Evaluation of the Antibacterial Effect of Silver Nanoparticles on Guided Tissue Regeneration Membrane Colonization - An *in Vitro* Study

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Abstract

Objective: The aim of this *in vitro* study was to investigate the colonization and penetration of specific bacteria on nanosilver-impregnated GTR (guided tissue regeneration) membranes.

Methods: Three sets of GTR membranes were used in this study: 1) GTR-C: Plain GTR membrane as a negative control; 2) GTR-NS: GTR membrane impregnated with silver nanoparticles as the test group; 3) GTR-DOX: GTR membrane impregnated with 25% (w/w) doxycycline hydrochloride acting as a positive control. Stress–strain characteristics were calculated to determine the physical properties of the control and impregnated membranes. Qualitative observation of microbial adherence and bacterial penetration through GTR membranes were performed by using four organisms (*Streptococcus mutans, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum and Porphyromonas gingivalis*) reported to have strong adherent capabilities to collagen membranes.

Results: The mean bacterial adherence scores were significantly greater ($p < 0.001$) in the GTR-C group when compared to GTR-DOX and GTR-NS groups. GTR-NS showed lower adherence scores than GTR-DOX across all four microorganisms; this difference, however was not statistically significant. The difference in colony forming units (CFUs) was highly significant ($p < 0.001$), suggesting greater penetration in GTR-C membranes when compared to GTR-NS and GTR-DOX groups. Though the mean CFUs were lower in GTR-DOX than in GTR-NS across all four microorganisms, this difference was statistically significant only for *S. mutans* and *F. nucleatum*.

Conclusion: The incorporation of silver nanoparticles may be of value when controlling membrane-associated infection. Studies with different nanosilver particle sizes should be conducted to further evaluate the beneficial properties of nanosilver against periodontal pathogens.

Key words: Periodontitis, nanoparticles, silver, guided tissue regeneration

Introduction

The concept of periodontal regeneration necessitated the exclusion of epithelial and connective tissue cells of the gingiva from the wound site leading to the development and application of guided tissue regeneration (GTR) membranes (Melcher, 1976). GTR procedures using non-absorbable and bioabsorbable membranes have been used to successfully and predictably treat various types of intrabony defects in terms of regeneration (Cristina and David, 2010). However, bacterial contamination of the wound site and the GTR membranes are known to significantly compromise the desired outcome (Rossa *et al.*, 2006). Nowzari and Slots (1994) emphasized the importance of controlling or eliminating periodontal pathogens on barrier membranes in order to gain new attachment. Specific bacterial species (Smith MacDonald *et al.*, 1998), bacterial counts (Ling *et al.*, 2003) and areas of bacterial contamination (Nowzari *et al.*, 1995) present on the GTR membrane are some of the factors that may affect the outcome of a GTR procedure at the time of surgery or during the period of healing.

Numerous protocols have been advocated in controlling or eliminating periodontal pathogens during GTR procedures (Machtei *et al.*, 1994). Systemic antibiotic therapy (Demolon *et al.*, 1993), local application of

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antibiotics in the form of antibiotic gel (Sander *et al*., 1994), antibiotic fiber (Sbordone *et al*., 2000), and irrigation with antibiotic solution (Machtei *et al*., 1993) have led to improved outcomes during GTR therapy. Chung *et al.* (1997) impregnated polyglycolic acid membranes with tetracycline and flurbiprofen and showed better bone formation in antibiotic-loaded membranes than in controls. Antibiotics such as amoxicillin (Chen *et al*., 2013), doxycycline (Chang and Yamada, 2000) and metronidazole (Dowell *et al*., 1995; Xue *et al.,* 2014) have been loaded on GTR membranes for successful treatment outcomes.

Silver has been known to be a disinfectant for several centuries and has been widely used in the treatment of clinical diseases, including newborn eye prophylaxis, topical burns and open wounds (Monafo and Freedman, 1987). Because silver therapy is of significant clinical benefit in the control of bacterial infections, various forms of medical, biological and pharmaceutical preparations containing silver ions have been developed (Hans and Lowman, 2002). Silver ions released from silver nitrate have been shown to be effective against Gram-negative periodontal pathogens and Gram-positive streptococci (Spacciapoli *et al*., 2001).

Silver nanoparticles are particles of silver ranging between 1 nm and 100 nm in size (Lok *et al*., 2005; Thangapandiyan and Prema, 2012). Because of their unique properties they are widely used in catalysis, chemical sensing, biosensing, photonics, electronics, pharmaceuticals and biomedicine, especially as antibacterial and antiviral agents (Thangapandiyan and Prema, 2012). Compared to conventional antibiotics, silver nanoparticles can exert superior activity by better contact with microorganisms, higher interaction with bacterial membranes and DNA, inhibitory effects on the respiratory chain in bacterial mitochondria and genesis of free radicals, enhancing their bactericidal activity (Lok *et al*., 2005). Studies have reported the inhibitory properties of silver nanoparticles against human immunodeficiency virus-1 (HIV-1; Elechiguerra *et al*., 2005), *Staphylococcus aureus* (Cho *et al*., 2005) and *Escherichia coli* (Sondi and Salopek-Sondi, 2004). The incorporation of silver nanoparticles is also known to effectively enhance the antibacterial activity against lyophilized *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Prevotella intermedia* (Bahador *et al*., 2014). Lotfi *et al.* (2011) compared the antimicrobial efficacy of nanosilver, sodium hypochlorite and chlorhexidine gluconate against *Enterococcus faecalis*, and found nanosilver to be superior.

In light of the advantages and effects of nanosilver as reported by previous studies, the aim of the present *in vitro* study was to evaluate the effect of silver nanoparticles when used to impregnate GTR membranes, in terms of bacterial adherence to GTR membranes and specific bacterial penetration through GTR membranes.

