Gabriela Leite de Souza

Síntese, caracterização e avaliação de viabilidade celular de nanocristais de óxido de zinco, óxido de zinco dopados com cálcio e hidróxido de cálcio.

Synthesis, caracterization and cell viability evaluation of zinc oxide, calcium doped zinc oxide and calcium hydroxide nanocrystals.

Dissertação apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, para a obtenção do Título de Mestre em Odontologia na Área de Clínica Odontológica Integrada.

Uberlândia, 2019.

Gabriela Leite de Souza

Síntese, caracterização e avaliação de viabilidade celular de nanocristais de óxido de zinco, óxido de zinco dopados com cálcio e hidróxido de cálcio.

Synthesis, caracterization and cell viability evaluation of zinc oxide, calcium doped zinc oxide and calcium hydroxide nanocrystals.

Dissertação apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, para a obtenção do Título de Mestre em Odontologia na Área de Clínica Odontológica Integrada.

Orientadora: Profa. Dra. Camilla Christian Gomes Moura

Banca Examinadora:
Profa. Dra. Camilla Christian Gomes Moura
Profa. Dra. Ana Paula de Lima Oliveira
Profa. Dra. Virgínea Oliveira Crema

Uberlândia, 2019.

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenação do Programa de Pós-Graduação em Odontologia
Av. Pará, 1720, Bloco 4L, Anexo B, Sala 35 - Bairro Umuarama, Uberlândia-MG, CEP 38400-902
Telefone: (34) 3225-8115/8108 - www.ppgoufu.com - copod@umuarama.ufu.br

Timbre

Ata

Ata da defesa de DISSERTAÇÃO DE MESTRADO junto ao Programa de Pós-graduação em Odontologia da Faculdade de Odontologia da Universidade Federal de Uberlândia.

Defesa de: Dissertação de Mestrado COPOD

Data: 27/02/2019

Discente: **Gabriela Leite de Souza (11712ODO011)**

Título do Trabalho: ***"Síntese e avaliação de nanocristais de óxido de zinco, óxido de zinco dopado com cálcio e hidróxido de cálcio na citotoxicidade e liberação de espécies reativas de oxigênio de células pulpares"***

Área de concentração: Clínica Odontológica Integrada.

Linha de pesquisa: Propriedades físicas e biológicas dos materiais e das estruturas dentais

Projeto de Pesquisa de vinculação: Propriedades físicas e biológicas dos materiais e das estruturas dentais

As **nove horas** do dia **vinte e sete de fevereiro de 2019** no Anfiteatro Bloco 4T, Campus Umuarama da Universidade Federal de Uberlândia, reuniu-se a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em janeiro de 2019, assim composta: Professores Doutores: Ana Paula Lima de Oliveira (UFU); Virginia Oliveira Crema (UFTM); e o orientador(a) do(a) candidato(a): **Camilla Christian Gomes Moura.**

Iniciando os trabalhos o(a) presidente da mesa ***Dra. Camilla Christian Gomes Moura*** apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de argüição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos (às) examinadore (as), que passaram a argüir o(a) candidato(a). Finalizada a argüição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais.

Em face do resultado obtido, a Banca Examinadora considerou o(a) candidato(a) (A)provado(a).

Esta defesa de Dissertação de Mestrado é parte dos requisitos necessários à obtenção do título de Mestre. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar foram encerrados os trabalhos às 11 horas e 40 minutos. Foi lavrada a presente ata que após lida e achada conforme foi assinada eletronicamente pela Banca Examinadora.

---

logotipo Documento assinado eletronicamente por **Camilla Christian Gomes Moura**, **Professor(a) do Magistério Superior**, em 27/02/2019, às 11:51, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Ana Paula de Lima Oliveira**, **Professor(a) do Magistério Superior**, em 27/02/2019, às 11:54, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **VIRGÍNIA OLIVEIRA CREMA**, **Usuário Externo**, em 27/02/2019, às 11:55, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

A autenticidade deste documento pode ser conferida no site https://www.sei.ufu.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **0986981** e o código CRC **D6F06CE6**.

Referência: Processo nº 23117.005960/2019-72 SEI nº 0986981

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da UFU, MG, Brasil.

S729s
2019

Souza, Gabriela Leite de, 1994
Síntese, caracterização e avaliação de viabilidade celular de nanocristais de óxido de zinco, óxido de zinco dopados com cálcio e hidróxido de cálcio = Synthesis, caracterization and cell viability evaluation of zinc oxide, calcium doped zinc oxide and ca [recurso eletrônico] / Gabriela Leite de Souza. - 2019.

Orientadora: Camilla Christian Gomes Moura.
Dissertação (mestrado) - Universidade Federal de Uberlândia, Programa de Pós-Graduação em Odontologia.
Modo de acesso: Internet.
Disponível em: http://dx.doi.org/10.14393/ufu.di.2019.1262
Inclui bibliografia.
Inclui ilustrações.

1. Odontologia. 2. Polpa dentária. 3. Óxido de zinco. 4. Nanocristais. I. Moura, Camilla Christian Gomes, 1979, (Orient.) II. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Odontologia. III. Título.

CDU: 616.314

Angela Aparecida Vicentini Tzi Tziboy – CRB-6/947

DEDICATÓRIA

Aos meus pais, pelo amor, compreensão e apoio inesgotável e incondicional que me concedem todos os dias. Essa obra não seria possível sem vocês.

AGRADECIMENTOS

Agradeço primeiramente à Deus, que me proporcionou discernimento e sabedoria para concluir mais uma fase da minha vida. Meu muito obrigada pelas provas diárias de amor e por ter colocado pessoas tão especiais ao meu lado, sem as quais certamente não teria conseguido concluir esse trabalho.

A meus pais, Adolfo e Maria do Rosário, meu infinito agradecimento. Sempre acreditaram em minha capacidade e me acharam “a melhor de todas”, mesmo não sendo. Isso só me fortaleceu e me fez tentar, não ser "a melhor" mas a fazer o melhor de mim. Obrigada pelo amor incondicional.

A minha avó, Maria Perpétua, por todo amor e paciência. Agradeço por sempre acreditar no meu potencial e pelas orações feitas por mim durante esse período. Tenho certeza que foram fundamentais para minha proteção e fortalecimento da minha fé.

A meu namorado, Diego, por ser tão importante na minha vida. Sempre a meu lado, me pondo para cima e me fazendo acreditar que posso mais que imagino. Devido a seu companheirismo, amizade, paciência, compreensão, apoio, alegria e amor, este trabalho pôde ser concretizado.

À Profª Camilla que acreditou no meu potencial de uma forma que eu não acreditava ser capaz de corresponder. Sempre disponível, disposta a ajudar, e me incentivando a aproveitar cada segundo dentro do mestrado para absorver algum tipo de conhecimento, afinal de contas “conhecimento não ocupa espaço”. Você me proporcionou a realização de alguns de meus sonhos e não foi somente orientadora, mas também conselheira, mãe e amiga. Você é uma referência profissional e pessoal para meu crescimento. Obrigada por estar a meu lado e acreditar tanto em mim. Serei sempre grata.

A Profª Ana Paula, Profª Anielle e Prof. Marcelo que com ensinamentos, orientações e amizade, me ajudaram ativamente ou passivamente neste projeto. Vocês também são referências para mim.

A minha turma de mestrado, por ser sempre tão solícita e pela partilha diária de informações, vendo um ao outro como futuros colegas de trabalho, e não como

concorrentes. Em especial, agradeço a Dani, Marcela, Simone, Luciana, Luís e Brenda, pela dedicação nos trabalhos em grupo e pelo aprendizado que adquiri com vocês.

Aos amigos do laboratório Camilla, Lílian, Milena, Luís, João, Jéssica e Amanda, obrigada por tornar os meus dias leves, por sempre confiarem no meu trabalho e me acalmarem quando eu pensava que estava tudo desmoronando. Vocês foram essenciais em cada momento e levarei a amizade de vocês em meu coração.

Aos técnicos do laboratório, Bruno, Eliete e Jhon, e secretárias da pós-graduação pela disponibilidade, simpatia e gentileza. Obrigada pela ajuda.

Ao Programa de Pós-Graduação, Faculdade de Odontologia, e ao laboratório CPbio por abrirem as portas para que eu pudesse realizar este sonho. Proporcionaram-me mais que a busca de conhecimento técnico e científico, mas uma lição de vida. Agradeço, também, à CNPq pelo apoio financeiro durante esses dois anos de trabalho.

Obrigada a todos, nada seria possível sem vocês!

"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar.
Mas o mar seria menor se lhe faltasse uma gota".

Madre Teresa de Calcutá

SUMÁRIO

Resumo

O objetivo deste trabalho foi sintetizar e carazterizar nanocristais de óxido de zinco (ZnO), óxido de zinco dopado com diferentes porcentagens de cálcio (ZnO : X Ca; X = 0.7, 1.0, 5.0, 9.0) e hidróxido de cálcio ($Ca(OH)_2$ - NC) e avaliar os nanocristais de ZnO e Zno:XCa na viabilidade de diferentes modelos celulares Os nanocristais foram sintetizados e analisados pelo método de Difração de Raios-X (DRX) e microscopia eletrônica de varredura (MEV) com espectrometria de energia dispersiva de Raios-X (EDS). Os nanocristais de ZnO e ZnO : X Ca foram solvatados e avaliados nas concentrações de 10 µg/ml, 50 µg/ml e 100 µg/ml, na viabilidade de RAW264.7 e SAOS-2, pelos métodos MTT formazan e Alamar Blue. Todos os dados obtidos foram estatisticamente analisados por análise de variância em fator único, seguido por teste de Tukey e Dunnett (α=0.05). Os dados obtidos pelas análises de DRX, MEV e EDS, mostraram que os nanocristais sintetizados foram característicos de ZnO, ZnO : X Ca e $Ca(OH)_2$, variando entre 20 e 80 nm. De acordo com os métodos de caracterização, todos os nanocristais foram sintetizados com sucesso, sendo que os nanocristais de ZnO, ZnO : 0.7 Ca e ZnO : 1.0 Ca e a concentração de 10 µg/ml foram os que menos afetaram a viabilidade de SAOS-2 e RAW264.7.

Palavras-chave: Citotoxicidade, Hidróxido de Cálcio, Nanocristais, Óxido de Zinco.

Abstract

The objective of this research was to synthesize and characterize zinc oxide (ZnO), zinc oxide doped with different percentages of calcium (ZnO: X Ca; X = 0.7, 1.0, 5.0, 9.0) and calcium hydroxide ($Ca(OH)_2$ - NC) nanocrystals. Furthermore, to evaluate ZnO and ZnO : X Ca on the viability of different cell models. The nanocrystals were synthesized and analyzed by the X-ray Diffraction (XRD) method and scanning electron microscopy (SEM) with energy dispersive X-Ray spectrometry (EDS). ZnO and ZnO : X Ca were solvated and evaluated at the concentrations of 10 μg/ml, 50 μg/ml and 100 μg/ml on the viability of RAW264.7 and SAOS-2 by MTT formazan and Alamar Blue. All data were statistically analyzed by One-Way ANOVA, followed by Tukey and Dunnett tests (α= 0.05). The data obtained by the XRD, SEM and EDS analyzes showed that the synthesized nanocrystals were characteristic of ZnO, ZnO:XCa and $Ca(OH)_2$, with the size ranging from 20 to 80 nm. According to the characterization methods, all nanocrystals were successfully synthesized. ZnO, ZnO : 0.7 Ca and ZnO : 1.0 Ca presented the highest percentages of cell viability.

Key Words: Calcium Hydroxide, Cytotoxicity, Nanocrystals, Zinc Oxide.

INTRODUÇÃO E REFERENCIAL TEÓRICO

A terapia pulpar vital (TPV) tem como objetivos manter a vitalidade da polpa, função e posição do dente no arco (Cohenca *et al.* 2013), sendo indicada no tratamento de injúrias pulpares em casos de exposição pulpar durante a remoção do tecido cariado ou fratura traumática (Gandolfi *et al.* 2014, Kim *et al.* 2018). Contrariamente, a terapia pulpar não vital, envolve a remoção do tecido pulpar inflamado ou necrótico e eliminação de microrganismos e seus subprodutos, através da limpeza do canal radicular promovida durante o preparo químico-mecânico (Saleh *et al*. 2004).

