BJMHR
British Journal of Medical and Health Research
Journal home page: www.bjmhr.com

Potential Preventive Effect of Artemisinin in Ovariectomy-induced Osteoporosis in Rats

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ABSTRACT

Osteoporosis is associated with inflammation and increased oxidative stress, artemisinin was documented to have anti-inflammatory and anti-oxidant effects. In the present study we evaluated the potential prophylactic effects of artemisinin on ovariectomy induced osteoporosis in rats. Thirty two sprague-dawley female rats aged 3 months old were classified into 4 equal groups (8 rats each), 1st group was left without ovariectomy, 2nd group was subjected to ovariectomy operation and left without treatment for 3 months, 3rd group was subjected to ovariectomy operation then started oral alendronate (1 mg/kg/d) next first day after the operation and continued for 3 months, 4th group was subjected to ovariectomy operation and then started oral artemisinin (75mg/kg/d) next day after the operation and continued for 3 months. Serum samples were tested for tumor necrosis factor alpha (TNF-α) and cross linked telopeptides of type I collagen (CTX-I). Rat femurs of each group were subjected to histopathological examination by haematoxyline and eosin (H&E) staining. Ovariectomized group showed increased serum level of both TNF-α and CTX-I. Alendronate treatment for 3 months reduced serum CTX-I, failed to decrease the increased serum level of TNF-α in ovariectomized rats and improved bone mineral density (BMD) as demonstrated by histopathological examination in comparison to control ovariectomized rat. Artemisinin treatment for 3 months reduced serum TNF-α, CTX-I and improved BMD as demonstrated by histopathological examination when compared to control ovariectomized rat. So that, artemisinin has a potential prophylactic effect against osteoporosis in ovariectomized rats which is comparable to that of alendronate effect.

Keywords: Ovariectomy**,** inflammation**,** osteoporosis, Artemisinin, Alendronate, TNF-α.

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Received 10 September 2022, Accepted 22 October 2022

Please cite this article as: Aladeeb N *et al*., Potential Preventive Effect of Artemisinin in Ovariectomy-induced Osteoporosis in Rats. British Journal of Medical and Health Research 2022.

INTRODUCTION

Osteoporosis is a skeletal bone disease characterized by progressive loss of bone mass resulting in increased bone fragility and a higher fracture risk[1]. Osteoporosis is the most common disease of bone, affecting about 75 million people all-over the world[2]**.** Sudden drop in estrogen level occurring at menopause or after ovariectomy induces bone loss by increasing TNF-α producing T lymphocytes[3]. TNF-α produced by these T lymphocytes is the most important effecter in estrogen deficiency-induced osteoporosis which increases the number of osteoclasts, increases their anti-resorptive capacity and increases their life span[4]**.** TNF-α induces RANKL release which is the main osteoclastogenic cytokine that target receptor activator nuclear factor kappa B (RANK) on the surface of pre-osteoclasts. This activates the maturation of osteoclast progenitor cells into functioning osteoclasts[5]. Artemisinin possesses anti-inflammatory, anti-oxidant, anti-adipogenic, anti-cancer and anti-microbial activities[6]**.** Pharmacological therapies used in treatment of osteoporosis unfortunately are not without side effects. Bisphosphonates are very effective in prevention and treatment of osteoporosis more over have undesirable side effects especially on long term use there for search for new drugs for the management of osteoporosis which are more safe, effective and suitable for chronic use is important. This present study was designed to test the anti-resorptive effect of artemisinin in ovariectomy induced osteoporosis in rats in comparison with alendronate which is the most widely used drug in treatment of osteoporosis.

MATERIALS AND METHOD

Animals

Thirty-two sprague-dawley 3 months old female rats, weighing about 200 grams each were used throughout this study. They were obtained from the medical experimental research center, faculty of medicine, Mansoura University. Animals were kept in cages at a room with controlled temperature 26˚ C and on a 12-h light– dark cycles with available food and water. The Institutional Research Board, faculty of medicine, Mansoura university animal ethics committee have approved in all experimental procedures.

