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Study of Cytotoxicity and Genotoxicity of "Nanoamorphus Calcium Phosphate (NACP)" In Macrophage RAW264 cell line: An In-vitro Study

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Abstract

Background: The aim of study was the biological assessment of cultured RAW264 macrophage exposed to nano amorphous calcium phosphate particles by analyzing of cytotoxicity and genotoxicity tests.
Methods: Nanoamorphus Calcium Phosphate particles were produced by sol-gel method, then particle size and hemogenicity was analyzed by XRD (X ray Diffraction). Cytotoxicity of nanoparticles was determinated with mouse RAW264 macrophage. The cells cultured in 37°c in DMEM medium 96 parts plates with concentration of 10000 cells in each part for 72 hours, then the medium removed and the second medium added to cells containing different concentrations of Nano particle (0, 200 and 400µg/ml). After 24 hours of incubation, MTT assay and Annexin V were used for assessing cell viability and apoptosis.
Results: The apoptosis insignificantly increased in macrophages with 200 and 400µg/ml NACP and for cytotoxicity, cell viability for control, 200µg and 400µg groups were 100,107,103 percent.
Discussion: NACP has no cytotoxicity and genotoxicity, so it can be used as non-toxic and beneficial material for clinical use.
Keywords: Cytotoxicity, Genotoxicity, Nanoamorphus Calcium Phosphate

1. Introduction

Nanotechnology has undergone many changes in various sciences, including dentistry, and the materials available as nanoparticles have very different properties to their larger size particles, and this distinguishes nanoparticles. One of these particles is Nano-amorphous calcium phosphate (NACP), which can decrease the amount of cariogenic bacteria in the mouth and increase pH of the mouth, and also in the long term, it can prevent reduced mineral content of the tooth and antibacterial properties without interference to mechanical properties of the material are unique features of this material [1-3].

NACP is used in many dental materials, including composites. The composite containing NACP is considered "smart" so that at acidic pH the release rate of calcium and phosphate ions drastically increases, the use of nanotechnology in the manufacture of composites has allowed more space for the filler to be added, resulting in a higher filler percentage. This has increased the composite's resistance to acid corrosion [4]. Also luster and refinement of the composite have also been improved [5, 6].

Since nanoparticles enter the bloodstream of the human body and are transported to various organs and so far, there is a lack of serious information about the relationship between the impact of nanoparticles on their

organisms and their size, surface structure and environment, degree of adhesion, chemical structure, degree of oxidation or crystalline phase, due to variation in toxicological methods [7]. The aim of this study was to investigate the cytotoxicity and genotoxicity of Nano-amorphous calcium phosphate particles at two concentrations of 200 and 400 μg after 24 h incubation of macrophage cells.

2. Methods and materials

The present study is an in vitro study of cell culture and adheres to a specific protocol of cytotoxicity and gene testing.

2.1.1. NACP preparation method

Hydrous calcium nitrate Ca(NO3)24H2O and polyethylene glycol (PEG, 10,000 MW) were dissolved in distilled water (solution 1). Ammonium hydrogen phosphate (NH4)2HPO4 was also dissolved in distilled water (solution 2). Then solution 2 was added dropwise to solution 1 at 5 ° C. It precipitates by adding solution 2 to 1 calcium phosphate solution. At the end of the reaction the precipitate was separated and washed several times with water and ethanol and separated by centrifugation. The particles were then dried in a vacuum oven. XRD (X-Ray Diffraction) was performed to confirm the amorphous size of the nanoparticles, and TEM (Transmission Electron Microscope), which is the best way to see particle size, was performed to evaluate NACP size (Figure 1).

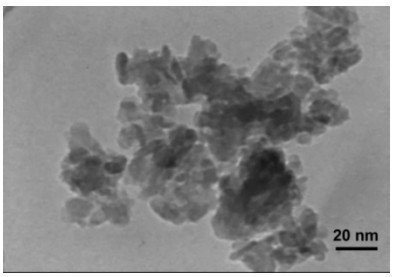


Figure 1.

Provide culture medium and other required solutions

2.1.2 Preparation of sterile media:

The cells studied need maintenance within the growth medium for their growth and survival. The culture medium of the selected cells contained DMEM (Dulbecco Modified Eagle Medium) and 10% FCS (Fetal Calf Serum) and 1% antibiotic 10000U / ml (penicillin and 10 mg / ml streptomycin), which was sterilized by filter 0.2 μ was used (8).

