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THE VALIDITY OF NANO-CHITOSAN/ NANO-HYDROXYAPATITE AS A PROMOTER OF BONE HEALING IN OVARIECTOMIZED RATS

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ABSTRACT

Background: Nano-hydroxyapatite (nHA) is a biomaterial successfully used in bone tissue engineering. As well, Nano-chitosan (nCTS) is one of the natural polymers used in tissue engineering research. The unique properties of nCTS/nHA composite materials have attracted research interest especially in bioapplications. Estrogen deficiency is responsible for osteoporosis which is a metabolic bone disease characterized by delayed bone healing.

Aim of study: to investigate the putative effect of nCTS/nHA combination on the healing of bony defects in experimental postmenopausal hypoestrogenic rat model.

Materials and Methods: Forty eight, 6 month old, virgin female rats were randomly allocated into four equal groups; ovariectomy (OVX), Sham, OVX- nCTS/nHA treated, and Sham- nCTS/nHA treated groups. Sixty days following OVX or Sham surgery, a critical-sized defect on the right side of mandible was created in all groups then filled with nCTS/nHA hydrogel only in the third and fourth groups. Two and four weeks following bony defect surgery, six animals per group were euthanized and bone samples were processed for light microscopic (LM) examination, X-ray elemental microanalysis and scanning electron microscopic (SEM) examination.

Results: LM and SEM examinations of OVX group revealed delayed bone healing during both experimental periods in comparison with other groups. Interestingly, application of nCTS/nHA revealed improvement in bone healing process. X-ray elemental microanalysis of OVX group depicted marked significant decrease in calcium level below those of other groups. However, using nCTS/nHA revealed significant increase in calcium level suggesting its augmenting role in bone mineralization.

Conclusion: Estrogen deficiency impaired bone healing process. However, the synergistic effect of nCTS / nHA has the ability to improve the impaired bone healing in rats with OVX-induced osteoporosis.

KEY WORDS: Nano-hydroxyapatite, Nano-chitosan, Ovariectomy, Estrogen deficiency, Bone healing

Abbreviations: nHA: Nano-hydroxyapatite; nCTS: Nano-chitosan; OVX: ovariectomy; LM: light microscope; SEM; scanning electron microscope.

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INTRODUCTION

Nanotechnology is a rapidly emergent field regarded as the ability to manufacture materials at the nanoscale level [1]. Nanomaterials refers to materials that have been synthesized to have a size with at least one dimension in the range of approximately 1 to 100 nanometers and exhibit unique properties determined by their size which make them able to be used in a wide range of innovative applications[2,3]. Interestingly, nanotechnology represents one of the outstanding strategies significantly progress the field of bone tissue engineering and resolve existing limitations of conventional approaches including insufficient mechanical strength of scaffolds, impaired cellular proliferation and differentiation and inadequate production of extrinsic factors necessary to optimize osteogenesis [4].

Nano-hydroxyapatite (nHA) is chemically and structurally very similar to the inorganic component of bone and it has been successfully applied for bone tissue engineering due to its excellent biocompatibility, bioactivity, osteoconductivity which means it is able to support the proliferation and attachment of bone cells as well as osteoinductivity which means its ability to cause stem cells to differentiate into osteoblasts [5]. The advantages of such nanostructured material in comparison to traditional bulk material are high number of molecules on its surface, quick resorption and its close contact with surrounding tissues [6]. However, the usage of pure nHA is limited due to its brittleness. In consequence, wide ranges of attempts have been focused on improving HA's properties, to compensate this problem, by incorporating biopolymers such as chitosan [7, 8].

Chitosan (CTS) is a natural polycationic linear polysaccharide derived from deacetylation of chitin and is composed of N-acetyl glucosamine and is structurally similar to the glycosaminoglycans in the extracellular matrix. Chitin is the structural element in the exoskeleton of insects, crustaceans (mainly shrimps and crabs) and cell walls of fungi, and the second most abundant natural polysaccharide after cellulose [9,10]. Since CTS contains a number of free amine groups, nano-chitosan (nCTS) using ionic cross-linking [11]. CTS has unique bioactive, biodegradable, biocompatible, nontoxic, low-antigenic and anti-bacterial properties which enable it to be used in several biomedical and pharmaceutical applications [12]. It is reported to have biological properties such as antitumor, antimicrobial and antioxidant activities [13-15]. Moreover, it can be used in pharmaceutical excipient or drug carrier, obesity treatment and as a scaffold for tissue engineering [16-18]. CTS has been extensively used in bone engineering since it was shown to promote cell growth and mineral-rich matrix deposition by osteoblasts cells in vitro [19]. However, researches depicted that CTS itself has limited osteoconductive effect and addition of ceramic materials to chitosan is required to provide sites for calcification which in turn improve its osteoconductivity and mechanical strength [20].

nCTS/nHA composite materials based on combinations of biodegradable polymers and bioactive ceramics exhibit tailored physical, biological, mechanical properties and predictable degradation behavior as well as cytocompatibility besides displaying promise in mimicking the organic portion in addition to the inorganic portion of natural bone thus permitting exciting alternatives in the design of prosthesis and suggesting its potential for bone tissue engineering applications [8,21].