Drugs used

Artemisinin as raw material from Holly pharmaceutical, USA (white powder, not soluble in water) for oral administration. Alendronate as raw material (white powder, soluble in water) for oral administration from Sigma Aldrich Chemicals Co. St. Louis, MO, USA. Carboxy methyl cellulose (0.5%) as raw material (white powder)

Experimental design

Eight rats were used as a negative control group. Ovariectomy operation was performed on

twenty-four rats according to[7], then were divided into three equal each of 8 rats/group and treated from the first day after the ovariectomy operation as following:

- Group 1 (negative control group): was left without ovariectomy. Received the vehicle (0.5% carboxy methyl cellulose) by oral gavage daily and once daily for 3 months.
- Group 2 (positive control group): was subjected to ovariectomy operation. Received the vehicle (0.5% carboxy methyl cellulose) by oral gavage daily and once daily for 3 months.
- Group 3 (Alendronate treated group): was subjected to ovariectomy operation. Received alendronate 1 mg/kg/day suspended in a base of 0.5% carboxy methyl cellulose orally by oral gavage daily and once daily for 3 months[8].
- Group 4 (Artemisinin treated group): was subjected to ovariectomy operation. Received artemisinin 75 mg/kg/day suspended in a base of 0.5% carboxy methyl cellulose orally by oral gavage daily and once daily for 3 months[9].

At the end of the 3 months rats were sacrificed using an over-dose of thiopental sodium (75 mg/kg body weight) intra-peritoneal[10], for studying the potential preventive effect of artemisinin and alendronate on ovariectomy induced osteoporosis in rats.

Blood samples were collected by cardiac puncture and allowed to be precipitated for two hours at room the temperature. Serum was separated by centrifugation to be tested for Anti-TNF-α antibody, enzyme-linked immunosorbent assay (ELISA) kit from RayBioRat, USA and anti-CTX-I antibody ELISA kit from Cusabio Co., China. The femurs were dissected then prefixed in 4% para-formaldehyde for 24 h. Samples were decalcified according to[11], then samples were subjected to routine dehydration and paraffin embedding. The histological sections were performed and stained by (H/E).

RESULTS AND DISCUSSION

Three months after surgical induction of osteoporosis by ovariectomy operation resulted in a significant rise in the serum level of TNF-α in the positive control ovariectomized non-treated group (group 2) **(**22.99±3.41 pg/ml) as compared to the negative control non-ovariectomized group (group1) (10.24 ±1.45 pg/ml). Alendronate treated group (1 mg/kg/d) for 3 months (group 3) showed a significant rise in the serum level of TNF-α (24.6±5.23 pg/ml) as compared to the negative control non-ovariectomized group (group 1) (10.24 ±1.45 pg/ml). This increased level of TNF-α was non–significant as compared to positive control ovarictomized group (group2) (22.99±3.41 pg/ml).Artemisinin treated group (75 mg/kg/d) for 3 months (group 4) resulted in a non-significant change in serum TNF-α (11.75±3.37 pg/ml) as compared to the negative control group (group 1) (10.24 ±1.45 pg/ml), but showed a significant reduction in the serum level of TNF-α when compared to the positive

control ovariectomized group (group 2) (22.99±3.41 pg/ml).This reduction in serum TNF-α also was significant as compared to the alendronate treated group (group 3) (24.6±5.23 pg/ml) (Table 1&figure 1).

Three months after surgical induction of osteoporosis by ovariectomy operation resulted in a significant increase in the serum level of CTX-I in the positive control ovariectomized group (group 2) (50.53±2.32 pg/ml) as compared to the negative control group (group 1) (31.04±4.53 pg/ml). Alendronate treated group (1 mg/kg/d) for 3 months (group 3) produced a non- significant change in serum CTX-I (34.69±2.54 pg/ml) as compared to the negative control group (group 1) (31.04±4.53 pg/ml), but produced a significant reduction in the serum level of CTX-I as compared to the positive control ovariectomized group (group 2) (50.53±2.32 pg/ml). Artemisinin treated group (75 mg/kg/d) for 3 months (group 4) resulted in a significant increased serum CTX-I (44.51±2.86 pg/ml) as compared to the negative control group (group1) (31.04±4.53 pg/ml), but showed a significant reduction in the serum CTX-I as compared to the positive control ovariectomized non-treated group (group 2) (50.53±2.32 pg/ml), and showed a significant increased serum CTX-I as compared to the alendronate treated group (group 3) (34.69±2.54 pg/ml) (Table 1 & Figure 2).

