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Original Article

Sub-acute Sytemic Toxicity Evaluation of Phase Pure Hydroxyapatite in *Mus musculus*

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To prevent a toxic or harmful effect, the biocompatibility of wear debris particles produced by implants and their coatings is particularly crucial. As a follow-up to our previously published work on pilot-scale HA synthesis, the current research looked at the toxicological features of phase pure HA in a mouse animal model. Preclinical biological effects of intraperitoneally administered particles of HA up to a concentration of 100 mg/kg in swiss albino mice were studied over a 28-day period, according to ISO 10993-part 11 and part 12 guidelines. Particle impacts on serum biochemistry levels and full hemogram were investigated. Histopathological examinations were performed on spleen, heart, lung, kidney, and liver tissues. The current study found that all of the experimental mice, both male and female, lived well with a gradual increase in body weight and no notable changes in complete hemogram or blood biochemistry. The lack of any tissue-level toxicity signal by injected HA particles was verified by thorough histological investigation. When compared to commercial HA powders, these good results show the non-toxic nature of phase pure HA powders. This novel class of HA powders may be beneficial in terms of extended usage for joint arthroplasties, enhancing patient compliance and reducing implant failure.

Introduction

Because of structural and compositional features similar to real bone tissues, hydroxyapatite (HA) based bioceramics belonging to the calcium phosphate family have long been explored for orthopaedic and dental applications [1]. Calcium phosphates are also a bioactive substance that can attach directly to host tissues, allowing them to be used as non-load bearing bone transplants in the clinic. Several processing strategies, including solid-state synthesis, wet chemical precipitation, hydrolysis, and hydrothermal synthesis, are being investigated in order to synthesize HA with superior bioactivity, biocompatibility, and osteoconductivity [19]. The wet precipitation route is the least rigorous and most popular method of synthesizing phase-pure HA. There are only a few requirements for rigorous setup and instrumentation for this method, making it the most straightforward and economically scalable [19].

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HA is used as a bone filler or coating to help with the fixation of metallic implants, such as the femoral stem/acetabular socket in total hip replacement or dental implants. According to certain research, HA coatings may create nanoparticle debris/wear particulate that triggers inflammation in vital organs [2]. Nanoparticulate debris or foreign material aggregation surrounding the implant is unavoidable in most articulating joints. Therefore, their toxicity assessment in the biological system is quite important.

For the assessment of detailed toxicity in a biological system, animal research is required since this permits tissue level toxicity of nanoparticulate debris to be assessed [3]. There is no evidence in the literature that offers information on critical accumulation and particle size that would be harmful if discharged *in vivo* [4].

In vivo, nanoparticles will translocate to other tissues and organs along the blood circulation [3]. Because of their nano size and vast surface area, they have an active group or inherent toxicity [3]. At the cellular level, they may potentially offer long-term health hazards [3]. When contemplating nanoparticles for therapeutic usage, their toxicological consequences must be thoroughly investigated. In the literature, several research on the *in vivo* toxicity

of nanoparticles such as HA, TiO_2, $BaTiO_3$, ZnO, CoO, and gold nanoparticles have been published [2, 3, 5-10] [11, 12] [13-17]. For implantation in subcutaneous or femoral lesions in animal models, several of these older investigations utilised biomaterials in particle or bulk form. The published investigations included a variety of experimental animals, including rats, mice, and rabbits. Some of the early studies showed that particle injections given intraperitoneally, subcutaneously, intravenously, or intraarticularly in mice might produce toxicity to varying degrees depending on the animal type. For example, Pujari-Palmer et al. [2] found that subcutaneous implantation of hydroxyapatite nanoparticles to fibers and dots was related with a high capsule thickness [2]. Nanosized breakdown products, in particular, might cause an inflammatory response, resulting in implant failure. The composition, size, and charge of these nanoparticles have been shown to alter *in vivo* responsiveness. However, nothing is understood about how the shape of the particle might trigger inflammation. This shows that the shape of nanoparticles can have a major impact on inflammatory responses [2]. Another factor to consider is that the majority of published *in vivo research* do not meet international standards. For example, ISO 10993 part 12 outlines the procedures for preparing biomaterial suspensions, while ISO 10993 part 11 outlines the protocols for studying sub-chronic toxicity in animal models.

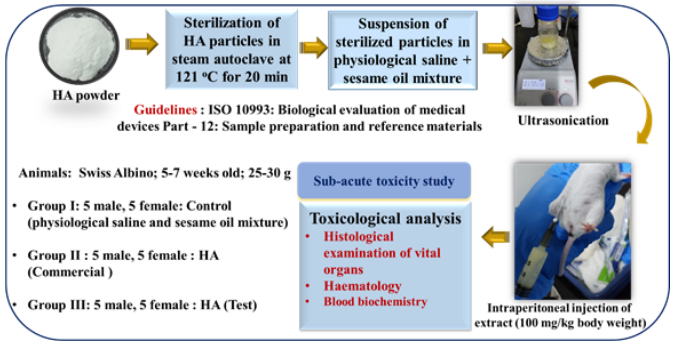

Figure 1: Experimentation design of repeated intraperitoneal dose toxicity (*in vivo)* of HA particulates in mice

Considering the foregoing, the sub-chronic toxicity of HA nanoparticles at a concentration of 100 mg/kg bodyweights was studied using ISO standards 10993-11 and 10993-12. To investigate the particles' toxicological effects, Swiss albino mice were injected intraperitoneally with particle eluates of HA under clean and aseptic

