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SISTEMA ADESIVO ODONTOLÓGICO COM NANOCÁPSULAS CONTENDO FÁRMACOS

Bruna Genari

Tese apresentada como requisito obrigatório para a obtenção do título de **Doutora em Odontologia** na área de concentração em Clínica Odontológica.

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“A menos que modifiquemos a nossa maneira de pensar, não seremos capazes de resolver os problemas causados pela forma como nos acostumamos a ver o mundo.”

Albert Einstein

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RESUMO

O objetivo da presente tese foi desenvolver um adesivo com nanocápsulas (NCs), contendo indometacina, e um sistema adesivo com nanocápsulas (NC), contendo indometacina e triclosan, e avaliar suas propriedades. As NCs foram produzidas por meio do método de deposição de polímero, secas e caracterizadas quanto ao tamanho de partículas, à forma, quantidade de fármaco encapsulado e citotoxicidade. Uma resina adesiva foi formulada. Foram adicionadas ao adesivo 1%, 2%, 5% e 10% de NCs em massa, e um grupo permaneceu sem NC. As NCs, contendo indometacina e triclosan, foram também incorporadas no *primer* comercial a 2% em peso e um grupo permaneceu sem NCs. Os adesivos foram avaliados quanto ao GC imediato e tardio, à degradação em solvente, liberação dos fármacos, difusão de indometacina pela dentina e resistência de união. O adesivo com 10% de NCs, contendo indometacina, foi também avaliado quanto à ação anti-inflamatória em modelo animal. O *primer* e o adesivo com as diferentes concentrações de NCs, contendo indometacina e triclosan, foram avaliados quanto à liberação dos fármacos, difusão de indometacina pela dentina, ao efeito antimicrobiano, grau de conversão (GC) *in situ*, ângulo de contato e à resistência de união à microtração. Os dados foram analisados por ANOVA, Tukey e teste t. As NCs apresentaram forma esférica e viabilidade celular acima de 80%. As NCs, contendo indometacina, apresentaram diâmetro médio de 165 nm e as NCs, contendo indometacina e triclosan, 159 nm. O adesivo, contendo 10% de NCs com indometacina, apresentou efeito anti-inflamatório. A incorporação de NCs não alterou o GC, que variou de 63,63 ± 1,01% a 70,50 ± 2,08%. A degradação em solvente não foi alterada com 2% de NCs. Tanto os adesivos quanto o *primer* apresentaram liberação controlada. A indometacina permeou através da dentina. O adesivo e *primer* também apresentaram efeito antimicrobiano. A incorporação de NCs no *primer* e no adesivo não influenciou o GC *in situ* nem a resistência de união imediata, em comparação aos materiais sem NCs. O uso concomitante do *primer* e adesivo com NCs aumentou o ângulo de contato e diminuiu a resistência de união longitudinal. Conclui-se que o uso do adesivo com a incorporação de NCs tem potencial para proporcionar ações terapêuticas à adesão dentinária.

Palavras-chave: Nanocápsula. Adesivo. *Primer*. Indometacina. Triclosan.

ABSTRACT

The aim of the present thesis was to develop an adhesive with nanocapsules (NCs) containing indomethacin and an adhesive system with nanocapsules containing indomethacin and triclosan and to evaluate their properties. NCs were prepared by the interfacial deposition of preformed polymer technique, dried and characterized regarding particle size, encapsulated drug content and cytotoxicity. Adhesive resin was produced. Concentrations of 1%, 2%, 5% and 10% of NCs were added in the adhesive and a group was maintained with no NCs. Indomethacin and triclosan-loaded NCs were also incorporated into a commercial primer in a concentration of 2% and a group was maintained with no NCs. Adhesives were evaluated regarding immediate and late degree of conversion (DC), degradation in solvent, drug release, indomethacin diffusion through dentin and bond strength. The adhesive with 10% of NCs containing indomethacin was also evaluated regarding the anti-inflammatory effect in an animal model. Primer and adhesive with different concentrations of NCs containing indomethacin and triclosan were evaluated regarding drug release, indomethacin diffusion through dentin, antimicrobial effect, *in situ* degree of conversion, contact angle and bond strength. Data were analyzed through ANOVA, Tukey *post-hoc* and t-test. NCs presented a spherical shape and cell viability higher than 80%. NCs containing indomethacin presented an averaged size of 165 nm and NCs containing indomethacin and triclosan, 159 nm. The adhesive with 10% of NCs containing indomethacin presented anti-inflammatory effect. The incorporation of NCs presented no alteration of DC, varying from 63.63 ± 1.01% a 70.50 ± 2.08%. Degradation in solvent suffers no influence of NCs with 2% of NCs. Adhesives and primer presented controlled drug release. Indomethacin diffused through dentin. Adhesive and primer also presented an antimicrobial effect. The incorporation of NCs in adhesive and primer showed no influence on *in situ* DC and immediate bond strength compared to materials with no NCs. The use in combination of primer and adhesive with NCs resulted in higher contact angle and lower longitudinal bond strength. It is possible to conclude that the use adhesive with incorporation of NCs has potential to provide therapeutic effects on dentin adhesion.

Palavras-chave: Nanocapsule. Adhesive. *Primer*. Indomethacin. Triclosan.

SUMÁRIO

1 INTRODUÇÃO

A era da odontologia adesiva, resultante de esforços da pesquisa para o desenvolvimento de técnicas e materiais adesivos, está baseada principalmente no binômio desmineralização da estrutura do dente e infiltração de materiais resinosos. Dentre as descobertas importantes e precursoras no atual paradigma restaurador adesivo, estão o condicionamento ácido do esmalte, descrito por Buonocore, em 1955, o condicionamento da dentina, descrito por Fusayama, em 1979, e a caracterização da camada híbrida, apresentada por Nakabayashi *et al.*, em 1982, entre outros. Analogamente ao que ocorre no processo carioso, em que há difusão de produtos bacterianos pela dentina, os agentes de adesão também podem se difundir através do tecido dentinário, podendo provocar reação pulpar inflamatória (Ricketts, 2001; Huang *et al.*, 2005; Kassa *et al.*, 2009). Além disso, o tratamento restaurador não garante a eliminação de microrganismos, principalmente devido à estrutura dentinária, nem a possibilidade de reinfecções, em função da constante formação de biofilmes orais e eventuais falhas de adesão (Wang *et al.*, 2014). Nesse contexto, o desenvolvimento de sistemas adesivos com capacidade de auxiliar no controle do processo inflamatório pulpar e da contaminação bacteriana tem sido objeto de estudos (Hiraishi *et al.*, 2010; Cheng *et al.*, 2013; Melo *et al.*, 2013).

Em relação à reação pulpar, seu nível é influenciado pela profundidade da lesão cariosa, tornando-se relevante a 0,75 mm da polpa e, mais intenso, a partir de 0,25 mm desse tecido (Reeves & Stanley, 1966; Kassa *et al.*, 2009). Devido ao risco de comprometer a vitalidade pulpar, quando há condição pulpar inflamatória reversível, procedimentos conservadores têm sido utilizados (Weber *et al.*, 2011), como, por exemplo, o capeamento pulpar indireto com hidróxido de cálcio, devido a sua capacidade de indução de remineralização, a sua biocompatibilidade e sua alcalinidade (Mohammadi & Dummer, 2011). Embora essa intervenção tenha mostrado resultados satisfatórios (Oliveira *et al.*, 2006; Maltz *et al.*, 2007), há estudos que

demonstram que as taxas de sucesso a longo prazo não diferem de procedimentos restauradores com sistema adesivo e resina composta (Falster *et al.*, 2002; Casagrande *et al.*, 2008; Casagrande *et al.*, 2010). Além disso, o hidróxido de cálcio apresenta baixa resistência à compressão (Mohammadi & Dummer, 2011) e pode ser degradado pelo condicionamento ácido do sistema adesivo (Heitmann & Utiterbrink, 1995). Portanto, essa alternativa para tratamento de lesões profundas pode não resultar em melhores taxas de sucesso.

Além de o comprometimento pulpar constituir uma falha no procedimento restaurador, a cárie secundária é uma das principais causas de substituição de restaurações. Cerca de 50% a 70% das trocas são em função de reincidência do processo carioso (Deligeorgi *et al.*, 2001; Kopperud *et al.*, 2012; Pallesen *et al.*, 2014). A cada substituição, mais estrutura dentária é perdida, aumentando o número de faces envolvidas, podendo comprometer a vitalidade pulpar (Brantley *et al.*, 1995). Estudos vêm sendo desenvolvidos no sentido de adicionar agentes antimicrobianos aos sistemas adesivos (Hiraishi *et al.*, 2010; Cheng *et al.*, 2013; Cheng *et al.*, 2013; Melo *et al.*, 2013). Em sistemas convencionais de três passos, há a possibilidade de incorporação de agentes antimicrobianos ao *primer* (Hiraishi *et al.*, 2010; Cheng *et al.*, 2013), que possui capacidade de infiltrar e imbricar de 5 a 8 μm na camada de fibras colágenas expostas pelo condicionamento ácido (Pashley *et al.*, 2011). Além do *primer*, é possível a incorporação ao adesivo (Cheng *et al.*, 2013; Melo *et al.*, 2013), cuja importância justifica-se pelo contato direto com a restauração (Pashley *et al.*, 2011; Cheng *et al.*, 2013). Componentes, como amônio quaternário (Cheng *et al.*, 2013), prata (Melo *et al.*, 2013) e clorexidina (Hiraishi *et al.*, 2010), já foram adicionados em sistemas adesivos. De uma maneira geral, há a ação antimicrobiana de curta duração. Além disso, o aumento da concentração desses agentes, em materiais resinosos, melhora a ação antimicrobiana; no entanto, pode afetar suas propriedades mecânicas (Syafiuddin *et al.*, 1997; Beyth *et al.*, 2006; Wiegand *et al.*, 2007; Hiraishi *et al.*, 2010).

Na prática odontológica, ocorrem, com frequência, processos inflamatórios e infecciosos, associados a doenças orais (Krasner *et al.*, 1986; Walton & Chiappinelli, 1993). Considerando situações inflamatórias, a indometacina é um agente anti-inflamatório não esteroide (AINE), cujo mecanismo de ação é a inibição da síntese de prostaglandinas, por bloqueio reversível da ação das cicloxigenases 1 e 2 (COX-1 e COX-2) (Summ & Evers, 2013). Apesar do seu valor terapêutico, o uso sistêmico de AINEs é cauteloso, por possuir ação não seletiva das enzimas COX-1 e COX-2, o que pode causar efeitos adversos, principalmente intestinais e gástricos (Chen & Dragoo, 2013).

Em situações de contaminação bacteriana, o triclosan é um agente antimicrobiano, com ação em bactérias gram-positivas e gram-negativas (Bhargava & Leonard, 1996; Jones *et al.*, 2000). Essa ação ocorre pela difusão do agente pela parede celular bacteriana e rompimento da membrana, resultando na inibição da multiplicação ou morte dos microrganismos (McMurry *et al.*, 1998; Jones *et al.*, 2000). Considerando a peculiaridade anatômica e a fisiologia dos dentes, em situações de inflamação e/ou infecção do tecido pulpar, o uso tópico de fármacos não encapsulados, como agentes anti-inflamatórios e/ou antimicrobianos, tem sido utilizado como alternativa aos fármacos de ação sistêmica (Nagle *et al.*, 2000). Além disso, a aplicação tópica de fármacos é interessante, visto que promove concentração terapêutica no tecido alvo, mas com baixos níveis séricos, geralmente insuficientes para a ocorrência de efeitos adversos (Flores *et al.*, 2012).

A Farmacologia tem sido beneficiada pelo uso de nanotecnologia. A capacidade de encapsulação de fármacos em dimensões nanométricas apresenta vantagens, como melhorar a estabilidade do fármaco (Ourique *et al.*, 2008), diminuir efeitos adversos (Bernardi *et al.*, 2009), apresentar biocompatilibilidade com os tecidos, pela utilização de materiais biocompatíveis (Guinebretière *et al.*, 2002), e possuir liberação controlada do fármaco (Hernandez *et al.*, 2013; Sun *et al.*, 2014). As nanocápsulas são compostas por uma parede polimérica, ao redor de um núcleo oleoso,

contendo o fármaco (Mora-Huertas *et al.*, 2010). A liberação do fármaco das nanocápsulas ocorre por dessorção do agente presente na superfície da nanopartícula, pela difusão da substância através da parede polimérica da nanocápsula, erosão da parede de polímero da partícula ou, ainda, combinação dos processos de difusão e erosão (Soppimath *et al.*, 2001). Essa liberação ocorre de acordo com o tipo de partícula, formulação e método de preparo e está relacionada com as condições do meio receptor (Mora-Huertas *et al.*, 2010). As nanocápsulas têm sido estudadas e aplicadas para o tratamento de diferentes doenças (Dalençon *et al.*, 1997; Barratt, 2000; Mora-Huertas *et al.*, 2010; Youm *et al.*, 2011; Saxena *et al.*, 2012; Figueiró *et al.*, 2013; Yang *et al.*, 2013; Moysan *et al.*, 2014). Na odontologia, nanocápsulas, contendo triethylene glycol dimethacrylate (TEGDMA), já foram incorporadas ao adesivo, visando seu auto-reparo (Ouyang *et al.*, 2011). Entretanto, até o momento, que se tenha conhecimento, não há sistemas adesivos, contendo nanocápsulas, capazes de controlar a liberação de fármacos para o controle de reações inflamatórias da polpa e de reincidência de cárie.

2 OBJETIVO

O objetivo do presente estudo foi desenvolver um adesivo resinoso com nanocápsulas, contendo indometacina, e um sistema adesivo com a incorporação de nanocápsulas, contendo indometacina e triclosan, e, posteriormente, a caracterização das propriedades dos materiais resultantes.

Dessa forma, em um primeiro momento, objetivou-se:

- preparar e caracterizar nanocápsulas contendo indometacina;
- desenvolver um adesivo, com a incorporação de nanocápsulas contendo indometacina, e avaliar suas propriedades;

Conhecendo a potencialidade de um adesivo contendo nanocápsulas com indometacina, objetivou-se:

- preparar e caracterizar nanocápsulas contendo indometacina e triclosan;
- incorporar nanocápsulas contendo indometacina e triclosan a um *primer* comercial e avaliar suas propriedades;
- desenvolver um adesivo, com a incorporação de nanocápsulas, contendo indometacina e triclosan, e avaliar suas propriedades.

3 MANUSCRITOS

A presente tese é composta por quatro manuscritos. O manuscrito I que foi submetido para a Clinical Oral Investigations e encontra-se publicado (doi:10.1007/s00784-016-1810-7) (Genari *et al.*, 2016). O manuscrito II que foi submetido para a Archives of Oral Biology. Os manuscritos III e IV que foram submetidos para a Journal of Dentistry.

Os manuscritos, formatados de acordo com os requisitos dos periódicos aos quais foram submetidos, encontram-se a seguir.

3.1 MANUSCRITO I

Effect of indomethacin-loaded nanocapsule incorporation in a dentin adhesive resin

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Abstract

Objectives: The aim of this study was to produce indomethacin-loaded nanocapsules (IndOH-NCs) and evaluated the influence of their incorporation into an adhesive resin.

Materials and Methods: Indomethacin was encapsulated by the deposition of preformed polymer. IndOH-NCs were caracterized by laser diffractometry, Fourier transformed infrared spectrometer, transmission electron microscopy (TEM), scanning electron microscopy, high-performed liquid chromatography (HPLC) and MTT assay. Nanocapsules (NCs) were incorporated into adhesive in concentrations of 1%, 2%, 5% and 10%. The addition was visualized by TEM and drug release was evaluated by HPLC until 120 h of immersion in simulated body fluid (SBF). Drug diffusion through dentin was tested using a Franz diffusion cell apparatus and quantified by HPLC. The degree of conversion (DC), softening in ethanol and microtensile bond strength (μTBS) were evaluated to determine whether the nanocapsules influenced the adhesive. Data were analyzed using two-way ANOVA and Tukey's post hoc test for DC, one-way ANOVA and Tukey's post hoc test for softening in ethanol, μTBS and cytotoxicity and paired t-test for comparison between the initial and final Knoop microhardness.

Results: IndOH-NCs, with a spherical shape and a mean diameter of 165 nm, were incorporated into adhesive. Indomethacin content was 7 mg drug/g powder. IndOH-NCs maintained high cell viability. At 120 h, an amount of 13.83% of indomethacin was release and, after seven days, 7.07% of this drug was diffused through dentin for an adhesive containing 10% of nanocapsules. No alteration in the DC, softening in ethanol and μTBS resulted from NCs addition.

Conclusions: IndOH-NCs may be incorporated into adhesive systems, without compromising properties, to add an anti-inflammatory controlled release for restorative procedures in deep cavities.

Clinical Significance: Here is the first step toward the goal of providing agents to act at inflammatory process of pulp tissue through dental adhesives via encapsulation of drug.

Keywords: Non-Steroidal Anti-Inflammatory Agents, Methacrylates, Drug Carriers, Dental Bonding, Drug Release, Dental Caries

1. Introduction

Dental pulp tissue injuries are promoted by deep caries lesions [1], bur preparation [2] and traumatic crown fracture [3]. At the first stages, hyperemia occurs, evolving into acute or chronic inflammation [4]. Pulp necrosis may be prevented by incomplete caries removal and indirect pulp capping. The most used materials for indirect pulp capping are calcium hydroxide cement [5] and glass ionomer cement [6]. Calcium hydroxide cement promotes reparative dentin formation at the expense of high solubility [7] and no adhesion to the tooth and to composite materials [8]. Modified glass ionomer cement adheres to the tooth structure [9] and is chemically compatible with composite [10]. Its application under restorations increases success rate of treatment [11]. However, the currently used indirect pulp capping materials require addicional step to restorative procedure and have no anti-inflammatory properties [12]. To prevent the initial stages of pulp inflammation without compromising adhesion to tooth structures, anti-inflammatory drugs may be suitable for incorporation into adhesive resins.

Indomethacin belongs to the non-steroidal anti-inflammatory class of drugs, inhibiting prostaglandins by reversibly blocking cyclooxygenases [13]. In contact with dental pulp cells, indomethacin prevents the production of proinflammatory cytokines and antioxidant defense enzymes [14]. However, high levels of drugs, to assure effectivity, added to methacrylate-based materials compromise the properties of the material since the drug is not well entrapped in a polymeric matrix [15,16]. An alternative strategy could be the use of polymeric nanocapsules (NCs) that contain drugs, such as indomethacin, promoting effective controlled drug release [17]. Improved drug efficacy and bioavailability are obtained from nanoencapsulation even with lower doses [18,19]. Besides, anti-inflammatory action occurs even using sub-therapeutic doses [17].

The aim of the present study was to incorporate indomethacin-loaded nanocapsules (IndOH-NCs) into a dental adhesive resin and determine their drug release and influence on related properties. The null hypothesis is that

the addition of IndOH-NCs will not influence the experimental adhesive resin properties.

