



## ORIGINAL ARTICLE

Jesús Rodríguez<sup>1</sup>.  
Rosa Sánchez<sup>1</sup>.  
Marianela Garza<sup>1</sup>.  
María Nakagoshi<sup>1</sup>.  
Juan Solís<sup>1</sup>.  
Katiushka Arévalo<sup>2,3</sup>.  
Emmanuel Garza<sup>1</sup>.

1. Facultad de Odontología, Universidad Autónoma de Nueva León. México.
2. Facultad de Ciencias Biológicas, Universidad Autónoma de Nueva León. México.
3. Instituto de Biotecnología, Universidad Autónoma de Nueva León. México.

**Corresponding author:** Emmanuel Garza. Depto de Histología, Facultad de Odontología, Universidad Autónoma de Nuevo León. México. E. Aguirre Pequeño y Silao, Mitras Centro, Monterrey, N.L. México, CP 64460. Phone: 52 81 83294000 Extensión UANL: 3300, 3161. E-mail: emmanueljc4@hotmail.com - emmanuel.garza@uanl.mx

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## Salivary stimulation by prolonged release of pilocarpine using films in diabetic rats.

**Abstract:** Introduction: The local use of prolonged drug delivery in the oral cavity provides many advantages, i.e., it increases pharmacologic actions in the desired local site, allows smaller doses and reduces adverse effects. Pilocarpine is a cholinergic drug approved by the FDA for treating glandular hypofunction; however, the adverse effects associated with it limit its use. Objective: To evaluate cytotoxicity of films in adherent fibroblasts and their ability to release pilocarpine in vivo for a prolonged time in the oral cavity of diabetic rats and its effect on salivary flow. Methods: Chitosan and Hydroxypropylmethylcellulose (Methocel K4MCR) films were prepared in 1% acetic acid and pilocarpine was added under magnetic stirring. Cytotoxicity of films was evaluated in adherent fibroblasts HS27 and assessed by neutral red technique. The sialogogue effect of films was evaluated on the floor of the mouth of diabetic rats. Later, histopathological analysis was performed using hematoxylin and eosin and Masson's trichrome stains. Results: Films were biocompatible and had 96% cell viability. It was possible to increase stimulation of salivary flow in diabetic rats ( $6.36 \pm 0.987$  mg/hr) compared to the control group ( $0.5 \pm 0.06$  mg/hr). The histopathological analysis did not show inflammatory infiltrate in the area where films were placed. Conclusion: Films were biocompatible and had high cell viability. Also, they considerably increased salivary flow in diabetic rats, without triggering an inflammatory infiltrate in the area which indicates that it is a biocompatible product for sustained release and safe for pilocarpine administration.

**Keywords:** *Hyposalivation, cytotoxicity, rats, pilocarpine, films.*

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### INTRODUCTION.

It is estimated that between 12 and 47% of elderly and 10 to 19.3% of people younger than 30 suffer from hyposalivation<sup>1</sup>. It has been demonstrated that multiple causes lead to hyposalivation, including Sjogren's syndrome, diabetes mellitus, sarcoidosis, hepatitis C, adverse effects of pharmacological agents<sup>2</sup>, external beam radiation therapy, patients on radioiodine therapy, tumors and trauma in salivary glands, nutritional deficiencies, and eating disorders such as anorexia and bulimia<sup>3,4</sup>.

Studies have shown that 38.9 -53% of type 1 and 14-62% of the type 2 diabetic patients<sup>1</sup> have experienced dry mouth with decreased salivary flow (<0.1-0.2ml/min of saliva secreted at rest or <0.4-0.7ml/min of total stimulated saliva)<sup>5</sup> and therefore have an increased risk of dental caries, gingivitis, periodontitis, soft tissue injury, loss of alveolar bone and dental organs<sup>6</sup>, as well as infections of *Candida albicans*<sup>7-8</sup>.

Pilocarpine is a cholinergic agonist that stimulates muscarinic receptors of the salivary glands and increa-

ses salivary secretion<sup>9</sup> and is now commercially obtained from *Pilocarpus Microphyllus*<sup>10</sup>. It was the first drug approved by the FDA (Federal Drug Administration) for treating deterioration of salivary glands<sup>11</sup>. However, it has many adverse effects such as sweating, rhinitis, nausea, increased urinary frequency, as well as increased gastrointestinal secretion of hydrochloric acid<sup>3,12</sup>. Besides, it is contraindicated for asthma, acute iritis, glaucoma, obstructive lung diseases<sup>5</sup>, not controlled peptic ulcer, hypertension and the interaction with adrenergic  $\beta$ -blockers<sup>13</sup>.

