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Induction of oxidative stress by restraint stress and corticosterone treatments in rats

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Chronic exposure to psychological stress in humans and restraint stress in experimental animals results in increased oxidative stress and resultant tissue damage. To study the contribution of stress hormones towards stress-induced oxidative processes in the brain, we investigated the response of important free-radical scavenging enzymes toward chronic administration of two doses of corticosterone (low dose: 10 mg/kg/day, high dose: 40 mg/kg/day) in rodents. After a 21-day experimental period, a significant decline in both superoxide dismutase and catalase was observed in both stressed and stress hormone-treated animals. The brain levels of glutathione as well as the activities of glutathione-S-transferase and glutathione reductase were also significantly decreased, while lipid peroxidation levels were significantly increased in comparison to controls. A direct pro-oxidant effect of stress hormones in the brain during physical and psychological stress was observed, indicating important implications for oxidative stress as a major pathological mechanism during chronic stress and a consequent target option for anti-stress therapeutic interventions.

Keywords: Oxidative stress, Restraint stress, Antioxidant enzymes, GSH, Lipid peroxidation, Corticosterone, Stress hormone

Stress is a state of disturbed homeostasis due to internal or external sources like physical or psychological stimuli known as stressors. It results in enhanced release of catecholamines and gluco-corticoids due to the activation of sympathoadrenal and hypothalamic-pituitary-adrenal (HPA) axes[1]. Chronic exposure to these mediators of the stress response has damaging effects, in which oxidative stress plays a major role[2,3].

It has been shown that neurodegeneration caused by glucocorticoid stress hormones under stress conditions may be linked to an increase in the generation of reactive oxygen species (ROS), which can directly damage cellular proteins, DNA and lipids[4]. However, reports suggesting a direct association between induction of oxidative damage and exposure to these stress hormones are still limited. It is well established that oxidative stress induces many damaging processes in stress disorders such as mitochondrial dysfunction[5], disruption of energy pathways[6], neuronal damage, impairment of neurogenesis[7] and induction of signalling events in apoptotic cell death[8]. In fact, evidences indicating that oxidative stress is integral, not only to neuro-degenerative processes, but also in many stress disorders, are becoming increasingly compelling.

In the present study, the contributory role of glucocorticoids has been investigated on alteration of oxidative processes by studying the response of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione reductase (GR) in brain of experimental animals (rats) after chronic treatment with the stress hormone corticosterone. Non-enzymatic antioxidants brain glutathione levels (GSH) and serum glucose have also been assayed. Lipid peroxidation has been examined as a secondary consequence of oxidative stress. These effects have been compared to the prooxidant status developed in a parallel group of rats submitted to restraint stress. Restraint as a stress model combines both emotional and physical components of stress, without any painful stimulation, in addition to producing robust increases in basal oxidative stress[9-12].

Materials and Methods

Animals and experimental protocol

Swiss albino rats weighing 100-125 g were housed under standard laboratory conditions of temperature (25 ± 5°C) and natural 12-h light/dark cycle with free access to standard pellet chow (Ashirwad Industries,

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Chandigarh, India) and drinking water *ad libitum*. All experimental procedures were carried out within the light period of the light/dark cycle. The experimental protocol was in strict accordance with regulations and prescribed animal ethical procedures outlined by the Institutional Research Committee.

All animals were habituated to maintenance conditions for a week, prior to experimental manipulations. During this time, they were randomly assigned to weight-matched groups of six animals each and their body weights were monitored every 7 days till the end of the experimentation. The following groups were constituted: Group I: control; Group II: restraint stress was given 3 h daily for 21 days; Group III: low dose of corticosterone (10 mg/kg/day) was given p.o. for 21 days; and Group IV: high dose of corticosterone (40 mg/kg/day) was given p.o. for 21 days.

Restraint stress was accomplished by immobilizing animals in wire mesh restrainers as described elsewhere[9]. Animals of this group were exposed to stress for 3 h each day at random times during the light period of the light/dark cycle to avoid habituation during the experimental period of 21 days. Low and high doses of corticosterone (Himedia, India) were administered via oral route to animals as 10 and 40 mg of corticosterone per kg of body weight per day for 21 days.

Collection of serum and preparation of tissue homogenates

At the end of the experimental period, all animals were sacrificed under ether anesthesia for biochemical studies. Blood was collected for separation of serum. Brain tissues were quickly removed and washed with ice-cold sterile physiological saline (0.9%). A 10% homogenate was prepared in 0.1 *M* sodium phosphate buffer, *p*H 7.4, centrifuged at 10,000 *g* (at 4ºC for 15 min) to remove cellular debris and the supernatant was used for further studies.

