Evaluation of the Effect of Omega 3 Fatty Acid (N-3) on Socket Healing in Orchiectomized Rats

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Abstract: Although osteoporosis has been long considered a disease of post-menopausal women, it is now increasingly being recognized among the growing population of elderly men as a source of substantial morbidity and even mortality in the aging male. It is firmly established that androgen withdrawal induced by orchiectomy (ORX) results in decreased bone mass in animal models especially in rodents that may be associated with an increase of bone resorption and fracture risk. The current research was aimed to determine the effect of fish oil supplementation on socket healing and bone structure and composition in orchiectomized rat model. Thirty male albino rats were randomized into two groups: sham-operated (Control) group (n=10) or bilaterally orchiectomized (ORX) group (n=20). The ORX groups were divided equally among the following treatment: orchiectomy and orchiectomy + 10% fish oil (omega 3). Four weeks after surgery, the right 1st molars were extracted. Twelfth weeks after surgery, rats were sacrificed and right mandibular bone was evaluated by light, scanning electron microscope and elemental analysis. Light microscopic examination of group II revealed osteoporosis that was evident as wide intercommunicating marrow spaces and many discontinuous trabeculae with an isolated trabecula were observed. Also, irregular, resorped outer cortical surface with many osteoclasts in their Howship's lacunae were observed. However, almost restoration of bone microarchitecture was observed in group III rats (Omega 3). SEM of group II showed significant morphological changes (pore formation, fissures, disintegrated bone architecture, reduced compactness and exposure of collagen fibers). However, in Group III sockets there were significant restoration of bone morphology. The X-ray microanalysis of Group II revealed a decrease in calcium ratio and an increase in phosphorous ratio as compared to Group I. However, Group III that received omega 3 showed higher calcium level and lower phosphorus level when compared to Group II. It is concluded that fish oil supplementation has a positive effect on socket healing and bone structure and composition in orchiectomized rats.
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1. Introduction

Osteoporosis is a systemic skeletal disorder characterized by increased bone fragility associated with decreased bone mass and deterioration of bone quality (microarchitecture and intrinsic material properties) **(Poole and Compston, 2006; Kanis, 2008).** It is considered a public health issue threatening a large portion of the population above 50 years of age **(Watts, 2004)**. Often presenting as a silent disease, it generally occurs asymptomatically and the afflicted individuals will be only diagnosed after the occurrence of some kind of fracture **(Boonen *et al.*, 2004).**

Osteoporosis is generally thought of as a "women's disease" because the prevalence of osteoporosis and the rate of fractures are much higher in postmenopausal women than in older men. However, according to the National Institutes of Health's Osteoporosis and Related Bone Diseases Resource Center, approximately 10 million people are affected by osteoporosis each year, and 20% of these are men **(National Institute of Health, 2006).**

Age and hypogonadism are both considered major risk factors for osteoporosis **(Kahn *et al.*, 2007).** In males with hypogonadism (whether induced by orchiectomy, androgen deprivation therapy (ADT), hyperparathyroidism, or other causes), both testosterone and estrogen levels fall, shifting the balance of bone turnover toward resorption **(Perez *et al.*, 2006; Higano, 2008).** ADT is increasingly being prescribed both for men with locally advanced or high-risk nonmetastatic prostate cancer and for those with recurrent disease **(Meng *et al.*, 2002; Sharifi *et al.*, 2005).** With this increased exposure to ADT, clinicians have seen the emergence of longer-term treatment complications, including osteoporosis and osteopenia. Prostate cancer itself is associated with bone resorption and osteoporosis, even among ADT-naïve patients without metastatic disease **(Boyle *et al.*, 2003).** There is also other risk factors for osteoporosis, including lifestyle (smoking) and dietary factors (Excessive intake of alcohol or caffeine, inadequate dietary calcium intake, weight), diseases associated with bone loss (Chronic obstructive pulmonary disease, malabsorption syndrome, hyperparathyroidism, hyperthyroidism,

rheumatoid arthritis, renal insufficiency, vitamin D deficiency). In addition to ADT there are treatments associated with bone loss including anticonvulsants, heparin, systemic glucocorticoids (duration > 3 months) **(Brown and Josse, 2002; Greenspan, 2008).**

