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COMPREHENDING THE ROLE OF AAS ON THE AChE ACTIVITY AND ANTIOXIDANT SYSTEM IN THE BRAIN OF POSTNATAL FEMALE MICE

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ABSTRACT

Abuse of Anabolic-androgenic steroids (AAS) by professional and recreational athletes for endurance performance is increasing globally. Supraphysiological doses of AAS exert profound effects on mental state and behavior viz., depression, anxiety, and oxidative stress. Present investigation elucidates chronic impact of one of the AAS Stanozolol (ST) on acetylcholinesterase (AChE) enzyme activity and oxidative stress in the brain of postnatal female mice *Mus musculus*. 20 animals (21-days-old) were randomly assigned into four experimental groups (n=5) and ST was administered subcutaneously for 30 days: [Low-dose (LD)-0.5mg/kg, medium-dose (MD)-5.0mg/kg and high-dose (HD)-7.5mg/kg bwt or 1% alcohol-baseline control]. Acetylcholinesterase enzyme activity was determined by Ellman's method. Oxidative stress marker Malondialdehyde (MDA), enzymatic antioxidant Superoxide Dismutase (SOD) and non-enzymatic antioxidant Glutathione (GSH) were assayed, and absorbance was recorded. Results demonstrate a notable reduction in the AChE enzyme activity in the entire treatment group, subsequently leading to the accumulation of ACh level in synaptic clefts. This results in impairment of neuronal transmission, which in turn affects the efficiency of cholinergic neurotransmission. An elevation in oxidative stress marker MDA level (in MD & HD) and a decline in anti-oxidant levels of SOD (in MD & HD) and GSH (in HD) ST-treated groups were observed. The results reveal that ST causes an imbalance between antioxidants and free radicals, which may lead to oxidative damage by attenuating antioxidant enzymes. It is inferred that Prolonged ST-treatment interferes with neuronal transmission, subsequently resulting in neuronal disorders, viz., stress-related anxiety, memory loss, and other cognitive behavioral abnormalities. Prolonged ST-treatment elevates oxidative stress marker MDA and a decline in antioxidants, leading to an imbalance in the local antioxidant system thereby disrupting cellular redox balance in the brain.

KEYWORDS: AChE activity, anti-oxidant levels, brain, stanozolol, Mus musculus.

INTRODUCTION

Anabolic-androgenic steroids (AAS) are testosterone derivatives that were once developed to encompass some androgenic properties and enhance anabolic efficacy for the treatment of a variety of therapeutic ailments, such as male hypogonadism, endometriosis, renal and hepatic failure, aplastic anemia, as well as serious burns and post-surgical recovery.[1-3] Approximately 100^{+} kinds of AAS have been synthesized to date and among them more than 60 different AAS are available in market.[4] Due to the anabolic efficiency of AAS, which can boost muscle mass and strength, they are now primarily being abused at supraphysiological doses by recreational athletes and sportsmen to enhance performance, strength, and endurance. Regrettably, these AAS compounds are now being abused as ergogenic aids globally by non-athletes also and, consequently, result in various secondary pathophysiological effects such as altered cholesterol and liver enzyme profile, impairment in cardiovascular, reproductive, immune response, renal functioning.[5-14]

Neuronal function is greatly influenced by the cholinergic system, which plays a key role in

transmitting information peripheral status to the central nervous system (CNS) and vice versa. The central players of this system are acetylcholine (ACh) and its enzyme acetylcholinesterase (AChE). Acetylcholine-mediated neurotransmission is fundamental to the functioning of the nervous system in mammals.[15,16] Acetylcholinesterase (AChE) is a critical enzyme in the neurotransmission of vertebrates, mainly found at neuromuscular junctions and cholinergic synapses.[17] During neurotransmission, ACh is released from the presynaptic neuron into the synaptic cleft and binds to ACh receptors on the post-synaptic membrane,[18] and AChE is one of the efficient primary cholinesterases that terminates acetylcholine-mediated neurotransmission at cholinergic synapses by rapidly hydrolyzing acetylcholine (ACh) into acetate and choline.[19-20] As AAS easily crosses the blood-brain barrier, they have an impact on the central nervous system and neurotransmission.[21] Relevant research shows that steroid hormones are known to affect neurotransmission via direct effects on the cellular membrane, modulation of synthesis and degradation of neurotransmitters by altering their metabolism.[22] They modulate memory processes by altering the functionality of several neurotransmitters such as acetylcholine by regulating acetylcholine transferase and acetylcholinesterase activities.[22] Furthermore, steroid hormones are known to affect neurotransmission through direct effects on the cellular membrane and modulation of the synthesis and degradation of neurotransmitters, such as acetylcholine, by regulating the activities of acetylcholine transferase and acetylcholinesterase.[22] In addition, chronic usage of AAS can affect mental health conditions, including anxiety, depression, and aggressive behavior.[23-28]

