The Effect of Omeprazole on Bone Mineral Density in Wistar Rats

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ABSTRACT

This study examined the association between use of Omeprazole and risk of osteoporosis in rat sample. For this study, 180 adult male Wistar rats were assigned to 3 groups with 60 rats in each group. Group I (a, b) (controls) was given intraperitoneal (i.p.) saline over the same durations as the test groups. Groups II and III (a, b) were given i.p. Omeprazole at 5 and 10 mg/kg/day, respectively, for 4 and 8 weeks. Ten rats from each subgroup were left without treatment for the following 4 weeks to detect any reversals in the effects of the drug. Intraperitoneal mode of delivery of drug is chosen for this study because intraperitoneal injection is suitable for smaller species like laboratory rodents for which intravenous access is challenging and it can be used to administer large volumes of fluid safely. Further, the bone mineral density and bone mineral content decreased in a dose and time dependent method, with recovery. The serum calcium and phosphate decreased in the 10 mg/kg for 8 weeks subgroup, with recovery of calcium, but not phosphate. The parathyroid hormone level increased, with no recovery. The Tartrate-Resistant Acid Phosphatase type 5b (TRAP5b) increased in the 5 and 10 mg for 8 weeks subgroups, with no recovery, and the Insulin-Like Growth Factor-1 (IGF-1) decreased in a dose and time dependent method, with recovery in only the 5 mg for 4 weeks subgroup. Diverse deteriorated histopathological changes were observed according to the dose and time of omeprazole treatment, which were more apparent in 10 mg for 8 weeks group with recovery in low dose. The use of high doses of omeprazole for 8 weeks durations could adversely affect bone homeostasis, and leads to decrease in Bone Mineral Density (BMD) resulting in the increased risk of subsequent fractures.

Keywords: Omeprazole, bone mineral density, osteoporosis, parathyroid hormone, calcium, phosphorus.

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1. Introduction

According to Sermet-Gaudelus et al. (2011), bone maintains its optimal structure, strength and mineralisation through a dynamic process that senses load, stress and repair areas of micro-damage. Further, bone remodelling cycle mediates this homeostatic process, in which old bone is replaced by newly formed one. The basic multicellular unit of bone remodelling is comprised of osteocytes, osteoclasts and osteoblasts (Clarke, 2008; Seeman and Delmas, 2006). Osteoporosis is a skeletal disease that approximately affects the entire skeleton, in which there is an imbalance between bone formation and bone resorption during the bone remodelling cycle. This imbalance results in bone micro-architectural deterioration and reduction of BMD (Milas-Ahic, Prus, Kardum, & Kovacevic, 2014). It is also observed that bone demineralisation disease does not become clinically apparent until a fracture occurs (Drake et al., 2015). Osteoporotic fractures account an immense public health problem among the elderly people worldwide (Cummings and Melton 2002). This problem may reduce the quality of life and raise the risk of morbidity and mortality (Johnell and Kanis 2006).

Some studies have pointed out a relationship between the long use of Proton Pump Inhibitors (PPIs) and elevated risk of osteoporosis-related fractures (Targownik, Lix, Leung, Leslie, 2010 & Ito and Jensen 2010). Niv, (2011) identified that PPIs are powerful drugs for gastric acid suppression, and are effective against acid-related diseases. They inhibit H^+/K^+ Adenosine Triphosphatase irreversibly in the gastric parietal cells, leading to effective gastric acid secretion suppression (Segawa, Nakazawa, Tsukamoto, Chujoh, Yamao, & Hase, 1987; Cui, Syversen, Zhao, Chen, &Waldum, 2001; & Shin, Vagin, Munson, Kidd, Modlin, & Sachs, 2008). These drugs show few adverse effects when administered correctly, so in clinical practice they are used for both acute symptoms and for long term purposes, although such indications are highly questionable (Yang, 2012). Despite outstanding efficacy and

negligible short-term adverse effects, increased interest is shown regarding the side effects of the chronic use of PPIs (Thomson, Sauve, Kassam, & Kamitakahara, 2010; Vestergaard, 2012 & Reimer, 2013). Omeprazole, which is one of the most important PPIs, in a dose of 20 mg/day is able to decrease BMD significantly as instigated by Yang, Lewis, Epstein, Metz, (2006).

However, studies conducted byYu et al., (2008) suggested that omeprazole diminishes bone resorption and blocks the progression to osteoporosis. So, the relation between the PPIs use and bone demineralisation and the increased risk of fractures associated with prolonged use of Omeprazole remains obscure (Targownik, Lix, Leung & Leslie, 2010). Due to the recurrent nature of acid-related diseases, many patients require long-term or lifelong therapy with PPIs (Scholten, 2007). Thus, the safety of this famous class of medications is of great public health concern. The widespread use of PPIs worldwide for almost two decades has gradually increased concerns from physicians and the public with regard to their benefits being compromised by a diversity of risks that, till now, have received little attention. The aim of the present work was to study the association between the use of Omeprazole with two different dosages and durations and BMD and Bone Mineral Content (BMC) together with bone turnover markers and bone histopathology.

2. Material and Methods

2.1. Test Drugs

This study used Omeprazole injections from a 40 mg vial (Losec; AstraZeneca UK Limited), ketamine-HCl and xylazine hydrochloride (Sigma-Aldrich, MO, USA).

2.2. Animals

For this study, 180 adult male Wister rates were selected each 10 weeks old. The subjects were weighed at 275±25 g (King Fahd Research Centre, KAU, Jeddah). The rats were housed in standard polypropylene cages and maintained in a 45 percent to 55 percent humid and controlled environment at 23±2 degree celsius with 5 rates per cage. Further, rats were exposed to a 12/12-hour modified dark-light cycle with transmission of light from 7:00 am to 7:00 pm. Moreover, before two weeks, the rats were adjusted to water and food conditions where in food was provided *ad libitum* (normal rodent diet contains 1.35 percent Calcium and 1.04 percent Phosphorus) (Smith et al., 2012). All the ethical considerations concerning the safety and harm issue related to laboratory animals were conducted in accordance with the ethically approved Umm al-Qura University Committee on the Ethics of Animal Experiments guidelines (HAPO-02-K-012-2015-05-117), which comply with the national, and international laws and policies in experimental field. It was also ensured that all the animals were treated efficiently and best possible measures were taken into account to reduce their sufferings.

2.3. Study Design

The rats were weighed and assigned to 3 groups of 60 rats per category. In group I (a, b), the controls were administered i.p. saline over the same durations as the test drug. Intraperitoneal mode of delivery of drug is chosen for this study because intraperitoneal injection is suitable for smaller species like laboratory rodents for which intravenous access is challenging and it can be used to administer large volumes of fluid safely. Group II (a, b) was given 5 mg/kg/day i.p. Omeprazole for 4 and 8 weeks respectively at 10 a.m. daily till the time of DXA. Group III (a, b) was given 10 mg/kg/day i.p. omeprazole for the same time periods (Segawa et al., 1987). At the end of the treatment, 20 rats from each subgroup (Ia, Ib, IIa, IIb, IIIa and IIIb) were weighed again and anaesthetised through the i.p. administration of

ketamine-HCl (80 mg/kg) and xylazine hydrochloride (10 mg/kg) (Kumar, Ramaswamy, & Nath Mallick, 2013) for the radiological analyses of the BMD and BMC in the head, leg, spine and the total. Blood samples were withdrawn from retroorbital venous plexus for biochemical analysis. It is essential to note that the subjects were sacrificed and both femurs from the body segments, the connective tissues and muscles were extracted and the samples were prepared for histological mounting. The remaining 10 rats from each subgroup were left without treatment for the next 4 weeks to detect the reversal of the effects of the drug (Figure 1).

