Biocompatibility of Intravenous Nano Hydroxyapatite in Male Rats

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Abstract: This study was carried out to evaluate the potential impact of nano hydroxyapatite (nano-HAp) intravenous injection with different concentrations on survival rates and the reaction affecting heart, liver and thyroid functions as well as the level of circulating nitric oxide. An inorganic-organic complexion route was selected for the synthesis of nano-HAp which was characterized by fourier transform infrared, x-ray diffraction, transmission electron microscope, and analyzed using inductively coupled plasma mass spectrometry. The synthesized nano HAp was thermally sterilized and prepared with different concentrations (150, 300, 600, 1200, 1800 and 2400 mg/kg b.w.) to be injected intravenously into different groups of male rats. Mortality percentage was recorded throughout the experiment. Blood samples were collected at time intervals of ½, 3, 6, 12, 24 and 48 hours after injection. The present results revealed that intravenous injection of nano- HAp at a dose of 2400 mg/kg b.w. was determined as lethal dose, where all the animals in this group died within ten minutes after injection. On the other hand, the amount of 150 mg/kg b.w. nano-HAp did not affect on the investigated parameters but the amount of 300 mg/kg b.w. appeared slightly variable changes in CKMB, LDH, AST and T_3 recovered after 24 hours. The animals injected with 600, 1200 1800 mg/kg b.w. appeared significant increase in serum CKMB, LDH, AST and thyroxin returned to approximate the normal level after 48 h. This experiment was repeated for two years under the same environmental conditions as well as the animals strain and the results were almost the same. [Nature and Science 2010;8(9):60-68]. (ISSN: 1545-0740).

KEYWORDS: Nano hydroxyapatite, Intravenous injection, thyroxin, cardiac enzymes, liver enzyme, NO

1. Introduction

With the development of material processing technology, nanomaterial is being widely used. Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ is a bioactive ceramic with a crystal structure similar to native bone and teeth minerals due to its structural similarity to bone mineral, enamel and dentin (Fua et al., 2001, LeGeros et al.,2003, Nelea et al., 2004, Kim et al., 2004 and Fouda et al., 2009). Nanoparticles of HAp are low crystalline particles with highly active surface. The studies of nano-HAp as a biological medicine have been the most important aspect bothering the investigators. Since the early studies on this subject, up to now, the most important biological end point has been the various uses of HAp in medical field especially hepatic tumor (Hu et al., 2007). It have been frequently used as genetic carrier (Bauer,et al., 2008), drug delivery (Hu et al., 2007), repairing material of bone defect in clinic (Suchanek and Yoshimura, 1998, Nakano et al., 2002). However, HAp nanoparticles have stronger anti-tumor effect than macromolecules with minimal side effect and could bring strong cooperative effect with chemotherapy medicine which, in turn, will result in reducing the toxicity of it (Pezzatini et al., 2006 and Hu et al., 2007). The invaginations in the cell membrane before nanoparticle uptake suggested endocytic pathways as internalization mechanism. When HAp nanoparticles were added to cell culture medium, the particles immediately became instable and formed agglomerates with a size of about 500–700 nm. The TEM showed internalized HAp captured by vacuoles in the cytoplasm of the hepatocellular carcinoma cells (Bauer et al., 2008).

Recently, some studies showed that HAp nanoparticles have biotoxicity which is affected by the diameter of the particles, exposure dose and contact way (Xu et al., 2009) and cautions should be exercised before using these nanoparticles as the size morphology, and concentration of nano-HAp have significant effect on the biological response (Hussain et al., 2009). However, optimal biological performance has not been established yet.

Therefore, in the present study, it could be synthesized nano-HAp in spherical smaller particles size to avoid the previous mentioned side effects and investigated the potential impact of intravenous injection of made nano-HAp with various concentrations, up to lethal dose, on survival rate and nitric oxide level as well as thyroid, heart and liver functions. This experiment was continued for two years on the same animals strain under the same environmental conditions to confirm the obtained data of made nano-HAp in biological applications.