Table 1: Effect of alendronate and artemisinin treatment on the serum level of TNF-α and CTX-I

Animal groups	Serum TNF-α (pg/ml)	P value for TNF-α	Serum CTX-I (pg/ml)	P value for CTX-I
Group 1 Negative control group	10.24±1.45		31.04±4.53	
Group 2 Positive control group	22.99±3.41	P1<0.001	50.53±2.32	P1=<0.001
Group 3 Alendronate treated group (1mg/kg/d for 3 months).	24.6±5.23	P1<0.001 P2=0.81	34.69±2.54	P1=0.12 P2=<0.001
Group 4 Artemisinin treated group (75 mg/kg/d for 3 months)	11.75±3.37	P1=0.83 P2<0.001 P3<0.001	44.51±2.86	P1=<0.001 P2=0.004 P3=<0.001

Data are presented as means ± SD (n=8) and were tested by one-way ANOVA followed by Tukey post hoc test and significant change was reported at $p< 0.05$.

SD: standard deviation.

P: probability.

Significant: ($p< 0.05$).

Non-significant: ($p> 0.05$).

P1: significance relative to group1.

P2: significance relative to group2.

P3: significance relative to group3.

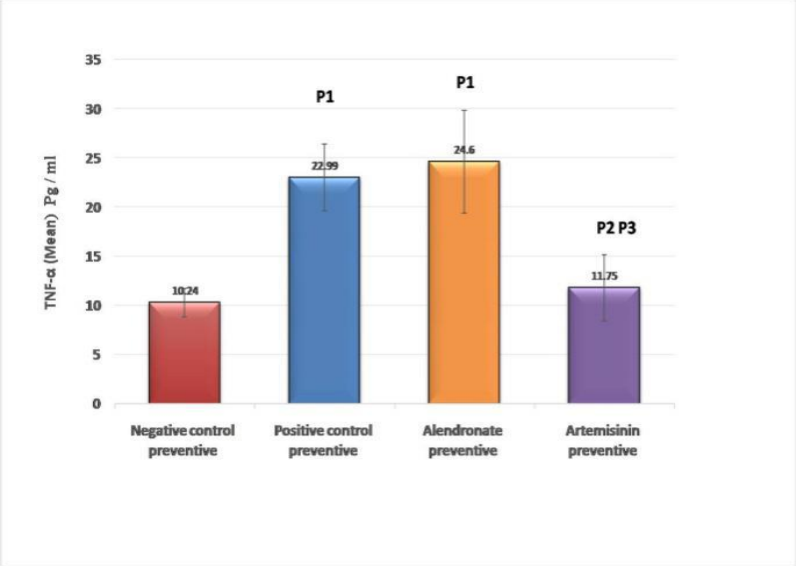

Figure 1: Effect of alendronate and artemisinin treatment on the serum level of TNF-α

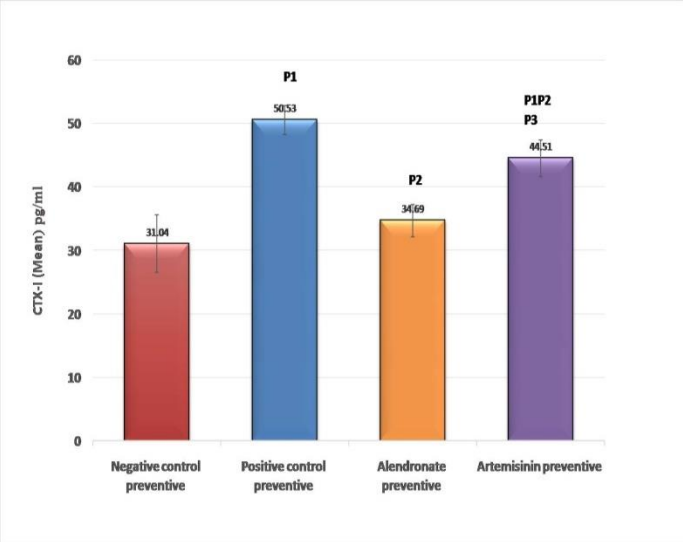

Figure 2: Effect of alendronate and artemisinin treatment on the serum level of CTX-I

P1: Significance in relation to negative control group.

