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**AMELIORATIVE EFFECT OF RESVERATROL ON HPA AXIS
MODULATED CHRONIC RESTRAINT STRESS IN RATS**

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ABSTRACT

Stress leads to threatened body homeostasis which causes biochemical and physiological changes that result in serious health risks. The aim of present study was to evaluate anti-stress effects of resveratrol, a polyphenol compound in an animal model of chronic restraint stress (CS). The effect of resveratrol was studied on CS-induced perturbations in behavioral, biochemical and brain oxidative stress status. The rats were subjected to restraint stress in an adjustable cylindrical plastic tube for 3 h once daily for ten consecutive days. Resveratrol (20 and 40 mg/kg, p.o.) and diazepam (1 mg/kg) were administered 60 min prior to the stress procedure for 10 days. Behavioral parameters (ambulations and rearing); biochemical parameters like serum glucose, creatinine, corticosterone levels and total leucocyte count were measured. On day 10, the rats were sacrificed and biochemical assessment of superoxide dismutase (SOD), lipid peroxidation (LPO), catalase (CAT), and glutathione reductase (GSH) in whole rat brain were performed. Exposure to chronic restraint stress produced significant reduction of ambulations and rearings in open field test. Pretreatment with resveratrol normalized the behavioral parameters. Chronic restraint stress produced elevation in serum glucose (136 ± 0.2 mg/dl), creatinine (3.34 ± 0.01 mg/dl), corticosterone (17.2 ± 0.09 μ g/dl) levels and total leucocytes (8033 ± 10.4 cells/mm³). Administration of resveratrol significantly reversed these changes. Restraint stress also causes significant reduction in CAT, SOD, GSH levels and increased lipid peroxidation in rat brain. Resveratrol significantly restored brain oxidative stress indices. This proved that resveratrol reversed oxidative damage to brain and possesses promising anti-stress activity.

Keywords: Catalase, Chronic restraint stress, HPA axis, Lipid peroxidation, Restraint stress, Resveratrol.

INTRODUCTION

Stress can be defined as physical and psychological modifications that disrupt the homeostasis and the balance of organisms [1]. Restraint stress is an easy and convenient method to induce both psychological (escape reaction) and physical stress (muscle work) resulting in restricted mobility and aggression. Restraint is a preferred means of stressing animals, largely because it is straightforward and painless method [2]. Immobilization has been used extensively as a stressor for the study of stress-related biological, biochemical and physiological responses in animals [3]. It has been postulated that integration of the stress signals originating from different

regions in the body occurs in the hypothalamus [4]. There are evidences that the sympathetic nervous system has a key role in producing stress-induced responses. It has been found that, increasing the duration of restraint stress leads to increase in the global activation of the sympathetic nervous system [5]. Complex interactions between various stress inputs and the hypothalamo-pituitary adrenal axis (HPA) along with several components of the visceral system determine the outcome of the stress response [6]. A rise in plasma corticosterone and adrenocorticotropic hormone (ACTH) level has been reported to be associated with immobilization induced stress [7]. Also, the cognitive impairments developed faster as a result of chronic immobilization stress.

Stress is a leading cause of stimulation of numerous intracellular pathways leading to the increased generation of reactive oxygen species (ROS). Various

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reports have proved that restraint stress results in imbalance of antioxidant status leading to increased oxidative stress [8]. The role of oxidative injury is important in stress because therapy with agents that scavenge reactive oxygen species and augment endogenous antioxidant capacity may prove useful in therapeutic modulation of stress-induced devastating neurological conditions.

Resveratrol belongs to the stilbene family of compounds and present in red grapes and red wine [9]. Many investigators have proved that it can slow down the progression of a various diseases in animal models and humans. Resveratrol shows diverse biological actions, suggesting its complex mode of action and it has been of great interest to researchers in nutrition, pharmacology, and clinical medicine [10]. Resveratrol has been reported to be a good antioxidant against the peroxidation of low-density lipoproteins [11]. It has been suggested that dietary resveratrol may act as an antioxidant and may promote nitric oxide production. There are evidences for its inhibitory action on platelet aggregation, thereby serving as a cardio-protective agent [12], but no major investigations were carried out reporting its anti-stress activity especially against chronic restraint stress-induced neurodegeneration and milk induced leucocytosis in rats. Therefore, in this study, an attempt has been made to investigate ameliorative potential of resveratrol in chronic restrain stress model and in milk induced leucocytosis to unravel its mechanism of action with respect to biochemical imbalances.