2 - Materials and Methods

Chemicals:

The chemicals used were calcium nitrate tetrahydrate (Ca $(NO_3)_2.4H_2O$, Mwt. 236.15g/mole, Merk, Germany), di ammonium hydrogen ortho phosphate anhydrous ($(NH_4)_2HPO_4$, 132.06g/mole, S.D. Fine Chem. Ltd. Mumbai, India), poly vinyl alcohol (PVAL) (Mwt. ≈160000 g/mole), and ammonium hydroxide (NH_4OH, Mwt. 35.5g/mole, May & Baker, England). All chemicals were used in the experimental work without further purification.

Synthesis and characterization of nano HAp:

5% of PVAL solution was prepared in 1-L flat bottom flask using deionized water while stirring and heated at 80˚C for 30 min. Then calcium nitrate ($Ca(NO_3)_2.4H_2O$) was added. Finally, diammonium hydrogen ortho phosphate ($(NH_4)_2HPO_4$) was added to the mixture with Ca/P atomic ratio 1.67 while stirring and heating at 80˚C under pH control. The formed was dried at 100°C for24 h and characterized using FTIR, XRD and TEM in order to prove the formation of hydroxyapatite structure. Brunauer-Emmett-Teller absorption technique was used to determine the specific surface area of the formed powder by PMI's instrument model 201A made in USA using nitrogen gas technique. The synthesized nano HAp was thermally sterilized using muffle furnace at 700^{o}C for 7 h.

Experimental Design:

Preliminary experiments were carried out to determine the lethal dose of nano-HAp. Firstly, a group of male rats was intravenous injected with prepared nano-HAp at a dose of 160mg/kg b.w. which was previously reported by Aoki et al., (2000) as lethal dose for rats. Secondly, a group of rats was intravenous injected with 200 mg/kg of nano-HAp which was previously determined by Liu et al (2005) as lethal dose for rabbit. Then, the concentration of nano-HAp increased gradually to other groups till reach to lethal dose (all animals died). The experiment was repeated under the same environmental conditions and the same animals strain to confirm the obtained data.

Based on the preliminary study, sixty male rats with average weight of 140 g, were divided into six groups, each of ten. Then, each animal group injected intravenously with a single dose of nano-HAp saline solution at different concentrations (150, 300, 600, 1200, 1800 and 2400 mg/kg b.w.). Control rats received the same dose of saline solution. The mortality rate was recorded in each group and died animals were compensated to maintain the animals number in each group (n=10). Blood samples were collected from orbital venous plexus of all rats at fixed time intervals of ½, 3, 6, 12, 24 and 48 hours after injection to investigate the effect of nano-HAp on nitric oxide (NO) level as well as heart, liver, thyroid functions. It was be difficult to collect samples from the animals that received 2400 mg/kg of nano-HAp because all the animals died within a time ranged from 5 to 10 min. after injection.

Biochemical and hormonal assays:

Nitric oxide was measured using Griess reagent, colorimetric Kit, according to method of Green et al. (1982). Creatine phosphokinase isoenzyme (CK-MB) activity was estimated using auto analyzer, Boehringer Mannheim GmbH, Germany according to techniques originated by Klauke et al., (1988). Serum level of lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed using kinetic kits (Stanbio Laboratory, Boeme, Texas) based on a technique originated by Buhl and Jackson (1978) for LDH and AST and ALT according to of Breuer (1996). The thyroid hormones, tetra-iodothyroxine (T_4) and Tri-iodothyroxine (T_3) were assayed by solid phase radioimmunoassay techniques using commercial kits purchased from (Siemens Medical Solutions Diagnostics, Los Angeles, USA) based on the technique described by Tietz (1995).

Statistical analysis:

Data were expressed as the mean ± S.E. Means were compared by T-test to identify differences treated and control groups. A value of $P< 0.05$ and 0.01 were accepted as significant.

3. Result

Characterization of the formed HAp:

The FTIR analysis of the formed HAp dried at 100˚C for 24h. Figure (1) showed the characteristic bands of HAp $Ca_{10}(PO_4)_6(OH)_2$ which appeared clearly. The two bands at 630 and 3570 cm^{-1} belonged to the vibration of hydroxyl OH gradually appeared. Those bands at 1036, 1091 and 963 cm^{-1} are the characteristic bands of phosphate PO_4^{3-} stretching vibration, while the bands at 603 and 565 cm^{-1} were due to phosphate bending vibration (Lee and Kim, 2002).

