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# Stanozolol Induces Alterations in Antioxidant cascade and AChE Activity in Brain and Renal Function in Female Mice, *Mus musculus*

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## ABSTRACT

Anabolic androgenic steroids (AAS) are derivatives of testosterone being abused by athletes and non-athletes. The present investigation aims to evaluate the impact of one of the AAS compounds Stanozolol (ST) on antioxidant system, AChE activity in brain and histopathology of kidney in female mice. Adult female mice were assigned to four experimental groups (n = 5) and different doses of ST (0.5, 5.0 and 7.5 mg/kg bwt, respectively) were administrated s.c. for 30 days. On 30$^{th}$ day the brain and kidney were removed, frozen and stored at -80 °C, for antioxidant and AChE enzyme activities. Kidney was processed for histopathological analysis. A significant decrease in superoxide dismutase (SOD) as well as glutathione (GSH) and augmented level of malondialdehyde (MDA) activity in brain and kidney homogenates of medium and high dose treated groups were noticed as compared to control. Significant reduction in AChE activity was perceived in the brains of entire treatment group as compared to control. The degree of impact of ST is more pronounced in brain in contrast to kidney. Histopathological analysis of kidney encountered changes in histoarchitecture of kidney that include shrunken glomeruli with minimized capillary tuft and increased glomerular space. At certain regions enlarged glomeruli with infiltration of endothelial cells leading to hyperplasia, hemorrhage and tubular necrosis were noticed in high dose treatment group. It is inferred that ST induces alterations in local redox antioxidant cascade in brain and kidney in dose dependent fashion. Besides, it alters neurotransmitter activity and impairs renal function in female mice.

**Keywords:** Anabolic-androgenic steroids, Stanozolol, AChE activity, Stress biomarkers, Histopathology, Brain and Kidney.

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## INTRODUCTION

The Anabolic androgenic steroids (AAS) are analogs of testosterone originally synthesized for therapeutic purpose. The classical therapeutic uses of these analogs include aplastic anemia, male hypogonadism, inoperable breast cancer, trauma, burns, kidney failure, osteoporosis, etc[1-5]. Nevertheless, AAS compounds are being abused as ergogenic resources at supraphysiological doses by professional athletes, body builders and non-athletes to enhance muscle mass, athletic performance, physique, strength and endurance despite their secondary adverse effects. Consequentially, the potential benefits of these AAS are often associated with various secondary adverse effects such as altered cholesterol and liver enzymes profile, impairment in renal, cardiovascular, reproductive, musculoskeletal, endocrine and immunological functioning[6-13].

Oxidative stress is the imbalance between pro-oxidant compounds (generation of reactive oxygen species; ROS) and those responsible for the removal of ROS, the so-called antioxidant cascade[14,15]. Antioxidants act in wide range of cellular processes and maintain the optimum level of ROS. The induction of oxidative stress among AAS abusers by altering stress biomarkers in different tissues, like cardiovascular, kidney, liver, brain, etc**.** have been reported[12,16-19]. However, additional systematic studies on quantification of antioxidants in brain and kidney as well as on the histopathology of kidney are needed involving use of AAS compounds with different doses and duration before arriving at conclusive assessment.

A previous study from our laboratory reported that administration of stanozolol (ST) lead to acceleration of granulopoiesis and stimulates immune response (at physiological level only), though it alters the lipoprotein profile in mice[9]. Further, the use of AAS is becoming increasingly popular among the adolescent girls and women athletes, yet the actual influence of these compounds on many of the physiological aspects is yet to be established clearly. Among many AAS, ST is one of the non-aromatizable androgen and is commonly used either clinically or abused by athletes and teenagers. Hence, the present study on female Swiss albino mice, *Mus Musculus* is aimed to know:

i. The efficacy of ST on oxidative stress and AChE activity in brain and
ii. To examine the effect of this compound on antioxidant system/cascade and histopathology of kidney.

## MATERIALS AND METHOD

### Animal model and experimental protocol

Two and half month old healthy female mice of Swiss albino strains were obtained from the mice colony, maintained in the Department of Zoology, Karnatak University, Dharwad. They were housed in individual cages at room temperature (27 ± 1 $^0$C) with natural- cum- artificial light for 12-14 h and were fed with a pelleted diet (Goldmohur, Lipton, India) and water *ad libitum*. Mice exhibiting normal Estrous cycles and weighing 25-30 g were used for the experiment.

ST was obtained from Sigma Chemical Co., USA. The mice were weighed and allocated into four experimental groups (each group consisting of 5 animals). Doses of ST administrated in the experiment were so chosen as to stimulate the range of doses taken by female users. The first group that received 1% alcohol acted as a base line (control). The 2$^{nd}$, 3$^{rd}$ and 4$^{th}$ groups received ST (0.5, 5.0, 7.5 mg/kg, respectively) for 30 days, by s.c. injection in 1% alcohol. On thirty-first day, mice were sacrificed by the inhalation of ethyl ether.

**Histological analysis**

The kidneys were dissected out, fixed in Bouin's fluid and processed for standard histological procedures. Sections (5 µm) of the kidney were taken on microtome (Leica RM 2255) stained with Harris's haematoxylin and eosin were subjected for observation (Nikon microscope 90E with ACT 2U software).

**Antioxidant enzymes activities**

Quantitative analysis of the antioxidant markers viz., the Glutathione (GSH), Superoxide dismutase (SOD), Malondialdehyde (MDA), Acetylecholinesterase (AChE) activity were carried out in the homogenates of brain and kidney. Both brain and kidney samples were homogenized in 5 mM Tris-HCl buffer (pH 7.4), containing 0.9% NaCl (w/v) and 1 mM EDTA, followed by centrifugation at 750×g for 10 min at 4 $^0$C. The supernatant aliquots were stored at -80 $^0$C, until assay.