On the molecular level, studies demonstrated that the conditional ablation of androgen receptors in mice caused bone loss that was associated with increased osteoblast expression of RANKL, with significant increase in its levels in bone marrow plasma and cells following orchiectomy **(Li *et al.*, 2009).** Androgen effects on bone may be also indirectly mediated by regulation of cytokines and growth factors expressed locally in bone. Androgens upregulate transforming growth factor (TGF)β and insulin-like growth factors (IGFs), which stimulate bone formation **(Kasperk *et al.*, 1997)**, and down regulate interleukin (IL)-6, which stimulates osteoclastogenesis **(Pilbeam *et al.*,1990).** Androgens inhibit parathyroid hormone (PTH)- or IL-1-induced prostaglandin (PG)E2 production **(Pilbeam *et al.*, 1990).** Androgens stimulate IL-1β production and enhance the mitogenic effect of fibroblast growth factor (FGF) in cultured osteoblasts **(Pivirotto *et al.*, 1995)**.

Long chain polyunsaturated fatty acids (FAs) and their metabolites, best known for their role in regulating bone metabolism **(Lavie *et al.*, 2009)** and may potentially play a role in the prevention of osteoporosis. Several epidemiological studies have shown a lower incidence of bone fractures in Mediterranean countries **(Kannus *et al.*, 1996)** and have suggested an association between Mediterranean diet, which is rich in omega-3 FA (n 3)and a lower rate of bone loss in older adults **(Puel *et al.*, 2007).** Fish oil (FO) is rich in omega-3 FA and has been shown to have beneficial effects on bone **(Poulsen *et al.*, 2007)** and to prevent bone loss in ovariectomized rodents **(Fernandes *et al.*, 2003; Sun *et al.*, 2003).** It has been proposed that omega-3 FAs could prevent age-related bone loss by inhibiting osteoclastogenesis while improving osteoblast differentiation and function **(Coetzee *et al.*, 2007).** Moreover, omega-3 FAs enhance calcium absorption by modifying the lipid composition of the intestinal cell membrane and decreasing intestinal calcium loss **(Kruger *et al.*, 2005).**

The healing events in the tooth extraction socket culminate in the formation of woven bone, which ultimately remodels, thus resulting in the restoration of the defect **(Devlin and Sloan, 2002).** Formation of blood clot is a fundamental step for sockets repair **(Yugoshi *et al.*, 2002).** The fibrin network formation provides a matrix to support lymphocytes, macrophages and neutrophils migration, characterizing the acute inflammatory response. Granulation tissues containing fibroblasts, inflammatory cells and newly formed capillaries were found at the bottom of the extraction wound at day 2 after extraction **(Kanyama *et al.*,2003; Hosokawa *et al.*, 2010).** Bone resorption is an important process in bone crests in the beginning of healing, while bone deposition is observed in the sockets **(Kurihashi *et al.*, 2002).** The periosteum outside the extraction wound began thickening at day 1 after tooth extraction, and some new bone formation by osteoblasts was observed at day 3 (**Hosokawa *et al.*, 2010)**. Mesenchymal cells that differentiate into osteoblast originated from remained periodontal ligament and medullar bone **(Devlin and Sloan, 2002)**. Finally, socket healing events result in a thick bone trabecular network containing small medullary spaces at 21 days **(Teo´ filo *et al.*, 2001; Devlin and Sloan, 2002).**

The present study was designed to test the effect of fish oil supplementation on healing of tooth extraction sockets in orchiectomized rats. Specifically, socket healing and bone structure and composition was evaluated by light, scanning electron microscope and elemental analysis.

