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# Antimicrobial efficacy of neutral super-oxidized electrolyzed gel versus chlorhexidine digluconate 0.12% in biofilm formation on orthodontic mini-implants: An *in vitro* study

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**The objective of this study was to compare the antimicrobial efficacy and cytotoxicity in fibroblasts of neutral super-oxidized electrolyzed gel and chlorhexidine digluconate. Cultures were prepared with *Streptococcus intermedius*, *Porphyromonas gingivalis* and a mixture of both. Thirty sterile orthodontic mini-implants were impregnated with neutral super-oxidized electrolyzed gel and chlorhexidine digluconate for 10 min, then immersed in culture media and incubated at 37°C for 24 h. Samples were taken for colony forming units (CFU), turbidity tests to determine bacterial absorbance and concentration, and for cytotoxicity testing in fibroblasts. Neutral super-oxidized electrolyzed gel had a lower inhibitory effect on *S. intermedius* with a statistically significant bacterial absorbance and CFU (P = 0.012 and P = 0.000, respectively). This was also observed for *P. gingivalis* with a statistically significant CFU (P = 0.000). A similar behavior was found with the mixture of *S. intermedius* and *P. gingivalis* with a significant bacterial absorbance and CFU (P = 0.003 and P = 0.000, respectively). Chlorhexidine digluconate showed no bacterial growth and a greater cell cytotoxicity (22.08% cell viability), compared with super-oxidized gel (97.16%). Super-oxidized gel inhibited bacterial growth around the mini-implant. Chlorhexidine digluconate acted as a bactericide. Chlorhexidine digluconate had a greater cytotoxic effect when compared with neutral super-oxidized electrolyzed gel.**

**Key words:** Mini-implants, chlorhexidine, neutral super-oxidized electrolyzed gel, bactericide.

## INTRODUCTION

Current orthodontics has systematized the placement of mini-implants as part of a treatment plan, primarily to obtain and improve skeletal anchorage and to reduce the need for patient co-operation. Peri-implantitis is an inflammation of the mucosa that surrounds the implant and has clinical and radiographic evidence of bone loss, bleeding upon probing, suppuration, epithelium infiltration and progressive mobility, which affects the soft tissue and supporting bone around a functioning implant (Kravitz et al., 2007). Chlorhexidine is a positively ionized bisbiguanide

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that absorbs different negative charges from the site where it is used, such as the mucus membrane, the Salivary film on teeth, and titanium surfaces, as well as the different components of the biofilm on the tooth surface (bacteria, extracellular polysaccharides and glycoproteins) (Zanatta et al., 2007). Chlorhexidine is used after mini-implant placement as an antibacterial and to minimize tissue inflammation. It promotes a slow epithelialization and may reduce the possibility of increased soft tissue around the mini-implant (Kravitz et al., 2007).

*In vitro* studies (Zanatta et al., 2007; Longworth, 1964; Xie, 2000; Kozlovsky, 2006) have shown that at low concentrations, chlorhexidine causes cell membrane damage and leakage of low molecular weight molecules of microorganisms. In contrast, at high concentrations, it causes protein precipitation and coagulation in the cytoplasm of the exposed microorganisms. These properties interfere with biofilm formation and prevent its growth.

Gianelli et al. (2008) found that chlorhexidine affects cell viability depending on the time of exposure, particularly in osteoblasts. Its toxic effect is the induction of apoptosis and autophagocytosis of dead necrotic cells involving damage of mitochondrial function, an increase in intracellular $Ca^{+2}$, and cell oxidation. This suggests that chlorhexidine is highly cytotoxic *in vitro* and that precaution is necessary when used as an antiseptic in surgical procedures of the oral cavity.

