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Original Article

Effect of Long-Term Treatment with Fluoxetine, Clomipramine and St. John' S Wort Extract on Bone Turnover in Female Irradiated Rats.

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A B S T R A C T

Aim: The study aimed at evaluating the bone turnover rate in irradiated female rats treated daily with fluoxetine, clomipramine or St. John's wort extract for 8 weeks.

Material and Methods: 40 rats were randomly classified into 5 experimental groups: normal, irradiated control, irradiated fluoxetine, irradiated clomipramine and irradiated St. John's wort extract treated groups. The irradiated animals were exposed to a total dose of 15 Gy, fractionated over 5 weeks to small doses each of 1 Gy (day after day). Bone turnover rate biomarkers [serum osteocalcin (OC), urinary hydroxyproline/ creatinine ratio (Hpr/ Cr), urinary calcium/creatinine ratio (Ca/Cr)], hypothalamic pituitary adrenal [serum corticosterone], thyroid activities [serum thyroxin (T4) and thyrotrophin (TSH)], antioxidant [serum total antioxidant capacity (TAC) and malondialdehyde (MDA)] and pro-inflammatory biomarkers [serum tumor necrosis factor-alpha (TNF-α)] were done after 8 weeks from the 1st exposure to radiation. Histopathological investigations were also performed.

Results: The present results revealed that irradiation induced a significant decrease in serum OC by 43.6%, and a significant increase of Hpr/ Cr and Ca/Cr by 186.4% and 192.4%, respectively. Irradiated rats showed also a significant increase in serum corticosterone, TNF-α and MDA as well as a significant decrease in serum T4, TSH levels and TAC. Treatment of irradiated rats with St. John's wort extract, fluoxetine or clomipramine ameliorated most of the changes caused by bone-irradiation. The latter findings were confirmed by histological examination of bone tissue.

Conclusions: St. John's wort extract offers a therapeutic potential on bone comparable to traditional antidepressants like fluoxetine or clomipramine in irradiated female rats.

Key Words: St. John's wort extract, fluoxetine, clomipramine, bone turnover rate, irradiated rats.

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1. INTRODUCTION

Radiotherapy is critical in improving survival rates in cancer patients *(Bentzen, 2006)*. However, patients increasingly suffer from the long-term side effects of radiotherapy such as osteoporosis and increased bone fracture risk *(Baxter et al., 2005)*.

Radiation damage to bone results from many factors, including rapid activation of osteoclasts *(Willey et al., 2008)*, osteoblasts loss, vasculature damage *(Hopewell, 2003)*, increase in inflammatory bone resorbing cytokines [tumor necrosis factor-alpha (TNF-α), interleukin-1, interleukin-6] *(Neta, 1997; Park et al., 2004)*, oxidative stress *(Ozgocmen et al., 2007)*, thyroid dysfunction *(van Santen et al., 2005; Williams, 2009)* and increased serum cortisol level *(Girinsky et al., 1994)*.

Although depression is considered a risk factor for osteoporosis *(Bab and Yirmiya, 2010)*, some studies consider that antidepressants can increase the risk of osteoporosis *(Halbreich et al., 1995)*, other studies limited such effect to selective serotonin reuptake inhibitors (SSRIs) *(Diem et al., 2007)*.

Antidepressant drugs are widely used, not only for the management and treatment of stress and stress-related depression and anxiety *(Skolnick et al., 2003)*, but also for the management of chronic pain in patients suffering from osteoporosis *(Hibi et al., 2007)* and low back pain *(Maizels and McCarberg, 2005)*. Antidepressants could regulate thyroid hormones *(Eker et al., 2007)* and corticotrophin releasing hormone *(Stout et al., 2002)*. Moreover, some antidepressants have antioxidant *(Zafir and Banu, 2007; Grundmann et al., 2010)* and anti-inflammatory properties; thus they may significantly reduce effects of inflammatory resorbing cytokines *(Roumestan et al., 2007)*.

The use of antidepressants to minimize radiation-induced bone loss is still a matter of debate. The present study aimed

to evaluate the possible effects of three different types of antidepressants, fluoxetine as one of SSRIs, clomipramine as a tricyclic antidepressant (TCAs) and St. John's wort extract as a natural herbal antidepressant product on bone turnover in female irradiated rats.