MATERIALS AND METHODS

Materials

Resveratrol (Zenith Nutritions, Bangalore, India); diazepam (Ranbaxy Laboratories Ltd, Baddi, India); thiobarbituric acid (TBA) (Research-Lab, Fine Chem Industries, Mumbai, India); nitrobluetetrazolium chloride (NBT) (Himedia Laboratories Pvt. Ltd., Mumbai, India); 5,5'-dithiobis-2-nitro benzoic acid (DTNB) (Alfa Aesar, A Johnson Mathey Company); Bovine serum albumin (Spectrochem Pvt. Ltd., Mumbai, India). The diagnostic kits for serum glucose and creatinine (Span Diagnostics, Gujarat, India). All the chemicals used were of analytical grade and purchased from standard manufacturers.

Animals

Male Wistar strain rats (200-230 g) were used for the study. Animals were housed in polypropylene cages and maintained under the standard laboratory environmental conditions; temperature 25 ± 2 °C, 12: 12 h L: D cycle and 50 ± 5 % RH with free access to food and water *ad libitum*. Animals were acclimatized to laboratory conditions before the test. All the experiments were carried out during the light period (08:00-16:00 h). The studies were carried out in accordance with the guidelines given by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) New Delhi (India). The Institution Animal Ethical Committee of M.V.P.S College of

Pharmacy, Nashik approved the protocol of the study (IAEC/2013/1a).

Experimental design

Animals were randomly assigned into 4 groups, each group consisted of 5 animals (n = 5). Group I – Vehicle (0.2% PEG [polyethylene glycol] in distilled water p.o.); Group II – Chronic restrain stress (CS); Group III – Diazepam (1mg/kg, p.o); Group IV – Resveratrol (20 mg/kg, p.o); Group V – Resveratrol (40 mg/kg, p.o).

Induction of chronic restraint stress

Chronic restraint stress was induced by restraining the animals inside an adjustable cylindrical plastic tube. The rats were confined individually and exposed continuously to restrain stress for 3 h once daily for ten consecutive days. Resveratrol (20 mg/kg and 40 mg/kg, p.o) and diazepam (1 mg/kg, p.o.) per day was administered 60 min prior to the stress procedure [13].

Behavioural testing

Open field test

Locomotor activity was evaluated in an open field paradigm. The open field was made up of plywood and consisted of floor (96 × 96 cm) with high walls (60 × 60 cm). Entire apparatus was painted black except for 6mm thick white lines that divided the floor into 16 squares. Each animal was placed at one corner of the apparatus and was observed up to 5 min for number of ambulations (number of squares crossed), number of rearings and latency to come at centre of the open field. The test was performed on day 1, 5 and 10 [14].

Biochemical estimation

Dissection and Homogenization

On the 10th day immediately after behavioural assessments, the animals were killed by cervical decapitation. The brain was removed, rinsed with isotonic saline and weighted. A 10 % (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The post nuclear fraction for catalase assay was obtained by centrifugation (Remi - C - 30, Remi Industries Ltd. Mumbai, India) of the homogenate at $1000 \times g$ for 20 min at 4 °C; for other enzyme assays, centrifugation was at $12000 \times g$ for 60 min at 4°C. A bio-spectrophotometer (Elico BL-200) was used for subsequent assays [15].

Catalase activity (CAT)

Catalase activity was assessed by the method of Luck (1971), where the breakdown of H₂O₂ was measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H₂O₂ phosphate buffer (0.0125 M H₂O₂) and 0.05 ml of supernatant of brain homogenate and the change in the absorbance was measured at 240 nm. The enzyme activity was calculated using the millimolar extension coefficient of

H₂O₂ (0.07). The results were expressed as μ moles of H₂O₂ decomposed per minute per milligram of protein [16].

