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Increasing Bacteriostatic Effect of Coating Stainless Steel Orthodontic Bands with Single and Multiple Layers of 10 Mg/Ml of Graphene Oxide against *Streptococcus Mutans*

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Abstract: **Orthodontic fixed appliances increase the risk of dental caries, often caused by *Streptococcus mutans*. Graphene Oxide (GO) exhibits antibacterial properties, and the thickness of nanomaterials may enhance their antimicrobial effectiveness. This study aimed to determine the bacteriostatic effect of a single and multiple layers of GO coating on stainless steel orthodontic bands on the inhibition of *S. mutans*. Sixty (60) Orthodontic stainless steel bands were divided into four groups - control group (uncoated brackets); experimental group 1 (treated with one (1) layer of GO coating); experimental groups 2 and 3 (treated with two (2) layers and three (3) layers of GO coatings respectively). Each experimental group was dipped in a 10mg/ml GO solution for two minutes per layer. The inactivation of *S. mutans* when exposed to the different layers of GO coating was assessed through modified agar well diffusion method. The results were analyzed using Guevara et al.,2005 index. Experimental groups 2 and 3 have shown a very active antibacterial effect against *S.mutans* with mean values of 38.00 and 21.5 mm respectively (very active > 19 mm). The least antibacterial activity against *S. mutans* was recorded in group 1 with a mean value of 9.43 mm (may be expressed as inactive < 10 mm). No zone of inhibition was observed with the control group of uncoated molar bands. The zone of inhibition diameter was compared and analyzed using Kruskal-Wallis test. Statistically, there was a significant difference among the groups with P value 0.000 <0.05.**

Keywords: **Graphene Oxide, Streptococcus mutans, Bacteriostatic effect, stainless steel orthodontic bands.**

I. INTRODUCTION

Orthodontics is the branch of dentistry that straightens the teeth and addresses improper alignment and malposition of teeth and jaws. Tooth movement is achieved with the use of orthodontic attachments (i.e., brackets, molar bands, etc.) and wires. The duration of treatment may vary from six months to two years (or more) depending on the diagnosis and the level of malocclusions and dental problems present. Placement of complex orthodontic appliances such as orthodontic brackets, molar bands, wires, and additional attachments makes the smooth surfaces of banded teeth vulnerable to colonization of cariogenic bacteria which causes mineral loss and may weaken the tooth structure.

White spot formation under orthodontic molar bands can be caused by many factors, among which are improper placement or loose bands, poor oral hygiene, and the nature of the cement used (Adriaens et al., 1990). The weak retention of the bands due to the improper adaptation to the tooth surface leads to a decreased physico-chemical bond between the metal and enamel. This creates areas between the band and the molar that are prone to food accumulation and bacterial growth which cause demineralization and white spot lesions eventually (Hodges et al., 2001).

Enamel demineralization is a process in which mineral ions are removed from the hydroxyapatite crystals of hard tissues such as enamel or dentin. It may appear as white eroded areas and is a common consequence of wearing braces for extended periods of time. These are early indicators of caries development and are readily observed by trained dental practitioners including orthodontists.

The prevalence of enamel demineralization after orthodontic treatment can be as high as 50% of the patients (Ogaard, B., 1989). Gorelick et al. (1982) found that fixed orthodontic appliances hastened the rapid growth in volume of dental plaque especially on the vulnerable surfaces of teeth and around molar bands. It has also been observed that white spot lesions caused by decalcification that occur around the banded teeth persisted even after completion of treatment. These consequences of the placement of molar bands are exacerbated by the increased number of retentive sites (cervical, buccal, and interproximal areas). Due to these added retentive sites, it may also compromise the mechanical removal techniques of plaque after band placement (Demling et al., 2010). Furthermore, Chatterjee and Kleinberg (1979) showed that dental plaque in the oral cavity had an average range of pH less than that of non-orthodontic areas. This acidic medium increases the susceptibility of the teeth to dental caries.

After completion of orthodontic treatment, defective enamel surfaces and damaged tooth structures resulting from constant white spot lesions can be observed in the long run (Ogaard, B., 1989). Enamel structure degradation can be caused by acidophilic microorganisms such as *S. mutans*, one of the main causative microorganisms of dental caries. The presence of fermentable carbohydrates like sucrose and fructose have also been considered as contributory factors to the progress of caries (Bradshaw and Lynch, 2013). Different techniques have been adopted in controlling the loss of enamel structure during active orthodontic treatment. Other than the traditional application of fluoride, pit and fissure sealants, patient education techniques have also been employed for better oral hygiene maintenance. However, there is still a need to establish sophisticated means to prevent the adhesion of these bacteria on the orthodontic appliances especially molar bands.

