Lipoic acid and pentoxifylline mitigate nandrolone decanoate-induced neurobehavioral perturbations in rats via re-balance of brain neurotransmitters, up-regulation of Nrf2/HO-1 pathway, and down-regulation of TNFR1 expression

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TNFR1

A B S T R A C T

Behavioral perturbations associated with nandrolone decanoate abuse by athletes and adolescents may be attributed to oxidative stress and inflammation. However, the underlying mechanisms are not yet fully explored. On the other hand, the natural antioxidant lipoic acid can pass the blood brain barrier and enhance Nrf2/HO-1 (nuclear factor erythroid-2 related factor 2/heme oxygenase-1) pathway. In addition, the phosphodiesterase-IV inhibitor xanthine derivative pentoxifylline has a remarkable inhibitory effect on tumor necrosis factor-alpha (TNF-α). Therefore, this study aimed at investigation of the possible protective effects of lipoic acid and/or pentoxifylline against nandrolone-induced neurobehavioral alterations in rats. Accordingly, male albino rats were randomly distributed into seven groups and treated with either vehicle, nandrolone (15 mg/kg, every third day, s.c.), lipoic acid (100 mg/kg/day, p.o.), pentoxifylline (200 mg/kg/day, i.p.), or nandrolone with lipoic acid and/or pentoxifylline. Rats were challenged in the open field, rewarded T-maze, Morris water maze, and resident–intruder aggression behavioral tests. The present findings showed that nandrolone induced hyperlocomotion, anxiety, memory impairment, and aggression in rats. These behavioral abnormalities were accompanied by several biochemical changes, including altered levels of brain monoamines, GABA, and acetylcholine, enhanced levels of malondialdehyde and TNF-α, elevated activity of acetylcholinesterase, and up-regulated expression of TNF-α receptor-1 (TNFR1). In addition, inhibited catalase activity, down-regulated Nrf2/HO-1 pathway, and suppressed acetylcholine receptor expression were observed. Lipoic acid and pentoxifylline combination significantly mitigated all the previously mentioned deleterious effects mainly via up-regulation of Nrf2/HO-1 pathway, inhibition of TNF-α and down-regulation of TNFR1 expression. In conclusion, the biochemical and histopathological findings of this study revealed the protective mechanisms of lipoic acid and pentoxifylline against nandrolone-induced behavioral changes and neurotoxicity in rats.

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Introduction

Androgenic anabolic steroids (AAS) are synthetic testosterone derivatives that are widely used by athletes, body builders and adolescents for improvement of performance and physical appearance (Tanner et al., 1995). Nevertheless, abusers may use AAS to provide them with a false sensation of braveness and fearlessness (Kindlundh et al., 1998). Notably, AAS doping has been associated with several behavioral alterations from delusions and manic episodes to outbursts of aggression, rage and brutal violence (Clark and Henderson, 2003). The latter is often elicited by the slightest provocation and is characterized by high intensity and long duration leading to vicious crimes (Choi and Pope, 1994). In addition, progressive cognitive impairment, especially in visuospatial memory, has been observed in illicit steroid users (Kanayama et al., 2013).

Nandrolone decanoate or 17b [(1-Oxodecyl) oxy]-est-4-en-one is a widely used AAS due to its anabolic, weak androgenic and non-virilizing effects (Wood, 2004). Its therapeutic implications include osteoporosis, aplastic anemia, cancer cachexia and weight loss in human immunodeficiency virus (HIV) and chronic obstructive pulmonary disease (COPD) patients (Seewald et al., 1989; Geusens, 1995; Storer et al., 2005). The common clinical dose range is 50–100 mg every 3–4 weeks (Hickson et al., 1989). However, nandrolone decanoate abusers may take doses that are 10–100 times higher than these prescription doses (Versalis and Bahrke, 1995). Supraphysiological doses of nandrolone decanoate may cause brain injury (Kurling et al., 2005; Novaes Gomes et al., 2014). Experimentally, nandrolone decanoate
has induced various paradoxical behavioral changes in rats including anxiogenic, anxiolytic, aggressive, depressant and memory impairment effects (Steensland et al., 2005; Rocha et al., 2007; Magnnsson et al., 2009). Several mechanisms are involved in nandrolone decanoate-induced behavioral changes including oxidative damage, inflammation, imbalance in brain neurotransmitter systems, modulation of nerve growth factor and neuronal apoptosis (Pieretti et al., 2013; Tanekhar et al., 2013; Rainer et al., 2014). However, the exact mechanistic pathways underlying nandrolone decanoate-induced neurobehavioral deterioration have not yet been explored. In addition, there is a persistent need for protective pharmacological interventions against nandrolone decanoate-induced behavioral alterations and neuronal injury.

Lipoic acid, thiotic acid, or 1,2-dithiole-3-pentonic acid, is a natural organo-sulfur compound that plays an essential role in the cellular oxidative metabolism by acting as a cofactor for mitochondrial citric acid cycle enzymes, namely α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (Yanagawa and Egami, 1975). Lipoic acid is found in various types of food e.g. kidney, liver, heart, yeast extract, broccoli and spinach. However, dietary sources contain little amounts of covalently bound lipoic acid resulting in poor bioavailability (Durrani et al., 2007). Therefore, exogenous lipoic acid is commonly available in the form of synthetic food supplements (Sitton et al., 2004). Endogenous lipoic acid is synthesized in the mitochondria from its precursors cysteine and octanoic acid (Bliska and Wolde, 2005). Lipoic acid is reduced to its biologically active form dihydrolipoic acid in the CNS and almost all other tissues (Packer et al., 1997). It readily passes across the blood brain barrier and provides outstanding defense against tissue damage induced by free radicals (Panigrahi et al., 1996). Either lipoic acid or its reduced form can suppress oxidative stress via redox regeneration of reactive oxygen species, suppression of NF-αB activation, or by chelation of transition metals such as iron and copper (Bast and Haenen, 2003; Ying et al., 2011). It is noteworthy that copper and iron may aggravate the progression of Alzheimer’s disease by inhibition of acetylcholinesterase enzyme (Sayre et al., 2000; Pohanka, 2014). Experimentally, lipoic acid has demonstrated neuroprotective effects in animal models of mania, hyperlocomotion, convulsions, and age-induced dementia (Liu, 2008; Macêdo et al., 2012; Kim et al., 2014). Interestingly, lipoic acid enhanced HO-1 and Nrε2 expression in human monocytic cells (Ogborne et al., 2005). However, the beneficial effects of lipoic acid against nandrolone-decanoate induced anxiety, aggression, memory impairment and neurochemical imbalance are still to be investigated.

Pentoxifylline or 1-(5-oxohexyl)-3,7-dimethylxanthine is a xanthine derivative and a non-specific type 4 phosphodiesterase inhibitor. It is indicated in the treatment of intermittent claudication due its direct vasodilatory and platelet aggregation inhibitory effects (Gupta et al., 1996). However, experimental studies have reported ameliorative effects of pentoxifylline in cognitive impairment, hypoxic encephalopathy, and ischemia (Vakili et al., 2011; Kalay et al., 2013; Ahmad et al., 2014). Pentoxifylline showed immunomodulatory properties on various cytokines but most prominently on TNF-α production via inhibition of TNF-α gene transcription and mRNA expression (D’Hellencourt et al., 1996; Schmidt-Choudhury et al., 1996). In the brain, TNF-α is produced by microglial cells and astrocytes in response to stress and may induce demyelination and neuronal death (Hartung et al., 1992). Interestingly, behavioral disorders including schizophrenia, panic, anxiety, and Alzheimer’s disease are partly influenced by inflammatory cytokines due to their interruption with normal neurotransmission (Spener-Unterweger, 2005). Pentoxifylline enhances brain serotonin synthesis and release, and suppresses its uptake (Kitatani et al., 1993). Serotonin deficiency has been correlated with anxiety and panic disorders (Coplan et al., 1999). Moreover, xanthine derivatives may selectively inhibit acetylcholinesterase enzyme activity and thereby may be effective in dementia (Mohamed et al., 2013).