Estimation of reduced glutathione (GSH)

Reduced glutathione (GSH) in the brain was assayed according to the method of Ellman (1959). Aliquot of 0.1 ml homogenate was precipitated with 0.75 ml of 4% sulphosalicylic acid. The assay mixture contained 0.5 ml supernatant and 4.5 ml of DTNB [5-5'-dithiobis (2-nitrobenzoic acid)] in 0.1 M phosphate buffer, pH 8.0. The yellow colour developed was read immediately at 412 nm. The results were expressed as micro moles of GSH per milligram of proteins [17].

Superoxide dismutase activity (SOD)

Superoxide dismutase activity was assayed according to the method of Kono (1978), where in the reduction of nitrobluetetrazolium chloride (NBT) was inhibited by the superoxide dismutase and measured at 560 nm spectrophotometrically. Briefly the reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and post nuclear fraction of brain homogenate. Results were expressed as percentage inhibition of reduction of NBT [18].

Lipid peroxidation assay (LPO)

The quantitative measurement of lipid peroxidation in brain was done by the method of Wills (1966). The amount of malondialdehyde (MDA) formed was measured by reaction with thiobarbituric acid at 532 nm. The results were expressed as nanomoles of MDA per milligram of protein, using the molar extension coefficient of chromophore ($1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$) [19].

Protein estimation

The protein content was measured according to the method of Lowry *et al.*, (1951), using bovine serum albumin as standard and expressed as μ g protein / mg of tissue [20].

Estimation of serum glucose levels

Serum glucose levels were determined using a standard glucose oxidase-peroxidase kit [21].

Estimation of serum corticosterone levels

The quantitative measurement of serum corticosterone was done [22].

Estimation of serum creatinine levels

Assessment of serum creatinine was carried out by alkaline picrate method. To 0.5 ml of serum sample solution, 0.5 ml of purified water and 3 ml of picric acid was added. It was kept in boiling water bath for 1 minute and cooled immediately under running tap water and then centrifuged. To the supernatant obtained, 0.5 ml of 0.75 N sodium hydroxide solution was added. Further, it was

allowed to stand for 20 minutes at room temperature and absorbance was measured immediately at 520 nm [23].

Milk-induced leucocytosis

Subcutaneous injection of milk is known to produce an infection like condition by acting as an antigen and increasing the leukocyte count. Anti-stress activity can be evaluated on the basis of the capacity of the drug to prevent this stress induced increase in white blood cell count. For this study, blood samples were collected from retro-orbital sinus. Blood was sucked in WBC pipette up to mark and further diluted with WBC diluting fluid. Pipette was shaken for few seconds and kept aside for 5 min. Neubaur's chamber was charged with above fluid and total leukocyte count was expressed as number of cells/cu.mm. Total leukocyte count was done in each group before drug administration and 24 h after milk injection (boiled and cooled; 4 ml/kg s.c.) [24].

Statistical analysis

The results are expressed as mean \pm SEM. Data was subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test. Probability level less than 0.05 was considered statistically significant.

RESULTS

Effect of resveratrol on behavioral parameters in the open field test

Chronic restraint stress produced significant ($P < 0.05$) reduction in number of ambulations and rearings on day 5th and 10th day as compared to vehicle group. Latency to reach to the central region in the open field was also significantly ($P < 0.05$) increased on day 5th and 10th day in restraint stress group. Animals treated with diazepam (1 mg/kg) showed significant improvement in number of ambulations, rearings and reduce in latency to reach to the central region as compared to restraint stress group on 5 and 10th day. Administration of resveratrol (20 and 40 mg/kg) showed promising effects on behavioural parameters. Treatment with resveratrol showed significant ($P < 0.05$) reduction in latency to reach to the central region and improvement in both number of ambulations and rearings as compared to restraint stress group at both 20 and 40 mg/kg dose (Fig. 1A,B and C).