Osteoporosis is a metabolic bone disease characterized by deteriorated bone mass, disturbed bone structure and subsequently enhanced bone fragility and increased the risk of fracture [22,23]. Previous researches had verified altered and delayed healing in osteoporotic bone especially in old individuals [24,25]. The incidence of osteoporosis is higher in postmenopausal women whose estrogen levels are lowered resulting in imbalance between

bone formation and bone resorption [26]. Noteworthy, ovariectomy (OVX) in rats provides the most popular animal model of osteoporosis which can mimic conditions in postmenopausal women with estrogen deficiency [27-29].

Hence, it is of prime importance to study the proficiency of nCTS/nHA combination as an advocate for bone healing in experimental postmenopausal hypoestrogenic rat model.

MATERIALS AND METHODS

Forty eight virgin female Sprague–Dawley rats (6 month in age and approximately 250-300 g in weight) were used. Animals were housed in separate cages at faculty of Medicine-Zagazig University and received a standard diet for rodents and tap water ad libitum with constant temperature at 22 degrees Celsius. The light cycle was fixed at 12 h. All animal experiments were carried out in accordance with the guidelines of the National Institutes of Health (NIH) for the care and use of laboratory animals (NIH Publication, Number 85-23, Revised 1985).

After one week acclimatization to the new laboratory environments, rats were assigned randomly into four equal groups as the following:

Group I: Rats of this group underwent bilateral OVX operation. Sixty days following OVX, a critical-sized defect on the right side of mandible was created.

Group II: Rats of this group underwent sham operation of OVX. A critical-sized defect on the right side of mandible was created sixty days following the shame operation by the same technique of group I.

Group III: Rats of this group underwent bilateral OVX operation. Sixty days following OVX, a critical-sized defect on the right side of mandible was created similar to group I then filled with nCTS/nHA hydrogel (Nanostream, Egypt).

Group IV: Rats of this group underwent sham operation of OVX. A critical-sized defect on the right side of mandible was created then filled with nCTS/nHA hydrogel similar to group III.

Surgical procedure of bilateral ovariectomies:

An osteoporosis animal model was carried out by bilateral OVX under sterile conditions with a minimally invasive surgical technique. The animals were anesthetized with intraperitoneal injection of pentobarbital sodium (15 mg/kg body weight) for the surgical procedure. In the ovariectomized groups, bilateral OVX were performed by the dorsal approach as described previously by Kalu et al. [30]. The sham-operated groups underwent a similar surgical procedure, exposing the ovaries and replacing them in the same position. The success of ovariectomy was confirmed by the analysis of serum estradiol level (E2) in El-Borg laboratory, Zagazig branch.

Surgical procedure of mandibular bone defect:

Sixty days following OVX surgery and after confirmation the success of ovariectomy, the animals were anesthetized and the skin and the muscle were incised and the soft tissues were dissected then a 5x5-mm full thickness critical defects were created in the right side of the body of the mandible in each animal with a slow-speed dental drill under constant normal saline irrigation to prevent overheating. After the defect was created, the area was plentifully irrigated with normal saline to eliminate residual bone chips. In group III and IV, the critical-sized defects of mandible were subsequently filled with nCTS/nHA hydrogel. The soft tissues above the defect, in all groups, were sutured in layers with 4-0 Vicryl sutures (Ethicon, Lenneke Marelaan, Belgium). Postoperatively, penicillin (40,000 IU/ml, 1 ml/kg) was injected intramuscularly for 3 days [31,32].

Euthanasia and sample collections

Two and four weeks following mandibular bony defect surgery, six animals from each group

were sacrificed with an overdose of pentobarbital sodium, confirmed with cervical dislocation and their mandibles were dissected and their right halves were collected and immediately fixed with 4% buffered formalin solution. At each experimental period, three samples per group were prepared for light microscopic (LM) examination and three other samples per group were prepared for X-ray elemental microanalysis and scanning electron microscopic (SEM) examination.

Light microscope (LM):

After fixation, the specimens were decalcified in 5% formic acid. After complete decalcification, specimens were washed in distalled water, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin. Serial sections (5µm) were cut and stained with hematoxylin and eosin (H&E). Afterward, the histological sections were examined by LM (Leica ICC50 HD) at Faculty of Medicine, Tanta University.

X-ray elemental microanalysis:

After fixation, the samples treated with 5% sodium hypochlorite (commercial bleach), for 1 hour, to remove the organic material. After washing in distilled water, specimens were dehydrated in ethanol then air-dried and examined by energy dispersive x-ray analysis (EDAX) attached with the SEM unit which is designed to analyze the inorganic constituents (mainly calcium level) of the specimens.