Attempts have been made to find an alternative way for administration of pilocarpine in xerostomia using mouthwashes of pilocarpine in saline solution, which had a good result with 2% pilocarpine. However, it was not found to be effective 90 minutes after administration of mouthwash<sup>3</sup>.

In comparison with other therapeutic agents for hyposalivation, such as cevimeline, it has been found that salivary flow increased with administration of pilocarpine during 4 weeks (8.96 ml/5min), compared with a dose of 30 mg of cevimeline (7.05ml/5min) and presents less adverse effects as well<sup>14</sup>. In patients with primary Sjögren's syndrome, it has been found that the rate for therapy discontinuation due to adverse effects with pilocarpine is 61% compared to 32% of patients using cevimeline<sup>15</sup>.

The system of HPMC (Hydroxypropylmethylcellulose) and chitosan polymeric matrices for controlled drug release facilitates mechanical properties for the desired application<sup>16-18</sup>. Additionally, it must comply with the requirements: to be biocompatible, biodegradable and avoid inflammatory, immune or cytotoxic responses<sup>19</sup>. We have studied physical-chemical properties (pH, tension-elongation, uniformity of drug diffusion) of controlled release systems using films containing chitosan and HPMC for transdermal and oral, animal and human models, and they have proved to be beneficial for future applications<sup>20-22</sup>.

The objective of this study was to evaluate cytotoxicity of films made of HPMC/chitosan biopolymers in adherent fibroblasts and their ability to *in vivo* release pilo-

carpine locally on floor of the mouth and ventral side of the tongue of diabetic rats. It was also meant to assess the effect on salivary flow and carry out a histopathological analysis in the area where the films were placed.

## MATERIALS AND METHODS.

### Preparation of chitosan/HPMC films with pilocarpine

Chitosan with 75% degree of deacetylation, obtained from Sigma-Aldrich Chemical Company (St. Louis, Missouri, US); Hydroxypropylmethylcellulose (Methocel K4M CR), donated by Colorcon from Mexico (Cuajimalpa, Edo. Of Mexico) and pilocarpine from GLOBAL Laboratory (Monterrey, N. L., Mexico) were used.

In an aqueous solution containing 1% acetic acid, a formulation of chitosan/ HPMC (0.5gr of each /100ml) was prepared with the addition of a usual dose of pilocarpine in rats (0.15gr/mL). The formulation became homogeneous under magnetic agitation at 70°C for one hour. Subsequently, it was emptied into 15ml Petri dishes and was left to air dry for 24 hours to be finally removed.

### Evaluation of the cytotoxic effect of Ch/HPMC/P films in adherent fibroblast cell line HS27

The test was conducted in adherent fibroblast cell line HS27. The cells were kept in boxes of cell culture with MEM (minimum essential medium), where they were poured and trypsin was added. Subsequently, they were incubated in an atmosphere containing 5% CO<sub>2</sub> at 37°C. Once the cells detached and were observed in the optical microscope, MEM supplemented with blood serum, pyruvate and 1% solution of antibiotics (penicillin 10 000IU/streptomycin 10 000 mg/ml) were added.

The cells were collected and maintained at a concentration of 5x10<sup>4</sup> cells/well in 12-wells plates for incubation during 24 hours prior to treatment with Ch/HPMC/P films. After a 24 hour-treatment period and with 1cm<sup>2</sup> of biofilm, the culture medium was removed and the solution of neutral red (50 mg/ml) was added and, after 3 hours of incubation *in situ*, the plates were washed with phosphate buffered saline (PBS), and a destaining solu-

tion (50% ethanol, 49% of distilled H<sub>2</sub>O and 1% acetic acid) was added to measure the absorbance for each well at 490nm using a plate reader (Biotek Instruments). The tests were conducted in triplicate.