**GSH assay**

The quantitative determination of the total amount of glutathione (GSH + GSSG) employs the enzymatic method. Briefly, the reaction of GSH with Elman's reagent (5, 5'-dithiobis-2-nitrobenzoic acid (DTNB)) gives rise to a product that can be quantified spectrophotometrically at 412 nm. This reaction is used to measure the reduction of GSSG to GSH. The rate of the reaction is proportional to the GSH and GSSG concentration. One (1) ml of tissue homogenate supernatant was mixed with 2 ml of tris buffer and 50 µl of DTNB[20]. This mixture was centrifuged at 1000 rpm for 10 min and the absorbance was measured at 412 nm using spectrophotometer (UV-VIS Spectrophotometer, Hitachi, and U-2800).

**SOD assay**

100 µl of the tissue supernatant (kidney and brain, respectively) is mixed with 0.8 ml of carbonate buffer (pH 10.2), incubated at 25 $^0$C for 15 min, before adding 100 µl of adrenaline solution[21]. The change in the absorbance was recorded at 295 nm using UV-VIS Spectrophotometer, Hitachi, U-2800.

**MDA assay**

Calorimetric MDA estimation was carried out in brain and kidney tissue extract by using thiobarbutric acid reactive substance. 0.5 ml of tissue extract was treated with 2 ml of TBA-TCA-HCL (1:1:1) reagent (Thiobarbituric acid 0.5%, 0.25 N HCL and 20% TCA) and heated in a water bath for 15 min and then cooled. The absorbance was recorded at 532 nm. The MDA content was calculated on the basis of the molar extinction coefficient of malondialdehyde and expressed as µmol MDA/ mg protein[22].

**AChE enzyme assay**

Fresh brain from treated as well as control mice, immediately after autopsy, were homogenized in phosphate buffer (pH 7.6) and centrifuged for 10 min @ 3000 rpm. The supernatant was used as the source of the AChE activity. The enzyme activity was estimated as per the procedure described elsewhere[23,24]. The incubation mixture comprised of 0.2 ml of 20 mM phosphate buffer (pH 7.6), 0.1 ml of 8 mM acetylcholine iodide and 40 ml of tissue supernatant. The incubation was carried out at room temperature for 30 min. The reaction was terminated by adding 1.8 ml of 5', 5'-dithiobis nitro-benzoate (DTNB) reagent. The absorbance was measured immediately at 412 nm using UV-VIS spectrophotometer.

**Statistical analysis**

Data were expressed as mean ± SE. Comparison of normally distributed variables across groups was made using one-way ANOVA followed by *post-hoc* test (Tukey). The level of statistical significance was set at $p < 0.05$ and $p < 0.01$. All the statistical analyses were performed using SPSS (version 16.0 for Windows) and the recorded values are summarized in Figure 1- 4.

## RESULTS AND DISCUSSION

**Organ weight**

An insignificant decrease in the weight of the brain ($p > 0.05$) and a significant increase in the weight of kidney ($p < 0.001$) were observed in the entire ST treated mice groups as compared to the control (Figure 1).

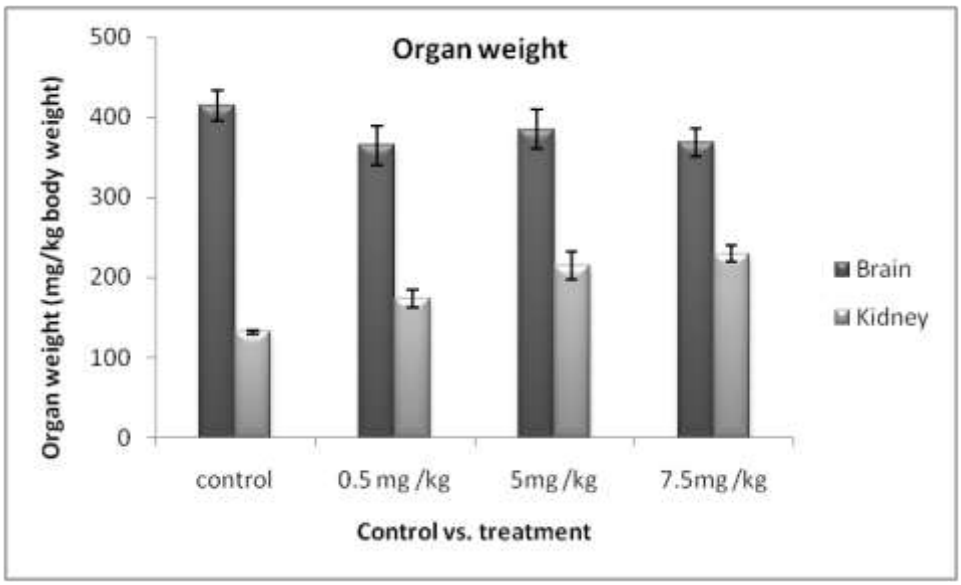
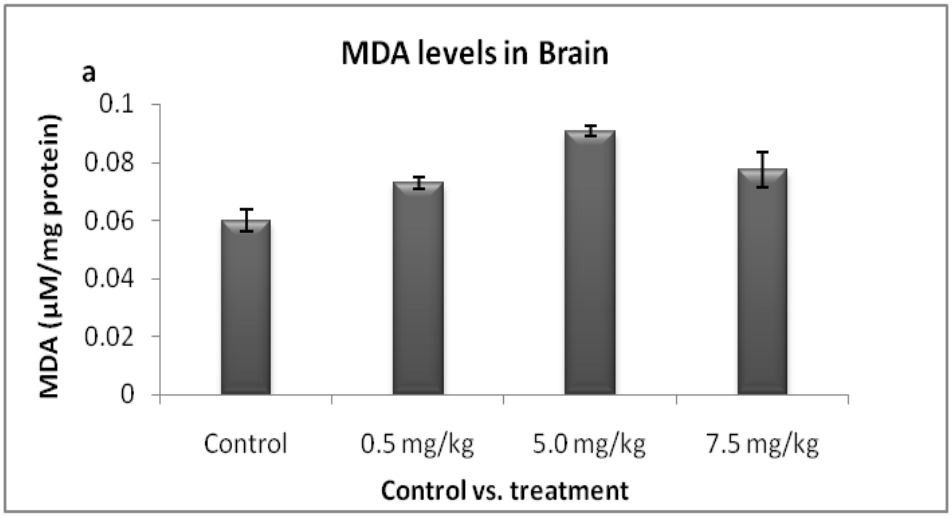