Interestingly, some studies revealed that coating the orthodontic appliance, molar bands in particular, with antibacterial coating materials such as silver nanoparticles (SNPs) have bacterial inhibiting effect against gram positive bacteria, and hence prevents the accumulation of dental plaque (Prabha et al., 2016).

There are several nanomaterial coatings that have the potential to minimize bacterial growth in the oral cavity. One of these more promising ones is Graphene Oxide (GO). Graphene was discovered by a Dutch-British physicist working in England in the School of Physics and Astronomy. It was first produced and recognized in 2004, by Andre Geim and Konstantin Novoselov.

Graphene is considered one of the more popular nanomaterials that could possibly have a wide antimicrobial spectrum effect because of its varied properties. Graphene oxide, when it is oxidized, becomes a dynamic material with wide applications in the field of science including orthodontics. It has been demonstrated to improve the mechanical and biological properties of orthodontic bonding adhesive materials. In dentistry, various studies have been made to determine its benefits including those for tissue engineering. It has been scrutinized as a multi- property agent, a shield coating for prosthodontic implants, and as a boosting agent for dental adhesives. Its antimicrobial activity was just newly discussed and is now being examined. It can be a promising antibiotic-free alternative for controlling bacterial infections while using a low amount of antimicrobial agent.

A recent study by Krystejan et al., (2021) proved a pronounced bacteriostatic effect of starch/Graphene oxide nanocomposites against various strains of facultative anaerobic pathogenic bacteria. Also, He et al., (2015) reported that GO had a strong antibacterial inhibitory effect on dental pathogens including *S. mutans*, the main etiological factor in the initiation and development of cariogenic biofilm. The inhibiting mechanism for bacteria occurs with a noticeable inhibition of *E. coli* and *S. mutans* proliferation by promoting hydrophilicity and creation of a physical aqueous barrier that prevents the adhesion and growth of bacteria on the surface (Zhao et al., 2020).

GO can be in extreme active form and well energized on the surface with sufficient amount of oxygen. A study on graphite (multiple layers of graphene) as well concluded that layering increases the active surface area by boosting or maintaining sufficient levels of oxygen on the surface (Ng et al., 2009). Graphene has very high electron mobility and, like graphite, is a good electrical conductor, due to the occurrence of a free pi (p) electron for each carbon atom. Graphite and graphene belong to the same mineral and they share the same atomic layer and interaction to the oxygen level. Due to these similarities between graphene and graphite, increasing the active surface area may likewise contribute to enhancing the efficacy of the antibacterial property of the GO nanoparticles.

There are different concentrations of GO depending on the purpose of the usage. However, there are established values such as MIC (minimal inhibitory concentration) which is determined by using the broth mini-concentration technique recommended by laboratory criteria in different Institutions (CLSI). Minimal Inhibitory Concentration (MIC) reported in mg/ml, is defined as the lowest level of an antimicrobial that will inhibit growth of a microorganism after incubation period. It had been found to be 10 mg/ml for graphene oxide (Kimiagar and Rashidi, 2015).

Several studies have been conducted in different fields on how to apply graphene oxide: chemical coating, spraying, and electrodeposition. In theory, dip coating is very simple, doable, and affordable compared to other coating techniques. The sample is perpendicularly dipped into a solution containing the nanoparticles. The sample should stay immersed in the solution to give some time for nanoparticle deposition, then the sample is withdrawn from the solution to dry.

The study of Ivanova et al. (2018) used electrostatic deposition technique. It basically applied electrostatic depositions of oppositely charged poly-ions without the need for chemical coating technique to achieve the ultra-thin multilayer coatings (adopting layer by layer deposition with nanoparticle-coated samples). This layer by layer deposition technique was also assessed in a separate study. It was established that the increase of ZnO coating thickness reaching 600 nm increased the active surface area of the column's length (coatings thickness). This would increase production of oxidative stress and/or bacterial membrane disruption and, therefore, cause bacterial inhibition (Wang et al., 2007). Interestingly, thinner samples (50 and 100 nm) presented a less pronounced antibacterial activity than the thickest ones (200–600 nm) (Carvalho et al., 2014) suggesting a possible link between the amount of coating thickness with its antimicrobial effect that may be true for GO as well.

II. MATERIALS AND METHODS

A. Band Preparation

A stainless steel orthodontic metal band material was cut 8mm in length and 6mm in height to form a band following the 6mm requirement of the agar well diffusion method. Each band was spot welded 1.5mm away from the upper (first welding point) and the lower (second spot welding point) margins of the band material to form a customized molar bands. These newly fabricated molar bands were placed in acetone for ultrasonic cleaning at 40 khz for 20 minutes. The clean bands were then kept in acetone until the experiment. Prior to GO coating, the bands were subjected to a 60°C drying oven for 15 minutes.