Studies report that the mechanism of action of super-oxidized gel is the oxidation of sulfhydryl and amino groups of the bacterial wall, which affects the respiration and nutrition process of microorganisms, resulting in oxidation of respiratory components, inhibition of protein synthesis, and altered cell metabolism with decreased production of high energy phosphates (adenosine phosphate), regardless of the breakage of chains and repression of RNA synthesis (Esteripharma Mexico, 2012). Super-oxidized solutions have been found effective in wound care and *in vitro* studies have demonstrated activity against different bacteria, viruses, and spores (Gutierrez, 2006; Tanaka et al., 1996). The potential toxicity of superoxide solution has also been studied in fibroblast cultures comparing hydrogen peroxide versus pH-neutral superoxide solution. It was found that superoxide solution is significantly less cytotoxic than antiseptic hydrogen peroxide (Gonzalez-Espinoza et al., 2007).

It was considered important to conduct a prospective experimental, longitudinal and comparative *in vitro* study of the efficacy of neutral super-oxidized electrolyzed antimicrobial gel (EsteripHarma Mexico, SA de CV, Mexico City, Mexico) and chlorhexidine digluconate 0.12% (Farmacia Morlan, Toledo, Spain) in biofilm formation and their cytotoxic effect on fibroblasts, because there are no previous reports in the literature comparing the antimicrobial and cytotoxic properties of super-oxidized gel and chlorhexidine digluconate 0.12%.

## MATERIALS AND METHODS

### Bacterial inoculation of samples

A prospective, experimental, longitudinal and comparative study was conducted of 30 orthodontic mini-implants in three different bacterial culture media (*Streptococcus intermedius, Porphyromonas gingivalis* and a bacterial mixture of *S. intermedius* and *P. gingivalis*). To determine the sample size and the experimental methodology with regard to obtaining bacterial cultures, we used the previous experience with bacterial cultures of Ferraz et al. (2007) and Chin et al. (2007).

Sterile titanium orthodontic mini-implants (Ancoragem Ortodôncica®, Neodent, Curitiba, Brasil), tripticasein culture medium, and sterile chemicals products (neutral super-oxidized electrolyzed gel and chlorhexidine digluconate 0.12%) were used. Titanium orthodontic mini-implants that were contaminated or damaged or that had contaminated culture medium, or contaminated chlorhexidine digluconate and super-oxidized gel solutions were excluded.

The variables studied were bacterial absorbance, bacterial cell concentration, colony forming units (CFU), and cytotoxicity. Bacterial cultures of *S. intermedius*, *P. gingivalis*, and a mixture of *S. intermedius* and *P. gingivalis* were prepared. Once bacterial cultures were active, we proceeded to conduct the experiment in 50 sterile Eppendorf tubes. Samples were divided into 6 groups and controls were included.

### Bacterial count analysis

The bacterial suspension was pipetted onto a microscope slide, through the edge of the cover slip, filling the counting chamber by capillarity. Within minutes, the cells precipitated to the bottom and counting began. Bacterial cells from each of the 25 larger squares drawn on the slide were counted. The bacteria from several large squares were counted and means were obtained. The number present in the largest square multiplied by 25 is the number present in 0.02 $mm^3$. The number multiplied by 50 is the number in 1 $mm^3$. This number times 1000 is the number in 1 ml. If the number of cells in a small square is counted, this number is multiplied by a factor of 16. Example: 16 × 25 × 50 × 1000 = 20,000,000.

#### *Group 1*

The study started with 5 Eppendorf tubes to which 50 µl of bacterial broth of *S. intermedius* (Si) was added. Subsequently, 1 sterile mini-implant was deposited in each of the tubes for 1 min to impregnate them with bacteria. Following this, we impregnated the mini-implants with neutral super-oxidized antiseptic gel for 1 min and then carefully removed the gel irrigating with sterile double distilled water. Finally, each mini-implant was placed in an Eppendorf tube containing 1000 µl of sterile broth culture and samples were incubated at 37°C for 16 h.

#### *Group 2*

The study started with 5 Eppendorf tubes to which 50 µl of bacterial broth culture of *P. gingivalis* (Pg) were added; afterwards, the same procedure as for group 1 was conducted.