**Figure 1: The effect of Stanozolol treatment on the organ weight (brain and kidney) in female mice. Data is represented as the mean ± SE, $p < 0.01$; $n =$ 5 mice/group.**

**MDA, GSH, SOD and AChE activity in brain**

A significant increase in the lipid peroxidation marker namely, malondialdehyde (MDA) levels, was observed in the entire ST treated groups ($F_{3, 16} = 302.79$, $p < 0.001$; Figure 2a). Besides, a noticeably higher ($p < 0.001$) MDA activity was observed in II group when comparison was made among the groups following Tukey's *post-hoc* test. However, all the ST treated groups exhibited a significant decrease in GSH ($F_{3, 16} = 267.14$, $p < 0.001$; Figure 2b) and SOD ($F_{3, 16} = 182.33$, $p < 0.001$; Figure 2c) enzyme activities.



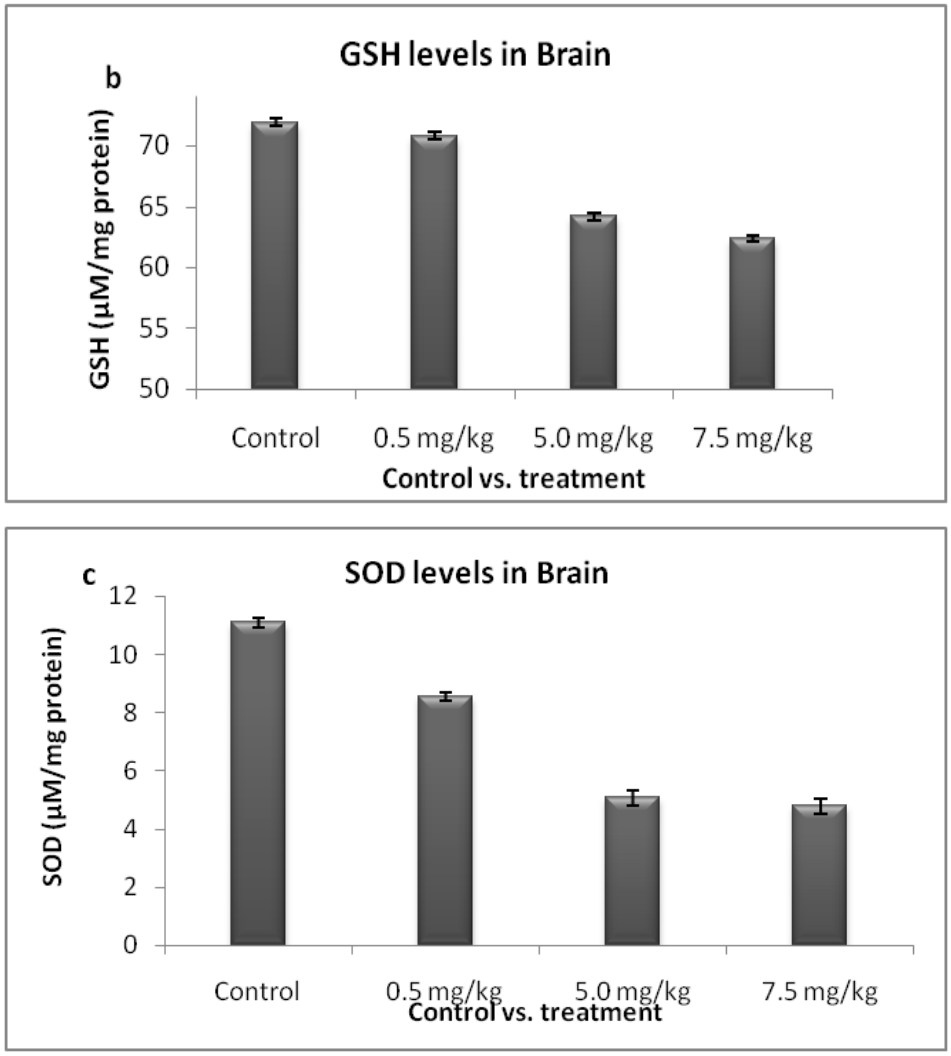



**Figure 2: The impact of ST treatment on MDA (a), GSH (b), and SOD (c) levels in brain homogenate of female mice. Data is represented as the mean ± SE, $p < 0.01$; $n = 5$ mice/group.**

The neurotransmitter, *AChE* activity in the brain tissue was found to be reduced ($F_{3, 16} = 57.04$, $p < 0.001$) in all ST treated groups (Figure 3).

