Colloids and Surfaces B: Biointerfaces 112 (2013) 204–212

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

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

C.S. Geetha [a], N.S. Remya [a], K.B. Leji [a], S. Syama [a], S.C. Reshma [a], P.J. Sreekanth [b], H.K. Varma [b], P.V. Mohanan [a,b,*]

[a] Toxicology Division, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram 695 012, Kerala, India
[b] Bioceramic Laboratory, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram 695 012, Kerala, India

ARTICLE INFO

*Article history:*
Received 21 February 2013
Received in revised form 19 July 2013
Accepted 29 July 2013
Available online 8 August 2013

*Keywords:*
Delayed hypersensitivity
HANP
LPO
Antioxidant enzymes
8-OHdG

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.

© 2013 Elsevier B.V. All rights reserved.

## 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].

* Corresponding author at: Toxicology Division, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram 695 012, Kerala, India. Tel.: +91 471 2520266; fax: +91 471 2341814.
*E-mail addresses:* mohanpv10@gmail.com, mohanpv@sctimst.ac.in (P.V. Mohanan).

0927-7765/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.colsurfb.2013.07.058

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 $2 \times 10^4$ 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 $24 \pm 1$ 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

concentration (12.5, 25, 50 and 100 μg/mL) of HANP for 3 h independently at 37 °C in a shaking water bath and then centrifuged at 3500 rpm for 10 min at 4 °C. The resultant supernatants was maintained in an ice bath until used for the estimation of total protein, lipid peroxidation (LPO), glutathione reductase (GR), reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and 8-OHdG using procedures mentioned below [22].

### 2.8. Delayed hypersensitivity assay

Adult healthy Guinea pigs, weighing 300–500 g were used for the study (10 for test and 5 for control). Prior to each application period, hair was clipped on the upper back, on either side of the vertebral column of each animal and swabbed with 70% alcohol.

Paste of HANP (80 mg/animals) in physiological saline was applied topically on the clipped upper back region of test animals (10 animals) in the induction phase. Similarly a patch of four ply gauze saturated in physiological saline (control) was applied to the control animals (5 animals). This application site was covered with occlusive dressings and kept for 6 h. Procedure was repeated thrice a week for three weeks. The challenge phase was fourteen days after the last application of the induction phase. During this stage, all the test and control animals were challenged with the HANPs. Hair was removed from the flank area of all the animals (untested area) and lightly swabbed with 70% alcohol and HANP was applied as in the induction phase. The site of application was secured with occlusive dressings. The dressings and patches were removed after 6 h. Following this the skin sensitization potential was evaluated at 24, 48 and 72 h [23].

At the end of observation period blood was collected for analysis of routine hematological and biochemical parameters. The animals were sacrificed and their liver and brain rapidly excised, washed in normal saline, and immediately placed in an ice bath. 10% of tissue homogenate was prepared in phosphate buffer (0.1 M, pH 7.4). These homogenates were analyzed for Total protein, LPO, GSH, GR, GPx, SOD and 8-OHdG.

### 2.9. Blood parameters

#### 2.9.1. Hematology

Blood was collected in EDTA vials and analyzed for routine hematological parameters such as Hemoglobin (Hb, g/dl), total count (WBC $\times 10^3/mm^3$), red blood corpuscles count (RBC $\times 10^6/mm^3$), platelet count (PLT $\times 10^3/mm^3$), using automated Vet ABC Animal blood counter (ABX Diagnostics, France).

#### 2.9.2. Biochemistry

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).

### 2.10. Preparation of tissue 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 (*in vitro* studies). Similarly, the liver and brain from the Guinea pigs used for delayed hypersensitivity assay were collected. 10% of tissue homogenate was prepared in phosphate buffer (0.1 M, pH 7.4) using an ice-chilled glass homogenizing vessel in a rotor stator homogenizer at 900 rpm [22].

Rat liver homogenate was incubated with increasing concentration (12.5, 25, 50 and 100 μg/mL) of HANP for 3 h at 37 °C in a shaking water bath. At the end of incubation period, it was centrifuged at 3500 rpm for 10 min at 4 °C. The liver and brain homogenate of Guinea pigs used for delayed hypersensitivity was centrifuged at 3500 rpm for 10 min at 4 °C. The resultant supernatants were maintained in an ice bath until used for the estimation of total protein, LPO, GR, GSH, GPx, SOD and 8-OHdG using standard protocols with slight modifications.

### 2.11. Total protein

Total protein in rat liver homogenate (*in vitro*), liver and brain homogenate of Guinea pigs used for delayed hypersensitivity were estimated by the method of Lowry et al. [24] using bovine serum albumin as standard.

### 2.12. Lipid peroxidation (LPO)

The extent of LPO in rat liver homogenate (*in vitro*), liver and brain homogenate of Guinea pigs were determined as the concentration of malondialdehyde (MDA) generated by the thiobarbituric acid reactive substances (TBARS), as described by Ohkawa et al. [25]. The amount of malondialdehyde (MDA) formed was measured spectrophotometrically at 532 nm and expressed in nmol/mg protein.

### 2.13. Glutathione reductase (GR)

Glutathione reductase activity in rat liver homogenate (*in vitro*), liver and brain homogenate of Guinea pigs were determined by measuring the reduction of GSSG in the presence of NADPH as described by Mize and Langdon [26]. Briefly, this assay measures the rate of NADPH oxidation to $NADP^+$, which is accompanied by a decrease in absorbance at 340 nm and can be monitored spectrophotometrically and expressed as units/mg protein. Thus, one GR unit is defined as the reduction of 1 μM of GSSG per minute at 25 °C and pH 7.6.

### 2.14. Reduced glutathione (GSH)

The level of GSH in rat liver homogenate (*in vitro*), liver and brain homogenate of Guinea pigs used for delayed hypersensitivity was determined by the method of Moron et al. [27], with slight modifications in which Ellman's reagent or DTNB (5,5′-dithiobis-(2-nitrobenzoic acid), reacts with GSH to form a spectrophotometrically detectable product at 412 nm. The change in absorbance is a linear function of the GSH concentration in the reaction mixture and is based on the reaction of GSH with DTNB to give a compound that absorbs at 412 nm. The amount of GSH was expressed as nmol/mg protein.

