

# Reversible Assessment of Restrained Stress after Withdrawal in Male Albino Rats

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## Abstract:

This study was carried out to determine the influence of restrained stress and its withdrawal possible alteration on gravimetry, organ weight, hematological parameters and level of serum cortisol in male Wistar albino rats. Adult male rats weighing about 175 to 225g were taken for the study and were divided at random into three groups of six animals each. Group I (control), kept undisturbed in the metabolic cage throughout the 42 days experimental period. Group II (stress) rats were kept in a wire mesh restrainer for 6 hr/day for 42 days. Group III (stress+ withdrawal) rats were stressed for 21 days and withdrawal of stress premeditated for remaining 21 days (total 42 days). At the end of the study period, all the experimental rats were sacrificed and gravimetric, organ weight, hematological parameters and serum cortisol level were evaluated. Results revealed that restrained stress being a psychological stress significant ( $P \leq 0.05$ ) change in the gravimetry, organ weight, hematological parameters and serum cortisol level. On the other hand, Group III of withdrawal of stress exhibits subsequent improvement in these alterations may probably neutralize restrained stress induced damage that lead to oxidant antioxidant balance and alter hypothalamic-pituitary- adrenal (HPA) axis.

**Key words:** Stress, Withdrawal, Gravimetry, Hematology, Cortisol, Rats

## INTRODUCTION

Stress has an impact to induce alteration in various anatomical, physiological, biological responses include psycho-physiological reactions. Stress related biological responses include psycho- physiological reactions, such as sweating, muscle tension as well as changes in action of various brain regions. During stressful events, our bodies release chemicals (adrenaline and cortisol) into the bloodstream that then trigger what is known as the 'fight-or-flight' response. In response to stressors, a series of behavioral, neurochemical, and immunological changes occur that ought to serve in an adaptive capacity (Brown, 1993; Anisman, 1999). Stress produces life threatening events in early development and can result in long term effects on organ development and may lead to pathological conditions. Effects of stress on different organs of the body have been extensively investigated in both humans and experimental animals and study revealed that severe stress lasting weeks or months can impair cell communication in the brain's region (McEwen, 2007).

Stress can be explained as any stimulus that creates an imbalance in the homeostasis processes (Oxington, 2005). Oxidative damage leads to mood and anxiety disorder and is a definite outcome of stress (Núñez *et al.*, 2011). The resultant disturbances may vary depending upon type, intensity, and the duration of a particular stressor and the strain/sex differentiation of the subjects (Kioukia-Fougia *et al.*, 2002). The blood is one of the major homeostatic systems of the body supporting normal viability, integrity and adaptive responses. The functional state of the blood systems changes dynamically according to the nature, strength and duration of exposure to external factors. Many studies performed on humans and

animals have provided evidence for significant shifts in physiological and biochemical processes during the modeling of emotional tension. The nervous and blood systems are the quickest to react to emotional stimuli. Stress and emotional reactions affect the immune system is by complete blood counts, including the hematopoietic system, leukocyte profile or biochemical markers of stress. In addition to this other studies elucidated that serum cortisol levels are a reliable indicator of stress responses in animals (Elizabeth *et al.*, 2004; Jaggi *et al.*, 2011).

Extensive research has been done on the effects of stress on the levels of various hormones in the blood but information about reversible assessment of restrained stress after withdrawal in rats is lacking. Hence, this study was aimed to assess the effect of restrained stress and its withdrawal possible alteration on gravimetry, organ weight, haematological parameters and serum cortisol level in male albino rats.

## MATERIAL AND METHODS

### Animals and Ethics

Colony bred healthy adult male albino rats (Wistar strain) weighing 175-225 g was utilized from Central Animal Facility, Indian Institute of Science, Bengaluru for the experiments. Wister rats fed with laboratory stock diet (Hindustan lever, Mumbai, India) and water *ad libitum*. They acclimatized a week to the laboratory conditions at 22-24°C and a 12 h light: dark (circadian) cycle. All the animals were sacrificed at the end of the last day after an overnight fast. All the experimental procedures followed were performed in accordance with the approval of the Institutional Animal Ethics Committee (IAEC/477/2016) under strict compliance of Committee for the Purpose of Control and Supervision of Experiments on

Animals (CPCSEA) guidelines for the experimental studies.

