Adaptogenic potential of Oxitard in experimental chronic stress and chronic unpredictable stress induced dysfunctional homeostasis in rodents

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1. Introduction

Now-a-days, stress has turned into an essential part of human life. Furthermore creatures are continuously exposed to stressful events which lead to various physiological changes. Stressful stimuli are all around archived to trigger the central monoaminergic systems and hypothalamic–pituitary–adrenal (HPA) axis [1]. Hypothalamic paraventricular nucleus surges the release of corticotrophin-releasing hormone upon activation of the HPA axis, which leads to the secretion of adrenocorticotropic from the anterior pituitary, and ultimately adrenal cortex discharges glucocorticoids [2,3]. Stress, a non-specific response of the body characterized as physical and psychological alterations that disturb the homeostasis and the balance of the organism resulting in various neuronal, endocrine and visceral dysfunction [4,5]. Physical stressor like loud noise, big crowds and cluttered surrounding causes stress. According to previous reports, glucocorticoids released by CS disrupt the useful homeostasis of the body and are also associated in the etiopathogenesis of a diversity of disease conditions like coronary heart disease, hypertension, diabetes, gastric ulcers, mental depression, immunosuppression and memory loss [6–9]. No such medicine is mentioned in current pharmacopoeia which could be utilized as a treatment for stress. Nevertheless, a few plants in folklore medication such as Bacopa monniera, Panax ginseng, Emblica officinalis, Withania somnifera,
2.2. Oxitard

1088/PO/Re/S/2007/CPCSEA). Dark cycle. The experimental protocol was duly approved by the ethical committee (Kisan Feeds Ltd., Chandigarh, India) and having free access to water and libitum.

2. Materials and methods

2.1. Animals

2.2. Oxitard is one more herbal antioxidant formulation comprising the extracts of *W. somnifera*, *Mangifera indica*, *Glycerrhiza glabra*, *Daucus carota*, *Vitis vinifera*, powders of *Syzygium aromaticum*, *Yashada bhasma* and *E. officinalis*; and oils of *Triticum sativum* [18]. Previous reports indicate the beneficial role of Oxitard in oral submucous fibrosis [19]. According to general hypothesis, intelligence (I) energy (E), and organization (O), the three fundamental elements control the strength of an individual [20], and adaptogens adjust these battleaxes of healthiness by providing energy, informational and organizational aid to cell arrangements establishing the body [21]. Non-ideal prescriptions perform by improving maybe a couple components, but concurrently destroy the residual one(s), leading to adverse effects [22]. Nevertheless, adaptogens activate bidirectional alteration by triggering and re-establishing the balance of all three factors in an ideal way [22,23]. Animals are exposed to the similar kind of stressor for diverse phases to induce CS experimentally. Previous reports state that animals have a tendency to adjust to exposure to a similar kind of stressor in CS. To overcome this, the animals are exposed to flexible stressors of diverse amount and time in the form of CUS [24,25]. Additionally, CUS model shows high level of stress related outcomes as compared to CS. Taking into account these findings, in the present study the two models (i.e., CS and CUS) were utilized to assess the adaptogenic effects of Oxitard (a polyherbal formulation), as there are no reports found about the adaptogenic potential of Oxitard till date. The current study was therefore designed to explore the adaptogenic potential of Oxitard in CS and CUS-induced dysfunctional homeostasis.

2.2. Oxitard

Composition: Each Oxitard capsule contains extracts of *W. somnifera* (Solanaceae, 71 mg), *G. glabra L.* (Papilionaceae, rhizome, 29 mg), *Mangifera indica L.* (Anacardiaceae, bark, 94 mg), *V. vinifera L.* (Vitaceae, fruit, 12 mg), *D. carota L.* Daucus vulgaris (*Umbelliferae*, root, 47 mg), and powders of *S. aromaticum* L. Merr. and L.M. Perry (*Myrtaceae*, flower bud, 29 mg), *E. officinalis* L. (*Euphorbiaceae*, fruit, 141 mg), Yashada bhasma (2.5 mg) and oil of *T. sativum* (Poaceae, 6.5 mg).