Therefore the present study aimed at exploration of nandrolone decanoate-induced behavioral changes in rats, elucidation of the associated neurochemical and biochemical disturbances, and investigation of the involvement of Nrf2/HO-1 pathway and TNFRI in the deleterious effects of this anabolic steroid on brain. In addition, the target of this work extended to study the possible protective mechanisms by which the potent antioxidant lipoic acid and/or the TNF-α inhibitor pentoxifylline may mitigate nandrolone decanoate-induced behavioral changes and neurotoxicity in rats.

**Materials and methods**

**Animals**

Seventy adult male Wistar albino rats (160–180 g) were used in this study. Rats were kept in plastic cages on a 12 h light/12 h dark cycle, at a controlled temperature of 23 ± 1 °C and provided free access to standard food pellets and water. The animals were allowed one week for acclimatization before the experiment. The experimental protocol was compatible with the international guidelines for care and use of laboratory animals and approved by the local institutional Research Ethics Committee.

**Chemicals**

Nandrolone decanoate was obtained as Nandurabolin ampoules (Nile Company for Pharmaceuticals and Chemical Industries, Egypt). Lipoic acid and pentoxifylline were purchased from Sigma-Aldrich Chemical Co. (USA). All other chemicals and reagents were of analytical grade.

**Experimental design**

Rats were randomly allocated into seven groups of ten animals each. The first one served as the control group and was administered drug vehicles; peanut oil/benzyl alcohol as 90:10 v/v, s.c. every third day as nandrolone decanoate vehicle (Hassan and Kamal, 2013) and 0.9% NaCl, p.o. and i.p. daily as lipoic acid and pentoxifylline vehicles, respectively. Rats of the second and third groups were administered lipoic acid (100 mg/kg/day, i.p.) (Macêdo et al., 2012), or pentoxifylline (200 mg/kg/day, i.p.) (Cunha et al., 2000), respectively. Rats of the fourth group were treated with nandrolone decanoate (15 mg/kg, s.c.) every third day (Magnnsson et al., 2009). The last three groups were treated with nandrolone decanoate and either lipoic acid, pentoxifylline or both. The dose of nandrolone decanoate was matched with the literature and was selected because it corresponds to heavy abuse of nandrolone decanoate and was found to elicit neurochemical and behavioral changes in rats (Pope and Katz, 1988; Johansson et al., 2000). In addition, the estimated half-life of nandrolone decanoate is 5.4 days therefore this injection regimen will produce a depot effect (Minto et al., 1997). All treatments were continued for 30 days. Behavioral experiments were performed in the period from the 24th till the 29th day of the experiment and during the light cycle from 8 a.m. till 5 p.m. (de Almeida et al., 2010). After the last dose on day 30, all rats were weighed then sacrificed by decapitation. Brains were rapidly excised on ice and stored at −70 °C till they were properly processed as indicated per each assay.

**Methods**

**Open field behavioral test**

This test measures the exploratory activity and changes in rat emotionality in a novel mild stressful condition. Increased locomotion in the quadrants near the wall relative to the central ones is an indication of increased anxiety (Cunha and Masur, 1978). The open field apparatus is a wooden box opened from its top, with red sides and white floor. The latter has black lines that divide it into equal squares (4 × 4). The dimensions of the box were 80 × 80 × 40. At the start of the experiment, each
rat was placed in the center of the box and allowed 5 min. to move and behave freely under careful monitoring. Ambulation and rearing frequencies were recorded as indicators of changes in exploratory activity, whereas grooming frequency was monitored as a marker of altered emotionality.

**Rewarded T-maze task**

The T-maze is a simply constructed, sensitive behavioral test for detection of cognitive dysfunction in rats. The paradigm consists of a black-painted wooden T-shaped enclosed apparatus that is placed horizontally. The start arm is 50 cm (length) \(\times\) 16 cm (width) \(\times\) 35 cm (height) while each of the goal arms was 50 cm (length) \(\times\) 10 cm (width) \(\times\) 35 cm (height). Two food wells are present at the ends of the goal arms (2 cm diameter \(\times\) 2 cm depth), in which reward food pellets are placed. The maze was cleaned with 10% ethanol between each trial to minimize olfactory interferences. Following the method of Deacon and Rawlins (2006), rats were adapted to the apparatus and food reward for 4 days, after which they were trained for three trials per day. The experiment starts with placement of rats in the start area for 10 s. In the sample trial, rats were forced to select the goal arm containing the food pellet reward at its end by lowering the barrier of the other arm. The rat was allowed 30 s to stay in the arm and eat the reward then it was returned back to the start area. The arm in the forced trial was randomly changed whether left or right so that rats were equally subjected to both directions and care was taken that the same arm was not visited more than 2 consecutive times. In the choice trial, each animal was allowed to choose freely between both goal arms but given 2 food pellets as a reward if it alternated successfully (entered into the previously unvisited arm). If the rat failed to choose an arm within 2 min, then it was returned back to the start area for another trial. The inter-trial time interval was kept at 2 min. The average latency time was calculated (the average time elapsed since the barrier was raised in the start area till the rat enters the arm with food reward).

**Morris water maze test**

This test detects changes in spatial learning and memory. The method of Morris (1984) was followed. Briefly, a circular black pool (150 cm in diameter and 60 cm in height) was filled with tap water \(22 \pm 1 ^{\circ}C\) to a depth of 30 cm and located in a room with different cues on the walls. The pool was divided into 4 quadrants and a platform was hidden 1.5 cm under the water surface in the target (southwest) quadrant. The platform position remained constant over acquisition days. Each session consisted of four trials. The rats were placed facing the pool wall with random starting positions and quadrants. Each rat was allowed a maximum of 60 s to find the platform. At the end of each trial, the rat was allowed to stay on the platform for 30 s to recognize the platform well. If the rat failed to find the platform within 60 s it was gently guided there by hand. Escape latency (time in seconds taken by the rat to find the submerged platform) was recorded per rat in the 4 training trials then the average escape latency of the group was calculated. A single probe trial was done 24 h after the last acquisition one, where the rats were allowed to swim for 60 s in the absence of the platform. The animals were allowed to start from the northeast quadrant. Latency in the target zone was monitored (time spent in the target quadrant in seconds).

**Resident/intruder test**

The resident–intruder test is used to estimate aggression based on the territorial behavior of rats. It has the advantage of detecting even mild aggression with minimal injury to the animal. This behavioral model determines the mean composite aggression score as an indicator of the aggressive attacks of an animal resident in its home cage against a novel intruder male (Pohorecky et al., 1999). The method described by Long et al. (1996) was followed and the test was carried out under dim red light. Resident rats were housed individually in transparent plastic cages. After adaptation for 15 min a male intruder rat was placed into the resident’s cage for 10 min then the intruder was returned back to its original cage. Aggressive behaviors including number of lateral and lung attacks, number of bites, duration of attack, duration of mounting and piloerection were monitored and scored. Being more consistent in measuring aggression than individual data, composite aggression score was calculated by adding the previously mentioned scores for each rat then a mean composite aggression score was calculated by dividing the sum of individual composite aggression scores in each group by the number of rats per group.

**Determination of dopamine (DA), nor-adrenaline (NA), serotonin (5-HT) and GABA concentrations in the brain tissue of rats by HPLC analysis**

Following the method of Zagrodzka et al. (2000), brain homogenates were prepared in 0.1 M perchloric acid and 0.4 mM sodium metabisulphite and then centrifuged at 10000 \(\times\) g for 25 min. at 4 \(^{\circ}\)C. The supernatants were then collected and filtered. The filtrate was injected into an HPLC system which consisted of ODS-2 C18, 4.6 \(\times\) 250 mm analytical column. The electrochemical detector was adjusted at +0.65 V for monoamines and +0.50 V for GABA. The mobile phase used for monoamines determination was composed of 0.15 M sodium dihydrogen phosphate, 0.1 mM EDTA, 0.5 mM sodium octanesulphonic acid, 10–12% methanol \((\nu/\nu)\) and 5 mM lithium chloride at pH 3.4 and 32 \(^{\circ}\)C and the flow rate was set at 1.4 ml/min. On the other hand, the mobile phase for GABA determination was 0.1 mM sodium acetate buffer with 0.1 mM EDTA and 5 mM lithium chloride in 25% \((\nu/\nu)\) methanol at pH 5.5. Samples were incubated for 2 min with o-phthalaldehyde thiol reagent for derivatization of GABA before injection into the HPLC system. The flow rate was adjusted at 1.3 ml/min at 34 \(^{\circ}\)C. The concentrations of monoamines and GABA in each sample were calculated from the peak areas of the corresponding chromatogram.