P2: Significance in relation to positive control group.

P3: Significance in relation to alendronate treated group.

Examination of H&E-stained slides under light microscopy from the negative control group (group 1) showed normal bone trabeculae (Figure 3) while the positive control ovariectomized group (group 2) showed loss of bone mass with thin bone trabeculae (Figure 4), alendronate treated group (group 3) showed partial restoration of bone mass and the thickness of bone trabeculae (Figure 5). Also, artemisinin treated group (group 4) showed partial restoration of bone mass and the thickness of bone trabeculae (Figure 6).

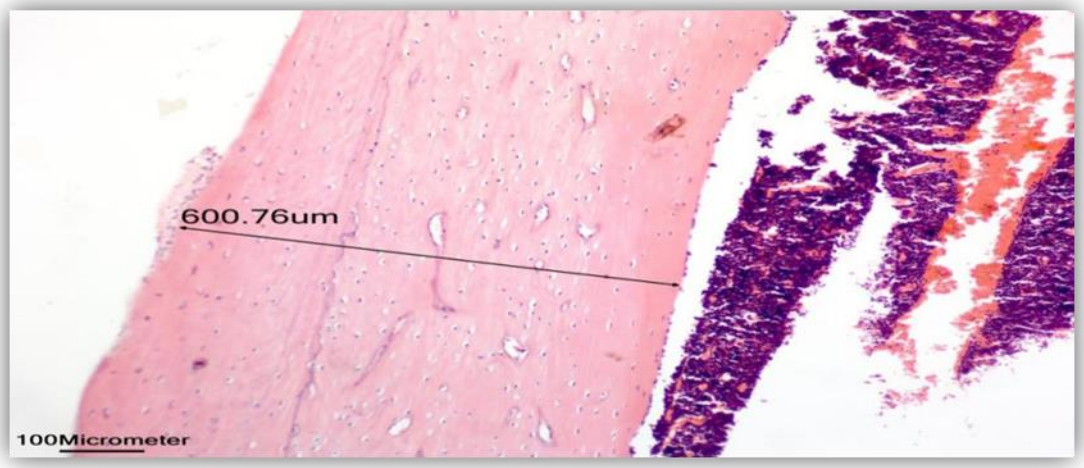

Figure 3: Negative control group (group 1): Routine H&E-stained slides revealed normal bone trabeculae with no detected microscopic abnormalities under light microscopy (100 x).

