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Evaluation of Cytotoxicity of A Nanoparticle Incorporated Root Canal Sealer – An In-Vitro Study

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ABSTRACT

The objective of a root canal therapy is to remove the infected pulpal tissue and fill the enlarged canal spaces in such a way that a 3-D hermetic seal is obtained. For the obturation of the root canal space, an obturating core and a sealer are used. The sealer must be as biocompatible as possible with low cytotoxicity and genotoxicity and additional antibacterial properties. The aim of the current study was to evaluate the cytotoxicity of a silver nanoparticle incorporated root canal sealer using MTT Assay against human periodontal ligament cells. MTT Assay is a colorimetric analysis for testing the cytotoxicity of drugs in-vitro. It was observed that the cytotoxicity of the nanoparticle incorporated sealer was concentration dependent with the maximum cytotoxicity at a concentration of 10% of silver nanoparticles within the original sealer. It can be concluded that the nanoparticle incorporated sealer has more cytotoxicity when compared to the original composition and this is directly proportional to the concentration of the nanoparticles. More studies need to be done to analyse the clinical use of this nanoparticle incorporated sealer as evaluation of cytotoxicity levels alone does not qualify for a material to be used as an endodontic sealer.

KEY WORDS: ANTIBACTERIAL; CYTOTOXICITY; MTA; NANOPARTICLES; SEALER; SILVER.

INTRODUCTION

The success or failure of a root canal therapy depends on the complete elimination and disinfection of the root canal system. It has been proven from time to time that inability to remove the microbial biofilms has caused root canal therapies to fail (Ramachandran Nair, 1987; Lin, Skribner and Gaengler, 1992). The role of cleaning and disinfection using irrigants and medicaments cannot be overlooked. However, a three dimensional seal of the canal system that includes the root canal, its accessory canals and abnormal anatomy, if any is extremely important and forms a goal of the root canal therapy. Root canal sealers in conjunction with solid core filling materials aid in the sealing of the canal system three dimensionally (Kumaravadivel and Pradeep, 2016). Sealers help overcome the limitations of Gutta percha by filling minute microscopic space between the dentinal wall and gutta percha.

Sundqvist and Figdor (Orstavik, 2020) described three major functions of the root canal sealer as to seal canal systems against bacterial ingrowth from oral cavity, entombment of leftover viable microorganisms and complete obturation of the canal system at a submicroscopic level to prevent stagnant fluid from

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accumulation and serving as bacterial nutrition. Root canal sealers that possess superior sealing ability and antimicrobial activity would hence be beneficial in clinical aspects (Branstetter and von Fraunhofer, 1982) as they can not only prevent bacteria from re-entering and re-infecting the canal system but also inactivate the remaining viable bacteria of the canal system post-obturation .

The ideal properties of sealers include establishment of hermetic seal, minimal cytotoxicity to periodontal ligament cells, tackiness when mixed to provide adhesion, radiopacity to be seen on a radiograph, lack of shrinkage and staining of tooth structure (ØRstavik, 2005). Traditional root canal sealers belong to the categories of zinc oxide eugenol (ZOE), epoxy resin (ER) and calcium hydroxide (CH). This categorization is based on the basic composition of the sealers (Shin, Lee and Lee, 2018). Calcium silicate based cement consisting of added metal oxides lead to the development of Mineral Trioxide Aggregate (MTA) that has bioactive properties (Torabinejad and Chivian, 1999). Apart from being extremely biocompatible, it is seen to stimulate tissue repair and induce mineralisation (Camilleri et al., 2005; Parirokh and Torabinejad, 2010). These reasons make MTA a suitable material to be used as a root canal sealer as it fulfils most of the criteria for a material to be called an ideal root canal sealer.

