Haloperidol affects bones while clozapine alters metabolic parameters - sex specific effects in rats perinatally treated with phencyclidine

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Abstract

Background: The presentation of schizophrenia (SCH) symptoms differs between the sexes. Long-term treatment with antipsychotics is frequently associated with decreased bone mineral density, increased fracture risk and metabolic side effects. Perinatal phencyclidine (PCP) administration to rodents represents an animal model of SCH. The aim of this study was to assess the effects of chronic haloperidol and clozapine treatment on bone mass, body composition, corticosterone, IL-6 and TNF-α concentrations and metabolic parameters in male and female rats perinatally treated with PCP.

Methods: Six groups of male and six groups of female rats (n = 6-12 per group) were subcutaneously treated on 2nd, 6th, 9th and 12th postnatal day (PN), with either PCP (10 mg/kg) or saline. At PN35, one NaCl and PCP group (NaCl-H and PCP-H) started receiving haloperidol (1 mg/kg/day) and one NaCl and PCP group (NaCl-C and PCP-C) started receiving clozapine (20 mg/kg/day) dissolved in drinking water. The remaining NaCl and PCP groups received water. Dual X-ray absorptiometry measurements were performed on PN60 and PN98. Animals were sacrificed on PN100. Femur was analysed by light microscopy. Concentrations of corticosterone, TNF-α and IL-6 were measured in serum samples using enzyme-linked immunosorbent assay (ELISA) commercially available kits. Glucose, cholesterol and triacylglycerol concentrations were measured in serum spectrophotometrically.

Results: Our results showed that perinatal PCP administration causes a significant decrease in bone mass and deterioration in bone quality in male and female rats. Haloperidol had deleterious, while clozapine had protective effect on bones. The effects of haloperidol on bones were more pronounced in male rats. It seems that the observed changes are not the consequence of the alterations of corticosterone, IL-6 and TNF-α concentration since no change of these factors was observed. Clozapine induced increase of body weight and retroperitoneal fat in male rats regardless of perinatal treatment. Furthermore, clozapine treatment caused sex specific increase in pro-inflammatory cytokines.

Conclusion: Taken together our findings confirm that antipsychotics have complex influence on bone and metabolism. Evaluation of potential markers for individual risk of antipsychotics induced adverse effects could be valuable for improvement of therapy of this life-long lasting disease.

Keywords: Schizophrenia, Bone mineral density, Haloperidol, Clozapine, Corticosterone, Phencyclidine, Interleukin
Background
Schizophrenia (SCH) is a severe neuropsychiatric illness characterized by presence of positive (hallucinations, delusions, thought disorders) and negative (flattened affect, social withdrawal, attention deficit) symptoms and cognitive dysfunction (problems with attention, working memory and executive functions) [1–3].

Long-term treatment with “typical” antipsychotics such as haloperidol, or with “atypical” antipsychotics such as clozapine, is often required for treating the symptoms. Typical antipsychotics, that primarily inhibit dopaminergic pathways, are more likely to produce extrapyramidal side effects due to dopamine blockade in the basal ganglia. “Atypical” antipsychotics have both anti-dopaminergic and anti-serotonergic activity. These drugs have greater anti-psychotic efficiency and fewer extrapyramidal side effects [4]. However, treatment with antipsychotics, especially atypical is frequently associated with distinctive metabolic side effects including obesity [5], type 2 diabetes and dyslipidaemia that contribute to overall morbidity and mortality [6]. Decreased bone mineral density (BMD) and increased fracture risk are noticed in SCH patients, who receive long-term antipsychotic therapy [7]. Changes of bone mass [8] and metabolic parameters [9] are also found in drug naive or SCH patients minimally exposed to antipsychotics. It is not clear whether the disease per se or a lifestyle (smoking, sedentary, nutrition, vitamin D deficiency, etc) together with antipsychotics is the cause of observed bone and metabolic changes.

Several studies reported that long-term use of antipsychotics may lead to a decrease in BMD and osteoporosis in relation to hyperprolactinemia [10–12] and currently, it is a generally accepted approach to categorize antipsychotics according to their effects on prolactin levels as prolactin-raising (PR) and prolactin-sparing (PS) antipsychotics [13–15]. However, recent meta-analysis of Stubbs et al. [16] has included nineteen studies and found that the hyperprolactinemia and taking of PR, as well as smoking, duration of illness and body mass index (BMI) were not related to the prevalence of osteoporosis. Significant correlation was seen with older age and a higher percentage of males indicating that men suffering from SCH are more vulnerable to osteoporosis and osteopenia than women. Recent review of Chen et al. [17] has also pointed out the gender differences in the effects of antipsychotics on BMD in patients with schizophrenia and has suggested that gender specific risk factors should be considered for intervention on bone loss. The authors have also found the evidences from previous studies, regarding the effects of antipsychotic-related hyperprolactinemia on BMD, inconclusive mainly due to cross-sectional study design or lack of adequate control groups.

Beside antipsychotic induced hyperprolactinemia and lifestyle, several other factors including increased activity of some interleukins and hypercortisolaemia could be responsible for accelerated osteoporosis seen in SCH patients [18]. Elevated levels of cortisol, the end hormone produced by the activation of the hypothalamic–pituitary–adrenal (HPA) axis, and of inflammatory markers, such interleukin (IL)-6, have been consistently shown in first-episode, drug-naive schizophrenia patients [19–21]. Also, evidences suggest that the levels of both cortisol and interleukins are affected by antipsychotic medications [22, 23].

Attempts to elucidate the causal mechanisms, identify psychosis-specific biomarkers of SCH, and to understand the effects of drugs, have led to the development of animal models [24]. One of the actual pharmacological animal models of this disease is perinatal phencyclidine (PCP) administration to rodents [25, 26].

PCP is a non-competitive antagonist of glutamatergic N-methyl-D-aspartate (NMDA) receptors, capable to produce a broad spectrum of effects in healthy human volunteers that resemble SCH. PCP may induce positive symptoms including agitation, audiovisual hallucinations and paranoid delusions, negative symptoms represented as blunting of affect and apathy, as well as, cognitive disorders [27, 28].

Schizophrenia like symptoms have been repeatedly found in rats perinatally treated with phencyclidine including deficit in the prepulse inhibition of acoustic startle response, a measure used in the assessment of sensorimotor gating deficits in schizophrenia, increased locomotor activity that is accepted as an index of positive symptoms and disturbances in working memory that are a core feature of the cognitive dysfunction [26, 29–33]. Disturbance of baseline temperature [34] as well as decreased glutathione levels and altered antioxidant defence [25, 35], decreased number of several classes of interneurons [36] and alterations of mitochondria and apoptosis and autophagy processes [37] have also been reported.