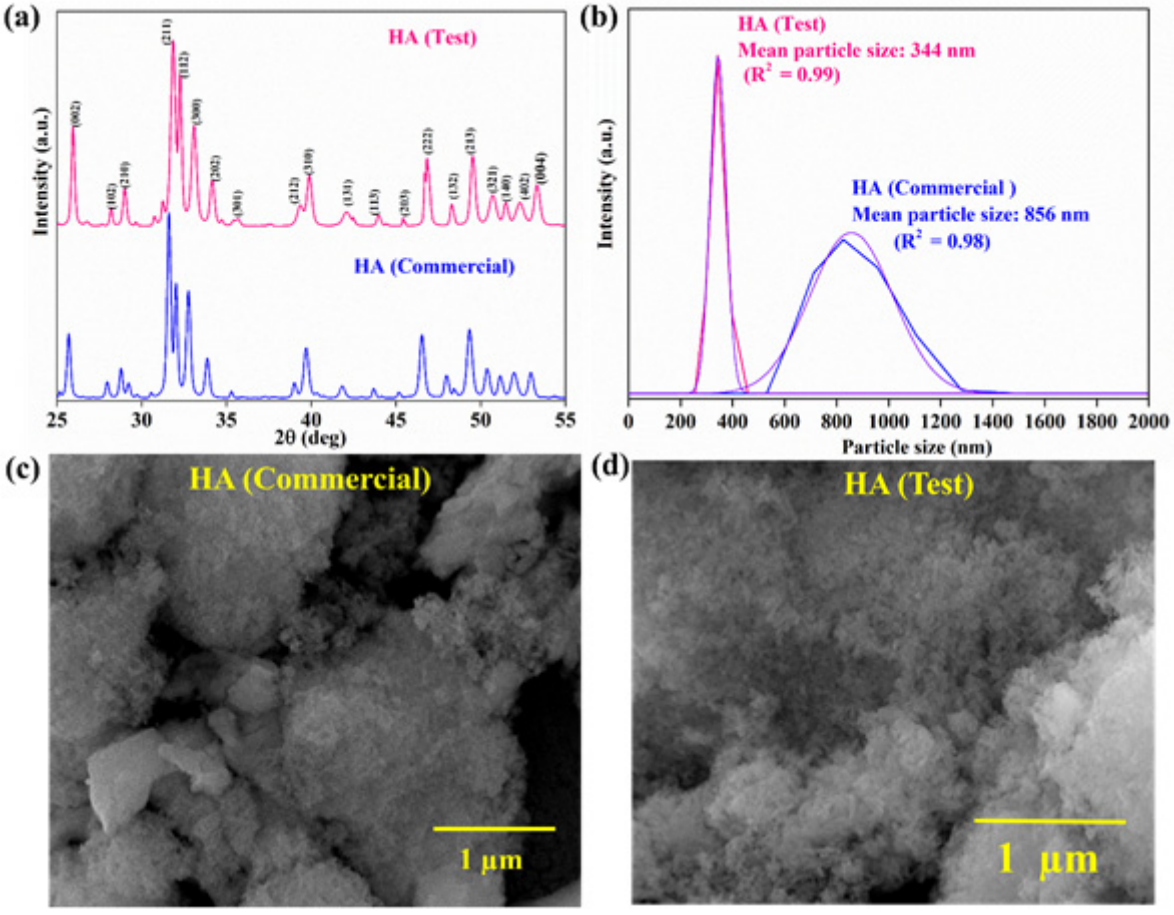

Figure 2: (a) XRD patterns and (b) particle size analysis determined using DLS for test HA(HA:Test) and commercial HA (HA: commercial) powders. Scanning electron microscopy images of (c) commercial HA and (d) test HA powders, which were used to prepare biomaterial suspensions for *in vivo* study, as per ISO 10993- part 11 guidelines

conditions, every alternate day over the period of 28 days. After 28 days of suspension administration, the animals were euthanized. The blood and tissue samples were retrieved from each animal and studied for complete hemogram and Blood biochemistry analysis and histopathological preparation, respectively.

Materials and Methods

Powder preparation and characterization

The wet chemical synthesis route was used to process the HA powders, and the detailed procedure has been reported in our previous article [10]. Ball milling was carried out for 15 hours at 300 rpm with HA powder using a planetary ball mill (Fritsch, Germany), with ethanol as milling media and agate jar and balls (Dia:10 mm). Powder to ball weight ratio was maintained at 1:4. The milled powder was dried overnight, then crushed. The presence of phases was determined by X-ray diffraction (PANalytical, Xpert Pro). Dynamic Light Scattering (DLS) (Zetasizer, Nano-ZS) was used to determine the distribution of particle size of HA powders. The synthetic HA particle suspension's polydispersity index (PDI) was measured at 0.402 and the commercial HA suspensions at 0.351. Both suspensions used for DLS were stable. The morphological analysis of the HA powders was studied by scanning electron microscopy (SEM). For benchmarking comparison purposes, commercial HA powders (Sigma-Aldrich) were used. The compositional analysis of hydroxyapatite was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES).

Sub-chronic toxicity study (ISO guidelines: 10993 11 & 12)

The present preclinical trial used Swiss albino mice (*Mus musculus*) from the Central Animal Facility at the Indian Institute of Science in Bangalore, which was 6-8 weeks old and weighed 25–35 g. In ventilated animal rooms, mice were housed in stainless steel cages with sterile rice husk as bedding. They were acclimated in a conventional controlled environment (22°C ambient temperature, 60% relative humidity, and a 12-hour light/dark cycle) with free access to water and a complete commercial laboratory diet. All animal experiments followed the guidelines set forth by the 'Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India) and were preapproved by the Indian Institute of Science's Institutional Ethical Committee of Laboratory Animals (Approval No. CAF/Ethics/809/2021).

Presently, thirty mice were randomly assigned into three treatment groups (10 mice per group with 5 male and 5 female) for the administration of different test particle suspensions as follows: The first group was treated as a control (without any injection of extract) and the second and third groups were injected intraperitoneally with 100 mg/kg body weight of commercial HA (Sigma-Aldrich) particles and test hydroxyapatite HA, respectively [20].

Preparation and injection of biomaterial suspension

The ball-milled HA particle were steam autoclaved for 20 minutes at 121°C. The different HA particles were suspended in a solution of physiological saline (0.9 percent NaCl) and sesame oil after being sterilised. The suspension was ultra-sonicated to guarantee uniform powder particle dispersion. The first group received only suspension injections, whereas the second and third groups received 100mg/kg body weight of commercial HA (Sigma-Aldrich) and test HA particles intraperitoneally, respectively. The suspensions were ultrasonicated for 15–20 minutes before injection, and injections were given intraperitoneally every other day for the course of the 28-day study period under clean and aseptic circumstances. The body weights of mice were assessed every other day throughout the time of study, and their daily activities, evident behaviour and survival inside the cage were documented.