Silver has gained a fame of being an antimicrobial agent and is being incorporated to check for its antimicrobial efficiency against various species of microorganisms important as per the dental perspective (Noronha et al., 2017). We have numerous highly cited publications on well designed clinical trials and lab studies (Govindaraju, Neelakantan and Gutmann, 2017; Azeem and Sureshbabu, 2018; Jenarthanan and Subbarao, 2018; Manohar and Sharma, 2018; Nandakumar and Nasim, 2018; Teja, Ramesh and Priya, 2018; Janani and Sandhya, 2019; Khandelwal and Palanivelu, 2019; Malli Sureshbabu et al., 2019; Poorni, Srinivasan and Nivedhitha, 2019; Rajakeerthi and Ms, 2019; Rajendran et al., 2019; Ramarao and Sathyanarayanan, 2019; Siddique and Nivedhitha, 2019; Siddique et al., 2019; Siddique, Nivedhitha and Jacob, 2019). This has provided the right platform for us to pursue the current study. Our aim was to evaluate the cytotoxicity of a MTA based sealer; MTA Fillapex, developed by Angelus (Londrina/Parana/ Brazil) against the human periodontal cell lines by MTT Colorimetric Assay in vitro.

MATERIAL AND METHODS

Silver nanoparticles (10nm), MTT(1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan) and DMSO purchased from Sigma Aldrich was used in this study. Silver nanoparticles 10 nm: AgNP 20 µg/mL solution (nanoparticles, 10 nm particle size), 0.02 mg/mL in aqueous buffer, contains sodium citrate as stabilizer. The endodontic sealer MTA-Fillapex, developed by Angelus (Londrina/Parana/ Brazil) and launched commercially in 2010 was mixed with 0.5%, 1.0%, 2.5%, 5%, and 10% of silver nanoparticles. Samples were prepared by mixing the silver nanoparticles percentages (by weight), proportionally incorporated into the base paste of the endodontic sealer and weighed. The pH value of the product was measured as 7.4. The concentration of the stock solution was 0.2 mM. The solution was stored at 4°C. Serial dilutions from the (100%) were made to obtain dilutions of 0.5%, 1.0%, 2.5%, 5%, and 10% in supplemented DMEM medium.

Human periodontal ligament cell lines were purchased from NCCS Pune, India.The PDL Cells were cultured in a humidified atmosphere at 37 °C in the cell growth DMEM medium with 10% fetal bovine serum, L–glutamine,1% penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified CO2 (5%) chamber and 95% air. The cells were detached using 0.25% EDTA Trypsin. Neutralization of the Trypsin was achieved using DMEM containing 10% FBS and PSGF, and cells were mechanically separated using a pipette. There were 96-well plastic culture plates filled with 200µl of medium containing in each well. The plates were then incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air for 24 h to permit attachment of the cells to the plates.

Testing groups: The testing groups include negative control- only the medium without any test compound; sealer control - only the sealer without nanoparticles; sealer+silver nanoparticles - (0.5, 1.0, 2.5, 5, 10%) of silver nanoparticles mixed with the sealer. The testing groups include negative control- only the medium without any test compound; sealer control - only the MTA without nanoparticles; MTA + silver nanoparticles - (0.5, 1.0, 2.5, 5, 10%) of silver nanoparticles mixed with the sealer.

Cell viability assay: The PDL cells were seeded at the density of (1 × 103 cells/ml) were plated on into well plates and the cells were permitted to adhere for 24 hours, and the growth medium (MEM) removed using micropipette and the monolayer of cells washed twice with MEM without FBS to remove dead cells and excess FBS. Then treated with 100µl of different concentrations of MTA + AgNps and MTA alone in respective wells for 24 h. Cell culture medium (DMEM) was used as a negative control for assessment of cell viability. 200 µl of MTT (5 mg/ml in PBS) were added to each well, and the cells incubated for a further 6-7 hrs in 5% CO2 incubator. After removal of the medium, 1ml of DMSO was added to each well. The effect of the MTA + AgNps (0.5, 1.0, 2.5, 5, 10%) and sealer control on cell growth inhibition was assessed as percent cell viability, where vehicle-treated cells were taken as 100% viable.

The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. The plates were placed on a shaker for 15

min and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm. From the values obtained, the percentage cytotoxicity (IC50 value) was calculated. Each experiment was carried out in triplicate and the half maximal inhibitory concentration (IC50) of the test samples as the percentage survival of the cells was calculated according to the formula provided below:

Percentage of viable cell concentration was calculated thus:

Viability (%) = (Mean test OD/Control OD) ×100

Statistical analysis: Results were expressed as mean ± Standard error of Mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference test. P values less than 0.05 were considered significant.