Although, there are several studies describing the effects of antipsychotics on bone [38–40] and metabolic parameters [41–43] in animals, there is a lack of investigations concerning effects of antipsychotics on these parameters in experimental animal model of SCH. Since our recent investigation has revealed reduced bone mass in male rats in PCP animal model of SCH and protective effect of atypical antipsychotic risperidone [44], the aim of this study was to assess the effects of chronic haloperidol (belonging to PR antipsychotics) and clozapine (belonging to PS antipsychotics) treatment on bone mass, body composition, corticosterone, IL-6 and TNF-α concentrations and metabolic parameters in male and female rats perinatally treated with PCP.
Methods

Animals

Twelve timed pregnant Wistar rats were obtained at day 14 of pregnancy. The animals were housed individually in wire-hanging cages located within a temperature-controlled animal vivarium maintained under a 12:12-h light/dark schedule (lights on at 07:00 h). Food and water were available ad libitum throughout the experiment. All experiments were carried out according to the Directive of the European Parliament and of the Council (2010/63/EU) and approved by The Ethical Committee of the University of Belgrade and Ministry of Agriculture and Environmental Protection Serbia (Permission No 323-07-09403/2015-05/2).

Within 12 h of parturition, the pups from dams were cross fostered and then randomly assigned to one of lactating dams. Day of birth was considered to be postnatal (PN) day 0. The animals were treated on 2nd, 6th, 9th and 12th PN days, with either phencyclidine (PCP, six groups) or saline (NaCl 0.9%, six groups). PCP (Sigma, St. Louis, MO) was dissolved in 0.9% physiological saline (0.001 g/ml) and injected subcutaneously (s.c.) in the interscapular region (10 mg/kg). The dose and time course of the treatment were selected according to published studies [25, 26, 34–37, 44–46]. The vehicle control was saline alone injected s.c. in the same volume as PCP. The pups were kept in the same litters and weaned at PN day 30, when they were separated from the dams and classified according to the sex. In the present study both male and female rats were included.

Treatment groups

Six groups of male and female rats were studied:

1) NaCl group (control): animals were perinatally treated with NaCl (11 males and 6 females); from PN 35 acetic acid (final concentration 1 mM) was added to drinking water in an equivalent concentration as it was used for dissolving of antipsychotics.

2) PCP group: animals were perinatally treated with PCP (7 males and 6 females); from PN 35 acetic acid was added to drinking water as in group 1.

3) NaCl–H group: animals perinatally treated with NaCl (6 males and 10 females); from PN 35 have started to receive haloperidol (H) (Krka, Slovenia) at dose 1 mg/kg/day.

4) PCP–H group: animals perinatally treated with PCP (7 males and 13 females); from PN 35 received haloperidol therapy in the same way as group 3.

5) NaCl–C group: animals perinatally treated with NaCl (10 males and 6 females); from PN 35 have started to receive clozapine (C) (Sandoz, Germany) at dose 20 mg/kg/day.

6) PCP–C group: animals perinatally treated with PCP (6 males and 12 females); from PN 35 received clozapine therapy in the same way as group 5.

Haloperidol and clozapine dosage were based on molecular, in vitro and in vivo, occupancy studies in adult animals [47, 48]. Drug dosing was based on the weight of the animals and the average daily fluid consumption. The drugs were administered orally in drinking water until PN 100. The antipsychotics were dissolved in 0.1 M acetic acid and subsequently diluted (1:100) for daily drug administration in drinking water [49, 50].

Bone density and body composition measurements by DXA

Dual X-ray absorptiometry (DXA) scans were performed at PN day 60 and 98 to measure areal BMD, bone mineral content (BMC), fat and lean using the Lunar Prodigy Advance DXA (GE Healthcare Lunar Corp., Madison, USA) with software EnCore2007 and option Small animal body analysis. Anaesthesia consisted on an intra-peritoneal injection of a solution of thiopental sodium (40 mg/kg bodyweight, Sigma, St. Louis, MO). Measurements were obtained in standard mode, area 20 × 30 cm, voltage 76.0 kV, dose 1.9 μGy. BMD (mg/cm²) and BMC (mg/cm) were measured in regions of interest (femur, spine and total body which besides spine and femur have included head, trunk and ribs). Fat and lean tissue quantities (g) were presented as legs, trunk and total content.

Tissue collection

Body weight was measured at PN 100 and animals were sacrificed by cervical dislocation and decapitation without anaesthesia. Rats were fasted for 12 h before sacrifice. Blood samples were collected and serum was obtained by centrifugation (15 min, 3000 rpm). Serum samples were stored at ~80 °C. Retroperitoneal and peri-epididymal adipose tissues were isolated and weighed. The femur was dissected and further processed for light microscopic pathology analysis.

Determination of IL-6, TNF-α and corticosterone concentration

Concentrations of corticosterone, TNF-α and IL-6 were measured in serum samples using enzyme-linked immunosorbent assay (ELISA) commercially available kits. Concentrations of corticosterone and TNF-α were measured in serum samples (dilution applied was 1:10 and 1:2 respectively), using Corticosterone EIA Kit, IDS and Rat TNF-α ELISA Kit, Invitrogen. Concentrations of IL-6 were measured in non-diluted serum samples, using Rat IL-6 ELISA Kit, Novex, Life technologies, following the manufacturer’s instructions. Absorbance was read at 450 nm using a microplate
reader. The results were calculated in comparison with the standard curve. Each sample was run in duplicate and the average was calculated.

For the corticosterone ELISA Kit the calculated intra-assay % CV was 4.9 for the concentration of 4.6 ng/ml and 3.8 for the concentration of 45.7 ng/ml and the calculated inter-assay % CV was 7.8 for the concentration of 4.7 ng/ml and 7.7 for the concentration of 45.2 ng/ml. For the TNF-α ELISA Kit the calculated intra-assay % CV was 6.9 for the concentration of 130.7 pg/ml and 4.3 for the concentration of 912.1 pg/ml while inter-assay % CV was 9.0 for the concentration of 135 pg/ml and 7.8 for the concentration of 970.9 pg/ml. For the IL-6 ELISA Kit the calculated intra-assay % CV was 5.8 for the concentration of 49.2 pg/ml and 3.5 for the concentration of 845.6 pg/ml while the inter-assay % CV was 8.8 for the concentration of 51.6 pg/ml and 6.3 for the concentration of 865.8 pg/ml.