### 2.15. Glutathione peroxidase (GPx)

Activity of GPx from both *in vitro* (rat liver homogenate) and *in vivo* (liver and brain homogenate of Guinea pigs) experiment was assayed by the method described by Rotruck [28]. The remaining GSH after the enzyme catalyzed reaction was complexed with 5,5′dithiobis 2-nitrobenzoic acid (DTNB) that absorbs at maximum wavelength of 412 nm. Enzyme activity was expressed as units/mg protein.

### 2.16. Superoxide dismutase assay (SOD)

Assay of superoxide dismutase in rat liver homogenate (*in vitro*), liver and brain homogenate of Guinea pigs was done using modified

pyrogallol autooxidation method, which can be spectrophotometrically measured at 420 nm (Marklund and Marklund [29]). It is expressed in units/mg protein.

### 2.17. 8-Hydroxyl-2-deoxyguanosine (8-OHdG) detection by ELISA

Genomic DNA was extracted from Guinea pig liver using standard protocol by Genelute mammalian genomic DNA miniprep kit, from Sigma Aldrich. 8-OHdG in liver DNA digests was determined by a competitive Immunosorbent assay using High sensitive 8-OHdG check (Japan Institute for the Control of Aging, Fukuroi, Japan). Samples were pretreated by incubating at 95 °C for 5 min and rapidly chilling on ice, digested with sodium acetate, magnesium chloride and DNase I for 10 min at room temperature. This was then centrifuged at 12,000 rpm for 1 min and the supernatants were used for 8-OHdG ELISA.

Assay was carried out as per the manufacturer's instruction. Briefly, digested DNA and primary antibody were added to the 8-OHdG coated microplate, mixed and incubated at 4 °C, overnight. Different concentrations of standards were also used in the experiment. At the end of incubation period, the wells were washed to remove the excess antibody. The enzyme linked secondary antibody was added to the wells and incubated at room temperature for 1 h followed by washing. Then the chromatic solution was added and again incubated at room temperature for 15 minutes in dark. The reaction was terminated by the addition of terminating solution and the absorbance was read at 450 nm using a microplate reader (Asys Expert Plus, Austria). The standard curve was used to determine the amount of 8-OHdG present in the samples. Results were expressed in nanograms per milliliter.

## 3. Data analysis

Statistical analysis was done by Student '*t*' test. The results were expressed as mean ± SD. The '*p*' value less than 0.05 were considered as significant.

## 4. Results

### 4.1. Synthesis and physico-chemical characterization of HANP

HANPs were synthesized by wet chemical method. These particles were characterized by Transmission electron microscopy (TEM), X-ray diffraction analysis (XRD), Fourier transform infrared spectral analysis (FT-IR), zeta potential measurements and SEM/EDS.

Fig. 1A shows the transmission electron microscopic data. It was found that the size of synthesized HANP particle was below 50 nm. The X-ray diffraction pattern of the HANP matches with the standard pattern of the hydroxyapatite (ICDD NO-PDF00-009-0432). Thus the phase purity of the HANP was found to be good (Fig. 1B). The Fourier transform infrared spectral analysis of HANP showed that the hydroxyl stretching was observed at 3568 $cm^{-1}$ and the different phosphate vibrations were observed at 1090, 1036, 962 and 476 $cm^{-1}$ and thus the presence of phosphate and hydroxyl group confirms the chemical nature of hydroxyapatite (Fig. 1C). The zeta potential of the HANP in buffer was found to be high (−13.4 mV) indicating that it is stable in buffer. The HANP was found to be unstable in water (zeta potential was low 3.19 mV). The results of SEM/EDS of HANP in contact with the L 929 cells (Fig. 2A) shows live cells with small white particles attached. This portion was spot analyzed by EDS which confirmed it to be hydroxyapatite with corresponding calcium and phosphorus peaks. The spectrum had the background signals of silica (from cover slip) as well as sodium and chloride from the media (Fig. 2B).

**Table 1**
Observation of Guinea pigs treated with HANP.

| Animal group | Erythema | | | Edema | | |
|---|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| Test (10 animals) | 0 | 0 | 0 | 0 | 0 | 0 |
| Control (5 animals) | 0 | 0 | 0 | 0 | 0 | 0 |

### 4.2. Cytotoxicity

The study investigated the effect of HANP on L929 cell morphology and viability. These cells have typical spindle shaped morphology. It was observed that after 24 h exposure to HANP at varying concentrations, the spindle shaped morphology was preserved. Different concentrations (10, 25, 50, 100, 200, 400, 600 and 700 μg/mL) of HANPs were tested for their cytotoxicity on L-929 fibroblast cells using MTT assay and Fig. 3A and B indicates that, more than 80% cells were viable up to a concentration of 600 μg/mL.

### 4.3. Delayed hypersensitivity

The Guinea pigs used for the study did not show any abnormalities during the experimental period. The appearance of the challenge skin sites of test and control Guinea pigs were observed at 24 h, 48 h and 72 h after removal of dressings and patches. The result of the delayed hypersensitivity assay is shown in Table 1 and it was found that the HANP did not elicit any skin sensitization in Guinea pigs when compared to control animals. The skin irritation score designated for the HANP treated and control areas are '0'. The skin reactions for erythema and edema were scored and the numerical grading was recorded as per ISO 10993-10 [23].

### 4.4. Blood parameters

#### 4.4.1. Hematological parameters

Table 2 indicates that the WBC count of HANP treated animals were well comparable with the control and was within the normal range. There was a significant increase in RBC count, hemoglobin and hematocrit level and a significant decrease in platelet count was observed in HANP treated group when compared to control animals.

#### 4.4.2. Biochemical parameters

The animals treated with HANP showed a slight decrease in SGOT, GLU and urea than that of control animals. The variations observed in SGPT and ALP values were within the normal range. Cholesterol value in test and control groups were lower than the normal range and are not significant. All other biochemical parameters were found to be comparable with control (Table 3).

