Scientific Research Publishing

Doxycycline Ameliorates Schizophrenia-Like Behaviors in Experimental Models in Mice by Targeting Underlying Oxidative Stress

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Abstract

Current evidences support the inhibition of oxidative and inflammatory signaling mechanisms in the treatment of schizophrenia; as cure for this disease still remains limited. Doxycycline is a tetracycline antibiotic (a minocycline congener) with strong antioxidant and anti-inflammatory properties, and better pharmacokinetic profiles. Preclinical evidence indicates that minocycline possesses antipsychotic properties. This present study was designed to evaluate the effect of doxycycline on schizophrenia-like behaviors, as well as biomarkers of oxidative stress in mice brains. Novelty-induced rearing (NIR) behavior was used to evaluate the tranquilizing effect of doxycycline (25 - 200 mg/kg). The acute antipsychotic effects of doxycycline were assessed using apomorphine-induced stereotypy, ketamine-induced stereotypy, hyperlocomotion and enhanced immobility in forced swim test (FST). Catalepsy test was also employed to evaluate the extrapyramidal adverse effect of doxycycline in mice. The chronic antipsychotic effect of doxycycline was evaluated following oral administration of doxycycline in combination with ketamine (100 mg/kg) intraperitoneally for 10 days. Twenty four hours after the last administration, positive (locomotor activity), cognitive (Y-maze) and negative (FST) symptoms were assessed. Thereafter, levels of biomarkers of oxidative stress were evaluated in mice brains. Doxycycline significantly ($P < 0.05$) decreased NIR, inhibited stereotypy induced by apomorphine and ketamine. Additionally, doxycycline significantly ($P < 0.05$) prevented ketamine-induced hyperlocomotion, cognitive deficit and reduced enhanced-immobility by ketamine in mice. Furthermore, doxycycline decreased malondialdehyde concentrations in a dose-related manner. Moreover, doxycycline significantly ($P < 0.05$) prevented the decrease in glutathione, and increased activities of superoxide dismutase and catalase in brain tissues. The results from this study suggest that doxycycline

ameliorated schizophrenic-like behaviors via mechanisms related to attenuation of oxidative stress in mouse brain.

Keywords

1. Introduction

Psychosis (e.g., Schizophrenia) is a heterogeneous neuropsychiatric disorder characterized by distorted or non-existent sense of reality [1]. It affects about 1% of the world's population [2]. Schizophrenia is characterized by positive (e.g., hallucinations), negative (e.g., social isolation) and cognitive (e.g., executive and memory dysfunction) symptoms [3]. While the positive symptoms results from hyperdopaminergic activity in the mesolimbic pathways, the negative and cognitive deficits emanate from hypodopaminergic system of the prefrontal cortex [4].

Although first-generation antipsychotics are responsive in reducing the positive symptoms, these agents have been relatively less effective in ameliorating the severity of negative and cognitive deficits, and are also limited by their high tendency to produce extrapyramidal side effects, due to excessive blockade of dopaminergic D_2 receptors [5]. In contrast, the second-generation antipsychotics are effective in ameliorating all groups of symptoms with lesser extra-pyramidal side effects, but in turn hold greater risk of cardiovascular diseases, diabetes, agranulocytosis etc. [6]. Moreover, regular intake of these agents may also increase oxidative stress and further enhance the progression of the disease [7]. Notably, Pazvantoglu *et al.* [8] demonstrated that the severity of the symptoms depended on the total antioxidant levels. The brain has been reported to be more vulnerable to oxidative stress, because it is the most metabolically active tissue in the body and so generates a high load of reactive oxygen moieties, which triggers lipid peroxidation that leads to several behavioral perturbations [7]. Hence, the need for newer antipsychotic agents with multipronged mechanisms of action that could target various aspects of the pathologies of schizophrenia has become imperative [7] [8]. To this end, the second-generation tetracycline antibiotic drugs (e.g., minocycline, doxycycline) have recently been attracting much attention in neuropsychiatric preclinical researches due to their antioxidant and anti-inflammatory/immunomodulatory mechanisms of action [9].

Doxycycline is a long acting second-generation tetracycline antibiotic which is rapidly absorbed and penetrates well into the brain, with very low toxicity profile [10]. Globally, doxycycline has remained one of the most commonly used inexpensive broad-spectrum antibiotics and is included in the list of Essential Medicine of the World Health Organization (WHO) [10]. Similar to minocycline, doxycycline possesses strong neuroprotective effect and this is closely attributed to its anti-inflammatory, antioxidant, anti-apoptotic and neurotrophic properties [9] [11] [12].

During the last decade, the neurotherapeutic potentials of minocycline and doxycycline in the treatment of mental disorders have reasonably increased [7] [12] [13]. However, there is a lack of preclinical studies investigating the psychotropic effects of doxycycline as a therapeutic agent for schizophrenia. Indeed, recent findings from preclinical studies suggest possible beneficial effects of minocycline as adjunctive therapy for the treatment of negative and cognitive symptoms of schizophrenia [13] [14]. In this context, the therapeutic properties of doxycycline relative to that of minocycline, suggests that it may also have clinical application in schizophrenia symptomology, most especially against the negative and cognitive symptoms of the disease [15] [16]. Therefore, this study was designed to evaluate the antipsychotic property of doxycycline by specifically investigating its effects on: 1) positive schizophrenia-like behavior, 2) negative schizophrenia-like behavior, 3) cognitive schizophrenia-like behavior and 4) biomarkers of oxidative stress in mouse brain.

2. Materials and Methods

2.1. Experimental Animals

Male Swiss mice (20 - 25 g; 6 weeks old) were obtained from the Central Animal House, Delta State University, Abraka. The animals were housed five per plastic cage (42 × 30 × 27 cm) at room temperature (25˚C ± 1˚C) with a 12:12 hr light/dark cycle. They were fed with standard rodent pellet food and water *ad libitum* throughout the experimental period. They were acclimatized for at least 1 week prior to commencement of the experiments. The experiments were performed according to the National institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85 - 23, revised 1985). Also, efforts were made to minimize the suffering of the animals by careful handling, treatments and euthanization of the animals.

2.2. Drugs and Chemicals

Doxycycline (DOX) (Hovid Pharmaceutical industry, Malaysia), apomorphine (APO) (Sigma-Aldrich, St. Louis, USA), ketamine (KET) hydrochloride (Sigma-Aldrich, St. Louis, USA), haloperidol (HLP) (Sigma-Aldrich, St. Louis, USA), risperidone (RIS) (Sigma-Aldrich, St. Louis, USA), trichloroacetic acid (TCA) (Burgoyne Burbidges & Co., Mumbai, India), thiobarbituric acid (TBA) (Guanghua Chemical Factory Co. Ltd., China), Ellman Reagent [5',5'-Dithiobis-(2-nitrobenzoate) DTNB] (Sigma-Aldrich, St. Louis, MO, USA), hydrogen peroxide (H_2O_2) (BDH Chemicals Ltd., Poole, England) and adrenaline (Sigma-Aldrich, St. Louis, USA) were used in the study.

2.3. Drug Preparation

Doxycycline, risperidone and haloperidol were dissolved in distilled water immediately before use and administered *per oral* (p.o.). Apomorphine was also dissolved in distilled water immediately before use and administered intraperitoneally (i.p.). Ketamine was diluted with distilled water and administered (i.p.). The doses of Doxycycline used in this study were selected based on the results from preliminary investigations. Halo-

peridol was used as the positive control for apomorphine model, while risperidone was used as the positive control for ketamine models, since ketamine-induced model of schizophrenia has been demonstrated to be more responsive to atypical antipsychotic [17]. Appropriate vehicle (VEH)-controlled groups were also assessed simultaneously *per oral* with 10 mL/kg of distilled water.

