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Effects of tamoxifen alone and in combination with risperidone on hyperlocomotion, hippocampal structure and bone in ketamine-induced model of psychosis in rats

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Abstract

Background and aim of the work: Protein kinase C activation with subsequent increase in oxidative stress (OXS) and reduction in brain derived neurotrophic factor (BDNF) are implicated in the pathophysiology of psychotic disorders and in osteoporosis. Accordingly PKC inhibitors such as tamoxifen could be a novel approach to psychotic illness and may reduce progression of osteoporosis. Since current antipsychotics such as risperidone have inconsistent effects on OXS and BDNF, combination with tamoxifen could be beneficial. Accordingly in this work, tamoxifen was used to investigate the impact of changes in OXS and BDNF on behavioral, hippocampus structural changes in a ketamine induced model of psychosis in rats. The impact of tamoxifen on the antipsychotic effects of risperidone and on its bone damaging effects was also determined.

Ketamine was chosen, because it is a valid model of psychosis. Hippocampus was chosen, since hippocampal overactivity is known to correlate with the severity of symptoms in psychosis. Hippocampal overactivity contributes to hyperdopaminergic state in ventral tegmental area and increase in DA release in nucleus accumbens, these are responsible for positive symptoms of schizophrenia and hyperlocomotion in rodents. Hyperlocomotion is considered a corelate of positive symptoms of psychotic illness in rodents and is considered primary outcome to assess manic-like behavior.

Methods: Rats were divided into seven groups (ten rats each (**1**) non-ketamine control and (**2**) ketamine treated groups (**a** ketamine control, **b** risperidone/ketamine, **c** tamoxifen/ketamine, **d** Risp/Tamox/ketamine risperidone, tamoxifen/risperidone) to test if TAM exhibited behavioral changes or potentiated those of risperidone); (**e** clomiphene/ketamine and **f** clomiphene/risperidone/ketamine) to verify that estrogen receptor modulators do not exhibit behavioral changes or potentiates those of risperidone. In addition, thus, the effects of tamoxifen are not due to estrogen effects but rather due to protein kinase c inhibition. Drugs were given for 4 weeks and ketamine was given daily in the last week. Effects of drugs on ketamine-induced hyperlocomotion (open field test) and hippocampus and bone biochemical (MDA, GSH, BDNF) and histological changes (Nissel granules, GFAP positive astrocytes in hippocampus were determined).

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Electron microscopy scanning of the femur bone was done. Histomorphometric parameters measuring the: **1**. Trabecular bone thickness and **2**. The trabecular bone volume percentage.

Results: Tamoxifen reduced hyperlocomotion, and improved hippocampus structure in ketamine-treated rats, by reducing OXS (reduced malondialdehyde and increased glutathione) and increasing BDNF. These effects might be related to (PKC) inhibition, rather than estrogen modulation, since the anti-estrogenic drug clomiphene had no effect on hyperlocomotion. Tamoxifen enhanced the beneficial effects of risperidone on hippocampal OXS and BDNF, augmenting its effectiveness on hyperlocomotion and hippocampal structure. It also reduced risperidone-induced OXS and the associated bone damage.

Conclusions: PKC inhibitors, particularly tamoxifen, might be potential adjuncts to antipsychotics, by reducing OXS and increasing BDNF increasing their effectiveness while reducing their bone damaging effects.

Keywords: Tamoxifen, Risperidone, Ketamine, BDNF, OXS, PKC

Introduction

Changes in brain redox state and in brain-derived neurotrophic factor (BDNF), with altered neuronal plasticity, may contribute to the pathophysiology of psychotic disorders [1–3].

Changes in redox state and in BDNF levels have also been shown to affect bone mineral density [4–6]. Indeed, an increased risk of osteoporosis has been reported in psychotic patients and has been attributed to the mental illness and/or to the antipsychotic medication [7].

Current antipsychotic agents, such as risperidone, have been reported to increase oxidative stress (OXS) [8, 9]. Increase in OXS could reduce the effectiveness of the antipsychotic and increase the risk of osteoporosis [10–12]. In addition, the effects of antipsychotics on BDNF are quite inconsistent. Antipsychotic agents have been reported to induce no significant effect [13], insignificant reduction [14] or increase [15] in BDNF level. Accordingly, psychotropic agents with more consistent beneficial effects on OXS and BDNF are required as alternatives or adjuncts to current antipsychotic agents.

Excessive activation of protein kinase C (PKC) has been recently implicated in the increase in OXS and decrease of BDNF in bipolar disorder [16, 17]. This finding implies that PKC inhibitors might be potential novel therapeutic agents in bipolar disorder [18]. Tamoxifen, the hormonal therapy used in the treatment of breast and endometrial cancer, also is a selective central nervous system PKC inhibitor [19–21]. It was shown to reverse and prevent amphetamine-induced behavioral changes and the associated oxidative stress in rats [22]. Similar anti-manic effects were reported experimentally and clinically [19, 20, 23–25].

In the present study, tamoxifen—the selective PKC inhibitor—was utilized to investigate the impact of changes in OXS and BDNF, following PKC inhibition on the behavioral (hyperlocomotion) and hippocampus structural changes in a ketamine-induced model of psychosis in rats. The impact of tamoxifen on the antipsychotic effects of risperidone and on its bone damaging effects was also determined. The effects of tamoxifen on hyperlocomotion were also compared to those of clomiphene, an estrogen receptor modulator [26]. This was done to confirm that the estrogen receptor modulating effect of tamoxifen does not contribute to its effects on hyperlocomotion and hence validate the use of tamoxifen as an experimental tool to study the effects of PKC inhibition.

The rationale was that if the present study confirms the efficacy of tamoxifen in ketamine-induced model of psychosis, this will be of clinical significance. Such findings will add further support to the involvement of PKC activation in the pathophysiology of psychotic disorders, as recently suggested. It will also underscore the efficacy of PKC inhibitors in such disorders as alternatives to current antipsychotics. PKC inhibitors could represent novel agents in psychotic disorders with more consistent beneficial effects on BDNF and oxidative stress, compared to current antipsychotics while simultaneously exerting positive effects on bone.

In this study ketamine was chosen, because it is a valid model of psychosis. Hippocampus was chosen, since hippocampal overactivity is known to corelate with the severity of symptoms in psychosis. Hippocampal overactivity contributes to hyperdopaminergic state in ventral tegmental area and increase in DA release in nucleus accumbens, these are responsible for positive symptoms of schizophrenia and hyperlocomotion in rodents. Hyperlocomotion is considered a corelate of positive symptoms of psychotic illness in rodents and is considered primary outcome to assess manic-like behavior.