Spectrophotometric measurement of glucose, cholesterol, triacylglycerol, calcium and phosphate concentration
Concentrations of glucose, cholesterol and triacylglycerol were measured spectrophotometrically using a commercially available enzymes assay kits (GL 364, Randox, Crumlin, UK, for glucose; CH 200, Randox, Crumlin, UK for cholesterol and TR 1697, Randox, Crumlin, UK for triacylglycerol). Concentrations of calcium and phosphate in serum were measured spectrophotometrically (using Arsenazo III method for calcium and UV for phosphate) with automatic biochemical analyzer (Biosystem A15; Biosystems, Spain).

Light microscopic analysis of femoral metaphysis
Analysis was performed by an investigator, blinded to the treatments. For tissue collection, the leg was disarticulated at the hip. For microscopic histological evaluation, femurs were removed and immediately fixed in 10% neutral-buffered formalin. The femur was cleaned of soft tissue, placed in decalcifying solution (8% HCl from 37% (v/v) concentrate in PBS) for ~24 h at 37 °C, dehydrated in graded ethanol, and then embedded in paraffin. Three 5-μm-thick paraffin-embedded horizontal bone sections were cut from the proximal end of the diaphysis, dyed with a haematoxylin–eosin, von Kossa and Masson’s trichrome stains and analysed by light microscopy and microscope Nikon Eclipse Ci with control unit DS-L3 and pre-installed software.

Statistical analysis
All results are presented as mean values with standard error of the mean (SEM) and were analysed using the one way ANOVA with Fisher’s post hoc test. \( p \) value less than 0.05 was considered statistically significant.

Results
Bone density and body composition measurements by DXA
Changes of BMD were more pronounced in male rats treated with PCP and/or antipsychotics
Effects of perinatal PCP treatment, haloperidol and clozapine on BMD are presented in Fig. 1.
Changes in bone mineral density were more prominent on PN 98 in male rats (Fig. 1a–e). ANOVA showed a significant changes of femur \( [F(5,41) = 7.06, p < 0.001] \), spine \( [F(5,41) = 2.84, p < 0.05] \) and total \( [F(5,41) = 5.26, p < 0.001] \) BMD between investigated groups. Post hoc analysis with Fisher’s test showed significantly lower femur BMD in PCP \( (p < 0.05) \), NaCl-H \( (p < 0.01) \), NaCl-C group \( (p < 0.05) \) and PCP-H and PCP-C group \( (p < 0.001) \) compared to controls. Spine BMD was significantly lower in NaCl-H and PCP-C group \( (p < 0.05) \), as well as, in PCP-H \( (p < 0.01) \) group while total BMD was reduced in NaCl-H and PCP-C group \( (p < 0.01) \) and PCP-H group \( (p < 0.001) \) compared to controls.

In female rats (Fig. 1b–f) the only observed change of BMD on PN 98 was a significant decrease of spine BMD \( [F(5,47) = 3.27, p < 0.01] \) in PCP-H \( (p < 0.05) \) compared to control and PCP animals.

Haloperidol unlike clozapine further promoted PCP induced changes in BMC in male rats
Effects of perinatal PCP treatment, haloperidol and clozapine on bone mineral content are presented in Fig. 2.
Changes of BMC in treated groups were more prominent on PN 98. In male rats ANOVA showed a significant changes of femur \( [F(5,41) = 5.89, p < 0.001] \), spine \( [F(5,41) = 3.74, p < 0.01] \) and total \( [F(5,41) = 8.45, p < 0.001] \) BMC between investigated groups (Fig. 2a–e). Post hoc analysis showed significantly lower femur BMC in PCP and NaCl-H group \( (p < 0.05) \), PCP-H \( (p < 0.001) \) and PCP-C group \( (p < 0.01) \) compared to control. Spine BMC was significantly reduced in PCP-H \( (p < 0.01) \) and PCP-C group \( (p < 0.05) \) and total BMC in PCP \( (p < 0.01) \), NaCl-H \( (p < 0.001) \), PCP-H \( (p < 0.001) \) and PCP-C group \( (p < 0.001) \) compared to control.

On PN 98 in female rats, ANOVA showed a significant changes of spine \( [F(5,47) = 3.53, p < 0.01] \) and total \( [F(5,47) = 8.9, p < 0.001] \) BMC between investigated groups (Fig. 2b–f). Post hoc analysis with Fisher’s test showed significantly lower spine BMC in PCP-H and PCP-C group \( (p < 0.01) \) while total BMC was significantly reduced in PCP \( (p < 0.05) \), PCP-H \( (p < 0.001) \) and PCP-C group \( (p < 0.05) \) compared to control.

PCP and/or antipsychotics have different effects on fat and lean contents in male and female rats
Effects of perinatal PCP treatment, haloperidol and clozapine on fat content are presented in Additional file 1:
Table S1. On PN 98 in male rats, ANOVA showed a significant changes of legs \([F(5,41) = 4.37, p < 0.01]\) and total \([F(5,41) = 4.25, p < 0.01]\), while there were no differences in trunk fat content \([F(5,41) = 2.76, p > 0.05]\) between investigated groups. Post hoc analysis with Fisher's test has shown significantly increased legs fat content in PCP \((p < 0.01)\) and NaCl-C group \((p < 0.05)\) compared to control.

On PN 98 in female rats, ANOVA showed a significant changes of trunk \([F(5,47) = 7.15, p < 0.001]\) and total \([F(5,47) = 5.70, p < 0.001]\), while there were no changes in legs fat content between evaluated groups \([F(5,47) = 1.84, p > 0.05]\). Post hoc analysis with Fisher's test has shown significantly decreased trunk fat content in PCP \((p < 0.001)\), NaCl-H \((p < 0.05)\), PCP-H \((p < 0.001)\) and PCP-C group \((p < 0.01)\) and total fat content in PCP and PCP-H group \((p < 0.001)\), as well as, in PCP-C group \((p < 0.05)\) compared to control.