2.4. Experimental Design

For acute studies, novelty-induced rearing (NIR) behavior was employed. Single dose of apomorphine (1 mg/kg, i.p.) [18] administration was used to induced stereotypy behavior. Different doses of ketamine were used in this study: Single dose of ketamine (10 mg/kg, i.p.) [17] administrations was used to induce stereotypy and hyperlocomotion (positive symptoms) respectively; ketamine (30 mg/kg, i.p./day) [19] was used for enhancement of immobility time (negative symptoms) following 5 days treatment and thereafter animals were treated with single administration of doxycycline 24 hr after the last treatment with ketamine, followed by behavioral despair assessment in the forced swim test. Furthermore, catalepsy test was also employed for the assessment of cataleptic behavior (extrapyramidal side effect) (**Figure 1**).

For sub-chronic study, repeated ketamine (100 mg/kg, i.p./day) [20] for 10 days was used to induced schizophrenia-like behaviors and oxidative alteration in mice brains. Mice were pretreated with either vehicle (10 mL/kg, p.o.), standard antipsychotic agents (risperidone, 0.5 mg/kg, p.o.) or doxycycline (25, 50, 100 and 200 mg/kg, p.o.) 1 hr prior to the administration of ketamine (100 mg/kg, i.p.) for 10 days. Animals were evaluated for behavioral phenotypes 24 hr after the last administration of doxycycline and ketamine respectively, on days 9, 10 and 11: 1) hyperlocomotor activity (open field test) (representing *positive symptoms*) on the 9th day, 2) Y-maze test (representing
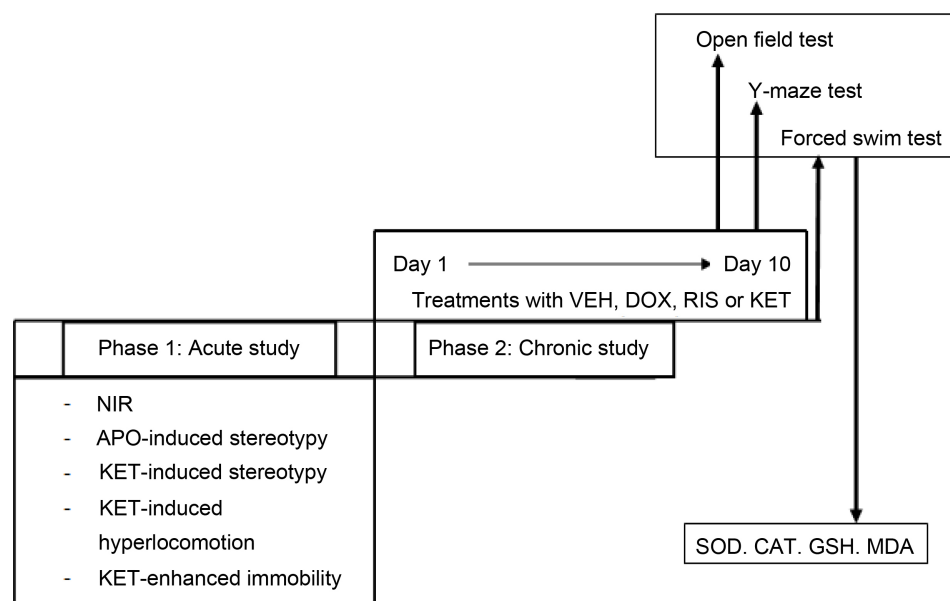

Figure 1. Experimental protocol.

cognitive symptoms) on the 10th day and 3) forced swim test (representing *negative symptoms*) was carried out 24 hr after the last administration (on the 11th day). Immediately after the behavioral tests, the animals were euthanized and the supernatant of the brain tissues were used to assay for levels of biomarkers of oxidative stress including superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and malondialdehyde (MDA) as presented in Figure 1.

2.5. Acute Study

2.5.1. Assessment of Novelty-Induced Rearing (NIR) Behavior in Mice

Novelty-induced rearing behavior was employed to evaluate the central excitatory/ inhibitory rearing behavior in mice [21]. The animals were divided into six treatment groups (n = 5). The group 1 received vehicle (distilled water) (10 mL/kg, p.o.), groups 2 - 5 were treated with DOX (25, 50, 100 and 200 mg/kg, p.o.), while the 6th group received RIS (0.5 mg/kg, p.o.), respectively. One hour later, mice were individually placed in a transparent Plexiglas chamber (45 cm × 25 cm × 25 cm) and the rearing frequencies (number of times the animal stood on its hind limbs or with its fore limbs against the walls of the observation box or free in the air) were recorded for 5 min. The arena was cleaned with 70% ethanol to eliminate olfactory bias (residual odour) after each session [17].

2.5.2. Apomorphine-Induced Stereotypy

The antipsychotic effect of DOX was assessed using the Apomorphine-induced stereotyped behavior as previously described by Bourin *et al.* [18]. The mice were randomly divided into six treatment groups (n = 5). Group 1 received vehicle (10 mL/ kg,p.o.) (serving as negative control), while groups 2 - 5 were pretreated with DOX (25, 50, 100 and 200 mg/kg, p.o.) and the 6th group was pretreated with HLP (1 mg/kg, p.o.) as positive control. Sixty minutes later, each animal in groups 1 - 6 received i.p. injection of APO (1 mg/kg) and mouse was placed immediately in a transparent observation chamber (20 cm × 20 cm × 23 cm). Thereafter, stereotype behaviors were observed for 2 min at 10, 15, 30, 45 and 60 min after APO injection. Stereotype behaviors were scored as: 0 = absence of stereotype behavior; 1 = presence of stereotype movements of the head; 2 = intermittent sniffing; 3 = chewing; 4 = intense licking. The observation chamber was cleaned with 70% ethanol after each test session [17].

2.5.3. Ketamine-Induced Stereotypy

Ketamine-induced psychosis was also employed to screen for the antipsychotic effect of DOX according to the method described by Ben-Azu *et al.* [17]. The mice were randomly divided into six treatment groups (n = 5). Group 1 received vehicle (10 mL/kg, p.o.) (Group 1 serving as negative control) while groups 2 - 5 were pretreated with DOX (25, 50, 100 and 200 mg/kg, p.o), and 6th group was pretreated with RIS (0.5 mg/kg, p.o.) as positive control. Sixty minutes later, each animal in groups 1 - 6 received i.p. injection of sub-anaesthetic dose of KET (10 mg/kg) and mouse was placed immediately in a transparent observation chamber (20 cm × 20 cm × 23 cm) and ste-

reotypy was observed for 2 min at 10, 15, 20, 30 and 45 min respectively. Stereotyped behaviors were scored as described above. The observation chamber was also cleaned with 70% ethanol after each test session [17].

2.5.4. Ketamine-Induced Hyperlocomotion

Ketamine-induced hyperactivity was also used to screen for the antipsychotic effect of DOX as previously described by Ben-Azu *et al.* [17]. The mice were randomly divided into six treatment groups (n = 5). Group 1 received vehicle (10 mL/kg, p.o.), while groups 2 - 5 were pretreated with DOX (25, 50, 100 and 200 mg/kg, p.o.) and the 6th group was pretreated with RIS (0.5 mg/kg, p.o.) as positive control. Sixty minutes thereafter, groups 1 - 6 received i.p. injection of sub-anaesthetic dose of KET (10 mg/kg). Thereafter, the animals were individually placed at the center of an open field chamber (35 × 30 × 23 cm). The duration of ambulation and number of line crossed were recorded for 5 min. The observation chamber was also cleaned with 70% ethanol as earlier described [17].