Methods

Animals

70 Adult male wistar rats (250–300 g) were kept on a 12-h light–dark cycle (lights on at 07:00), at a temperature of 23 °C ± 1 °C with food and water ad libitum, and the container was cleaned daily. During cleaning time, rats were

placed in a dry cage for 5 min, where they stayed awake (grooming and exploratory behavior). 60 rats were caged in groups of 2–3 in a 41 × 34 × 16 cm cage. Control rats (home-caged controls, CC) remained in their home cages (2–3 rats per cage) for the duration of the experiment. All rats were allowed to acclimate for at least 1 week prior to experiments and were gently handled three times before behavioral testing. All behavioral data were recorded in a calm room by an experienced observer. The observer was in the room, where experiments were performed and was blind to the animal condition. All procedures were carried out according to the guidelines of animal care and the scientific research ethical committee.

Ethical statement

The study was approved by the local research committee. Reporting of the results adheres to the ARRIVE and BJP guidelines.

Diet

Rat chow: obtained from Meladco for Animal Food, Egypt and composed of (20% proteins, 10% fat and 70% carbohydrates).

Drugs

The study used tamoxifen citrate 2 mg/kg (AstraZeneca, UK) risperidone 2.5 mg/kg (Janssen-Cilag, USA) and clomiphene 10 mg/kg (Sanofi-Aventis, France). Doses of the drugs were based on previous studies [27–29]. All drugs were dissolved in saline solution (NaCl 0.9%) and were given intraperitoneally in volumes of 1 ml/kg daily for 4 weeks.

Ketamine solution for injection as injectable vials; 50 mg/ml purchased from Hamlen ltd, injected at a volume of 0.5 ml/kg of body weight.

Animal groups and study design

See Additional file 1: Fig. S1 for graphical study design. Seventy rats were divided into seven groups (ten rats each): control non-ketamine treated group receiving saline; and ketamine groups: (1) control, (2) tamoxifen, (3) risperidone, (4) tamoxifen plus risperidone, (5) clomiphene, and (6) Clomiphene plus risperidone, treated groups.

Rats were treated with the test drugs for 4 weeks. Control rats received saline.

Ketamine was administered intraperitoneally (i.p.), once a day in the last week in a sub anesthetic dose of 25 mg/kg [30–32].The effects of drugs on locomotor activity was assessed on the last day, using the open-field test, 30 min after the administration of the last dose of the drugs.

Rats were then sacrificed, and decapitated. Blood was withdrawn from the rats from lateral tail vein into a tube and was left for 30 min to clot then centrifuged for 20 min to obtain serum samples. The hippocampus structure was manually dissected on ice, rapidly frozen on dry ice and stored at − 70 °C until assayed. The femur bones were isolated for biochemical and immuno-histochemical studies and 3D scanning.

Only male animals were used to avoid the potential confound of antiestrogenic effects of tamoxifen on female hormonal physiology [26, 28, 33, 34].

Outcome measures

Behavioral studies

Effects of drugs on hyperlocomotion induced by ketamine were tested using the open-field test [35]. Rats were allowed to adapt to the test room 1 h before the experiment was conducted. Each rat was placed individually in the center of the quadrangular arena (60 × 60 cm) which was divided into 16 equal squares, each rat was tested for 5 min. The number of crossed squares (with at least three paws) was counted manually after the test was video recorded. The arena was cleaned by 10% alcohol after each rat.

The points investigated were the effects of tamoxifen alone and in combination with risperidone on hyperlocomotion induced by ketamine. The effects of tamoxifen on hyperlocomotion were compared to those of clomiphene, an estrogen receptor modulator. This was done to confirm that the estrogen receptor modulating effect of tamoxifen does not contribute to its effects on hyperlocomotion and hence validate the use of tamoxifen as an experimental tool to study the effects of PKC inhibition.

Biochemical studies

The level of BDNF was measured in hippocampus tissue using Rat BDNF ELISA estimation kit (Kamiya Biomedical company Cat. No. KT-8 575). Oxidative stress markers, reduced glutathione (GSH) and MDA were measured spectrophotometrically in serum and in bone, and hippocampus tissue using rat Kits for serum sample and tissue sample (Oxford biomedical research, USA; and OxiSelect, Cell Biolabs, USA).

Histological studies

Dissection of hippocampus [36]

Hippocampus was reached from the medial side after division of the brain at the mid sagittal plane into right and left hemispheres [37]. The right hippocampus was manually dissected on ice, rapidly frozen on dry ice and stored at − 70 °C until assayed. The left hippocampus was immediately fixed in 10% formalin, processed and stained

with H&E, toluidine blue and glial fibrillary acidic protein (GFAP).

Dissection of femur

The distal part of right femur was dissected out, trimmed and was immediately divided longitudinally into two halves using scalpel. One half from each femur was processed for preparation of decalcified specimen, and the other half was processed for preparation of scanning electron microscopic study (SEM).

Preparation of decalcified specimen

Specimens were fixed in neutral buffered formaldehyde for 2 days. Decalcification was performed by chelating agent ethylene–diamine–tetra–acetic acid in the form of its disodium salt [38]. An ample volume of decalcifying solution was used. The solution was changed every day. The time required for decalcification was 4 weeks. Then the decalcified specimens were dehydrated and processed to form paraffin blocks. Serial longitudinal sections, five micrometers thickness were prepared. Then, sections were stained with hematoxylin and eosin stain (H&E) [38].

Preparation of SEM [36]

Longitudinal section of distal end of femur was perfused thoroughly with saline to remove the bone marrow. Specimens were fixed immediately in 1.5% gluteraldehyde in phosphate buffer saline for 2 h then gradual dehydration was done. Alcohol substitution was done using acetone. The fixed dehydrated pieces of bone were dried using Critical Point Dryer, using liquid carbon dioxide. The tissue specimens were mounted on brass studs using double adhesive tape and coated with 20 nm layer of gold in a JFC-ion sputter (JEOL, Tokyo, Japan) Using sputter coated SCD/0γ15. The specimens were then examined with an XL30 SEM (PHILIPS, Amsterdam, and Netherlands) operated at 30 kV. All photographs were taken in the second electron mode with the beam incident to the surface of the tissue.

Morphometric studies

This was performed using a Leica DM2500 microscope (Wetzlar, Germany) and the image analyzer Leica Q win V.3 program. Stained sections of hippocampus in CA3 region from each subgroup were examined. Five high power fields/sections were chosen to measure: Nissl's granules density in the pyramidal cells' cytoplasm stained by toluidine blue and the area percentage of astrocytes stained by GFAP immune stain. The sections were examined using ×40 objective lens (final magnification ×400).

