stress, a reaction in response to aversive stimuli seems to be a conception that is not easy to describe fully as its understanding ought to vary in specific categories. In 1974 Hans Seyle defined stress as ‘‘the non-specific response of the body to any demand imposed upon it” **1**. This definition of stress was later modified in 1992 by Chrousos and Gold and the term ‘‘non-specific” replaced by the hypothesis that above a threshold intensity any stressor would elicit the ‘‘stress syndrome” **2**. Stress can occur in a variety of forms: physical, psychological, acute or chronic. Responses to stress often fall into two categories: acute and chronic.

The acute stress response is composed of adaptive physiological and behavioral responses to a noxious stimulus, or ‘‘stressor”. If a stressor persists or a series of acute stressors initiate multiple consecutive stress responses, an animal becomes chronically stressed **3**. In a chronically-stressed animal, the short-term physiological and/or behavioral changes crucial for alleviating or ameliorating the acute stressor no longer aid in survival and instead become detrimental, potentially leading to pathological conditions **4**. In other words, the stress response itself precipitates other problems. For example, the fight-or-flight response system, when chronically stimulated, can have a negative impact on cardiovascular health through excess catecholamines exposure leading to hypertension, myocardial infarction, increased cardiac output and arrhythmias **5**. In addition, because the fight-or-flight response is the animal’s first line of defense to an acute stressor, such as the approach of a predator, changes in cardiac function due to chronic stress **6** may alter the animal’s ability to mount the appropriate response that is crucial to survival. The adaptive aspects of the GC response also can prone maladaptive under chronic stress.

An important component of the tightly regulated HPA cascade depends on the stress-induced elevation of glucocorticoids (GCs) to exert a negative feedback signal that inhibits prolonged axis activation **6, 7**. In chronically-stressed animals, this GC negative feedback signal is disrupted **8** meaning that individuals experience prolonged elevation of GC at stress-induced concentrations **9**. Overall, chronic stress disrupts its own response system, which can lead to the detrimental physiological and behavioral consequences.

Free radicals are highly reactive moieties having one or more unpaired electrons in its valency shell which make them more reactive and play an important role in health and disease **10, 11**. The processes like oxygen “activation” by physical irradiation or metabolic process results in the formation of superoxide anion from molecular oxygen which further interact to form the “secondary” ROS, by either direct method or through enzyme or metal catalyzed processes **11, 12**.

Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) constitute the most complex interactive system aimed at maintaining homeostasis. The brain is in particular vulnerable to free radical damage because of its high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes compared with other tissues.

In the recent years oxidative stress has been implicated in the pathophysiology of several human diseases **13** and since reactive oxygen and nitrogen species (ROS/RNS) may be simultaneously generated during various pathophysiological states, the balance between ROS and RNS may play a vital role in initiation and progression of various disease states **14**. Hence the present study was designed to evaluate the pharmacological and biochemical modulation of stress markers by L-NAME and L-Ascorbic Acid in chronic restraint model in rats

**2. Materials and Methods**

**2.1 Experimental Subject**

**Animals**

The *in vivo* study was carried out in inbred wistar rats (200-250g) of either sex. Rat chow and tap water were allowed ad-libitum regularly. The animals were housed in standard laboratory conditions of light-dark cycle (12h light-12h dark) and at a temperature of 22 ± 2oC. Throughout the experiment the animals had free access to food and water. Each experimental group comprised of 6-10 animals. The animals care were taken as per guidelines in care and use of animals in scientific research prepared by the Indian National Science Academy (INSA), New Delhi and the study protocol had the approval of the Institutional Animal Ethical Committee (IEAC). In addition, maximize efforts were made to curtail the distress of the rodents and also simultaneously to limit the number of the animals being used.

**2.2 Experimental Procedure**

**Immunization**

On first day (day 0) different experimental animal groups were immunized with an intraperitoneal injection of ovalbumin (10 mg per rat) adsorbed to 10 µg of aluminum hydroxide. After treatments from day 0 to day 21 followed by stress procedure, blood samples were collected on day 21 under light ether anesthesia.