### 4.5. LPO

Fig. 4A indicates the lipid peroxidation induced in rat liver. The figure represents MDA production in the rat liver homogenate, incubated with the different concentrations of HANP. It was found that the level of MDA induced in the control and HANP treated

**Table 2**
Hematological values of HANP treated animals (mean ± SD, $n = 5$).

| Parameters | Test | Control |
|---|---|---|
| WBC ($10^3/mm^3$) | 7.88 ± 2.23 | 8.18 ± 1.19 |
| RBC ($10^6/mm^3$) | 7.67 ± 0.39* | 4.66 ± 0.28 |
| Hb (g/dL) | 23.04 ± 1.13* | 14.18 ± 0.58 |
| HCT (%) | 62.68 ± 3.14* | 39.62 ± 1.38 |
| PLT ($10^3/mm^3$) | 189.2 ± 43.04* | 426.6 ± 60.33 |

* indicates statistically significant

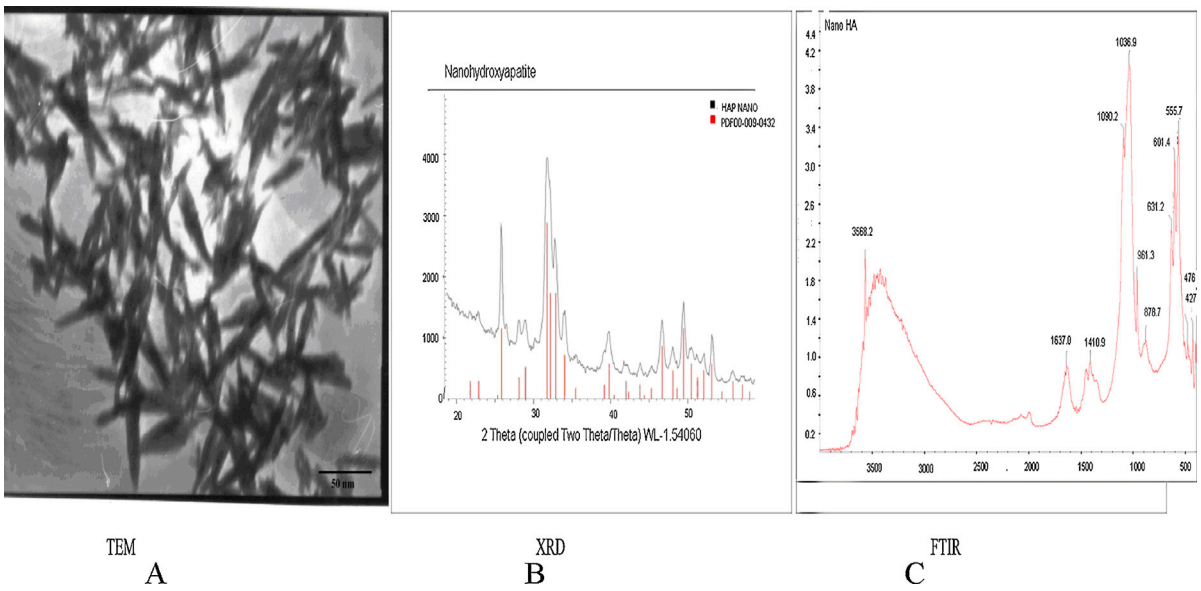


**Fig. 1.** (A) TEM image of HANP, (B) XRD spectra of HANP, (C) FT-IR spectrum of HANP.

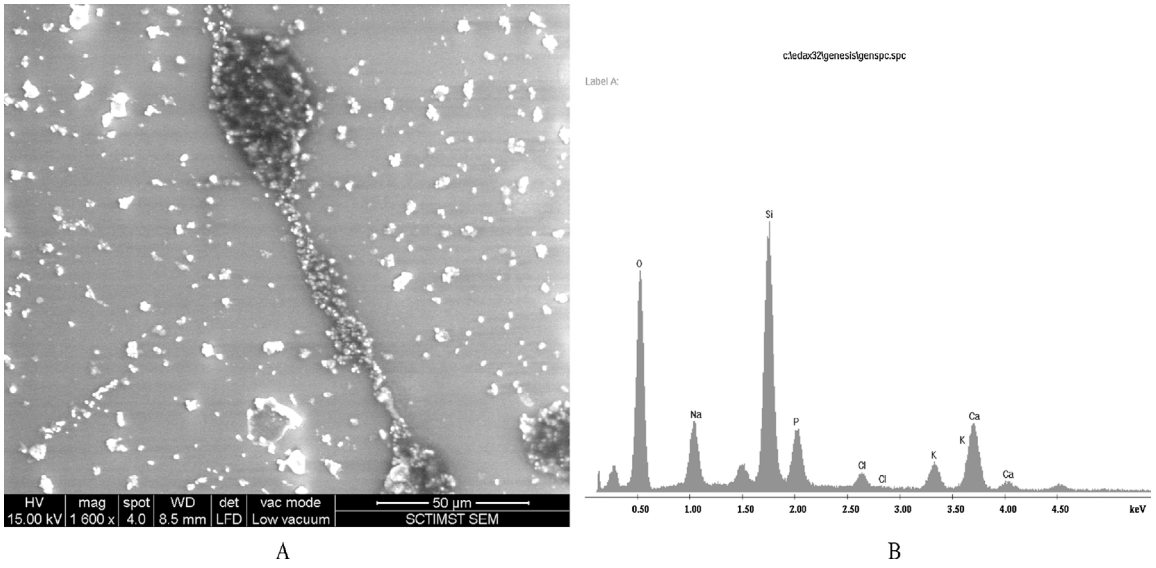


**Fig. 2.** (A) SEM analysis of cells with HANP, (B) spot analysis (EDS) of HANP in cell suspension.

**Table 3**
Biochemical values of HANP treated animals (mean ± SD, $n = 5$).

| Parameters | Test | Control |
|---|---|---|
| Urea (mg/dL) | 55.4 ± 9.83 | 65 ± 4.74 |
| SGOT (IU/L) | 116.4 ± 5.85 | 132.2 ± 25.72 |
| SGPT (IU/L) | 81.8 ± 13.68* | 63.8 ± 9.67 |
| ALP (IU/L) | 121.6 ± 15.3* | 80 ± 22 |
| GGT (IU/L) | 21 ± 5.7 | 16.4 ± 2.07 |
| GLU (mg/dL) | 145.4 ± 6 | 187.4 ± 32.21 |
| CHO (mg/dL) | 27.6 ± 4.39 | 25.6 ± 2.45 |
| TG (mg/dL) | 105.4 ± 32.8 | 87.8 ± 14.7 |
| TP (g/dL) | 5.5 ± 0.46 | 5.3 ± 0.22 |
| ALB (g/dL) | 3.12 ± 0.04 | 3.16 ± 0.32 |
| CAL (mg/dL) | 10.46 ± 0.49 | 10.3 ± 1.03 |
| PHO(mg/dL) | 6.9 ± 1.29 | 6.9 ± 1.35 |
| CHLO (mmol/L) | 105 ± 0.7 | 105 ± 2 |
| BIL. T (mg/dL) | 0.03 ± 0.02 | 0.06 ± 0.02 |
| CRE (mg/dL) | 0.77 ± 0.05 | 0.73 ± 0.09 |

