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Effect of *Pueraria tuberosa* on cold immobilization stress induced changes in plasma corticosterone and brain monoamines in rats

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Abstract

The study was carried out to establish the anti-stress effect of *Pueraria tuberosa* in relation to the levels of plasma corticosterone and norepinephrine (NE), dopamine (DA) and 5-hydroxytryptamine (5-HT) in whole brain and hypothalamus and compared with *Withania somnifera* (adaptogen). Adult male Wistar rats pretreated with 70% hydroethanolic extract of *P. tuberosa* tuber root (PTE) at the doses of 50, 100, 200 and 400 mg/kg, for 5 consecutive days. *W. somnifera* rhizome extract (WSE) at 100 mg/kg was used as reference drug. The rats were subjected to cold immobilization stress (IS) for 2 h. Thereafter, the animals were sacrificed and ulcer formation in gastric mucosa was noted. Weights of adrenals and spleens were also taken. Further, plasma corticosterone levels were estimated by spectrophotoflurometrically. Simultaneously, brain monoamines like, NE, DA and 5-HT were determined in whole brain and hypothalamus region HPLC electrochemical detector. IS for 2 h damaged the gastric mucosal layers, enhanced plasma corticosterone levels and increase adrenal glands and spleen weight. Further, IS elevated NE, DA and 5-HT levels both in whole brain and hypothalamus. PTE significantly protected the gastric mucosa, lowered corticosterone level in blood and negated the hypertrophy of adrenals and spleen. PTE (200 and 400 mg/kg) and WSE (100 mg/kg) significantly decreased IS elevated NE, DA and 5-HT both in whole brain and hypothalamus. These data suggest that anti-stress effect of *Pueraria tuberosa* may be modulated by monoaminergic system.

Key words: Stress, corticosterone, monoamines, HPLC, *Pueraria tuberosa*, *Withania somnifera*.

1. Introduction

It has consistently been shown that individuals experiencing stress have impaired physical and mental functioning and increased use of healthcare services [1]. The hypothalamic-pituitary-adrenal (HPA) axis is activated to prepare the body for adaptation during stressful conditions [2,3]. Corticotrophin releasing hormone (CRH) is the major physiological

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regulator of the secretion of adrenocorticotrophic hormone (ACTH) and is the predominant chemical messenger by which the CNS controls the activity of the HPA axis and is therefore, ultimately responsible for orchestrating the endocrine response to stress [4]. Central neurotransmitters are important mediators implicated in physiological and behavioral responses to stress [5]. Among these central neurotransmitters, norepienhrine (NE), dopamine (DA) and 5-hydroxytryptamine (5-HT) are studied extensively and their role is well established [6,7,8]. Dysfunction of these monoamines due to prolonged stressful conditions has been associated with a wide range of central and peripheral disorders like depression, anxiety, obsessive compulsive disorder, eating and sleeping disorders, hyperglycemia, and decrease immune response [9,10,11].

The drugs of plant origin are gaining importance and are being investigated for remedies of a number of disorders including stress. Since the introduction of adaptogen concept several plants have been investigated, which were used earlier as tonics due to their adaptogenic and rejuvenating properties in traditional medicine [12]. *Pueraria tuberosa* (Roxb. ex Willd. DC) (family: Fabaceae) is a perennial climber found throughout the Indian subcontinent in wet and damp areas [13]. The tuberous roots of this plant are used in Indian traditional medicine in general debility, nervous breakdown, spermatorrhoea, burning sensation, heart diseases, intrinsic hemorrhage, tuberculosis etc [14]. The chemical constituents have been identified as puerarin, diadzein, daidzin, β-sitosterol and sigmasterols [15,16]. Puerarin (isoflavones) has been reported to possess anti-fertility [17], anti-hypertensive [18], anti-hyperglycemic [19], nootropic [20] and neuroprotective effects [21,22]. In our previous studies, we identified the potential actions of *P. tuberosa* as an antioxidant that normalizes the hypoxic stress induced elevation of lipid peroxides and catalase [23] and swim stress induced depression; however the detailed mechanisms for its adaptogenic activity are yet to be explored. Hence, it is necessary to explore the effect of *P. tuberosa* on altered HPA axis activity and levels of monoamines in brain during stressful conditions. The aim of the present study is to establish the anti-stress effect of *Pueraria tuberosa* in relation to the levels of plasma corticosterone and norepinephrine, dopamine and 5-hydroxytryptamine in whole brain and hypothalamus and compared with *Withania somnifera*, a known adaptogen.

