RESEARCH ARTICLE

Histopathological and Ultrastructure Implication of Calcium Nanoparticles in Wistar Rats

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Abstract

The present study was conducted to observe the histopathological and ultrastructure alteration of the cell organelle if any using calcium nanoparticles (80 nm) in 35 Wistar rats. Calcium nanoparticles were administered at dose of 1000 mg/kg body weight in test group (G2, n=20), and control group (G1, n=15) was provided *ad-libitum* feed and water only. The dose rate selected was a no observed adverse effect level (NOAEL) dose, apparently possessing no harm to the visceral organs. However, over a period of 90 days the calcium nanoparticle was suggestive of producing cytotoxic actions on liver, kidneys and spleen. Tissue samples were taken from rats at every 30 days interval and were subjected to histopathological procedures to observe the alteration induced by calcium nanoparticles. Subsequently, transmission electron microscopy (TEM) was also carried for the tissue samples to record the ultrastructural alterations, if any. The histopathological and TEM showed the cytotoxic effect of nano calcium on the visceral organs. Therefore, the application of nanocalcium in biomedical sciences should be strictly regulated depending on the size of the nanoparticles used.

Key words: Calcium, Histopathology, Nanoparticles, Transmission Electron Microscopy.

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Introduction

Calcium is the 5th most abundant element in human body and the 3rd most abundant metal. Its requirement is vital in diversified processes; in muscle contraction, cell signaling through synapses, hormonal secretion and a major component in bones and teeth around 99%, and less than 1% of the total body calcium is necessary for metabolism (Lee *et al.,* 2016). Concentration of calcium is very crucial in the living system as decrease in calcium level may cause de-mineralization of bones exposing individual to certain disease conditions. Any increase in calcium concentration leads to its deposition in tissues and vascular system and causes cell necrosis (Sung *et al.,* 2014). Calcium is commonly found in the form of calcium carbonate ($CaCO_3$), the increased dissolution, bioavailability and absorption (Lee *et al.,* 2016; Hassim and Rachmawati, 2010) of nanocalcium has been a topic of interest for many researchers because of its small size and larger surface area. Nanosized calcium compound forms protein corona, this interaction increase oral calcium bioavailability and are actually responsible for absorption and metabolism of the nanoparticles (Aggarwal *et al.,* 2009; Yoshioka *et al.,* 2014; Tenzer *et al.,* 2020).

Nanocalcium has surpassed other nanoparticles in biomedical applications, being less inflammatory and used in drug delivery, vaccine adjuvants, in colorectal cancer treatment. However, recently, protective functioning against tumor is found to be debatable, which might be suggestive of tumor inducing ability. The research led by Som *et al.* (2019) states that administration of nanocalcium carbonate may help in decreasing the pH of the tumor affected region, but upon withdrawal an increase in acidic pH is noticed demonstrating a metabolic reprogramming of the tumor cells. However, the researchers found out that consistent therapy with calcium nanoparticles reduces clearance and might be effective in inhibiting metastasis. Another experiment by Senchukova *et al.*, (2019) reported that nanocalcium might play role in tumor induction as they witnessed increase in size of the tumor cells after treatment with nanocalcium. With all these relevant information on calcium nanoparticle, the present study was designed to assess the toxic effect of calcium nanoparticles on the visceral organs of Wistar rats.

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Materials and Methods

The study was conducted at small house facility of College of Veterinary and Animal Sciences, Pantnagar (Uttarakhand, India) during the month of December to February 2018-19.

The experiment was designed using 35 Wistar rats of 6 weeks age of either sex with prior approval from the Institutional Animal Ethical Committee (IAEC). The study was designed for a period of 90 days. The calcium nanoparticles were procured from Siesco Research Ltd with average size of 80 nm with a shelf-life of 60 days.

The rats were divided into control (G1, n=15) and treatment (G2, n=20) groups. The control group was provided *ad-libitum* feed and water only, whereas calcium nanoparticles were administered through oral gavaging at no observed adverse effect level (NOAEL) dose of 1000 mg/kg body weight in group G2. The rats were sacrificed following the standard protocols at 30, 60 and 90 days post-treatment (5 each) and were subjected to detailed postmortem examination including collection of all the vital organs (liver, lungs, heart, kidneys intestine, spleen). The organs were analyzed for presence of lesions if any and the samples were collected for histopathological and ultrastructure analysis according to standard protocols. The organs for histopathology were collected in 10% neutral buffer formalin (fixative for tissue) and sections were processed using standard procedures and stained with Hematoxylin & Eosin staining (H&E) and special staining (Von Kossa) technique (Luna, 1968).

