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# Cytotoxicity and Effects of a New Calcium Hydroxide Nanoparticle Material on Production of Reactive Oxygen Species by LPS-Stimulated Dental Pulp Cells

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ABSTRACT

**Introduction:** The aim of this study was to synthesize and characterize calcium hydroxide (CH) nanoparticles [CH-NP] and compare the cytotoxicity of these materials with that of mineral trioxide aggregate (White MTA) in human dental pulp mesenchymal cells (hDPMCs) stimulated by lipopolysaccharide (LPS). **Methods and Materials:** The CH-NP were synthesized by the co-precipitation method, and the physical properties were investigated through X-ray diffraction, scanning electron microscopy (SEM) and energy dispersive x-ray spectrometry (EDS). LPS-stimulated hDPMCs were placed in contact with different dilutions of culture media previously exposed to CH-NP and white MTA for 24 h. The groups were tested for cell viability by MTT formazan and Alamar Blue assays, the production of nitric oxide (NO) by Griess method and the production of reactive oxygen species (ROS) by means of the fluorescent oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Control groups for viability test were maintained in DMEM (not LPS-stimulated). For NO and ROS production, negative control group was cells in DMEM, and positive control was cells stimulated by LPS. The results were statistically analyzed by two-way ANOVA, Tukey's test and Dunnett's test ($a$=0.05). **Results:** The results showed that the cell viability remained above 50% in all materials, independent of the dilution in MTT formazan and Alamar Blue tests. MTA showed a reduction in NO production at dilutions of 1:4 to 1:32 compared with the positive control group ($P$<0.05). The tested materials exhibited lower ROS production by DPMCs than that by cells in the positive control group ($P$<0.05), and similar ROS production to the negative control group ($P$>0.05). **Conclusion:** The outcomes of present *in vitro* study showed that MTA and [CH-NP] were not cytotoxic materials, with MTA closer to the results of control group (DMEM). MTA and [CH-NP] reduced ROS production at basal levels, with MTA inhibiting NO production at higher dilutions.

**Keywords:** Calcium Hydroxide; Cell Survival; Lipopolysaccharides; Mineral Trioxide Aggregate; Nanoparticles

## Introduction

Vital pulp therapy (VPT) comprises procedures indicated to maintain pulp vitality in cases of exposure during caries excavation or as a result of trauma [1,2]. VPT corresponds to pulp cappings/pulpotomies and requires use of a covering biomaterial to form a protective layer over exposed vital pulp [1]. The success of VPT depends on the presence of pulp vitality without inflammation, control of hemorrhage or application of a nontoxic capping biomaterial [3]. Early interventions on pulp tissue is necessary to prevent increased inflammation, which can lead to pulp necrosis [4]. The presence of lipopolysaccharide causes pathologic changes; inducing pulp inflammation, excessive intracellular reactive oxygen species (ROS) [4] and nitric oxide (NO) [5].

In recent years, mineral trioxide aggregate (MTA) has emerged as a new class of pulp capping biomaterials. MTA is a calcium silicate-based hydraulic cement with excellent properties, *i.e.* good biocompatibility, bioactivity and osteoconductivity [6]. Recently, it has been reported that MTA has a higher success rate than calcium hydroxide (CH) when used as pulp capping biomaterial in permanent human teeth, resulting in less pulpal inflammation and more predictable formation of a hard dentinal bridge [7]. However, there are disadvantages regarding MTA, mainly tooth discoloration [8, 9] and the difficulty in handling [6, 10].

CH has a long track record of clinical success and thus, has been considered the "gold standard" of direct pulp capping materials in permanent teeth for many years [11]. CH is a strong base with a pH of ~12, which could provide excellent antibacterial properties [12] and induce mineralization [13]. However, CH has a number of limitations in its practical use; including poor sealing ability and lack of adhesion to dentine [14, 15]. Furthermore, its use in pulpotomy has been related to internal resorption in deciduous teeth [16]. However, CH-based materials continue to be widely studied, *via* variations in their formulation [17, 18] and combinations with other materials [16, 19].