2.5.5. Ketamine-Enhanced Immobility in Forced Swim Test

The antipsychotic effect of DOX was also screened using ketamine-enhanced immobility in forced swim test that is predictive of the negative symptoms of schizophrenia; which is reflected as a state of despair in mice as described by Chindo *et al.* [19] and Chatterjee *et al.* [20], with brief modifications. The reduction in the immobility time serves as a specific and selective index of antidepressant activity that can be used to alleviate the negative symptoms of schizophrenia. The mice were randomly divided into seven (7) treatment groups (n = 5). Group 1 was pretreated with vehicle (10 mL/kg, i.p.) once daily for 5 days while groups 2 - 6 were pretreated with a sub-anaesthetic dose of KET (30 mg/kg, i.p.) once daily for 5 days. After which, each mice were placed in a transparent glass cylinder (height 46 cm, diameter 20 cm) containing water at 25˚C to a depth of 30 cm and was forced to swim for 5 min for habituation (pretest session) 1 h after the last treatment (5th day) with ketamine. Twenty four hours after the last treatment (6th day) with vehicle or KET respectively, group 2 received vehicular treatment (10 mL/kg, p.o.) as a negative control, groups 3 - 6 were treated with DOX (25, 50, 100 and 200 mg/kg, p.o), while group 7 was treated with RIS (0.5 mg/kg, p.o.) as positive control. Sixty minutes later, each animal was placed again in the water for 6 min and the immobility time was recorded for a period of 5 min with a stopwatch (test session) after discarding activity in the first 1 min, during which the animal tries to escape. After each session, the mice were removed immediately from the cylinder, dried with a towel and kept in an open space until completely dried before returning the mice to their home cages.

2.5.6. Catalepsy Test on DOX

The cataleptic effect of the DOX was investigated according to the modified version previously described by Omogbiya *et al.* [21] and Chatterjee *et al.* [20]. The animals were divided into six treatment groups (n = 5). The group 1 was treated with vehicle

(10 mL/kg, p.o.) while groups 2 - 5 were treated with DOX (25, 50, 100 and 200 mg/kg, p.o) and the sixth group was treated with HLP (1 mg/kg, p.o.) 60 min before testing for catalepsy. The test was done by gently placing the fore limbs of each animal on an inclined horizontal plane wood surface (H = 6 cm; W = 4 cm; L = 16 cm) and the duration of akinesia (period of time the animal remained on an imposed posture, before initiating any active movement) in seconds was recorded.

2.6. Sub-Chronic Study: Sub-Chronic Treatment of Ketamine-Induced Schizophrenia-Like Behavior and Oxidative Damage in Mouse Brain

The model described by Chatterjee *et al.* [20] was adapted with little modification. Briefly, mice were divided into 7 groups (n = 5). Group 1 was pretreated with distilled water (10 mL/kg, i.p.) as vehicle for 10 days, group 2 was treated with ketamine (100 mg/kg, i.p.) only for 10 days, while groups 3 - 6 were pretreated with DOX (25, 50, 100 and 200 mg/kg, p.o.) one hour prior to the administration of KET (100 mg/kg, i.p.) for 10 days, while group 7 received risperidone (0.5 mg/kg, p.o.) one hour prior to KET (100 mg/kg, i.p.) administration for 10 days.

2.6.1. Behavioral Tests
Behavioral tests were performed 24 hr after the last administration of DOX and ketamine on days 9, 10 and 11, respectively. Each animal was evaluated for behavioral phenotypes as previously by Chatterjee *et al.* [20], *i.e.*: 1) hyperlocomotor activity (representing *positive symptoms*) on the 9th day, 2) Y-maze test (representing *cognitive symptoms*) on the 10th day and 3) forced swim test (representing *negative symptoms*) was carried out 24 hr after the last administration (on the 11th day).

1) Open-field test (OFT)

Locomotor behavior was monitored using the open field test apparatus with brief modifications. The open field apparatus consisted of a wooden box measuring 35 × 30 × 23 cm with visible lines drawn to divide the floor into 36 (20 cm × 20 cm) squares with a frontal glass wall, and placed in a sound free room. The animals were placed in the rear left square and left to explore it. The parameter measured includes number of lines crossed for 5 min using a stopwatch [21].

2) Y-maze test (YMT)

The effect of DOX on spontaneous alternation performance was also assessed using YMT which allows for the evaluation of cognitive searching behavior, as an index of spatial working memory dysfunction of schizophrenia. Animals were gently placed individually in the Y-maze apparatus, which consisted of three identical arms (33 × 11 × 12 cm each) in which the arms were symmetrically separated at 120°. Each mouse was placed at the end of arm A and allowed to explore all the three arms (labeled A, B, C) freely for 5 min, taking the following parameters: the number of arm visits and sequence (alternation) of arm visits visually. The percentage of alternations was calculated as total of alternations/(total arm entries-2). After each test session, the observation chamber was cleaned with 70% ethanol to remove residual odour [22].

3) Forced swim test (FST)

Briefly, each mouse was placed in a standardized transparent glass cylinder (Height 46 cm, diameter 20 cm) containing water at 25˚C to a depth of 30 cm and was forced to swim for 5 min (pretest session) 1 hr after the last treatment (10th day) with ketamine for habituation. Twenty four hours after the pretest session (on the 11th day), each animal was placed again in the same transparent Plexiglas cylinder containing water at 25˚C to a depth of 30 cm. They were then forced to swim for 6 min and the immobility time (which is the time the animal floats in the water in an upright position and made only slight movements to prevent sinking) was recorded for a period of 5 min with a stopwatch after discarding activity in the first 1 min, during which the animal tries to escape. After each session, the mice were removed immediately from the cylinder, dried with a towel and kept in an open space until completely dried before returning them to their home cages [20].

2.6.2. Biochemical Assays

1) Preparation of brain tissues for biochemical assay

Immediately after the behavioral tests, the animals were decapitated under ether anaesthesia and the brains were immediately removed, weighed and kept in the refrigerator in the refrigerator with ice block for 30 min. Thereafter, the whole brains were homogenized with 5 mL of 10% w/v phosphate buffer (0.1 M, PH 7.4). Each brain tissue homogenates were centrifuged at 10,000 g for 10 min at 4˚C, the pellet was discarded and the supernatant was immediately separated into various portions for the different biochemical assays.

2) Determination of superoxide dismutase (SOD) activity

The level of SOD activity was measured by the method described by Misra and Fridovich [23]. This method is based on the inhibition of superoxide dependent adrenaline auto-oxidation in a spectrophotometer adjusted at 480 nanometer (nm). Brain supernatant of 1 mL was diluted in 9 mL of distilled water to make a 1 in 10 dilution. An aliquot of 0.2 mL of the diluted sample was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction was started by the addition of 0.3 mL of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette (Blank) contained 2.5 mL buffer, 0.3 mL of substrate (adrenaline) and 0.2 mL of distilled water. The increase in absorbance at 480 nm was monitored for 30 s for 150 s. A unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline [23].

3) Determination of catalase (CAT) activity

CAT activity was assayed by the method of Sinha [24], which was based on the disappearance of Hydrogen peroxide (H_2O_2) in the presence of an enzyme source (catalase). Brain supernatant of the sample homogenate (1 mL) was mixed with 19 mL of distilled water to give a 1:20 dilution. Then, 1 mL of this was added to 5 mL of phosphate buffer (pH 7.0) and 4 mL of H_2O_2 solution (800 μmoles). The reaction mixture was mixed by a gentle swirling motion at room temperature. Then, 1mL of this portion of the reaction mixture was withdrawn and added into 2 mL dichromate/acetic acid

reagent. The absorbance was measured using spectrophotometer at 570 nm and change in absorbance at 60 s interval. The catalase activity was expressed as µmoles of H_2O_2 decomposed per minutes per mg protein.

