SILVER NANOPARTICLES IN THE TREATMENT OF CARIES IN DECIDUOUS DENTITION: ALTERNATIVES TO DIAMINO SILVER FLUORIDE AND GENETIC IMPACTS

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ABSTRACT

The treatment of dental caries in the deciduous dentition can be carried out using products that control the carious process by reducing bacterial activity. Products based on silver nanoparticles have been studied to stop caries disease, without causing tooth darkening. The effects of nanoparticles on the environment and human health are not fully understood, so the aim of this study is to describe the role of diamino silver floride and experimental karyostats and their effects on the expression of BMP-7, TNF-α and TGF-β genes.

Keywords: Kariostatic. Tooth decay. Deciduous tooth. Gene expression.

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INTRODUCTION

The treatment of dental caries in the deciduous dentition can be carried out using products that control the carious process by reducing bacterial activity, while waiting for the patient's inadequate hygiene and nutrition habits to change (DITTERICH *et al*., 2006). In this context, dental materials must be easy to apply (ALMEIDA; CAVALCANTI; VALENÇA, 2011) given the difficulty of managing the child, with few side effects, high efficiency, and low cost (BURGESS; VAGHELA, 2018).

Taking these aspects into account, silver diamino fluoride (DFP) has been used all over the world (BURGESS; VAGHELA, 2018) as a solution that stops the carious process, in addition to being a preventive agent for the development of future lesions (ROSENBLATT; STAMFORD; NIEDERMAN, 2009). The effectiveness of this silver-based product in the treatment of carious lesions is supported by scientific literature (ROSENBLATT; STAMFORD; NIEDERMAN, 2009, GAO, *et al*., 2016a, CHENG, 2017).

Despite the various benefits, the main disadvantage of PFD is the darkening of the teeth, causing aesthetic damage (BURGESS; VAGHELA, 2018). Thus, products based on silver nanoparticles have been studied (GUZMÁN; DILLE; GODET, 2009, CHALOUPKA; MALAM; SEIFALIAN, 2010, SANTOS, *et al.,* 2014, TARGINO, *et al.,* 2014, ZHANG, *et al.,* 2015, GUO, *et al.,* 2016b, SCARPELLI, *et al.,* 2017, SCHWASS, *et al.,* 2018, NAGIREDDY, *et al*., 2019, XU, *et al.,* 2019) as an alternative to the use of PFD, as they are efficient in paralyzing caries disease, without causing the aesthetic inconvenience of tooth darkening (SANTOS, *et al.,* 2014). Silver nanoparticles (AgNps) have gained significant interest due to their remarkable antimicrobial properties (SANTOS, *et al.,* 2014), with broad spectrum and some concerns about safety and toxicity issues.

The effects of nanoparticles on the environment and human health are not fully understood (ATALAY; ÇELİK; AYAZ, 2018). Most studies have placed greater emphasis on the cytotoxicity of AgNPs, demonstrating that concentrations between 0.05-0.70% did not show cytotoxicity against human cells (MARTINEZ-GUTIERREZ, *et al.,* 2013). However, studies on the characterization of this material under the aspect of gene modulation are scarce. The aim of the present study was to evaluate the expression of BMP-7, TNF-α and TGF-β genes of an experimental karyostatic based on silver nanoparticles in L929 mouse fibroblast cells.

LITERATURE REVIEW

DIAMINO FLUORETO DE PRATA

Dental caries can be defined as a chemical dissolution of hard dental tissues, caused by acidic by-products from the metabolic processes of the biofilm that cover the affected tooth surface (SELWITZ; ISMAIL; PITTS, 2007). Currently, it is considered a complex sucrose-dependent biofilm disease, as sugar from the diet plays a decisive role in the carious process (SHEIHAM; JAMES, 2015). Traditionally, the most indicated treatment for this disease consists of the removal of the demineralized tissue and restoration of the cavity (BURGESS; VAGHELA, 2018).

Historically, in Japan, married women used a solution called ohaguro, which pigmented their teeth in a dark hue, characterizing their marital status. This solution had iron ions in its composition, and after some time, a lower rate of carious lesions was observed in these women (MACIEL, 1988, JUNIOR; SOUZA; ROSENBLATT, 2012). In 1969, the diamino silver fluoride solution was introduced into dental practice by Yamaga and Yokomizo, for the prevention and stoppage of dental caries (COUTINHO, 2002).