Haematological and serum biochemical analysis

After 28 days of suspension administration the animals were euthanized. A standard orbital sinus blood collection technique was used to extract blood for haematological analysis (EDTA collection tube). Red blood cell count (RBC), haemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, neutrophils, lymphocytes, monocytes, basophils, eosinophils, RDW- CV (Red blood cell distribution width-coefficient of variation), RDW- SD (Red blood cell distribution width-standard deviation), MPV (mean platelet volume), PDW (platelet distribution width) and PCT (procalcitonin) were all measured using a haematological

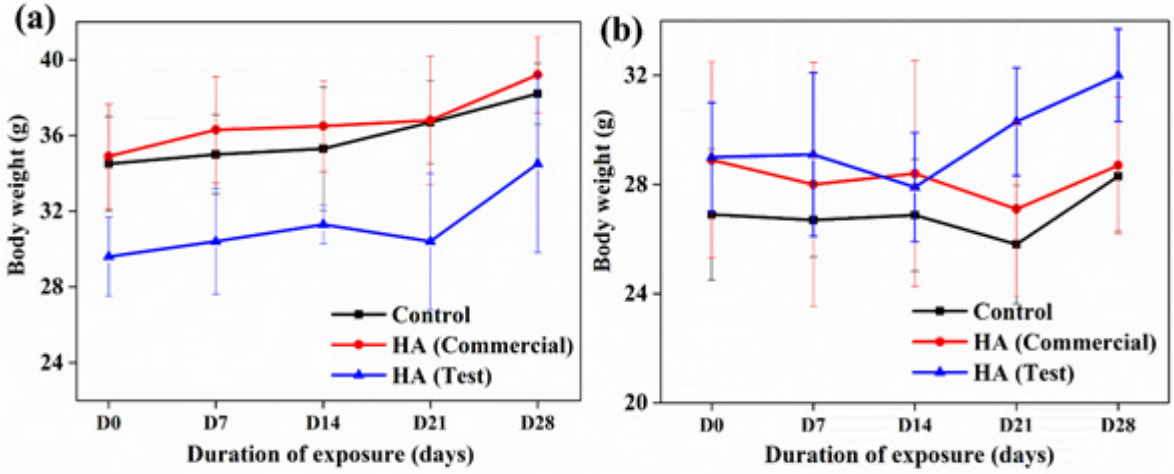

Figure 3: Evaluation of mouse body weight during the *in vivo* toxicity study of HA particles (a) male groups and (b) female groups of mice who received HA (commercial) and HA (test) particles intraperitoneally, as per ISO 10993-12 guidelines

autoanalyzer (Sysmex KX-21N™, USA).

To separate serum, whole blood (0.5-0.8 ml) was obtained through orbital sinus or cardiac puncture and centrifuged twice at 3000 rpm for 10 minutes. Blood biochemical analysis was performed using a biochemical autoanalyzer (Erba Mannheim, Erba Diagnostics, Inc. USA) to measure serum levels of total protein and total cholesterol, which are appropriate parameters for assessing the influence of oxidative stress generated by any toxic agent. To assess liver function, total bilirubin (TBIL), alanine aminotransferase (ALT/SGPT), aspartate aminotransferase (AST/SGOT), and alkaline phosphatase (ALP) were tested. To test nephrotoxicity, the levels of blood urea nitrogen (BUN) and creatinine (CR) were measured. To assess the cardiac activity, triglycerides, lactate dehydrogenase (LDH) and creatine phosphokinase was quantified. Albumin (ALB) and Globulin was measured as parameters of tissue damage or inflammation.

Histological preparation and examination

A small tissue section of all the vital organs such as the heart, liver, kidney, spleen and lung were recovered at the end of day 28 of particulate suspension administration, fixed in 10% neutral buffer formalin (NBF), dehydrated using an ascending ethanol series (70,

