

Acute Oral Toxicity Analysis of Nano-Hydroxyapatite-Gelatin Suspension in Albino Wistar Rats

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Abstract

Hydroxyapatite (HAp), a calcium phosphate-based bio-ceramic was prepared from waste ostrich bone using the thermal decomposition method, and the nano-HAp was segregated by the water-in-oil microemulsion technique. A single acute dose of oral toxicity of nano-HAp in gelatin suspension was tested in 11 female Albino Wistar rats following the OECD 420 guidelines. The rats were divided into three groups: three for control, three for group I, and five for group II. The first group was given 300 mg of nano-HAp in gelatin suspension per kg of body weight, while group II was given 2000 mg per kg of body weight. Results show that no signs or symptoms of toxic effects were seen in the group during the 14-day study period. Furthermore, no significant change in their average body weight or other physical behaviors such as autonomic, respiratory, or somatomotor effects were observed in the rats. The macroscopic examination of internal organs and bodyweight observation have shown no symptoms of toxicity in either group. It could be concluded that the nano-HAp suspension in gelatin does not show any acute toxic effect. The lethal 50% dose (LD₅₀) of the nano-HAp-Gel suspension has been estimated to be more than 2000 mg/kg of the body weight, suggesting that nano-HAp extracted from ostrich bone is safe to use for calcium supplements and other biomedical applications.

Keywords: *Nano-hydroxyapatite, gelatine suspension, acute toxicity test, Albino Wistar rat, OECD-420 guidelines.*

1. Introduction

Natural bone comprises hydroxyapatite (HAp), an inorganic calcium phosphate (CaP) mineral that is primarily embedded in the collagen matrix [1]. It is one of the main parts of vertebrate bones and other mineralized tissues [1-5]. The nano-HAp is well known for its excellent biomedical properties such as osteoconductivity, osteoinductivity, and biocompatibility. It has been used for various biomedical purposes such as implant coating, bone tissue engineering, drug/protein/gene delivery, bone grafting, dental fillings, catalysis, sensing, and chromatographic separation [6-11]. Recent studies have shown that it can be used as an oral source of calcium and phosphorous for bone health and growth as well as the prevention of osteoporosis [12,13]. In the studies of Hanh *et al.* [12] and Bezerra & Donangelo [14], it has been reported that using nano-HAp extracted from biogenic sources as a calcium supplement for pregnant and lactating mothers promotes the bone health of the fetus and lactation. Furthermore, taking an appropriate dose of nano-HAp during pregnancy can help to improve mesoscale bone density at the terminal portion of the legs and hands while also protecting them from hypocalcemia [12, 15,16]. Among the various CaP supplements, nano-HAp has been found to have a high potential for bone strength regeneration [16]. So, before using this as a calcium supplement or for other biomedical applications, it is important to perform the acute toxicity test [17–20].

Generally, the acute toxicity test is suggested for drugs that are chemically synthesized or extracted

from plants [21]. Then, the test compound is orally gavaged to the animals and the symptoms afterwards are monitored using an OECD guideline [22, 23]. Hanh *et al.* [12], Remya *et al.* [21], Mohanan *et al.* [24], Parayanthala Valappil *et al.* [25], and Mosa *et al.* [26] performed the acute toxicity tests of chemically synthesized HAp in animal models. However, similar studies of nano-HAp extracted from biogenic sources have not been reported yet. This work aims to conduct the acute toxicity tests of nano-HAp extracted from ostrich bone biowaste on the albino Wistar rats.

2. Materials and Methods

2.1. Materials

The analytical grade chemicals were used for all the experiments without further purification. Gelatin type-A (approx. 300 bloom) was obtained from Fisher Scientific, India. Normal saline was purchased from Otsuka Pharmaceutical Company, India. The nano-HAp was extracted from ostrich femur bones as reported in our earlier studies [27, 28].

2.2. Preparation of Oral Gavage Suspension

The nano-HAp used in this study is a natural bioceramic that does not dissolve in water. But it can be dispersed in water using a magnetic stirrer. However, it is segregated from the dispersion medium after some time after removing the stirrer force. So, to solve this problem, an aqueous gelatin solution was used. Due to its nontoxic nature and crosslinking ability, gelatin can bind the nano-HAp and mitigate the problems

associated with segregation. For this purpose, 300 mg of nano-HAp as per the body weight of rats, was blended with 15 wt.% gelatin solution at 500 rpm for 6 hours. Before oral gavage, the suspension was again sonicated for 15 minutes.