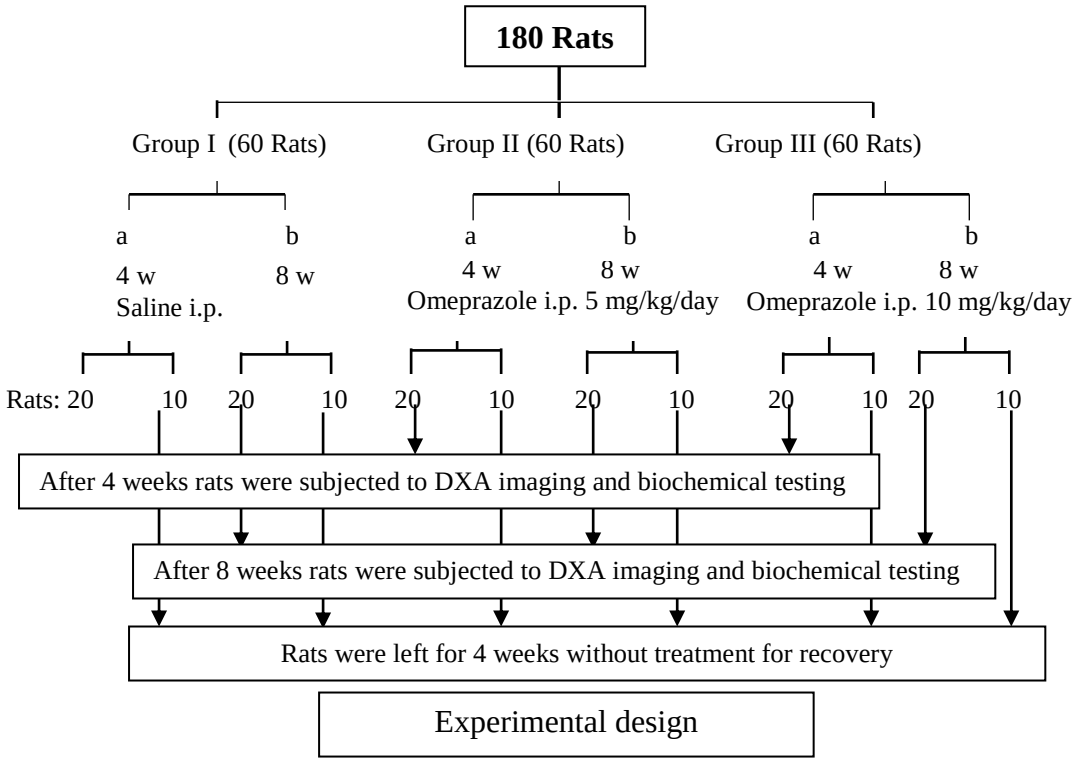

Figure 1: Experimental study design

2.4. The following parameters were measured

2.4.1. Bone Mineral Density (BMD) and Bone Mineral Content (BMC)

The BMD and BMC were measured in head, spine, legs and total through dual-energy x-ray absorptiometry (DXA) using a Hologic QDR-1000 (San Francisco, USA) adapted for the measurement of small animals (Katikaneni, Ponnapakkam, Miller, Ponnapakkam, Gensure, 2009). Later, the DXA software calculates the BMD automatically using the equation (BMC/bone area). DXA were performed by the same technician and further the anatomic regions typically scanned in the rat include the lumbar spine (L_1-L_4), total femur, and tibia (Griffin, Kimble, Hopfer, & Pacifici, 1993). Total femur was measured including all areas of interest the neck, trochanter and the diaphysis (Rosen et al., 1995). The anesthetised rat is placed on the densitometer, usually in ventral or dorsal recumbency, with the legs secured in place with tape. The hip, knee, and ankle typically are flexed at 90 (Rozenberg et al., 1995). The scan is performed and lasts about 7 minutes depending on the size of the rat.

2.4.2. Measurement of Bone Turnover Markers

Blood samples were drawn from the retroorbital venous plexus 24 hours after DXA measurements at 10 a.m. Samples were collected in vacutainer tubes and centrifuged for 15 min at 1500 x g. The resulting supernatant serum was aliquoted and stored at -80 ^{o}C until the time of analysis. The bone resorption marker, TRAP5b was measured using an ELISA test (Bone TRAP, SBA). In addition, to gain insight into the mechanisms behind the increase or decrease in the bone formation markers, the IGF-1 was also measured via ELISA Kits.

2.4.3. Measurement of serum calcium, phosphorus and parathyroid hormone

Serum Calcium and Phosphorus assays were performed by means of diagnostic kits supplied by ELISA. Parathyroid hormone was measured by immunoassay using ELISA kits supplied by Sigma-Aldrich (Mybiosource Inc., San Diego, California, USA).

2.5. Bone histopathological procedures

Bones are excised completely from the soft tissue and is placed in a sealed containers containing 10 percent phosphate buffered formalin solution (An YH 2003). Bone samples for mounting were prepared for histopathology according to the method mentioned in a study by Goldschlager, Abdelkader, Kerr, Boundy, Jenkin, 2001). The right femur from each rat was cut from distal ends, with measurements from 5 mm proximally to the articular surface of medial condyle. Later, the cut samples were mounted in the Neutral Buffered Formalin (NBF) solution for 48 hours, demineralised in 7.5 percent ethylene diaminetetraacetic acid (EDTA) in 0.1 mol/l cacodylate buffer over a period of 1 week, rinsed in buffer, and then stored in 70 percent ethanol. The specimen were dehydrated by a series of ethanol and acetone perfusions and later immersed in a solution of paraffin wax. For this mounting, 5 μm thick frontal sections were cut from femurs using a rotatory microtome (Leica pharmaceutical company, Germany) and then uploaded on glass slides. Specimen sections and samples were stained with hematoxylin and eosin (H&E) for routine histological analysis (Morrisett, Jope, Snead, 1987).

2.6. Statistical Analysis

The analysis of data and information collected were carried out by utilising the commercially available software program SPSS 18.0 (SPSS Inc.), and the results and findings were presented in the form of the mean ± standard deviation. One-way analysis of variance (ANOVA) test was used to evaluate the results. The difference was considered to be statistically significant when $p \leq 0.05$ was obtained.

3. Results

3.1. Body weight

None of the animals died during the experiment. By the end of 4 weeks and 8 weeks durations animals were weighed. The body weights of the rats increased significantly in the 8 week treatment groups, when compared to the 4 week treatment groups ($p<0.05$). Omeprazole 5mg/kg and 10 mg/kg administered daily did not change the body weight significantly compared to the corresponding control of untreated groups (Table 1).

Table 1: Effect of omeprazole treatment on rat body weight (g)

	Body weight day (1)	**Body weight (4 weeks) (g)**			**Body weight (8 weeks) (g)**		
		Control	**5mg/kg**	**10mg/kg**	**control**	**5mg/kg**	**10mg/kg**
Treatment	210±10.5	235±5.4	232±6.5	229±10	265±7.6	260±10.8 [d]	259±17.6 [d]
4w after Withdrawal of the drug	240±4.9	260±6.7	255±8.1 [b]	258±6.2 [b]	288±17.0	285±10.1 [d]	282±4.9 [b, d]

Data are presented as mean ±SD and analyzed using ANOVA. Significant level $p\leq0.05$ a: $p < 0.05$ compared to control. b: $p < 0.05$ compared to treatment group. c: $p < 0.05$ compared to 5 mg/kg dose. d: $p <0.05$ compared to 4 weeks duration

3.2. Effect of Omeprazole on the BMD and BMC

Table 2 shows the BMD and BMC results in the heads, legs and spines of the rats given 5 and 10 mg/kg/day of omeprazole for 4 and 8 weeks. The total BMD and BMC after 4 weeks of treatment were reduced significantly ($p< 0.05$) when compared to the controls for

both doses. After 4 weeks from withdrawal of omeprazole, the BMD and BMC in the 5 mg/kg group showed significant improvement when compared to the end of treatment ($p< 0.05$), but the 10 mg/kg group showed no significant changes in the BMD or BMC when compared to the end of treatment values. The coefficient of variation for BMD measurements was 1.5 percent and 2 percent at femur spine and head, respectively. The total BMD and BMC results after 8 weeks of treatment were significantly and dose-dependently decreased when compared to the controls ($p< 0.05$). Recovery was observed in the BMD and BMC for both doses when compared to the end of treatment values.

