



Modulation Of The Neurochemical And Behavioral Changes Induced By Nandrolone Decanoate And Nicotine Combination In Rats Using Grape Seed Extract



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Abstract

Nandrolone decanoate is a synthetic anabolic steroid used by both athletes and non-athletes extensively, especially in combination with nicotine in smokers. The goal of the current study was to investigate the neurochemical and behavioral alterations of nandrolone decanoate combined with nicotine and the potential modulation effect of grape seed extract. Rats were daily treated with nandrolone (Nan 15 mg/kg s.c), nicotine (Nic 0.75 mg/kg, i.p.), grape seed extract (GSE 100 mg/kg, i.p.), Nan/Nic, Nan/Nic/GSE 50 mg/kg or Nan/Nic/GSE 100 mg/kg in combination for 14 days. Oxidative stress biomarkers (malondialdehyde, glutathione and nitric oxide), peroxisome proliferator-activated receptor alpha and acetylcholinesterase in brain homogenate were measured. Open field, defensive aggression, irritability, novel object recognition paradigms and brain histopathology were done. The control group received arachis oil subcutaneously and i.p. saline. Nandrolone alone increased anxiety, defensive aggression, and irritability compared to the normal group. Exposure to Nic increased locomotion, rearing, and defensive aggression compared to the normal group. When Nan/Nic were given together they had no significant effect on locomotion, rearing, or anxiety compared with the normal group. In contrast, defensive aggression and irritability increased with the Nan/Nic combination. Memory performance (object recognition) was decreased by Nan and Nan/Nic coadministration yet increased after Nic treatment. Grape seed treatment especially the high dose normalized irritability and defensive aggression in comparison to the Nan/Nic group. Nandrolone or Nic being given alone or in combination significantly increased lipid peroxidation of the brain (malondialdehyde) and nitric oxide, while decreased reduced glutathione and peroxisome proliferator-activated receptor alpha (PPAR- α) levels were decreased. These biochemical alterations were markedly alleviated by treatment with GSE. In addition, degenerated neurons were observed in the brains of the Nan, Nic or Nan/Nic groups but not in the Nan/Nic/GSE combination groups. Our results suggest that GSE can modulate the behavioral, biochemical, and histopathological alterations in rats exposed to nandrolone and nicotine.

Keywords: Anabolic steroids; Nandrolone; Nicotine; Grape seed extract

1. Introduction

Anabolic androgenic steroids (AASs), such as nandrolone decanoate, are synthetic testosterone compounds with anabolic and androgenic properties (Van Amsterdam et al. 2010). AASs are absorbed rapidly from the small intestine and metabolized by 5 α -reductase enzyme in the liver (Monda et al. 2017).

AASs are popularly abused indiscriminately by adolescents (mainly adolescent males) and adults, athletes and non-athletes, and bodybuilders to improve their physical abilities and increase their muscle mass (Woerdeman et al., 2010). The World Anti-Doping Agency and Olympic committees prohibited and sanctioned the use of AASs as they were declared

controlled substances (García-Arnés and García-Casares, 2022). These agents are being used in supraphysiological doses by athletes causing a number of undesirable physiological, behavioral as well as psychological effects ranging from marked aggression to depression, increased anxiety and irritability (Bertozzi et al., 2018). During the past two decades, it was observed in several countries that adolescents misuse AASs to become braver, gain weight as well as boost physical and sports performances (Kindlundh et al., 2004).

On the other hand, these drugs are in use for the treatment of many disorders such as hypogonadism (Boje, 1939), growth retardation (Basaria et al., 2001),

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renal insufficiency, hereditary angioedema (Shahidi, 2001; Gerez et al., 2005), sarcopenia (Tan and Scally, 2009) and in chemotherapy (especially breast cancer) (C  lerier et al., 2003; Clark and Henderson, 2003). AAS acts directly on certain receptors known as androgen receptors (ARs) while indirectly on others such as the estrogen receptors (ER) (Penatti et al., 2009). ARs exhibit a broad expression in the male locus coeruleus nuclei and the rat dorsal raphe (Hamson et al., 2004). Outstandingly, the pathophysiology of depression is directly influenced by these two monoaminergic nuclei (Belmaker and Agam, 2008).

Nicotine (Nic), being the most active chemical compound contained in tobacco, is a natural alkaloid that is chiefly found in the *Nicotiana tabacum* plant, family Solanaceae L (Henningfield and Zeller, 2006). Nicotine acts on nicotinic acetylcholine receptors (nAChRs) which are ligand-gated ion channels that are extensively spread in the brain both pre- and post-synaptically (Grady et al., 2007; El-Hiny et al., 2019). By modifying the flux across cell membranes of the cation, nAChRs adjust neuronal excitability as well as the release of different neurotransmitters affecting numerous behavioral and physiologic pathways such as motor function, synaptic plasticity, memory, learning and attention (Bertrand and Terry, 2018). The nAChRs abnormalities have been associated with neurologic disorders pathophysiology involving epilepsy, Parkinson's and Alzheimer's diseases in addition to psychological disorders including depression, schizophrenia and anxiety (Terry et al., 2023).