2. Materials and Methods

2.1. Preparation and characterization of IndOH-NCs

Indomethacin-loaded NCs were prepared by the interfacial deposition of preformed polymer technique [20]. All reagents were purchased from Sigma Chemical (St. Louis, USA). The organic phase was prepared with Eudragit® S100, poly (MMA-co-MAA) (0.50 g), indomethacin (0.05 g), medium chain triglycerides (0.81 mL) and sorbitan monostearate (0.19 g) dissolved in acetone (125 mL). Under magnetic stirring at 25° C, organic phase was added through a funnel to an aqueous phase containing polysorbate 80 (0.385 g) and water (250 mL). Acetone and water excess were eliminated using a rotary evaporator (Rotavapor II, Buchi, Flawi, Switzerland), a B-740 recirculating chiller (Buchi, Flawi, Switzerland) and a U-700 vacuum pump (Buchi, Flawi, Switzerland). The IndOH-NC suspension was spray dried (B-290, Buchi, Flawi, Switzerland) using hydrophilic fumed silicon dioxide (Aerosil® 200) as an adjuvant, in amount of 3% of the suspension content. The inlet temperature at the drying chamber was maintained at approximately 150 ± 4 °C, and the outlet temperature was 107 ± 4 °C.

The mean sizes ($d_{4.3}$) of the IndOH-NC suspension and spray-dried IndOH-NCs were measured by laser diffractometry, in the wet and dried states, respectively (Mastersizer 2000, Malvern, Worcestershire, United Kingdom). The distribution of the particle size (span) values was calculated by $(d_{0.9} - d_{0.1})/d_{0.5}$, where $d_{0.9}$, $d_{0.5}$, and $d_{0.1}$ are the particle diameters determined, respectively, at the 90th, 50th, and 10th percentile of particles.

The dried IndOH-NCs were analyzed using a Fourier transform infrared spectrometer (Vertex 70, Bruker Optics, Ettlingen, Germany) coupled with a diamond crystal of ATR device (Platinum ATR-QL, Bruker Optics, Ettlingen, Germany). Five co-addition scans were made at 3 seconds velocity. The morphological analysis of suspension was conducted with transmission electron microscopy (TEM, JEM 1200 Exll, Jeol, Tokyo, Japan) at 80 kV. The IndOH-NC suspension at a dilution of 1:10 (20 µl) was deposited in Formvar-Carbon support film on a specimen grid and negatively stained with uranyl acetate solution (2% m/v). Dried IndOH-NCs (0.01 g) were processed using

gold-sputter-coating and submitted to scanning electron microscopy (SEM, JSM 6060, Jeol, Tokyo, Japan) at an accelerating voltage of 10 kV and 3.5-nm resolution.

2.2 Determination of drug content for dried IndOH-NCs

The dried IndOH-NCs (20 mg) were dissolved in acetonitrile (10 mL) under 30 min of ultrasound stirring. The solution was filtered using a 0.45-µm (Millipore) filter, and free indomethacin was measured using high performance liquid chromatography (HPLC, Shimadzu LC 10-A Shimadzu, Kyoto, Japan) with injector S-200, a UV/visible detector (λ = 280 nm), a guard-column and Nova-Pak® C18 3.9 x 150 mm (4 µm) Waters column. The mobile phase (60/40 v/v acetonitrile/water solution, pH 4.5, adjusted with acetic acid) was filtered and pumped at a constant flow rate of 1mL min^{-1}. After injection of 20 µL, the indomethacin was detected at 280 nm with a retention time of 3.1. The method was previously validated, showing linearity between 1.24 and 20.13 µg/mL (r = 0.99), an accuracy of 0.92%, a precision of 0.8%, a quantification limit of 1.34 µg/mL and a detection limit of 0.45 µg/mL.

2.3 Cytotoxicity

Cell viability was assessed by direct contact using fibroblast cells (L929, BCR, batch no. 000604, Rio de Janeiro, Brazil) according to the procedures described in ISO 10993-5. The MTT test was performed in sextuplicate. Cells were incubated at 37 °C in an atmosphere containing 5% CO_2. Cells (L929) were seeded in a 96-well tissue culture plate at a concentration of 10^4 cells/well in a medium volume of 100 µL. After 24 h, the IndOH-NCs suspension (5 µL/mL) and 1:10 dilutions were added to wells and incubated for 24 h. The medium alone was used as a negative control, and cells cultured with dimethylsulfoxide (DMSO) were used as a positive control. Medium was removed, and 50 µL of MTT (1 mg/mL) was added to each well. Formazan salts were diluted in 100 µL of DMSO, and the absorbance was measured at 570 nm (Multiskan EX Microplate Reader, MTX Lab Systems, Vienna, USA).

2.4 Formulation of the adhesive resin with indOH-NCs

Experimental dental adhesives were produced using 50/25/25 wt% bisphenol A glycol dimethacrylate (BisGMA)/triethylene glycol dimethacrylate (TEGDMA)/ 2-hydroxyethyl methacrylate (HEMA). Camphoroquinone (CQ), ethyl 4-dimethylaminobenzoate (EDAB) and diphenyliodonium hexafluoroantimonate were added at a concentration of 1 mol% and 0.01 wt% of butylated hydroxytoluene (BHT) as a photoactivation system. The dried IndOH-NCs were added at 1, 2, 5 and 10 wt%. As a control, one group had no addition of particles. All formulations were mixed and ultrasonicated (CBU 100/1 LDG, Plana, São Paulo, Brazil) for thirty minutes.

2.5 Morphological characterization

The adhesive with 10% of dried IndOH-NCs was analyzed by transmission electron microscopy at 80 kV. The monomeric adhesive was diluted (1:10) and prepared as described above.

2.6 Drug release

Two discs with 100 μg of adhesive containing 10% of nanocapsules were immersed in volumetric flasks with 10 mL of simulated body fluid (SBF; Kokubo & Takadama, 2006) under magnetic stirring at 37^{o}C. After 2, 6, 24, 48, 72, 96 and 120 hours, 1 mL of released medium was collected and fresh SBF was replaced. The aliquots were filtered using a 0.45-μm (Millipore) filter and analyzed using HPLC method previously validated and described above, in section 2.2.

2.7 Drug diffusion through dentin

A total of 8 healthy premolar teeth (n=2), which were extracted for orthodontic purpose were used in this study. Prior to the study, patients were informed and consented about the extracted teeth would be used in an *in vitro* study. After the removal of the soft tissues, a plane parallel dentin section, with a thickness of 0.75 (± 0.05) mm right after the end of pulp horns, was

obtained from each tooth using a low-speed diamond saw with water coolant. A side of the dentin discs was ground with 600-grit SiC abrasive paper under water for 30 s to create a standardized smear layer. Each dentin disc was fixed in a Teflon disc (outer diameter of 27 mm de diameter, inner diameter of 4 mm and thickness of 2 mm) using cyanoacrylate adhesive. The role (diameter of 4 mm) in the center of Teflon disc exposed a part of dentin that was etched with phosphoric acid for 15 s, washed for 15 s and dried. Primer (Scotchbond multi-purpose, 3M ESPE, St Paul, USA) was vigorously applied, and the solvent was evaporated for 10 s. The adhesive containing nanocapsules was applied in an amount of 0,08 g (2 mm of thickness) using a microbrush and photoactivated for 20 s using a light-emitting diode (Radii cal, SDI, Bayswater, Australia). A composite build-up was performed (Z350, 3M ESPE, St Paul, USA) in an increment of 2 mm and photoactivated.

Each Teflon disc containing a dentin disc was fixed in the effective diffusion area of a Franz diffusion cell apparatus [21,22]. The donor and receptor compartments were filled with 1 mL and 2.5 mL of simulated body fluid [23], respectively. After seven days, an amount of 1 mL of the content of receptor compartment was collected, filtered and analyzed by high-performance liquid chromatography as described above, in section 2.2. The results were calculated in percentage of diffused drug and drug diffusion in gram per square millimeters of area of restoration.

2.8 Degree of conversion (DC)

DC was evaluated with an ATR-FTIR spectrometer [24]. A disk (5.0 mm diameter and 1.5 mm thick) from each sample (n = 5) was photoactivated for 20 s by a light-emitting diode with an irradiance value of 1200 mW/cm^2 (Radii cal, SDI, Bayswater, Australia). Absorbance spectra were obtained before and immediately after light polymerization. The DC was calculated for the intensity (peak height) of the aliphatic carbon-carbon double bond stretching vibration at 1635 cm^{-1} and aromatic ring at 1610 cm^{-1} from the polymerized and unpolymerized samples. DC measurements were repeated after four months of adhesive resin storage at 21 ± 2 °C, in an eppendorf

protected from light.

2.9 Softening in ethanol

Specimens (5.0 mm diameter and 1.5 mm thick; n=5) prepared as described in Section 2.6 were embedded in acrylic resin and polished through a series of silicon carbide (SiC) papers (400-, 600-, 800- and 1200-grit) for 2 minutes each. Surface microhardness was measured using a microhardness tester (HMV-2, Shimadzu, Kyoto, Japão) and Knoop indenter at a load of 25 g for 15 s, before and after immersion in absolute ethanol for two hours, and percent reduction was calculated. Three indentations were performed on each sample [25].

2.10 Microtensile bond strength (μTBS)

One hundred bovine permanent incisors (n=20) were horizontally sectioned below the dentin-enamel. A 600-grit SiC paper was used under wet conditions to create a smear layer on the dentine surface. The dentine surface was etched with phosphoric acid for 15 s, washed and dried. Primer (Scotchbond multi-purpose, 3M ESPE, St Paul, USA) was vigorously applied, and the solvent was evaporated for 10 s. The adhesive was applied and photoactivated for 20 s using a light-emitting diode (Radii cal, SDI, Bayswater, Australia). A composite build-up was performed (Z350, 3M ESPE, St Paul, USA) in two increments of 2 mm.

After storage in distilled water at 37 °C for 24 h, the teeth were sectioned into 4-6 beams (area of 0.5 mm^2) with a slow-speed saw. Specimens were fixed to a microtensile device and tested on a mechanical testing machine (DL-2000, EMIC Equipments and Systems for Essay Ltda, São José dos Pinhais, Brazil) at a crosshead speed of 0.5 mm/min until failure. Fractographic failure mode analysis was performed using an optical microscope and classified as cohesive, mixed or adhesive.

2.11 Statistical analysis

Statistical analysis was performed using one-way ANOVA (nanocapsule concentration) for DC, softening in ethanol, the microtensile test and cytotoxicity. The paired t-test was used for comparison between the initial and final Knoop microhardness. All tests were performed at $\alpha=0.05$.

3. Results

The macroscopic aspect of the IndOH-NC suspension was homogeneous and presented with a milky white aspect and the Tyndall effect [25]. The production of nanoparticle exclusivity was confirmed by laser diffraction analysis. The IndOH-NCs in suspension presented a $d_{4.3}$ of 165 nm, d_{10} of 95 nm, d_{50} of 161 nm, d_{90} of 241 nm and a span value of 0.91. The measurements of the dried IndOH-NCs were $d_{4,3}$ of 4.58 µm, d_{10} of 1.04 µm, d_{50} of 3.89 µm, d_{90} of 9.12 µm and a span value of 2.08 µm. FTIR spectra showed an aliphatic carbon-hydrogen bond (2920 cm^{-1}), carbon oxygen double bond (1720 cm^{-1}), carbon-hydrogen bond (1450 cm^{-1}) and silicon-oxygen bond (1070 cm^{-1}) (Figure 1), which are present in the copolymer of methacrylic acid and methyl methacrylate, polysorbate 80 and silicon dioxide, respectively [26,27,28]. In TEM images (Figures 2a and 2b), the spherical shape of IndOH-NCs in suspension can be observed. The morphological analysis of dried IndOH-NCs by SEM showed nanostructures with spherical morphology on the surface of the silicon dioxide (Figure 2c). The indomethacin content was 7 mg drug/g powder. Indomethacin-loaded NCs maintained cell viability higher than 80% (Figure 3).

The incorporation of dried IndOH-NCs into the adhesive maintained the spherical shape of the particles (Figure 2d). Indomethacin release profile from nanocapsules into adhesive is presented in Figure 4, as an exponential model. At 120 h, an amount of 13.83% of indomethacin was release and, after seven days, 7.07% of this drug was diffused through dentin for an adhesive containing 10% of nanocapsules. After 7 days, indomethacin was diffused through dentin in similar amounts for all concentrations of IndOH-NCs in adhesive, as can be observed with results in g/mm^2. However, these amounts had different correspondence in percentage for each concentration of IndOH-NCs (Figure 5).

The incorporation of dried IndOH-NCs in the adhesive resin showed that there was no influence on immediate DC ($p=0.054$) or on DC after four months of storage ($p=0.071$) (Table 1). The results of softening in solvent showed no difference with up to 5% IndOH-NC addition (Table 1). The

addition of 10% of IndOH-NCs significantly increased the softening effect compared to 1%, 2% and 5%; however, it was not different from the 0% group. The μTBSs of groups were not significantly different (Table 1). Table 1 also summarizes the percentage failure modes of debonded specimens according to groups. Mixed fractures were frequently identified in all groups.

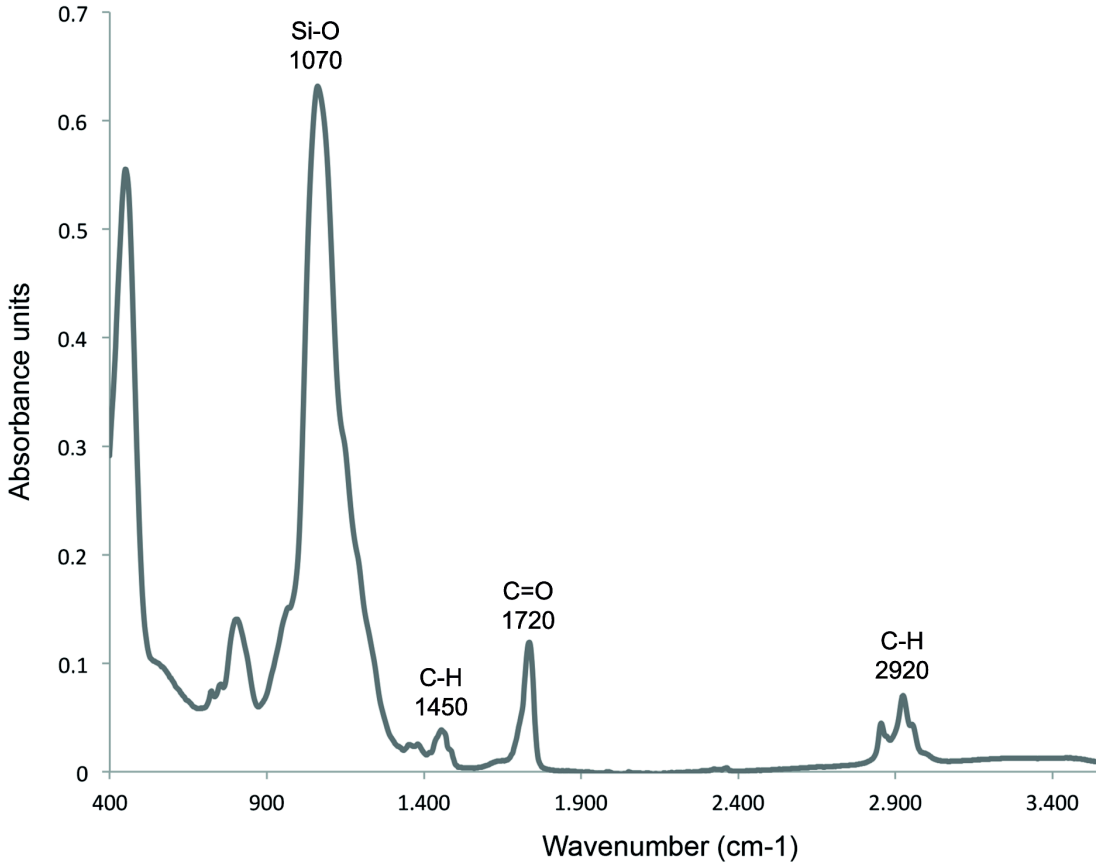

Fig. 1 FTIR spectra of dried indomethacin-loaded nanocapsules (IndOH-NCs) show peaks corresponding to the chemical bonds of NC/SiO_2 powder components

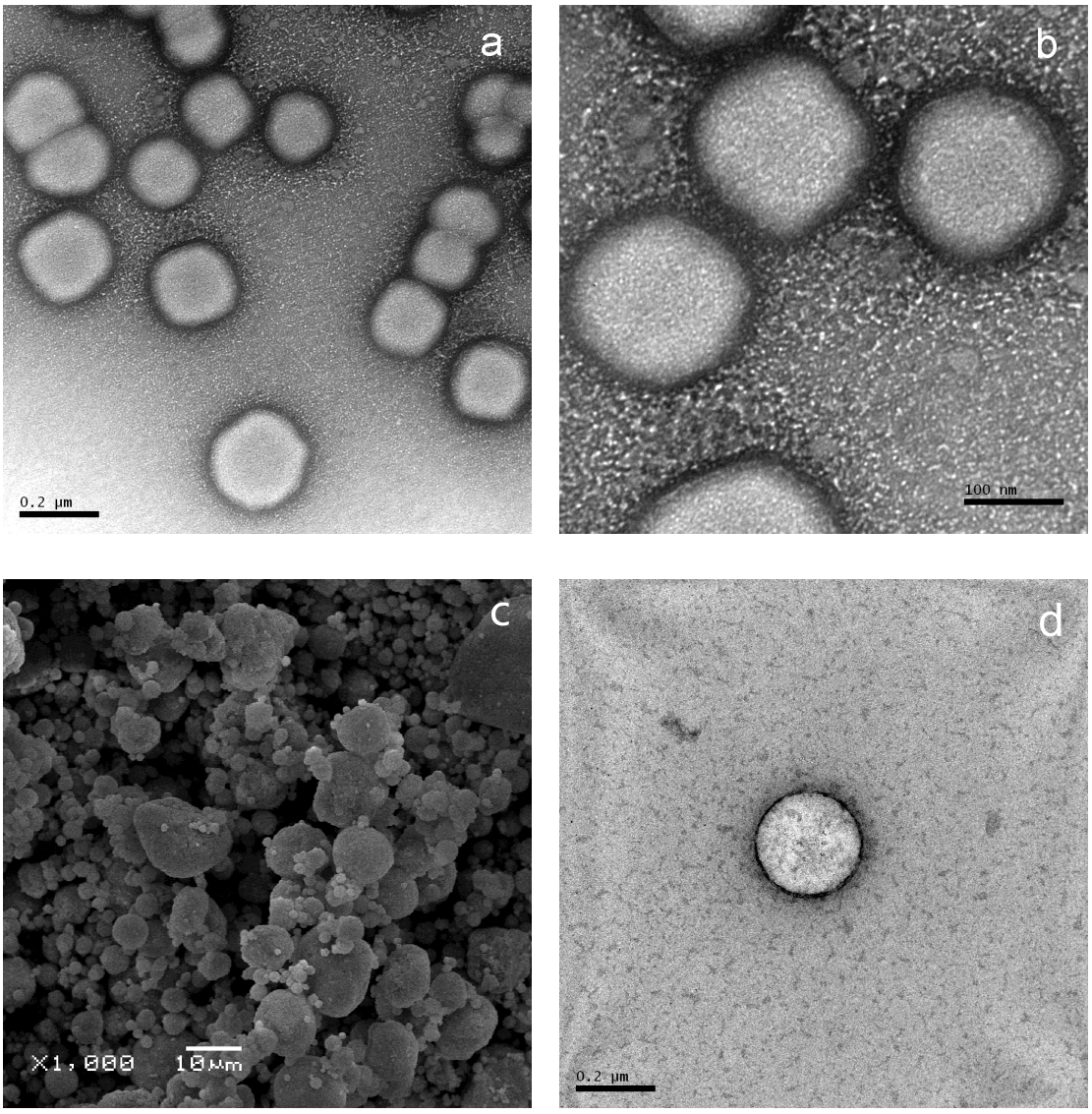

Fig. 2 SEM and TEM images. **(a, b)** TEM images of an indomethacin-loaded nanocapsule (IndOH-NC) suspension, with 100,000x and 250,000x magnifications, respectively. Indomethacin-loaded NCs in suspension have spherical morphology and nanometric dimensions. It is possible to see a polymeric capsule around IndOH-NCs. **(c)** SEM image of dried IndOH-NCs with a magnification of 1,000x. Dried IndOH-NCs are spherical and agglomerate on the silicon dioxide surface, resulting in clusters with micrometric dimensions. **(d)** TEM image of unpolymerized adhesive resin containing 10% dried IndOH-NCs, with a magnification of 100,000x, showing IndOH-NC with a spherical shape and nanometric dimensions into the adhesive