### Experimental study

The acclimatized animals divided into three groups of six animals each and three animals were kept in each metabolic wire cage (60cm x 30cm x 20cm). Group I (untreated control) rats were healthy controls, kept undisturbed in the metabolic cage throughout the experimental period for 42 days. Group II (stress induced) rats were stressed in wire mesh restrainer for 6h/day for 42 days (Kumar *et al.*, 2007). Group III (stress + withdrawal) rats were stressed for 21 days by keeping them inside mesh restrainer and withdrawal of stress intended for remaining 21 days by keeping animals in normal cages (total 42 days).

### Stress Procedure

Rats were subjected to restrained stress in a wire mesh restrainer for 6 hours per day for 42 days. The wire mesh restrainer had a wooden base and stainless steel wire mesh restrainer hinged to the base. The restrainer having the dimensions of 8 cm (L) x 4 cm (B) x 4 cm (H) was used for the experiments. A pad lock and latch helped to secure the rat in the restrainer (Kumar *et al.*, 2007).

### Gravimetry

The body weight (BW) of all the rats was recorded on the day 1 of stress alternate 10<sup>th</sup> day and the day of sacrifice (i.e. 42<sup>nd</sup> day). Percent BW gain was determined in experimental groups of the rats with a ratio of final BW to the initial BW. Organ somatic index was determined by the ratio of organ weight to BW of rat before sacrifice (final BW). All the animals were sacrificed by cervical dislocation and followed by decapitation at the end of the last dose after an overnight fast and relative organs such as brain, liver, kidney, adrenal gland and testis were dissected out, blotted free of mucus and weighed to the nearest milligram.

### Hematological parameters and serum cortisol

The blood collected from retro orbital method. Blood was collected in centrifuge tubes, kept at room temperature for about 2 h and centrifuged at 1500×g for 15 min to collect serum. Serum was then used for analyzing the hematological parameters such as PCV, Hb, RBC, MCV, MCHC, MCH, TWBC, Platelets, Neutrophils, Lymphocytes, Eosinophils and Monocytes (using SYS MAX-35 automated cell counter machine) and estimations of cortisol level by the Enzyme-linked Immunosorbent Assay (ELISA) kit (DRG, USA) method (Jameel *et al.*, 2014).

### Statistical analysis:

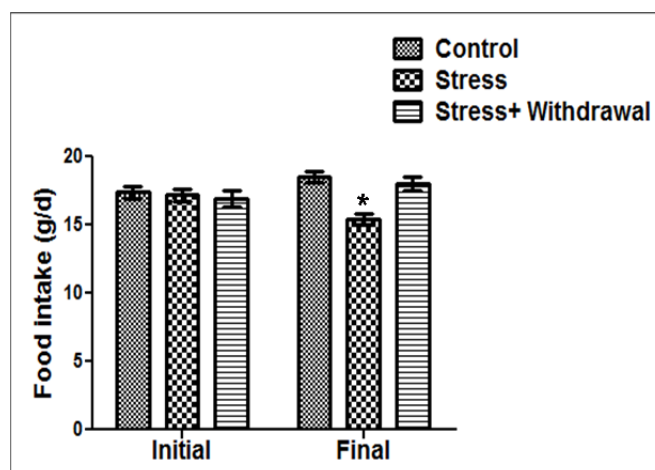
Data were analyzed using one way analysis of variance (ANOVA) using the Graph Pad Prism software method, followed by Dunnet test by comparing all treated groups against controls. Values represented are mean ± SEM (n=6). P ≤ 0.01 is considered to indicate a significant difference between experimental and controls.