Table 2: Intra and inter-assay coefficients

Bone mineral density (g/ cm^2)	**Experimental groups**	**Control group**	**p-value**
Spine (L1-L4)	1.1.68±0.15	1.165±0.15	0.96
Femoral neck	1.029±0.17	1.061±0.17	0.49
trochanter	0.870±0.13	0.923±0.13	0.03
Total	1.051±0.15	1.111±0.15	0.04

Intra and inter-assay coefficients of variations of the measurements for BMD & BMC

Table 3: Effect of administration of omeprazole for 4 and 8 weeks on rat BMD & BMC

		Control untreated	**5 mg/kg/d**	**10 mg/kg/d**	**Control untreated**	**5 mg/kg/d**	**10 mg/kg/d**
site			**BMD (g/cm^2)**			**BMC (g)**	
Head	**4w** treatment	0.24± 0.01	0.23 ± 0.01 [a]	0.22 ± 0.01 [a]	2.3 ±0.14	2.05 ±0.25 [a]	1.91 ±0.17 [a]
	4w after Withdrawal	**0.25±0.01**	0.24 ± 0.03 [b]	0.24± 0.13 [b]	2.3±0.2	2.23 ± 0.16 [b]	2.16 ± 0.2 [a,b]

	8w Treatment	0.25± 0.03	0.22 ± 0.01[a,d]	0.20 ± 0.01[a,d]	2.4 ±0.17	2.04 ± 0.49 [a]	1.84 ± 0.20 [a]
	4w after Withdrawal	**0.26±0.02**	0.24 ± 0.01	0.24±0.01	2.4±0.2	2.14 ± 0.32	2.05 ±0.30 [a]
Femur	**4w** Treatment	0.13±0.009	0.13±.0.05	0.12 ± 0.01	1.86±0.11	1.51± 0.23 [a]	1.50 ± 0.46 [a]
	4w after Withdrawal	**0.14±0.01**	0.13 ±0.002	0.13 ±0.07	1.9±0.2	1.70 ±0.37	1.65±0.20
	8w Treatment	0.14±0.02	0.12 ± 0.01 [a]	0.12 ± 0.005 [a]	1.88±0.12	1.50 ± 0.52 [a]	1.55 ± 0.21 [a]
	4w after Withdrawal	**0.15±0.01**	0.13 ± 0.007 [b]	0.13 ± 0.009 [b]	2.01±0.1	1.60± 0.33	1.65± 0.41
Spine	**4w** Treatment	0.15±0.015	0.13 ±0.013 [a]	0.12±0.007 [a, c]	1.77±0.34	1.24 ±0.13 [a]	1.31 ±0.31 [a]
	4w after Withdrawal	**0.15±0.02**	0.14 ±0.001 [b]	0.13±0.008[a,b,c]	1.80±0.3	1.50 ±0.10 [a, b]	1.31 ±0.19 [a, c]
	8w Treatment	0.16±0.015	0.12± 0.02 [a]	0.11 ± 0.012 [a]	1.79±0. 4	1.22 ± 0.28 [a]	1.20 ± 0.19 [a]
	4w after Withdrawal	**0.16±0.01**	0.13 ± 0.15 [a]	0.12 ± 0.01[a,c]	1.82±0.1	1.14 ± 0.38 [a]	1.13+ 0.33 [a]
Total	**4w** Treatment	0.15±0.007	0.14 ±0.04 [a]	0.14 ±0.007 [a]	9.17±1.16	7.24 ±0.66 [a]	7.53 ±1.15 [a]
	4w after Withdrawal	**0.15±0.01**	0.15 ± 0.00 [b]	0.14 ±0.004 [a, c]	9.2±2.1	8.02 ± 0.52	8.23 ±0.58 [a, c]
	8w	0.16±0.007	0.14 ±	0.13± 0.008 [a,	9.19±1.13	7.10 ±	7.07 ±

Treatment		0.01 [a]	[c]	2.26 [a]	0.95 [a]
4w after Withdrawal	**0.17±0.001**	0.15 ± 0.06 [a,b]	0.14±0.009 [a, b]	9.2±3.2	8.22 ± 1.62 [a,b]	8.25 ±0.3 [a, b]

BMD: Bone mass density; BMC: Bone mass content; Data are presented as mean ±SD and analyzed using ANOVA. Significant level p≤0.05 a: p< 0.05 compared to control. b: p< 0.05 compared to treatment group. c: p< 0.05 compared to 5 mg/kg dose. d: p<0.05 compared to 4 weeks duration

3.3. Effects of Omeprazole on the Serum Concentrations of Calcium, Phosphate and PTH

The administration of Omeprazole at 5 and 10 mg/kg for 4 weeks did not affect the serum Calcium (Ca^{+}), Phosphate (PO4) or Parathyroid Hormone (PTH) levels when compared to the controls. After 8 weeks of treatment with Omeprazole at 10 mg/kg, the serum concentrations of the Ca^{+} and PO4 were lower than those of the controls ($p< 0.05$). The Ca^{+} level was restored after the discontinuation of therapy, but the PO4 level was not restored. On the other hand, after 8 weeks of 5 and 10 mg/kg of Omeprazole treatment, the serum PTH level was significantly increased when compared to the controls and to 4 weeks treatment ($p< 0.05$), but the PTH level after the discontinuation of therapy did not recover (Table 3).

3.4. Effect of Omeprazole on Bone Markers

The administration of 5 and 10 mg/kg of Omeprazole for 8 weeks significantly increased the TRAP5b when compared to the controls and 4 weeks treatment ($p<0.05$), with no recovery. The 5 and 10 mg/kg dosages of Omeprazole for 4 and 8 weeks reduced the IGF-1 when compared to the controls ($p< 0.05$), but after the discontinuation of the 5 mg/kg of omeprazole for 4 weeks, the IGF-1 was recovered ($p<0.05$) (Table 3).