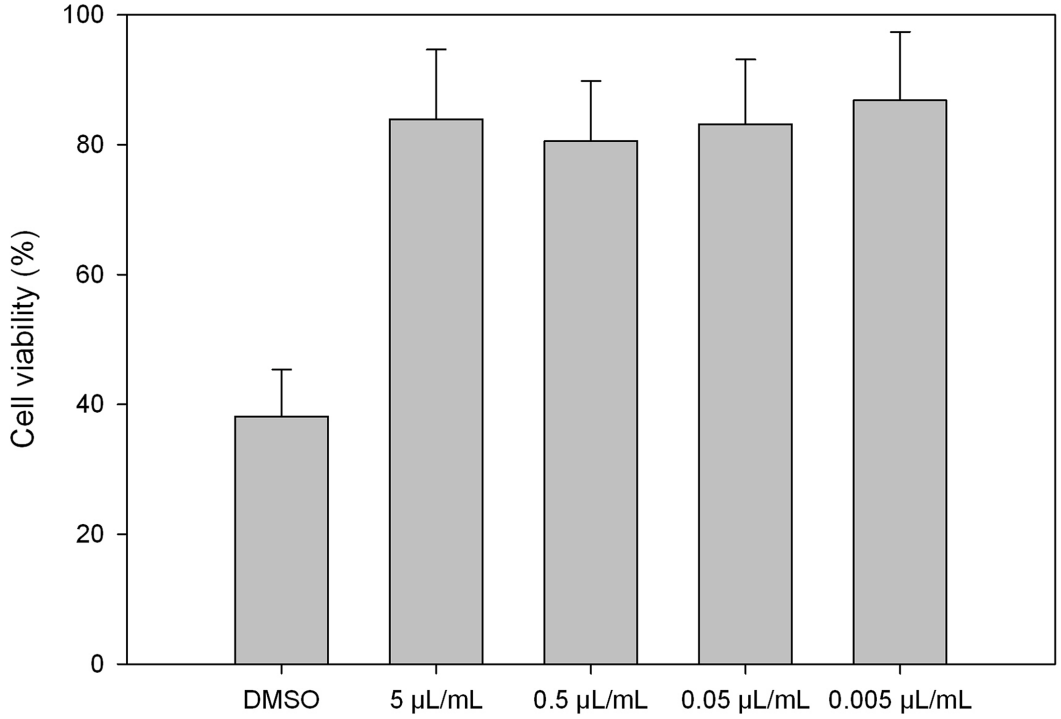

Fig. 3 Viable L929 cells (in percent, %), by MTT test, on direct contact, during 24 h, to an indomethacin-loaded nanocapsule (IndOH-NC) suspension at 5 µL/mL, its dilutions (0.5, 0.05 and 0.005 µL/mL) and dimethylsulfoxide (DMSO, positive control). The percentages of viable cells in contact with the IndOH-NC suspension in different dilutions were higher than 80%. For cells in contact with DMSO, the viability was lower than 40%

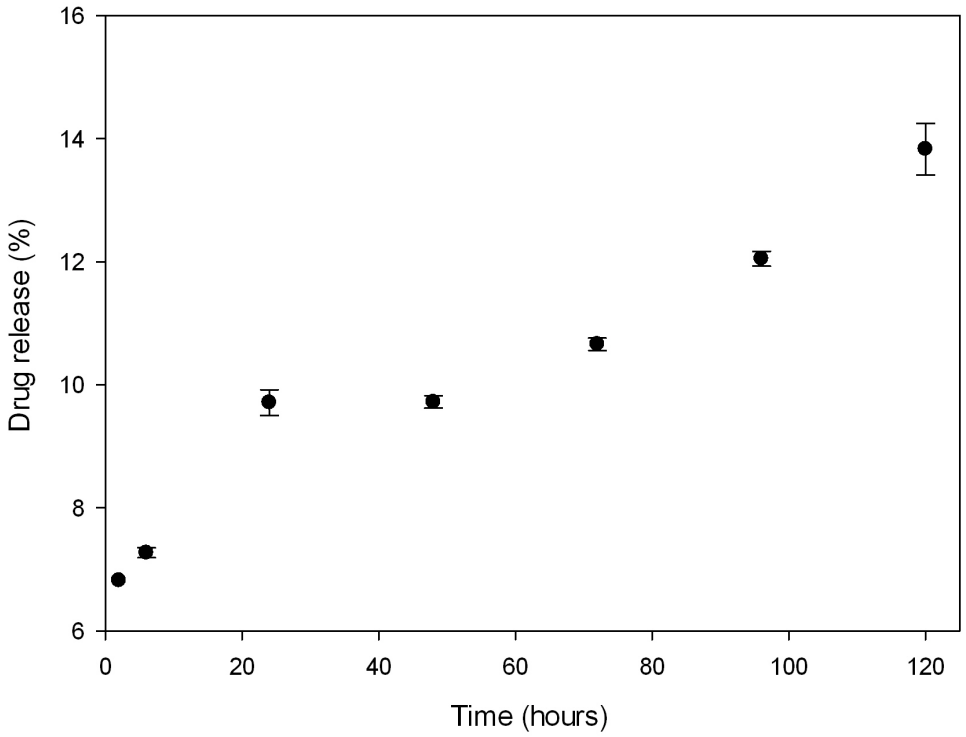

Fig. 4 The release profile of indomethacin from the adhesive in SBF immersion until 120 hours. Drug release (in percent, %) was an exponential model and is presented in function of time (hours). Error bars indicate standard deviation

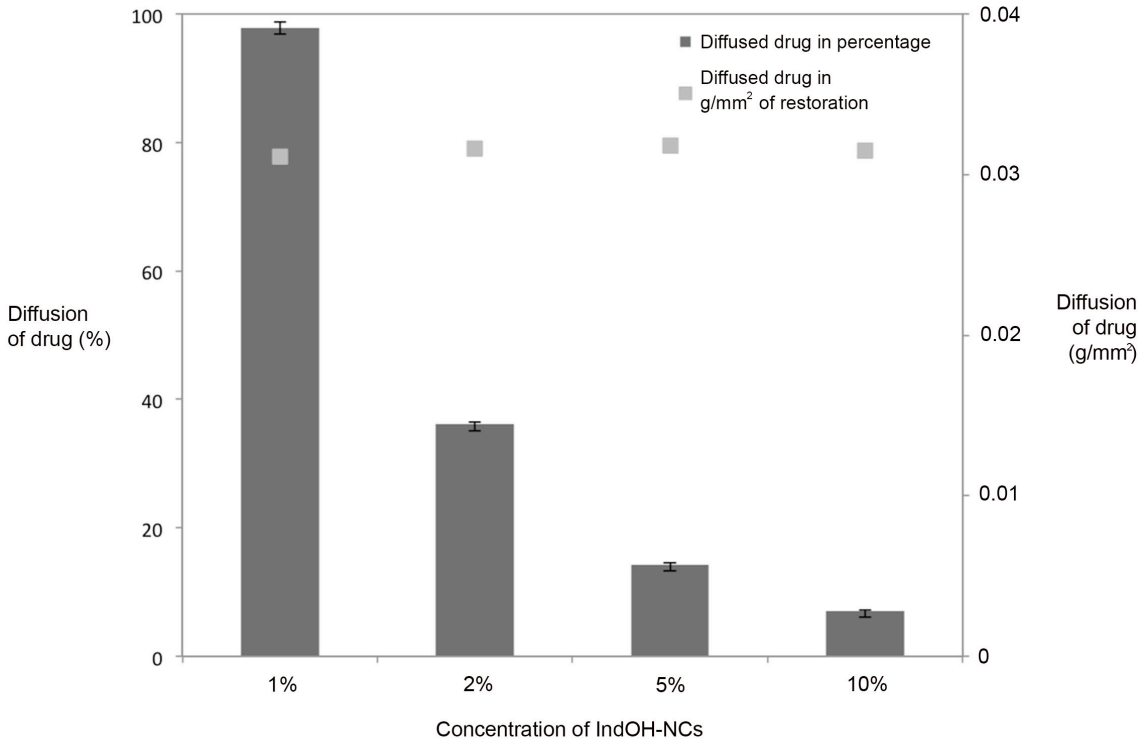

Fig. 5 Drug diffusion through dentin from adhesives containing different concentrations (1%, 2%, 5% and 10%) of IndOH-NCs used in restorations, after seven days in Franz diffusion cell apparatus with SBF. In columns, results presented in percentage, according left axis. In scatter plot, results in gram per mm^2 of restoration, according right axis. It was not possible to show the standard deviation of data in g/mm^2 in chart, because the values were lower than 0.0006

Table 1. Degree of conversion (DC), in percent (%), immediately and after a storage period; initial (KHN1) and final (KHN2) microhardness; softening in ethanol, in percent (Δ%); microtensile bond strength (μTBS) to dentin in megaPascals (MPa) and mode of failure of the adhesive with different concentrations of indomethacin-loaded nanocapsules (IndOH-NCs)

	DC						Mode of Failure (%)		
Groups	Immediate	4 months	KHN1	KHN2	Δ%	μTBS	A	M	C
0%	70.02 ± 1.28^{A}	68.39 ± 2.21^{A}	25.07 ± 2.68^{a}	14.33 ± 3.15^{b}	43.18 ± 8.41^{AB}	50.99 ± 13.26^{A}	22.81	66.67	10.53
1%	67.89 ± 0.75^{A}	70.50 ± 2.08^{A}	25.87 ± 1.74^{a}	15.59 ± 1.69^{b}	39.49 ± 8.00^{A}	43.52 ± 9.90^{A}	17.02	63.83	19.15
2%	67.21 ± 1.28^{A}	70.12 ± 1.24^{A}	25.08 ± 1.44^{a}	15.22 ± 2.17^{b}	38.95 ± 11.16^{A}	48.21 ± 7.57^{A}	16.67	71.43	11.90
5%	69.03 ± 2.57^{A}	71.68 ± 1.42^{A}	24.45 ± 2.05^{a}	15.14 ± 1.08^{b}	37.79 ± 5.99^{A}	47.05 ± 14.89^{A}	11.11	71.11	17.77
10%	67.34 ± 1.54^{A}	71.41 ± 1.94^{A}	23.99 ± 0.48^{a}	10.19 ± 0.52^{b}	57.50 ± 2.03^{B}	46.67 ± 9.56^{A}	21.05	66.67	12.28

A, adhesive; M, mixed; C, cohesive. Identical capital letters in the same column denote statistically significant equivalences. Values followed by identical lower case letters in the same row denote no significant difference ($p > .05$).

4. Discussion

Nanocapsules with indomethacin were successfully loaded into adhesive resin. In this study, we first tested the drug release profile of an anti-inflammatory-loaded NCs incorporated in an adhesive resin and properties of this adhesive to be an alternative for protecting pulp tissue in deep carious lesions. The clinical use of an adhesive with potential to inhibit an inflammatory reaction could eliminate the use of an indirect pulp-capping agent in conservative treatments. The null hypothesis was accepted because addition of IndOH-NCs did not influence adhesive resin properties.

The size of nanocapsules was in accordance with the method of preparation and the nature and concentration of the components that was previously described [25,29]. Mass transfer between two liquids and the Gibbs–Marangoni effect, stabilized by surfactants, explain the NC conformation in the formulation [20]. To remove water and stabilize the NCs, spray drying with silicon dioxide as an adjuvant was performed [25,29]. The drying adjuvant avoid the nanocapsules adhere to equipment, allowing the process [25,29]. The drying process resulted in IndOH-NC agglomeration on the silicon dioxide surface (Figure 2c) through bonds between the carboxylic acids of polymeric capsules and hydroxyl groups of the silica surface [30]. The size of the clusters achieved an almost 5 μm mean diameter. However, after IndOH-NC incorporation into the adhesive resin, these linkages were undone (Figure 2d), returning approximately to their baseline size before drying (165 nm).

The results of this study provide the first step toward the goal of providing agents that act on the inflammatory process of pulp tissue, through dental adhesives, via drug encapsulation. Indomethacin is predominantly adsorbed in polymeric wall of NCs due to its log D [31]. The release occurred by desorption of the drug from the NC surface, diffusion of the substance through the polymeric wall, erosion of the polymeric capsule or the combination of erosion and diffusion [32]. Different phases occur in exponential curve of drug release due to distribution of indomethacin in nanocapsules: outside, adsorbed in polymeric wall and in the core [31]. According our anti-inflammatory drug release profile, adhesive with

indomethacin-loaded nanocapsules has potential to control the early and intermediate stages of pulp-dentin complex responses. Mild inflammation and hyperemia start until 24 hours after a restorative procedure in a deep cavity [30], which is same time that release has been already initiated. Progression of inflammatory process extremely occurs during first 30 days [33,34], which can be accompanied by the controlled drug release. The anti-inflammatory effect of IndOH-NCs was reported in a previous study [17] in acute and chronic inflammation processes even in sub-therapeutic doses. In that study, the indOH-NCs were inserted in subcutaneous tissues, acting directly at the inflammation site.

The combination of processes of drug release and dentin diffusion results in the potential of nanocapsules of releasing indomethacin through dentin toward pulp tissue, and to have a local anti-inflammatory effect in that tissue. The dentin tubule diameter and the movement of substances from a region of high concentration to low concentration allow the diffusion of therapeutic agents through dentin-pulp complex [35]. According results of drug diffusion, indomethacin permeated through dentin in a thickness that simulates a deep lesion, with short distance from pulp tissue, in which could occur a severe inflammatory response in vivo situation [36] and this diffusion was within a crucial period that could prevent the development and evolution of this process [33,34]. In the present study, the concentration of drug in adhesive did not influence the diffused drug mass through dentin in seven days, contrary to Passos et al. (2015) [35], probably due to the drug encapsulation. It is possible to consider that the diffusion occurred even in a great challenged situation, because it was tested without pressure adapter and using a phosphate buffered saline, which could decrease permeability capacity [37]. The influence of simulated pulpal pressure in permeability tests and the ideal values of pressure to be equivalent to in vivo situation are controversial [38,39,40]. The influence of pressure is dependent of other several factors [38,39,40] and the value of clinical pulpal pressure is variable, decreasing during a local anestesia, for example [41]. The diffusion through an affected dentin can be tested in future studies. Due to proximity to pulp tissue, the formulation with IndOH-NCs must not be cytotoxic. Generally, nano-size particles tend to present with greater cell damage due to their larger

catalytic surface [42]. However, according to ISO 10993-5 (lower than 20%), the IndOH-NC formulation had a minimal effect on cell viability.

The degradation process of adhesive resin could lead to a plasticizing effect into polymer networks, breaking ester linkages [43]. The addition of IndOH-NCs did not influence hardness reduction after solvent immersion. Furthermore, the high degree of conversion of formulated adhesive resins (up to 71%), which could lead to a long-term performance of adhesive. These may due to IndOH-NCs were mainly between polymeric chains. Chemical affinity between Eudragit® S100 capsules (methacrylate co-polymer) and the adhesive resin polymeric methacrylate-based matrix also favors reliable properties [44,45]. The addition of up to 10% of IndOH-NC did not influence the bond strength in spite of the increased viscosity, which could interfere with resin diffusion along the demineralized dentin and result in lower bond strength [46,47,48]. Most of the NCs among silica particles in adhesive resin can diffuse through dentin tubules, but not in the limited interfibrillar space widths of the hybrid layer [49]. The reduced dimensions of the particles, organic nature of the capsules and spherical shape of the NCs favored the homogeneous distribution of stress throughout the resin, maintaining bonding ability compared to the control group [50,51]. Furthermore, the predominance of mixed failure of all groups suggests that the stress concentration at failure start was similar for all groups. The results of bond strength were in accordance with the *in vitro* results for the best commercial adhesive systems [52]. Based on the μTBS, DC and KHN data obtained, we forecast reliable long-term performance for the IndOH-NC adhesive resin.

5. Conclusion

The present investigation demonstrated successful loading of indomethacin in methacrylate nanocapsules that were subsequently incorporated into an adhesive resin. The IndOH-NC adhesive resin presented reliable bond strength, degree of conversion, degradation in solvente, drug diffusion through dentin and drug release without compromising the polymeric matrix. The adhesive resin showed no negative effects from incorporation of the nanocapsules. The adhesive resin, with drug loaded NCs, may be useful for improving therapeutic action for adhesives to be used in restorative

procedures of deep cavities.

Compliance with Ethical Standards

Conflict of Interest: Author BG declares that he has no conflict of interest. Author VCBL declares that he has no conflict of interest. Author DSJ declares that he has no conflict of interest. Author MC declares that he has no conflict of interest. Author ARP declares that he has no conflict of interest. Author SSG declares that he has no conflict of interest. Author SMWS declares that he has no conflict of interest. Author FMC declares that he has no conflict of interest.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: For this type of study, formal consent is not required.

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3.2 MANUSCRITO II

Anti-inflammatory effect of an adhesive resin containing indomethacin-loaded nanocapsules

Running title: Anti-inflammatory adhesive resin

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Abstract

Objective: To analyze the anti-inflammatory and analgesic effects of an adhesive resin containing indomethacin-loaded nanocapsules in rat model.

Design: Adhesive resin disks with or without indomethacin-loaded nanocapsules were subcutaneously implanted into right hind paw of rats. A week after surgical procedure, 2% formalin solution was intradermally injected into plantar surface of paw. Nociceptive and inflammatory responses were evaluated by formalin test. Paw edema by pletismometer and mechanical hyperalgesia by von Frey test were performed on day 2, day 4, day 6, day 8, day 10 and day 12 after surgery. IL-6, IL-10, and lactate dehydrogenase (LDH) serum levels were determined by ELISA-sandwich test.

Results: Group containing indomethacin-loaded nanocapsules (NC) presented lower edema in the right hind paw at 24 h after formalin injection than those of the control group (CT) (P<0.01). NC group showed decrease in the nociceptive response in phase I (neurogenic pain) compared to CT group (NC - 66.86 ± 22.83s X CT - 130.17 ± 35.83s, P<0.001). NC group presented supporting higher intensity of stimulus on days 8 and 12 (24 h and 72 h after formalin injection) (P<0.01 and P<0.02 respectively). The IL-6 serum level was also significantly higher in the NC group than CT group (p<0.001).