2.3. Experimental Rats

For the study, nine healthy Albino Wistar rats (*Ratus norvegicus*), each approximately 10 weeks old and weighing 100-133g, were purchased from the animal breeding center, Department of Plant Resources, Ministry of Forest, and Environment (MoFE) Thapathali, Kathmandu. The experiments were conducted from January 15 to October 8, 2020. All rats were treated in compliance with Nepal's ethical guidelines for animal care and utilization in health research [23]. The moral approval for the toxicity test was obtained from the Nepal Veterinary Council (NVC) (Ref. No. Ethical.17/2077/78).

For the toxicity test, the rats were randomly selected and kept in clean polypropylene cages for seven days before dosing with the nano-HAp-Gelatin suspension to allow them to acclimate to the laboratory environment. The temperature and the relative humidity of the room were maintained at 23–25 °C and 50-65%, respectively, throughout the experiment. Artificial lighting was provided with twelve-hour light and twelve-hour dark cycles according to OECD 420 guidelines [29]. Each rat was handled with proper attention and care and without causing any physical stress, as per animal handling guidelines [29]. The animals were settled on a clean paddy husk bed that was

replaced twice a week and were fed with a commercially available standard diet available in the market and distilled water. Each animal was marked on its tail with a color marker for its identification.

2.4. Acute Toxicity Tests

The toxicity tests were conducted using a fixed-dose method as prescribed by the OECD 420 guidelines which offers information about the substance under investigation according to the principles of the Globally Harmonized System (GHS). The guideline recommends the tests on single-sex animals whereby females have been usually considered more sensitive than males [29]. For the tests, rats were divided into 3 groups (Table 1) and orally gavage with 10 mL of the nano-HAp-Gelatin suspension per kg body weight of each rat [30].

Table 1. Grouping of rats for acute toxicity test of nano-HAp-Gelatin suspension

Group I	Group II	Group III
Control	Medium dose (300 mg/kg body weight)	High dose (2,000 mg/kg body weight)
3 Rats	3 Rat	5 Rats

According to guidelines, the control group was gavage with a 15% w/v gelatin solution. Group II, was given 300 mg/kg (medium dose) of the nano-HAp-Gel suspension, and group III was gavage with a dose of 2000 mg/kg (high dose) of body weight. The starting dosage was selected at 300 mg/kg bodyweight due to the lack of preclinical toxicity evidence for the nano-HAp. Before oral

gavage, the rats were made to fast overnight, but distilled water was given sufficiently.

2.5. Observations

After administration of the dosage, the rat in Group I was kept under observation to monitor the clinical symptoms of toxicity for the first critical 4 hours. As expected, no signs or symptoms of toxicity were observed. Following that, a lethal dose of (2000 mg/kg bodyweight) suspension was gavage to one Group III rat, and clinical symptoms were monitored as usual. This dose also did not show any toxicological signs or symptoms within the critically monitored period. Then, the same dose was administered to all the remaining rats, and the toxic symptoms were observed as usual for the first critical 4 hours. Afterward, the symptoms were monitored every day at 10 a.m. and 3 p.m. during the 14-day study period. Most of the behavioral and physical evaluations, autonomic effects, sensory reactions and responses, pulmonary effects, ability to grasp activity, and time of death, if any, were all seriously observed and documented. Similarly, the change in body weight was regularly noted from the starting day on and continuously for the 2nd, 4th, 6th, 7th, 8th, 10th, 12th, and 14th days, respectively. The percentage of body weight change was calculated using the method used by Venkatasubbu *et al.* [31].

$$\text{Percentage body weight change} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Initial body weight}} \times 100 \quad (1)$$

Finally, the experimental rats were allowed to fast overnight on the 13th day of the experiment, and

then on the 14th day, their weight was recorded, and they were slaughtered under a high dosage of ether anaesthesia. The dead bodies were subjected to an autopsy examination. The actual mass of the major organs (like the lungs, liver, kidneys, ovaries, heart, brain, spleen, etc.) was determined by excising, soaking in saline solution, blotting dry, and finally weighing them. Afterward, an autopsy and a total morphological examination of the delicate organs were carried out. The organs were examined under an optical microscope for any abnormalities and lesions.

2.6 Statistical analysis

All the numerical data of the experimental findings of this study were expressed by the standard deviation and standard error of the mean (SEM) in the MS Excel 2007 sheet for statistical analysis. The so-called "two-tailed student's test" was used to determine statistical significance. The P values with a magnitude less than 0.05 were regarded as being statistically significant.