Effects of perinatal PCP treatment, haloperidol and clozapine on lean content are presented in Additional file 2: Table S2. On PN 98 in male rats ANOVA showed a significant changes of legs \([F(5,41) = 8.1, p < 0.001]\), trunk \([F(5,41) = 8.35, p < 0.001]\) and total lean content \([F(5,41) = 11.73, p < 0.001]\). Post hoc analysis with Fisher's
test has shown significantly increased legs lean in PCP ($p < 0.05$) and significantly reduced in PCP-H group ($p < 0.01$) compared to control. Trunk lean was significantly reduced in NaCl-H ($p < 0.05$), PCP-H ($p < 0.001$) and PCP-C ($p < 0.05$), while total lean was significantly reduced in PCP-H ($p < 0.001$) and PCP-C group ($p < 0.05$) compared to control.

On PN 98 in female rats, ANOVA showed a significant changes of legs [F(5,47) = 5.11, $p < 0.001$], trunk [F(5,47) = 5.72, $p < 0.001$] and total lean content [F(5,47) = 5.56, $p < 0.001$]. Post hoc analysis with Fisher’s test has shown significantly increased legs lean content in NaCl-H group ($p < 0.01$) and significant decrease of trunk ($p < 0.001$) and total ($p < 0.01$) lean content in PCP-H group compared to control.

**Light microscopic analysis of the femoral metaphysis**

Light microscopic analysis of the femoral metaphysis is presented in Table 1. ANOVA showed significant changes of trabecular area in males [F(5,41) = 313.54, $p < 0.001$] and females [F(5,47) = 24,388.04, $p < 0.001$].

Representative pictures showing optic microscopy of cortical and trabecular structure of proximal femur in male and female rats are presented on Figs. 3 and 4.
Table 1: Light microscopic pathology analysis of femur

|                      | NaCl     | PCP      | NaCl-H   | PCP-H    | NaCl-C   | PCP-C    |
|----------------------|----------|----------|----------|----------|----------|----------|
| Trabecular area (μm²)| **       | **       | **       | **       | **       | **       |
| M                    | 25,825 ± 837 | 14,916 ± 689 | 16,754 ± 1590 | 5875 ± 1897 | 35,615 ± 2661 | 35,624 ± 2755 |
| F                    | 34,734 ± 1073 | 17,380 ± 714   | 35,802 ± 2171 | 25,140 ± 1441 | 35,292 ± 1018 | 37,212 ± 977   |

Effects of perinatal phencyclidine (PCP) treatment, haloperidol (H) and clozapine (C) on trabecular area of the femoral metaphysis on PN 100 in male (M) and female (F) rats. Results are presented as mean values with standard error of the mean (SEM)

* p < 0.05; ** p < 0.01 - comparing to control group
*** p < 0.001 - comparing to PCP group

Fig. 3: Comparative review of proximal femoral metaphysis in male rats (haematoxylin and eosin, original magnification ×40; arrows point to trabecular area). Histology of the femur in: **A** control animals (NaCl); **B** animals perinatally treated with phencyclidine (PCP) showing a significant reduction of trabecular area; **C** animals perinatally treated with NaCl followed by NaCl-H showing a reduction of trabecular area; **D** animals perinatally treated with PCP-H showing a more profound reduction of trabecular area compared to both NaCl and PCP animals; **E** animals perinatally treated with NaCl followed by treatment with clozapine (NaCl-C) showing a significant increase of trabecular area compared to control animals; **F** animals perinatally treated with PCP-C showing a significant increase of trabecular area compared to both NaCl and PCP animals.
Body weight, retroperitoneal and periepididymal fat content

Effects of perinatal PCP treatment, haloperidol and clozapine on body weight, retroperitoneal and periepididymal fat content are presented in Table 2. ANOVA detected significant body weight changes in both male $[F(5,41) = 14.75, \ p < 0.001]$ and female $[F(5,47) = 4.9, \ p < 0.001]$ rats. Post hoc analysis with Fisher’s test in male rats has shown a significant decrease of body weight in PCP-H ($p < 0.001$) compared to control and PCP group and a significant increase in NaCl-C group ($p < 0.01$) compared to control. In female rats, body weight was significantly lower in PCP ($p < 0.05$) and PCP-H group ($p < 0.01$) compared to control.

ANOVA showed a significant changes of retroperitoneal fat $[F(5,41) = 2.92, \ p < 0.05]$ in male rats. Post hoc analysis with Fisher’s test has shown significantly increased retroperitoneal fat in NaCl-C and PCP-C groups compared to control ($p < 0.01$) in male rats. There were no differences in retroperitoneal fat content between investigated groups $[F(5,47) = 2.54, \ p > 0.05]$ in female rats. Also, no differences in periepididymal fat content between investigated groups $[F(5,41) = 1.24, \ p > 0.05]$ were found.
Changes of corticosterone, IL-6 and TNF-α concentration in the serum were sex and drug specific

Effects of perinatal PCP treatment, haloperidol and clozapine on corticosterone, TNF-α and IL-6 concentration are presented in Fig. 5.

In male rats ANOVA showed a significant changes of corticosterone concentration \( [F(5,34) = 53.79, p < 0.001] \) between investigated groups. Post hoc analysis with Fisher’s test showed significantly decreased corticosterone concentration in PCP-H group \( (p < 0.05) \) compared to PCP group and a significantly increased corticosterone concentration in PCP-C group \( (p < 0.001) \) compared to both control and PCP group. In female rats \( [F(5,32) = 19.6, p < 0.001] \) corticosterone concentration was significantly decreased in PCP, PCP-H and PCP-C group compared to control \( (p < 0.001) \) (Fig. 5a and b).

ANOVA showed a significant changes of TNF-α concentration \( [F(5,34) = 2.84, p < 0.05] \) between investigated groups in male rats. Post hoc analysis with Fisher’s test showed significantly increased TNF-α concentration in NaCl-C group \( (p < 0.05) \) compared to control. Also, TNF-α concentration was significantly increased in PCP-C group compared to both control \( (p < 0.01) \) and PCP group \( (p < 0.05) \). In female rats \( [F(5,34) = 2.67, p < 0.05] \) the only observed change was a significant increase of TNF-α concentration in PCP-C \( (p < 0.05) \) group compared to PCP group (Fig. 5c and d).

ANOVA showed a significant changes of IL-6 concentration \( [F(5,33) = 2.11, p < 0.05] \) between investigated groups in male rats. Post hoc analysis with Fisher’s test showed significantly decreased IL-6 concentration in PCP group \( (p < 0.01) \) compared to control and a significantly increased IL-6 concentration in PCP-H group \( (p < 0.05) \) compared to PCP group. In female rats \( [F(5,33) = 2.55, p < 0.05] \) there was a significant increase of IL-6 concentration in NaCl-C \( (p < 0.01) \) and PCP-C \( (p < 0.05) \) group compared to control (Fig. 5e and f).

