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Liaising Immune System Alteration in Wistar Rats by Calcium Nanoparticles

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ABSTRACT

Nanoparticles being a modern technology are being explicitly exploited for their application in medical sciences such as gene targeted drug delivery, vaccine delivery, adjuvants, and medicines. Metal nanoparticles studied by far have associated adverse effect except the calcium nanoparticle. Nanoforms of calcium are much acceptable and induce minimum inflammatory response. Albeit, the literature regarding calcium nanoparticle is not well cited and lacks immunological and cytotoxic studies on calcium nanoparticles. Henceforth, the effect of calcium nanoparticle on immunity was taken into focus in the present study. Wistar rats were administered nanocalcium at dose rate of 1000 mg/kg b.wt. for a period of 90 days *per-os*. Immunopathological alterations were assessed based on T-cells and B-cells proliferation assays, ELISA (Enzyme Linked Immunosorbent Assay), Haemagglutinition inhibition, macrophage function test (MFT) and gamma-globulin levels. Calcium nanoparticles at low doses improve the immune responses as they elicit the immune response levels at initial phase of the study. However, prolonged nanoparticle administration induces immune-alteration effect. Thus, for prolong use of nanoparticle in oncology, gene therapy, implantation and others should be assessed.

HIGHLIGHTS

- Calcium nanoparticles have abilities to induce immunopathological alteration.
- Calcium nanoparticles given at rate of 1000 mg/kg body weight to wistar rats over period of 90 days.
- The result showed decreased T and B cells during blastogenesis assay and increased gamma globulin levels.

Keywords: Calcium, nanoparticles, Immunopathology, T and B-cells decrease, cytotoxicity

Nanoparticles typically are particles with measurements in the range between 1-100 nm (Laurent *et al*., 2008). However, incalculable inference of nanoparticles has been expressed, which says that nanoparticles need the entirety of its measurements to be in the scope of nanoscale (ISO Standards-TC229). However, widely accepted definition is given by European Commission (2011) which states that nano-object needs just one of its measurements in the scope of nano-scale to be classified as nanoparticle. Nanoparticles can be distinctively arranged as: (1) *Ecological*, produced from backwoods flames and remains; (2) *Non-engineered*, speak to side-effects of human action including power plants and incinerators; and (3) *Engineered*, Engineered nanoparticles are refined from mass materials to offer phenomenal collaborations (Aggarwal *et al*., 2009). Engineered materials can be blended in different shapes and sizes which can be conjugated to various bioactive atoms, making a practically limitless number of varieties with a boundless potential for organic applications (Kawai *et al*., 2011). Engineered nanoparticles have created a potential impact on various fields like medicine, immunology, cardiology, endocrinology, ophthalmology, oncology as anti-cancer therapy, (Bhatia, 2016; Shin-Woo *et al*., 2013) drug delivery, gene therapy, imaging and disease diagnosis

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(Gwinn and Vallyathan, 2006) using biochips, food safety through identity preservation, breeding, and preservation using nanotubes implantation (Patil *et al*., 2009) . Metal nanoparticles studied by far (copper, silica, zinc, silver, gold) have associated adverse effect except the calcium nanoparticle. Nanoparticles being smaller in size have a bigger surface volume which expands their reactivity and makes them conceivably toxic. (Fu *et al*., 2014) Principle mechanism of nanotoxicity is the generation of reactive oxygen species (ROS), (Gonzalez *et al*., 2008) creation of ROS which at last outcomes into cytotoxicity. (Fu *et al*., 2014) Nanoforms of calcium are much acceptable and induce minimum inflammatory response. Calcium is one of the most significant mineral component of the living framework. Research studies have discovered calcium effective in treatment of colon cancer (Bai *et al*., 2017; Urbanska *et al*., 2019) and other therapeutic effect (Bisht and Jha, 2017; Dizaj Maleki *et al*., 2015)

Calcium being an integral body component shows less inflammatory reaction making itself applicable choice amongst all nanoforms available in biomedicals. Calcium nanoform is presently used in toothpastes, as anti-microbial agent in food (Roy *et al*., 2013) vaccine administration (He *et al*., 2000) drug delivery and medicines (Sawai and Igarashi, 2002).