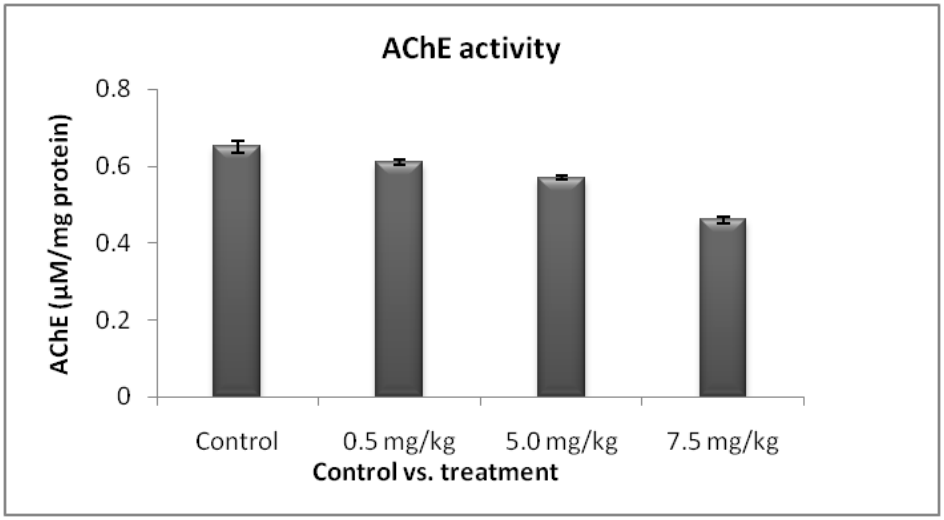
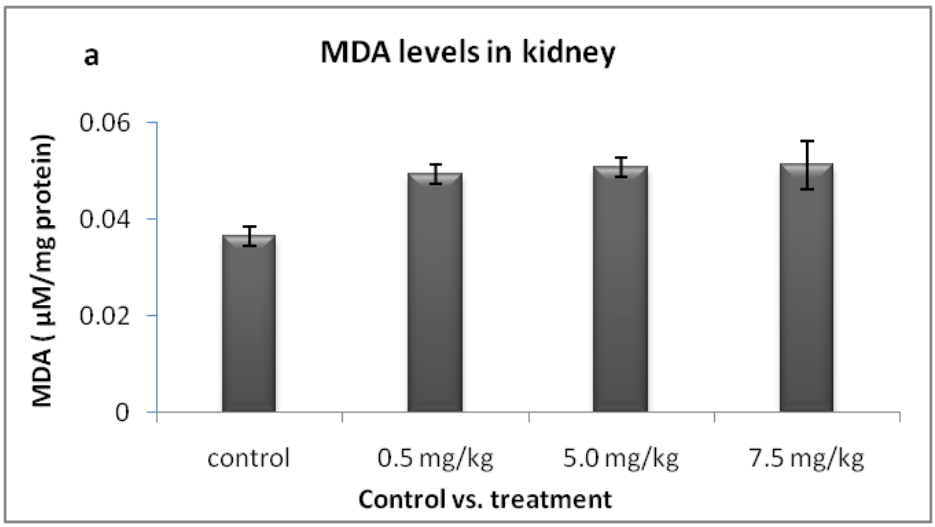



**Figure 3: The effects of ST treatment on the AChE activity in mice brain. Data is represented as the mean ± SE, $p < 0.01$; $n$= 5 mice/group.**

**MDA, GSH and SOD activity in kidney**

A significant increase in MDA level ($F_{3, 16} = 1.782$, $p < 0.001$) was observed in all the ST treated groups (Figure 4a). In contrast to MDA activity, a reduction in the activities of first line redox defense enzyme SOD ($F_{3, 16} = 177.49$, $p < 0.001$; Figure 4b) and GSH activity ($F_{3, 16} = 1360$, $p < 0.001$; Figure 4c) was noticed in medium and high dose treatment groups with the exception of low dose treated group.



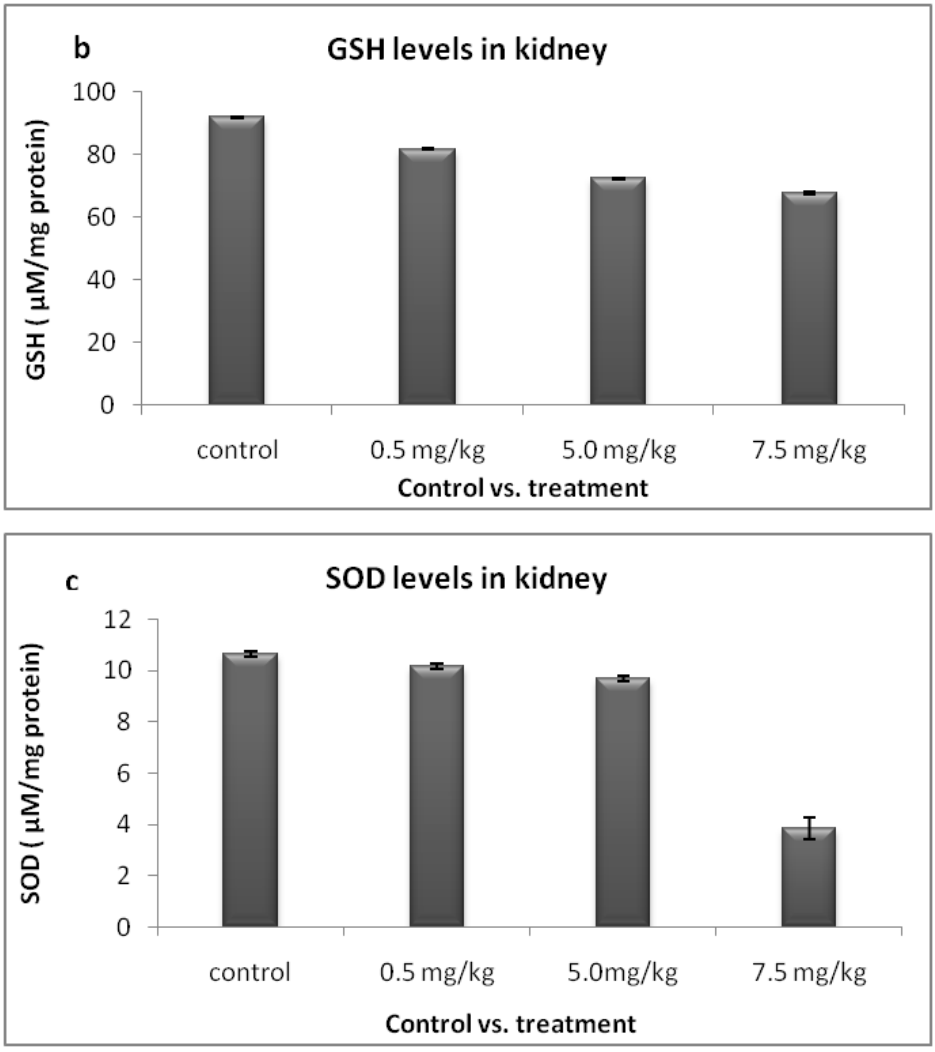



**Figure 4: The effects of ST treatment on MDA (a), GSH (b), and SOD (c) content in the kidney in mice. Data is represented as the mean ± SE, $p < 0.01$ and $p < 0.05$; $n = 5$ mice/group.**