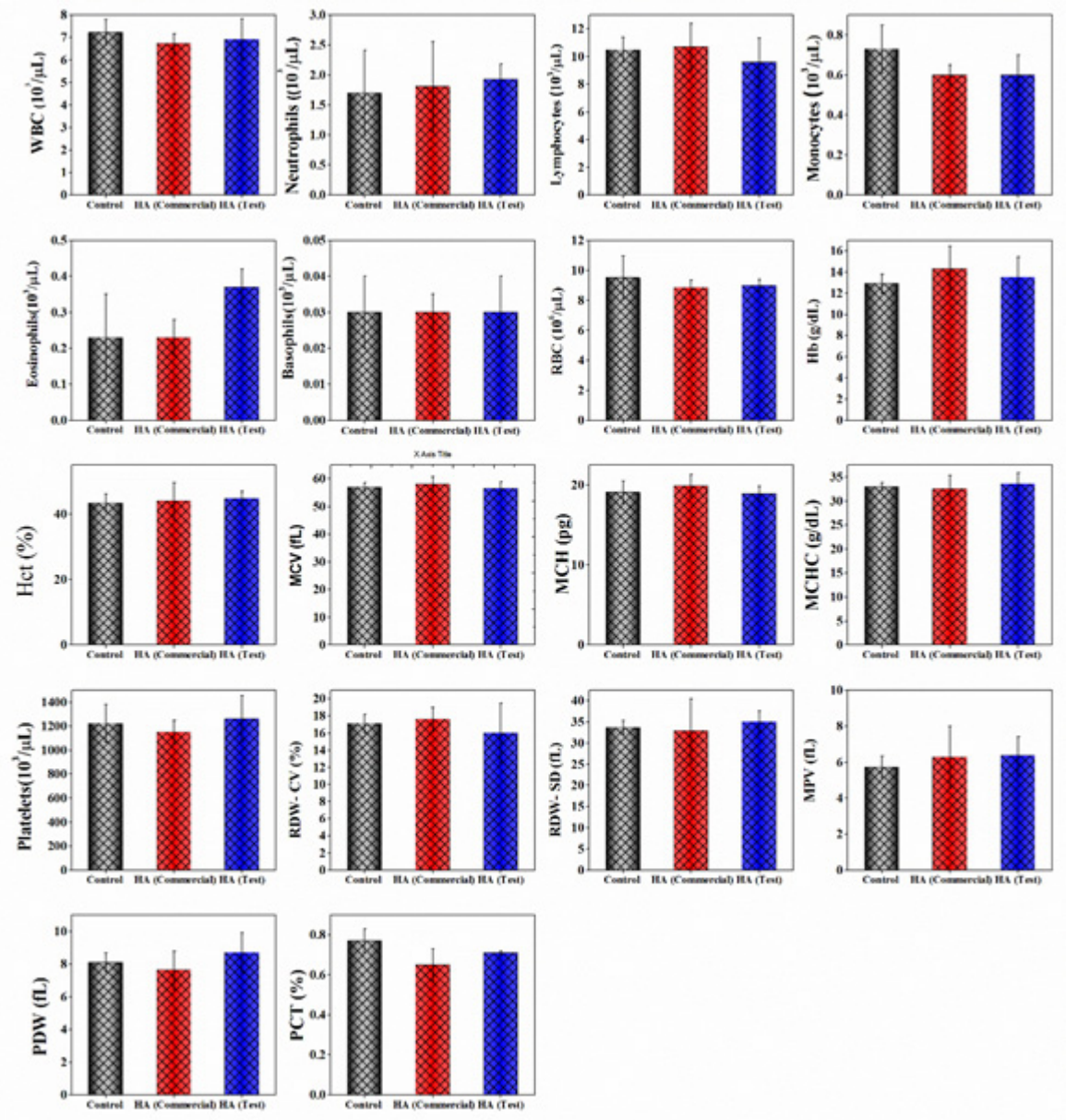

Figure 4: Haematology results from male mice treated with control, commercial HA and test HA particulates, 28 days after intraperitoneal injection at the dose of 100 mg/kg. Bars represent mean ± standard deviation (n=5). Abbreviations: Red blood cell count (RBC), haemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, neutrophils, lymphocytes, monocytes, basophils, eosinophils, RDW- CV (Red blood cell distribution width-coefficient of variation), RDW- SD (Red blood cell distribution width-standard deviation), MPV (mean platelet volume), PDW (platelet distribution width) and PCT (procalcitonin)

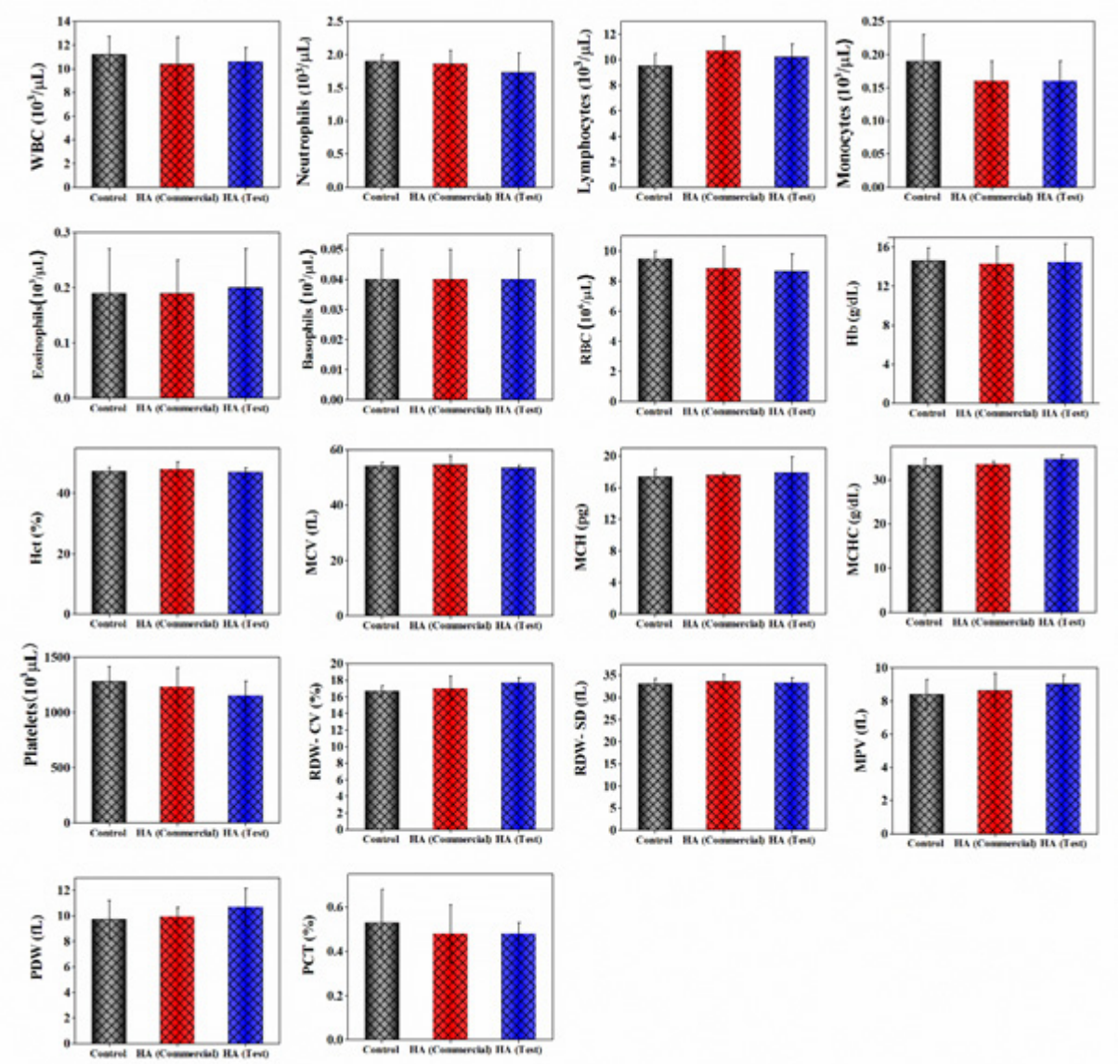

Figure 5: Haematology results from female mice treated with control, commercial HA and test HA particulates, 28 days after intraperitoneal injection at the dose of 100 mg/kg. Bars represent mean ± standard deviation (n=5). Abbreviations: Red blood cell count (RBC), haemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, neutrophils, lymphocytes, monocytes, basophils, eosinophils, RDW- CV (Red blood cell distribution width-coefficient of variation), RDW- SD (Red blood cell distribution width-standard deviation), MPV (mean platelet volume), PDW (platelet distribution width) and PCT (procalcitonin)

80, 90, and 100 percent), embedded in paraffin blocks, sectioned into 5–7 m thickness, and mounted on glass microscope slides using standard histological techniques. Hematoxylin and Eosin stain were used to stain the sections and light microscopy was used to analyse it. Gross and histopathological evaluation of all the tissue was done by Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala India.

Statistical analysis

The data were presented as a mean ± standard deviation (S.D.). The experimental values were compared to the appropriate control values for statistical analysis. The significant difference between the experimental and control groups was demonstrated using a one-way analysis of variance (ANOVA) with post-hoc Tukey HSD calculated in SPSS software. *p* d" 0.05 and *p* d" 0.01 were used to determine whether a difference was significant.