Materials and methods

Sample size estimation

Sample size was calculated according to the formula ($n \geq [z\alpha/2]^2S^2)/d^2$ where n is the sample size, z is the normal distribution tabled value, d is the detection level considered important and S is the standard deviation from the pilot data. To detect a 0.1 log10 difference in inner tube cultures, a minimum of 14 membranes per organism per time frame were required assuming $z\alpha/2 = 2.5$, $S = 0.1453$ from pilot culture studies and $d = 0.1$. In the present study, 17 membranes per organism per time interval were used.

GTR membranes

Because guided tissue regeneration is commonly accomplished by using biodegradable collagen membranes, collagen was the substrate of choice for nanosilver and antibiotic impregnation. Three sets of GTR membranes (PerioCol®-GTR, Eucare, Chennai, India) were used in this study and were grouped as GTR-C: Plain GTR membrane as a negative control; GTR-NS: GTR membrane impregnated with silver nanoparticles as the test group; and GTR-DOX: GTR membrane impregnated with 25% (w/w) doxycycline hydrochloride acting as a positive control.

Preparation of GTR-NS and GTR-DOX membranes

Membranes were cut into 6 mm diameter collagen discs and 15 x 12 mm collagen sheets in a sterile environment. Silver nanoparticles as a colloidal solution (0.1 mg/mL) in water (PlasmaChem GmbH, Berlin, Germany; 100 mL; average particle size: 10 nm) was utilized in this study to prepare GTR-NS membranes. Doxycycline hydrochloride (25%, w/w) was used to coat GTR-DOX membranes as described in a previous study (Chang and Yamada, 2000). Silver nanoparticles were applied by immersion of the collagen membranes in the colloidal solution for 3 hours at 30ºC. A readymade solution of 100 mg/mL doxycycline HCl (Sigma-Aldrich, Hyderabad, India) was reduced to a concentration of 25%, and the membrane was coated with this solution by the immersion technique for 3 hours. The prepared membranes were freeze-dried according to the method described by Li *et al.* (2011) in a commercially available laboratory freeze dryer (Lyophilization Systems Pvt Ltd, Hyderabad, India). Control membranes were immersed in distilled water for 3 hours and were then freeze-dried. The treatment gives the membranes light to dark yellowish hues upon completion *(Figure 1)*. Freeze drying retains the physical structure and preserves the material for storage or transport. At the same time, freeze-dried materials can be reconstituted quickly and easily, without compromising the original physical or biological properties of the material (Patel and Amiji, 1996). Freeze drying doxycycline (Phaechamud and Charoenteeraboon, 2008) and nanosilver (Yazdimamaghani *et al*., 2014) does not appear to affect the drug release profile and sustainable antibacterial activity when used in controlled-delivery systems.

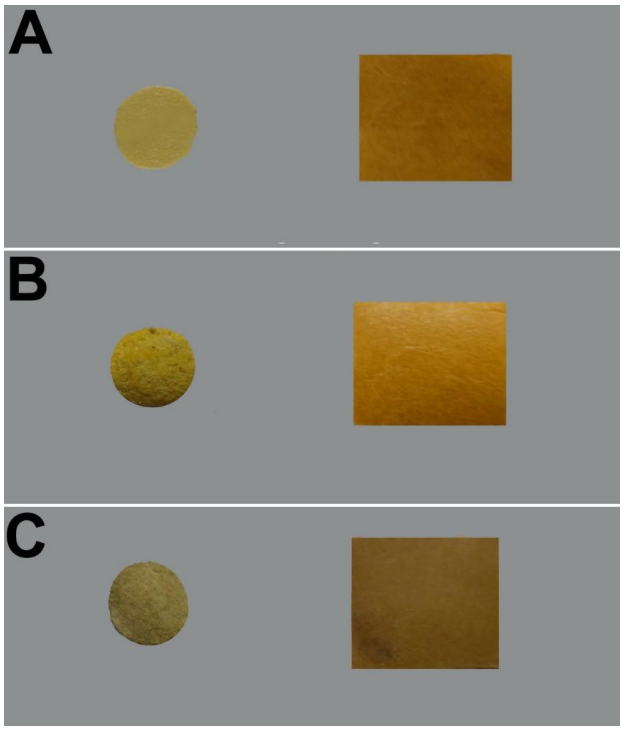

Figure 1. A) Collagen disc (6 mm) and 15 x 12 mm collagen sheet; B) after doxycycline and C) silver nanoparticle impregnation.

Stress–strain behaviour of treated membrane samples

Deposition of extraneous agents on collagen membranes can lead to alterations in collagen membrane architecture resulting in changes of physical properties. Mechanical properties of the collagen membranes were studied as per the protocol described by Charulatha and Rajaram (2003). Seventeen membranes per group were loaded in a tensile tester (TTM-SS®, Simpletech Instruments, Bangalore, India) in a range of 0.1 g to 0.5 kg. Tensile testing was performed at a standard extension rate of 5 mm/min. Tensile strength was calculated from the breaking load and area of cross section and the percentage extension was calculated from the ratio of increase in length to original length.

Bacteria and culture conditions

The bacterial strains used in this study were *Streptococcus mutans* (ATCC 25175), *Aggregatibacter actinomycetemcomitans* (ATCC 29523), *Fusobacterium nucleatum* (ATCC 25586) and *Porphyromonas gingivalis* (ATCC 33277). All four organisms were reported in previous studies to have strong adherence to collagen membranes (Wang *et al.*, 1994). *S. mutans* and *A. actinomycetemcomitans* were cultured in brain heart infusion (BHI) agar/broth at 37°C and incubated in an anaerobic environment with an atmosphere of 75% N_2, 5% CO_2 and 20% H_2 at 37°C. *F. nucleatum* was cultured in modified chopped meat medium and *P. gingivalis* was cultured in supplemented tryptic soy broth/agar; both were incubated in an anaerobic environment with an atmosphere of 80% N_2, 10% CO_2 and 10% H_2 at 37°C.