In the last decade, the synthesis and characterization of new biomaterials for medical and dental purposes have advanced [20, 21]. Nanomaterials are products consisting of particles that have at least one dimension between 1nm and 100 nm, which results in better physicochemical properties than those of bulk materials [22]. In dentistry, several nanomaterials have been developed with better antimicrobial activity, mechanical reinforcement, aesthetics and therapeutic effects than those of traditional products. However, the production technique and toxicity are considered challenges regarding these materials [23].

CH nanoparticles [CH-NP] have higher levels of antimicrobial activity than conventional nanoparticles [17]. Although some microorganisms, such as *Enterococcus faecalis (E. faecalis)* , are resistant to conventional CH, CH-NP have efficiently eliminated *E. faecalis* from human root dentine, demonstrating the beneficial use of nanoparticles over traditional methods [17, 20]. Furthermore, CH-NP better can better penetrate and remain longer in dentinal tubules, causing a probable increase in the duration of their effects [17, 18]. The size, shape, chemical composition and presence of impurities of the nanoparticles influence the cellular response [24], and physical characterization and cytotoxicity evaluation of the new nanoparticle materials are essential. Furthermore, due to the need to avoid pulp tissue irritation by the contact of pulp capping materials, the evaluation of biocompatibility is of great importance.

The purpose of this *in vitro* study was to synthesize and characterize CH nanoparticles [CH-NP] and compare the cytotoxicity of the nanoparticles with that of MTA (White MTA) in human dental pulp mesenchymal cells (hDPMCs) stimulated by lipopolysaccharide (LPS). The null hypothesis was that the materials tested did not affect hDPMC responses.

## Materials and Methods

### *Synthesis and characterization of CH-NP*

CH-NP were synthesized via coprecipitation according to Silva *et al.* [25] using aqueous solutions of calcium chlorite (1 M) (Sigma-Aldrich, St. Louis, MO, USA) and sodium hydroxide (2 M) (Sigma-Aldrich, St. Louis, MO, USA). The X-ray diffraction (XRD) measurements were performed on a SHIMADZU XDR-6000 diffractometer (Shimadzu, Barueri, São Paulo, Brazil) operated at 20 kV and 2 mA with CuKα radiation (λ=1.5406 Å) in the powder samples [25]. Scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDS) were performed using an electronic microscope (Zeiss EVO MA10; Zeiss, Jena, Germany) and Oxford model 51-ADD0048 detector.

### *Preparation of extracts*

In this study, white MTA (MTA) (Angelus, Londrina, Paraná, Brazil), and CH-NP were used to prepare the extracts. MTA samples were prepared in 24-well plates according to the manufacturer's recommendations. CH-NP samples were prepared according to the same recommendations as the MTA samples by adding 0.28 g of the CH-NP to 3 mL of distilled water. Next, all materials were incubated at 37 °C for 24 h immediately after mixing [26]. Then, the specimens were covered with 2.5 mL of cell culture in Dulbecco's modified Eagle medium (DMEM) (Vitrocell Embriolife, Campinas, SP, Brazil) and incubated at 37 °C for 24 h in the dark. The original extracts (1:1) were prepared according to ISO 10993-5 recommendations [27]. After incubation, the original extracts were serially diluted in cell culture medium to a dilution of 1:32 before testing [26].

### *DPMC culture*

Primary hDPMC cultures were donated from the "Faculty of Dentistry, Federal University of Uberlândia" (UFU) (Ethics Committee protocol number 09016219.1.0000.5152). Two healthy deciduous teeth ($n$=2), close to their time of exfoliation, were collected/placed inside a conical tube with DMEM (Vitrocell) and immediately taken to the "Biomaterials and Cell Biology Laboratory". Then, the pulp was extracted from the interior of the pulp chamber using a sterilized sharp dentine spoon and immersed for 1 h in a solution consisting of 3 mg/mL collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) and 4 mg/mL dispase (Sigma-Aldrich St. Louis, MO, USA). The solution was centrifuged at 1200 rpm for 2 min and re-suspended

in basal medium. The obtained cells were plated in 25-$cm^2$ flasks and incubated at 37 °C for 4 days with 5% $CO_2$. The culture medium was first replaced after 3 days of incubation, and thereafter, it was changed twice a week. The cells were expanded up to the 4th passage and frozen for later experimental use [5].