Conclusions: These results indicate that an adhesive resin containing indomethacin-loaded nanocapsules has anti-inflammatory and nociceptive activities in a chemical model of acute inflammation. The present investigation confirms an adhesive resin with drug-loaded nanocapsules may be useful for improving therapeutic effect for adhesives.

Keywords Non-steroidal anti-inflammatory agents, Indomethacin, Drug carriers, Dental bonding, Drug release

Introduction

Deep carious lesions promote inflammation process proportionally to the cavity depth (Kassa et al., 2009; Reeves & Stanley, 1966; Wanachantararak et al., 2016), which involves cytokines IL-2, IL-6, IL-8, IL-10 (Elsalhy, Azizieh, & Raghupathy, 2013). Indirect pulp capping using calcium hydroxide is commonly applied to prevent the pulpal inflammatory progress and irreversibility through dentin repair (Weber, Alves, & Maltz, 2011). However, indirect pulp capping showed no improvement in long-term success rate (Casagrande et al., 2008; Casagrande et al., 2010; Falster et al., 2002). Further, calcium hydroxide presents low compressive strength (Mohammadi & Dummer, 2011) and high solubility, including degradation by acid etching (Heitmann & Utiterbrink, 1995).

Previous studies have been sought for modifying capping agents to achieve anti-inflammatory effect (Liu et al., 2013; Louwakul & Lertchirakarn, 2012). Notwithstanding, an adhesive resin containing indomethacin-loaded nanocapsules has demonstrated controlled release of the non-steroidal anti-inflammatory (NSAID) and permeability of indomethacin through dentin (Genari et al., 2016).

The anti-inflammatory agents commonly used include steroidal and no steroidal drugs (NSAID). One representative of NSAID agents is indomethacin. The anti-inflammatory effect of NSAID is mainly mediated by the inhibition of cyclooxygenases 1 and 2 (COX-1 and COX-2) and it decreases the formation of prostaglandins (Summ & Evers, 2013). NSAIDs oral administration also demonstrated analgesic effect decreasing the endodontic treatment pain (Lapidus et al., 2016). NSAIDs have potential in the depressing sensory responses of the nociceptive system by central action (Bustamante et al., 1996; Jurna & Brune, 1990). The depression of the afferent C-fiber reflex is the main antinociceptive path of NSAIDs (Bustamante et al., 1996; Jurna & Brune, 1990). NSAID analgesic and the anti-inflammatory effects are directly related to its concentration at the required site of action. Pharmacokinetic properties, such as half-life rates and time taken to deliver active metabolites, are imperative for NSAIDs effects (Bustamante et al., 1996).

The non-specific distribution of drug leads to high concentration in no target sites, leading to low effect and toxicity (Soppimath et al., 2001). One method of restricting the drug to the required site is to employ a carrier system. Nanoparticles have received considerable attention as potential drug delivery vehicles over the last few years. Related to controlled release, the use of a carrier system modifies the bioavailability of drugs. NSAIDs-loaded nanocapsules have presented an increase of efficacy, associated to adverse effects reduction (Bernardi et al., 2009; Guterres et al., 2001).

Animal models of acute inflammation are commonly used to assess the anti-inflammatory properties of agents (McCarson, 2015, Blattes et al., 2017). Intradermal formalin injection into the rat hindpaw is widely used as a nociceptive stimulus in the rat. It represents a classical and valid model employed to study acute inflammation and pain for different inflammatory diseases (Barth et al., 2016; Bernardi et al., 2009; Dubuisson & Dennis, 1977; Kawamura et al., 2000; McCarson, 2015). The injection of agent causes a rapid formation of edema and an exacerbated sensitivity to stimuli (McCarson, 2015; Rocha et al., 2006). Thus, formalin-induced rat paw edema and responses to mechanical and thermal stimuli are widely used to characterize the mechanisms of action of new anti-inflammatory drugs or formulations, including NSAIDs (Bernardi et al., 2009; Kawamura et al., 2000; McCarson, 2015).

Therefore, the purpose of this study was to analyze the anti-inflammatory and analgesic effects of a resin containing indomethacin-loaded nanocapsules in acute inflammatory animal model.

Materials and Methods

Animals

A total of 20 adult male Wistar rats (55–65 days old; weight 200–250 g) were used. The animals were randomized by weight and housed in groups of three per polypropylene cage (49 cm x 34 cm x 16 cm) with sawdust-covered flooring. All animals were maintained in a controlled environment (22±2°C) under a standard light-dark cycle (lights-on at 7 a.m. and lights-off at 7 p.m.), with water and chow (Nuvital, Porto Alegre, Brazil) ad libitum. All experiments and procedures were approved by the institutional Animal Care and Use Committee (UFRGS protocol no. 28648) and performed in accordance with the Guide for the Care and Use of Laboratory Animals, 8th ed, 2001 and law 11794 (Brazil). The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines (Kilkenny et al., 2010). The experiment used the number of animals necessary to produce reliable scientific data.

Experimental design

Rats were acclimated to maintenance room for 1 week before the experiment began. Animals were divided into two groups: adhesive resin disk without nanocapsules (control – CT), and adhesive resin disk containing indomethacin-loaded nanocapsules (nanocapsule – NC). Each animal surgically received a disk of resin (3 x 1 mm, 0.7 g) with or without nanocapsules into plantar surface of the right hind paw. A week after surgical procedure, 0.17 ml/kg of a 2% formalin solution, Formaldehyde P.A.® (Sigma-Aldrich, São Paulo, Brazil) diluted in 0.9% NaCl (saline), was intradermally injected into plantar surface of the right hind paw. Rats were killed by decapitation 13 days after surgery. The experimental design of study is in Figure 1. For all procedures, examiner was blinded to group of rats being tested.

Preparation of nanocapsules

Indomethacin-loaded NCs were prepared by the interfacial deposition of preformed polymer technique [20]. All reagents were purchased from Sigma Chemical (St. Louis, USA). The organic phase was prepared with

Eudragit® S100, poly (MMA-co-MAA) (0.50 g), indomethacin (0.05 g), medium chain triglycerides (0.81 mL) and sorbitan monostearate (0.19 g) dissolved in acetone (125 mL). Under magnetic stirring at 25 ^{o}C, organic phase was added through a funnel to an aqueous phase containing polysorbate 80 (0.385 g) and water (250 mL). Acetone and water excess were eliminated using a rotary evaporator (Rotavapor II, Buchi, Flawi, Switzerland), a B-740 recirculating chiller (Buchi, Flawi, Switzerland) and a U-700 vacuum pump (Buchi, Flawi, Switzerland). The IndOH-NC suspension was spray dried (B-290, Buchi, Flawi, Switzerland) using hydrophilic fumed silicon dioxide (Aerosil® 200) as an adjuvant, in amount of 3% of the suspension content. The inlet temperature at the drying chamber was maintained at approximately 150 ± 4 °C, and the outlet temperature was 107 ± 4 °C.

Formulation of the resin disk

Experimental dental adhesives were produced using 50/25/25 wt% bisphenol A glycol dimethacrylate (BisGMA)/triethylene glycol dimethacrylate (TEGDMA)/ 2-hydroxyethyl methacrylate (HEMA). Camphoroquinone (CQ), ethyl 4-dimethylaminobenzoate (EDAB) and diphenyliodonium hexafluoroantimonate were added at a concentration of 1 mol% and 0.01 wt% of butylated hydroxytoluene (BHT) as a photoactivation system. The dried IndOH-NCs were added at 10 wt% (0.07 g). As a control, one group had no addition of particles. All formulations were mixed and ultrasonicated (CBU 100/1 LDG, Plana, São Paulo, Brazil) for thirty minutes. Disks (3.0 mm diameter and 1.0 mm thick; n=10) from each resin disk (with and without nanocapsules) were photoactivated for 20 s by a light-emitting diode with an irradiance value of 1200 mW/cm^2 (Radii cal, SDI, Bayswater, Australia).

Surgical procedure

For surgical procedure, rats were anesthetized by 3% isoflurane through inhalation and placed in the dorsal position for right thigh hair shaving and skin antisepsis with 2% iodine-alcohol. Incision was performed according Brennan model, subsequently the adhesive resin disk with or without nanocapsules was subcutaneously implanted and plantar paw was sutured with Mononylon 4.0. All procedures were performed in the same day period

and by the same investigator on all rats. Following surgery and anesthetic recovery, the animals were returned to their cages, where they remained until the day of death. Dypirone (10 mg/kg) was administrated intraperitoneally immediately after anesthesia recovery.

Paw edema

Volume of right hind paw was measured using a caliper rule on day 2, day 4, day 6, day 8, day 10, and day 12 after surgical procedure. The measurements were performed in width and height of paw of each animal by the same experimenter.

Formalin test

The formalin test was performed as previously described (Tai et al., 2006; Tjølsen et al., 1992) with minor modifications. Twenty-four hours before the test, animals were placed in the individual chamber for ten minutes to familiarize them with the procedure, since the novelty of the apparatus itself can induce antinociception. A week after surgery, as described above, at section 2.4, the animals were injected intradermally on the plantar surface of the left hindpaw with 0.17 ml/kg of a 2% formalin solution (Formaldehyde P.A.®, obtained from Sigma-Aldrich, São Paulo, Brazil) diluted in 0.9% NaCl (saline). Each animal was observed in a varnished polymeric cage (60 × 40 × 50 cm). Nociceptive response was recorded for a total period of 30 min. Test produces two distinct phases of nociceptive behavior: an early, transient phase (phase I, up to 5 min after the injection) and a late, persistent phase (phase II, up to 30 min after the injection). Thus, the first part of observation was for 5 min and the second part was during 15 min. A break of 10 min was taken between phases I and II. Time (in seconds) spent in licking, biting, and flicking of right hind paw was recorded in phases I and II. Phase I has been considered to reflect direct stimulation of primary afferent fibers, predominantly C-fibers (neurogenic pain), whereas phase II is dependent on peripheral inflammation (inflammatory pain) (Tjølsen et al., 1992).

Von Frey test

Mechanical hyperalgesia was assessed on day 2, day 4, day 6, day 8,

day 10, and day 12 after surgical procedure using an automatic von Frey aesthesiometer (Insight, São Paulo, Brazil). This device automatically records the pressure intensity after paw withdrawal. Test was performed in polypropylene cages (12 × 20 × 17 cm) with wire grid flooring. Rats were habituated to cages for 10 minutes 24 hours prior to test and 5 minutes daily before test to prevent analgesia induced by apparatus novelty. For testing, a polypropylene tip was inserted perpendicularly from underneath the floor grid and applied to plantar side of right hind paw at gradually increasing pressure. A tilted mirror below the grid provided a clear view of the animal's hind paw. The intensity of the stimulus supported up to paw withdrawal, in grams (g), was automatically recorded. Three successive readings were measured between interval periods of 5 s and averaged.

Serum collection

The animals were killed by decapitation by trained practitioner 13 days after the surgery and blood were collected. Trunk blood was drawn, and blood samples were centrifuged in plastic tubes for 5 min at 5000 × g, at room temperature. This method was used to enable the collection of large volumes of blood serum for analysis. Serum was obtained and frozen at −80 °C until assays were performed.

Biochemical assays

Lactate dehydrogenase (LDH) serum level was determined by ELISA using a commercial LDH assay kit (Abcam Inc, Cambridge, MA, USA). Procedures were performed in accordance to the manufacturer's protocol. Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for quantifying IL-10 (n=6) and IL-6 (n=6) cytokines were performed using monoclonal specific antibodies (R&D Systems, Minneapolis, United States) and 96 well cell flat bottom plates. Standard curves for IL-10 and IL-6 were obtained. Optical density was measured using an ELISA reader at wavelength of 450 nm.

Statistical analysis

Outliers were removed according analyses performed by Statistical Package for the Social Sciences (SPSS) 20.0. T-test was used for

comparison between the groups. The data were expressed as the mean ± standard error of the mean (S.E.M) and considered significant at p≤ 0.05. SPSS 20.0 for Windows was used for the statistical analysis.

Figure 1. Experimental design. : Paw edema and Von Frey test. : Interventions - surgery and formalin injection. : Death.

Results

Paw edema

Indomethacin-loaded nanocapsules group presented lower edema in the right hind paw on day 8 (24 h after formalin injection) than control group (p=0.001) (Figure 2a). There was no difference in height dimensions (Figure 2b).

Formalin test

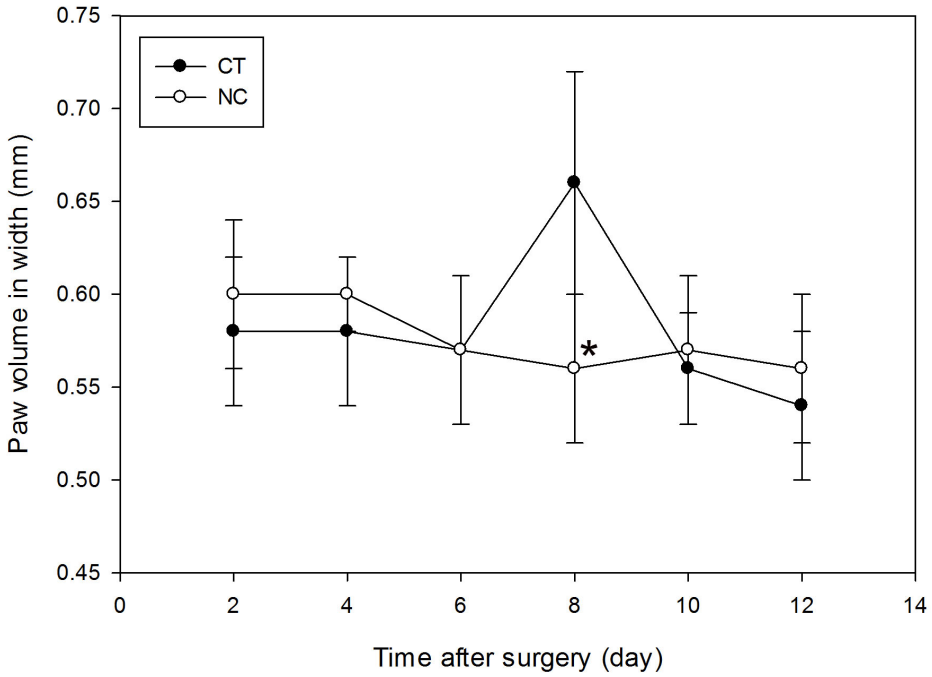
After formalin injection, the group with adhesive resin disk containing indomethacin-loaded nanocapsules showed smaller nociceptive response in phase I compared to control group (NC- 66.86 ± 22.83s X CT- 130.17 ± 35.83s; *P*<0.001). No difference was observed in phase II (Figure 3).

Von frey test

Indomethacin-loaded nanocapsules group presented supporting higher intensity of stimulus on days 8 and 12 (24 h and 72 h after formalin injection) (NC- 60.38 g ± 16.00 X CT- 40.82 ± 9.70 g, *P*<0.01 and NC- 61.53 g ± 21.06 X CT- 37.00 ± 11.00 g, *P*<0.02 respectively) in the von Frey test (Figure 4).

Biochemical assays

There is no difference between groups regarding to LDH levels (NC- 561.66 ± 77.71 pg/mL X CT- 489.52 ± 57.53 pg/mL) and IL-10 (NC- 34.47 ± 4.03 pg/mL X CT- 37.80 ± 2.43 pg/mL) (Table 1). Group containing indomethacin-loaded nanocapsules presented higher IL-6 level (NC- 356.81 ± 7.51 pg/mL X CT- 327.19 ± 0.01 pg/mL; *P*<0.001).



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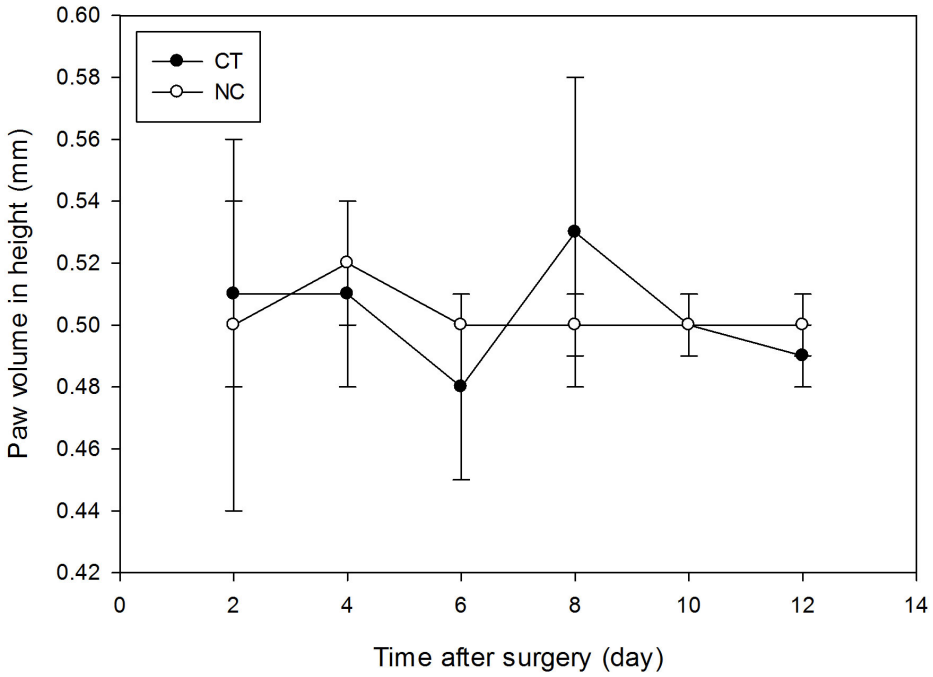

B.

Figure 2. Effect of resin disk containing indomethacin-loaded nanocapsules on rat paw oedema. (A) Paw volume in width; (B) paw volume in height. CT corresponds to control group. NC represents group with resin disk containing indomethacin-loaded nanocapsules. Asterisk (*) in NC group denote significant difference in comparison to respective control ($p<0.05$).

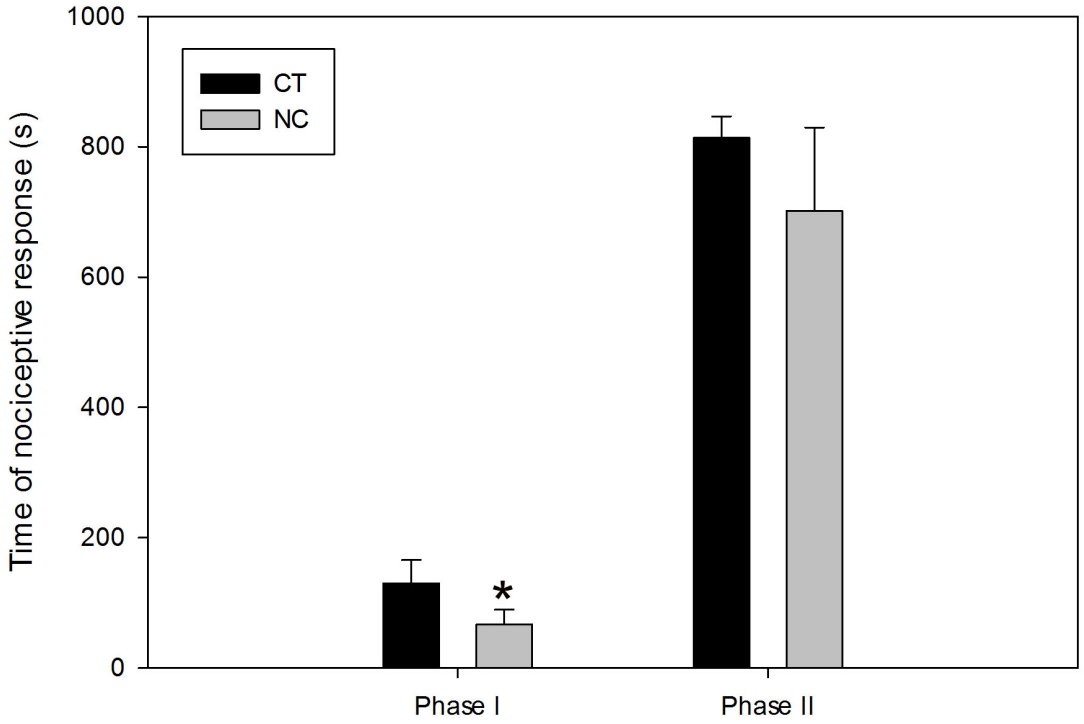

Figure 3. Nociceptive response after formalin-induced in early and inflammatory phases on paw containing resin disk with and without indomethacin-loaded nanocapsules. CT corresponds to control group. NC represents group with resin disk containing indomethacin-loaded nanocapsules. Asterisk (*) in NC group denote significant difference in comparison to respective control ($p<0.05$).