The histomorphometric parameters of bone were defined according to the report by the American Society for Bone and Mineral Research Committee [39] measuring the following: (1) Trabecular bone thickness indicating the thickness of trabeculae in the cancellous bone. Bone trabeculae were measured at their midpoint away from their branching areas; and (2) The trabecular bone volume percentage as the percentage of cancellous bone area occupied by trabeculae.

Statistical analysis

All values in the results is expressed as mean ± standard error of the mean (SEM). Statistical difference among groups was determined using two way analysis of variance; ANOVA followed by Tukey's multiple comparison test. p values < 0.05 will be considered statistically significant. Statistical analysis was carried out using Graph pad prism, software program, version 5.0. (2007). Inc., CA, USA.

Results

Behavioural results

As shown in Table 1, control ketamine rats showed a significant increase in the number of crossed squares, compared to rats in the control non-ketamine treated group. Tamoxifen/Ketamine treated rats showed significant reduction in number of crossed squares, compared to control ketamine rats. In contrast, clomiphene treatment had no significant effect on Ketamine induced hyperlocomotion and did not augment the effects of risperidone when combined with it. Risperidone/ketamine rats showed significant decrease in number of crossed squares, compared to control ketamine rats; reaching significantly lower levels than control non-Ketamine treated rats. Combination of risperidone with tamoxifen resulted

Table 1 Effects of test drugs on the number of crossed squares in the open field test in ketamine treated rats

Animal groups $n = 10$ each	Open field test (no. of crossed squares)
Control non-ketamine treated	51.3 ± 6.7
Control ketamine treated	83.4 ± 14.2*
TAM/ketamine treated	49.6 ± 6.4#
Risp/ketamine treated	33 ± 3*,#
TAM/Risp/ketamine treated	18 ± 2.5*,#,ǂ
Clomiphene/ketamine treated	84 ± 2.6*
Clomiphene/Risp/ketamine treated	30 ± 3*,#

Data are expressed as mean ± SEM, *n* number of rats, two-way ANOVA followed by Tukey multiple comparison tests

Tam tamoxifen, *Risp* risperidone

*$p < 0.05$ significant compared to control non-ketamine treated

# $p < 0.05$ significant drug treated ketamine compared to control ketamine treated rats

ǂ $p < 0.05$ combination compared to risperidone/ketamine treated

in a significant decrease in number of crossed squares, compared to control ketamine and to risperidone/ketamine rats; reaching levels significantly lower than control non-Ketamine treated rats.

Biochemical results

As shown in Table 2, control ketamine treated rats showed significantly lower hippocampus BDNF as well as lower GSH and higher MDA levels, in hippocampus and bone, compared to control non-ketamine treated rats. Tamoxifen/ketamine rats showed significantly higher hippocampus BDNF as well as higher glutathione (GSH) and lower malondialdehyde (MDA) levels in hippocampus and bone, compared to control ketamine treated rats. Risperidone/ketamine treated rats showed significantly higher hippocampus BDNF as well as higher GSH and lower MDA levels, in hippocampus, compared to control/ketamine treated rats. In contrast, significantly lower GSH levels and higher MDA levels were noted in bone tissue. Tamoxifen/risperidone/ketamine rats showed significantly higher hippocampus BDNF as well as higher GSH and lower MDA levels, in hippocampus and bone, compared to control/ketamine treated rats, and to risperidone/ketamine treated rats.

Histological results

Control ketamine treated rats showed many shrunken pyramidal cells with dark cytoplasm and pyknotic nuclei a significant reduction in hippocampal Nissl's granules density and an increase in area % of GFAP positive astrocytes compared to control non-ketamine treated rats. Rats treated with either tamoxifen or risperidone showed a significant improvement in these parameters compared to control ketamine treated rats. Rats receiving combined treatment showed a significant improvement in these parameters compared to risperidone treatment alone $p < 0.05$. Table 3 photographs of H&E, toluidine blue and glial fibrillary acidic protein (GFAP) stained sections of hippocampus (CA3) region showing these changes are presented in Fig. 1, 2, and 3

Rats treated with risperidone showed a significant reduction in trabecular bone thickness and in percentage of trabecular bone volume compared to control ketamine treated rats. Rats receiving combined treatment showed a significant improvement in these parameters compared to risperidone treatment alone $p < 0.05$ (Table 3 photographs of H&E stained sections of the lower femur showing these changes are presented in Figs. 4, 5, 6, 7, and 8

Scanning electron microscopic examination of rat femur of control/non-ketamine treated group showed cancellous bone as branching and anastomosing bone trabeculae. Collagen fibres were seen on surface of bone (Fig. 9A, B). Control/Ketamine treated group were similar to control (Fig. 9C). In the tamoxifen/ketamine treated group minimal cracks were seen on the surface of bone trabeculae (Fig. 9D, E). In contrast, risperidone/ketamine treated group showed extensive bone damage as cracks were frequently seen. Thin tapering bone trabeculae were seen and were sometimes seen fractured. Focal areas of disorganized collagen fibers were seen (Fig. 9F–H). These changes were less marked in tamoxifen/risperidone/ketamine treated group (Fig. 9I, J).

Table 2 Effect of test drugs on BDNF in hippocampus and oxidative stress markers in hippocampus and femur bone, of ketamine treated rats

Animal groups $n = 10$	BDNF hippocampus ng/g tissue	Oxidative stress markers			
		Hippocampus		Bone	
		GSH pg/g tissue	MDA ng/g tissue	GSH pg/g tissue	MDA ng/g tissue
Control non-ketamine treated	10.2 ± 0.31	10.55 ± 0.42	1.6 ± 0.2	6.5 ± 0.35	1.49 ± 0.15
Control/ketamine	1.94 ± 0.1*	1.54 ± 0.18*	10.93 ± 0.5*	3.28 ± 0.19*	5.2 ± 0.25*
TAM/ketamine	6.35 ± 0.34*,#,ŧ	5.5 ± 0.25*,#,ŧ	4.5 ± 0.5*,#,ŧ	5.66 ± 0.29#,ŧ	2.26 ± 0.1#,ŧ
Risp/ketamine	4.8 ± 0.22*,#	3.6 ± 0.22*,#	7.03 ± 0.48*,#	0.88 ± 0.15*,#	7.5 ± 0.29*,#
TAM/Risp/ketamine	7.66 ± 0.25*,#,ŧ	7.8 ± 0.33*,#,ŧ	4.6 ± 0.25*,#,ŧ	5.01 ± 0.3*#,ŧ	3.19 ± 0.133*,#,ŧ