O tratamento da polpa exposta em dentes permanentes tem sido controverso (Awawdeh *et al.* 2018). Porém, atualmente, realizar o tratamento endodôntico convencional somente porque ocorreu exposição pulpar, não deveria ser rotineiro (Awawdeh *et al.* 2018), uma vez que a preservação da vitalidade conserva o mecanismo de defesa da polpa e capacita a formação de dentina (Whitherspoon *et al.* 2008). Além disso, o dente tratado endodonticamente possui maior incidência de fratura radicular, por ser mais frágil e requerer mais carga para registrar uma resposta proprioceptiva, comparado a um dente vital (Awawdeh *et al.* 2017). Sendo assim, a TVP é uma alternativa conservadora de tratamento que mantem a vitalidade da polpa (Cohenca *et al.* 2013, Trope 2008). Na prática clínica, esse termo corresponde aos procedimentos de capeamento pulpar direto ou indireto, no qual um material de cobertura é administrado sobre a polpa exposta ou dentina profunda, respectivamente, ou pulpotomia, o qual envolve a remoção da polpa coronária seguida pela cobertura do tecido pulpar remanescente (Cohenca *et al.* 2013).

O material considerado ideal para capeamento pulpar deveria aderir ao substrato dental, manter um selamento suficiente, ser insolúvel aos fluidos teciduais, dimensionalmente estável, não reabsorvível, não tóxico, não carcinogênico, não genotóxico, radiopaco e exibir biocompatibilidade e bioatividade (Camilleri *et al.* 2006, Roberts *et al.* 2008, Torabinejad *et al.* 2010). Entretanto, nenhum dos biomateriais atualmente disponíveis tem sido capaz de satisfazer todos os requisitos de um agente ideal para TVP (Roberts *et al.* 2008, da Rosa *et al.* 2017).

Desde a evolução da odontologia, vários materiais dentários têm sido desenvolvidos para obter máximo potencial e gerar melhor resposta tecidual. Com o avanço do entendimento acerca do reparo do complexo dentino-pulpar e processo de regeneração, novos biomateriais têm sido propostos para manutenção da vitalidade pulpar, priorizando a odontologia minimamente invasiva (Mente *et al.* 2010, Bergenholtz *et al.* 2013). Nesse sentido, a nanotecnologia tem capacitado a produção de materiais dentários em escala nanométrica, com melhores propriedades físico-quimicas (Vilela Teixeira *et al.* 2017, Zanjani *et al.* 2018). A nanotecnologia é um campo que está crescendo rapidamente, o qual é definido como: pesquisa e desenvolvimento da tecnologia dos níveis atômico, molecular e macromolecular usando uma escala de aproximadamente 1-100 nm em qualquer dimensão (Das *et al.* 2009). Este campo mostra aplicações promissoras, criando materiais com novas propriedades e atributos que os materiais em escala normal não apresentam (Das *et al.* 2009).

A síntese do tamanho e formato controlados da nanoestrutura do material, é muito importante no controle das propriedades físicas e químicas para sua potencial aplicação (Haja Hameed *et al.* 2013). As propriedades ópticas das nanopartículas têm sido extensivamente estudadas nos últimos anos. Como o tamanho do material se torna menor e a "banda gap" maior, as suas propriedades elétricas são modificadas (Haja Hammed *et al.* 2013). Sabe-se que as propriedades biológicas das nanopartículas estão intimamente relacionadas às suas propriedades físicas, como formato e tamanho da estrutura da nanopartícula (Bae *et al.* 2005, El Mir *et al.* 2007). O tamanho nanométrico das partículas resulta em maior superfície de contato com os componentes biológicos permitindo melhor biodisponibilidade e absorção (Huang *et al.* 2017, Landisiedel *et al.* 2012). Isso também amplia seu espectro de ação e efetividade antibacteriana (Louwakul *et al.* 2016), observando-se que, enquanto alguns materiais já apresentam essa característica em sua forma natural, outros apresentam apenas na sua forma nanoparticulada (Seil & Webster 2012). Uma das formas de mudar as propriedades ópticas e elétricas das nanopartículas, e, consequentemente seus efeitos biológicos é a técnica de dopagem (Bae *et al.* 2005, Xia *et al.* 2011). A dopagem consiste no processo pelo qual se introduzem impurezas em uma rede cristalina de um semicondutor de maneira a modificar adequadamente suas propriedades físicas, com a intenção de melhorar suas funcionalidades ou reduzir propriedades indesejáveis (Adeleye *et al.* 2018).

O óxido de zinco (ZnO) tem sido amplamente utilizado na odontologia em combinação com o eugenol, como material obturador endodôntico em dentes decíduos (Pilownic *et al.* 2017) e material de cobertura em pulpotomias (Gonzalez-Lara *et al.* 2011). Esse material é frequentemente usado devido a suas propriedades sedativas e paleativas em casos de dor pulpar, entretanto, diversos efeitos tóxicos tem sido reportados (Hui Derksen *et al.* 2013). Independente se os cimentos com base em ZnO são julgados ser biocompatíveis ou não, essa determinação poderia ser considerada não confiável dado que o ZnO geralmente é testado na presença de outras substâncias como o eugenol, formaldeído, corticosteróides entre outros (Gulati *et al.* 1991, Gerosa *et al.* 1996, Geurtsen *et al.* 1997).

ZnO tem mostrado reduzir naturalmente a atividade de uma ampla variedade de bactérias (principalmente gram-positivas) (Soderberg *et al.* 1990). A implementação da nanotecnologia aumentou ainda mais as propriedades antibacterianas do ZnO (Seil & Webster 2012, Zhang *et al.* 2007). Na odontologia, estudos *in vitro* tem mostrado que nanopartículas de ZnO apresenta propriedades e ação antibacterianas dose-dependente, reduzindo a população de *Enterococcus faecalis* nos túbulos dentinários e biofilme por mais de 90 dias. Efeitos similares também foram identificados contra *Escherichia coli*, o qual se manifestou como dano a membrana celular da bactéria, resultando em liberação dos componentes intracelulares e morte da bactéria (Liu *et al.* 2009).

O hidróxido de cálcio ($Ca(OH)_2$) tem uma longo histórico de sucesso clínico, e é considerado o "padrão ouro" entre os materiais de capeamento pulpar direto (Hilton *et al.* 2009). $Ca(OH)_2$ é uma base forte, com pH de aproximadamente 12, o que providencia excelentes propriedades antibacterianas (Chen & Suh 2017) e tem a propriedade de indução a mineralização (Wu *et al.* 2018). Três tipos de $Ca(OH)_2$ tem sido usados para capeamento pulpar direto, incluindo $Ca(OH)_2$ de uma pasta, $Ca(OH)_2$ auto-regulável de duas pastas e $Ca(OH)_2$ modificado por resina (Chen & Suh 2017). Recentemente, a eficácia do $Ca(OH)_2$ em forma de nanopartícula foi avaliada na eliminação de *Enterococcus faecalis* na dentina radicular humana, demonstrando benefícios quando comparado aos agentes tradicionais (Louwakul *et al.* 2016). Além disso, o $Ca(OH)_2$ nanoparticulado penetra melhor dentro do túbulo dentinário e permanece por mais tempo, prolongando o seu efeito (Roy & Bhattacharya 2010, Dianat *et al.* 2015).

Considerando que o tamanho, formato, composição química e presença de impureza das nanopartículas influenciam a resposta celular (Hussain *et al.* 2014, Huang *et al.* 2017), é essencial a caracterização de novos nanomateriais, bem como a análise da citotoxicidade. Nesse contexto, para caracterizar os materiais nanoparticulados, diferentes técnicas são usadas. Dentre elas o método de Difração de Raios-X e Espectroscopia de Energia Dispersiva de Raios-X, para as propriedades estruturais, microscopia eletrônica de varredura, microscopia eletrônica de transmissão para as propriedades morfológicas (El Mir *et al.* 2017) e degradação de azul de metileno para análise da atividade catalítica de cada material (Wei *et al.* 2018). Nas análises biológicas, técnicas de cultura celular são úteis para a avaliação da biocompatibilidade de diferentes materiais (Peters *et al.* 2013). Adicionalmente, ensaios *in vitro* com culturas celulares são comumente usados para elucidar o mecanismo envolvido em diferentes respostas biológicas e para investigar o comportamento celular em situações específicas. Apesar da importância das avaliações *in vitro*, é importante ressaltar que os resultados não podem ser imediatamente extrapolados para condições clínicas em humanos, embora representam um modelo apropriado para a triagem correta de diferentes propriedades dos materiais odontológicos, bem como para avaliar seus potenciais riscos para a saúde (Pires *et al.* 2016).

Diante do exposto acima, este trabalho teve por objetivo sintetizar e caracterizar nanocristais (nanopartículas cristalinas) de óxido de zinco (ZnO), óxido de zinco dopadas com cálcio (ZnO:XCa) e hidróxido de cálcio (Ca(OH)2) e avaliar os nanocristais de ZnO puros e dopados com diferentes porcentagens de íons Ca^{2+} (0.7%; 1.0%; 5.0%; 9.0%) na viabilidade celular de células de linhagem de osteocarcoma humano SAOS-2, células de linhagem de macrófágos murinos RAW 264.7 .

CAPÍTULO 1

Effects of Zinc Oxide and Calcium doped Zinc Oxide Nanocrystals on cell viability of different cell culture models.

Souza GL, Silva ACA, Dantas NO, Turrioni APS, Bonvicini JFS, Moura CCG.

Effects of Zinc Oxide and Calcium doped Zinc Oxide Nanocrystals on cell viability of different cell culture models.

Gabriela Leite de Souza[1], Anielle Christine Almeida Silva[2], Noelio Oliveira Dantas[2], Ana Paula Silveira Turrioni[3], Jéssica Fernanda Sena Bonvicini[3], Camilla Christian Gomes Moura[1].

[1] Department of Endodontics, School of Dentistry, University of Uberlândia, Uberlândia, MG, Brazil; [2] New Nanostructured and Functional Materials Laboratory, Physics Institute, Federal University of Alagoas, Maceió, AL, Brazil ; and [3] Department of Pediatric, School of Dentistry, University of Uberlândia, Uberlândia, MG, Brazil

Running title: ZnO and ZnO:Ca nanocrystals on cytotoxicity

Key Words: Calcium Oxide, Cytotoxicity, Nanocrystals, Zinc Oxide, synthesis and characterization

***Corresponding Author**

Prof. Camilla Christian Gomes Moura

Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil.

Av Pará 1720, Campus Umuarama, Block 4L, annex A, Uberlândia, MG, Brazil.

Zip-Code: 38405-328

E-mail address: camillahistologia@yahoo.com.br Phone number: +55 34 3225-8119

Abstract

Aim: To synthesize and to characterize pure ZnO and Ca^{2+} doped ZnO (ZnO : Ca) nanocrystals (NCs) (ZnO : 0.7 Ca, ZnO : 1.0 Ca, ZnO : 5.0 Ca and ZnO : 9.0 Ca), and evaluate the cytotoxicity in human osteosarcoma SAOS-2 and leukemia macrophage murine RAW 264.7 cell lines.

Methodology: The NCs were synthesized by coprecipitation method. The physical properties were investigated through X-Rays Diffraction, scanning electron microscopy images and degradation of methylene blue. ZnO, ZnO : 0.7 Ca, ZnO : 1.0 Ca, ZnO : 5.0 Ca and ZnO : 9.0 Ca were cultivated in contact with SAOS-2 and RAW 264.7 at 10, 50 and 100 µg/ml concentrations. Cell viability were analyzed at 24 h using MTT formazan and Alamar Blue. Data were submitted to one-way ANOVA, Tukey and Dunnett tests ($\alpha = 0.05$).

Results: The X-Ray diffractograms show Bragg diffraction peaks characteristic of ZnO and ZnO : X Ca. SEM demonstrated that the diameter obtained were: 50 nm (ZnO), 30 nm (0.7 Ca), 20 nm (1.0 Ca), maintaining the same value for samples 5.0 and 9.0 Ca. MB degradation founded were 59, 61, 37, 29 and 37% to samples of ZnO, 0.7, 1.0, 5.0 and 9 wt.% Ca, respectively. Regarding the cell viability, ZnO, ZnO : 0.7 Ca and ZnO : 1.0 Ca at 10 µg/ml were not cytotoxic to SAOS-2 and RAW 264.7.