Amidst, considerable number of focal points, the hostile impacts of calcium has stayed covered up for long. Albeit, the literature regarding calcium nanoparticle is not well cited and lacks immunological and cytotoxic studies on calcium nanoparticles. Ongoing researches have uncovered its effect in tumor cell reprogramming (Senchukova *et al*., 2019) (Som *et al*., 2019) likewise calcium and its relative immunopathology have not been given genuinely necessary consideration.

MATERIALS AND METHODS

The research was conducted with due approval from the Institutional Animal Ethical Committee (IAEC). The research was conducted using no observed adverse effect level dose (NOAEL) of calcium nanoparticles at the rate of 1000 mg/kg body weight (Sung *et al*., 2015). Preformed Nano-calcium powder of 80 nm particle size were procured from the SIESCO Research Laboratory, Mumbai, having molecular weight of 100.09 and 98% assay with shelf life of 60 days. The nanoparticles were administered through oral gavaging to wistar rats daily for a period of 90 days. Rats of either sex of 6 weeks age were divided into two groups of control rats (G1 group) and second of nano- calcium treated rats (G2 group). After every 30 days interval blood sample and tissues were collected from rats. The blood was collected to measure various hematological and biochemical patterns for alterations due to calcium nanoparticles administration. Various criteria were evaluated including Total leucocyte count (TLC), Absolute lymphocyte count (ALC), Enzyme linked immune-sorbent assay (ELISA), Serum Globulin, Lymphocyte blastogenesis test (LST) and Macrophage function test (MFT).

Lymphocyte blastogenesis assay

For lymphocyte blastogenesis assay, spleen was collected from the nano calcium treated rats in phosphate buffered saline (PBS). Then the splenocytes were prepared from the spleen by trituration. Supernatant was discarded and the cells were washed in RPMI-1640 (basal culture media). After this the cells were subjected to trypan blue dye for assessing the live and dead lymphocytes. After the live and dead cell count, following the protocol the cell suspension was prepared as 1×10^7 cells/ml and then poured into 96 wells plate along with the mitogen. For T- cells, Con-A (5 µg/ml) and PHA (5 µg/ml) mitogens were used and for B- cells LPS (5 µg/ml) was used. Triplicate wells were prepared and incubated in CO_2 incubator for 68 hours. Following the procedure 10 microlitre of MTT dye (5mg/ml) was added to the entire plate and then incubated for 4 hours. Addition of the MTT dye leads to the formation of the formazon crystal and the yellow color of the dye changes to purple. The crystals formed are then diluted using acid isopropanol or DMSO to all the wells and mixed properly. After few minutes the OD was taken at 570 nm in microplate spectrophotometer reader (Mosmann, 1983) (Rai-el-Balhaa *et al*., 1985).

Macrophage function test

Macrophage function test evaluates alteration in the concentration and activity of the macrophages upon treatment with calcium nanoparticles. For this purpose mineral oil was injected peritoneally to induce inflammatory reaction and then the following day the sample was collected intraperitoneally. To the sample collected equal

amount of RPMI-1640 was added. In another centrifuge tube equal volume of histopaque 1119 (procured from Hi-media as GranuloSep TM GSM 1119) was poured and sample mixture containing RPMI -1640 was overlaid slowly without mixing the content. Centrifugation of the tube at 400g (10- 15 minute) was done, an opaque layer was formed this opaque layer was collected. This layer is then subjected to nitroblue tetrazolium (NBT) test. A mixture was prepared containing 0.3 ml of 0.2% NBT in PBS, 0.2 ml of cell suspension and 0.1 ml activated plasma (activated plasma is prepared by mixing 1 ml plasma + 15 microlitre of LPS). The mixture was then incubated in water bath at 37°C. The reaction was stopped by adding cold PBS. Centrifugation of cell suspension was done at 500g for 5 minute and supernatant was discarded. The cells were re-suspended in 0.5 ml PBS. Smear from the cell suspension was made and air dried and methanol fixed. Counter stain using 0.5% safranin for 2 minute was done. Slides were air dried and viewed (Chauhan, 1998).

