Safety evaluation of needle-like hydroxyapatite nanoparticles in female rats

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Objective
The present study was designed to evaluate the safety of synthesized needle-like hydroxyapatite (HAp) nanoparticles ranging from 3 to 7 nm in diameter and from 27 to 46 nm in length when administered in female rats orally or subcutaneously at different concentrations.

Methods
Animals in different treatment groups were maintained on their respective diets as follows: group 1, untreated control; group 2, treated orally with HAp (300 mg/kg body weight) for 3 weeks; group 3, treated orally with a low dose of HAp (150 mg/kg body weight) for 3 weeks; and group 4, implanted subcutaneously with HAp (600 mg/kg body weight) once and left for 5 weeks. At the end of the experimentation period, blood samples were collected from all animals for biochemical analysis (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, uric acid, urea, and creatinine). After sacrifice, histopathological examination of the liver and kidney was carried out.

Results and conclusion
The biochemical results showed an increase in alanine aminotransferase and aspartate aminotransferase in the groups treated orally and those treated subcutaneously. There was an increase in alkaline phosphatase only in the group receiving the high oral dose; however, animals treated with the low dose or those treated subcutaneously were comparable with the control group. All the rats showed normal kidney function because of normal levels of creatinine, urea, and uric acid. The histopathological results indicated that the liver and kidney of all rats treated with HAp (oral or subcutaneously) had a normal structure. The previous results confirmed the safety of the synthesized nanoneedle HAp when administered orally or subcutaneously at the suggested dose.

Keywords:
hydroxyapatite nanoparticles, kidney, liver, rats, safety evaluation

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Introduction

Nanotechnology is known to be a revolutionary manufacturing technology of the 21st century involving multidisciplinary research issues that rely on the understanding and control of substances at the nanoscale length of around 1–100 nm. It has been established that nanotechnology offers a unique approach to overcome the limitations of many conventional materials. From nanomedicine to nanofabrics, this promising technology has encompassed almost all fields of human life. Hydroxyapatite (HAp) $Ca_{10}(PO_4)_6(OH)_2$ is one of the most important materials affected by nanotechnology. One of the main reasons for the intense focus on HAp nanoparticles is because of its structural and compositional similarity to the mineralized matrix of natural bone, enamel, and dentin [1–4].

Synthetic HAp nanoparticles are increasingly being used in medical applications as a bioresorbable carrier material for controlled drug delivery in the treatment of diseases such as cancer [5], osteoporosis [6], osteomyelitis [7], and diabetes. Also, HAp nanoparticles have excellent biocompatibility with soft tissues such as skin, muscle, and gums, making them an ideal candidate for orthopedic and dental implants or as components of implants. It has been used widely in the repair of hard tissues, and common uses including bone repair, bone augmentation, as well as coating of implants or as fillers in bone or teeth [8,9]. Furthermore, it has been found to have an obvious inhibitory function on the growth of many kinds of tumor cells, and its nanoparticle exerts a stronger antitumor effect than macromolecule microparticles [10]. Because of the previously mentioned benefits of HAp nanoparticles, the current study was carried out to evaluate the safety of the prepared needle-like HAp nanoparticles when administered in Sprague–Dawley female rats orally or subcutaneously (s.c) at different concentrations.

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Subjects and methods

Chemicals

Kits of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, uric acid, and creatinine were purchased from Biodiagnostic Co. (Cairo, Egypt).

Experimental animals

One-month-old Sprague–Dawley female rats (100–120 g, purchased from the animal house colony, Giza, Egypt) were maintained on a standard lab diet (protein: 160.4; fat: 36.3; fiber: 41 g/kg, and metabolizable energy 12.08 MJ) obtained from Meladco Feed Co. (Aubor City, Cairo, Egypt). Animals were housed in a room free from any source of chemical contamination, artificially illuminated and thermally controlled, at the Animal House Lab (National Research Centre, Dokki, Cairo, Egypt). After an acclimatization period of 1 week, the animals were divided into four groups (seven rats/group) and housed in filter-top polycarbonate cages. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Center.

