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Effect of Clodronate Alone or in Combination with Vitamin E on Demineralization Induced by Whole Body Gamma Irradiation in Rats

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BISPHOSPHONATES usually have been chosen in cancer treatment protocols for the management of fractures risk and controlling metastatic bone pain following chemo- and radiotherapy. This study aims at investigating the beneficial effect of clodronate alone or in combination with vitamin E in irradiated rats exposed to 1Gy 3 alternating days/week for 5 weeks. Calcitonin was also studied individually and in combination with vitamin E in order to investigate its possible effect on bone mass in irradiated rats. Irradiated rats treated with clodronate (5mg/kg, s.c., twice a week) or calcitonin (10 IU/kg, s.c., 48hrs) showed significant decreases in urinary hydroxyproline (Hpr), calcium (Ca) and significant increases in urinary phosphorus (Ph) and serum total antioxidant capacity (TAC) levels, while combination with vitamin E normalized most of measured parameters. It could be concluded that vitamin E could synergize the anti-osteoporotic effect of clodronate or calcitonin especially in γ- irradiated cases.

Keywords: Clodronate, Calcitonin, Vitamin E, γ- irradiation.

Introduction

Exposure to gamma radiation has deleterious effects on bone tissue and corresponding blood vessels. Several studies recorded increases in fracture incidences following radiotherapy treatments protocols (Wenxi et al., 2015; Shanmugarajan et al., 2017).

Most physicians recommended bisphosphonates as a first choice for management and treatment of osteoporosis and other metabolic bone diseases as Paget's, osteogenesis imperfecta and metastatic bone diseases, especially those associated with breast and prostate cancer (Reyes et al., 2016). Bisphosphonate, which is a synthetic pyrophosphate analog, suppresses osteoclast activities by binding to hydroxyapatite and concentrating in the bones (Diamond et al., 2004).

Although 49% of patients diagnosed with osteoporosis were prescribed with bisphosphonates, intranasal calcitonin was preferred in cases of gastrointestinal intolerance (Modi et al., 2015). The calcitonins are 32 amino acids, naturally occurring calcium-regulating peptide hormones produced by the thyroid glands in mammals, they have antiresorptive properties by binding to specific G protein-coupled receptors on osteoclasts, resulting in non-apoptotic inhibition of osteoclastic activity (Chesnut et al., 2008).

Reactive oxygen species (ROS) are one of several pathogenic factors in osteoporosis. It was reported that ROS inhibits the osteogenic differentiation of pluripotent mesenchymal precursors and stimulates osteoclast formation and bone resorption (Sharma et al., 2015). Most experimental and clinical studies revealed the beneficial effect of vitamin E on bone health either in normal or osteoporotic cases (Holvik et al.,

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2014; Kasai et al., 2015). However, Hamidi et al. (2012) demonstrated that only gamma-tocopherol initiates bone formation in post menopausal women.

The aim of this work is to study the possible role of vitamin E as a promising supplement with anti-osteoporotic agents (clodronate or calcitonin) to counter act the deleterious effect on bone mass in whole body gamma irradiated female rats.

Materials and Methods

Animals

Female Wistar albino rats, matched for age (3-6 months) and weight (180-200g), bred in the animal house of the National Center for Radiation Research & Technology (Cairo, Egypt) were used. 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 (revised directive 86/609/EEC).

Drugs

Clodronate, was purchased from Bayer Schering Pharma, Cairo, Egypt, in the form of ampoules (Bonefos®). Each 5ml ampoule contains 300mg anhydrous disodium clodronate. It was given subcutaneously at a dose level of 5mg/kg twice a week according to Kippo et al. (1995) for 5 weeks.

Calcitonin, purchased from Novartis pharmaceuticals Company, Cairo, Egypt, in the form of ampoules (Miacalcic®), was given subcutaneously at a dose level of 10 IU/kg/48 hours for 5 weeks according to Kavuncu et al. (2003).

Vitamin E was purchased from Pharco Co., Cairo, Egypt, in the form of soft gelatin capsules (Vitamin E 1000®), and given orally in a dose of 60 mg/Kg (body weight)/day for six days a week (5 consecutive weeks) according to Mohamad et al. (2012).

Irradiation

Rats were exposed to 15Gy whole body γ-irradiation fractionated over 5 weeks (3Gy/ week, 1Gy for 3 alternating days/week) (Moulder et al., 1990; Ramadan et al., 2011) 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 0.46Gy/min.