A premixed solution containing GO (0.5-0.7 microns) dispersed in water with a concentration of 10mg/ml was utilized in this study. This aqueous GO suspension was prepared for dipping technique via sonication for two hours. For the first coating cycle, the pre-treated bands were immersed in the GO suspension. After two minutes, the bands were raised out from GO and dried at 37°C in an incubator for 24 hours. With this, a single layer of GO film was formed. This coating was done on each surface of the band). An adhesive tape was used to cover the surfaces that will not be coated per dipping process to control unwanted double coating. This coating process was repeated twice for Group 2 and thrice for Group 3. The coated bands were disinfected by an ultraviolet unit for five minutes with wavelength of 260nm, before exposing the bands to the bacterial culture.

B. Preparation of Agar Plates and Inoculum

Mueller-Hinton (MH) agar plates were prepared by creating a suspension from 38gm of MH agar powder and 1000ml of distilled water with a pH of 7.2-7.4. It was poured into petri dishes up to a depth of 6mm and stored at temperatures between 4-8ºC. The MH agar plates were placed in an incubator maintained at 25ºC to prevent drying before actual exposure to *S. mutans* inoculum. Each plate was labelled properly according to its designated group.

S. mutans ATCC 25175 in lyophilized form were activated according to manufacturer instructions by cracking the ampoule to allow the hydrating fluid to submerge the pellet and form homogenous suspension. It was then cultured with 5ml of Brain Heart Infusion (BHI) broth. This bacterial suspension was incubated for 48 hours at 37°C in an anaerobic unit. The viability of the culture was assessed by plating 1 microliter of bacterial suspension solution into one petri dish. Once the viability is confirmed, the *S. mutans* culture was serially diluted with BHI solution (9 mL of pure BHI with 1 mL of bacterial suspension) to get a 0.5 McFarland standard which is equivalent to a bacterial suspension containing between 1×10^8 and 2×10^8. Following the result of the pilot test where the suspension was deemed too thick for spreading, the inoculum had to be diluted twice with 1:10000 bacterial suspension containing 1×10^{10} CFU/ml after 0.5 McFarland to achieve a favourable suspension.

C. Inoculation of MH plates and Exposure of coated molar bands

Prior to spreading, the inoculum was vortexed to ensure dispersion of the *S. mutans* in the culture medium. The dried surface of the MH agar plate was inoculated at its centre with 0.1 ml of the bacterial suspension. This was dispersed in the agar plate by moving a sterile glass spreader three (3) times over the entire agar surface and rotating the petri dish approximately 45^{o} each time to ensure an even distribution of the inoculum.

Six (6) millimeter wells were cut into the agar plate. Using a sterile bracket holder, the coated orthodontic bands were placed carefully in the MH agar well according to their corresponding groups. There must be a 50 millimeter centre spacing between the wells to ensure easy measurement of the zones. The bands must be in full contact with the agar surface to prevent irregular zone shapes. These plates were then incubated anaerobically at 37°C and examined after 48 hours. Following incubation, the zones were measured to the nearest millimeter using a digital calliper.

III. RESULTS

TABLE 1: The Means and Standard Deviations of the Control Group and Experimental Groups on ZOI diameter:

Descriptive Statistics				
Group	N	Mean	Std. Deviation	Std. Error of Mean
1 LAYER	15	17.53	3.50	0.90
2 LAYERS	15	22.67	2.44	0.63
3 LAYERS	15	33.67	4.50	1.16

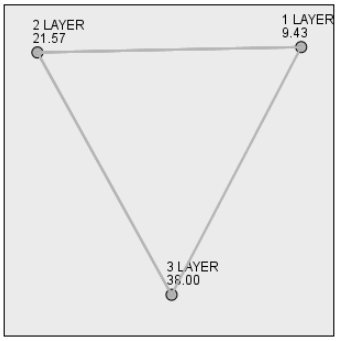
TABLE 2: Comparison of means of single and multiple layers of GO coated bands

Ranks			
	Group	N	Mean Rank
ZOI diameter	1 LAYER	15	9.43
	2 LAYERS	15	21.57
	3 LAYERS	15	38.00
	Total	45	

Test Statistics[a,b]	
	ZOI diameter
Kruskal-Wallis H	35.89
df	2
Asymp. Sig.	0.00

a. Kruskal Wallis Test
b. Grouping Variable: Group

Table 3: Pairwise comparison between groups showing significant difference between group 1 and group 2 with P value 0.034 <0.05.