Transmission electron microscopy (TEM) was conducted wherein the tissue samples were cut upto a size of 1 mm and put in 2.5% glutaraldehyde solution. After preservation the samples were kept in Sorensen's buffer and sent for further processing of transmission electron microscopy at AIIMS, Delhi. Samples were then processed and viewed using Tecnai EM preference, and photomicrographs were taken (image processing software (TEM and TIA).

Results and Discussion

The gross pathological study of experimental rats in different groups sacrificed at every 30 day interval till 90th day post-treatment revealed no gross or apparently visible lesions.

Histopathology

Histopathological examination of liver in nanocalcium treated rats revealed congestion of central veins, vacuolar degeneration of the hepatocytes, areas with coagulative necrosis and infiltration of mononuclear cells (MNCs) and neutrophils around the capillaries and central vein and passive hyperemia in the sinusoidal spaces (Fig. 1). Lungs revealed congestion, places of emphysema and compensatory atelectasis with thickening of alveolar septa due to infiltration of neutrophils and MNCs. Emphysematous lung was quiet evident in nanocalcium treated rats. Nodule formation was seen with infiltration MNCs. Degeneration and necrotic changes were recorded around the bronchiolar epithelium in few rats with desquamation of the bronchiolar epithelium and proliferation of the fibroblast (Fig. 2). Areas of necrosis along with depletion of lymphoid cells and mild hemosiderosis were evident on microscopic examination of spleen in nanocalcium treated rats (Fig. 3). Nanocalcium treated kidneys of Wistar rats revealed degeneration of the tubular epithelium. Kidneys revealed atrophy of glomeruli, decrease in Bowman's space, and constriction of the lumen due to infiltration of the mononuclear cells along with coagulative necrosis of the kidney tubular epithelium (Fig. 4). Histopathology of the intestine showed shortening of villi with goblet cell hyperplasia suggestive of catarrhal inflammation along with infiltration of MNCs. Histopathology of heart revealed insignificant changes including congestion and haemorrhages and mild myocardial fiber necrosis (Fig. 5). The changes observed were similar to one observed in studies conducted by Sung *et al.* (2014), Guo *et al.* (2015), Navolokin *et al.* (2012) contrary to result observed by Liu *et al.* (2022).

To visualize the accumulation of nanoform of calcium in the tissue special staining of the tissue sections using Von Kossa procedure was conducted. The organs subjected to special staining for calcium included liver, lung, heart and kidney. However, on light microscopy the sections were

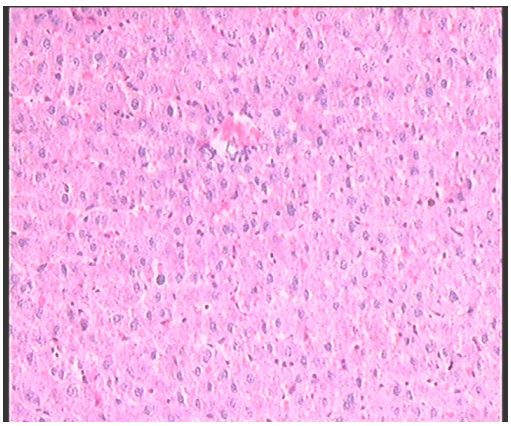

Fig. 1: Photomicrograph of liver showing congestion, coagulative necrosis, necrotic and degenerative changes of hepatocytes and and congestion in sinusoidal spaces at 90th DPT (Group II-Treated, H& E × 100.

