ISSN 1996-3351

Asian Journal of Biological Sciences

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OPEN ACCESS Asian Journal of Biological Sciences

ISSN 1996-3351
DOI: 10.3923/ajbs.2018.89.97

Research Article
Neuroprotective Potential of Metformin against Forced Swimming Induced Neurodegeneration Wistar Albino Rats

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Abstract

Background and Objective: Metformin is the first line medication for the treatment of type 2 diabetes. It is also used in the treatment of polycystic ovary syndrome (PCOS). It is reported to have a neuroprotective effect due to inhibition of apoptosis in neuronal cortical cells. Exposure to chronic stress is an important factor of neurodegeneration. The present study aimed to evaluate the effect of metformin on the behavioral, histological and antioxidant status of rats exposed to force swimming for 30 min daily for 28 days. **Materials and Methods:** Adult Wistar albino rats (150-200 g) of both sexes were used. The time of fall in rotarod, locomotor activity in actophotometer, number of correct entries in radial maze, superoxide dismutase (SOD) level and the malondialdehyde (MAD) content along with prominent tissue degradation in brain and pancreas were measured. **Results:** Significant alterations in behavioral, histological and antioxidant status were found following 28 days of forced swimming. Metformin (200 mg kg^{-1}) showed significant protection against forced swimming induced alterations. Metformin-treated animals showed an increase in the level of SOD (superoxide dismutase) and a decrease in MDA (malondialdehyde) level, which may contribute to its antioxidant status. The protection against tissue degeneration in case of histological studies further confirms its neuroprotective potential. **Conclusion:** Metformin prevented the alteration in behavioral, biochemical and histology due to chronic forced swimming-induced stress which may be attributed to its neuroprotective effect.

Key words: Metformin, stress, forced swimming test, neurodegeneration, neuroprotective, swimming-induced stress, polycystic ovary syndrome

Citation: Manas Kumar Das, Prashant Tiwari, Shakti Ketan Prusty and Pratap Kumar Sahu, 2018. Neuroprotective potential of metformin against forced swimming induced neurodegeneration wistar albino rats. Asian J. Biol. Sci., 11: 89-97.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Stress induces disturbance in physiological and neuro behavioral homeostasis[1]. The central nervous system regulates the stress responses and complex neurochemical pathways[2]. Continuous, as well as periodic stress, activates sympatho-adrenal medullary system and hypothalamic-pituitary-adrenal axis respectively[3]. Stressful life exhibits different alteration like cardiovascular, metabolic and neuropsychiatric illness[4].

Furthermore, prolonged exposure to stress is degenerated central nervous system by producing reactive oxygen species (ROS). This results in changing the neurochemical, immunological and behavioral pattern. The brain is highly affected to ROS due to high utilization of oxygen but a relatively low anti-oxidant defense mechanism. Oxidative damage due to the stressful condition in the brain leads to neuro degeneration and central nervous system (CNS) disorders[5,6]. Free radicals are major pathological factors in neuro-degenerative disorder, pulmonary disorders, cardiovascular disorders, autoimmune disorders, metabolic disorders, cancer and aging[7,8]. Different type of stressful stimuli targets to the brain as it is sensitive to stress-induced neuro degeneration and alters the antioxidant status, protein oxidation and lipid peroxidation[9,10]. Patients suffering from depression have remarkably increased plasma levels of peroxide as compared to normal which is regarded as an indication of oxidative stress[11,12]. Chronic stress degenerate hippocampus and medial prefrontal cortex followed by dendritic hypotrophy[13,14].