Data are expressed as mean ± SEM, *n* number of rats. Two way ANOVA followed by Tukey multiple comparison tests

Tam tamoxifen, *Risp* risperidone, *BDNF* brain derived neurotrophic factor, *GSH* reduced glutathione, *MDA* malondialdehyde

*$p < 0.05$ significant compared to control non-ketamine treated

# $p < 0.05$ significant drug treated/ketamine compared to control/ketamine

ŧ $p < 0.05$ significant compared to Risp/ketamine treated

Table 3 Showing mean ± SD of Nissl granules density, area % of GFAP positive astrocytes, trabecular bone thickness and percentage of trabecular bone volume in different groups

	Nissl's granules density	Area % of GFAP positive astrocytes	Trabecular bone thickness (μm)	Percentage of trabecular bone volume
Control/non-ketamine treated	9 6 ± 1.1	1.86 ± 0.81	151.9 ± 5.1	46.7 ± 3.3
Control/ketamine treated	66.4 ± 1.51*	34.78 ± 1.83*	143.5 ± 4.3	39.5 ± 4.4
TAM/ketamine treated	81.6 ± 1.1*,#	17.76 ± 1.67*,#	139.7 ± 5.7	36.7 ± 3.1
RISP/ketamine treated	88 ± 1.6*,#	12.34 ± 0.91*,#	79.4 ± 3.2*,#	12.4 ± 1.4*,#
TAM/RISP/ketamine treated	93.6 ± 1.2#,ǂ	7.21 ± 0.68*,#,ǂ	113.5 ± 4.4*,#,ǂ	30.7 ± 3.6*,#,ǂ

Tam tamoxifen; *Risp* risperidone; *GFAP* glial fibrillary acidic protein

*$p < 0.05$ significant compared to control non-ketamine treated

# $p < 0.05$ significant drug treated ketamine compared to control/ketamine

ǂ $p < 0.05$ significant compared to Risp/ketamine treated

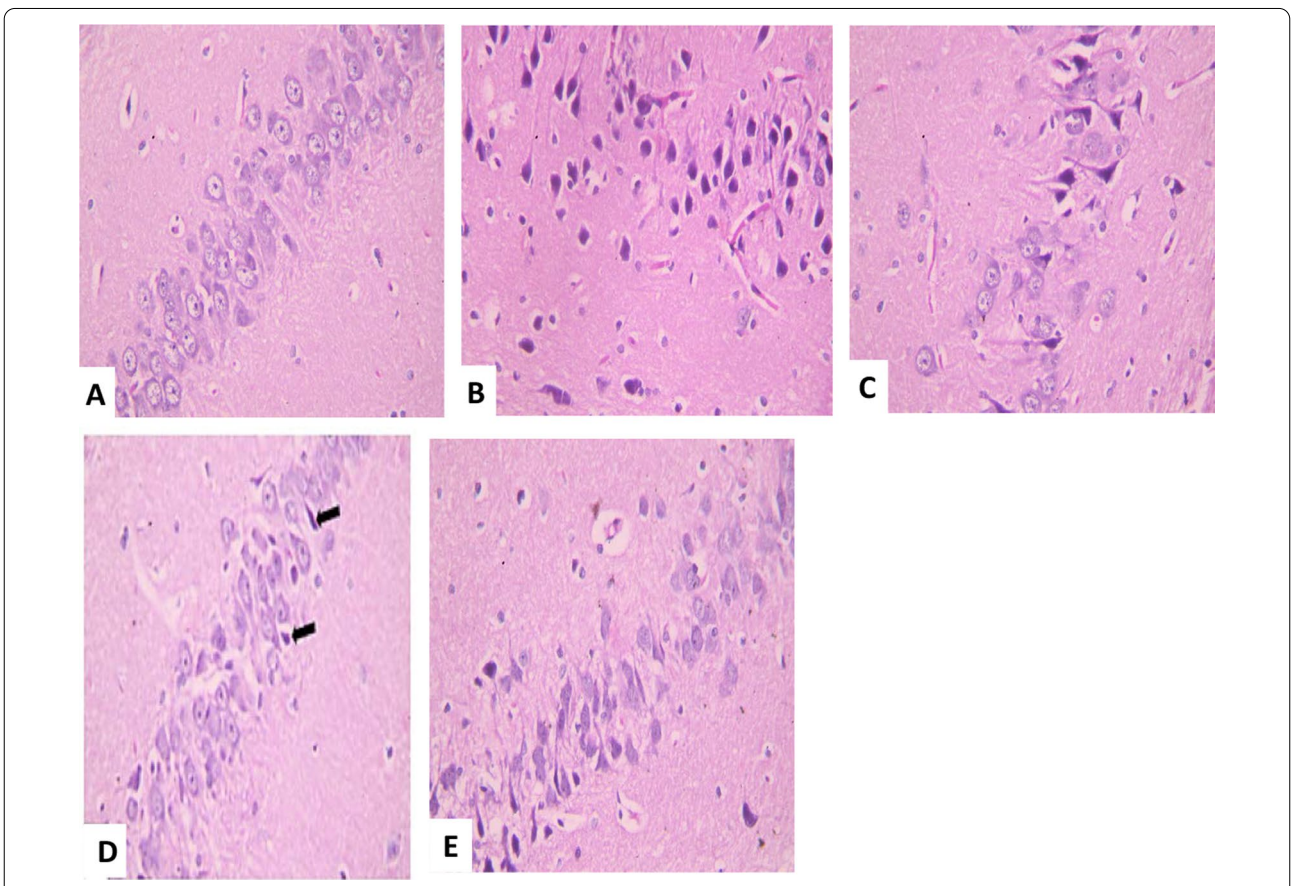

Fig. 1 H&E stained sections of hippocampus (CA3) region. **A** Control non ketamine treated rats showed pyramidal layer is formed of large pyramidal cells having vesicular nuclei. **B** Ketamine treated rats showed many shrunken pyramidal cells with dark cytoplasm and pyknotic nuclei. **C** Tamoxifen/ketamine treated group showed some pyramidal cells were shrunken with pyknotic nuclei. **D** Risperidone/ketamine treated rats showed very few cells having dark pyknotic nuclei. **E** Tamoxifen/risperidone/ketamine treated rats showed most pyramidal cells were nearly comparable to control

