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Cells–nano interactions and molecular toxicity after delayed hypersensitivity, in Guinea pigs on exposure to hydroxyapatite nanoparticles

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ABSTRACT

The aim of the study was to evaluate the cells–nanoparticle interactions and molecular toxicity after delayed hypersensitivity in Guinea pigs, exposed to hydroxyapatite nanoparticles (HANP). The study focuses on synthesizing and characterizing HANPs and gaining an insight into the cytotoxicity, molecular toxicity, hypersensitivity and oxidative stress caused by them *in vitro* and *in vivo*. HANP was synthesized by chemical method and characterized by standard methods. Cytotoxicity was assessed on L929 cells by MTT assay and *in vitro* studies were carried out on rat liver homogenate. *In vivo* study was carried out by topical exposure of Guinea pigs with HANP, repeatedly, and evaluating the skin sensitization potential, blood parameters, oxidative stress in liver and brain and DNA damage (8-hydroxyl-2-deoxyguanosine: 8-OHdG) in liver. The results of the study indicated that there was no cytotoxicity (up to 600 μg/mL) and oxidative damage (up to 100 μg/mL), when exposed to HANPs. It was also evident that, there was no skin sensitization and oxidative damage when HANP were exposed to Guinea pigs.

1. Introduction

Nanotechnology focuses on the manipulation of materials and its exploitation in various fields, such as biology, medicine, pharmacology and electronics for the benefit of mankind. Advances in nanotechnology led to the exposure of humans to engineered nano materials and hence it became necessary to evaluate the potential human health effects before these materials are fully exploited. Nanoparticles are diverse class of small-scale (<100 nm) substances with novel properties like small size, large surface area, particular shape and surface activity. Nanomaterial toxicology is an important sub discipline of nanotechnology, which deals with the possible toxic effects of nanomaterials [1]. Toxicity of nanoparticles depends on their size, composition, surface functionalization *etc.* The major toxicological issue associated with the manufactured nanomaterials is that some of them are redox active and can be transported across cell membranes and then they can interact with subcellular organelles. As a consequence, nanoparticles can interact directly with individual target cells, either at the external membrane or the cytoplasm. Nanoparticles can be used for drug delivery either as the drug itself or as a drug carrier [2]. Delivery of nanomaterials through skin has potential applications in drug delivery due to its large surface area and can be challenging as the skin acts as a barrier.

Delayed hypersensitivity or allergic contact dermatitis is a sensitive immunological response caused by substance when it comes in contact with the cutaneous layer. The delayed hypersensitivity reactions are mediated by T cells and monocytes/macrophages rather than by antibodies. Major lymphokines involved in delayed hypersensitivity reactions include monocyte chemotactic factor, interleukin-2, interferon-γ and Tumor Necrosis Factor α [3]. When an allergen comes in contact with the skin, the epidermal langerhan cells internalize and process it. The cells that contain these antigens move from epidermis to draining lymph nodes. They undergo functional maturation and become immunostimulatory dendritic cells which are able to present antigen to T lymphocytes [4]. The T cells become activated and proliferate and are able to recognize and respond to the same allergen on subsequent exposure. The activated T lymphocytes will release inflammatory cytokines that will initiate cutaneous inflammatory response [5].

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Delayed hypersensitivity normally includes two phases, *i.e.*, induction phase and elicitation phase. In the induction phase, the skin is exposed to a substance repeatedly. After topical exposure, the particle absorption can follow transcellular, intercellular or transappendageal pathways [6–8]. On repeated application the body recognizes the substance as foreign and an immune response is evoked. Here, the substance induces proliferation and clonal expansion of allergen responsive T lymphocytes and provides immunological memory [9–12]. On subsequent exposure with the substance on skin, the memory T lymphocytes elicit an increased immune response, normally within 24–48 h, which is known as elicitation phase [13]. The clinical manifestations in animals vary from erythema, edema to necrosis.

Experimental evidence has shown that engineered nanoparticles may induce DNA damage and apoptosis through reactive oxygen species (ROS) generation and oxidative stress [4,8]. Due to the high reactivity of ROS, most cellular components are likely to be targets of oxidative damage: lipid peroxidation, protein oxidation, GSH depletion and DNA single strand breaks. All of these events ultimately lead to cellular dysfunction and injury [5]. For this reason antioxidant enzymes are vital markers for oxidative stress induced in the body. Aerobic organisms possess antioxidant defense systems that deal with the removal of ROS [14–16]. As long as there exist a balance between oxidative stress and antioxidant defense system, the body is maintained at optimal health [17,18]. Free radicals have an affinity to damage the DNA bases leading to their modifications. Of these, 8-OHdG with a hydroxyl group at the eighth position of guanine is formed easily and abundantly by oxidative stress [19,20]. 8-OHdG is considered a universal oxidative stress marker in the body. This biomarker is sensitive and proportionally corresponds to the degree of oxidative stress caused in the body. The present study focuses on the synthesis, characterization and bio-distribution of an in-house synthesized HANP, both *in vitro* and *in vivo.*

2. Materials and methods

2.1. Chemicals

Thiobarbituric acid (TBA), reduced glutathione (GSH), oxidized glutathione (GSSG), and dithio-bis-2-nitrobenzoic acid (DTNB) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Pyrogallol (PG), diethylene triamine penta acetic acid (DTPA), trichloro acetic acid (TCA) and other chemicals and reagents used were of analytical grade.