Scanning electron microscope (SEM):

After determination of their elemental composition, the samples were prepared for SEM examinations. They were vacuumed, coated with gold through Blazers' SCD-050 sputter that converted electrically non-conductive samples into conductive ones hence enabled a tightly focused electron beam to be scanned across the sample surface by SEM (JEOL JSM-636 OLA at an accelerating voltage of 15kv) at National Research Center, Cairo, Egypt.

Statistical Analysis

Statistical analyses of the calcium levels were performed using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test to reveal statistical significance of difference among groups. Statistical significance was considered at $P < 0.05$ and the calcium levels were expressed as the mean ± standard error (SE). All statistics were performed with SPSS (statistical package for social sciences) version 11.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

Histological findings:

Histological examination of bone healing two weeks after induction of bone defect in OVX group revealed a predominance of soft tissue composed of a blend of hematoma and immature granulation tissue with inflammatory cells infiltration and numerous vacuoles (Fig. 1-A). In sham group, the bone defect area was filled with granulation tissue, angiogenesis and irregular fibrous callus with noticeable newly formed woven bone (Fig. 1-B). On the other hand, OVX- treated group revealed granulation tissue characterized by inflammatory cells infiltration with some vacuoles and scanty bone trabeculae were noticeable (Fig. 1-C). In sham- treated group, there was obvious woven bone formation (Fig. 1-D). Histological examination of bone healing four weeks after induction of bone defect in OVX group showed granulation tissue which, in some areas, was consecutively replaced by more mature connective tissue characterized by angiogenesis and inflammatory infiltrate reduction along the increased bone formation while other areas still showed granulation tissues with inflammatory cells infiltration and some vacuoles (Fig. 2-A). However, sham group revealed bone trabeculae with well-defined medullary spaces (Fig. 2- B). In OVX- treated group, the bone defect site was almost filled with new bone trabeculae except for

minimal area containing granulation tissue (Fig. 2-C). On the other hand, sham- treated group showed well-formed bone trabeculae and medullary spaces (Fig. 2- D).

X-Ray Elemental Microanalysis

Representative spectra of the mandibular bone defect areas of the different groups revealed variation in their elemental composition during different experimental intervals. Statistical analysis demonstrated that two weeks after induction of mandibular bony defect there was a significant difference in bone calcium level between OVX group and all other groups including sham, OVX-treated & sham- treated groups. On the other hand calcium level of OVX- treated group simulated that of shame group. Moreover, there was significant increase in calcium level of sham-treated group in comparison with those of shame and OVX- treated groups (Table 1 & Fig. 3). Statistical analysis of bone calcium level four weeks after induction of bone defect revealed significant differences among all groups (Table 2 & Fig. 4). Furthermore, comparison of bone calcium level between the two periods of each group revealed significant increase in OVX- treated group four weeks compared with the same group two weeks post bone defect induction. Additionally sham- treated group showed significant increase four weeks compared with the same group two weeks post bone defect induction. (Table 3 & Fig. 5).