liver (100 μg/mL) is 25.21 ± 1.29 and 31.35 ± 3.68 nmol/mg protein respectively and is significant. The MDA generated in the liver and brain of Guinea pigs exposed to HANPs is shown in Fig. 4B. The level of MDA in HANP (liver: 17.01 ± 3.36, brain: 17.67 ± 1.32) treated group was similar to that of control (liver: 16.38 ± 1.06, brain: 18.69 ± 1.87) group.

### 4.6. GSH

GSH values are explained in Fig. 5A and it was found that there was a decrease in the level of GSH in the liver homogenates incubated with increasing concentration (12.5, 25, 50 and 100 μg/mL) of the HANP. The level of GSH produced in *in vitro* conditions using liver homogenate was 0.732 ± 0.151 (control) and 0.602 ± 0.120 nmol/mg (HANP, 100 μg/mL) respectively. The level of GSH in liver and brain of Guinea pigs treated with HANP was determined and the results are shown in Fig. 5B. It was found that the level of GSH in HANP treated animal was comparable to control animals. Following are the GSH values in control (liver: 1.82 ± 0.49

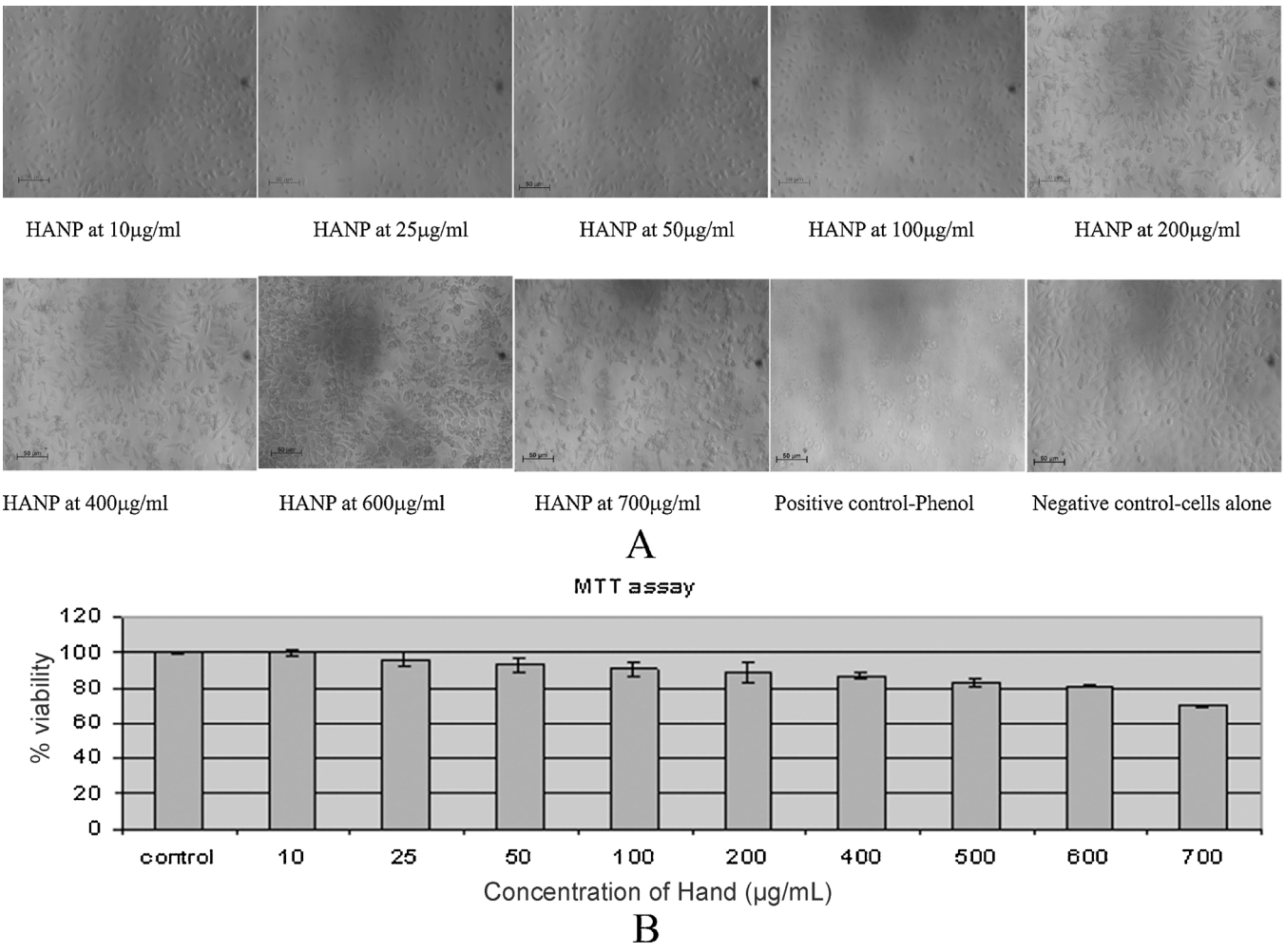


**Fig. 3.** (A) Photomicrograph of L 929 cells treated with HANP (µg/mL), (B) viability of L 929 fibroblast cell line (MTT assay) incubated with the HANP.

and brain: 1.46 ± 0.27) and HANP treated (liver: 1.52 ± 0.38, brain: 1.82 ± 0.14) groups.