Oxidative stress is defined as an imbalance between the generation of reactive oxygen species (free radicals-ROS) and their removal by the cascade of antioxidant system of an organism.[29] The cells constantly generate free radicals as a result of both enzymatic and non-enzymatic processes, and antioxidants act in a wide range of cellular processes to maintain the optimum level of ROS.[30] The induction of oxidative stress among AAS abusers by altering stress biomarkers in different tissues, like cardiovascular, kidney, liver, brain, etc. have been reported.[31-35] Global reports on AAS-induced neurodegeneration confirm that oxidative stress and apoptosis are the main causes of AAS neurotoxicity.[35] AAS administered at supraphysiological levels has the potential apoptotic impact on neurons including other cell types.[36] Nevertheless, additional systematic studies on the quantification of antioxidants in the brain are required, utilizing AAS compounds at varying dosages and duration.

The growing brain is particularly susceptible to neuroactive substances that trigger or block neurotransmitter responses during the rapid brain growth, from the early embryonic stage to adolescence. Acetylcholine, as a neurotransmitter, performs multitasking roles.[37]

However, there is no evidence available on the chronic abuse of one of the AAS (Stanozolol-17β-hydroxy-17α-methyl-androstano (3, 2-c) pyrazole) on the AChE activity and oxidative stress in the brain of postnatal female mice. Stanozolol is a non-aromatizable androgen that is frequently used in therapeutics as well as abused by athletes and teens. Hence, it is essential to comprehend how ST affects the brain of postnatal female Swiss albino mice, *Mus musculus*, aiming to determine:

i. the impact of ST on AChE activity in the post-natal brain.
ii. the efficacy of ST on the oxidative stress in the post-natal brain of mice.

MATERIALS AND METHODS

All protocols used in this experiment adhered to the CPCSEA guidelines for the Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committee No. 639/GO/02/a/CPCSEA, at the Department of Zoology, Karnatak University, Dharwad. 21 days-old female mice were obtained from the mice colony, maintained in the Department of Zoology, Karnatak University, Dharwad, just after weaning. They were housed in individual cages at room temperature (27±1°C) with natural-cum-artificial light for 12–14 h and fed with a pelleted diet (Gold Mohur, Lipton, India) and water ad libitum.

Hormone treatment

Stanozolol was obtained from Sigma-Aldrich, USA. The mice were randomly assigned to four experimental groups, each group containing 5 animals. ST was dosed subcutaneously [0.5 mg/kg bwt (low); 5.0 mg/kg bwt (medium); 7.5 mg/kg bwt (high-dose); or 1% alcohol (baseline control); $n = 5$] for 30 consecutive days. On the 31st day, animals were sacrificed.

Separation of different parts of the brain

The brain was isolated from the skull and rinsed out in cold phosphate-buffered saline (pH 7.4) to remove the peripheral blood. Prior to the separation, whole brain was kept on a cold metal plate, which was placed on the ice blocks. After confirming its hardness, precisely separated the different brain parts, viz., forebrain, midbrain and hindbrain. Each brain part was homogenized and subjected to the enzyme assay.

Quantification of AChE enzyme activity

Fresh brains (immediately after the isolation) from the treated and control mice were homogenized in 0.1M phosphate buffer (pH 7.6) and centrifuged for 10 min at 3000 rpm at 4 ºC (Thermo Scientific^{TM} – Sorvall ST 8R Centrifuge). The supernatant was used as the source of the AChE activity. The enzyme activity was estimated as per the procedure described elsewhere.[38-39] 100µl of brain homogenate was mixed with the incubation mixture, followed by the addition of 20µl of the substrate

8mM Acetylthiocholine Iodide in a 96 microplate well (Sigma Aldrich, USA). The incubation period was set for 30 minutes at room temperature. The enzyme activity was terminated by the addition of 180µl of the chromogen - 5', 5'-dithiobis nitro-benzoate reagent (DTNB; Sigma Aldrich, USA). The absorbance at 412 nm was measured immediately using a spectrophotometer (Thermo Scientific™ Multiskan Sky Microplate Spectrophotometer, USA). Subsequently, Ellman's formula was used to convert the change in absorbance into enzyme activity.