2. MATERIAL AND METHODS

2.1. Animals:

Female Wistar albino rats, matched for age and weight (180-200 g), bred in the animal house of the National Centre for Radiation Research & Technology (Cairo, Egypt) were used. Animals were housed under standard laboratory conditions (room temperature 23 ± 2◦C; relative humidity 55 ± 5%, 12 h light/ dark cycle). They had free access to food (standard pellet diet) and water ad libitum. The study was carried out according to the guidelines of the ethical committee in Faculty of Pharmacy, Cairo University.

2.2. Drugs:

- Fluoxetine hydrochloride, purchased from Amoun Pharmaceutical Company (Egypt), it was dissolved in distilled water and used in a dose of 10 mg/kg, p.o. *(Huang et al., 2007)*.
- Clomipramine hydrochloride purchased from Novartis Pharmaceutical Company (Egypt), it was dissolved in distilled water and used in dose of 10 mg/kg, i.p. *(Gur et al., 1999)*.
- St. John's wort extract, purchased from Nature Answer Inc. Hauppage. NY, USA. The drug was dissolved in distilled water and used in a dose of 500 mg/kg, p.o. *(Butterweck et al., 2001)*.

2.3. Irradiation:

Rats were exposed to 15 Gy whole body γ-irradiation fractionated over 5 weeks (3 Gy/ week, 1Gy day after day) using the facilities provided by the National Center for Radiation Research and Technology (NCRRT) using Cesium-137 irradiation unit (Gamma cell-40) produced by the Atomic Energy of Canada Limited at a dose rate of 0.46 Gy/min.

2.4. Experimental design:

A total of 40 rats were used, 32 rats were irradiated for 5 weeks followed by 3 weeks without irradiation. The remaining 8 rats were not exposed to γ-radiation and served as normal group.

During the 8 week experimental period, rats were classified into 5 groups each of 8 rats as follows:

Animals of the 1st group were injected daily with saline for 8 weeks and served as normal group. Those of the 2 nd group were injected with saline daily and served as control irradiated group. Groups from 3-5 were treated daily with fluoxetine, clomipramine or St. John's wort extract, respectively.

2.5. Sampling:

By the end of 8 weeks, 24 h urine samples of fasting rats' were collected using metabolic cages. To collect blood samples, rats were anesthetized with urethane (1 g/kg; i.p.) *(Guedes and de Vasconcelos, 2008)* then decapitated. Serum samples were stored in aliquots at -20°C till use. For estimation of serum corticosterone, blood samples were collected after last stressor exposure *(Krame and Sothern, 2001)*, i.e. after 5 weeks from 1st dose of irradiation. Rats' femurs were isolated for histopathological examinations.

2.6. Biochemical investigations:

2.6.1 Bone turnover biomarkers:

Serum osteocalcin (OC) was determined using ELISA reagent kit (DIA Source, Belgium) to reflect bone formation rate. Urinary hydroxyproline to creatinine (Hpr/ Cr) and calcium to creatinine (Ca/ Cr) ratios were measured to evaluate bone resorption rate. Hydroxyproline was determined according to the method described by Woessner *(1961)*, while calcium and creatinine were measured using colorimetric reagent kits (Biodiagnostic, Egypt).

2.6.2. Hypothalamic pituitary adrenal and thyroid biomarkers:

Serum corticosterone was measured using ELISA kits (IBL International GMBH, Germany) whereas total thyroxin (T4) and thyrotropin (TSH) were measured in serum using ELISA kits purchased from Monobind, Inc. (USA).

2.6.3. Pro- inflammatory and oxidative stress biomarkers:

Serum TNF- was assayed using ELISA reagent kit (Ani Biotech, Finland). Total antioxidant capacity (TAC) was determined in serum using colorimetric reagent kit (Biodiagnostic, Egypt). Serum malondialdehyde (MDA) level was determined according to the method described by Yoshioka et al. *(1979)*.