The XRD analysis of the dried powder at 100°C for 24 h identified the presence of HAp crystal structure (Mc Clune, 1982) with no any other calcium phosphate structure as shown in figure (2). The surface area of the dried powder was 405 m^2/g with total pore diameter 0.4621 cc/g. the average pore diameter was 45.63Å. The synthetic of HAp in polymeric media (polymer matrix) without any chemical interaction between organic–inorganic

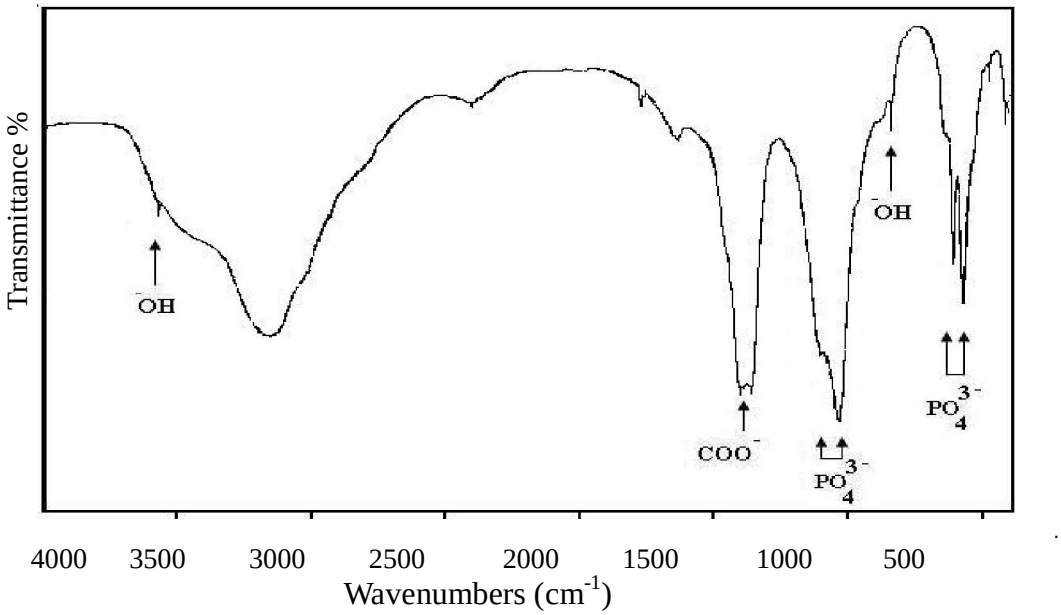

Fig. 1: IR spectra of HAp.