Conclusions: According to the characterization methods, the nanocrystals were successfully synthesized. ZnO, ZnO : 0.7 Ca and ZnO:1.0 Ca presented the highest percentages of cell viability.

Introduction

Vital pulpal therapy (VPT) is an alternative to endodontic treatment to maintain pulpal vitality (Trope 2008, Cohenca *et al.* 2013), which may be indicated for permanent vital teeth independent of the signs and symptoms of irreversible pulpitis (Linsuwanont *et al.* 2017, Taha & Abdulkhader 2018). VPT preserves the vitality of the pulp remaining and promotes a new formation of dentin to cover the exposed pulp. In clinical practice, the term VTP corresponds to direct or indirect pulp capping procedures, in which a cover material is administered in the exposed pulp or dentin, respectively; or pulpotomy, involving the removal of the coronary pulp followed by direct coverage of the pulp tissue remaining (Cohenca *et al.* 2013). The ideal agent should contain some main characteristics, among them be bactericidal; be innocuous to the pulp and periodontal tissues, to induce formation of mineralized tissue, promote the healing of the root pulp (Gonzalez-Lara *et al.* 2016). However, until this time, no material containing all these features has been found.

Considering the current trend of minimally invasive dentistry, which promotes the preservation of natural tissues, the development of new agents for VPT represents new ways of maintaining pulp vitality. In this sense, nanotechnology has enabled the production of dental materials on a nanometric scale, with better physicochemical properties (Vilela Teixeira *et al.* 2017, Zanjani *et al.* 2018). The development of nanoparticles for medical purposes has been widely investigated, since when the size is greatly reduced at the nanometric scale, the reason surface/volume increase generating new and interesting properties, such as a greater ability to absorb drugs, probes and proteins (Landsiedel *et al.* 2012, Silva *et al.* 2018**).** In addition to size and shape, a crystalline nanoparticle (NPs) alters as both physical and biological properties. The size reduction provides better bioavailability compared to bulk material, improving the absorption of nanoparticles in biological systems (Das *et al.* 2009). In relation the crystalline structure, Reis Éde *et al.* 2015 have shown that amorphous nanoparticles are more genotoxic than crystalline (nanocrystals). In addition, a crystal phase in which nanocrystals (NCs) themselves also enables

physical and biological properties (Silva *et al.* 2018). Nanomaterials can interact with cellular components and induce cytotoxic effects, since proteins and cellular parts range between 5 and 50 nm (Valdiglesias *et al.* 2015).

Zinc oxide (ZnO) have been widely used as endodontic filling material (Pilownic *et al.* 2017) and pulpotomy (Gonzalez-Lara *et al.* 2016) for primary teeth. ZnO has been shown, in its natural form, the ability to reduce the activity of a wide variety of bacteria (Soderberg *et al.* 1990), and its nanoparticulate form improves this profile (Lipovsky *et al.* 2011). It is known that the properties of the nanoparticles are strictly related to the shape and size of the nanoparticle structure (Bae *et al.* 2005, El Mir *et al.* 2007). In addition to size and shape, the crystallinity of nanoparticles influences biological results, for example, Reis Éde *et al.* 2015 have shown that amorphous nanoparticles are more genotoxic than crystalline (nanocrystals).

One of the ways to change the properties of nanoparticles, and therefore their environmental implications, is the doping technique (Xia *et al.* 2011, Silva *et al.* 2018). Doping consists of the intentional modification of the composition of the nanoparticles by the addition of dopants (impurities), with the intention of improving their functionalities or reducing undesirable properties (Adeleye *et al.* 2018). Many agents have been suggested in the doping process of ZnO nanoparticles (Silva *et al.* 2018). Among them, it has been reported the calcium doping in ZnO (ZnO:Ca), that relaxes strain in the unit cell (Karthikeyan *et al.* 2011) and exhibit antibacterial activity (Haja Hameed *et al.* 2013). Considering that the calcium hydroxide ($Ca(OH)_2$), is a regenerative material widely used in VPT, responsible to inducing the formation of mineralized tissue (Aguilar & Linsuwanont 2011, Tsukibosh *et al.* 2017), it would be interesting to evaluate the association of calcium to zinc oxide, in the nanoparticulate form, using the doping technique.

This study synthesized pure ZnO and Ca^{2+} doped ZnO (ZnO:xCa) NCs in different percentages of Ca^{2+} (x= 0.7%; 1.0%; 5.0%; 9.0%), evaluated the chemistry and physical properties and cell viability in human osteosarcoma cell line SAOS-2 and leukemia macrophage murine cell line RAW 264.7.

Materials and Methods

Preparation of the pure ZnO and Ca-doped ZnO

ZnO NCs were synthesized via coprecipitation based on the reference of Silva *et al.* 2018. The ZnO:xCa samples were synthesized by incorporating a solution of calcium chloride (≥99.9%) (Sigma-Aldrich, St. Louis, MI, USA) in the concentration of xwt. % of Ca in relation to zinc (x= 0.7%; 1.0%; 5.0%; 9.0%).

Characterization

The X-Ray Diffractometer (XRD) measurements were performed on a SHIMATZU diffractometer XDR-6000 operated at 20kV and 2mA with CuK radiation (λ=1.5406 Å) in the powder samples. Scanning electron microscopy (SEM) images were performed by electronic microscope (Zeiss EVO MA10) in the powder samples. Photocatalytic activities of samples were tested in degradation of methylene blue (MB) under artificial light irradiations, with a 300 W Xenon lamp as the light source. In details, 8 mg photocatalyst was added into 50 mL MB solution (0.02 mmol/L) in a cylindrical reactor. A magnetic stirrer was used to ensure the homogeneous dispersion of the photocatalyst during the reaction. The photocatalytic reactor was positioned 15 cm below the light source and the photocatalytic reaction was initiated by adding the catalyst into the reactor and switching on the lamp. In controlled intervals, 1.0 mL suspension was collected for analysis. Adsorption experiments were conducted in the same conditions without the Xenon light irradiations. MB concentration was measured by a UV-VIS-NIR spectrophotometer (Shimadzu) at the wavelength of 663 nm. The percentage of discoloration was calculated by the formula 1: % Discoloration = $1 – (A/A_o) \times 100$ (1), where A is the absorbance of the solution at time $t > 0$, and A_o is the initial absorbance.

RAW 264.7 / SAOS-2 cell culture and nanocrystals treatment

RAW 264.7 and SAOS-2 were obtained from Cell Bank of Rio de Janeiro (ATCC, Rio de Janeiro, RJ, Brazil). Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Vitrocell Embriolife, Campinas, SP, Brazil) supplemented with 10% heat inactivated FBS

(Gibco, Langley, OK, USA) and 1% penicillin-streptomycin (Sigma-Aldrich), in a humid atmosphere of 5% CO_2 and 37 °C, until confluence. Quintuplicate of RAW 264.7 and SAOS-2 were plated on 96-well plates (2×10^4 cells/well) and were allowed to adhere overnight. Then the cells were stimulated with autoclaved ZnO and ZnO : Ca solutions at concentrations of 10 μg/mL, 50 μg/mL and 100 μg/mL, in a fresh medium, for 24 h. The control group contained cells maintained in culture medium (DMEM). After the incubation period, the cells were prepared for cell viability analysis by MTT and Alamar blue viability assays.

Cell Viability Assays

In all cell models, the cell viability was evaluated 24h after the treatment with NCs. MTT solution (Sigma-Aldrich) (5 mg/ml) was added to each well, and cells were incubated at 37ºC for 4 h. Supernatants were removed and then 100 μl dimethyl sulfoxide (DMSO) (LGC Biotecnologia, Cotia, SP, Brazil) was added. After, the optical density (OD) at 570 nm was measured using a microplate reader (Biochrom, Cambridge, UK). The 10% Alamar blue solution (InvitroGen, Karlsruhe, BW, Germany) was added to each well, and cells were incubated at 37ºC for 24 h. After,the OD at 570-600 nm was measured using a microplate reader. In both viability assays, the absorbance value readings were normalized to untreated control cultures (DMEM-100%), and differences between mean values were statistically analyzed.

Statistical Analysis

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

Results

Characterization of pure and Ca doped ZnO Nanocrystals

X-Ray Diffraction (XRD)

The structural properties changes in crystal lattice, type and percentage of crystals were performed by X-ray diffractograms of the samples and is shown in Figure 1. The X-Ray diffractograms show Bragg diffraction peaks characteristic of ZnO in the wurtizite structure (JCPDS: 36-1451), confirming the formation of ZnO NCs (Figure 1A). As the increase of the concentration of Ca we observe a shift of the diffraction peak (100) to smaller angles (Figure 1B). This displacement is related to the substitutional incorporation of the Zn by Ca ions in the crystalline structure of ZnO, since the ionic radius of Ca (0.99 Å) is higher than Zn (0.74 Å). Moreover, the presence of Bragg diffraction peaks characteristic of CaO NCs (JCPDS: 00-037-1497) is observed from the 0.7 Ca concentration and intensifies with (Figure 1C) the increase in the concentration of Ca in the samples increases from 3.8% to 6.7% for samples 0.7 to 9.0 Ca (Figure 1D). In the samples that occur the formation of NCs of CaO is denominated as nanocomposite (Ca doped ZnO + CaO NCs) illustrated in Figure 1C.

Scanning electron microscopy (SEM)

The incorporation of ions into the crystalline structure can change the morphology, so the morphological properties of the samples were investigated by scanning electron microscopy (SEM) with energy dispersive X-Ray spectrometry results (EDS) (Figure 2). SEM images show that ZnO NCs (Figure 2A) in its greater quantity spherical morphology with small amount in rod-shaped. However, the incorporation of Ca in the ZnO structure favors growth in rod length (Figure 2B-E). The increase in rod length ranges from 160, 200, 300, 400 and 350 nm for the samples, ZnO, 0.7 Ca, 1.0 Ca, 5.0 Ca and 9.0 Ca, respectively. This result indicates that Ca ions are being incorporated into the sides of the crystal favoring growth in length. Regarding the diameter, the following values were obtained: 50 nm (ZnO), 30 nm (0.7 Ca), 20 nm (1.0 Ca), maintaining the same value for samples 5.0 and 9.0 Ca. These results reinforce the results of XRD (Figure 1), confirming the incorporation of Ca ions into the crystalline structure of ZnO. In relation to NCs of spherical shape, it is observed that their quantity increases, however the average size remains

around 50 nm. In the EDS results observed only the Zn and O atoms (Figure 2F). There were no Ca atoms of the results of EDS due to the low percentage of CaO formed in the sample, as demonstrated in the XRD results (Figure 1). The presence of C and Au atoms is related to the sample port and metallization, respectively. It can be stated that the formation of the CaO compound occurred on the basis of the variation of the percentage of Zn by O, for example, in the sample of ZnO the ratio between Zn for O is one confirming the formation of ZnO, since an ion of Zn is required for one of O. Already in the doped samples, observed that there was an increase in the percentage of O indicating that there is the formation of some compound containing O, which by the results of XRD is CaO.

Methylene blue degradation (MB)

The photocatalytic activities and % Discoloration of these samples were examined in MB degradation aqueous solution under 25 °C and UV–vis light irradiation and is shown in Figure 3. After UV-Vis irradiation for 5 h, the MB degradation is found to be 59, 61, 37, 29 and 37% to samples of ZnO, 0.7, 1.0, 5.0 and 9 wt.% Ca, respectively.

Biologic Assays

SAOS- 2 Cytotoxicity

MTT Formazan Assay

The cytotoxicity of NCs on human osteoblast cancer cell line by MTT formazan assay is shown in Figure 4. At 10 µg/ml concentration, ZnO, ZnO : 0.7 Ca and ZnO : 1.0 Ca had the highest percentage of cell viability ($p < 0.05$) (Figure 4A). At 50 µg/ml the ZnO : 1.0 Ca had higher viability compared with all experimental groups ($p < 0.05$) (Figure 4B). At 100 µg/ml, ZnO : 0.7 Ca and ZnO : 1.0 Ca, presented higher values of cell viability compared with ZnO : 5.0 Ca and ZnO : 9.0 Ca ($p < 0.05$) (Figure 4C). Compared with the control group, ZnO : 5.0 Ca and ZnO : 9.0 Ca had lower percentage of cell viability at 10 µg/ml ($p < 0.05$) (Figure 4A). All groups

presented lower percentage of viability at 50 µg/ml (Figure 4B) and 100 µg/ml (Figure 4C) than the control group ($p < 0.05$), except ZnO : 1.0 Ca at 50 ug/ml that presented similar values ($p > 0.05$).