**Determination of acetylcholine (Ach) concentration in the brain tissue of rats**

This assay was performed using a rat specific ELISA kit (CUSABIO, PRC). The instructions of the manufacturer were strictly followed. The absorbance was measured by a microplate reader adjusted at 450 nm and the concentration of acetylcholine in each sample was calculated from a plotted standard curve.

**Determination of acetylcholinesterase (AchE) activity in the brain tissue of rats**

For AchE activity, 3 ml of brain homogenate in ice-cold saline was centrifuged at 6000 \(\times\) g for 10 min. The supernatant was collected and used for colorimetric determination of AchE activity (Ellman et al., 1961). In brief, 0.4 ml of brain homogenate supernatant was added to 3 ml of 0.1 M phosphate buffer (pH 8.0) and 100 µl of 0.01 M 5-dithio-bis-nitrobenzoic acid (DTNB). The mixture was incubated for 2 min at 25 \(^{\circ}\)C and the reaction was then started by the addition of 20 µl of 0.075 M acetylthiocholine iodide (ATC), the change in absorbance was determined at 412 nm at 2 min interval for 10 min.

**Determination of lipid peroxides as malondialdehyde (MDA) level in the brain tissue of rats**

Brain MDA level was measured following the method of Mihara and Uchiyama (1978). The assay is based on the reaction of MDA and thiobarbituric acid in the ratio 1:2 at low pH and 95 \(^{\circ}\)C to yield a pink adduct that is extractable by n-butanol and can be colorimetrically detected at 535 nm.
Determination of catalase activity in the brain tissue of rats

A colorimetric kit (Cell Biolabs, Inc., USA) was used for this assay. The procedure was followed as per the manufacturer’s instructions. Absorbance was measured at 520 nm and a standard curve was plotted for calculation of the enzymatic activity in samples.

Determination of heme oxygenase-1 (HO-1), TNF-α receptor-1 (TNFR1) and Ach receptor (AchR) mRNA expression in the brain tissue of rats by real time PCR (RT–PCR)

Total RNA was extracted from brain tissue by RNeasy Purification Reagent (Qiagen, USA) according to the manufacturer’s instructions. RNA purity (A260/A280 ratio) and concentration were assessed spectrophotometrically (Gene Quant 1300, Uppsala, Sweden). RNA quality was confirmed by gel electrophoresis. The first-strand cDNA was synthesized from 4 μg of total RNA using an Oligo-(dT)–12–18 primer and Superscript™ II RNase Reverse Transcriptase (SuperScript Choice System, Life Technologies, Netherlands). This mixture was incubated at 42 °C for 1 h. Real time PCR amplification was performed using amplification mixture (10 μl) containing Power SYBR Green PCR Master Mix (Applied Biosystems, USA), 3 μl of cDNA, and 300 nM of primers. An ABI PRISM 7900 HT detection system was used (Applied Biosystems, USA). The PCR reactions consisted of 1 cycle at 95 °C for 10 min, followed by another one at 94 °C for 15 s, then 40 cycles at 60 °C for 1 min. Analysis and quantification of data was carried out by ABI Prism Sequence Detection Software (v.1.7, PE Biosystems, USA). All values were normalized to GAPDH gene. Relative expression of the investigated genes was determined by the comparative threshold cycle method (Livak and Schmittgen, 2001). The used PCR primer pairs were of the following gene sequence:

AchR
Forward 5’-TATAGAACCTCACCACACAC-3’
Reverse 5’-CCGTAAGGCTCTCTGTC-3’

TNFR1
Forward 5’-CGCCCTACAA CGG TGG AAG TC-3’
Reverse 5’-CAGCT CCC CCT TTJ CA-3’
HO-1
Forward 5’-CAGAAAGGCT AAGACCGCT-3’
Reverse 5’-TTCTGTCCTGTGTTCTCGTCA-3’

GAPDH
Forward 5’-CTCCCATCCCTCCACCTTG-3’
Reverse 5’-CAGTCTGTACGATCTTGCGC-3’

Determination of Nrf2 (nuclear factor erythroid-2 related factor 2) expression in the brain tissue of rats by western blot analysis

According to the method of Stewart et al. (2003), brain tissue (50 mg) was homogenized in 1.5 ml cold lysis buffer that consisted of 50 mM/l Tris–HCl (pH 8.0), 150 mM/l NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and 0.5 mM/l phenyl methyl sulfon fluoride. The homogenate was centrifuged for 20 min at 4 °C and the supernatant was collected. After boiling at 95 °C for 5 min, samples (50 μg) were electrophoresed on 7% SDS–PAGE gel (Invitrogen, Thermo Fisher Scientific, USA). After separation, gels were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, USA). The membranes were blocked for 2 h at room temperature in TBST (0.05% Tween 20 in Tris buffered saline) and 7.5% (w/v) non-fat dry milk and then incubated overnight at 4 °C with the primary specific antibody against Nrf2 at 1:1000 dilution (Cell Signaling Technology, USA). The membranes were washed before incubation for 1 h at room temperature with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody) at 1:25000 dilution (Bio-Rad, USA), followed by additional washing. Protein visualization was carried out by enhanced chemiluminescence ECL Plus System (Amersham Biosciences, USA). Quantification was done using densitometry and Molecular Analyst Software (Bio-Rad, USA). Nrf2 protein expression was determined relative to β-actin.

Determination of brain tumor necrosis factor-alpha (TNF-α) level

TNF-α level was determined by ELISA kit (R&D Systems, USA). The procedure was followed as indicated in the manual. The absorbance was determined using a microplate reader at 450 nm. TNF-α values were expressed as picogram per milligram protein.

Histopathological examination

Rat brain autopsy samples were fixed in 10% formal saline for 24 h, rinsed by water then dehydrated by serially diluted methanol, ethanol and absolute ethanol. Samples were cleared by xylene and embedded for 24 h in paraffin (at 56 °C). Brain tissue sections (4 μm thick) were prepared from paraffin bees wax blocks by a slide microtome, collected on glass slides, deparaffinized and stained with H&E. Routine examination was performed using light electric microscope (Banchroft et al., 1996).

Statistical analysis

Data was expressed as mean ± S.E.M. ANOVA and Tukey’s post hoc test were carried out for statistical analysis of all data except composite aggression score which was statistically analyzed by Kruskal–Wallis test followed by Dunn’s post hoc test. GraphPad prism software, v.3.0 (USA) was used. Values of p < 0.05 were considered significant. Eta squared ($\eta^2$) was calculated for data analyzed by ANOVA while Cohen’s d was estimated for pair-wise comparisons (Cohen’s d value of 0.2 was considered a small, 0.5 a medium and 0.8 or more a large effect size). Cohen’s d was calculated using the online effect size calculator at “http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php”.

Results

Enhancement of body weight of rats by nandrolone decanoate either alone or in combination with lipoic acid and/or pentoxifylline

There was a significant treatment effect on the body weight of rats ($F_{(6,63)} = 6.174$, $p < 0.0001$, $\eta^2 = 0.3707$). As demonstrated in Fig. (1), administration of nandrolone decanoate to rats either alone or in combination with lipoic acid, pentoxifylline or both drugs induced a

![Fig. 1. Effect of nandrolone decanoate either alone or in combination with lipoic acid and/or pentoxifylline on the body weight of rats. LA (lipoic acid), P (pentoxifylline), ND (nandrolone decanoate). Data is expressed as mean ± SEM, n = 10. ANOVA and Tukey’s post hoc test were used for statistical analysis. *Significantly different from the control group at p < 0.05.](image-url)
significant increase in body weight as compared to the control group (p < 0.05, Cohen’s d = 1.6205, p = 0.05, Cohen’s d = 1.6955, p < 0.05, Cohen’s d = 1.3881, and p = 0.05, Cohen’s d = 1.6386), respectively.