Metformin due to its several neuro-protective as well as antioxidant potential has been in clinical use over 50 years[15]. Besides its glucose metabolizing effect, metformin prevents the learning and memory impairment in insulin-resistant rats[16]. Kickstein *et al.*[17] have demonstrated that metformin can reduce Aβ production and tau Phosphorylation. Parkinson's disease (PD) is a neurodegenerative disorder which is signalized by a loss of neuron in substantia nigra applying an effect on striatum along with target cortical and limbic regions[18]. Adedeji *et al.*[19] have advocated the anti-parkinsonian mechanism of metformin in haloperidol initiated catalepsy model. Metformin reduces MPTP (a mouse model for PD) induced oxidative stress, dopaminergic degeneration and motor abnormalities[20]. However, Li *et al.*[21] have investigated that metformin suppresses Alzheimer's disease-like neuropathology in obesity. Hsu *et al.*[22] have also revealed that the incidence of dementia in diabetes is reduced by the use of metformin. Metformin has shown protective action in scopolamine-induced cognitive impairment in rats[23]. Guo *et al.*[24] have investigated that use of metformin for 24 weeks remarkably enhance cognitive function and decreases depression in T2DM patients.

Forced swimming test (FST) is a stressor which causes different neurobiological alterations[25-27]. Previous reports suggested that forced swimming test (FST) is a viable test in rodents to calculate antidepressive potential[28-30]. Chronic forced swimming stress (FST) causes a remarkable alteration in psychophysical, physiological and biochemical parameters in albino rats[31].

The main objective of this study was to evaluate the protective effect of metformin against forced swimming stress-induced neuro degeneration and it's possible ameliorating effect against oxidative stress. In addition, the experimental effect of met form in action on motor coordination as well as learning and memory was investigated.

MATERIALS AND METHODS

Chemical: Metformin was obtained as gift sample from Mercury Laboratory. Ltd., Vadodara, India and other solvents and chemicals used were of analytical grade.

Animals: Wistar albino rats of both sexes (150-200 g) were used for the experiment. The animals were procured from animal house of School of Pharmaceutical Sciences, Siksha Oanus and han (Deemed to be University), Bhubaneswar, Odisha, India. They were acclimatized for 48 h till the experimentation and housed in 12 h day-night cycle. Food and water were provided *ad libitum*. All the experimental protocols were approved by IAEC of School of Pharmaceutical Sciences, SOA Deemed to be university (1171/c/08/CPCSEA).

Grouping of animals: The animals were divided in three groups. In each group 6 rats were taken:

Control (Group I) = Animals were treated as control
FST (Group II) = Animals were subjected to forced swimming daily for 30 min for 28 days
FST+MET (Group III) = Animals were administered met form in 200 mg kg^{-1} p.o. daily for 28 days, 1 h before forced swimming

Forced swimming induced stress: Rats were exposed to forced swimming for 30 min in polypropylene tank (height 45 cm and diameter 35 cm) containing water up to 30 cm at 25°C for 28 days. The swimming was carried out in morning

between 09:00 and 11:00 am to prevent the effect of variation in plasma corticosterone levels. After swimming the rats were dried and allowed to access food and water[32].

Behavioral assessment

Locomotor activity: Actophotometer (INCO) was used to study locomotor activity. Each rat was allowed to move and explore the environment inside the instrument for 10 min. The movement of rat was counted digitally[32].

Motor co-ordination: Rotarod apparatus (INCO) was used for assessing motor co-ordination test. Each rat was placed on the rotating rod at a speed of 20 rpm and the time taken to fall down was noted in case of control, stress group, stress+ metformin group[33].

Elevated plus maze: The transfer latency (time taken by the rat to enter to the closed arm after placing on the maze) was recorded on an elevated plus maze (INCO, India). The rat was allowed to move freely in the maze regardless of open and closed arms for 5 min. Additionally, the time spent in open arm was observed within 5 min. All the animals were placed individually on the elevated plus maze[34].

Radial arm maze: Behavioral alteration was studied by using radial arm maze (INCO) consisting of 8 arms. Each rat was placed on the centre and allowed to enter arms freely for 5 min. Entry into an arm which the rat had not visited previously recorded as a correct response whereas re-entry was counted as an error. The number of correct responses before committing the first error was calculated as the index of radial arm maze performance[32].