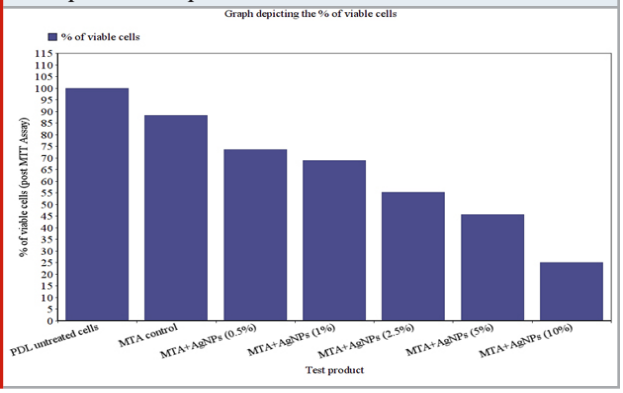
Table 1: Table showing % of cell viability after MTT Assay was performed. Values are expressed as Mean ± Standard error of Mean (SEM). (n=3); MTA + AgNPS treated PDL cells showed statistically significant difference *P<0.05; **P<0.01; ***P<0.001 as compared with Negative control. MTA alone treated PDL cells showed statistically significant difference #P<0.001 as compared with Negative control. The IC50 of the MTA + AgNPS is 4.52%.

S. No	Treatment	Conc (%)	Absorbance 570nm	% of cell viability
1.	PDL untreated cells		0.427 ± 0.11	100 ± 7.6
2.	MTA Control	1.0	0. 376 ± 0.25	88.3 ±2.7*
3.	MTA+AgNPs	0.5	0.315 ± 0.14	73.7 ± 3.8*
4.	MTA+AgNPs	1.0	0.295 ± 0.19	69.0 ± 1.9**
5.	MTA+AgNPs	2.5	0.236 ± 0.11	55.2± 2.4***
6.	MTA+AgNPs	5.0	0.195 ± 0.23	45.6 ± 1.5***
7.	MTA+AgNPs	10.0	0.107 ± 0.11	25.0 ± 1.2#

RESULTS AND DISCUSSION

In the current study cytotoxicity analysis was done using MTT Assay which is a colorimetric test. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based upon the conversion of MTT to formazan crystals that determine the mitochondrial activity of living cells (van Meerloo, Kaspers and Cloos, 2011). The formazan crystals presence in the cells can be evaluated by spectrophotometer. For most cell populations' total mitochondrial activity is related to the number of viable cells . MTT assay, hence broadly is used to measure the in vitro cytotoxic effects of drugs and dental materials on cell lines or primary cells lines. As seen in the results (Table 1 and Figure 1), the incorporation of nanosilver particles increases the cytotoxicity of the PDL cells and decreases the cell viability proportionally as the increase in the concentration of the silver nanoparticles is seen. Minimum cytotoxicity is seen in the original product while maximum is seen when 10% of silver nanoparticles are added. The mechanism of cytotoxicity of the silver nanoparticles in vitro to PDL cells reportedly is due to the interaction of the nanoparticles with mitochondria and other cell organelles after they have phagocytized into the cells. This results in cells' apoptosis or necrosis. (Wei et al., 2010).

Figure 1: Bar graph depicting the % of viable cells. X-axis denotes the nature of the test product used and Y-axis denotes the % of periodontal cells viable after the performance of MTT Assay. It can be inferred that maximum % of the periodontal cell viability was seen when MTA with no silver nanoparticles were added. The % of cell viability is concentration of silver nanoparticles incorporation dependent.



Since ancient times, the silver ion has been known to be effective against a broad range of microorganisms. Silver ions are used to control bacterial growth in a variety of medical applications, including dental work, catheters, and the healing of burn wounds. The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfhydryl) groups and it has a deadly effect on bacterial enzymes, bacterial growth and cell division and result in damage of bacterial cell wall and contents (Jung et al., 2008). The addition of nanoparticles to the endodontic sealers help in an interaction at the molecular level. Silver particles are extremely efficient when delivered in particles of nano ranges. The nano range comprises particles with a size of 1 to 100 nm range. The silver nanoparticles are stored in a liquid medium to prevent agglomeration and entrapment of the particles within the matrix.

