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Investigation of chronic toxicity of hydroxyapatite nanoparticles administered orally for one year in wistar rats

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ABSTRACT

Although the toxicity/biocompatibility of hydroxyapatite nanoparticles (nano HA), a prospective nano biomaterial is extensively studied, its interaction on biological systems following chronic exposure is less exploited. In the present study, Wistar rats were given various concentrations of nano HA in the diet to determine the chronic toxicity and potential carcinogenicity. Altogether 140 rats were used for the study under various administration dosages along with control. The animals were sacrificed after 12 months of controlled continuous dosing. All in-life parameters, including body weight, food consumption, clinical observations, survival, biochemical and hematology, were unaffected by the chronic exposure of nano HA orally. Similarly, gross and histopathological evaluation was also unchanged following exposure to nano HA. No evidence of nano HA-related lesions or Nano HA-induced neoplasia was suggested in this rodent bioassay study.

1. Introduction

Nanomaterials as defined as materials with at least one external dimension in the size range from approximately 1–100 nm have revolutionized the present era in almost all aspect of human life starting from nano electronic devices, paints, cosmetic and food industries as well as health care systems. Owing to the versatile properties of nano scale materials when compared to their bulk form, they have emerged as an excellent area of applied research. Hydroxyapatite nanoparticles (nano HA), a prospective biomaterial is used in various biomedical areas such as drug delivery, tissue engineering, bone grafts, dental implants and fillings, *etc.* [1–4].

Hydroxyapatite is a natural constituent of human bone and is biocompatible and biodegradable, has got osteo-conductive, osteo-inductive and osteo-integrative properties and hence it has found wide application as a bone substitute. Even though the nanoscale form Hydroxyapatite (nano HA) are documented to be biocompatible [5–7], it is unknown whether they are safe when applied in medical scenarion due to their nano scale particle size. Nano particles owing to their nano scale size can be internalized by the cells where they can interact with the biological molecules altering the cell response which could affect the cells in a deleterious manner leading to toxicological response [8]. The acute toxicity studies of nano HA *in vivo* [9], sensitization studies [10] *in vitro* interaction studies with various cell sources [11], have already been documented in several references, whereas those of chronic toxicity and carcinogenicity *in vivo* were not so thoroughly investigated.

The safety and toxicity of nano HA are of growing concern despite their promising potential in many biomedical applications [12]. The biological activity and bio kinetics of the proposed bio nanomaterial are dependent on many parameters such a size shape, chemistry, charge and surface modification [13]. Chronic toxicity/carcinogenicity studies are therefore essential in identifying the carcinogenic properties of a chemical, primarily its potential to induce neoplastic lesions, and its toxicological responses due to chronic exposure. Identification of target organs is feasible as nanoparticles are carried by the bloodstream to be lodged in various target organs that could elicit cumulative toxicological effects. Chronic toxicity studies are usually conducted in rodent species where the test compound is administered over >90 days, and the animals are observed periodically [14]. The cumulative/long term effect of a test substance is inferred from such a study that could be extrapolated to its clinical translation. A combined chronic toxicity and carcinogenicity study often provides an added advantage such that the experimental animals could be monitored for the development of tumors due to the toxicity of test compound under consideration [15].

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Hence the present study provides information on the possible health hazards likely to emerge following repeated exposure of nano HA over the majority of lifespan in rodents which could be extrapolated for its safe use in humans.

2. Methodology

2.1. Synthesis and characterization of hydroxyapatite nano materials

In-house synthesized Nano HA was used for the study [16]. Nano HA was synthesized by wet chemical method where calcium phosphate was precipitated from the aqueous solution of calcium nitrate tetrahydrate (Ca (NO3)2·4H2O) and ammonium dihydrogen orthophosphate (NH4H2PO4). The chemicals were procured from Sigma-Aldrich. After aging, freeze drying and calcination, particles of size 50 nm was obtained. The particles were characterized for their particle size by Transmission Electron Microscopy (TEM) [Hitachi H-600]. The chemical composition of nano HA particles were compared with standard material using Nicolet Impact 410 FT-IR spectroscopy (Fourier Transform Infrared Spectrometry) and X-ray diffraction (XRD) spectrum was recorded in a diffractometer (Siemens D5005) for phase purity.