DFP, whose chemical formula is represented by Ag(NH3)2F, is a colorless alkaline solution that contains silver and fluoride in its composition, forming a complex with ammonia (CHU; LO, 2008). Ammonia aims to maintain a constant concentration of the solution for a certain period (ROSENBLATT; STAMFORD; NIEDERMAN, 2009, MEI; IT; CHU, 2016). Silver compounds have long been used in both medicine and dentistry for their antimicrobial properties (ROSENBLATT; STAMFORD; NIEDERMAN, 2009, PENG; BANKS; MATINLINNA, 2012). The fluoride in the solution produces fluorohydroxyapatite (MEI, *et al.,* 2013), used in dentistry in various ways to prevent caries disease (CHU; LO, 2008). Therefore, due to the combination of these characteristics, it has been suggested that silver and fluorides have the ability to interrupt the progression of carious lesions and simultaneously prevent the development of new caries lesions (ROSENBLATT; STAMFORD; NIEDERMAN, 2009).

(Ag(NH3)2F) acts in the inorganic portion, through the reaction of sodium fluoride with hydroxyapatite, and also in the organic part of proteins through sodium nitrate (NISHINO; MASSLER, 1977, RODRIGUES; OLIVE TREE; ANDO, 1989, ROCHA *et al.*, 1999). In 1976, Shimizu and Kawagoe (SHIMIZU; KAWAGOE, 1976) described the mechanism of action of DFP, dividing it into three, where the first occurs from the obstruction of the dentin tubules, altering its morphology in relation to the decrease in caliber, in addition to having silver inside. This obliteration makes it difficult for bacteria to invade and diffuse their acids. The second mechanism is characterized by its karyostatic

action, involving the DFP and the mineral component of the substrate; The action of fluoride provides an increase in resistance to the dentin tissue, consequently reducing acid penetration into deeper layers of the dental element. The third, due to the action of silver diamino fluoride with dentin protein, which causes greater resistance to collagenase and trypsin. (OPPERMANN; JOHANSEN, 1980)

Silver has great variability of action, and can act with living organisms, intracellular regions or even in vital activities of cells. Studies carried out by Lansdown (2002; 2006) (LANSDOWN, 2002, LANSDOWN, 2006) demonstrate the interaction of silver with sulfhydryl groups of proteins and DNA, modifying hydrogen bonds, preventing vital cellular processes, such as respiration, division and synthesis (LANSDOWN, 2002, LANSDOWN, 2006). In addition, silver has the property of inhibiting the formation of biofilm. (WU et al., 2007)

Minimally invasive techniques have shown increasing acceptance (KIDD, 2004) allowing treatments with few resources (SCHWASS, *et al.,* 2018), enabling care in places of low socioeconomic level, becoming a simple way to solve the caries problem (GAO, *et al.*, 2016b). In addition, less invasive techniques are more effective in patients who are poorly cooperative (SCHWASS, *et al.,* 2018).

Although it is a widely used material and considered easy to apply and minimally invasive, it does not have a completely established standardized protocol (CONTRERAS et al., 2017, FRANCELINO, et al., 2019). It is recommended to carry out a previous prophylaxis, perform well-performed relative isolation and protect the adjacent soft tissues with petroleum jelly. Then, the product should be applied with the help of a microbrush for 3 minutes, and then wash the element in question (SANTOS *et al.,* 2008). Investments made every six months are more effective than when made annually (CLARK; SLAITON, 2014; FUNG et al., 2016; FUNG et al., 2018). Despite this evidence, a consensus on the frequency and number of applications has not yet been established. (GAO et al., 2016a).

Ag(NH3)2F is able to effectively stop the carious process (GAO, *et al.*, 2016b). It is easily applied to the teeth, requiring no great training from the operator (BURGESS; VAGHELA, 2018), with low cost and few side effects (SCHWASS, *et al.*, 2018). In addition, it does not characterize an invasive procedure (NAGIREDDY, *et al*., 2019), as it eliminates the use of rotating instruments, reducing the risk of disease transmission (NAGIREDDY, *et al*., 2019) by infectious aerosols through the air (JONES; BROSSEAU, 2015), in addition to providing greater acceptance in pediatric patients (SCHWASS, *et al.*, 2018).