### 4.7. GR

The level of GR was not altered when exposed to different concentrations of HANPs in rat liver homogenate (*in vitro*). The GR induction is 0.328 ± 0.026 and 0.336 ± 0.026 in control and 100 µg/mL of HANP respectively (Fig. 6A). In the liver and brain of Guinea pigs exposed to HANP, the level of GR (liver: 0.526 ± 0.180, and brain: 0.586 ± 0.071) was well comparable with control group (liver: 0.371 ± 0.021 and brain: 0.665 ± 0.160) (Fig. 6B).

### 4.8. GPx

In the results of the study mentioned in Fig. 7A it was found that the level of GPx is 0.019 ± 0.002 and 0.027 ± 0.003 in control and 100 µg/mL of HANP treated group respectively (*in vitro*). It was shown that there was an increase in GPx when compared to control values and is not significant. GPx was evaluated in the liver

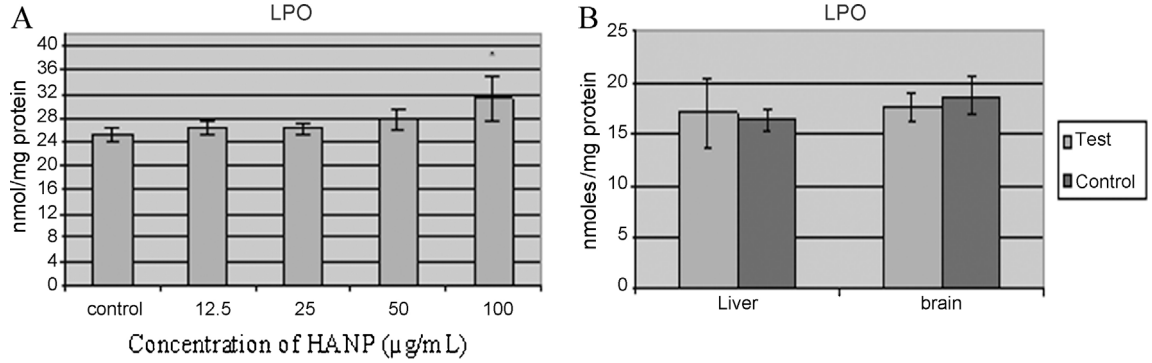


**Fig. 4.** (A) Level of malonedialdehyde in rat liver homogenate exposed to HANP (*in vitro*), (B) LPO of liver and brain of HANPs treated Guinea pigs (values are mean ± SD, $n = 3$, *in vitro* and $n = 5$, *in vivo*).

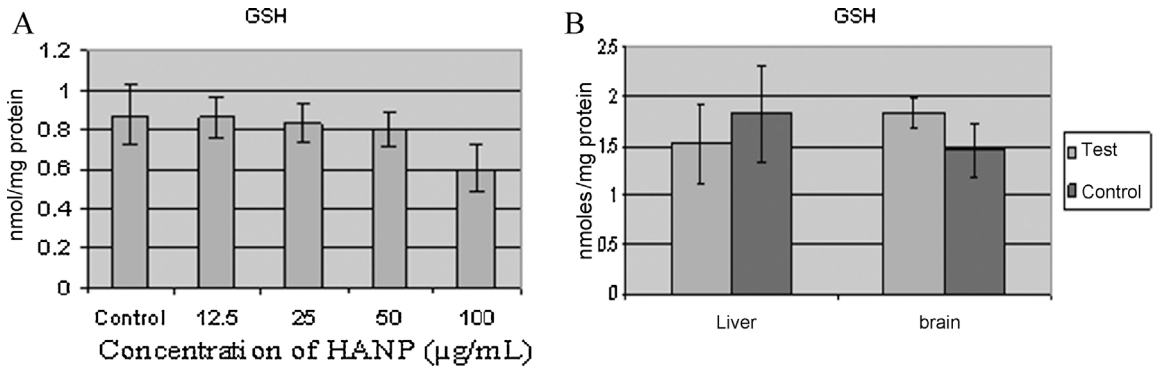


**Fig. 5.** (A) Level of GSH in rat liver homogenate exposed to HANP (*in vitro*), (B) GSH of liver and brain of HANPs treated Guinea pigs (values are mean ± SD, $n = 3$, *in vitro* and $n = 5$, *in vivo*).

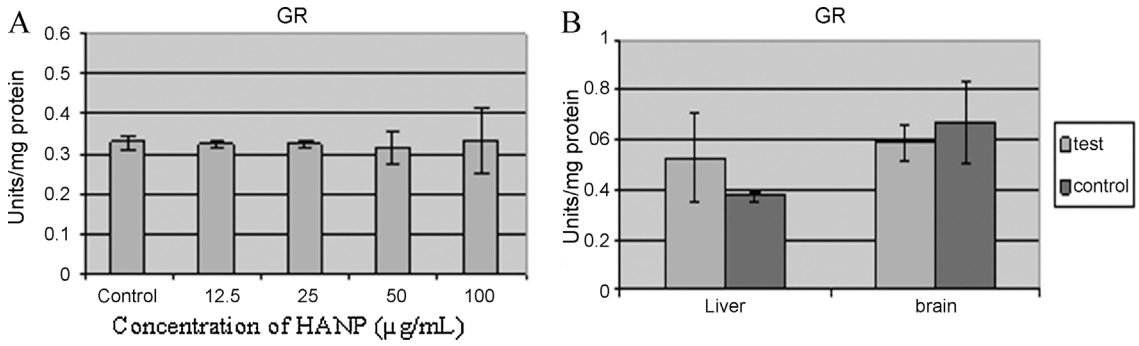


**Fig. 6.** (A) Level of GR in rat liver homogenate exposed to HANP (*in vitro*), (B) GR of liver and brain of HANPs treated Guinea pigs (values are mean ± SD, $n = 3$, *in vitro* and $n = 5$, *in vivo*).

and brain of Guinea pigs exposed topically to the HANP and the results are shown in Fig. 7B. The results of the study indicated that there was no alternations in the level of GPx in liver and brain tissues of HANP treated group when compared to control group (liver: test – 0.034 ± 0.007, control – 0.047 ± 0.004 and brain: test – 0.111 ± 0.047, control – 0.103 ± 0.036).