Y-maze: Y-maze was used to evaluate the spatial working memory through the spontaneous alteration behavior in rats. The animals were individually placed at the centre of the Y-maze and allowed to explore for 5 min. The successive entries to different arm were observed and expressed as alternation (%). Alternation is defined as the number of successive entries in to the three arms on overlapping triplet sets. The percentage alternation was calculated as the ratio of actual to possible alternations[33]:

$$\text{Spontaneous alternation (\%)} = \frac{\text{Number of 3 out of 3 choices}}{\text{Total number of entries-degree of freedom}} \times 100$$

where, degree of freedom is 2.

Anti-oxidant study

Superoxide dismutase assay: The SOD assay was done by using JASCO (V-630) UV spectrophotometer. A blank was prepared by adding 0.5 mL of EDTA (1 mM) to 1.5 mL of Tris buffer (0.05 M) whereas 1 mL of Pyrogallol (0.2 mM) was added to the same blank preparation as control. The test preparation consisted of reagent blank and 50 µL of serum or brain homogenate in a separate test tube. Variation in absorbance was recorded against blank at 420 nm. The percentage protection was calculated from the following equation:

$$\text{Protection (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

The SOD content was determined by putting each protection (%) in the standard curve ($Y = 56.53 \times -0.1198$, $R^2 = 0.99$) and expressed[32] as mmol L^{-1}.

Lipid peroxidation activity: Malondialdehyde (MDA) an end product of lipid peroxidation was estimated by UV spectrophotometer. Blank solution was prepared by mixing 2 mL of Trichloroacetic acid (15%), 2 mL of Thiobarbituric acid (0.37%) and 2 mL of 0.25 N HCl. Test solutions were prepared by adding 100 µL of plasma or brain homogenate to the blank solution separately. The reaction mixture was heated for 60 min at 90°C over water bath followed by gradual cooling and centrifuged at 3000 rpm for 15 min. Absorbance of supernatant was measured at 532 nm against a reagent blank. Extent of MDA content was determined from the standard curve and expressed[32] as nmol L^{-1}.

Histopathology: On the 28th day, the animals were anaesthetized with light ether anesthesia and sacrificed. Brain and pancreas were isolated for histopathological study. Fixed tissues were dehydrated in different mixture of ethanol and water followed by cleaning with xylene. Then cleaned tissues were embedded in paraffin and prepared 5-6 µm thick sections. This was further stained with hematoxylin and eosin dyes followed by mounting in DPX medium for microscopic observations[35].

Statistical analysis: The statistical analyses were performed by using One-way ANOVA followed by Dunnet's-t test and level of significance is $p<0.05$. The statistical analysis of the experiment data was presented as Mean±SD.

RESULTS

Pharmacological studies

Locomotor activity: Forced swimming for 28 days significantly ($p<0.05$) decreased locomotor score (#) but met form in treated animals showed significant increase ($p<0.05$) in locomotor activity (*) as compared to animals exposed to FST (Fig. 1).

Rotarod test: Stress treated group showed significant ($p<0.05$) decrease in falling time after 30 min daily exposure to forced swimming for 28 days compared to control (#) (Fig. 2). However, metformin treated animals have shown significant increase ($p<0.05$) in falling time as compared to FST treated animals (*).

Elevated plus maze: Forced swimming for 28 days increases the transfer latency (time taken to enter the closed arm) ($p<0.05$) as compared to control. But this was significantly ($p<0.05$) reduced by metformin treated animals (Fig. 3). Similarly, FST increased time spent in open arm ($p<0.05$) as compared to control whereas metformin significantly ($p<0.05$) reduced the time spent in open arm (Fig. 4).