The DFP is widely used in public and private health services (PENG; BANKS; MATINLINNA, 2012) and is indicated for pediatric patients who do not cooperate with

complex restorative procedures and with high caries activity in primary teeth (CHU; LO, 2008, SHAH, *et al.,* 2014).

Silver diamino fluoride is available on the market in concentrations of 10%, 12%, 30% and 38%. (JUNIOR; SOUZA; ROSENBLATT, 2012). However, studies indicate that the 38% solution is more effective in the preventive treatment of dental caries (LO; CHU; LIN, 2001, CHU; IT; LIN, 2002, LLODRA, *et al.,* 2005, SANTOS, *et al.,* 2014, BURGESS; VAGHELA, 2018). This concentration is more efficient in inhibiting collagenase activity and preventing collagen degradation than in low concentrations (SHARMA; PURANIK; K.R., 2015, ZHAO, *et al.,* 2018).

Despite presenting several benefits, the main disadvantage of silver diamino fluoride is the darkening of decayed teeth in shades of dark brown to black (HIRAISHI, *et al.,* 2010), causing aesthetic damage and dissatisfaction to patients and guardians (BURGESS; VAGHELA, 2018). For this reason, during the treatment, a free and informed consent must be attached to the treatment record informing that the dental structures affected by the disease will be darkened (BURGESS; VAGHELA, 2018).

This pigmentation is caused by the oxidation of ionized silver into metallic silver, limiting the use of this solution in patients who are more demanding in terms of aesthetics. In addition to this inconvenience, the material has an unpleasant metallic taste. Also, it can produce transient gingivitis, and contact with soft tissue should be avoided by using rubber dams, cotton rollers or protecting the gingival tissue with petroleum jelly (MEI; IT; CHU, 2016).

SILVER NANOPARTICLES

In the search for a product that adds advantages to silver diamino fluoride (DFP) without aesthetic harm, silver nanoparticles (AgNPs) have been gaining prominence (PENG; BANKS; MATINLINNA, 2012). A karyostatic agent based on silver nanoparticles has been studied, where evidence suggests that it does not cause a change in the color of teeth affected by the disease after its application (SANTOS, *et al.,* 2014, TARGINO, *et al.,* 2014, ESPINDOLA-CASTRO, *et al.*, 2020). This agent has shown low toxicity to living cells and has antibiotic efficacy similar to DFP against *Streptococcus mutans* (TARGINO, *et al.,* 2014).

Compounds that have silver in their composition have long been applied in dentistry due to their antimicrobial activity. At first, they were used in their ionic form, and more recently, in the form of nanoparticles (PENG; BANKS; MATINLINNA, 2012). AgNPs are antimicrobial agents, and due to their physical and chemical properties, such as shape,

hydrophobicity and loading surface, they are used in many fields, such as in the treatment of infections and the production of dental materials (MORONES, *et al.,* 2005, QING, *et al.,* 2018). These nanoparticulate metals and metal oxides have important bactericidal effects on dental caries (NGUYEN; HIORTH, 2015, SCARPELLI, *et al.*, 2017).

Studies indicate that the mechanism of action of AgNPs depends on their ability to penetrate the bacterial cell wall (SANTOS, *et al.*, 2014, DURAN, *et al.,* 2016). The passage of AgNPs smaller than 10 nanometers (nm) in diameter into the cytoplasmic matrix may cause interference in a series of cellular processes (MORONES, *et al.,* 2005). This penetration into the cells may result in direct and indirect lipid peroxidations that destroy the cell membrane, leading to the interruption of DNA replication, and inhibiting cellular respiration (TARGINO, *et al.,* 2014, DURAN, et al., 2016).

The fundamental characteristics of metallic nanoparticles depend on their shapes, sizes, configurations, crystallinity, and structure (AHMED, *et al.,* 2017). They can be synthesized in a variety of ways, including forms of chemical reduction, photochemical, aerosol laser irradiation techniques (EVANOFF JR; CHUMANOV, 2005), or taking advantage of natural biological processes (VAIDYANATHAN, *et al.,* 2009), and the mode of production will influence the size and shape of AgNPs.