Table 4: Effect of administration of omeprazole for 4 and 8 weeks on biochemical parameters

			4weeks		8 weeks	
		Control	**5 mg/kg/d**	**10 mg/kg/d**	**5 mg/kg/d**	**10 mg/kg/d**
Calcium (mg/dl)	Treatment	9.23 ±0.73	9.17 ±0.34	9.26 ±0.42 [d]	8.92 ±0.83	8.40 ±0.32 [a]
	4w after Withdrawal	**9.3 ±0.60**	9.25 ±0.37	9.26 ±0.64	9.60 ±1.47	9.34 ±0.79 [b]
PO4 (mg/dl)	Treatment	7.017 ±0.76	6.97 ±0.59	6.72 ±0.97	5.95 ±0.95 [c]	5.88 ±0.54 [a]
	4w after Withdrawal	**6.9 ±0.65**	6.81 ±1.09	6.33 ±0.99	6.33 ±0.99	5.97 ±1.11
PTH (pg/ml)	Treatment	15.27 ±0.21	15.2 ±0.03	15.19 ±0.34	17.08 ±0.58 [a, d]	18.91 ±0.57 [a , d]
	4w after Withdrawal	**14.95 ±0.3**	15.32 ±0.26	15.2 ±0.01	17.01 ±0.58	18.53 ±0.60
1GF-1 (ng/ml)	Treatment	233.07 ±19.35	191.80 ±33.14 [a]	162.46 ±6.10 [a, c]	173.55 ±8.86 [a]	143.86 ±26.52 [a, c]
	4w after Withdra	**230.9 ±20.5**	229.53 ±12.6 [b]	185.33± 26.92 [a]	180.33 ±6.9 [a]	156.53 ±14.85 [a]

	wal					
TRAP5b (U/L)	Treatment	0.57± 0.04	0.54 ±0.04	0.57± 0. 01	0.61 ±0.01 [a, d]	0.74 ±0.20 [a, d]
	4w after Withdra wal	**0.65± 0.06**	0.50 ±0.02	0.54 ±0.04	0.60 ±0.02	0.60 ±0.84

PO4: Phosphate; PTH: Parathyroid hormone; 1GF-1: Insulin-like growth factor 1; TRAP5b: Tartrate resistant acid phosphatase 5b; Data are presented as mean ±SD and analyzed using ANOVA. Significant level p≤0.05 a: p< 0.05 compared to control. b: p< 0.05 compared to treatment group. c: p< 0.05 compared to 5 mg/kg dose. d: <0.05 compared to 4 weeks duration

2.5. Histopathological results

Examination of bone sections from control groups revealed its usual appearance, which is formed of outer compact bone (CB) and inner spongy bone (SP). The CB showed normal sized Haversian canals and regular arrangement of concentric lamellae and interstitial lamellae. Osteocytes with their darkly stained nuclei inside their lacunae were seen between different lamellae. Moreover, the covering periosteum showed subperiosteal bone deposition appearing as a distinct basophilic line. The endosteal surface was lined with osteoblastic cells and osteoclasts in their Howship's lacunae. The inner spongy bone consisted of branching and anastomosing thick bony trabeculae separated by interconnecting spaces containing bone marrow. These trabeculae consisted of irregular and basophilic bone lamellae and osteocytes within their lacunae in between the lamellae (Figure 2).

Examination of bone sections from different omeprazole treated groups (5 mg/kg/day/4w group, 10 mg/kg/day/4w group, 5 mg/kg/day/8w group, 10 mg/kg/day/8w

group), diverse deteriorated histopathological changes were observed according to the dose and time of omeprazole treatment, which were more apparent in the groups of more dose and longer time of treatment. In general, thinning of the CB with presence of many cavities, tunnels and lightly stained areas were seen. The Haversian systems appeared irregular and shrunken with some Haversian canals were dilated. Many osteocytes appeared darkly stained and also, some empty lacunae were observed. In several sections, there was hypertrophy of the periosteum especially in its fibrogenic layer. Moreover, the endosteum had irregular surface with multiple notches and increase in number of osteoclasts. The trabeculae of the inner spongy bone appeared thin and discontinuous with loss of their normal architecture and widening of the interconnecting bone marrow spaces (Figures 3 and 4). Examination of bone sections from different recovery groups (5 mg/kg/day/4w/Recovery, 10 mg/kg/day/4w/Recovery, 5mg/kg/day/8w/Recovery, 10mg/kg/day/8w/Recovery) presented varied results where there was nearly complete to partial recovery. Almost complete recovery took place in 5 mg/kg/day/4w/Recovery and 5mg/kg/day/8w/Recovery groups while the other two groups showed an incomplete recovery. In general, there was a relative increase in the thickness of the CB. The outer and inner bone surfaces appeared more or less regular. The bone matrix presented some deeply stained areas indicating bone repair. The Haversian systems with regular bone lamellae were seen. The inner spongy restored its normal appearance which was comparable to that of the control group (Figures 5 & 6).

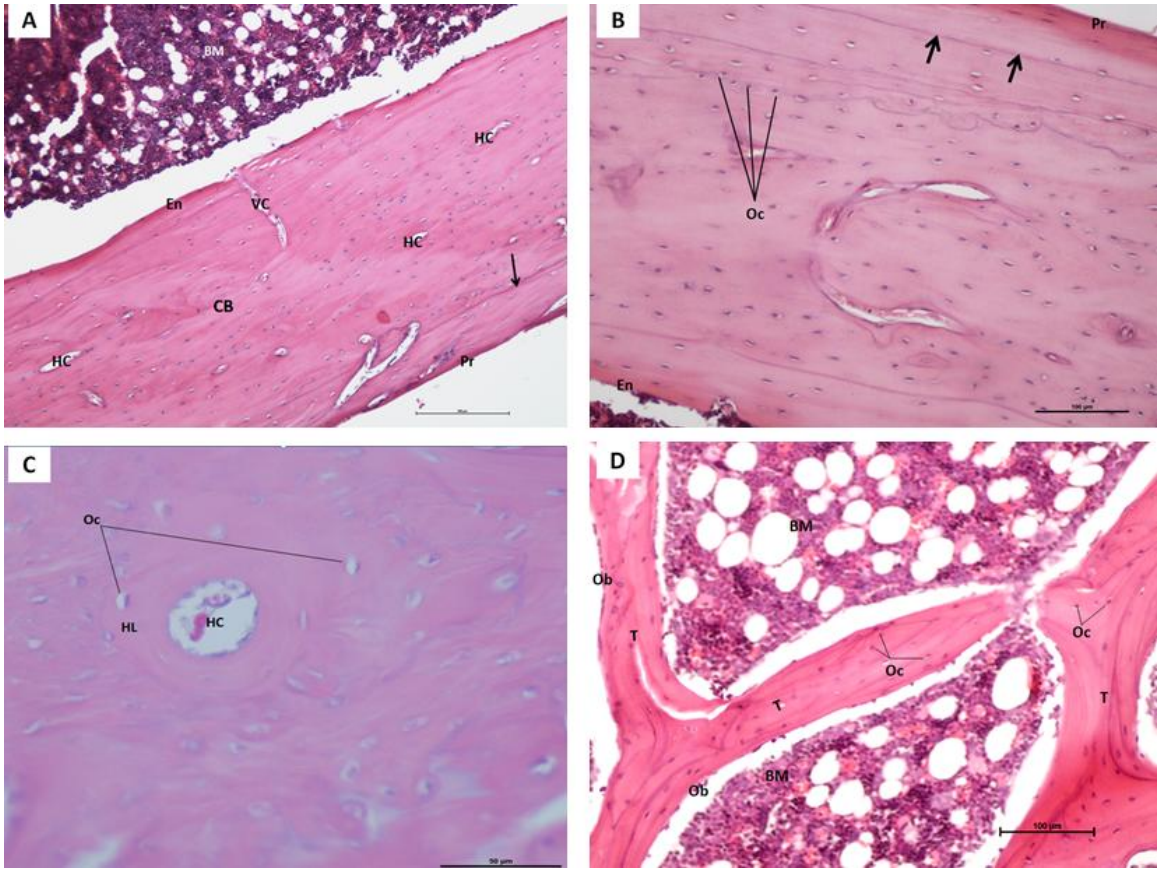

Figure 2: A photomicrographs of different bone sections (A, B, C, D) from rats of control group showing outer compact bone (CB) with normal appearance. The bone is covered from outside by the periosteum (Pr) and from inside by smooth endosteal surface (En). Many lacunae containing osteocytes (Oc) were shown in between the bone lamellae. Notice subperiosteal bone deposition appearing as distinct basophilic line (arrow). The compact bone tissue is well organised, showing Haversian lamellae (HL) arranged around the Haversian canals (HC). Also, the inner spongy bone was formed of branching and anatomizing thick trabeculae (T) with bone marrow spaces (BM) in between. These trabeculae were lined with cuboidal osteoblasts (Ob). VC: Volkmann's canals. (A: X100, B & D: X 200, C: X 400).