2.6.4. Histopathological examination:

A total of 20 rats were used, 16 rats were irradiated for 5 weeks followed by 3 weeks without irradiation (they were divided into 4 groups; irradiated control, irradiated fluoxetine, irradiated clomipramine and irradiated St. John`s wort extract), the remained 4 rats formed the normal group.

Femurs were removed and immediately fixed in 10% neutral-buffered formalin. The femur was cleaned from soft tissue, placed in decalcifying solution (8% hydrochloric acid (37% v/v) and 10% formic acid (89% v/v) in phosphate-buffered saline) for about 24 h at 37 °C, this was followed by dehydration in 95% (v/v) ethanol and then embedding in paraffin. Three 5-mm-thick paraffin-embedded horizontal bone sections were cut from the proximal end of the diaphysis, stained with hematoxylin–eosin and examined with light microscope after 5x magnification.

2.7. Statistical analysis:

All values are expressed as means ± S.E. Data were analyzed using one way ANOVA followed by Tukey-Kramer multiple comparison test. The p value was considered significant at $P < 0.05$. Graphpad software instat (version 2) was used to carry out these statistical tests.

3. RESULTS

The present results showed a significant decrease in serum OC in control irradiated female rats by 43.6%

(Figure 1-A). No significant changes in serum OC were observed after treatment with either fluoxetine or St. John's wort extract; while clomipramine treatment significantly increased serum OC by 52.2% as compared to control irradiated group (Figure 1-A).

Results showed a significant increase of Hpr/Cr and Ca/Cr in control irradiated female rats by 186.4% and 192.4%, respectively. Clomipramine treatment in irradiated rats significantly decreased Hpr/Cr and Ca/Cr by 17.5% and 29%, respectively as compared to control irradiated group (Figures 1B and 1C). Moreover, irradiated rats treated with St. John's wort extract exhibited a significant decrease in Hpr/Cr and Ca/Cr by 49.2% and 35.5%, respectively as compared to control irradiated group (Figures 1B and 1C).

Irradiated female rats exhibited a significant increase in serum corticosterone concentration by 11.32% as compared to normal values (Table 1). Daily treatment of irradiated female rats either with fluoxetine or clomipramine significantly decreased serum corticosterone level by 5.6% and 5.25%, respectively as compared to control irradiated female rats. Moreover, treatment with St. John's wort extract significantly normalized serum corticosterone level in irradiated rats (Table 1).

Irradiation resulted in a significant decrease of serum T4 concentration by 53.7% in female irradiated rats after 3 weeks from last irradiation dose as compared to normal rats (Table 1). Daily treatment with fluoxetine or clomipramine in irradiated rats resulted in significant increase in serum T4 by 80% and 72.8%, respectively as compared to control irradiated rats (Table 1). In addition, irradiated female rats treated daily with the natural St John's wort extract exhibited normal values of serum T4.

Data in Table (1) showed that fractionated doses of γ-radiation significantly increased serum TSH concentration by 30.1%. Daily administration of fluoxetine, clomipramine and St. John's wort extract in irradiated rats significantly decreased serum TSH concentration in irradiated female rats by 8.7%, 25.34% and 24.8%, respectively as compared to control irradiated female rats (Table 1).

Our results exhibited a significant surge in serum TNF-α level 3 weeks post irradiation. Treatment of irradiated rats with fluoxetine, clomipramine and St John's wort extract significantly decreased serum TNF-α level by 77.8%, 71.5% and 77.6%, respectively as compared to control irradiated group (Table 2).

Irradiation induced a significant decrease in serum TAC by 19.8%. Both fluoxetine and St John's wort extract significantly normalized serum TAC in irradiated rats (Figure 2A). Irradiation induced a significant increase in serum MDA by 232.6%. Treatment with fluoxetine in irradiated rats significantly reduced serum MDA level by 36.2%, while treatment with St John's wort extract significantly normalized serum MDA levels in irradiated rats (Figure 2B).