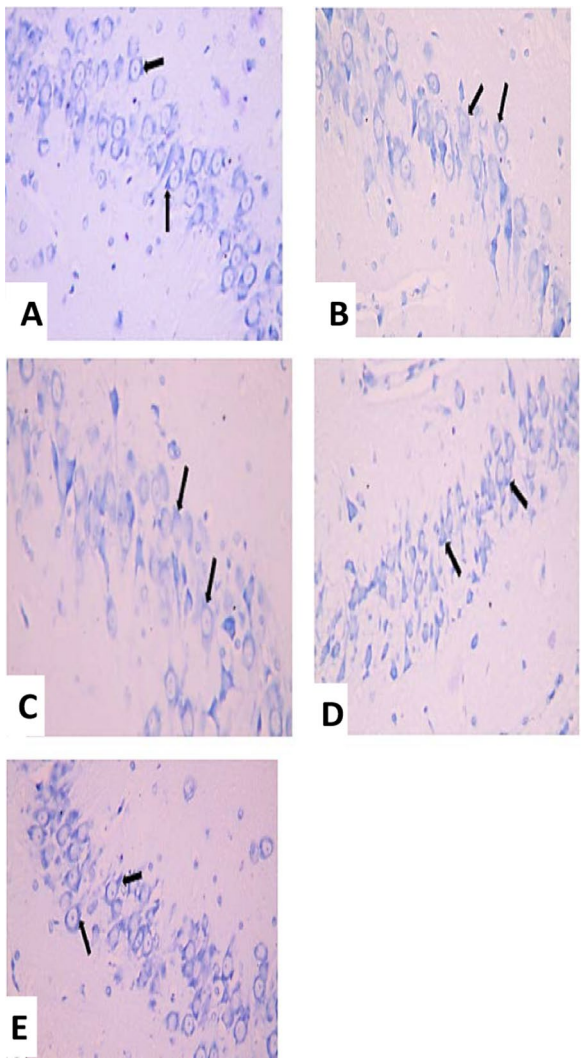

Fig. 2 Toluidine blue stained sections of hippocampus (CA3) region. **A** Control non ketamine treated group showed pyramidal cells studded with Nissl's granules. **B** Ketamine treated rats shows Nissl's granules of pyramidal cells were less in number compared to control non ketamine treated group. **C** Tamoxifen/ketamine treated rats appeared studded with more Nissl's granules compared to control ketamine treated group. **D** Risperidone/ketamine treated rats appeared studded with more Nissl's granules compared to control ketamine treated group. **E** Tamoxifen/risperidone/ketamine treated rats shows Nissl's granules were significantly increased compared to ketamine treated and risperidone/ketamine treated group

Discussion

In the present study, tamoxifen, was used to investigate the impact of changes in OXS and BDNF on hyperlocomotion and hippocampus structural changes, in a ketamine-induced model of psychosis in rats. The hippocampus was chosen, since hippocampal over-activation is known to correlate with the severity of symptoms in psychosis [40, 41]. Hippocampal over-activity contributes to the hyperdopaminergic state in the ventral tegmental area and increases dopamine release in the nucleus accumbens [42]. These changes are responsible for the positive symptoms of schizophrenia in humans [43] and hyperlocomotion in rodents. Hyperlocomotion is considered a putative correlate of positive symptoms of psychotic illness in rodents [44, 45] and is considered a primary outcome to assess manic-like behavior [46].

In the present study, chow-fed rats treated with a sub-anesthetic dose of ketamine, exhibited hyperlocomotion. In pre-clinical studies, low doses of ketamine (5–10 mg/kg) induce antidepressant effects [47]. However, in moderate doses (10–50 mg/kg), ketamine induces hyperlocomotion, impaired cognitive function and cellular dysfunction [30, 31]. Ketamine treatment is reported to induce oxidative stress and an inflammatory response with increase in pro-inflammatory cytokines including TNF alpha [48, 49]. Oxidative stress induces neuroinflammation [50, 51] and activation of microglial cells that increases the release of inflammatory cytokines, reinforcing the effects of oxidative stress on neuronal toxicity [52, 53].

The hyperlocomotion induced by ketamine was associated with histopathological changes in CA3 region of hippocampus together with increase in hippocampal oxidative stress (OXS) and reduction in BDNF in hippocampus tissue.

According to Lin et al. [54], ROS release after central nervous system injury, leads to astrocytes activation and increase in their number, size, and expression of GFAP, thus causing hippocampus structural abnormalities. ROS release following glutamate excitotoxicity has also been reported to be responsible for disappearance of Nissl's granules in many pathological conditions indicating decreased neuron function [55]. This could explain the impairment and significant decrease in Nissl's granules detected in the ketamine group compared to control non ketamine group.

In the present work, tamoxifen/ketamine-treated rats displayed a reduction in hyperlocomotion together with moderate improvement in the structure of (CA3) region in the hippocampus. Risperidone/ketamine-treated rats also showed noticeable improvement compared to control ketamine-group. Ketamine-rats treated with risperidone and tamoxifen combination showed a further reduction in hyperlocomotion and more significant improvement in the structure of (CA3) region in the hippocampus compared to control ketamine-group and to the risperidone treated group. This was evident by a significant increase in the Nissl granules content of the pyramidal cells and a significant decrease in immune reactive astrocytes with GFAP, when compared to control ketamine group.