2. Material and Methods:

2.1 Animals and experimental design:

Thirty Wister male albino rats (20 weeks old, 150–200 g) were obtained from Physiology Department, Alexandria University. The rats were housed under controlled laboratory conditions (room temperature 23 ± 2 °C, relative humidity 60 ± 5%, with light–dark cycle of 12 h each). After one week of acclimatization, the animals were divided into two groups: a control (Sham) group (n = 10) and ORX groups (n = 20). Rats were anaesthetized with sodium pentobarbitone (35 mg/kg, *i.p.*), and bilateral orchiectomy were done aseptically. Sham operation was done in the same manner but only exposing the testis. The animals were given prophylactic ampicillin (4000 IU/kg, i.p.) for 3 days and coloplast paste (Humlebaek, Denmark) applied locally. The ORX rats were randomly distributed equally amongst two groups and assigned to one of the following treatments: orchiectomy (ORX) (group II) and orchiectomy + 10% fish oil (omega 3) (group III). One percent corn oil was added to the fish oil to prevent essential fatty acid (EFA) deficiency. Fresh diet was prepared daily. Four weeks after the surgery the right 1st molars were extracted. Twelfth week after the surgery rats were sacrificed and right halves of mandibular bone were carefully dissected for light microscopic examination, electron microscopic examination as well as elemental analysis at Faculty of Science, Alexandria University. The experiment

was carried out in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publication Number 85-23, Rev. 1985). The protocol was examined and approved by the research ethics committee, Faculty of Pharmacy, Cairo University, Egypt.

2.2 Tissue preparation for light microscopic examination:

Bone was fixed in 10% formol-buffer for 24 hrs, decalcified in EDTA (15%) for about 20 days, dehydrated in alcohol, cleared in xylol and embedded in paraffin wax (56–58° C mp). Sections (4-6 µm) were cut with rotary microtome (Weswox Optik, India), stained with haematoxylin–eosin, and observed under light microscope.

2.3 Tissue preparation for scanning electron microscopic examination and x-ray microanalysis:

Bone was fixed (2.5% glutaraldehyde for 72 hrs and post-fixation in 1% OsO_4 for 24 hrs), then dehydrated in graded ethanol, dried, mounted on stubs and coated with gold using a sputter coater. The processed bone was then analysed at 25 kV accelerating voltage by scanning electron microscope. The calcium to phosphorous ratio was determined by using x-ray analysis system attached to SEM with the aid of a software system.

2.4 Statistical analysis of the results

Data were collected, coded and analyzed using SPSS software under windows VISTA, descriptive analysis were applied followed by inferential statistics, using ANOVA and Paired t-test. Level of significance of 0.05 was considered to control for alpha error.

3. Results

3.1. Evaluation of the weight:

Evaluation of the weight at the start of the study revealed that there was no statistical significant difference between the three groups regarding the weight at the baseline. In all orchiectomy animals, the increase in body weight was lower by about 10% during the first two months following orchiectomy compared to sham operated animals and remained decreased throughout the 3-months period post-orchiectomy (Table 1). Analysis of data shows that there were statistical significant difference in weight after 2 months in each group ($P<0.01$). There was no evidence of appetite suppression by any of the three groups.

3.2. Histological results:

The mandibular socket histology of Group I rats (sham) showed normal bone microarchitecture, as compared with Group II (Fig. 1). Light microscopic examination of Group II sockets revealed osteoporosis that was evident as wide intercommunicating marrow spaces and many thin fanned woven bone in the top and base of the socket were observed (Fig. 2). Also, irregular, resorped outer cortical surface with many osteoclasts in their Howship's lacunae were observed (Fig. 2). However, almost restoration of bone microarchitecture was observed in Group III mandibular sockets (Omega 3). There were relatively thick woven bone at the top of the socket, thick lamellar bone in the base of the socket and several reversal lines (Fig. 3).

3.3. Results of x-rays Microanalysis:

The elemental composition of calcium, phosphorus and calcium to phosphorus ratio (Ca/P) is recorded. Analysis of variance (ANOVA) was used to compare Ca and P levels as well as Ca/P ratio in the selected mandibular areas among the three groups (Table 1)

The representive spectra of selected mandibular areas in Group I rats revealed normal calcium, phosphorus and calcium to phosphorus ratio (Fig. 4-A). The representive spectra of selected mandibular areas in Group II (ORX) rats revealed a decrease in calcium ratio and an increase in phosphorous ratio as compared to Group I (sham operated) rats ($P <0.01$). The Ca/P ratio was also found to be higher in Group I rats when compared to Group II rats ($P <0.01$) (Fig. 4-B). Elemental microanalysis of Group III that received omega 3 showed higher calcium level and lower phosphorus level when compared to Group II. The Ca/P ratio was less than control group but higher than ORX group (Fig. 4-C).