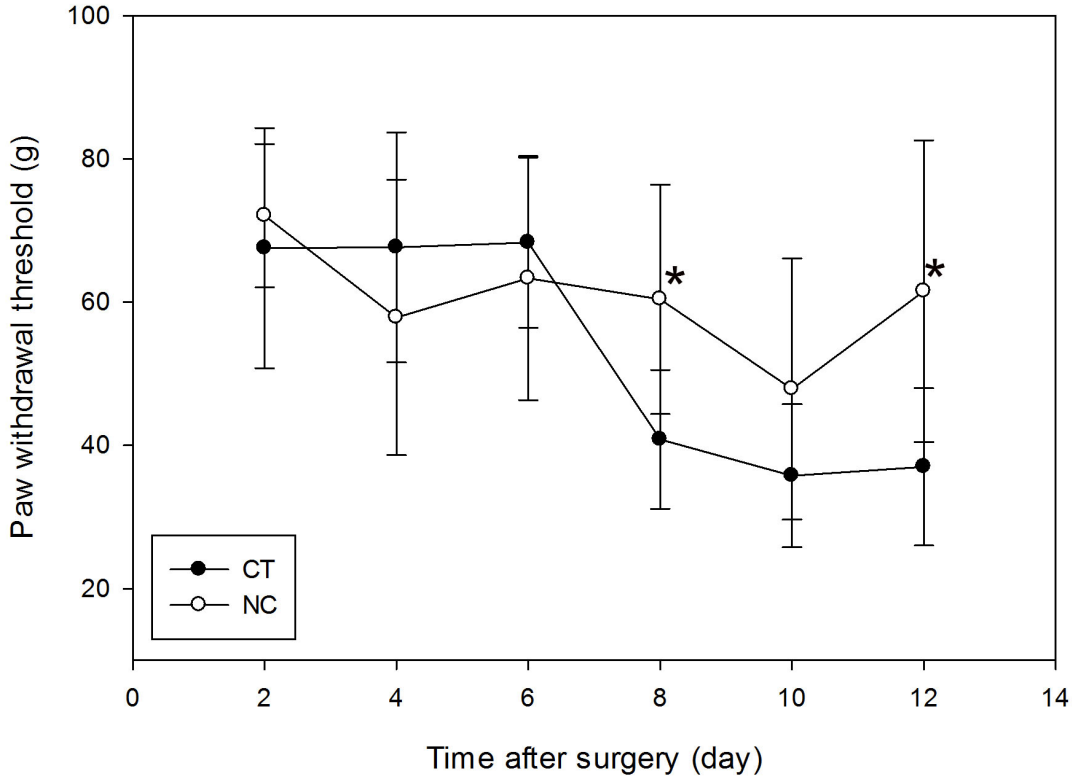

Figure 4. Mechanical hyperalgesia presented through paw withdrawal threshold. CT corresponds to control group. NC represents group with resin disk containing indomethacin-loaded nanocapsules. Asterisk (*) in NC group denote significant difference in comparison to respective control ($p<0.05$).

Table 1. LDH, IL-10 and IL-6 levels.

Groups	LDH (U/L)	IL-10 (pg/mL)	IL-6 (pg/mL)
CT	489.52 ± 57.53^{A}	37.80 ± 2.43^{A}	327.19 ± 0.01^{A}
NC	561.66 ± 77.71^{A}	34.47 ± 4.03^{A}	356.81 ± 7.51^{B}

CT corresponds to control group. NC represents group with resin disk containing indomethacin-loaded nanocapsules. Significant differences between groups are denoted with different capital letters.

Discussion

This study demonstrates that the resin containing indomethacin-loaded nanocapsules showed successfully anti-inflammatory and nociceptive activities in a chemical model of acute inflammation. In addition to previous results (Genari et al., 2016), the clinical use of a dentin adhesive with potential to inhibit an inflammatory reaction in deep cavities is promising, being an alternative instead of using indirect pulp-capping agent.

Anti-inflammatory effect of indomethacin occurs through inhibition of prostaglandins as a consequence of reversible block of cyclooxygenase 1 e 2 (Summ & Evers, 2013). In addition, the analgesic effect is a result of depressing of sensory responses of nociceptive system, mainly the afferent C-fiber reflex (Bustamante et al., 1996; Jurna & Brune, 1990). In the present study, prompt effect of anti-inflammatory drug post formalin injection could be expected, since more than 13% of indomethacin had already been released from resin disk after seven days from the surgical procedure (Genari et al., 2016). Controlled bioavailability of indomethacin in a deep cavity could be relevant since triggering of inflammation of pulp tissue occurs 24 hours after damage and progresses within 30 days (Alves & Sobral, 2015).

No significant visually inflammatory process was promoted by adhesive resin disk when comparing to paws without resin disk contact (data not show). It can indicate an *in vivo* biocompatibility of the resin. Therefore, induced inflammation model using formalin injection was required to promote inflammatory process as a challenge for resin disk containing indomethacin-loaded nanocapsules. The present inflammatory model can be useful for other dental materials.

Changes in microcirculation characterize the first line of defense against an injury and involve the exudation of fluid and migration of cells, resulting in acute signals like edema (Matsuo et al., 2016). Formalin injection (on day 7) promoted an increased paw volume in width, which was reverted by indomethacin-loaded nanocapsules. No difference in height was noticed by inflammation in addition to a volume alteration due to presence of adhesive resin disk into paw. In addition to edema, inflammation process involves pro and anti-inflammatory mediators and nociceptive responses.

Cytokines are involved in the development and regulation of

inflammatory (Zhang & An, 2007). The LDH is considered an injury peripheral marker, associated to cell damage and oxidative stress (Berthier et al., 2002), but in the present study, there was no difference between groups in LDH serum levels. Systemic blood collection can be a too hard challenge to represent an anti-inflammatory effect through LDH levels. The function of Interleukin-10 is to limit inflammatory responses and its role is dependent of the moment and area of its measurement (Moor et al., 2001; Mosser & Zhang, 2008). In the present study, there was no difference between groups in the IL-10 serum levels. This equivalence probably occurred due to the moment of IL-10 collection. This cytokine was collected 13 days after surgery, which is considered late to measure an acute expression, but also early to represent a chronic process (Mosser & Zhang, 2008). Further, IL-6 is associated with classical mediation of anti-inflammatory effect, leading to the attraction of neutrophils, monocyte recruitment, B, and T cells activations (Gadient & Otten, 1997; Kawasaki et al., 2008; Scheller et al., 2011). Systemic levels of IL-6 had already been correlated to its local levels (Lattimer et al., 2016). In the present study, high level of IL-6 in NC group indicates the activation of anti-inflammatory pathways on target cells by indomethacin released from NC (Gadient & Otten, 1997; Medeiros et al, 2015; Scheller et al., 2011). Higher levels of IL-6 were also observed for free indomethacin administration (Gentile et al., 2015).

During inflammatory process, nociceptive response is a consequence of fibers regulation (Dubin & Patapoutian, 2010). In formalin test, animals present a biphasic nociceptive response (Dubuisson and Dennis, 1977). The early phase (first 5 min) is a reflex of activation of nociceptive sensory afferent receptors, predominantly C-fibers, and the tonic phase (from 15 to 30 min) is an inflammatory response (Dubuisson and Dennis, 1977). In the present investigation, the group containing indomethacin presented a decrease in the hyperalgesia in the first phase. Previous studies also showed NSAID, including indomethacin, suppressed C-fibers and consequently attenuated nociceptive response in first phase and in acute models of hyperalgesia (Bustamante et al., 1997). At the same time, C-fibers participate of Aβ fibers activation, which mediates spinal reflex, complex behaviors, and supra spinal integration (Millan, 1999). In this study the mechanical hyperalgesia evaluated

in the von Frey test showed that NC group presented an increased mechanical withdrawal threshold 24 h and 72 h after formalin injection. Thus, we can suggest that peripheral nociceptive fibers were inhibited by local released indomethacin. It is important to note that our formalin and von Frey results suggested modulation of C and Aβ fibers by indomethacin. Similarly to this model, dental pulp nociception involves C and A fibers (Jain, Gupta, & Meena, 2013). Thus, we can suggest that adhesive resin containing indomethacin-loaded nanocapsules has also potential to modulate pulpal nociceptive response.

Conclusions

These results indicate that an adhesive resin containing indomethacin-loaded nanocapsules has anti-inflammatory and nociceptive activities in a chemical model of acute inflammation. The present investigation confirms an adhesive resin with drug-loaded nanocapsules may be useful for improving therapeutic action for adhesives.

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3.3 MANUSCRITO III

Antimicrobial and anti-inflammatory adhesive system containing nanocapsules

Short title: Antimicrobial and anti-inflammatory adhesive system

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Abstract

Objectives: To incorporate indomethacin and triclosan-loaded nanocapsules into primer and adhesive, and evaluate its properties.

Methods: Indomethacin and triclosan were encapsulated by deposition of preformed polymer and subsequently characterized regarding morphology, particle size, drug content and cytotoxicity. Nanocapsules (NCs) were incorporated into primer at 2% and into adhesive at 1%, 2%, 5%, and 10% concentrations. Degree of conversion (DC) and softening in ethanol of the adhesive were evaluated. Drug release and drug diffusion through dentin was quantified by high performance liquid chromatography. Antimicrobial test was performed until 96 h.

Results: Spherical and biocompatible NCs presented mean size of 159 nm. Drugs content was 3 mg indomethacin/g powder and 2 mg triclosan/g powder. Incorporating NCs in adhesive showed no influence in DC ($p=0.335$). The addition of 2% of NCs showed no influence in softening in ethanol ($p>0.05$). After 120 h, 93% of indomethacin and 80% of triclosan were released from primer, 20% of indomethacin and 17% of triclosan were released from adhesive with 10% of NCs. Indomethacin showed diffusion through dentin. In 24 h, adhesive containing 2% and 5% of NCs using primer with NCs showed antimicrobial effect. In 96 h, adhesives containing different concentration of NCs promoted antimicrobial effect.

Conclusions: Indomethacin and triclosan-loaded nanocapsules were successfully incorporated into primer and adhesive, promoting controlled drugs release, indomethacin diffusion through dentin and antimicrobial effect without compromising its physicochemical properties.

Clinical Significance: Indomethacin and triclosan-loaded nanocapsules have potential to prevent recurrent caries and be used in deep cavities controlling pulpar inflammatory process.

Keywords: Indomethacin, Triclosan, Drug rele
ase**,** Drug carriers, Dental bonding

Introduction

The failure rate of composite resin restorations as a result of recurrent caries was reported between 40% and 70% [1,2,3]. The increase of bacterial oral colonization around restorations leads to a pH decrease and consequently recurrent desmineralization process [4]. Post-operative sensitivity is another reason of failure with an occurrence rate around 10% [3]. The sensitivity occurs in deep cavities with a pulpal involvement that can lead to an acute or chronic inflammation or necrosis of pulp tissue [5,6].

In order to reduce failures of restorations, antibacterial agents have been added to adhesive systems [7,8,9]. Overall, agents are effective but limited for a short time of action. Further, the increase of concentration of agents can negatively affect the properties of material [7,10,11,12]. Regarding pulp sensitivity, indirect pulp capping using calcium hydroxide is the most widely applied treatment to prevent progress of pulp inflammation in deep cavities through dentin repair [13]. However, calcium hydroxide as pulp capping resulted no improvement in long-term success rate of restorations [14,15,16]. Therefore, new alternatives have been developed to improve the prevention of pulp inflammatory progress [17,18,19]. Nonetheless, there is no study focused on development of adhesive system promoting antimicrobial and anti-inflammatory effects.

An alternative strategy to improve bioavailability and efficacy is drug-loaded nanocapsules [20]. Due to encapsulation, drug release is controlled, effect occurs even in sub-therapeutic doses, and adverse effects are sparse [21,22]. In addition to carrier systems, the selection of drugs is essential. Triclosan presented broad-spectrum of antimicrobial activities through structural perturbations resulting a loss of permeability-barrier functions [23]. Due to its ability to inhibit membrane enzymes and glycolysis of *S. mutans* in biofilms, triclosan is considered an anti-caries agent [24]. Indomethacin has presented successful anti-inflammatory effect, including in pulp tissue [25], due to inhibition of prostaglandins by reversibly blocking cyclooxygenases [26]. Further, indomethacin has been showed high effective nanoencapsulation [22].

Therefore, the aim of this study was to incorporate indomethacin and triclosan-loaded nanocapsules into a primer and an adhesive resin and evaluate its properties.

Materials and Methods

Preparation of indomethacin and triclosan-loaded NCs

Indomethacin and triclosan-loaded NCs were prepared by the interfacial deposition of preformed polymer in a miniemulsion. The reagents were obtained from Sigma Chemical (St. Louis, USA). An organic phase was composed by polymer (MMA-co-MAA), Eudragit® S100 (0.50 g), indomethacin (0.025 g), triclosan (0.025 g), medium chain triglycerides (0.81 mL), sorbitan monostearate (0.19 g) and acetone (125 mL). An aqueous phase contained polysorbate 80 (0.385 g) and water (250 mL). The organic phase was added through a funnel to aqueous phase under magnetic stirring at 25° C. Acetone and water excess were eliminated using a rotary evaporator (Rotavapor II, Buchi, Flawi, Switzerland), a B-740 recirculating chiller (Buchi, Flawi, Switzerland) and a U-700 vacuum pump (Buchi, Flawi, Switzerland). The suspension containing NCs was spray dried (B-290, Buchi, Flawi, Switzerland) using hydrophilic fumed silicon dioxide (Aerosil® 200) in amount of 1.5% of the suspension content as an adjuvante to avoid the aggregation on internal wall of equipment. The inlet temperature at the drying chamber was approximately 150 ± 4 °C, and the outlet temperature was 107 ± 4 °C.

Characterization of indomethacin and triclosan-loaded NCs

The mean size ($d_{4.3}$) of indomethacin and triclosan-loaded NCs were measured by laser diffractometry (Mastersizer 2000, Malvern, Worcestershire, United Kingdom), in wet and dried states for NCs in aqueous suspension and dried NCs respectively. The distribution of the particle size (span) values was calculated by $(d_{0.9} - d_{0.1})/d_{0.5}$, where $d_{0.9}$, $d_{0.5}$, and $d_{0.1}$ were the particle diameters at the 90th, 50th, and 10th percentile of particles. Zeta potential of the suspension was determined using a Zetasizer nano-ZS ZEN 3600 model (Malvern Instruments, Malvern, Worcestershire, United Kingdom). The samples were then diluted with 1 mM NaCl aqueous solution. The measurements were made in triplicate to assure accuracy.

The morphological analysis was realized using a transmission electron microscopy (TEM, JEM 1200 Exll, Jeol, Tokyo, Japan) at 80 kV. An amount of 20 µl of NCs suspension at a dilution of 1:10 was deposited in a Formvar-

Carbon support film on a specimen grid and negatively stained with uranyl acetate solution (2% m/v). Dried NCs (0.01 g) were processed using gold-sputter-coating and submitted to scanning electron microscopy (SEM, JSM 6060, Jeol, Tokyo, Japan) at an accelerating voltage of 10 kV.

Determination of drugs content for dried indomethacin and triclosan-loaded NCs

The dried NCs (20 mg) were dissolved in acetonitrile (10 mL) under 30 min of ultrasound stirring. The solution was filtered using a 0.45-µm (Millipore) filter, and free drugs was measured using high performance liquid chromatography (HPLC, Shimadzu LC 10-A Shimadzu, Kyoto, Japan), a UV/visible detector (λ = 280 nm) and Nova-Pak® C18 3.9 x 150 mm (4 µm) Waters column. A mobile phase (60:40 acetonitrile:water solution in volume, pH 4.5, adjusted with acetic acid) was pumped at a constant flow rate. The method was previously validated with linearity (y=159387x + 62555 and r^2=0.99822 for indomethacin, y=73937 + 31383 and r^2=0.98672 for triclosan) and a precision of 1.02% for indomethacin and 1.00% for triclosan.

Cytotoxicity

Cell viability was tested by direct contact using fibroblast cells (L929, BCR, batch no. 000604, Rio de Janeiro, Brazil) according to ISO 10993-5. The MTT test was performed in sextuplicate. Cells were incubated at 37 °C and atmosphere containing 5% CO_2. Cells (L929) were seeded in a 96-well tissue culture plate at a concentration of 10^4 cells/well (100 µL). After 24 h, the Indomethacin and triclosan-loaded NCs suspension (5 µL of nanocapsules/ mL in well) and its 1:10 dilutions were added to wells and incubated for 24 h at 37 °C. The medium alone was used as a negative control, and, as positive control, cells were cultured with dimethylsulfoxide (DMSO). A volume of 50 µL of MTT (1 mg/mL) was added to each well. Formazan salts were dilssolved in DMSO (100 µL), and the absorbance was measured at 570 nm (Multiskan EX Microplate Reader, MTX Lab Systems, Vienna, USA).

Formulation of the adhesive resin and primer containing indomethacin and triclosan-loaded NCs

Experimental dental adhesives were formulated using 66/33 wt% bisphenol A glycol dimethacrylate (BisGMA)/2-hydroxyethyl methacrylate (HEMA). Camphoroquinone (CQ) and ethyl 4-dimethylaminobenzoate (EDAB) were added at a concentration of 1 mol% as a photoactivation system. The dried indomethacin and triclosan-loaded NCs were added at 1, 2, 5 and 10 wt%. One group had no addition of particles, as control. An amount of 2 wt% of indomethacin and triclosan-loaded NCs were incorporated into a comercial primer (Scotchbond MP, 3M-ESPE, St Paul, MN, USA). All formulations were mixed and ultrasonicated (CBU 100/1 LDG, Plana, São Paulo, Brazil) for thirty minutes.

Morphological characterization

The adhesive containing 10% of dried indomethacin and triclosan-loaded NCs was analyzed by transmission electron microscopy at 80 kV. The monomeric adhesive was diluted (1:10) and prepared as described above, in “Characterization of indomethacin and triclosan-loaded NCs”.