3. Results and Discussion

3.1. Observation of Physical Signs, Behavioural Effects, and Sensory Parameters

The physical signs and symptoms of all the rats were recorded after the oral gavage of suspension. The results show that the physical signs of the rats in Group III's eating and drinking habits didn't improve until 4 hours after they were given the gavage. However, the behavioral properties such as alertness and reaction to changes in the surroundings were also found to

decrease in the first 30 minutes in comparison to the control group, possibly due to the stress of the gavage but not the toxic effect of suspension. Throughout the study period, all rats showed normal behavior after 30 minutes (shown in Table 2).

On the other hand, grooming, which is an important behavior in rats, was found to be completely absent in the first critical hour because of the stress of gavage. This behavior became completely normal after that. Similarly, the central nervous system (CNS)-related excitation was also totally absent. However, a slight increase in sedation and pain responses was observed after 30 minutes. The effect remained for 2 hours, which might have been caused by the physical stress of gavage.

It might be assumed that the suspension of 2000 mg/kg bodyweight contains a greater amount of nano-HAp than the suspension with a lower dose, which might increase the viscosity of the

suspension. For these reasons, higher pressure had to be applied to the syringe piston of the gavage needle, which might be the cause of pain. However, all the other noted physical, behavioral, sensory, and respiratory signs and symptoms were found to be normal after 2 hours and throughout the study periods. Also, no autonomic, respiratory, or somatomotor effects were observed in experimental rats. Therefore, significant alterations were not observed in the parameters used to evaluate the acute toxicity in this study. Hanh *et al.* [12] and Venkatasubbu *et al.* [31] showed similar results in their studies. All observed physical signs, behavioral effects, autonomic effects, sensory responses, respiratory effects, and somatomotor effects are indexed in Table 2.

Table 2. Observation variables of rats administered nano-HAp-Gelatin suspension at a dosage of 2000mg/kg body mass (n=5)

A. Physical observations	Times								
	0 m	30 m	1h	2h	4h	8h	24h	7D	14D
1. Skin colour	N	N	N	N	N	N	N	N	N
2. Fur colour	N	N	N	N	N	N	N	N	N
3. Eyes colour	N	N	N	N	N	N	N	N	N
4. Urine colour	N	N	N	N	N	N	N	N	N
5. Mucous secretion	N	N	N	N	N	N	N	N	N
6. Eating and drinking habits	N	D	D	D	D	N	N	N	N
7. Itching	A	A	A	A	A	A	A	A	A
8. Mortality	A	A	A	A	A	A	A	A	A
B. Behavioural effects									
(I) Mood									
9. Alertness exploratory activities	N	D	N	N	N	N	N	N	N
10. Eye opened/closed	N	C	C	N	N	N	N	N	N

11. Grooming	N	A	A	N	N	N	N	N	N
12. Restlessness	A	A	A	A	A	A	A	A	A
13. Reactivity towards environment	N	D	N	N	N	N	N	N	N
(II) CNS excitation	A	A	A	A	A	A	A	A	A
14. Tremors	A	A	A	A	A	A	A	A	A
15. Twitches	A	A	A	A	A	A	A	A	A
16. Convulsions	A	A	A	A	A	A	A	A	A
(III) CNS depression	A	A	A	A	A	A	A	A	A
17. Sedation sleep	A	P	P	P	A	A	A	A	A
18. Catatonia	A	A	A	A	A	A	A	A	A
19. Ataxia	A	A	A	A	A	A	A	A	A
20. Coma	A	A	A	A	A	A	A	A	A
C. Autonomic effects									
21. Faces consistency	N	N	N	N	N	N	N	N	N
22. Lacrimation/Tearing	A	A	A	A	A	A	A	A	A
23. Urination	N	N	N	N	N	N	N	N	N
24. Salivation	N	N	N	N	N	N	N	N	N
25. Piloerection	A	A	A	A	A	A	A	A	A
26. Emesis	A	A	A	A	A	A	A	A	A
27. Diarrhoea	A	A	A	A	A	A	A	A	A
D. Sensory responses									
28. Touch response	N	N	N	N	N	N	N	N	N
29. Pain response	N	P	P	P	A	A	A	A	A
E. Respiratory effects									
30. Apnea	A	A	A	A	A	A	A	A	A
31. Dyspnea	A	A	A	A	A	A	A	A	A
F. Somatomotor effects									
32. Abnormal gait	A	A	A	A	A	A	A	A	A
33. Righting reflex/response	N	N	N	N	N	N	N	N	N
34. Body position	N	N	N	N	N	N	N	N	N
35. Limb position	N	N	N	N	N	N	N	N	N