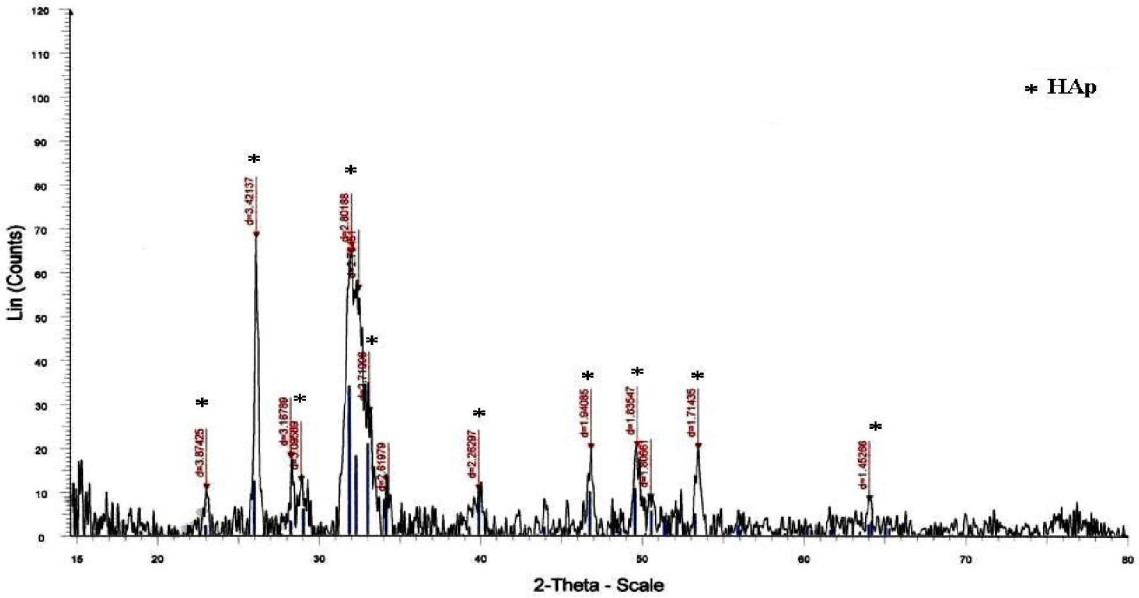

Fig.2 : XRD patterns of the HAp dried at 100°C for 24 h.

interface leads to the formation of HAp in nano scale with high surface area (Mizushima et al., 2006). The advantages of this technique showed to have: low processing temperature, high molecular level homogeneity, improved purity, morphology, texture, and a scope to tailor the made compound.

The transmission electron microscope showed nano-HAp of ultra small crystals distributed in matrix as shown in figure (3) with average grain size of 40 nm. TEM micrograph depicted the precipitation of hydroxyapatite aggregates in porous poly (vinyl-alcohol)–gelatin matrix. TEM studies showed a uniform distributed of HAp with self-assembled and aggregates of uniform size and morphology. Due to the very fine size of precipitated particles present in the aggregates, they could not be individually resolved, however, their crystalline nature and phase identification could be ascertained through selected area diffraction pattern (fig.3) which confirm the formation of nano hydroxyapatite phase.

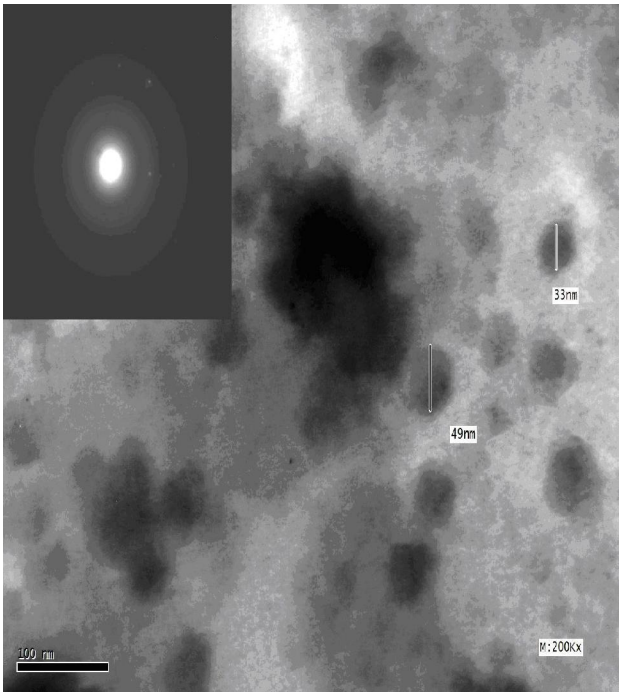

Fig.3: TEM micrograph analysis of nano-HAp dried at 100°C for 24 h with its diffraction pattern.

Determination of the mortality rate:

The lethal dose of intravenous nano-HAp was determined as 2400 mg/kg b. w., where at this dose all the rats died (100%) within 10 min (fig. 4). About 50 % of the animals died when injected with nano-HAp at a dose of 1200 mg/kg (LT_{50}).

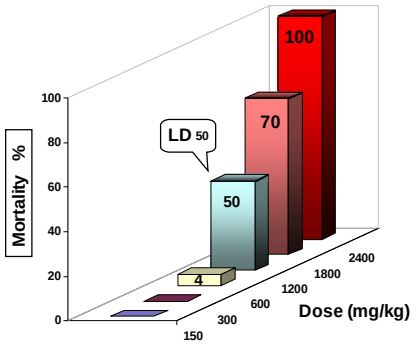

Fig.(4): Mortality percentage of intravenous nano-HAp with different concentrations.

Biochemical results:

Figure.(5) revealed that intravenous injection of nano-HAp into rats with different concentrations of 150, 300, 600, 1200 and 1800 mg/kg b.w. had a slightly non significant effect on NO level through 48 h after injection as compared to control value.

