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In vitro analysis of antimicrobial activity in exposed collagen membranes applying chitosan-metronidazole in topical gel

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Abstract
Guided Bone Regeneration through the use of a membrane has been a Gold standard to obtain the best results, in turn, being a sensitive technique whose main complication is its exposure and infection by pathogenic periodontium bacteria.
Objective: To observe the antimicrobial effect of topical chitosan-metronidazole on *Porphyromona gingivalis*.
Materials and Methods: Topical Chitosan-Metronidazole was prepared with which it is antimicrobial sensitivity was observed in the crop medium of Müller-Hinton agar with the well diffusion method after having applied 50 microliters of *P. gingivalis*, 8mm was extracted with a punch and filled with chitosan-metronidazole, the inhibitory halo was analyzed to obtain results.
Results: The inhibitory halo was observed and measured in the two samples which is 20 mm for both, obtaining a sensitive result.
Conclusion: Topical chitosan-metronidazole was shown to have a good effect on *P. gingivalis*.

Keywords: Chitosan, metronidazole, *Porphyromona gingivalis*

Introduction
Different strategies, such as bone grafting techniques, alveolar distraction, and guided bone regeneration (GBR), have been applied to restore lost bone and allow the implant to fully integrate and be maintained during functional loading [1]. The regeneration procedure can promote healing of the bone defect after 6 to 10 months [2].
Resorbable membranes demonstrate some advantages over non-resorbable devices: there is no need to remove the membrane, the surgical procedure is simpler, there are fewer complications with lower patient morbidity, and they are less expensive. However, when defects are not sufficient, bone regeneration using resorbable materials has drastically reduced effectiveness. In these "critical" bone defects, non-resorbable devices were found to have better capabilities to achieve successful regeneration thanks to membrane stiffness, controlled time of barrier function, and lack of reabsorption process of the device.

- A horizontal bone defect within the bone contour (horizontal critical size defect): implant placement with simultaneous alveolus augmentation using a resorbable membrane associated with a composite graft (made of autogenous bone graft and bovine bone mineral).
- A horizontal bone defect that extends outward from the bone contour (de novo formation): implant placement with simultaneous contour augmentation using a non-resorbable membrane associated with a composite graft
- A combined horizontal and vertical bone defect (de novo formation): guided bone regeneration with a non-resorbable membrane associated with a composite graft and delayed implant placement (6 to 9 months) [3].

Despite scientific evidence demonstrating that ROG with a PTFE membrane with titanium mesh is a successful and predictable technique for horizontal as well as vertical regeneration.

Using a barrier has several potential drawbacks. The most common complication is premature exposure of the membrane to the oral environment due to wound dehiscence, infection, and its sequelae [3, 4].

Exposed membranes accumulate bacterial plaque associated with inflammation of adjacent gingival tissue within approximately one month of exposure [8, 21].

Limited bacterial invasion through membrane thickness begins after the second week of exposure [8].

Chitosan is a copolymer of D-glucosamine and N-acetyl-D-glucosamine, it is commonly derived by the N-diacetylation of chitin, found in crustaceans and shelled insects [22, 23]. The effects of chitin and chitosan on wound healing have had a focus of great attention since Prudden *et al*. 1970, published a study indicating that N-acetylglucosamino polymers have effects on the acceleration of healing, it has also been shown to have bioactive properties such as antimicrobial, hemostatic, active tissue regeneration ligating osteoblasts that eventually in the wound increase osteo conductivity and neovascularization, followed by acceleration of bone increase [23, 44, 45].

Metronidazole is a compound of nitroimidazole, it was developed in France during the 50's for the treatment of protozoan infections. Metronidazole has been seen effective against *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, Bacteroides forsythus, Treponema, *Actinobacillus actinomycetemcomitans*, Campylobacter rectus, Enterobacterias, Pseudomonas, *Actinomyces israelii*, Staphylococcus sanguis, S. mutans, S. mitis, S. intermedius [18, 32]. A 25% metronidazole dental gel has been developed for local application within the periodontal pocket [34].

In one study indicate that a mixture of 1.5% chitosan with 25% metronidazole gel is thermoreversible, mucoadhesive, injectable and releases the drug slower up to 48 hours. The efficacy of the drug in situ gel on Gram + and Gram- microorganisms, cocci and bacilli has also been observed [18].