Experimental design

A total of 56 rats were used, 48 rats were pre-treated with saline or the selected treatment for 1 week followed by irradiation for the next 5 weeks. The remaining 8 rats were not exposed to γ-radiation and served as the normal group.

During the 6 weeks experimental period, rats were classified into 7 groups each of 8 rats as follows :

Animals of the 1st group were injected daily with saline for 8 weeks and served as the normal group. Those of the 2nd group were injected with saline daily and served as the control irradiated group. Groups from 3-7 were treated daily with either Clodronate, Calcitonin, Clodronate+ vitamin E, Calcitonin+ vitamin E or vitamin E alone, respectively.

Sampling

By the end of 6 weeks, 24hrs urine samples of fasting rats were collected using metabolic cages. To collect blood samples, rats were anesthetized with urethane (1g/kg; i.p.) (Guedes & de Vasconcelos, 2008), and then decapitated. Serum samples were stored in aliquots at -20°C till use. Right femurs were isolated for histopathological examination.

Biochemical investigations

Bone turnover biomarkers

Serum osteocalcin (OC) level was determined using ELISA reagent kit (DIA Source, louvain- la-Neuves, Belgium) to reflect bone formation rate. Urinary hydroxyproline (Hpr), calcium (Ca) and phosphorus (Ph) levels were measured to evaluate bone resorption rate. Hydroxyproline was determined according to the method described by Wossner (1961), while calcium and phosphorus were measured using colorimetric reagent kits (Biodiagnostic Co., Cairo, Egypt).

Oxidative stress biomarkers

Total antioxidant capacity (TAC) was determined in serum using colorimetric reagent kit (Biodiagnostic Co., Cairo, Egypt). Serum malondialdehyde (MDA) level was determined according to the method described by Yoshioka et al. (1979).

Pain perception

Thermal hypersensitivity to heat was evaluated as previously described by Eddy & Leimbach (1953). Animals were placed in a hot plate set at 50±1ºC and the nociception was recorded as the latency time to withdrawal, shaking or licking both or either paws. A cut-off time of 20sec was used to avoid tissue damage.

Histopathological examination

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 saline for about 24hrs 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 using light microscope at 16 x magnifications (Bitto et al., 2008).

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 6) was used to carry out these statistical tests.

Results

Irradiated rats treated with clodronate showed a significant decrease in urinary Hpr and urinary Ca by 63.47% and 26.21%, respectively and showed normal urinary Ph level (Fig. 1, 2). Moreover, serum OC level did not change in the irradiated rats treated with clodronate (Fig. 1).

Combined treatment of vitamin E with clodronate (Fig. 1, 2) improved serum OC level by 12.36% compared with the irradiated control rats. In addition, this combination significantly decreased urinary Ca level in irradiated rats by 67.35%, while clodronate alone induced a significant decrease by 26.13% compared to the control irradiated rats.

Irradiation induced a significant increase of 144.1% in serum MDA and a significant decrease of 25.2% in TAC. Treatment of irradiated rats with clodronate alone or in combination with vitamin E induced significant decreases in serum MDA levels by 47.7% and 43.9%, respectively, and induced significant increases in serum TAC by 28.88% and 28.34%, respectively (Fig. 3).

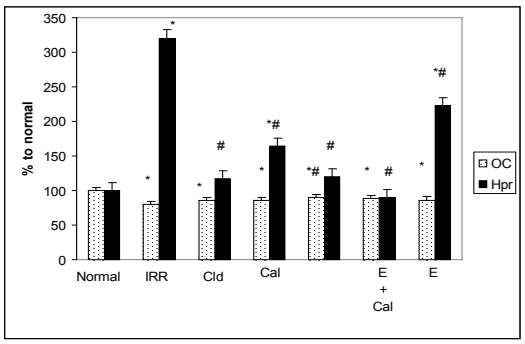

Fig. 1. Effect of six weeks treatment of clodronate (5mg/kg body weight) and calcitonin (10 IU/kg body weight) each alone or in combination with vitamin E (60mg/kg body weight) on serum osteocalcin (ng/ml) and urinary hydroxyproline (mg/dl) in ϒ- irradiated female rats [All values are expressed as means ± S.E.M of 8 rats, data were analyzed by one way ANOVA followed by Tukey-Kramer as a post ANOVA test, *: Significantly different from normal group at P≤0.05, #: Significantly different from irradiated group at P≤0.05. IRR= Irradiated group, Cld= Clodronate treated group, Cal= Calcitonin treated group, E= vitamin E treated group].