**Histopathology of kidney**

The histological features of kidney in control group revealed prominent numerous Bowman's capsule with peripheral squamous epithelium; glomeruli with optimum urinary space and normal convoluted tubules thus exhibiting normal cellularity (Figure 5 A & C). While the kidney of medium and high dose ST treated mice exhibited shrunken glomeruli with minimized capillary tuft and increased urinary space (Figure 5B & D). Further, at certain regions enlarged glomeruli with infiltration of endothelial cells leading to hyperplasia, hemorrhage and tubular necrosis were also noticed in high dose treatment group. Also, at some regions dilated convoluted tubules and hemorrhage were noticed (Figure 6B, C & D).

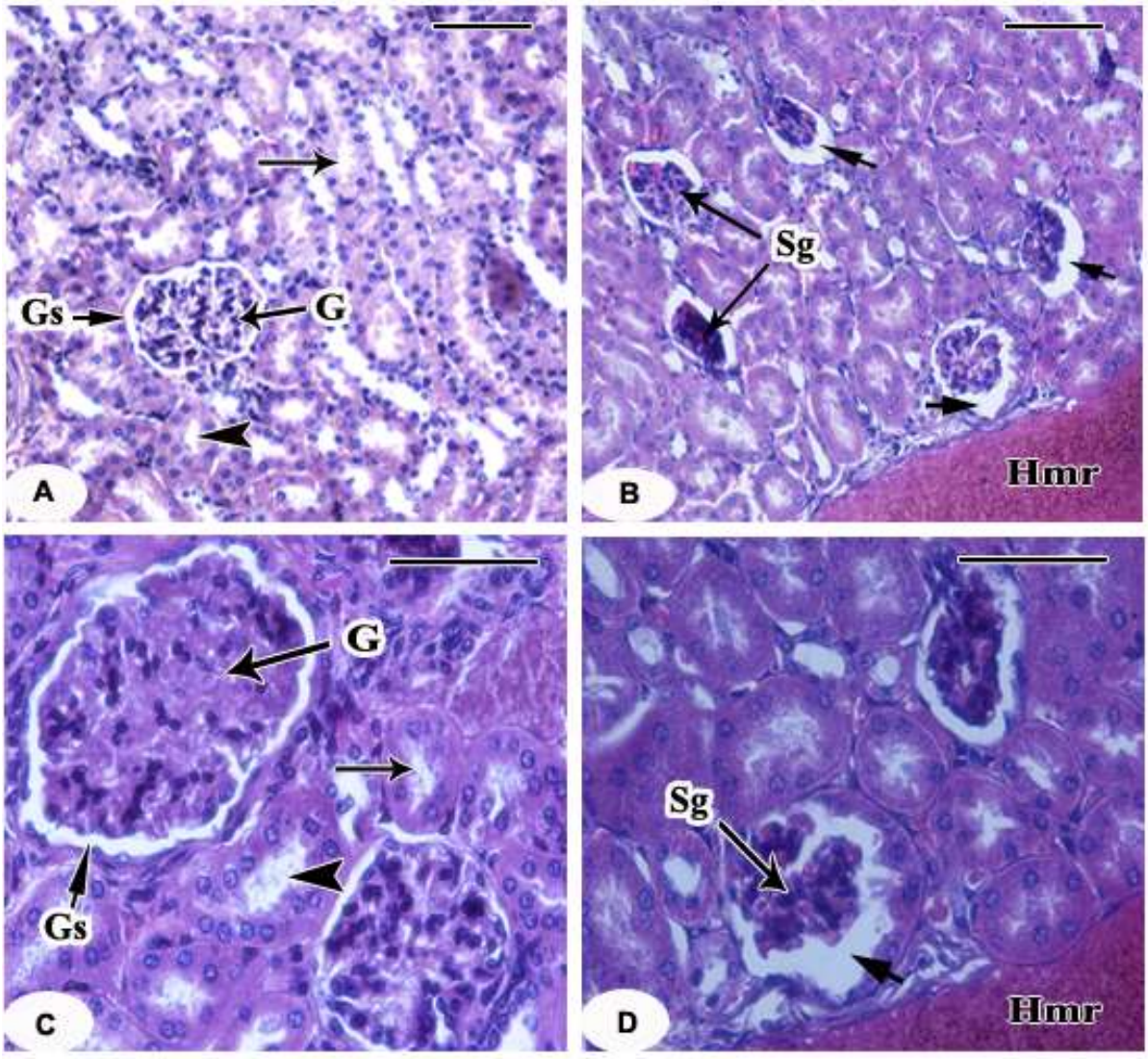



**PLATE- 1: (A – D) Sections of kidney treated with stanozolol (Daily) for 30 days, showing histopathological description of kidney**

**1A –** T.S. of the kidney of control mice showing prominent Bowman's capsule with peripheral squamous epithelium, glomeruli (G), revealing optimum glomerular space (Gs) and normal convoluted tubules (arrow head and arrows) thus exhibiting normal cellularity. (40x)

Scale line – 40 µm

**1B –** T.S. of the kidney of Stanozolol treated mice showing shrunken glomeruli (Sg) with minimized capillary tuft and increased glomerular space (arrows). Hmr - haemorrhage. (40x)

Scale line – 40 µm

**1C –** Magnified view of Fig. 1A revealing T. S. of the kidney of control mice.

G – Glomerulus ; Gs - Glomerular space; Arrow heads and arrows – Convoluted tubules. (20x)