Serum gamma globulin

For estimation of serum gamma globulin; a solution of ammonium sulfate (19.5%) and sodium chloride (2.03%) was prepared. 5.7 ml of this solution was then put in a centrifuge tube and overlaid with 0.3 ml of clear serum. The mixture was then mixed gently and kept on ice bath for 15 minutes. Followed by centrifugation of the mixture at 1250g for 10 minutes. Precipitate obtained, was dissolved in 0.2 ml of normal saline solution and process of precipitation was repeated. Finally, all the precipitates were dissolved in 2 ml of normal saline solution. Finally, 5 ml of biuret reagent was added. The mixture was then kept at room temperature for 10 minutes and then OD was read at 555 nm in UV-Vis spectrophotometer (Chauhan, 1998).

RESULTS AND DISCUSSION

The values obtained over the period of 90 days were recorded and was then assessed for alteration in the hematological and immunological parameters. The recorded data is presented with mean ± SE and statistically analyzed with two way analysis of variance (ANOVA) using SPSS software with $p<0.05$.

For hematological evaluation, TLC showed decrease in the count in nano-calcium treated rats from 30th to 90th day of treatment (16.19±1.86; 15.05±1.09; 14.87±0.97) as compared with the control rats (Fig. 1).

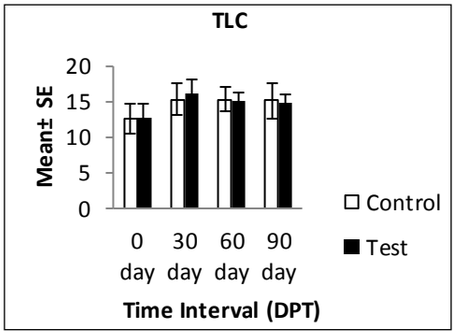

Fig. 1: Mean Total Leucocyte Count (TLC, 103/microlitre) in different groups of experimental rats at different time intervals of the experimental study

The TLC was found to increase in nano calcium treated rats at 30th day post treatment; however, subsequent decrease in the TLC count was noted after 60th and 90th day post treatment with calcium nanoparticles. Following the decrease in TLC value the decrease in particular cell count was observed, as the lymphocyte and neutrophils consists of leucocyte in large numbers. Therefore, absolute lymphocyte count (ALC) was recorded and evaluated for 90 days period. It was observed that ALC showed similar pattern as that of TLC. ALC increased initially at 30th day post treatment (13.62± 1.56); however, a decrease in ALC was noticed both within the group at 60th (12.72± 1.89) and 90th day (12.49± 2.12) post treatment with the calcium nanoparticles. When compared in between the 2 groups the ALC was found to reduce in nano-calcium treated group (Fig. 2).

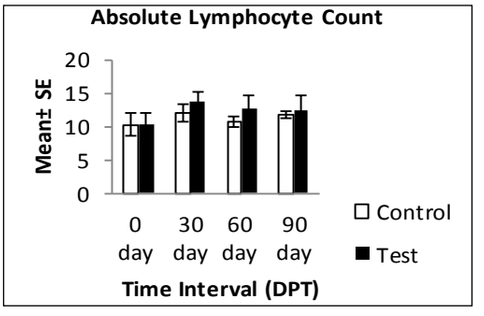

Fig. 2: Mean absolute lymphocyte count (ALC, 103/µl) in different groups of experimental rats at different time intervals of the experimental study

The absolute neutrophil count (ANC) was estimated for effect on the phagocytic activity due to calcium nanoparticle (Fig. 3). Initially the neutrophil count was increased at 30th day (1.96± 0.28) and then gradual fall in the count was observed at 90th day (1.24±0.33) post nano-calcium treatment.

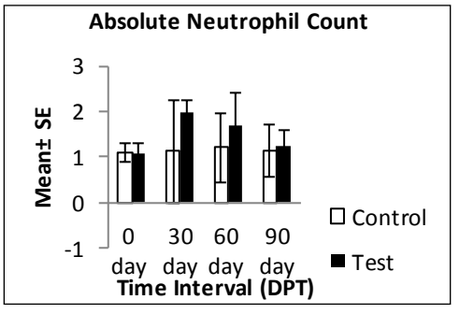

Fig. 3: Mean absolute lymphocyte count (ALC, 103/µl) in different groups of experimental rats at different time intervals of the experimental study