3.4. Scanning Electron Microscopic results:

Scanning electron microscopy of mandibular bone of the Group I (sham rats) showed normal compact and cancellous bone architecture as compared with Group II rats (Fig. 5). In group II there were significant morphological changes (pore formation, fissures, disintegrated bone architecture, reduced compactness and exposure of collagen fibers) were observed (Fig. 6). However, in Group III rats there were significant restoration of bone morphology as reduced pore formation, improved bone architecture and increased compactness, as compared with Group II (Fig. 7).

Table 1: Effects of orchiectomy on rat weight and elemental composition of mandibular bone in the three groups.

Variables	Group I	Group II	Group III	P-Value
Weight/ gm.	168±15.84	170±16.15	167±14.57	>0.05 (NS)
Weight after 2 months	214±14.1	190.5±16.9	191.5 ±16.3	<0.01 **
Ca (elemental %)	68.4±1.26	42.75±1.2	59.1±0.81	<0.01 **
P (elemental %)	30.16±0.24	58.84±0.77	39.43±0.98	<0.01**
Ca/ P ratio	2.26±0.053	0.77±0.028	1.5±0.48	<0.01 **

All values are expressed as mean ± SD, *(n=10)*, P<0.01 ** as compared to sham operated animals.

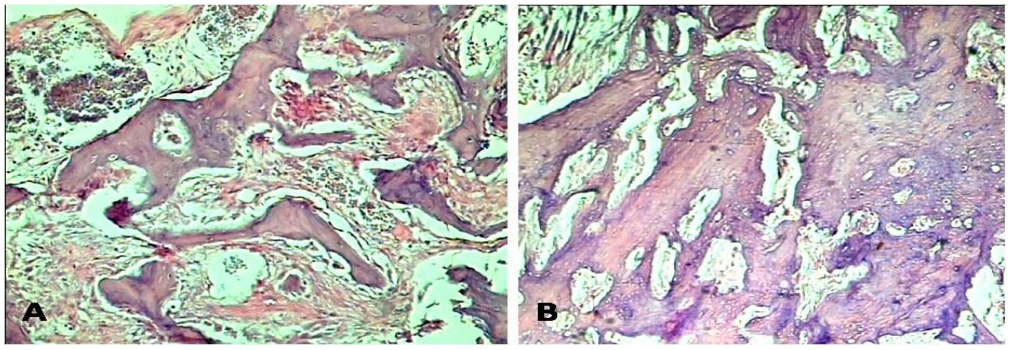

Fig. 1. H&E stained section of group I showing 1st molar socket healing. **A**: Normal healing with trabeculae of lamellar bone (X100). **B**: Thick bone trabeculae at the side and base of the socket separated by thin bone marrow (X200).