### 4.9. SOD

Fig. 8A shows the production of SOD when different concentrations of HANP were exposed to rat liver homogenate. It was found that there was a slight alternation in the production of SOD when treated with different concentrations of HANP (control and test values) in *in vitro* conditions and is not significant. The SOD activity analyzed in liver and brain tissues of Guinea pigs treated with HANP is depicted in Fig. 8B. The values are well comparable with the control both in liver (test – 0.251 ± 0.073, control –0.276 ± 0.070) and brain tissues (brain: test – 0.640 ± 0.069, control – 0.528 ± 0.025).

### 4.10. 8-OHdG

Fig. 9A shows the level of 8-OHdG when different concentrations of HANP were exposed to liver homogenate. It was found that the level of 8-OHdG in 100 µg/mL of HANP (22.48 ± 1.23) exposed group was comparable to control values (16.58 ± 2.10) in *in vitro* conditions.

It was also found that the level of 8-OHdG on exposure to HANP (liver – 8.09 ± 0.62) were slightly increased when compared to control (liver – 7.62 ± 0.97) (Fig. 9B) and is not significant, indicating that no oxidative stress was developed in liver of Guinea pigs.

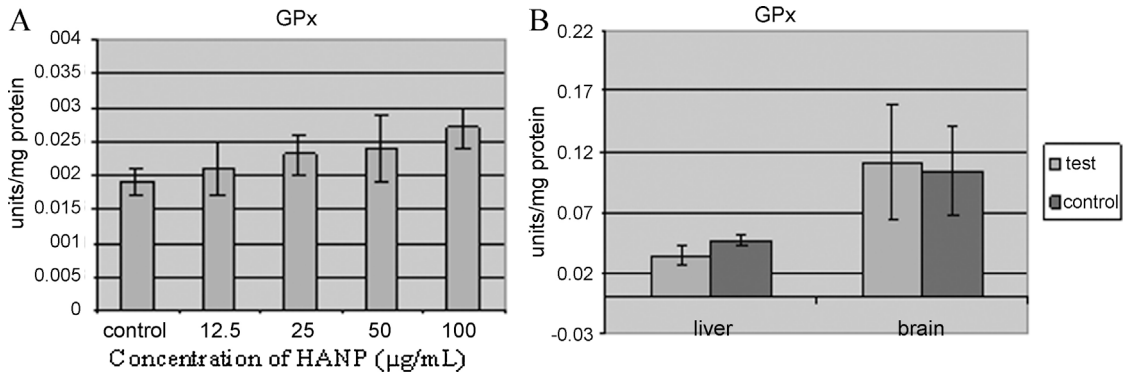


**Fig. 7.** (A) Level of GPx in rat liver homogenate exposed to HANP (*in vitro*), (B) GPx of liver and brain of HANPs treated Guinea pigs (values are mean ± SD, $n = 3$, *in vitro* and $n = 5$, *in vivo*).

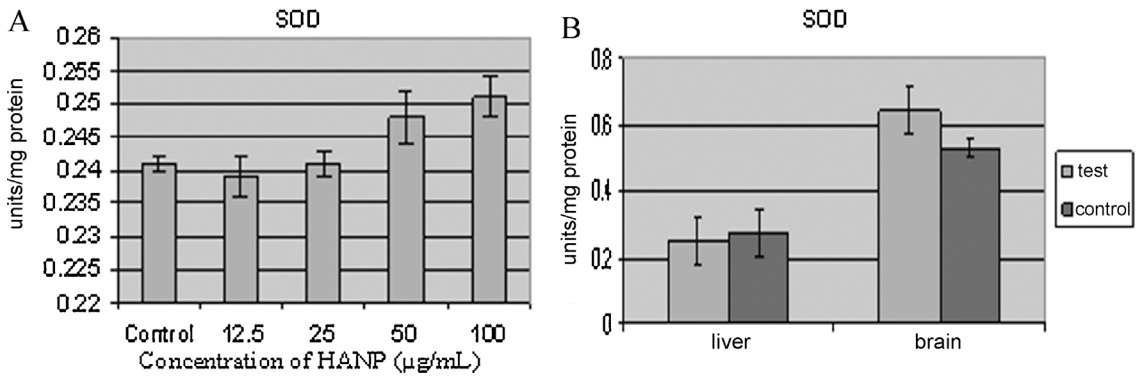


**Fig. 8.** (A) Level of SOD in rat liver homogenate exposed to HANP (*in vitro*), (B) SOD of liver and brain of HANPs treated Guinea pigs (values are mean ± SD, $n = 3$, *in vitro* and $n = 5$, *in vivo*).

## 5. Discussion

The molecular level toxicity studies aim to provide the biological plausibility of health effects of nanomaterials and identify the cascades of molecular pathways that get activated leading to a transition from pathological to a pathophysiological status resulting in the development of chronic disease. There was shortage of published information on the delayed hypersensitivity or skin allergenicity of these particles and hence the present study investigated these parameters both *in vitro* and *in vivo* using in house synthesized HANP.

HANPs were subjected to characterization by transmission electron microscope for particle size analysis, X-ray diffraction technique for phase purity analysis, Fourier transform infrared spectral analysis and Malvern Zeta sizer (zeta potential) for stability analysis. The results of the study indicated that the synthesized HANP is a stable authentic particle with a size less than 50 nm. The SEM/EDS reports indicated that the HANP was stable and co existed with cell suspension, without distortion or recrystallization.

The results of zeta potential measurements revealed that the HANP was unstable in water and stable in buffer. In *in vitro* and in *in vivo* conditions the HANP are coming in contact with buffer. Yuan et al. [30] reported that the hydroxyapatite nanoparticles with different sizes entered inside the L-02 cells when incubated with it. It was also reported that the pH of the cell culture medium will interfere with the dispersal of hydroxyapatite nanoparticles and it will result in the reversible agglomeration of nanoparticles [31]. Many researchers reported that if agglomeration occurs also the HANP will enter inside cells by mechanism like endocytosis devoid of its size [31].

It was found that the synthesized HANPs are non toxic up to 600 µg/mL on L 929 fibroblast cells and was confirmed by MTT assay.