Scale line – 30 µm

**1D –** Magnified view of Fig. 1B showing T. S. of the kidney of Stanozolol treated mice.

Sg - Shrunken glomeruli; Arrows - Glomerular space; Hmr - Haemorrhage. (20x)

Scale line – 30 µm

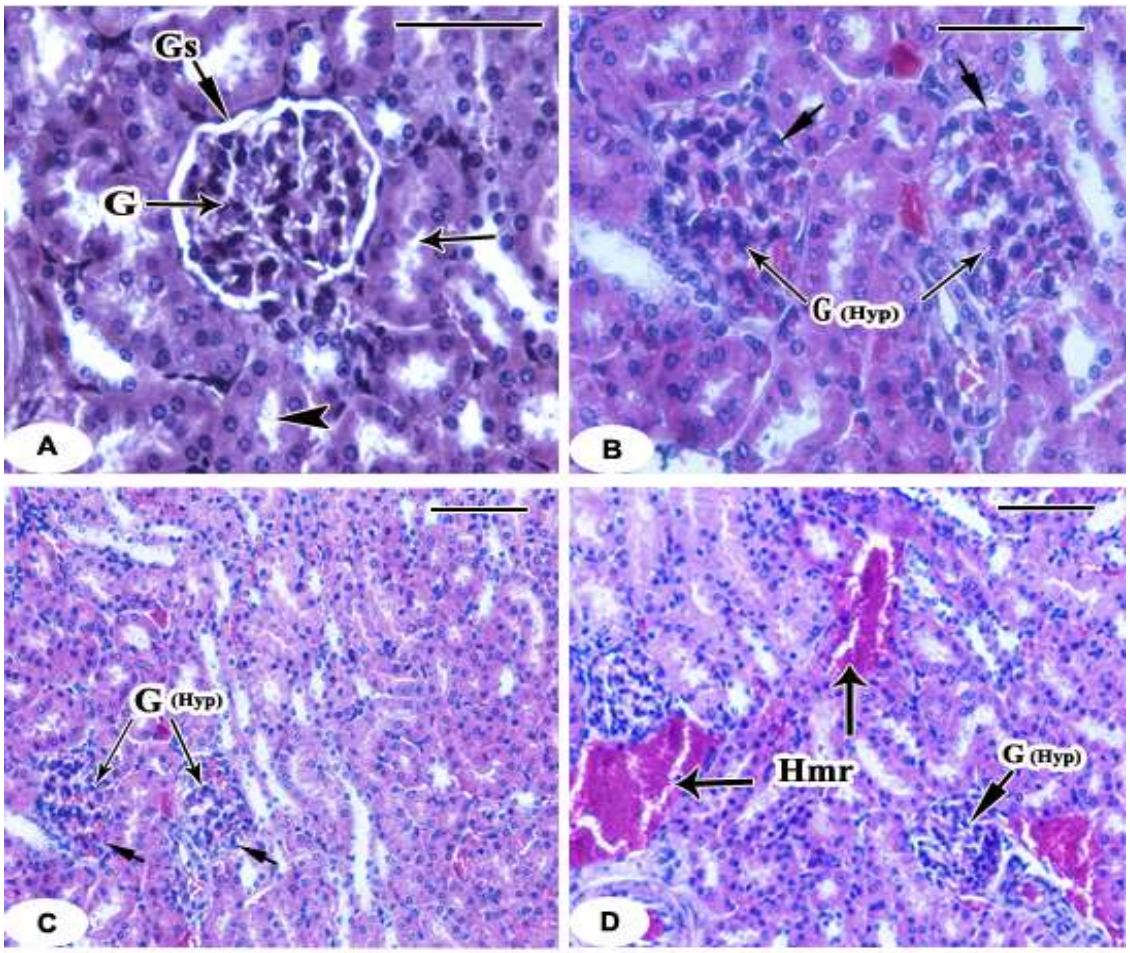



**PLATE- 2: (A – D) Sections of kidney treated with stanozolol (Daily) for 30 days, showing histopathological details of kidney**

**2A** – T.S. of the kidney of control mice showing prominent Bowman's capsule, normal glomeruli (G), revealing optimum glomerular space (Gs) and normal convoluted tubules (arrow head and arrows) thus exhibiting normal cellularity. (20x)

Scale line – 30 µm

**2B** – T.S. of the kidney of mice treated with Stanozolol (high dose) showing enlarged glomeruli with infiltration of endothelial and blood cells leading to hyperplacia $G_{(Hyp)}$ of the glomerulus. Note the narrowing of glomerular space (arrows). (20x)

Scale line – 30 µm

**2C** – T.S. of the kidney of mice treated with Stanozolol (high dose) at low magnification revealing hypertrophied glomeruli - $G_{(Hyp)}$ with narrowing glomerular space (arrows). (40x)

Scale line – 40 µm

**2D** – T.S. of the kidney of mice treated with Stanozolol (high dose) revealing Haemorrhage (Hmr) at certain places. $G_{(Hyp)}$ - Hypertrophied glomeruli. (40x)

Scale line – 40 µm

## DISCUSSION

Androgens induce oxidative stress and up regulate components of ROS leading to imbalance between pro-oxidant components and anti-oxidant defense[17,18].

**Brain- antioxidant enzyme and AChE enzyme activity:**

In the present investigation, a significant decrease in the activity of SOD and GSH in ST treated mice brain homogenate divulges an increased superoxide radical production and other ROS. This suggests that ST induces imbalance between free radicals and antioxidants which might have resulted in attenuation of antioxidant enzymes leading to oxidative damage. Besides an augmented level of lipid peroxidation marker, MDA enzyme activity in the medium and high dose treatment groups suggests the up regulation of lipid peroxidation. The observed result indicates that ST disrupts the cellular redox balance there by leading to local oxidative stress in mice brain, in a dose-dependent manner. Parallel to our results, chronic use of ST significantly affected brain monoamines leading to neurochemical modifications which possibly involved in depression and stress-related states in Wister rats [25]. On the contrary, the high intensity exercise reduces the AAS (single dose) mediated negative effect on the brain redox status of male rat[16]. Similarly, testosterone induces a preset cellular adaptation to radiation therapy in prostate cancer cell lines[26]. Generally chronic AAS abuse results in different patterns of pathological alterations which depend on dose, duration, frequency and mode of use.