2. Materials and Methods

2.1 Animals

Male Wistar rats (120-150 g) adult in age were obtained from Central Drug Laboratories, Kolkata, Govt. of India and maintained according to recommended guidelines for the care and use of the animals (NIH publication No. 85-23, revised 1985). The institutional animal ethics committee approved all the experimental protocols. Animals were allowed to take food pellets and water *ad libitum*.

2.2 Drugs and reagents

3,4-Dihydroxybenzylamine hydrobromide, dopamine hydrochloride, norepinephrine hydrochloride,5-hydroxytryptamine hydrochloride, heptasulphonic acid, hydroxycorticosterone, were obtained from Sigma Chemicals (St. Louis, , USA). HPLC grade citric acid, sodium citrate, EDTA, glacial acetic acid, tetrahydrofuran, ethanol, methanol, dichloromethane, hydrochloric acid, sulphuric acid. were purchased from E.Merck (India).

2.3 Preparation of extract

Tuberous roots of *P. tuberosa*and rhizomes of

*W. somnifera*were collected from the herbal garden of medicinal plants near Kolkata, identified by the experts of Botanical Survey of India, West Bengal and the voucher specimens (DB/NSU/01 and DB/NSU/02) kept in the departmental herbarium. The shade dried tuberous roots of *P. tuberosa* and rhizomes of *W. somnifera* were powdered and extracted with 70% ethanol in a soxhlet apparatus for 24h. Thereafter, the extract was concentrated under reduced pressure below 40ºC by rotary evaporator (Hahn Shin Science Co., Korea) and lyophilized (Vertis, Italy) under freezing condition. [24] The yield of the ethanolic extract of *P. tuberosa* (PTE) was 14-18% (w/w) and *Withania somnifera* (WSE) was 8-12% (w/w). HPTLC analyses reported 0.62% puerarin on PTE.

2.4 Treatment schedule

Male Wistar rats (120-150 g) were divided into seven groups, consisting of eight animals in each. Group I served as normal, group II as positive control, while group III-VII as test drug treated. PTE was given to rats of groups III-VI at the doses of 50, 100, 200 and 400 mg/kg respectively, while group VII treated with WSE (100 mg/kg) as reference drug. Group II (stress control) received only equivalent volume of distilled water (0.5 ml/100 g) orally. All the drugs were given orally once/day for five consecutive days. The last dose was given 1 h before study.

2.5 Cold immobilization stress

On day 5, all animals (except group I) were subjected to stress by forced cold immobilization [6,7]. The fore and hind limbs were tied separately and then together and were individually put in restraint chamber in a well ventilated refrigerator at 4°C for 2 h [25]. Thereafter, the rats were sacrificed by cerebral dislocation. Gastric ulcer formation was scored [25]. Adrenal glands and spleen weight were measured using high precision electronic balance [26]. Plasma corticosterone was estimated spectrophotoflurometrically (Hitachi, Japan) as described by Sur and Bhattacharyya [27].

2.6 HPLC analysis of DA, NE and 5-HT in whole brain and hypothalamus

Levels of NE, DA and 5-HT were using HPLC electrochemical detector [28,29]. In brief, the whole brain and hypothalamus were collected and homogenized in 10 nM HCl. Homogenates were then centrifuged (Haureas, Germany) at 4ºC. Supernatant of volume 20ml was injected into a reverse phase HPLC-C18 column (3.9x150 mm, Novapack, USA) and via HPLC pump (Waters - 515 pump) coupled to a pulse electrochemical detector (Model 464, Waters Assoc. USA, Millennium software). The mobile phase consisted of citric acid, sodium citrate, EDTA, heptasulphonic acid, glacial acetic acid, tetrahydrofuran, methanol and water (pH 4.9). The flow rate of HPLC pump was set at 0.6 ml/ min and retention time was 25 min. The contents of monoamines were estimated against known standards and internal standards. The results were expressed as µg/g wet tissue.