4) Determination of glutathione (GSH) concentration

GSH concentration was assayed by the method described by Jollow *et al.* [25], which was based upon the development of a relatively stable (yellow) colour when 5', 5'-di-thiobis-(2-nitrobenzoic acid) (DTNB) is added to sulfhydryl compounds. Brain homogenates of 0.4 mL was added to 0.4 mL of 20% trichloroacetic acid (TCA) and mixed by a gentle swirling motion and then centrifuged in a cold (4°C) centrifuge at 10,000 rpm for 20 min. Then, 0.25 mL of the supernatant was added to 2 mL of 0.6 mM DTNB and the final volume of the solution was made up to 3 mL with phosphate buffer (0.2 M, pH 8.0). Absorbance was read at 412 nm against blank reagent-[2 mL of 0.6 mM DTNB + 1 mL phosphate buffer (0.2 M, pH 8.0)] using a spectrophotometer. The concentration of reduced GSH in the brain tissues was expressed as nanomoles per gram tissue (nmol/g tissue).

5) Estimation of brain level of malondialdehyde (MDA)

The brain level of MDA was measured according to the method described by Okhawa *et al.* [26]. This assay principle is based on the fact that lipid peroxidation generates unstable lipid peroxides that decompose to form a complex series of compounds including reactive carbonyl compounds. The polyunsaturated fatty acid peroxides produced generate MDA upon decomposition. MDA forms a 1:2 adduct with thiobarbituric acid (TBA) that gives rise to a pink color product when heated in acidic pH, with a maximum absorbance of 532 nm. In line with this, an aliquot of 0.4 mL of the sample was mixed with 1.6 mL of Tris-potassium chloride (Tris-KCl) buffer to which 0.5 mL of 30% trichloroacetic acid (TCA) was added. Then, 0.5 mL of 0.75% TBA was added and placed in a water bath for 45 min at 80°C. This was then cooled in ice at 4°C and centrifuged at 3000 rpm for 15 min. The clear supernatant was collected and absorbance was measured against a reference blank of distilled water at 532 nm. The MDA concentration was calculated using a Molar extinction coefficient of 1.56×10^5 M^{-1} CM^{-1} and the value was expressed as nanomole of MDA per gram tissue (nmol/g tissue).

6) Protein content estimation

This assay was done according to method described by Gornall *et al.* [27], using the Biuret method. 1 mL of the diluted sample was taken and added to 3 mL of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 min after which the absorbance was read at 540 nm using distilled water as blank. Bovine serum albumin (1 mg/mL) was used as standard and was measured in the range of 0.01 - 0.1 mg/mL [28].

2.7. Statistical Analysis

Statistical analysis was done using Graph Pad Prism software version 5.0 and data were expressed as Mean ± S.E.M (standard error of mean). Following a normality test, data were analyzed using one-way analysis of variance (ANOVA) followed by post-hoc test

(Newman-Keuls) for multiple comparisons where appropriate. A level of $P < 0.05$ was considered as statistically significant for all tests.

3. Results

3.1. Effect of DOX on Novelty-Induced Rearing (NIR) Behavior in Mice

Pretreatment with DOX (50, 100 and 200 mg/kg, p.o.) [F (5, 24) = 79.76, $P < 0.0001$] significantly ($P < 0.05$) reduced the NIR in a similar manner to RIS compared with the control; however, DOX (25 mg/kg, p.o.) showed no significant ($P > 0.0.5$) reduction in NIR. Peak inhibition of NIR was observed at 200 mg/kg (**Figure 2**).

3.2. Effect of DOX on Apomorphine-Induced Stereotypy

In this study, apomorphine (1 mg/kg, i.p.) administration significantly ($P < 0.05$) elicited marked stereotyped behaviors characterized by head movements, intermittent sniffing, chewing and intense licking, which are reflected in schizophrenic patient as a form of positive symptom (**Figure 3**). Pretreatment with DOX (100 and 200 mg/kg, p.o.) significantly ($P < 0.05$) prevented apomorphine-induced stereotyped behaviors [F (5, 24) = 50.28, $P < 0.0001$] (**Figure 3**). However, treatment with the lower doses of DOX (25 and 50 mg/kg, p.o.) failed prevent this stereotypic behavior in the animals compared to APO-treated control group. Similar effects were also observed in animals treated with HLP (1 mg/kg, p.o.), as it significantly ($P < 0.05$) prevented the manifestations of stereotyped behaviors induced by APO (**Figure 3**).

3.3. Effect of Acute Administration of DOX on Ketamine-Induced Stereotypy

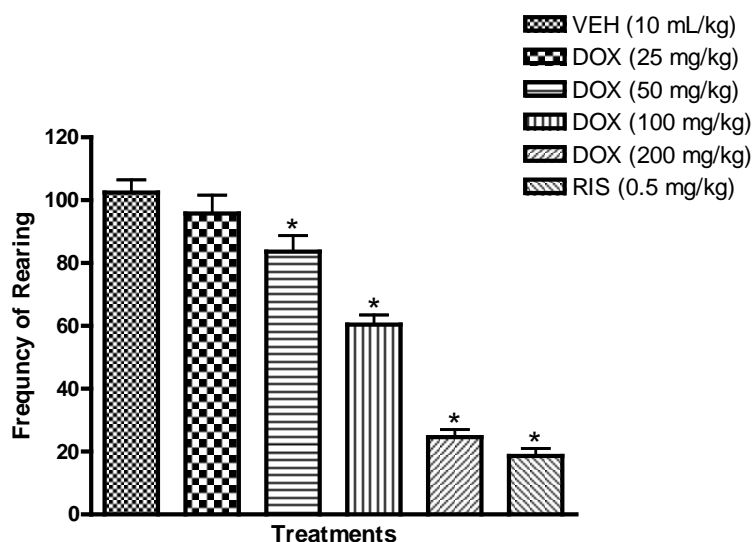Treatment with ketamine (10 mg/kg, i.p.) demonstrated significant ($P < 0.05$) increase

Figure 2. Effect of DOX on novelty-induced rearing behavior in mice. Values represent the mean of 5 animals/group. *Denotes $P < 0.05$ compared with vehicle group. VEH = Vehicle, DOX = Doxycycline, RIS = Risperidone.

in stereotyped behaviors compared to vehicle (Figure 4). The study showed that pretreatment with DOX (25 and 50 mg/kg, p.o.) failed to demonstrate significant ($P > 0.05$) protection against ketamine induced stereotypic behavior whereas, treatment with higher doses of DOX (100 and 200 mg/kg, p.o.) demonstrated significant ($P < 0.05$) protection against these stereotypic dispositions caused by ketamine in a similar manner to RIS [$F(5, 24) = 50.28$, $P < 0.0001$] (Figure 4).