**Stress:**

Restraint stress (RS) for 1 hour at room temperature was used as an experimental stressor.

For RS, rats were immobilized in specific Plexiglas restrainer (INCO, Ambala). This method involves minimum pain and minimum movement of tail. This restraint or immobilization technique is widely validated animal model of stress because it induces activation of sympatho adrenal meduallary system, the HPA axis, elevation of blood pressure and heart rate **15**. Multiple sessions (X21) of RS were employed depending upon experimental protocol. After completion of RS the animals were anaesthetized under light ether anesthesia and blood samples were collected for various biochemical.

**2.3 Drugs**

Ovalbumin, L-Ascorbic acid and L-NAME were procured from the Sigma Aldrich Chemical Co. USA. L-Ascorbic acid and L-NAME were dissolved in distilled water. The drugs were prepared freshly and administered intraperitoneally (*i.p.*) in a ratio of 2 ml/kg body weight.

**2.4 Experimental Methods**

Blood samples were collected at various time intervals from different experimental groups. Protein was estimated by Lowry’s method **16** and following parameters were assayed:

**2.4.1 Estimation of MDA levels 17**

Malondialdehyde (MDA), a marker of lipid peroxidation was determined according to the method of Okhawa et al (1979). Briefly the reaction mixture consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid and 0.1 ml of sample. The mixture was made upto 4 ml with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 5 ml of n-butanol and pyridine (15:1 v/v) and 1 ml of distilled water was added and centrifuge. The organic layer was separated out and its absorbance was measured at 532 nm using a UV-VIS spectrophotometer (UV 5740 SS, ECIL, India) and results were expressed as μM/ml/mg of protein.

**2.4.2 Estimation of GSH levels 18**

Reduced glutathione (GSH) was measured according to the method of Ellman. An equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5’5-dithiobis (2nitrobenzoic acid) and 0.4 ml of double distilled water was added. The mixture was vortexed and absorbance read at 412 nm within 15 min using UV-VIS spectrophotometer (UV 5740 SS, ECIL, India). The concentration of reduced glutathione was expressed as μM/ml/mg of protein.

**2.4.3 Estimation of Superoxide Dismutase (SOD) levels 19**

The SOD level was estimated by using the method of Nandi and Chatterjee. Briefly 2.860 ml of tris buffer (50mM) pH 8.5 mixed with 0.1 ml of EDTA (30mM) followed by 0.02 ml of sample and 0.02-0.08 ml of pyrogallol, The increase in the absorbance at 420 nm was recorded on spectrophotometer (UV 5740 SS, ECIL, India) from 30 second to 3 min, the lag period of 30 second was allowed for steady state or auto-oxidation of pyrogallol to be attained. The concentration of pyrogallol was so adjusted that the rate of change of absorbance was approximately 0.025 to 0.030 per minute. The increase in the absorbance at 420 nm after addition of pyrogallol was inhibited by presence of SOD. One unit of SOD was defined as the amount of the enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 3 ml of assay mixture, and the results were expressed in units/mg of protein.

**2.4.4 Estimation of Catalase levels 20**

Activity of this enzyme was carried out in blood using hydrogen peroxide as a substrate by the method of Aebi et al. The assay was carried out by taking 0.1 ml of sample and 1.9 ml of PBS pH 7.0 in a 3 ml cuvette and the reaction was started by addition of 1 ml of H2O2 (30 mM/l). Decrease in the absorbance was recorded at 240 nm for 30 second using UV-VIS spectrophotometer (UV 5740 SS, ECIL, India). The activity of catalase was expressed in units/mg of protein.

**2.5 Statistical Analysis**

All the statistical analysis was performed using SPSS version 20. The data were expressed as Mean ± SEM. For statistical significance, data were analyzed by using Kruskal–Wallis one way ANOVA followed by Tukey’s test for post-hoc comparisons. The 5 % probability level was considered as statistically significant i.e. P < 0.05.

**3. Results**

**3.1 Effect of chronic (RSx21) restraint stress and its modulation by L-NAME and L-AA on MDA and GSH levels in blood in rats**.