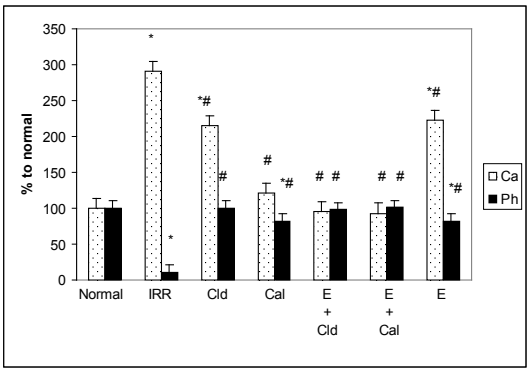

Fig. 2. Effect of six weeks treatment of clodronate (5mg/kg body weight) and calcitonin (10 IU/kg body weight) each alone or in combination with vitamin E (60mg/kg body weight) on urinary calcium (mg/dl) and phosphorus (mg/dl) in ϒ- irradiated female rats [All values are expressed as means ± S.E.M of 8 rats, data were analyzed by one way ANOVA followed by Tukey-Kramer as a post ANOVA test, *: Significantly different from normal group at P≤0.05, #: Significantly different from irradiated group at P≤0.05. IRR= Irradiated group, Cld= Clodronate treated group, Cal= Calcitonin treated group, E= vitamin E treated group].

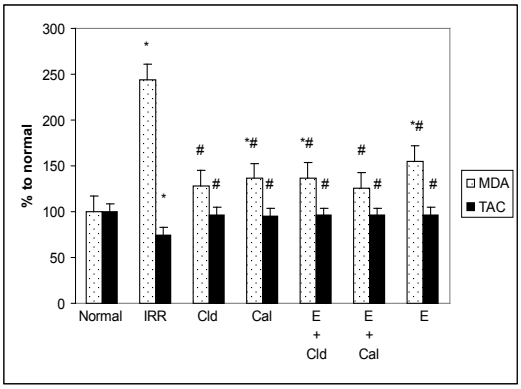

Fig. 3. Effect of six weeks treatment ofclodronate (5mg/kg body weight) and calcitonin (10 IU/kg body weight) each alone or in combination with vitamin E (60mg/kg body weight) on serum MDA (ng/ml) and serum total antioxidant capacity (mM/l) in ϒ-irradiated female rats [All values are expressed as means ± S.E.M of 8 rats, data were analyzed by one way ANOVA followed by Tukey-Kramer as a post ANOVA test, *: Significantly different from normal group at P≤0.05, #: Significantly different from irradiated group at P≤0.05. IRR= Irradiated group, Cld= Clodronate treated group, Cal= Calcitonin treated group, E= vitamin E treated group].

Calcitonin given to the irradiated female rats in a dose of 10 IU/ 48hrs induced significant decreases in urinary Hpr and Ca levels by 48.87% and 58.39%, respectively, and normalized urinary Ph level. However, combination with vitamin E induced normalization in both urinary Hpr and Ca levels (Fig. 1, 2). Figure 3 exhibits the antioxidant activity of calcitonin in irradiated rats; it induced a significant decrease in serum MDA level as well as a significant increase in serum TAC by 44.26% and 27.8%, respectively. However, calcitonin combination with vitamin E normalized TAC measured in serum of irradiated rats.

Although fractionally irradiated rats (15Gy) treated with vitamin E showed significant decreases in urinary Hpr, Ca and serum MDA levels as well as a significant increase in urinary pH level by 30.6%, 23.6%, 36.6% and 63.5%, they were significantly different from normal values (Fig. 1-3).

Table 1 shows that clodronate, calcitonin or vitamin E have analgesic activities exhibited by significant increases in pain perception times by 94.85%, 112%, and 137.14%, respectively.

Treatment of the irradiated rats with vitamin E showed that resorption was noticed in the bony trabeculae of the epiphysis associated with narrow articular cartilaginous surface, while the metaphysic and the cortex were intact. Olive oil the vehicle of vitamin E showed a wide zone of cartilaginous surface replaced the resorption in the epiphyseal trabeculae. There was no histopathological alteration in the metaphysis. Focal fibrous osteodystrophy was detected in the compact cortex. Furthermore, treatment with calcitonin showed a wide zone of articular cartilaginous surface associated with resorption in the underlying epiphyseal bony trabeculae while the metaphysic and cortex compact bone were intact. Treatment with calcitonin with vitamin E showed resorption, only noticed in the epiphyseal trabeculae. Clodronate as well as clodronate with vitamin E did not show any histopathological alterations (Fig. 4).