Nicotine has a profound effect on brain neurochemistry such as altering the release and reuptake of various neurotransmitters leading to short-term cognitive enhancements and long-term consequences on brain functions as it acts on the reward-related brain regions. Also, it evokes behavioral changes such as increased locomotion, alertness, improved concentration and reduced anxiety (Heishman et al., 2010).

The incident of concomitant use of AASs and nicotine had been world-wide implicated in many health, behavioral and social hazards. Henceforth, the combined use of nandrolone and nicotine could produce complex effects, resulting from functional interactions that alter the brain neurochemistry and behavioral outcomes (Patel et al., 2021; McNealy et al., 2023).

Grape seed extract is a naturally abundant source of bioactive phenolic compounds, mainly flavonoids (Jara-Palacios et al., 2016) that are extracted from the deciduous Climber *Vitis vinifera* seeds family Vitaceae (Jayamathi et al., 2011; Vitalini et al., 2011). Grape seeds have the highest concentration of phenolics as their extractable percentage is 60–70%. GSE has potent antioxidant properties as well as

beneficial effects against oxidative injury, anti-inflammatory, antithrombotic, antineoplastic, lipid-lowering, and cardioprotective properties (Foshati et al., 2021; Tofolean et al., 2016; Jambi and Khattab, 2019). Moreover, it was also found to improve motor and memory performances in cognitive and neuronal function with aging (Krikorian et al., 2012).

The purpose of the present study is to investigate the neurochemical and behavioral changes consequent to the combined nandrolone decanoate with nicotine and their possible modulation by GSE. Biochemical data were combined, oxidative stress biomarkers and PPAR- α were measured where its activation can inhibit the expression and the release of pro-inflammatory cytokines, chemokines and inflammatory enzymes. Also, PPAR- α activation has been implicated in increased locomotor activity and motor coordination as well as in the regulation of irritability or aggressive behavior in rats.

2. Material and methods

2.1 Animals

The National Research Center (NRC; Giza, Egypt) animal house colony provided adult male (180-200 g; 5-7 weeks old) Sprague-Dawley rats. The animals were kept in a natural environment for the duration of the investigation, under a 12-hour light/dark cycle, and were on a standard diet and tap water ad libitum. Animal procedures abide by the guidelines of the Institute ethics committee and the Guide for Care and Use of Laboratory Animals by the U.S. National Institutes of Health for the use of animals in experimental studies (Publication No. 85-23, revised 1985).

2.2 Drugs and chemicals

Nandrolone decanoate was obtained from the Nile Company for Pharmaceuticals and Chemical Industries, Egypt. Grape seed extract, which contained approximately 95% standardized proanthocyanidins was diluted in isotonic (0.9% NaCl) saline solution just before usage, was bought from the Arab Company for Pharmaceuticals and Medicinal Plants (MEPACO, Egypt). Nicotine was purchased from Sigma-Aldrich, MO, USA. Kits used for biochemical analyses were obtained from Biodiagnostic (Egypt).

2.3 Experimental design

After randomly dividing the rats into 7 groups, each group was comprised of 10 rats. They received daily injections for 14 successive days.

- Group 1: received arachis oil (0.1 ml s.c) and saline (0.2 ml i.p) served as the normal group
- Group 2: received grape seed extract (100 mg/kg i.p; Rabiei et al., 2016)
- Group 3: received Nan (15 mg/kgs.c; El-Shamarka et al., 2020)
- Group 4: received 0.75 mg/kg i.p Nic (Anshu and Flora, 2012)
- Group 5: received Nan (15 mg/kg s.c) in combination with Nic (0.75 mg/kg i.p)

- Group 6: received Nan in combination with Nic and GSE at 50mg/kg
- Group 7: received Nan in combination with Nic and GSE at 100mg/kg

2.4 Behavioral assessment

At the end of the experimental period, open field, defensive aggression and irritability tests were conducted to evaluate exploratory behaviors and motor coordination, offensive and defensive aggression as well as irritability and anxiety. While novel object recognition was done to evaluate long-term memory and cognition.

2.4.1 Open field test

The open field test was conducted in a wooden red-walled arena with white squares on the floor (80 x 80 x 40 cm), which were divided into a total of 16 equal squares by black lines. To avoid any external stimuli, the test was carried out in a quiet room with white light. The observation period lasted for 5 min where behaviors such as ambulation, rearing, and anxiety frequencies were recorded (Markel et al., 1989).