Experimental design

Needle-like HAp nanoparticles ranging from 3 to 7 nm in diameter and from 27 to 46 nm in length were synthesized according to the technique of Sabry [11]. Animals in different treatment groups were maintained on their respective diets as follows: group 1, untreated control; group 2, treated orally with HAp (300 mg/kg body weight) for 3 weeks; group 3, treated orally with a low dose of HAp (150 mg/kg body weight) for 3 weeks; and group 4, implanted s.c. with HAp (600 mg/kg body weight) once and left for 5 weeks. Body weight was recorded weekly during the experimental period. At the end of the experimentation period, blood samples were collected from all animals from the retro-orbital venous plexus for the determination of ALT and AST [12], ALP [13], uric acid [14], urea [15], and creatinine [16]. After the collection of blood samples, all animals were sacrificed and samples of the liver and kidney from all animals from different treatment groups were excised and fixed in 10% neutral formalin, followed by dehydration in ascending grades of alcohol, clearing in xylene, and embedding in paraffin wax. Liver and kidney sections (5 µm thickness) were stained with hematoxylin and eosin for histological examination [17]. The histopathological study was carried out in a clinical pathology private lab.

Statistical analysis

All data were analyzed statistically using the General Linear Model Procedure of the Statistical Analysis System [18]. The significance of the differences among the treatment groups was determined using the Waller–Duncan k-ratio [19]. Significance was determined on the basis of P less than 0.05.

Results and discussion

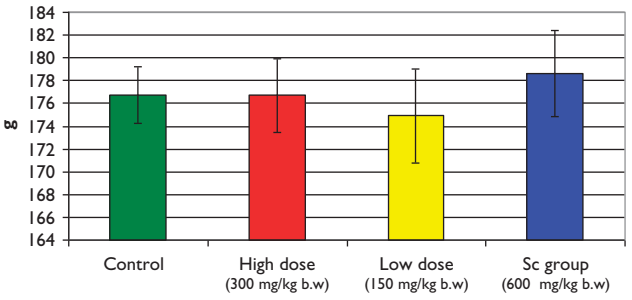
During this study, no animal died in any of the treatment groups and all animals appeared healthy during the entire treatment period. No significant changes were observed in the body weight of the animals (Fig. 1).

The biochemical results indicated that animals treated with HAp showed an increase in liver function parameters (Table 1). ALT showed a significant increase in the groups treated orally at the two tested doses or those treated s.c. with HAp (Fig. 2a). This increase was more pronounced in the group treated with the high dose and reached 92.4%, whereas it reached 60.19 and 29.9% in the group received the low oral dose and the group treated s.c. with Hap, respectively.

AST showed a significant increase (Fig. 2b) in the orally treated groups. This increase reached 48.9% in the group that received the high dose and reached 20.4 and 23.46% in the group that received the low dose and the s.c. treated group, respectively. ALP also showed the same trend of increase (Fig. 2c) only in the group that received the high oral dose, reaching 20.8%. However, animals treated with the low dose or those treated s.c. with HAp were comparable with the control group.

The current results showed that HAp did not exert any significant effects on kidney function tests (Table 2). No significant differences were found between the control group and the groups treated orally or s.c. with HAp. Uric acid tended to increase insignificantly in the group treated orally with the high dose and the group treated s.c. with HAp (Fig. 3a). The same trend was observed in