Biochemical investigations

Antioxidant investigations

Brain tissue homogenates were used for the estimation of the following antioxidant enzymes: SOD activity was assayed[13] by monitoring the inhibition of auto-oxidation of pyrogallol (0.05 *M* Tris succinate buffer, *p*H 8.2) at 420 nm. One enzyme unit was defined as the amount of enzyme required to cause 50% inhibition of the rate of pyrogallol auto-oxidation.

CAT activity was measured[14] in 0.05 *M* phosphate buffer (*p*H 7.0) by following the decrease in absorbance at 240 nm due to decomposition of 30 m*M* hydrogen peroxide (H_2O_2). One enzyme unit was defined as the amount of enzyme decomposing 1 m*M* H_2O_2 per min at 25°C.

GST activity was assayed[15] in 0.2 *M* phosphate buffer (*p*H 6.5) after adding 1 m*M* 1-chloro 2, 4 dinitrobenzene (CDNB) and 1 m*M* GSH in the reaction mixture and following the increase of absorbance at 340 nm due to formation of the CDNB-GSH conjugate. One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 m*M* product per min under the specific assay conditions.

GR activity was assayed[16] by monitoring the oxidation of 0.1 m*M* NADPH as a decrease in absorbance at 340 nm due to NADPH-dependent reduction of 1.0 m*M* oxidized glutathione disulphide to glutathione by the catalytic action of GR (0.1 *M* phosphate buffer, *p*H 7.6). One unit of enzyme activity was defined as the amount of enzyme catalysing the 1 m*M* NADPH per minute under assay conditions.

Concentrations of non-enzymatic antioxidant GSH[17] were determined in brain homogenates using the classical thiol reagent 5, 5´-dithiobis-2-nitro-benzoic acid (DTNB) (0.1 *M* phosphate buffer, *p*H 7.4). The yellow colour developed by the reaction of GSH with DTNB was read at 412 nm.

Glucose estimation

Estimation of glucose in serum was performed using a specific commercial kit (Span Diagnostics Ltd., Surat, India). The assay principle is based on oxidation of the aldehyde group of glucose by glucose oxidase to form gluconic acid and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide is coupled with phenol and 4-aminoantipyrine to form the coloured quinoneimine dye, detectable at 505 nm, and directly proportional to glucose concentration in the sample. 0.02 ml serum was mixed with the reagents and incubated at 37 ºC for 10 min, following which absorbance at 505 nm was recorded against a reagent blank.

Lipid peroxidation estimation

Lipid peroxidation was assessed by determining MDA (a thiobarbituric acid reactive species: TBARS)

spectrophotometrically, following the thiobarbituric acid (TBA) test for the formation of TBARS[18]. The pink chromogen formed by MDA-TBA complex was detected at 535 nm and quantified using an extinction coefficient of 1.56×10^5 *M*/cm.

Protein content was estimated by the method of Lowry *et al.*[19] using bovine serum albumin as standard.

Statistical analysis

Data were expressed as mean ± S.E.M. of six values and analyzed by One-way ANOVA for differences among controls and treatment groups. *P* values less than 0.05 were considered statistically significant.

Results and Discussion

The results depicted in Figs 1A-D showed that chronic administration of both the doses of corticosterone elicited a significant decrease ($P<0.05$) in the levels of antioxidant enzymes SOD, CAT, GST and GR, in comparison to controls. Restraint stress also significantly decreased enzymatic antioxidant status in a manner, similar to that of corticosterone treatment ($P<0.05$). As shown in Table 1, there was a significant decrease in brain GSH levels ($P<0.05$) and serum glucose concentrations ($P<0.05$) by corticosterone as well as restraint stress, in comparison to control animals. A significant increase in lipid peroxidation was observed, as indicated by the accumulation of malondialdehyde (MDA) in brain tissues of animals treated with corticosterone (Fig. 2; $P<0.05$). Exposure to restraint stress also produced significant increase in MDA levels in comparison to controls ($P<0.05$).

Fig. 3 depicts influence of increasing doses of corticosterone on body weights of animals measured every 7 days beginning from the day of experimental manipulations. In contrast to controls, both doses of exogenous corticosterone markedly decreased weight gain ($P<0.05$) over the treatment period of 21 days.