Metal-based nanoparticle exhibits cellular toxicity through Fenton-type responses and ROS production (Manke *et al*., 2013) (Risom *et al*., 2005) (Thannickal and Fanburg, 2000) as observed in the result mentioned above. However, this increase in intracellular calcium or the cytoplasmic calcium due to nanoparticle administration directs the lymphocyte multiplication in the peripheral blood mononuclear cells at initial phase of low dose. Subsequently, higher dose/ prolong use however may cause increase in calcium concentration there by activating calcineurin and decreasing the lymphocyte count by activation of TGF β and also due to prolonged use of nanoparticles ROS and nitrogen species are generated hampering the phagocytic cell count gradually. Likewise, calcium nanoparticle also exhibit similar pattern in the lymphocyte viz., ALC in the rodents treated with the calcium nanoparticles uncover that calcium additionally acts in the strategies as portrayed for other metal nanoparticles (Petrarca *et al*., 2015) (Manke *et al*., 2013).

For immunological alteration various tests were employed to record and evaluate the effect of calcium nanoparticles. These include serum globulin, lymphocyte blastogenesis test/ assay (LST), macrophage function test (MFT), haemagglutinition inhibition assay (HI) and enzyme linked immune sorbent assay (ELISA).

LST was done to evaluate the effect of calcium nanoparticle on T-cells and B-cells population as they play role in both cellular and humoral immunity. For this 3 mitogens were made use of CON-A and PHA- M for T cells and LPS for B-cells respectively. The value for CON-A was found to decrease from 30th (0.90±0.55) to 90th (0.76± 0.17) day of study with $p<0.05$ at 30th and 60th day of experiment (Fig. 4) and PHA-M was decreased (1.06±0.13; 0.83±0.02; 0.80± 0.11) at 30th, 60th and 90th days of study respectively with $p<0.05$. (Fig. 5) and LPS (Fig. 9) in calcium nanoparticle treated rats was also found to decrease from 30th (0.79± 0.41) to 90th (0.75± 0.01) day post treatment with calcium nanoparticles.

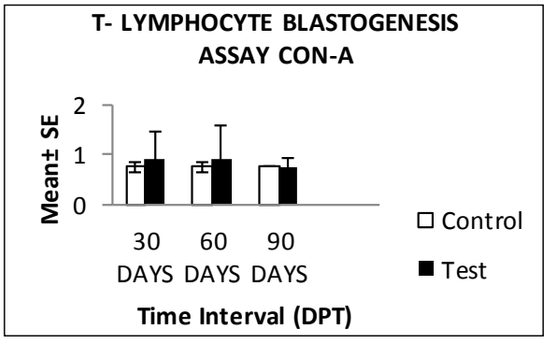

Fig. 4: T-lymphocyte blastogenesis in different group of rats using Con-A mitogen at different time intervals of the experimental study

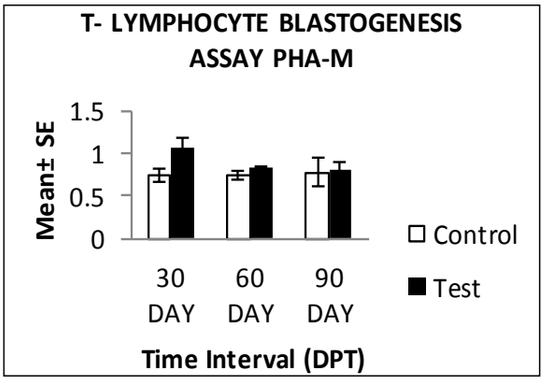

Fig. 5: T-lymphocyte blastogenesis in different group of rats using PHA-M mitogen at different time intervals of the experimental study

Further, the phagocytic activity was checked with macrophage function test (Fig. 6), to access the effect on

the phagocytic ability of macrophages. An initial increase in the macrophages was observed at 30th day followed by a slow fall in the count by 90th day post nano-caclium treated rats.