2.3. Chronic stress (CS)

CS was induced according to the method devised by Kvetnansky and Mikulai [26]. Rats were immobilized in inclined position by fixing each of the four limbs on the immobilization board with sticky tape. Animals were immobilized in this position for 2 h daily for 10 days to induce CS.

2.4. Chronic unpredictable stress (CUS)

CUS was induced in the mice according to a method described by Ortiz et al. [27] which involved acquaintance to numerous stressors in adjustable schedules (Table 1).

2.5. Treatment

Albino Wistar rats (n = 30) were divided into five groups. CS was induced in all the groups excluding the control group (0.1% carboxy methyl cellulose (CMC)] after treatment with the standard drug and Oxitard. Buspirone (10 mg/kg) and Oxitard (200 and 400 mg/kg) were administered orally. All the drug solutions were prepared in 0.1% CMC. Stress was induced by CS 30 min after administration of each drug. Dose of Oxitard was decided after dose deciding pilot study (data not shown here).

Animals (Swiss albino mice) induced with CUS were randomly assigned to 4 groups [vehicle, buspirone (10 mg/kg) and Oxitard (200 and 400 mg/kg); n = 6 for each group] excluding control group. CUS was induced in all the groups excluding the control group (0.1% carboxy methyl cellulose (CMC)] after treatment with the standard drug and the Oxitard. Buspirone (10 mg/kg) and Oxitard (200 and 400 mg/kg) were administered orally. All the drug solutions were prepared in 0.1% CMC. Stress was induced by CUS 30 min after administration of each drug.

2.6. Hematological parameters

Following anesthesia, blood was collected by retro-orbital plexus to estimate hematological parameters viz. RBC and WBC counts [28].

2.7. Neurochemical analysis

Following behavioral studies, animals were sacrificed; skull cut open and the whole brain was dissected out and stored at –80 °C for further procedure. Homogenization buffer (10 ml/g of brain tissue) with the subsequent composition (12.5 mM sodium phosphate buffer pH 7.0 and 400 mM NaCl) was used to homogenize the brains using glass Teflon homogenizer. After homogenization, the homogenates were centrifuged at 1000 × g for 10 min at 4 °C. The supernatant was collected and utilized for the enzyme assay. Reduced glutathione (GSH) assay [29], thiobarbituric acid reactive substances (TBARS) assay [30] and catalase assay [31] were performed for the measurement of GSH and MDA activities respectively.

2.8. Reduced glutathione (GSH)

GSH was analyzed in the brain homogenate. In brief, equal volumes of 20% trichloroacetic acid (TCA) and tissue homogenate were mixed in equal parts and added to 20% trichloroacetic acid (TCA) and tissue homogenate.

| Day                      | Stress type and schedule                                  |
|--------------------------|----------------------------------------------------------|
| 1                        | 1900 h (earlier night), kept on moist sawdust, overnight; 1000 h restriction, 60 min |
| 2                        | 1500 h, cold (4 °C) segregation, 60 min; 1900 h, kept on lights, overnight |
| 3                        | 1200 h, kept on dark, 180 min; 1500 h, stress induced by swim, 4 min |
| 4                        | 0700 h, kept on moist sawdust, all day; 1900 h, kept deprived of food/water, overnight |
| 5                        | 1300 h, stress induced by swim, 3 min; 1900 h, sequestration housing, overnight |
| 6                        | 1400 h, isolated at cold (4 °C), 15 min; 1900 h, kept in dark, 120 min |
| 7                        | 1900 h, kept on moist sawdust in dark, overnight |
| 8                        | 1900 h, isolated with deprived of food/water, overnight |
| 9                        | 1600 h, restriction, 60 min; 1900 h, kept in lights, overnight |
| 10                       | 0900 h, stress induced by swim, 4 min; 1000 h, restriction, 60 min |

Table 1

The procedure used in CUS.
2.9. Lipid peroxidation potential (LPO)

LPO in brain was measured by estimating MDA levels. In brief, 2.0 ml of the tissue homogenate (supernatant) was mixed with 2.0 ml of freshly prepared 10% v/v TCA and the resulting mixture was kept in ice bath for 15 min. Later, centrifugation was done to separate the precipitate. 2.0 ml of obtained clear supernatant solution was mixed with 2.0 ml of freshly prepared thiobarbituric acid (TBA) (0.67% v/v). The resulting solution was heated in boiling water bath for 10 min and then instantaneously cooled on ice bath for 5 min. The colour so developed was measured at 532 nm against the reagent blank. Standard MDA (0–23 nM concentrations) were used to obtain the standard graph. Values were expressed as nM of MDA/mg of protein.