Qualitative observation of microbial adherence to GTR membranes

Membranes were cut into circular portions of 6 mm in diameter in a sterile environment. Membranes were placed in Eppendorf tubes containing 2 mL of appropriate media for the growth of organisms. All the tubes were arranged on the same horizontal plane. The tubes were inoculated with log-phase pure cultures [3 x 10^4 colony forming units (CFU)] of test organisms grown in broth and incubated under appropriate conditions at 37°C. *S. mutans* and *A. actinomycetemcomitans* were incubated in the CO_2 jar, while *P. gingivalis* and *F. nucleatum* were inoculated in the anaerobic jar. Bacterial adherence to the membranes was scored at the end of 1, 3, 5 and 7 days. Seventeen membranes were used per organism, per time interval across all the three groups in this study. Examination of the membranes was performed by a single blinded examiner (SR) under an inverted binocular microscope using the following visual assessment criteria (Wang *et al.*, 1994); 0 - no visible adherence, 1 - adherence on less than 100% of the surface area of the membrane, 2 - adherence on 100% of the surface area of the membrane and 3 - adherence on 100% of the membrane surface and extending out from the edges of the membrane.

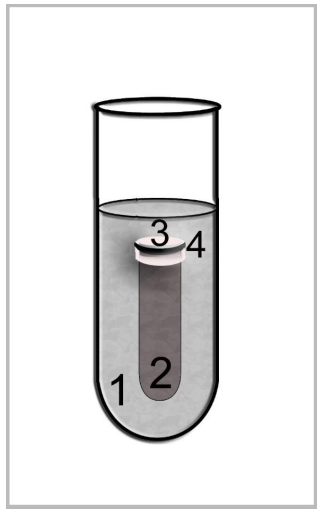

Figure 2. Schematic diagram of the in vitro experimental setup for bacterial permeability assay. 1) Outer tube with growth media inoculated with fixed quantity of bacteria; 2) inner tube (with growth media) placed in the outer tube; 3) guided tissue regeneration (GTR) membrane; and 4) silicon O-ring.

Bacterial penetration through GTR membranes

The permeability experiments were performed under a laminar flow hood in a device as described by Cheng *et al.* (2009). Each membrane was positioned over an inner glass tube filled with the specific growth media for each of the four strains used in this study. The inner glass tubes were sealed with silicon O-rings and were placed in the outer tube *(Figure 2)*. A fresh culture of the microorganisms (3 x 10^4 CFU) in 3 mL of respective

growth media was introduced into the outer bottle and the devices were incubated in the required environment. Over specific time intervals (1, 3, 5, and 7 days), the bacterial counts in the inner tubes were measured by counting on the appropriate agar plates. The presence of colonies in the outer tube were also confirmed on agar plates to avoid false negative and positive results. Colonies of *A. actinomycetemcomitans* and *S. mutans* were counted on BHI agar plates. Colonies of *F. nucleatum* and *P. gingivalis* were counted on blood agar plates.

Statistical analysis

Intragroup comparison was performed using repeated measures analysis of variance (ANOVA) followed by multiple comparisons using Bonferroni correction. One-way ANOVA followed by the post hoc test was used for intragroup and intergroup comparison. A p value of ≤ 0.05 was considered statistically significant, and a p value of ≤ 0.001 was considered as highly significant.

Results

In this study, the microbial adherence and penetration of the four selected microorganisms in their respective membrane groups were evaluated over a period of four time intervals. Seventeen membranes were used per organism, per time interval across the three groups for both microbial adherence and microbial penetration.

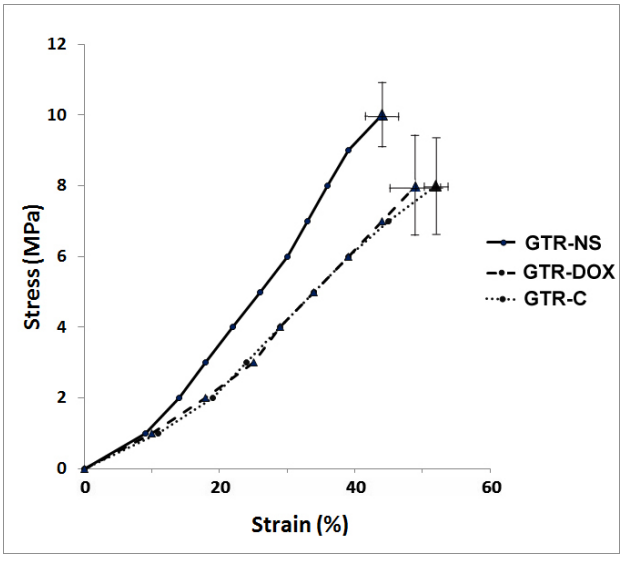

Figure 3. Stress–strain characteristics of control (GTR-C) and doxycycline- (GTR-DOX) and silver nanoparticle (GTR-NS)-impregnated collagen guided tissue regeneration (GTR) membranes. The error bars represent SD across both axes. Silver nanoparticle coating resulted in an increase in the tensile strength while the percentage elongation showed an opposite trend, resulting in a harder and more brittle collagen membrane.

Stress–strain characteristics

Stress–strain curves for control and impregnated collagen membranes are shown in *Figure 3*. As a result of coating, the nanosilver curve sloped more towards the stress axis. GTR-NS showed significantly higher tensile strength (10.01 ± 0.92 MPa; $p \leq 0.001$) over GTR-DOX (8.01 ± 1.4 MPa) and GTR-C (8.02 ± 1.2 MPa). The strain at the point of breakage measured in percentage elongation was maximum for uncoated GTR-C membranes (52.2 ± 2.4%) followed by GTR-DOX (49.17 ± 3.9%) while GTR-NS showed significantly lower strain values (44.01 ± 3.1%; $p \leq 0.001$). No significant differences were found in tensile strength and percentage elongation values between the GTR-C and GTR-DOX groups. The fracture energy was maximum for GTR-NS (2.20 ± 0.05 MJ/m^3) and lowest for GTR-DOX (1.96 ± 0.06 MJ/m^3). The increase in the fracture energy for GTR-NS was highly significant compared to GTR-DOX ($p \leq 0.001$) and significant compared to GTR-C (2.20 ± 0.05; $p \leq 0.05$).