Figure 1



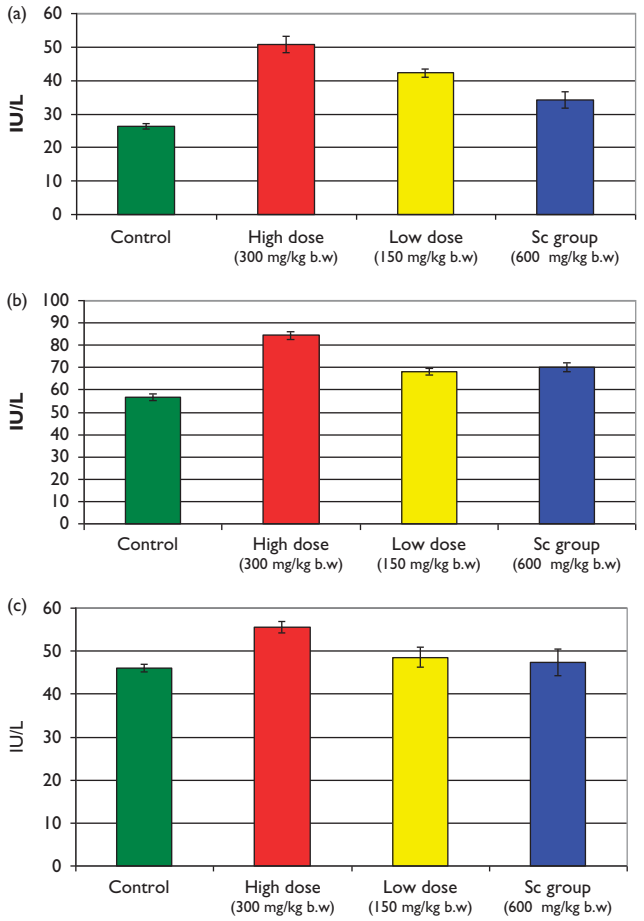
Effect of oral or subcutaneous (s.c.) treatment with hydroxyapatite on body weight in rats.

Table 1 Effect of oral or subcutaneous treatment with hydroxyapatite on liver function tests in rats

	Parameters		
Groups	ALT (IU/l)	AST (IU/l)	ALP (IU/l)
Control	26.4 ± 0.82^{a}	56.7 ± 1.45^{a}	45.97 ± 0.88^{a}
High dose	50.8 ± 2.49^{b}	84.4 ± 1.72^{b}	55.54 ± 1.26^{b}
Low dose	42.29 ± 1.27^{c}	68.29 ± 1.54^{c}	48.52 ± 2.34^{a}
Subcutaneous group	34.29 ± 2.41^{d}	70.0 ± 2.0^{d}	47.39 ± 3.19^{a}

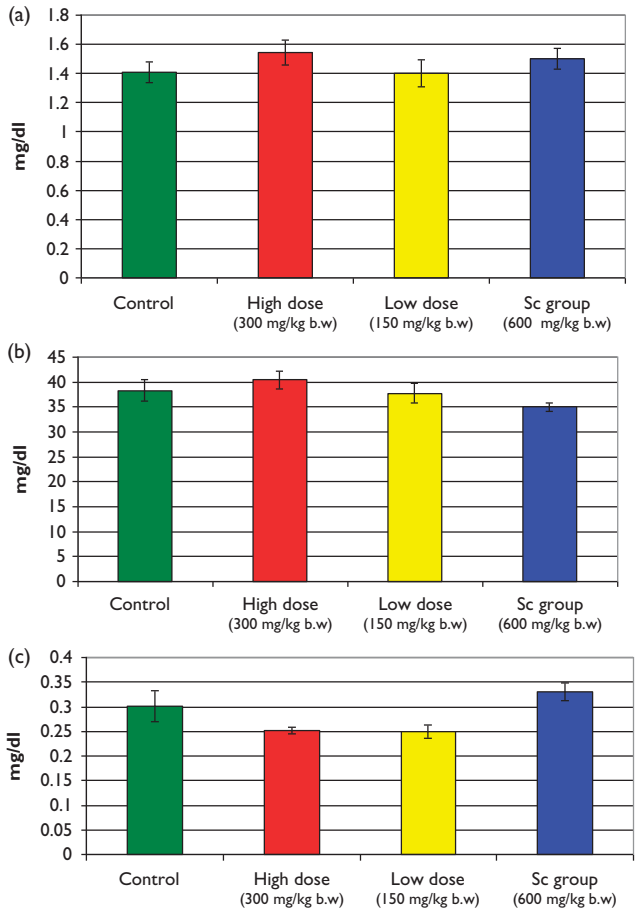
Within each column, means with different letters are significantly different ($P < 0.05$).
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Figure 2



Effect of oral or subcutaneous (s.c.) treatment with hydroxyapatite on: (a) alanine aminotransferase, (b) aspartate aminotransferase, and (c) alkaline phosphatase activity in rats.