**Modulation of nandrolone decanoate-induced alterations in the behavior of rats challenged in the open field test by lipoic acid and/or pentoxifylline**

There was a significant treatment effect on the ambulation frequency of rats in the quadrants near the walls in the open field test (F(6,63) = 8.109, p = 0.0001, η² = 0.4358). As shown in Table (1), administration of nandrolone decanoate to rats resulted in a significant elevation of ambulation frequency in the quadrants near the walls (p < 0.01, Cohen’s d = 2.5036) as compared to the control group. Rats treated with lipoic acid, pentoxifylline or both drugs in combination with nandrolone decanoate showed a significant reduction in the same parameter (p < 0.01, Cohen’s d = 1.7430, p < 0.05, Cohen’s d = 1.2677, and p < 0.01, Cohen’s d = 2.1988) as compared to the ND group, respectively. Similarly, there was a significant treatment effect on the rearing frequency of rats in the open field test (F(6,63) = 7.342, p < 0.0001, η² = 0.4115). Rearing frequency was significantly increased in ND-treated rats (p < 0.01, Cohen’s d = 1.9854) as compared to the control group, whereas it was significantly decreased upon treatment of rats by lipoic acid, pentoxifylline or both drugs in combination with nandrolone decanoate (p = 0.01, Cohen’s d = 1.4242, p < 0.05, Cohen’s d = 1.0567, and p < 0.01, Cohen’s d = 2.163) as compared to the ND group, respectively. Likewise, grooming behavior showed a significant treatment effect (F(6,63) = 8.312, p < 0.0001, η² = 0.4418). Alterations in grooming behavior showed similar pattern, with a significant increase in ND-treated rats (p < 0.01, Cohen’s d = 2.0678) as compared to the control group. On the other hand, administration of lipoic acid, or lipoic acid plus pentoxifylline in combination with nandrolone decanoate significantly decreased the mentioned parameter (p = 0.05, Cohen’s d = 1.1925, and p < 0.01, Cohen’s d = 1.5727) as compared to the ND group, respectively.

**Effect of lipoic acid and/or pentoxifylline on nandrolone decanoate-induced increase in latency time of rats in the rewarded T-maze test**

There was a significant treatment effect on the latency time of rats in the rewarded T-maze test (F(6,63) = 11.86, p < 0.0001, η² = 0.5922). As shown in Table (2), rats treated with ND showed a significant increase in latency time (p < 0.01, Cohen’s d = 2.5496) as compared to the control group, while rats treated with lipoic acid, or both lipoic acid and pentoxifylline in combination with ND showed a significant decrease in latency time (p < 0.01, Cohen’s d = 1.7580, p < 0.01, Cohen’s d = 2.1897) as compared to the ND group, respectively. Administration of pentoxifylline to ND-treated rats significantly elevated latency time (p < 0.05, Cohen’s d = 1.5389) as compared to the control group and significantly decreased latency time (p < 0.05, Cohen’s d = 1.1338) compared to the ND group.

**Effect of nandrolone decanoate either alone or in combination with lipoic acid and/or pentoxifylline on the behavior of rats in the Morris water maze test**

There was a significant treatment effect on the latency time spent by rats in the Morris water maze test during the 3rd and 4th trial days (F(6,63) = 5.404, p < 0.0001, η² = 0.3398 and F(6,63) = 6.996, p < 0.0001, η² = 0.3999, respectively). As shown in Table (3), treatment of rats with ND significantly increased latency time on the 3rd and 4th trial days (p < 0.01, Cohen’s d = 1.9288) as compared to the control group, respectively. Administration of lipoic acid or pentoxifylline to ND-treated rats significantly reduced latency time on the 4th trial day (p < 0.01, Cohen’s d = 1.4779, and p < 0.05, Cohen’s d = 1.2230) as compared to the ND group, respectively. Interestingly, rats treated with both lipoic and pentoxifylline concurrent to ND showed significantly reduced latency time on the 3rd and 4th trial days (p < 0.01, Cohen’s d = 1.5133, and p < 0.01, Cohen’s d = 1.7161) as compared to the ND group, respectively. Similarly, a significant treatment effect was obvious on the mean time spent in the target quadrant by rats in the Morris water maze test.

### Table 1

<table>
<thead>
<tr>
<th>Ambulation frequency (counts/5 min)</th>
<th>Rearing frequency (counts/5 min)</th>
<th>Grooming frequency (counts/5 min)</th>
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<tbody>
<tr>
<td>Control</td>
<td>25.80 ± 2.80</td>
<td>31.20 ± 2.03</td>
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<tr>
<td>LA</td>
<td>28.50 ± 2.47</td>
<td>32.00 ± 2.03</td>
</tr>
<tr>
<td>ND</td>
<td>45.90 ± 2.93</td>
<td>13.60 ± 0.95</td>
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<tr>
<td>LA + ND</td>
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<td>11.00 ± 0.76</td>
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<tr>
<td>P + ND</td>
<td>34.70 ± 2.65</td>
<td>12.60 ± 0.97</td>
</tr>
<tr>
<td>LA + P + ND</td>
<td>28.00 ± 2.17</td>
<td>12.60 ± 0.97</td>
</tr>
</tbody>
</table>

LA (lipoic acid), P (pentoxifylline), ND (nandrolone decanoate). Data is expressed as mean ± SEM, n = 10.

ANOVA and Tukey’s post hoc test were used for statistical analysis. **Significantly different from the control group at p < 0.05.*** Significantly different from the control group at p < 0.001.

### Table 2

<table>
<thead>
<tr>
<th>Average latency time (s)</th>
<th>Baseline time (0 weeks)</th>
<th>End of the experiment (4th week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.13 ± 5.33</td>
<td>49.00 ± 4.13</td>
</tr>
<tr>
<td>LA</td>
<td>50.00 ± 4.49</td>
<td>47.88 ± 3.93</td>
</tr>
<tr>
<td>P</td>
<td>49.75 ± 4.32</td>
<td>43.50 ± 4.27</td>
</tr>
<tr>
<td>ND</td>
<td>47.75 ± 2.53</td>
<td>101.30 ± 9.39***</td>
</tr>
<tr>
<td>LA + ND</td>
<td>51.75 ± 4.50</td>
<td>62.63 ± 5.73***</td>
</tr>
<tr>
<td>P + ND</td>
<td>53.50 ± 3.97</td>
<td>74.50 ± 7.18**</td>
</tr>
<tr>
<td>LA + P + ND</td>
<td>52.75 ± 4.58</td>
<td>56.50 ± 4.07***</td>
</tr>
</tbody>
</table>

LA (lipoic acid), P (pentoxifylline), ND (nandrolone decanoate). Data is expressed as mean ± SEM, n = 10.

ANOVA and Tukey’s post hoc test were used for statistical analysis. ***Significantly different from the control group at p < 0.001.

### Table 3

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>LA</th>
<th>P</th>
<th>ND</th>
<th>LA + ND</th>
<th>P + ND</th>
<th>LA + P + ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.50 ± 2.33</td>
<td>24.80 ± 2.41</td>
<td>52.60 ± 2.63</td>
<td>29.00 ± 2.89</td>
<td>31.90 ± 3.75</td>
<td>29.30 ± 3.25</td>
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</tr>
<tr>
<td>2</td>
<td>21.00 ± 1.53</td>
<td>19.00 ± 1.89</td>
<td>22.30 ± 1.59</td>
<td>24.20 ± 2.38</td>
<td>27.40 ± 2.92</td>
<td>23.40 ± 2.43</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16.00 ± 1.07</td>
<td>14.80 ± 1.27</td>
<td>17.30 ± 0.84</td>
<td>19.90 ± 1.59</td>
<td>21.50 ± 2.06</td>
<td>23.40 ± 1.04</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9.60 ± 0.75</td>
<td>8.80 ± 0.87</td>
<td>10.30 ± 0.99</td>
<td>15.90 ± 1.16**</td>
<td>12.00 ± 1.23</td>
<td>16.70 ± 1.04**</td>
<td></td>
</tr>
</tbody>
</table>

LA (lipoic acid), P (pentoxifylline), ND (nandrolone decanoate). Data is expressed as mean ± SEM, n = 10.