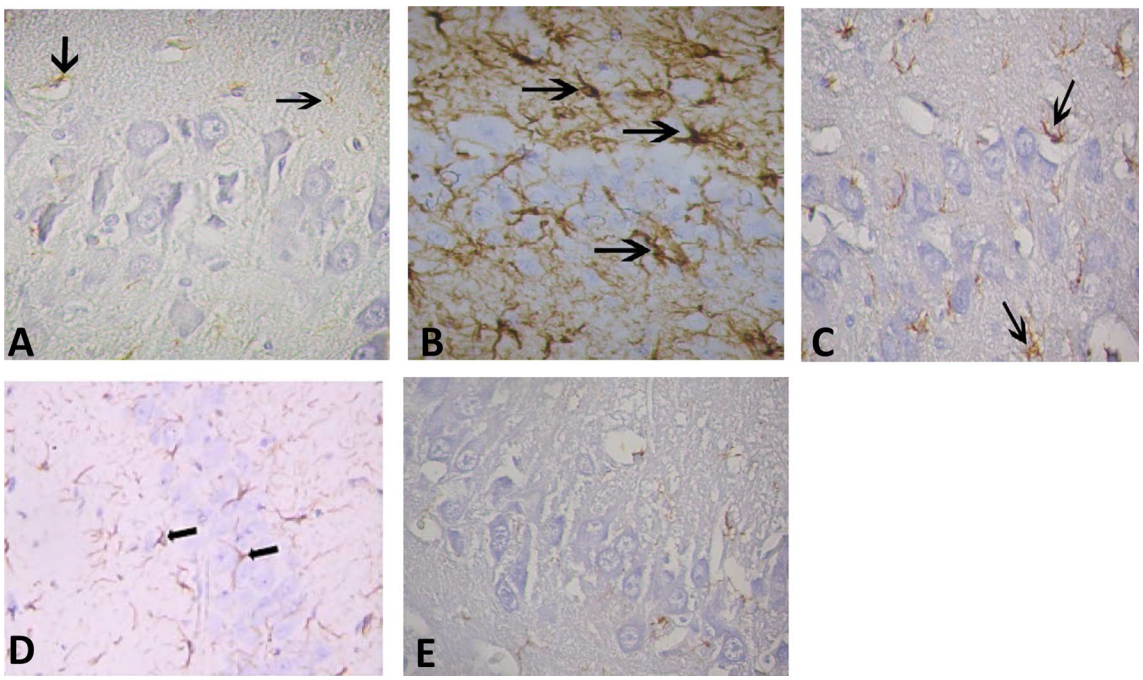

Fig. 3 Glial fibrillary acidic protein (GFAP) stained sections of hippocampus (CA3) region. **A** Control non ketamine treated rats showed few **GFAP** positive astrocytes. **B** Ketamine treated rats showed substantial number of **GFAP** positive astrocytes with many tall processes compared to control non ketamine treated GROUP. **C** Tamoxifen/ketamine treated rats showed some positive astrocytes with tall processes. **D** Risperidone/ketamine treated rats showed few **GFAP** positive astrocytes with tall processes. **E** Tamoxifen/risperidone/ketamine treated rats showed positive astrocytes were significantly decreased compared to ketamine treated and risperidone/ketamine treated groups comparable to control

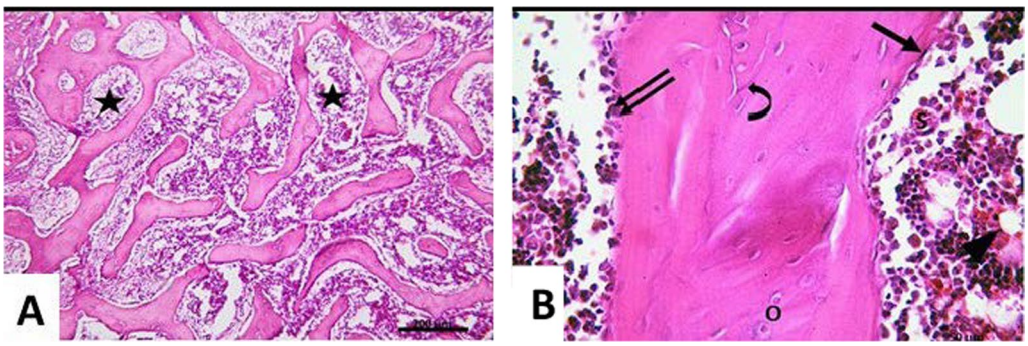

Fig. 4 H&E stained Sections of the lower femur of control non ketamine treated rat showed cancellous bone formed of branching and anastomosing bone trabeculae with bone marrow spaces in between (**A** magnification H&E ×100). Bone trabeculae were covered with endosteum containing osteoprogenitor cells and osteoblasts. Osteocytes were seen inside their lacunae. Bone marrow was formed of haemopoietic cells, blood sinusoids and fat cells (**B** magnification H&E ×400)

The beneficial effects of tamoxifen on hyper locomotion and hippocampus structure in the ketamine-rats may be related to the reduction in OXS and increase in BDNF, compared to control ketamine-group. These findings are in accordance with previous studies [19, 22] and [23].

The greater improvement in hyperlocomotion and hippocampus structure, in risperidone/tamoxifen/Ketamine treated rats, compared to risperidone/ketamine treated rats, might be related to the greater reduction in OXS and increase in BDNF in risperidone/tamoxifen/ketamine treated rats.

The reduction on OXS and increase in BDNF and the subsequent reduction in hyperlocomotion induced by tamoxifen are probably related to its PKC inhibitory effects [19]. Indeed, excessive activation of PKC has been

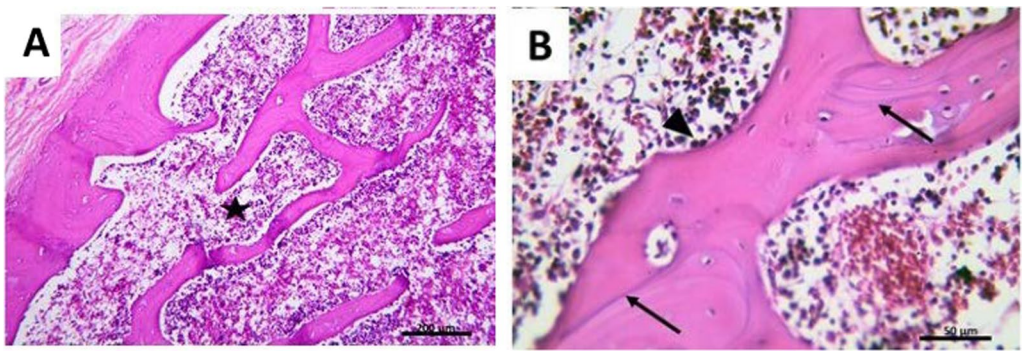

Fig. 5 H&E stained Sections of the lower femur of ketamine treated rat appear similar to that of control (**A** H&E ×100, **B** H&E ×400 magnification)

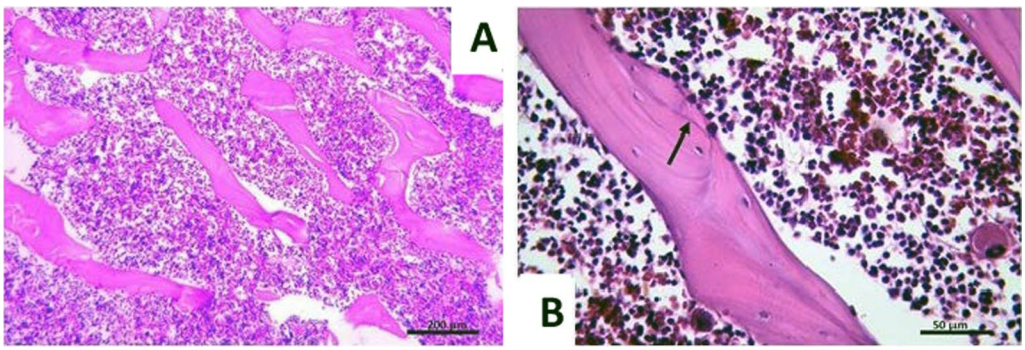

Fig. 6 H&E stained sections of the lower femur of tamoxifen/ketamine treated rat appear similar to that of control (**A** H&E ×100, **B** H&E ×400 magnification)