3.4. Effect of Acute Administration of DOX on Ketamine-Induced Hyperlocomotion

The effect of DOX on ketamine-induced hyperlocomotion in mice, as assessed in the open-field test (OFT) is shown in Table 1. The administration of ketamine (10 mg/kg, i.p.) induced hyperlocomotion compared to vehicular (10 mL/kg) control group, as indexed by the increase in the number of line crossings and reduction in the duration
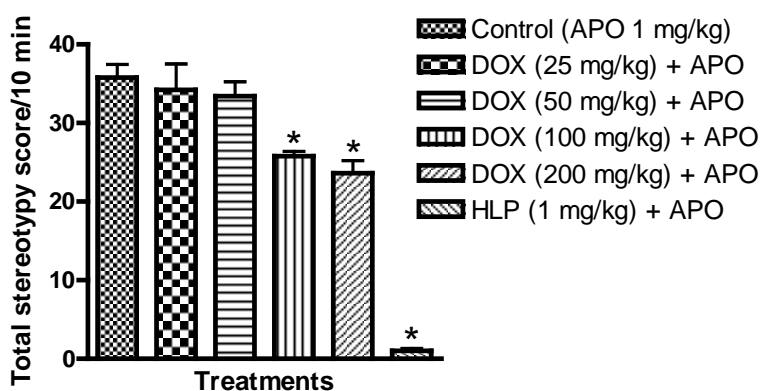

Figure 3. Effect of DOX on apomorphine-induced stereotypy. Value represents the mean ± S.E.M of 5 animals/group. *Denotes $P < 0.05$ compared with APO control group (ANOVA followed by Newman-Keuls test). APO = Apomorphine, DOX = Doxycycline, HLP = Haloperidol.
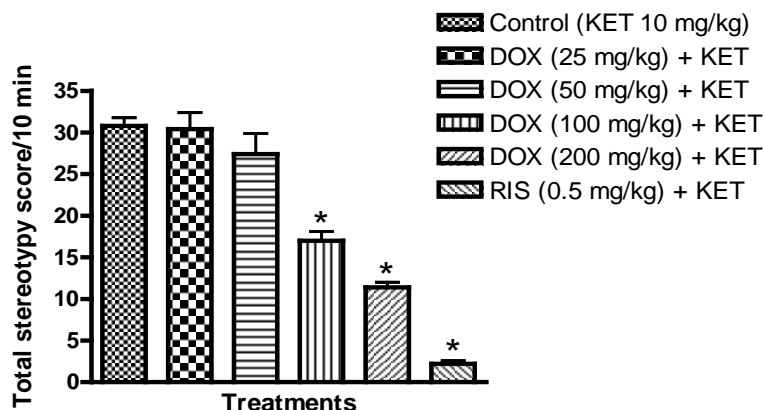

Figure 4. Effect of acute administration of DOX on ketamine-induced stereotypy. Value represents the mean ± S.E.M of 5 animals/group. *Denotes $P < 0.05$ compared with KET control group (ANOVA followed by Newman-Keuls test). KET = ketamine, DOX = Doxycycline, RIS = Risperidone.

Table 1. Effect of acute administration of DOX on ketamine-induced hyperlocomotion.

Treatments	Dose (mg/kg)	Number of line crossing (s)	Duration of ambulation
KET	10	129.0 ± 4.77	96.40 ± 6.78
DOX	25	125.0 ± 3.53	94.20 ± 8.59
DOX	50	123.6 ± 2.08	92.80 ± 3.85
DOX	100	105.6 ± 2.59	88.6 ± 10.80
DOX	200	90.40 ± 4.14*	184.2 ± 7.98*
RIS	0.5	32.20 ± 4.99*	223.6 ± 9.33*

Value represents the mean ± SEM of 5 animals/group. *Denotes $P < 0.05$ compared with KET control group (ANOVA followed by Newman-Keuls test). KET = ketamine, DOX = Doxycycline, RIS = Risperidone.

of ambulation in the OFT. DOX pretreatment (200 mg/kg, p.o.) significantly ($P < 0.05$) prevented hyperlocomotion induced by ketamine (10 mg/kg, i.p.) as indicated by the decrease in the number of line crossings [$F_{(6, 28)} = 69.38$, $P < 0.0001$] and increase in duration of ambulation of the animals [$F_{(6, 28)} = 42.58$, $P < 0.0001$] in the OFT similar to RIS compared to KET-treated group. However, treatments with lower doses of DOX (25, 50 and 100 mg/kg, p.o.) did not show any significant protection against hyperlocomotion induced by ketamine in mice.

3.5. Effect of Acute Administration of DOX on Ketamine-Enhanced Immobility in Forced Swim Test in Mice

Effect of the acute (single dose of drug) administration of DOX and risperidone on ketamine-induced enhanced immobility in forced swim test is shown in Figure 5. Repeated ketamine administration (30 mg/kg, i.p./day) for 5 days significantly ($P < 0.05$) enhanced the duration of immobility in the forced swim test compared to the group treated with vehicle (10 mL/kg, i.p.) in mice. However, single treatment with RIS (0.5 mg/kg, p.o.) significantly ($P < 0.05$) reduced enhancement in immobility time by ketamine. Meanwhile, acute treatment with DOX (50, 100 and 200 mg/kg, p.o.) but not with 25 mg/kg, p.o 24 hr post-treatment with ketamine also showed significant ($P < 0.05$) restoration against ketamine-induced immobility similar to RIS [$F_{(6, 28)} = 25.47$, $P < 0.0001$] compared to ketamine-treated group (Figure 5).

3.6. Effect of DOX on Catalepsy Test

The DOX (25 - 200 mg/kg, p.o.) showed no significant ($P > 0.05$) prolongation in the duration of akinesia compared with the group that received vehicle (10 mL/kg, p.o.). However, HLP (1 mg/kg, p.o.) significantly ($P < 0.05$) prolonged the duration of akinesia when compared to vehicle treated group (Figure 6).

3.7. Prevention of Sub-Chronic Ketamine-Induced Hyperlocomotion, Alteration in Working Memory and Behavioral Despair by Doxycycline

3.7.1. Effect of DOX on Ketamine-Induced Hyperlocomotion
Our data show that sub-chronic administration of ketamine (100 mg/kg, i.p./day) for
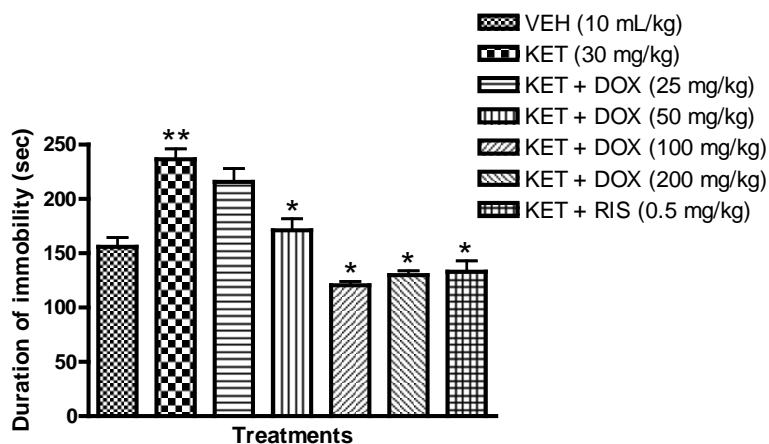
Figure 5. Effect of acute administration of DOX on ketamine-enhanced immobility in forced swim test in mice. Value represents the mean ± S.E.M of 5 animals/group. **Denotes $P < 0.05$ compared with vehicle control group, *Denotes $P < 0.05$ compared with KET treated group (ANOVA followed by Newman-Keuls test). KET = ketamine, DOX = Doxycycline, RIS = Risperidone.
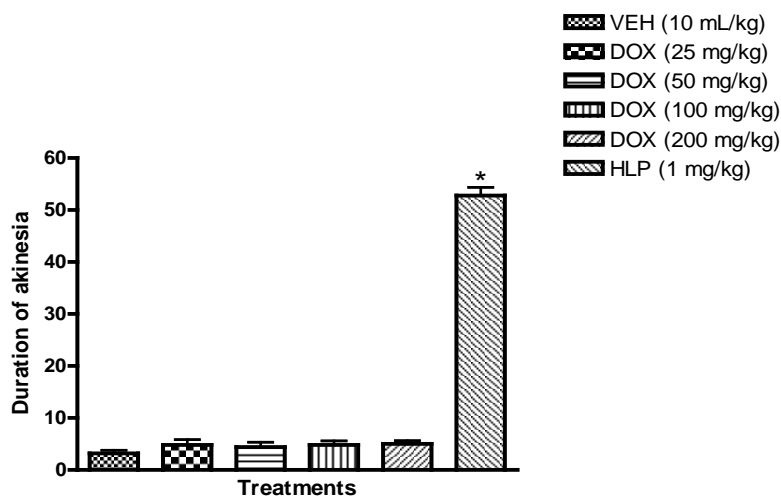

Figure 6. Effect of DOX on catalepsy test.