## RESULTS AND DISCUSSION

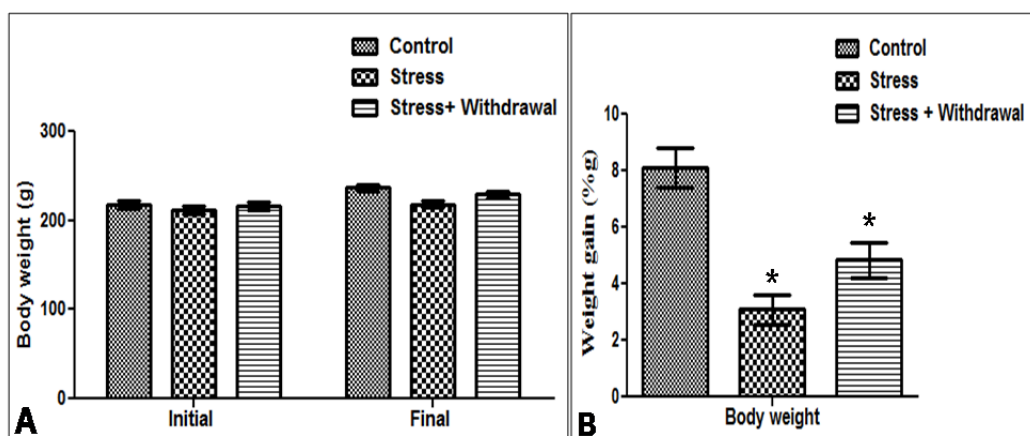
### Gravimetry

In the present study, no adverse reactions were observed in any of the experimental groups and remained active and healthy with normal feeding behavior (Figure 1). Rats in the control group had a normal weight accumulation, whereas the weight gain in the restrained stress group (chronic moderate stress, Group I) was significantly lower when compared to groups of control (Group I) and stress withdrawal (Group III). Stress induced rats (Group II) were found to be lethargic and their BWs decreased remarkably which is indicated by the mean percentage of BW gain of 14.07%, as compared to their mean initial BW gain (25.06%). However, Group III (stress withdrawal) rats had a non-significant increase in mean initial percentage of BW gain (23.86%) as compared to untreated controls (Figure 2A and B).

The exposure to restrained stress for 6 hours per day for 42 days resulted in a significant reduction of BW may be due to low food consumption, hormonal imbalance and protein metabolism in the rats under the influence of stress. Several studies have demonstrated that chronic exposure to restraint stress reduces the BW and food intake of rodents (Marti *et al.*, 1994; Harris *et al.*, 1998; Gamaro *et al.*, 2003; Jeong *et al.*, 2013). However, the mechanisms underlying these restraint-induced changes in BW and food intake remain to be elucidated. Evidences shown the decrease in BW could be due to the direct effect of stress on the food intake behavior of the rats (First *et al.*, 2011), metabolic processes are mediated by glucocorticoids (Bhatnagar *et al.*, 2006), increase in metabolic demands, reduced digestion, and increased adrenal steroid secretion (Nayanatara *et al.*, 2012) and stress might have increased the protein catabolism and hampered the utilization of food consumed during the stress period, thereby causing decrease in BW (Ng *et al.*, 2008).



**Figure 1:** Effect of restrained stress (6 hr/day for 42 days) induced alteration on mean daily food intake in male albino rats. Values are mean ± SEM (n=6). P ≤ 0.05 (asterisk) is considered to indicate a significant difference between experimental and controls.



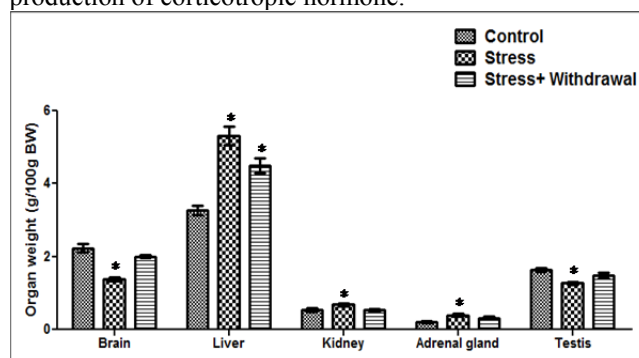
**Figure 2:** Effect of restrained stress (6 hr/day for 42 days) induced alteration on BW (A) and percentage gain of BW (B) in male albino rats. Values are mean  $\pm$  SEM (n=6).  $P \leq 0.05$  (asterisks) is considered to indicate a significant difference between experimental and controls.