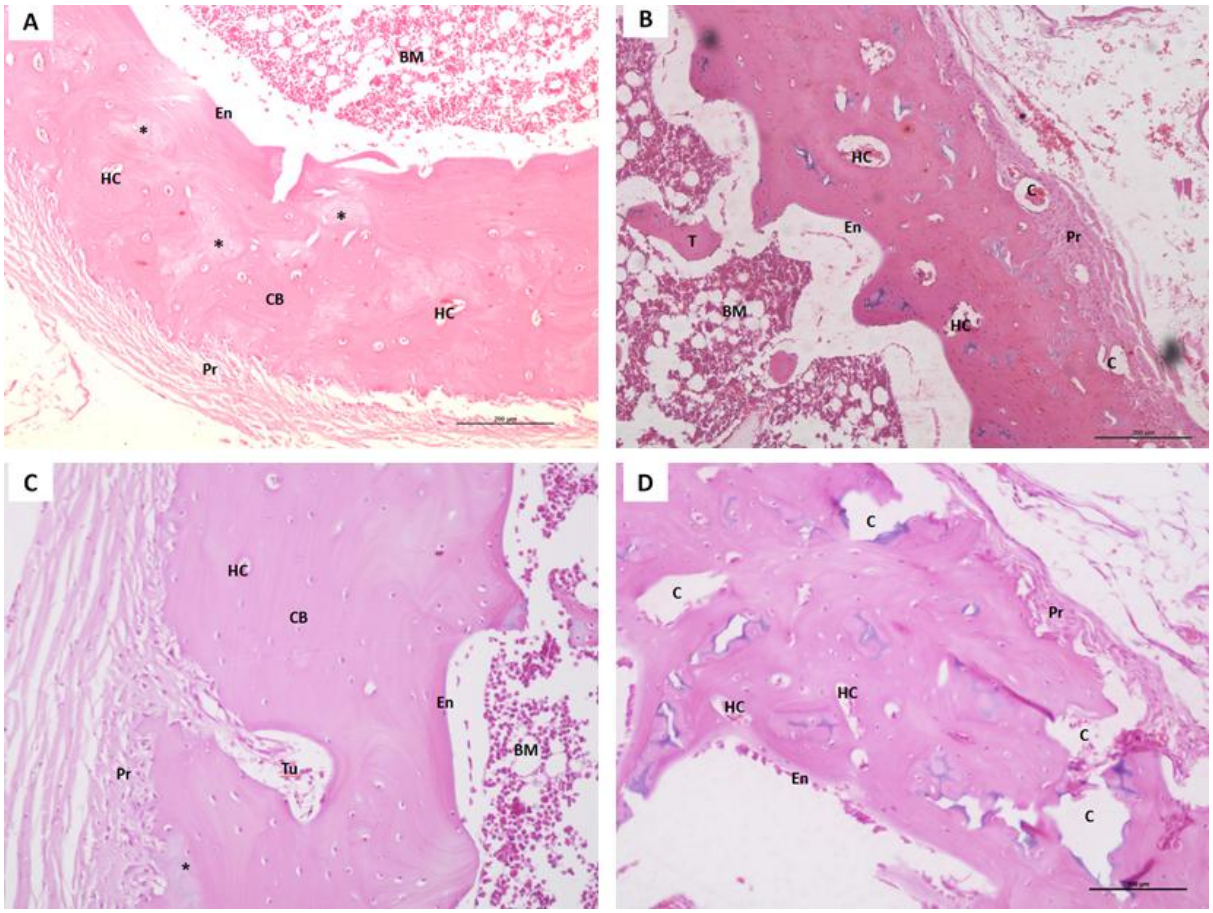

Figure 3: A photomicrographs of different bone sections from omeprazole treated groups (A: 5 mg/kg/daily/4w group, B: 10 mg/kg/daily/4w group, C: 5 mg/kg/daily/8w group, D: 10 mg/kg/daily/8w group) showing thinning of the compact bone (CB) with absence of subperiosteal bone deposition, many cavities (C), tunnel formation (Tu) and scattered pale areas (*). The periosteum (Pr) displays different degrees of thickness and the endosteal surface (En) is irregular with many eroded areas. (A & B: X100, C & D: X 200).

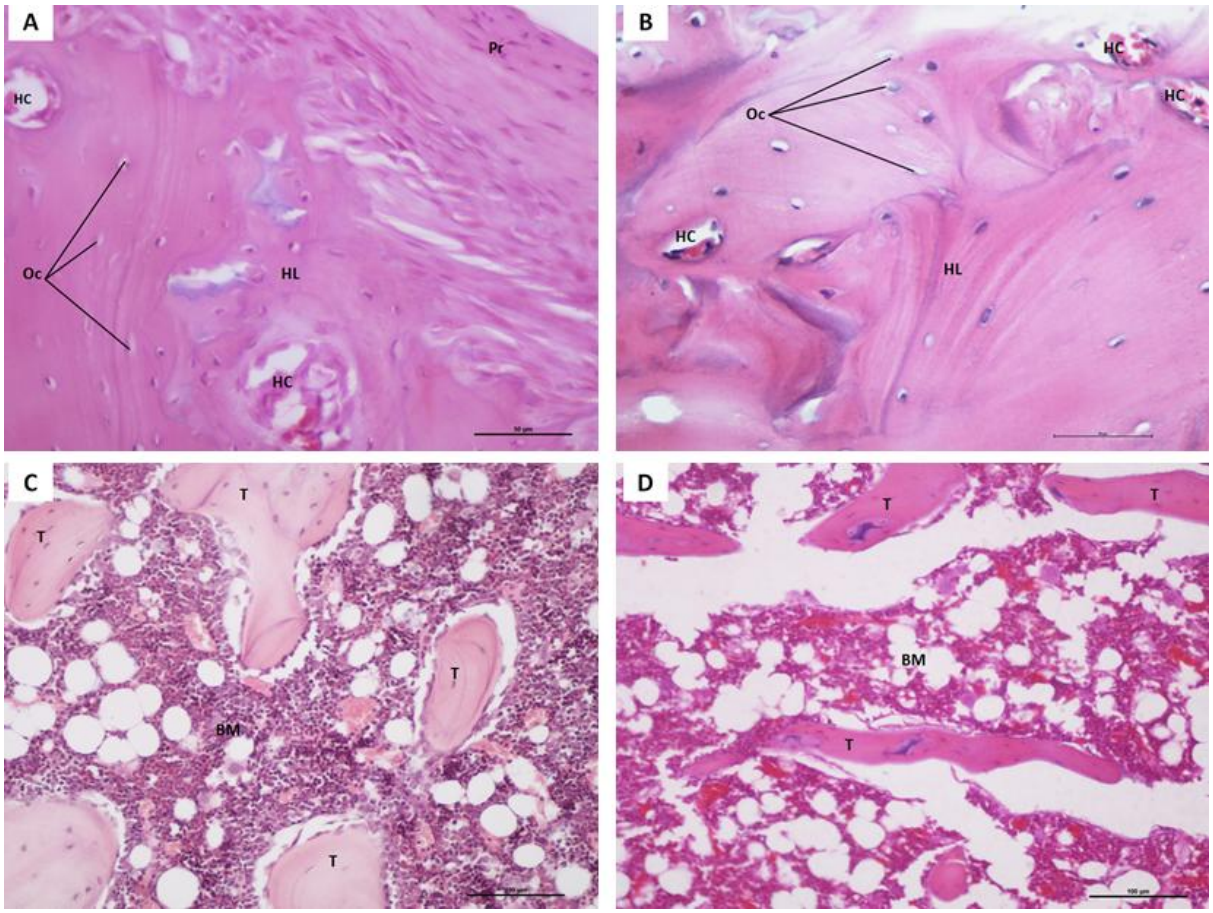

Figure 4: A photomicrographs of different bone sections from omeprazole treated groups (A & C: 5 mg/kg/daily/8w group, B & D: 10 mg/kg/daily/8w group) showing Haversian systems with irregular lamellae (HL), dilated Haversian canals (HC) and little number of osteocytes (Oc) inside empty lacunae in A and B. Also, notice that number, size, and density of trabeculae (T) decreased with the appearance of widely separated bony spicules. BM= bone marrow, especially in D. (A & B: X 400, C & D: X 200).