### Evaluation of the sialogogue effect of films in diabetic rats

Six Wistar female rats from 3 to 4 months of age and with an average weight of 250 to 300 gr were selected. Three of them were taken to the experimental group and 3 for the control analysis. (Committee on Bioethics SPSI - 010613, Folio:0018)

### Induction of diabetes in Wistar rats

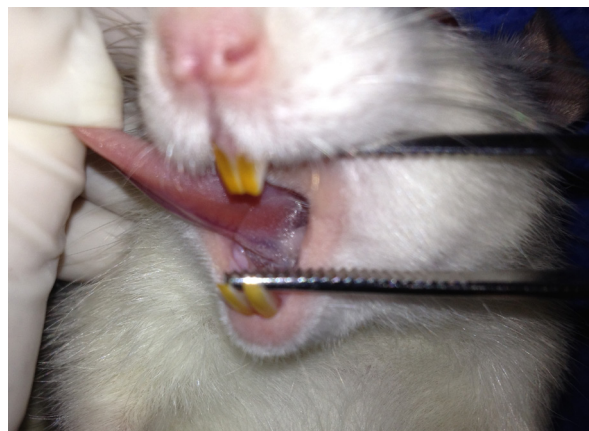
With the objective to evaluate the effect of films in salivary flow of rats, diabetes was induced using a single dose of streptozotocin (STZ), a drug capable of inducing hyposalivation in animal models. The dose was 60 mg/kg dissolved in 0.1 M sodium citrate buffer and it was administered intraperitoneally (IP). Only those rats which showed glucose levels higher than 250 mg/dl 30 days after the induction of diabetes were included in the study.

### Evaluation of the sialogogue effect of films

With the purpose of evaluating the effect of films in salivary flow, it was proceeded to induce general anesthesia using a dose of 3.5 to 5 mg/kg of body weight of sodium pentobarbital (Pisabental, PISA) intraperitoneally. In the experimental group, 1cm<sup>2</sup> of Chitosan / HPMC/ P biofilm (with a thickness of 0.053±0,011 μ) was placed in the floor of the mouth and ventral side of the tongue of the rats (Figure 1) along with small cotton balls which were previously weighted in an analytical balance (Explorer, Ohaus, Mexico D. F) (Figure 2) in such a way that they weighted the same with the purpose of collecting saliva secreted by the rats for one hour<sup>6</sup>, which was the period of time the anesthesia provided. The rats were sacrificed by cervical dislocation, and then the area of placement of films was dissected and histological sections were performed for their evaluation. The tissues were fixed in 5% formaldehyde and embedded in paraffin to perform staining with hematoxylin and eo-

sin, and Masson's trichrome stain for histopathological analysis. The control group was considered as a structural base to evaluate the presence of surrounding inflam-

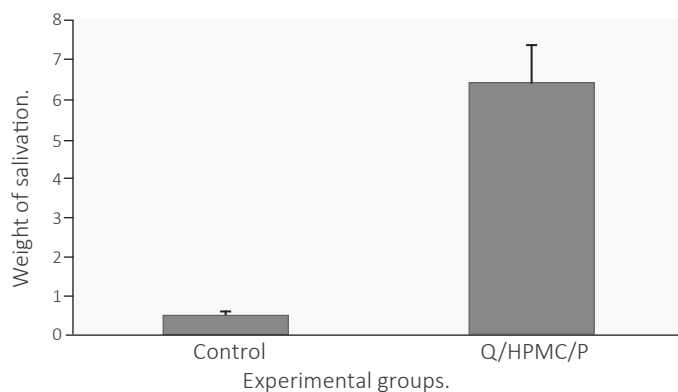
**Figure 1.** Placement of 1cm<sup>2</sup> biofilm Ch/HPMPC/P on the floor of the mouth of a diabetic rat.



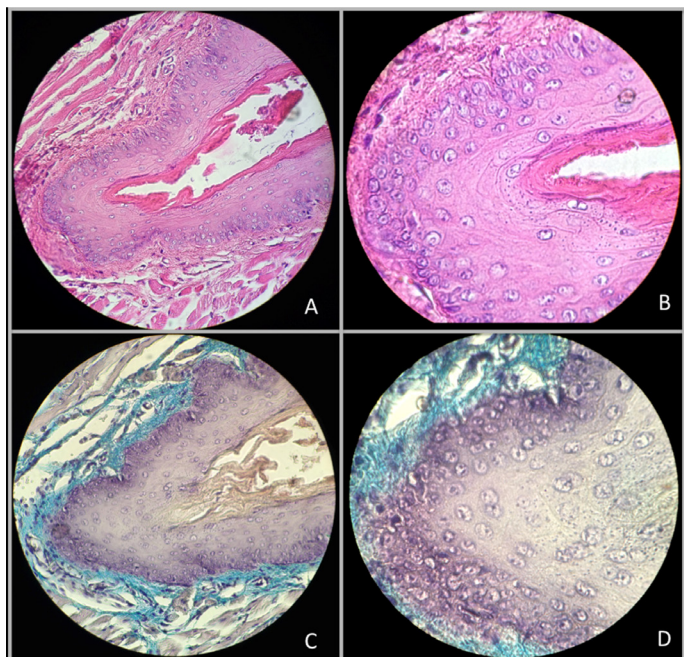
**Figure 2.** Placement of cotton for saliva in the rat