It is known that the smaller the nanoparticles are, the smaller their surface area and the greater their reactivity in relation to ionic silver. That is, this characteristic increases exponentially when the particle size is decreased (PAL; TAK; SONG, 2007, GUZMÁN; DILLE; GODET, 2009, BURGESS; VAGHELA, 2018, SCHWASS, *et al.,* 2018). In addition, as these particles decrease, in the range of 6 to 9 nm, they exhibit a pale yellow color, conveniently close to the color of dentin (GUZMÁN; DILLE; GODET, 2009).

Therefore, the use of karyostatic based on silver nanoparticles will not cause the darkening of the demineralized dental tissue caused by silver precipitation (SANTOS, *et al.,* 2014, TARGINO, *et al.,* 2014, ESPINDOLA- CASTRO, *et al.,* 2020). This product adds known advantages of PFD, such as interrupting the clinical progression of carious lesions, postponing definitive restorative care in non-compliant children (SCHWASS, *et al.,* 2018), antimicrobial effect, and remineralizing action (SCARPELLI, *et al.,* 2017). In addition, it has a simple application protocol, requiring no complete dental equipment or clinical environment, also leading to a decrease in the risk of infection (NAGIREDDY, *et al.,* 2019). It should be noted that this product does not cause lesions in oral soft tissues (NAGIREDDY, *et al.,* 2019) and does not have a metallic taste (SANTOS, *et al.*, 2014, NAGIREDDY, *et al.,* 2019).

Although the karyostatic based on silver nanoparticles shows promise, they do not yet present the same literary consolidation compared to the DFP, especially with regard to the biological properties of this material. For this reason, there is a need for new studies addressing this theme.

REAL-TIME PCR

The Polymerase Chain Reaction (PCR) was developed in 1983 by Kary B. Mullis, and is considered a great advance in molecular biology techniques (MULLIS, 1990). In 1993, the researcher received the Nobel Prize in chemistry due to the reach achieved by the technique. PCR is based on large-scale *in vitro* replication of the DNA molecule, allowing several analyses to be performed from a template fragment (WENG; RUBIN; BRISTOW 2006, YANG; ROTHMAN, 2004).

The PCR reaction made it possible to expand the analysis of genetic material, expanding its applicability to various sectors such as medicine, biotechnology, dentistry, among others. To obtain several copies of a specific nucleic acid sequence, cycles are carried out with thermal alterations, replicating physiological conditions of the organism. For the duplication process to occur, it is necessary to use a template strand, nucleotides, primers and DNA polymerase enzyme (YANG; ROTHMAN, 2004).

The conventional PCR technique is basically divided into three stages: denaturation, pairing, and extension. The cycles are repeated countless times, and their evaluation is later performed through agarose gel electrophoresis. A biotechnological advance has made it possible to use real-time PCR (qPCR), a technique that has greater sensitivity, specificity and speed, allowing results in 2 to 3 hours. The biggest difference between the two tests is that qPCR makes use of fluorescent probes, which allow the visualization of the results in real time. During the assay, these probes promote the emission of fluorescence, which increases in proportion to the amplification of the DNA. (PAIVA-CAVALCANTI; LORRAINE; GOMES, 2008)

An example of a probe that can be used during qPCR is the hydrolyzable probe system such as TaqMan (Applied Biosystems, Perkin-Elmer Corp.), which is specifically targeted to the region where the amplification is to be performed. During the assay, the probe degrades and emits a fluorochrome, which absorbs energy and releases light (YANG; ROTHMAN, 2004). From this light signal, a detector and signal amplifier are read, which draw a graph with the information obtained after each qPCR cycle (MORTARINO, *et al.,* 2004).