ANOVA and Tukey’s post hoc test were used for statistical analysis. **Significantly different from the control group at p < 0.01.

***Significantly different from the control group at p < 0.001.

**Significantly different from the nandrolone decanoate group at p < 0.05.

***Significantly different from the nandrolone decanoate group at p < 0.001.
test \(F_{(6,63)} = 6.343, p < 0.0001, \eta^2 = 0.3766\). As illustrated in Fig. (2), rats of the ND group showed significant decrease in the mean time spent in the target quadrant \((p < 0.01, \text{Cohen’s } d = 2.2518)\) vs. the control group, whereas rats treated with ND and lipoic acid only or combined with pentoxifylline showed significantly elevated mean time spent in the target quadrant \((p < 0.05, \text{Cohen’s } d = 1.8385, \text{and } p < 0.001, \text{Cohen’s } d = 3.8830)\) as compared to the ND group, respectively.

### Significance

**Nandrolone decanoate-enhanced aggression of rats in the resident–intruder test is attenuated by lipoic acid and pentoxifylline**

As shown in Table (4), administration of ND to rats either alone or combined with lipoic acid, or pentoxifylline resulted in a significant elevation of the mean composite aggression score by 534.0% \((p < 0.0001), 114.0\% \((p < 0.05), \text{and } 152.0\% \((p < 0.01)\) as compared to the control group, respectively. On the other hand, administration of both lipoic acid and pentoxifylline to ND-treated rats significantly reduced the mentioned score by 75.7% \((p < 0.01)\) as compared to the ND group.

### Effect of lipoic acid and/or pentoxifylline on nandrolone decanoate-induced alterations in the levels of brain dopamine, nor-adrenaline, serotonin, GABA and acetylcholine in rats

Significant treatment effect on the levels of brain dopamine \((F_{(6,63)} = 5.081, p < 0.0003, \eta^2 = 0.3261)\), nor-adrenaline \((F_{(6,63)} = 5.083, p < 0.0003, \eta^2 = 0.3262)\), serotonin \((F_{(6,63)} = 5.328, p < 0.0002, \eta^2 = 0.3366)\), GABA \((F_{(6,63)} = 7.155, p < 0.0001, \eta^2 = 0.4053)\) and Ach \((F_{(6,63)} = 5.725, p < 0.0001, \eta^2 = 0.3528)\) was revealed. As shown in Table (5), ND significantly elevated brain dopamine and nor-adrenaline levels \((p < 0.01, \text{Cohen’s } d = 1.9286, \text{p} < 0.01, \text{Cohen’s } d = 1.7854)\), and reduced brain serotonin, GABA, and Ach levels \((p < 0.001, \text{Cohen’s } d = 2.3858, p < 0.01, \text{Cohen’s } d = 1.9557, \text{and } p < 0.001, \text{Cohen’s } d = 2.5165)\) as compared to the control group, respectively. Administration of lipoic acid to ND-treated rats significantly reduced brain dopamine level \((p < 0.05, \text{Cohen’s } d = 1.4229)\), and elevated brain serotonin and Ach levels \((p < 0.01, \text{Cohen’s } d = 2.8017, \text{and } p < 0.05, \text{Cohen’s } d = 2.1043)\) as compared to the ND group, respectively. Treatment of rats with pentoxifylline in combination with nandrolone decanoate significantly reduced brain nor-adrenaline level \((p < 0.05, \text{Cohen’s } d = 1.9286)\), and elevated brain serotonin and Ach levels \((p < 0.05, \text{Cohen’s } d = 1.7633, \text{and } p < 0.05, \text{Cohen’s } d = 1.7427)\) as compared to the ND group, respectively.

**Table 4**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean composite aggression score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>LA</td>
<td>0.58 ± 0.05</td>
</tr>
<tr>
<td>P</td>
<td>0.64 ± 0.05</td>
</tr>
<tr>
<td>ND</td>
<td>3.17 ± 0.18***</td>
</tr>
<tr>
<td>LA + ND</td>
<td>1.07 ± 0.10</td>
</tr>
<tr>
<td>P + ND</td>
<td>1.26 ± 0.09**</td>
</tr>
<tr>
<td>LA + P + ND</td>
<td>0.77 ± 0.08**</td>
</tr>
</tbody>
</table>

**LA** (lipoic acid), **P** (pentoxifylline), **ND** (nandrolone decanoate). Data is expressed as mean ± SEM, \(n = 10\).

Kruskal–Wallis and Dunn’s multiple comparison test were used for statistical analysis:

* Significantly different from the control group at \(p < 0.05\).

** Significantly different from the control group at \(p < 0.01\).

*** Significantly different from the control group at \(p < 0.001\).

** Effect of nandrolone decanoate-induced changes in brain acetylcholinesterase activity (AChE) and acetylcholine receptor (AChR) expression by lipoic acid and/or pentoxifylline

There was a significant treatment effect on brain acetylcholinesterase activity in rats \((F_{(6,63)} = 7.577, p < 0.0001, \eta^2 = 0.4191)\). As illustrated in Fig. (3A), administration of ND to rats significantly enhanced brain AChE activity \((p < 0.001, \text{Cohen’s } d = 2.0514)\) as compared to the control group, while administration of lipoic acid alone or in combination with pentoxifylline to ND-treated rats significantly inhibited AChE activity \((p < 0.05, \text{Cohen’s } d = 1.3679, \text{and } p < 0.01, \text{Cohen’s } d = 1.6279)\) as compared to the ND group, respectively.

Likewise, a significant treatment effect was revealed on rat brain AchR expression \((F_{(6,63)} = 15.14, p < 0.0001, \eta^2 = 0.5905)\). As demonstrated in Fig. (3B), treatment of rats with ND alone or in combination with lipoic acid or pentoxifylline significantly down-regulated AchR expression \((p < 0.001, \text{Cohen’s } d = 3.3917, p < 0.001, \text{Cohen’s } d = 2.556, \text{and } p < 0.05, \text{Cohen’s } d = 1.4425)\) as compared to the control group, respectively. On the other hand, administration of pentoxifylline alone or in combination with lipoic acid to ND-treated rats significantly up-regulated brain AchR expression \((p < 0.05, \text{Cohen’s } d = 2.1642, \text{and } p < 0.05, \text{Cohen’s } d = 2.3746)\) as compared to the ND group, respectively.

### Attenuation of nandrolone decanoate-induced increase in brain lipid peroxides level (measured as malondialdehyde) and inhibition in catalase activity by lipoic acid and/or pentoxifylline

There was a significant treatment effect on brain MDA level in rats \((F_{(6,63)} = 6.422, p < 0.0001, \eta^2 = 0.3795)\). As illustrated in Fig. (4A), administration of ND to rats significantly elevated brain MDA level \((p < 0.001, \text{Cohen’s } d = 1.8926)\) in comparison to the control group. Administration of lipoic acid, pentoxifylline or both to ND-treated rats significantly reduced brain MDA level \((p < 0.01, \text{Cohen’s } d = 1.393, \text{and } p < 0.05, \text{Cohen’s } d = 1.7074, \text{and } p < 0.001, \text{Cohen’s } d = 1.773)\) as compared to the ND group, respectively.