It has been implied that the particle shape, size, distribution and agglomeration are important characteristics of nanoparticles (Lara et al., 2011). These factors determine the distribution of the particles in vivo, their biological fate, toxic effects, and targeting ability. Silver nanoparticles are non-toxic at low concentrations and have a broad spectrum of antimicrobial activity that includes many gram positive, gram negative and antibiotic drug resistant species like MRSA (Morones et al., 2005). The antimicrobial mechanism of action of silver nanoparticles can be attributed to its greater ability of binding to the negatively charged part of the bacterial cell wall causing the rupture of the cell membrane and subsequent leak in the cytoplasmic

contents of the microorganisms. Furthermore, the nanoparticles infiltrate within the cytoplasm and interact with the nuclear content causing bacterial cell death (Ibrahim et al., 2017) . This was the idea behind inclusion of silver nanoparticles in a MTA based sealer.

MTA Fillapex, developed by Angelus (Londrina/Parana/ Brazil) and launched commercially in 2010, is seen to comprise natural resin, salicylate resin, diluting resin, bismuth trioxide, nanoparticulated silica, MTA and pigments (Bin et al., 2012). There have been claims that MTA Fillapex has a good antimicrobial efficiency (Rahman et al., 2017; Faria-Júnior et al., 2013; Kuga et al., 2013; Ustun et al., 2013; Hasheminia et al., 2017). The product claims to provide high alkalinity that favours hard tissue remineralisation and offers good antimicrobial activity (Kuga et al., 2011; Borges et al., 2014). Physical properties of MTA Fillapex when compared to commonly used epoxy resin based sealers are acceptable and MTA Fillapex has shown lowest values of flow, shorter working and setting times, lesser solubility and water absorption and good radiopacity, making it a clinically acceptable and desirable endodontic sealer (Vitti et al., 2013).

In the current scenario, epoxy resin based sealers are seen to possess very good physical properties, excellent apical sealing ability and biocompatibility (Singh et al., 2015). AH 26 releases formaldehyde hence its use has been questionable and this resin based sealer is being replaced with AH Plus which is a more biocompatible option (Spångberg, Barbosa and Lavigne, 1993; Huang et al., 2002). Zinc oxide eugenol based sealers have released potentially cytotoxic concentrations of eugenol that affect the viability of periodontal cells (Jung et al., 2018). Calcium hydroxide based sealers promote calcification but dissolution over a period of time remains a challenge (Khashaba, Chutkan and Borke, 2009).

Calcium silicate based sealer MTA Fillapex, developed by Angelus (Londrina/Parana/ Brazil), is reported to have a similar or greater cytotoxicity when compared to conventionally used sealers as reported by few authors when cytotoxicity studies on various cell lines like human periodontal cell lines, human gingival fibroblasts and primary human osteoblasts were performed (Bin et al., 2012; Scelza et al., 2012; Silva et al., 2013; Zhou et al., 2015; Collado-González et al., 2017; Poggio et al., 2017; Saygili et al., 2017) . A time- and dose-dependent response has been reported when MTA Fillapex was tested in-vitro for cytotoxicity analysis (Yoshino et al., 2013; Jafari et al., 2017). In our study, hence it is evident that with the addition of silver nanoparticles, though there may be an increase in the antibacterial activity of the sealer, there definitely is an increase in the cytotoxicity of the dental material which is dose dependent in nature. The decision of using this nanoparticle incorporated root canal sealer needs to be made after balancing all the desired properties and requirements. More tests need to be carried out for the cytotoxic and genotoxic analysis of this nanoparticle incorporated sealer. However, an endodontic sealer that is ideal for use appears as a myth even today.

CONCLUSION

Nanoparticulate dental materials are the future of dentistry. The in-vitro study gives us a perspective of nanosilver particle modified MTA based sealers to be cytotoxic to the human periodontal cells in a concentration dependent fashion. However, more clinical oriented studies need to be done to check for other aspects like toxicity, discoloration or microleakage in the canal of this nanosilver modified sealer.

Conflict of Interest: The authors deny any conflict of interests related to this study.

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