Antioxidant enzyme activities

The Oxidative stress marker, Malondialdehyde (MDA), and the potential antioxidants, Superoxide Dismutase (SOD) and Glutathione (GSH) were quantified. The fresh brain samples were hand homogenized in a 5 mM Tris-HCl buffer (pH 7.4) solution that contained 0.9% NaCl (w/v) and 1 mM EDTA. This mixture was subjected to centrifugation at 750×g (Thermo Scientific™ – Sorvall ST 8R Centrifuge) for 10 minutes at 4 °C. Aliquots of the supernatant were kept at -80°C until the test.

i. The Oxidative stress marker-Malondialdehyde (MDA) assay

The aliquoted brain samples were subjected to calorimetric MDA estimation utilizing a thiobarbituric acid reactive substance. The 0.5 ml of the supernatant brain sample was mixed with 2 ml of TBA-TCA-HCL (1:1:1) reagent (which contains 0.5% thiobarbituric acid, 0.25 N HCL, and 20% TCA) and heated in a water bath for 15 minutes, and then cooled. The absorbance at 532 nm was measured immediately using a spectrophotometer (Thermo Scientific™ Multiskan Sky Microplate Spectrophotometer, USA). The molar extinction coefficient of malondialdehyde was used to compute the MDA content.[40]

ii. Enzymatic antioxidant: Superoxide Dismutase (SOD) assay

100 µl of the brain sample was mixed with 0.8 ml of carbonate buffer (pH 10.2) followed by incubation at 25 ^{0} C for 15 min. To this mixture, 100 µl of adrenaline solution,[41] was added. The UV-VIS Spectrophotometer, Hitachi, U-2800, was used to record the changes in absorbance at 295 nm.

iii. Non-Enzymatic antioxidant: Glutathione (GSH) assay

The total amount of glutathione (GSH + GSSG) was determined quantitatively by the enzymatic method. In short, the GSH reacts with Ellman's reagent [5,5'-dithiobis-2 nitrobenzoic acid (DTNB)] and forms a product that can be measured at 412 nm using spectrophotometer. This reaction was used to measure the reduction of GSSG to GSH. The rate of the reaction is proportional to the GSH and GSSG concentration. The one (1) ml of supernatant brain sample was mixed with 2 ml of tris buffer, and 50 µl of DTNB20. This mixture was centrifuged at 1000 rpm for 10 minutes, and the absorbance at 412 nm was measured with a spectrophotometer (UV-VIS Spectrophotometer, Hitachi, and U-2800).

STATISTICAL ANALYSIS

All statistical tests were performed using SPSS version 20. Difference in the initial and final body weight was compared using paired t-test. Data were expressed as mean ± SE. Enzyme and antioxidant concentration levels between treatment and control groups were evaluated using a one-way analysis of variance (ANOVA) to determine the significant difference between different treated groups. The multiple comparison test (Tukey's HSD post-hoc test) was carried out to determine which treatment group significantly differed from the control group. Numerical values are presented as the mean ± standard error (SE). Both statistical tests were two-sided with a 5%, 1% & 0.1% levels of significance ($P < 0.05$, $P < 0.01$ & $P < 0.001$).

RESULTS

To investigate the impact of one of the AAS Stanozolol on neuronal function and antioxidant cascade, the postnatal female mice were examined for AChE, MDA, SOD and GSH activity. The results reveal that all the ST-treated groups low-dose (LD), medium-dose (MD) and high-dose (HD) showed a significant increase in the forebrain weight ($p < 0.001$; $F_{3,16}= 23.468$); midbrain weight (LD and MD- $p < 0.001$; HD $p < 0.05$; $F_{3,16}= 33.908$) and hindbrain weight ($p < 0.001$; $F_{3,16}= 47.111$) when compared to the control was apparent (Fig. 1. A, B & C).