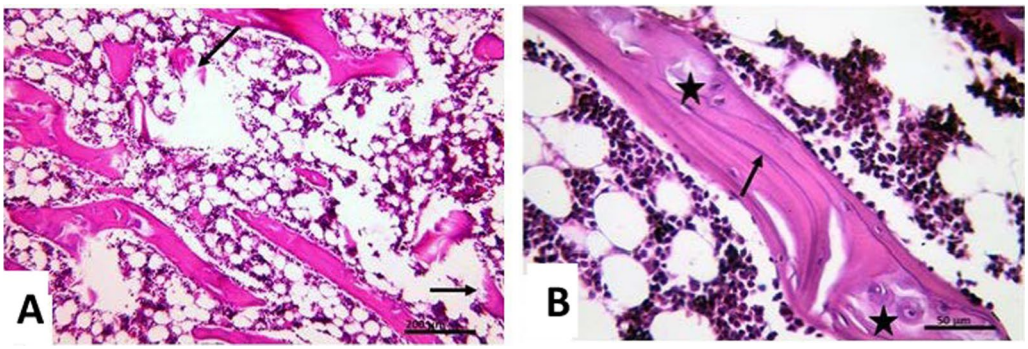

Fig. 7 H&E stained sections of the lower femur of risperidone/ketamine treated rat shows bone damage. Cancellous bone appeared as thin widely separated bone trabeculae with small fragmented areas of bone. Widening of bone marrow spaces and increase number of fat cells were also seen. Localized areas of faint staining bone trabecula with no osteocytes were frequently noticed (**A** H&E ×100, **B** H&E ×400 magnification)

suggested to be involved in mania [16] via an increase in OXS [56] and a decrease in BDNF [57, 58].

The inhibitory effects of tamoxifen on hyperlocomotor activity are not probably related to its estrogen modulatory effects, since the anti-estrogenic agent clomiphene failed to induce such an effect when given alone and did not enhance the effects of risperidone when combined with it. Similar results were reported by several investigators. Acute and chronic tamoxifen but not clomiphene or medroxyprogestrone blocked methylphenidate-induced hyperlocomotion in mice [26]. Similarly, in a study by Sabioni et al. [33], tamoxifen and chelerythrine

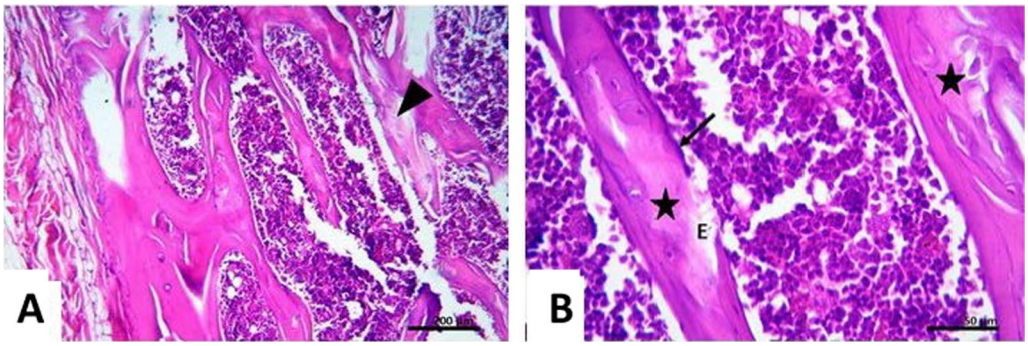

Fig. 8 H&E stained sections of the lower femur of tamoxifen/risperidone/ketamine treated rat shows localized areas of faint staining of bone matrix and eroded areas of bone could be seen which shows improvement compared to risperidone/ketamine treated group (**A** H&E ×100 & **b** H&E ×400 magnification)