#### *Group 3*

We started with 5 Eppendorf tubes to which 50 µl of bacterial broth

culture of *S. intermedius* and *P. gingivalis* were added, carrying out the same procedure as for groups 1 and 2.

***Group 4***

The same procedure was performed as for group 1 using chlorhexidine digluconate 0.12%.

***Group 5***

The same procedure was performed as for group 2 using chlorhexidine digluconate 0.12%.

***Group 6***

We carried out the same procedure as for group 3 using chlorhexidine digluconate 0.12%.

***Negative controls***

Negative controls were carried out to confirm the sterility of the culture medium, of the mini-orthodontic implant, and of the study chemicals. Samples were incubated at 37°C for 24 h.

***Positive controls***

These controls were performed to verify that there was bacterial growth in the different bacteria broth cultures used in the study. Samples were incubated at 37°C for 24 h.

After 24 h, real-time polymerase chain reaction (PCR) was performed for DNA extraction to identify bacterial colonies. This was done to confirm that the bacteria had been inoculated by first intention, and were present at the end of the experiment. Likewise, spectrophotometry was carried out to identify turbidity of the culture medium in order to quantify absorbance and concentration of each of the samples.

**Turbidity test by absorbance**

A Smart Spec™ Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA) was used, in which a cuvette with a concentration of 50 µl of sterile double distilled water for 45 µl of bacterial sample was placed. Absorbance and concentration were then measured to confirm inhibition of bacterial growth around the mini-implant when neutral super-oxidized electrolyzed gel and chlorhexidine digluconate were used.

**Cytotoxicity measurement in fibroblasts**

To assess the cytotoxic effects of neutral super-oxidized electrolyzed gel and chlorhexidine digluconate, normal human cell lines (ATCC Hs68) were used. We placed 50,000 cells per well in a 16 chambered slide containing different concentrations of chlorhexidine and super-oxidized electrolyzed gel in media culture. These were grown for 24 h. After incubation new media was added to wash the cells and eliminate the drugs. Phosphate buffered saline (PBS) was used to replace media culture and perform morphologic analysis. Buffered formalin (4%) was used as a fixative, and an autofluorescence inductor were mixed in the chamber with PBS. Ten minutes later, cells were washed with PBS again and the remaining autofluorescence inductor was eliminated. Slides were mounted with fluoroshield and analyzed with a Zeiss LSM 710 confocal microscope using a 360 nm line laser to excite 4',6-diamidino-2-phenylindole (DAPI) and a 514 one to excite autofluorescence of cytoplasm induced by the fixative. Morphologic analysis to evaluate the effect of drugs on the cytoplasm was implemented. Images of negative controls and cell cultures exposed to different drug concentrations were acquired in the same conditions.

**Absorbance, concentration and CFU**

To determine differences in absorbance, concentration, and CFU relative to the bacterial dilution between treatments we applied simple linear regression and then the slopes obtained were compared for the following groups: (1) Si + super-oxidized gel, (2) Si + chlorhexidine digluconate, (3) Pg + super-oxidized gel, (4) Pg + chlorhexidine digluconate, (5) Si + Pg + super-oxidized gel, (6) Si + Pg + chlorhexidine digluconate.

**Statistical analysis**

Simple linear regression was used to correlate absorbance to the logarithm of the dilution and concentration, and also to the logarithm of CFU with the logarithm of dilution.

## RESULTS

### Cytotoxicity

A clear difference in the toxicity of chlorhexidine was observed. The test was carried out for only 2 h, because if cells were left longer, there would be no living cells to count and make a comparison.

Chlorhexidine digluconate 0.12% showed a high degree of cytotoxicity causing a considerable decrease in cell viability in comparison to neutral super-oxidized electrolyzed gel, which had a lower percentage (Figures 1 to 6).

### Results for absorbance, concentration and CFU explained by treatment

Simple linear regression was used to correlate absorbance to the logarithm of dilution, and concentration and also, the logarithm of CFU with the logarithm of dilution (Table 1).

