Int. J. Pharm. Med. Res. 2015; 3(5):267-272 **ISSN: 2347-7008**

International Journal of Pharmaceutical and Medicinal Research

Journal homepage: **www.ijpmr.org**

Research Article

Evaluation of Antimicrobial Efficacy of Graphene Silver Composite Nanoparticles against *E. faecalis* as Root Canal Irrigant: An *ex-vivo* study

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ARTICLE INFO:

Article history:
Received: 07 October, 2015
Received in revised form: 16 October, 2015
Accepted: 25 October, 2015
Available online: 30 October, 2015

Keywords:
Antimicrobial
E. faecalis
Graphene Silver Composite Nanoparticles
Sodium hypochlorite

ABSTRACT

Aim: The aim of this *in vitro* study was to evaluate the antimicrobial effectiveness of Graphene Silver Composite Nanoparticles as an endodontic irrigation solution. **Methodology:** Thirty caries-free, single-rooted, mandibular premolar human teeth were prepared in a similar method to that used for *in vivo* root canal treatment. The root canals were inoculated with a suspension containing *E. Faecalis* bacteria. The teeth were then randomly divided into three groups. Each group was irrigated with one of the following solutions: Saline (control), Graphene Silver Composite Nanoparticles and Sodium hypochlorite (3%). Antimicrobial effectiveness was evaluated immediately after irrigation and again after 3 days, by counting colony forming units on blood agar plates**. Results:** The percentage reduction of *E. Faecalis* in Saline was 21.64 %, with Sodium hypochlorite it was 80.40% and the maximum reduction was observed in Graphene Silver Composite Nanoparticles with 86.85%. **Conclusion:** Within the confines of this study Graphene Silver Composite Nanoparticles demonstrated maximum antimicrobial effectiveness against *E. Faecalis* bacteria.

1. Introduction

Elimination of microorganisms from the root canal plays an important role in accomplishing long term success in endodontic treatments. This task is done by biomechanical preparation along with irrigation with various antibacterial agents. Canal irrigation solutions should possess characteristics such as low toxicity, low surface tension, lubrication, substantively and odorless. Chlorhexidine, sodium hypochlorite, EDTA, MTAD or tetracycline isomer are among the commonly used root canal irrigation solutions**[1]**. Chlorhexidine is a popular antimicrobial agent but it is not capable of dissolving pulp tissue. Sodium hypochlorite has a wide range of antimicrobial activity and is able to kill various bacteria. It also has disadvantages such as toxicity and risk of tissue destruction, bad taste, inability to eliminate all the microorganisms present in infectious canals**[2]** and risk of physically changing the structure of dentinal canal walls.

Antimicrobial effects of silver have long been recognized. The ability to produce silver as nanocrystalline structure has greatly enhanced its biological and antimicrobial values**[3]**. Silver nanoparticles provide a greater contact surface compared to mass silver; which increases its antimicrobial efficacy. Therefore, a tiny amount of silver nanoparticles is required to exert an antimicrobial effect similar to that of mass silver**[4]**.

Various nanosilver-coated products have been manufactured such as the wound dressings, contraceptive devices, surgical tools and skeletal prosthesis. At the same time, many researchers have assessed the possibility of using nanosilver products in endodontic therapy**[5]**. In addition to bacteria, nanosilver has cidal effects on a wide range of fungi, protozoa, and even viruses**[6]**.

Graphite-based materials, mainly graphite oxide, have been shown to be strongly cytotoxic toward bacteria, and the antimicrobial actions caused by these nanoparticles because both membrane and oxidation stress**[7]**. Furthermore, several studies have shown that nanoparticles, mainly metal oxides, activated carbons and graphene-based materials, can disrupt and kill bacteria via the oxidation of glutathione, an important cellular antioxidant**[8]**. These nanomaterials act as conducting bridges that extract electrons from glutathione molecules thereby releasing them into the external environment, but the effect of membrane-disruption disappears after four hours of incubation.

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The aim of the present study is to evaluate the antimicrobial efficacy of graphene silver composite nanoparticles and sodium hypochlorite against *E.faecalis* as root canal irrigant.

2. Materials and Method

Graphene Oxide (GO) was synthesized by modified Hummers' method involving three steps. Initially 5 g of graphite powder was taken in a solution of 7.5 mL of conc. H_2SO_4, 2.5 g of $K_2S_2O_8$ and P_2O_5 at 80°C. 5 g of oxidized graphite powder was placed in cold (0°C) of conc. H_2SO_4 (115 mL). 15g of $KMnO_4$ was added with stirring, cooled and maintained at < 20°C. The mixture was then stirred at 35 °C for 2 h, and 230 mL of DI water was added. To terminate the reaction, large amount of DI water, 10% of 12.5 mL H_2O_2 solution, were added over 15 min, once the color changes into bright yellow it is finally washed with 1 M HCl. After the unexploited graphite in the resulting mixture was removed by centrifugation, as-synthesized graphene oxide (GO) was dispersed into individual sheets in distilled water at a concentration of 0.5 mg/mL with the aid of ultrasound for further use.