Similarly, a significant treatment effect was revealed on brain catalase activity in rats \((F_{(6,63)} = 6.187, p < 0.0001, \eta^2 = 0.3708)\). As
demonstrated in Fig. (4B), ND significantly inhibited catalase activity in the rat brain (p < 0.01, Cohen’s d = 2.1842), as compared to the control group. Administration of lipoic acid either alone or in combination with pentoxifylline to ND-treated rats significantly enhanced brain catalase activity (p < 0.05, Cohen’s d = 1.6284, and p < 0.01, Cohen’s d = 1.8569) as compared to the ND group, respectively.

Effect of nandrolone decanoate either alone or in combination with lipoic acid and/or pentoxifylline on brain Nrf2 protein and HO-1 gene expression in rats

There was a significant treatment effect on brain Nrf2 protein expression in rats (F(6,63) = 8.829, p < 0.0001, η² = 0.4568). As shown in Table 5, the effect of nandrolone decanoate either alone or in combination with lipoic acid and/or pentoxifylline on brain neurotransmitters level in rats.

**Table 5**

<table>
<thead>
<tr>
<th>Dopamine (ng/g wet tissue)</th>
<th>Nor-epinephrine (ng/g wet tissue)</th>
<th>Serotonin (ng/g wet tissue)</th>
<th>GABA (μg/g wet tissue)</th>
<th>Acetylcholine (ng/g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>420.10 ± 34.32</td>
<td>288.80 ± 20.55</td>
<td>582.31 ± 43.27</td>
<td>117.40 ± 9.52</td>
</tr>
<tr>
<td>LA</td>
<td>414.60 ± 36.87</td>
<td>275.11 ± 20.61</td>
<td>577.52 ± 45.02</td>
<td>121.00 ± 9.12</td>
</tr>
<tr>
<td>P</td>
<td>434.91 ± 36.95</td>
<td>266.42 ± 22.00</td>
<td>586.60 ± 44.15</td>
<td>138.60 ± 10.42</td>
</tr>
<tr>
<td>ND</td>
<td>637.70 ± 36.99</td>
<td>431.51 ± 29.25</td>
<td>315.60 ± 25.05</td>
<td>69.28 ± 5.52</td>
</tr>
<tr>
<td>LA + ND</td>
<td>480.22 ± 32.90</td>
<td>354.30 ± 28.38</td>
<td>538.00 ± 42.57</td>
<td>96.52 ± 9.19</td>
</tr>
<tr>
<td>P + ND</td>
<td>536.93 ± 33.57</td>
<td>314.22 ± 27.12</td>
<td>527.00 ± 47.41</td>
<td>88.58 ± 7.44</td>
</tr>
<tr>
<td>LA + P + ND</td>
<td>459.81 ± 36.34</td>
<td>300.50 ± 28.83</td>
<td>573.11 ± 41.68</td>
<td>106.81 ± 7.89</td>
</tr>
</tbody>
</table>

LA (lipoic acid), P (pentoxifylline), ND (nandrolone decanoate).

Data is expressed as mean ± SEM, n = 10. ANOVA and Tukey’s post hoc test were used for statistical analysis.

* Significantly different from the control group at p < 0.05.
** Significantly different from the control group at p < 0.001.
*** Significantly different from the control group at p < 0.0001.

* # Significantly different from the nandrolone decanoate group at p < 0.05.
** # Significantly different from the nandrolone decanoate group at p < 0.01.
*** # Significantly different from the nandrolone decanoate group at p < 0.001.
in Figs. (5A, B), administration of ND to rats either alone or in combination with pentoxifylline significantly reduced Nrf2 protein expression ($p < 0.001$, Cohen's $d = 3.0941$, and $p < 0.01$, Cohen's $d = 1.8766$) as compared to the control group, respectively. On the other hand, rats treated with lipoic acid, or both drugs in combination to ND showed significantly elevated brain Nrf2 protein expression ($p < 0.01$, Cohen's $d = 2.202$, and $p < 0.01$, Cohen's $d = 2.2328$) as compared to the ND group, respectively.

In a similar manner, there was a significant treatment effect on brain HO-1 gene expression in rats ($F(6,63) = 8.463$, $p < 0.0001$, $\eta^2 = 0.4463$). As demonstrated in Fig. (5C), rats treated with ND showed significantly reduced brain HO-1 gene expression ($p < 0.001$, Cohen's $d = 3.3001$) as compared to the control group. Administration of lipoic acid alone or with pentoxifylline to ND-treated rats significantly elevated brain HO-1 gene expression ($p < 0.001$, Cohen's $d = 2.7725$, and $p < 0.001$, Cohen's $d = 2.8257$) as compared to the ND group, respectively.

Modulatory effects of lipoic acid and/or pentoxifylline on nandrolone decanoate-induced increase in brain TNF-α level and TNF-α receptor-1 (TNFR1) expression

There was a significant treatment effect on brain TNF-α level in rats ($F(6,63) = 23.45$, $p < 0.0001$, $\eta^2 = 0.6907$). As illustrated in Fig. (6A), administration of ND to rats significantly reduced TNFR1 expression ($p < 0.001$, Cohen's $d = 3.3001$, $\eta^2 = 0.4463$). As demonstrated in Fig. (6B), administration of ND to rats significantly increased brain TNFR1 expression ($p < 0.01$, Cohen's $d = 2.7725$, and $p < 0.01$, Cohen's $d = 2.8257$) as compared to the ND group, respectively.

Similarly, a significant treatment effect was obvious on brain TNF-α receptor-1 (TNFR1) expression in rats ($F(6,63) = 21.25$, $p < 0.0001$, $\eta^2 = 0.6693$). As demonstrated in Fig. (6B), administration of ND to rats significantly increased brain TNFR1 expression ($p < 0.001$, Cohen's $d = 2.6865$) as compared to the control group. Administration of lipoic acid to ND-treated rats induced a significant increase in brain TNF-α level ($p < 0.05$, Cohen's $d = 1.538$) as compared to the control group, and a significant reduction in the same parameter ($p < 0.01$, Cohen's $d = 1.3856$) as compared to the ND group. On the other hand, rats treated with ND and pentoxifylline alone or combined with lipoic acid showed significantly reduced brain TNF-α level ($p < 0.001$, Cohen's $d = 2.0912$, and $p < 0.001$, Cohen's $d = 2.4934$) as compared to the ND group, respectively.

Administration of ND to rats significantly elevated brain TNF-α level ($p < 0.001$, Cohen's $d = 2.6865$) as compared to the control group. Administration of lipoic acid to ND-treated rats induced a significant increase in brain TNF-α level ($p < 0.05$, Cohen's $d = 1.538$) as compared to the control group, and a significant reduction in the same parameter ($p < 0.01$, Cohen's $d = 1.3856$) as compared to the ND group. On the other hand, rats treated with ND and pentoxifylline alone or combined with lipoic acid showed significantly reduced brain TNF-α level ($p < 0.001$, Cohen's $d = 2.0912$, and $p < 0.001$, Cohen's $d = 2.4934$) as compared to the ND group, respectively.
1.9492, and \( p < 0.001 \), Cohen’s \( d = 2.7334 \) as compared to the ND group, respectively.

**Attenuation of nandrolone decanoate-induced brain histopathological alterations by lipoic acid and/or pentoxifylline**

As illustrated in Fig. (7), brain sections of the control, lipoic acid and pentoxifylline groups were devoid of any histopathological alterations and revealed normal histological structure of the meninges, cerebral cortex and cerebrum striatum. Sections of the nandrolone decanoate group showed eosinophilic plaques formation as well as excessive gliosis in the cerebrum striatum. Moreover, cerebral blood vessels dilatation and congestion, as well as neuronal degeneration in the cerebrum striatum were apparent. On the other hand, brain sections of rats treated with nandrolone decanoate and lipoic acid showed few glial cells proliferation in the cerebrum striatum, whereas brain sections of rats...
treated with nandrolone decanoate and pentoxifylline demonstrated mild gliosis and mild neurodegeneration. Notably, maximum neuroprotection was apparent in the brain sections of rats treated with nandrolone decanoate and both lipidic acid and pentoxifylline where diminished giall cells proliferation was observed with absence of neuronal degeneration.