Figure 3



Effect of oral or subcutaneous (s.c.) treatment with nano-hydroxyapatite on: (a) uric acid (b) urea, and (c) creatinine level in rats.

Table 2 Effect of oral or subcutaneous treatment with hydroxyapatite on kidney function tests in rats

	Parameters		
Groups	Uric acid (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Control	1.41 ± 0.07^{a}	38.30 ± 2.16^{a}	0.30 ± 0.03^{a}
High dose	1.54 ± 0.09^{a}	40.41 ± 1.74^{a}	0.25 ± 0.007^{a}
Low dose	1.40 ± 0.09^{a}	37.76 ± 1.98^{a}	0.25 ± 0.01^{a}
Subcutaneous group	1.50 ± 0.07^{a}	34.99 ± 0.84^{b}	0.33 ± 0.02^{a}

Within each column, means with different letters are significantly different ($P < 0.05$).

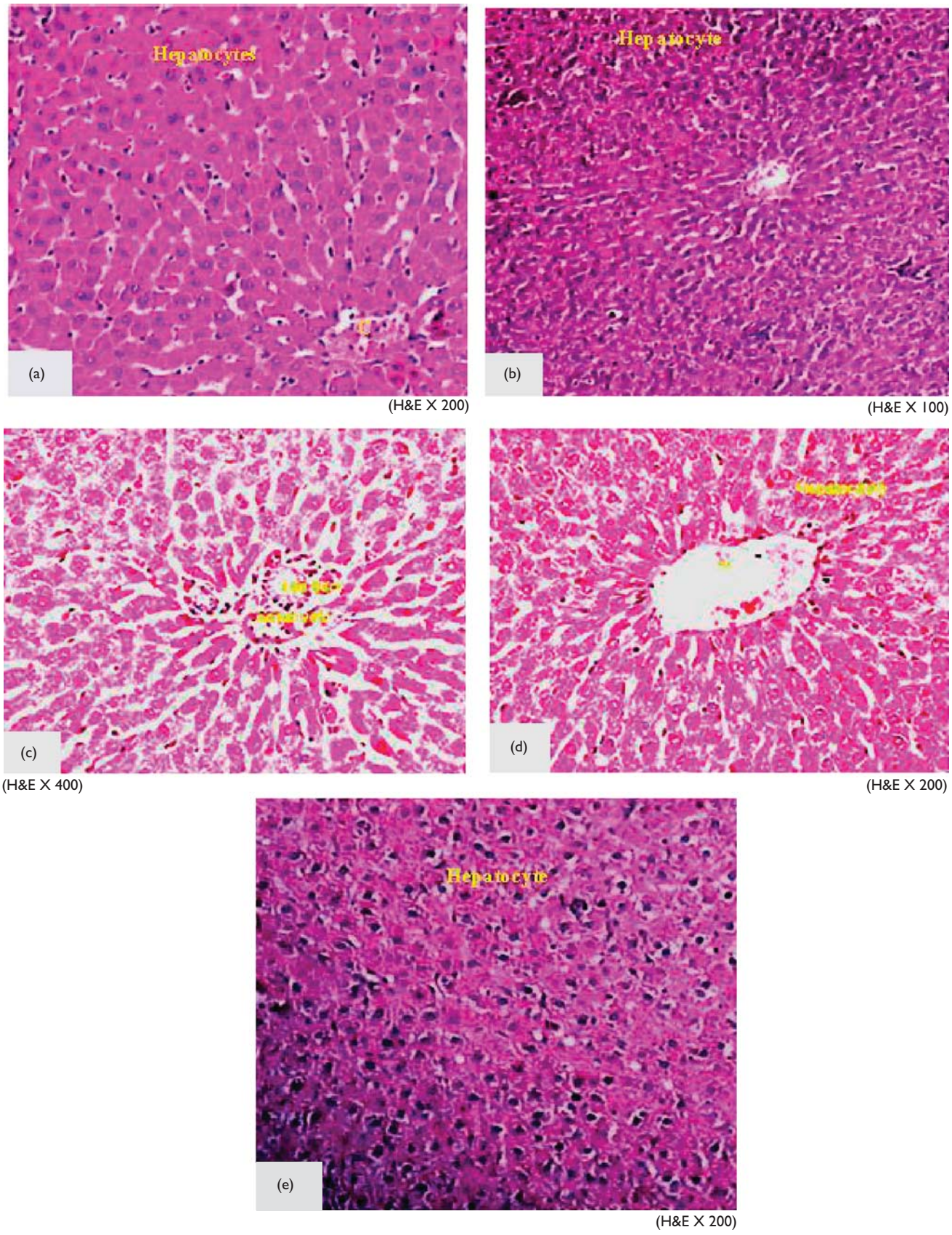
urea (Fig. 3b). No significant differences were observed in the creatinine level between animals treated orally with the high and the low dose, although these groups were decreased insignificantly compared with the control group and the s.c. treated group (Fig. 3c).