Bone histology revealed a marked decrease in trabecular bone area and to less extent cortical area accompanied by vasculature bleeding in proximal end of diaphysis of irradiated rats femur stained with hematoxylin–eosin after 5x magnification with light microscope (Figure 3- R). Samples of irradiated female rats treated daily with fluoxetine exhibited a significant trabecular and cortical tissue damage accompanied by vasculature bleeding (Figure 3- FR). In case of clomipramine treatment, significant heavy vasculature damage was recorded without affecting bone density (Figure 3- CR). St John's wort extract maintained a normal trabecular and cortical tissue with little non-significant hemorrhage (Figure 3- SR).

Table 1: Effect of 8 weeks daily treatment with fluoxetine (10 mg/kg, p.o.), clomipramine (10 mg/kg, i.p.) and St. John's wort Extract (500 mg/kg, p.o.) on serum corticosterone, total thyroxin (T4) and thyrotrophin (TSH) in irradiated female rats.

Parameters	Normal	Irradiated Control	Irradiated Fluoxetine	Irradiated Clomipramine	Irradiated St. John's wort extract
Serum corticosterone (nmol/l)	135.20 ± 0.91	150.50* ± 1.04	142.10*# ± 0.25	142.60*# ± 0.46	138.60# ± 1.05
Serum tT_4 (µg/dl)	8.82 ± 0.46	4.08* ± 0.32	7.05*# ± 0.19	6.42*# ± 0.47	9.71# ± 0.22
Serum TSH (µIU/ml)	0.67 ± 0.01	0.88* ± 0.01	0.80*# ± 0.01	0.65# ± 0.02	0.66# ± 0.01

Drugs were administered daily for 8 weeks. Irradiation was done by exposing of all rats except the normal group to 15 Gy fractionated over 5 weeks (1 Gy doses administered day after day). Blood samples were collected at the end of the experiment except corticosterone samples which were collected 2 h after last radiation exposure.

All values are expressed as means ± S.E.M of 8 rats. Data were analyzed by one way ANOVA followed by Tukey-Kramer as a post ANOVA tests.

*Significantly different from normal group at $P \leq 0.05$.

#Significantly different from irradiated group at $P \leq 0.05$.

Table 2: Effect of daily fluoxetine (10 mg/kg, p.o.), clomipramine (10 mg/kg, i.p.) and St. John's wort extract (500 mg/kg, p.o.) on the tumor necrosis factor- alpha (TNF-α) in irradiated female rats.

Groups	Serum TNF-α (Pg/ml)
Normal	7.80 ± 0.63
Irradiated Control	81.20* ± 4.95
Irradiated Fluoxetine	18.60*# ± 0.31
Irradiated Clomipramine	23.20*# ± 1.81
Irradiated St. John's wort extract	18.20*# ± 1.28

Drugs were administered daily for 8 weeks. Irradiation was done by exposing all rats except the normal group to 15 Gy fractionated over 5 weeks (1 Gy doses administered day after day). Blood samples were collected at the end of experiment.
All values are expressed as means ± S.E.M of 8 rats. Data were analyzed by one way ANOVA followed by Tukey-Kramer as a post ANOVA tests.
*Significantly different from normal group at $P \leq 0.05$.
#Significantly different from irradiated group at $P \leq 0.05$.