2.10. Catalase activity

Catalase activity in the brain was measured. Briefly, 2.0 ml of the homogenate was taken and 1.0 ml of hydrogen peroxide (30 mM) was added in the sample to initiate the reaction. The blank was set by mixing 2.0 ml of the diluted sample (similar dilution) with 1.0 ml of phosphate buffer (50 mM; pH 7.0). The reduction in absorbance was measured at 240 nm. Catalase level was expressed as units/mg of protein.

2.11. Organ weight

The weight of organs (liver, spleen, adrenal gland and testes) after washing with ethanol was recorded per 100 g body weight.

2.12. Ulcer index

The stomach was separated out and opened beside the greater curvature by applying cut for evaluating the occurrence of ulcer. The ulcer index was scored according to the technique described by Takagi and Okabe [32]. The technique can be used to assess the ulcer index as well as the severity of gastric lesions:

0 = no lesion
1 = mucosal edema and petechiae
2 = one to five small lesions (1–2 mm)
3 = above five small lesions or one intermediate lesion (3–4 mm)
4 = two to more intermediate lesions or one gross lesion (>4 mm)
5 = perforated ulcers

The ulcer index is given by the following equation:

\[
\text{Ulcer Index (UI)} = \frac{\text{Total ulcer score}}{\text{Number of animals ulcerated}}
\]

2.13. Statistical analysis

GraphPad InStat software (version 5.00, San Diego, CA) was used for data analysis. All data are expressed as mean ± SD. The mean significant difference in the experimental groups was analyzed using one way ANOVA followed by Bonferroni test. Values of \( p < 0.05 \) were judged statistically significant.

3. Results

3.1. Effect of Oxitard on oxidative stress markers

CS and CUS produced a significant decrease in GSH and CAT levels and increase in MDA levels as compared to the control group. Oxitard (200 mg/kg p.o.), significantly improved the oxidative parameters in the CS and CUS models. To evaluate whether Oxitard exerts antioxidative effect, brain tissues of rats treated with Oxitard were subjected to colorimetric estimation to establish its antioxidative properties in the brain. The results showed that CS and CUS significantly \( (p < 0.001) \) increased the brain MDA levels (11.10 ± 1.09 and 5.627 ± 0.142 nM/mg protein) compared to the control groups (5.397 ± 0.602 and 3.320 ± 0.195 nM/mg protein). Treatment with buspirone (10 mg/kg, p.o.) and Oxitard (200 mg/kg, p.o.) significantly decreased brain MDA levels (Table 2; Figs. 1a and 2a) [Buspirone (6.363 ± 0.578 and 3.723 ± 0.127) and Oxitard (7.250 ± 0.547 and 4.217 ± 0.189) nM/mg protein] compared to the corresponding CS and CUS groups (Table 2; Figs. 1a and 2a). Further, CS and CUS significantly \( (p < 0.001) \) decreased the brain GSH level (1.607 ± 0.051 and 1.510 ± 0.096 μg/mg protein) compared to the control groups (2.953 ± 0.055 and 2.487 ± 0.081 μg/mg proteins). Treatment with buspirone (10 mg/kg, p.o.) and Oxitard (200 mg/kg, p.o.) significantly increased brain GSH levels (Table 2; Figs. 1b and 2b) [Buspirone (2.813 ± 0.051 and 2.403 ± 0.112), and Oxitard (2.287 ± 0.065 and 2.303 ± 0.111) μg/mg protein] compared to the corresponding CS and CUS groups (Table 2; Figs. 1b and 2b).