Cholinergic neurotransmission in the mammalian central nervous system is regulated predominantly by the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) which catalyzes the hydrolysis of the neurotransmitter acetylcholine to choline and acetate[27,28]. In the present investigation a concurrent decrease in AChE activity in the brain of ST treated mice may suggest the accumulation of AChE at synaptic junctions which in turn would result in the impairment of neuronal transmission. Since the regulation of cholinergic system through acetylcholine hydrolysis has been largely attributed to AChE activity, 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, warranting further investigation (a study in progress). Similar increase in AChE activity following treatment of Nandrolone decanoate (ND) suggested impairment in

neurotransmission and cholinergic modulation[29]. Likewise the supraphysiological levels of testosterone initiates apoptotic cascade in neuronal cells of human neuroblastoma cell lines[30].

**Kidney-antioxidant enzyme activity and histopathology:**

The present investigation demonstrates that prolonged treatment of ST leads to significant increase in MDA level and de crease in antioxidant enzyme activities (SOD and GSH) in kidney homogenates suggesting its diminished ability to scavenge toxic hydrogen peroxide and lipid peroxides. It is also evident from the results that increase in lipid peroxidation, as normal levels of antioxidant produced could not quench the excess free radicals generated by ST. Also, fall in the GSH level may suggest its usage to reduce lipid peroxidation. However, the impairment in the antioxidant biomarkers in low dose treatment group is not significant. The observed findings are in agreement with those observed by Tsitsimpikou et al[31]. Wherein prolonged treatment of ND leads to an increase in MDA and decrease in GSH levels in male rabbits. Similarly long term administration of ND leads to an increased MDA, reduced glutathione peroxidase (GPx) and glutathione reductase (GR) activity in kidney of CD1 mice[12]. Overall, it is inferred that altered renal function is closely associated with considerable dose and duration related oxidative stress.

In the present investigation, ST treated mice shows a significant enhancement in the weight of the kidney when compared to brain. Our finding is in accordance with earlier reports that treatment of AAS such as testosterone and ND lead to the increase in weight of the kidney[32,33]. Also, Hoseini et al. reported an increase in the volume of the renal cortex and its two main parts[32] followed by ND treatment in mice.

The histopathology of medium and high dose ST treated mice kidney encountered with shrunken glomeruli with minimized capillary tuft and increased Bowman's space leading to alteration in the histoarchitecture of the kidney, indicating impairment in the renal function. Besides, in the chronic dose ST treated mice, certain cortical regions of the kidney reveal enlarged glomeruli with infiltration of endothelial cells leading to hyperplasia. It may be due to partially obstructed glomerular capillary loops by a mixture of activated endothelial cells and mesengial cells. Similar results were observed in the kidney of rabbit[34] and guinea pig[35] following the treatment of the AAS compounds, Boldenone and Sustenone, respectively. Likewise administration of ND led to glomeruli fragmentation, focal tuft collapse with hypertrophy and hyperplasia in mice[12]. The end stage of renal dysfunctions portraying nephrosclerosis is reported in bodybuilders abusing chronic dose of AASs[36].

Overall, our results reveal that long term treatment of ST induces local antioxidative stress in brain and kidney in female mice. Besides it alters AChE activity which may lead to impairment

in neuronal transmission and/or some neurological disorders warranting further investigation (a study in progress). This may attribute to the fact that the ST (a 17 α-alkylated compound) is a non-aromatizable compound and does not serve as precursor for estrogen production leading to the increased level of androgen. This increase in androgen level perhaps affects the neuronal activity in the brain and possibly inducing renal dysfunction.

## CONCLUSION

The degree of impact of ST on impairment in antioxidants defense system is significantly more pronounced in brain when compared to kidney.

1. ST treatment leads to noteworthy alterations in terms of reduction in AChE activity levels in the brain which may lead to impairment in neuronal transmission.
2. Histopathological analysis in medium and high dose treatment group of kidney encountered changes in its histoarchitecture. Therefore, it is inferred that the prolonged administration of ST induces alterations in local antioxidant cascade in brain and kidney thereby disrupting cellular redox balance in dose dependent fashion.

## ETHICAL APPROVAL

All experiments were conducted in accordance with the regulations of CPCSEA guidelines and the Institutional Animal Ethical Committee No. 639/GO/02/a/CPCSEA of the Department of Zoology, Karnatak University, Dharwad, and Karnataka, India.

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## REFERENCES

1. Basaria S, Wahlstrom JT, Dobs AS. Clinical review 138: anabolic-androgenic steroid therapy in the treatment of chronic diseases. J Clin Endocrinol Metab 2001; 86(11): 5108-5117.
2. Kochakian CD. Anabolic androgenic steroid: A historical perspective and definition, in: C.E.Yesalis (Ed.), anabolic steroids in sports and exercise. Champaign, IL: Human kinetics publishers, Inc 1993; 3-33.
3. Lukas SE. CNS effects and abuse liability of anabolic-androgenic steroids. Annu Rev Pharmacol Toxicol 1996; 36: 333-357.