Alamar Blue Assay

The cytotoxicity of NCs on human osteoblast cancer cell line by Alamar Blue assay is shown in Figure 5. At 10 µg/ml and 50 µg/ml concentrations, the ZnO : 1.0 Ca and ZnO : 0.7 Ca groups presented the highest percentage of cell viability ($p < 0.05$) (Figure 5A). At 50 µg/ml the ZnO : 1.0 Ca and ZnO : 0.7 Ca had higher viability compared with all experimental groups ($p < 0.05$) (Figure 5B). At 100 µg/ml, the experimental groups did not present statistical differences among them ($p > 0.05$) (Figure 5C). Compared with the control group, ZnO and ZnO : 5.0 Ca had lower percentage of cell viability at 10 µg/ml ($p < 0.05$) (Figure 5A) and 50 µg/ml ($p < 0.05$) (Figure 5B). In addition, ZnO : 9.0 Ca also had lower viability compared with the control at 50 µg/ml ($p < 0.05$) (Figure 5B). All groups presented lower percentage of viability at 100 µg/ml than the control group ($p < 0.05$) (Figure 5C).

RAW 264.7 Cytotoxicity

MTT Formazan Assay

The cytotoxicity of NCs on murine macrophage cell line by MTT formazan assay is shown in Figure 6. All groups evaluated had similar viability percentage among themselves ($p > 0.05$) and similar values compared with the control ($p > 0.05$), regardless of the concentration evaluated (Figure 6A, 6B and 6C), except ZnO group at 100 µg/ml (Figure 6C), which presented lower cell viability compared with the control ($p > 0.05$).

Alamar Blue Assay

The cytotoxicity of NCs on murine macrophage cell line by Alamar Blue assay is shown in Figure 7. All groups presented similar absorbance levels at 10 µg/ml, except the ZnO : 5.0 Ca,

that presented the lowest viability ($p < 0.05$) (Figure 7A). At 50 µg/ml (Figure 7B) and 100 µg/ml (Figure 7C), the percentage of cell viability was similar among all groups tested ($p > 0.05$). All groups presented lower cell viability compared with the control at the concentrations of 50 µg/ml ($p < 0.05$) (Figure 7B) and 100 µg/ml ($p < 0.05$) (Figure 7C). At 10 µg/ml all groups presented similar values of cell viability compared with the control ($p > 0.05$), except ZnO : 5.0 Ca which presented lower percentage ($p < 0.05$) (Figure 7A).

Discussion

The results of the physical characterizations confirmed that ZnO NCs were synthesized and that with the increase of Ca doping concentration the formation of CaO NCs occurs. The SEM images confirmed that ZnO NCs and its greater quantity spherical morphology with small amount in rod-shaped. The increase in doping concentration favored the increase in the rod length while those of spherical morphologies remained the same size. All results confirmed the incorporation of Ca ions into the crystalline structure of ZnO and formation of CaO to samples doped with Ca.

The treatment with the NCs evaluated did not affect cell viability in lower concentration, but at the concentrations of 50 µg/ml and 100 µg/ml, the viability was reduced. However, it was possible to observe that the results varied according to the type of test performed. Studies have demonstrated different toxic effects associated to nanomaterials, including mitochondrial damage (Babele *et al.* 2018). Both the MTT formazan and the Alamar Blue methods are based on mitochondrial cell activity (Gonzalez & Tarlloff 2001). MTT assay is based on the conversion of yellow tetrazolium salt into dark blue formazan crystals by mitochondrial dehydrogenases activity (Mosmann, 1983). The Alamar Blue assay involves the conversion of resazurin (oxidized form) a non-fluorescent, blue state, into resorufin, which is fluorescent, pink state, by mitochondrial reductases. However, the Alamar Blue method also involves the action of enzymes located on the cytoplasm, also displaying cytosolic and microsomal activity, which increases the

detection of cytotoxic effects in the cells (O'Brien *et al.* 2000). The combination of these methods in the study increase the chances of detecting any possible cytotoxic effects related to NCs concentration in cell culture assays, increasing the validity of the studies.

Regarding the cells models used to test the NCs, the cell models selected to the first stage of this study was macrophage RAW 264.7 and osteoblast SAOS-2 cell lines. Controversy exists regarding the choice of permanent cell lines or primary cell cultures as study models (Honegger, 2001). Although primary culture cells are obtained directly from the organism and therefore exhibit more faithful behavior (Honegger, 2001), the use of immortalized cell cultures in a preliminary study assessing new materials is recommended in the literature (Das *et al.* 2018; Zhang *et al.* 2018), and are useful complementary systems for studying normal cells.

In general the results of both cell viability tests indicated that the less cytotoxic NCs were ZnO, ZnO: 0.7 Ca and ZnO : 1.0 Ca at 10 µg/ml, and cells treated with NCs containing the highest percentage of calcium in their composition (ZnO: 5.0 Ca and ZnO: 9.0 Ca) showed the lowest values of cell viability. This can be justified by the different percentages of calcium present in each evaluated nanocrystal. Calcium ions (Ca^{+2}) play important roles in cellular functions, acting as secondary messengers that control cellular responses and mediate physiological pathways, including cell death pathways (Wojcik-Piotrowicz *et al.* 2016). The scientific literature states that when the mechanism of homeostasis of calcium ions signals are disturbed, the cells can die by apoptosis or necrosis (Berridge *et al.* 2003, Wojcik-Piotrowicz *et al.* 2016). An overload of extracellular Ca^{+2} may increase its intracellular concentration, but the cell response can differ depending on whether the calcium signal develops as a local or global change (Wojcik-Piotrowicz *et al.* 2016).

Another mechanism related to the cytotoxicity of cells treated with ZnO : 5.0 Ca and ZnO : 9.0 Ca, is the highest percentage of doping of these NCs. The increase in doping percentage alters physical properties such as zeta potential and dielectric constant. These properties can lead

to a greater internalization of the doped NCs inside the cell and the interaction with proteins and biomolecules related to the production of ROS and cellular apoptosis (Das *et al.* 2018), inducing a higher cytotoxicity, as observed in the results of the present study. The greater number of ROS is attributed mainly to the larger surface area, an increase in oxygen vacancies and the diffusion ability of the reactant molecules (Haja Hameed *et al.* 2013). It was reported that oxygen vacancies can acts as electron capturing center, which will restrain the combination of photogenerated electrons (e^- CB) and holes (h^+ VB) and promotes photocatalytic ability (Meng *et al.* 2018). Ca into the B site of $BaTaO_2N$, i.e. $BaCa_{x/3}Ta_{1-x/3}O_{2+y}N_{1-y}$ ($0 \leq x, y \leq 1$) was proposed, and successfully gained control over its defects levels (e.g. Ta^{4+} species) and band gap improving the photocatalysis (Wei *et al.* 2018). Another study reported the excellent degradation dye activities of Ca (II) doped In_2S_3 are mainly attributed to the large amount of effectively reactive species like h^+ and O_2^- (Yang *et al.* 2017).

The oxygen vacancy acted as active center and was the main reason for causing the difference in photocatalytic activities for varied morphologic Mg, Ca and Y-doped ZnO/La_2O_3 samples (Li *et al.* 2014). Thus, based on the literature is justified highest photocatalytic activity of 0.7 wt.% Ca sample. Nevertheless, introducing more high level of Ca (more than 0.7 wt.% Ca) has negative effects to both photocatalytic activity and stability, probably due to rapid descending of visible light absorbance and ascending of defects levels (Wei *et al.* 2018), the excess of defects can act as the recombination center of the electrons and holes pairs. The radicals oxidative species, such as OH^- and O_2^-, generated on the surface of catalysts are responsible for degradation of MB. The 0.7 wt.% Ca sample shows MB degradation of 61 %, being greater than that of ZnO (59%), indicating that the synergism effect between NCs may intensify the photocatalytic activity. The diminution of MB degradation of 1.0, 5.0 and 9.0 wt. % Ca samples, in relation of ZnO, of 37, 29 and 37% to is due percentage of CaO relatively higher than in the other samples that disfavored photocatalysis.

Despite the limitation of the present research, it is important to emphasize that the NCs used in this study have never been evaluated before. Therefore, this is an important preliminary study to define the specific physical characteristics of these NCs and the cytotoxic effect on the cellular models used in contact with these materials. However, more studies should be performed using another models of cell cultures and molecular analyzes, to complement the present findings.

Conclusions

The results show that the synthesis methodology produced pure, homogeneous and uncontaminated nanocrystals. The results of XRD and EDS investigated the composition of the nanocrystal and confirmed the formation of ZnO and ZnO:XCa nanocrystals. In relation to the cytotoxicity ZnO, ZnO : 0.7 Ca and ZnO : 1.0 Ca presented the highest percentages of cell viability.

Conflict of Interest

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

Acknowledgments

This project was developed on CPBio - Biomechanics, Biomaterials and Cell Biology Research Center, Multiuser laboratory of the Institute of Chemistry and Faculty of Chemical Engineering, both of the Federal University of Uberlândia. The research was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, FAPEMIG and CNPq.

References

Adeleye AS, Pokhrel S, Mädler L, Keller AA (2018) Influence of nanoparticle doping on the colloidal stability and toxicity of copper oxidenanoparticles in synthetic and natural waters. *Water Research* **132**, 12-22.

Aguilar P, Linsuwanont P (2011) Vital pulp therapy in vital permanent teeth with cariously exposed pulp: a systematic review. *Journal of Endodontics* **37**, 581-7.

Babele PK, Thakre PK, Kumawat R, Tomar RS (2018) Zinc oxide nanoparticles induce toxicity by affecting cell wall integrity pathway, mitochondrial function and lipid homeostasis in Saccharomyces cerevisiae. *Chemosphere* **213**, 65-75.

Bae SY, Na CW, Kang JH, Park J (2005) Comparative structure and optical properties of Ga-, In-, and Sn-doped ZnO nanowires synthesized via thermal evaporation. *The Journal of Physical Chemistry B* **109**, 2526-31.

Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nature Reviews Molecular Cell Biology* **4**, 517-29.

Cohenca N, Paranjpe A, Berg J (2013) Vital pulp therapy. *Dental Clinics of North America* **57**, 59-73.

Das M, Saxena N, Dwivedi PD (2009) Emerging trends of nanoparticles application in food technology: Safety paradigms. *Nanotoxicology* **3**, 10-18

Das BK, Verma SK, Das T *et al.* (2018) Altered electrical properties with controlled copper doping in ZnO nanoparticles infers their cytotoxicity in macrophages by ROS induction and apoptosis. *Chemico-Biological Interactions* **297**, 141-54.

El Mir L, Ben Ayadi Z, Saadoun M, Von Bardeleben HJ, Djessas K, Zeinert A (2007) Optical, electrical and magnetic properties of transparent, n-type conductive $Zn_{0.90-x}V_{0.10}Al_xO$ thin films elaborated from aerogel nanoparticles. *Physica Status Solidi* **204**, 3266-77.

Gonzalez RJ, Tarloff JB (2001) Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity. *Toxicology In Vitro* **15**, 257-9.

Gonzalez-Lara A, Ruiz-Rodriguez MS, Pierdant-Perez M, Garrocho-Rangel JA, Pozos-Guillen AJ (2016) Zinc Oxide-Eugenol Pulpotomy in Primary Teeth: A 24-Month Follow-up. *Journal of Clinical Pediatric Dentistry* **40**, 107-12.

Haja Hameed AS, Karthikeyan C, Senthil Kumar V, Kumaresan S, Sasikumar S (2013) Impact of alkaline metal ions Mg2+, Ca2+, Sr2+ and Ba2+ on the structural, optical, thermal and antibacterial properties of ZnO nanoparticles prepared by the coprecipitation method. *Journal of Materials Chemistry B* **1**, 5950–5962.

Honegger P (2001) Overview of cell and tissue culture techniques. *Current Protocols in Pharmacology* **4**, 12.1.1-12.1.12.

Karthikeyan B, Pandiyarajan T, Mangaiyarkarasi K (2011) Optical properties of sol-gel synthesized calcium doped ZnO nanostructures. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **82**, 97-101.

Landsiedel R, Fabian E, Ma-Hock L *et al.* (2012) Toxico-/biokinetics of nanomaterials. *Archives of Toxicology* **86**, 1021-60.