2.4.2 Defensive aggression test

In this test the reactivity of rats toward provocative stimuli is measured, in which the rat was lifted by its tail and allowed to habituate for 30 sec in a Plexiglas cage (60x31x41 cm dimensions). The rats' reaction was then assessed towards four different stimuli including a moving wooden rod that touched the rat's snout, an air puff at the back is blown by a 50 ml syringe, prodding the flanks with a wooden rod and grabbing with a gloved hand. The averages of the total scores from each individual stimuli test were recorded (Steenland et al., 2005).

2.4.3 Irritability test

Typically, the reaction of each rat was tested for 60 sec towards a pencil that was located 1 inch from the nose. The rating of the response was as follows; 3 (the pencil is bitten); 2 (the pencil is touched); 1 (no response) and 0 (moves away from the pencil). The rat was then held by the neck for an additional 60 sec while being rated for urination, defecation behaviors "3 (both drops and urine); 2 (only urine); 1 (only drops); 0 (none)" and vocalization "4 (continuous); 3 (intermittent); 2 (medium); 1 (few); 0 (no vocalization)". The total of all the above-mentioned scores was used to signify irritability. (Keleta et al., 2007).

2.4.4 Novel object recognition test (NOR)

The fundamental tenet of the experiment is predicated on rats' intrinsic propensity to study novel objects more thoroughly than familiar ones (Ennaceur, 2010). It is used to evaluate long-term cognition and memory (Nalivaeva et al., 2012). Three phases of the test are completed over the course of three days. Every rat was given 10 minutes to become acclimated to its surroundings (in a wooden box measuring 60 x 60 x 60) during the habituation phase of the experiment (Botton et al., 2010). On the second day, the second phase, referred to as the training phase, was carried

out. Two identical wooden pieces, constructed of non-toxic materials and having the same size, shape, and color, were used for familiarization. The two items were positioned inside the wooden box in opposite corners, 2 cm away from the walls (Hammond et al., 2004). For ten minutes, each rat was permitted to distinguish between the two items (Botton et al., 2010). The final day was the testing phase, during which one of the two identical objects was changed for a brand-new object with another size, shape, and color. The two objects were then available for each rat to investigate for 5 minutes (Goulart et al., 2010). After every rat, both the objects and the arena were thoroughly cleaned with 70% ethanol to counteract the impact of odor cues. By dividing the amount of time spent investigating a novel object by the total amount of time spent investigating a novel object and a familiar object (novel /novel+ familiar investigation), the recognition index (RI) was calculated (Nalivaeva et al., 2012).

2.5 Preparation of samples

Rats were anesthetized 24 hr after the last dose was administered for the purpose of blood collection. They were euthanized, by cervical dislocation, in order to homogenize (Heidolph, Germany) then centrifuge (Hermle, Germany) their dissected brains using a pH 7.4 ice-cold 0.1 M phosphate buffer saline as 20% w/v homogenate.

2.6 Biochemical assessment

2.6.1 Determination of brain malondialdehyde (MDA) content

The method is based on the reaction of one mole of the degradation product of lipid peroxides, with two moles of thiobarbituric acid in an acidic medium (pH 2-3) at high temperature. The resultant pink product was extracted in n-butanol and measured at wavelengths 532 and 520 nm and the difference in absorbance was calculated between the two determinations (Uchiyama and Mihara, 1978).

2.6.2 Determination of glutathione (GSH) content

The glutathione content of brain homogenate was measured according to the colorimetric method of Ellman (1959) which was modified by Bulaj et al., (1998). The method depends on the reduction of Ellman's reagent (5, 5' -dithiobis-2-nitrobenzoic acid) (DTNB) by sulfhydryl (SH) group in GSH to form an intense stable yellow color of 2-nitro-5-mercaptobenzoic acid which was measured colorimetrically at 412 nm using a double beam spectrophotometer.

2.6.3 Determination of brain nitric oxide (NO) content

The method of Montgomery and Dymock (1961) was used for the determination of total nitrate/nitrite as an index of NO production in brain homogenates, where Griess reagent determines NO content by forming a chromophore; from the diazotization of sulfanilamide by acidic nitrite, followed by coupling with N-(1-naphthyl)-ethylenediamine (bicyclic amine).

2.6.4 Determination of peroxisome proliferator-activated receptor alpha (PPAR- α)

Total RNA tissue extraction was performed using a Qiagen tissue extraction kit according to the manufacturer's protocol. Total RNA (0.5-2 μ g) was converted to cDNA using a high-capacity cDNA reverse transcription kit (Fermentas, USA). PCR primers were designed from RNA sequences in GenBank using Gene Runner Software (Hasting Software, Inc., Hasting, NY). The annealing temperature was calculated as 60°C for all primer sets. The reaction volume (25 μ l) of quantitative RT-PCR consisted of 2 \times SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer and 2 μ l cDNA. Amplification conditions were set for 2 min at 50°C, 10 min at 95 °C as well as 40 cycles of denaturation for 15 sec and annealing/extension for 10 min at 60°C. The data calculated from real-time assays was carried out with the Applied Biosystems Software (CA, USA), followed by the comparative threshold cycle method to evaluate the relative expression of the studied gene. The results were then normalized to the beta-actin gene (Assaf et al., 2020).