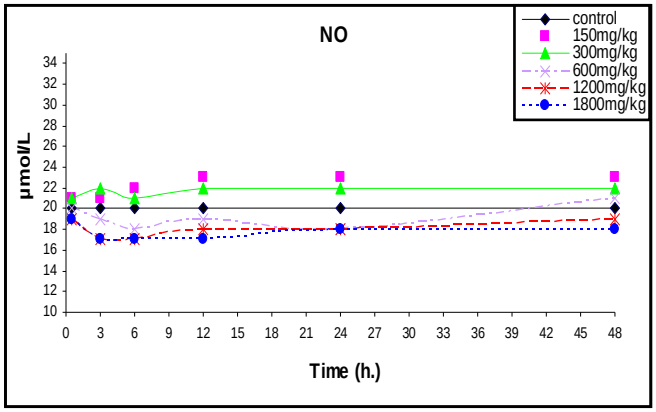

Fig. 5: NO pattern after intravenous injection of different concentrations of nano-HAp.

As shown in figure (6), intravenous injection of nano-HAp at a dose of 150 and 300 mg/kg b. w. did not affect on the value of creatine phosphokinase isoenzyme (CK-MB) to the control value. Whereas, the animals received 600, 1200 and 1800 mg/kg b.w. of nano-HAp intravenously revealed significant increase in CK-MB value after 3 h of injection. This increase recovered to approximate the normal value after 24 h. The changes in CK-MB pattern was obviously related to nano-HAp concentrations and time progression.

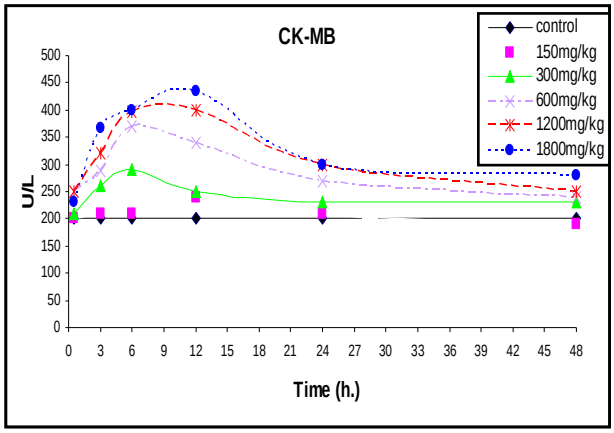

Fig. 6: CK-MB pattern after intravenous injection of different concentrations of nano-HAp.

Animals received 150 mg/kg b.w. of nano-HAp showed non significant changes in LDH value as compared to control. But, LDH value showed significant increase at 6 and 12 h after nano-HAp injection. In contrary, the value of LDH showed progressive significant increase in 30 min., reaching the peak at 12 h. later in animals received 600 mg/kg of nano-HAp and after 6 h. in animals received 1200 and 1800mg/kg. However, LDH value returned to normal 48 h. after injection in all animals as shown in figure (7).

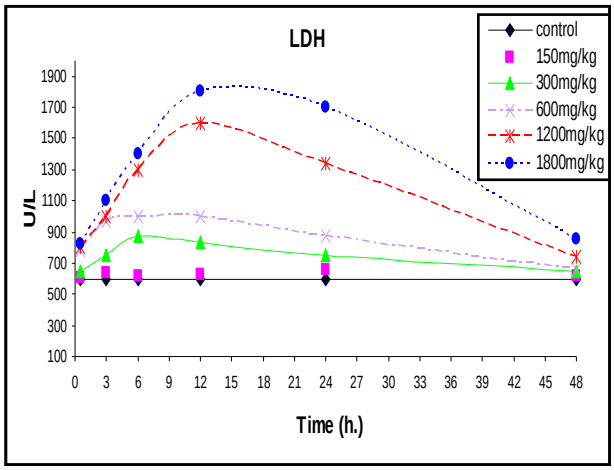

Fig. 7: LDH pattern after intravenous injection of different concentrations of nano-HAp.

AST value apparently had variable changes according to the nano-HAp concentrations. Since, intravenous injection of 150 mg/kg b. w. nano-HAp did not show significant changes in AST value at all time intervals. Whereas, the animals injected with 300 mg/kg b.w. recorded significant increase at 12 and 24 h after injection. On the other hand, the animals received 600, 1200 and 1800 mg/kg of nano-HAp b.w. revealed significant increase in AST value after 3 h as compared to control value (fig. 8).