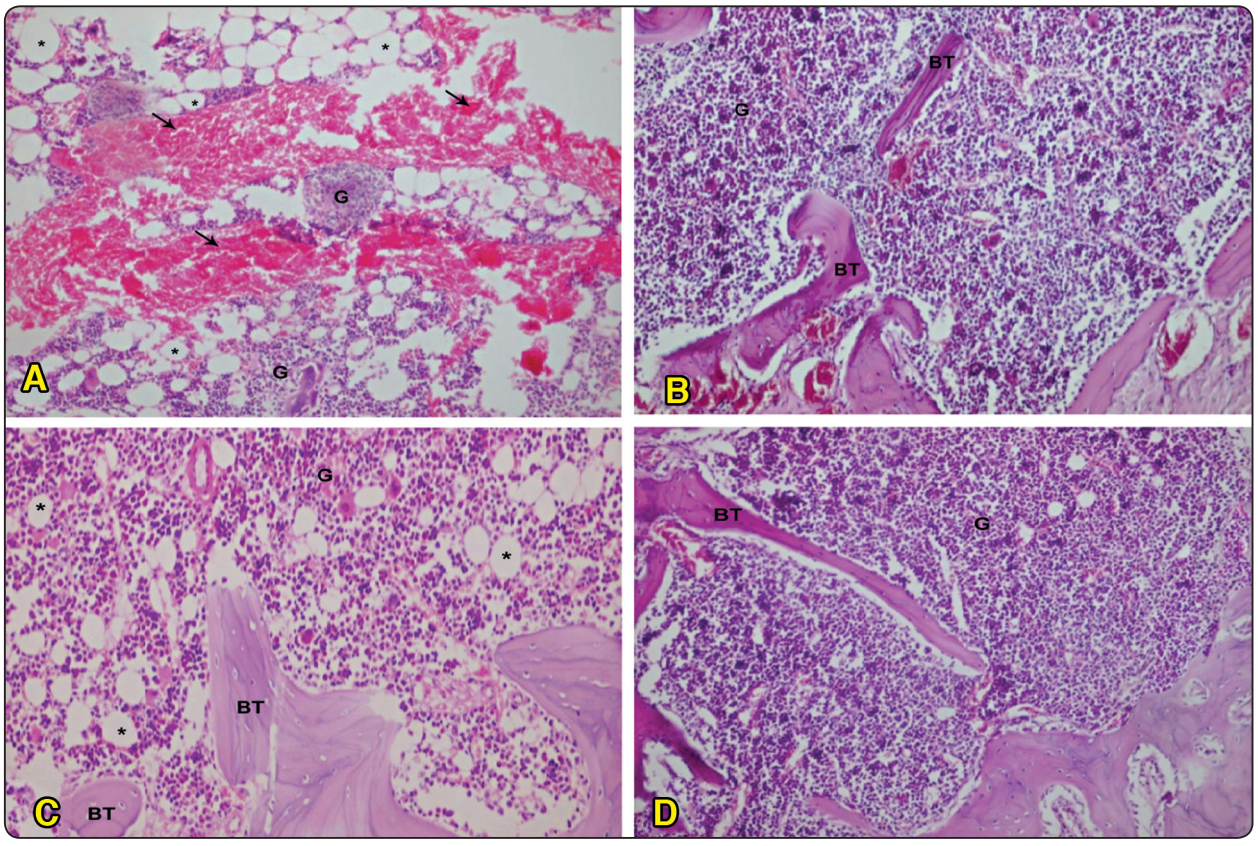

Fig. (1) Decalcified H&E stained sections in rat mandibular bone two weeks after induction of bone defects: (A) OVX group. (B) Sham group. (C) OVX- treated group. (D) Sham– treated group. Bone trabeculae (BT), granulation tissues (G), hematoma (arrows), vacuoles (*) (Original Magnification; A-D X 400)

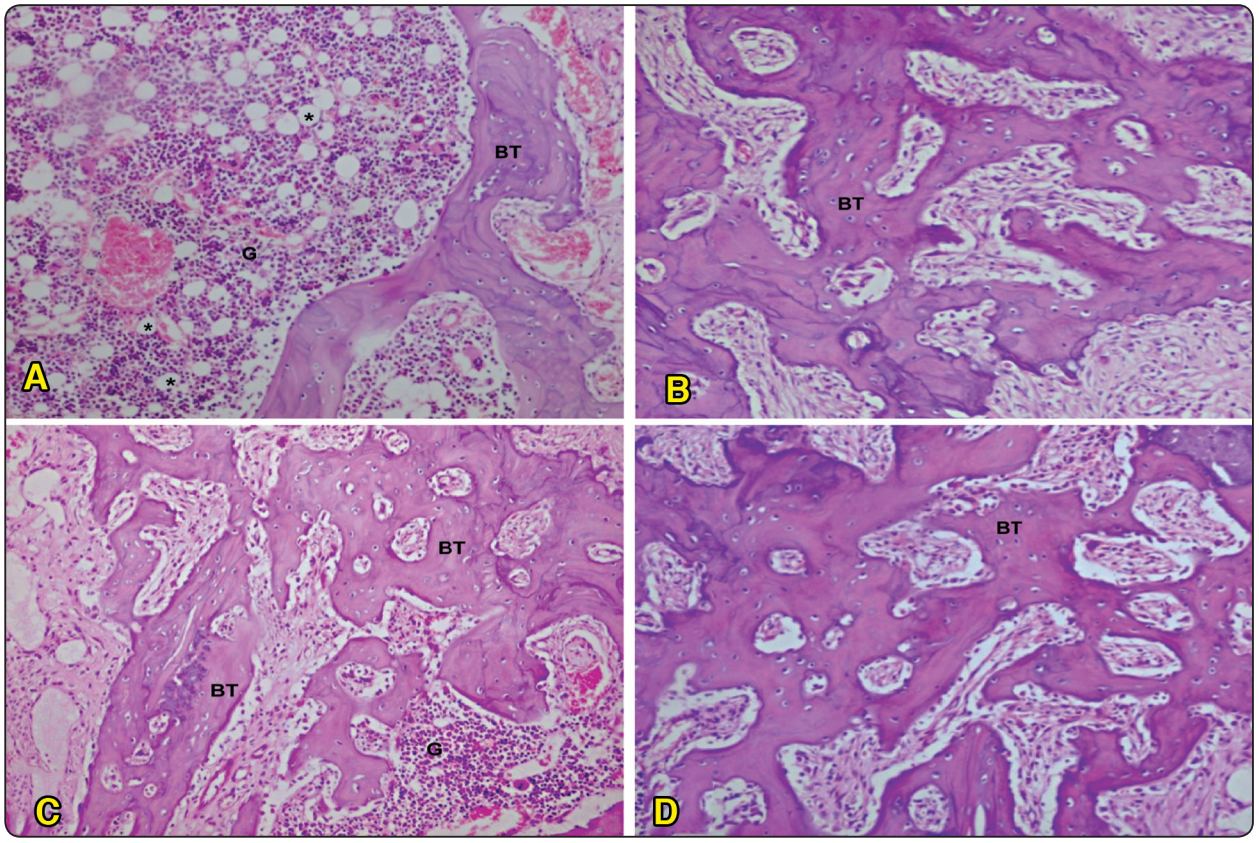

Fig. (2) Decalcified H&E stained sections in rat mandibular bone four weeks after induction of bone defects: (A) OVX group. (B) Sham group. (C) OVX- treated group. (D) Sham– treated group. Bone trabeculae (BT), granulation tissues (G), vacuoles (*) (Original Magnification; A- D X 400)