Table 2: Effect of Oxitard on oxidative stress and hematological parameters in control and CS and CUS-induced stress in rodents.

| Groups          | Oxidative stress parameters | Hematological parameters |
|-----------------|-----------------------------|--------------------------|
|                 | MDA level (nM/mg protein)   | GSH level (μg/mg protein) | CAT level (U/mg protein) | RBC (× 10^6/μl) | WBC (× 10^3/μl) |
| Control in CS   | 5.397 ± 0.602               | 2.953 ± 0.055            | 3.970 ± 0.320             | 5.36 ± 0.29     | 4.20 ± 0.22     |
| Control in CUS  | 3.320 ± 0.195               | 2.487 ± 0.081            | 1.340 ± 0.087             | —                | —               |
| CS              | 11.10 ± 1.09***             | 1.607 ± 0.051***         | 2.467 ± 0.208***          | 7.71 ± 0.42***  | 6.47 ± 0.42***  |
| CUS             | 5.627 ± 0.142**             | 1.510 ± 0.096***         | 0.283 ± 0.085***          | —                | —               |
| Buspirone + CS  | 6.363 ± 0.578***           | 2.813 ± 0.051***         | 3.720 ± 0.154***          | 5.51 ± 0.33***  | 4.30 ± 0.26***  |
| Buspirone + CUS | 3.723 ± 0.127***           | 2.403 ± 0.112***         | 1.200 ± 0.046**           | —                | —               |
| Oxitard (200 mg/kg) + CS | 7.250 ± 0.547*** | 2.287 ± 0.065*** | 3.573 ± 0.097*** | 5.18 ± 0.21*** | 4.28 ± 0.29***  |
| Oxitard (200 mg/kg) + CUS | 4.217 ± 0.189**       | 2.303 ± 0.11**            | 1.153 ± 0.045             | —                | —               |
| Oxitard (400 mg/kg) + CS | 8.60 ± 0.957***       | 2.02 ± 0.089**            | 3.133 ± 0.10**            | 6.32 ± 0.25**   | 4.76 ± 0.21**   |
| Oxitard (400 mg/kg) + CUS | 4.32 ± 0.126***       | 1.62 ± 0.08**             | 0.820 ± 0.061**           | —                | —               |

Values are mean ± SD (n = 6); CS—Chronic stress, CUS—Chronic unpredictable stress. Significant values were compared with ***p < 0.001 vs CS/CUS groups, **p < 0.01 vs CS, ***p < 0.001 vs CS and CUS groups & ###p < 0.001 vs control groups. Hematological parameters were not evaluated in CUS model.
Fig. 1. Effect of Oxitard on *ex vivo* antioxidant properties in CS model. Values are expressed as mean ± SD. (n = 6) Significant values were compared with ***p < 0.001 vs CS group, **p < 0.01 vs CS group & ###p < 0.001 vs control group.

Fig. 2. Effect of Oxitard on *ex vivo* antioxidant properties in CUS model. Values are expressed as mean ± SD. (n = 6) Significant values were compared with ***p < 0.001 vs CUS group, **p < 0.01 vs CUS group & ###p < 0.001 vs control group.

Fig. 3. Effect of Oxitard on hematological parameters in CS induced stress in rats. Values are expressed as mean ± SD. (n = 6) Significant values were compared with ***p < 0.001 vs CS group, **p < 0.01 vs CS group & ###p < 0.001 vs control group.
addition, CS and CUS significantly (p < 0.001) decreased the brain CAT level (2.467 ± 0.051 and 2.083 ± 0.085 U/mg protein) as compared to the control groups (3.970 ± 0.32 and 1.340 ± 0.087 U/mg proteins). Treatment with buspirone (10 mg/kg, p.o.) and Oxitard (200 mg/kg, p.o.) significantly increased brain CAT levels (Table 2; Figs. 1c and 2c) [Buspirone (3.720 ± 0.154 and 1.20 ± 0.045), and Oxitard (3.573 ± 0.097 and 1.153 ± 0.045) U/mg protein] compared to the corresponding CS and CUS groups (Table 2; Figs. 1c and 2c). Oxitard (400 mg/kg, p.o.) showed less antioxidant activity as compared to Oxitard (200 mg/kg, p.o.). This may be due to saturated dose of Oxitard.

