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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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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. **Aims and 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. **Material and 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

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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.

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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.

MATERIALS AND METHODS

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 ± 2°C. 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.

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.

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.

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:

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.

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 (2 nitrobenzoic 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.

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.

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 H_2O_2 (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.

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. $P < 0.05$.

RESULTS

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 levelin 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 1).

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 2).

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]

Table 1: Effect of chronic restraint stress (RS×21) 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)	5.4±0.29*	10.9±1.2*
L-NAME (10) + RS (x21)	8.1±0.52[b]	13.5±0.57
L-NAME (50) + RS (x21)	9.7±0.61[b]	10.7±1.2
L-AA (100) + RS (x21)	3.9±0.17	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], [b]P<0.01 [compared to respective vehicle + RS (x21) group]

Table 2: Effect of chronic restraint stress (RS×21) 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)	2.66±0.07*	2.25±0.07*
L-NAME (10) + RS (x21)	2.26±0.04	2.68±0.08
L-NAME (50) + RS (x21)	2.8±0.14	2.60±0.09
L-AA (100) + RS (x21)	3.14±0.14	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]

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 50mg/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 H_2O_2 into H_2O 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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REFERENCES

1. Selve H. Stress without Distress. Toronto: McClelland and Steward Ltd.; 1974.
2. Chrousos GP and Gold PW. The concept of stress and stress system disorders. JAMA 1992; 267:1244-1252.
3. Wingfield JC and Romero LM, Adrenocortical responses to stress and theirmodulation in free-living vertebrates. In: McEwen BSand Goodman HM (Eds.), Handbook of Physiology; Section 7: The Endocrine System, Coping with theEnvironment: Neural and Endocrine Mechanisms, vol. IV. Oxford Univ. Press, New York, 2001; pp. 211–234.
4. McEwen BS. Seminars in medicine of the Beth Israel Deaconess medicalcenter: protective and damaging effects of stress mediators. New England Journal of Medicine 1998; 338:171–179.
5. Rupp H. Excess Catecholamine Syndrome: Pathophysiology and Therapy.Annals of the New York Academy of Sciences 1999; 881: 430–444.
6. Cyr NE, Dickens MJ and Romero LM. Heart rate and heart rate variabilityresponses to acute and chronic stress in a wild-caught passerine bird. Physiological and Biochemical Zoology 2009; 82:332–344.
7. Jacobson L. Hypothalamic-pituitary–adrenocortical axis regulation. Endocrinology and Metabolism Clinics of North America 2005; 34:271–292.
8. Sapolsky RM, Romero LM and Munck AU. How do glucocorticoids influence stress responses?Integrating permissive, suppressive, stimulatory, and preparative actions.Endocrine Reviews 2000; 21:55–89.
9. Dallman MF, Akana SF, Scribner KA, Bradbury MJ, Walker CD, Strack AM, et al. Stress, feedback and facilitation in the hypothalamo-pituitary–adrenal axis. Journal of Neuroendocrinology 1992; 4:517–526.
10. Romero LM. Physiological stress in ecology: lessons from biomedicalresearch. Trends in Ecology and Evolution 2004; 19,249–255.
11. Miller DM, Buettner GR and Aust SD. Transition metals as catalysts of "autoxidation" reactions. Free Radic Biol Med 1990; 8:95–108.
12. Valko M, Morris H and Cronin MTD. Metals, toxicity and oxidative stress. Curr Med Chem 2005; 12:1161–1208.
13. Flora SJ. Role of free radicals and antioxidants in health and disease. Cell Mol Biol (Noisy-le-grand) 2007; 15:53(1):1-2.
14. Vaziri N and Khan M. Interplay of reactive oxygen species and nitric oxide in the pathogenesis of experimental lead-induced hypertension. Clin Exp Pharmacol Physiol 2007; 34(9):920-925.
15. Pare WP and Glavin GB. Restraint stress in biomedical research: a review. Neurosci Biobehav Rev 1986; 10:339-370.
16. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193:265- 275.
17. Okhawa H, Ohisi N and Yagi K. Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95:351-358.
18. Ellman GI. Tissue sulfydryl group. Arch Biochem Biophys 1959; 82:70-77.
19. Nandi A and Chatterjee IB. Assay of superoxide dismutase in animal tissues. J Biosci 1988; 13:305-315.
20. Aebi H. Catalase in vitro Methods. Enzymol 1984; 105:121-126.
21. Wong DL and Tank AW. Stress-induced catecholaminergic function: transcriptional and post-transcriptional control. Stress. 2007; 10:121-130.
22. Gulati K, Chakraborti A and Ray A. Modulation of stress induced neurobehavioral and brain oxidative injury by nitric oxide (NO) mimetics in rats. Behav Brain Res 2007; 183:226-230.

23. Halliwell B and Gutteridge JMC. Oxygen radicals and nervous system. Trends Neurosci 1985; 8:22-26.
24. Pal G, Jena J, Swain TR and Ray A. Possible involvement of free radicals and anti-oxidant in acute stress induced inflammation and immunity in rats. Indo American Journal of Pharmaceutical Research 2014; 4(11).
25. Raghavendra V and Kulkarni SK. Possible antioxidant mechanism in melatonin reversal of aging and chronic ethanol induced amnesia in plus maze and passive avoidance memory tasks. Free Radical Biology and Medicine. 2001; 30 (6): 595-602.
26. Farooqui AA and Horrocks LA. Lipid peroxides in the free radical pathophysiology of brain diseases. Cell Mol Neurobiol 1998; 18:599-608.
27. Dabrowiecki Z, Gordon-Majszak W and Lazarewicz J. Effects of lipid peroxidation on neurotransmitters uptake by rat synaptosomes. Pol J Pharmacol Pharm 1985; 37:325-331.

Authors Contribution:
GP - Concept and designed the study, reviewed the literature, experimental research work, acquisition of data, analysis and interpretation of data; drafting of the manuscript and critical revision; **VR -** Conception and design of the study, analysis and interpretation of data, drafting the manuscript and revising it critically for important intellectual content, final approval of the version to be published; **RA -** Drafting the manuscript; **TB -** helped in preparing first draft of manuscript and intellectual contribution from time to time; **SB -** drafting the manuscript, intellectual contribution in acquisition of data; **TRS -** Drafting the manuscript and intellectual contribution; **MI -** Contribution in the statistical analysis; **JJ -** Has made substantial contributions to conception and design of the study.

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