TABLE (1) Diagram showing comparison of calcium level between the different groups two weeks after induction of bone defects

Calcium level	**Group I**	**Group II**	**Group III**	**Group IV**
Range	7.4 – 10.5	13.2 – 16.4	13.6 – 19.6	19.8 – 25.9
Mean ±SD	9.22 ± 1.33	14.52 ± 1.25	15.98 ± 2.32	23.42 ± 2.26
F test	49.673			
P value	0.001*			

Group I&II	**Group I&III**	**Group I&IV**	**Group II&III**	**Group II&IV**	**Group III&IV**
0.001*	0.001*	0.001*	0.232^{NS}	0.001*	0.001*

P value< 0.05 ******* ***significant*** ***NS*** ***not significant***

TABLE (2) Diagram showing comparison of calcium level between the different groups four weeks after induction of bone defects

Four weeks	**Group I**	**Group II**	**Group III**	**Group IV**
Range	8.6 – 10.9	14.1 – 18.8	17.5 – 21.80	25.10 – 27.2
Mean ±SD	9.72 ± 0.86	15.88 ± 1.87	19.74 ± 2.01	26.36 ± 0.88
F test	104.578			
P value	0.001*			

Group I&II	**Group I&III**	**Group I&IV**	**Group II&III**	**Group II&IV**	**Group III&IV**
0.001*	0.001*	0.001*	0.029*	0.001*	0.001*

P value< 0.05 **** significant*** ***NS not significant***

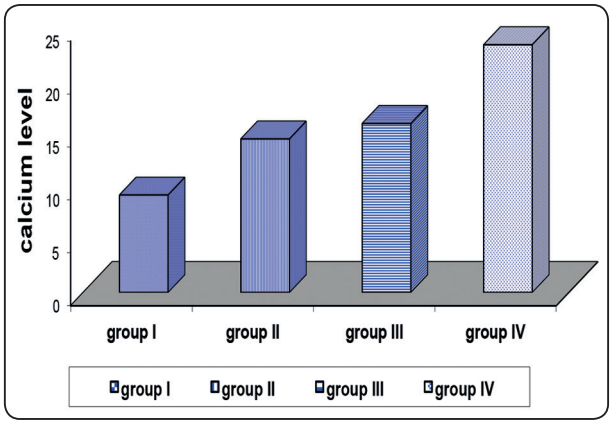

Fig. (3) Bar chart shows the mean calcium level among the different study groups two weeks after induction of bone defects

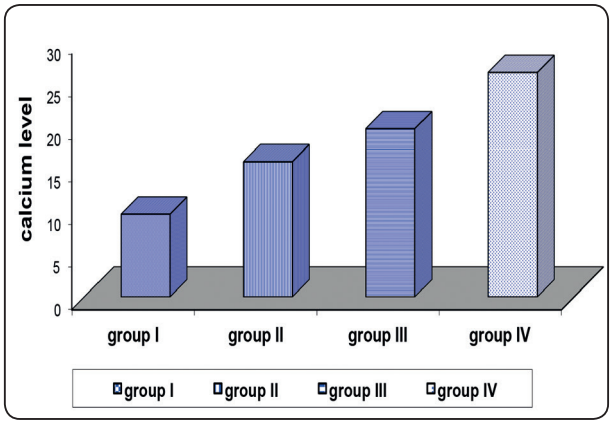

Fig. (4) Bar chart shows the mean calcium level among the different study groups four weeks after induction of bone defects

TABLE (3) Diagram showing comparison of calcium level between two and four weeks after induction of bone defects in various groups

		Two weeks	Four weeks	T test	P value
Group I	Range	7.4 – 10.5	8.6 – 10.9	0.496	0.501^{NS}
	Mean ±SD	9.22 ± 1.33	9.72 ± 0.86		
Group II	Range	13.2 – 16.4	14.1 – 18.8	1.826	0.214^{NS}
	Mean ±SD	14.52 ± 1.25	15.88 ± 1.87		
Group III	Range	13.6 – 19.6	17.5 – 21.80	6.307	0.025*
	Mean ±SD	15.98 ± 2.32	19.74 ± 2.01		
Group IV	Range	19.8 – 25.9	25.10 – 27.2	7.363	0.027*
	Mean ±SD	23.42 ± 2.26	26.36 ± 0.88		