***DPMC lipopolysaccharide-induced stress and exposure to extracts***

Cells were cultured in DMEM (Vitrocell, Embriolife) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) and 1% penicillin-streptomycin (Sigma-Aldrich) in a humid atmosphere of 5% $CO_2$ at 37 °C until confluence. The hDPMCs were plated on 96-well plates ($2\times10^4$ cells/well) and allowed to adhere for ~12 h. Then, the cells were incubated with 200 µL of tested material original extracts and serial dilutions with lipopolysaccharide (LPS, Ultrapure grade, *Escherichia coli* O111: B4; InvitroGen, San Diego, CA, USA) at a concentration of 10 µg/mL. After an incubation period of 24 h, the cells were immediately tested for i) viability by MTT formazan and Alamar Blue assays, ii) release of nitric oxide (NO) by Griess method [30], and iii) reactive oxygen species (ROS) by a fluorescent probe. The control group was maintained in DMEM (not LPS-stimulated) for MTT formazan and Alamar Blue tests. For NO and ROS production, the positive control group was stimulated by LPS, and the negative control group was maintained in DMEM (not LPS-stimulated). This study was repeated two times; using five samples for each group at every time period.

***MTT formazan and Alamar Blue***

The cell viability was evaluated 24 h after treatment with the extracts. MTT solution (Sigma-Aldrich) (5 mg/mL) was added to each well, and the cells were incubated at 37 °C for 4 h. Supernatants were removed, and then, 100 µL of dimethyl sulfoxide (DMSO) (LGC Biotecnologia, Cotia, SP, Brazil) was added. Next, the optical density (OD) at 570 nm was measured using a microplate reader (Biochrom, Cambridge, UK) [28]. A 10% Alamar blue solution (InvitroGen, Karlsruhe, Germany) was added to each well, and the cells were incubated at 37 °C for 24 h. Afterwards, the OD at 570-600 nm was measured using the same microplate reader [29]. The mean values obtained for the control group were considered 100% cell viability. Cell viability evaluation was performed proportionally to absorbance and expressed as a percentage of viable cells. Cells of control group were maintained in DMEM (not LPS-stimulated).

***Nitrite assay***

The NO production in the supernatants of hDPMCs was quantified spectrophotometrically 24 h after treatment with the extracts by means of the diazotization reaction with Griess reagent. In this method, nitrite is first treated with sulfanilamide in acidic media, followed by the addition of N-naphthyl-ethylenediamine (NED). In a 96-well plate, 100 µL aliquots of the supernatant of each of the samples were placed in quintuplicate, with the same quantity of Griess reagent. After 10 min of incubation at room temperature, the absorbance was determined using a microplate reader with a 540 nm filter (Biochrom). The absorbance of this reaction at 540 nm is linearly proportional to the nitrite concentration in the samples. Cells of positive control group was stimulated with LPS, and the negative control group was maintained in DMEM (not LPS-stimulated).

***Reactive oxygen species production***

Reactive oxygen species (ROS) production was quantified by means of the fluorescent oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, InvitroGen) [5]. After 24 h of cell incubation within the extracts, the medium was removed, and a solution, containing the fluorescent probe in PBS (5 µmol /L), was added (200 µL). After 45 min, the free radical release was measured using a multimode plate reader (GloMax® Discover Multimode Microplate Reader, Promega, SP, Brazil). Cells of positive control group were stimulated with LPS, and the negative control group was maintained in DMEM (not LPS-stimulated). The mean values obtained for the positive control group were considered 100% ROS production.

***Statistical analysis***

All data were analysed for normality using Kolmogorov-Smirnov's test. Two-way ANOVA and Tukey's tests were used to compare data between the treated groups. Dunnett's test was used to compare the experimental groups with the control groups. Statistical significance was set at $\alpha=0.05$. Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA, USA).