Degree of conversion (DC)

DC was evaluated with an ATR-FTIR spectrometer [27]. A disk (5.0 mm diameter and 1.5 mm thick) from each sample (n = 5) was photoactivated for 20 s by a light-emitting diode with an irradiance value of 1200 mW/cm^2 (Radii cal, SDI, Bayswater, Australia). Absorbance spectra were obtained before and immediately after light polymerization. DC was calculated for the intensity (peak height) of the aliphatic carbon-carbon double bond stretching vibration at 1635 cm^{-1} and aromatic ring at 1610 cm^{-1} from the polymerized and unpolymerized samples. DC measurements were repeated after one month of adhesive resin storage at room temperature, in an eppendorf protected from light.

Softening in ethanol

Disk specimens (5.0 mm diameter and 1.5 mm thick; n=5) prepared as described in “Degree od conversion” were embedded in acrylic resin and polished through a series of silicon carbide (SiC) papers (400-, 600-, 800- and 1200-grit) during 2 minutes each. Surface microhardness was measured

using a microhardness tester (HMV-2, Shimadzu, Tokyo, Japan) and Knoop indenter at a load of 25 g for 15 s, before and after immersion in absolute ethanol for two hours, and percent reduction was calculated. Three indentations were performed on each sample surface.

Indomethacin and triclosan release

Discs of adhesive (10 x 1.5 mm) containing 1%, 2%, 5% and 10% of indomethacin and triclosan-loaded NCs, prepared as described in “Degree of conversion“, and 1 mL of primer containing 2% of NCs into a dialysis bag were used to measured drugs release, both in triplicate. The specimens were immersed in 10 mL of simulated body fluid (SBF) [28] under magnetic stirring at 37 °C. After 6, 12, 24, 48, 72, 96, and 120 h, 1 mL of released medium was collected and fresh SBF was replaced. The aliquots were filtered using a 0.45-μm (Millipore) filter and analyzed using HPLC method previously validated and described above, in ”Determination of drugs content for dried indomethacin and triclosan-loaded NCs” section.

Drug diffusion through dentin

A total of 24 healthy premolar teeth (n = 3), which were extracted for orthodontic purpose, were used in this study for eight groups, combining primer with or with no NCs and adhesive with 1%, 2%, 5%, and 10% of NCs. Prior to the study, patients were informed and consented about the extracted teeth would be used in an in vitro study. After the removal of the soft tissues, a plane parallel dentin section, with a thickness of 0.75 (± 0.05) mm right after the end of pulp horns, was obtained from each tooth using a low-speed diamond saw with water coolant. A side of the dentin slabs was ground with 600-grit silicon carbide (SiC) abrasive paper under water for 30 s to create a standardized smear layer. Each dentin slab was fixed in a Teflon disc (outer diameter of 27 mm, inner diameter of 4 mm, and thickness of 2 mm) using cyanoacrylate adhesive. The role (diameter of 4 mm) in the center of Teflon disc exposed a part of dentin that was etched with phosphoric acid for 15 s, washed for 15 s, and dried. Primer (Scotchbond multi-purpose, 3M ESPE, St Paul, USA) with or with no NCs was vigorously applied, and the solvent was evaporated for 10 s. The adhesive containing nanocapsules was applied in an

amount of 0.08 ± 0.01 g and photoactivated for 20 s using a light-emitting diode (Radii cal, SDI, Bayswater, Australia). A composite build-up was performed (Z350, 3M ESPE, St Paul, USA) in an increment of 2 mm and photoactivated.

Each Teflon disc containing a dentin slab was fixed in the effective diffusion area of a Franz diffusion cell apparatus. The donor and receptor compartments were filled with 1 and 2.5 mL of SBF, respectively. After seven days, an amount of 1 mL of the content of receptor compartment was collected, filtered, and analyzed by HPLC as described above, in ”Determination of drugs content for dried indomethacin and triclosan-loaded NCs” section. The results were calculated in percentage of diffused drug and drug diffusion in gram per square millimeters of area of restoration.

Antimicrobial test

For antibacterial activity evaluation of adhesive, six specimens (3.0 x 2.0 mm) of each group were fixed on teflon matrixes (two samples on each side of the matrixes) on the lid of a 48-well plate and sterilized by hydrogen peroxide gas plasma. Antimicrobial test was performed according [29]. To evaluate primer and adhesive together, disks were made using one third portion in weigth of primer and two thirds of adhesive, also polymerized during 20s, fixed on teflon matrixes and sterilezed. In the sterile 48-well plate, 900 µl of brain heart infusion (BHI) broth (Sigma-Aldrich, St Louis, MO, USA) with 0.5% sucrose and 90 µl of a suspension of an overnight broth culture of *Streptococcus mutans* UA 159, adjusted to optical density of 0.3 (550 nm) were added to each one of the wells. The plate was closed and incubated at 37°C for 24, 48, and 96 hours. The samples from each group were then removed from the lid's teflon matrixes and placed inside a micro-tube containing 900µl of saline and vortexed. Dilutions were made up to 10^{-6}. Two 25 µl-drops of each dilution were platted in BHI agar Petri dishes and incubated for 48 hours at 37°C. The number of colony forming units (CFU) was visually counted by optical microscopy and transformed to logCFU/ml.

Statistical analysis

Statistical analysis was performed using two-way ANOVA (nanocapsule concentration and time) and Tukey's post hoc test for DC. One-way ANOVA (groups) was performed for cytotoxicity, softening in ethanol, drug diffusion through dentin, and antibacterial test in each time. The paired t-test was used for comparison between the initial and final Knoop microhardness. All tests were performed at $\alpha=0.05$.

Results

Indomethacin and triclosan-loaded NCs in suspension presented mean size of 159 nm ($d_{4.3}$), d_{10} of 68 nm, d_{50} of 134 nm, d_{90} of 266 nm and a polydispersity index of 1.47. The dried NCs with silicon dioxide presented $d_{4,3}$ of 4.03 µm, d_{10} of 1.34 µm, d_{50} of 3.35 µm, d_{90} of 7.80 µm and a polydispersity index of 1.93. The zeta potential of NCs was -17.1 mV. The microscopic analysis showed spherical morphology of NCs in suspension, as it can be observed in Figure 1a. The dried particles are nanocapsules on surface of silicon dioxide (Figure 1b). Drugs content was 3 mg indomethacin/g powder and 2 mg triclosan/g powder. The suspension of indomethacin and triclosan-loaded NCs maintained cell viability higher than 90% (Figure 2).

In resin, NCs were dispersed and maintained at spherical shape (Figure 2c). The incorporation of dried indomethacin and triclosan-loaded NCs in adhesive resin results no influence on immediate DC ($p=0.335$) or on DC after one month of storage ($p=0.195$) (Table 1). The DC after one month did not decrease. The addition of 2% of NCs did not influence softening in solvent comparing to control (Table 1).

In 120 h, 93% of indomethacin and 80% of triclosan were released from primer (Figure 3a), 20% of indomethacin and 17% of triclosan were released from adhesive with 10% of NCs (Figure 3b). Indomethacin diffused through dentin (Figure 4). For groups using primer with NCs and adhesive containing 1%, 2%, 5%, and 10% of NCs, 0.04 ± 0.0001 g of drug per cm^3 diffused in seven days, which was higher than using primer with no NCs with adhesive containing 1% and 2% of NCs. The percent of drug diffusion decreased as total encapsulated drug in adhesive system increased, except for adhesive containing 10% of NCs, whose drug diffusion in percent presented no different with primer containing or not NCs.

In 24 h, groups of adhesives containing 2% and 5% of NCs with primer also containing NCs presented antimicrobial effect (Figure 5). In 48h, there is no group presented antimicrobial effect. In 96 h, adhesives containing different concentration of NCs promoted antimicrobial effect.

Table 1. Degree of conversion (DC) and softening in ethanol (Δ%) of the adhesive with different concentrations of indomethacin and triclosan-loaded nanocapsules (NCs). DC, in percent (%), immediately and after one month of storage. Initial (KHN1) and final (KHN2) microhardness and softening in ethanol in percent (Δ %).

	DC (%)				
Groups	Immediate	1 month	KHN1	KHN2	Δ%
0%	65.47 ±1.54^{Aa}	62.34 ±2.21^{Aa}	20.62 ±1.16^{a}	9.70 ±1.67^{b}	52.90 ±7.86^{A}
1%	63.63 ±1.01^{Aa}	65.39 ±3.62^{Aa}	21.09 ±1.08^{a}	7.43 ±0.71^{b}	64.73 ±3.23^{B,C}
2%	64.73 ±0.96^{Aa}	65.11 ±1.40^{Ab}	21.55 ±1.09^{a}	8.97 ±1.16^{b}	58.20 ±6.50^{A,B}
5%	63.66 ±1.16^{Aa}	64.08 ±1.58^{Aa}	21.92 ±0.81^{a}	7.63 ±1.95^{b}	65.38 ±7.84^{B,C}
10%	63.93 ±1.30^{Aa}	67.73 ±3.68^{Ab}	18.49 ±1.48^{a}	5.12 ±0.97^{b}	72.33 ±4.37^{C}

Mean ± standard deviation, n = 5. Comparison of means of degree of conversion was performed by two-way analysis of variance (ANOVA) and Tukey *post hoc* test. Comparison between initial and final microhardness was performed by paired t-test. Comparison between means of softening in ethanol between groups was performed by one-wat ANOVA and Tukey *post hoc* test. Identical capital letters in the same column denote significant equivalence.. Values followed by same lowercase letters in a row represent no

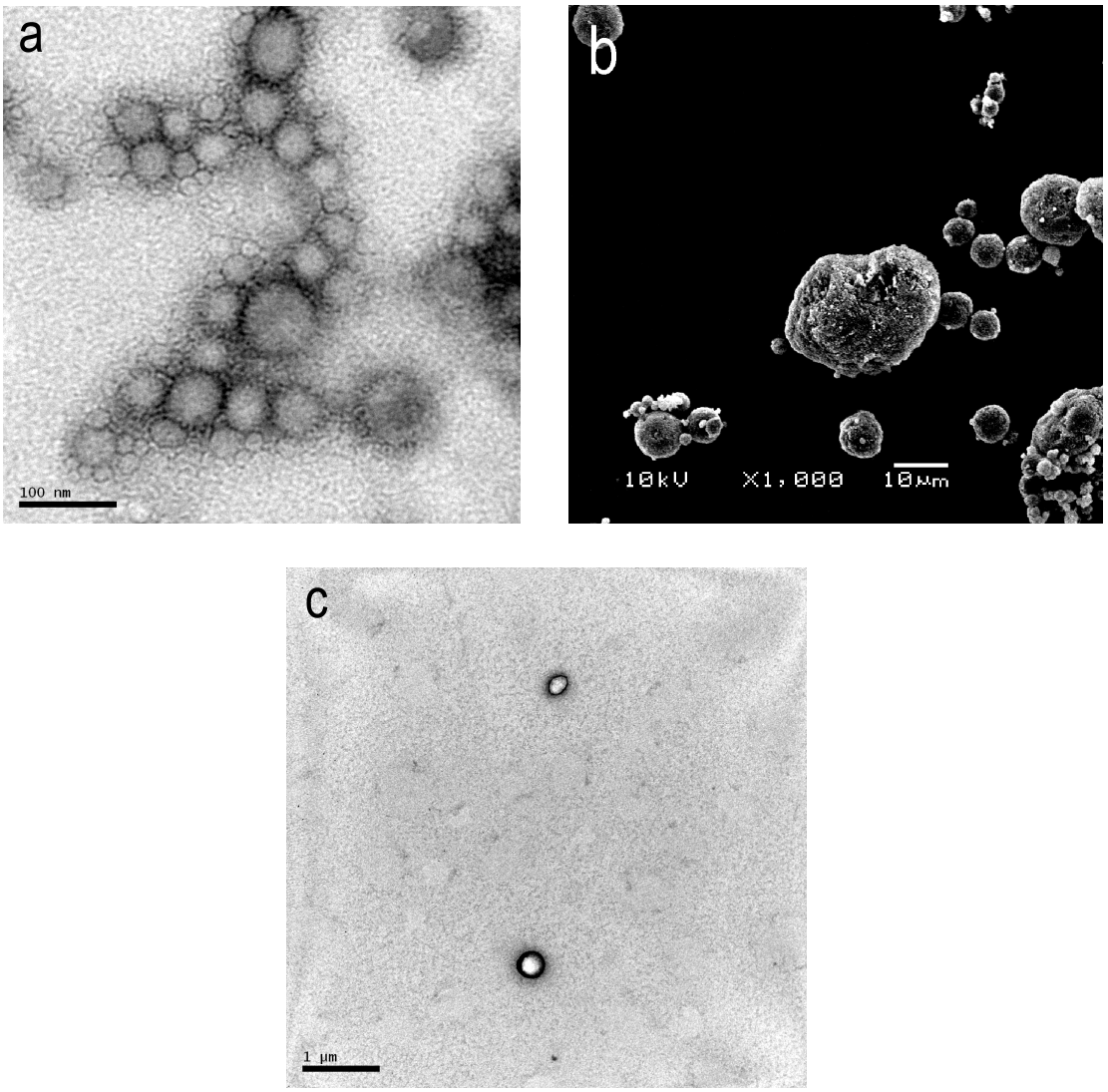


Figure 1. Transmission electronic microscopy (TEM) and scanning electron microscopy (SEM) images of indomethacin and triclosan-loaded nanocapsules (NCs). (a) NCs in suspension, by MET, with 250,000x magnification, showing spherical morphology and nanometric dimensions of NCs. (b) Dried NCs, by MEV, with 5,000x magnification, showing NCs onto silica particles surface. (c) Unpolymerized adhesive resin containing 10% of NCs in 1:10 dilution, with 20,000x magnification, showing dispersed NCs with maintained size and morphology.

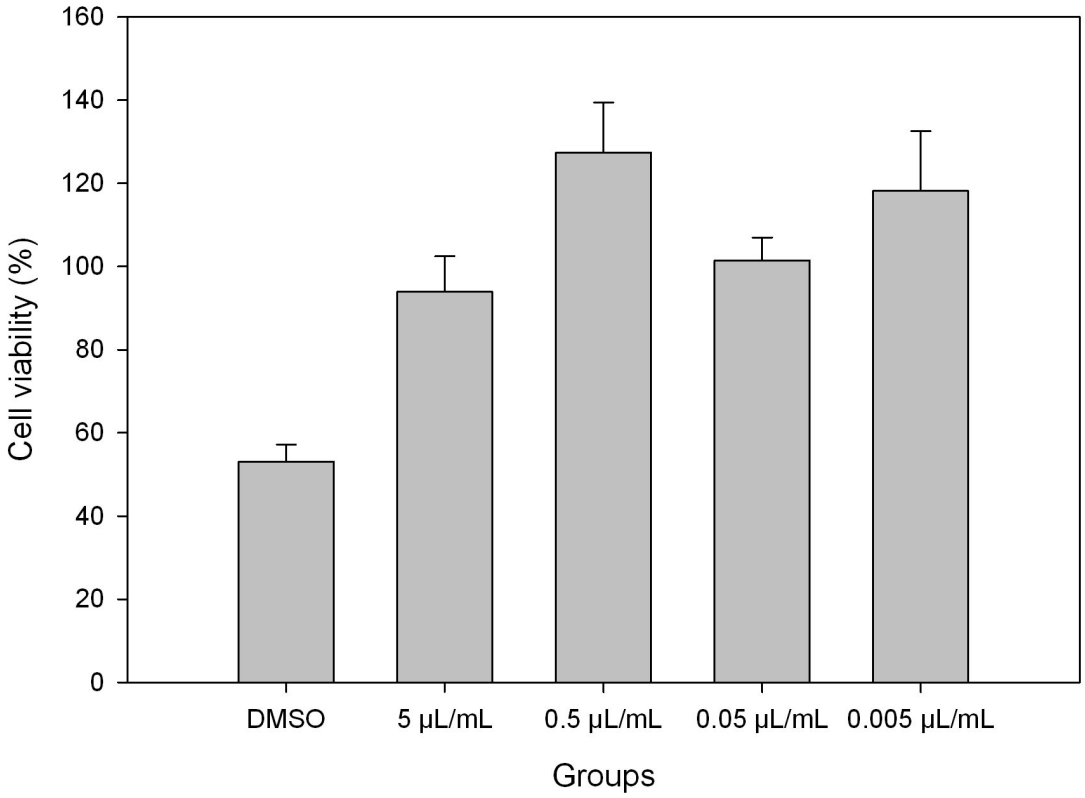
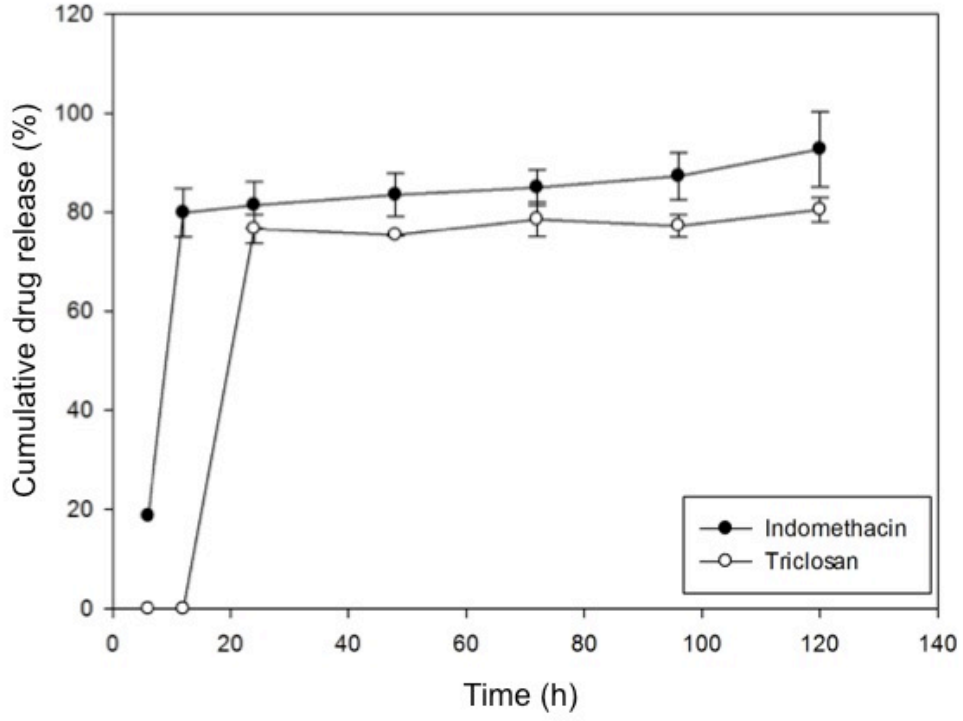


Figure 2. Viable L929 cells (in percent, %), by MTT test, on direct contact, during 24 h, to indomethacin and triclosan-loaded nanocapsules (NCs) suspension at 5 µL/mL, its dilutions and dimethylsulfoxide (DMSO, positive control). The percent of viable cells on contact to IndOH-NCs suspension in different dilutions were higher than 90%. On contact to DMSO, the viability was lower than 40%.