P value< 0.05 **** significant*** ***NS not significant***

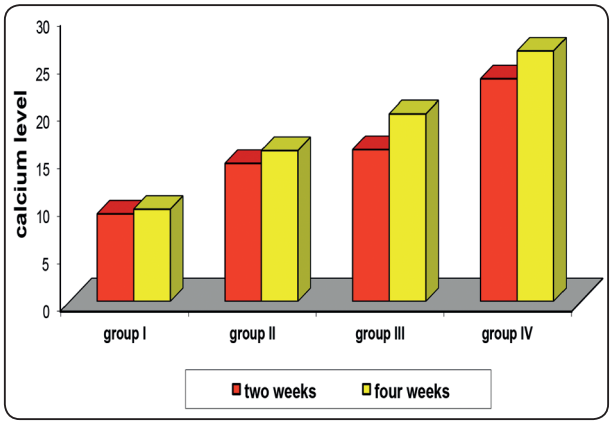

Fig. (5) Bar chart shows the mean calcium level among the different study groups both two and four weeks after induction of bone defects

Scanning electron microscopic findings

Topographical examination of bone defect areas two weeks after their induction in OVX group revealed obvious defect area almost devoid of bone (Fig. 6-A). Sham and OVX-treated groups showed bone defect areas filled by numerous new bone trabeculae (Fig. 6- B & C). However, Sham-treated group revealed bone defect packed with thicker new bone trabeculae (Fig. 6- D). Four weeks after induction of bone defects, OVX group appeared filled with many bone trabeculae (Fig. 7- A). However, Sham, OVX-treated and Sham-treated groups showed complete closure of bone defect areas with almost normal bone surface that was best seen in sham- treated group (Fig. 7- B, C& D).

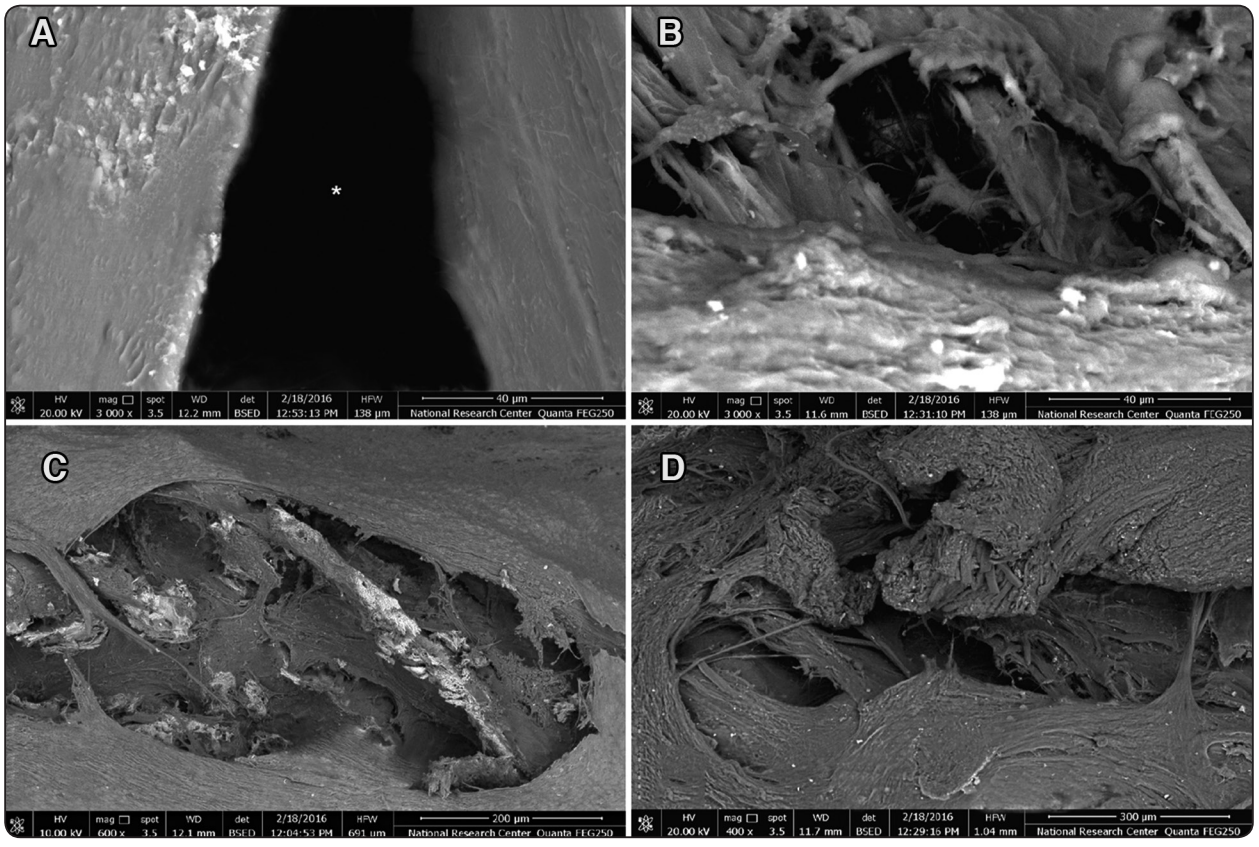

Fig. (6) Scanning electron micrographs of rat mandibular bone two weeks after induction of bone defects: (A) OVX group. Defect area without bone (*) (B) Sham group. (C) OVX- treated group. (D) Sham– treated group