### *Group 1*

For Group 1 (Si + super-oxidized gel), taking into consideration zero dilution, it was found out that although there was a certain bacterial growth, absorbance was not statistically significant (P = 0.156).

When compared with the same treatment eliminating the zero dilution (that is, the experiment), it was found that there was bacterial growth, which reflected in a statistically significant absorbance level (P = 0.012). The

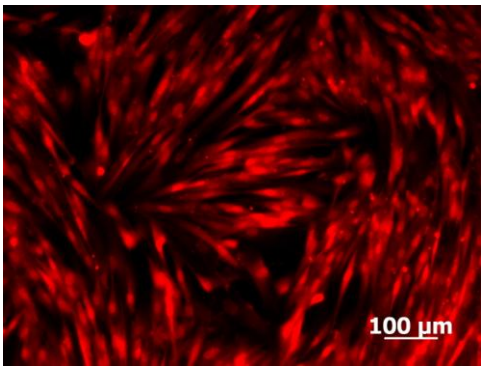


**Figure 1.** Mesenchymal cells of human dental pulp without treatment in culture medium, control group (scale bar 100 µm).

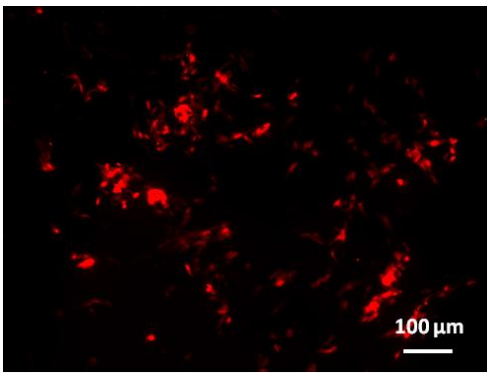


**Figure 2.** Mesenchymal cells of human dental pulp after exposure to chlorhexidine 0.12% for 30 s (scale bar 100 µm).

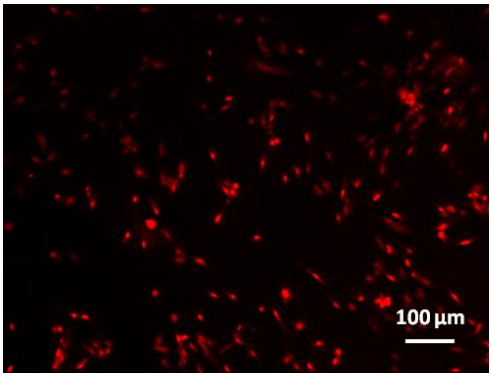


**Figure 3.** Mesenchymal cells of human dental pulp after exposure to chlorhexidine 0.12% for 1 min (scale bar 100 µm).

concentration for this treatment, although it showed some bacterial growth, was not statistically significant (P = 0.318). Instead, the CFU bacterial count was highly significant (P = 0.000), demonstrating that super-oxidized

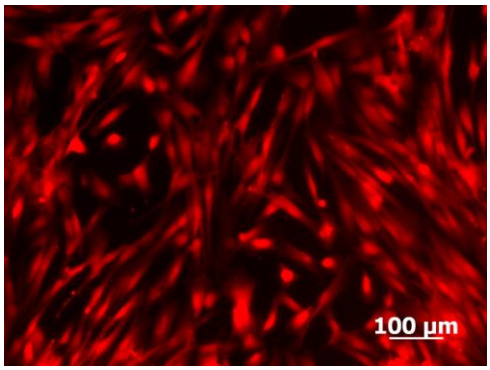


**Figure 4.** Mesenchymal cells of human dental pulp after exposure to neutral super-oxidized electrolyzed gel 15 ppm for 30 s (scale bar 100 µm).