10 days significantly ($P < 0.05$) increased locomotion (hyperlocomotion) in the OFT compared to vehicle group, as indexed by the increase in the number of line crossings (Figure 7). Increased locomotion induced by ketamine was significantly ($P < 0.05$) prevented by DOX (100 and 200 mg/kg, p.o./day) in a dose dependent manner following treatment for 10 days [$F(6, 28) = 31.67$, $P < 0.0001$]; however, treatment with DOX (25 and 50 mg/kg, p.o./day) failed to antagonize ketamine-induced hyperlocomotion. Furthermore, pretreatment with risperidone prior to ketamine treatment once daily for 10 days significantly reduced hyperlocomotion by ketamine (Figure 7).

3.7.2. Effect of DOX on Ketamine-Induced Alteration in Working Memory
The effect of DOX on ketamine-induced alteration in working memory as an index of

cognitive impairment was assessed based on the sequence of arm entry and number of arm entries in the YMT (Figure 8). Sub-chronic ketamine (100 mg/kg, i.p./day) treatment for 10 days significantly ($P < 0.05$) decreased percentage alternations in YMT compared to vehicle-treated group. The administration of risperidone protected the animals against the cognitive deficits induced by ketamine. Also, pretreatment with DOX (100 and 200 mg/kg, p.o./day) in combination with ketamine for 10 days demonstrated significant ($P < 0.05$) prevention against the alteration in working memory by ketamine as shown by the increase in the percentage alternations in the YMT [$F(6, 28) = 9.798$, $P < 0.0001$] compared to the ketamine treated group. However, treatment with
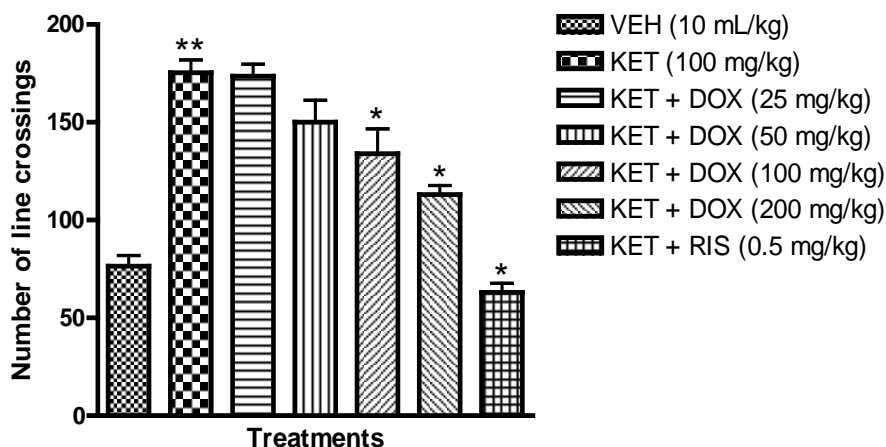

Figure 7. Effect of sub-chronic administration of DOX on ketamine-induced hyperlocomotion. Value represents the mean ± S.E.M of 5 animals/group. **Denotes $P < 0.05$ compared with vehicle group. *Denotes $P < 0.05$ compared with ketamine group (ANOVA followed by Newman-Keuls test). VEH = Vehicle, KET = Ketamine, RIS = Risperidone, DOX = Doxycycline.
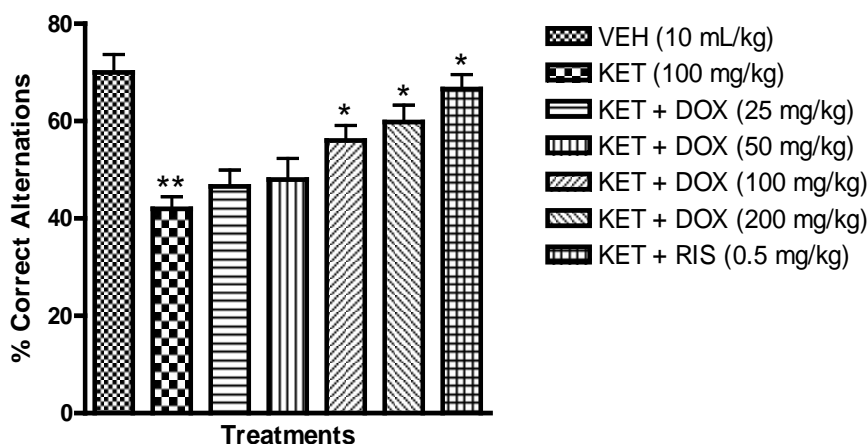

Figure 8. Effect of DOX on ketamine-induced alteration in working memory. Value represents the mean ± S.E.M of 5 animals/group. **Denotes $P < 0.05$ compared with vehicle group. *Denotes $P < 0.05$ as compared with ketamine group. (ANOVA followed by Newman-Keuls test). VEH = Vehicle, KET = Ketamine, RIS = Risperidone, DOX = Doxycycline.

lower dose of DOX (25 and 50 mg/kg, p.o./day) failed to demonstrate significant ($P >$ 0.05) effect against ketamine-induced alteration in working memory (Figure 8).

3.7.3. Effect of DOX on Ketamine-Enhanced Immobility (Behavioral Despair) in Forced Swim Test in Mice

Furthermore, the effect of sub-chronic pretreatment of DOX (25, 50, 100 and 200 mg/kg, p.o./day) for 10 days against ketamine-induced enhancement of immobility as an index of behavioral despair (negative symptoms) was demonstrated as shown in Figure 9. Ketamine (100 mg/kg, i.p./day) administration for 10 days significantly ($P <$ 0.05) enhanced the duration of immobility in the forced swim test (FST) compared to vehicle control group. Pretreatment with DOX (25, 50, 100 and 200 mg/kg, p.o./day) prior to ketamine administration significantly ($P <$ 0.05) decreased immobility time in a dose dependent manner [$F (6, 28) = 23.66$, $P <$ 0.0001] compared to ketamine treated group. Similarly, effect was also observed in the group pretreated with risperidone (0.5 mg/kg, p.o./day)prior to ketamine administration, as it also significantly ($P <$ 0.05) prevented the increase in immobility time of the mice compared to ketamine treated group (Figure 9).