Results and Discussion

Because of the rising worldwide burden of disease associated with osteoarthritis and inflammatory rheumatoid arthritis in younger patients, the number of total hip arthroplasties has gradually climbed in recent years. Total hip and knee arthroplasties, in which the patient's joint is replaced by an implant, have helped to slow the ageing process as human life expectancy has increased. However,

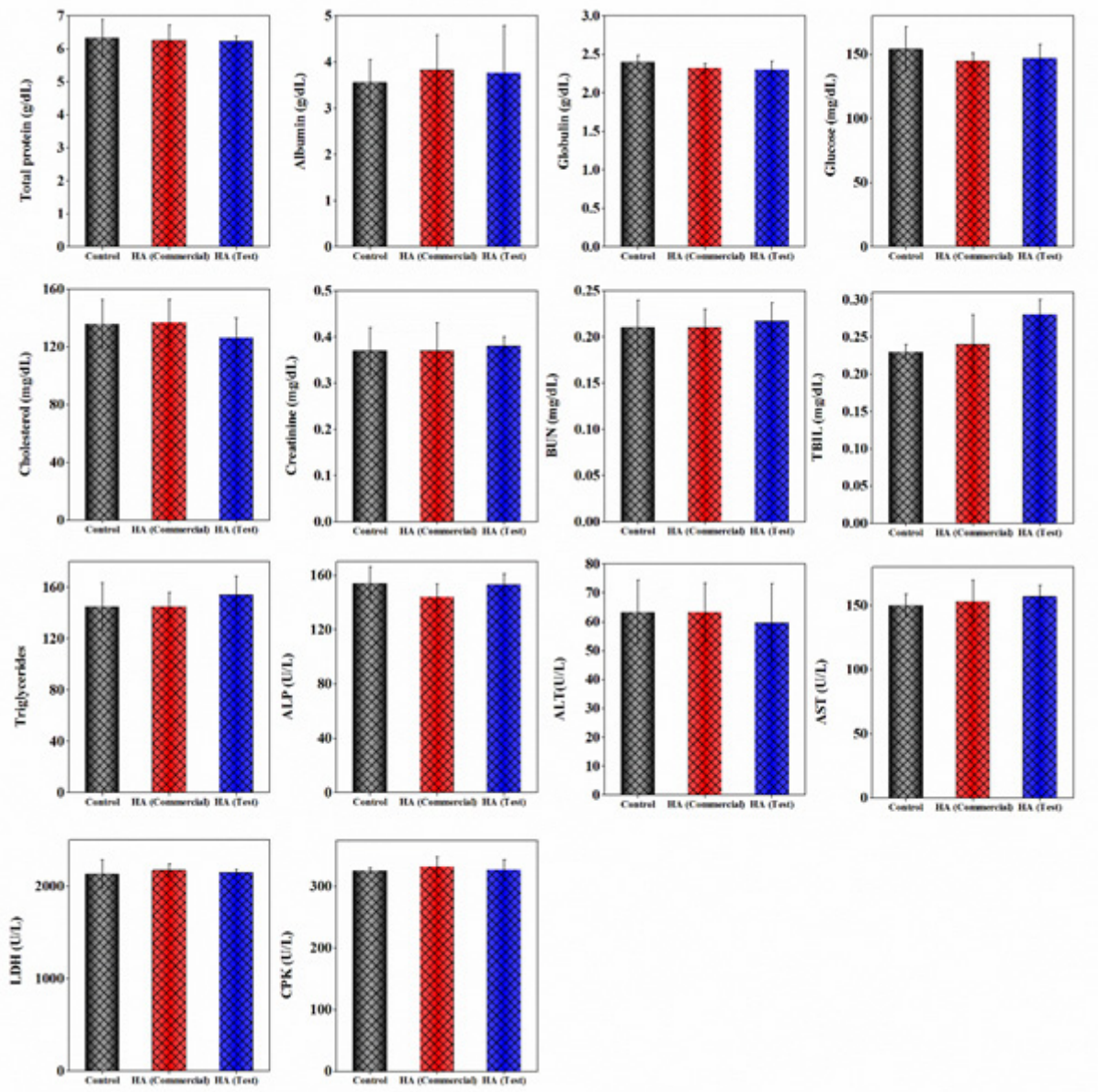

Figure 6: Biochemical results from male mice treated with control, commercial HA and test HA particulates, 28 days after intraperitoneal injection at the dose of 100 mg/kg. Bars represent mean ± standard deviation (n=5). Abbreviations: TBIL: total bilirubin; BUN: blood urea nitrogen; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase and CPK: creatine phosphokinase

because to a complex problem of aseptic loosening caused by an unfavourable cellular inflammatory reaction to wear particles formed largely at articulating surfaces, the longevity and performance of these orthopaedic implants cannot be guaranteed beyond 12 to 15 years [1]. These particles cause periprosthetic bone resorption (osteolysis), which causes mechanical instability between the implant and the surrounding tissue, eventually leading to implant failure. Therefore, to extend the service life of these orthopaedic prostheses, it's critical to develop and offer a variety of innovative materials and designed surfaces with equivalent qualities and lower wear rates that reduce the danger of osteolysis [1, 2].

The present study focuses on hydroxyapatite, which is widely used in the bone tissue engineering field because of its osteogenic capabilities. Here we describe the immune responses induced by phase hydroxyapatite particles developed by wet chemical process. We addressed particle-induced *in vivo* inflammatory responses triggered following intraperitoneal injection of test HA in mice. The measure of immunological as well as systemic biodistribution effects caused by submicron/nano sized HA particulates were then compared with commercially available HA particles which enabled us to understand and measure the potential toxicity of associated in house developed HA particles. To evaluate the potential injury in vital organs, we observed changes in the hematologic and serum biochemical parameters along with effects on the ultrastructure of liver, kidney, lung, heart and spleen collected after 28 days of experimental time period.