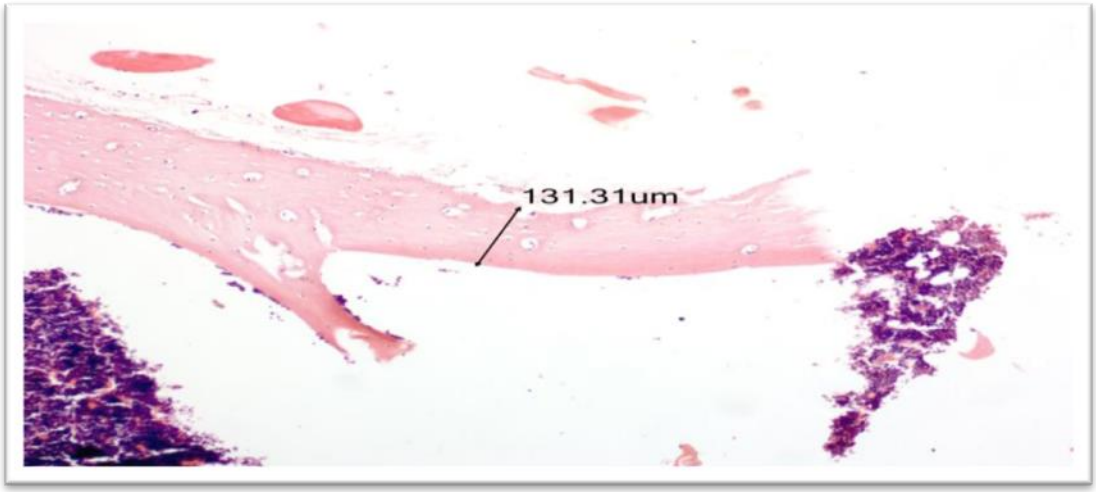

Figure 4: Positive control ovariectomized group (group 2): Routine H&E stained slides revealed loss of bone mass with thin bone trabeculae under light microscopy (100 x).

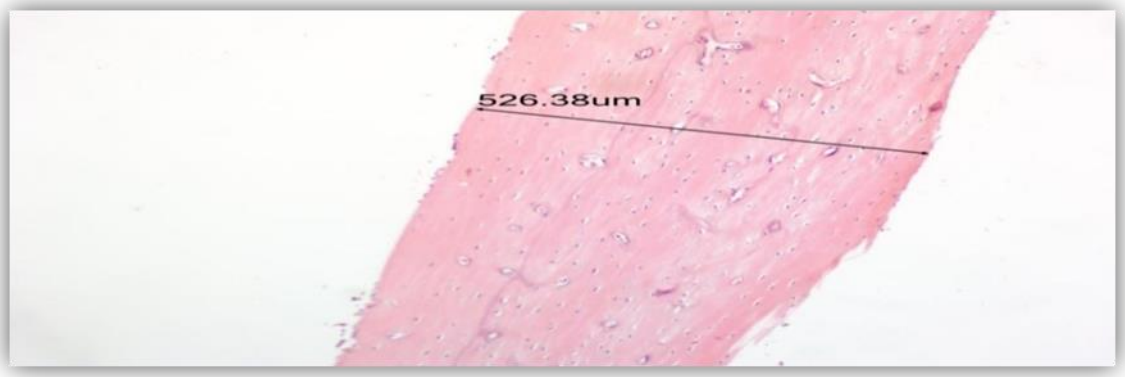

Figure 5: Alendronate treated group (group 3): Routine H&E stained slides revealed partial restoration of bone mass and the thickness of bone trabeculae under light microscopy (100 x).

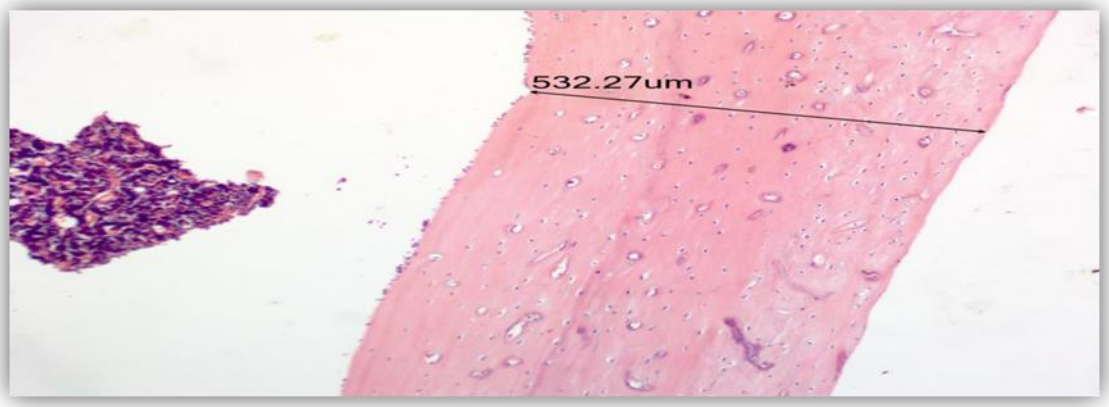

Figure 6: Artemisinin treated group (group 4): Routine H&E stained slides revealed partial restoration of bone mass and the thickness of bone trabeculae under light microscopy (100 x).