Intragroup comparison of bacterial adherence

For all groups, the increase in mean adherence score at the end of days 3, 5, and 7 was highly significant when compared to that of day 1 ($p = 0.000$; *Table 1*). In the GTR-C group, the increase in mean adherence score at the end of days 3, 5, and 7 was highly significant when compared to that of day 1 ($p = 0.000$) with respect to *S. mutans*. The increase in mean adherence scores by the end of days 5 and 7 was highly significant when compared to day 1 ($p = 0.000$) with respect to *F. nucleatum, P. gingivalis* and *A. actinomycetemcomitans*. However, the increase in mean adherence score on day 3 when compared to day 1 was not significant with respect to these three organisms. In the GTR-NS and GTR-DOX groups, the increase in mean adherence score at the end of days 3, 5, and 7 was highly significant when compared to that of day 1 ($p = 0.000$) with respect to all four microorganisms (*Table 1*).

Intergroup comparison of bacterial adherence

The mean bacterial adherence scores were greater in the GTR-C group when compared to the GTR-NS group for all four microorganisms at all four time intervals. This difference in adherence scores was highly significant ($p \leq 0.001$). The mean bacterial adherence scores were greater in the GTR-C group when compared to the GTR-DOX group at all four time intervals with respect to all four microorganisms; these differences in adherence scores were statistically significant ($p \leq 0.001$). Though the mean bacterial adherence scores were greater in GTR-DOX than in GTR-NS across all time intervals with respect to adherence of all microorganisms, this difference in adherence scores was not statistically significant (*Figure 4*).

Table 1. Intragroup comparison of bacterial adherence scores.

Group (n = 17 per time interval)	**Day**	**Adherence Score (mean ± SD)**			
		S. mutans	***F. nucleatum***	***P. gingivalis***	***A. actinomycetemcomitans***
GTR-C	1	1.11 ± 0.33	1.00 ± 0.00	1.11 ± 0.33	1.00 ± 0.85
	3	2.05 ± 0.24*	1.05 ± 0.24	1.23 ± 0.56	1.05 ± 0.00
	5	2.88 ± 0.33*	2.17 ± 0.39*	2.11 ± 0.48*	3.00 ± 0.24*
	7	2.11 ± 0.33*	3.00 ± 0.00*	2.94 ± 0.24*	3.00 ± 0.00*
		F = 91.119	F = 294.529	F = 68.463	F = 1497.000
		p = 0.000	p = 0.000	p = 0.000	p = 0.000
GTR-NS	1	0.05 ± 0.04	0.05 ± 0.04	0.05 ± 0.04	NG
	3	1.11 ± 0.22*	1.11 ± 0.48*	1.11 ± 0.48*	1.05 ± 0.24*
	5	2.88 ± 0.33*	1.00 ± 0.61*	.88 ± 0.38*	1.64 ± 0.78*
	7	1.58 ± 0.79*	1.58 ± 0.79*	1.64 ± 0.78*	1.47 ± 0.79*
		F = 102.194	F = 21.454	F = 29.065	F = 28.171
		p = 0.000	p = 0.000	p = 0.000	p = 0.000
GTR-DOX	1	0.17 ± 0.09	0.17 ± 0.09	0.11 ± 0.03	0.17 ± 0.09
	3	1.17 ± 0.52*	1.11 ± 0.33*	1.23 ± 0.66*	1.23 ± 0.56*
	5	3.00 ± 0.00*	.88 ± 0.33*	1.05 ± 0.42*	1.76 ± 0.36*
	7	2.00 ± 0.00*	1.82 ± 0.39*	1.70 ± 0.58*	1.76 ± 0.43*
		F = 225.989	F = 59.259	F = 27.937	F = 34.560
		p = 0.000	p = 0.000	p = 0.000	p = 0.000

*Highly significant as compared to the levels at day 1 using Tukey HSD/multiple comparisons. GTR, guided tissue regeneration; C, control; NS, nanosilver; DOX, doxycycline