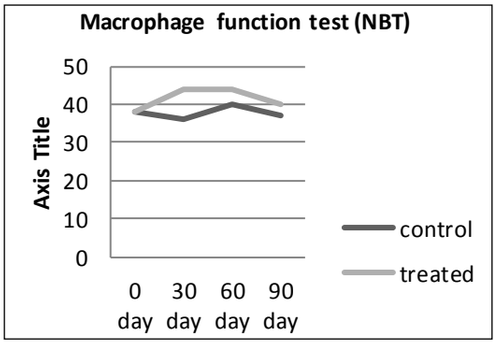

Fig. 6: NBT positive cells (%) on pooled blood samples in different groups of experimental rats at different time intervals of the experimental study

Nanoparticles besides mechanism of ROS generation also form protein corona or bio-interface on interaction with biological fluid, (Barbero *et al*., 2017) (Lundqvist *et al*., 2011) (Tenzer *et al*., 2013) which decides the liaison of nanoparticles (Lynch *et al*., 2007) (Mahmoudi *et al*., 2011) thereby inciting inflammatory responses through MAPK and NF-κB signaling pathways that control transcription of inflammatory genes such as IL-1β, IL-8, and TNF-α (Thannickal and Fanburg, 2000) (Borm *et al*., 2006) (Hsin *et al*., 2008). The inflammation causes cascade of response, which prompts increment in the T cell population. Initially, encounter with the calcium nanoparticles, are viewed as remote and this prompts increment in the immature T-cell population as mentioned above.

Further, alteration in the B-cell population was checked by serum gamma globulin level. The alteration in immunoglobulin level was checked with increase in gamma globulin levels (Fig. 7). The gamma globulin levels were found to increase in nano calcium treated rats at 30th (0.68 ±0.06; $p<0.05$), 60th (1.3 ±0.16; $p<0.05$) and 90th (1.32 ± 0.07; $p<0.05$) day post treatment.

The ascent in the serum gamma globulin level in rodents treated to calcium nanoparticle is attributed to possible damage to visceral organs (Fu *et al*., 2014) (Andre *et al*., 2006). Nanoparticles through ROS causes oxidative stress and instigate arrival of oxidized glutathione (Fenoglio *et al*., 2008) thereby enhancing the adverse effect of nanoparticle and causing cell death via apoptosis (Rai-el-Balhaa *et al*., 1985) (Fenoglio *et al*., 2008). Similarly, the working of NADPH dependent enzymes is likewise influenced. This NADPH is utilized by the phagocytic cells to direct the procedure of phagocytosis through myeloperoxidase compound. Because of increment in the oxidative pressure and diminishing in the fixation or non-attendance of the NADPH oxidase hinder the utilization of defensive instrument utilized by neutrophils and macrophages to utilize and change over free radical into hydrogen peroxide particles. Accordingly, it brings about debilitating of the phagocytic system and result in death of influenced macrophages and neutrophils (Eom and Choi, 2010) (Figueiredo *et al*., 2018) (Sanfins *et al*., 2018).

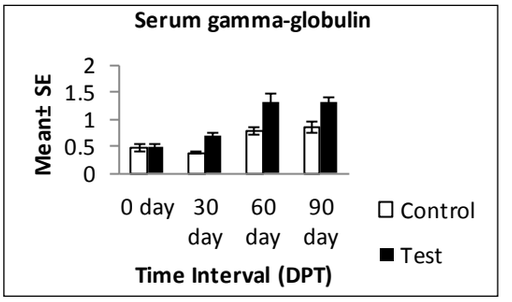

Fig. 7: Mean Serum gamma globulin (g/dl) in different groups of experimental rats at different time intervals of the experimental study

At first, the low concentration of the nanoparticles builds the creation of neutrophils and macrophages however; with the ascent in convergence of the nanoparticles the system is hindered there by influencing the insusceptibility (Sanfins *et al*., 2018) (Roy *et al.,* 2013). The outcomes of MFT and ANC in present study might clarify and bolster the mechanism of activity by which the calcium nanoparticle incites the alteration.

The HI titer values were used for assessing the effect of calcium nanoparticles on the humoral immunity. The HI titers were increased at 30th (8.2±0.39), 60th (8.2±0.37), and 90th (8.4±0.25) day (Fig. 8). However, these alterations were insignificant in between the groups and within the groups statistically. ELISA values were calculated for measuring the effect of calcium nanoparticles on IgG antibodies in serum. The mean ELISA values were also found to increase at 30th, 60th, and 90th, day in between the

groups. However, these alterations were insignificant (Fig. 10). When compared within the group the values were found to increase at 30th, 60th and 90th day (0.923±0.087; 1.216± 0.09; 1.266±0.15) post treatment. The increase in values suggests increased B-cell activation in the peripheral blood lymphocytes. Increase in T cell population indirectly influences B-cell population (Manke *et al*., 2013).