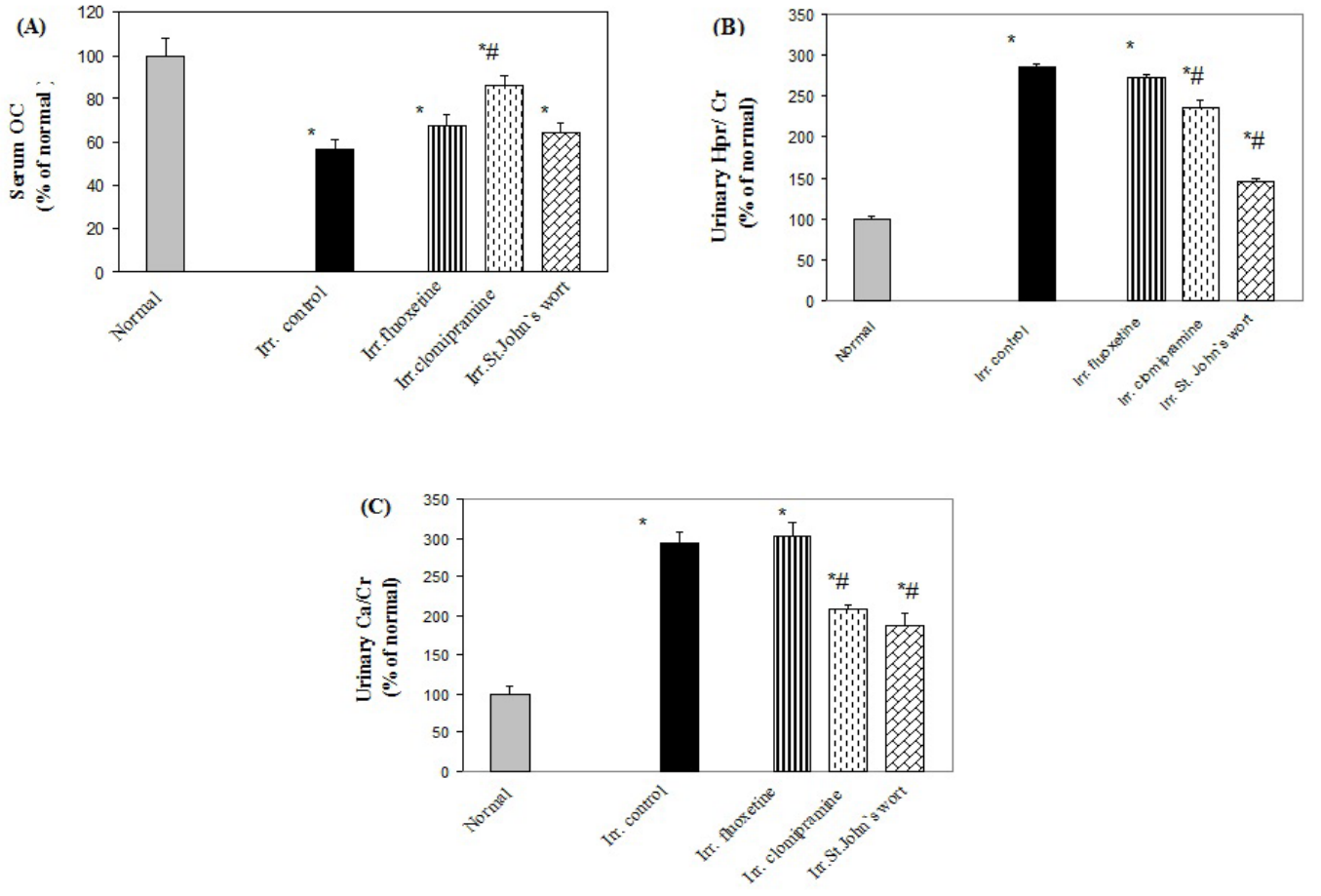

Figure 1: Effect of fluoxetine (10 mg/ kg, p.o.), clomipramine (10 mg/kg, i.p.) and St. John's wort extract (500 mg/kg, p.o) on: A. serum osteocalcin (OC), B. urinary hydroxyproline to creatinine ratio (Hpr/Cr) and C. calcium to creatinine ratio (Ca/Cr) in irradiated (Irr.) female rats.
Drugs were administered daily for 8 weeks. Irradiation was done by exposing all rats except the normal group to 15 Gy fractionated over 5 weeks (1 Gy doses administered day after day). Blood and urine samples were collected at the end of the experiment.
All values are expressed as means ± S.E.M of 8 rats. Data were analyzed by one way ANOVA followed by Tukey-Kramer as a post ANOVA tests.
*Significantly different from normal group at $P \leq 0.05$.
#Significantly different from irradiated group at $P \leq 0.05$.