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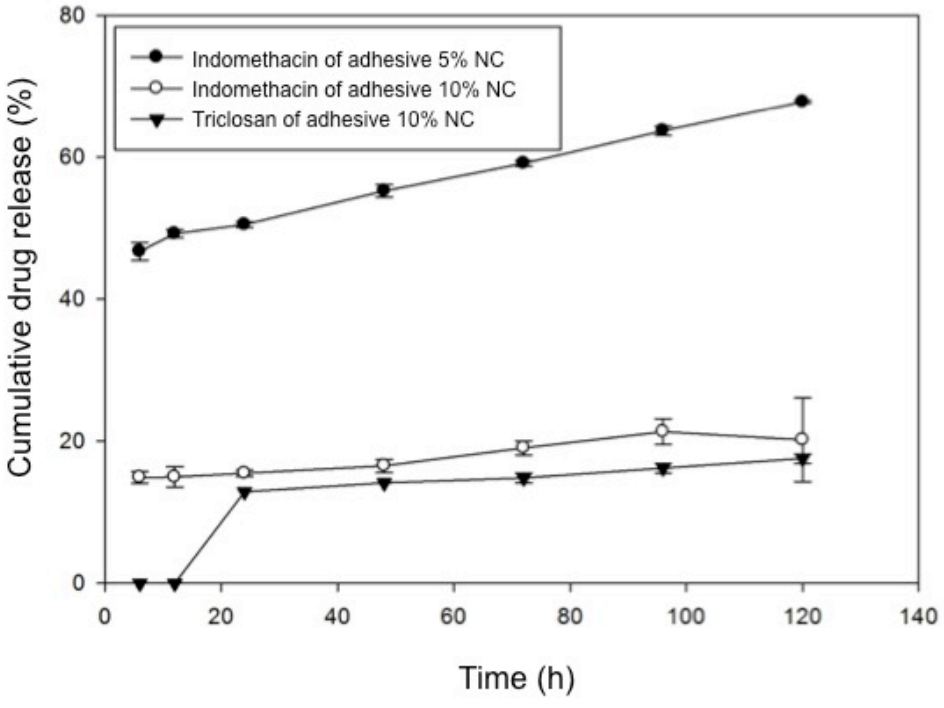

b

Figure 3. Indomethacin and triclosan release profiles from adhesive system. a) Drugs released from primer, showing a controlled release. b) Drugs released from adhesive, showing slower release as result of polymeric matrix. Amount of

released drugs from adhesive with other concentrations of NCs were lower than detection limit.

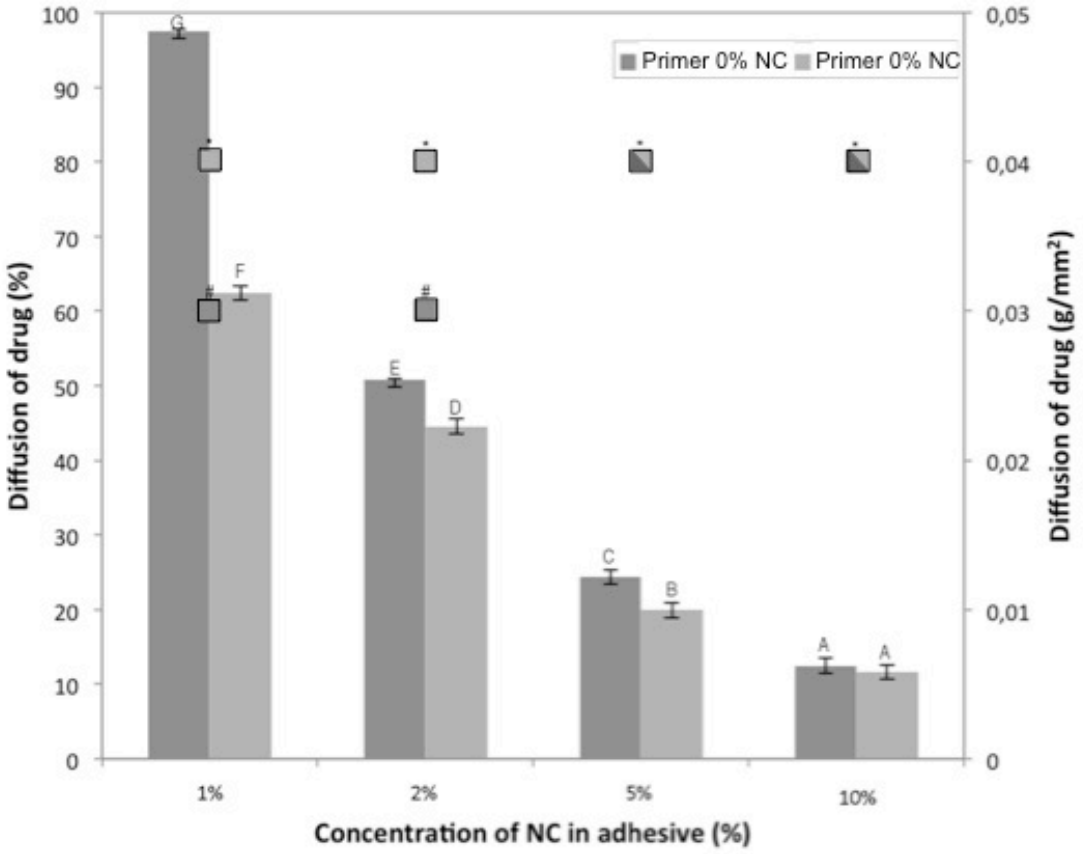

Figure 4. Indomethacin diffusion through dentin after seven days. Amounts of permeated indomethacin through dentin slabs restored with primer containing or not nanocapsules (NCs) and adhesive with diferent concentrations of NCs immersed in simmulated body fluid (SBF) until seven days were quantified by high performance liquid chromatography (HPLC).

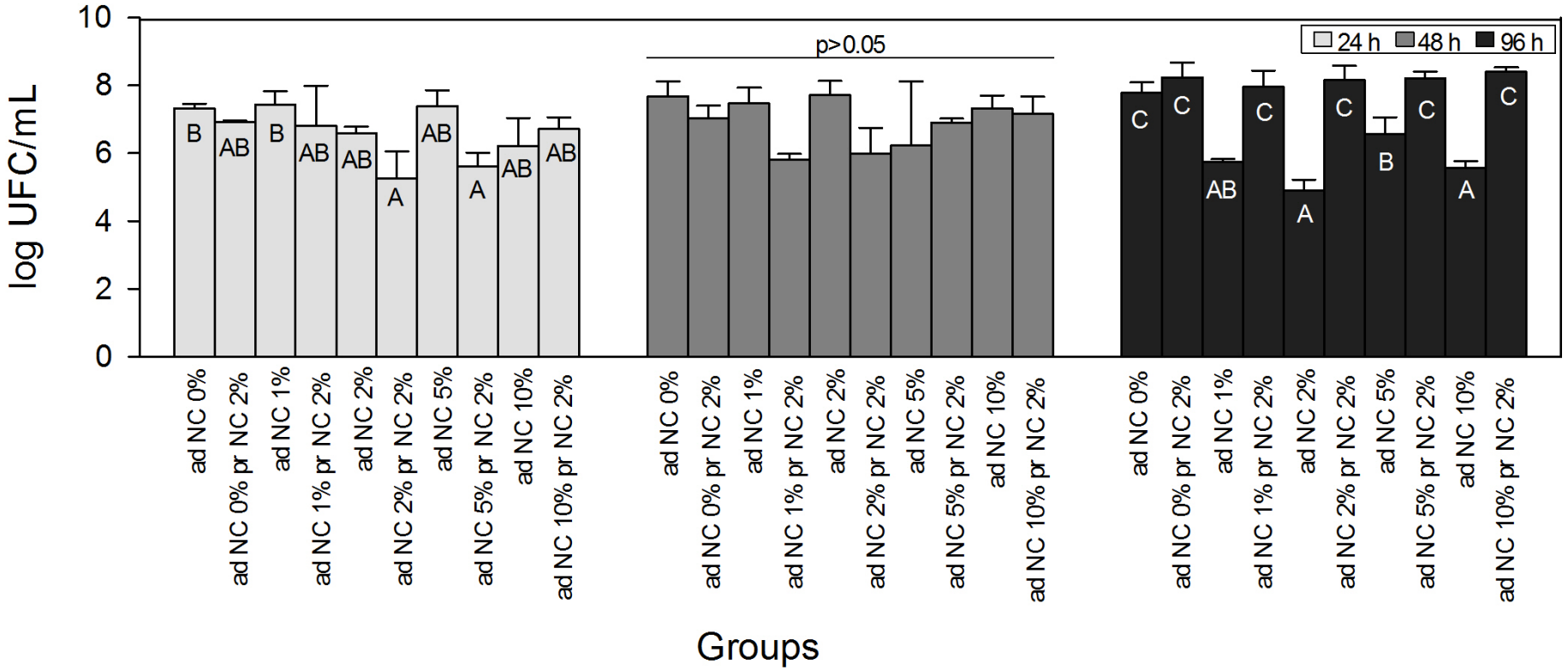

Figure 5. Antimicrobial effect of triclosan released from primer and adhesive by dilution in broth until 96 h. Groups with primer (pr) containing indomethacin and triclosan-loaded nanocapsules (NCs) present earlier effect, while groups only with adhesive (ad) containing NCs showed late antimicrobial effect.

Discussion

The present study introduces a novel approach of drug delivery to an adhesive system. Nanocapsules containing more than one drug were formulated and incorporated in dental materials. Nanocapsuled indomethacin and triclosan were added to primer and adhesive, resulting an adhesive system with antimicrobial effects, without compromising its physicochemical properties.

The method of preparation used in the present study was based in self-assembly principle [30] and resulted nanoparticles, which favors incorporation due to their small dimensions [31]. Potential zeta depends on the chemical nature of polymer and chemical nature of stabilizing agent on certain preparation conditions that determine molecular organization of oil core, re-precipitated polymeric capsule and stabilizing agents [33]. In the present study, NCs were prepared from methacrylate derivates using non-ionic stabilizing agents, which results a negative zeta-potential value due to presence of polymer terminal carboxylic groups [33]. Dispersion of nanocapsules was visualized (Figure 1c) due to weak links between silica and polymer of NCs [19,32] and steric impediment promoted by surfactant.

Well-dispersed particles and spherical shape avoid stress concentration, favoring the properties of polymeric materials [34]. The incorporation of NCs into resin did not alter the degree of conversion due to the no reactivity of polymeric capsule [33], since stabilizing agent is adsorbed onto NC surface. Chemical affinity between NCs and adhesive resin also contribute to favorable results. Maintenance of NCs conformation provides a constant degree of conversion after months. The good filler-polymer interaction also favors the results of degradation in solvent. Polar portion in polymer network is prone to hydrolytic degradation, reducing the properties as hardness [35]. Hydrolytic process can also influence the degradation of polymeric capsule. Further, diffusion of drug occurs across polymer network. Although processes of hydrolytic degradation and releasing of drug from adhesive occur, the incorporation of certain concentration of NCs showed no influence in the results of degradation of adhesive, as previous study [19].

Although polymeric capsule suffers long-term degradation, drug release from capsule with no trigger is mainly conducted by diffusion of drugs

across capsule polymer network, influenced by osmotic pressures [33,36]. Drug release from NCs suspension presented a classical release profile [33] while release is difficult when NCs are into a polymeric material. The imprisonment of NCs in polymer network implicates diffusion of drugs between network of polymeric material in addition to diffusion through capsule. Drug cumulative release of adhesive presented similar profile compared to previous studies also with NC into an adhesive resin [19,37]. Indomethacin is firstly released due to its adsorption onto NCs surface, indicated by log D value of 2.31 while triclosan is mainly distributed in oil core with a log D value of 4.9 [38]. Independently from time, both releases presented a controlled profile.

Controlled release improves bioavailable and consequently effect of drug [20]. Nanoencapsulated triclosan presented an antimicrobial effect in lower concentration than minimum inhibitory concentration (MIC) for *S. mutans* AU159 [39]. In 24 h, groups with primer containing NCs presented antimicrobial effect due to increased amount of released triclosan in first hours (Figure 3a). However, the increased release modifies osmotic pressure and may influence the amount of drug releasing later [33.36]. Thus, in 48h, there was no sufficient amount of released triclosan to promote effect. Finally, in 96 h, relatively slow release of triclosan from adhesive was enough to promote an antimicrobial effect. Using an adhesive system with NCs, an early and prolonged antimicrobial effect is expected related to controlled release of triclosan from primer and adhesive.

The nanometric dimensions of particles allows drug achieve reduced spaces [40,41]. Indomethacin released from primer and adhesive presented the potential of permeating through dentin in a thickness that simulates a deep lesion [5] to achieve pulp tissue, allowing it to perform anti-inflammatory effect. Nanocapsules in adhesive resin can diffuse into dentin tubules, inside the interconnected dentinal tubules branches and dentin collagen interfibrils spaces [34,37]. Indomethacin released from adhesive with nanocapsules containing also triclosan presented similar permeation profile (Figure 4) than nanocapsules with only indomethacin [19]. No significant differences in amount of diffused drug in g/mm^2 also comparing different concentration of NCs in adhesive and primer with NCs. The difference is related to percent,

which also occurs in drug release. Longer indomethacin release through dentin can be expected using high concentration of NC in adhesive and primer with nanocapsules (Figure 4) due to high amount of drug [33,36]. According to present results and previous studies [32,42,43,unpublished results], different combinations of primer and adhesives have potential to promote an anti-inflammatory effect in initial pulp reactions after deep cavity treatment.

Being applied in deep lesions and permeating through dentin, NCs must present low toxicity. Since resins were similar to the commonly used, NCs were singly evaluated in estimated concentrations that would be used in restorative treatments. NCs maintained cell viability higher than 90%, which is according ISO recommendations (ISO 10993-5). It also agrees with one of the most relevant advantages of using NCs, which is the decrease of adverse effects and toxicity of drugs when nanoencapsulated [21,22,33]. High cell viability confirms the incorporation of drugs-loaded NCs may have wide applicability to other dental materials, as composites, sealants, and cements.

Conclusions

Based on present study, it is possible to conclude that indomethacin and triclosan-loaded nanocapsules were successfully produced and incorporated into a primer and an adhesive resin, promoting controlled drugs release, permeability of indomethacin through dentin and antimicrobial effect without compromising physicochemical properties of adhesive resin with 2% of NCs.

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3.4 MANUSCRITO IV

Effect of indomethacin and triclosan-loaded nanocapsules into an adhesive system

Short title: Nanocapsules into an adhesive system

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Abstract

Objectives: The aim of this study was to evaluate *in situ* degree of conversion, contact angle, and immediate and long-term bond strength of primer and adhesive containing indomethacin and triclosan-loaded nanocapsules.

Methods: Indomethacin and triclosan-loaded nanocapsules (NCs) were incorporated into primer at 2% and into adhesive at 1%, 2%, 5%, and 10% concentrations. *In situ* degree of conversion (DC, n=3) was evaluated by micro-Raman spectroscopy. The contact angle of primer and adhesive on dentin surface (n=3) was performed by optical tensiometer. For μTBS test (n=12), stick-shaped specimens were tested under tensile stress immediately and after water storage for 1 year. Data were analyzed using ANOVA and Tukey's post hoc tests with $\alpha=0.05$.

Results: The use of NCs-loaded primer and adhesive resulted no influence on *in situ* degree of conversion and immediate bond strength in comparison to control. The values of DC varied from 75.07 ± 8.83% to 96.18 ± 0.87%. The contact angle of primer presented no difference comparing with or with no NCs. The use of both primer and adhesive with NCs resulted higher contact angle of adhesive. Longitudinal μTBS was inversely proportional to NCs concentrations into adhesive system. However, the use of adhesive with NCs up to 5% of concentration did not decrease longitudinal μTBS.

Conclusions: It is possible to conclude that adhesives containing up to 5% of nanocapsules can be an alternative to combine bond performance and therapeutic effects.

Clinical Significance: Adhesive with nanocapsules containing indomethacin and triclosan maintains long-term bond performance.

Keywords: Indomethacin, Triclosan, Drug carriers, Dental bonding

Introduction

Nanocapsules, composed by a polymeric capsule and an oil core containing drug, promote controlled drug release [1]. Consequently, bioavailability for encapsulated drug lasted twice and promoted higher efficiency over time comparing to free drug [2]. Other advantages of encapsulated drugs are deeper penetration of drug in narrow spaces, lower adverse effects, and higher drug stability due to nanometric size of particles and low reaction of encapsulated drug with media [3,4,5].

Applied on dentin, nanocapsules into water formulation, primer or adhesive promoted drug diffusion up to 0.75 mm through dentin [6, 7, unpublished results]. In hybrid layer, nanocapsules incorporated in adhesive were seen dispersed over the intertubular dentin surface and penetrated into dentinal tubules [6,8]. Stress concentration is avoided in adhesive with adequate dispersion of particles and spherical shape of NCs, resulting favorable mechanical properties [8]. The chemical affinity between polymer of NCs and monomers of adhesive system leads to reliable physicochemical properties [6,7,8]. The diffusion of nanocapsules in hybrid layer also resulted no decrease of immediate bond strength [7,8]. Nonetheless, the influence of nanocapsules on long-term bond strength is not yet elucidated.

Therefore, the aim of this study was to evaluate *in situ* degree of conversion, contact angle, immediate and long-term bond strength of primer and adhesive containing indomethacin and triclosan-loaded nanocapsules.

Materials and Methods

Preparation of indomethacin and triclosan-loaded NCs

Indomethacin and triclosan-loaded NCs were prepared by interfacial deposition of preformed polymer technique, obtaining particles with an averaged size of 159 nm (unpublished results). The reagents were obtained from Sigma Chemical (St. Louis, USA). An organic phase was composed by polymer (MMA-co-MAA), Eudragit® S100 (0.50 g), indomethacin (0.025 g), triclosan (0.025 g), medium chain triglycerides (0.81 mL), sorbitan monostearate (0.19 g) and acetone (125 mL). An aqueous phase contained polysorbate 80 (0.385 g) and water (250 mL). The organic phase was added through a funnel to aqueous phase under magnetic stirring at 25° C. Acetone and water excess were eliminated using a rotary evaporator (Rotavapor II, Buchi, Flawi, Switzerland), a B-740 recirculating chiller (Buchi, Flawi, Switzerland) and a U-700 vacuum pump (Buchi, Flawi, Switzerland). The suspension containing NCs was spray dried (B-290, Buchi, Flawi, Switzerland) using hydrophilic fumed silicon dioxide (Aerosil® 200) in amount of 1.5% of the suspension content as an adjuvant to avoid the aggregation on internal wall of equipment. The inlet temperature at the drying chamber was approximately 150 ± 4 °C, and the outlet temperature was 107 ± 4 °C.

Formulation of the adhesive resin and primer containing indomethacin and triclosan-loaded NCs

Experimental dental adhesives were formulated using 66/33 wt% bisphenol A glycol dimethacrylate (BisGMA)/2-hydroxyethyl methacrylate (HEMA). Camphoroquinone (CQ) and ethyl 4-dimethylaminobenzoate (EDAB) were added at a concentration of 1 mol% as a photoactivation system. The dried indomethacin and triclosan-loaded NCs were added at 1, 2, 5 and 10 wt% (groups AD1%, AD2%, AD5%, and AD10%). One group had no addition of particles, as control (group AD0%). An amount of 2 wt% of indomethacin and triclosan-loaded NCs were incorporated into a comercial primer (Scotchbond MP, 3M-ESPE, Seefeld, Germany) and one group was maintained with no NCs (groups PR0% and PR2%). All formulations were mixed and ultrasonicated (CBU 100/1 LDG, Plana, São Paulo, Brazil) for 30 minutes.