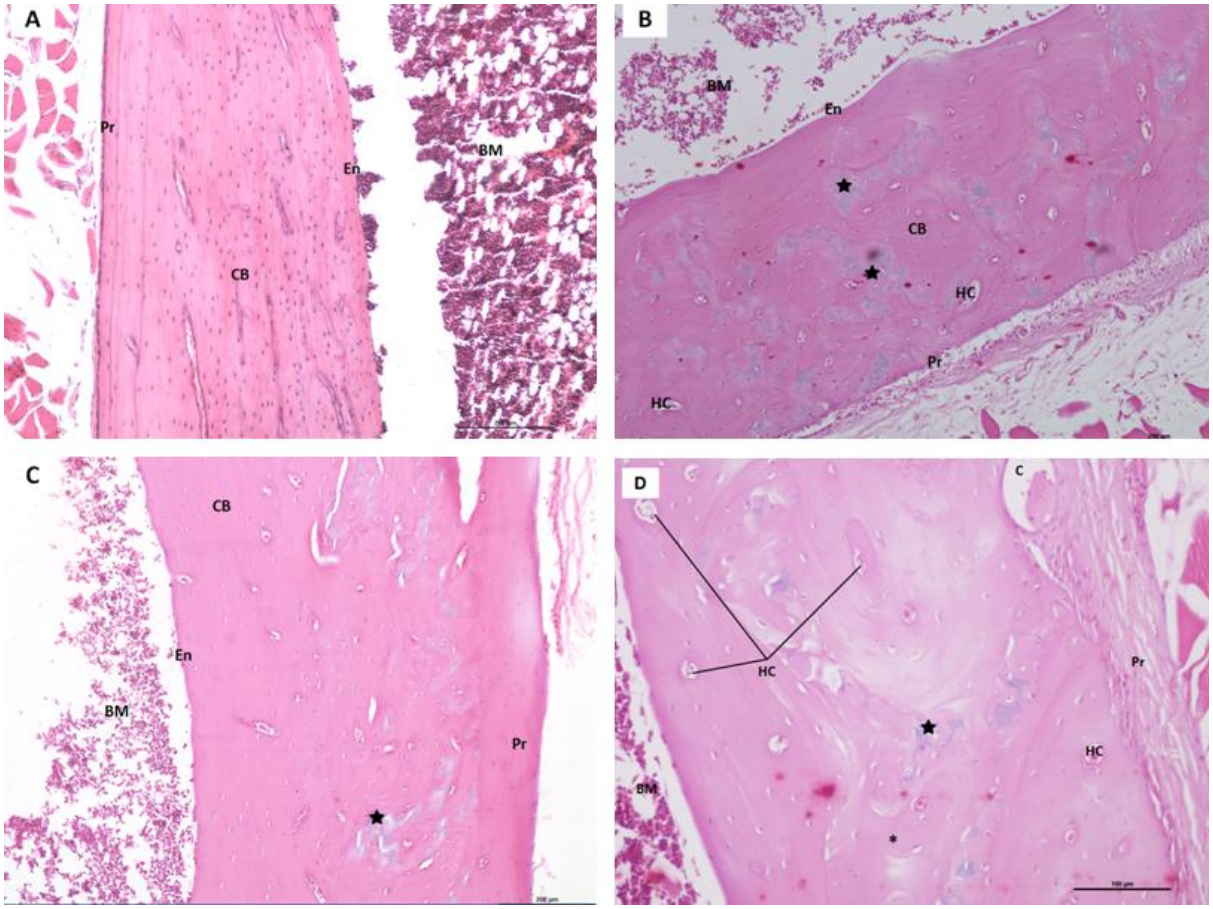

Figure 5: A photomicrographs of different bone sections from different recovery groups after omeprazole treatment (A: 5 mg/kg/daily/4w/ recovery group, B: 10 mg/kg/daily/4w/ recovery group, C: 5 mg/kg/daily/8w/recovery group, D: 10 mg/kg/daily/8w/ recover group) showing nearly normal structure with increased thickness of the outer compact bone (CB) to be comparable with the control and the appearance of subperiosteal distinct basophilic line and the presence of many basophilic areas (★) in A & B. Notice the existence of some cavities and hypertrophied periosteum in C & D. BM= bone marrow, En = endosteum, HC = Haversian canal, Oc = osteocyte (A & B: X100, C & D: X 200).