To prepare Ag Nanoparticles Modified Graphene Oxide **(**AgNP/RGO), an aqueous dispersion of (10 mL) of GO mixed with 200 µL of aniline and 5mL of 0.01 M AgNO3. Then, 15 mL of DMF was added to the reaction mixture and allowed then stirring for 3 hr. The precipitate was collected by centrifugation and washed with water twice and then dried. Suspension was prepared by dissolving 0.5mg of graphene silver composite in per ml of saline.

Thirty extracted non-carious, single rooted mandibular premolar which extracted for orthodontic reasons were used in the present study. Calculus and tissue tags were removed using hand and Ultrasonic scaling. The teeth were soaked in 5% NaOCl for 30 minutes to remove any remaining residual loose tissue and debris from the root surface. The teeth were stored in gauze soaked sterile saline till use to prevent dehydration. All the teeth were marked and then sectioned 14 mm from the apex with a carborundum disc using a low speed straight hand piece, so as to standardize roots of all the teeth approximately to the same length.

An ISO #15 K file was used to determine the working length. The root segments were mounted in wax bases for ease of instrumentation. All root canals were instrumented, using the step back technique and the circumferential filing motion upto K file #45. During cleaning and shaping, sterile distilled water was used after each instrument size. The segments were then removed from the wax bases.

Finally, the canals were flushed with 5 mL of distilled water to remove any debris. The root apices were sealed with type II GIC and coated with two coats of nail varnish to prevent bacterial leakage. Each tooth was sterilized in steam autoclave for 30 minutes under 15 psi pressures at 121°C.

The bacterial strains used in this study are *Enterococcus faecalis* (MTCC 439). The primary culture was raised by inoculating *Enterococcus faecalis* (MTCC 439) in the Brain heart infusion (BHI) broth after incubation at 37°C for 24 hrs. The canals of the experimental teeth were cautiously inoculated using a

micropipette with 20 µL of the freshly prepared suspension of the organisms and for this a sterile #15 K file was used to carry the bacterial suspension to the entire root canal length. The teeth were then incubated at 37°C for 72 hours.

After incubation, 30 contaminated roots were divided into 3 groups (n=10) according to the irrigation regimen used.

Group A **:** Graphene silver composite (10 teeth)
Group B **:** 3% NaOCl (10 teeth)
Group C **:** Saline positive control (10 teeth)

All the teeth were handled with sterile gloves and sterile tweezers to prevent contamination. A sterile 5 mL syringe with 26 gauge needle was used to deliver 0.5 ml of irrigant into the canal for three minutes. All experimental teeth were then flushed with distilled water to prevent potential carry-over of irrigants.

A small amount of distilled water was introduced into the canal, and an endodontic hand file was used in a filing motion to a level approximately 1 mm short of the root apex. The canal contents were aspirated and then placed into appendorf tubes containing 1 mL of sterile saline. A 30 number paper point was then placed into the canal at the working length for 30 seconds each and also used to soak up the canal contents. Paper points were transferred to the same tubes containing 1 mL saline and agitated in vortex for 1 minute. Aliquots of 500 µl of the appropriate dilutions were cultured into BHI agar plates. All plates were cultivated at 37°C in a micro-aerophilic environment in 5% CO_2 for 48 hours. The colonies were identified on the basis of their morphology and counted using a digital colony counter. Confirmation was performed under light microscopy after staining a heat fixed smear slide. Microbial counts were expressed as colony-forming units (CFU) per ml of sample. The laboratory staff and clinicians evaluating the culture plates were blinded to the subject's group assignment.

$$\textbf{COLONY FORMING UNIT /ml} = \frac{\textbf{Number of colonies obtained X Dilution Factor}}{\textbf{Volume of sample inoculated}}$$

3. Results

Large numbers of bacteria were present in the canals of teeth irrigated with saline (Table 1). Pre and post irrigation comparison using paired t test showed a significant difference within the groups as shown in Table 2. However the percentage reduction of *E. Faecalis* in Saline was 21.64 %, Sodium hypochlorite with 80.40% and the maximum reduction was seen with Graphene Silver Composite Nanoparticles with 86.85% (Table 3). No significance difference was observed in bacteria

reduction following irrigation with NaOCl and Graphene Silver Composite Nanoparticles (Table 4).