2.6.5 Determination of acetylcholinesterase activities

The determination of acetylcholinesterase activity in serum is a modification described by Gorun et al. (1978) of the Ellman et al. (1961) method. The principle is based on the measurement of thiocholine produced upon the hydrolysis of acetyl thiocholine where the color was read at 412 nm immediately.

2.7 Brain histology

Brain samples were dissected immediately after death for histopathological examination. The specimens were then fixed in 10 % neutral-buffered formalin saline for at least 72 hours. All the specimens were dehydrated in ascending grades of alcohol, cleared in xylene and finally embedded in paraffin wax. Serial sections of 6 μ m thick were cut and stained with haematoxylin and eosin for histopathological investigation.

2.8 Statistical analysis

Data were presented as means \pm standard error of the means (SEM) or median (minimum–maximum). Comparison between groups was carried out using the parametric one-way ANOVA test followed by Tukey multiple comparison test except for the open field test where nonparametric Kruskal-Wallis one-way ANOVA was carried out followed by Dunn multiple comparison test. Differences were considered significant when $P < 0.05$. Graph-pad Prism8.00 for Windows software (CA, USA) was used to plot graphs and carry out these statistical tests.

3. Results

3.1 Open field test (OFT)

The Nic-treated group showed an increase in ambulation by 60% compared to the Nan/Nic group which was normalized by the combination of

Nic/Nan/GSE at 50 and 100 mg/kg (Fig 1). While Nan/Nic group showed a decrement in ambulation relative to the normal group by 29%. On the other hand, Nan decreased ambulation and rearing by 44% and 41%, respectively, relative to the normal group (Fig 1). However, Nan and Nan/Nic/GSE100 groups increased grooming by 38% and 53%, respectively in comparison to the Nan/Nic group (Fig 1).

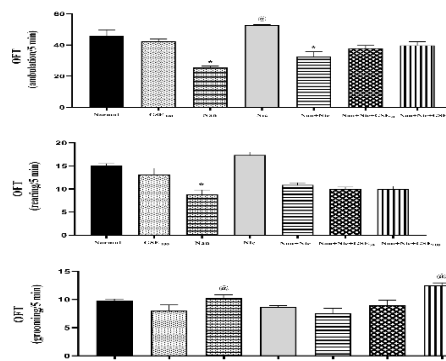


Fig (1): Effect of GSE and/or Nan, Nic administration on OFT (5 mins) in rats. Values are expressed as mean \pm SEM (n = 10). Statistical analysis was carried out by non-parametric Kruskal-Wallis one-way ANOVA followed by Dunn multiple comparison test. *, @ $P < 0.05$, compared to normal and Nan/Nic groups respectively

3.2 Defensive aggression test

The administration of Nan, Nic and Nan/Nic had a significant increase in defensive aggression by 61%, 58% and 64%, respectively, relative to the normal group. Nevertheless, GSE at 100 mg/kg group induced a significant decrease in defensive aggression by 43% compared to Nan/Nic group (Fig 2).

3.3 Irritability test

Compared with the normal group, treatment with Nan, Nan/Nic and Nan/Nic/GSE50 resulted in a significant increase in the irritability scores by 53%, 73% and 53%, respectively (Fig 2). On the other hand, GSE at 100 mg/kg group decreased irritability by 46% relative to the Nan/Nic group (Fig 2).

3.4 Novel object recognition test

The administration of Nan and Nan/Nic impaired memory performance in object recognition compared to the normal group by 30% and 12%, respectively. While the administration of GSE at 100 mg/kg and Nan/Nic/GSE100 increased memory performance relative to the normal group by 26% and 13%, respectively. Similarly, GSE at 100 mg/kg, Nic, Nan/Nic/GSE50 and Nan/Nic/GSE100 groups showed increased memory performance in relation to Nan/Nic group by 43%, 21%, 23% and 28%, respectively. However, decreased memory performance by 20% was observed in Nan group in relation to Nan/Nic group (Fig 2).

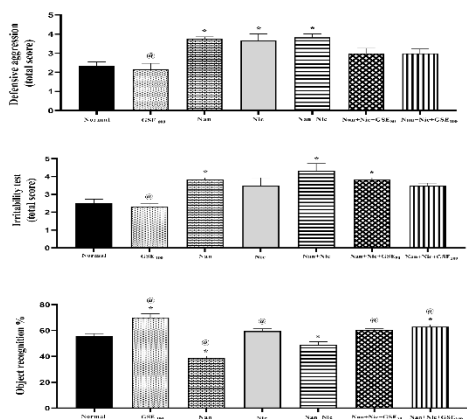


Fig (2): Effect of GSE and/or Nan, Nic administration on defensive aggression, irritability and novel object recognition tests in rats. Values are expressed as mean \pm SEM (n = 10). Statistical analysis was carried out by one-way ANOVA followed by Tukey multiple comparison test. *, @ P < 0.05, compared to normal and Nan/Nic groups respectively