Fig. A. Forebrain Weight

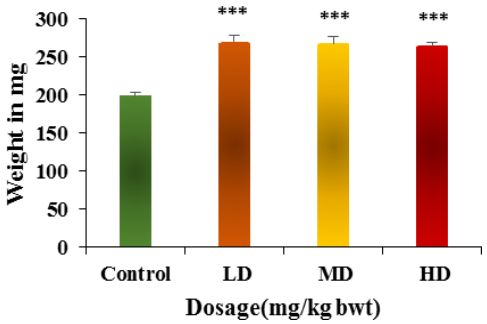

Fig. B. Midbrain Weight

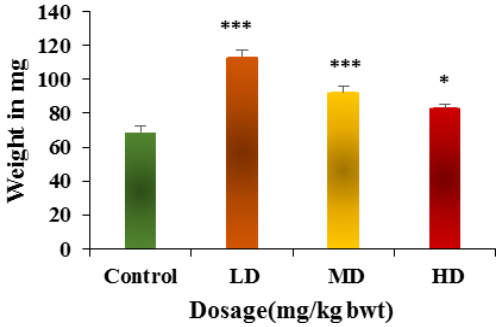

Fig. C. Hindbrain Weight

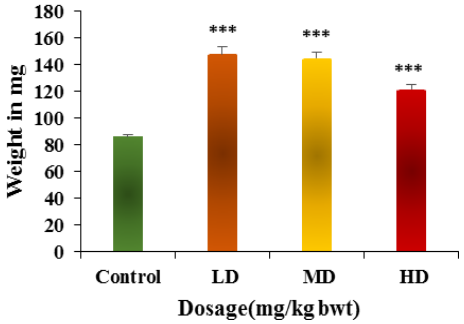

Fig. 1: Administration of ST for 30 days leads to increase in brain weight.

(A) A significant increase ($p < 0.001$) in forebrain weight of all the ST-treated groups. **(B)** There was a significant rise in midbrain weight of LD, MD ($p < 0.001$) and HD ($p < 0.05$) treated groups. **(C)** A significant rise ($p < 0.001$) in hindbrain weight of all the ST-treated groups.

Following the termination of drug administration, the activity of Acetylcholinesterase (AChE) enzyme was determined in all three regions of the brain. The results disclosed that the entire ST-treated group revealed a substantial decline in AChE activity in the forebrain ($F_{3,16}= 119.85$; $p < 0.001$), midbrain ($F_{3,16}= 35.074$; $p < 0.001$) and hindbrain ($F_{3,16}= 153.47$; $p < 0.001$) regions of mice in comparison to controls. The observed results signify profound alterations in AChE activity in all three regions of the brain (Fig. 2. A, B & C).

Fig. A. AChE concentration in Forebrain

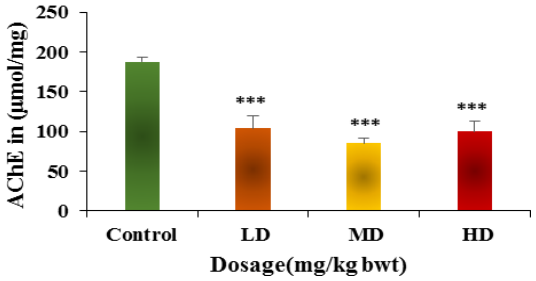

Fig. B. AChE concentration in Midbrain

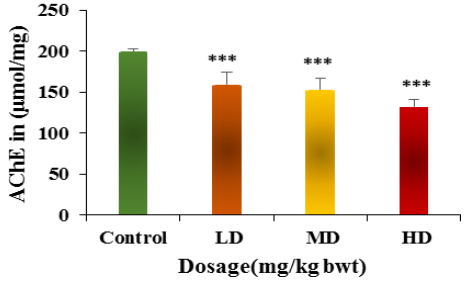

Fig. C. AChE concentration in Hindbrain

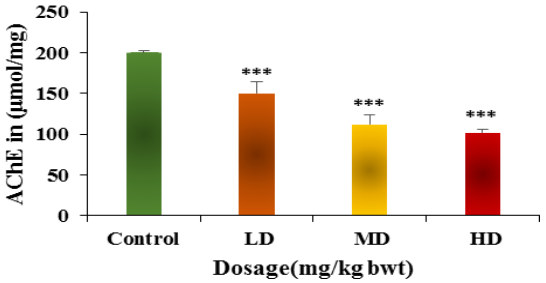

Fig. 2: Administration of ST for 30 days leads to decline in the brain AChE activity.

A significant reduction in **(A)** forebrain ($p < 0.001$ ($p < 0.001$), **(B)** midbrain ($p < 0.001$) and **(C)** hindbrain ($p < 0.001$) AChE activity of all the ST-treated groups.