## Results

***Characterization***

Figure 1 presents the results of XRD, SEM and EDS. The XRD (Figure 1A) showed Bragg diffraction peaks, expressing characteristics of portlandite [CH, JCPDS: 72-0156] and confirming the formation of CH nanocrystals. The SEM image (Figure 1B) demonstrated the presence of crystals with hexagonal structures. The right inset shows a magnified image, reinforcing the hexagonal morphology of the crystals and indicating the size (D=~80 nm). The left inset shows the results of EDS, confirming the amounts/types of atoms that constituted the nanocrystals, reinforcing the XRD results on the formation of CH. It should be noted that hydrogen was not observed in EDS, however, as the percentage of O in relation to Ca was double, the formation of CH was presumed.

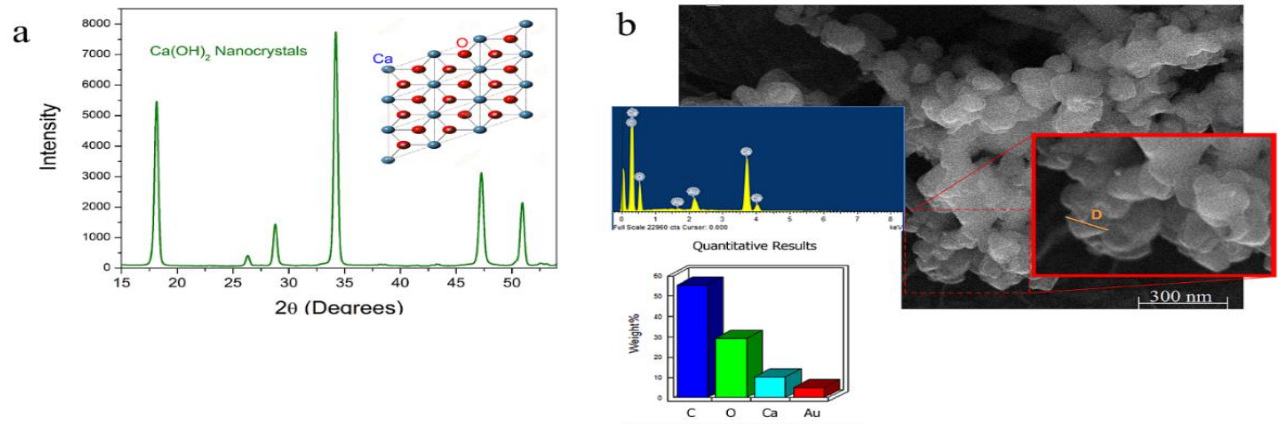


***Figure 1.*** Investigation of the type, morphology and composition of crystals by X-ray diffraction (XRD) and scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDS). (A) Bragg diffraction peaks characteristic confirming the formation of CH nanocrystals, (B) SEM image of CH nanocrystals. The left inset shows the EDS results.

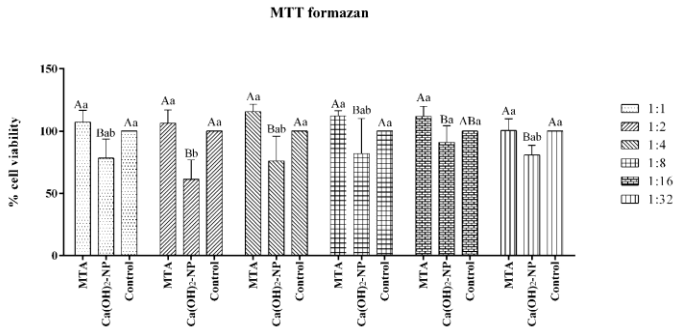


***Figure 2.*** Cell viability percentage of DPMCs after exposure to extracts, according to the material tested and the dilution by the MTT formazan method. Original extracts (1:1) were serially diluted in fresh media as indicated, the cell cultures were exposed for 24 h, and the cellular survival was determined in quintuplicate. Data are presented as mean±standard deviation. Capital letters indicate comparisons between different materials and between the materials and control group (DMEM). Lowercase allows comparisons of a material among the different dilutions.