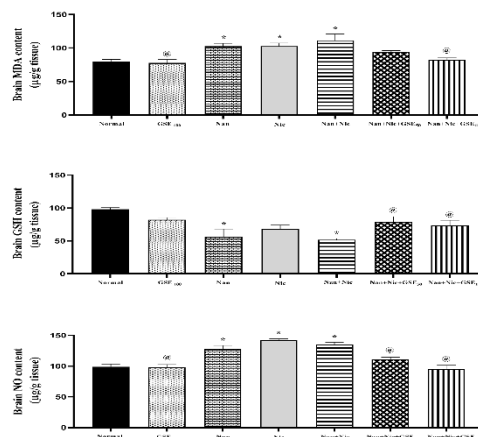


Fig (3): Effect of GSE and/or Nan, Nic administration on oxidative stress biomarkers in rats. Values are expressed as mean \pm SEM (n = 10). Statistical analysis was carried out by one-way ANOVA followed by Tukey multiple comparison test. *, @ P < 0.05, compared to normal and Nan/Nic groups respectively

3.5 Oxidative stress markers

Nan, Nic and Nan/Nic groups elevated MDA by 29%, 29% and 40%, respectively, relative to the normal group. The administration of GSE at 100 mg/kg exhibited decreased lipid peroxidation in relation to the Nan/Nic group (Fig 3). Correspondingly, the addition of GSE at 100 mg/kg to the Nan/Nic decreased lipid peroxidation by 26% in relation to the Nan/Nic group (Fig 3).

The administration of Nan and Nan/Nic increased oxidative stress by 43% and 47%, respectively, which was obvious in determining the content of reduced glutathione (GSH) as they decreased GSH in comparison to the normal group. On the other hand, groups of GSE at 50 and 100 mg/kg combinations modulated the GSH content almost back to normal in comparison to the Nan/Nic group (Fig 3).

The administration of Nan, Nic and Nan/Nic exhibited increased brain NO content relative to the normal group by 30%, 44% and 37%, respectively (Fig 3). On the contrary, GSE at 100 mg/kg, Nan/Nic/GSE50 and Nan/Nic/GSE100 improved brain NO content in comparison to Nan/Nic group by 27%, 18% and 30%, respectively (Fig 3).

3.6 PPAR- α level and AChE activity

Nan, Nic and Nan/Nic groups exhibited decreased PPAR- α levels by 67%, 54% and 55% relative to the normal, respectively. Nevertheless, GSE at 100 mg/kg and GSE at 50 and 100 mg/kg combinations showed increments in PPAR- α levels comparative to Nan/Nic group by 110%, 84% and 76%, respectively (Fig 4).

Though all treatments did not alter AChE activity, Nan increased AChE activity by 82% and 70%, respectively, compared to the normal and Nan/Nic groups (Fig 4).

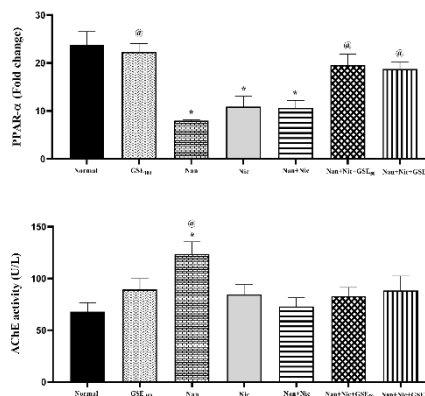


Fig (4): Effect of GSE and/or Nan, Nic administration on AChE activity in rats. Values are expressed as mean \pm SEM (n = 10). Statistical analysis was carried out by one-way ANOVA followed by Tukey multiple comparison test. *, @ P < 0.05, compared to normal and Nan/Nic groups respectively

3.7 Histopathological results

In the current study, no histopathological changes were observed in the cerebral cortex tissue, hippocampal or substantia nigra areas of normal rats treated with arachis oil (Fig 5A, 7A&9A, respectively).

Although rats receiving GSE at 100mg/kg showed no histopathological changes in the cerebral cortex tissue and hippocampal area; the substantia nigra area showed large neurons with cytoplasmic pigmented granules. These neurons had large vesicular nuclei and well-defined nucleoli (Fig 5B, 7B & 9B, respectively).

In groups receiving Nan, the cerebral cortex tissue exhibited neurons with small darkly stained bodies (Fig 5C). On the other hand, the hippocampal area appeared to have atrophied dark neurons (Fig 7C). Additionally, decreased number and size of pigmented neurons were found in the substantia nigra area (Fig 9C).