Delayed hypersensitivity or skin sensitization or allergic contact dermatitis is a heightened immunological response caused by any substance when it comes in contact with the cutaneous layer. Delayed hypersensitivity normally includes two phases, the induction phase and elicitation phase. When human body encounters a foreign substance, an immune response is evoked. During induction phase, the chemical induces selective proliferation and clonal expansion of allergen responsive T lymphocytes and provides immunological memory [9]. In elicitation phase, on subsequent exposure of the substance to skin, the memory T lymphocytes elicit an increased immune response normally within 24–48 h [13]. The clinical manifestation in animals varies from erythema to edema to necrosis. In the delayed hypersensitivity assay, the skin irritation grades of 1 or greater in the test group generally indicates sensitization, provided that grades of less than 1 are seen on control animals. If grades of 1 or greater are noted in control animals, then the reactions of test animals that exceed the most severe control reaction are presumed to be due to sensitization. If the response is equivocal, a re-challenge is recommended to confirm the results from the first challenge. In the present study it was found that the HANP did not have potential to induce skin irritation in Guinea pigs after a challenge dose and hence it is considered as non allergic or non skin irritant.

The results of the blood parameters show that there was no significant alteration in the hematological and biochemical parameters, indicating the normal physiological functions in Guinea pigs

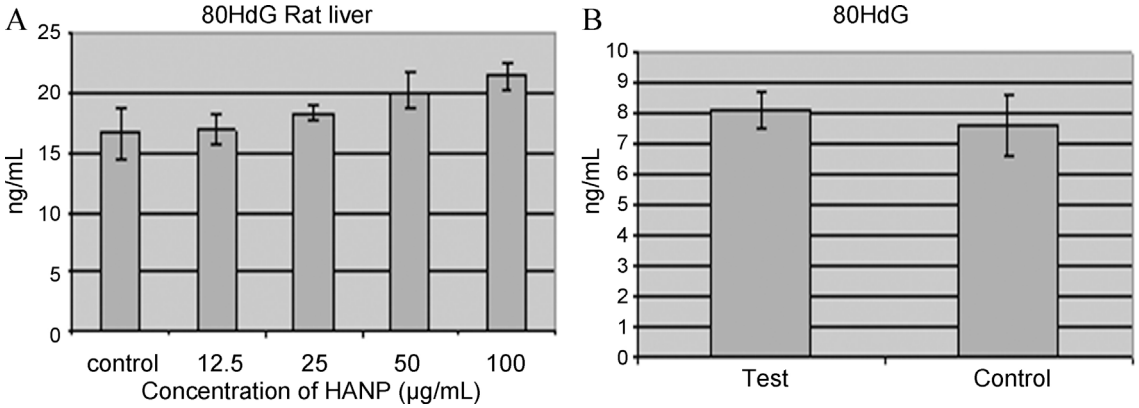


**Fig. 9.** (A) Level of 8OHdG (ng/mL) in rat liver exposed to HANP (*in vitro*), (B) level of 8OHdG (ng/mL) in liver of HANPs treated Guinea pigs (values are mean ± SD, $n = 3$, *in vitro* and $n = 5$, *in vivo*).

treated with HANP. The alterations in few of the parameters were under normal range.

The penetration and accumulation of nanoparticles in the organs may cause oxidative stress in the cells. The factor determining the protection of cells against oxidative stress is nothing but the balance between rate of reactive oxygen species (ROS) production and their elimination by different antioxidant systems. In order to determine the deleterious effects of a material, it is imperative to measure ROS generation. The ROS has a short half-life and its effects are determined indirectly by analyzing the biomarkers of oxidative stress including lipid peroxidation or the antioxidant status such as GSH levels and GR activity [19]. Lipid peroxidation occurs in the cells by the action of hydroxyl radical and hydrogen peroxide. The peculiarity of lipid peroxidation is that it is a self-propagating chain-reaction and the initial oxidation of a few lipid molecules may lead to significant tissue damage [32]. In the present study, it was observed that, the HANP treated liver homogenate (*in vitro*) showed increased LPO production only at higher concentration (100 μg/mL). In *in vivo*, the LPO level was found to be slightly increased in brain which was not significant.

GSH is the main endogenous antioxidant present in cells and it protects the cells from oxidative stress. Most of the glutathione present in the body are in the reduced form and it can be generated from the oxidized glutathione [32,33]. The results of the study indicated that the level of GSH was not significantly affected by the exposure of different concentrations of HANP on rat liver homogenate. Similarly the level of GSH was not influenced in the liver and brain homogenate of Guinea pigs used for delayed hypersensitivity. So it is evident that HANP did not cause any oxidative damage.

GR is an enzyme that reduces oxidized glutathione (GSSG) to reduced glutathione GSH [34,35]. It was found that the level of GR in different concentrations of HANP in rat liver homogenate was not influenced when compared to control values. Similarly, the level of GR did not significantly differ either in liver or brain homogenates of Guinea pigs exposed to HANP, when compared to control values. This indicated that the HANP did not inhibit the conversion of oxidized glutathione to reduced glutathione.

GPx is a protective enzyme and the biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water [28]. It was found that there was a slight increase in the level of GPx induction, when exposed to different concentration of HANP *in vitro*. In the liver and brain of Guinea pigs exposed to HANP a reduction in the GPx level was observed. The reduction in GPx level was not significant when compared to control values.

SOD is considered as the first line of defense against the deleterious effects of oxygen radicals in the cells and it scavenges ROS by catalyzing the dismutation of superoxide to $H_2O_2$ [36]. The results of the study indicate that, there was no increase in the SOD production, when different concentrations of HANP were exposed to rat liver homogenate. Similarly the SOD level was comparable to control values in liver and brain homogenates of Guinea pigs used for delayed hypersensitivity. This indicated that HANP does not affect the dismutation of superoxide to $H_2O_2$ by SOD, both in *in vitro* and *in vivo* conditions.

Oxidative stress can be measured in terms of DNA damage. 8-OHdG is a common marker to measure oxidative DNA damage. Oxidative DNA damage can be commonly caused by ROS, radiation and chemicals [37]. It was observed that there was no alterations in the level of 8-OHdG under *in vitro* or *in vivo* conditions. This indicated that different concentrations of HANP did not induce any oxidative stress in rat liver genomic DNA. Similarly, there was no oxidative stress in the liver genomic DNA of Guinea pigs used for delayed hypersensitivity assay.