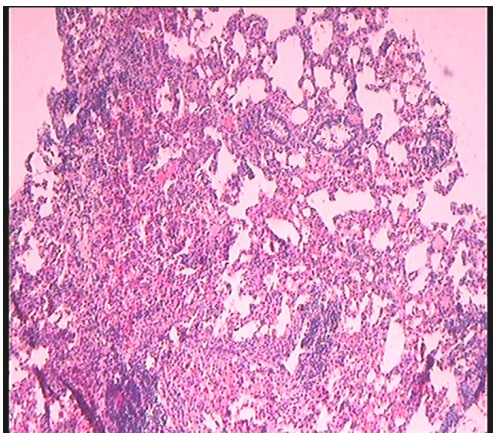

Fig. 2: Photomicrograph of lungs showing thickening of interalveolar septum due to accumulation of mononuclear cells along with congestion at 90th DPT (Group II-Treated, H& E × 40)

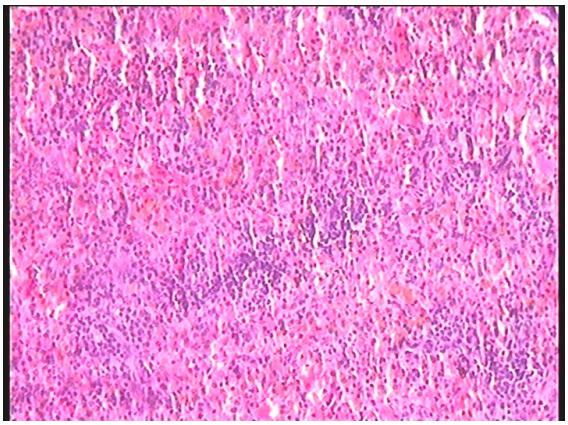

Fig. 3: Photomicrograph of spleen showing mild hemosiderosis and lymphoid depletion at 90th DPT (Group II-Treated, H& E × 100).

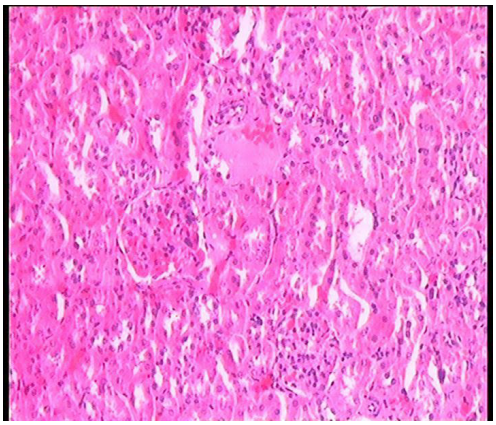

Fig. 4: Photomicrograph of kidney showing necrosis of tubular epithelium, hyalinization, constriction of the lumen of glomeruli due to neutrophils and mononuclear cells at 90th DPT (Group II-Treated, H& E × 200)

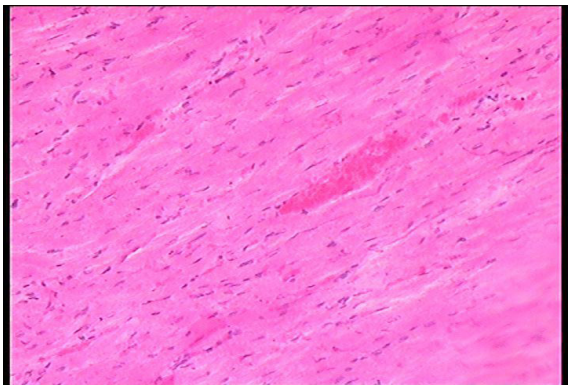

Fig. 5: Photomicrograph of cardiac muscle showing congestion, hemorrhage and necrosis of the mycordial fibre at 90th DPT (Group II-Treated, H& E × 100)

found negative for Von Kossa staining with no accumulation of calcium in the visceral organs.

Ultrastructural Pathology

Research based on various other nanoparticles (NPs) have revealed the toxic effect of nanoparticles on target organs and therefore the transmission electron microscopy of the target organs, liver, kidneys, lungs and spleen were undertaken in the present study to observe the ultrastructural alterations caused due to nanocalcium administration.

Transmission electron microphotography (TEM) of liver at 90 days revealed mild degenerative changes such as swelling of the mitochondria with partial and complete loss of cristae and mitochondrial degeneration. The hepatocytes were deformed along with loss of nuclei, rough endoplasmic reticulum was found to submerge with other cell organelles (Fig. 6). TEM of kidney also revealed similar degenerating changes as in liver in addition to degeneration of kidney tubules, increased thickness of basal lamina of the tubules, disruption of brush-border epithelium and presence of few amyloid granules (Fig. 7). Visualization of TEM of spleen revealed phagocytic phenomenon of leucocytes. Presence of foreign material identified as electron dense particles suggestive of calcium nanoparticle was observed adhered to surface membrane of leucocyte (Fig. 8, 9).