Materials and Methods

Preparation of the topical of chitosan-metronidazole

The preparation of the chitosan-metronidazole topical and the sensitivity test by the well diffusion agar method were carried out in the laboratories of the Faculty of Biological Sciences Torreón Unit, of the Autonomous University of Coahuila.

Materials

1. Beaker 250 ml (1)
2. Glass rod (1)
3. Spatula (1)
4. Magnetic stirrer (1)
5. Beaker 100 ml (1)
6. Test tube 100 ml (1)
7. Piseta (1)
8. Pipette 5 ml (2)
9. Syringe (2)

Team

1. pHmeter.
2. Electric autoclave.
3. Analytical balance.
4. Thermoshaker

Reagents

1. Glacial acetic acid
2. Sodium hydroxide 0.5 ml
3. Saline solution 98%
4. Deionized water 20ml

Experimental development

Cleaning and preparation of the material

All the glassware material that would be used for the elaboration of the product was washed and rinsed, followed by the preparation and placement inside the preheated electric autoclave to make the sterilization of the equipment more efficient. After being purged for the first time to remove the cold air, we waited for it to reach 15lb of pressure and it remained for 15 minutes. Completed in time, the material was purged and allowed to cool for use.

Metronidazole pretreatment

After having defined the percentage of concentration of the drug to be used in the sample, the calculations were made to determine the number of tablets required. Once the number (18 tablets) was obtained, it was diluted with 20 ml of 98% saline in a 100 mL beaker.

Pretreatment of chitosan

In the same way as the drug, the percentage of the desired concentration was determined, and the calculations were made. 3g of chitosan was weighed in an analytical balance, gradually diluted in 30 mL of distilled water in a 250 mL beaker and with the support of a glass rod it was mixed until homogenized as best as possible.

Elaboration of the pasta

After diluting the Chitosan in distilled water, it was placed in a thermo agitator to execute the homogenization of the sample by raising the temperature to 40°C and the magnetic stirrer was introduced to the sample to continue with the mixing process. As even after a time exposed to heat and constant agitation there were residues of Chitosan, 1mL of acetic acid was added to acidify the sample and thus achieve greater dilution and homogeneity of the Chitosan.

When the ideal texture was achieved, the metronidazole already diluted to the Chitosan was poured, the mixing and homogenization process was continued, then 20 ml of distilled water are added, obtaining an ideal consistency for its application.

Neutralization

For this procedure they used 9mL of Sodium Hydroxide (NaOH) to neutralize the paste. (Were the remaining milliliters of the total sample volume).

Note

The estimated total volume of preparation was 60 ml, 20ml more bidistilled water was added, which caused dilution of the concentration of metronidazole and chitosan (Fig. 1 and Fig. 2) The topic is finalized (Fig. 3)

In 80mL: Chitosan 3.75% and Metronizadole 11.25%

The pH was to be 7-7.2, however, it was at a pH of 6.5-6.8 due to the total volume.

Fig 1: Mixture of bidistilled water with metronidazole and chitosan

Fig 2: Mix result

Fig 3: Mix result

Crop preparation

A culture of *P. gingivalis* was performed in a medium of Müller-Hinton Agar, using a well diffusion method since it is one of the methods that the National Committee for Clinical Laboratory Standards (NCCLS) recommends for the determination of bacterial sensitivity to antimicrobials.

Two Petri dishes were used, in which agar solution with a thickness of approximately 4mm was placed, then incubated for 5 days (Fig. 4).

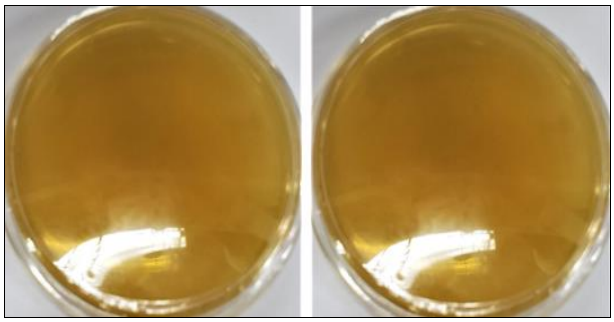

Fig 4: Petri dishes with agar

Then 50 microliters of *Porphyromona gingivalis* were placed in each box (Fig. 5). With the help of 6mm crystal spheres (Fig. 6) a uniform distribution of the bacterium throughout the culture medium was initiated, when observing a good distribution, a portion of agar with a sterile punch of approximately 8mm was extracted to the bottom of the Petri dish to create a well (Fig. 7).