2.1.3 Preparation of PBS (Phosphate Buffered Saline)

To prepare the PBS solution, the following materials were first dissolved in deionized water and diluted to one liter with a Whitman filter paper. Then the pH of the solution was adjusted to 7.2 and 7.4 using NaOH and HCl concentrated and kept in the refrigerator after autoclaving. This solution is used at various stages of cell culture to prepare MTT cell staining buffer [8].

Na_2HPO4	2.16 g
KH_2PO4	0.20 g
NaCl	8.00 g
KCl	0.20 g

2.1.4 *Preparation of trypan blue 0.2% solution for staining of cells*

0.2 g of trypan blue dye was dissolved in 100 ml of physiological serum and then sterilized with 0.2 μl filter.

2.1.5 Preparation of MTT solution

0.25 g of MTT powder, chemically named 3-4 and 5-dimethyl-2 and 5-diphenyl tetrazolium bromide, was dissolved in PBS and the solution was dissolved in a 50 ml jujube balloon to volume with MTT solution at a concentration of 5 g/ml was obtained. Since MTT is a toxic and mutagenic substance, it should be carefully avoided Body surface when working with it [8].

2.1.6. Preparation of Cell Staining Buffer Solution Required for Genotoxicity Testing

Cell staining buffer=PBS+10% FCS

2.2.1. Cell line used

In this study, we provided RAW 264 macrophage cells in logarithmic growth phase to the Pasteur Institute of Tehran for the cytotoxicity and gene toxicity tests and sent by air courier. The cells were cultured in the cell culture and immunology laboratory of BouAli campus of Mashhad University of Medical Sciences. Cells selected for growth were

incubated in DMEM, 10% Fetal Calf Serum and 1% antibiotic in incubator with 5% CO_2 at 37 ° C. Before the cells were adjacent to the test material, they were cultured and passaged to obtain sufficient numbers of cells.

2.2.2. Cell culture method

Live cells were cultured in small cell culture flasks, incubated with 5% CO_2 at 37 ° C and if the color of the medium changes, every two days the medium is changed to allow the cells to cover the flask floor in a mono layer, when cells reached a density of 70 to 80%, passage was performed [8].

2.2.3. Cell passage method

The flask contents were poured into a sterile valve centrifuge tube and centrifuged at 1900 rpm for 5 minutes. After washing the cells with PBS, to the cell pellet at the bottom of the centrifuge tube was added a fresh culture medium and vortex.

After cell suspension, 2 ml of it was counted (about 401 ml 1 ml) in culture flasks and kept in a CO_2 incubator [8]. In the present study, the passage number of all cell cultures used in this study was not more than 5 passages.

2.2.4. The Cryo Method of Cells

For macrophage cell proliferation, the passage is performed and for long-term storage, cell cryo-action is performed on a flask. In this study, media containing 20% DMEM, 10% DMSO and 70% FCS were used.

The cells must first be counted, and after each wash with PBS dissolved in freezing medium, transferred to freeze-dried sterile vials. The type of cell and date of preparation should be indicated on the vial. First, the vials were stored at 20 ° C for 4–3 h and then placed in a freezer at -80 ° C overnight. The next day, the vials were transferred from a 80 ° C freezer to a nitrogen tank [8].

2.2.5. Defrost cells (freeze)

Extract the vial containing the frozen cell from the nitrogen tank carefully and with safety considerations (using cold resistant gloves and helmet) and by cleaning the vial by 70 degree alcohol they are transported under a sterile hood. As soon as the cell mass is removed from the vial wall, it is slowly added to the FCS and Peptage. The cell mass was suspended in DMEM medium with 20% FCS and transferred to flasks and then incubated for adhesion. After cell adhesion, In the case of a yellow medium that needs replacing or passage, the medium with 10% FCS was used [8].

2.3. Cell counting method

For cell counting, from the cell suspension, 100 µl of the sample was taken with a sampler and discharged into an open vial. To the vial were added 100 µL of trypan blue dye and 300 µL of culture medium and with the sampler the vial was Peptage several times until a homogeneous mixture was obtained. The resulting suspensions were transferred to a homocytometer with a micropipette and counted under live microscope at 10 times magnification. The blue cells of the dead are separated from the living cells. Using a homocytometer is a very routine, accurate and effective way of counting cells from which the cell content in ml of suspension can be calculated. This method requires at least 104 cells / ml for accurate counting.