As shown in Table (6), brain sections of rats of the control, lipidic acid, and pentoxifylline groups were devoid of any histopathological alterations. On the contrary, brain sections of rats administered nandrolone decanoate showed severe plaque formation, gliosis and neuronal degeneration as well as moderate blood vessels congestion. On the other hand, brain sections of rats treated with nandrolone decanoate and pentoxifylline revealed mild neuronal degeneration, whereas brain sections of rats co-administered nandrolone decanoate with lipidic acid and/or pentoxifylline showed mild gliosis.

### Discussion

The present study showed that lipidic acid and pentoxifylline may attenuate nandrolone-induced behavioral changes and neurotoxicity in rats without affecting its anabolic activity. Similarly, previous reports revealed that the anabolic steroid, nandrolone decanoate, may increase muscle mass and body weight (Hartgens and Kuipers, 2004). Nandrolone-treated rats showed elevated ambulation, rearing, and grooming behavior in the open field test. These behavioral changes were correlated with elevated exploratory activity and emotionality (Edinger and Frye, 2005). Elevated emotional levels and enhanced locomotion of rats in the quadrants along the walls in the open field test revealed nandrolone ability to induce anxiety (Rocha et al., 2007; Ambar and Chiavegatto, 2009). Anxiety is usually associated with aggression, diminished learning ability and accelerated memory impairment (Patki et al., 2013; Zhang et al., 2014).

On the other hand, the resident intruder paradigm is commonly used to assess inter-male social hostility and aggression (Steensland et al., 2005). In harmony with the present findings, McGinnis (2004) reported that androgenic anabolic steroids may sensitize rats to their surroundings and lower aggression threshold. The crucial role played by androgens as mediators of aggression is emphasized by eliminated aggression in castrated rats (Bergvall and Hansen, 1990; Vohe et al., 2012). Rats are normally known to defend their territory in violence against intruders. However, nandrolone decanoate-treated rats were reported to be more sensitive to environmental stimuli such as sensory or behavioral signals from their cage mates (Steensland et al., 2005). They demonstrated promoted dominance and competitive behavior associated with long aggressive attacks (Lindqvist and Fahlike, 2005).

Interestingly, the present study showed that nandrolone decanoate may impede normal behavior of rats challenged in the Morris water maze and rewarded T-maze tests reflecting defects in long term spatial and working memories (Tanehkhar et al., 2013; Karamian et al., 2015). In addition, increased ambulation and rearing frequencies observed in the open field test may reveal impaired habituation memory (Barichello et al., 2014).

### Table 6

<table>
<thead>
<tr>
<th></th>
<th>Plaques formation</th>
<th>Gliosis</th>
<th>Congestion</th>
<th>Neuronal degeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>LA</td>
<td>−</td>
<td>−</td>
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<td>−</td>
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<tr>
<td>P</td>
<td>−</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>ND</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>LA + ND</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>P + ND</td>
<td>−</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>LA + P + ND</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

LA (lipidic acid), P (pentoxifylline), ND (nandrolone decanoate).
− (Nil), + (mild), ++ (moderate), ++++ (severe).

Behavioral changes usually reveal brain neurotransmitters imbalance. For instance, aggressive attitude and elevated ambulation and rearing frequencies were attributed to elevated dopamine and reduced serotonin levels in brain (Abililo et al., 1999; Van Erp and Miczek, 2000). Moreover, the balance between nor-epinephrine and cholinergic systems may influence social behaviors and aggression (Zarrindast et al., 2012; Coura et al., 2013). Therefore, enhanced aggression observed after nandrolone treatment may be attributed to its potency to elevate adrenergic amines in the hypothalamus (Tamaki et al., 2003). Other plausible explanations include nandrolone-induced enhancement of dopamine biosynthesis (Kurling et al., 2005), over-expression of dopaminergic receptors (Birgner et al., 2008a), and inhibition of dopamine-metabolizing enzymes activity (Birgner et al., 2008b). In addition, nandrolone decanoate may enhance anxiety behavior in rats by stimulation of brain excitability secondary to down-regulation of serotonin receptor mRNA expression (Ambar and Chiavegatto, 2009). Increased firing of nor-adrenergic neurons and suppressed firing of serotnergic ones was observed after nandrolone administration (Rainer et al., 2014). Diminished serotoninergic neurotransmission may induce locomotor hyperactivity, memory impairment and learning deficits (Adams et al., 2008). It is noteworthy that serotonin normally suppresses neuronal excitability by activation of the inhibitory GABAergic neurons (Rainnie, 1999). Interestingly, this study showed diminished GABA levels in the brain of rats treated by nandrolone decanoate. GABA controls behaviors related to anxiety and aggression (Henderson et al., 2006). GABA analogs revealed anxiolytic effects while inhibition of GABAergic neurotransmission induced anxiety (Nutt, 2001; Stahl, 2004). The excitatory effect of nandrolone may be further mediated by enhancement of glutamatergic neurotransmission secondary to inhibition of glutamate uptake (Kaline et al., 2014).

The current enhancement of AchE activity and the associated reduction in acetylcholine level by nandrolone decanoate may account for the observed memory impairment in rats. AchE is capable of acetylcholine breakdown leading to alterations in its synaptic availability and interference with its postsynaptic effects (Grisaru et al., 1999). Acetylcholine plays a crucial role in cognitive functions including memory and learning (Eidi et al., 2003; Easton et al., 2012). Therefore, enhanced AchE activity and suppressed expression of AchR may mediate nandrolone-induced dementia (Wright et al., 1993).

In the current study, administration of both lipidic acid and pentoxifylline to nandrolone-treated rats attenuated anxiety, hyperactivity, aggression and dementia. It seems this is the first study to elucidate the protective effects of lipidic acid against aggression and anxiety in rats. Previously, it was shown that lipidic acid may reduce hyperlocomotion, elevate brain serotonin and GABA levels, enhance dopamine breakdown, and down-regulate dopamine receptors (Bist and Bhatt, 2009; Macêdo et al., 2012; Deslauriers et al., 2014). Interestingly, hyper-responsiveness and aggression in rats were efficiently attenuated by suppression of dopaminergic and enhancement of serotoninergic neurotransmission (de Almeida et al., 2005). Lipidic acid-induced improvement of cognition and spatial memory in the water maze test may be attributed to suppressed activity of AchE and enhanced synthesis of brain acetylcholine via stimulation of choline acetyltransferase activity (Packer et al., 1997; Zhao et al., 2015).

In a similar manner, pentoxifylline has previously shown anxiolytic effects and spatial memory enhancement in rats (Kitatani et al., 1993; Bruno Rde et al., 2009). These effects may be mediated through acceleration of brain nor-epinephrine metabolism, enhancement of brain serotonin level, and suppression of oxidative stress-induced neuronal injury (Cardinali, 1978; Tariq et al., 2008). It is reasonable that pentoxifylline by virtue of its bulky structure may inhibit both catalytic and peripheral anionic sites of AchE (Mohamed et al., 2013). Interestingly, caffeine, another xanthine derivative, may induce up-regulation of acetylcholine receptors expression in mice brain (Shi et al., 1993).

In this study, nandrolone decanoate inhibited catalase activity and enhanced MDA production. Catalase enzyme is mainly responsible of
extinguishing hydrogen peroxide radicals (Cecchini et al., 1990). The brain is normally deficient in catalase and further inhibition of the enzyme activity may result in oxidative stress, membrane lipid peroxidation and neuronal death (Dringen et al., 2005). The ability of nandrolone to induce oxidative stress and disturb normal antioxidant defenses was previously reported (Cecel et al., 2003; Ahmed, 2015). This oxidative potential may be attributed to increased brain dopamine levels by nandrolone and production of hydrogen peroxide radicals as a result of dopamine metabolism by monoamine oxidase (Berman and Hastings, 1999). Similarly, the auto-oxidation of nor-epinephrine may generate superoxide free radicals and other neurotoxic products (Louis et al., 1992). Accumulation of reactive oxygen species may degrade DNA and impair cellular functions especially under impaired catalase activity (Berman and Hastings, 1999).