Biochemical Estimations

Effect of resveratrol on chronic restraint stress-induced alterations in rat brain SOD, CAT and GSH

Chronic stress significantly ($P < 0.001$) decreased the levels of SOD, CAT and GSH as compared to vehicle group. Pre-treatment with resveratrol (20 and 40 mg/kg) significantly ($P < 0.001$) increased the levels of SOD, CAT and GSH in a dose dependant manner as compared to restraint stress group. Diazepam also showed significant increase in the levels of SOD, CAT and GSH (Table 1).

Effect of resveratrol on chronic restraint stress-induced alterations in rat brain on LPO

Chronic stress led to significant increase in LPO level. Pretreatment with resveratrol significantly ($P < 0.001$) reduced elevated level of LPO at both 20 and 40 mg/kg dose. Diazepam also significantly decreased LPO level ($P < 0.001$) (Table 1).

Effect of resveratrol on chronic restraint stress-induced alterations in serum glucose, creatinine and corticosterone levels

Exposure to chronic stress significantly ($P < 0.05$) increased the serum glucose, creatinine and corticosterone levels as compared to vehicle treated group. Pretreatment with resveratrol (20 mg/kg) significantly ($P < 0.05$) decreased glucose, creatinine and corticosterone levels as

compared to chronic stress group. Resveratrol (40 mg/kg) showed highest significant effect on serum glucose; creatinine and corticosterone levels. The serum glucose, creatinine and corticosterone levels were also significantly decreased in the diazepam treated group (Table 2, 3 and 4).

Effect of resveratrol on chronic restraint stress-induced alterations in milk-induced leucocytosis

Subcutaneous injection of milk in a dose of 4 ml/kg produced a significant ($P < 0.001$) increase in the leucocyte count after 24 h of its administration which was elevated till the last day of the experiment after induction of chronic stress as compared to vehicle group. Resveratrol dose dependently reversed this rise in the leucocyte count ($P < 0.001$). Diazepam also significantly inhibited the rise in total leucocyte count (Table 5).

Table 1. Effect of resveratrol on chronic restraint stress-induced alterations in rat brain CAT, GSH, SOD and LPO levels

Treatments	CAT (μ moles of H_2O_2 decomposed/mg protein/min)	GSH (μ moles of GSH/mg protein)	SOD (% inhibition of reduction of NBT)	LPO (n moles of MDA/mg protein)
Vehicle	7.13 \pm 0.7	7.57 \pm 0.2	74.41 \pm 0.1	6.96 \pm 0.2
Chronic stress (CS)	5.07 \pm 0.1 ^{###}	2.79 \pm 0.1 ^{###}	59.82 \pm 3.5 ^{###}	16.38 \pm 0.71 ^{###}
Diazepam (1 mg/kg, p.o.)	5.74 \pm 0.2 ^{***}	6.05 \pm 0.2 ^{***}	72.73 \pm 0.3 ^{***}	12.87 \pm 0.4 ^{***}
Resveratrol (20 mg/kg, p.o.)	6.92 \pm 0.1 ^{***}	7.17 \pm 0.1 ^{***}	75.35 \pm 0.1 ^{***}	10.48 \pm 0.2 ^{***}
Resveratrol(40 mg/kg, p.o.)	7.22 \pm 0.39 ^{***}	7.74 \pm 0.62 ^{***}	75.47 \pm 0.41 ^{***}	7.38 \pm 0.29 ^{***}

Each value represents mean \pm SEM (n = 5).

[#]Compared with vehicle treated group. ^{*} Compared with chronic restraint stress (CS) group.

^{###}, ^{***} $P < 0.001$. (One-way ANOVA followed by Dunnett’s test).

Table 2. Effect of resveratrol on chronic restraint stress-induced alterations in serum glucose level

Treatments	Serum Glucose (mg/dl)		
	Day 1	Day 5	Day 10
Vehicle	86 \pm 0.2	88 \pm 0.36	87 \pm 0.34
Chronic Stress (CS)	91 \pm 0.10 [#]	126 \pm 0.12 [#]	136 \pm 0.23 [#]
Diazepam (1 mg/kg)	87 \pm 0.56 [*]	99 \pm 0.84 [*]	102 \pm 0.16 [*]
Resveratrol (20 mg/kg)	85 \pm 0.59 [*]	94 \pm 0.80 [*]	83 \pm 0.18 [*]
Resveratrol (40 mg/kg)	88 \pm 0.40 [*]	92 \pm 0.70 [*]	79 \pm 0.91 [*]

Each value represents mean \pm SEM (n = 5).