The vast majority of the metal-based NP exhibit toxicity through Fenton-type responses and ROS production (Sanfins *et al.,* 2018). Nanoparticles have been accounted to impact intracellular calcium fixations, actuate translation factors, and adjust cytokine creation by means of free radicals (Urbanska *et al.,* 2019). Due to prolonged use of nanoparticles ROS and nitrogen species are generated hampering the phagocytic cells gradually. Many researchers observed that metallic nanoparticles in general discharge their metal in its ionic structure, which prompts the subsequent harm to the body (Zolinik *et al.,* 2010). Likewise, calcium whenever discharged in ionic form (Ca++) will build the grouping of calcium extracelluarly. Raised calcium fixation prompts activation of calcineurin, a calcium dependent serine-threonine protein phosphatase. Calcineurin causes resulting increment in transforming growth factor β (TGF-β), which elevates ROS and nitrogen oxide creation, which causes mitochondrial damage which diminishes the development of lymphocytes (Zolnik *et al.,* 2010), thus causing cell death due to apoptosis. The phenomenon of cells undergoing necrosis was well observed under histopathological examination of lungs, liver, kidneys and spleen.

Besides organelle damage, nanoparticles upon ROS production cause oxidative stress and instigate conversion of glutathione to glutathione disulfide (Figueiredo *et al.,* 2018). In addition, working of NADPH dependent enzymes is likewise influenced. Constant increase in the oxidative pressure reduces fixation of the NADPH oxidase and subsequently causing hindrance in utilization of phagocytic mechanism of neutrophils and macrophages to convert free radical into hydrogen peroxide particles. Thus, disturbances in the phagocytic system results in death of macrophages and

neutrophils (Fenoglio *et al.,* 2008; Hsin *et al.,* 2008; Manke *et al.,* 2013; Sanfins *et al.,* 2018). Thereby enhancing the adverse effect of nanoparticle and causing cell death via apoptosis (Risom *et al.,* 2005; Figueiredo *et al.,* 2018).

The same mechanism of action of the calcium nanoparticles was recorded in the present study. The organs on histopathological examinations at initial stages showed congestion and increased infiltration of mononuclear cells. Initially, nanoparticles contribute towards the proliferation of the phagocytic cells, but as the nanoparticle is continued over a time-period decrease in the phagocytic activity of the cell is seen because the nanoparticles cause decrease in the myeloperoxidase enzyme, which derives the energy for phagocytosis, thus hampering the protective mechanism of the body. In intestine prolonged exposure to nanoparticles causes catarrhal inflammation leading to the shortening of the villi and increase in the goblet cells. As nanoparticles are relatively smaller in size as compared to the macromolecule and thus they get absorbed into the body and trespass the immunological barrier. This results into kidney damage causing glomerulonephritis and necrosis of the kidney tubular epithelial cells and liver damage inducing necrosis in liver with mononuclear cells infiltration. However, the lesions observed in histopathology and ultrastructural pathology are of mild to moderate in nature, and may be severe in case of prolonged administration.

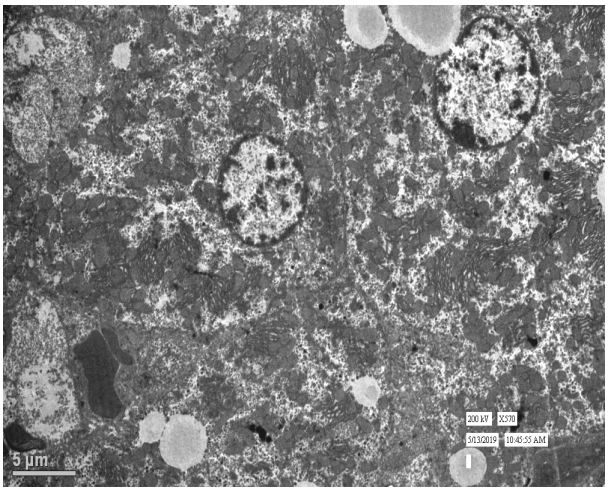

Fig. 6: Transmission electron micrograph of liver showing nucleus (euchromatin and heterochromatin), vacuoles, an area with swollen mitochondria at 90th DPT 570x (scale bar 5 µm)