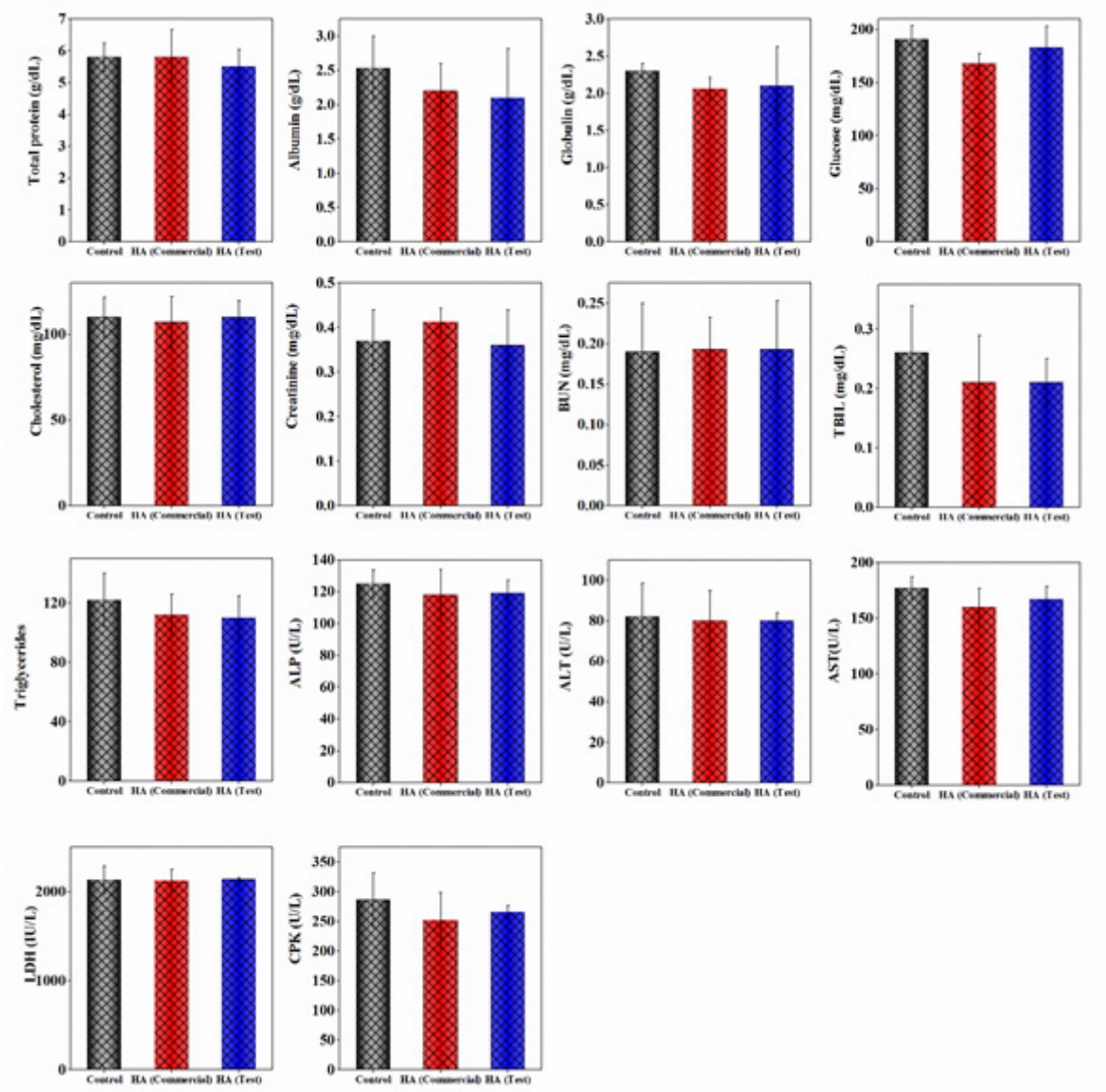

Figure 7: Biochemical results from female mice treated with control, commercial HA and test HA particulates, 28 days after intraperitoneal injection at the dose of 100 mg/kg. Bars represent mean ± standard deviation (n=5). Abbreviations: TBIL: total bilirubin; BUN: blood urea nitrogen; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase and CPK: creatine phosphokinase

Physicochemical analysis of biomaterials

The XRD patterns commercial and test HA particles are shown in figure 2(a). The presence of phase pure HA in both powders was confirmed by XRD measurements, and relevant peaks were indexed using the standard (ICDD-09-0432) files. The particle size distribution for commercial and test HA powders, as measured by DLS analysis, is shown in figure 2(b). The average particle size of the test HA is 344 nm, with a narrow dispersion in the range of 251-454 nm (figure 2d). Commercial HA particles are somewhat bigger, about 856 nm, with a wide size variability between 536 and 1280 nm (figure 2c). Iron with a concentration of 148.85 ppm and manganese with a concentration of 35.64 ppm are present in the hydroxyapatite together with Ca and P without changing its crystal structure, according to ICP-OES analysis.

Biocompatibility assessment, *in vivo*

Animal behaviour, symptoms and mortality

At the end of 28 days, 100 mg/kg intraperitoneal treatment of vehicle, commercial HA, and test HA resulted in 1/10, 0/10, and 1/10 mortality, respectively. The weight of each mouse was measured every week throughout this investigation, and the data of weight change collected over the course of four weeks is represented in figure 3. Figures 3 (a) and (b) illustrate the fluctuation in mice body weights over time with HA particle administration in male and female control, HA (commercial), and HA (Test) groups, respectively. In both the control and particle injection groups, there was no significant difference in body weight. During gross inspection, there is no inflammation at the injection site on mice. Furthermore, the mice's activity and survival for the whole 28-day