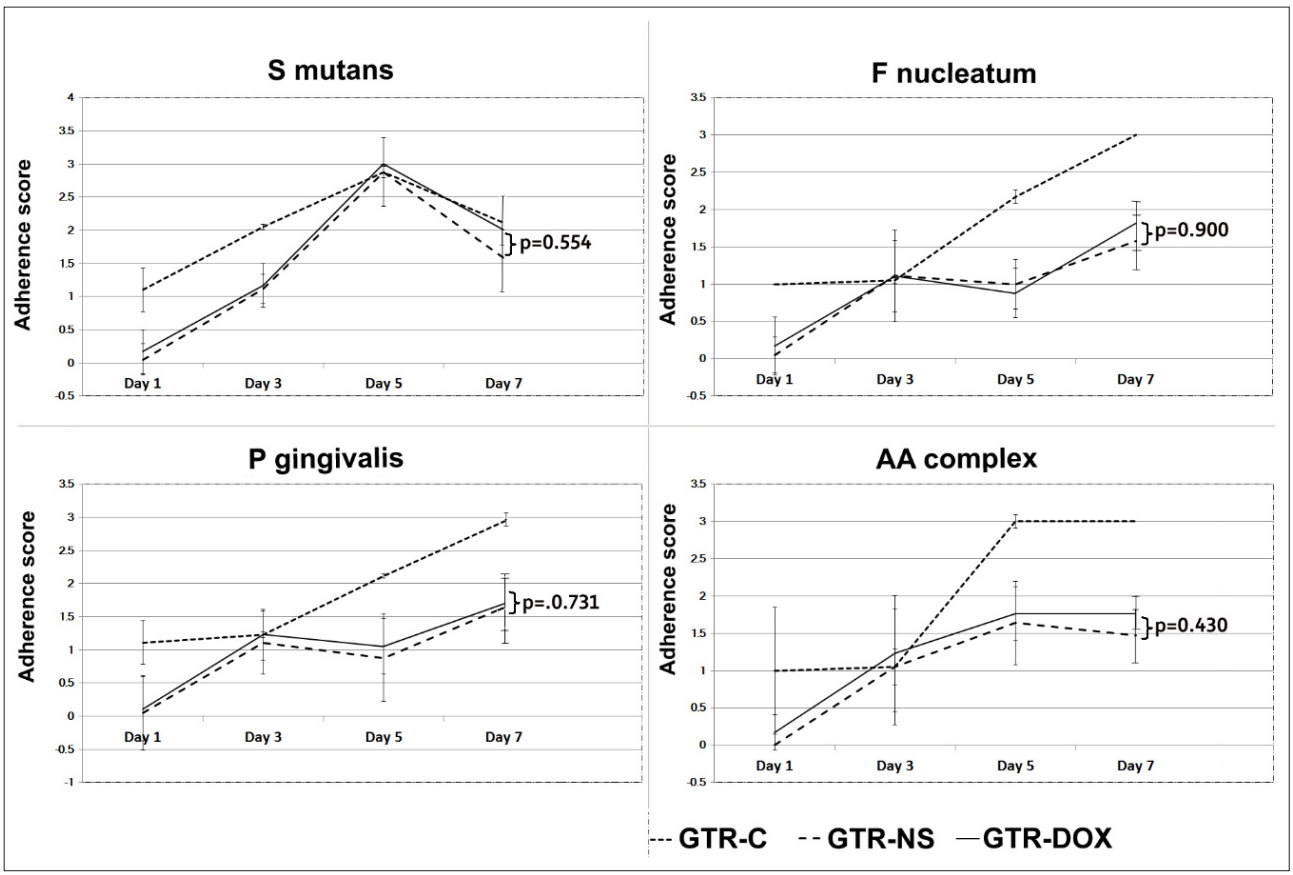

Figure 4. Intergroup comparison of bacterial adherence to control (GTR-C) and doxycycline- (GTR-DOX) and silver nanoparticle (GTR-NS)-impregnated collagen guided tissue regeneration (GTR) membranes. The mean bacterial adherence scores were significantly greater (p < 0.001) in the GTR-C group when compared to GTR-DOX and GTR-NS groups. GTR-NS showed lower adherence scores than GTR-DOX across all four microorganisms, but the difference was not statistically significant.

Table 2. Intragroup comparison of bacterial penetration.

Group (n = 17 per time interval)	Day	CFU/mL x 10^3 (mean ± SD)			
		S. mutans	***F. nucleatum***	***P. gingivalis***	***A. actinomycetemcomitans***
GTR	**1**	250.70 ± 2.93	20.00 ± 0.00	90.00 ± 0.00	121.17 ± 3.86
	3	281.00 ± 2.87*	55.0588 ± 0.55*	199.64 ± 0.99*	199.76 ± 0.66*
	5	252.17 ± 12.15	289.17 ± 8.33*	300.00 ± 0.00*	236.17 ± 60.86*
	7	179.58 ± 7.34*	279.82 ± 4.53*	198.82 ± 3.30*	280.23 ± 3.83*
		F = 580.253	F = 15464.194	F = 42045.205	F = 82.604
		$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$
GTR-NS	1	180.00 ± 0.00	75.00 ± 0.00	NG	15.00 ± 0.00
	3	176.52 ± 6.91	57.94 ± 3.11*	98.70 ± 3.33*	0.94 ± 0.24*
	5	149.76 ± 8.56*	50.05 ± 10.18*	52.17 ± 6.26*	257.94 ± 17.78*
	7	254.47 ± 10.31*	177.82 ± 16.62*	71.47 ± 4.93*	NG*
		F = 587.385	F = 614.350	F = 1580.465	F = 3440.873
		$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$
GTR-DOX	**1**	252.52 ± 7.50	NG	NG	2.00 ± .00
	3	74.76 ± 2.07*	NG	50.94 ± 4.16*	30.47 ± 2.03*
	5	99.17 ± 2.67*	NG	NG	205.88 ± 15.44*
	7	149.64 ± 3.12*	16.47 ± 2.45*	90.00 ± 1.22*	NG
		F = 5445.409	F = 766.748	F = 6903.944	F = 2728.619
		$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$

*Highly significant as compared to the levels at day 1 using Tukey HSD/ multiple comparisons. GTR, guided tissue regeneration; C, control; NS, nanosilver; DOX, doxycycline; NG, no growth

Intragroup comparison of bacterial penetration

The number of CFU/mL cultured from the inner tube on days 3, 5 and 7 were compared with the CFU/mL cultured from the inner tube on day 1 *(Table 2)*. In the GTR-C group, within group comparison across all time intervals revealed statistically highly significant differences in colony forming units ($p = 0.000$) with respect to all four microorganisms. For *S. mutans*, though the number of CFU/mL on day 3 and 5 increased when compared to day 1, the increased difference seen on day 3 only was highly significant ($p \leq 0.001$). The number of CFU/mL on day 7 decreased when compared to day 1; this difference was highly significant ($p < 0.001$). For *F. nucleatum, P. gingivalis* and *A. actinomycetemcomitans,* the increase in the number of CFU/mL of these organisms cultured from the inner tube on days 3, 5 and 7 was highly significant ($p \leq 0.001$) when compared to that of day 1.