Postmenopausal osteoporosis is a common condition resulting in fragility fractures. It is associated with significant morbidity and mortality as well as health care[1]. Estrogen prevents bone loss by blocking the production of pro-inflammatory pro-resorptive cytokines such as TNF-α, interlukin-1 (IL-1), IL-8, IL-6, IL-17 and RANKL on the contrary increasing the production of anti-resorptive cytokines such transforming growth factor beta (TGF-B), interferon gamma (IFN-g) and osteoprotegren (OPG)[12].

In the present study; serum TNF-α was significantly increased in ovariectomized non-treated group as compared to the negative control group. This is in agreement with previous studies[13, 14, 15].

TNF-α levels increase dramatically at menopause as serum estrogen levels decline rapidly. This is complicated by the low systemic inflammatory status associated with the onset of menopause and synchronizes with the course of aging characterized by an inflammatory condition termed as ‘‘inflamm-aging’’[16]. TNF-α induced bone loss by up regulating colony stimulating factor receptor-1 (c-fms) expression which is essential for maturation of bone marrow progenitor cells into functioning osteoclasts, TNF-α induces osteoclast precursors and marrow stromal cells to produce osteoclastogenic cytokines such as IL-1, RANKL and macrophage colony stimulating factor (M-CSF) activating osteoclasts after its maturation. TNF-α also inhibits the osteoblast progenitor differentiation in to new osteoblasts by down regulating the expression of the Runt-related transcription factor-2 (RUNX-2) gene which is responsible for osteoblast maturation and function[17].

Alendronate treatment failed to correct the increased serum level of TNF-α induced by ovariectomy. This is in agreement with previous studies as described previously[13]. Most nitrogen containing bisphosphonates (NBPs) including alendronate have inflammatory adverse effects such as increased acute phase proteins, ophthalmic inflammation,

gastrointestinal disturbance, fever and a serious flu-like reaction in children treated with alendronate for osteogenesis imperfecta [18]. Alendronate caused inflammatory actions via inhibition of the component of mevalonate pathway as the inhibition of farnesyl pyrophosphate synthase resulting in accumulation of isopentenyl pyrophosphate, which is a strong natural stimulant of T-cells and macrophages producing pro-inflammatory cytokines as IL-1and TNF-α [19].

Artemisinin treated group showed a significant decrease in TNF-α as compared to ovariectomized non treated group and alendronate treated group. This is in agreement with previous studies as described by[13, 14].

TNF-α is released mainly from activated macrophages, also other cell types can produce it as monocytes, activated T cells, endothelial cells, polymorphonuclear leukocytes, epithelial cells, fibroblasts, bone-lining cells and osteoblasts [17]. The main source of TNF-α during inflammation is the macrophages; this effect is under the influence of nuclear factor kappa B (NF-κB) signaling pathway [20]. Artemisinin produced a significant inhibition of the NF-κB canonical pathway activation [21]. Also, artemisinins inhibits T-cell proliferation and prevent immune response related to it. This inhibited T-cell can't produce TNF-α and IL-2 [22]. In human monocytes, artemisinin suppresses the expression and the production of TNF-α and IL-1b via regulating NF-κB signaling pathway [23].

Ovariectomy operation increased the serum level of CTX-I in control ovariectomized non-treated group as compared to the negative control group. This is the same finding as found in previous studies [24, 25, 26].

Estrogen suppresses bone resorption [27] by blocking new osteoclasts formation, modulating RANK signaling in osteoclasts [28] and inducing osteoclasts death [29]. Estrogen deficiency leads to a progressive increase in bone resorption [30], inducing bone loss [27] and causing a rapid rise in the levels of markers of bone resorption [31]. Also, estrogen deficiency decreases defense against oxidative stress. Reactive oxygen species create a more oxidized bone microenvironment charring in the pathogenesis of postmenopausal osteoporosis [32]. The over expression of antioxidant enzyme in the osteoclast abolishes new osteoclast formation [33]. Estrogen deficiency at menopause inhibits osteoblastogenesis and stimulates osteoclastogenesis [34] by increasing oxidative stress [33].