Table 1: CFU in each Sample in different groups against *Enterococcus faecalis*

Group	S No	Pre-Irrigation	Post-irrigation	% age reduction
Group A	1	2.54×10^6	0.58×10^6	77.17
Group A	2	1.98×10^6	0.195×10^6	90.15
Group A	3	1.43×10^6	0.085×10^6	94.06
Group A	4	1.75×10^6	0.21×10^6	88.00
Group A	5	1.79×10^6	0.195×10^6	89.11
Group A	6	1.88×10^6	0.27×10^6	85.64
Group A	7	1.95×10^6	0.21×10^6	89.23
Group A	8	1.48×10^6	0.28×10^6	81.08
Group A	9	1.68×10^6	0.17×10^6	89.88
Group A	10	1.83×10^6	0.29×10^6	84.15
Group B	1	2.275×10^6	0	100.00
Group B	2	3.525×10^6	0.125×10^6	96.45
Group B	3	2.675×10^6	0	100.00
Group B	4	1.375×10^6	0.415×10^6	69.82
Group B	5	1.875×10^6	0.875×10^6	53.33
Group B	6	2.52×10^6	0.12×10^6	95.24
Group B	7	1.12×10^6	0.42×10^6	62.50
Group B	8	1.01×10^6	0.4×10^6	60.40
Group B	9	2.52×10^6	0.112×10^6	95.56
Group B	10	1.12×10^6	0.328×10^6	70.71
Group C	1	6.605×10^6	5.1×10^6	22.79
Group C	2	5.9×10^6	4.3×10^6	27.12
Group C	3	3×10^6	2.575×10^6	14.17
Group C	4	3.9×10^6	3.775×10^6	3.21
Group C	5	1.875×10^6	1.2×10^6	36.00
Group C	6	4.27×10^6	3.1×10^6	27.40
Group C	7	5.7×10^6	4.1×10^6	28.07
Group C	8	3.2×10^6	2.375×10^6	25.78
Group C	9	4.1×10^6	3.575×10^6	12.80
Group C	10	2.1×10^6	1.7×10^6	19.05

Table 2: Pre and post irrigation comparison within group using paired t test

		N	Mean ($x10^5$)	Std. Deviation ($x10^5$)	'p' value*
Group A	***E. faecalis* Pre-Irrigation**	10	18.31	3.08	<0.001
	***E. faecalis* Post-irrigation**	10	2.49	1.31	
Group B	***E. faecalis* Pre-Irrigation**	10	20.02	8.39	<0.001
	***E. faecalis* Post-irrigation**	10	2.80	2.67	
Group C	***E. faecalis* Pre-Irrigation**	10	40.65	16.02	<0.001
	***E. faecalis* Post-irrigation**	10	31.80	12.22	

P<0.05 – significant

Table 3: *E. faecalis* Percentage Reduction in different groups

	N	Mean (%)	Std. Deviation
Group A	10	86.85	4.94
Group B	10	80.40	18.66
Group C	10	21.64	9.48

Table 4: Comparison of mean percentage reduction among three different groups using Post Hoc test

Group	Group	'p' value*
Group A	**Group B**	0.773
	Group C	0.00
Group B	**Group A**	0.773
	Group C	0.00
Group C	**Group A**	0.00
	Group B	0.00

Tukey HSD P < 0.05 significant, <0.01 highly significant

4. Discussion

The main cause of endodontic failure is the persisting infections in the root canal system. The primary endodontic infections are polymicrobial with predominantly anaerobic species with equal proportion of gram-positive and gram-negative bacteria. The secondary or persistent infection on the other hand are mono infections, with predominantly gram-positive microorganisms (equal proportion of facultative and obligate anaerobes), which are less susceptible to antimicrobial agents**[9]**. Most studies show that there is a high prevalence of *Enterococci* species in persistant root canal infections**[10]**.

The significant characteristics of *Enterococci* include their ability to grow in the range of 10°C-45°C and to survive around 30 min at 60°C; and at high salt concentrations of 6.5% saline as well as at extremely alkaline pH of upto 11.5**[11]**. *E.faecalis* endures prolonged period of nutritional deprivation. It binds to dentin and proficiently invades dentinal tubules**[12]**. It alters the host response and suppresses the action of lymphocytes. It possesses lytic enzymes, cytolysin, aggregation substance, pheromones and lipoteichoic acid**[13]**. It utilizes serum as the nutritional source. It resists intracanal medicaments i.e. calcium hydroxide by maintaining pH haemostasis.

In this study, the apices of all sampled teeth were sealed with glass ionomer cement followed by nail varnish application to prevent any contamination from the outer tooth surface during the sampling procedure. To eliminate the variable effects of mechanical instrumentation and smear layer removal in reducing bacterial count, both were accomplished before sterilization and inoculation of sample.