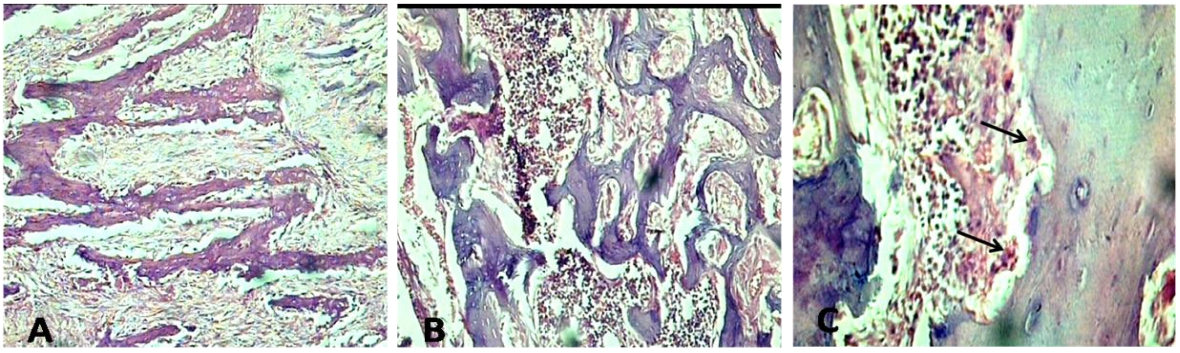

Fig. 2. H&E stained section of group II socket. **A**: Very thin fanned woven bone at the top of the socket (X 200). **B**: Woven bone at the base of the socket (X 200). **C**: Thick lamellar bone at the socket's wall with osteoclasts in their Howships' lacunae (arrows) (X 400).

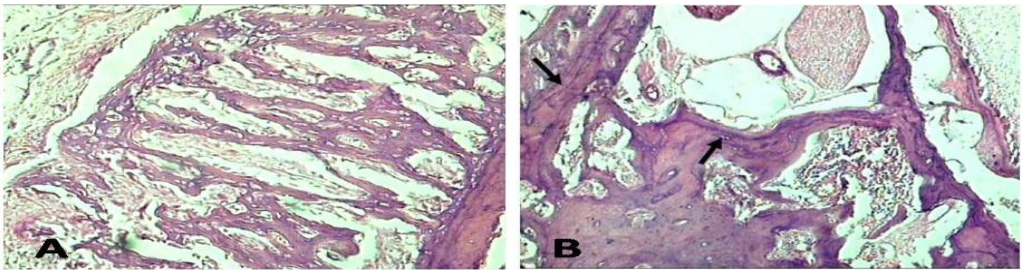

Fig. 3. H&E stained section of group III socket. **A**: Relatively thick woven bone at the top of the socket (X 200). **B**: Almost normal healing of the socket with thick bone trabeculae and thin bone marrow spaces at the sides and the base of the socket with several reversal lines (arrows) (X 200).

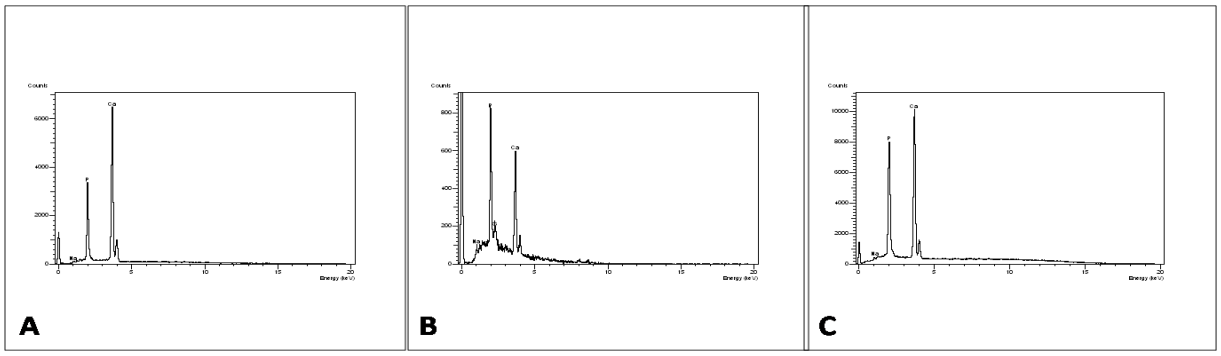

Fig.4. Representive spectra of elemental composition of selected mandibular areas. **A:** Group I showed Ca level of (69.5%), Phosphorus level of (30%) with Ca/P ratio of 2.31. **B:** Group II (ORX) showed declined Ca level of (43%) and higher Phosphorus level of (54.5%) with greatly lower Ca/P ratio of 0.78 than control group. **C:** Group III (omega 3) showed Ca level of (59.6%), Phosphorus level of (39.8%) with Ca/P ratio of 1.49 that was lesser than control group but higher than group II.