The microscopic examination of the liver tissues in the control group showed normal central vein and hepatocyte architecture (Fig. 4a). The liver of animals treated with the high dose of HAp nanoparticles showed a normal histological structure of the central vein and surrounding hepatocytes (Fig. 4b). Rats treated with the low dose had a normal structure of hepatocytes, portal vein, and bile ducts (Fig. 4c). The same group showed a normal structure of hepatocytes and the central vein (Fig. 4d). Histopathological investigation of the liver of rats treated s.c. with HAp nanoparticles showed a normal structure of hepatocytes as shown in Fig. 4e.

The biochemical results were in good agreement with the histopathological studies of kidney tissue. The microscopic examination of the kidney tissue of the control group showed normal appearance of parenchyma, glomeruli with mesangial cells, normal Bowman's space and capillaries, normal tubules with normal lining cells, and normal interstitium (Fig. 5a). No differences were found between the control group and those treated orally (high and low dose) or s.c. with HAp nanoparticles during the histological examination of kidney tissues as shown in Fig. 5b–d.

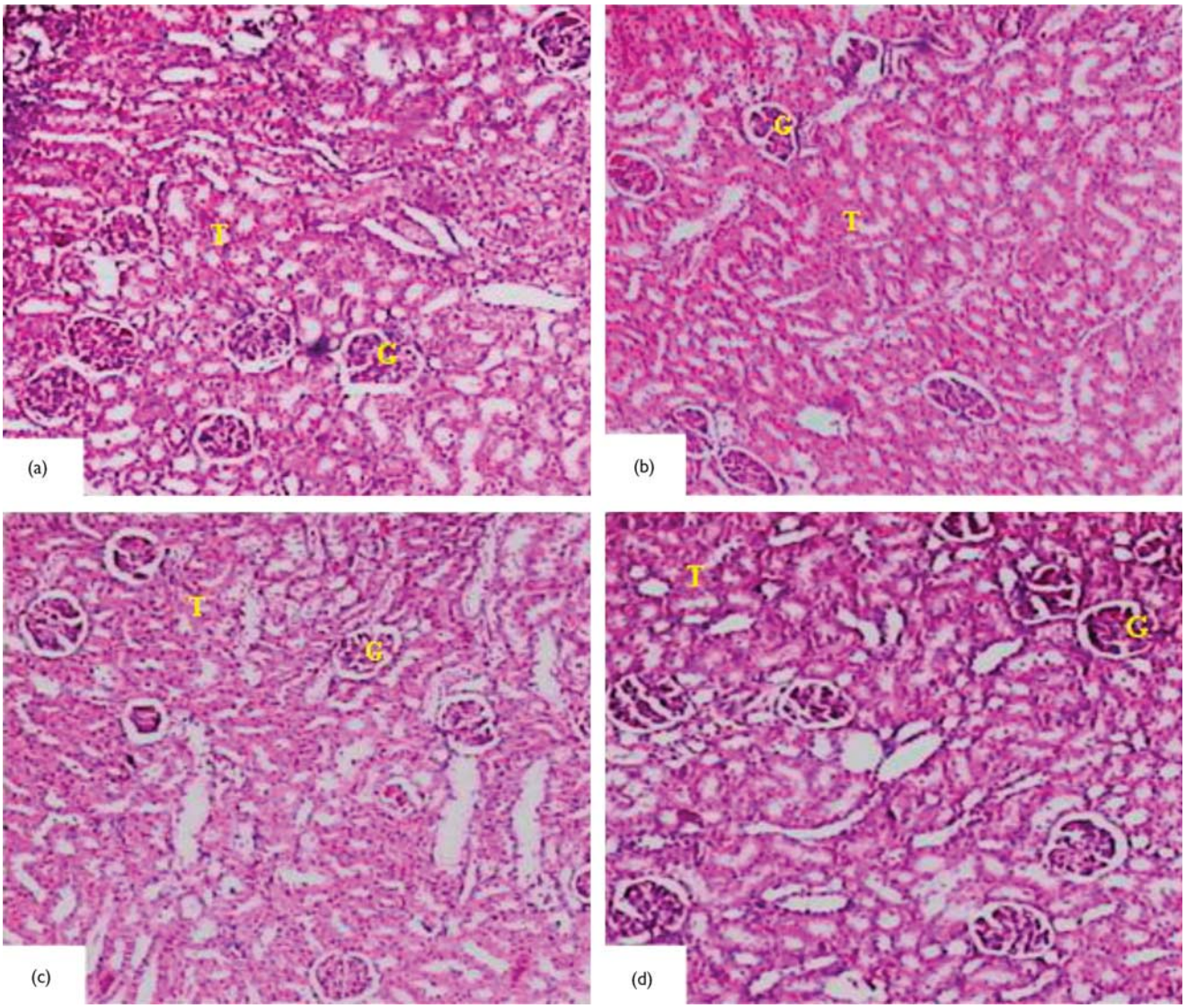
The results indicated that the synthesized HAp nanoparticles exerted no toxic effects on the kidney as indicated by kidney function tests (uric acid, urea, and creatinine) and histological examination. However, it exerted a significant effect on the liver function ALT and AST; this shows that the metabolism of HAp nanoparticles

Figure 4



A photomicrograph in the liver of (a) the control group showing normal central vein and hepatocyte architecture; (b) rats treated orally with a high dose of nano-hydroxyapatite (Hap) (300 mg/kg body weight) showed a normal histological structure of the central vein and surrounding hepatocytes; (c, d) rats treated orally with a low dose of nano-HAp (150 mg/kg body weight) showed a normal structure of hepatocytes, portal vein, and bile ducts; (e) rats treated subcutaneously with nano-HAp (600 mg/kg body weight) showed a normal structure of hepatocytes. H&E, × 100.