[#]Compared with vehicle treated group. ^{*} Compared with chronic restraint stress (CS) group.

[#], ^{*} $P < 0.05$. (One-way ANOVA followed by Dunnett’s test).

Table 3. Effect of resveratrol on chronic restraint stress-induced alterations in serum creatinine level

Treatments	Serum Creatinine (mg/dl)		
	Day 1	Day 5	Day 10
Vehicle	0.47 \pm 0.07	0.52 \pm 0.05	0.58 \pm 0.34
Chronic Stress (CS)	0.84 \pm 0.06 [#]	2.14 \pm 0.13 [#]	3.34 \pm 0.01 [#]
Diazepam (1 mg/kg)	0.46 \pm 0.05 [*]	0.79 \pm 0.02 [*]	1.21 \pm 0.10 [*]
Resveratrol (20 mg/kg)	0.41 \pm 0.11 [*]	0.53 \pm 0.12 [*]	0.62 \pm 0.05 [*]
Resveratrol (40 mg/kg)	0.48 \pm 0.13 [*]	0.54 \pm 0.03 [*]	0.57 \pm 0.15 [*]

Each value represents mean \pm SEM (n = 5).

[#]Compared with vehicle treated group. ^{*} Compared with chronic restraint stress (CS) group.

[#], ^{*} $P < 0.05$. (One-way ANOVA followed by Dunnett’s test).

Table 4. Effect of resveratrol on chronic restraint stress-induced alterations in serum corticosterone level

Treatments	Serum Corticosterone (µg/dl)		
	Day 1	Day 5	Day 10
Vehicle	10.6 ± 0.10	9.5 ± 0.02	10.0 ± 0.11
Chronic Stress (CS)	10.9 ± 0.16 [#]	13.6 ± 0.12 [#]	17.2 ± 0.09 [#]
Diazepam (1 mg/kg)	9.9 ± 0.06 [*]	12.2 ± 0.12 [*]	10.7 ± 0.10 [*]
Resveratrol (20 mg/kg)	10.3 ± 0.03 [*]	10.5 ± 0.12 [*]	9.8 ± 0.06 [*]
Resveratrol (40 mg/kg)	9.20 ± 0.91 [*]	9.56 ± 0.81 [*]	8.90 ± 0.22 [*]

Each value represents mean ± SEM (n = 5).

[#]Compared with vehicle treated group. ^{*} Compared with chronic restraint stress (CS) group.

[#], ^{*}P < 0.05. (One-way ANOVA followed by Dunnett’s test).