### *MTT formazan*

Figure 2 presents the cell viability evaluated by MTT formazan assay after contact between hDPMCs and extracts, serially diluted to 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32. MTA presented similar cell viability among all dilutions ($P$>0.05), and between the dilutions and the control groups ($P$>0.05). CH-NP had similar percentages of cell viability among the dilutions ($P$>0.05), except between 1:2 and 1:16 dilutions, at which 1:16 presented higher values than 1:2 ($P$=0.0012). CH-NP had lower percentages of cell viability between all dilutions and the control groups ($P$>0.05), except for the 1:16 dilution, which had similar values ($P$=0.4182). When the materials were compared in the same dilution, MTA presented a higher percentage of viable cells than CH-NP at all dilutions ($P$<0.05). In addition, it could be observed, by the cell survival mean values, that all materials maintained cell viability levels above 60% for all dilutions.

### *Alamar Blue assay*

Figure 3 presents the cell viability evaluated by Alamar Blue assay after contact between hDPMCs and extracts serially diluted to 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32. MTA presented the highest value of cell viability at 1:2 dilution ($P$<0.05), and the lowest values at 1:1, 1:8 and 1:32 dilutions ($P$<0.05). CH-NP did not present significant differences in the percentage of cell viability amongst the dilutions evaluated ($P$>0.05). MTA presented higher percentages than control group at all dilutions ($P$<0.05). CH-NP presented lower values to those of the control group ($P$>0.05) in all dilutions, except for CH-NP at 1:4 and 1:16 dilutions, which showed lower viability ($P$<0.05). Comparing the materials at the same dilutions, CH-NP had lower viability than MTA at all dilutions ($P$<0.05). Cell survival mean values indicated that all materials maintained cell viability levels above 80% at all dilutions. Considering that a threshold of 70% cell viability is considered cytotoxic according to ISO 10993-5 guideline [27], the materials tested were not classified as cytotoxic.

### *Nitrite assay*

Figure 4 presents the amounts of NO produced by hDPMCs after contact between the cells and extracts serially diluted to 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32. The cells treated with MTA extracts had higher values of NO production at a 1:1 dilution than at 1:4 ($P$=0.0003), 1:8 ($P$=0.0119), 1:16 ($P$=0.0002), and 1:32 ($P$=0.0021) dilutions and then that of the negative control group (DMEM) ($P$=0.0148). The cells had lower NO production than the positive control group containing LPS, independent of the dilution evaluated ($P$<0.05), except for the 1:1 ($P$=0.5187) and 1:2 ($P$=0.0781) dilutions, which presented similar values. There was no statistically significant difference in NO production by hDPMCs treated with CH-NP extracts amongst the dilutions

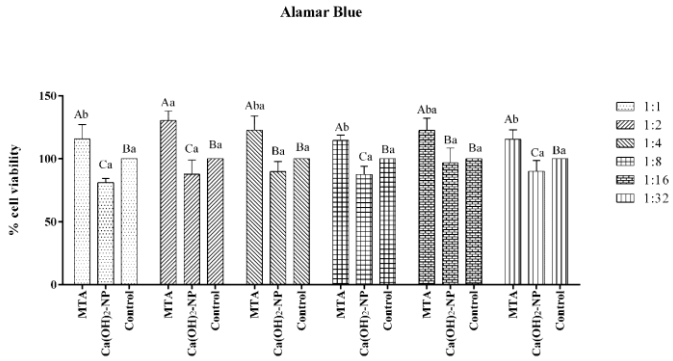


***Figure 3.*** Cell viability percentage of DPMCs after exposure to extracts, according to material tested and the dilution by the Alamar Blue method. Original extracts (1:1) were serially diluted in fresh media as indicated, the cell cultures were exposed for 24 h, and the cellular survival was determined in quintuplicate. Data are presented as mean ± standard deviation. Capital letters indicate comparisons between different materials and between the materials and control group (DMEM). Lowercase allows comparisons of a material among the different dilutions.

