—Regular Articles—

Studies on Effect of Stress Preconditioning in Restrain Stress-induced Behavioral Alterations

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Stress preconditioning has been documented to confer on gastroprotective effects on stress-induced gastric ulcerations. However, the effects of prior exposure of stress preconditioning episodes on stress-induced behavioral changes have not been explored yet. Therefore the present study was designed to investigate the ameliorative effects of stress preconditioning in immobilization stress-induced behavioral alterations in rats. The rats were subjected to restrain stress by placing in restrainer (5.5 cm in diameter and 18 cm in length) for 3.5 h. Stress preconditioning was induced by subjecting the rats to two cycles of restraint and restrain-free periods of 15 min each. Furthermore, a similar type of stress preconditioning was induced using different time cycles of 30 and 45 min. The extent and severity of the stress-induced behavioral alterations were assessed using different behavioral tests such as hole-board test, social interaction test, open field test, and actophotometer. Restrain stress resulted in decrease in locomotor activity, frequency of head dips and rearing in hole board, line crossing and rearing in open field, and decreased following and increased avoidance in social interaction test. Stress preconditioning with two cycles of 15, 30 or 45 min respectively, did not attenuate stress-induced behavioral changes to any extent. It may be concluded that stress preconditioning does not seem to confer any protective effect in modulating restrain stress-induced behavioral alterations.

Key words——restrain stress; stress preconditioning; behavior; social interaction

INTRODUCTION

Stress has been described as a sum total of all the reactions of the body that disturb the normal physiological equilibrium and result in a state of threatened homeostasis. Stressful events trigger changes in different organ systems such as cardiovascular,[1] gastrointestinal,[2] and central nervous system.[3] Stress has been associated with post-traumatic stress disorders, major depression, schizophrenia, and neurodegenerative diseases.[1,3–5] Although stress is widespread, yet no specific medicine is available that can qualify as therapy for stress management. There has been a long quest for finding an effective way to enhance the body's resistance against stress and associated pathological changes.

Preconditioning is an endogenous protective mechanism activated by a mild insult that makes the tissue more resistant to subsequent insults of greater magnitude and intensity. The concept of preconditioning has been applied to render tissues such as heart,[6] brain,[7] kidney,[8] and liver[9] more resistant to ischemic insult by subjecting these tissues to shorter durations of ischemic insults. Our own laboratory has documented the protective effects of ischemic preconditioning in heart[10,11] and brain.[12,13] This concept has been expanded to include pharmacological preconditioning,[14,15] remote preconditioning,[16,17] and ischemic post-conditioning.[18] The concept of ischemic and pharmacological preconditioning has been extrapolated to investigate the ameliorative role of stress preconditioning on stress-induced pathological changes and there have been reports suggesting the gastroprotective effects of stress preconditioning on cold, alcohol, restrain, and water immersion-induced gastric lesions in rats.[2,19,20] However, the effects of prior exposure of stress preconditioning episodes on stress-induced behavioral changes have not been explored yet. Therefore the present study was designed to investigate the ameliorative effects of stress preconditioning in restrain stress-induced behavioral alterations in rats.

MATERIALS AND METHODS

Animals Albino Wistar rats (Punjabi Agriculture University, Ludhaina, Punjab, India) of female sex, weighing 150–200 g, were employed in the present study. The activity of pituitary-adrenal axis is sex-dependent and this activity is comparatively low in male rats. Furthermore, female rats show a high

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emotionality as compared with male rats, when exposed to chronic stress situations.[21] Therefore female rats were employed in the present study. The animals were fed on standard laboratory diet and water *ad libitum*. They were housed in the departmental animal house and exposed to natural cycles of light and dark. The experimental protocol was approved by the Institutional Animal Ethics Committee and care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Reg. No.-107/1999/CPCSEA).

Induction of Restrain Stress Restrain stress was induced by placing the rats individually in a semi-cylindrical, acrylic restrainer, 5.5 cm in diameter and 18 cm in length for 3.5 h.[22]

Stress Preconditioning Stress preconditioning was given immediately before subjecting the animals for restrain stress. Before subjecting the rats for sustained restrain stress, brief exposure of restrain stress was given to the animals in the form of two cycles of restraint and restrain-free period of variable duration *i.e.*, 15, 30, and 45 min. For stress preconditioning protocol with 15 min of restraint and restrain-free period, the rats were restrained for 15 min followed by 15 min of restrain-free period. The same cycle of restraint and restrain-free period was repeated immediately after completion of the first cycle. Similarly, for stress preconditioning protocol with 30 and 45 min, the rats were subjected to two cycles of restraint and restrain-free period for 30 and 45 min each, respectively.