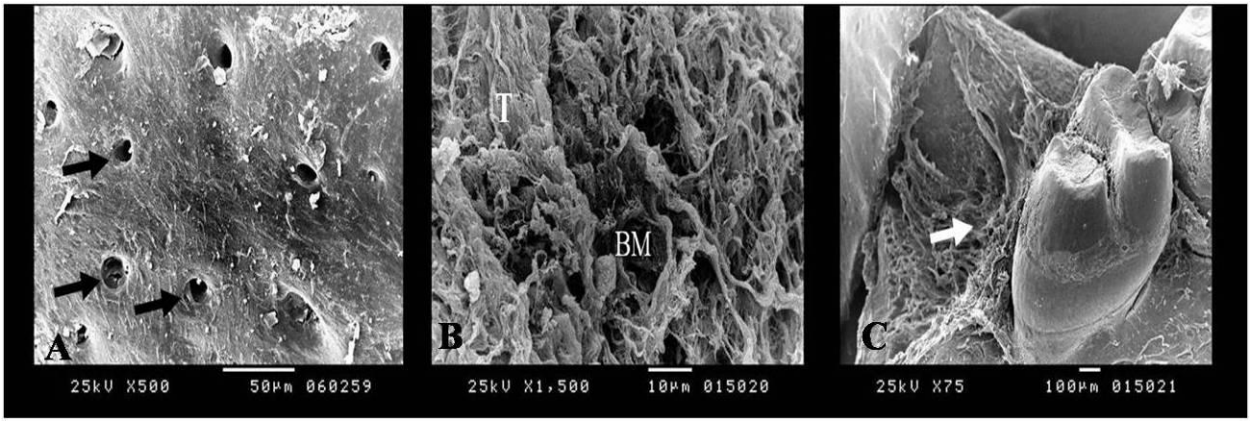

Fig. 5. SEM photograph of mandibular bone of group I showing in **A:** normal smooth surface of compact bone with openings of neurovascular canals (arrows).
B: Cancellous bone of group I with large and small marrow spaces (BM) separated by thick bone trabeculae (T).
C: Lamellar bone of socket healing of 1st molar (arrow)

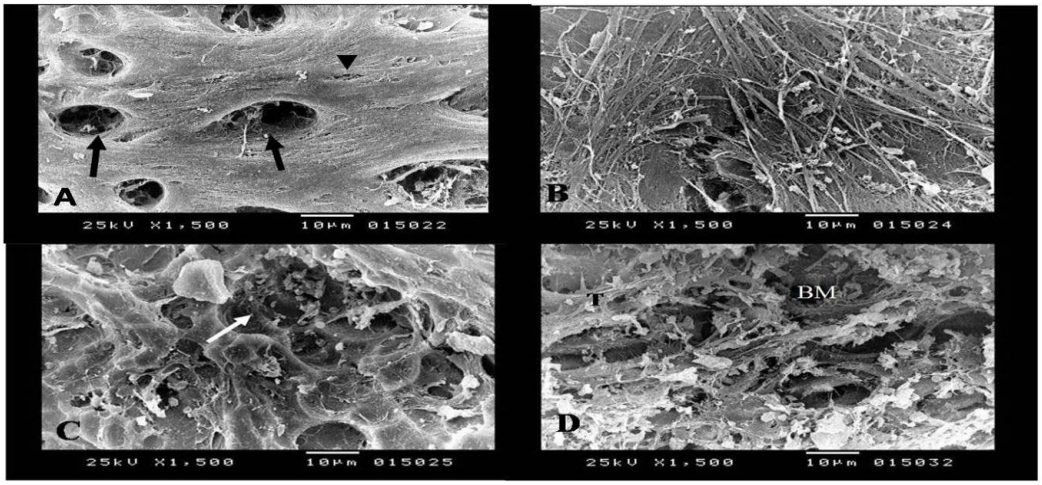

Fig.6. SEM photograph of mandibular bone of group II. **A:** Compact bone with smooth surface and wide openings of neurovascular canals (arrows). Some fissures in between (arrow head) can be seen. **B:** Compact bone showing obvious rough and disintegrated bone surface associated with exposure of some collagen fibers and bundles. **C:** Compact bone with rough surface and coalesce neurovascular openings (Arrow). **D:** Cancellous bone showing intercomunicating large marrow spaces (BM) separated by thin bone trabeculae (T).