Li C, Hu R, Zhou T *et al*. (2014) Special morphologies of Mg, Ca, and Y-doped ZnO/ La 2 O 3 composite for photocatalysis. *Materials Letters* **124**, 81-84.

Lipovsky A, Nitzan Y, Gedanken A, Lubart R. (2011) Antifungal activity of ZnO nanoparticles--the role of ROS mediated cell injury. *Nanotechnology* **22**, 105101.

Linsuwanont P, Wimonsutthikul K, Pothimoke U, Santiwong B (2017) Treatment Outcomes of Mineral Trioxide Aggregate Pulpotomy in Vital Permanent Teeth with Carious Pulp Exposure: The Retrospective Study. *Journal of Endodontics* **43**, 225-230.

Meng F, Liu Y, Wang J *et al.* (2018) Temperature dependent photocatalysis of g-C 3 N 4 , TiO 2 and ZnO: Differences in photoactive mechanism. *Journal of Colloid and Interface Science* **532**, 321- 330.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods* **65**, 55-63.

O'Brien J, Wilson I, Orton T, Pognan F (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry* **267**, 5421-6.

Pilownic KJ, Gomes APN, Wang ZJ *et al.* (2017) Physicochemical and Biological Evaluation of Endodontic Filling Materials for Primary Teeth. *Brazilian Dental Journal* **28**, 578-586.

Reis Éde M, de Rezende AAA, Santos DV *et al* (2015) Assessment of the genotoxic potential of two zinc oxide sources (amorphous and nanoparticles) using the in vitro micronucleus test and the in vivo wing somatic mutation and recombination test. *Food and Chemical Toxicology* **84**, 55-63.

Silva ACA, Zóia MAP, Correira LIV et al. (2018) Biocompatibility of Doped Semiconductors Nanocrystals and Nanocomposites. In: Celik TA, ed. *Cytotoxicity*, 1st edn; pp. 149-161. London, UK

Söderberg TA, Sunzel B, Holm S, Elmros T, Hallmans G, Sjöberg S (1990) Antibacterial effect of zinc oxide in vitro. *Scandinavian journal of plastic and reconstructive surgery and hand surgery*. **24**, 193–197.

Taha NA, Abdulkhader SZ (2018) Full Pulpotomy with Biodentine in Symptomatic Young Permanent Teeth with Carious Exposure. *Journal of Endodontics* **44**, 932-937.

Tsukiboshi M, Ricucci D, Siqueira JF Jr (2017) Mandibular Premolars with Immature Roots and Apical Periodontitis Lesions Treated with Pulpotomy: Report of 3 Cases. *Journal of Endodontics* **43**, S65-S74.

Trope M (2008) Regenerative potential of dental pulp. *Journal of Endodontics* **34**, S13-7.

Valdiglesias V, Kiliç G, Costa C *et al.* (2015) Effects of iron oxide nanoparticles: cytotoxicity, genotoxicity, developmental toxicity, and neurotoxicity. *Environmental and molecular mutagenesis* **56**, 125-48.

Vilela Teixeira AB, Vidal CL, de Castro DT *et al.* (2017) Effect of incorporation of a new antimicrobial nanomaterial on the physical-chemical properties of endodontic sealers. *Journal of conservative dentistry* **20**, 392-397.

Wei S, Zhang G, Xu X (2018) Activating BaTaO 2 N by Ca modifications and cobalt oxide for visible light photocatalytic water oxidation reactions. *Applied Catalysis B: Environmental* **237**, 373–381.

Wójcik-Piotrowicz K, Kaszuba-Zwoińska J, Rokita E, Thor P (2016) Cell viability modulation through changes of Ca(2+)-dependent signalling pathways. *Progress in biophysics and molecular biology* **121**, 45-53.

Xia T, Zhao Y, Sager T *et al.* (2011) Decreased dissolution of ZnO by iron doping yields nanoparticles with reduced toxicity in the rodent lung and zebrafish embryos. *ACS applied nano materials* **5**, 1223-35.

Yang S, Xu CY, Zhang BY, Yang L, Hua SP, Zhen L (2017) Ca(II) doped □-In 2 S 3 hierarchical structures for photocatalytic hydrogen generation and organic dye degradation under visible light irradiation. *Journal of Colloid and Interface Science* **491,** 230–237.

Zhang R, Liu X, Xiong Z *et al.* (2018) The immunomodulatory effects of Zn-incorporated micro/nanostructured coating in inducing osteogenesis. *Artificial cells, nanomedicine, and biotechnology* **8**, 1-8

Zanjani VA, Tabari K, Sheikh-Al-Eslamian SM, Abrandabadi AN (2018) Physiochemical Properties of Experimental Nano-hybrid MTA. *Journal of medicine and life* **11**, 51-56.

Figures and Legends

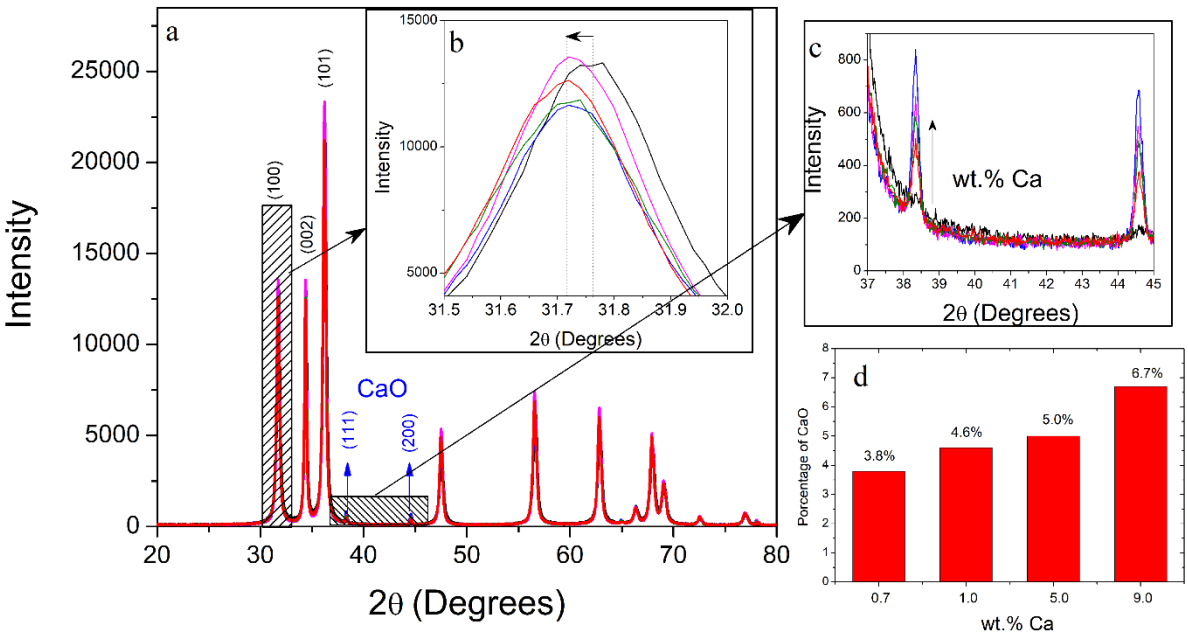

Figure 1. X-ray diffractograms of the ZnO:xCa nanocrystals. X-ray diffraction patterns of pure and Ca doped ZnO nanocrystals (A), variation in angular distance confirming the incorporation of Ca ions into ZnO nanocrystals (B), CaO nanocrystals patterns observed from the 0.7 wt.% Ca (C), increase in the concentration of CaO with the Ca doping from 3.8% to 6.7% for samples 0.7 to 9.0 wt% (C).

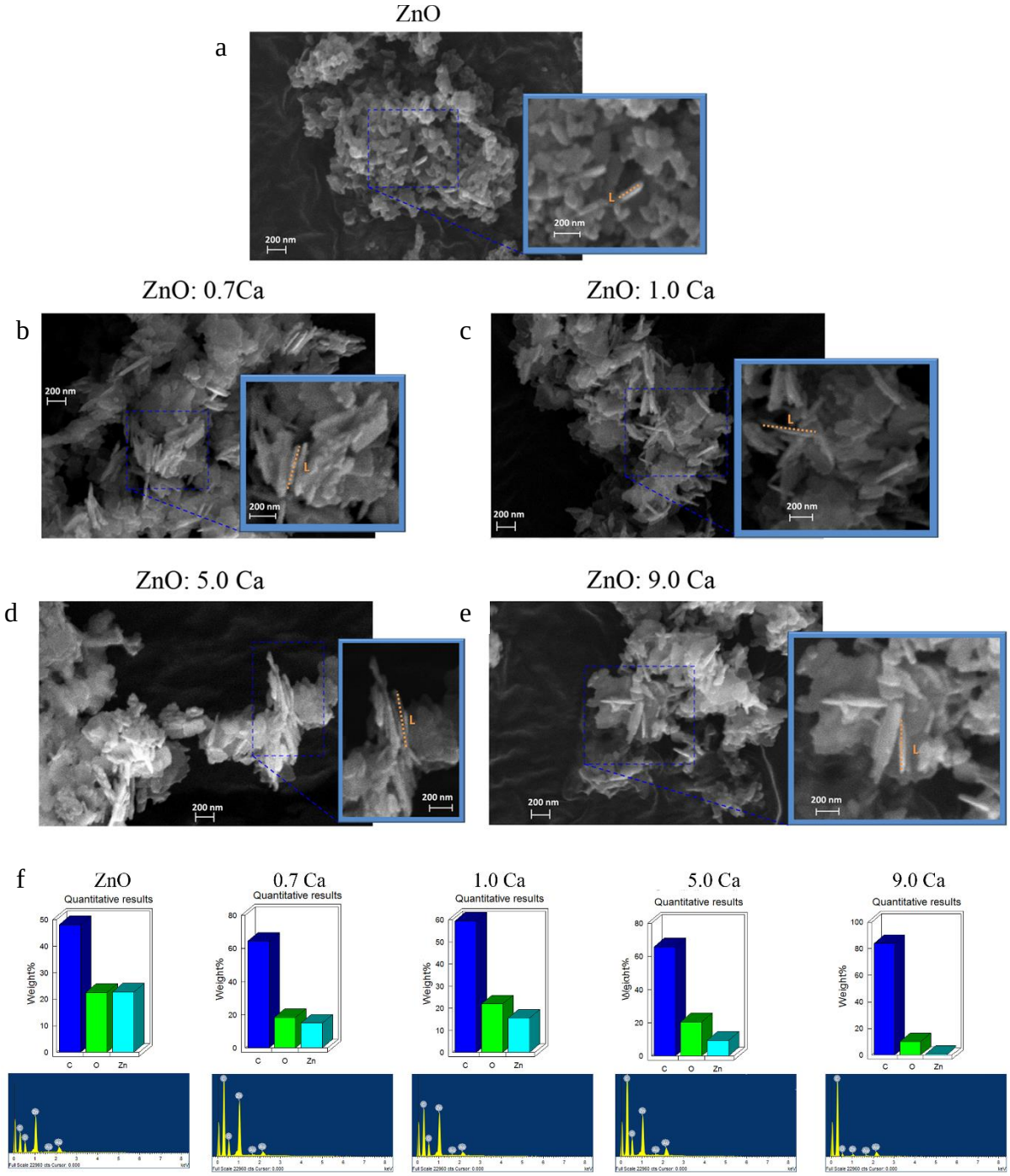

Figure 2. Scanning electron microscopy (SEM) and EDS results of the ZnO:xCa nanocrystals. ZnO nanocrystals (A) with spherical morphology and in rod-shaped, growth in rod length of ZnO nanocrystals with the incorporation of Ca (B-E). In the EDS results observed only the Zn and O atoms (F).