Behavioral Measurements Immediately after completion of restrain stress protocol, the battery of behavioral tests was performed in animals with the sequence of actophotometer, hole board, open field, and social interaction test. There was a time gap of 5 min between the successive behavioral tests.

Actophotometer The locomotor activity has been used as an index of wakefulness (alertness) of mental activity and is assessed by actophotometer. The animals were placed in actophotometer for 5 min and their activity was assessed in terms of counts per 5 min.[22,23]

Hole Board Test The hole board test has been employed to assess the exploratory behavior of animals. The hole board consisted of a wooden, grey box measuring 68×68 cm. The walls were 40 cm high, and the box was raised 28 cm above the ground on a metal stand. Four holes (4 cm in diameter) were cut into the floor of the apparatus: each hole was 28 cm from a corner of the box along the diagonal from the corner to the centre. The floor of the box was marked out into four outer areas and one central area using black masking tape. The central area was delineated by four lines of tape each 20 cm from one of the walls, while the four outer areas were marked out by diagonal lines of tape running from the corners of the floor to the corners of the central square. The four holes were thus located at the corners of the central area. The apparatus was located in a small testing room with dimmed white lighting. The animals were assessed for 10 min during which the following behavioral patterns were recorded:[24]

* Head dips: The animal places its head into one of the holes, to a minimum depth such that the ears were level with the floor of the apparatus. The low levels of head dipping reflect high anxiety state level in animal.
* Rear: The animal is stationary on its back paws and raises its forepaws off the ground, extending its body vertically. The number of rearing represents exploration in novel surroundings.

Open Field Test The open field test has been employed to assess the spontaneous activity, general exploration, and ambulation of rodents.[25,26] The open field consisted of a wooden box 90.0×90.0×38.0 cm positioned in a dimly lit room. The walls were painted black, while the floor was painted white and was divided by 1 cm wide black lines into 25 squares 17.0×17.0 cm (16 peripheral squares and 9 central squares). The rats were placed in the centre of the open field. For the following 10 min, the number of line crossings and the time spent in the peripheral and central areas were recorded.

Social Interaction Test The social interaction test has been carried out according to the method described previously.[27,28] After performing open field test, the social interaction test was performed in the same box. During the 10-min test, following and avoidance of two animals to each other was assessed and expressed in seconds.

EXPERIMENTAL PROTOCOL

Group I (Normal Control) Rats were not subjected to any type of stressor and subsequently, the locomotor, exploratory and social interaction activi-

ties were noted in these normal rats.

Group II (Stress Control) Rats were subjected to restraint stress for 3.5 h and subsequently the different behavioral tests were employed as described in group I.

Group III (Stress Preconditioning with Two Cycles of 15 min) Rats were subjected to stress preconditioning with two cycles of restraint and restrain free period of 15 min each, respectively. Thereafter, rats were restrained for three and half hours, and subsequently the different behavioral parameters were assessed as described in group I.

Group IV (Stress Preconditioning with Two Cycles of 30 min) Rats were subjected to stress preconditioning with two cycles of restraint and restrain-free period of 30 min each. Thereafter, rats were restrained for 3.5 h and subsequently the different behavioral parameters were assessed as described in group I.

Group V (Stress Preconditioning with Two Cycles of 45 min) Rats were subjected to stress preconditioning with two cycles of restraint and restrain-free period of 45 min each. Thereafter, rats were restrained for 3.5 h and subsequently the different behavioral parameters were assessed as described in group I.

Statistical Analysis The results are expressed as mean±standard error of means (S.E.M.). The results were analyzed by one-way ANOVA followed by *post-hoc* analysis using Tukey's multiple comparison test. $p<0.05$ was considered statistically significant.

RESULTS

Effect of Restrain Stress and Stress Preconditioning on Locomotor Activity In restrain stress subjected rats, locomotor activity was decreased significantly as compared with normal control rats ($p<0.05$; F=25.09). Stress preconditioning with two cycles of 15, 30, and 45 min did not ameliorate restraint-induced decrease in locomotor activity. On the other hand, stress preconditioning protocols aggravated restrain stress induceddecrease in locomotor activity as compared with stress control group ($p<0.05$; F=25.09) (Fig. 1).