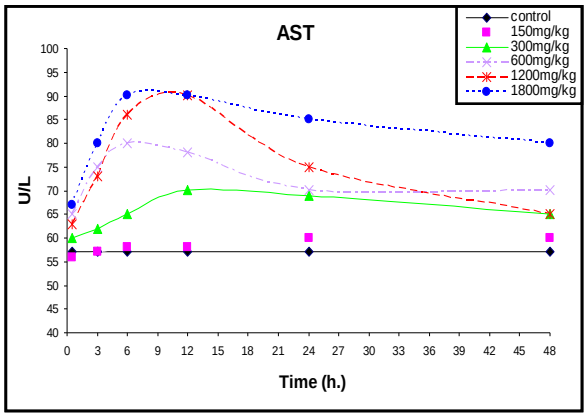

Fig. 8: AST pattern after intravenous injection of different concentrations of nano-HAp.

With respect to ALT values, it recorded variable non significant changes through the experimental period in animals injected with different concentrations of nano-HAp as compared to control value (fig. 9).

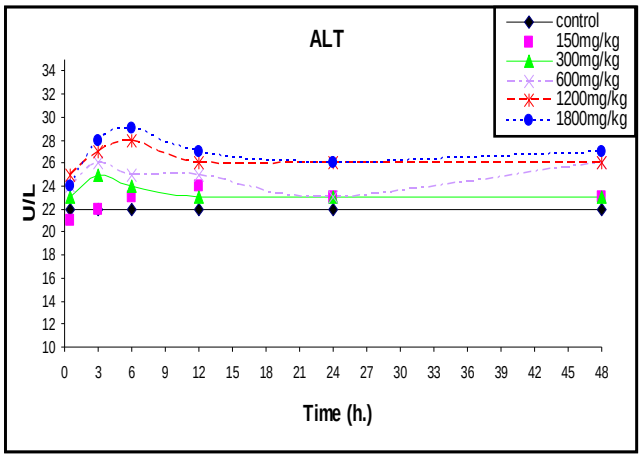

Fig. 9: ALT pattern after intravenous injection of different concentrations of nano-HAp.

Figure (10), clarified that intravenous injection of nano-HAp at a dose of 150 mg/kg b.w. into rats did not affect T_3 level at all investigated times as compared to control. On the other hand, when nano-HAp at a dose of 300 mg/kg was injected, the level of T_3 showed significant increase at 6 and 12 h, and then returned to normal value after 24 h of injection. It was observed that the level of T_3 increased in 3 h in animals receiving nano-HAp at doses of 600, 1200 and 1800 mg/kg nano-HAp then recovered to normal after 24 h. of injection.

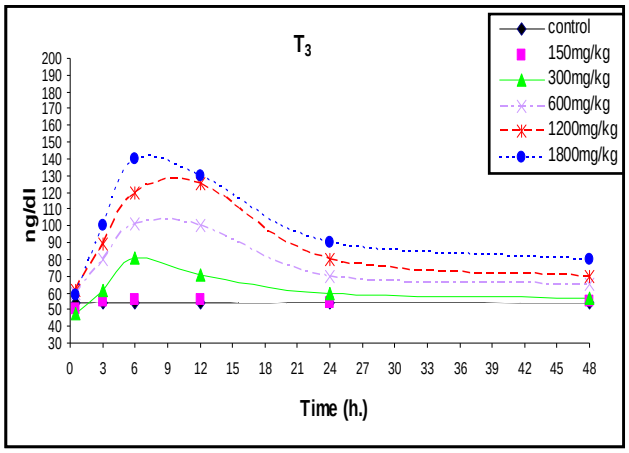

Fig. 10: T_3 pattern after intravenous injection of different concentrations of nano-HAp.

As shown in figure (11), when the animals received 150 and 300 mg/kg of nano-HAp intravenously, they showed non remarkable changes in the circulating level of T_4. When the animals injected with 600,1200 and 1800 mg/kg of nano-HAp showed remarkable increase in T_4 value in 30 min till 12 h after injection, then it returned to normal value at 24 and 48 h after injection.