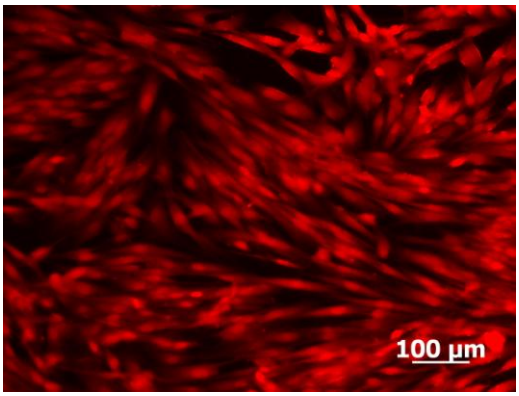


**Figure 5.** Mesenchymal cells of human dental pulp after exposure to neutral super-oxidized electrolyzed gel 15 ppm for 1 min (scale bar 100 µm).

gel did not inhibit *S. intermedius* cell growth in the same measure as chlorhexidine, therefore, it was concluded that there was some bacterial growth with super-oxidized gel.

### *Group 2*

In Group 2 (Pg + super-oxidized gel), taking into account the zero dilution, it was found that despite the observed bacterial growth, absorbance (P = 0.119) was not statistically significant.

When compared with the same treatment eliminating the zero dilution, a statistically significant absorbance was not found (P = 0.095). This means that there was growth of *Pg* with super-oxidized gel. However, the bacterial count of CFU was highly significant (P = 0.000), demonstrating that with super-oxidized gel, growth of *P. gingivalis* was not inhibited (Figure 7).

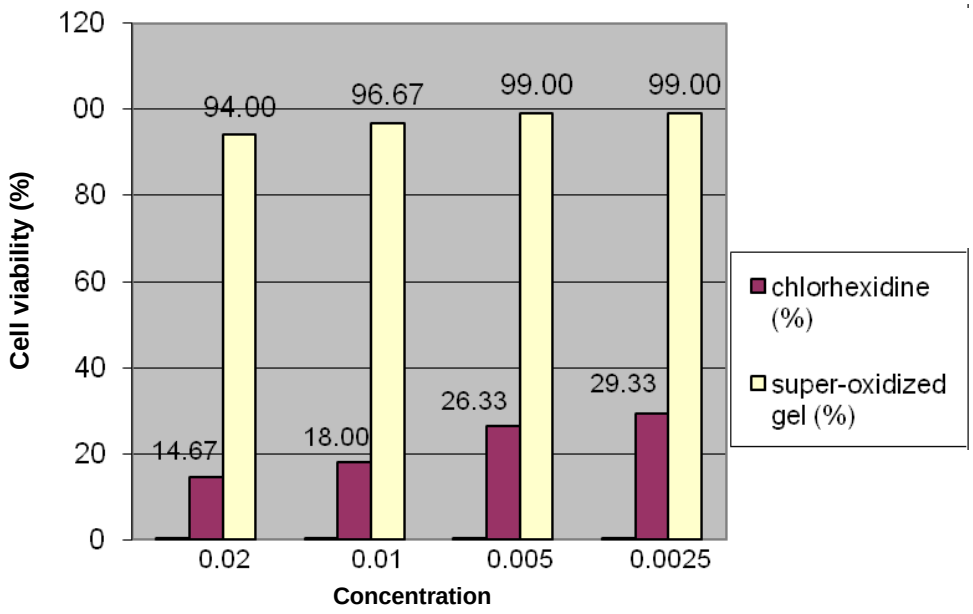


**Figure 6.** Comparison of cell viability. Chlorhexidine has greater toxicity than neutral super-oxidized electrolyzed gel since there is a greater percentage reduction in cell viability with chlorhexidine with a range of 79.33 with regard to the greater concentration and of 69.67 with regard to the lesser concentration of both products.