Preparation of teeth

To perform *in situ* degree of conversion, contact angle and microtensile bond strength, bovine permanent incisors were transversally sectioned since exposition of dentin surface. A 600-grit SiC paper was used for 30 s under wet conditions to create a smear layer on dentin surface. The dentin surface was etched with phosphoric acid for 15 s, washed, and slightly dried using absorbent paper. Primer, with or with no NCs, was applied, and the solvent was evaporated for 10 s. The adhesive with different concentrations of NCs was applied and photoactivated for 20 s using a light-emitting diode (Radii cal, SDI, Bayswater, Australia). A composite build-up was

performed (Z350, 3M ESPE, St Paul, USA) in two increments of 2 mm.

In situ degree of conversion (DC)

Thirty bovine incisors (n=3) were prepared as described in section "Preparation of teeth". Restored teeth were transversely cutted and analyzed by micro-Raman spectroscopy (SENTERRA, Bruker Optics, Ettlingen, Germany) device. The spectra were acquired, starting from the dentin, and the appearance of peaks associated with the adhesive. Raman spectra of each uncured adhesive were also collected to identify the reference and reaction peaks needed for degree of conversion calculations, comparing these spectra with the spectra of cured adhesive on dentin. The phenyl C=C peak at 1610 cm^{-1}, which was stable and unmodified during polymerization, was selected as a reference peak, while the vinyl C=C 1640 cm^{-1} peak was selected as a reaction peak. The DC of the adhesive within the hybrid layer was calculated using the ratio between the reaction and the internal reference peak areas of polymerized over ratio of unpolimerized adhesive.

Contact angle

Thirty bovine incisors (n=3) were embedded in acrylic resin and prepared as described in section "Preparation of teeth". Teeth were mounted on optical tensiometer Theta (Biolin Scientific, Stockholm, Sweden). Primer (3 µL) and adhesive (3 µL) with different concentration of NCs was dropped in a speed of 60 mm/min

through a microsyringe on dentin surface. Images were captured in a speed of 12 frames/s until 20 s after drop deposition, using a microvideo system. OneAttension software (Biolin Scientific, Stockholm, Sweden) provided the values of contact angle 10 s after drop deposition by Young's equation.

Microtensile bond strength (μTBS)

Two hundred and forty bovine permanent incisors were divided into 20 groups (n=12), depending on concentration of NCs into primer and adhesive, and the length of evaluation (24 hours or 12 months). Teeth prepared as described in section "Preparation of teeth". After storage in distilled water at 37 °C for 24 h, teeth were sectioned into four to six beams (area of 0.5 mm^2) with a slow-speed saw. After 24 hours or 12 months, specimens were fixed to a microtensile device and tested on a mechanical testing machine (DL-2000, EMIC Equipments and Systems for Essay Ltda, São José dos Pinhais, Brazil) at a crosshead speed of 0.5 mm/min until failure. Fractographic failure mode analysis was performed using an optical microscope and classified as adhesive and mixed, cohesive in dentin or cohesive in resin. Premature failures were also reported.

Statistical analysis

Statistical analysis was performed using one-way ANOVA (nanocapsule concentration) and Tukey *post-hoc* tests for *in situ* degree of conversion and contact

angle. Two-way ANOVA (time and nanocapsule concentration) and Tukey *post-hoc* tests were performed for microtensile test. All tests were performed at $\alpha = 0.05$.

Results

The use of NCs-loaded primer and adhesive resulted no influence on *in situ* degree of conversion in comparison to control group. The values of DC varied from 75.07 ± 8.83% to 96.18 ± 0.87% (Table 1).

The contact angle of primer presented no difference comparing samples with or with no NCs. The contact angle value of primer with NCs was 24.09 ± 5.62 degrees and 23.20 ± 4.91 degrees for primer with no NCs. Regarding contact angle of adhesive, no significant difference between different concentrations of NCs when primer with no NCs was used. Primer containing NCs also resulted no influence on contact angle when adhesive with no NCs was used. The use of both primer and adhesive with NCs resulted higher contact angle of adhesive ($p < 0.05$), reaching from 50.71 ±15.23 to 54.69 ± 5.19 (Table 1).

Microtensile bond strength results and mode of failure are presented in Table 1. NCs resulted no influence on immediate μTBS. Longitudinal μTBS was inversely proportional to NCs concentrations into adhesive system. Groups of primer with no NCs and adhesive containing 10% of NCs, and primer with NCs and different concentration of NCs presented decreased microtensile bond strength after 12 months ($p < 0.05$). Mixed fractures were frequently identified in all groups (Table 2).

Table 1. Degree of conversion (DC, in percent, %) *in situ*, contact angle, immediate (24 hours) and longitudinal (12 months) microtensile bond strength (μTBS, in MPa)

Groups	DC *in situ* (%)	Contact angle (degrees)	μTBS (24 hours)	μTBS (12 months)
PR 0% AD 0%	80.58 ±6.58^{AB}	23.35 ±3.50^{A}	36.71 ±10.36^{Aa}	31.30 ±11.34^{ACa}
PR 0% AD 1%	96.02 ±1.11^{A}	24.74 ±5.31^{A}	33.26 ±4.12^{Aa}	35.05 ±4.25^{ABa}
PR 0% AD 2%	92.61 ±0.55^{A}	25.19 ±2.47^{A}	31.96 ±4.50^{Aa}	30.31 ±8.39^{BCa}
PR 0% AD 5%	93.54 ±0.47^{A}	22.40 ±5.52^{A}	36.83 ±9.35^{Aa}	40.63 ±10.66^{Aa}
PR 0% AD 10%	94.57 ±0.44^{A}	21.23 ±7.85^{A}	39.10 ±6.45^{Aa}	29.78 ±14.03^{BCDb}
PR 2% AD 0%	75.07 ±8.83^{B}	22.97 ±4.04^{A}	38.79 ±7.36^{Aa}	28.37 ±14.30^{BCDb}
PR 2% AD 1%	92.84 ±3.75^{A}	54.69 ±5.19^{B}	31.19 ±8.45^{Aa}	17.49 ±5.70^{DEb}
PR 2% AD 2%	88.26 ±5.79^{A,B}	50.71 ±15.23^{B}	30.09 ±5.75^{Aa}	20.36 ±4.22^{CEb}
PR 2% AD 5%	93.85 ±0.61^{A}	53.27 ±16.49^{B}	29.23 ±2.07^{Aa}	17.11 ±6.41^{DEb}
PR 2% AD 10%	96.18 ±0.87^{A}	53.56 ±8.54^{B}	30.05 ±4.28^{Aa}	13.92 ±6.92^{Eb}

Identical capital letters in the same column denote statistically significant equivalences. Values followed by identical lower case letters in the same row denote statistically significant equivalences. PR – primer; AD - adhesive.

Table 2. Mode of failure distribution (in percent, %) of immediate and longitudinal microtensile bond strength specimens

Groups	Mode of failure n (%)							
	A/M immediate	A/M longitudinal	CD immediate	CD longitudinal	CR immediate	CR longitudinal	PF immediate	PF longitudinal
PR 0% AD 0%	35 (76.09)	32 (71.11)	6 (13.04)	10 (22.22)	4 (8.70)	3 (6.66)	1 (2.17)	0 (0.00)
PR 0% AD 1%	28 (63.64)	30 (65.22)	6 (13.64)	11 (23.91)	10 (22.72)	5 (10.87)	0 (0.00)	0 (0.00)
PR 0% AD 2%	26 (55.32)	39 (68.42)	10 (21.28)	14 (24.56)	10 (21.28)	4 (7.02)	1 (2.13)	0 (0.00)
PR 0% AD 5%	25 (67.57)	34 (69.39)	11 (29.73)	14 (28.57)	1 (2.70)	1 (2.04)	0 (0.00)	0 (0.00)
PR 0% AD 10%	21 (52.50)	37 (71.15)	12 (30.00)	13 (25.00)	5 (12.50)	2 (3.85)	2 (5.00)	0 (0.00)
PR 2% AD 0%	27 (75.00)	34 (72.34)	7 (19.44)	11 (23.40)	2 (5.55)	2 (4.25)	0 (0.00)	0 (0.00)
PR 2% AD 1%	25 (65.79)	24 (60.00)	6 (15.79)	13 (32.50)	5 (13.16)	3 (7.50)	2 (5.26)	0 (0.00)
PR 2% AD 2%	29 (67.44)	21 (58.33)	12 (27.91)	12 (33.33)	2 (4.65)	3 (8.33)	0 (0.00)	0 (0.00)
PR 2% AD 5%	29 (69.05)	23 (60.53)	11 (26.11)	10 (26.32)	2 (4.76)	5 (13.16)	0 (0.00)	0 (0.00)
PR 2% AD 10%	23 (57.50)	32 (71.11)	8 (20.00)	6 (13.33)	5 (12.50)	7 (15.55)	4 (10.00)	0 (0.00)

PR – primer; AD - adhesive. A/M – adhesive/mixed fracture mode; CD – cohesive fracture mode in dentin; CR – cohesive fracture mode in resin; PF – premature failures.

Discussion

Nanocapsules may be added to an adhesive system in order to promote antimicrobial and anti-inflammatory to restorations through controlled drug release. Nanocapsules must maintain reliable long-term bond performance. In the present study, NCs-loaded primer and adhesive resulted no influence on *in situ* degree of conversion and immediate bond strength. Nonetheless, the use in combination of primer and adhesive with NCs increased contact angle and decreased bond strength after 1 year of aging.

Networks with high cross-linking density have low hydrolytic degradation due to less available ester groups for attack and reduced free volume, avoiding water sorption [9]. Adhesive with NCs maintain high polymeric cross-linking density [7,10,11,12,13]. Due to no altered DC of adhesive with NCs, NCs allow light reach bulk of resin. *In situ* analysis is correspondent to the most challenging condition, which is inside hybrid layer [11,14,15] and can predict adhesive performance *in vivo* [16], due to a positive correlation with μTBS [14]. Therefore, analyzing the results of *in situ* DC in the present study, it is possible to predict low hydrolytic degradation.

In adhesion, thermodynamic equilibrium between liquid and solid surface is expressed by contact angle [17]. Firstly, acid etching increased wettability about 35% [18], subsequent primer presented high wettability (results described in section 3) and its application also increases wettability of dentin surface prior to application of adhesive [19]. However, primer and adhesive containing NCs presented higher

contact angles due to the interference of high massive organic content in the interface of both media. NCs in adhesive system resulted high interfacial energy, as result of low free surface energy of primed dentin due to using of NCs in primer and high liquid surface free energy of adhesive with NCs, leading to low infiltration of adhesive in dentin [20]. The incorporation of NCs only into adhesive resulted no alteration on the contact angle. Low contact angle indicates the equilibrium between phases with high surface energy and wettability on dentin, leading to high bond strength [16,21], which occurred for NCs only in adhesive.

Measuring immediate and long-term bond strength is required in prediction of long-term clinical performance [22,23]. Incorporating NCs into primer and adhesive have no compromised immediate bond strength as in previous studies [7,8], indicating reliable distribution of NCs in hybrid layer [6,8]. Previous studies, testing commercial and experimental adhesives, showed similar bond strength results [24,25].

Water storage satisfactorily mimics the clinically restoration degradation [22,26], since it infiltrates, promotes swelling and reduction of forces between polymer chains [9], causing decrease of mechanical properties, elution of monomers and consequent weakening of bond [9,22,26]. Up to 5% of NCs into adhesive promotes no influence on longitudinal bond strength. A decrease of bond strength around 17% can be expected for other three-step systems, but it could present no significance [24,25]. The increased strength to hydrolytic degradation is due to the

final, separate and more hydrophobic resin layer [25]. High content of NCs in both primer and adhesive decreased longitudinal bond strength, due to possible higher water uptake by outer hydrophilic portion of polymeric capsule of NCs [27]. Further, the altered thermodynamic equilibrium between adhesive and dentin using both primer and adhesive containing NCs (expressed by higher contact angles) may have influenced longitudinal bond strength. Lower surface energy and wettability on dentin surface allow lower penetration of adhesive, resulting higher portion of non-protected dentin whose collagen fibrils are susceptible to enzymatic degradation and porosity in the hybrid layer leading to higher hydrolytic degradation [28]. Therefore, the incorporation of NCs only into adhesive showed better adhesion performance.

Failure analysis in μTBS is similar to *in vivo* failure patterns, showing relation to deproteinization of collagen fibrils in demineralized non-protected dentin and degradation of polymeric matrix of adhesive [22,26]. The distribution of fracture pattern in the present study was very similar to previous results [7,14,29]. The stress concentration at failure mainly starts at interface for all groups, indicating degradation of collagen fibrils and polymeric matrix in hybrid layer.

Conclusions

Based on results of the present study, it is possible to conclude that adhesives containing up to 5% of nanocapsules presented no influence on *in situ* degree of conversion, contact angle, immediate and long-term bond strength. Therefore, NCs-

loaded adhesive can be an alternative to combine bond performance and therapeutic effects.

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4 CONSIDERAÇÕES FINAIS

A busca por materiais que contribuam para o controle das falhas restauradoras e manutenção da vitalidade pulpar tem sido objeto de estudos (Cheng *et al.*, 2013; Melo *et al.*, 2013; Hiraishi *et al.*, 2010; Aljandan *et al.*, 2012; Louwakul & Lertchirakarn, 2012). A fim de manter a vitalidade pulpar, quando há condição inflamatória reversível, em cavidades profundas, o uso de agentes anti-inflamatórios incorporados a capeadores pulpares tem sido testados (Aljandan *et al.*, 2012; Louwakul & Lertchirakarn, 2012). O objetivo é que os materiais tenham a capacidade de propiciar efeito terapêutico anti-inflamatório ao tecido pulpar, contendo a reação inflamatória e reduzindo desconforto pós operatório. Além disso, agentes antimicrobianos, como amônio quaternário (Cheng *et al.*, 2013), prata (Melo *et al.*, 2013) e clorexidina (Hiraishi *et al.*, 2010), têm sido incorporados a sistemas adesivos. Esses materiais são alternativas para auxiliar o controle de uma das principais causas de substituição de restaurações, que corresponde à reincidência de cárie (Deligeorgi et al., 2001; Kopperud et al., 2012; Pallesen et al., 2014). No entanto, para garantir a durabilidade restauradora, é fundamental que os materiais restauradores possuam propriedades físico-químicas e mecânicas adequadas a longo prazo.

No presente estudo, um adesivo com nanocápsulas, contendo indometacina, e um sistema adesivo com nanocápsulas, contendo indometacina e triclosan, foram desenvolvidos. Os objetivos propostos na presente tese foram contemplados em quatro manuscritos, de forma que o manuscrito I descreve a obtenção e caracterização de um adesivo com a incorporação de nanocápsulas, contendo indometacina. No manuscrito II, a ação anti-inflamatória do adesivo com nanocápsulas, contendo indometacina, em modelo animal está apresentada. O manuscrito III descreve e caracteriza o sistema adesivo desenvolvido com a incorporação de nanocápsulas, contendo indometacina e triclosan, cuja resistência de união a longo prazo e características associadas à adesão estão descritas no manuscrito IV.

A incorporação de fármacos encapsulados a sistemas adesivos mostrou viabilidade no desenvolvimento de materiais com ação terapêutica para o controle das falhas restauradoras através da possível interferência na manutenção da vitalidade pulpar e redução do desconforto pós operatório. Por meio da liberação controlada dos fármacos pela encapsulação, a biodisponibilidade e ação terapêutica apresentam-se por maior tempo (Groo *et al.*, 2015). No presente estudo, as nanocápsulas, incorporadas em primer e adesivo, promoveram liberação controlada dos fármacos, o que resultou em ação anti-inflamatória após 12 dias e antimicrobiana após 96 h, que foram os períodos avaliados. Além disso, por estar encapsulado, o fármaco apresenta diminuição de efeitos adversos (Ourique *et al.*, 2008; Paese *et al.*, 2009; Bernardi *et al.*, 2009), o que, no presente estudo, pode ser observado pela baixa citotoxicidade das nanocápsulas. Devido ao tamanho nanométrico, o fármaco penetra em espaços estreitos (Bernardi *et al.*, 2012). Este estudo demostrou a difusão da indometacina pela dentina até 0,75 mm.

As nanocápsulas, no presente estudo, foram formuladas a partir de polímero a base de metacrilado, apresentando afinidade química com os materiais nos quais foram incorporadas. O adesivo com nanocápsulas, contendo indometacina, apresentou grau de conversão, degradação em solvente e microtração imediata inalterados, comparado ao adesivo sem nanocápsulas. O adesivo com nanocápsulas, contendo indometacina e triclosan, não mostrou diferença quanto ao grau de conversão e microtração imediata, e a degradação em solvente não aumentou com a incorporação de 2%. Embora a incorporação de 5% de nanocápsulas, contendo indometacina e triclosan, tenha resultado em maior degradação em solvente, apresentou resistência de união inalterada após 1 ano de envelhecimento.

Já o *primer* com nanocápsulas, contendo indometacina e triclosan, apresentou uma taxa de liberação mais rápida. Esse perfil de liberação proporcionou efeito antimicrobiano com início em menor tempo. Além disso, a taxa de difusão do *primer* com adesivo contendo 1% e 2% de nanocápsulas foi semelhante aos adesivos, contendo 5% e 10%, com *primer* sem nanocápsulas. O uso do *primer* com

nanocápsulas não influenciou o grau de conversão *in situ*. No entanto, o uso do *primer* com nanocápsulas aumentou o ângulo de contato do adesivo sobre a dentina e diminuiu a resistência de união longitudinal.

O uso de adesivo com nanocápsulas pode ser uma alternativa que alia efeito terapêutico com liberação controlada de fármacos e propriedades físico-químicas adequadas que garantem durabilidade do desempenho adesivo. Com a presente tese, buscou-se o desenvolvimento de conhecimento científico, com a produção de artigos baseados no uso de nanocápsulas, contendo fármacos, para o desenvolvimento de materiais de matriz polimérica. Os materiais desenvolvidos aliam-se à nanotecnologia, que é uma área de fronteira de conhecimento em crescimento nos últimos anos. Nesse caso, o uso de nanotecnologia controla a liberação dos fármacos e facilita que eles atinjam sítios específicos de ação pelo tamanho reduzidos das partículas. O processo de produção de nanocápsulas já vem sendo utilizado na indústria de cosméticos e medicamentos, o que garante a disponibilidade de insumos e tecnologias para a sua fabricação em larga escala no mercado. A tecnologia disponível pode tornar viável o desenvolvimento de outros materiais com potencial terapêutico para a Odontologia, como, por exemplo, cimentos endodônticos, forradores de prótese total para tratamento de estomatite prótetica, e resinas compostas.

Considerando todas as combinações de concentrações de nanocápsulas, contendo diferentes fármacos, que foram incorparadas aos componentes do sistema adesivo e os ensaios realizados nesta tese, é possível concluir que a associação do uso do adesivo com 5% de nanocápsulas contendo indometacina e triclosan, associado a um primer sem nanocápsulas, apresentou os melhores resultados no sentido de proporcionar efeitos terapêuticos sem comprometer a adesão dentinária a longo prazo.

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