Quantification values are expressed as fold change. It has been proposed to calculate the value of the relative expression without efficiency correction, called the "comparative Cq method". This method does not use the correction of the reaction efficiency, because it is based on the requirement that both the gene of interest (GDI) and the internal control reference gene (GDR) have an efficiency ~100% and similar between them, with tolerance limits of 5-10% (LIVAK; SCHMITTGEN, 2001). It is worth mentioning that the Cq is the number of the cycle where the fluorescence crosses an established threshold, being used as a quantification value of the reaction. For this calculation, a sample, cell line, normal tissue equivalent, or group value (e.g., mean of the control group) is established as a calibrator (value 1). The calculation is performed using equation (1.5):Fold change = 2-ΔΔCq (1.5)Where ΔΔCq = [(Cq GDI sample – Cq GDR sample) - (Cq GDI calibrator – Cq GDR calibrator)] and the number 2 represents an efficiency of 100%.

qPCR has several advantages such as reproducibility, quantification capacity and speed of the technique, since this modality eliminates the stage of agarose or polyacrylamide gel electrophoresis, conventionally used during conventional PCR (SUNDSFJORD, *et al.,* 2004, YANG; ROTHMAN, 2004). In addition, it allows real-time monitoring of the product being amplified during the technique.

TRANSFORMING GROWTH FACTOR BETA (TGF-ß)

Transforming growth factor beta (TGF-ß) is an extracellular protein essential for survival, as it plays important roles in cellular activities, such as proliferation, development, inflammation and host immunity. (CLARK; COKER, 1998, MORIKAWA; DERYNCK; MIYAZONO, 2016)

TGF-ß binds to specific receptors on the cell membrane, inducing the generation of new tissues, acting as molecular signaling that modulates the behavior of cells. After the beginning of this intracellular cascade, they can act in an autocrine or paracrine manner, being altered by the extracellular matrix, neighboring cells and other cytokines, acting directly on the target cells (CLARK; COKER, 1998, KIM *et al.,* 2012)

This growth factor belongs to the TGF-ß1 Superfamily, which also aggregates activins, myostatins, and bone morphogenetic proteins (BMPs) (SEERGER; MUSSO; SOZZANI, 2015), are of fundamental importance in tissue repair, and are highly evaluated due to their therapeutic potential (BARRIENTOS *et al*., 2008). TGF-ß is of great prominence, as it participates in the repair of tissue injuries, attracting macrophages and fibroblasts to the site of injury (ROUSSELLE; BRAYE; DAYAN, 2018).

TGF-ß1 has five different forms, encoded by 23 different genes (BURT; LAW, 1994), and only three isoforms are expressed in mammals, called TGF-ß1, ß2, ß3. These are responsible for signaling inflammation, angiogenesis, re-epitheliation, and granulation tissue formation (WERNE; GROSE, 2003).

Growth factors such as TGF-β and BMPs (bone morphogenetic proteins) have been evaluated in their ability to induce and mediate the differentiation of stem cells into pre-odontoblast cells (GANESH; MASSAGUÉ, 2018). TGF-β has chemotactic action and guides cell differentiation and extracellular matrix synthesis (GALLER *et al.,* 2015).

PROTEÍNAS MORFOGENÉTICAS ÓSSEAS (BMPS)

Bone morphogenetic proteins (BMPs) are multifunctional growth factors (JIN *et al.,* 2003), regulators of cartilage and bone formation, belonging to the superfamily of transforming growth factor ß (TGF-ß) (YAMASHIRO *et al.,* 2003). These proteins are present in vertebrates and invertebrates, and their evolutionary conservation indicates a direct action on the proper development of animals. (GONÇALVES; GUIMARÃES; GARCIA, 1998).

The analysis of the identity of BMPs allows them to be classified into subgroups according to their sequential identities. BMP-2 and BMP-4 have 80% homology to each other, while BMP-5, BMP-6, BMP-7 and BMP-8 have 78% of similar amino acids (RENGACHARY, 2002). BMP-3 alone forms a subgroup, with 45% of the homologous sequence to BMP-2. BMP-1 differs from the rest because it does not belong to the TGF-ß superfamily, and is considered to be procollagen C proteinase. (GONÇALVES; GUIMARÃES; GARCIA, 1998).

These proteins play a major role in cell differentiation, acting on the growth and differentiation of various tissue types (COOK; RUEGER, 1996). They are found in the dentin and bone organic matrix, and can also be synthesized from recombinant gene therapy, using a viral vector (GONÇALVES; GUIMARÃES; GARCIA, 1998). Studies have determined that the biological properties of BMPs or TGF- can induce the development of reparative dentin tissue (SIX; LASFARGUES; GOLDBERG, 2002)

BMPs are responsible for the morphogenesis of dental and supporting tissues, including gingiva, alveolar bone, periodontal ligament, and cementum. When implanted in an extraosseous site, it can stimulate bone neoformation, enabling postnatal osteogenic repair (GONÇALVES; GUIMARÃES; GARCIA, 1998). In addition, when applied directly to pulp tissue, they are able to stimulate the regeneration of dentin tissue (NAKASHIMA; REDDI, 2003; NAKASHIMA; AKAMINE, 2005).