Table 5. Effect of resveratrol on total leucocyte count

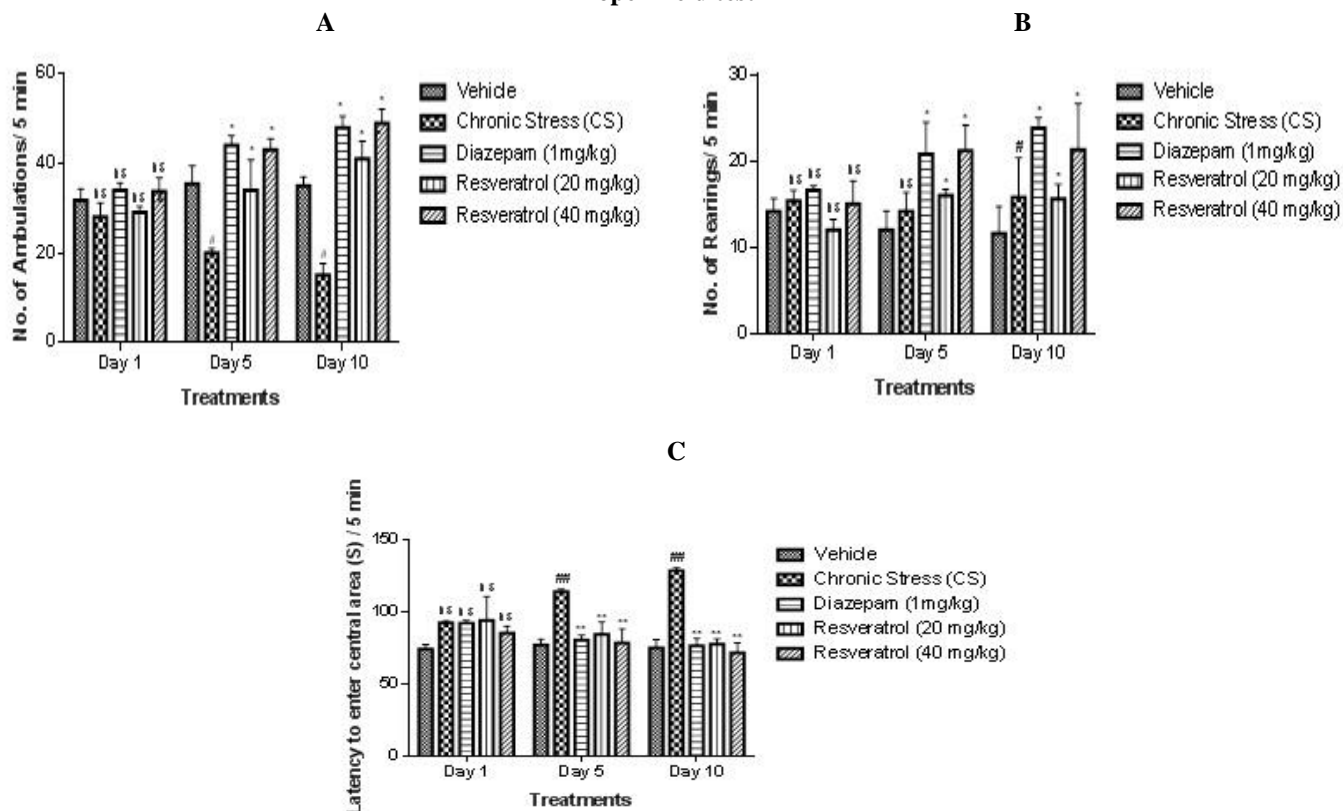
Treatments	Difference in leucocyte count (cells/ mm ³)
Vehicle	8033.10 ± 109.42
Diazepam (1mg/kg)	3449.60 ± 363.92 ^{***}
Resveratrol (20 mg/kg)	2136.10 ± 115.48 ^{***}
Resveratrol (40 mg/kg)	1012.66 ± 22.56 ^{***}

Each value represents mean ± SEM (n = 5).

^{***}Compared with vehicle treated group.

^{***}P < 0.001. (One-way ANOVA followed by Dunnett’s test).

Figure 1. Effect of resveratrol on number of ambulations, number of rearings and latency to enter central area in the open field test



Each value represents mean ± SEM (n = 5).

[#]Compared with Vehicle group. ^{*} Compared with chronic restraint stress (CS) group.

^{ns}Non-significant, [#], ^{*}P < 0.05, ^{##}, ^{###}, ^{***}P < 0.01. (One-way ANOVA followed by Dunnett’s test).

DISCUSSION

A variety of stress situations have been employed in animals to evaluate anti-stress agents. Immobilization has been the ideal choice for the induction of stress responses in animals and more specifically, for the investigation of drug effects, on typical stress-related neuro-endocrine and immunological pathology [25]. The distinct advantage of using immobilization as a stressor lies in the fact that it produces both physical as well as inescapable psychological stress. Stress cause significant reduction in the motor activity, motivation and acquisition in animals [26].

The results of the present study demonstrate that resveratrol improves the ability to withstand the stressful stimuli, in chronic restraint stress model. The data of the present study clearly indicated that administration of resveratrol significantly ameliorated restraint stress-induced alterations in behavioural parameters in open field test. Chronic restraint stress produces significant reduction in number of ambulations and rearings with increase in latency to reach to the central region in the open field test. Resveratrol showed significant effect on behavioural parameters. Resveratrol produced significant reduction in latency to reach to the central region and increased both number of ambulations and rearings, suggesting improvement in stress related behavioural alterations. Diazepam also showed significant increase in number of ambulations, rearings and reduce in latency to reach to the central region.