In the GTR-NS group, within group comparison across all time intervals revealed statistically highly significant differences in colony forming units ($p = 0.000$) with respect to all four microorganisms. For *S. mutans*, the number of CFU/mL on days 3 and 5 decreased when compared to day 1, though the difference seen on day 5 only was highly significant ($p \leq 0.001$). The number of CFU/mL on day 7 increased when compared to day 1; this difference was highly significant ($p < 0.001$). For *F. nucleatum,* the decrease in the number of CFU/mL cultured from the inner tube on days 3 and 5 was highly significant ($p \leq 0.001$) when compared to day 1, and a highly significant ($p < 0.001$) increase in the number of CFU/mL cultured from the inner tube on day 7 was observed when compared to day 1. For *P. gingivalis,* no growth was observed in the culture from the inner tube on day 1. The increases in the number of CFU/mL cultured from the inner tube on days 3, 5 and 7 were highly significant ($p \leq 0.001$) when compared to day 1. For *A. actinomycetemcomitans*, the decrease in the number of CFU/mL cultured from the inner tube on day 3 was highly significant ($p \leq 0.001$) when compared to day 1. The increase in the number of CFU/mL cultured from the inner tube on day 5 was highly significant ($p \leq 0.001$) when compared to day 1. No growth was seen in the culture from the inner tube on day 7.

In the GTR-DOX group, within group comparison across all time intervals revealed statistically highly significant differences in colony forming units ($p = 0.000$) with respect to all four microorganisms. For *S. mutans*, the decreases in the number of CFU/mL cultured from the inner tube on days 3, 5 and 7 were highly significant ($p \leq 0.001$) when compared to day 1. For *F. nucleatum*, no growth was observed in the culture from the inner tube on days 1, 3 and 5. The number of CFU/mL cultured from the inner tube on day 7 was highly significant ($p \leq 0.001$) when compared to that of day 1. For *P. gingivalis*, no growth was observed in the culture from the inner tube on days 1 and 5. The numbers of CFU/mL cultured from the inner tube on days 3 and 7 were highly significant ($p \leq 0.001$) when compared to that of day 1. For *A. actinomycetemcomitans*, the increase in the number of CFU/mL cultured from the inner tube on days 3 and 5 was highly significant ($p \leq 0.001$) when compared to day 1. No growth was seen in the culture from the inner tube on day 7 *(Table 2)*.

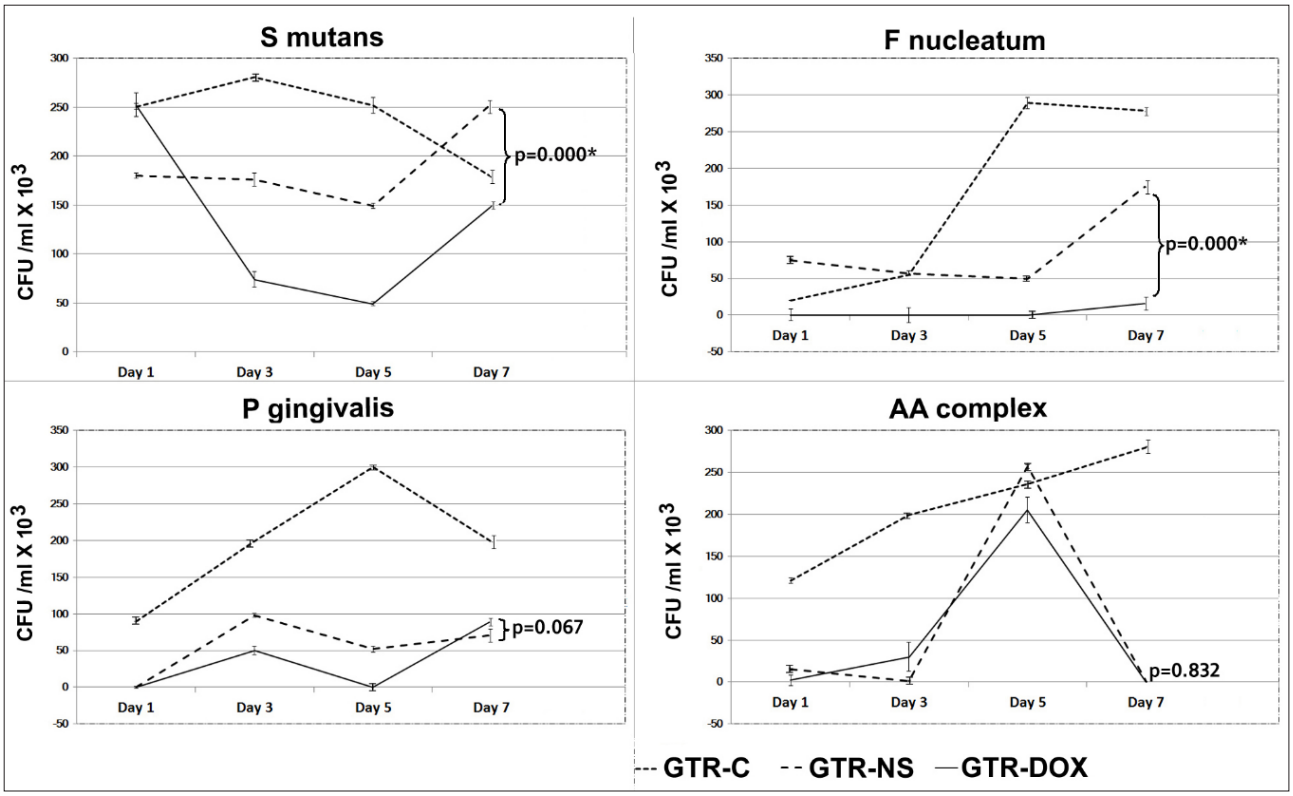

Figure 5. Intergroup comparison of bacterial penetration of control (GTR-C) and doxycycline- (GTR-DOX) and silver nanoparticle (GTR-NS)-impregnated collagen guided tissue regeneration (GTR) membranes. The difference in colony forming units (CFUs) was highly significant (p < 0.001), suggesting greater penetration in GTR-C membranes compared to GTR-NS and GTR-DOX groups. Though the mean CFUs were lower in GTR-DOX than in GTR-NS across all four microorganisms, the difference was statistically significant only with respect to S. mutans and F. nucleatum.