Discussion

Cancer patients are often exposed to a high risk of bone fragility either due to metastatic bone or due to the treatment protocol including radiotherapy (Bonarigo & Rubin, 1967; Chandra et al., 2018). In current study; female rats exposed to fractionated doses of gamma radiation resulted in significant increases in urinary Ca and Hpr as well as a decrease in urinary Ph and serum OC levels. In addition, histopathological manifestations of the bone of irradiated rats showed degeneration in chondroblast of articular cartilaginous surface, associated with resorption in the bone of metaphysis in addition to resorption in the compact bone of the cortex compared with the normal bone.

The deleterious effects of ionizing radiation on bone have been largely attributed to arrested proliferation of osteoblast precursors and inhibition of the expression of osteogenesis- related genes (Li et al., 2014) as well as increases in osteoclasts number and activities (Willey et al., 2008).

Generation of free radicals damages bone vasculature and retard bone growth (Cao et al., 2011), they accelerate osteoclasts activities causing destruction in normal calcified bone tissues (Callaway & Jiang, 2015). In this study, a decrease in serum TAC and increase in serum MDA level in the irradiated rats were observed reflecting oxidative stress state in the irradiated rats.

TABLE 1. Effect of six weeks treatment of calcitonin (10 IU/kg body weight) and clodronate (5mg/kg body weight) each alone or in combination with vitamin E (60mg/kg body weight) on pain perception (seconds) in ɣ-irradiated female rats.

Groups		Pain perception (time in seconds)
Normal (non-irradiated)		14.56 ± 1.47
Irradiated (15Gy/5 weeks)	Irradiated **(control)**	3.58* ± 0.12
	Clodronate (**5mg/kg)**	6.80*# ± 0.34
	Calcitonin (**10 IU/kg)**	7.42*# ± 0.37
	Clodronate+ vitamin E	9.12*# ± 0.50
	Calcitonin+ Vitamin E	10.04*# ± 0.82
	Vitamin E (**60mg/kg**)	8.29*# ± 0.78

- 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 test.
- *: Significantly different from normal group at P≤0.05.
- #: Significantly different from irradiated group at P≤0.05.

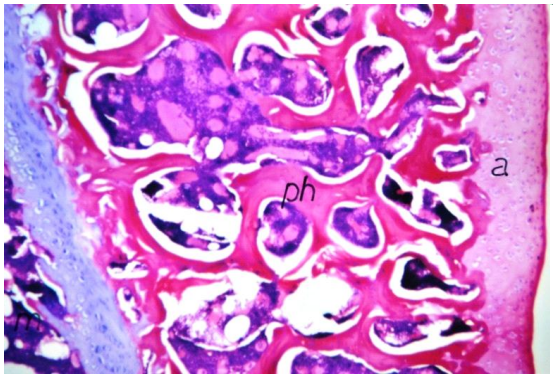

Fig. 4.a. (H&E ×16) Femur bone of rat in Normal group showing normal histological structure of articular cartilaginous surface (a), epiphysis bone (ph) and metaphysis (m).

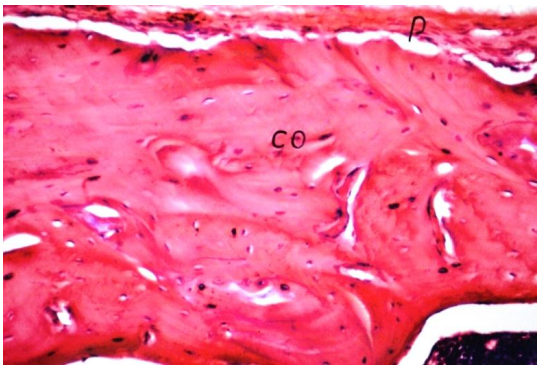

Fig. 4.b. (H&E ×40) Femur bone of rat in Normal group showing normal histological structure of periostium (p) and cortex of bone (co).