Further, the Oxidative stress marker, specifically the MDA level, was insignificantly elevated in LD-treated mice ($p > 0.05$). Whereas, a significant increase ($p < 0.01$) in its level was witnessed in MD and HD-treated groups when comparison was made among the groups following Tukey's post-hoc test ($F_{3,16}= 8.771$). An enzymatic antioxidant analysis revealed an insignificant reduction in SOD concentration in LD-treated ($p > 0.05$); and a noteworthy fall in MD and HD-treated groups ($p < 0.001$); ($F_{3,16}= 18.426$) was observed when compared to control. Similarly, the quantification of a non-enzymatic antioxidant – Glutathione (GSH) in the brain showed an insignificant decline in LD-and MD-treated mice ($p > 0.05$). Whereas, there was a noticeable reduction was observed in HD-treated groups ($p < 0.05$) compared to control ($F_{3,16}= 11. 671$); (Fig. 3. A, B & C).

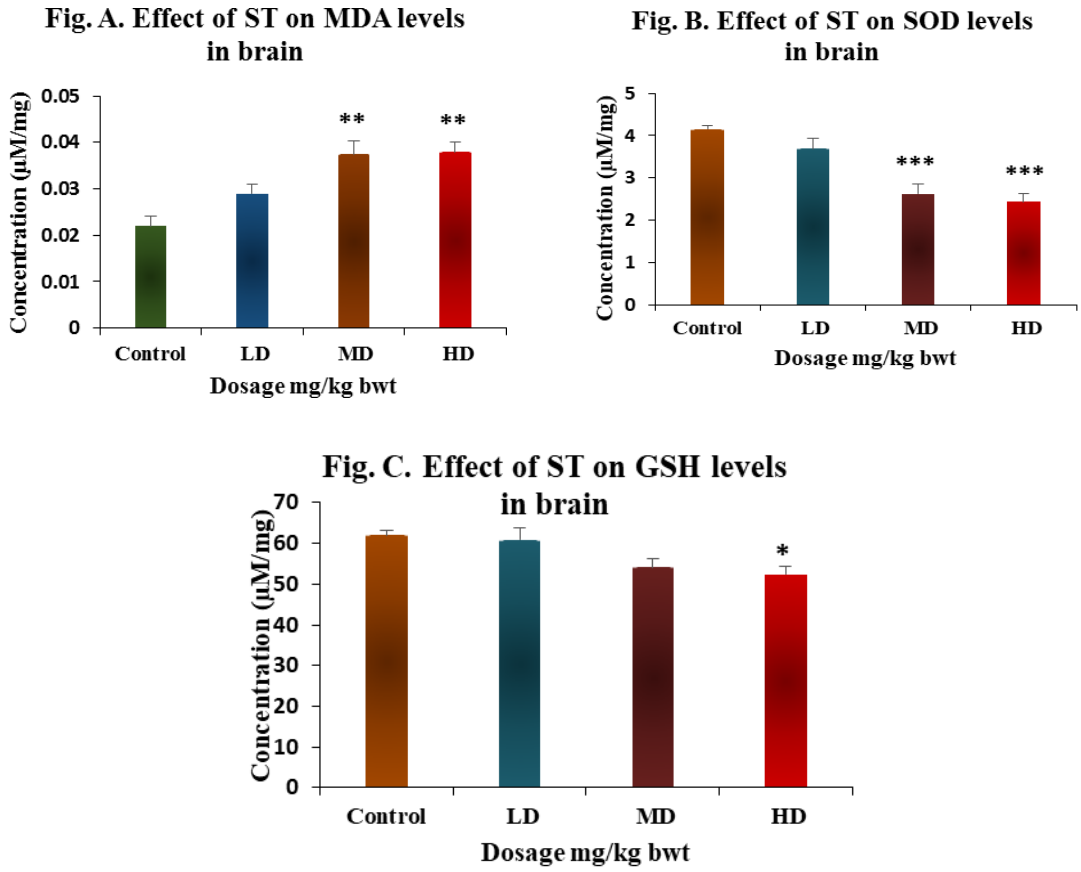

Figure 3: The impact of ST treatment on MDA (A), SOD (B), and GSH (C) levels in brain homogenate of female mice.

(A) There was an insignificant increase of MDA ($p > 0.05$) in LD. Whereas, a significant upregulation was observed in MD & HD ($p < 0.01$) ST-treated groups. **(B)** There was an insignificant down-regulation of SOD ($p > 0.05$) in LD. Whereas, a significant reduction was observed in MD & HD ($p < 0.001$) ST-treated groups. **(C)** There was an insignificant down- regulation of GSH ($p > 0.05$) in LD & MD. Whereas, a significant reduction was observed in HD ($p < 0.05$) ST-treated groups.