2.7 Statistical analysis

The results were expressed as mean ± SEM. The statistical significance was determined by one-way ANOVA followed by Newman-Keul's multiple comparison tests. $P<0.05$ was considered to be statistically significant.

3. Results

3.1 Gastric ulcer score, spleen and adrenal gland weight

Forced cold immobilization (IS) damaged gastric mucosal layers resulting in ulcer formation, and significantly increase adrenal glands and spleen weight. Pretreatment with PTE protected gastric mucosa dose dependently

Table 1: Effects of *P. tuberosa* (PTE) and *W. somnifera* (WSE) on cold immobilization stress (IS) induced changes of gastric ulcers, adrenal and spleen weight and plasma corticosterone level (data represents mean ± SEM)

Groups n=10	Ulcer Score	Adrenal gland (mg/100g)	Spleen (mg/100g)	Plasma Corticosterone (µg/dl)
Control	-	18.05±1.06	0.315±0.04	30.15±1.20
IS	14.25±1.90	30.58±2.56(a)***	0.382±0.02(a)	64.05±2.18 (a)***
IS+PTE-50	12.80±1.06(b)	28.27±1.96(b)	0.365±0.07(b)	58.09±2.18(b)
IS+PTE-100	10.39±1.15(b)*	26.54±1.82(b)**	0.358±0.05(b)	52.75±2.45(b)**
IS+PTE-200	9.50±1.08(b)***	24.09±1.78(b)***	0.355±0.01(b)	46.97±2.38(b)***
IS+PTE-400	8.25±0.86(b)***	23.27±1.65(b)***	0.342±0.03	40.80±2.37(b)***
IS+WSE-100	6.50±0.58(b)***	20.68±1.15(b)***	0.328±0.02*	35.25±2.18(b)***

*P<0.05, **P<0.01 and ***P<0.001 and (a) and (b) means when compared to control and IS group correspondingly.

Table 2: Effects of *P. tuberosa* (PTE) and *W. somnifera* (WSE) on cold immobilization stress (IS) induced changes of neurotransmitters in brain and hypothalamus (data represents mean±SEM).

Groups n=10	Whole Brain (µg/g tissue)			Hypothalamus (µg/g tissue)		
	NE	DA	5-HT	NE	DA	5-HT
Control	0.32±0.02	2.45±0.09	1.35±0.02	3.45±0.08	2.05±0.04	4.82±0.16
IS	0.78±0.08(a)***	4.80±0.12(a)***	3.90±0.28(a)**	6.20±0.26(a)**	4.65±0.15(a)***	.25±0.14(a)***
IS+PTE-50	0.70±0.07(b)	4.38±0.18(b)	3.75±0.34(b)	5.87±0.39(b)	4.10±0.64(b)	8.75±0.42(b)
IS+PTE-100	0.69±0.04(b)	4.15±0.21(b)	3.14±0.45(b)	5.54±0.46(b)	3.95±0.36(b)	7.62±0.58(b)
IS+PTE-200	0.54±0.06(b)*	3.82±0.15(b)*	2.90±0.12(b)**	5.12±0.24(b)**	3.64±0.32(b)**	.95±0.66(b)***
IS+PTE-400	0.48±0.08(b)**	3.16±0.14(b)**	2.72±0.16(b)***	4.80±0.38(b)**	3.28±0.45(b)**	.54±0.45(b)***
IS+WSE-100	0.40±0.02(b)***	2.72±0.12(b)**	1.58±0.18(b)**	4.26±0.84(b)***	2.90±0.27(b)***	5.84±0.68(b)***

*P<0.05, **P<0.01 and ***P<0.001 and (a) and (b) means when compared to control and IS group correspondingly.

from IS induced ulcer formation and prevented its severity. PTE treatment also alleviated the hypertrophy of adrenal glands and spleen. Pretreatment with WSE (known adaptogen) supported these results (Table 1)

3.2 Plasma corticosterone

Plasma corticosterone level was changed by IS which corroborate stress induced HPA axis activation.Both PTE and WSE individually attenuated elevation of plasma corticosterone by IS (Table 1).