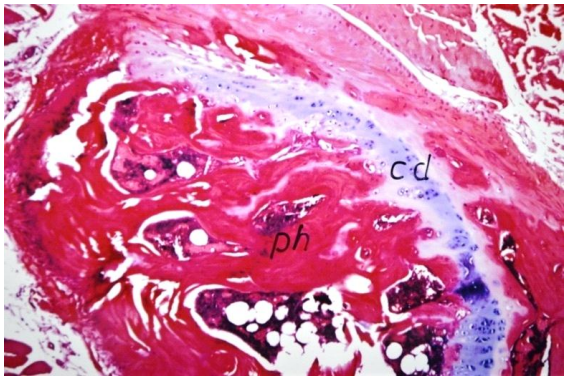

Fig. 4.c. (H&E ×16) Femur bone of rat in Irradiated group showing degeneration in articalar cartilaginous surface (cd).

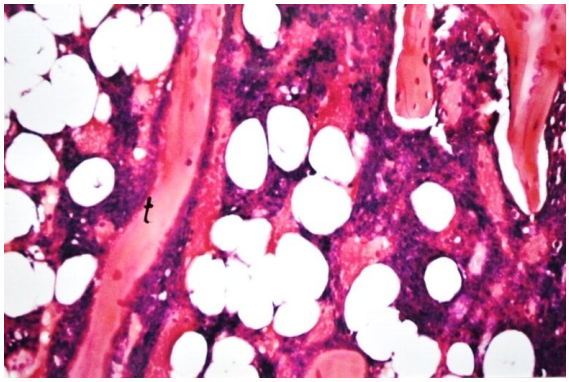

Fig. 4.d. (H&E ×40) Femur bone of rat in Irradiated group showing resorption in the metaphyseal bony trabeculae (t).

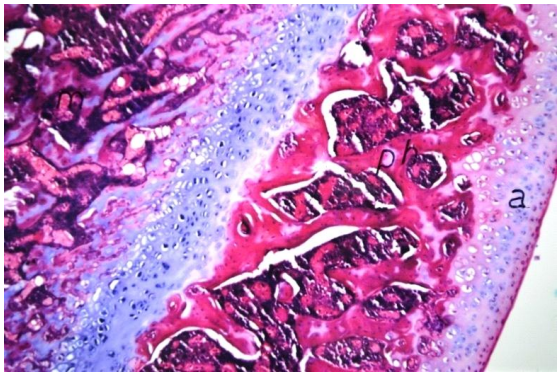

Fig. 4.e. (H&E ×16) Femur bone of rat in Clodronate group showing normal histological structure.

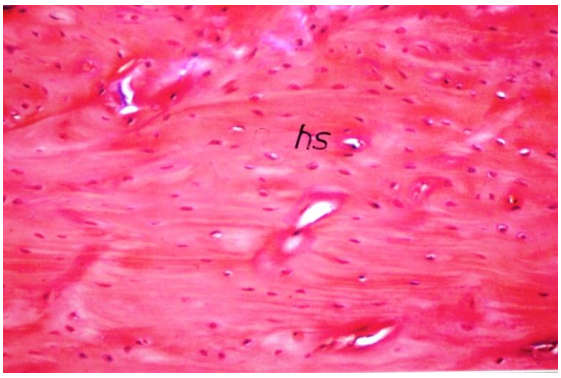

Fig. 4.f. (H&E ×40) Femur bone of rat in Clodronate group showing normal histological structure of haversian system (hs).

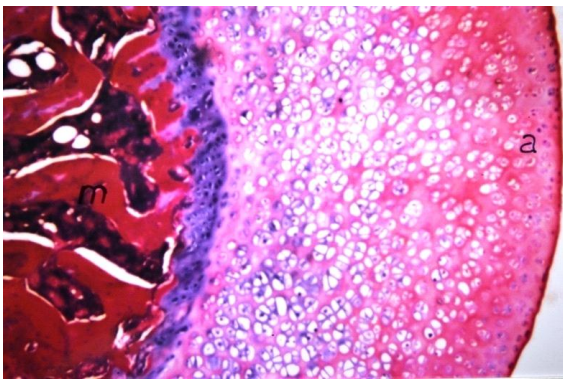

Fig. 4.g. (H&E ×16) Femur bone of rat in Calcitonin group showing wide zone of articular cartilaginous surface (a) with epiphyseal resorption.

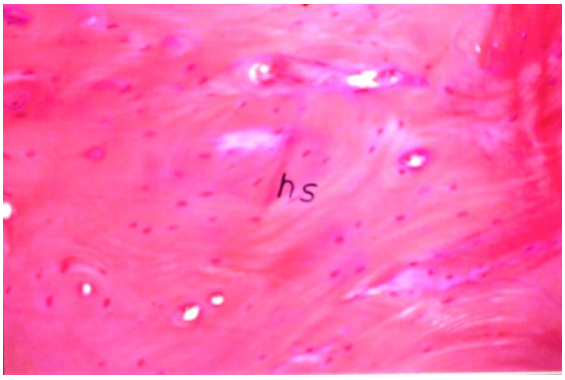

Fig. 4.h. (H&E ×40) Femur bone of rat in Calcitonin group showing normal haversian system in cortex of bone (hs).