Figure 5



A photomicrograph in rats' kidney of: (a) the control group; (b) rats treated orally with a high dose of nano-hydroxyapatite (Hap) (300 mg/kg body weight); (c) rats treated orally with a low dose of nano-HAp (150 mg/kg body weight); and (d) rats treated subcutaneously with nano-HAp (600 mg/kg body weight) showing the normal appearance of parenchyma, glomeruli (G) with mesangial cells, normal Bowman's space and capillaries, normal tubules (T) with normal lining cells, and normal interstitium. H&E, $\times$ 100.

was mainly through the liver as reported by Hou *et al.* [20]. These results were in agreement with those of Abdel Gawad *et al.* [21], who reported that the animals injected with 300 and 600 mg/kg body weight showed a significant increase in serum AST and reverted to almost the normal level after 48 h. It is well documented that HAp particles are converted into Ca^{2+} and PO_4^{3-} ions by a natural metabolic process and eliminated over a period of 6 weeks [22,23]. Moreover, Xie [24] has reported that the maximum concentration of intravenous nano-HAp was detected in the liver and spleen at 1 h after administration and decreased significantly after 72 h, which explains the increase in liver function in the treated rats. However, all the animals had normal ALP levels, indicating that bone metabolism was not disturbed with nano-HAp. The ALP level is known to be indicative of hepatobiliary disease [25,26] or a mild hepatocellular injury [27].

Taken together, the current results showed that HAp is safe when administered orally or s.c. in Sprague–Dawley female rats at different concentrations (150, 300, and 600 mg/kg body weight). Similar to the current observation, Hu *et al.* [10] reported that HAp is safe and could induce inhibition of implanted hepatic VX2 tumor growth in rabbits and cell p53/c-Myc protein expression. Moreover, Abdel Gawad *et al.* [21] have reported that the liver and kidney in animals treated with HAp showed a normal structure and HAp could restore most of the normal structure of liver and kidney after treatment with lead nitrate.

Conclusion

The current study showed that the synthesized HAp nanoparticles ranging from 3 to 7 nm in diameter and from range 27 to 46 nm in length was safe when

administered orally or s.c. in Sprague–Dawley female rats at different concentrations (150, 300, and 600 mg/kg body weight). The biochemical results showed an increase in liver function, whereas kidney function was normal as shown by biochemical results as in the control group. The histopathological examination indicated that liver and kidney tissues of all rats treated with HAp nanoparticles (orally or s.c.) showed a normal structure compared with the control group.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

References

1 Fu L, Aik Khor K, Peng Lim J. The evaluation of powder processing on microstructure and mechanical properties of hydroxyapatite (HA)/yttria stabilized zirconia (YSZ) composite coatings. Surface Coatings Technol 2001; 140:263–268.

2 LeGeros RZ, Lin S, Rohanizadeh R, Mijares D, LeGeros JP. Biphasic calcium phosphate bioceramics: preparation, properties and applications. J Mater Sci Mater Med 2003; 14:201–209.

3 Nelea V, Pelletier H, Mille P, Muller D. High-energy ion beam implantation of hydroxyapatite thin films grown on TiN and ZrO_2 inter-layers by pulsed laser deposition. Thin Solid Films 2004; 453-454:208–214.

4 Fouda MFA, Nemat A, Gawish A, Baiuomy AR. Does the coating of titanium implants by hydroxyapatite affect the elaboration of free radicals. An experimental study. Aust J Basic Appl Sci 2009; 3:1122–1129.

5 Génin FY, Luo P, Dash AK. Hydroxyapatite based drug delivery implant for cancer treatment. 2004; US Patent 6767550.

6 Fernández-Pareja A, Hernández-Blanco E, Pérez-Maceda JM, Riera Rubio VJ, Palazuelos JH, Dalmau JM. Prevention of osteoporosis: four-year follow-up of a cohort of postmenopausal women treated with an ossein-hydroxyapatite compound. Clin Drug Investig 2007; 27:227–232.

7 Rauschmann MA, Wichelhaus TA, Stirnal V, Dingeldein E, Zichner L, Schnettler R, Alt V. Nanocrystalline hydroxyapatite and calcium sulphate as biodegradable composite carrier material for local delivery of antibiotics in bone infections. Biomaterials 2005; 26:2677–2684.