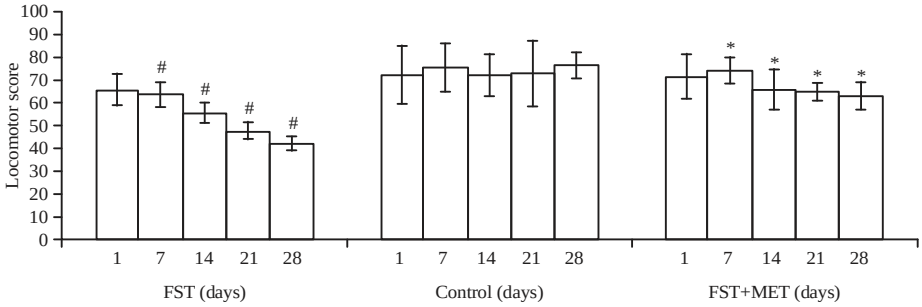

Fig.1: Effect of metformin on locomotor score in FST induced rats

#Significant effect of forced swimming stress at $p<0.05$, *Significant effect of metformin at $p<0.05$

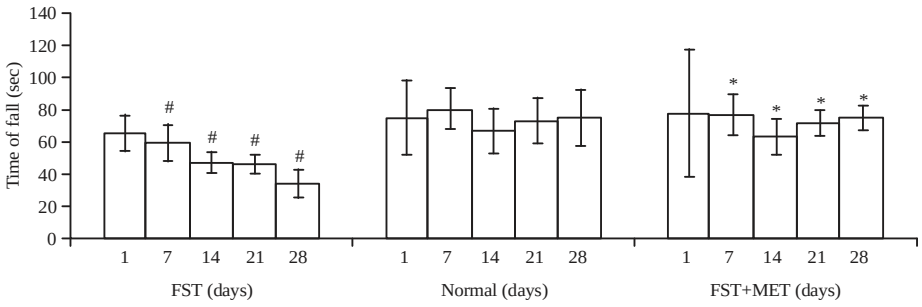

Fig. 2: Effect of metformin on time of fall in FST induced rats

#Significant effect of forced swimming stress at $p<0.05$, *Significant effect of metformin at $p<0.05$

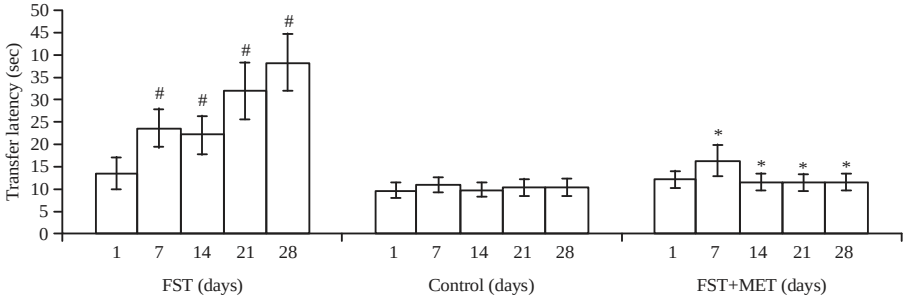

Fig. 3: Effect of metformin on the transfer latency in elevated plus maze

#Significant effect of forced swimming stress at $p<0.05$, *Significant effect of metformin at $p<0.05$

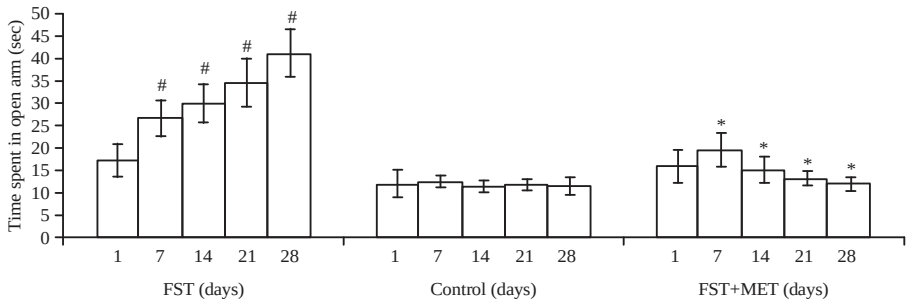

Fig. 4: Effect of metformin on the time spent in open arm in elevated plus maze
#Significant effect of forced swimming stress at p<0.05, *Significant effect of metformin at p<0.05