### Organ weight

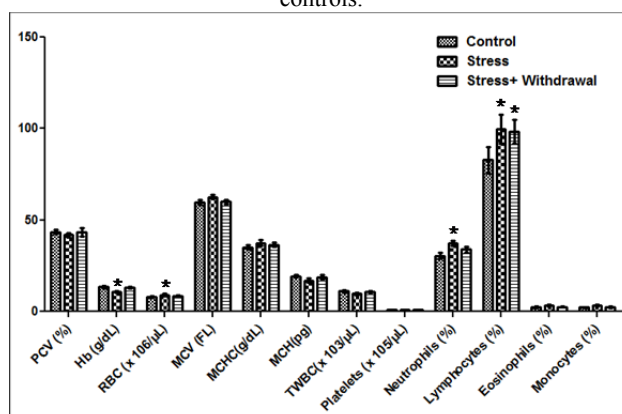
Organ weight can be the most sensitive indicator of an effect of an experimental compound, as significant differences in organ weight between treated and untreated (control) animals may occur in the absence of any morphological changes (Bailey *et al.*, 2004). In our study (Figure 3), the animals were subjected to restrained stress, every day for 42 days produces a greater a psychological stress (Tecoma and Hncy, 1985). Stress induced (Group II) rats had a significant decrease in whole brain weight and testis weight. Decrease in the weight of brain could result from either decline of neuronal pathways or from myelination of the already existing ones (Koob and Bloom, 1985; Meyer, 1987). Since the restrained stress in our study was prolonged for 6 hours per day for 42 days, we observed a significant change in the weight of testes. Prolonged and severe stress lowers LH release (Rivier and Vale, 1985). CRH inhibits the central release of GnRH which mediates the deleterious action of stress on reproductive organs (Rivier and Plolsky, 1986). Increased weight of the liver during stress could be due to the increased secretion of the stress hormones, which are known to increase the metabolic activities and mRNA levels in the hepatic cells (Nayanatara *et al.*, 2005). Changes in the homeostatic mechanism such as increased cardiac output and blood pressure during stress might have contributed to the increased kidney weight after stress (Chang *et al.*, 1995; Nagaraja *et al.*, 2006). Strong stimulation of the adrenal glands during prolonged stress situations is known to cause adrenal hyperplasia and hypertrophy (Marti *et al.*, 1993; Tuli *et al.*, 1995).

Studies show that hyperactivity of adrenals in stressed animals is due to the stress induced adrenomedullary response leading to increased production of corticotropin hormone that leads to increase in weight of adrenals (Lakshmi and Sudhakar, 2009; Jiban *et al.*, 2011). A significant increase in weight of adrenal glands in restrained stress animals indicate adrenalin in turn stimulates  $\beta_2$ -receptors on the pituitary gland causing greater release of ACTH (Reisine *et al.*, 1983). In this study, restrained stress being a psychological stress could cause greater release of adrenalin which in turn leads to

greater increase in weight of adrenal gland compare to control and withdrawal groups. However, withdrawal of stress used in favor of remaining 21 days has significantly reduced the weight of liver, kidney, brain and adrenal glands might be due to the subsequent recovery of stress induced adrenomedullary response and hence decrease production of corticotropin hormone.



**Figure 3:** Effect of restrained stress (6 hr/day for 42 days) induced alteration on relative organs weight of male albino rats. Values are mean  $\pm$  SEM (n=6).  $P \leq 0.05$  (asterisks) is considered to indicate a significant difference between experimental and controls.



**Figure 4:** Effect of restrained stress (6 hr/day for 42 days) induced alteration on hematological parameters in male albino rats. Values are mean  $\pm$  SEM (n=6).  $P \leq 0.05$  (asterisks) is considered to indicate a significant difference between experimental and controls.