Index: A = Absent, N = Normal, P = Present, D = Decrease, C = Close

3.2. Observation of Nano-HAp-Gelatin Suspension Induced Body Weight Loss

The body weight of the experimental rats slightly decreased after the second day of gavage,

which might be due to the pain caused by the gavage needle. Afterward, there was a slow increment of body weight in all groups. Figure 1 shows that the average body weight increases on

the 4th and 7th days of oral gavage. The study of Mosa *et al.* [32] revealed that orally gavage chemically synthesized HAp nanoparticles were disseminated throughout the body through blood circulation but mostly accumulated in the kidney and stomach, causing apoptosis. It serves crucial functions in physiology and can be triggered by a variety of stimuli, including ischemia and hypoxia. However, no such symptoms of apoptosis have been seen in our study, due to which no statistically significant difference in average body weight was noticed among the control and treated groups throughout the investigations ($p > 0.05$).

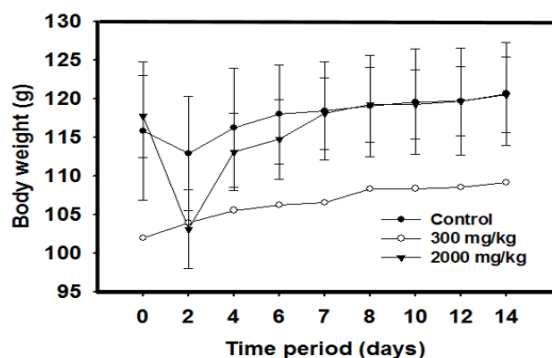


Figure 1: Effect of nano-HAp-Gelatin suspension on the average body weight of the rats subjected to acute toxicity tests. The data of mean values are expressed.

Figure 2 shows the effect of nano-HAp-Gelatin suspension on body weight gain on the 7th and 14th days. The data are averaged across the different numbers (n) of experimental rats used. The bodyweight of the rats was found to increase remarkably in both kinds of groups during the investigation. It was found that rats given the medium dosage (Group II) had a greater improvement in body weight gain than the rats given the high dosage (Group III). This might be

due to the lack of rivalry for food and water. It should be noted that group II kept only three rats, while the high-dose group III accommodated five rats (Table 1).

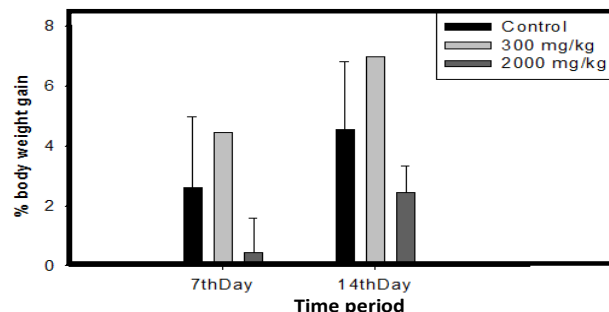


Figure 2: Effect of nano-HAp-Gelatin suspension on percentage body weight gain. The data are expressed as an average (Control n=3, 300 mg/kg n=1, 2000 mg/kg n=5, $p > 0.05$).

Regardless of the cage setting and accommodations, the percentage of body weight gain is determined by a variety of factors, including physical activity, food intake frequency, dietary preferences, and the rat's overall health. The health issue is directly related to the nature of the substance being gavage. Presumably due to the nontoxic nature of bio-source nano-HAp, the rats in the high and low-dose groups showed weight gain on the 7th and 14th days of oral gavage, respectively.