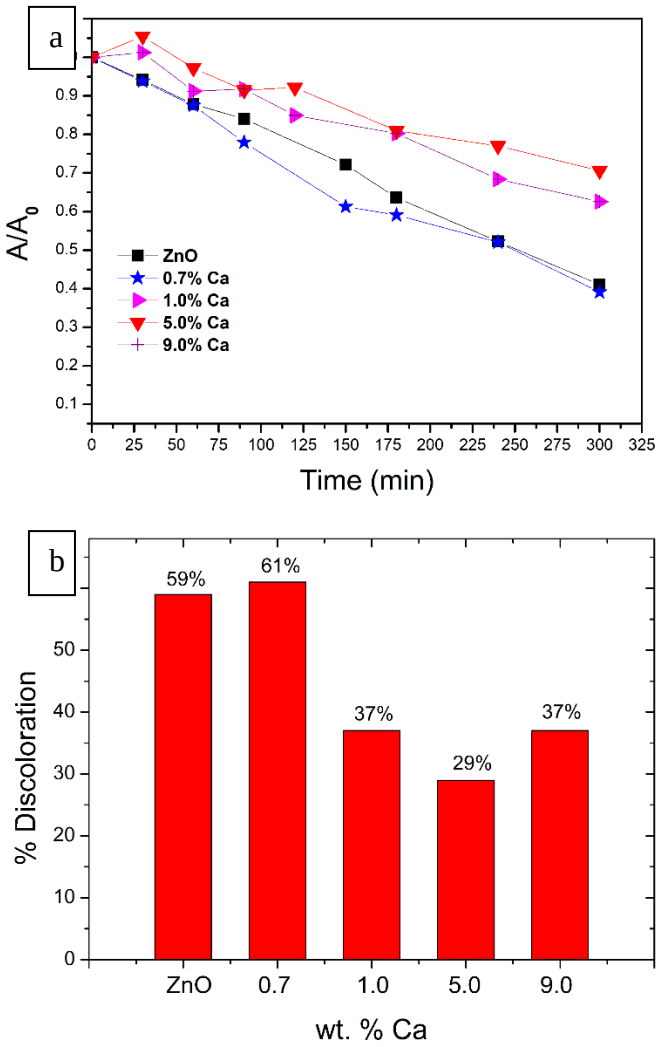

Figure 3. MB degradation aqueous solution and UV–vis light irradiation of the ZnO:xCa nanocrystals . Photocatalytic activities (A) and % Discoloration by MB degradation (B).

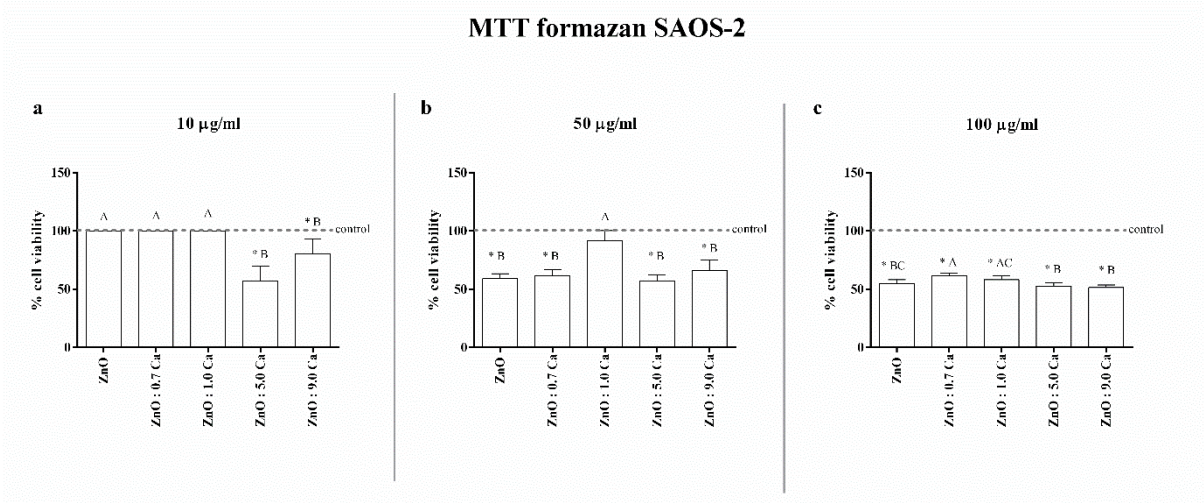

Figure 4. Cell viability determination of SAOS-2 after treatment with ZnO, ZnO : 0.7 Ca, ZnO : 1.0 Ca, ZnO : 5.0 Ca and ZnO : 9.0 Ca by MTT formazan assay. Effects of nanoparticles at the concentrations of 10 µg/ml (A), 50 µg/ml (B) and 100 µg/ml on SAOS-2 viability. Capital letters allow comparison among different nanocrystals. * allow comparison among the different nanocrystals and the control group.

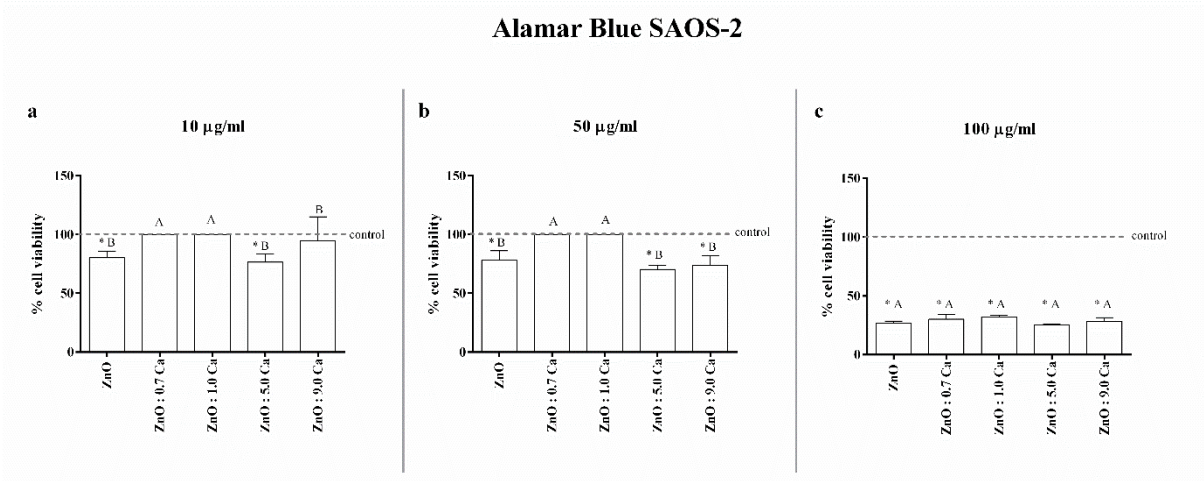

Figure 5. Cell viability determination of SAOS-2 after treatment with ZnO, ZnO : 0.7 Ca, ZnO : 1.0 Ca, ZnO : 5.0 Ca and ZnO : 9.0 Ca by Alamar Blue assay. Effects of nanocrystals at the concentrations of 10 µg/ml (A), 50 µg/ml (B) and 100 µg/ml on SAOS-2 viability. Capital letters allow comparison among different nanocrystals. * allow comparison among the different nanocrystals and the control group.

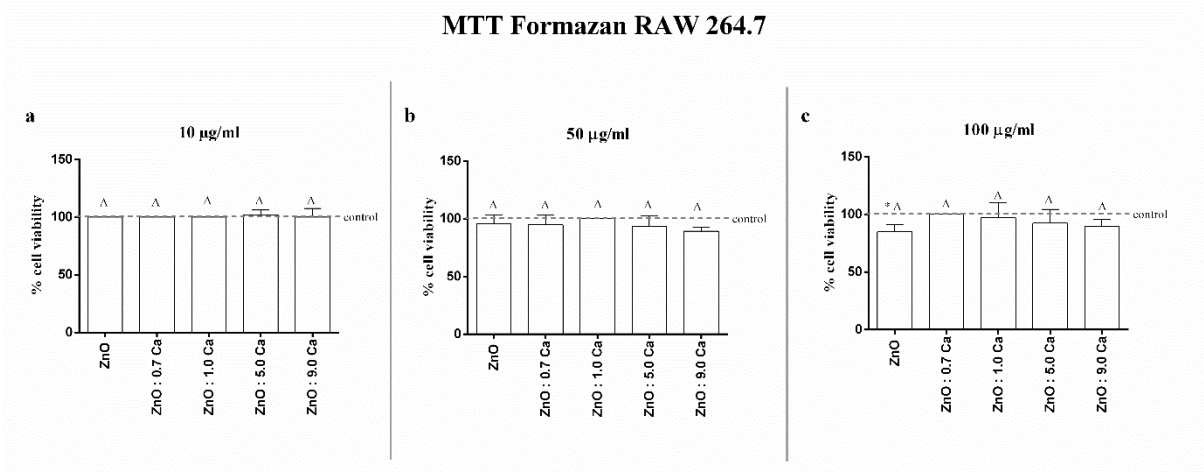

Figure 6. Cell viability determination of RAW 264.7 after treatment with ZnO, ZnO : 0.7 Ca, ZnO : 1.0 Ca, ZnO : 5.0 Ca and ZnO : 9.0 Ca by MTT formazan assay. Effects of nanocrystals at the concentrations of 10 µg/ml (A), 50 µg/ml (B) and 100 µg/ml on SAOS-2 viability. Capital letters allow comparison among different nanocrystals. * allow comparison among the different nanocrystals and the control group.

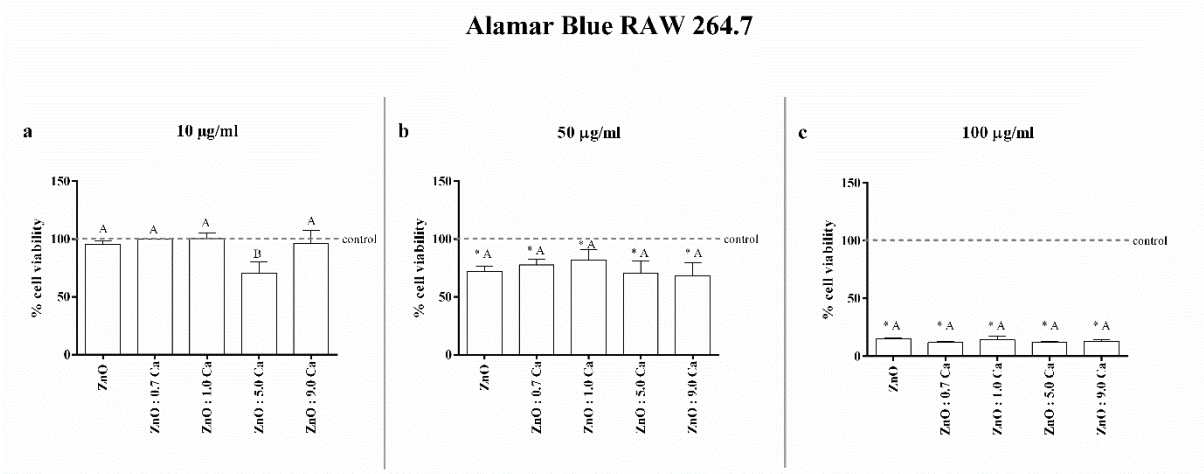

Figure 7. Cell viability determination of RAW 264.7 after treatment with ZnO, ZnO : 0.7 Ca, ZnO : 1.0 Ca, ZnO : 5.0 Ca and ZnO : 9.0 Ca by Alamar Blue assay. Effects of nanocrystals at the concentrations of 10 µg/ml (A), 50 µg/ml (B) and 100 µg/ml on SAOS-2 viability. Capital letters allow comparison among different nanocrystals. * allow comparison among the different nanocrystals and the control group.

CAPÍTULO 2

Synthesis and caracterization of a new calcium hydroxide nanoparticulated.

Souza GL, Silva ACA, Dantas NO, Turrioni APS, Moura CCG.

Synthesis and caracterization of a new calcium hydroxide nanoparticulated.

Gabriela Leite de Souza[1], Anielle Christine Almeida Silva[2], Noelio Oliveira Dantas[2], Ana Paula Silveira Turrioni[3], Camilla Christian Gomes Moura[1].

[1] Department of Endodontics, School of Dentistry, University of Uberlândia, Uberlândia, MG, Brazil; [2] New Nanostructured and Functional Materials Laboratory, Physics Institute, Federal University of Alagoas, Maceió, AL, Brazil ; and [3] Department of Pediatric, School of Dentistry, University of Uberlândia, Uberlândia, MG, Brazil

Running title: $Ca(OH)_2$ nanoparticulated, characterization, synthesis.

Key Words: Calcium hydroxide, Nanocrystal, White MTA.

***Corresponding Author**

Prof. Camilla Christian Gomes Moura

Department of Endodontics, School of Dentistry, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil.