In this study, counting lamellae were placed on a homocytometer and about 20 µL of the colored suspension of cells was inserted into the space between the lamellae and the lamellae. Under the microscope, all 5 slides were counted and cells were counted on the outermost margins of the house. Only the cells on the upper and right barriers were counted. Cells that did not receive trypan blue were counted as living cells. Since the cell suspension was diluted 5 times, the total of 5 cells was equal to the number of cells (n) present in the cell suspension. The number of cells per ml is 104 n [8].

2.4. Inclusion and exclusion criteria

As a general rule, during the logarithmic phase, cells are generally in their healthiest state, so it is only natural to use logarithmic phase cells when studying. In the logarithmic phase the percentage of dividing cells may be 90 to 100% and at a given time the cells are randomly observed in different cell division states. In the Plato phase, cell division is balanced by cell death, and the rate of cell division is slowed and reduced. Cells are often vulnerable in this phase and therefore should not be in the plato phase while studying. Cells from each well were observed under a 96-well plate under a microscope, and bacterial contamination of the well was not reported by cytotoxicity and excluded [9].

2.5. How to calculate the number of cells

$$n = \frac{c}{v} = \frac{a}{1 \times 10^{-1} mm^3} = \frac{a}{1 \times 10^4 cm^3} = a \times 10^4 \, cell/ml$$

To ensure accuracy of counting, cell counts were also counted with the Cell counter.

Stages of treatment of macrophage cells with NACP particles to evaluate cytotoxicity

1- Cells were counted with neobar lam and trypan blue staining.
2- Cell suspension with a concentration of $2\times10^5 cell/ml$ prepared and plated in a 96-well U-shaped lid in the following order:

In the first row of 8 (Al-HI) and in the last row of 8 the amount of $160\mu l$ sterile distilled water was considered to prevent evaporation in the incubator.

In the second row, $200\mu l$ of culture medium was poured and considered as blanks (the solution blanks used to calibrate the apparatus).

In the other wells, 200 μl cell suspension with a concentration of $2\times10^5 cell/ml$ was poured.

3. The plate was incubated for 24 hours So that the cells while adhering to the bottom To adapt to the environment.

4- After 24 hours, the supernatant was extract and the cells were exposed to nano-amorphous calcium phosphate at concentrations of 200 and 400 µg/ml. Negative control of the culture medium was added to the wells.

5- After treatment with NACP at the desired time (24 hours) culture medium was evacuated and the cells were washed with PBS.

6- 250µl of culture medium plus 25µl of MTT solution at 5mg/ml concentration were added to the wells.

7. Cells were incubated at 37 ° C and 5% CO_2 for 3h.

8. Discharge media and 160µL of DMSO were added and Peptage to dissolve Formazan crystals.

9- The solution was read at 540 nm with ELISA and the percentage of living cells was calculated according to the following formula [10]:

$$\frac{\text{The average absorption of wells corresponding to a specific concentration of a given compound - Mean Blank Absorption}}{\text{Negative control group mean absorption - Mean Blank Absorption}}$$

In this negative control formula, the cell was in the culture medium, and the positive control (the cell containing the culture medium containing the drug mitomycin) was not necessary due to the absence of the cancer cell.

Triplicated experiments were repeated three times for each sample to ensure reproducibility.

Finally, the toxicity was measured in accordance with Table 1.

Table 1. Cell Life Classification by Sjogren Method

The percentage of cell life	Toxicity
More than 90%	Non-toxic
Between 60% and 90%	Mild Toxicity
Between 30 to 59%	Moderate Toxicity
Less than 30%	Severe toxicity

Stages of treatment of macrophage cells with nano-amorphous calcium phosphate, for evaluation of genotoxicity

The FITC Annexin V Apoptosis Detection Kit with PI kit was used for the evaluation of genotoxicity and DNA damage to induce apoptosis. During programmed death or apoptosis, phosphatidyl serine is transferred from the inner surface to the outer surface of the cell membrane, where Annexin binds to the phosphatidyl serine present on the outer surface of the cell and is detected by flowcytometry. Propidium Iodine also binds to the fragmented DNA of the cell nucleus and is detected by flowcytometry.