2.2. Experimental animals

Healthy Wistar rats weighing 150–180 g were used for the study. They were maintained in a 12 h light/dark cycle at a constant temperature of 22 ± 3 °C and provided with commercially available feed and filtered fresh drinking water *ad libitum*. Individual animals were identified with picric acid marks. In addition to this, each animal cage was identified by labels having details such as experiment number, name, animal number(s) and date of experiment. All animals were handled humanely, without causing pain or distress and with due care. The care and management of the animals were in compliance with the regulations of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Govt. of India. The animal experiments were carried out after prior approval from Institutional Animal Ethics Committee and approved institutional protocol.

2.3. Experimental design

There were 5 groups and each group consisted of 15 male and 15 female rats. The animals were exposed to nano HA in rat feed at concentrations of 0, 25, 50 and 100 mg/kg body wt. for 90 weeks. Table 1 shows corresponding nano HA concentrations. Feed intake was recorded daily. Animals were weighed initially, and weekly till the end of the studies. Animals were observed twice daily and clinical findings were recorded at 4-week intervals.

After 78 weeks the animals on groups1 to 4 were sacrificed and processed for various analyses, while group 5 animals were again observed after providing standard diet without nano HA treatment for a period of 4 weeks.

Table 1
Experimental groups.

Groups	Animals	Average daily ingested dose (mg/kg)
1	Control (15♂ + 15♀)	0
2	Low (15♂ + 15♀)	25
3	Medium (15♂ + 15♀)	50
4	High (15♂ + 15♀)	100
5	High recovery (10♂ + 10♀)	100

Table 2
Common clinical signs and observation.

Clinical observation	Observed sign	Involved system (s)
Respiratory	Dyspnea (abdominal breathing, gasping), aponoea, cyanosis, tachypenea, nostril discharges	CNS, pulmonary, cardiac
Motor	Decrease/increase somnolence, loss of righting, anesthesia, catalepsy, ataxia, unusual locomotion, prostration, tremors, fasciculation	CNS, somatomotor, sensory, neuromuscular, automatic respiratory
Convulsion	Clonic, tonic, tonic-clonic, asphyxial opisthotonos	CNS, neuromuscular, autonomic, respiratory
Reflexes	Corneal, righting, myotact, light, startle reflex	CNS, sensory, autonomic, neuromuscular
Ocular signs	Lacrimation, miosis, mydriasis, exophthalmos, ptosis, opacity, iritis, conjunctivitis, chromodacryorrhea, relaxatrion of nicititating membrane	Autonomic, irritation
Cardiovsacular signs	Bradycardia, tachycardia, arrhythmia, vasodialation, vasoconstriction	CNS, autonomic, cardiac, pulmonary
Salivation	Excessive	Autonomic
Piloerection	Rough hair	Autonomic
Analgesia	Decrease reaction	CNS, sensory
Muscle tone	Hypotonia, hypertonia	Autonomic
Gastrointestinal	Soft stool, diarrhea, emesis, diuresis, rhinorrhea	CNS, sensory, GI motility, Kidney
Skin	Edema, Erythema	Tissue damage and irritation

2.4. Ante mortem observations

The animals were observed daily for general appearance, behavior, signs of morbidity and mortality (Table 2). Each week, all animals were given a detailed physical examination that included palpation for the presence of tissue masses. Individual body weights and feed intake were determined biweekly for the duration of the study.

2.5. Biochemical and hematological parameters

Blood from optical plexus was collected in EDTA vials and analyzed for routine hematological parameters such as Hemoglobin (Hb, g/dl), total count (WBC × 10^3/mm^3), red blood corpuscles count (RBC × 10^6/mm^3), platelet count (PLT × 10^3/mm^3), using automated Vet ABC Animal blood counter (ABX Diagnostics, France). For biochemical analysis blood was collected and the serum was analyzed for biochemical parameters such as urea, Serum Glutamic Oxaloacetic transaminase (SGOT), Serum Glutamic Pyruvate Transaminase (SGPT), Alkaline Phosphatase (ALP), Gamma-Glutamyl Transferase (GGT), glucose (GLU), cholesterol, triglycerides, total protein, albumin, calcium, phosphorus, chloride, total bilirubin and creatinine using automated biochemistry analyzer, ERBA Manheim XL 300 (ERBA, Mannheim, Germany).

2.6. Postmortem examination

All animals were euthanized using carbon dioxide chamber using accepted protocol and subjected to a postmortem examination. At necropsy, all organs and tissues were examined for grossly visible lesions, and the required tissues were fixed and preserved in 10% neutral buffered formalin, for microscopic examination. For all paired organs (*e.g.*, adrenal gland, kidney, ovary, lung, eyes), samples from each organ were examined. The entire gastrointestinal tract was