It was done to take in volume the chitosan-metronidazole to place it inside the well that was created, until a uniform volume was observed and similar to the other (Fig. 8). The petri boxes were sealed and placed in the incubator at 37º C (± 1ºC).

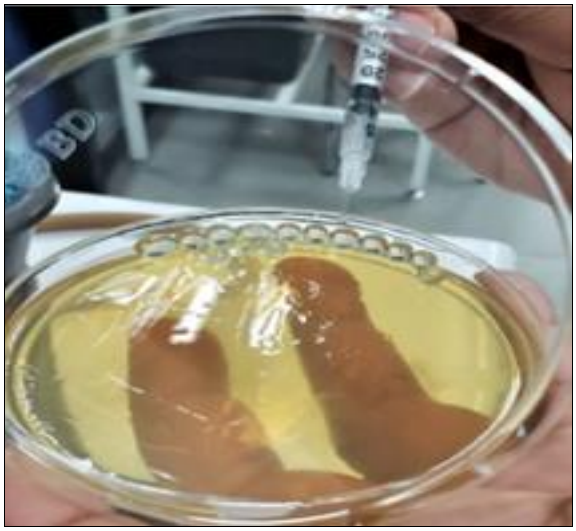

Fig 5: Porphyromonas gingivalalis (50 microliters) placed in each box

Fig 6: Crytsa spheres (6 min)

Fig 7: Extraction of agar (8 mm)

It was kept incubated for a period of 48 hours, then removed from the incubator to interpret the results of bacterial sensitivity by observation and measurement of the inhibition halo around each well.

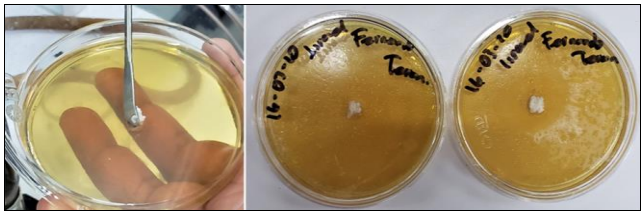

Fig 8: Volume measurement chitosan-metronidazole

Results

After 48 hours the observation and measurement of the halo created around the well was carried out (Fig. 9 and 9a), the halo was measured with a calibrated ruler, taking into account the parameters of the Clinical and Laboratory Standards Institute (CLSI), where it tells us that there are three results: sensitive, intermediate and resistant, according to the inhibition halo observed in the Petri dish.