3.7.4. Amelioration of Biomarkers of Oxidative Stress in Mice Brain

The effect of DOX on the levels of antioxidant biomarkers of mice brains was done to assess for protection against oxidative stress following co-administration of DOX (25 - 200 mg/kg, p.o.) and ketamine (100 mg/kg, i.p.) for 10 days (Table 2). Ketamine administration significantly decreased SOD and CAT activities in the whole brain of mice compared with the vehicle-treated group (Table 2). Pretreatment with DOX (50, 100 and 200 mg/kg, p.o./day) prior to the administration of ketamine significantly ($P <$ 0.05) increased SOD [$F (6, 28) = 44.81$, $P <$ 0.0001] and CAT [$F (6, 26) = 39.29$, $P <$ 0.0001] activities compared to ketamine treated group. RIS, in turn, ameliorated the decrease in SOD and CAT activity by ketamine. Meanwhile, treatment with lower dose of DOX (25 mg/kg, p.o.) failed to significantly protect the animals against ketamine induced
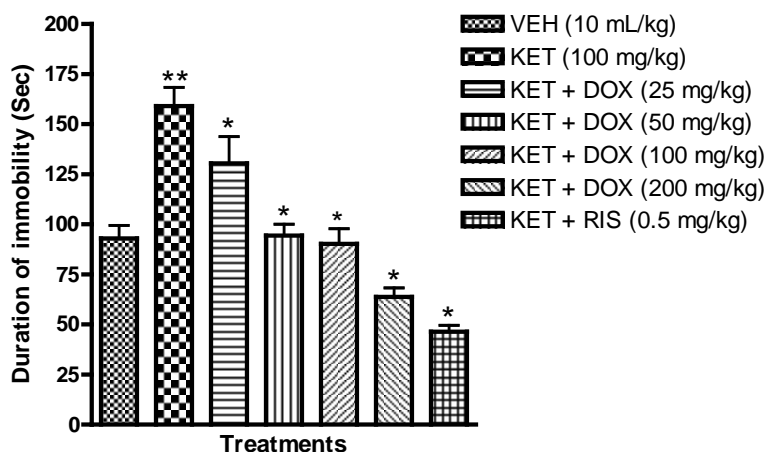
Figure 9. Effect of Sub-chronic administration of DOX on ketamine-enhanced immobility in forced swim test in mice.

Table 2. Prevention of alterations of biomarkers of oxidative stress in mice whole brain subjected to sub-chronic ketamine treatment by doxycycline.

Treatments	SOD (Unit/mg protein)	CAT (Unit/mg protein)	GSH (nmole/g tissue)	MDA (nmole/g tissue)
VEH (10 mL/kg)	6.76 ± 0.35	4.64 ± 0.22	140.0± 5.40	16.64 ± 0.78
KET (100 mg/kg)	2.34 ± 0.27**	1.24 ± 0.24**	60.4 ± 5.51**	33.20 ± 2.51**
DOX (25 mg/kg) + KET	3.70 ± 0.20	1.74 ± 0.12	106.8 ± 6.05*	29.20 ± 1.53
DOX (50 mg/kg) + KET	4.28 ± 0.19*	3.84 ± 0.22*	146.2 ± 10.07*	10.42 ± 1.23*
DOX (100 mg/kg) + KET	7.46 ± 0.23*	4.60 ± 0.32*	178.8 ± 6.95*	11.78 ± 0.85*
DOX (200 mg/kg) + KET	7.56 ± 0.41*	5.66 ± 0.40*	173.6 ± 7.39*	9.00 ± 1.30*
RIS (0.5 mg/kg) + KET	6.58 ± 0.39*	5.02 ± 0.26*	159.6 ± 7.69*	13.58 ± 1.36*

Value represents the mean ± S.E.M of 5 animals/group. **Denotes $P < 0.05$ compared with vehicle group, *Denotes $P < 0.05$ compared with KET treated group (ANOVA followed by Newman-Keuls test). VEH = Vehicle, KET = Ketamine, RIS = Risperidone, DOX = Doxycycline.

decrease in SOD and CAT enzyme activities compared to ketamine treated group. Furthermore, the administration of ketamine also significantly decreased the GSH concentration in the brain of the animals (Table 2) compared with the vehicle treated group. However, pretreatment with DOX (25, 50, 100 and 200 mg/kg, p.o.) and RIS (0.5 mg/kg, p.o.) significantly prevented ($P < 0.05$) the decrease in GSH concentration [$F_{(6, 28)} = 33.92$, $P < 0.0001$] compared to ketamine treated group.

Moreover, the pretreatment with DOX (50, 100 and 200 mg/kg, p.o./day) but not with DOX (25 mg/kg, p.o.) prior to the administration of ketamine (100 mg/kg, i.p./day) significantly ($P < 0.05$) protected the animals against ketamine induced lipid peroxidation as indexed by the decrease in malondialdehyde (MDA) [$F_{(6, 28)} = 42.81$, $P < 0.0001$] levels in the whole brain of the animals (Table 2). Meanwhile, 10 days administration of ketamine alone significantly increased lipid peroxidation as the consequences of decrease in the endogenous antioxidants (SOD, CAT and GSH) compared to vehicle treated group (Table 2).

4. Discussion

Current evidences support oxidative and nitrosative stress, as well as inflammatory responses resulting from microglial hyperactivation as signaling mechanisms, outside the long believed dopamine hypothesis in the pathogenesis of schizophrenia [29] [30]. The results of this study revealed that single and repeated administration of doxycycline ameliorated schizophrenia-like behaviors and reduced the increased brain levels of biomarkers of oxidative stress induced by repeated administration of ketamine.

Neurobehavioral effects of DOX on novelty-induced rearing (NIR) in mice were assessed. Rearing is a vertical locomotor activity involving an animal standing on its hind limbs while raising up with its forearms in the air or placed on the wall of the cage [31]. It is an indication of an increase in exploratory behavior, which is a measure of central nervous system excitation [32]. It is important to note that biochemical data have

shown that neurotransmitter like dopamine is implicated in central nervous system excitation [16]. Therefore, the decrease in NIR in this study by DOX is suggestive of possible interference with central dopaminergic neurotransmission and tranquilizing effect. Animal models using blockade of stereotypy behaviors, the degree of which are usually heightened when induced with apomorphine [18] or ketamine [20] is a valid tool for screening agents with suspected neurolepic activity. The stereotypic effects of apomorphine and ketamine have been shown to be mediated through the stimulation of dopamine receptors [33] and noncompetitive blockade of NMDA glutamate receptors [34], thereby indirectly decreasing the glutamate-dopamine pathway [33], respectively. Therefore, inhibition of these behavioral perturbations induced by apomorphine and ketamine herein by DOX suggests a neuroleptic activity. The observation from this study thus corroborates other studies that have demonstrated antipsychotic-like activity using inhibition of apomorphine- and ketamine-induced stereotypy in mice [17] [35].

The accentuation of locomotor activity has been majorly linked to the dopaminergic hyperactivation in the striatal areas of the brain [36]. Because the systemic administration of dopamine antagonists counteracts the hyperlocomotion induced by the administration of NMDA receptor antagonist [37], it is therefore; suggestive that dopamine neurotransmission is involved in the motor activating effects of ketamine-induced hyperlocomotion [16]. The inhibition of acute and sub-chronic ketamine-induced hyperlocomotion following single (200 mg/kg, p.o.) and repeated (100 and 200 mg/kg, p.o.) administration of DOX respectively, further suggest that the drug possesses at least, some mild to moderate ability to ameliorate some form of positive symptoms of schizophrenia. The observation from this study on ketamine-hyperlocomotion is also in agreement with previous findings showing antipsychotic-like activity via antagonism of ketamine-induced hyperlocomotion [17] [20] [38]. Indeed, extrapyramidal symptoms are believed to develop from abnormal decreased dopamine activity in the striatum [20]. Therefore, preferential action of a novel neuroleptic agents against dopamine agonist-induced hyperactivity or stereotypy has been shown by previous studies, as an indicator of little or no extrapyramidal symptoms by novel antipsychotic agents [18] [21]. Hence, the test for extrapyramidal symptoms on catalepsy test in this study, demonstrated that DOX is devoid of cataleptic behavior which suggest the absence of extrapyramidal side effects.