### 3.2. Effect of Oxitard on hematological parameters

Exposure to CS elevated the RBC and WBC count (Table 2; Fig. 3a and b). Treatment with Oxitard (200 and 400 mg/kg; p.o.) showed significant decline in RBC and WBC count as compared to stressed animals (Table 2; Fig. 3a and 3b).

### 3.3. Effect of Oxitard on organ weight

The weights of the organs like liver and adrenal gland were increased, while the weight of spleen and testis was reduced in

### Table 3

Effect of Oxitard on organ weight and ulcer index in control, CS and CUS-induced-stress in rodents.

| Groups               | Liver (gm/100 gm body weight) | Spleen (gm/100 gm body weight) | Adrenal (gm/100 gm body weight) | Testis (gm/100 gm body weight) | Ulcer Index |
|----------------------|-------------------------------|--------------------------------|---------------------------------|--------------------------------|-------------|
| Control in CS        | 4.943 ± 0.04                  | 0.80 ± 0.002                   | 0.48 ± 0.004                    | 2.57 ± 0.02                    | 0           |
| Control in CUS       | 0.847 ± 0.06                  | 0.690 ± 0.054                  | 0.107 ± 0.07                    | 0.197 ± 0.002                  | 0           |
| CS                   | 8.83 ± 0.5***                 | 0.32 ± 0.004***                | 0.85 ± 0.008***                 | 0.109 ± 0.012***               | 14 ± 0.64***|
| CUS                  | 1.29 ± 0.6***                 | 0.349 ± 0.05***                | 0.228 ± 0.006***                | 0.081 ± 0.007***               | 13 ± 0.64***|
| Buspirone + CS       | 6.09 ± 0.1***                 | 0.50 ± 0.006***                | 0.56 ± 0.006***                 | 0.973 ± 0.015***               | 8 ± 0.62*** |
| Buspirone + CUS      | 1.06 ± 0.06***                | 0.576 ± 0.02***                | 0.140 ± 0.005***                | 0.140 ± 0.006***               | 7 ± 0.65*** |
| Oxitard (200 mg/kg) + CS | 6.44 ± 0.03***               | 0.64 ± 0.007***                | 0.58 ± 0.004***                 | 0.670 ± 0.007***               | 8 ± 0.64*** |
| Oxitard (200 mg/kg) + CUS | 1.02 ± 0.03***               | 0.542 ± 0.04***                | 0.155 ± 0.005***                | 0.146 ± 0.005***               | 8 ± 0.55*** |
| Oxitard (400 mg/kg) + CS | 7.18 ± 0.04**                | 0.51 ± 0.003**                 | 0.63 ± 0.005**                  | 0.653 ± 0.021**                | 12 ± 0.77** |
| Oxitard (400 mg/kg) + CUS | 1.11 ± 0.06**                | 0.461 ± 0.04**                 | 0.175 ± 0.004**                 | 0.122 ± 0.004**                | 11 ± 1.30** |

Values are mean ± SD (n = 6). CS= Chronic stress, CUS- Chronic unpredictable stress. Significant values were compared with ***p < 0.001 vs CS/CUS groups, **p < 0.01 vs CS/CUS groups & ###p < 0.001 vs control groups.

![Fig. 4. Effect of Oxitard on organ weight in CS induced-stress rats. Values are expressed as mean ± SD. (n = 6) Significant values were compared with ***p < 0.001 vs CS group, **p < 0.01 vs CS group & ###p < 0.001 vs control group.](image-url)
stressed animals (Table 3; Figs. 4a–d and 5a–d). Treatment with Oxitard (200 mg/kg and 400 mg/kg; p.o.) significantly reduced the weight of the liver, and adrenal gland, and significantly increased the weight of the spleen and testis as compared to stressed animals (Figs. 4a–d and 5a–d).

3.4. Effect of Oxitard on pathological changes

CS and CUS produced significant increase in ulcer index as compared to the control groups (Table 3; Fig. 6a and b). Treatment with Oxitard (200 and 400 mg/kg p.o.) significantly attenuated the stress-induced rise in ulcer index (Table 3; Fig. 6a and b).