## 6. Conclusion

The results of the present study demonstrated that the synthesized HANP has a particle size less than 50 nm and is non cytotoxic on L 929 fibroblast cells. The result of the delayed hypersensitivity assay suggests that the HANPs failed to induce any skin allergenicity reactions such as erythyma or edema. The HANPs did not significantly alter the level of lipid peroxidation, reduced glutathione, glutathione reductase, glutathione peroxidase, superoxide dismutase or oxidative stress related DNA damage. Hence, it can be concluded that the in-house synthesized HANP is non cytotoxic, non irritant and non toxic at the molecular level.

## Acknowledgments

Authors are thankful to Prof. K. Radhakrishan, Director and Head, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology for providing the facilities to carry out the work. We also acknowledge the technical assistance of Mr. Shaji S. and Mr. Harikumar G. The work was financially supported by The Nanomission, Dept. of Science and Technology (Govt. of India), New Delhi (Grant No. SR/NM/NS-90/2008).

## References

[1] L. Hovgaard, H. Brondsted, Crit. Rev. Ther. Drug Carrier Syst. 13 (1996) 185.
[2] M.T. Zhu, Y. Wang, W.Y. Feng, B. Wang, M. Wang, H. Ouyang, Z.F. Chai, J. Nanosci. Nanotechnol. 10 (12) (2010) 8584.
[3] I. Kimber, M. Cumberbatch, Toxicol. Appl. Pharmacol. 117 (1992) 137.
[4] O. Lunov, T. Syrovets, B. Büchele, X. Jiang, C. Röcker, K. Tron, Biomaterials 31 (2010) 5063.
[5] A. Sutton, G.E. Harrison, T.E. Carr, D. Barltrop, Intl. J. Rad. Biol. 19 (1971) 79.
[6] S. Kim, Y.T. Lim, E.G. Soltesz, A.M. De Grand, J. Lee, A. Nakayama, J.A. Parker, T. Mihaljevic, R.G. Laurence, D.M. Dor, L.H. Cohn, M.G. Bawendi, J.V. Frangioni, Nat. Biotechnol. 22 (1) (2004) 93.
[7] K.G. Desai, H.J. Park, Drug Deliv. 13 (5) (2006) 375.
[8] M.J. Akhtar, M. Ahamed, S. Kumar, S. Siddiqui, G. Patil, M. Ashquin, I. Ahmad, Toxicology 276 (2010) 95.
[9] I. Kimber, D.A. Basketter, K. Berthold, M. Butler, J.L. Garrigue, L. Lea, C. Newsome, R. Roggeband, W. Steiling, G. Stropp, S. Waterman, C. Wiemann, Toxicol. Sci 59 (2001) 198.
[10] H. Zinsser, J.H. Mueller, J. Exp. Med. 41 (1925) 159.
[11] J. Freund, J. Immunol. 11 (1926) 383.
[12] K. Landsteiner, M.W. Chase, Exp. Biol. Med. 49 (1942) 688.
[13] D.A. Basketter, P. Cadby, Contact Dermatitis 50 (1) (2004) 15.
[14] D. Bonnefont-Rousselot, J.P. Bastard, M.C. Jaudon, J. Delattre, Diabetes Metab. 26 (2000) 163.
[15] A. Telci, U. Cakatay, S. Salman, I. Satman, A. Sivas, Diabetes Res. Clin. Pract. 50 (2000) 213.
[16] H.M. Turk, A. Sevinc, C. Camci, A. Cigli, S. Buyukberber, H. Savli, N. Bayraktar, Acta Diabetol. 39 (2002) 117.
[17] D. Giugliano, A. Ceriello, G. Paolisso, Diabetes Care 19 (1996) 257.
[18] J.W. Baynes, S.R. Thorpe, Curr. Opin. Endocrinol. 3 (1999) 277.
[19] M. Arun, P.K. Silja, L.E. Sheeja, C.S. Geetha, P.V. Mohanan, Toxicol. Environ. Chem. 94 (4) (2012) 760.
[20] A. Pilger, H.W. Rudiger, Intl. Arch. Occup. Environ. Health 80 (2006) 1.
[21] V.D. Loosdrecht, J. Immunol. Methods 174 (1994) 311.
[22] P.V. Mohanan, H.A. Yamamoto, Toxicol. Lett. 129 (2002) 99.
[23] ISO 10993-10:2010 (E), Biological evaluation of medical devices – Part 10: Test for irritation and skin sensitization. Clause: 7.6: Closed patch test.
[24] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randal, J. Biol. Chem. 193 (1) (1951) 265.
[25] H. Ohkawa, M. Ohishi, K. Yagi, Anal. Biochem. 95 (1979) 351.
[26] C. Mize, R.G. Langdon, J. Biol. Chem. 37 (1962) 1589.
[27] M.S. Moron, J.W. Depierre, B. Mannervik, Biochim. Biophys. Acta 582 (1979) 67.
[28] J.T. Rotruck, Science 179 (1972) 588.
[29] S. Marklund, G. Marklund, Eur. J. Biochem. 47 (3) (1974) 469.
[30] Y. Yuan, L. Changsheng, Q. Jiangchao, W. Jing, Z. Yuan, Biomaterials 31 (2010) 730.
[31] Mei-Zhen Yin, Xian-Ying Cao, Shi-Pu Li, Front Mater. Sci. China 4 (4) (2010) 376.
[32] C. Mylonas, D. Kouretas, In Vivo 13 (3) (1999) 295.
[33] J.B. Owen, D.A. Butterfield, Methods Mol. Biol. 648 (2010) 269.
[34] A. Meister, J. Biol. Chem. 263 (33) (1988) 17205.
[35] B. Mannervik, Biochem. Soc. Trans. 15 (4) (1987) 717.
[36] A. Okado-Matsumoto, I. Fridovich, J. Biol. Chem. 276 (2001) 38388.
[37] C.G. Fraga, M.K. Shigenaga, J.W. Park, P.B. Degan, N. Ames, Proc. Natl. Acad. Sci. 87 (1990) 4533.