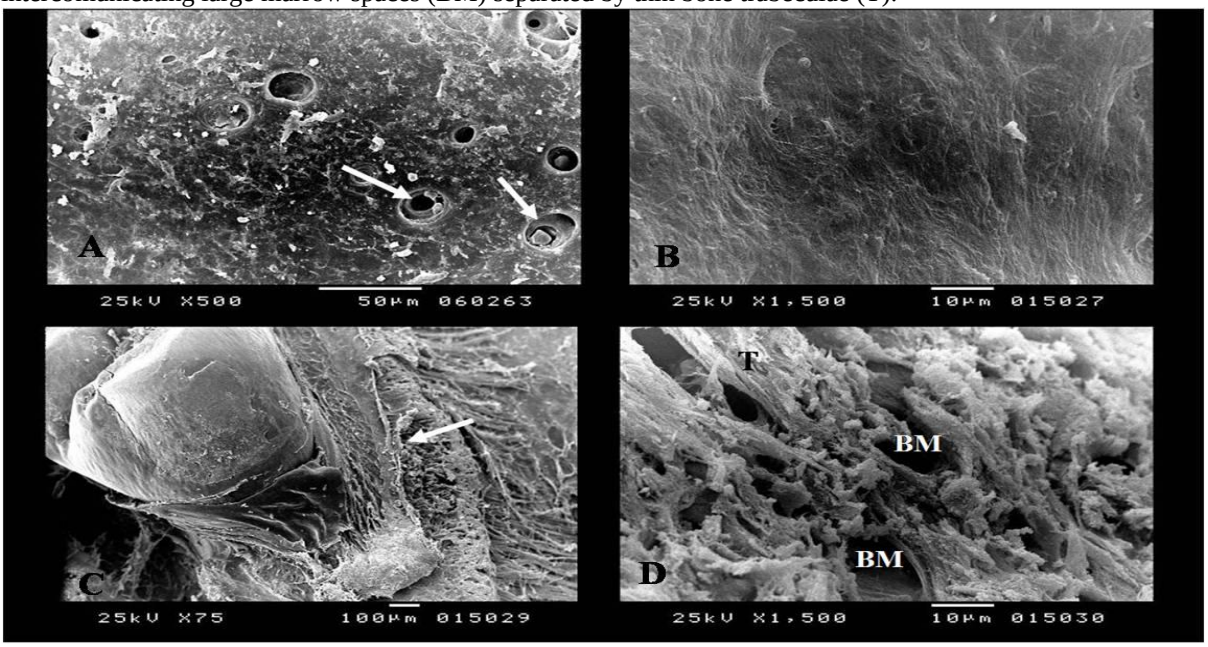

Fig.7. SEM photograph of mandibular bone of group III. **A:** Compact bone showing almost smooth surface with narrow openings of neurovascular canals. There were no fissures in between as compared with group II. Notice new bone formation within the neurovascular bundle (arrows). **B:** Compact bone showing almost smooth surface with covered collagen bundles. **C:** Lamellar bone of socket healing of 1st molar (arrow). **D:** Cancellous bone showing separate small marrow spaces (BM) separated by thick bone trabeculae (T).

4. Discussion

In the present study, osteoporosis model was established in male Wister albino rats by bilateral orchiectomy. The orchiectomy was proposed to simulate male osteoporosis due to hypogonadism **.** Adult and mature rats were used to avoid modeling and growth effects **(Libouban *et al.*, 2008).** Alveolar wound healing following tooth extraction is a sensitive indicator of bone damage under different experimental conditions **(Elsubeihi and Heersche, 2004)** and is used in our study as a model for the study of bone formation in rats.

The elemental analysis of the current study pointed up on calcium (Ca) and phosphorus (P) which are the main constituents of hydroxyapatite crystals [$(Ca_{10}$ $(PO_4)_6$ $(OH)_2$] **(Avery and Bernick, 1994)**. Additionally, Ca/P ratio was used in the current study since bone does not make a good subject because of its organic and water contents (even after drying) along with its porosity. This porosity means that the total weight ratio is always less than 100%. The balance between organic content and porosity will almost certainly shift from point to point in the sample and it is impossible to determine how much of the missing weight is due to each of these factors. Accordingly, the best measurements are those that involve ratios, such as Ca/P, as these systematic errors will cancel **(Coats *et al.*, 2003)**.