The cell treatment steps were performed according to the kit protocol as follows:

1. Preparation of cell suspension with concentration (1×10^6 cell/ml) in Falcon tubes
2. Proximity of 1 ml of nano-amorphous calcium phosphate with negative control group for 24 hours
3. Wash the cells twice with Cell staining buffer and then dissolve the cell precipitate in Annexin V binding buffer at a concentration of $2\times10^5 cell/ml$
4. Transfer 100µl of cells dissolved in binding buffer to a 5 ml flow cytometer tube
5. Add 5µl of FITC Annexin V solution
6. Add 10µl of Propidium Iodine solution
7. Vertex cells and place the tubes for 15 minutes at room temperature at 25 ° C in the dark
8. Add 400 µl of Annexin V binding buffer and flow cytometer analysis
9. Data analysis using Flowjo 7.6 software to calculate apoptosis induction percentage

Triplicated experiments were repeated three times for each sample to ensure reproducibility.

Statistical Analysis

The test for normality distribution of the data was done using Shapiro-Wilk test. Also, for the analysis of the cytotoxicity and genotoxicity of NACP, One-way ANOVA and post-hoc Tukey were used for statistical analysis using SPSS (Statistical package for social sciences, version 16.0; SPSS Inc., Chicago, IL). P-value < 0.05 was considered statistically significant.

3. Results

Based on the Shapiro-Wilk test the data had a normal distribution (P=0.827). The findings of the present study include two parts: determination of cytotoxicity and genotoxicity of nano-amorphous calcium phosphate on RAW264 macrophages.

3.1. Cytotoxicity

In this group, negative control cell with culture medium, in other words, cell viability was compared to negative control group and growth rate in negative control group was 100%.

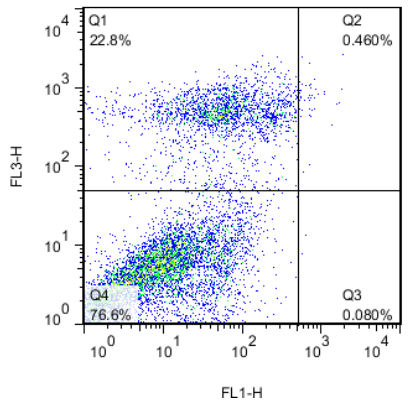
The data in Table 2 and Figure 3 indicate that the mean number of living cells and the percentage of cell survival tested in the three stages, after 24 hours incubation in the 200 µg group had the highest rate, followed by 400 µg in the second category, with the lowest cell viability and the lowest percentage of cell survival in the control group.

The percentage of cell survival is directly related to the average number of living cells.

3.2. Genotoxicity

To evaluate the genotoxicity of macrophages, RAW264 macrophages were exposed to Nano-amorphous calcium phosphate for 24 hours.

Geneotoxicity test data indicate that apoptosis percentage increases with increasing concentration of nano-amorphous calcium phosphate in macrophages, and the data in Table 3 indicate that in the control group we have the lowest apoptosis and in the 400 µg group the highest apoptosis, but this increase is not statistically significant. On the other hand, the percentage of live cells (Figure 2.b) in this experiment had the highest (76.6%) in the 200 µg group (column 2), 75.9% in the 400 µg group (column 3) and the lowest in the control group (column 1) that is 74.4%.



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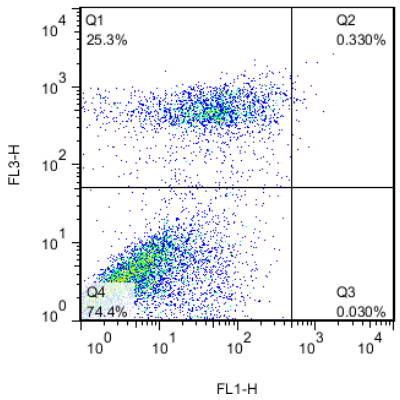