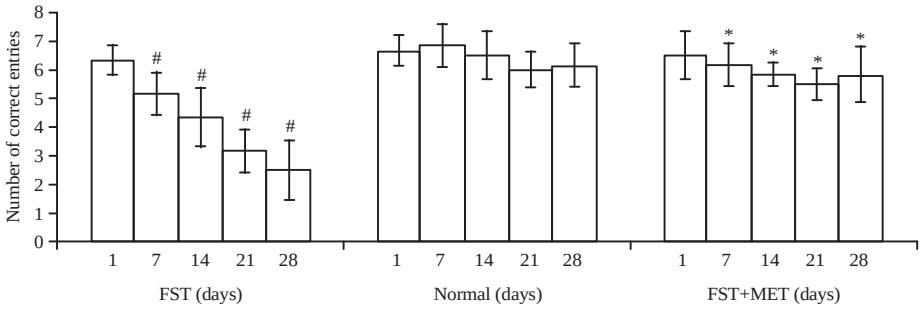

Fig. 5: Effect of metformin on number of correct entries in FST induced rats
#Significant effect of forced swimming stress at p<0.05, *Significant effect of metformin at p<0.05

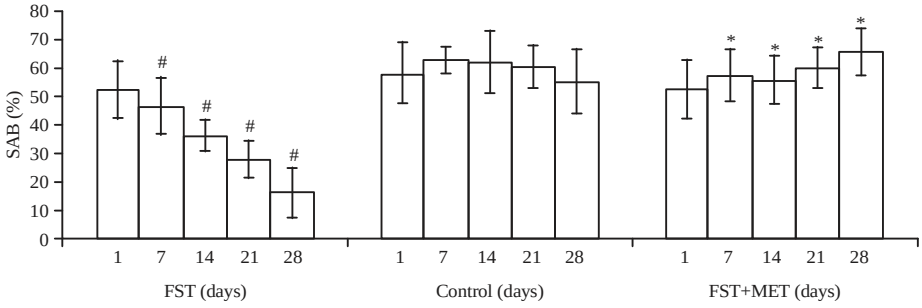

Fig. 6: Effect of metformin on SAB (%) in FST induced rats
#Significant effect of forced swimming stress at p<0.05, *Significant effect of metformin at p<0.05

Radial maze test: After 28 days of exposure to forced swimming test for 30 min daily, FST treated animals showed significant decrease (p<0.05) in number of correct responses comparing to control group (#). However, significant (p<0.05) increases in number of correct responses in metformin treated group were found when compared to FST treated animals (*) (Fig. 5).

Y maze: Forced swimming for 28 days significantly (p<0.05) decreases SAB (%) compared to control animal (#). But met form in treated animals improved SAB (%) significantly (p<0.05) as compared to animals exposed to FST (*) (Fig. 6).

Enzymatic antioxidant status: After 28 days of exposure to forced swimming test impairment of antioxidant status with decrease in SOD level was found compared to control group. However, animals treated with met form in significantly (p<0.05) increased SOD level after 28 days of exposure (Fig. 7a).

Also, the MDA content was significantly increased in FST group in comparison to control group after 30 min of daily exposure to forced swimming test for 28 days (p<0.05) but met form in treated animals showed significantly (p<0.05) low level of lipid peroxidation as compared to FST group (Fig. 7b).