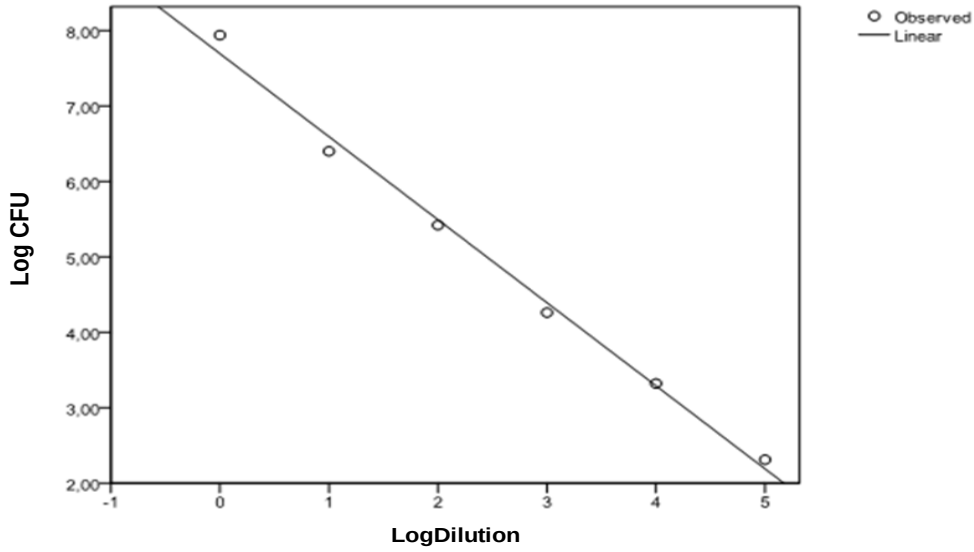


**Figure 7.** Number of colony forming units for Group 2.
Equation: Log = 7810 1082 (log dilution), F = 4854,898, P = 0.000.

### *Group 3*

In Group 3 (Si + Pg + super-oxidized gel), taking into account the zero dilution, it was found that, notwithstanding bacterial growth, absorbance was not statistically significant (P = 0.083) (Figure 8). When compared with the same treatment eliminating the zero dilution, it was found out that growth of the bacterial mixture was such that a highly significant absorbance (P = 0.003) was found. Despite the observed bacterial growth, the concentration for this treatment was not statistically significant (P = 0.639). However, the CFU bacterial count was highly significant (P = 0.000), which represents a lack of effectiveness of super-oxidized gel

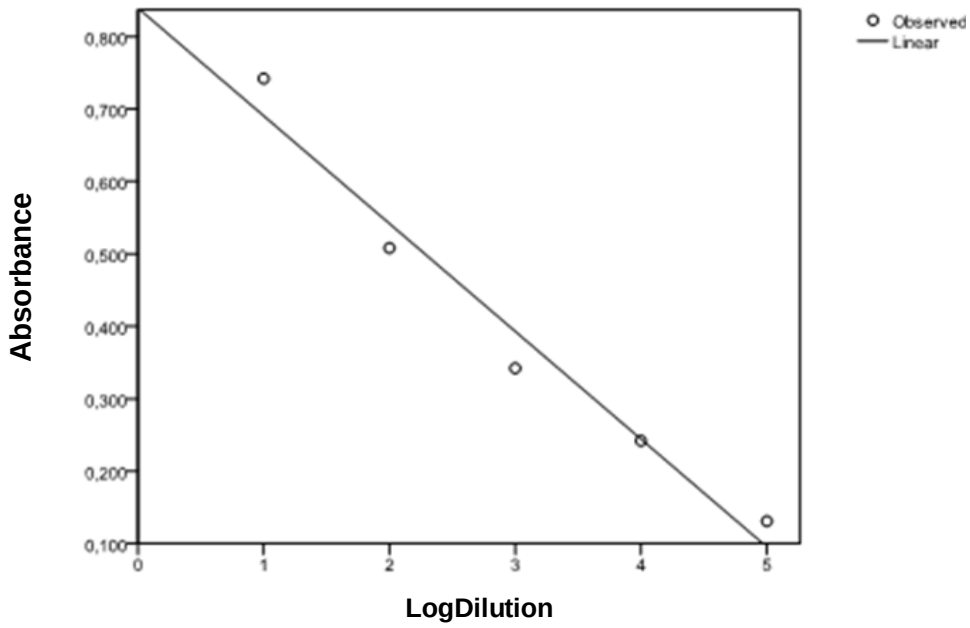


**Figure 8.** Results of absorbance for Group 3 eliminating the zero dilution.
Equation: Absorbance = 0042 - 0004 (LogDilution), F = 29 547, P = 0.012.