Analysis of blood biochemical data of OVA- immunized and challenged rats revealed that there were significant effects [ F (6,48) = 50.1, P < 0.001 for MDA;F (6,48) = 19.4, P< 0.001 for GSH] on RS(x21) induced alteration of oxidative markers in the blood (one-way ANOVA). Intergroup comparisons revealed that RS(x21) enhanced MDA levels while suppressing plasma GSH level in chronic stress situation.

Pretreatment with L-NAME enhanced serum MDA levels in RS(x21) exposed animals compared to vehicle treated RS group (P<0.01 for L-NAME in both doses). While pretreatment with L-NAME (10 and 50 mg/kg) failed to reverse RS(x21) induced changes in plasma GSH levels (P>0.05). Antioxidant L-AA (100 and 200 mg/kg) reversed RS(x21) induced changes in these oxidative stress markers in blood, changes were not statistically significant (P>0.05 in each case), compared to vehicle treated RS group [Table-I].

**Table I: Effect of chronic restraint stress (RSx21) and its modulation by L-NAME and L-AA on MDA and GSH levels in rat blood**

|  |  |  |
| --- | --- | --- |
| **Treatment Group**  **(mg/kg)** | **MDA**  **(μM/ml/mg of P)** | **GSH**  **(μM/ml/mg of P)** |
| Vehicle | 3.7±0.24 | 25.4±1.3 |
| Veh + RS(x21)  L-NAME(10) + RS(x21) | 5.4±0.29\*  8.1±0.52b | 10.9±1.2\*  13.5±0.57 |
| L-NAME(50) + RS(x21)  L-AA(100) + RS(x21) | 9.7±0.61b  3.9±0.17 | 10.7±1.2  13.0±1.26 |
| L-AA(200) + RS(x21) | 3.7±0.34 | 14.1±1.22 |

All data are expressed as mean± SEM

Veh: Vehicle; L-NAME: N-nitro-L-arginine-methyl ester; L-AA: L-Ascorbic acid

\*P<0.01 [compared to respective vehicle]

bP<0.01 [compared to respective vehicle + RS(x21) group]

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| --- | --- |
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**3.2 Effect of chronic (RSx21) restraint stress and its modulation by L-NAME and L-AA on SOD, Catalase levels in blood in rats.**

Analysis of blood biochemical data of repeated (RSx21) stress induced changes in the antioxidant enzymes SOD and catalase in plasma were found significant effects [F (6, 48) = 61.3; P< 0.001 for SOD; F (6,48) = 10.4, P< 0.001for catalase ] as compared to vehicle group on stress induced alteration of these oxidative markers (one-way ANOVA).

RS(x21) significantly suppressed these oxidative stress markers levels (P<0.01 in each case for SOD, catalase levels) compared to non-stressed control group.

On the other hand NO-synthase inhibitors L-NAME (10 and 50 mg/kg) aggravated the stress effects (P>0.05 in each case).

The antioxidant L-AA (100 and 200 mg/kg) reversed these RS induced changes in oxidative parameters in dose dependent manner (P>0.05 for SOD and Catalase levels compared to non-stressed control group. [Table-II]

**Table II: Effect of chronic restraint stress (RSx21) and its modulation by L-NAME and L-AA on SOD and Catalase levels in blood in rats**

|  |  |  |
| --- | --- | --- |
| **Treatment Group**  **(mg/kg)** | **SOD**  **(U/mg of Protein)** | **Catalase**  **(U/mg of Protein)** |
| Vehicle | 3.92±0.08 | 3.28±0.18 |
| Veh + RS(x21)  L-NAME(10) + RS(x21) | 2.66±0.07\*  2.26±0.04 | 2.25±0.07\*  2.68±0.08 |
| L-NAME(50) + RS(x21)  L-AA(100) + RS(x21) | 2.8±0.14  3.14±0.14 | 2.60±0.09  2.40±0.07 |
| L-AA(200) + RS(x21) | 3.21±0.07 | 2.46±0.05 |