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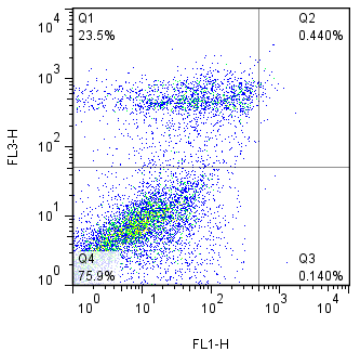
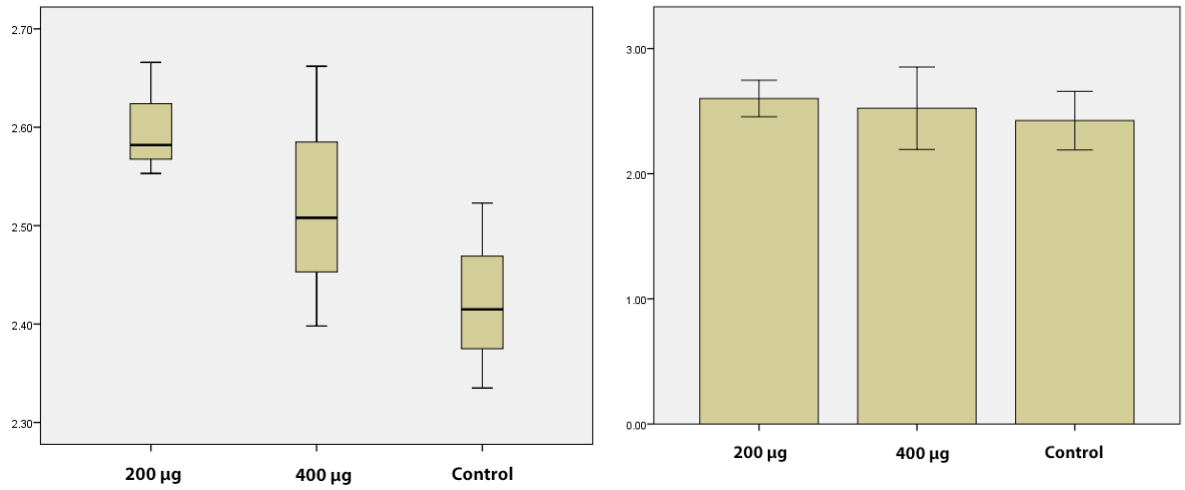


C

Figure 2.



A B

Figure 3.

Table 2. Mean number of living cells and percentage of cell survival after 24 hours of incubation

concentration	Normal	200µg	400µg	Significancy
Average live cells	$2.42 \pm 0.11 \times 10^5$	$2.60 \pm 0.08 \times 10^5$	$2.52 \pm 0.20 \times 10^5$	
The percentage of cell survival	100% ± 4.1	107% ± 7.6	103% ± 5.2	P=0.21

Table 3. Percentage of macrophage apoptosis at different concentrations after 24 h incubation

concentration	Normal	200µg	400µg	Significancy
Percentage of apoptosis	0.03	0.08	0.14	P=0.58

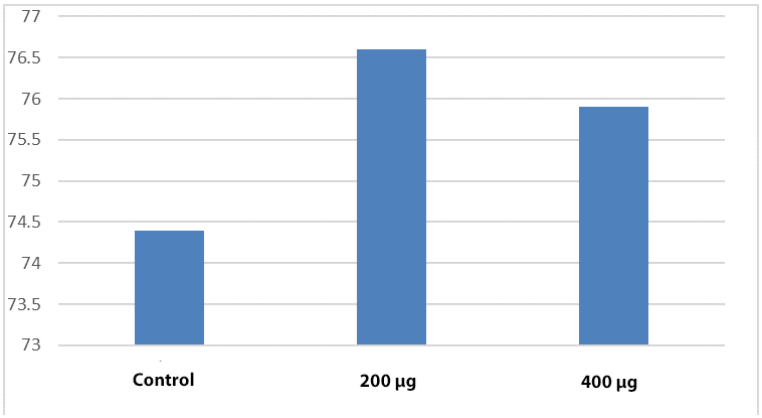


Figure 4.

4. Discussion

The results of this study showed that after 24 hours incubation of nanoparticles and macrophage cells, the mean number of viable cells and percentage of cell survival were highest in 200µg group and then 400µg is in the second category, with the lowest cell viability and the lowest percentage of cell survival were observed in the control group.

The percentage of cell survival is directly related to the average number of living cells. In this experiment, nano-amorphous calcium phosphate particles at two concentrations of 200 and 400µg lack cytotoxicity and increase percentage of cell survival and increase the number of viable cells.

Genotoxicity was assessed by Fitc_Annexin V / PI method which was evaluated by evaluation of apoptosis induction in three groups. Genetoxicity testing data indicate that apoptosis percentage increases as in concentration of nano-amorphous calcium phosphate that macrophages increased and the data in Table 3 and Figure 4 indicate that in the control group we have the lowest apoptosis and in the 400 µg group the highest apoptosis. But overall, the rate of apoptosis increase is statistically insignificant and

negligible. The highest number of healthy living cells in the experiment was observed in the 200 and 400μg groups, indicating that not only was the effect of genotoxicity very low but also an increase in the number of normal and healthy cells in the 200 and 400 μg groups compared to the control group [11].