### Hematological parameters

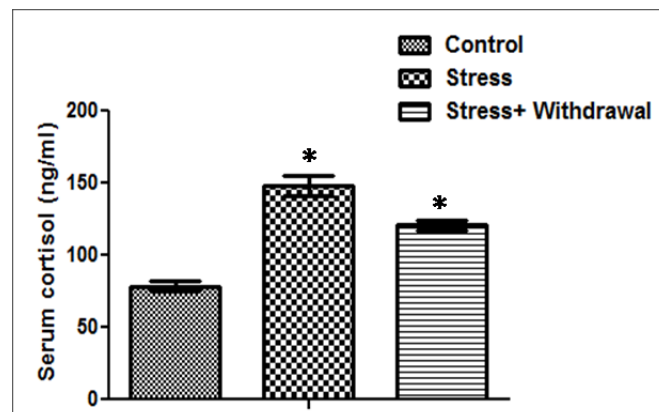
Stress is thought to impair immune function (Zorrilla *et al.*, 2001; Herbert and Cohen, 1993) through emotional or behavioral manifestations such as anxiety, fear, tension, cognition, anger, and sadness and physiological changes of heart rate, blood pressure, glucose metabolism, and sweating. Stress induced changes in hematological parameters clearly showed anemia, and it may be due to release of immature RBCs in circulation. However, in our study (Figure 4), the decrease in hemoglobin concentration, RBC count, total WBC count and MCV may be due to non-regenerative anemia arising from stress induced disorder of hematopoietic stem cells resulting in decreased erythrocyte, leukocyte and platelet count (Table 2). Similar to this study, other experimental studies have been shown by the significant reduction in hematological parameters when exposed to immobilization stress (Caporossi *et al.*, 2010; Sarumathi and Saravanan, 2013) and restrained stress animals (Kori *et al.*, 2016, 2017).

Decreased in the level of hematological parameters in the stressed group and at the same time withdrawal of stress meant for remaining 21 days were shown near normal values as control rats might be due to the reversal of stress induced antioxidant, anti-lipidperoxidation, immunostimulation and anticonvulsant response. Results revealed that exposure to restraint stress induced peripheral oxidative stress, which is defined by an increase in the generation of reactive oxygen species (ROS) in peripheral blood lymphocytes, granulocytes and monocytes. A recovery phenomenon of leucocytes from oxidative stress was observed in withdrawal of stress animals indicating that alleviation of oxidative damage induced by psychological stress on the peripheral immune system. Our results are in concurrence with other studies showing that the production of ROS by immune cells might be influenced by psychological stress. However, the available results in reference to the influence of psychological stress on the production of ROS are contradictory (Ng *et al.*, 2008; Patki *et al.*, 2013), while others have shown a decreased ROS production (Atanackovic *et al.*, 2003; McCarthy *et al.*, 1992).

### Serum Cortisol

Cortisol, a stress hormone, serves as a key controller for neurohumoral responses in turn leads to behavioral adaptations. Statistically significant increase in levels of serum cortisol after restrained stress suggests that hypothalamic-pituitary-adrenal (HPA) axis is activated. This feedback mechanism is extremely important for survival with stress (Lupien *et al.*, 2007). Many studies showed that HPA axis is activated by new or unpredictable situations (Attia *et al.*, 2014; Lupien *et al.*, 2007). In this study, the stress induced exhibit significant increase in the level of serum Cortisol and the percent change chart in showed 46.99% increased serum Cortisol level as compared to group I (Figure 5). Whereas, withdrawal of stress meant for remaining 21 days has partially or gradual recovery this change by reducing the Cortisol level. Statistically significant decrease in serum cortisol after

withdrawal from the stress in group III rats may be due to reduction of stress response via HPA axis without involvement of internal tissues.



**Figure 5:** Effect of restrained stress (6 hr/day for 42 days) induced alteration on serum Cortisol level in male albino rats. Values are mean  $\pm$  SEM (n=6).  $P \leq 0.05$  (asterisks) is considered to indicate a significant difference between experimental and controls.

### CONCLUSION

The study therefore concluded that exposure to restrained stress resulted alter the levels of serum cortisol and hematological parameters, Gravimetry and organ weights in male albino rats. Withdrawal of stress designed meant for remaining 21 days may probably neutralize restrained stress induced damage that lead to oxidant antioxidant balance and alter hypothalamic-pituitary-adrenal (HPA) axis. However, the degrees of changes depend on the stress duration. Further research needs to be done with stress duration and recovery period to reveal the possible biochemical mechanism and other tissue damage by restrained stress.

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