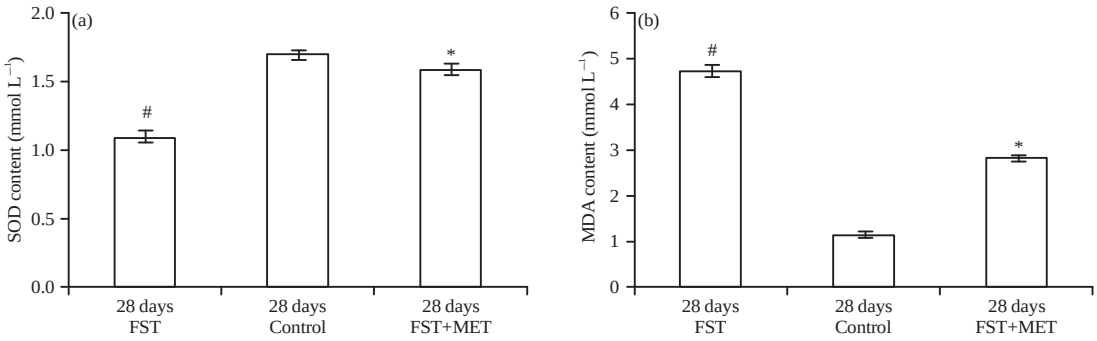

Fig. 7(a-b): Effect of metformin on antioxidant status in brain (a) Superoxide dismutase (SOD) and (b) Activity in brain MDA content

#Significant effect of forced swimming stress at $p<0.05$, *Significant effect of metformin at $p<0.05$

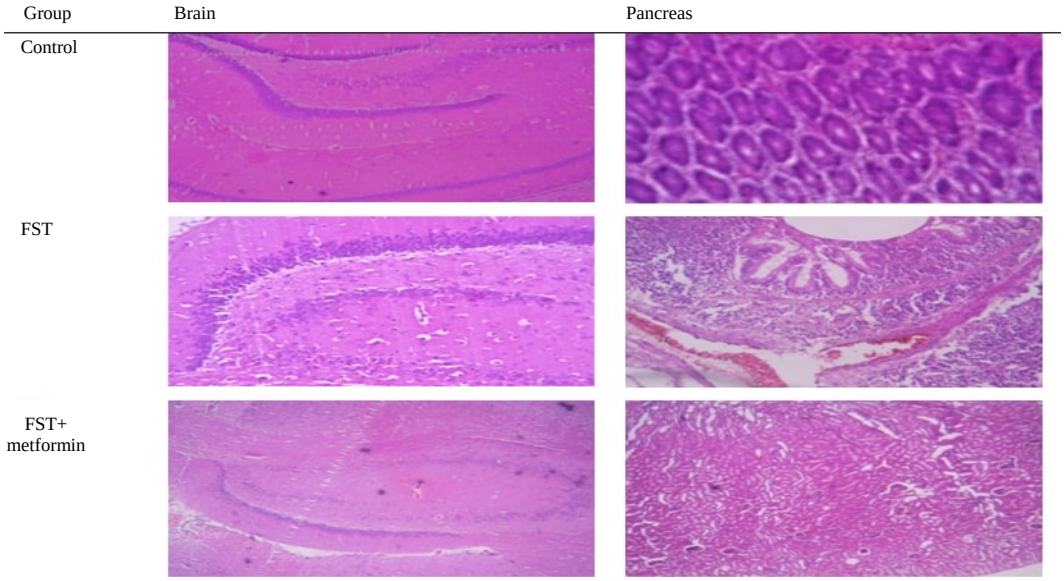

Fig. 8: Histopathology photos of brain and pancreas

Histopathology

Brain: Forced swimming for 30 min for 28 days degenerated the normal architecture of neuroglial cells along with pyknosis and necrosis of hippocampal neurons. Also, vacuolization in few areas were observed. However, metformin (200 mg kg^{-1}) showed a normal texture of the neuroglial cells. There is no pyknosis and necrosis of neuroglia was found (Fig. 8).