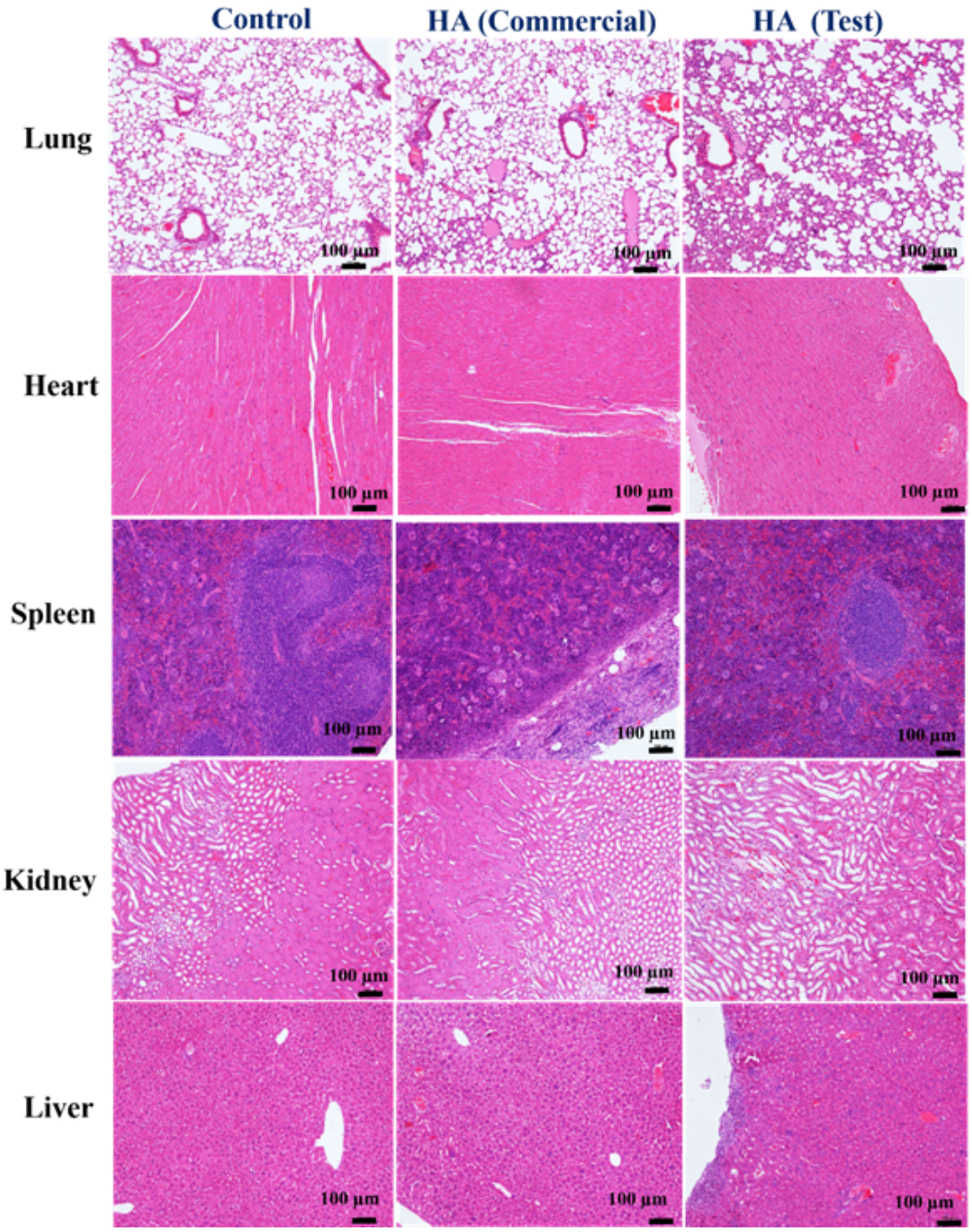

Figure 8: Translocation of particles and their toxicity can cause changes in tissue architecture. Representative histology images (hematoxylin/eosin-stained) reveal the characteristic tissue architecture of the male mice's vital organs i.e., lung, heart, spleen, kidney, and liver sections, at 28 days' post-exposure. Results exhibit no indication of toxicity in any tissue/ sections of organs

period were typical and uneventful, with no negative effects on their growth (figure 3). There were no symptoms of congestion, atrophy, or inflammation, and the size, color, and form of the tissue were all unaltered.

Haematological and serum biochemical analysis

The toxicity of smaller particulates can cause changes in animal metabolism, which can be detected by a comprehensive hemogram analysis. Any substantial difference in the counts of the hemogram parameters in blood samples implicates general animal vitality and identifies illnesses such as leukemia, anemia, and infection. Therefore, the haematological analysis were done to assess changes on the levels of RBC, Hb, Hct, MCV, MCH, MCHC, platelet count, neutrophils, lymphocytes, monocytes, basophils, eosinophils, RDW- CV, RDW- SD, MPV, PDW and PCT. Representative complete blood count results indicated that measured factors were within normal ranges and none of these parameters showed any statistical difference ($p \geq 0.05$) between the control and the HA particulate