Although the amorphous calcium phosphate nanoparticles are widely used in dental materials, especially composites, to date, no studies have been performed to evaluate the toxicity and adverse effects of nano-amorphous calcium phosphate particles on cells. Various experiments have been performed on the effects of other nanoparticles, including the effect of SiO2 and Al2O3 nanoparticles on RAW264 murine macrophages, which were cells similar to ours, conducted by Hashimoto and to investigate cellular toxicity of bioavailability 8-WST cells and Hoechst / PI apoptosis were used. DNA was damaged by exposure to both types of nanoparticles and cytotoxicity and genotoxicity were well correlated in this study [12]. Al2O3 particles in E.Radziun study were also described using SEM and BET analysis and these nanoparticles penetrated into BJ, L929 cells but unlike Hashimoto's study, there was no significant increase in apoptosis and no decrease in cell viability [7]. Al2O3 nanoparticles in the study of A.L. Virgilio were also examined on the hamster ovary. After treatment with 1-25 μg of Al2O3, no induction of sister chromatid alteration was found. In conclusion, the findings showed the effects of Al2O3 cytotoxicity and gene expression on K1-CHO cells [13].

TiO2 nanoparticles were investigated in A.L. Virgilio's research on Chinese hamster ovaries and in the study of Heravi et al. On rat L929 gingival fibroblasts in a titanium oxide composite form. The cytotoxicity test of Hervey et al. Was similar to our study of MTT and the results showed even less toxicity in the TiO2-containing composite group [14]. But the result of A.L.Virgilio's study using a neutral red technique, Contrary to the results of the study by Hervey et al., They show the effects of TiO2 cytotoxicity and genotoxicity on K1-CHO cells [13].

ZnO nanoparticles were studied in Pandurangan study on alanine transaminase (ALT), transparate (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) and mRNA enzyme modes in 12 C2C cells. The end result was an increase in all of these enzymes [15].

The toxicity of amorphous calcium phosphate particles was investigated in C. G. Simon J by microscopic phase shift and enzymatic evaluation for mitochondrial enzyme activity on osteoblast-like MC3T3-E1 cells. The results showed no change in cell morphology and cell survival. This indicates that non-toxic amorphous calcium phosphate is similar to nano-amorphous calcium phosphate [16].

The limitations of cell culture and gene and cell toxicity tests are time and costly. In addition to the potential limitations of laboratory studies in this study, therefore, its results cannot be generalized to clinical practice and there is a need for similar clinical studies. Among the limitations of the in vitro study of cell toxicity are the inability to keep cells alive after the testing process and the inability to detect and diagnosis the type of cell death. In addition, the organism has defense mechanisms and a lymphatic system that helps eliminate toxic released substances. However, these tests can be an estimate of survival, proliferation, and cell metabolism compared to the control group.

It is recommended to perform other bioassay tests including Comet Tunel, WST, DNA covalent binding assay and qRT-PCR analysis to complete the cytotoxicity and gene toxicity tests.

1. Due to differences in the performance of different cell lines, the effect of nanoparticle on other cell lines should also be investigated.
2. Other properties of nano-amorphous calcium phosphate should be investigated by in-vitro studies and, if desired, other clinical trials of this compound should first be evaluated in animal studies and then in clinical trials.
3. Evaluate the cytotoxicity and genotoxicity of nano-amorphous calcium phosphate at successive times with different concentrations.

Conclusion

Cytotoxicity and gene toxicity are important processes to evaluate the potential of risk with dental materials. The results of both cytotoxicity and genotoxicity tests in this study show that calcium nano-amorphous has no toxic effects, plus an increase in survival and healthy cell count. The results of this study support the use of nano-amorphous calcium phosphate in dental materials such as composite and glass ionomer.

Figures Legend

Figure 1. XRD sample of amorphous calcium phosphate synthesized.

Figure 2. a. Flowcytometry histogram of 200 µg group b. Flowcytometry histogram of control group c. Flowcytometry histogram of 400 µg group

Figure 3. a. Optical absorption status in the study groups. b. Mean optical absorption (OD) and its 95% confidence interval by study groups

Figure 4. Percentage of normal living cells after 24 h of incubation.

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