Changes of glucose, cholesterol and triacylglycerol concentration in serum are more pronounced in male rats

Concentrations of glucose, cholesterol, triacylglycerol, calcium and phosphate are presented in Table 3.

Glucose serum concentration in all experimental groups was in the reference range that is for male 5.0-12.2 mmol/L and for female 5.6-10.2 mmol/L rats. Statistically significant difference of glucose concentration between experimental groups \( [F(5,30) = 2.25, p < 0.05] \) was seen only in male rats where post hoc analysis has revealed significant reduction in PCP comparing to control group \( (p < 0.05) \).

The concentration of cholesterol in the tested groups was in the normal ranges both in males (1.8-3.6 mmol/L) and females (1.1-4.1 mmol/L). However, the significant differences between groups were seen in both males \( [F(5,30) = 6.48, p < 0.001] \) and females \( [F(5,30) = 2.41, p < 0.05] \). It is interesting that in all experimental groups of males cholesterol concentrations were significantly increased \( (p < 0.05 \) for PCP group, \( p < 0.01 \) for NaCl-H, PCP-H, NaCl-C and PCP-C groups) compared to control, while in female rats the concentrations were significantly decreased in PCP \( (p < 0.05) \), PCP-H \( (p < 0.01) \) and PCP-C \( (p < 0.05) \) groups compared to control.

The concentrations of triacylglycerol in all experimental groups were also in the reference range for males \( (0.7-5.12 \text{ mmol/L}) \) and females \( (0.4-5.92 \text{ mmol/L}) \). Significant difference in the concentration of triglycerides between tested groups was seen only in male rats \( [F(5,30) = 2.78, p < 0.05] \) where by applying post hoc analysis, a statistically significant reduction was demonstrated in the PCP-H group \( (p < 0.05) \) and a highly statistically significant reduction in the NaCl-C and PCP-C groups \( (p < 0.01) \) compared to the control group.

ANOVA did not show significant changes of calcium concentration in male rats \( [F(5,30) = 0.67, p > 0.05] \) nor in female rats \( [F(5,30) = 0.37, p > 0.05] \) between investigated groups. Also, concentration of phosphate was not changed in male rats \( [F(5,30) = 1.09, p > 0.05] \) and female rats \( [F(5,30) = 1.97, p > 0.05] \) between investigated groups.

### Table 2

|                | NaCl | PCP | NaCl-H | PCP-H | NaCl-C | PCP-C |
|----------------|------|-----|--------|-------|--------|-------|
| **BW (g)**     |      |     |        |       |        |       |
| M              | 354.2 ± 6.1 | 361.3 ± 6.7 | 338.5 ± 7.9 | 305 ± 7 | 386 ± 5 | 342.4 ± 6.5 |
| F              | 256.7 ± 2.3 | 229 ± 8 | 262 ± 6 | 222.4 ± 8 | 260 ± 12 | 241.5 ± 5.6 |
| **RPF (g)**    |      |     |        |       |        |       |
| M              | 2.5 ± 0.1 | 3.3 ± 0.1 | 2.9 ± 0.3 | 2.8 ± 0.1 | 3.7 ± 0.4 | 3.6 ± 0.4 |
| F              | 2.79 ± 0.18 | 2.63 ± 0.37 | 2.51 ± 0.24 | 2.34 ± 0.26 | 3.43 ± 0.28 | 3.03 ± 0.14 |
| **PEF (g)**    |      |     |        |       |        |       |
| M              | 2.5 ± 0.3 | 2.7 ± 0.2 | 2.6 ± 0.5 | 2.3 ± 0.2 | 2.8 ± 0.4 | 2.7 ± 0.4 |

Effects of perinatal phencyclidine (PCP) treatment, haloperidol (H) and clozapine (C) on body weight (BW), retroperitoneal (RPF) and periepididymal (PEF) fat content in male (M) and female (F) rats. Results are presented as mean values with standard error of the mean (SEM).

* \( p < 0.01 \); ** \( p < 0.001 \) comparing to control group

**Discussion**

In this study, the influence of haloperidol and clozapine treatment on the bone, body composition,
corticosterone, proinflammatory cytokines and metabolic parameters was investigated in male and female rats perinatally treated with PCP. The DXA measurements were done at PN 60 and PN 98 and the dynamic of changes caused by the treatment with antipsychotics was followed. Changes in BMD and BMC in treated groups were more pronounced on PN 98.

Reduction of BMD and especially BMC, as a more precise indicator for osteoporosis and risk factor for bone fractures [51], clearly indicates that perinatal use of PCP in rats, representing an animal model of schizophrenia, has long-term effects on bones. This is in accordance with findings in patients with schizophrenia. Recent meta-analyses have suggested that BMD is significantly lower in schizophrenia patients than in healthy controls [52] and that people with schizophrenia are at increased risk of developing fractures [53]. The finding of decreased bone density in

**Fig. 5** Effects of perinatal phencyclidine (PCP) treatment, haloperidol (H) and clozapine (C) on serum corticosterone, TNF-α and IL-6 concentration in male (a, c and e) and female (b, d and f) rats. Results are presented as mean values with standard error of the mean (SEM). *p < 0.05; **p < 0.01; ***p < 0.001 - comparing to control group. &p < 0.05; &&p < 0.001 – comparing to PCP group
our PCP perinatally treated rats indicate that complex changes in the brain, that are the basis for schizophrenia pathophysiology, could be responsible for observed changes of bones. It is known that bone remodelling which is essential for bone acquisition and bone maintenance is regulated systemically by many hormones and locally by cytokines, but recently the regulation through a central – beta adrenergic system relay [54] has been suggested. Also, the role of brain serotonin, in the modulation of sympathetic nervous system outflow to the skeleton and regulation of bone remodelling in experimental animals has been suggested [55, 56]. Our previous results showing normal prolactin levels in PCP perinatally treated rats [57], as well as, the changes of corticosterone or IL-6 and TNF-α concentration in this study, indicate that these factors are not responsible for the changes observed on bones. Also, findings of normal serum concentration of calcium and phosphates in our animals indirectly indicate normal levels of hormones involved in calcium homeostasis.