The observed changes in the rat weight in ORX groups may be explained by several previous studies. They reported that orchiectomy of male Fischer 344 rat produced catabolic effects including a decrease in body weight and muscle mass, along with increases in adiposity and bone resorption **(Borst and Conover, 2006).** On the other hand, **Villarreal *et al.*, 2007** and **Deyhim *et al.*, 2008** reported no effect of orchiectomy on body weights of ORX rats. Clinically, the decreased gonadal sex steroid levels in men following surgical or chemical orchiectomy is associated with rapid bone loss as well as decreased lean body mass and muscle mass **(Smith, 2002).**

Light microscopic examination of group II revealed an alteration in post extraction bone healing that was evident as wide intercommunicating marrow spaces and many discontinuous trabeculae with

isolated trabeculae were observed. These findings were coincident with that of **Filipovic *et al.*, 2007 and Villarreal *et al.*, 2007.** They reported marked bone loss and changes in bone structure of tibiae of ORX rats. This bone loss was accompanied with a remarkable decrease in cancellous bone area, trabecular thickness and trabecular number, with increased trabecular separation**.** The detected wide cancellous bone marrow spaces with loss of complete trabeculae in group II could be attributed to ORX-induced bone loss through increased osteoclastic resorption and decreased bone formation **(Libouban *et al.*, 2008).** Orchidectomy induced rapid bone loss is also associated with greater increases in biochemical markers of bone resorption than bone formation **(Smith *et al.*, 2002).**

However at SEM examination, the detected exposure of some collagen fibers of cortical bone of group II could be due to increased osteoclastic activity that dissolve inorganic component of bone as well as decreased Ca/P ratio of ORX rats, compared to Sham operated rats, as proved by the results of elemental analysis. All the degenerative changes that appeared in SEM of group II may be explained by androgen effects on skeletal cells that appear to be capable of regulating osteoclastogenesis both directly and indirectly.

In accordance with our histological as well as SEM results of group II, the x-ray analysis data revealed a decrease in Ca and increase in P composition while Ca/P ratio was decreased. This agreed with findings of **Nagareddy and Lakshmana, 2005** in femurs of rats. The observed decrease in Ca ratio in Group II could result in reduced bone density and may be explained by increased fecal and urinary excretion of calcium and reduced calcium absorption following ORX supporting testosterone mediated calcium absorption **(Deyhim *et al.*, 2008; Mandadi *et al.*, 2009).**

The most significant finding of our study shows that omega 3 fatty acids exert beneficial effects on post extraction bone healing in osteoporotic male rats. Group III rats treated with omega 3 showed obvious improvement in bone architecture at LM and SEM levels evidenced as less intratrabecular space, increased mineralization and trabecular components as well as increased bone matrix. These observations were in agreement with previous studies that confirmed that omega 3 fatty acids is a novel dietary supplement that can improve conditions associated with osteoporosis **(Sun *et al.*, 2003; Watkins *et al.*, 2005).** The effects of omega-3 on bone resorption could be mediated by a decrease in inflammatory cytokines such as IL-6, IL1-β or TNF-1 **(Kang *et al.*, 2008).** It is now clearly emerging that omega 3 might exert its effects on inflammatory gene expression through direct actions on the intracellular signaling pathways leading to inhibition of NF-κB activation **(Rahman *et al.*, 2009)**.

Although the LM and SEM of Group III of the present study clearly denotes that omega 3 increased bone formation excessively, the bone showed a lower degree of Ca and a higher degree of P as compared with control , which may take longer time to reach normal levels. The x-ray microanalysis of Group III revealed higher Ca/P ratio as compared with Group II which means decreased bone osteoporosis. In summary, our results confirmed that fish oil supplementation has a positive effect on socket healing and bone structure and composition in orchiectomized rats.

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