Among these proteins, BMP-2, BMP-4 and BMP-7 were considered to induce reparative dentin. BMP-7 e is associated with osteogenic differentiation (FENG, *et al.,* 2013). When this gene is expressed in bone cells, (WU, *et al.,* 2008, YAGYUU, *et al*., 2010, HONDA *et al., 2011) it is suggested that these cells have the ability to produce mineralized matrix. Due to their high inducing and therapeutic potential for bone and dental tissue repair, BMPs have been the target of research in the area of bioengineering.*

FATOR DE NECROSE TUMORAL ALFA (TNF-A)

Tumor necrosis factor alpha (TNF-α) discovered in 1975 by Carswell *et al.* (1975), is an important cytokine related to inflammatory and immune processes, and can act in different parts of the body. It plays an important role in the regulation of bone homeostasis in chronic immune and inflammatory joint diseases (OSTA; BENEDETTI; MIOSSEC, 2014), being secreted by macrophages, lymphocytes and monocytes (IINO *et al.,* 1990). When released in low concentrations, this cytokine acts on cells

endothelial, providing vasodilation and consequent secretion of chemokines, enabling a local inflammatory process to combat infectious conditions (ABBAS; LICHTMAN; POBER, 1998). For this reason, the main physiological effect attributed to this cytokine is the promotion of an immune and inflammatory response, from the recruitment of neutrophils and monocytes to the site of infection, activating them (VITALE; RIBEIRO, 2007).

The immune response is controlled by several inflammatory processes, which includes the cytokine TNF-α. Its action occurs through several pathways, such as the activation of the nuclear factor kappa-B (NF-κB) involved in inflammation and apoptosis (AGGARWAL, 2003). In inflammatory bone diseases, its role is well established (BITON; BOISSIER; BESSIS, 2011, OSTA; BENEDETTI; MIOSSEC, 2014), regulating bone resorption, since TNF-α favors remodeling in this tissue (SASTRY *et al.,* 1999).

Studies carried out with TNF-α found that its expression occurs in cases of periodontitis (GARLET, 2010, NAPIMOGA *et al.,* 2014, da COSTA *et al.,* 2015). When the inhibitor of this cytokine was used, it was observed that TNF-α contributes to the loss of microvascular cells in diabetic retinopathy (BEHL *et al.,* 2010). In addition, it also accelerates cartilage loss during fracture healing in mice with type 1 diabetes (ALBLOWI *et al.,* 2009, KAYAL *et al.,* 2010, ALBLOWI *et al*., 2013, LIM *et al.,* 2017). These findings suggest that TNF-α is associated with alveolar bone loss in type 1 diabetes with periodontitis, and that treatment with a cytokine antagonist may attenuate alveolar bone loss (KIM *et al.,* 2017).

Tumor necrosis factor alpha is also present in pulp inflammatory processes, playing an important role in the immune response to infection (COTRAN; KUMAR; COLLINS, 2002). TNF-α provides immune responses in odontoblasts, fibroblasts and monocytes of pulp tissue (FOUAD; ACOSTA, 2010, HE *et al.,* 2012). The different concentrations and times of these cytokines can produce different effects on these cells. For example, when pulp cells remain exposed to TNF-α for more than three days, they can impair their ability to differentiate into odontoblasts (YANG *et al.,* 2012). In other words, TNF-α can inhibit the process of dentin and pulp repair and regeneration during inflammation (YANG *et al*., 2012). When exposure occurs in a shorter time interval (3 days), the action of this cytokine may be beneficial, inducing the expression of genes related to mineralization in pulp cells (PAULA-SILVA *et al.,* 2009, YANG *et al.,* 2012, HUANG *et al*., 2015). For this reason, it is presumed that this mineralization can aid in host defense, leading to the repair of pulp tissues with early inflammation (YANG *et al.,* 2012).

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