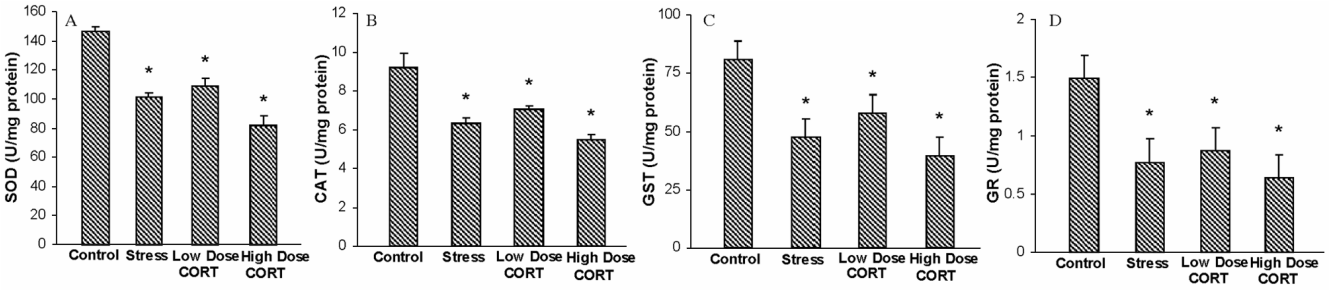

Fig. 1—Effects of chronic corticosterone administration (low dose CORT = 10 mg/kg and high dose CORT = 40 mg/kg CORT, p.o. for 21 days) and chronic restraint stress (3 h/day for 21 days) on brain antioxidant enzyme activities: (A) SOD, (B) CAT, (C) GST and (D) GR [Values shown are the group means ± S.E.M (n = 6). *P<0.05 compared to controls]

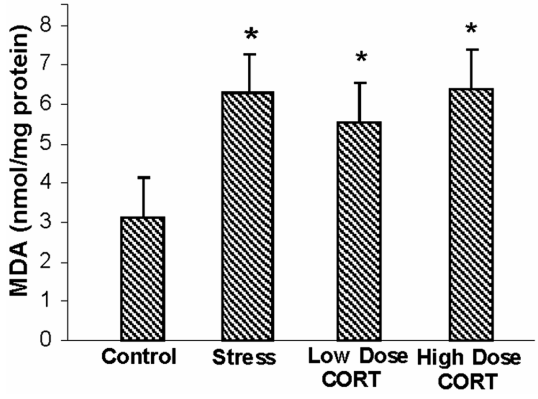

Fig. 2—Effects of chronic corticosterone administration (low dose CORT = 10 mg/kg and high dose CORT = 40 mg/kg CORT, p.o. for 21 days) and chronic restraint stress (3 h/day for 21 days) on brain lipid peroxidation [Values shown are the group means ± S.E.M (n = 6). *P<0.05 compared to controls]

Table 1 — Effects of chronic corticosterone administration (Low dose CORT: 10 mg/kg and high dose CORT: 40 mg/kg CORT, p.o. for 21 days) and chronic restraint stress (3 h/day for 21 days) on brain GSH levels and serum glucose concentrations

[Values shown are the group means ± S.E.M, n = 6)]

Group	Brain GSH (nmol/mg protein)	Serum glucose (mg/dl)
Control	17.05 ± 0.60	112.44 ± 1.60
Stress	10.98* ± 0.88	72.44* ± 6.98
Low dose CORT	12.18* ± 1.15	81.55* ± 2.91
High dose CORT	7.86* ± 0.93	65.55* ± 7.60

*$P<0.05$ compared to controls (ANOVA)

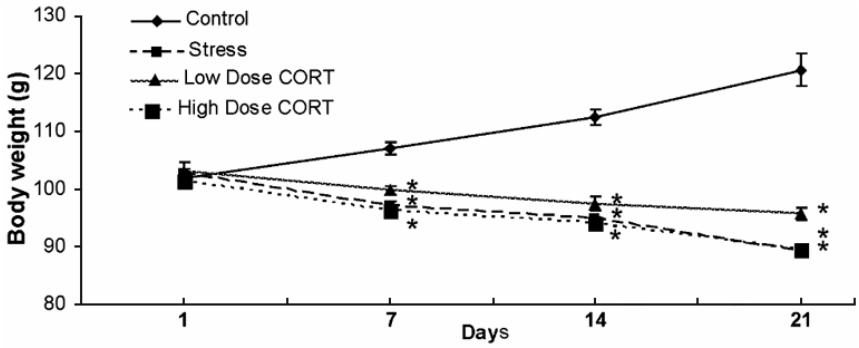

Fig. 3 — Effects of chronic corticosterone administration (low dose CORT = 10 mg/kg and high dose CORT = 40 mg/kg CORT, p.o. for 21 days) and chronic restraint stress (3 h/day for 21 days) on body weight of animals, measured every 7 days [Values shown are the group means ± S.E.M (n = 6). *P<0.05 compared to controls]

Restraint stress also induced a similar significant decrease (P<0.05) in body weight throughout the experimental period.