Pancreas: Forced swimming for 30 min for 28 days degenerated connective tissue of pancreas. Additionally, hemorrhages are found in islets of Langerhans. However, met form in (200 mg kg^{-1}) prevented the degeneration of pancreatic connective tissue with normal appearance of islets of langerhans (Fig. 8).

DISCUSSION

Chronic exposure to stress is more likely to induce biochemical, endocrine and immune changes than acute stress. Chronic stress causes in cognitive impairment, alteration in behavior, increases oxidative stress and suppresses immune system[9]. Several types of stressors are used including forced swimming test, tail shock, immobilization, cold and ether stress in laboratory[36].

Forced swimming test (FST) is a stressful condition which causes different neurobiological alterations[25-27]. Forced swim test (FST) is a sustainable test in rodents to calculate ant depressive effect[28-30]. Chronic or repeated forced swimming stress (FST) causes a remarkable alteration in behavioral,

biochemical and histological parameters in albino rats[31]. Prolonged exposure to stress leads to an increase in stimulation of extra-synaptic receptors resulting disturbance in mitochondrial function, alteration of cellular calcium homeostasis and increase production of (ROS) reactive oxygen species[5,6]. Chronic exposure to force swimming stress changes different enzymatic status like an increase in free radical production (lipid peroxidation) and decreases antioxidant activity (SOD) leading to oxidative stress with change in histology[37,32]. Validated models for forced swimming induce stress for 30 min for 15 days significantly changes the behavioral, histological and anti-oxidant status[32]. However, in this experiment, it have applied forced swimming stress for 30 min for 28 days.

Oxidative stress causes learning and memory deficit and induced brain cholinergic dysfunction[38]. Forced swimming stress for 28 days were reported to bring out cholinergic dysfunction, spatial memory loss and cognitive impairment[37,32]. Significant reduction in time of fall in rotarod and decrease in locomotor activity linked with loss of motor coordination and CNS depression[32,39]. However, Current outcome from radial arm maze and Y-maze are in concurrence with these studies and metformin-treated animals have improved the responses.

Forced swimming for 15 days significantly increases MDA content and decreases SOD level[32]. Additionally, this alteration in antioxidant status can also result from oxidative stress in brain Promoted by Type 2 Diabetes mellitus[40]. However, in the present study, it found that metformin (200 mg kg^{-1} p.o.) increases the SOD level and decreases the MDA level significantly following exposure to 28 days of forced swimming for 30 min daily.

Histopathological data revealed that there is a significant loss of hippocampal cells in rat brain tissue after 28 days of daily 30 min exposure. Hippocampus is a major stress track for the reason that of its contribution in the regulation of hypothalamo-pituitary-adrenocortical (HPA) axis activity and also for its hypersensitivity to stress[38,41].

The increase in locomotor score in case of actophoto meter and time of fall in case of rotarod experiment due to met form in administration may be regarded as CNS stimulation and enhancing motor coordination respectively. Additionally, an increase in spontaneous alternation behavior may be a contributing factor for increasing spatial memory. Again, metformin increases the number of correct entries in a radial maze and decreases the transfer latency as well as time spent in open arm, which suggests its nootropic potential[32,33,39]. Metformin-treated animals showed an increase in the level of SOD (superoxide dismutase) and a decrease in MDA (malondialdehyde) level, which may contribute to its antioxidant status. The protection against tissue degeneration in case of histological studies further confirms its neuroprotective potential[32,42].

CONCLUSION

Experimental data concluded that chronic forced swimming for 30 min daily for 28 days causes behavioral, biochemical and histological alteration which is a strong indication of the neurodegeneration. Metformin showed neuroprotective effect by preventing the alteration in behavioral, biochemical and histology due to chronic forced swimming-induced stress.

ACKNOWLEDGMENT

Authors thank the Department of Pharmacology, School of Pharmaceutical Sciences, Siksha O Anusandhan (Deemed to be University), Bhubaneswar for providing laboratory facilities and technical assistance.

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