Furthermore, the cerebral cortex tissue of rats receiving Nic showed many neurons smaller in size than normal with deeply stained nuclei (Fig 5D). In the hippocampal area, many elongated neurons with dark cytoplasm and small apoptotic nuclei were observed (Fig 7D). The substantia nigra showed a marked decrease in size and number of pigmented neurons (Fig 9D). These effects were much deteriorated in all three brain areas in the case of combining Nan and Nic together (Fig 6A, 8A & 10A).

The administration of GSE at 50mg/kg in combination with Nan and Nic resulted in much amelioration of most of the neurons in all the three brain areas. The cerebral cortex area appeared normal with few small deeply stained neurons and still dilated capillaries were observed (Fig 6B). There was a noticeable increase in the hippocampal thickness marked with a decrease in the affected neurons (Fig 8B). Although the substantia nigra area showed normal large neurons with well-defined granules, some neurons were still of small size (Fig 10B).

The administration of GSE at 100 mg/kg in combination with Nan and Nic resulted in the normal appearance of mostly all the neurons in the cerebral cortex tissue as well as the hippocampal area (Fig 6C & 8C, respectively). Nevertheless, the substantia nigra revealed large normal neurons with well-defined granules, yet some neurons were still small (Fig 10C).

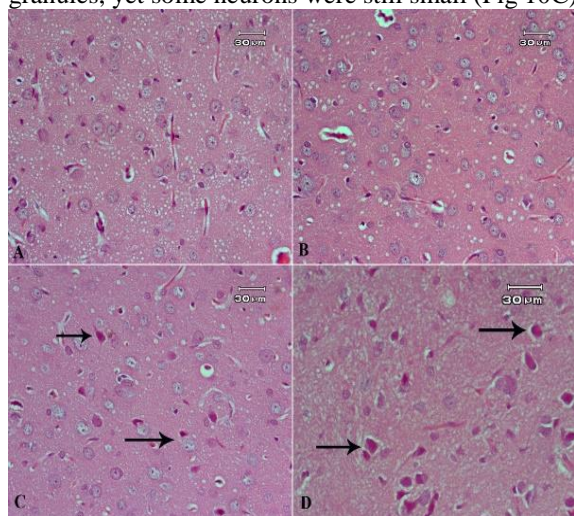


Fig 5: Photomicrographs of Hx & E-stained sections of rat cerebral cortex after treatment with (A) Saline showing normal appearance of neurons; (B) GSE at 100 mg/kg showing normal structure of cerebral cortical cells with large vesicular nuclei; (C) Nan alone showing some neurons with small darkly stained bodies in comparison to normal neurons (arrow); (D) Nic alone showing many neurons smaller in size than normal with deeply stained nuclei (arrow)

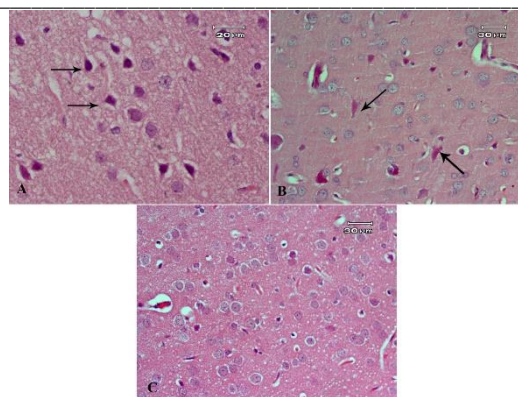


Fig 6: Photomicrographs of Hx & E-stained sections of rat cerebral cortex after treatment with (A) Nan/Nic showing an increase in affected neurons (small with deeply stained nuclei) and slightly dilated capillaries (arrow); (B) Nan/Nic/GSE 50 showing that most of the neurons appeared normal in appearance with few small deeply stained neurons (arrow). Dilated capillaries were still observed; (C) Nan/Nic/GSE 100 showed that mostly all the neurons appear normal

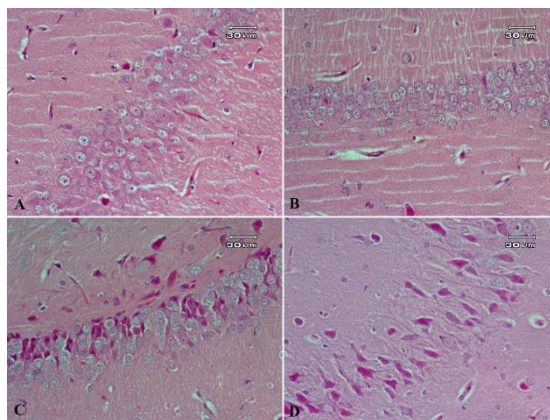


Fig 7: Photomicrographs of Hx & E-stained sections of rat hippocampal area after treatment with (A) Saline A normal rat showing the normal structure and orientation of neurons; (B) GSE (100 mg/kg) showed normal structure of the hippocampal area; (C) Nan showing some atrophied dark neurons among large normal neurons; (D) Nic showing many elongated neurons with dark cytoplasm and small apoptotic nuclei