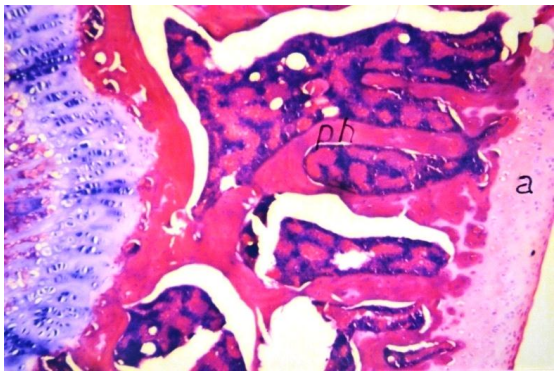

Fig. 4.i. (H&E ×16) Femur bone of rat in Clodronate +Vit.E group showing normal histological structure of articular cartilaginous surface (a), epiphyseal bone trabeculae (ph).

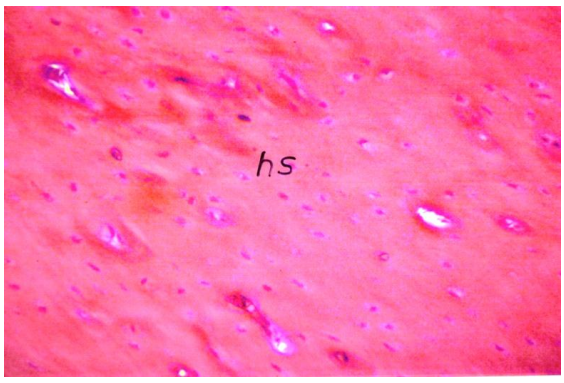

Fig. 4.j. (H&E ×40) Femur bone of rat in Clodronate +Vit.E group showing normal haversian system of the cortex.

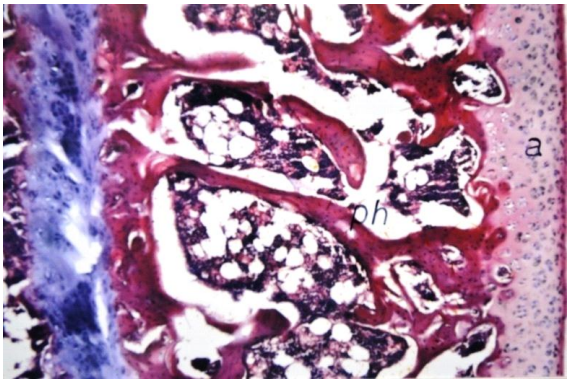

Fig. 4.k. (H&E ×16) Femur bone of rat in Calcitonin +Vit.E group showing resorption in epiphysis (ph).

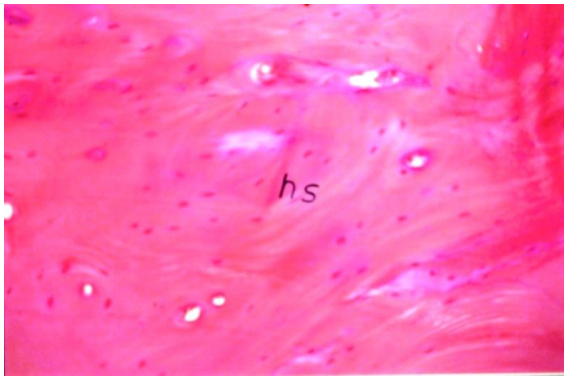

Fig. 4.l. (H&E ×40) Femur bone of rat in Calcitonin +Vit.E group showing normal haversian system in cortex of bone (hs)

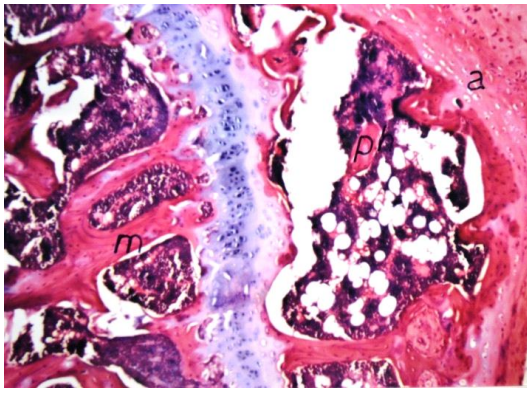

Fig. 4.m (H&E ×16) Femur bone of rat in Vitamin E group showing resorption in the bone trabeculae in epiphysis (p) with narrow cartilaginous articular surface (a).