The investigations of the influence of antipsychotics in our model have revealed sex differences in the effects of haloperidol and the absence of the effects of clozapine on bones regardless the sex, in control (NaCl perinatally treated) animals. Among these animals decrease of total BMD and BMC was only seen in male haloperidol treated rats. It seems that observed changes are not the consequence of the alterations of corticosterone, IL-6 and TNF-α concentration since no change of these factors was observed. Previously, we have demonstrated dose dependent effects of haloperidol treatment on prolactin levels in male rats perinatally treated either with NaCl or PCP [57] and that higher dose of haloperidol (3 mg/kg) markedly increased prolactin levels while dose of haloperidol (1 mg/kg) used in the present investigation did not cause changes in level of prolactin. Therefore, this hormone cannot be regarded as a causal factor for the bone changes observed in the present study. The effects of haloperidol on BMD and BMC were present in PCP perinatally treated animals both male and female rats. The haloperidol treatment was related to the further deterioration of bone density that was already significantly decreased in PCP group without any antipsychotic medication. However, this further decrease was more significant in male rats. Treatment with clozapine, on the other hand, did not cause further deterioration of BMD and BMC in comparison to PCP perinatally treated rats and even modest protective effect of this drug was seen by microscopic examination in animals of both sexes. Interesting are the findings of corticosterone and interleukin measurements in these animals. While haloperidol did not cause changes of corticosterone, IL-6 and TNF-α concentrations both in NaCl and PCP perinatally treated rats comparing to control groups, clozapine treatment caused the significant elevation of IL-6 in female rats and significant elevation of TNF-α in male rats in both NaCl and PCP perinatally treated animals (NaCl-C and PCP-C groups) compared to controls. Also, clozapine treatment caused significant elevation of corticosterone concentration in male and significant decrease in female PCP perinatally treated rats. Our previous investigation has revealed the absence of the effects of clozapine on prolactin concentration regardless of the treatment dose.

Gender differences of antipsychotic effects on bone [17] were frequently analyzed in patients with schizophrenia. Some studies have demonstrated the beneficial effects of PS antipsychotics [58] and the association of PR antipsychotics with hip fractures [59] in both genders. Lin et al. [60] have found dose-related bone-density protecting effect of clozapine in women with chronic schizophrenia. However, although the prevalence of hyperprolactinemia was more frequently seen in

| Table 3 Spectrophotometric measurement of glucose, cholesterol, triacylglycerol, calcium and phosphate concentrations |
|-----------------------------------------------|
|                                | NaCl   | PCP   | NaCl-H | PCP-H   | NaCl-C  | PCP-C  |
| Glucose (mmol/L)               |        |       |        |        |        |       |
| M                              | 8.34 ± 0.39 | 7.33 ± 0.19* | 7.73 ± 0.27 | 7.60 ± 0.32 | 8.61 ± 0.49 | 8.08 ± 0.13 |
| F                              | 7.46 ± 0.29  | 7.17 ± 0.30 | 7.68 ± 0.21 | 7.63 ± 0.33 | 7.74 ± 0.22 | 7.85 ± 0.33 |
| Cholesterol (mmol/L)           |        |       |        |        |        |       |
| M                              | 1.00 ± 0.02 | 1.18 ± 0.06* | 1.25 ± 0.05*** | 1.28 ± 0.05*** | 1.36 ± 0.04*** | 1.26 ± 0.06*** |
| F                              | 1.93 ± 0.17  | 1.65 ± 0.05* | 1.72 ± 0.07 | 1.53 ± 0.06** | 1.84 ± 0.07 | 1.68 ± 0.08** |
| Triacylglycerol (mmol/L)       |        |       |        |        |        |       |
| M                              | 1.98 ± 0.14 | 1.73 ± 0.09 | 1.73 ± 0.09 | 1.50 ± 0.15** | 1.37 ± 0.14** | 1.43 ± 0.18** |
| F                              | 1.24 ± 0.08  | 1.37 ± 0.12 | 1.39 ± 0.12 | 1.16 ± 0.11 | 1.16 ± 0.11 | 1.25 ± 0.11 |
| Calcium (mmol/L)               |        |       |        |        |        |       |
| M                              | 2.38 ± 0.05 | 2.4 ± 0.06 | 2.28 ± 0.06 | 2.32 ± 0.05 | 2.3 ± 0.07 | 2.33 ± 0.05 |
| F                              | 2.25 ± 0.06  | 2.27 ± 0.07 | 2.33 ± 0.07 | 2.33 ± 0.05 | 2.27 ± 0.05 | 2.28 ± 0.06 |
| Phosphate (mmol/L)             |        |       |        |        |        |       |
| M                              | 3.17 ± 0.09 | 3.42 ± 0.18 | 3.15 ± 0.04 | 3.32 ± 0.09 | 3.27 ± 0.17 | 3 ± 0.19 |
| F                              | 2.82 ± 0.17  | 3.05 ± 0.16 | 3.23 ± 0.14 | 2.9 ± 0.11 | 2.7 ± 0.18 | 2.73 ± 0.08 |

$^p < 0.05; ^* p < 0.01; ^** p < 0.001$ - comparing to control group
women taking PR antipsychotics [61–63] the meta-analysis done by Stubbs et al. [16], that has included nineteen studies and more than three thousands patients with schizophrenia, pointed out that men are more vulnerable to osteoporosis and osteopenia than women. This finding is in accordance with our results. Interestingly, Lee et al. [64] have suggested that decreased bone mineral density found in the male schizophrenia patients may be caused rather by the negative schizophrenia symptoms than the hyperprolactinemia due to the antipsychotics.

Sex specific effects of PCP on cognition, brain derived neurotrophic factor (BDNF), spine synapses and dopamine turnover in prefrontal cortex were found in the juvenile but not, adult monkey’s representing the primate PCP model of schizophrenia. Namely in male juvenile monkey’s grater loss of spine synapses, grater effects on cognition, lower level of dopamine turnover and lower level of BDNF were found after two weeks of treatment with intramuscular injections of PCP [65]. As the most straightforward explanation for observed sex-dependent effects of PCP the authors have suggested the findings of protective and reparative effects of estradiol and progestrone in the CNS [66, 67] including the finding of the presence of an estrogen response element-like motif in the BDNF gene that regulates BDNF expression levels [68]. In accordance with these results is also our finding that at the PN 60 (juvenile rats) the effects of PCP on BMD and BMC are present only in male rats.