Effect of Restrain Stress and Stress Preconditioning on Head Dips and Rearing in Hole Board Test Head dips in the hole board test are considered as an index of curiosity or exploration and the frequency of rearing reflects the exploration of novel surroundings. In restrain subjected rats, the frequency of head dips ($p<0.01$; F=14.53) and rearing ($p<0.01$; F=15.26) decreased significantly as compared with the normal control group. Stress preconditioning with two cycles of 15, 30, and 45 min did not ameliorate restrain stress-induced decrease in head dips and frequency of rearing. On the contrary, stress preconditioning protocols further aggravated restrain stress-induced decrease in the frequency of head dips and rearing as compared with stress control group ($p<0.05$; F=14.53 and $p<0.05$; F=15.26) (Fig. 2 and 3).

Effect of Restrain Stress and Stress Preconditioning on Total Motor Activity (Line Crossings) and Rearing in Open Field Test Line crossings are taken as an indicator of motor activity and the frequency of

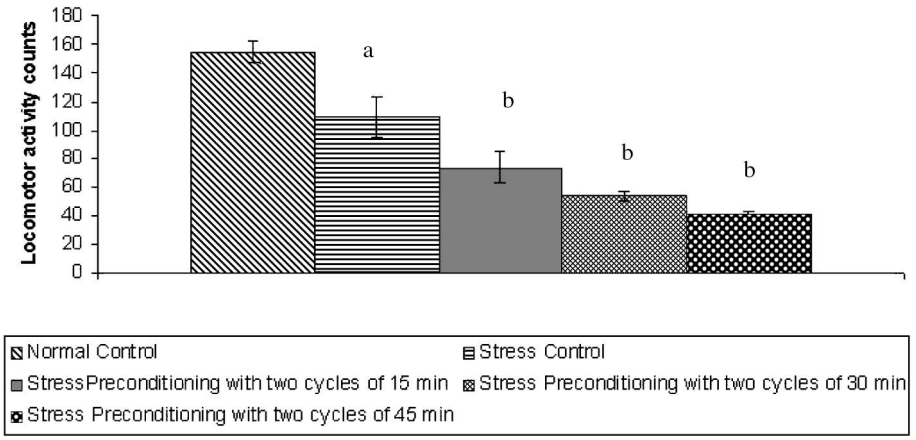

Fig. 1. Effect of Immobilization Stress and Stress Preconditioning of Variable Cycles on Locomotor Activity in Actophotometer
Values are expressed as mean±S.E.M. a=$p<0.05$ *versus* normal control, b=$p<0.05$ *versus* stress control.

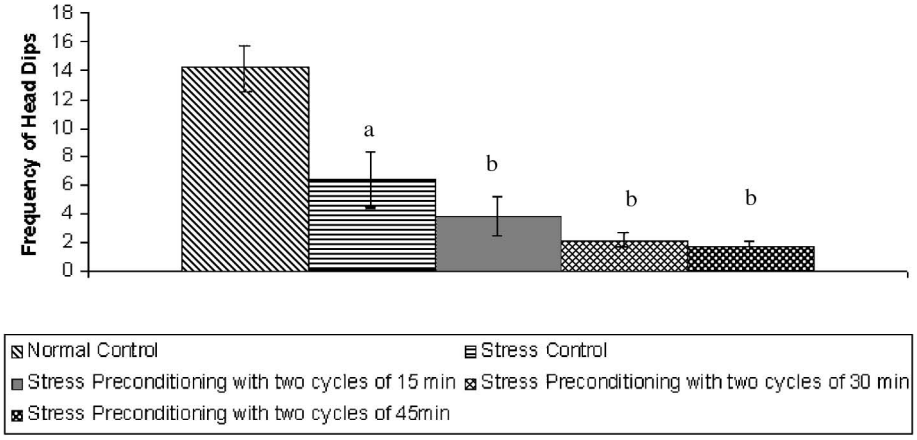

Fig. 2. Effect of Immobilization Stress and Stress Preconditioning of Variable Cycles on Frequency of Head Dips in the Hole Board Test

Values are expressed as mean±S.E.M. a=$p<0.05$ *versus* normal control, b=$p<0.05$ *versus* stress control.