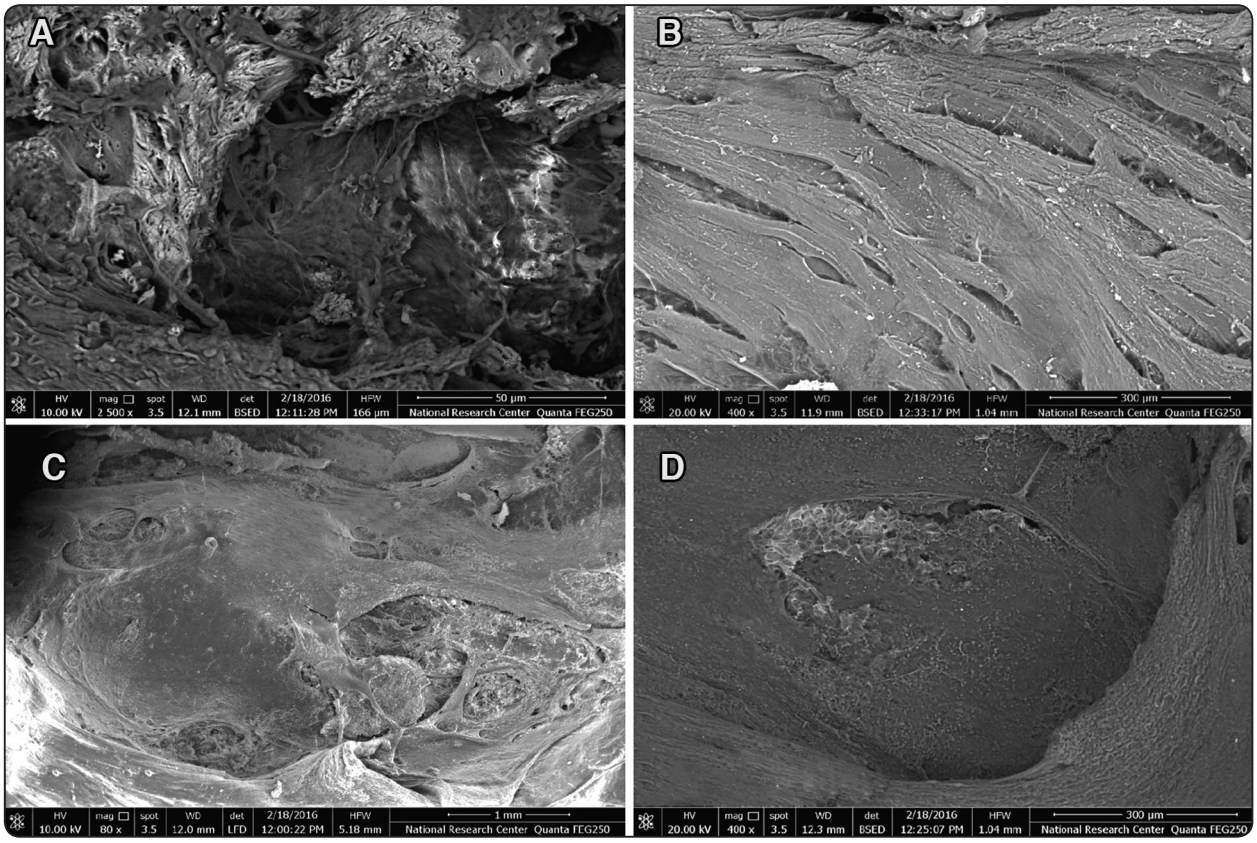

Fig. (7) Scanning electron micrographs of rat mandibular bone four weeks after induction of bone defects: (A) OVX group. (B) Sham group. (C) OVX- treated group. (D) Sham– treated group

DISCUSSION

The present study includes both quantitative X-ray elemental analysis and qualitative (LM and SEM) assessments of the effect of nano-chitosan/ nano-hydroxyapatite on bone healing in ovariectomized rats. Six- months old, virgin female rats were used in this study to avoid possible pregnancy and lactation related effects.