Recently two estrogen hypotheses in SCH have been proposed [69]. First one is the hypoestrogenism or deficiency hypothesis which describes gonadal dysfunction in women with SCH. The second is the protection hypothesis which states that estrogen exerts a relative protection against SCH in premenopausal women. Our results of selective sensitivity of PCP perinatally treated females to haloperidol treatment could be related to hypoestrogenism. It is possible that control (NaCl perinatally treated) females in our study are protected against effects of haloperidol on bones, seen in healthy male rats, by their normal level of estrogen and that PCP itself produces hypoestrogenism that is further pronounced by haloperidol treatment. Encouraging for this assumption is the finding that estrogen pre-treatment is effective in attenuation of a PCP-induced object recognition memory deficit in rats [70].

Effects of antipsychotics on experimental animals were investigated in several studies [38, 39]. To the best of our knowledge only one study assessed effect of risperidone in an animal model of schizophrenia showing protective effects of long-term treatment with atypical antipsychotic [44].

Study of Kunimatsu et al. [39] has indicated that chronic treatments with typical antipsychotics haloperidol or chlorpromazine induced a loss of trabecular bone of the femur in female rats. These results differ to results demonstrated in this study and could represent consequence of the applied doses as authors have used 2 and 10 mg/kg/day of haloperidol that highly exceed the doses applied in human treatment.

On the other hand, Costa et al. [38] have demonstrated, by both DXA and micro-computed tomography, that 6 weeks of clozapine, but not haloperidol treatment, decreases BMD in growing male rats. The authors have suggested that this effect may be mediated by direct actions of clozapine to decrease proliferation and differentiation of osteoblasts. Interestingly, our results show that clozapine treatment leads to normalization of the changes caused by perinatal use of PCP and, as we have previously mentioned, haloperidol is more harmful. The possible reason for the discrepancy between findings of these studies could be in the use of different protocol, especially the dose and duration of the antipsychotic treatment. In the study done by Costa et al. [38], the drugs were administered daily in the form of s.c. injection for 42 days, in the lower doses comparing to our protocol and this might have impact to antipsychotic metabolism and effects of the drugs. Also, the authors did not analyse the influence of antipsychotics in an animal model of schizophrenia.

Contrary to the effects on parameters measured on bones, the effects on biochemical parameters in serum were more pronounced after the treatment with clozapine. The corticosterone levels were higher in female than in male control (NaCl perinatally treated) rats and these finding is in accordance with the results of Ableson et al. [71] who have investigated plasma corticosterone levels during automated blood sampling in rats. Perinatal PCP treatment in our study was followed by the decrease of serum corticosterone concentration in female rats and decrease of IL-6 concentration in male rats. The study of Amani et al. [72] has indicated that neonatal PCP treatment did not alter baseline corticosterone levels both in male and female mice, but these authors have obtained experimental evidence showing that stress dose of PCP (10 mg/kg) increases corticosterone levels, anxiety- and depression-related behaviours in males, while decreases levels of anxiety without any significant effect on depression in female mice in adulthood.

The treatment with haloperidol in present study was followed by the decrease of corticosterone levels both in males and females perinatally treated with PCP, but clozapine has shown different effects in males and females. Highly significant increase of corticosterone level was seen in male rats while in female rats this drug did not have further influence on the changes already produced by PCP.
Elevated cortisol concentrations are found in first-episode, drug-naive schizophrenia patients without influence of antipsychotic medication, as well as in chronic medicated patients [19]. Also, patients with schizophrenia frequently display an impaired HPA axis response following an acute stress [73–77]. Both first and second generation antipsychotics have been associated with cortisol changes in patients with psychosis, but increasing evidences suggest that second generation antipsychotics reduce cortisol to a greater extent comparing to first generation [78–80]. The study, conducted in a small sample of healthy volunteers [81], has demonstrated no effect of the haloperidol on cortisol levels, and a reduction of cortisol levels by antipsychotics like quetiapine and risperidone. The effect of second generation antipsychotics on cortisol has been suggested to reflect differences in affinity and occupancy at the D2 and 5-HT receptor subtypes [82].

Increasing evidences indicate immune pathway dysfunction in schizophrenia. Autoimmune disorders and infections are associated with increased risk of developing schizophrenia [83, 84]. However, measurements of the levels of pro-inflammatory cytokines have obtained different results. Elevated levels are found in high risk and first episode psychosis patients [85, 86] and also during symptom exacerbations and stable phases of chronic illness [87, 88]. Dunjić et al. [89] have demonstrated higher IL-6 and lower TNF-α level in patients with schizophrenia in both exacerbation and remission phase in comparison to healthy controls and the absence of significant correlation between the levels of cytokines and sex, age, BMI, smoking habits, antipsychotic medication, duration of treatment and duration of illness. Chase et al. [90] have found that the levels of IL-6 mRNA in the peripheral blood mononuclear cells, in the absence of any other information, reliably discriminated between a diagnosis of schizophrenia and normal controls suggesting it as a useful and easily clinically accessible biomarker for the diagnosis of schizophrenia. Genome-wide association studies have identified immune system gene loci as among the leading associations with schizophrenia [91–94]. Chiappelli et al., [95] have found dysfunction in the homeostatic interactions between glucocorticoid and immune pathways. The applied stress paradigm has induced a rise in both cortisol and IL-6 in SCH patients, while in healthy controls a more robust acute cortisol response followed by a steeper decline of IL-6 that corresponds to the expected anti-inflammatory effects of cortisol, was seen. The opposite relationship demonstrated in SCH patients suggested a presence of an inability to down-regulate inflammatory responses to psychological stress. Our results have shown that perinatal PCP treatment does not cause significant changes in the measured pro-inflammatory cytokine concentrations. The only difference that has been noticed was the decrease of IL-6 in male rats. Treatment with haloperidol was not followed by significant changes of IL-6 and TNF-α both in male and female rats regardless of perinatal treatment, while clozapine caused significant elevation of TNF-α in male and significant elevation of both IL-6 and TNF-α in females regardless of perinatal treatment. In accordance with our findings is the study of Handley et al. [96] demonstrating that haloperidol, but not aripiprazole, lowers cortisol and IL-6 levels, and increases hippocampal regional cerebral blood flow within hours of drug administration. In the study of O’Connell et al. [97] increased levels of proinflammatory cytokines and BMI were found in female, but not male patients treated with clozapine compared to healthy controls. The authors have suggested that association of increased number of adipocytes may contribute to increased cytokine serum concentrations in females.