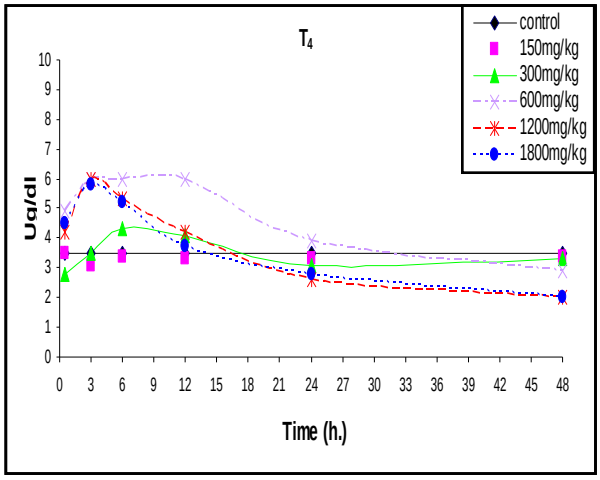

Fig. 11: T_4 pattern after intravenous injection of different concentrations of nano-HAp.

4. Discussion

Nano-HAp used for this research was synthesized by organic-inorganic reaction in polymeric matrix rout, having a good dispersive effect, its particles size with about 40 nm is spherical and very uniform, which meets the requirement for nano meter grade (0.1 nm-100 nm), having advantage of high surface energy, that can not exist for the large particle size of HAp.

In the current study, nano-HAp at a dose of 2400 mg/kg b.w. was determined as lethal dose. Such dose was greatly higher than the lethal dose which previously reported by Aoki et al., (2000) in rat and Liu et al., (2005) in rabbit. This variation was achieved mainly due to the very fine size particles of nano-HAp which used in this research. The features of the results of nano-HAp toxicity, regardless of few exceptions, showed that the most mortality of animals occurred when injected with doses higher than 600 mg/kg b.w. Besides, lethal time as well as number of died animals recorded through the experiment was nano-HAp dose dependent. This is because of the high proportion of phenomenon of decreasing O_2 and CO_2 partial pressure after nano-HAp intravenous injection with high concentration or due to temporarily blockage in the lung veins by excess concentration of crystals suspending in nano-HAp solution (Aoki et al 2000).

It is well established that overt hyperthyroidism induces a hyperdynamic cardiovascular state which is associated with a faster heart rate. However, the signs and symptoms referable to the cardiovascular system may be the only manifestation of overt thyroid dysfunction and persistent subclinical thyroid dysfunction may notably increase the cardiovascular risk (Fazio et al., 2004). Extensive evidences indicated that the cardiovascular system responds to the minimal but persistent changes in circulating thyroid hormone levels, which are typical of individuals with subclinical thyroid dysfunction (Klein and Ojamaa, 2001). In this context, it is important to recognize that thyroid hormone also modifies the expression of ion channels, such as Na+/K+-activated adenosine triphosphatase (ATPase), Na^+/Ca^{++} exchanger, and some voltage-gated K^+ channels, thereby coordinating the electrochemical and mechanical responses of the myocardium (Ojamaa et al., 1999). It is pertinent the endothelial dysfunction coexists with many disease states in cardiovascular system. The most important mechanism for endothelial dysfunction is the decrease in NO availability. The vascular flow and the shear stress caused by vascular flow induce NO synthesis by phosphorylation of nitric oxide synthesis (NOS). Nitric oxide synthesis catalyzes the reaction which converts L-arginine to citrulline and NO and requires help of calmodulin and pteridin tetrahydrobiopterin (BH_4) as cofactors (Cengel and Sahinarslan, 2006). The more important to mention that nitric oxide has both prooxidant and antioxidant activities in the endothelium; however, the molecular mechanisms involved are still a matter of controversy (Fouda et al., 2009). It could be regulate mitochondrial oxidative stress protection via the transcriptional coactivator PGC-1 (Borniquel et al.,

2004). However, when ROS production is increased, tetrahydrobiopterin (BH_4) generation is reduced, and eNOS produces superoxide ($\bullet O^{2-}$). Excess generation of $\bullet O^{2-}$ by different sources (NADPH oxidase, uncoupled eNOS, xanthine oxidase, myeloperoxidase, cyclooxygenase, mitochondria) will reduce NO bioavailability and convert NO into peroxynitrite ($ONOO^-$), which has deleterious effects (Joshua and Hare, 2004).