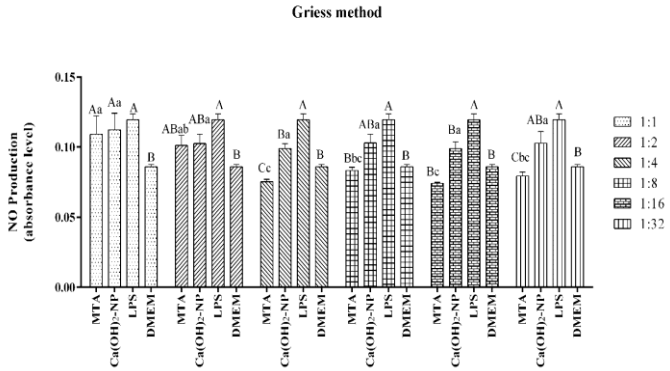


***Figure 4.*** Nitric oxide (NO) production by DPMCs after exposure to extracts, according to the material tested and the dilution. Original extracts (1:1) were serially diluted in fresh media as indicated, the cell cultures were exposed for 24 h, and NO production was determined in quintuplicate by the Griess method. Data are presented as mean±standard deviation. Capital letters indicate comparisons between different materials and between the materials with positive (LPS) and negative (DMEM) control. Lowercase allows comparisons of a material among the different dilutions

evaluated ($P$>0.05). The cells had similar NO production with positive (LPS) and negative (DMEM) control groups at all dilutions ($P$> 0.05), except for 1:1 dilution which presented higher values than the negative control (DMEM) ($P$=0.0040), 1:4 ($P$=0.0380) dilution and 1:16 ($P$=0.0380) dilution, showing lower values than the positive control (LPS) group. Comparing the materials at the same dilution, the NO production by hDPMCs was higher for CH-NP ($P$<0.05) than that of MTA, except for the dilutions at 1:1 ($P$=0.9745), 1:2 ($P$=0.9947) and 1:8 ($P$=0.0532), presenting similar values with MTA.

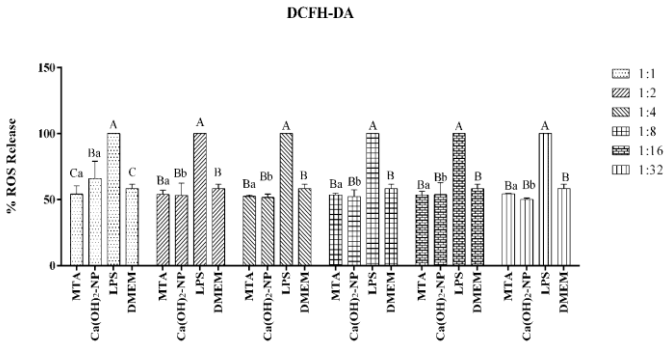


***Figure 5.*** Reactive oxygen species (ROS) production by DPMCs after exposure to extracts, according to the material tested and the dilution. Original extracts (1:1) were serially diluted in fresh media as indicated, the cell cultures were exposed for 24 h, and the ROS production was determined in quintuplicate by DCFH-DA probe. Data are presented as mean ± standard deviation. Capital letters indicate comparisons between different materials and between the materials with positive (LPS) and negative (DMEM) control. Lowercase allows comparisons of a material among the different dilutions

### *Reactive oxygen species (ROS) production*

Figure 5 presents the amounts of ROS produced by hDPMCs after contact between the cells and extracts serially diluted to 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32. There was no statistically significant difference in ROS release by the hDPMCs treated with MTA extracts amongst the dilutions evaluated ($P$>0.05). The groups treated with CH-NP extracts had the highest ROS release at 1:1 dilution ($P$<0.05). The percentage of ROS release by hDPMCs was similar for CH-NP and MTA; independent of the dilution ($P$>0.05), however, CH-NP had higher values than MTA at 1:1 dilution ($P$=0.0005). The cells had similar ROS production with the negative control group (DMEM) at all dilutions ($P$>0.05), except for CH-NP at 1:1, which had higher values ($P$<0.05). All groups presented lower percentages of ROS production compared with the positive control group (LPS) ($P$<0.05).