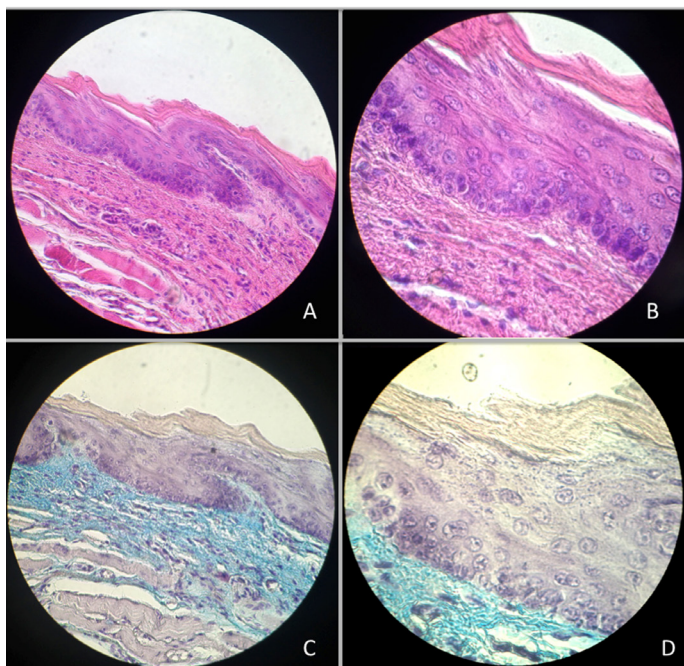
**Figure 3.** Evaluation of salivary flow in weight of diabetic rats using cotton balls (mg).



**Figure 4.** Histological sections in experimental rat, oral mucosa coating the floor of the mouth and ventral side of the tongue. (A) 40x HE. (B) 100x HE. (C) 40x Masson. (D) 100x Masson.



**Figure 5.** Histological sections in control rat, oral mucosa coating the floor of the mouth and ventral side of the tongue. (A) 40x HE. (B) 100x HE. (C) 40x Masson. (D) 100x Masson.



matory infiltrate. All the tests in the animal model were supervised by a certified veterinarian.

## RESULTS.

For analyzing the results of the cytotoxicity assessment and the influence of films in the salivary flow, we used statistical t-test (IBM SPSS statistics, Version 20, USA).

### Cytotoxic evaluation in adherent fibroblasts cell line HS27

The results of the cytotoxic effect of films in an adherent fibroblast cell line HS27 showed that Ch/HPMC/P films obtained 96% cell viability with an absorbance of  $0.326 \pm 0,032$  A compared with the control with 100% of cell viability and absorbance of  $0.337 \pm 0,028$  A.

### Evaluation of the sialogogue effect of films in rats

The evaluation of the biofilm with pilocarpine showed a significant increase in salivary flow. For the experimental group, a weight of  $6.4 \pm 0,987$  mg was observed, unlike the control group without placement of films ( $0.5 \pm 0.06$  mg) (Figure 3).

### Histopathological analysis

In the control group, the presence of non-keratinized stratified epithelium of the coating oral mucosa, with its basal, intermediate and superficial stratum, with chorionic papillae and epithelial peaks inside in the subepithelial area of connective tissue and bundles of skeletal muscle was seen. The experimental group presented the same structures without PMN cells involving the presence of inflammatory infiltrates (Figure 4 and 5).

## DISCUSSION.

In the present, few studies about the local use of pilocarpine in the oral cavity have been reported. Bernardi *et al.*, in 2001<sup>3</sup>, applied mouthwashes for one minute with different drug concentrations (2%, 1% and 0.5%). Additionally, they used kinetics for measuring salivation during periods of 15 minutes in healthy patients. The results showed increased salivary flow with the 2% pilocarpine rinse 45 minutes after using the mouthwashes. However, during the study, patients presented some alterations in systolic blood pressure, palpitations and symptoms of motion sickness, in addition to again pre-

senting dry mouth 90 minutes after rinsing. Coinciding with those results, in the present study, a significant increase in salivary flow was observed with the use of films with pilocarpine.