**Table 1.** Correlation of absorbance with the logarithm of dilution and concentration, and correlation of the logarithm of CFU with the logarithm of dilution.

| Group | Function or regression correlation | Condition | Significance |
|---|---|---|---|
| 1 | Absorbance = F (log of dilution) | With dilution = 0 | NS |
| | | Without dilution = 0 | * |
| | Concentration = F (log of dilution) | - | NS |
| | Log of CFU = F (log of dilution) | - | ** |
| 2 | Absorbance = F (log of dilution) | With dilution = 0 | NS |
| | | Without dilution = 0 | NS |
| | Concentration = F (log of dilution) | - | NS |
| | Log of CFU = F (log of dilution) | - | ** |
| 3 | Absorbance = F (log of dilution) | With dilution = 0 | NS |
| | | Without dilution = 0 | ** |
| | Concentration = F (log of dilution) | - | - |
| | Log of CFU = F (log of dilution) | - | - |

NS = not significant; * = significant ($P < 0.05$); ** = highly significant ($P < 0.01$)

on bacterial mixtures.

### *Group 4*

In Group 4 (Si + chlorhexidine), it was found that all the variables measured were zero, that is, there was no bacterial growth, which means that chlorhexidine digluconate had a bactericidal effect against *S. intermedius*.

### *Group 5*

In Group 5 (Pg + chlorhexidine), it was found that all the variables measured were zero, that is, there was no bacterial growth, which means that chlorhexidine digluconate

is effective in inhibiting proliferation of *P. gingivalis*.

### *Group 6*

For Group 6 (Si + Pg + chlorhexidine), it was found that all the variables measured were zero, that is, there was no bacterial growth.

## DISCUSSION

We compared the antimicrobial and cytotoxic properties of neutral super-oxidized electrolyzed gel and chlorhexidine digluconate 0.12% and found that neutral super-oxidized electrolyzed gel inhibits biofilm formation and acts as an antibacterial agent on the surface of orthodontic mini-implants.

Kravitz and Kusnoto (2007) determined that to prevent ulceration of tissue around the mini-implant and improve patient comfort, the use of chlorhexidine 0.12% (10 ml) is recommended. This is because ulceration can cause severe inflammation of tissue. This is why chlorhexidine is used after mini-implant placement. It is also used to minimize tissue inflammation, promote slow epithelization and reduce the possibility of increased soft tissue around the mini-implant. In this study, chlorhexidine digluconate 0.12% was employed around the surface of mini-implants and it was found out that its antibacterial properties were significantly greater (CFU = 0) when compared with neutral super-oxidized gel; however, super-oxidized gel was less cytotoxic. Our results showed that there was no bacterial growth in any of the mini-implants used. In contrast, Dennison et al. (1994) found that chlorhexidine had poor efficacy in removing bacteria from the implant surface treated with hydroxyapatite.

Järvinen et al. (1993) conducted a study that demonstrated the susceptibility of *Streptococcus mutans* to chlorhexidine and six other commonly used antibacterial agents such as amoxicillin, cefuroxime, penicillin, sulfamethoxazole, trimethoprim, tetracycline and erythromycin. It was found out that the bacteria exposed to various antimicrobial agents remained susceptible to all, and more importantly to chlorhexidine. The results obtained in the studies mentioned earlier agree with those obtained in this study with regard to chlorhexidine.