## Discussion

This study compared the cytotoxicity of CH-NP to that of MTA (White MTA) in hDPMCs stimulated with LPS. The tested materials affected the hDPMC responses, thus, the null hypothesis was disproved. Previous studies have evaluated the effects of MTA in pulp cells, demonstrating cytocompatibility, bioactivity [31,32], and biomineralization [31]. *In vivo* studies have shown that MTA does not induce an exacerbated inflammatory reaction [21,33], and has been considered to be the gold standard in primary pulpotomies [16]. As a result, MTA was chosen for comparison with the new nanocrystals evaluated in the present study. Additionally, a preliminary pilot study in triplicate was conducted

comparing CH-NP with commercially available CH powder, with no statistically significant differences for the lowest dilutions, justifying the need for no comparison in the current study. These results are in agreement with a previous study using L929 murine fibroblasts, in which the nanoparticulate CH showed cytotoxicity similar to that of the conventional CH [28].

The results showed that the chosen synthesis methodology produced pure, homogeneous and uncontaminated CH nanocrystals. The co-precipitation synthesis method has demonstrated adequate control over physical properties, *e.g.* dimension, structure, purity and stability, which are essential in biological tests [25,34]. The structure and composition of the synthesized material was evaluated using XRD, which is often used to characterize new materials. In this method, X-rays are directed into the crystalline material and reflect or diffract at various angles. Considering that the diffraction pattern is unique for each material, this method is appropriate for determining the components of materials, including dental materials. To complement the XRD results, SEM, which shows the microstructural and topographic features of the sample, and EDS, which allows the chemical analysis of the material with qualitative and quantitative information, were also performed. The XRD results confirmed the formation of CH nanocrystals, whereas the SEM images exhibited a hexagonal structure with a size of ~80 nm. Nanoparticle morphology is one of the most important characteristics amongst the factors affect the cytotoxicity of nanoparticles [35] and is directly related to the process of cell internalization based on endocytosis. The fact that nanoparticles are ~80 nm in size indicates that they can be internalized by both clathrin- and caveolae-mediated endocytosis [36]. Therefore, it is possible to speculate that internalization could be related to the cell viability parameters.

In literature, different dilutions of extracts are applied in cytotoxicity tests [26,37,38]. The dilution chosen in this study was based on Bin *et al.* [26] and in accordance with ISO 10993-5 recommendations [27]. This approach can be considered ideal for material evaluation, especially new materials *e.g.* CH-NP.It has been shown that the direct use of materials or undiluted extract on cells could lead to cell death. Furthermore, upon contact with tissue, leachable components are eliminated by extracellular fluid, and thus, their concentration decreases progressively [37].

hDPMCs are considered an applicable cell model to evaluate the outcomes of endodontic materials due to the physiological properties that are homologous to the primary tissue where the material is supposed to be in contact [39]. In addition to the already known pluripotency characteristics, this cellular model has shown to have i) a high proliferation capacity, ii) the ability to maintain the cellular phenotype for a long time, and iii) sensitivity to LPS [40]. To simulate the inflammatory environment often found in teeth submitted to pulpotomy, the cells were stimulated with *Escherichia coli* LPS; an outer membrane component of gram-negative bacteria that can stimulate the production of many cytokines in human dental pulp cells [2,5]. Furthermore, LPS-induced cellular stress results in increased ROS [5] and NO production [5,41], which have been used as nonspecific markers of *in vitro* induction of the inflammatory process [41].

In our study, biocompatibility was assessed using MTT assay and Alamar Blue cytotoxicity test, which determine the cell viability as a function of mitochondrial activity. The principle of MTT assay is the conversion of a tetrazolium salt into formazan crystals *via* mitochondrial dehydrogenases [42]. Alamar Blue assay involves the conversion of resazurin into resorufin by mitochondrial reductases and cytochromes [43]. The combination of these viability methods a) allows the evaluation of complementary parameters which could detect possible cytotoxic effects, and b) increases the validity of the results. This outcome is confirmed in the present study where slight differences in the results were observed between the tests, with special attention to the higher cell viability values found in MTA group *via* Alamar Blue test compared to the control group. MTA has been reported to induce proliferation of hDPMCs by elution components such as calcium ions [44]. The high proliferation of hDPMCs after the contact with MTA extracts corroborates previous studies [31,45]. Both tests indicated that the cells in contact with the MTA extract showed superior viability compared to those in contact with CH-NP at all dilutions. Since MTA is considered the gold standard for pulpotomies, similar results are desirable. The outcomes of the current investigation indicated that MTA presented less cytotoxicity than that of CH and agreed with previous studies [46,47]. In the related studies, calcium hydroxide-containing cements were prepared into transwell membranes [47], formed by direct contact [46], and were evaluated; however, avoiding comparisons due to methodological differences. It is possible that the high alkalinity of CH, which is in direct contact with pulpal tissue, could lead to the formation of a layer of coagulative necrosis and cause reduction in cell viability [47]. The lowest values of cell viability were related to CH-NP group. These results corroborate those found by Dianat *et al.* [28], where CH nanoparticles led to high cytotoxicity. One of the hypothesis could be related to the physicochemical characteristics of the biomaterials evaluated. Smaller particles have a larger specific surface area to interact with cellular components such as nucleic acids, proteins, fatty acids, and carbohydrates [24]. Furthermore, the small size of CH-NP makes it possible for these biomaterials