Mechanical debridement alone does not result in total or permanent reduction of bacteria. The use of irrigants with antimicrobial action has been recommended as an important adjunct to mechanical instrumentation so as to eliminate or at least reduce the numbers of microorganisms. The aim of this study was to determine the antimicrobial efficacy of Graphene Silver Composite Nanoparticles and NaOCl solutions against *E.faecalis*.

Historically, countless compounds in aqueous solutions have been suggested as root Canal irrigants. Of all the currently used substances, sodium hypochlorite appears to be the most ideal, as it covers more of the requirements for endodontic irrigant than any other compound. Various investigations have shown that NaOCl might irritate the periodontal and periapical tissues**[14]**. This has led researches to evaluate various antimicrobial properties.

Nanotechnology deals with processes that take place on the nanometer scale, that is, from approximately 1 to 100nm. It is believed that due to their large surface areas nanoparticles have more penetration powers into microorganisms and if the active plant extracts can be delivered into the 'interior' of the microbes more activity could be recorded. Many chemical methods exist for synthesis of nanoparticles but have been found to be toxic since nanoparticles could be used in humans and other animals or plants which may eventually end up in human system**[15]**.

The exact mechanism which silver nanoparticles employ to cause antimicrobial effect is not clearly known. There are however various theories of the action of silver nanoparticles on microbes to cause the microbicidal effect. Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of 'pits' on the cell surface, and there is accumulation of the nanoparticles on the cell surface**[4]**. Electron spin resonance spectroscopy studies suggested that there is formation of free radicals by the silver nanoparticles when in contact with the bacteria, and these free radicals have the ability to damage the cell membrane and make it porous which can ultimately lead to cell death**[16]**.

It has also been proposed that there can be release of silver ions by the nanoparticles**[17]**, and these ions can interact with the thiol groups of many vital enzymes and inactivate them**[18]**. The bacterial cells in contact with silver take in silver ions, which inhibit several functions in the cell and damage the cells. Then, there is the generation of reactive oxygen species, which are produced possibly through the inhibition of a respiratory enzyme by silver ions and attack the cell itself. Silver is a soft acid, and there is a natural tendency of an acid to react with a base, in this case, a soft acid to react with a soft base**[19]**. The cells are majorly made up of sulfur and phosphorus which are soft bases. The action of these nanoparticles on the cell can

cause the reaction to take place and subsequently lead to cell death. Another fact is that the DNA has sulfur and phosphorus as its major components; the nanoparticles can act on these soft bases and destroy the DNA which would definitely lead to cell death**[20]**.

Graphene Based Materials (GBMs) include few-layer graphene, graphene nanosheets, graphene oxide and reduced graphene oxide. Graphene comprising of single atom-thick sheets of sp2-bonded carbon. It is a typical two-dimensional material made of carbon atoms packed densely in a honeycomb crystal lattice**[21]**. Graphene Oxide (GO) is chemically modified graphene, containing hydroxyl, carbonyl and epoxy functional groups, which is obtained by synthesis of graphite with strong oxidizing agents**[22]**. Also it has been used as a promising material for preparing new composites**[23]**. It is well known that GO and its composites possess anti-microbial properties and have been used as anti-bacterial and antifungal agents**[24-25]**. The effect and interaction of GBMs on microbial cells structure, metabolism and viability has been shown to depend on the materials' concentration, time of exposure and physical-chemical properties, as well as on the characteristics of microorganisms used in the tests**[25-28]**. There are different mode of action of GBM into microbial cells some studies suggests disruption cell wall and membranes because of sharp edges of GO or because of generation of Reactive Oxygen Species (ROS) which may be fatal factor for microbial cells**[29]**.

Our goal was to evaluate if nanoparticles during endodontic treatment resulted in a cleaner root canal. In this study we used bacterial sampling to indicate the presence of infection in the canal. Of all the teeth treated with nanoparticles showed a positive reduction in bacterial growth. This study showed that there was no significant difference in reduction of bacterial count between graphene silver composite or 3% sodium hypochlorite.

Teeth treated with 3% sodium hypochlorite which showed equally good results. In fact some samples of this group have shown 100% reduction in bacterial count. However, Hypochlorite is acutely operator sensitive, requiring careful application during root canal cleaning to prevent seepage through the apex into bone or soft tissue, which can cause oedema, pain and tissue damage.

In the present study graphene silver composite nanoparticles showed antimicrobial potential as a root canal irrigants with less cytotoxic effect to the bone or soft tissues. However, further investigations are required to specify the conditions of size, concentration and ideal morphology of nanoparticles in general to optimize their antimicrobial effect mainly by acting against the resistant root canal microorganisms such as *E. faecalis*.

5. Conclusion

Within the limitation of this study, graphene silver composite nanoparticles solution has favorable antimicrobial properties and can be used as an alternative to other root canal irrigating solutions.

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Source of support: Nil, Conflict of interest: None Declared