Chronic exposure to stress is known to alter the prooxidant-antioxidant balance, which might lead to the development of various human pathological states[20]. The restraint stress model has been shown to produce robust increases in basal oxidative stress in our laboratory[9,11,12]. The present study has not only further confirmed this, but has also demonstrated that the effects of chronically administered corticosterone on induction of oxidative damage are comparable to that induced by a psychological stressor (restraint stress). This may demonstrate a contributory role of stress hormones in the oxidative route of damage induced by restraint stress. It implicates oxidative stress as a major pathological mechanism during exposure to chronic stress.

The glucocorticoids have been demonstrated to intensify the process of hippocampal neuron loss and associated degenerative markers[21]. It is important to note that such neuroendangerment and neurodegeneration mediated by glucocorticoids under stress conditions have been linked to an increase in the generation of ROS, which can directly damage cellular macromolecules, thereby affecting all cellular functions[4]. Glucocorticoids have been shown to decrease the activity of brain SOD and glutathione peroxidase (GPx), both basally and in the presence of an oxidative stressor[22,23]. The results of the present study further authenticate this finding by demonstrating that administration of corticosterone to rodents results in a coordinated decline of not only enzymatic antioxidant defense components SOD and CAT, but also elevation of the oxidative stress marker lipid peroxidation.

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms to neutralize excess ROS and reduce oxidative stress[24]. Inspite of this, some tissues, especially the brain are much more vulnerable to oxidative stress, because of their elevated consumption of oxygen and high content of polyunsaturated fatty acids (PUFAs)[25]. The consequent generation of large amounts of ROS can directly modify cellular DNA, proteins, and lipids, causing extensive oxidative damage to these critical molecules[26]. The enzymes that provide the first line of defense against superoxide and hydrogen peroxide include SOD, CAT and GPx. A simultaneous decline in the activities of both SOD and CAT by corticosterone thus damages this important avenue of enzymatic antioxidant defense.

Moreover, significant depletion in GSH, GST and GR by oral administration of corticosterone indicates damage to the second line of antioxidant defense as well. This probably further exacerbates oxidative damage by adversely affecting critical GSH-related processes such as free radical scavenging, detoxification of electrophilic compounds, modulation of cellular redox status and thiol-disulphide status of proteins, and regulation of cell signaling and repair pathways.

Similarly, glucose is among the major circulating non-enzymatic antioxidant molecules in circulation, and a scavenger of hydroxyl radicals[27]. Therefore, a significant decline in serum glucose in response to corticosterone treatment demonstrates a direct vulnerability to extensive damage by hydroxyl radicals which are the most aggressive member of the ROS family[26]. Furthermore, the results obtained in the present study show that corticosterone treatment

significantly increased lipid peroxidation in a manner similar to restraint stress. The peroxidation of PUFAs by hydroxyl radicals constitutes one of the most severe attacks on cellular integrity[28].

Peroxidation of membrane PUFAs may adversely affect many functionally important parameters, such as membrane fluidity, permeability, electrical potential, and controlled transport of metabolites across the membrane[29]. Lipid peroxidation products MDA and 4-hydroxynonenal have the additional ability to inactivate phospholipids, proteins, and DNA by bringing about cross-linking between these molecules[30]. These damaging events are of particular significance in the brain which is rich in oxidizable PUFAs. Glutamate, iron-catecholamine complexes, dopamine and ROS have been found involved in generation of oxidative stress in several diseases of the nervous system, including Parkinson's disease, schizophrenia and Alzheimer's disease[31]. Thus, exposure to stress hormones in the brain has immense potential to impair critical processes involved in normal functioning.

Furthermore, oxidative damage induced by corticosterone is found to be associated with decreased body weight, an organic correlate of stress effects[32]. Substantial weight loss during exposure to repeated stress has been frequently reported by other investigators, in addition to previous reports of dose-dependent decrease in body weight gain due to glucocorticoid administration[33,34]. Consistent with these findings, the results in this study also reveal a decline in body weight due to restraint stress as well as corticosterone, further emphasizing the contributory role of stress hormones toward oxidative processes during the stress response.

Therefore, the data presented here indicating a substantial decline in endogenous antioxidant status and rise in oxidative stress markers by corticosterone parallel to the effects of restraint stress points to a key role of stress hormones in initiating stress-induced oxidative damage. Consequently, targetting oxidative stress to restore optimal antioxidant status *in vivo* may be of considerable relevance for combating the deleterious and pathological outcomes of stress.

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