Av Pará 1720, Campus Umuarama, Block 4L, Uberlândia, MG, Brazil. Zip-Code: 38405-328

E-mail address: camillahistologia@yahoo.com.br Phone number: +55 34 3225-8119

Abstract

Aim: To synthesize and to characterize calcium hydroxide ($Ca(OH)_2$) nanoparticulated (CaOH-NC) .

Methodology: The $Ca(OH)_2$ nanocrystals were synthesized by coprecipitation method. The physical properties were investigated through X-Rays Diffraction and scanning electron microscopy (SEM) with energy dispersive X-Ray spectrometry (EDS).

Results: The XRD show Bragg diffraction peaks characteristic of $Ca(OH)_2$, confirming the formation of the nanocrystals. SEM image observed that crystals are hexagonal structure and that the size is around 80 nm.

Conclusion: The results of DRX, SEM and EDS analysis showed that the synthesis methodology produced pure, homogeneous and uncontaminated Ca $(OH)_2$ nanocrystals, with the size around 80 nm.

Introduction

Vital pulpal therapy (VPT) comprise procedures indicated to mantain the pulp vitality in cases of exposure during caries excavation or as result of traumatic fracture (Gandolfi *et al.* 2014, Kim *et al*. 2018). VTP corresponds to direct pulp cap or pulpotomies, and in general require the use of a cover material to form a protective layer over exposed vital pulp (Gandolfi *et al.* 2014). These approaches have a great success rate if (1) the pulp is vital and not inflamed, (2) hemorrhage controlled and (3) a nontoxic capping material is applied (Cohenca *et al.* 2013).

Calcium hydroxide ($Ca(OH)_2$) has a long track record of clinical success, and is considered the "gold standard" of direct pulp capping materials (Hilton 2009). $Ca(OH)_2$ is a strong base with pH of approximately 12, wich provides excellent antibacterial properties (Chen & Suh 2017) and has the property of induction of mineralization (Wu *et al.* 2018). However, $Ca(OH)_2$ in its practical use has a number of limitations, including high solubility in oral fluids, poor sealing ability and lack of adhesion to the dentin (Hilton 2009, Sangwan *et al.* 2013).

The development of nanoscience in the last decade has yielded advanced in material synthesis and characterization. Nanotechnology comprises the technology development at the atomic, molecular or macromolecular levels ang a length scale of approximately 1-100 nm in any dimension. This field has become increasingly utilized for medical and dental applications and is of great interest because of the broad spectrum of its antibacterial effectiveness (Louwakul *et al.* 2016).

The efficacy of $Ca(OH)_2$ nanoparticulate (CaOH-NP) habe been evaluated on the elimination of *Enterococcus faecalis* in human root dentin, demonstrating the beneficial use of nanoparticles over traditional agents (Louwakul *et al.* 2016), which may be caused by na increased surface area to contact with bacteria and chemical reactivity of the material or change in physical properties of traditional agents (Seil & Webster *et al.* 2012). Furthermore, CaOH-NP

penetrate better into the dentinal tubules and stay longer in dentinal tubules that make their effects last longer (Roy & Bhattacharya 2010, Dianat *et al.* 2015).

However, the size, shape, chemical composition and the presence of impurities of the nanoparticles influence the cellular response (Hussain *et al.* 2014, Huang *et al.* 2017), being essential the characterization tests in the new nanoparticulate materials that are developed. Therefore, the purpose of this *in vitro* study was to synthesize and to characterize CaOH-NP.

Materials and Methods

Preparation of the CaOH-NP

$Ca(OH)_2$ nanocrystals were synthesized via coprecipitation using aqueous solutions of calcium chlorite (1M) (Sigma-Aldrich, St. Louis, MI, USA) and sodium hydroxide (2M) (Sigma-Aldrich).

Characterization

The X-Rays Diffraction (XRD) measurements were performed on a SHIMATZU diffractometer XDR-6000 operated at 20kV and 2mA with CuK radiation (λ=1.5406 Å). Sanning electron microscopy (SEM) with energy dispersive X-Ray spectrometry (EDS) were performed by electronic microscope (Zeiss EVO MA10) and detector Oxford modelo 51-ADD0048.

Results

Characterization

The results of XRD and SEM with EDS are presented in Figure 1. The XRD show Bragg diffraction peaks characteristic of portlandite ($Ca(OH)_2$, JCPDS: 72-0156), confirming the formation of $Ca(OH)_2$ nanocrystals (Figure 1A). The SEM image (Figure 1B) observed that crystals are hexagonal structure. In the right inset show zoom reinforcing the hexagonal morphology of the crystals and that the size (D) is around 80 nm. In the left inset shows the results of EDS confirm the amount and types of atoms that constitute the nanocrystals and reinforce the

XRD results of the formation of Ca $(OH)_2$. It is worth noting that hydrogen is not observed in this technique, but as the percentage of O in relation to Ca is double this confirms the formation of Ca $(OH)_2$.

Discussion

The results show that the synthesis methodology produced pure, homogeneous and uncontaminated nanocrystals. This method has demonstrated the best control over physical properties, as dimension, structure, purity and stability, which are essential in biological tests (Song *et al.* 2010, Gandolfi *et al.* 2011, Gordon *et al.* 2011).

In order to investigate the type, morphology and composition of crystals formed were performed X-ray diffraction (XRD) and scanning electronic microscope (SEM) with energy dispersive X-Ray spectrometry (EDS). The X-Ray diffractograms show Bragg diffraction peaks characteristic of portlandite ($Ca(OH)_2$, JCPDS: 72-0156), confirming the formation of $Ca(OH)_2$ nanocrystals.

The SEM image observed that crystals are hexagonal structure. In the right inset show zoom reinforcing the hexagonal morphology of the crystals and that the size is around 80 nm. In the left inset shows the results of EDS confirm the amount and types of atoms that constitute the nanocrystals and reinforce the XRD results of the formation of Ca $(OH)_2$. It is worth noting that hydrogen is not observed in this technique, but as the percentage of O in relation to Ca is double this confirms the formation of Ca $(OH)_2$.

Despite the limitation of the present research, it is important to emphasize that the nanomaterial proposed in this study have never been evaluated before. Therefore, this is an important preliminary study to define the specific physical characteristics of these nanocrystals.

Conclusion

The results of DRX, SEM and EDS analysis show that the synthesis methodology produced pure, homogeneous and uncontaminated Ca $(OH)_2$ nanocrystals.

Conflict of Interest

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

Acknowledgments

This project was developed on CPBio - Biomechanics, Biomaterials and Cell Biology Research Center, Multiuser laboratory of the Institute of Chemistry and Faculty of Chemical Engineering, both of the Federal University of Uberlândia. The research was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, FAPEMIG and CNPq.

References

Chen L, Suh BI (2017) Cytotoxicity and biocompatibility of resin-free and resin-modified direct pulp capping materials: A state-of-the-art review. *Dental Materials Journal* **36**, 1-7.

Cohenca N, Paranjpe A, Berg J (2013) Vital pulp therapy. *Dental Clinics of North America* **57**, 59-73.

da Silva RA, Leonardo MR, da Silva LA, Faccioli LH, de Medeiros AI (2008). Effect of a calcium hydroxide-based paste associated to chlorhexidine on RAW 264.7macrophage cell line culture. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* **106**, e44-51.

Dianat O, Saedi S, Kazem M, Alam M (2015) Antimicrobial Activity of Nanoparticle Calcium Hydroxide against Enterococcus Faecalis: An In Vitro Study. *Iranian Endodontic Journal* **10**, 39–43.

Gandolfi MG, Taddei P, Siboni F, Modena E, Ginebra MP, Prati C (2011) Fluoride-containing nanoporous calcium-silicate MTA cements for endodontics and oral surgery: early fluorapatite formation in a phosphate-containing solution. *International Endodontic Journal* **10**, 938–49.

Gandolfi MG, Siboni F, Botero T, Bossù M, Riccitiello F, Prati C (2014) Calcium silicate and calcium hydroxide materials for pulp capping: biointeractivity, porosity, solubility and bioactivity of current formulations. *Journal of applied biomaterials & functional materials.* **13**, 43-60.

Gordon T, Perlstein B, Houbara O, Felner I, Banin E, Margel S (2011) Synthesis and characterization of zinc/iron oxide composite nanoparticles and their antibacterial properties. *Colloids and Surfaces a-Physicochemical and Engineering Aspects* **347**, 1–8.

Hilton TJ (2009) Keys to clinical success with pulp capping: a review of the literature. *Operative Dentistry* **34**, 615-25.

Huang YW, Cambre M, Lee HJ (2017) The Toxicity of Nanoparticles Depends on Multiple Molecular and Physicochemical Mechanisms. International journal of molecular sciences **18**, pii: E2702.

Hussain S, Garantziotis S, Rodrigues-Lima F, Dupret JM, Baeza-Squiban A, Boland S (2014) Intracellular signal modulation by nanomaterials. *Advances in experimental medicine and biology* **811,** 111-34.

Kim DH, Jang JH, Lee BN *et al*. (2018) Anti-inflammatory and Mineralization Effects of ProRoot MTA and Endocem MTA in Studies of Human and Rat Dental Pulps In Vitro and In Vivo. *Journal of Endodontics* **44**, 1534-41.

Louwakul P, Saelo A, Khemaleelakul S (2017) Efficacy of calcium oxide and calcium hydroxide nanoparticles on the elimination of Enterococcus faecalis in human root dentin. *Clinical Oral Investigation* **21**, 865-871.

Roy A and Bhattacharya J (2010) Synthesis of Ca(OH)2 nanoparticles by wet chemical method. *Micro & Nano Letters* **5,** 131-134.

Sangwan P, Sangwan A, Duhan J, Rohilla A (2013) Tertiary dentinogenesis with calcium hydroxide: a review of proposed mechanisms. *International Endodontic Journal* **46**, 3-19.

Seil JT, Webster TJ (2012) Antibacterial effect of zinc oxide nanoparticles combined with ultrasound. *Nanotechnology* **23**, 495101.

Song W, Zhang J, Guo J, Ding F, Li L, Sun Z (2010) Role of the dissolved zinc ion and reactive oxygen species in cytotoxicity of ZnO nanoparticles. *Toxicology* **3**, 389–97.

Torabinejad M, Hong CU, Pitt Ford TR, Kaiyawasam SP (1995) Tissue reaction to implanted super-EBA and mineral trioxide aggregate in the mandible of guinea pigs: a preliminary report. *Journal of Endodontics* **21**, 569-571.

Wu T, Xu C, DU R *et al.* (2018) Effects of silicate-based composite material on the proliferation and mineralization behaviors of human dental pulp cells: An in vitro assessment. *Dental Materials Journal* **37**, 889-96.

Figure and Legend

Figure 1

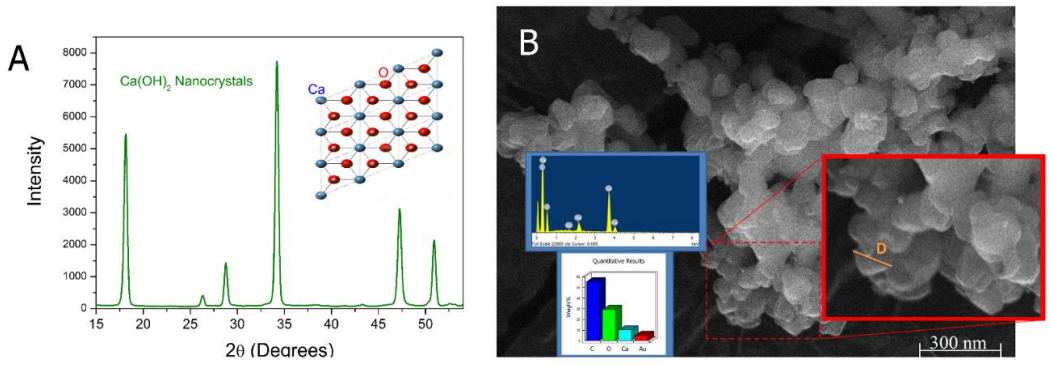

Figure 1. Investigation of the type, morphology and composition of crystals by XRD and SEM with EDS. (A) Bragg diffraction peaks characteristic confirming the formation of $Ca(OH)_2$ nanocrystals, (B) SEM image of $Ca(OH)_2$ nanocrystals. In the left inset shows the results of EDS.