Fonseka et al. [98] have proposed that antipsychotics with a high propensity for weight gain, such as clozapine and olanzapine, influence the expression of immune genes, and induce changes in serum cytokine levels to ultimately down-regulate neuroinflammation. Inflammatory cytokines are normally involved in anorexigenic responses and reduced inflammation has been shown to mediate changes in feeding behaviours and other metabolic parameters, resulting in obesity. Study on rats [99] however, has shown that clozapine induces an immune response with an increase in inflammatory cytokines such as IL-6 due to its oxidation to a reactive nitrenium ion that covalently binds to neutrophils leading to the increase of inflammatory cytokines.

Increase of body weight and retroperitoneal fat in male rats treated with clozapine, seen in our study, could be related to increased serum corticosterone. Hyperactivity of the HPA axis is generally associated with obesity [100–102]. However, the increase of corticosterone was seen only in male PCP-C group while changes of body weight and retroperitoneal fat were observed in both NaCl-C and PCP-C groups of males indicated that increase of corticosterone could not be the only factor responsible clozapine influence on these parameters. The sex specific effects of clozapine were also demonstrated by Baptista et al. [103] who have found that different doses of clozapine (from 0.5 – 20 mg) applied intraperitoneally for 21 days have effects on body weight only in male rats. On the contrary Ferno et al. [104] show that depot injections of 100–250 mg/kg olanzapine in male rats resulted in weight loss rather than weight gain. Investigation of the ability of clozapine to induce weight gain in female rats [41], with progressively lowered doses of the drug, have resulted in finding that clozapine did not induce weight gain despite the given
doses. Instead, clozapine induced weight loss without alterations in food intake and muscle mass or changes in glucose, insulin, leptin and prolactin levels. In clinical studies, substantial clozapine induced weight gain was observed in humans [105]. In the meta-analysis performed by Gressier et al. [106] a strong relationship between serotonergic genes polymorphism and clinical response to clozapine was seen without clear findings of influence of tested genes polymorphism on clozapine induced weight gain.

In this study, significant increase of cholesterol concentration in male rats compared to control animals was shown in all experimental groups. It is noteworthy to mention that although those significant differences were noticed, all values have remained within the reference range for this kind of laboratory animals. Sex-dependent metabolic alterations have been also manifested in rat liver after 12-week exposition to haloperidol or clozapine [107]. The stress of endoplasmic reticulum caused by applied drugs via changes in Ca\(^{2+}\) homeostasis, is thought to be responsible for observed changes. It seems that women were protected by their estrogen. A variety of preclinical and clinical studies have indicated that in animals and patients treated with clozapine and haloperidol, obesity and elevated cholesterol and triacylglycerol's concentrations are due to the changes in the expression of genes involved in lipogenesis [108, 109], as well as in disorders of hormones responsible for the metabolism of fat [107, 110]. Olanzapine applied as intramuscular injection in the dose of 200 mg/kg or above has induced significant elevation of plasma cholesterol levels and pronounced activation of lipogenic gene expression in the liver [104].

Limitations of the study: Antipsychotics were used in one dose and applied orally giving the possibility for various ingestions of drugs by animals. Although this can mimic the situation with schizophrenia patients that have various medication compliance, different doses and different route of application of antipsychotics are needed. Also, since the female rats were investigated, the limitation of this study lies in the fact that the phase of menstrual cycle is not monitored. Furthermore, advanced investigations should be directed to the molecular mechanism of antipsychotic influence including measuring of parathyroid hormone, vitamin D, calcitonin, RANK (receptor activator of nuclear factor kappa-B)/RANK ligand and osteoprotegerin molecules. Mechanistic approach should also include the haloperidol-treated group receiving an antiresorptive (e.g., bisphosphonates) or osteoanabolic agent (e.g., calcitonin).

Conclusions
To the best of our knowledge, this is the first study that investigated the effects of haloperidol and clozapine on bone mass and body composition in PCP animal model of schizophrenia. Our results have shown that perinatal PCP administration causes a significant decrease in bone mass and deterioration in bone quality in male and female rats indicating that disease per se could be related to the changes of bones. Haloperidol had deleterious, while clozapine had protective effect on bones. The effects of haloperidol on bones were more pronounced in male rats. It seems that observed changes are not the consequence of the alterations of corticosterone, IL-6 and TNF-\(\alpha\) concentration since no change of these factors was observed. Treatment with clozapine was followed by more prominent, mainly sex specific, changes of measured biochemical and metabolic parameters. Clozapine induced increase of body weight and retroperitoneal fat in male rats regardless of perinatal treatment. However, the increase of corticosterone, seen only in male PCP perinatally treated rats that received clozapine, indicate that corticosterone is not the main factor responsible for clozapine influence on body weight. Furthermore, clozapine treatment caused increase in pro-inflammatory cytokines with sex specific changes that can fit in the theory of ‘cytokine signature’. Male rats have reacted to all applied drugs by significant increase of cholesterol concentration.

Taken together our findings confirm that antipsychotics have complex influence on bone and metabolism. The use of animal models in this investigation is important since they offer a precise control of factors that are highly variable and hardly controlled in humans. Evaluation of potential markers for individual risk of antipsychotics induced adverse effects could be valuable for improvement of therapy of this life-long lasting disease.

Additional files

**Additional file 1: Table S1.** Fat content measured by DXA. (DOCX 20 kb)

**Additional file 2: Table S2.** Lean content measured by DXA. (DOCX 19 kb)

**Abbreviations**
ANOVA: The Analysis Of Variance; BMC: Bone mineral content; BMD: Bone mineral density; C: Clozapine; DXA: Dual X-ray absorptiometry; H: Haloperidol; HPA: Hypothalamic–pituitary–adrenal; II-6: Interleukin 6; NMDA: N-methyl-D-aspartate; NRG-1: Neuregulin-1; PN: Prenatal day; PCP: Phencyclidine; PN: Postnatal day; RANK: Receptor activator of nuclear factor kappa-B; s.c.: Subcutaneously; SEM: Standard error of the mean; SCH: Schizophrenia; TNF-\(\alpha\): Tumor necrosis factor alpha

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**Availability of data and materials**
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors’ contributions
Conceived and designed the experiments: NP, MP, JS, NVR. Performed the experiments: TN, MV, TS, GJ, MA, MN. Analyzed the data: TN, NP, JS, MP. Contributed reagents/materials/analysis tools: NP, MP, JS. Wrote the paper: TN, NP. All authors read and approved the final manuscript.

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Ethics approval and consent to participate
All experiments were carried out according to the Directive of the European Parliament and of the Council (2010/63/EU) and approved by The Ethical Committee of the University of Belgrade and Ministry of Agriculture and Environmental Protection Serbia (Permission No 323-07-09403/2015-05/2).

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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