3.3 DA, NE and 5-HT in whole brain and hypothalamus

Exposure to IS significantly elevated the concentrations of NE, DA and 5-HT both in whole brain and also in hypothalamus. Pretreatment of PTE significantly reduced the elevations of all monoamines in whole brain as also in hypothalamus by IS. PTE at the doses of 200 mg/kg and 400 mg/kg, while WSE at the dose of 100 mg/kg significantly decreased IS elevated NE, DA and 5-HT both in whole brain and hypothalamus (Table 2).

4. Discussion

Of all the experimental approaches undertaken to precipitate the non-specific stress-syndrome in the experimental animals, the process of restraining or immobilizing the animals from moving has been one of the most popular methods [3]. Painful stimuli are not involved in immobilizing stress (IS) and it is more akin to physiological stress as it combines emotional stress (escape reaction) and physical stress (muscle work) resulting in both restricted motility and aggression [6]. Recently, we observed that IS damaged gastric mucosal layers, depleted adrenal ascorbic acid concentration, enhanced plasma corticosterone levels and increase adrenal glands and spleen weight [26]. The results of the present study showed that the rats pretreated with PTE and WSE were able to overcome a variety of stressful situations. IS in cold condition resulted in increased ulcer formation in rats [25]. PTE protected gastric mucosa dose dependently from IS induced ulcer formation and prevented its severity. Recent studies revealed various neuroprotective actions of puerarin (active constituent of *P. tuberosa*), such as protection of cerebral cortical neurons from damage by glutamine and N-methyl-D-aspartic acid and nootropic activity [20,21,22]. The diversity in plants and their ability to synthesize complex mixtures of structurally diverse compounds with multiple health benefits make them promising source for drug leads. Most of the reported biological activities and active constituents of *P. tuberosa* may be related to its antioxidant nature.

IS affects the steady state concentration and turnover of all established as well as putative neurotransmitters, along with hormonal changes, in the same way, as visualized by researcher to be happening, in case of one ideal non-specific stress [8,27]. The ascending 5-HTergic neurons from raphe nuclei innervate hypothalamic and limbic sites and have an overall role in the secretion of ACTH during stress [30,6]. In this study, IS significantly enhanced plasma corticosterone level, a marker of HPA axis activation. Prior treatment of PTE and WSE for 5 days negated the increment of plasma corticosterone and reduced the adrenals and spleen weight following IS. These findings clearly indicated that PTE and WSE have potential HPA axis mediatory actions, particularly during acute IS in rats. Previous studies indicated that PTE has antioxidant and free radical scavenging activity, which might be responsible for the protection of the brain cell during hypoxia [31].

Central neurotransmitters are important mediators implicated in physiological and behavioral responses to stress. Among these central neurotransmitters, NE, DA and 5-HT are studied extensively and their role is well established in various stress- mediated disorders [1,3]. In previous study, rats exposed to acute

restraint stress showed an initial short-lived increase of NE in medial prefrontal cortex and DA in nucleus accumbens [32]. Acute stress (immobilization) resulted in increase in the levels of DA and 5-HT in cortex was also documented [33]. Shah *et al* (2005) [34] reported immobilization stress induced elevation in levels of brain catecholamine. In the present study, immobilization in cold environment significantly enhanced NE, DA and 5-HT levels in brain and hypothalamus which is in corroboration with the previous reports. Present study indicated that acute stressful conditions activated monoaminergic system leading to an increase in the levels of NE, DA and 5-HT in cortical and hypothalamic regions of brain. 5-HTergic, NEergic and DAergic systems are closely related anatomically and functionally, and the changes of activity in one system might contribute to alterations in the regulation of the other [1,8]. PTE at the doses of 200 mg/kg and 400 mg/kg, while WSE at the dose of 100 mg/kg significantly decreased IS elevated NE, DA and 5-HT both in whole brain and hypothalamus. Result suggested that PTE normalized stress mediated transient deregulation of plasma corticosteroids and monoamine changes in brain which might be one of the reasons for its adaptogenic activity and mild anti-stress effects respectively.

Thus our study projects PTE as a possible therapeutic candidate against stress mediated disorders and provides evidence that the observed anti-stress effects are due to normalization of adversely affected central monoamines.

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