Interestingly, anxiety has been postulated to be partly induced by oxidative stress and to be associated with diminished antioxidant defenses and enhanced expression of inflammatory markers (Rammal et al., 2008; Salim et al., 2010). Increased oxidative stress may damage brain serotonin and cholinergic neurons and hence contribute to aggression and memory impairment (Pathki et al., 2015; Xi et al., 2014). Experimentally, reduction of the brain malondialdehyde level and enhancement of catalase activity resulted in diminished anxiety and attenuated memory impairment in rats (Parr et al., 2003; Kamar et al., 2014).

Up to our knowledge, this study is the first one to attribute nandrolone-induced oxidative stress and neurobehavioral changes to inhibition of Nrf2/ HO-1 pathway. Suppressed Nrf2 expression leads to progressive neurotoxicity and increases the risk of Alzheimer’s disease and other neurodegenerative disorders (Ramsey et al., 2007). Nrf2 or nuclear factor erythroid-2 related factor 2 is a redox-sensitive transcription factor that translocates from the cytoplasm to the nucleus in response to oxidative stress insult (Venugopal and Jaiswal, 1998). In the nucleus, it binds to certain DNA segments called antioxidant response elements (ARE) to induce transcription of genes that code for antioxidant proteins such as glutathione, catalase and HO-1. These proteins constitute the major neuronal defenses against free radicals attack (Steele et al., 2013). HO-1 has been assigned a neuroprotective role owing to its ability to re-balance redox homeostasis and minimize inflammation by virtue of its antioxidant, anti-inflammatory and antiapoptotic activities (Paine et al., 2010). HO-1 is the key enzyme in free heme catabolism and is induced by a range of stimuli including pro-inflammatory cytokines (Spylin, 2008). By degrading heme, HO-1 produces carbon monoxide, iron and biliverdin. The latter is enzymatically converted to the powerful antioxidant bilirubin (Sarady-Andrews et al., 2005). In addition, HO-1 regulates cerebral hydrogen peroxide concentration and blocks neutrophil migration in acute inflammation (Freitas et al., 2006; Meng et al., 2014).

The present findings showed that nandrolone decanoate increased brain TNF-α level. Enhanced oxidative stress by nandrolone may trigger inflammatory responses and enhance TNF-α expression (Janssen et al., 1993). In addition, inhibition of Nrf2 expression by nandrolone may play a role since Nrf2 induces the expression of the anti-inflammatory cytokine IL-10 (Plantadosi et al., 2011). Another explanation may be attributed to nandrolone-induced inhibition of brain serotonin level because serotonin receptor activation has been associated with decreased TNF-α level (Nau et al., 2013). It is noteworthy that enhancement of TNF-α and other inflammatory cytokines may induce aggregation and contribute to neuronal apoptosis and memory impairment through the interaction with TNFR1 (Medina et al., 2002). Interestingly, TNFR1 knockout mice showed reduced anxiety, diminished aggression, and attenuated dementia (Patel et al., 2010; Calsavara et al., 2015).

The present results showed that both lipoic acid and pentoxifylline induced antioxidant and anti-inflammatory effects. Previous studies demonstrated that lipoic acid may cross the blood brain barrier, reduce MDA formation and enhance the activity of brain catalase and other antioxidant enzymes (Veskovic et al., 2015). In addition, lipoic acid is a thiol compound that possesses direct free radical scavenging activity (Shila et al., 2005). The current up-regulation of Nrf2 and HO-1 expression by lipoic acid was previously reported (Lee and Johnson, 2004; Lin et al., 2013). This effect may account for the protective effects of lipoic acid against nandrolone-induced memory impairment in rats since Nrf2 induction may attenuate oxidative stress-associated dementia in mice (Lee et al., 2013; Gault et al., 2015). Moreover, stimulated HO-1 expression may suppress glutamate-induced ROS production and protect against neuronal inflammation and death (Im et al., 2015). The observed reduction in nandrolone-induced TNF-α level by lipoic acid may be due to inhibition of oxidative stress following Nrf2 activation (Mazurek et al., 2008). It is noteworthy that Keap-1, the regulator protein of Nrf2 activation, can negatively regulate NF-κB signaling and reduce TNF-α production (Kim et al., 2010).

The present study confirmed previous reports about the inhibitory effect of pentoxifylline on TNF-α production (Siwa et al., 2002). The anti-inflammatory activity of pentoxifylline may attenuate brain neurotransmitters imbalance and suppress neuronal excitation since catecholamines are induced by over-expression of TNF-α (Guggilam et al., 2007; Kang et al., 2009). TNF-α interacts with its receptor TNFR1 to induce inflammatory responses. Therefore TNFR1 deficiency may attenuate neuronal inflammation, down-regulate apoptotic genes expression, and minimize brain damage (Shohami et al., 1996; Quintana et al., 2005).

The current histopathological examination revealed enhanced brain injury by nandrolone decanoate in rats. Combination of lipoic acid and pentoxifylline offered superior neuroprotection than any of the drugs alone. Similarly, it was previously reported that nandrolone decanoate may reduce neuronal density in the rat brain (Tugyan et al., 2013). The currently enhanced gliosis and scar formation by nandrolone decanoate may be attributed to increased TNF-α and oxidative stress (Quintana et al., 2005). Enhanced gliosis or hypertrophy of glial cells is a typical CNS response to damage. Gliosis hinders axon regeneration, induces the production of inflammatory mediators, causes scar formation and impede functional recovery (Sofroniew, 2009; Zhang et al., 2010). Excessive gliosis is associated with impaired memory and cognitive functions (Verkhratsky et al., 2012). On the other hand, nandrolone decanoate promoted the deposition of eicosanophilic plaques and increased brain susceptibility to beta amyloid fragments (Caraci et al., 2011). Aggregation of the latter fragments may induce reactive oxygen species, inflammatory cytokines, apoptosis, and neuronal mitochondrial dysfunction (Tiffany et al., 2001; He et al., 2011).

Histologically, antioxidants attenuated nandrolone decanoate-induced testicular injury in rats (Tahtamouni et al., 2010; Ahmed, 2015). Interestingly, lipoic acid decreased neuronal necrosis in rats with Alzheimer’s disease and attenuated glial cell scar formation (Ahmed, 2012; Rocamonde et al., 2012). Suppression of astroglisis may inhibit neuronal injury (Li et al., 2015). Likewise, pentoxifylline increased the number of viable neurons after cerebral ischemia (Bruno Rde et al., 2009).

In conclusion, the present study showed that nandrolone decanoate induced behavioral alterations namely anxiety, aggression, and dementia in rats. These behavioral disturbances were associated with imbalance in brain neurotransmitters, down-regulation of Nrf2/HO-1 pathway and over-expression of TNFR1. Nandrolone-induced deleterious effects were remarkably attenuated by a combination of lipoic acid and pentoxifylline without disturbing the anabolic effect of nandrolone decanoate. This was achieved by virtue of the potent antioxidant activity of lipoic acid and the remarkable anti-inflammatory effect of pentoxifylline. The underlying mechanisms of the protective effects of lipoic acid and pentoxifylline include maintenance of normal balance of brain neurotransmitters, inhibition of AchE activity, up-regulation of AChR, stimulation of Nrf2/HO-1 pathway, and down-regulation of TNFR1.

Further investigations are needed to elucidate the effect of nandrolone decanoate on neurotransmitters in individual brain regions. Quantitative histopathological studies by specialists are encouraged. Clinical
trials are recommended to assess the neurobehavioral protective effects of lipic acid and pentoxifylline in androgen-deplete causers. 

Conflict of interest

The authors deny any conflict of interest and declare equal contribution to this work.

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References


Dell’Elencl, C.L., Dlasci, D., Cornillet, P., Guenounou, M., 1999. Differential regulation of TNF alpha, IL-1 beta, IL-6, IL-8, TNF beta, and IL-10 by pentoxifylline. Int. J. Immunopharmacol. 21, 739–746.