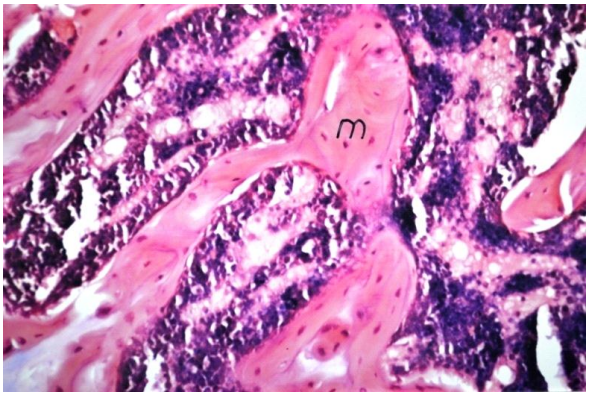

Fig. 4.n (H&E ×40) Femur bone of rat in Vitamin E group showing intact histological structure of the deep trabeculae of metaphysis (m).

Female irradiated rats showed a significant decrease in the time of pain perception at the end of this experiment. However, osteoporosis is usually accompanied by chronic pain (Hongo et al., 2015) due to upregulations in neuropeptides which could influence bone microstructures (Xiao et al., 2016). In 2006, Nagae et al. explained that acidosis is the main cause of pain in osteoporotic cases; they stated that bone minerals were degraded by osteoclasts secreting protons through the vacuolar H+-ATPase, creating acidic microenvironments causing hyperalgesia.

Clodronate was used in this study as a traditional, widely used treatment for osteoporosis, and despite the fact that it did not alter serum OC level in irradiated rats, it showed a significant decrease in bone resorption biomarkers: urinary Hpr and urinary Ca levels. In addition, histopathological findings reported normal bone outlook in femurs of the irradiated rats treated with clodronate. Clodronate has the capability of decreasing osteoclast metabolic activity by 90% and increasing calcium deposition by osteoblasts (Hayden et al., 2014). Moreover, treatment with clodronate normalized both MDA and TAC levels, however; bisphosphonates were able to alleviate oxidative stress by inhibiting microsomal lipid peroxidation activity due to iron chelating characteristics (Koçer et al., 2014).

Clodronate markedly increased the time needed for pain perception in ɣ-irradiated rats which is in accordance with (Rossini et al., 2015) who reported clodronate analgesic effect. A previous study suggested that bisphosphonates exert their long term analgesic effect probably due to osteoclast inhibition (Herrak et al., 2004).

Clodronate, compared to other bisphosphonates, blocks mevalonate pathway generating accumulation of pro-inflammatory cytokines (Frediani & Bertoldi, 2015). Furthermore, clodronate might have an inhibitory effect on the release of inflammatory cytokines (IL-1b, IL-6, TNF-α) and COX-2 activity (Liu et al., 2006).

However, combined treatment of vitamin E with clodronate normalized serum OC level. It is similar to the notion that vitamin E may synergize with clodronate (nitrogen free bisphosphonate) by antagonizing cytotoxic effect of clodronate which metabolized to ATP analogues (Dominguez et al., 2011).

Calcitonin is important in appreciating the totality of calcium homeostasis in the body. Two distinct but related roles for calcitonin, are firstly it protects the skeleton by regulating bone turnover, and secondly it maintains calcium homeostasis were known (Davey & Findlay, 2013).

The present finding of successfully counteracting changes of urinary Ca, Ph and Hpr in irradiated rats by calcitonin has been reported and confirmed by histopathological results which revealed that metaphysic and cortex compact bone were intact. Moreover, calcitonin treatment showed an increase in pain perception time of irradiated rats. A considerable amount of clinical evidence displayed a good analgesic effect of calcitonin, it was supposed that its antinociceptive action was exerted centrally not peripherally. These studies supposed that calcitonin could increase the release of an excitatory neurotransmitter by promoting calcium influx in the CNS (Barry et al., 2015).