Quantitatively, X-ray elemental microanalysis of calcium in bone of OVX group illustrated a marked significant decrease in bone calcium value below those of other groups through the two experimental periods. These results comparable with that of Rahnama and Światkowski [33] who reported that hypoestrogenism after OVX caused characteristic decrease in calcium level in mandible and incisors. This significant decrease in bone calcium level indicated that OVX could deteriorate bone mineralization indicating hypomineralization owing to the pathologically increased bone turnover where bone resorption exceeded bone formation[34] and proved the capability of OVX to induce potential changes in mineralization kinetics [35]. On the other hand, the present study illustrated a marked significant augmentation of the bone calcium level following application of nCTS/nHA at the different experimental periods which indicate that nCTS/ nHA could enhance bone mineralization. This is in agreement with Teng et al. [36] who found that CTS/HAp composite scaffolds possessed higher alkaline phosphatase activity, an important marker of bone formation and mineratization. Moreover, Lin et al. [37] demonstrated the ability of nHA to

induce expression of genes for alkaline phosphatase and osteocalcin which are necessary for matrix maturation and mineratization. Furthermore, OVX-treated group illustrated elevated calcium values which implies an almost absolute recovery and suggests the competence of nCTS/nHA to alleviate OVX- induced defect in mineralization.

The present qualitative investigation used both LM and SEM examinations. LM study was used demineralized sections to depict the bone matrix architecture during bone healing while SEM depicted the topographical features of un-demineralized bone in bone defect areas. In the current study, the histological findings in OVX group were attuned with the significant decrease in serum level of estrogen consecutive to ovariectomy. LM examination of bone defect areas in OVX rats of this study revealed disorganized and immature tissue and a lower amount of newly formed bone than other groups. Similarly, EM examination revealed obvious delay in healing and osteogenesis of bone defect area in comparison with other groups. These findings coincide with other researches that support the disturbing effect of OVX on the bone healing process [38-41]. Similarly, Meyer et al. [42] found a decrease in the biomechanical properties of the bone callus in OVX rats. In addition, Nikolaou et al. [43] reported a delay in bone consolidation in osteoporotic patients after a fracture. The worrisome effect of OVX- induced osteoporosis on bone healing explained by previous researches which suggest that in osteoporosis there is a decreased proliferative activity of progenitor cells of osteoblasts, decreased osteoblastic response and an imbalance between bone formation and resorption which may lead to a delay in bone healing [42,44,45]. Furthermore, many researches verified the fundamental role of estrogen in bone metabolism and homeostasis as it inhibits local factors that hinder bone formation and augments local factors that stimulate bone formation. Estrogen has the ability to increase levels of osteoprotegerin (OPG), a protein produced by osteoblasts which can inhibit bone resorption by preventing Receptor Activator of NF-kB Ligand (RANKL) from binding to nuclear factor kappa-B (RANK) receptors [46-48]. Therefore, estrogen plays an important role in down-regulating osteoclastogenesis process by modulating the production of osteoclastogenic cytokines and affecting the sensitivity of maturing osteoclast to RANKL [49]. Hence, estrogen deficiency is an important pathogenic factor in bone loss associated with osteoporosis because hypoestrogenism caused increased expression of RANKL and increased osteoclast activities leading to delayed bone healing [50-54]. Furthermore, angiogenic factors, such as vascular endothelial growth factor (VEGF), are required for bone formation during bone healing. OVX- induced hypoestrogenism caused lower VEGF expression in osteoblasts of OVX animals as compared to sham animals [55]. Moreover, Runx2, a key transcription factor associated with osteoblast differentiation and osteogenesis and and regulates the expression of various extracellular matrix protein, exhibited lower immunoexpression during osteoporosis that may be related to the decreased osteoblast activity [40].

Interestingly in the current qualitative study, application of nCTS/nHA in bone defect areas confirmed its capability to accelerate bone healing and improve the architecture and the topographical features of bone at the site of defects in comparison with shame group. These results could be related to constructive effects of nCTS/nHA on bone formation and mineralization as reported by several researches [56-62]. This beneficial effect of nCTS/nHA on bone healing could be explained by the results of Chen et al. [63] who demonstrated that CS/nHAC scaffolds increased RUNX-2, osteocalcin and COL-1 genes expression which enhanced better osteoblasts differentiation and promoted mineral deposition. In this regard, Lu., et al. [64] who reported that osteoinductive effect of nHA related to its ability to induce differential expression of a large

number of genes closely related with osteogenic differentiation and then activate some signaling pathways, such as TGF-β signaling pathway, MAPK signaling pathway, Notch signaling pathway and Wnt signaling pathways, which ultimately promoted the osteogenic differentiation of mesenchymal stem cells (MSCs). Also, Gua et al. [65] proved the osteoinductivity and osseointegrative capacity of n-HA composite scaffold as it induces osteoblast adhesion, proliferation and differentiation in vitro. More interesting in the current study, Ovx rats treated with nCTS/nHA demonstrated improved healing of bone defects in comparison with OVX-non treated group. This proved the reversibility of OVX- induced defective bone healing following nCTS/nHA application. The capability of various nanomaterials to improve the deteriorating effect of osteoporosis on bone healing and architecture was substantiated by many authors [66-68].

In conclusion, estrogen deficiency affects badly bone healing process. However, this study proved the synergistic effect of nCTS and nHA as the best bioactive biomaterials to alleviate the impaired bone healing in rats with OVX-induced osteoporosis.

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