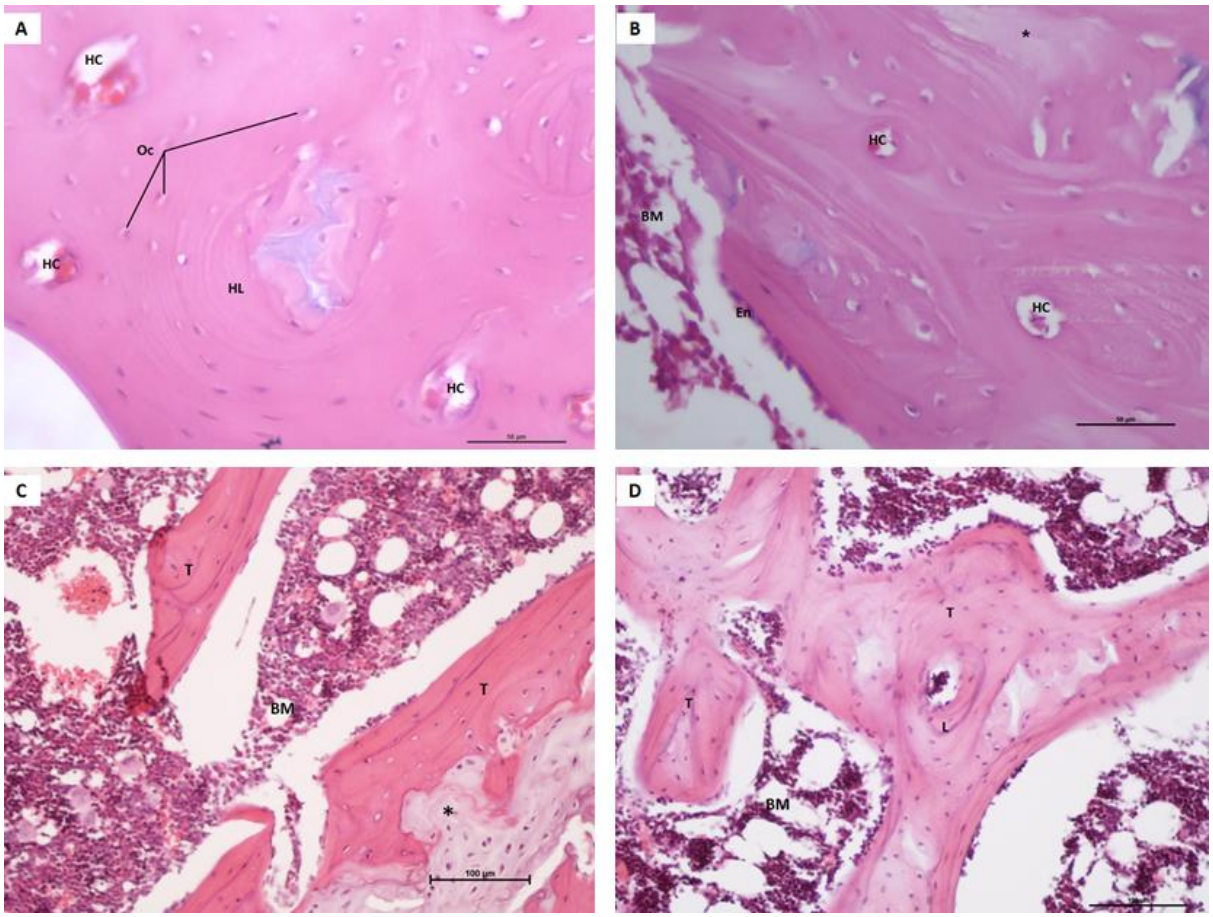

Figure 5: A photomicrographs of different bone sections from different recovery groups after omeprazole treatment (A: 5 mg/kg/daily/4w/ recovery group, B& D: 10 mg/kg/daily/8w/ recovery group, C: 5 mg/kg/daily/8w/ recovery group) showing some irregular bone lamellae around apparently normal Haversian canals (HC). Notice the presence of distinct deep basophilic areas in the core of the thickened trabeculae (T) of spongy bones with less apparent widening in the interconnecting bone marrow spaces (BM). Oc = osteocytes (A & B: X400, C & D: X 200).

4. Discussion

According to the results, the acid-suppressive drugs are widely used for the treatment of stomach acid-related disorders (Ali, Roberts & Tierney, 2009), but despite the outstanding efficacy and negligible short-term adverse effects, increased concerns have been raised with

regard to the side effects of chronic PPI use (Thomson et al., 2010; Vestergaard, 2012; and Reimer, 2013). In the present study, the BMD and BMC were decreased in a dose and time-dependent manner, with recovery after stopping the use of Omeprazole. The Ca^{+} and PO4 were decreased with higher dose and longer duration, with recovery of the Ca^{+} levels, but not the phosphate levels, after stopping Omeprazole. However, the PTH is increased when compared to the controls, with no recovery. The long-term effects of intake of Omeprazole (30mg/kg × 8weeks) are observed on bone turnover in a recent study by Al Subaie et al., (2016) in an experimental rat model with analysis of the signalling pathway involved in osteoclast differentiation, bone resorption, and alteration of trabecular bone microstructure. In our study, the mean PTH levels were reported as 15.27 for controls in relevance to 15.2±0.03 & 15.19±0.34 and 17.08±0.58 & 18.91±0.57 at 4 and 8 weeks, respectively. According to the findings, PTH levels were reported to be higher in 8 weeks group compared to 4 weeks group, with comparable low Ca^{+} levels (8.92±0.83 & 8.40±0.32 for 5 & 10 mg/Kg/day PPIs dose). Coinciding with these results, Assiri, Borham, Awad, Al-Maraghy, (2003); Yang, (2008); Yu et al., (2008) found that the use of PPIs, especially at high dosages and prolong time, was related to an increased risk of fracture owing to lower Ca^{+} and elevated PTH levels.

In addition, a case-controlled study performed by Chiu, Huang, Chang, Yang, (2010) and the results of Cai, Feng, and Jiang, (2015) showed that PPIs were linked to an increased fracture risk, especially hip fractures, in a dose-dependent way. Similar to our findings Krause et al., (2015) instigated, that the use of standard doses of PPIs for 3 months is associated with significant changes in serum markers of Ca^{+} homeostasis or bone turnover. Similarly, our results suggested that PO4 levels were reported at 6.97±0.59 & 6.72±0.97 at 4 weeks and 5.95±0.95 & 5.88±0.54 at 8 weeks interval signifying that the levels were decreased with the increasing dose and time of use of Omeprazole; however, the levels were

not retrieved on withdrawal of the drug (6.81±1.09 & 6.33±0.99 at 4 weeks; and 6.33±0.99 & 5.97±1.11 at 8 weeks). Similar results were obtained in a study by Malluche, Davenport, Cantor and Monier-Faugere, (2014), the lower intestinal phosphate absorption were observed in patients with higher TRAP5b level and it has been previously shown that intestinal calcium absorption was significantly decreased in patients treated with PPIs when compared with healthy controls. It is imperative that hypergastrinemia has been shown to have a stimulatory effect on the parathyroid glands. For example, experimental hypergastrinemia produced by antral exclusion in rats led to hyperparathyroidism and an increase in volume and weight of the parathyroid gland, due to parenchymal cells hyperplasia, resulting in increased bone resorption (Grimelius, Johansson, Lundqvist, Olazabal, Polak, Pearse, 1977). The mechanism that links acid-suppressive medication with the risk of fractures remains largely unknown (Cea-Soriano, Johansson, Garcia & Rodriguez 2013), although the effects of PPIs on calcium absorption and metabolism have recently taken much concentration.

The intake of omeprazole has been reported to inhibit the absorption of calcium and reduce the BMD in rat models (Chonan, Takahashi, Yasui, Watanuki, 1998). In addition (Corley, Kubo, Zhao, & Quesenberry, 2010) demonstrated that the use of PPIs has been correlated with calcium malabsorption and the loss of BMD, resulting in an increased risk of fractures. Hypochlorhydria or achlorhydria in humans caused by some conditions, including pernicious anaemia, gastrectomy and atrophic gastritis, are combined with an increased incidence of osteoporosis and bone fractures; it is supposed that this is secondary to the effects of the low gastric acid levels on calcium absorption (Yu et al., 2008; Lodato; Sipponen and Harkonen, 2010). Some preclinical and clinical studies have shown that gastric acid secretion can enhance the absorption of calcium and that the acidic medium in the stomach promote the production of ionised calcium from insoluble calcium salts. Calcium solubilisation is suggested to be crucial for calcium absorption. At the same time, gastric acid

suppression by a PPI can indirectly cause hypergastrinemia by suppressing the release of somatostatin, and may cause the malabsorption of calcium. Both hypergastrinemia and calcium malabsorption can negatively influence bone and mineral metabolism, at least in part, through the induction of hyperparathyroidism, thus leading to a reduction in bone mineral density (Insogna, 2009; Yang 2008 & 2012).

In the present study, the TRAP5b was increased in 0.54 ±0.04 & 0.57±0.01 after 4 weeks and 0.61±0.01 & 0.74±0.20 at 8 weeks, respectively. It is also observed that at 8 weeks significant increase was reported both at 5 and 10 mg/kg/day with no recovery after 4 weeks of withdrawal. From our findings, TRAP 5b showed a significant negative correlation with BMD and could be useful as a marker in the serum for bone resorptive activity in pathological conditions like osteoporosis (Halleen, Tiitinen, Ylipahkala, Fagerlund, Vaananen, 2006; Oddie et al., 2000). The results predicted that when Omeprazole was used for 8 weeks, the TRAP5b showed highest increment with no significant recovery after withdrawal, hence, this suggests that TRAP5b is time dependent and chronic use of Omeprazole may lead to significant rise in TRAP5b. It is interesting to note that TRAP5b is significantly correlated with markers of resorption as well as with PTH and serve as a marker of bone resorption in the assessment cases of osteodystrophy. With regard to the IGF-1, it was decreased in this study in a dose and time dependent manner, with recovery only for the low dose and short duration (4 weeks: 229.53±12.6 at 5 mg/kg/day & 185.33±26.9 at 10 mg/kg/day compared to 8 weeks: 180.33±6.9 at 5 mg/kg/day & 156.53±14.85 at 10 mg/kg/day).