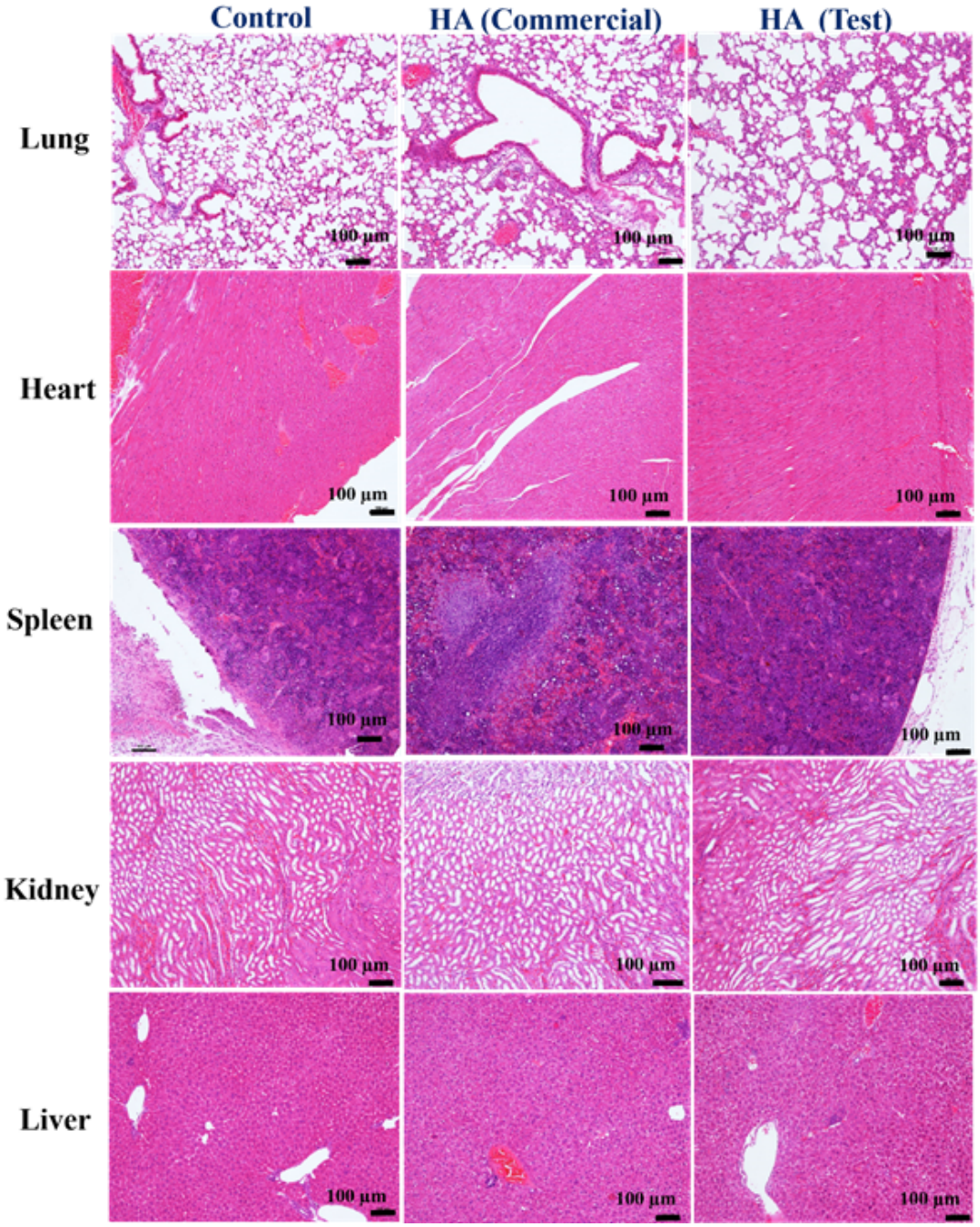

Figure 9: Translocation of particles and their toxicity can cause changes in tissue architecture. Representative histology images (hematoxylin/eosin-stained) reveal the characteristic tissue architecture of the female mice's vital organs i.e., lung, heart, spleen, kidney, and liver sections, at 28 days' post-exposure. Results exhibit no indication of toxicity in any tissue/sections of organs

treated groups (figures 4 and 5).

Additionally, to determine if particles of commercial HA and test HA produce any physiological/metabolic changes in the animals, different types of serum biochemical parameters were detected (figures 6 and 7). The level of total protein, glucose, albumin, globulin and cholesterol in the serum were assessed to know the effects of stressors on animal's metabolic pathway and both the parameters remain unaffected in all the animals. For renal toxicity, the levels of BUN and CR (metabolites associated with the functionality of the kidney) were detected. Whereas the levels of TBIL, ALT, AST and ALP in blood were tested to measure of hepatic and biliar functionality. A detailed analysis of all these metabolites of kidney and liver in serum of animals treated with commercial HA and test HA at dose level 100 mg/kg, showed no statistically significant differences ($p \geq 0.05$) as compared to controls

when sacrificed at the end of particulate administration at day 28. In all the experimental groups, the release of LDH, creatine phosphokinase and triglycerides in serum was also similar to that of control with no statistically significant difference. The assessment of complete blood count and serum biochemical parameters of animals reflect no interference of ceramic particles with important biochemical and enzymatic processes of metabolism which regulate the normal functioning of vital organs of treated animals.

Histological examination

At 28 days' post-exposure to HA particles, the tissues of heart, lung, liver, spleen and kidneys were excised out and processed for pathological examination to assess the potential translocation and toxicity. The histological examination of main viscera revealed that HA particles would not induce any pathological changes in appearance and micro morphology of vital organs at 100 mg/kg. The presence of HA particles could not be ascribed in any of the organs (figure 8 and figure 9). No evidences of atrophy, congestion and inflammation was noticed in any of the excised organ. The ultrastructural examination of the liver in all groups revealed a well-defined liver parenchyma with hepatic cords and sinusoids organised radially around the major vein. Hepatocytes have a polygonal shape with circular vesicular nuclei in the centre. The blood sinusoids were distinguished by their narrow branching, which separated the liver cell cords and anatomized with one another. A deeper examination of these sections revealed no pathological changes or particle aggregation that could be linked to liver exposure to particles.

Ultrastructural examination of kidney tissue revealed no signs of renal necrosis, and there was no discernible difference in the histology of control mice and animals treated with commercial HA and test HA particles. The normal tissue architecture of the renal corpuscle, renal tubules, and convoluted tubules could be seen in all histology photographs. The tissue architecture of nearly all of the straight portions of the proximal tubules of the kidney looked to be normal. Likewise, Natural parenchymal tissue made composed of red and white pulp was found in all of the spleen sections obtained from each group. A three-dimensional meshwork of vessels and threads distinguishes the crimson pulp. In the red pulp area, erythrocytes, leukocytes, hemocytoblasts, and megakaryocytes can all be seen. Lymphoma tissue is represented by the white pulp region, which is made up of lymphocytes, macrophages, plasma cells, dendritic cells, arterioles, and capillaries in a reticular framework. However, in most of the control and particulate treated animals, the spleen capsule was found to be thick with polymorphonuclear cells (PMNCS), plasma cells and mononuclear cell (MNC) infiltration. Lung tissue samples collected from control, commercial HA and test HA group showed normal appearance with myocardium and cardiomyocytes which are continuous striated heart muscle cells with one centrally located oval nucleus. Likewise, after 28 days of intraperitoneal injection, histological sections of the lungs showed no significant differences between the control, commercial HA, and test HA particle treated groups. They discovered typical tissue architecture in the alveolar, peribranchial, and perivascular sections of the lungs, as well as uniform alveoli and clear bronchioles lumen. When comparing the lung parenchyma of mice treated with any of these particles to that of controls, there was no hyperplasia or particulate build-up.

Taking into account the clinical uses of HA particles, the toxicity and biocompatibility of HA particles were examined by administering HA suspension up to a dose of 100 mg/kg intraperitoneally during a 28-day period in swiss albino mice. The findings revealed that HA particles had no substantial adverse effect on animal physiology, including serum biochemistry and full hemogram. We have also discovered that HA particle exposure had no deleterious effects on the histological architecture of the liver, kidney, heart, lung, and spleen. The lack of evidence of particle dissemination or any cellular level immunological response can be attributed to either their local removal via lymphatic systems or their rapid elimination by animal metabolic systems, involving biotransformation and excretion processes, following their systemic translocation to various organs. Furthermore, the existence of local macrophages or a variety of other immune cells that play a critical role in defence against foreign particles (ceramic) by removing the particles by phagocytosis may contribute to the lack of particle-mediated degenerative alterations [18].

Conclusions

In view of the anticipated concern over potential toxicological features of nanoparticles, this study aims to examine and compare the biological effects of test HA particles with commercially available HA particles *in vivo,* after repeated intraperitoneal administration of particulate for a period of 28 days. The following conclusion can be drawn from the study:

1. There was no indication of *in vivo* toxicity of test HA particles based on mortality, clinical characteristics, animal behaviour, and haematological and serum biochemical indicators.

2. There was no biodistribution or particulate accumulation in any of the vital organs. Due to translocation of commercial HA and test HA particles, the histological appearance of the heart, lung, liver, spleen, and kidney was normal, with no obvious clinical lesion or systemic immune response with atrophy, congestion and inflammation.

3. Together, these data demonstrate non-toxic nature of test HA ceramic particulates, *in vivo*, suggesting its usage for multiple biomedical application.

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AU Queries
1. Reference 19 is on 1st page. Check the reference order.