All data are expressed as mean± SEM

Veh: Vehicle; L-NAME: N-nitro-L-arginine-methyl ester; L-AA: L-Ascorbic acid

\*P<0.01[compared to respective vehicle]

# DISCUSSION

Research done in the past indicate the inability of the body of maintain the physiological homeostasis give rise to development of morbidities of the cardiovascular, gastrointestinal, immunological and central nervous system **2.** Since the introduction of the concept of ‘stress’ to biomedical research by Hans Selye, stress research has evolved considerably over the last few decades, and cellular and molecular concepts are being forwarded to explain stress responses **21.** Complex neurochemical pathways have been demonstrated to regulate such stress related responses and interactions between varieties of host factors are instrumental in deciding the nature and extent of the impact of such aversive inputs on the biological system. “stress system” is itself a complex system involving the wide array of interactions that occur between CNS, neuroendocrine, immune and visceral systems **22.**

Free radicals are highly reactive moieties that play mandate role in health and disease. Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) constitute the most complex interactive system involved in maintenance of homeostasis. The brain consumes a large amount of oxygen, has lipid content in the maximum amount and attenuated antioxidant enzymes in comparison with other tissues; thus making it susceptible to damage caused by free radicals **23.**

The level of SOD, catalase, GSH and MDA were estimated in rat blood for the screening of oxidative stress markers. Results from our study revealed that exposure to chronic restraint stress (RSx21) caused accentuation of MDA while attenuated the level of SOD, catalase and GSH levels. The amount of lipid peroxidation in blood was significantly increased as evidenced by elevated levels of MDA and significant decreased levels of GSH in chronic stress when compared to control (no stress) group. Glutathione plays a significant role in the antioxidant– redox cycle in the body. The role of glutathione in the immune regulation is found to be protective. Pretreatment with L-NAME (10 and 50 mg/kg) enhanced MDA levels and antioxidant L-Ascorbic acid reduced plasma MDA levels whereas both L-NAME and L-AA aggravated the plasma GSH levels in chronic stress group. The levels of antioxidant and oxidant balance determine the cause of disease progression. This balance if shifted towards oxidant, the oxidative stress may occur. The levels of antioxidant are essential for the counter balance of the free radicals. It is known that the role of free radicals during various aversive stimuli is found to be increased and regulate the progression of disease state. The antioxidant enzyme SOD and catalase which are endogenous antioxidant play a well-marked significant role in the regulation of generation of free radicals. In the present study we have found that excessive free radicals are generated during chronic stress as evidenced by decreased levels of SOD, catalase as compared to control (no RS) group. SOD catalyzes and neutralizes the super oxide radicals which are generated by the various metabolic pathways. The catalase catalyzes H2O2 into H2O and oxygen and act as an antioxidant enzyme. The NOS inhibitors L-NAME aggravated these RS induced effect in these oxidative stress markers. The antioxidant L-Ascorbic acid scavenge free radicals and tended to increase SOD, catalase levels of the blood as compared to RS treated vehicle group in both stress situations. The antioxidant has some protective effects during stress as evidenced by the result of our study **24.** The non-enzymatic scavenging of singlet oxygen and hydroxyl radicals provides an insight of anti-oxidant mechanisms operative in brain **25**. The depletion of GSH causes an impairment of the defense mechanism due to compromised removal of toxic metabolites. Consequent to lipid peroxidation cause neurons dysfunction and degeneration due to loss of polyunsaturated fatty acids and various abnormal alterations in the plasma membrane **26.**This also leads to an altered functioning of neurotransmitter uptake and depolarization dependent calcium channels **27.**

**Conclusion**

The role of chronic stress is highly crucial in causing various alterations in oxidative stress as evidenced by increased levels of MDA and reduced levels of GSH, SOD and catalase. The administration of L-NAME reversed chronic stress induced changes in oxidative stress marker, while L-Ascorbic acid also showed protective effects which corroborated with associated changes in oxidative stress markers and hence impart protective role against oxidative stress thereby maintaining the optimum balance required for survival of organisms and their associated health status.

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