The findings suggest the level of IGF-1 decreased when the dose of Omeprazole increased from 5 mg/kg/day to 8 mg/kg/day; and on withdrawal levels recovered in only 4 weeks group. This is an indication that IGF-1 is dependent on the chronic use of Omeprazole with depletion in concentration on long-term PPIs use. In a study by Courtland et al., (2013),

it is indicated that osteoclasts may react to the secreted IGF-1 from osteoblasts, which indicates that this molecule acts also as a coupling factor for the remodelling unit. In addition, IGF-1 is essential for normal osteoclast differentiation (Guntur and Rosen, 2013). Our results were supported by Maggio et al., (2014) that inferred that the use of PPIs shows an independent and negative association with IGF-1 levels. Moreover, acid-suppressive medications have been reported to affect bone remodelling, and account for the increased risk of fractures (Mizunashi, Furukawa, Katano, & Abe, 1993), while one multicentre cohort study with 9,423 participants suggested a modest risk of fracture in PPI users (Fraser, Leslie, Targownik, Papaioannou, Adachi, 2013). However, the studies assessing the association between acid inhibition and bone mineral density have reported different conclusions. For example, some studies found that a mildly reduced bone density was related to PPI use, but there was no significant change in the levels of bone mineral density between the PPI users and the controls in other studies (Yu et al., 2008). The authors further signified that there is a higher incidence of hip fractures in patients treated with high doses of PPIs, and the risk shows a progressive increase with the duration of the PPI treatment (Yang et al., 2006). Experimentally, evidence has pointed out that PPIs may inhibit osteoclastic proton transport system and affect bone resorption, which may alleviate the negative effect of the PPIs on osteoporosis by decreasing calcium absorption (Yang et al., 2006; Wright, Proctor, Insogna, Kerstetter, 2008; and Sheraly, Lickorish, Sarraf, & Davies, 2009).

The mechanism of this action focuses on bone turnover cells especially osteoclasts, as there is a difference between proton pumps in gastric parietal cells (H^+/K^+ ATPase) and osteoclasts (vacuolar H^+ ATPase) Jefferies, Cipriano, Forgac, 2008). However, omeprazole has been shown to block both H^+/K^+ ATPase and vacuolar H^+ ATPase (Xu et al., 2007). It is imperative to note that acid secretion through the H^+ ATPase in osteoclasts is important for bone resorption. Further, the hydrogen ions secreted cause decalcification of bone, and

activation of the proteolytic enzymes which cause bone matrix degradation. By blocking an important step in bone resorption, there should be an increase in the bone mineral density, leading to prevention or reduction of osteoporosis, and hence a decreased risk of bone fractures. However, PPIs block the vacuolar H^+ ATPase and inhibit the activity of osteoclasts (Yu et al., 2008). This effect, together with their effect on calcium homeostasis, mentioned previously, supports the assumption that PPIs can result in a condition resembling osteopetrorickets, as reported by Schinke et al., (2009). Diverse deteriorated histopathological changes were observed according to dose and time of omeprazole treatment, which were more apparent in the groups of increased dose and longer duration of treatment. There was nearly complete recovery in the low dose groups for both durations, while the high dose groups showed an incomplete recovery.

Our data suggest that high-dose of Omeprazole appears to primarily affect biomechanical strength parameters in metabolically active trabecular bone, owing to thinning of CB with absence of subperiosteal bone deposition and cavity formation in 10 mg/kg/daily/8w group. Further, this is likely to be due to degenerated bone quality, according to our histological data that indicate an increased content of Haversian systems with irregular and dilated Haversian canals (HC) in Omeprazole-treated animals versus controls. It is imperative to note that presence of cavities and hypertrophied periosteum denotes trabeculae containing relatively more cartilage, and consequently less mineralised bone and lose in bone strength. The microscopic examination of sections belonging to 10 mg/kg/daily/4w/ recovery group compared with sections from the control group show the presence of obvious changes and sometimes common ones but of different degrees, depending on the dose of drug administered and time duration. The changes occurred at both the epithelial level (increased thickness, different thicknesses, and appearance of subperiosteal distinct basophilic line) as well as decreased density of trabeculae with the appearance of widely separated bony

spicules. In our study, the histological findings reveal that demineralisation of bone occurs gradually; depending on the time elapsed since the start of Omeprazole administration. These subsequent changes appeared in 5 mg/kg/daily group and 10 mg/kg/daily group after about 4 weeks of treatment and continued until the 8th week. In the control group, there was no significant increase in tissue.

Additionally, results showed that BMC values 9.17±1.16 for control group; 7.24 ±0.66 for 4 weeks group and 7.53 ±1.15 for 8 weeks group has been reported in this study. These findings signified that BMC of 8.02 ± 0.52 & 8.23 ±0.58 for 4 weeks and 8 weeks, respectively, is due to higher serum PTH levels and poses an important threat to bone mass of the femur spine. In a study by Rhee et al., (2011), the excess PTH is reported to have anabolic effects on trabecular bone. Moreover, our study suggested that studies have demonstrated an increase in BMD in the femur head was reported as 0.15 ± 0.00 & 0.14 ±0.004 indicating that BMD loss in the femur in chronic use of PPIs has higher PTH levels. These findings were supported by studies by Sikjaer, Rejnmark, Rolighed, Heickendorff & Mosekilde; Siilin et al.; and Pallan & Khan, (2011) by depicting that hyperparathyroidism affects both cortical and trabecular bone leading to sclerotic thickening with increased PTH and simultaneously stimulating resorption in cortical bone with reductions in BMD.

Some limitations were found in the present study, where the experiment was done on adult male rats only. So, there were no data to point out if these results were related to gender. In addition, mechanical properties, bone strength, bone macrometric parameters (bone length, bone diameter of femur and tibia) were not measured. Further, duration of PPI therapy was a major limitation of our study, with PPI use for a shorter period of time (8 weeks). Significant duration of time is a need to observe delayed recovery effects of Omeprazole withdrawal in experimental samples.

5. Conclusion

The results of this study have demonstrated that the daily administration of Omeprazole in rats was associated with decreased in the BMD and BMC in a dose and time dependent manner, with recovery after stopping the use of the drug. These results were accompanied with decrease in the serum Ca^{+} and PO4 concentrations, and an increase in the PTH level. In addition, the TRAP5b was increased, with a decrease in the IGF-1 peptide. Deteriorated histopathological changes were observed according to the dose and time of omeprazole treatment, which were more apparent in the groups of increased dose and longer time of treatment with recovery in low dose groups and with shorter time. These results support the studies confirming that that chronic treatment of PPIs decreases the BMD and could increase the risk of hip, spinal and radial weakness and fractures in femur. Clinicians should keep in mind the risk of fracture when balancing the safety and efficacy of these medications.

Conflict of Interest

Layla Borham, Magda Hagras, Altaf Abdulkhaliq, Mohammed Badawood and Gamal Abd El Aziz declare that they have no conflict of interest.

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Statement of Animal Rights

The procedures involving the animals and their care were conducted in accordance with the ethically approved Umm al-Qura University Committee on the Ethics of Animal Experiments guidelines (HAPO-02-K-012-2015-05-117), which comply with the national and international laws and policies. Every effort was made to minimise the number of animals that were used, and their suffering.

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