3.3. Analysis of Absolute Mass of Body Organs

The absolute organ weights of both treated and control groups were recorded during necropsy, as shown in Table 3. The results show that the relative organ weight of all treated animals is comparable to that of the control groups (with $p > 0.05$). The delicate internal organs (such as those of the brain, lungs, heart, liver, spleen, kidneys, and ovaries) are sensitive and might be affected

by the metabolic activities of toxic substances that cause changes in their bodyweights within study periods. It is known that the internal organ weight correlates well with the physiological and pathological status of test rats, as reported by Venkatusubbu *et al.* [31]. During the necropsy toward the conclusion of this investigation, the overall macroscopic inspection of the organs of all test groups revealed no abnormalities, even when compared to those of the control groups in terms of color and texture. However, the change in hypertrophy of these organs might be an initial indication of organ toxicity, as reported by Mirza

and Panchal in their studies [33]. The relative organ weight of rats was found to be normal throughout the study period ($p > 0.05$). Similar types of results were also reported by Remya *et al.* [21] on the histopathological and organ body weight evaluation of chronic exposure to nano-HAp in their studies. Therefore, it could be concluded that the oral gavage of nano-HAp-Gelatin suspension hasn't shown any acute toxic effects up to a lethal dose of 2000 mg/kg body weight of the rats. Hanh *et al.* [12] reported similar findings in Swiss Albino rats regarding the acute toxicity of HAp-alginate suspension.

Table 3. The absolute organ weight of experimental animals which received nano-HAp-Gelatin suspension at a dose of 300 mg/kg (n=3), 2000 mg/kg (n=5) and control (n=3) in the acute oral toxicity studies

Gr.	Rats	Brain	Lungs	Heart	Liver	Spleen	R. Kidney	L. Kidney	R. Ovary	L. Ovary
I	1	1.44	0.81	0.45	5.25	0.40	0.42	0.42	0.03	0.03
	2	1.29	0.94	0.44	3.74	0.35	0.40	0.40	0.04	0.03
	3	1.53	1.16	0.46	4.11	0.46	0.37	0.37	0.04	0.04
	Mean	1.42	0.97	0.45	4.36	0.40	0.40	0.40	0.04	0.03
	SD	0.12	0.18	0.01	0.79	0.05	0.03	0.02	0.00	0.00
II	1	1.34	0.82	0.42	3.85	0.31	0.37	0.38	0.03	0.04
	2	1.37	0.81	0.44	3.85	0.30	0.39	0.39	0.04	0.04
	3	1.35	0.81	0.43	3.86	0.30	0.38	0.39	0.03	0.04
	Mean	1.35	0.81	0.43	3.85	0.30	0.38	0.39	0.03	0.04
	SD	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00
	t-test	0.42	0.19	0.09	0.32	0.09	0.33	0.51	0.67	0.22
III	1	1.33	1.05	0.42	3.40	0.49	0.41	0.40	0.04	0.04
	2	1.52	0.82	0.50	4.30	0.40	0.46	0.46	0.05	0.05
	3	1.49	0.83	0.50	3.48	0.37	0.45	0.47	0.02	0.03
	4	1.35	0.94	0.40	2.99	0.33	0.35	0.39	0.03	0.03
	5	1.41	0.87	0.50	4.85	0.36	0.54	0.51	0.04	0.04
	Mean	1.42	0.90	0.46	3.80	0.39	0.44	0.45	0.04	0.04
	SD	0.09	0.10	0.05	0.75	0.06	0.07	0.05	0.01	0.01
	t-test	0.96	0.49	0.70	0.35	0.73	0.32	0.17	0.73	0.47

4. Conclusion

The acute oral toxicity test of nano-HAp-Gelatin suspension was tested according to OECD 420 guidelines. The tests were performed on Albino Wistar rats using the fixed dosage method, with eight animals in the experimental groups and three in the control group. The test animals showed no acute toxicity or mortality. In comparison to the control groups, no significant differences were observed in average body mass, other physical parameters, or behavioral, autonomic, respiratory, and somatomotor effects in the experimental rats. The test animals showed no acute toxicity or mortality. In comparison to the control groups, no significant differences were observed in average body mass, other physical parameters, or behavioral, autonomic, respiratory, and somatomotor effects. Furthermore, a macroscopic examination of delicate internal organs revealed no signs of acute toxicity of the nano-HAp-Gelatin suspension. The tested HAp has a lethal 50% dose (i.e., LD₅₀) value exceeding 2000 mg/kg of body weight, which is consistent with GHS classification and labeling of chemicals. Hence, this study confirmed that ostrich bone-based nano-HAp can

be used as a safer source of calcium supplements and for other biomedical applications.

Limitation of the Study

This was a pilot study that solely aimed to assess the acute toxicity test of nano-HAp-Gelatin suspension in Albino Wistar rats. The studies only focused on the physical observations, changes in body weight, and comparative studies of the internal organ weights of the test and control groups. A suggestion for further investigation into histopathology and cytotoxicity may be allowed.

Declaration of Competing Interests

The authors do not have any kind of competing interests.

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