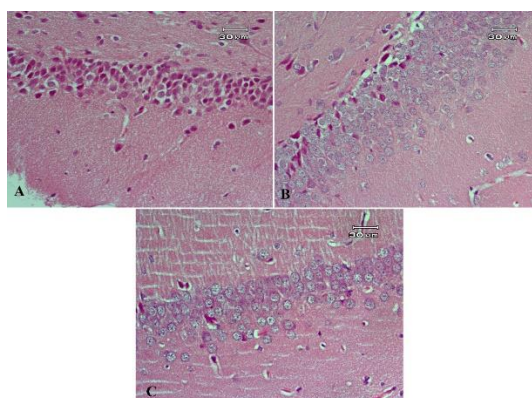


Fig 8: Photomicrographs of Hx & E-stained sections of rat hippocampal area after treatment with (A) Nan/Nic showing an increase of the damaged neurons; (B) Nan/Nic/GSE 50 showed marked decrease of affected neurons with a noticeable increase in hippocampal thickness; (C) Nan/Nic/GSE 100 showing normal neurons with large vesicular nuclei. Normal thickness of this area is observed

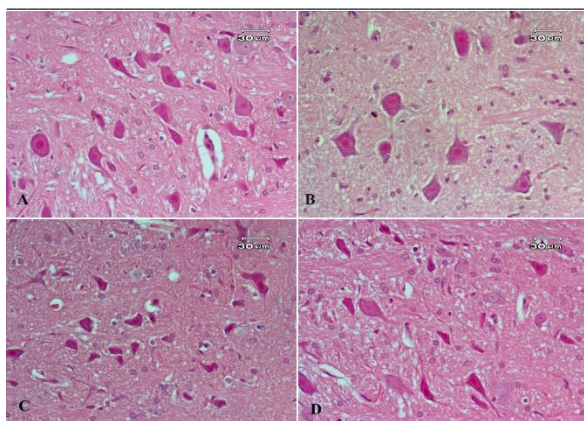


Fig 9: Photomicrographs of Hx & E-stained sections of rat substantia nigra after treatment with (A) Saline showing the normal structure of large, pigmented neurons; (B) GSE (100 mg/kg) showed large neurons with cytoplasmic pigmented granules, large vesicular nuclei and well-defined nucleoli; (C) Nan showing decrease in number and size of pigmented neurons; (D) Nic showing marked decrease in size and number of pigmented neurons

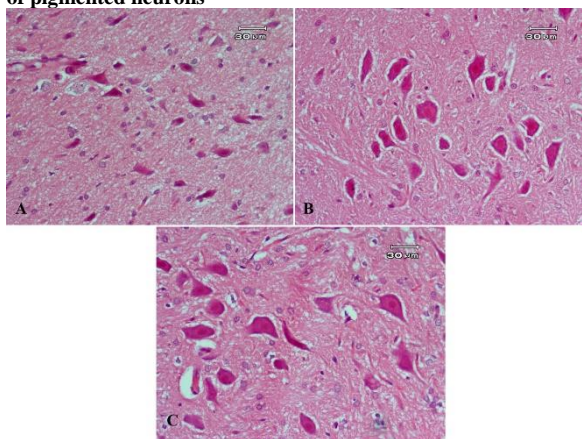


Fig 10: Photomicrographs of Hx & E-stained sections of rat substantia nigra after treatment with (A) Nan/Nic showing decrease in number of pigmented neurons that become flattened in shape with no well-defined granules; (B) Nan/Nic/GSE 50 showing many neurons with normal structure, although they are decreased in size; (C) Nan/Nic/GSE 100 showing normal large neurons with well-defined granules. Some neurons are still of small size

4. Discussion

The present study indicates that the co-administration of GSE with Nan and Nic could improve locomotion and memory, counteract aggression and irritability as well as attenuate the neurobiochemical and tissue damage caused by the administration of either Nan and/or Nic in a dose-dependent manner. GSE, known for its antioxidant and anti-inflammatory properties, has been shown to mitigate oxidative stress and protect against neurodegenerative disorders as confirmed by histopathological investigations. On the other hand, the administration of Nan and/or Nic induced more long-term deleterious neurochemical, cognitive as well as behavioral changes in rats, which are consistent with previous research (Niromand et al, 2021; Castro et al, 2023). There was a decrease in locomotion following the administration of Nan and

Nan/Nic which was supported by the observed effect found in substantia nigra.

Reactive oxygen species (ROS) play a crucial role in the pathology of the brain which results from its lipid-rich content and high oxygen consumption that increases its susceptibility to oxidative stress (Salim, 2017; Patel, 2016). Subsequently, several behavioral changes such as irritability, depression and memory impairments were reported to be linked to ROS (Maria Michel et al., 2012; Kanamaru et al., 2015; Black et al., 2017; Tanasawet et al., 2017).