4. Shahidi NT. A review of the chemistry, biological action and clinical applications of anabolic- androgenic steroid. Clin Ther 2001; 23(9): 1355-1390.
5. Wilson JD. Androgens abused by athletes. Endocr Rev 1998; 9(2): 181-199.
6. Lund BC, Perry PJ. Androgenic anabolic steroids: an overview for clinicians. Medscape Pharmocotherapy 2000; 2(2): 1-7.
7. Hoffmann U. Anabolic steroids – A problem in popular sports. *T+K* 2002; 69(3): 136.
8. Hartgens F, Kuipers H. Effects of androgenic-anabolic steroids in athletes. Sports Med 2004; 34(8): 513-554.
9. Inamdar (Doddamani) LS, Jayamma Y. Acceleration of neutrophil precursors' maturation and immunostimulation of CD3+, CD4+ lymphocytes by stanozolol in mice. J Steroid Biochem Mol Biol 2012; 129(3-4): 172-178.
10. Kicman AT. Pharmacology of anabolic steroids. Br J Pharmacol 2008; 154(3): 502-521.
11. Marshall-Gradsnik S, Green R, Brenu EW, Weatherby RP. Anabolic androgenic steroids effects on the immune system: a review. Cent Eur J Biol 2009; 4(1): 19-33.
12. Riezzo I, Turillazzi E, Bello S, Cantatore S, Cerretani D, Paolo MD et al. Chronic nandrolone administration promotes oxidative stress, induction of pro-inflammatory cytokine and TNF-α mediated apoptosis in the kidneys of CD1 treated mice. Toxicol Appl Pharmacol 2014; 280(1): 97-106.
13. Yavari A. Abuse of anabolic androgenic steroids. J Stress Physiol Biochem 2009; 5(3): 22-32.
14. Devasagayam TPA, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS, Lele RD. Free radicals and antioxidants in human health: current status and future prospects. JAPI 2004; 52: 794-804.
15. Sayre LM, Perry G, Smith MA. Oxidative stress and neurotoxicity. Chem Res Toxicol 2008; 21(1): 172-188.
16. Camiletti-Moiron D, Aparicio VA, Nebot E, Medina G, Martinez R, Kapravelou G, et al. High-intensity exercise modifies the effects of stanozolol on brain oxidative stress in rats. Int J Sports Med 2015; 36(12): 984-991.
17. Frankenfeld SP, Oliveira LP, Ortenzi VH, Rego-Monteiro ICC, Chaves EA, Ferreira AC, et al. The Anabolic androgenic steroid nandrolone decanoate disrupts redox homeostasis in liver, heart and kidney of male wistar rats. PLoS ONE 2014; 9(9): e102699.

18. Pay A, Saborido A, Blazquez I, Delgado J, Megias A. Effects of prolonged stanozolol treatment on antioxidant enzyme activities, oxidative stress markers, and heats shock protein HSP72 levels in rat liver. J Steroid Biochem Mol Biol 2003; 87(4-5): 269–277.
19. Pomara C, Neri M, Bello S, Fiore C, Riezzo I, Turillazzi E. Neurotoxicity by synthetic androgen steroids: oxidative stress, apoptosis and neuropathology: A review. Curr Neuropharmacol 2015; 13(14): 132-145.
20. Irfan R, Aruna K, Saibal KB. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nature Protocols 2007; 1: 3159-3165.
21. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969; 244(22): 6049-6055.
22. Yagi K. Simple procedure for specific assay of lipid hydroperoxides in serum or plasma. Free radical and antioxidant protocols 1998; 108: 101-106.
23. Ellman GL, Courtney KD, Andres VJr, Featherstone RM. A new and rapid colorimetric determination of acetyl cholinesterase activity. Biochem Pharmacol 1961; 7: 88-95.
24. Gorun V, Proinov I, Baltescu V, Balaban G, Barzu O. Modified Ellman procedure for assay of cholinesterases in crude enzymatic preparations. Anal Biochem 1978; 8: 324-326.
25. Tucci P, Morqese MG, Colaianna M, Zotti M, Schiavone S, Cuomo V. Neurochemical consequence of steroid abuse: stanozolol-induced monoaminergic changes. Steroids 2012; 77(3): 269-275.
26. Pinthus JH, Bryskin I, Trachtenberg J, Lu JP, Singh G, Fridman E, et al. Androgen induces adaptation to oxidative stress in prostate cancer: implications for treatment with radiation therapy. Neoplasia 2007; 9(1): 68-80.
27. Giacobini E, Pepeu G. [Eds.] In: The brain cholinergic system in health and disease. Abingdon [England]: Informa Healthcare 2006.
28. Silver A. The biology of cholinesterases. North-Holland, The Netherlands, 1974.
29. Martins DB, Mazzanti CM, Spanevello R, Schmatz R, Cargnelutti JF, Schmidt C, et al. Subchronic administration of nandrolone decanoate in acetylcholinesterase activity in wistar rats. Comp Clin Pathol 2012; 21: 256-268.
30. Estrada M, Varshney A, Ehrlich BE. Elevated testosterone induces apoptosis in neuronal cells. J Biol Chem 2006; 281(35): 25492-25501.

31. Tsitsimpikou C, Vasilaki F, Tsarouhas K , Fragkiadaki P, Tzardid M, Goutzourelas N, et al. Nephrotoxicity in rabbits after long-term nandrolone decanoate administration. Toxicol Lett 2016; 259: 21-27.
32. Hoseini L, Roozbeh J, Sagheb M, Karbalay-Doust S, Noorafshan A. Nandrolone decanoate increases the volume but not the length of the proximal and distal convoluted tubules of the mouse kidney. Micron 2009; 40: 226-230.
33. Mills NC, Bardin CW. New androgen-stimulated proteins in the kidney of female mice. Endocrinology 1980; 106(4): 1182-1186.
34. Tousson E, El-Gerbed MSA, Shaleby S. Effect of maturity on histopathological alteration after a growth promoter boldenone injection in rabbits. J Am Sci 2011; 7(12): 1074-1080.
35. Ahmed Bin Bisher AS. Histopathological evidences of the nephritic pathological alterations induced by the anabolic androgenic drug (Sustanon) in male guinea pigs (*Cavia porcellus*). J Biol Sci 2009; 6: 514-523.
36. Hurtaguan F, Gerth J, Funfstuck R, Grone HJ, Stein G. End-stage renal disease in a bodybuilder: a multifactorial process or simply doping? Nephrol Dial Transplant 2001; 16: 163-165.