In 2000, Miyazaki *et al.*<sup>23</sup> used tablets made of HPMC and pectin for diltiazem extended-release in Wistar rats. The tablets were placed in the sublingual area and blood samples were obtained to evaluate plasma concentration of the drug in the blood. After prolonged sublingual administration, diltiazem was found 2.5 times higher in comparison with oral administration, supporting the effectiveness of prolonged local release methods to treat oral pathologies.

In 2012, Romero *et al.*<sup>6</sup> stimulated salivation in diabetic rats by IP administration of pilocarpine (0.6mg/kg of body weight) and using small cotton balls to collect saliva. Salivary flow increased in the control group (35.9 $\mu$ l/min) and in the diabetic group (8.81 $\mu$ l/min). In our study, the local administration of pilocarpine in oral cavity increased salivary flow considerably, with an average rate of 6.4gr/hr, compared with the control group (0.5gr/hr). Perhaps this is due to the fact that prolonged administration of topical pilocarpine in

the site of the vascular plexus ranine can facilitate rapid absorption and maintain good bioavailability of the drug in the blood plasma to carry out its pharmacological action thus increasing the blood flow in an effective and constant manner.

To our knowledge, no studies evaluating cytotoxicity in fibroblast cell lines formulated with chitosan, HPMC and pilocarpine have been reported. However, the results reflected in the present study showed a high cell viability in these formulated films (96%), indicating that the studied product for prolonged release of pilocarpine is biocompatible and safe. This could be due to the fact that 1% acetic acid, which is used for preparing the formulations, evaporates when the films dry, leaving only the polymer mixture with pilocarpine, creating a less acid environment to preserve cell viability.

#### CONCLUSION.

Films with pilocarpine considerably increased salivary flow in diabetic rats, without triggering an inflammatory infiltrate in the area. In the present study, they showed a high cell viability (96%), indicating that the product used for prolonged drug release is biocompatible and safe for administration of pilocarpine.

#### Estimulación salival mediante biopelículas de liberación prolongada de pilocarpina en ratas diabéticas.

**Resumen:** Introducción: El uso local de administración prolongada de fármacos en la cavidad oral proporciona múltiples ventajas, aumentando la acción farmacológica en el sitio de acción, reducción de la dosis usual y disminución de los efectos adversos. La pilocarpina es una droga aprobada por la FDA para el tratamiento del deterioro de las glándulas salivales, sin embargo sus efectos adversos limitan su uso. Objetivo: Evaluar la citotoxicidad de las biopelículas en fibroblastos adherentes y su capacidad in vivo de liberar pilocarpina prolongadamente en la cavidad oral de ratas diabéticas y su efecto en el flujo salival. Metodología: Se elaboraron biopelículas de Quitosán y HPMC (Methocel K4MCR) adicionadas con pilocarpina en una solución de ácido acético 1%. Fue evaluada la citotoxicidad de las biopelículas en fibroblastos adherentes HS27 mediante la técnica de rojo neutro, además de ser evaluado

el efecto sialogogo de las biopelículas en el piso de boca en ratas diabéticas Wistar, realizando después un análisis histopatológico de la zona de colocación, mediante la tinción de Hematoxilina y Eosina y Tricrómico de Masson. Resultados: Las biopelículas resultaron ser biocompatibles con un 96% de viabilidad celular. Se logró aumentar la estimulación del flujo salival en las ratas diabéticas (6.36 $\pm$ 0.987 mg/hr), a comparación del grupo control (0.5 $\pm$ 0.06 mg/hr). El análisis histopatológico no reveló un infiltrado inflamatorio presente en la zona de aplicación de las biopelículas. Conclusión: Las biopelículas resultaron biocompatibles con alta viabilidad celular, además que lograron aumentar considerablemente el flujo salival en ratas diabéticas, sin desencadenar un infiltrado inflamatorio en la zona de aplicación, indicando que es un producto de liberación prolongada biocompatible y seguro para la administración de pilocarpina.

**Palabras clave:** Hiposalivación, Citotoxicidad, Diabetes, Pilocarpina, Biopelículas.

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