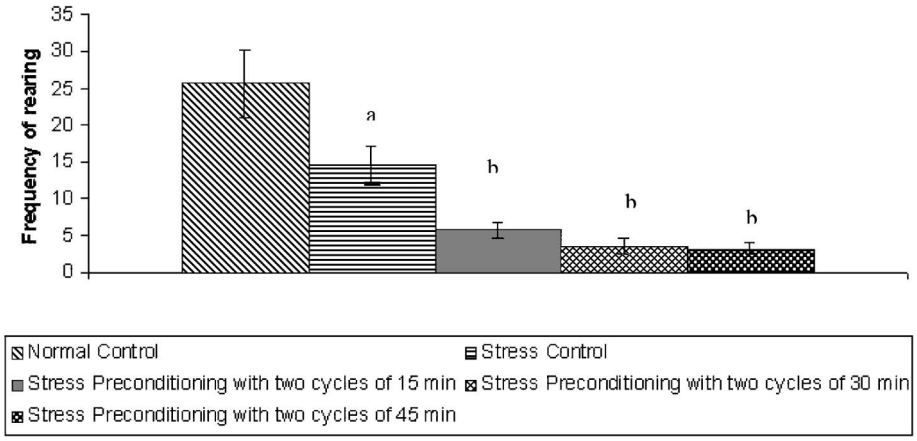

Fig. 3. Effect of Immobilization Stress and Stress Preconditioning of Variable Cycles on Frequency of Rearing in the Open Feld Test

Values are expressed as mean±S.E.M. a=$p<0.05$ *versus* normal control, b=$p<0.05$ *versus* stress control.

rearing reflects the exploration of novel surroundings. In restrain subjected rats, total line crossings ($p<0.01$; F=7.51) and rearing ($p<0.01$; F=22.08) decreased significantly as compared with the normal control group. Stress preconditioning with two cycles of 15, 30, and 45 min did not ameliorate restrain-induced decrease in the line crossings and rearing in open field. On the other hand, stress preconditioning aggravated restrain stress-induced decrease in total line crossings and frequency of rearing as compared with stress control group ($p<0.05$; F=7.51; $p<0.05$; F= 22.08) (Table 1).

Effect of Restrain Stress and Stress Preconditioning on Social and Non-Social Behavior in Social Interaction Test In restrain-subjected rats, non-social behavior (avoiding the partner) was predominantly shown as compared with the normal control group, which exhibited social behavior (following the partner) ($p<0.001$; F=1468.80). Stress preconditioning with two cycles of 15, 30, and 45 min did not ameliorate restrain stress-induced non-social behavior. Stress preconditioning protocols further aggravated non-social behavior; thus avoiding the partner ($p<0.05$; F=1468.80) (Table 1).

DISCUSSION

Exposure to stress stimuli induces various changes in the body including alteration in behavior, autonomic function, and secretion of multiple hormones including adrenocorticotropin hormone (ACTH) and corticosterone/cortisol.[29,30] Several different studies have reported the development of behavioral alterations such as decrease in locomotor activity, decrease in spontaneous activity, decrease in explora-

Table 1. Effect of Immobilization Stress and Stress Preconditioning of Variable Cycles on Line Crossings and Rearing in the Open Field Test; and Following and Avoidance (s) in the Social Interaction Test

Experimental Groups	Open Field Test		Social Interaction Test	
	Line Crossing	Rearing	Following (s)	Avoidance (s)
Normal Control	121.0±7.2	30.7±4.7	593.0±2.1	7.0±2.3
Stress Control	58.8±17.3[a]	15.7±1.1[a]	78.3±10.6[a]	521.7±10.6[a]
Stress Preconditioning with two cycles of 15 min	49.3±13.4[b]	8.0±1.1[b]	67.1±16.2[b]	532.9±16.2[b]
Stress Preconditioning with two cycles of 30 min	41.3±12.9[b]	5.2±1.3[b]	52.7±5.6[b]	547.3±5.6[b]
Stress preconditioning with two cycles of 45 min	37.7±9.2[b]	4.5±0.9[b]	49.0±8.5[b]	551.0±8.5[b]

Values are expressed as mean±S.E.M. a = $p<0.05$ *versus* normal control, b = $p<0.05$ *versus* stress control.

tory behavior, and decrease in social behavior as a consequence of stress. Furthermore, chronic exposure to stress has been well documented to induce a state of depression both in animal models[31,32] and humans.[1,3–5] In the present study also, restraint of female rats for 3.5 h resulted in induction of acute stress characterized by significant behavioral alterations including decreased locomotor activity, spontaneous activity, and orientational-investigating activity and altered social behavior. Restrain stress-induced stress has been one of the more commonly employed models for induction of acute stress in rats[23,33] and this type of physical stress is most useful for studying stress-induced neurodegeneration and post-traumatic disorders.[34,35]

Several different research groups have employed different time intervals of immobilization such as 1,[22,33] 2,[36] 2.5,[37] 3,[38] 4,[39] and 6 h[23,40] for the induction of variable degrees of acute stress. In the present study, rats were restrained for 3.5 h for inducing acute stress, since this time period was found to produce reproducible and optimum stress in rats during pilot studies.