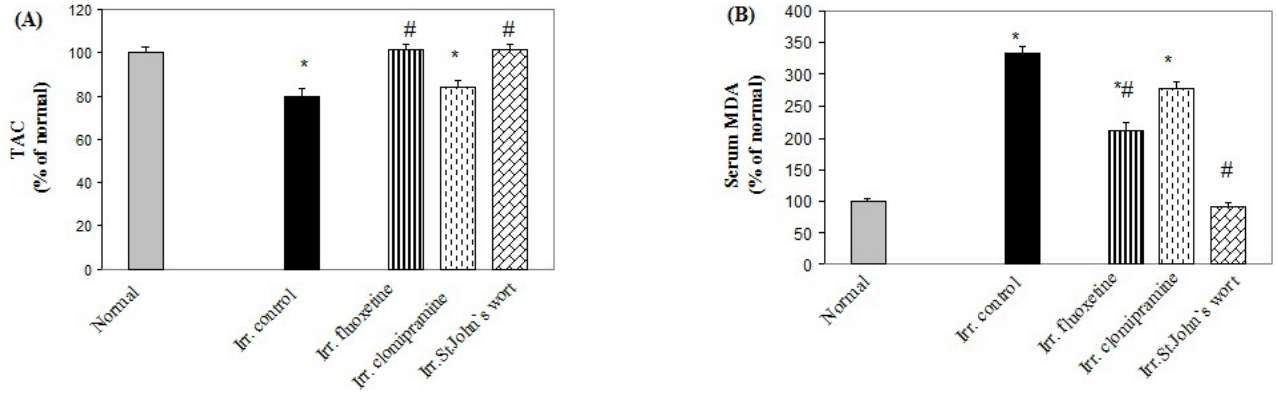

Figure 2: Effect of fluoxetine (10 mg/ kg, p.o.), clomipramine (10 mg/kg, i.p.) and St. John's Wort extract (500 mg/kg, p.o) on: **A.** serum total antioxidant capacity (TAC) and **B.** serum malondialdehyde level (MDA) in irradiated (Irr.) female rats.
Drugs were administered daily for 8 weeks. Irradiation was done by exposing all rats except the normal group to 15 Gy fractionated over 5 weeks to 1 Gy doses administered day after day. Blood were collected at the end of experiment.
All values are expressed as means ± S.E.M of 8 rats. Data were analyzed by one way ANOVA followed by Tukey-Kramer as a post ANOVA tests.
*Significantly different from normal group at $P \leq 0.05$.
#Significantly different from irradiated group at $P \leq 0.05$.