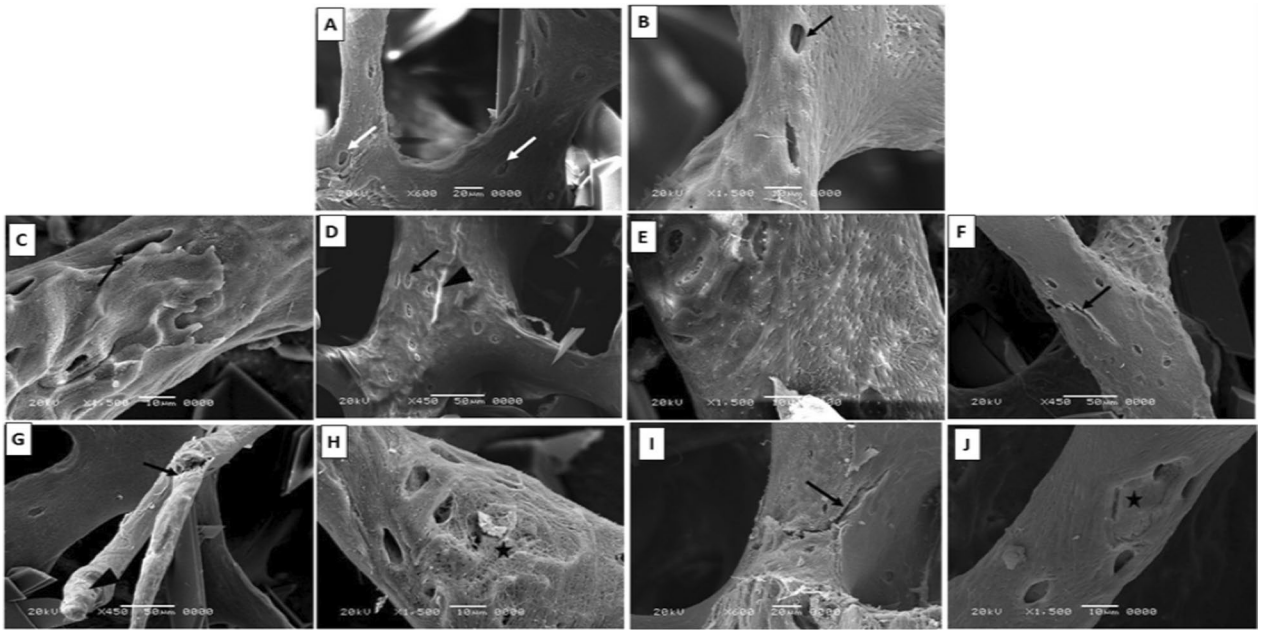

Fig. 9 Scanning electron microscopic examination of rat femur. Control group [A, B] **A** Branching and anastomosing bone trabeculae and osteocyte lacunae. **B** Collagen fibers are seen on the surface of bone trabecula. Osteocyte lacuna. *ketamine treated group* (**C**): Collagen fibers are seen on the surface of bone trabecula. Osteocyte lacunae (↑) are also seen. *Tamoxifen treated group* [D, E]: **D** A small crack is seen on the surface of bone trabecula. Osteocyte lacunae. **E** Collagen fibers on the bone surface. [F–H] *Risperidone treated group* (**F**) A crack is seen in a bone trabecula. **G** Thin tapering and fractured bone trabecula are seen. **H** Disturbed and disorganized collagen arrangement are seen on the surface of bone. [I, J] *Combination treated group*: **I** A crack is seen on the surface of bone. **J** Localized area of loss of collagen fibers

(a PKC inhibitor) completely blocked the hyperlocomotion induced by amphetamine, while an intermediate medroxyprogesterone dose partially reduced the amphetamine-induced hyperlocomotion.

In the second part of the study, bone examination revealed that risperidone/ketamine group exhibited marked thinning of bone trabeculae with wide separation and discontinuity of some bone trabeculae with areas of osteolysis. Histomorphometric findings revealed a significant decrease in trabecular bone thickness and percentage of trabecular bone volume. Indeed, risperidone was reported to increase bone resorption and reduce bone formation with elevation of all bone resorption parameters as, number of osteoclasts, osteoclastic surface, and eroded surface, probably due to elevated Rankl expression [59]. The effects of risperidone were more apparent

in inner cancellous bone trabeculae than in the outer cortical bone. This is in accordance with Takata and Yasui [60] who reported that bones with high proportion of cancellous bone were at the highest risk of osteoporosis. Wide bone marrow spaces with increased adipocytes were also observed in risperidone/ketamine group and might be attributed to the observed decrease in cancellous bone thickness.

The combination of tamoxifen with risperidone in ketamine treated rats was associated with lesser bone damage compared to risperidone/ketamine group. According to Zhong et al., [61] tamoxifen reduced osteoclast activity and bone turnover in humans, decreased bone resorption, increased femoral trabecular bone formation, increasing trabeculae number and thickness, bone volume with net bone gain at the distal femurs.

The bone damage seen in risperidone/ketamine group might be attributed to the increase in OXS in this group. Increased OXS has been reported to affect bone mineral density [5] and to be involved in the increased risk of bone fractures in patients on antipsychotic agents. [62].

The favorable effects of combination with tamoxifen on bone architecture might be attributed to the reduction in bone OXS seen in the tamoxifen/risperidone/ketamine group compared to risperidone/ketamine group.

An increase in central BDNF in the tamoxifen/risperidone group compared to risperidone alone might have also contributed to the favorable effect of combination with tamoxifen. Central BDNF levels have also been suggested to affect bone mineral density and BDNF receptors have been described in bone [4]. BDNF has been shown to play a role in osteogenesis and to be involved in osteoblast differentiation and in the increased mRNA expression of osteocalcin [63].

Although the estrogenic effect of tamoxifen is expected to be involved in its beneficial effects on bone structure, yet, its PKC inhibitory effects might have also contributed. In fact, PKC inhibition has been reported to block osteoclast functions and bone resorption [64, 65].

The findings of the present study have important implications. The greater reduction in OXS and increase in BDNF seen in the risperidone/tamoxifen treated group, compared to risperidone treatment alone is clinically significant. Risperidone has been reported to induce both pro-oxidative and antioxidative effects [8, 66]. Its effects on BDNF level are also quite inconsistent. Risperidone has been reported to induce, no significant effect, insignificant reduction, or an increase in BDNF level [13–15, 67]. Thus, the effects of risperidone on OXS and BDNF might be the net result of its positive and negative effects on OXS and BDNF. Indeed in our study although the effects of risperidone on OXS and BDNF were potentiated by combination with tamoxifen, yet the effects of tamoxifen were not potentiated by combination with risperidone. These findings suggest that the effects of tamoxifen on OXS and BDNF are more consistent than those of risperidone as suggested by other studies.

Hence, combination of tamoxifen with risperidone, may counteract any pro-oxidative effect or reduction in BDNF that might be induced by risperidone, increasing its beneficial behavioral effects while reducing its bone damaging effects.

These results may need to be replicated with other protein C kinase inhibitors and in larger samples and in combination with several toeher anti-psychotics besides risperidone. Measurement of protein C Kinase is a difficult process but will be needed in future studies to confirm the results. The real challenge is the search for a PKc inhibitor which unlike tamoxifen exhibits non-estrogenic properties, since unfortunately TAM is the only PKc inhibitor that can cross the BBB.

Conclusions

The PKC inhibitor tamoxifen reduced hyperlocomotion, and improved hippocampus structure in ketamine-treated rats when given alone and increased the effects of risperidone when combined with it. These effects were associated with reduction in OXS and increase in BDNF in hippocampus. The behavioral effects of tamoxifen might be related to its PKC inhibitory effects, rather than to its estrogen modulatory effects, since the antiestrogenic agent clomiphene had no effect on hyperlocomotion. Combination of tamoxifen with risperidone in ketamine-treated rats resulted in lesser bone damage with reduction in bone OXS compared to risperidone ketamine-treated rats. Accordingly, it could be postulated that PKC inhibitors, by reducing OXS and increasing BDNF level, might prove to be potential adjuncts to antipsychotic agents increasing their behavioral therapeutic effects while reducing their bone damaging effect.

Abbreviations

OXS: Oxidative stress; PKC: Protein kinase C; BDNF: Brain-derived neurotrophic factor; SEM: Scanning electron microscopic study; H&E: Hematoxylin and eosin stain; GFAP: Glial fibrillary acidic protein; GSH: Glutathione; MDA: Malondialdehyde; Tam: Tamoxifen; Risp: Risperidone.

Supplementary Information

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Additional file 1: Fig 1. S1. Graphical abstract of study design.

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Authors' contributions
AS, YM and KS conceived the idea, designed the study, performed the rat experiments, analyzed the data and prepared the manuscript. MR and GH, dissected the hippocampus and bone specimens, performed the histological studies and analyzed their results and contributed to the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate
This study was approved by the local research ethics committee of Ain Shams University, Cairo, Egypt (2017). Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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