DISCUSSION

In the present investigation, we evaluated the impact of one of the anabolic-androgenic steroids, ST on the AChE enzyme activity in the different brain regions of the post-natal female mice.

The results reveal that, there was a drastic rise in the weight of forebrain, midbrain and hindbrain in all ST-treated groups. This impact might be attributed to the androgenic to anabolic efficacy of ST. The vital enzyme AChE has been substantially implicated in regulating the cholinergic neurotransmission through acetylcholine hydrolysis (ACh).[42] In the present investigation, a remarkable reduction of AChE enzyme activity in all three brain regions across all the treatment groups (LD, MD & HD) was the most notable effect elicited by four-week ST-treatment.

The observed results demonstrate that stanozolol inhibits the AChE enzyme activity, subsequently leading to the accumulation of ACh level in the synaptic clefts, resulting in impairment of neuronal transmission, which in turn affects the efficiency of cholinergic neurotransmission. Whereas, sub-chronic treatment of nandrolone decanoate (ND) resulted in the augmented levels of AChE activity in cerebellum and striatum of rat brain, affecting the cholinergic system and consequently the CNS.[43] Whether elevated acetylcholine (ACh) levels have beneficial or detrimental effects, however, depends on the phase of memory formation at which cholinergic stimulation occurs. Since the regulation of CNS cholinergic system through acetylcholine hydrolysis has been largely attributed to AChE activity, and a significant reduction in its activity may lead to stress-related anxiety, memory loss, thus interfering with some cognitive and behavioral aspects in the mice. An excess level of ACh was shown to be neurotoxic while the opposite is also true since low levels of ACh in the synaptic junction may influence memory negatively.[16,44] A report on human imaging study suggests the elevation of acetylcholine (ACh) levels in patients who are actively depressed, as measured by occupancy of nicotinic receptors throughout the brain and remain high in patients who have a history of depression.[45] Likewise, ND treatment induced behavioral changes including oxidative damage, inflammation and imbalance in brain

neurotransmitter systems, modulation of nerve growth factor and neuronal apoptosis.[46-48] Likewise, the supraphysiological levels of testosterone initiate apoptotic cascade in neuronal cells of human neuroblastoma cell lines.[49] Accumulation of acetylcholine in the brain can cause headaches, sleep disturbances, dizziness, confusion, and drowsiness, and chronic exposure may lead to central depression, slurred speech, convulsions, and coma.[50] Similarly, rats exposed to AChE inhibitors during the active synaptogenesis phase of the postnatal period would be more susceptible to developing a variety of behavioral abnormalities, including defects in motor development and coordination.[51]

Oxidative stress is characterized by the decreased ability of endogenous systems to fend off the oxidative attack aimed at target biomolecules. It can arise from increased ROS/RNS formation or a decline in the antioxidant protective ability.[52,53] The cell has numerous antioxidant systems to either eliminate or to stop the production of ROS. Both internal and external (nutritional) sources are possible. Findings of the current study exhibited an augmented level of lipid peroxidation marker, MDA in medium and high-dose treated groups, revealing the upregulation of lipid peroxidation. Since malondialdehyde is one of the reactive aldehydes that are produced when lipids undergo an oxidative attack,[54] the results of the present investigation indicate greater oxidative stress.

Glutathione (GSH), vitamin C and E, glutathione reductase (GR), heme oxygenase (HO), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) are a few of the most essential antioxidants.[55] In the present investigation, a notable reduction in brain SOD levels of medium and high-dose ST-treated groups and significant decline in GSH of high-dose ST-treated groups divulges an increased superoxide radical production and other ROS. Without SOD, superoxide could cause cell injury. However, if SOD were present, all of these negative effects could be avoided.[56] This shows that ST causes an imbalance between antioxidants and free radicals, which may lead to oxidative damage by attenuating antioxidant enzymes.

CONCLUSION

- With low AChE levels, ACh accumulates and an excess of ACh concentration interferes with the regular transmission of nerve impulses and this condition may affect the cognitive functions and may lead to impairment in neuronal transmission subsequently resulting in neurological disorders.
- Prolonged ST-treatment elevates oxidative stress marker MDA, and a decline in anti- oxidants SOD and GSH levels in the brain indicates that ST causes an imbalance in the local antioxidant system, thereby disrupting cellular redox balance in the brain.

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