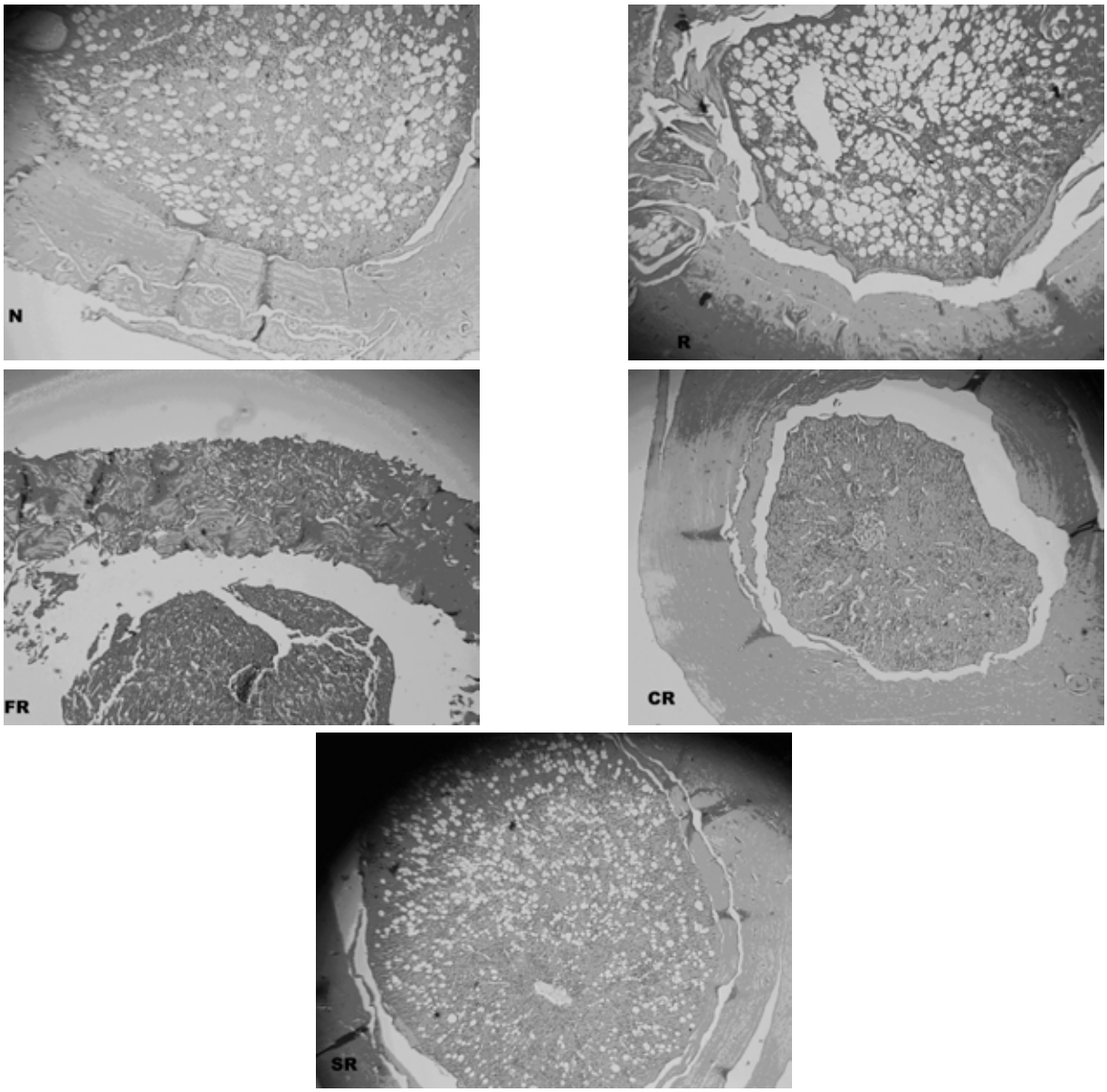

Figure 3: Light microscopy of the cortical and trabecular structure of the femur head. Effect of fluoxetine (10 mg/ kg, p.o.), clomipramine (10 mg/kg, i.p.) and St. John's Wort extract (500 mg/kg, p.o.). (Haemotoxylin and eosin, original magnification 5x).
N= normal rats, R= irradiated control rats, FR= fluoxetine irradiated rats, CR= clomipramine irradiated rats, SR= St. John's wort extract irradiated rats.

4. DISCUSSION

Exposure of female rats to γ-irradiation, in the present study, revealed a significant decrease in serum OC level which reflects bone formation rate coupled with a significant increase in both urinary Hpr/Cr and Ca/Cr which reflect bone resorption rate.

The deleterious effects of ionizing radiation on bone have been largely attributed to arrested proliferation of osteoblast precursors, osteoid production, and cell death *(Gal et al., 2000; Szymczyk et al., 2004)*, as well as increase in osteoclasts activity and number *(Willey et al., 2008)*.

Mechanisms underlying γ-irradiation - induced bone loss include direct effect on bone cells as well as alteration in the function of several organ systems that are important in bone homeostasis, such as the hypothalamic-pituitary adrenal (HPA) axis *(Willey et al., 2010)*.

In the current study, a significant increase in serum corticosterone level was detected in irradiated rats. Clinical *(Girinsky et al., 1994)* and experimental *(Cohen et al., 2011)* studies suggested that total body irradiation was capable of activating the HPA- axis since corticotropin-releasing factor and blood adrenocorticotropic hormone levels were increased. However, Velicković *et al. (2008)* suggested that the late response to gamma radiation is a hypo-suppressive state of the HPA axis.

The current study showed a significant decrease in serum T4 level as well as a significant increase in serum TSH in control irradiated rats. Such findings are in accordance with the study of van Santen *et al*. *(2005)* and this thyroid dysfunction could play a factor in enhancing bone turnover rate *(Williams, 2009)*.

The present study revealed a significant increase in TNF-α post irradiation. The role of gamma irradiation in increasing inflammatory resorbing cytokines is well known *(Neta, 1997)*, however, the increase in serum TNF-α may be strongly linked to osteoclastogenesis *(Azuma et al., 2000)*.