Following this important introductory notation, the results recorded in the current study are very consisting with biological rationality. Since, it showed that intravenous injection of nano-HAp (600, 1200 and 1800 mg/kg) caused temporarily increase in circulating thyroxin as well as myocardial enzymes level associated with non significant change in circulating NO level. Most of the studies carried out on HAp, dealt with the effect of treatment using this material on various medical therapy, however, there is a lack of data regarding to serum levels of thyroxin hormones under the effect of different doses of nano-HAp. The studies on its physiological effect might be useful in view of the use of HAp during critical period of rat development (Monika et al., 2005). These studies suggested that HAp caused disturbances in thyroid hormones through apatite ions that disrupts the trapping of iodide as well as facilitates the discharge of unorganified iodide from the thyroid gland. Based on these remarks, it could be assumed that the notably elevation in serum thyroid hormones (T_3 and T_4) of nano-HAp treated animals was reasonable for the increase in cardiac enzymes levels, leading to an under estimation of the extent to which the cardiac enzymes pattern contributes to improving thyroidal performance. On the other hand, nano-HAp had a biological function in tissues, cells and blood (Aoki et al., 2000) including motivation of cells activity leading to withdrawal of enzymes from cytoplasm into blood (Aoki et al., 2000). Since, injection of nano-HAp with high concentrations into blood may be allowed to vast amounts of its crystals entering into the cells by phagocytosis or led to environmental changes that occurred by the alteration of calcium, phosphorus, plasma bicarbonate ($PHCO_3$) and total carbon dioxide (TCO_2) values (Aoki et al., 2000 and Liu et al., 2005).

Interestingly, the elevation of cardiac enzymes was biologically considered as oxidative stress but, the level of NO unaffected in nano-HAp treated animals. It seemed that nano-HAp sustained endothelial survival without any cytotoxic effect and maintained the endothelial cells biochemical markers as healthy endothelium (Pezzatini et al., 2006). On the other hand, nano-HAp could be inhibits the formation of oxidative and nitrooxidative species (Fouda et al., 2009) by the following mechanism:

1- It favors the isomerization of peroxynitrous acid (ONOOH) to nitric acid.
2- It favors the non symmetrical cleavage of ONOOH yielding OH^- and NO^-, radicals.
3- It inhibits the symmetrical cleavage which leads to formation of OH and NO radicals.
4- It inhibits the transformation of ONOOH to $CO^{\cdot}{}_3^-$ and NO_2 via reaction with CO_2.

Among all nano-HAP treated animals, there was a decline in serum ALT enzyme in 30 min. In this aspect, several authors (Kunida et al., 1993, Liu et al., 2003 and Hu et al., 2007) had studied the effect of nano-HAp on hepatic tumor in vitro and in vivo. They reported that although nano-HAp inhibits the hepatic tumors growth, it had a little effect on physiological function of the organism with no obvious adverse reaction in the body.

5- Conclusion

Nano HAp (20-40nm) could be synthesized with average grain size of 20-40 nm by inexpensive simple technique. The advantages of this technique are: low processing temperature, molecular level homogeneity, improved purity, morphology. An intravenous lethal dose of made nano-HAp in rats was identified as 2400 mg/kg and the factors that could have the potential impact, lower molecular size level of synthesized nano-HAp with high surface area, well dispersed form without other toxic side products and speed of injection. This study was extended to examine the biocompatibility of nano-HAp through the follow up of heart and liver enzymes pattern and the activity of thyroxin hormones. These experiments were repeated under the same conditions for two years and the results obtained were similar. It must be mentioned in this respect that disturbance in enzymes and thyroid hormones activities after nano-HAp injection are irreproducible. This fact is also true for all concentrations of nano-HAp that used in this study. The only effect that was reproducible to a very far extent is animal death. From the current results, it could be highly recommended with the use of intravenous nano-HAp at a dose of 150 and 300 mg/kg in a single safely dose, absorbent into blood without side effects for biomedical applications.

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5/5/2010