Intergroup comparison of bacterial penetration

The mean CFUs cultured from the inner tube were greater in GTR-C group when compared to mean CFUs of the GTR-NS group at all four time intervals with respect to penetration for all four microorganisms *(Figure 5)*. This difference in CFUs was highly significant ($p \leq 0.001$), suggesting greater penetration in GTR-C membranes when compared to the GTR-NS group. Though the mean CFUs were lower in the GTR-DOX group than in the GTR-NS group at all time intervals with respect to all four microorganisms, this difference was statistically significant only with respect to *S. mutans* and *F. nucleatum*. The mean CFUs were greater in the GTR-C group when compared to the GTR-DOX group at all four time intervals with respect to penetration of all four microorganisms: these differences in penetration scores were statistically highly significant ($p \leq 0.001$; *Figure 5)*.

Discussion

The concentration of silver nanoparticles used in the current study was 100 μg/mL (0.1 mg/mL); this is above the minimum inhibitory concentration (MIC) of silver nanoparticles, which is approximately 0.04 mg/mL for *S. mutans* (Holla *et al*., 2012), 0.003 mg/mL for *F. nucleatum* (Bahador *et al*., 2013) and 0.5 mg/mL for *Actinomyces oris* (Eid and Taha, 2012). Collagen membranes were used as substrate for silver nanoparticle and doxycycline deposition in this study. It is reported that bacterial adhesion decreases as the hydrophobicity of biomaterials increases (Olsson *et al*., 1992). Being more hydrophilic, collagen has an increased propensity towards bacterial colonization by *S. mutans*, *A. actinomycetemcomitans, F. nucleatum* and *P. gingivalis* than other GTR membranes (Cheng *et al*., 2009).

Collagen is a viscoelastic material possessing high tensile strength and low extensibility (Olsson *et al*., 1992). However, improving the multifunctional properties of collagen biomaterial by crosslinking or by loading other nanomaterials may alter its basic physical properties (Charulatha and Rajaram, 2003). A previous study by Chang and Yamada (2000) observed 25% doxycycline-coated membranes used in their study to be harder and fragile. As evident from the stress–strain curve, the strain at the point of breakage was maximum for uncoated membranes (controls) and minimum for silver nanoparticles-coated membranes. At the same time, silver nanoparticle coating resulted in an increase in the tensile strength while the percentage elongation showed an opposite trend. Silver nanoparticles and doxycycline coatings also affected the fracture energies,

with the GTR-NS membranes recording the highest values. Increase in tensile strength with relative decrease in percentage elongation indicates "stiffness" of a material, while increase in fracture energy indicates the ability of a material to absorb energy and plastically deform without tearing or fracturing (toughness)(Charulatha and Rajaram, 2003). When manipulating the membranes for penetration tests, GTR-NS membranes felt harder and slightly brittle, while GTR-DOX and GTR-C membranes showed similar handling characteristics. An ideal GTR membrane must be clinically manageable, rigid and yet elastic at the same time (Cristina and David, 2010). In the event that silver nanoparticle-coated GTR membranes are approved for intraoral clinical use, it would be interesting to know how the alteration in mechanical property will affect its clinical manageability, space making, and eventually, its ability to promote periodontal regeneration.

Potential toxicity associated with the medical use of nanosilver must be given due consideration as well. In an oral toxicity study in rats (Kim *et al*., 2008), silver nanoparticles accumulated in multiple tissues without significant toxicity after oral administration of silver nanoparticles. A uniform consensus on nanosilver toxicity in humans seems to be lacking, as most toxicity investigations of silver nanoparticles are based on *in vitro* cellular experiments and relatively short-term animal experiments (Ge *et al*., 2014).

For periodontopathic organisms to colonize GTR membranes and affect the regenerating site, initial motility and adherence to the membrane and subsequent penetration through the degrading membrane are vital events (Nowzari *et al*., 1995). This study sought to replicate these events by evaluating adherence and penetration of the bacteria through silver nanoparticles- and doxycycline-loaded membranes. Cheng *et al.* (2009) compared bacterial penetration of *A. actinomycetemcomitans* and *S. mutans* through three types of GTR membranes – ePTFE membrane, collagen membrane, and glycolide fiber composite membrane which were impregnated with amoxicillin and tetracycline. The results showed that the inhibitory effect of tetracycline on *S. mutans* was greater than that of amoxicillin for all GTR membranes. Furthermore, the inhibitory effect of tetracycline on *A. actinomycetemcomitans* was lower than that of amoxicillin with the glycolide fiber membrane.

A literature search did not reveal bacterial adherence studies pertaining to specific antibiotic-loaded GTR membranes, making it difficult to compare our results with any other similar study. Within each group, the bacterial adherence results in our study showed a gradual increase in the mean bacterial adherence score over time for all four organisms. Adherence scores for *A. actinomycetemcomitans* and *P. gingivalis* at the end of day 3 in the GTR-C group of the present study are similar to a previous study by Wang *et al.* (1994), whereas the scores obtained for *S. mutans* and *F. nucleatum* were lower in the present study. Wang *et al.* (1994), however, initially placed membranes in tubes containing *S. mutans* and *F. nucleatum* at higher concentrations (1×10^8 *vs.* 3×10^4 CFU in this study), which might have resulted in lower adherence scores in our study.

The *in vitro* bacterial colonization on the plain collagen membranes observed in this study correlates to an *in vivo* supragingival bacterial adherence study on GTR membranes where the four organisms used in this study were found adhering to collagen membranes exposed to the oral environment (Chen *et al*., 1997). Sela *et al.* (1999) demonstrated the adherence of *A. actinomycetemcomitans* and *P. gingivalis* to plain collagen membranes at the end of 24 hrs. Though the present study also demonstrated adherence with respect to the same organisms, the results are not comparable, as estimation of adherence is different in both studies.