to enter into the cell, act on cell machinery related to cell-death control [24,48]. Future studies, which could evaluate the mechanism of internalization of these nanoparticles, are needed to confirm this hypothesis.

NO mediates the process of tissue inflammation through its pro-inflammatory capacity and the regulation of cell differentiation/growth [49]. It is enzymatically generated by NO synthases (NOS), in which L-arginine is oxidized to L-citruline [50]. Specifically, inducible NO synthases (iNOS) are activated though microbial products, such as LPS [49], as confirmed by the higher levels of NO in the LPS group (positive control) than in the DMEM group (negative control). It was expected that all biomaterials tested at different concentrations reduced cell-produced NO, presenting lower levels than the LPS treatment alone. However, the mentioned assumption was not observed in the groups containing CH-NP. These results contradict those found in an earlier study, where CH promoted decrease in NO release [51]. However, it is important to note that the latter study used RAW 264.7 macrophage cell line, not hDPMCs. Different cell lines may respond with different intensities to nanoparticles [35]. Regarding MTA, a reduction in cell-produced NO was observed, except for the lowest dilutions tested. MTA is composed of bismuth oxide which is added to provide radiopacity to the material. This Bismuth oxide induces iNOS expression and nitrite release in human dental pulp cells [41], which could justify the higher levels of NO in 1:1 and 1:2 dilutions, indicating a possible synergic effect of bismuth oxide with LPS. Moreover, LPS-induced cellular stress resulted in an increase in ROS production, which was in line with other studies [5]. ROS include oxygen-based free radicals; *e.g.* superoxide anion radicals ($O^{2-}$)/hydroxyl radicals ($OH^{-1}$), and nonradical oxidants, *e.g.* hydrogen peroxide ($H_2O_2$)/singlet oxygen ($^1O_2$), which are related to oxidative stress and cellular damage [52]. The down-regulation of ROS production in hDPMCs has been associated with an increase in odontoblastic differentiation [53,54]. As a result, biomaterials with intrinsic redox activity could be promising during odontoblastic differentiation of hDPMCs. Our results also demonstrated that the production of ROS was reduced by the application of extracts of the tested biomaterials, presenting levels near the basal level (unstimulated cells) and lower than those of the LPS-stimulated cells. Since that the biomaterials tested would be used in pulpotomies, this outcome is promising because the formation of mineralized tissue is desirable in these cases. However, further studies using CH-NP are needed to confirm this hypothesis.

Despite the limitations of the present research, it is important to emphasize that, to the best of our knowledge, the CH-NP proposed in this study have never been evaluated. The production methodology of the nanoparticles involved low cost and easy to obtain, which could allow its production on a commercial scale in emerging countries. Therefore, this study could be considered as an important preliminary investigation to define the specific physical characteristics of CH-NP and their biological behavior on pulp cells. Nevertheless, the results indicated a better performance of MTA, which remains the gold standard for pulpotomies.

## Conclusion

The cell viability results presented different complementary patterns, with cell viability percentages above 60% and 80%. Considering that the percentage of cell viability can be regarded optimal when the average value obtained is 70% or higher, the present results showed that MTA and CH-NP were not cytotoxic materials. CH-NP do not affect pulp cell NO production and reduce ROS production, making them a promising material for future studies.

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