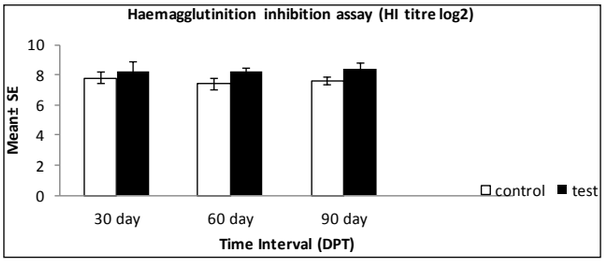

Fig. 8: Mean hemagglutination inhibition (HI) titre (log_2) in different groups of experimental rats at different time intervals

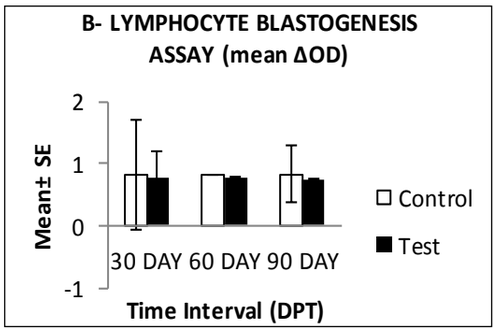

Fig. 9: B- lymphocyte blastogenesis in different group of rats at different time intervals of the experimental study

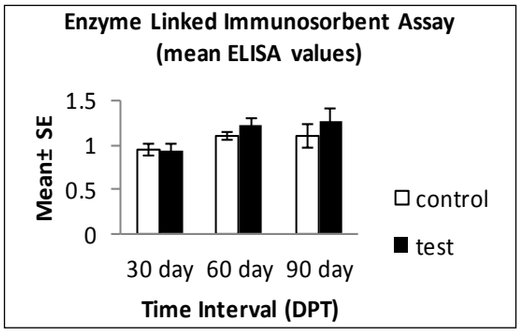

Fig. 10: Mean ELISA values in different group of rats at different time intervals of the experimental study at 90 days

The B cells upon initiation separate into immunoglobulin producing B cells and along these lines increase the immunoglobulin level in the peripheral blood. Similar outcomes could be found in the current investigation led with calcium nanoparticles as apparent with the rise in HI, ELISA (Brown *et al*., 1990) and serum gamma globulin levels. Research studies on different metallic nanoparticles expresses that metallic nanoparticle will in general discharge their metal in its ionic structure which prompts the subsequent harm to the body (Rahman, 2019). In this manner, reaching inference from the examination on different other metallic nanoparticles, calcium whenever discharged in ionic structure (Ca++) will build the grouping of calcium extracellularly. 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). Increment in TGF-β elevates ROS and nitrogen oxide creation, which causes mitochondrial damage which diminishes the development of lymphocytes **(**Zolnik *et al*., 2010**)** and influences insusceptibility. This relates with the possible decrease and altered immune response in the T cell and B cell population during LST involving calcium nanoparticle.

CONCLUSION

The present study on calcium nanoparticles however rectifies that at low dose improve the immune responses as they have been able to elicit the response through inflammatory receptors as supported by increase in neutrophils and macrophages and immunoglobulin levels at initial phase of the study. However, continued with the same dose of nanoparticle administration alteration in the immune system was noticed. Decrease in the level of T-cell and B- cell population was noticed. Thus, for prolong use of nanoparticle in certain biomedical conditions as in oncology, gene therapy; implantation etc., should be assessd. Also the cytotoxic and immunotoxic effect regarding calcium nanoparticle are not much cited in literature and hence more information in this regard is indeed necessary. Further, effect of nanoparticle varies depending on the size and shape and the purpose they are used for in biomedical sciences. The effect of a particular nanoparticle may differ at different sizes. A nanoparticle with a size of 10 nm will have more pronounced cytotoxic and immunotoxic effect as compared to the

same nanoparticle at 30 nm or at 50 nm range. Therefore, the toxic effect of nanoparticles should be measure at particular range for which they are to be used. Therefore much insight into the mechanism and functioning of the calcium nanoparticles need to be evaluated.

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