In an *in vitro* study conducted by De Baun (2008), in order to prove the antimicrobial properties of chlorohexidine digluconate against seven different bacterial samples, it was found out that this compound reduced bacterial content after 3 min of exposure and its efficacy continued. In this study, the antimicrobial effectiveness of chlorhexidine digluconate was compared only against neutral super-oxidized gel. The results showed that chlorhexidine had a better response by significantly reducing bacterial counts around the mini-implant surface. Other *in vivo* studies conducted by Persson et al. (2007) and by Paolantonio et al. (2008) also found that chlorhexidine has important antimicrobial properties.

Noiri et al. (2003) conducted an *in vitro* study that examined the effects of chlorhexidine on *P. gingivalis* biofilms, finding that the extracellular matrix of the latter was altered in the presence of this substance. This coincides with the results found in this research although when applying neutral super-oxidized gel, this bacterium had greater resistance.

The susceptibility to chlorhexidine of various types of bacterial samples was demonstrated in a study by McBain et al. (2003). The most susceptible was *Actinomyces naeslundii* followed by Gram-negative anaerobic bacteria such as *Prevotella nigrescens*, *P. gingivalis*, *S. mutans* and *Streptococcus sanguinis*. The results of this study are similar to those in the present study in which chlorhexidine showed a bactericidal effect on the microorganisms used.

Melsen (1986) conducted research that measured the reaction of periodontal and gingival tissues to intrusion forces applied to teeth, as well as the influence of oral hygiene in *Macaca fascicularis* monkeys, using chlorhexidine. The results showed that on the side of hygiene, there were clear signs that bone deposition remained present after the applied eruption forces, something that did not occur with hygiene. This suggests that chlorhexidine inhibits bacterial growth, as was demonstrated in our *in vitro* study.

The findings of this study with regard to cytotoxicity coincide with studies performed by Zanatta et al. (2007), and Kozlovsky et al. (2006) in which it was found that low concentrations of chlorhexidine cause cell membrane damage and the release of low molecular weight molecules of microorganisms. With regard to cytotoxicity, there is contradiction in the literature. Campos et al. (2010) showed that in all concentrations, chlorhexidine had a high direct cytotoxic effect on cell cultures. This is consistent with the results in this study, since chlorhexidine was shown to be cytotoxic with increasing concentrations. In contrast, Bonacorsi et al. (2004) reported that chlorhexidine did not have immunostimulatory activity and subtoxic concentrations did not affect macrophage response, a condition that we did not analyze in this study.

Definitely, one of the studies whose results are similar to those obtained in this investigation is that of Gianelli et al. (2008) who found that chlorhexidine affects cell viability depending on the time of exposure particularly osteoblasts; in our study, this condition was analyzed in fibroblasts. These authors explained the induction of apoptosis and autophagocytosis of necrotic cells as the cause of the cytotoxic effect and also implicated mitochondrial function, increased intracellular $Ca^{+2}$, and cellular oxidation. In our study, as in that of Gianelli, it was concluded that chlorhexidine is highly cytotoxic *in vitro* and dentists are urged to use caution in oral cavity

procedures.

The potential toxicity of super-oxidized solutions was studied by Gonzalez-Espinosa et al. (2007). These researchers compared the solution with hydrogen peroxide in dermal fibroblasts. They found out that hydrogen peroxide was more toxic. The super-oxidized solution had less effect on cell viability and genotoxicity. Cell viability in our study was >90% after 30 s and 1 min. This is in contrast with Gonzalez-Espinosa et al (2007) who found a viability of 75 and 70% after 5 and 30 min of exposure, respectively. This discrepancy is probably due to the difference in exposure times.

## Conclusions

Neutral super-oxidized electrolyzed gel inhibits biofilm formation on the surface of orthodontic mini-implants and acts as an antibacterial agent. In summary, super-oxidized gel offers a nonirritating inhibitory benefit in cells allowing a regenerative therapeutic effect, which is healthier since this promotes internal recovery.

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### Abbreviations

**CFU,** Colony forming units; **Si,** *Streptococcus intermedius*; **Pg,** *Porphyromonas gingivalis*.

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