Each node shows the sample average rank of Group.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
1 LAYER-2 LAYER	12.133-	4.787	2.535-	.011	.034
1 LAYER-3 LAYER	28.567-	4.787	5.968-	.000	.000
2 LAYER-3 LAYER	16.433-	4.787	3.433-	.001	.002

Table 4: Guevara interpretation Table which represents standard zones of inhibition and corresponding inferences (Guevara et al.,2005)

ZOI Diameter	**<10mm**	***10-13 mm***	***14-19mm***	***>19mm***
Inferences	*Maybe expressed as inactive*	*Partially active*	*Active*	*Very active*

*mm: millimeter for diameter size

Table 5: Mean zone of microbial growth inhibition (mm) (mean rank ± standard deviation provided by different layers of GO as interpreted using Guevara et al., 2005 *S. mutans* susceptibility range

Ranks				
	Group	N	Mean Rank	p-value
ZOI diameter	1 LAYER	15	9.43	0.00
	2 LAYERS	15	21.57	
	3 LAYERS	15	38.00	
	Total	45		

Classification: very active (>19 mm),active (14-19 mm), partially active (10-13 mm)

IV. DISCUSSION

Graphene oxide (GO) is considered to be a versatile nanomaterial in dentistry and tissue engineering due to its varied characteristics. In conservative dentistry, dental materials such as resin and adhesives which have been optimized with GO. Its use for surface modification of dental implants is expected to improve not only the antibacterial properties but also biocompatibility (QI et al.,2021).

The results of this study showed that the mean of bacterial inhibition zone in the experimental group 1 (graphene oxide single layer coated bands) is lower than the mean of bacteria inhibition zone in the experimental group 2 (bands coated with two layers of GO). The same applies to the mean of bacteria inhibition zone in the experimental group 3 (bands coated with three layers of GO) which showed the greatest inhibition mean diameter among the groups.

The mechanism between layering GO and S mutans as explained by He et al., 2015 was due to the increase in surface energy released from the reaction on the coated surface and oxidative stress under different levels causing disturbance in the bacterial outgrowth.

Multilayers of GO increased the surface energy and that improved the antibacterial efficacy of GO (Sztrum et al., 2005). This mechanism is interpreted by the way graphene oxide can fill a cube lattice of liquid, nanoparticles and vapor. Those variables are roughly proportional to the density of the solvent and graphene oxide. Each variable can equal to 0 (low density) or 1 (high density with multi-layers). Initially, with single layer, the lattice is partially filled with liquid and low density of graphene oxide nanoparticles. When additional nanoparticles occupy the surface, more reaction on the coated surface occur releasing metal ions which inhibit bacteria and causing disturbance in its outgrowth thickness.

Carvalo et al., (2014) evaluated layering in relation to thickness and it cited the thinner thickness samples (range from 50 to 100 nm) presented a less pronounced antibacterial activity than the thickest ones (200–600 nm).

In the results of this experiment, using the Guevara et al., 2005 *S. mutans* susceptibility range confirmed and classified the obtained results within the groups to be very active for the 2 and 3 layer group, 1 layer group may be expressed as inactive and zero bacteriostatic activity in the uncoated group in relation to zone of inhibition.

The single coated layer was described as having the least bacteriostatic effect can be explained by the fact that as a single oxide layer is formed with dip coating, it is disturbed with the fabrication of crystalline and nonhomogeneous oxides which reduce the activity of GO (Hussmann and Glaswerke, 1983). Low bonding strength to the substrate is also noticed because with single layer, the cube lattice is entirely filled with liquid and low density of nanoparticles (Sztrum et al., 2005). This may explain the inactivity of single coated layer.

With layering, molecules already dissolve most evaporated solvents and build polymolecules of GO. The films are transformed by drying and heating into solid homogeneous transparent oxide films.

High bonding strength to the surface is reached with layering. The rate of diffusion of the various oxides into each other maximize the efficacy of the nanoparticles towards its target bacteria (Hussmann and Glaswerke, 1983).

According to Henrique et al., (2018), film thickness can be controlled by adjusting the volume and concentration of the Graphene suspension, allowing the formation of films ranging from a single layer of graphene up to 10 layers. However, in this study as mentioned in the limitations, thickness evaluation was not assessed across the samples.

V. CONCLUSION

The varied characteristics of graphene oxide (GO) make it a promising nanomaterial in dentistry and tissue engineering. In conservative dentistry, GO is incorporated into dental materials like resin and adhesives for enhanced performance. Surface modification of dental implants with GO aims to improve antibacterial properties and biocompatibility. This in vitro study was done to assess the bacteriostatic effect of coating stainless steel molar bands with single and multiple layers of Graphene Oxide. The results showed that Graphene Oxide multi-layers coating affects the inhibition of *S.mutans*, is popularly known as one of the cariogenic bacteria that is responsible for the formation of dental caries typically seen in orthodontic patients. Layering facilitates the formation of GO polymolecules as molecules dissolve the evaporating solvents. Subsequent drying and heating convert these into solid, uniform, and transparent oxide films. This layering technique achieves strong surface adhesion and maximizes the diffusion of different oxides, enhancing the nanoparticles' antibacterial effectiveness.

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