In the present study, groups receiving Nan and/or Nic exhibited marked oxidative stress and impaired antioxidant defense mechanism indicated by the increase in the lipid peroxidation end-product MDA, as well as by the decrease in the antioxidant GSH in tissues and PPAR- α . There was also a significant and marked decrease in the brain levels of PPAR- α in rats treated with Nan, Nic, or their combination. These results are consistent with previous reports demonstrating the ability of Nan to induce oxidative stress and disturb normal antioxidant defenses (Ahmed and El-Awdan, 2015; Riezzo et al., 2014; Celec et al., 2003). Furthermore, Chang et al. (1995) reported that the enhancement of anabolic pathways by AAS increases oxidative stress. Calabrese et al. (2007) stated that NO is a highly reactive molecule that plays a key role in a variety of physiological and pathological processes, such as neuronal plasticity and neurotoxicity. The results showed that Nan, Nic and Nan/Nic treated groups exhibited elevated NO levels which was in line with other studies (Keser et al., 2013; El-Shamarka et al., 2020). GSE was shown to confer neuroprotection against brain induced chemical injury via inhibiting lipid peroxidation, the excessive release of NO and replenishing the depleted stores of reduced glutathione (Abdel-Salam et al., 2019). Nicotinic acetylcholine receptors (nAChRs), which are found throughout the brain, are activated by Nic resulting in an increase in calcium influx into the neurons. Calcium, in turn, activates neuronal nitric oxide (nNOS) which is responsible for NO production. Accordingly, the behavioral changes associated with the combined administration of Nan and Nic observed in the present work could be speculated to be partially attributed to oxidative stress. This was also evident in the results as an increase in aggression and irritability in groups receiving Nan and/or Nic that were normalized by the administration of GSE combinations.

Decreased PPAR- α expression can disrupt the normal breakdown of fatty acids and impair the body's ability to generate energy from fats. This can lead to an accumulation of lipids and contribute to oxidative stress leading to cellular damage and dysfunction. This could also be associated with neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases. Of note, our findings revealed that the combined treatment of GSE normalized oxidative stress biomarkers as well as the impaired

antioxidant and ameliorated tissue injury in a dose-dependent manner. It is worth mentioning that the brain relies heavily on efficient energy metabolism and protection against oxidative damage for optimal cognitive processes.

Furthermore, the administration of either Nic or GSE at 50 and 100 mg/kg combinations improved memory performance while Nan and Nan/Nic decreased it. This was in accordance with Magnusson et al. (2009) who stated that rats injected with Nan exhibited memory impairment. This was possibly due to the relationship between the androgen receptor and cognitive function which are mainly implicated with the hippocampus in the brain (Kerr et al., 1995; Daumas et al., 2007).

Although there is limited research on Nan and its effect on AChE our data complied with Ahmed and El-Awdan (2015) showing increased AChE activity suggesting that this might be related to the altered cholinergic neurotransmission associated with anabolic steroid use. AChE breaks down acetylcholine leading to changes in its synaptic availability and intervention with its postsynaptic outcome (Grisaru et al., 1999). Nan enhanced the AChE activity which may have resulted in the detected memory impairment as acetylcholine is important for memory and learning (Eidi et al., 2003; Easton et al., 2012; Wright et al., 1993).

The administration of Nan and/or Nic resulted in increased aggression and irritability. Rammal et al. (2008) and Salim et al. (2010) reported that anxiety has been suggested to be in part induced by oxidative stress and to be accompanied by reduced antioxidant defenses. The cholinergic neurons may be damaged by the increase in oxidative stress which might contribute to aggression and memory impairment (Patki et al., 2015; Xi et al., 2014).

The novel object recognition test evaluates long-term memory and cognitive functions hence, the observed behavioral changes imply the presence of cognitive dysfunction in Nan and Nan/Nic groups. Furthermore, these changes were accompanied by neurochemical alterations in the hippocampus with a significant decrease in the level of PPAR α . This was in line with the findings of Barker and Warburton (2011).

5. Conclusion

In conclusion, it appears that the administration of Nan in combination with Nic had deleterious neurochemical and behavioral effects that were obviously mitigated by GSE combinations. The improvement in neurobiochemical and behavioral aspects induced by GSE was indicated by the histopathological examination and was more prominent in the combined administration of GSE at the higher dose. Collectively, some parameters were worsened by Nan/Nic combination, hence it looks like Nan antagonized the Nic action. These data suggest

that the combined treatment of GSE may provide a new therapeutic strategy to ameliorate the side effects of Nan and/or Nic abuse. Our study, however, was limited to 14 days. Longer-term studies are still needed to delineate the neurobehavioral and neurobiochemical alterations consequent to the use of anabolic steroids in conjunction with nicotine and possible therapeutic remedies.

6. Conflict of interest

The authors declare that they have no conflict of interest.

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