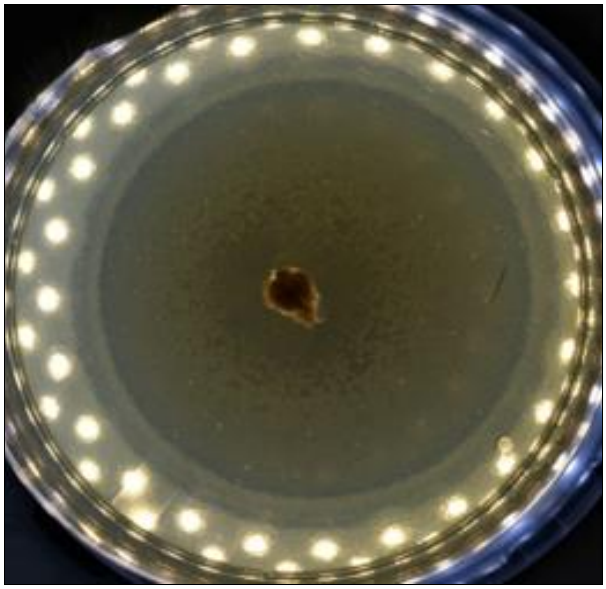

Fig 9: 48 hours the observation and measurement of the created

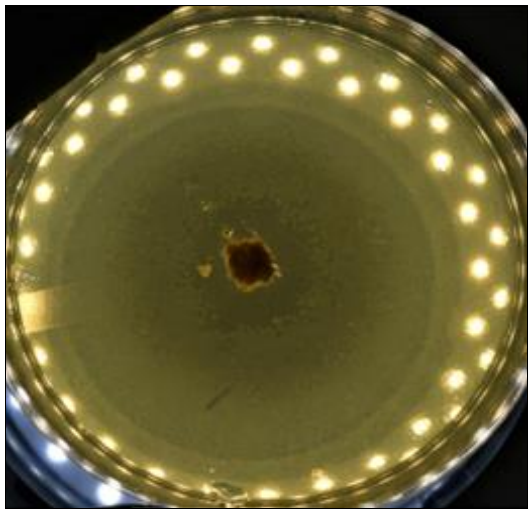

Fig 9a: 48 hours the observation and measurement of the created

In crop 1, an inhibition halo of 20mm was observed, in which CLSI mentions that the bacterium is sensitive to chitosan-metronidazole (Fig. 10).

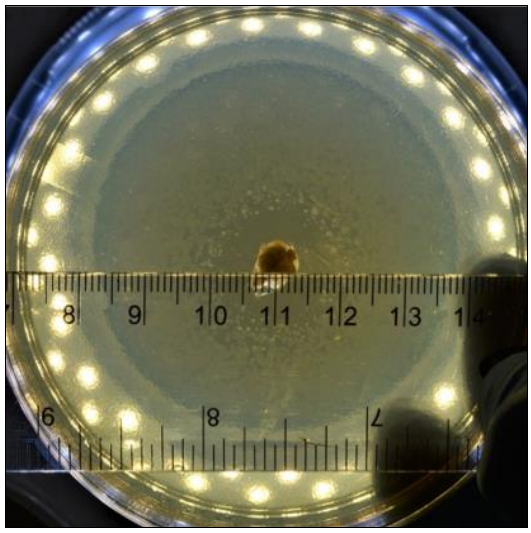

Fig 10: Crop 1 observed 20 min of inhibition

In crop 2, an inhibition halo of 20mm was observed.
With which the reproducibility of this method of agar diffusion in well is denoted, since the same result was obtained, which is sensitive (Fig. 11).

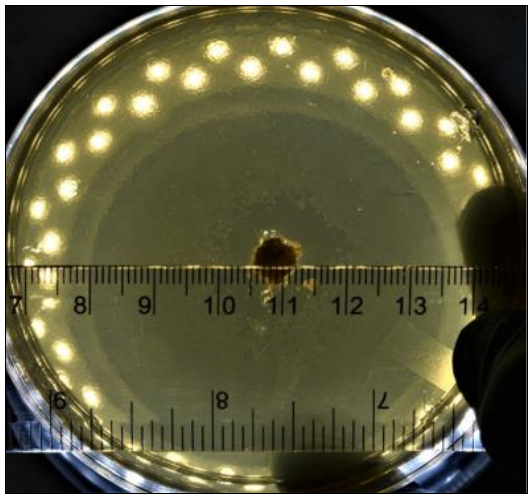

Fig 11: In crop 2 observed 20 mm of inhibition

Discussion

In our study this type of method was carried out since it is necessary to highlight the importance of choosing the techniques and methodologies to be used because the variation of the results depends on that, as well as their sensitivity and reproducibility according to the characteristics of the extracts. We observed in our study a reproducibility of the method in which in both we obtained the same results to sensitivity.

Due to the discrepancies of opinions that may exist due to the use of other methods and in order to obtain greater sensitivity in these tests, many researchers perform the well diffusion technique; as is the case of the study carried out by Panda *et al*. [41]. Those who used this method, this technique being more sensitive because the extract diffuses freely in the agar.

Rojas *et al*. [42], based on their study, recommend using the modified method of wells in agar to perform antimicrobial activity tests due to its high sensitivity; aspect that was confirmed in our study.

Conclusion

It was concluded that Phorpyromona *gingivalis* is sensitive to topical chitosan-metronidazole gel, which is reflected in the study using the well agar diffusion method. Being a sensitive and reproducible method.

Conflict of Interest

Not available

Financial Support

Not available

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