A significant decrease in TAC as well as a significant increase in serum MDA level was detected in the present study following exposure to γ-irradiation. This data was in accordance with the results of Cao *et al*. *(2011)* who suggested that irradiation induces bone injury by producing free radicals that adversely affect microenvironment for mesenchymal stem cells and damaging bone marrow blood vessels which slow bone fracture healing. We prospect that generation of free radicals following radiation exposure could damage bone vasculature and retard bone growth.

Examination of histology samples revealed a significant bone loss in irradiated rats manifested by trabecular bone loss and damage of bone vasculature. This was in accordance with the study of Willey *et al*. *(2010)* who showed that post irradiation, loss in trabecular bone was due to active bone resorption followed by reduction in bone formation. The present findings may be explained by osteoblasts cell death and vasculature damage post irradiation *(Hopewell, 2003)*.

In our results, irradiated female rats treated daily with fluoxetine showed high bone turnover rate, exhibited by a significant increase in urinary Ca/Cr and Hpr/Cr ratios, as well as, a significant decrease in serum OC. This was confirmed by histopathological changes recorded in bone architecture. The present finding may be partially attributed to the significant increase in serum corticosterone level *(Henneicke et al., 2011)*. However, Marx *et al*. *(2006)* reported that fluoxetine either in low or high doses induce a significant increase in serum deoxycorticosterone. On the other hand, Bab and Yirmia *(2010)* showed that bone loss accompanied with fluoxetine treatment is due to serotonin increase which restrained osteoblasts activity.

The clinical study by de Carvalho *et al*. *(2009)* concluded that fluoxetine adjusted all thyroid parameters within the euthyroid range and acted directly on relevant peripheral cells to decrease expression of inflammatory mediators probably by affecting their gene transcription. In our results, although fluoxetine induced significant changes in serum levels of T4, TSH and TNF-α compared with irradiated control rats but it didn't return them to the normal level. This could partly explain lack of improvement in bone architecture observed in fluoxetine-treated group.

Many studies revealed the antioxidant properties of fluoxetine *(Kirkova et al., 2010)*. In our results, although fluoxetine resulted in normalization of serum TAC, it was still accompanied by a significant increase in serum MDA level as compared to the normal group. This may partly account for the observed hemorrhage in trabecular bone area of fluoxetine-treated group.

In the current study, clomipramine improved thyroid function, serum corticosterone and TNF-α level. Moreover, histopathological results of irradiated rats treated with clomipramine demonstrated an improvement of bone state. Similar results were reported previously by Kadono *et al*. *(1995)* and Xia *et al*. *(1996)*.

The mechanism of St. John's wort is believed to involve inhibition of serotonin reuptake, much like the conventional SSRIs antidepressants *(Leuner et al., 2007)*. The major active antidepressive constituents in St. John's wort are thought to be hyperforin and hypericin *(Nahrstedt and Butterweck, 1997)*. Due to its SSRIs mechanism of action, St. John's wort couldn't increase OC level in irradiated female rats owing to osteoblasts activity suppression *(Bab and Yirmia, 2010)*. On the other hand, markers of bone resorption as Hpr/Cr and Ca/Cr were significantly decreased after St. John's wort treatment; this could be attributed to the significant improvements in serum levels of T4, TSH, corticosterone and TNF-α as compared to control irradiated group.

Besides its anti-stressful *(Kumar et al., 2010)* and anti-inflammatory properties *(Paterniti et al., 2010)*, St. John's wort has a powerful antioxidant activity, showed in this study

by normalizing serum TAC as well as MDA level. Sánchez-Reus *et al*. *(2007)* returned this powerful antioxidant property to quercetin content, while Crockett *et al*. *(2011)* returned that to xanthones contents.

In conclusion, the present study demonstrated that St. John's wort extract as a natural herbal agent possesses a therapeutic potential on bone comparable to traditional antidepressants like fluoxetine (SSRIs) and clomipramine (TCAs) in case of long-term exposure to γ-radiation, as it prevents high bone turnover rate induced by irradiation.

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