Chronic restraint stress was associated with increased oxidative stress as evidenced by raised lipid peroxidation level and depletion of endogenous enzymatic antioxidants. It has been found that the nervous system is extremely sensitive to oxidizable substrates and high oxygen tension. In this study, significant reduction in the levels of SOD, CAT and GSH with concomitant increase LPO in rat brains were found in animals subjected to chronic restraint stress for 10 consecutive days. Rise in lipid peroxidation might be due to depletion of intracellular GSH content which is considered as a first line of defence as an endogenous non-enzymatic antioxidant. Pre-treatment with resveratrol significantly reduced restraint stress-induced lipid peroxidation and increased the levels of SOD, CAT and GSH. Thus, resveratrol possesses antioxidant activity and showed protection against oxidative stress. The mechanistic approach of such protection against oxidative stress is mediated through the augmentation of the number of cellular antioxidants, such as SOD, CAT and GSH etc. The results of the present study indicated that decrease in CAT level after induction of chronic restraint stress. This decreased activity of catalase could be attributed as a result of increased H_2O_2 formation after chronic restraint stress. H_2O_2 production is reported to be enhanced after stress. These findings support that resveratrol has extensive antioxidant properties. Also, there are reports suggesting neuroprotective role of resveratrol against cerebral

ischemia-induced oxidative damage in terms of restoration in various antioxidant enzymes activity [27].

In our study, rise in plasma glucose level was observed in animals subjected to chronic stress. We propose that, this particular effect was observed because of release of glucocorticoids as a consequence of stimulation of hypothalamic–pituitary–adrenocortical (HPA) axis to compensate the initial demand of energy [28]. This hyperglycaemic response during stress has been associated with glucose intolerance [29]. The serum corticosterone levels of the animals in stress group were found to be significantly elevated on 5th and 10th day of study, indicating the activation of hypothalamic-pituitary adrenal (HPA) axis. Our results are in accordance with the previous studies depicting that; corticosterone has been one of the most useful indicators of stress [30]. Glucocorticoid hormones, mainly corticosterone in rats and cortisol in humans, are the final effectors of the HPA axis and participate in the control of homeostasis and the response of the organism to stressors. In this study, the protective effects of resveratrol in stress regimen can be attributed to the decreased activation of HPA axis as shown by the lowered levels of plasma corticosterone which is an immediate and chronic index of hyperactive HPA axis. The level of serum creatinine was also increased in chronic stress group. It has been already documented that, when ATP is rapidly depleted, creatine kinase catalyses the donation of a phosphate group from phosphocreatine to ADP, producing more ATP to buffer energy needs. Conversely, when energy is released, an individual phosphate group is cleaved from ATP and bound to creatine to rejoin the phosphocreatine pool. This reversible reaction causes a spontaneous by product-creatinine. The rise in creatinine is a direct consequence of rise in the levels of creatine kinase. Thus, our study supports the fact that, level of creatine kinase rises in stress regimen. The creatine kinase system is important in stabilizing the ATP levels and energy metabolism of the myocardium and other skeletal muscles of animals during stress. Diazepam also significantly decreased glucose, creatinine and corticosterone levels. Subcutaneous injection of milk produces an increase in leucocyte count [31]. Resveratrol significantly reduced leucocyte count, suggesting adaptogenic activity. Diazepam also decreased leucocyte count. Thus, our results proved that resveratrol showed protective effects against chronic restraint stress induced perturbations and milk induced leucocytosis.

CONCLUSION

We conclude that, resveratrol has significant anti-stress and antioxidant activities. Thus, the combination of anti-stress and antioxidant effect could be responsible for amelioration of several chronic stress-induced biochemical and neurobehavioral perturbations. Hence resveratrol may be useful as an adaptogenic agent in the treatment of stress related disorders.

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