Results indicating that calcitonin markedly increased the MDA level and maintained high TAC are supported by the study of Raddant & Russo (2014), who found that the superoxide anion production by osteoclasts cultured on bone was almost completely abolished by salmon calcitonin and indicated that, in adult rats, reactive oxygen species activated procalcitonin which is a precursor form of calcitonin that can act as a partial agonist at the calcitonin gene related peptide (CGRP) receptor by a paracrine regulatory mechanism.

Combined treatment of calcitonin with vitamin E showed resorption noticed only in the epiphyseal trabeculae. Vitamin E possibly by being antioxidant (Ahsan et al., 2014) potentiated the effect of calcitonin and clodronate to elevate serum osteocalcin.

Treatment of the irradiated rats with vitamin E exhibited an improvement in antioxidant defense system as well as a significant decrease in urinary Hpr level. Many studies showed the positive correlation between intakes of proper dose of α- tocopherol and bone health (Iwaniec et al., 2013). Studies showed that there are relationships between free radicals, inflammation, and bone loss which can lead to osteoporosis, and when vitamin E in the form of tocotrienols or α-tocopherol were supplemented to rats, IL-1 and IL-6 elevations were suppressed and osteoporotic changes were also inhibited (Norazlina et al., 2004; Nazrun et al., 2012). On the other hand, Mazière et al. (2010) demonstrated that oxidized LDL inhibits inorganic phosphate signaling responsible for mineralization of osteoblasts via generation of ROS and down regulates the expression of osteopontin (OPN) and receptor activator of nuclear factor-kappaB ligand (RANKL). It is well known that vitamin E has a powerful inhibitory effect on plasma LDL (Hwang et al., 2000).

On the other hand, although vitamin E significantly decreased urinary Hpr and Ca and significantly increased urinary Ph levels, all these parameters including serum OC level were significantly different from normal ranges. In addition, histopathological results showed that resorption was noticed in the bony trabeculae of the epiphysis associated with narrow articular cartilaginous surface. Although most studies revealed the beneficial effect of vitamin E (α-tocopherol) on bone density (Shi et al., 2016), other studies revealed that α- tocopherol in high doses carries a harmful effect on bone health (Guralp, 2014). The hypothesized mechanisms for the negative and null effects of alpha-tocopherols that vitamin E supplement suppress serum alpha and gamma-tocopherol levels have been previously reported (Zhao et al., 2015). In vivo studies have shown that serum tocopherols are able to produce nitric oxide (NO) which can uncouple bone resorption and formation, reducing bone resorption and stimulating bone formation. Furthermore, tocopherol metabolites may inhibit pro-inflammatory cytokines that induce osteoclast differentiation (Kim et al., 2012; Mah et al., 2015).

Conclusion

It could be concluded from this study the synergistic importance of vitamin E with anti-osteoporotic bisphosphonate and calcitonin in cancer patients receiving radiotherapy to stop bone mass deterioration after exposure to gamma radiation.

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تأثير الكلودرونات منفردا أو مدمجا مع فيتامين ه علي نقص المعادن المحدث بالتعرض الكلي للأشعاع الجامي في الجرذان.

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عادةً ما يتم اختيار البيسفوسفونات في بروتوكولات علاج السرطان من أجل السيطرة على مخاطر الكسور والحد من آلام العظام المنتشرة عقب العلاج الكيميائي والعلاج الإشعاعي. تهدف هذه الدراسة إلى دراسة التأثير المفيد للكلودرونات بمفرده أو بالاشتراك مع فيتامين (هـ) في الفئران المعرضة للأشعاع الجامي لثلاثة أيام بالتناوب في الأسبوع لمدة 5 أسابيع. أيضا تم دراسة الكالسيتونين بشكل فردي أوبالإشتراك مع فيتامين E من أجل التحقيق في تأثيره المحتمل على كتلة العظام في الفئران المشعة. أظهرت الفئران المشعة التي عولجت بالكلودرونات (5 مجم / كجم ،تحت الجلد، مرتين في الأسبوع) أو الكالسيتونين (10 وحدة دولية / كجم ، تحت الجلد، 48 ساعة) انخفاضات كبيرة في مستوي هيدروكسي برولين والكالسيوم وزيادة كبيرة في الفسفور في البول وارتفاع مستويات مضادات الأكسدة الكلية في الدم (TAC)، في حين أن الجمع مع فيتامين (ه) عزز معظم القياسات. يمكن أن نخلص إلى أن فيتامين (هـ) يمكن أن يتضافر مع تأثير الكلودرونات أو كالسيتونين في السيطرة على هشاشة العظام خاصة في الحالات المشعة.