At the end of study period, the mean adherence scores in the control group were higher than silver nanoparticles- and doxycycline loaded membranes, suggesting the possible effect of antibiotics in preventing the adherence of bacteria. When overall bacterial adherence among control, doxycycline and silver nanoparticle groups were compared, antibiotic coating resulted in significantly reduced adherence scores compared to controls, strongly suggesting positive effects of doxycycline and silver nanoparticles in inhibiting bacterial adherence. While silver nanoparticles did result in lower adherence values than doxycycline, the difference was not significant. A similar trend was seen in within-group comparisons of penetration values as well; there was a gradual increase in the mean bacterial CFU with time for all four organisms. However, when compared to silver nanoparticles, doxycycline inhibited the growth of *F. nucleatum* and *P. gingivalis* until the end of 5 days. When bacterial penetration among the three groups was compared, antibiotic coating resulted in significantly fewer CFUs, strongly suggesting positive effects of doxycycline and silver nanoparticles in inhibiting bacterial adherence. However, silver nanoparticles showed activity comparable to doxycycline only on *P. gingivalis* and *A. actinomycetemcomitans*.

In an *in vitro* study reported by Ricci *et al.* (1996), penetration of *P. gingivalis* through plain collagen membranes was reported at the end of 24 and 48 hrs. In the present study, the GTR-C group also showed penetration of *P. gingivalis* through the membranes at the end of day 1 and other time intervals. The CFUs/mL in the inner tube of the GTR-C group with respect to *A. actinomycetemcomitans* at the end of 3 days in the present study was 1.99×10^5. A similar value of 1.2×10^5 CFU/mL was reported for the same organism by

Hung *et al.,* (2002) at the end of day 3. The permeability of *S. mutans* too was observed in the same study, though higher CFUs than in the present study were reported at the end of day 1 in the GTR-C group. Only the values of the GTR-C group could be compared to the study of Hung *et al.* (2002), as they did not include antibiotic-loaded membranes as one of the study groups.

In a similarly designed comparative *in vitro* bacterial penetration study through amoxicillin- and tetracycline-loaded collagen GTR membranes, Cheng *et al.* (2009) evaluated the permeability of *A. actinomycetemcomitans* and *S. mutans*. No growth in the inner tube was demonstrated with respect to *A. actinomycetemcomitans* until the end of day 3 in both antibiotic groups. Doxycycline resulted in very minimal penetration at end of day 1, which increased substantially by day 3. No growth in the inner tube was demonstrated with respect to *S. mutans* for 8 hours in the amoxicillin group, and until day 4 in the tetracycline group. In our study, significant growth was demonstrated by the end of day 1.

Nanoparticles are known to have different modes of action on bacteria that make them more effective than conventional antibiotics (Holla *et al.*, 2012). However, in this study, silver nanoparticles showed a comparable, if not superior, action over doxycycline. Compared to silver nanoparticles, doxycycline has an extremely low MIC against periodontopathic bacteria. According to Park *et al.* (2014), the values are as low as 5×10^{-9} mg/mL to 2×10^{-8} mg/mL for *P. gingivalis*, 2.5×10^{-9} mg/mL to 9.7×10^{-7} mg/mL for *F. nucleatum*, and 3×10^{-8} mg/mL to 1.25×10^{-4} mg/mL for *A. actinomycetemcomitans*. The lower MIC might have contributed to the comparable effects seen between silver nanoparticles and doxycycline. In this study, the average silver nanoparticle size in the colloidal solution was 10 nm. In a study by Lu *et al.* (2013), 5 nm silver nanoparticles demonstrated higher antibacterial activity against *A. actinomycetemcomitans, F. nucleatum, Streptococcus mitis, S. mutans* and *Streptococcus sanguis* compared to 15 and 55 nm nanoparticles. A similar effect was also seen in a study by Elechiguerra *et al.* (2005) on HIV-1, where silver nanoparticles of size 1–10 nm showed increased binding to the gp120 glycoprotein knobs, inhibiting the virus from binding to host cells. The effect of this size-dependent interaction of silver nanoparticles on the overall effects on the tested organisms may be a contributory factor to the lower than expected results.

The present study has limitations that need to be considered when interpreting the results. Because a GTR membrane, when placed, is sandwiched between the bone and the gingival corium, the inherent toxicity of nanoparticles will become a deciding factor during the post-operative healing phase (Sondi and Salopek-Sondi, 2004). Elemental silver is cytotoxic and may directly damage DNA, denature proteins and enzymes and produce reactive oxygen species (ROS; Kim *et al.*, 2008). However, studies on dental materials incorporating silver have not reported cases or communications on the potential side-effects of nanosilver usage (Ge *et al.*, 2014). Thus, this study only determines the colonization and penetration of specific bacteria on nanosilver-impregnated GTR membranes and the results must be interpreted as such. While *in vitro* testing of microbial susceptibility can be an invaluable tool, an *in vitro* setup cannot replicate factors such as the host environment or the pharmacokinetics of the antimicrobial agent (Bahador *et al.*, 2013). However, *in vitro* bacterial studies are still preferred in situations studying the adhesion and penetration ability of bacteria (Jones *et al.*, 2004) and on this principle, an *in vitro* culture broth method was utilized in the current study.

Silver nanoparticles were as effective as doxycycline in controlling bacterial adherence to GTR membranes but showed a comparable activity to doxycycline in preventing penetration of *P. gingivalis* and *A. actinomycetemcomitans* only. From the present study, it can be concluded that incorporation of silver nanoparticles into the membrane may be of value when controlling membrane-associated infection during GTR therapy. Further studies with different particle sizes of silver nanoparticles are to be conducted to emphasize the beneficial properties of silver nanoparticles against periodontal pathogens.

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