Alendronate treated group showed a significant decrease in CTX-I as compared to ovariectomized non-treated group. This is in agreement with previous studies [26].

Alendronate prevents bone loss induced by estrogen deficiency in animals and is a strong inhibitor of the bone resorptive capacity of mature osteoclasts, it also induces osteoclasts apoptosis [35]. Alendronate which is a NBPs binds to and inhibit a key regulatory enzyme in the mevalonic acid pathway which is important in production of cholesterol and isoprenoid

lipids called farnesyl diphosphate synthase [36], inhibiting the prenylation of small guanosine triphosphate-binding proteins including Rac, Rab and Rho. These prenylation is essential for membrane ruffling and fiber assembly. Osteoclast can't survive without actin ring and ruffle border leads to cell failure and apoptosis in mature osteoclast [37]. The presence of a nitrogen group in bisphosphonate's structure as in the case of alendronate increases its anti-resorptive potency by 10,000 folds when compared to bisphosphonates without nitrogen group [38].

Artemisinin treated groups showed a significant decrease in serum level CTX-I when compared to the positive control ovariectomized non-treated groups. This is the same as described by [14, 39].

Osteoclast life span is controlled by RANKL and M-CSF [40]. M-CSF activates proliferation and differentiation of hematopoietic progenitors into preosteoclasts expressing RANK receptor on its surface [41]. TNF-α up regulates c-fms expression, which is the receptor for M-CSF [17]. Artemisinin produced a significant inhibition of TNF-α, IL-1b and IL-6 production in the macrophages by inhibiting NF-κB canonical pathway [21].

Binding of RANKL to its receptor RANK on the surface of osteoclast initiates TRAF-6 signaling which activates NF-κB activation and translocation to the nucleus inducing transcription of NFATc-1, the main regulator of osteoclastogenesis [42], and c-Fos which is a transcriptional protein necessary for osteoclast differentiation[14], leading to nuclear transcription of anti-apoptotic proteins preventing death of osteoclast [43], and promoting its maturation and differentiation [44]. In this study, artemisinin significantly decreased the RANKL expression when studied using immunohistochemistery. Artemisinin impair the activity of the transcriptional factor NF-κB, either by alkylating it or by preventing the degradation of its inhibitory protein NF-κB [21]. Also, in this study artemisinin significantly suppressed the expression of NF-κB when studied using immunohistochemistery. Also, western blot analysis showed that artemisinin treatment significantly inhibited the RANKL-induced expression of c-Fos and NFATc1 in a dose-dependent manner[14]. Artemisinin has anti-oxidant effect [6], which was approved by its ability to prevent mRNA expression of the inducible nitric oxide synthase and nitric oxide production [45]. In human astrocytoma T67 cells, artemisinin decreased the level of inducible nitric oxide synthase [46]. Artemisinin decreases the production of intracellular reactive oxygen species especially of the H2O2 by reacting with its endoperoxide moiety to form the carbon-based free radicals [47]. All the above-mentioned mechanisms may contribute to artemisinin supposed anti-resorptive effect.

Histological examination of H&E-stained sections of rat femurs of different groups demonstrated that; the negative control group showed normal bone trabeculae with no detected microscopic abnormalities. The ovariectomized untreated groups showed loss of bone mass with thin bone trabeculae. This result is in agreement with previous studies [15].

Regarding the alendronate treated group, it showed partial restoration of bone mass and the thickness of bone trabeculae. This result is in agreements with previous studies described previously [48]. Artemisinin treated group also showed partial restoration of bone mass and the thickness of bone trabeculae. This result is in agreement with pervious study described by [14].

CONCLUSION

Artemisinin has a potential prophylactic effect against osteoporosis in ovariectomized rats which is comparable to that of alendronate effect. Artemisinin has significantly decreased the serum level of tumor necrosis factor alpha (TNF-α) and cross linked telopeptides of type I collagen (CTX-I). So that, artemisinin is a promising drug for prevention of postmenopausal osteoporosis. But further studies are needed to confirm these beneficial experimental data, and to use artemisinin in clinical trials.

Conflict of Interest; there is no conflict of interest.

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