REFERÊNCIAS

1. Cohenca N, Paranjpe A, Berg J. Vital pulp therapy. Dent Clin North Am. 2013;57(1):59-73. https://doi.org/10.1016/j.cden.2012.09.004
2. Gandolfi MG, Siboni F, Botero T, Bossù M, Riccitiello F, Prati C. Calcium silicate and calcium hydroxide materials for pulp capping: biointeractivity, porosity, solubility and bioactivity of current formulations. J Appl Biomater Funct Mater. 2015;13(1):43-60. https://doi.org/10.5301/jabfm.5000201
3. Kim DH, Jang JH, Lee BN, Chang HS, Hwang IN, Oh WM. Anti-inflammatory and Mineralization Effects of ProRoot MTA and Endocem MTA in Studies of Human and Rat Dental Pulps In Vitro and In Vivo. J Endod. 2018;44(10):1534-1541. https://doi.org/10.1016/j.joen.2018.07.012
4. Saleh IM, Ruyter IE, Haapasalo M, Ørstavik D. Survival of Enterococcus faecalis in infected dentinal tubules after root canal filling with different root canal sealers in vitro. Int Endod J. 2004 Mar;37(3):193-8. https://doi.org/10.1111/j.0143-2885.2004.00785.x
5. Awawdeh L, Al-Qudah A, Hamouri H, Chakra RJ. Outcomes of Vital Pulp Therapy Using Mineral Trioxide Aggregate or Biodentine: A Prospective Randomized Clinical Trial. J Endod. 2018;44(11):1603-1609. https://doi.org/10.1016/j.joen.2018.08.004
6. Witherspoon DE. Vital pulp therapy with new materials: new directions and treatment perspectives in permanent teeth. J Endod. 2008;34:25–8. https://doi.org/10.1016/j.joen.2008.02.030
7. Awawdeh L, Hemaidat K, Al-Omari W. Higher maximal occlusal bite force in endodontically treated teeth versus vital contralateral counterparts. J Endod. 2017;43:871–5. https://doi.org/10.1016/j.joen.2016.12.028
8. Trope M. Regenerative potential of dental pulp. J Endod. 2008;34(7 Suppl):S13-7. https://doi.org/10.1016/j.joen.2008.04.001
9. Camilleri J, Montesin FE, Brady K, Sweeney R, Curtis RV, Ford TR. The constitution of mineral trioxide aggregate. Dent Mater. 2005;21: 297-303. https://doi.org/10.1016/j.dental.2004.05.010

10. Roberts HW, Toth JM, Berzins DW, Charlton DG. Mineral trioxide aggregate material use in endodontic treatment: a review of the literature. Dent Mater. 2008; 24: 149-164. https://doi.org/10.1016/j.dental.2007.04.007
11. Parirokh M, Torabinejad M. Mineral trioxide aggregate: a comprehensive literature review —Part III: Clinical applications, drawbacks, and mechanism of action. J Endod. 2010; 36: 400-413. https://doi.org/10.1016/j.joen.2009.09.009
12. da Rosa WLO, Cocco AR, Silva TMD, Mesquita LC, Galarça AD, Silva AFD *et al.* Current trends and future perspectives of dental pulp capping materials: A systematic review. J Biomed Mater Res B Appl Biomater. 2018;106(3):1358-1368. https://doi.org/10.1002/jbm.b.33934
13. Mente J, Geletneky B, Ohle M, Koch MJ, Friedrich Ding PG, Wolff D, et al. Mineral trioxide aggregate or calcium hydroxide direct pulp capping: An analysis of the clinical treatment outcome. J Endod. 2010;36:806–813. https://doi.org/10.1016/j.joen.2010.02.024
14. Bergenholtz G, Axelsson S, Davidson T, Frisk F, Hakeberg M, Kvist T, et al. Treatment of pulps in teeth affected by deep caries—A systematic review of the literature. Singapore Dent J. 2013;34:1–12. https://doi.org/10.1016/j.sdj.2013.11.001
15. Vilela Teixeira AB, Vidal CL, de Castro DT, da Costa Valente ML, Oliveira-Santos C, Alves OL *et al.* Effect of incorporation of a new antimicrobial nanomaterial on the physical-chemical properties of endodontic sealers. J Conserv Dent. 2017;20(6):392-397. https://doi.org/10.4103/JCD.JCD_266_17
16. Zanjani VA, Tabari K, Sheikh-Al-Eslamian SM, Abrandabadi AN. Physiochemical Properties of Experimental Nano-hybrid MTA. J Med Life. 2018;11(1):51-56.
17. Das M, Saxena N, Dwivedi PD. Emerging trends of nanoparticles application in food technology: Safety paradigms. Nanotoxicology. 2009; 3:10-18 https://doi.org/10.1080/17435390802504237
18. Haja Hameed AS, Karthikeyan C, Senthil Kumar V, Kumaresan S, Sasikumar S (2013) Impact of alkaline metal ions Mg2+, Ca2+, Sr2+ and Ba2+ on the structural, optical, thermal and antibacterial properties of ZnO nanoparticles prepared by the coprecipitation method. J Mater Chem B. 2013; 1:5950–5962. https://doi.org/10.1039/c3tb21068e

19. Bae SY, Na CW, Kang JH, Park J. Comparative structure and optical properties of Ga-, In-, and Sn-doped ZnO nanowires synthesized via thermal evaporation. J Phys Chem B. 2005;109(7):2526-31. https://doi.org/10.1021/jp0458708
20. El Mir L, Ben Ayadi Z, Saadoun M, Von Bardeleben HJ, Djessas K, Zeinert A. Optical, electrical and magnetic properties of transparent, n-type conductive $Zn_{0.90-x}V_{0.10}Al_xO$ thin films elaborated from aerogel nanoparticles. Phys. State Solidi 2007; 204:3266-3277 https://doi.org/10.1002/pssa.200723046
21. Huang YW, Cambre M, Lee HJ. The Toxicity of Nanoparticles Depends on Multiple Molecular and Physicochemical Mechanisms. Int J Mol Sci. 2017;18(12). pii: E2702. https://doi.org/10.3390/ijms18122702
22. Landsiedel R, Fabian E, Ma-Hock L, van Ravenzwaay B, Wohlleben W, Wiench K et al. Toxico-/biokinetics of nanomaterials. Arch Toxicol. 2012;86(7):1021-60. https://doi.org/10.1007/s00204-012-0858-7
23. Louwakul P, Saelo A, Khemaleelakul S. Efficacy of calcium oxide and calcium hydroxide nanoparticles on the elimination of Enterococcus faecalis in human root dentin. Clin Oral Investig. 2017;21(3):865-871. https://doi.org/10.1007/s00784-016-1836-x
24. Seil JT, Webster TJ. Antimicrobial applications of nanotechnology: methods and literature. Int J Nanomedicine. 2012;7:2767-81. https://doi.org/10.2147/IJN.S24805
25. Xia T, Zhao Y, Sager T, George S, Pokhrel S, Li N *et al.* Decreased dissolution of ZnO by iron doping yields nanoparticles with reduced toxicity in the rodent lung and zebrafish embryos. ACS Nano. 2011;5(2):1223-35. https://doi.org/10.1021/nn1028482
26. Adeleye AS, Pokhrel S, Mädler L, Keller AA. Influence of nanoparticle doping on the colloidal stability and toxicity of copper oxide nanoparticles in synthetic and natural waters. Water Res. 2018;132:12-22. https://doi.org/10.1016/j.watres.2017.12.069
27. Pilownic KJ, Gomes APN, Wang ZJ, Almeida LHS, Romano AR, Shen Y *et al.* Physicochemical and Biological Evaluation of Endodontic Filling Materials for Primary Teeth. Braz Dent J. 2017;28(5):578-586. https://doi.org/10.1590/0103-6440201701573
28. Gonzalez-Lara A, Ruiz-Rodriguez MS, Pierdant-Perez M, Garrocho-Rangel JA, Pozos-Guillen AJ. Zinc Oxide-Eugenol Pulpotomy in Primary Teeth: A 24-Month

Follow-up. J Clin Pediatr Dent. 2016;40(2):107-12 https://doi.org/10.17796/1053-4628-40.2.107

29. Hui-Derksen EK, Chen CF, Majewski R, Tootla RGH, Boynton JR. Reinforced zinc oxide-eugenol pulpotomy: A retrospective study. Pediatr Dent. 2013;35: 43–6.
30. Gulati N, Chandra S, Aggarwal PK, Jaiswal JN, Singh M. Cytotoxicity of eugenol in sealer containing zinc-oxide. Endod Dent Traumatol. 1991;7(4):181-5. https://doi.org/10.1111/j.1600-9657.1991.tb00205.x
31. Gerosa R, Borin M, Menegazzi G, Puttini M, Cavalleri G. In vitro evaluation of the cytotoxicity of pure eugenol.J Endod. 1996;22(10):532-4. https://doi.org/10.1016/S0099-2399(96)80012-4
32. Geurtsen W, Leyhausen G. Biological aspects of root canal filling materials--histocompatibility,cytotoxicity, and mutagenicity. Clin Oral Investig. 1997;1(1):5-11. https://doi.org/10.1007/s007840050002
33. Söderberg TA, Sunzel B, Holm S, Elmros T, Hallmans G, Sjöberg S. Antibacterial effect of zinc oxide in vitro.Scand J Plast Reconstr Surg Hand Surg. 1990;24(3):193-7. https://doi.org/10.3109/02844319009041278
34. Zhang L, Jiang Y, Ding Y, Povey M, York D. Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). J Nanopart Res. 2007;9(3):479–489. https://doi.org/10.1007/s11051-006-9150-1
35. Liu Y, He L, Mustapha A, Li H, Hu ZQ, Lin M. Antibacterial activities of zinc oxide nanoparticles against Escherichia coli O157:H7. J Appl Microbiol. 2009;107(4):1193-201. https://doi.org/10.1111/j.1365-2672.2009.04303.x
36. Hilton TJ. Keys to clinical success with pulp capping: a review of the literature. Oper Dent. 2009;34(5):615-25. https://doi.org/10.2341/09-132-0
37. Chen L, Suh BI. Cytotoxicity and biocompatibility of resin-free and resin-modified direct pulp capping materials: A state-of-the-art review. Dent Mater J. 2017;36(1):1-7. https://doi.org/10.4012/dmj.2016-107
38. Wu T, Xu C, Du R, Wen Y, Chang J, Huan Z *et al*. Effects of silicate-based composite material on the proliferation and mineralization behaviors of human dental pulp cells: An in vitro assessment. Dent Mater J. 2018;37(6):889-896. https://doi.org/10.4012/dmj.2017-328
39. Roy A, Bhattacharya J. Synthesis of Ca(OH)2 nanoparticles by wet chemical method. Micro Nano Lett. 2010;5:131-134. https://doi.org/10.1049/mnl.2010.0020

40. Dianat O, Saedi S, Kazem M, Alam M. Antimicrobial Activity of Nanoparticle Calcium Hydroxide against Enterococcus Faecalis: An In Vitro Study. Iran Endod J. 2015;10:39–43.
41. Hussain S, Garantziotis S, Rodrigues-Lima F, Dupret JM, Baeza-Squiban A, Boland S. Intracellular signal modulation by nanomaterials. Adv Exp Med Biol. 2014;811:111-34. https://doi.org/10.1007/978-94-017-8739-0_7
42. Huang YW, Cambre M, Lee HJ. The Toxicity of Nanoparticles Depends on Multiple Molecular and Physicochemical Mechanisms. Int J Mol Sci. 2017;18(12). pii: E2702. https://doi.org/10.3390/ijms18122702
43. El Mir L. Luminescence properties of calcium doped zinc oxide nanoparticles . J Lumin. 2017;186:98–102 https://doi.org/10.1016/j.jlumin.2017.02.029
44. Wei S, Zhang G, Xu X. Activating BaTaO 2 N by Ca modifications and cobalt oxide for visible light photocatalytic water oxidation reactions. Appl Catal B. 2018;237:373–381. https://doi.org/10.1016/j.apcatb.2018.06.017
45. PetersOA. Research that matters - biocompatibility and cytotoxicity screening. Int Endod J. 2013;46(3):195-7. https://doi.org/10.1111/iej.12047
46. Pires CW, Botton G, Cadoná FC, Machado AK, Azzolin VF, da Cruz IB *et al.* Induction of cytotoxicity, oxidative stress and genotoxicity by root filling pastes used in primary teeth. Int Endod J. 2016; 49(8):737-45. https://doi.org/10.1111/iej.12502