8 Suchanek W, Yoshimura M. Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants. J Mater Res 1998; 13:94–117.

9 Li L, Pan H, Tao J, Xu X, Mao C, Gu X, Tang R. Repair of enamel by using hydroxyapatite nanoparticles as the building blocks. J Mater Chem 2008; 18:4079–4084.

10 Hu J, Liu ZS, Tang SL, He YM. Effect of hydroxyapatite nanoparticles on the growth and p53/c-Myc protein expression of implanted hepatic VX 2 tumor in rabbits by intravenous injection. World J Gastroenterol 2007; 13:2798–2802.

11 Sabry R. 2012. Preparation of hydroxyapatite nanoparticles by using emulsion liquid membrane [PhD Thesis]. Egypt: Al-Azhar University.

12 Reitman S, Frankel S. Colorimetric method for aspartate and alanine tranferases. Am J Clin Pathol 1957; 28:56–63.

13 Roy AV. Rapid method for determining alkaline phosphatase activity in serum with thymolphthalein monophosphate. Clin Chem 1970; 16:431–436.

14 Haisman P, Muller BR. *Glossary of clinical chemistry terms*. London: Butterworth-Heinemann; 1974.

15 Fawcett JK, Scotte JE. A rapid and precise method for the determination of urea. J Clin Pathol 1960; 13:156–159.

16 Bartels H, Böhmer M, Heierli C. Serum creatinine determination without protein precipitation. Clin Chim Acta 1972; 37:193–197.

17 Drury RAV, Wallington EA. *Carltons histological techniques*. 5th ed. New York: Oxford University Press; 1980. p. 206SY.

18 SAS Institute Inc., *SAS user's guide: statistics*. Cary, NC: SAS Institute Inc.; 1982.

19 Waller RA, Duncan DB. A Bayes rule for the symmetric multiple comparison problems. J Am Stat Ass 1969; 64:1484–1503.

20 Hou CH, Hou SM, Hsueh YS, Lin J, Wu HC, Lin FH. The in vivo performance of biomagnetic hydroxyapatite nanoparticles in cancer hyperthermia therapy. Biomaterials 2009; 30:3956–3960.

21 Abdel Gawad EI, Awwad SA. In-vivo and in-vitro prediction of the efficiency of nano-synthesized material in removal of lead nitrate toxicity. J Am Sc 2011; 7:105–119.

22 Chinol M, Vallabhajosula S, Goldsmith SJ, Klein MJ, Deutsch KF, Chinen LK, *et al*. Chemistry and biological behavior of samarium-153 and rhenium-186-labeled hydroxyapatite particles: potential radiopharmaceuticals for radiation synovectomy. J Nucl Med 1993; 34:1536–1542.

23 Unni PR, Chaudhari PR, Venkatesh M, Ramamoorthy N, Pillai MR. Preparation and bioevaluation of 166Ho labelled hydroxyapatite (HA) particles for radiosynovectomy. Nucl Med Biol 2002; 29:199–209.

24 Xie JSG. Tissue distribution of intravenously administrated hydroxyapatite nanoparticles labeled with ^{125}I. Nanoelectronics Conference (INEC), 3rd International, Hong Kong, China, January 3-8, 2010. pp. 1415–1416.

25 Abdel-Wahhab MA, Omara EA, Abdel-Galil MM, Hassan NS, Nada SA, Saeed A, el-Sayed MM. Zizyphus spina-christi extract protects against aflatoxin B1-initiated hepatic carcinogenicity. Afr J Tradit Complement Altern Med 2007; 4:248–256.

26 Abdel Wahhab MA, Hassan NS, El Kady AA, Khadrawy YA, El Nekeety AA, Mohamed SR, *et al*. Red ginseng extract protects against aflatoxin B1 and fumonisins-induced hepatic pre-cancerous lesions in rats. Food Chem Toxicol 2010; 48:733–742.

27 Sharma A, Sharma V, Kansal L. Amelioration of lead-induced hepatotoxicity by Allium sativum extracts in Swiss albino mice. Libyan J Med 2010; 5:1–10.