Ketamine-induced enhancement of immobility in forced swim test (FST) following acute and sub-chronic administrations of DOX against ketamine, has been previously used as an animal model for behavioral despair in experimental mice; which is indicative of negative symptoms (anhedonia, social withdrawal) of schizophrenia [19] [39]. Acute DOX (50 - 200 mg/kg) and sub-chronic DOX (25 - 200 mg/kg) treatments demonstrated dose-dependent inhibition of ketamine enhanced immobility in FST comparable to risperidone. Of note, a recent study elsewhere demonstrated that risperidone reduced the enhanced duration of immobility by ketamine in experimental mice comparable to paroxetine, a selective serotonine reuptake inhibitor [19]. Thus, the effect of risperidone on the negative symptoms has been positively correlated to its 5-hydroxy-

tryptaminergic (5-HT) $5HT_{2A}$ receptor blocking action [20]. Besides, accumulating evidence shows that ketamine also bind to other allosteric sites in the brain, such as phencyclidine-binding site within the NMDA receptor channel complex [40] and dopamine-D_2 receptor binding sites in the hippocampus [41]. However, the findings from Chindo *et al.* [19] suggest that ketamine-enhanced immobility in the FST might be mediated, at least in part, through $5-HT_{2A}$ receptors, since phencyclidine- and ketamine-enhanced immobility time are inhibited by $5-HT_{2A}$ receptor antagonists such as ritanserin, clozapine, risperidone and paroxetine, respectively [19] [20] [42]. Although, the neurotransmitter changes following ketmaine-enhanced immobility and the acute/sub-chronic treatments with DOX respectively, in the present study have not yet been shown, the results of the present findings suggest that the mechanism of action of DOX against the negative symptoms of schizophrenia in ketamine-enhanced immobility may be in part, related to the modulation of 5-hydroxytryptaminergic system; meanwhile, the specific mechanism of action of this drug under this context is still under active investigation.

Also, in this study DOX (100 and 200 mg/kg, p.o.) significantly prevented the cognitive impairment following repeated administration of ketamine for 10 days, as evaluated from the YMT. YMT has been used previously to measure the influence of suspected antipsychotic agents on cognitive functions [13] [17]. This study demonstrated that DOX prevented the cognitive impairment induced by ketamine; as indexed by the increase in the percentage correct alternations in YMT compared to ketamine treated group. In point of fact, the negative and cognitive symptoms of schizophrenia have been previously correlated to microglial hyperactivation and inflammatory involvements [30]. Taken together, the effects demonstrated by DOX may at least, in part, be mediated from its antioxidant and anti-inflammatory actions [43] [44].

Evidence derived supports oxidative stress in the pathophysiology of schizophrenia [45]. In fact, accumulating body of evidences has reportedly demonstrated increase in the concentrations of oxidative stress parameters after treatment with classical antipsychotics such as, haloperidol [7] [8] [15] [46]; and that treatments with antioxidants like vitamin E lowered the levels of reactive oxygen species (ROS) and protected the cells. So, it is suggested that novel antipsychotic compounds with antioxidants potential might be useful in the treatment of oxidative stress pathology associated with schizophrenia [7]. Indeed, besides stimulating the schizophrenic phenotypes, chronic ketamine treatment is also known to induce oxidative aberrations which correlates with those described in postmortem brains of individuals with schizophrenia [47]. Also, based on the construct, aetiologic and predictive validity with respect to both clinical phenomena and responsiveness to antipsychotic drugs [13] [16], it was thus, chosen for the present study to evaluate the possible anti-oxidative effects of DOX in the repeated administration of ketamine-induced oxidative alterations.

Therefore, the results from the biochemical assays in this study revealed that DOX (50 - 200 mg/kg, p.o.) significantly decreased the biomarker of lipid peroxidation, as indexed by decreased MDA levels respectively, consequently to the increase in the an-

tioxidant activity compared to the ketamine treated group. Moreover, DOX (25 - 200 mg/kg, p.o.) significantly increased GSH levels and activities of antioxidant enzymes including SOD and CAT in the whole brains of the experimental animals respectively. In the context of the present study, it might be inferred that the mechanism involved in the antipsychotic behavioral phenotypes of DOX majorly against the negative and cognitive symptoms of the experimental animals may be mediated, in part, through increase in antioxidant defense capacity and decrease in lipid peroxidation. Our study also showed that risperidone, an atypical antipsychotic agent, demonstrated an increase in the antioxidant defense mechanisms with a significant decrease in MDA levels compared to ketamine treated animals, which is in line with previous findings [13] [38] [48], and thus, further supports the role of oxidative stress in the pathophysiology of schizophrenia. In another study elsewhere, minocycline, a tetracycline congener of doxycycline demonstrated significant increase (preventive and reversal) in antioxidant defense systems, decrease in MDA and nitrite levels [13] [15]. In view of this, our findings confirmed that the schizophrenia-like behaviors induced by ketamine was accompanied by increased oxidative stress, as demonstrated by the decreased antioxidant defense systems and increased levels of MDA, respectively. Therefore, the ability of DOX to prevent ketamine-induced schizophrenia-like behaviors in the experimental animals may suggest an action involving the inhibition of biomarkers of oxidative stress in the mice brains.

From the glutamate-cycle, prolonged oxidative stress and immune alterations reduces the capacity of astrocytes to import glutamate, facilitating an increased extracellular glutamate levels [15] [30] [45]. Oxidative stress increased glutamate levels can inhibit cystine uptake by the cysteine/glutamate exchange system thereby causing intracellular GSH depletion, and consequently poor NMDA surface expression that results in NMDA hypofunctionality [34]; leading to decrease glutamate-dopamine modulations, that is, at least partly, mediated by decreased GSH signaling [49]. Moreover, Behrens et al. [45] and Sorce et al. [50] previously reported increase in the level of the pro-inflammatory cytokine, interleukine-6 (IL-6) and superoxide producing enzyme, nicotinamide adenine denucleotide phosphate oxidase-2 (Nox-2) in the brain of rodents following repeated administration of ketamine, respectively. Therefore, the increased GSH concentration observed in this study by doxycycline might be mediated through prevention of microglial oxidative burst and inflammatory response elements, thereby enhancing microglial internalization of cysteine intracellularly [51], and cysteine uptake by the cysteine/glutamate exchange system to increase GSH synthesis [52]. The increase in GSH in this study, suggests enhanced glutamate uptake by the astrocytes, possibly leading to increase in the surface expression of NMDA receptor and glutamate binding for the modulation of dopamine neurotransmission [50]. Therefore, the positive effects of DOX on ketamine-induced schizophrenia-like behavioral phenotypes, further point to mechanisms of action involving antioxidant activities, demonstrated herein and the inherent anti-inflammatory properties [12] [44]. Although, additional preclinical studies are necessary to ascertain the precise mechanisms of action of

doxycycline against schizophrenia-like behaviors particularly against the negative and cognitive symptoms in experimental animals, the present findings suggest inhibition of biomarkers of oxidative stress in ketamine model of psychosis.

5. Limitations and Further Studies

Limitations of this study include the following: 1) lack of evidence of pro-inflammatory cytokines e.g., interleukin (IL)-6, IL-1β to evaluate the anti-inflammatory effect of doxycycline, 2) lack of neurotransmitters e.g., dopamine, glutamate, gamma-amino butyric acid (GABA) level determinations. Therefore, further studies will be focused on the determination of the above limitations presented herein, as well as other brain neurochemical and cellular alterations associated with schizophrenia.

6. Conclusion

Our studies showed that doxycycline ameliorated schizophrenia-like behaviors via mechanism related to inhibition of oxidative stress and did not produce extrapyramidal side effects in mice. Our data also suggest that doxycycline may be useful as an adjunctive neuroleptic drug for the treatment of schizophrenia-like behaviors particularly in patients with negative symptoms and cognitive deficits.

Acknowledgements

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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