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**Fig. 5. Effect of Oxitard on organ weight in CUS induced-stress rats**
Values are expressed as mean ± SD (n = 6) Significant values were compared with ***p < 0.001 vs CUS group, **p < 0.01 vs CUS group & ###p < 0.001 vs control group.

**Fig. 6. Effect of Oxitard on ulcer index CS and CUS induced stress in rats**
Values are expressed as mean ± SD (n = 6). Significant values were compared with ***p < 0.001 vs CS, CUS group, **p < 0.01 vs CS, CUS group & ###p < 0.001 vs control group.
4. Discussion

In the present study, CS and CUS brought about noteworthy rise of MDA (a marker of lipid peroxidation) and decrease in the levels of GSH (an endogenous anti-oxidant). According to previous reports, free radicals have been produced by stress [33], which further increase corticosterone secretion through hyper-actuation of the HPA axis [34]. On the other hand, HPA over-stimulation brought about by free radicals elevates corticosterone secretion by disrupting hippocampal neurons, which sustain the homeostasis of the HPA axis by negative feedback system [35]. Previous study signifies the key role of free radicals in stress-induced related pathological effects and biochemical imbalance [36]. Additionally, stress reduces the GSH level and prompts to elevated ROS levels in rodent tissues [37]. Strong evidence suggests that the GSH system facilitates an enzymatic antioxidant defense system against hydrogen peroxide (H2O2) [4,38]. Furthermore, an immobilization stress leads to lipid peroxidation by overproduction of free radicals, mainly in cell membranes [39]. Previous studies also showed a significant reduction of CAT level in stressed animals [40]. In the present study, pre-treatment with Oxitard improvised CS and CUS-associated oxidative stress parameters in terms of elevation of GSH, CAT and decrease in MDA levels, signifying that adaptogenic potential of Oxitard might be contributed by its free radical scavenging property.

Increased secretion of stress hormones, which are identified to increase the mRNA levels and metabolic activities in the hepatic cells, could be the reason behind increased weight of liver during stress. Furthermore, adrenal hyperplasia and hypertrophy is known to be caused by powerful stimulation of the adrenal glands throughout delayed stress conditions [41,42]. Stress induced adrenomedullary response leads to hyperactivity of adrenals in stressed animals which increases the production of corticotrophic hormone and ultimately results in increased weight of adrenals [43,44]. The result indicates that the decreased weight of liver and adrenal glands following pre-treatment with Oxitard might be due to the reversal of stress induced adrenomedullary response and consequently decreased production of corticotrophic hormone. Decreased weight of spleen during stress is due to its contraction which releases more amount of blood (RBC) into circulation [45]. Increased weight of spleen following pre-treatment with Oxitard might be due to the inhibition of recruitment of lymphocytes to blood from spleen. The weight of testis decreases because there is destruction of spermatogenesis and reduction of testosterone levels during stress [46]. Pre-treatment with Oxitard significantly increased weight of testis indicating its anti-stress property. Stress also causes alteration in hematological parameters like increase in total and differential WBC counts [43,47]. Significant reduction of RBC and WBC counts as compared to stressed rats following pre-treatment of Oxitard authenticated the adaptogenic potential of Oxitard. Since gastric mucosal blood flow plays an essential role in defensive mechanism of gastric mucosa, its disruption results in beginning of ulcers [48]. In our study, Oxitard treatment significantly diminished the occurrences and severities of ulcers caused by CS and CUS. This further validated the adaptogenic potential of Oxitard. Thus Oxitard could be a better candidate in the treatment of stress.

5. Conclusion

Oxitard showed antioxidant property and prevented the stress-induced alterations in body weight, organ weight, ulcer index and hematological parameters, indicating its protective effect against stress. Oxitard-facilitated antioxidant actions might be the reason behind its defensive effects in stress-induced dysfunctional homeostasis. However, further experiments are required to discover the mechanism behind the adaptogenic potential of Oxitard utilizing different animal models of stress.

Conflict of interest

The authors declare that there is no conflict of interest.

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