Preconditioning is a phenomenon of reduction in the severity of tissue damage on application of insults of shorter durations prior to prolonged and severe insult. The concept of preconditioning has been widely exploited for preventing ischemia-reperfusion injury to organs such as heart,[6] brain,[7] kidney,[8] and liver[9] by subjecting these tissues to shorter durations of ischemic insults. Our own laboratory has documented protective effects of ischemic preconditioning in heart[10,11] and brain.[12,13] Moreover, pharmacological preconditioning,[14,15] remote preconditioning,[16,17] and ischemic post-conditioning[18] have been documented to produce tissue-protective effects. In the present investigation, stress as an insult was analogous to the ischemic insult and hence, accordingly short episodes of stress were employed before sustained restrain stress, analogous to protocols employed to investigate the protective effects of ischemic reconditioning.

In the present study, animals were subjected to stress preconditioning comprising short durations of interrupted stress prior to being subjected to severe, prolonged, and inescapable stress so as to mimic the protocols of classical preconditioning *i.e.*, application of milder and interrupted stress before the commencement of severe and prolonged stress. Three different protocols of stress preconditioning each comprising two cycles of restrain stress of 15, 30, and 45 min duration were employed to explore the protective effects of preconditioning in attenuating stress-induced behavioral alterations. Unlike the protective effects of classical preconditioning, stress preconditioning was not found to ameliorate stress-induced decrease in locomotor activity, exploratory behavior, or social behavior in any type of stress preconditioning employed in the present study. On the contrary, stress preconditioning was observed to enhance restraint-induced behavioral alterations. The exact mechanism of preconditioning duration-dependent exacerbation of stress-induced behavioral changes is not clear. It may be possible that preconditioning stress does not exist and the repeated stress protocols actually enhance the sustained restraint stress-induced behavioral changes. However, the protective or deleterious effect of preconditioning is critically dependent on the number of cycles and time duration of previous shorter insults. It may be possible that the preconditioning protocol employed in the present study is non-optimal to exhibit the beneficial effect at the behavioral level and

might result in enhancement of sustained restraint stress-induced deleterious effect.

The present results are in contradiction to earlier reports documenting protective effect of stress preconditioning on cold, alcohol, or restrain and water immersion stress-induced gastric ulceration.[2,19,20,41] In earlier reports, protective effects of preconditioning stress were assessed in terms of reduction in gastric ulcerations on application of severe stress/alcohol and attributed the protective effects to induction of COX-1, COX-2, and PLA_2. However, the documented gastroprotective effects of preconditioning stress could not be extrapolated in the present study in attenuating the behavioral alterations associated with restrain stress. Since the gastroprotective effects of stress preconditioning have been well reported, therefore, in this study gastroprotection afforded by stress preconditioning was not evaluated. However, the noninclusion of gastroprotective parameters marks a limitation of the present study, which otherwise would have clearly demonstrated the lack of restoration of behavioral alterations by the preconditioning protocols affording gastroprotection. The contradictory response in the present study may possibly be due to differential role of COX in different parts of the body. Up-regulation of COX in the gastric mucosa has been associated with gastroprotective activity, whereas up-regulation in brain region produces deleterious effects.

It has been well documented that there is an increased level of COX expression and activity in the cerebral cortex[40,42] and hippocampus[23] of rats exposed to restrain stress. The enhanced COX-2 level after restraint stress results in the production of PGE_2, which promotes behavioral alterations.[42] Furthermore, it has been reported that inhibition of COX-2 using selective (rofecoxib) or non-selective COX-2 (naproxane) inhibitor[23] results in attenuation of anxiety-like responses and decreased locomotor activity provoked by a variety of neurogenic stressors.[40,42] Therefore the lack of preconditioning stress effect in attenuating stress associated behavioral alterations may be tentatively linked to deleterious effect of COX-2 expression in brain. However, the data so far are not sufficient to provide direct evidence of increased COX-2 expression in brain due to preconditioning stress.

The lack of attenuating effect may possibly be linked to an increased COX-2 expression in brain, which directly produces behavioral alterations and as a consequence the existence of preconditioning stress in modulating the behavioral alterations may be ruled out. However, it is also worth mentioning that stress preconditioning did not elicit beneficial effects in this experimental designed state. Further investigations with other strategies are still needed for revealing the real aspects of stress preconditioning and may provide insights into the formation of stress-related malfunctions. The designing of other types of stress preconditioning protocols with inclusion of a higher number of preconditioning cycles or by altering the time cycle of restrain stress (during preconditioning stress and sustained stress) may elicit beneficial effects in attenuating restrain stress-associated behavioral alterations. Nevertheless, it may be concluded that preconditioning stress does not modulate restrain stress-mediated behavioral alteration using three different preconditioning stress protocols of 15, 30, and 45 min in this study in rats.

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