2.2. Equipments

Spectrophotometer (Shimadzu, Japan), laminar air flow (Mark Air Particulars, India), incubator shaker (New Brunswick Scientific, USA), biophotometer (Eppendorf, Germany) and steam sterilizer (Nat Steel, India).

2.3. Experimental animals

Albino Guinea pigs (Hartley) and Albino rats (Wistar) were procured from the Division of Laboratory Animal Sciences of Biomedical Technology Wing, SCTIMST, Trivandrum. The Guinea pigs were housed in the anodized aluminum cages (individually). Albino rats were housed in individually ventilated cages. They were maintained in a 12 h light and dark cycle at controlled environmental conditions of temperature ($22 \pm 2\,^{\circ}C$) and humidity (30–70%). Commercially available feed and distilled water were provided *ad libitum*. The experiments were performed after obtaining prior approval from the Institutional Animal Ethics Committee and as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

2.4. Synthesis of hydroxyapatite nanoparticles (HANPs)

HANPs were synthesized by wet chemical method where calcium phosphate was precipitated from the aqueous solution of calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) and ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$) (Rankem, India). Precipitation was carried out at a pH of 11 and at $0\,^{\circ}C$ for 2 h. After aging for 24 h, the precipitate was washed in distilled water, freeze-dried and calcined at $300\,^{\circ}C$. The calcined precipitate was ball milled and sieved to collect particle of size below 50 nm.

2.5. Physico-chemical characterization of HANPs

The synthesized HANPs were physico-chemically characterized using standard techniques. Transmission electron microscopy (TEM) was performed to obtain the particle size using TEM (H-600). The Infra Red spectrum of HANPs was compared with standard material using Nicolet Impact 410 FT-IR spectroscopy and X-ray diffraction (XRD) spectrum was recorded in a diffractometer (Siemens D5005) for phase purity. The zeta potential of the HANP in buffer and water was analyzed using a Malvern Zeta sizer. In order to establish the stability of HANP in the cell suspension, the sample was freeze dried (in cell suspension) and observed in EDS and scanning electron microscopy (SEM) analysis.

2.6. Cytotoxicity studies

Cytotoxicity assay of the synthesized HANP was carried out by MTT assay (direct contact method). This assay is a simple non-radioactive, colorimetric assay used to measure cytotoxicity, cell proliferation or viability. MTT is a yellow, water-soluble tetrazolium salt. Metabolically active cells are able to convert this dye into a water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring [21]. Formazan crystals, can be dissolved in an organic solvent, dimethylsulphoxide (DMSO) and quantified by measuring the absorbance of the solution at 540 nm, and the resultant value is related to the number of living cells. To determine cytotoxicity, the L929 Fibroblast cells were plated at a density of 2×10^4 cells/well in a 96-well plate at $37\,^{\circ}C$ in 5% CO_2 atmosphere. Briefly, HANP at different concentrations (10, 25, 50, 100, 200, 400, 600 and 700 μg/mL) in triplicates were added on to a confluent monolayer of L 929 mouse fibroblast cells. After incubation of cells with HANP at $37 \pm 1\,^{\circ}C$ for 24 ± 1 h, cell culture was examined microscopically for morphological changes and quantitated by MTT assay. Phenol served as positive control and untreated cells served as negative control. 20 μL of MTT dye solution (5 mg/mL in phosphate buffer pH 7.4) was added to each well. After 4 h of incubation the MTT was removed and formazan crystals were solubilized with 200 μL of DMSO. The absorbance of each well was read on a microplate reader (ELx 808 IU ultra microplate reader, Bio-Tek Instruments, USA) at 540 nm. The relative cell viability (%) with that of control wells, containing cell culture medium without nanoparticles, was calculated.

2.7. In vitro assessment of oxidative stress

The *in vitro* assessment of oxidative stress on exposure to HANPs was evaluated using rat liver homogenate. Wistar rats (200–250 g body weight) were sacrificed by cervical dislocation and their liver was rapidly excised, washed in normal saline and immediately placed in an ice bath. 10% freshly prepared (in 0.1 M phosphate buffer, pH 7.4) liver homogenate was incubated with increasing