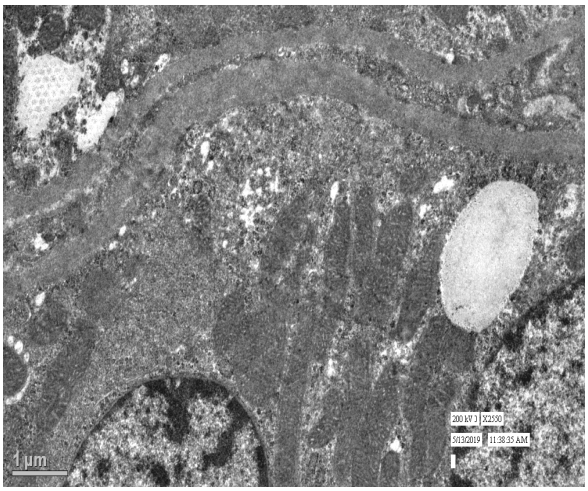

Fig. 7: Transmission electron micrograph of kidney tubule cells showing swollen mitochondria and thickening of the basal lamina at 90th DPT 4000x (scale bar 1 µm)

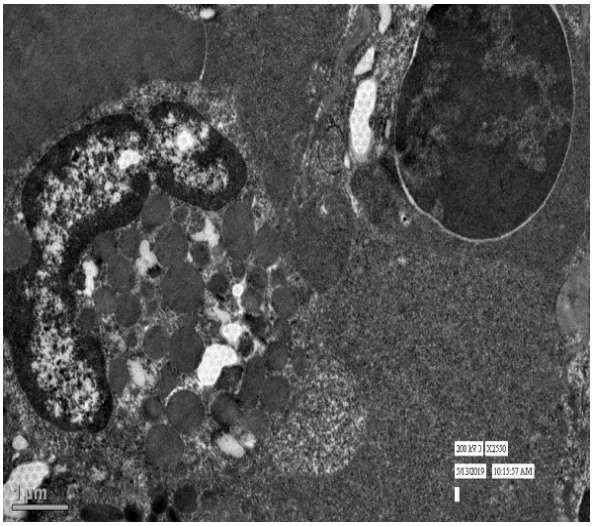

Fig. 8: Transmission electron micrograph of a neutrophil showing specific granules and azurophilic granule and phagocytosis of electron dense particles at 90th DPT 2550x (scale bar 1 µm)

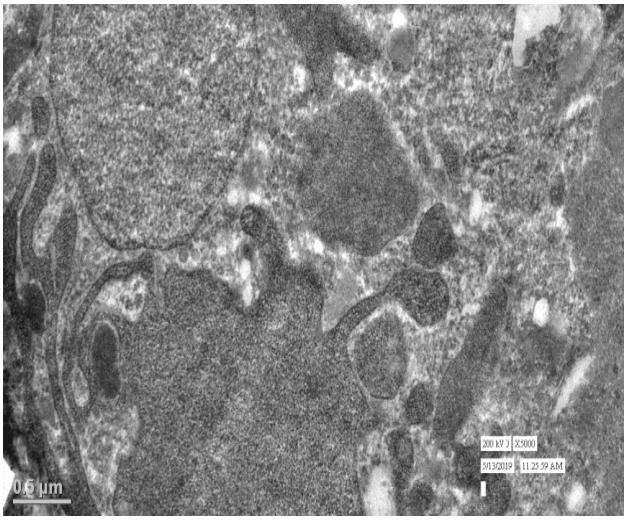

Fig. 9: Transmission electron micrograph of leucocyte showing attachment of a foreign body consisting electron dense area on leucocyte (scale bar 1 µm) phagocytosis phenomenon extension of cell membrane in shape of pseudopodia for engulfment of foreign material (scale bar 0.5 µm)

Conclusion

The present study concludes the cytotoxic effect of calcium nanoparticles even at NOAEL dose which apparently produces no observable lesions. However, the tissues undergo cytotoxic inflammatory reaction relative to nanoparticles size, the smaller the size greater the effect. Therefore, further study of calcium nanoparticles relative to its size needs to be extensively done, so as to alleviate its hazardous effect in the living system.

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