Experimental model of traumatic ulcer in the cheek mucosa of rats

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ABSTRACT

Purpose: To establish an experimental model of traumatic ulcer in rat cheek mucosa for utilization in future alternative therapy studies.

Methods: A total of 60 adult male rats (250 – 300g) were used. Ulceration of the left cheek mucosa was provoked by abrasion using a nº 15 scalpel blade. The animals were observed for 10 days, during which they were weighed and their ulcers were measured. The histological characteristics were analyzed and scored according to the ulcer phase. In the statistical analysis, a value of p<0.01 was considered a statistically significant response in all cases.

Results: During the five first days, the animals lost weight (Student \( t \) test, \( p<0.01 \)). The ulcerated area receded linearly over time and was almost completely cicatrized after 10 days (ANOVA, Tendency post-test, \( p<0.0001 \)). Groups on days 1, 2 and 3 days displayed similar results, but a decrease in scores were observed after the 4th day.

Conclusion: The proposed cheek mucosa ulcer model in rats can be considered an efficient, low-cost, reliable, and reproducible method.

Key words: Ulcer. Mouth Mucosa. Wound Healing. Rats.

RESUMO

Objetivo: Estabelecer um modelo experimental de úlcera traumática em mucosa jugal de ratos para utilização em futuros testes de terapias alternativas. Métodos: Foram utilizados 60 ratos, adultos, machos, pesando entre 250 a 300g. A ulceração na mucosa jugal esquerda foi provocada por meio da abrasão desta com uma lâmina de bisturi número 15. Os animais foram observados por um período de 10 dias, sendo estes pesados e suas escoriações mensuradas. As características histológicas foram analisadas e descritas adotando escores para comparação dos estágios da fase da úlcera. Na análise estatística um valor de \( p<0.01 \) foi considerado uma resposta estatisticamente significante em todos os casos. Resultados: Durante os cinco primeiros dias os animais perderam peso (\( t \) Student \( p<0.01 \)). A área da úlcera regrediu linearmente com o tempo, estando quase que completamente cicatrizada ao final de dez dias (ANOVA, pós-teste de Tendência \( p<0.0001 \)). Os grupos do 1º, 2º e 3º dias tiveram comportamento semelhante havendo uma diminuição dos escores a partir do 4º dia. Conclusão: O modelo de úlcera na mucosa jugal de ratos proposto pode ser considerado eficaz, apresentando reprodutibilidade confiável e baixo custo.

Introduction

Traumatic ulcers in the oral cavity are relatively frequent and are usually a result of mechanical injuries. Traumatic ulcers are usually found in non-keratinized surfaces such as the cheek mucosa, edge of the tongue, lips, gingiva and hard and soft palates. Lesions in the gums and cheek mucosa can be caused by other forms of irritation such as brushing, which causes linear erosion along the free gingival margin and is sometimes related to areas of hyperkeratosis. These ulcers are usually ovoid and painful, being limited to the area subjected to the damage. They can present as a white-yellowish necrotic center surrounded by an erythematous halo and pain. The repair stage happens when the lesion assumes a crateriform aspect. In this stage, the ulcer is covered with a superficial circular membrane, erythematous halo and pain. The third is the ulcerative stage when the membrane is whitish and necrotic, which happens during the period from days 1 to 3. A whitish-yellow fibrinous exudate is formed on the ulcer floor; then the erythematous halo persists, and the lesion assumes a crateriform aspect. In this stage, the ulcer reaches its developmental peak, and the pain recedes, becoming merely a sensation of discomfort. The repair stage happens spontaneously.

From the microscopic point of view, the mucosa that covers the mouth is represented by an external layer of epithelial cells that is supported by connective tissue of variable constitution. This mucosa is a protective barrier for the organism because it does not allow for penetration of any external agent that can be harmful to health. The ulcerations present discontinuity of the epithelial surface and are covered by a fibrinopurulent membrane mixed with neutrophils. Granulation tissue can be observed at the base of the ulcer, which supports an inflammatory infiltrate—a mixture of lymphocytes, histiocytes, neutrophils and occasionally plasmocytes. The constitution of this infiltrate will vary according to the evolution phase of the ulcer. In the initial phases after the injury, neutrophils predominate, performing phagocytosis. After undergoing apoptosis, the neutrophils are progressively substituted by monocytes. In addition, chemotactic factors for neutrophils and monocytes are activated during the different periods of inflammation, maintaining the migration of monocytes after the migration of neutrophils has ceased. Approximately 3 days after the histological profile of the ulcer starts to present alterations, the cellular infiltration becomes predominantly lymphoplasmacytic, which is characteristic of chronic inflammation. It is in this phase that the proliferation of blood vessels and fi broblasts occurs, signaling the beginning of repair. Although neutrophils are characteristic of acute inflammation, they can also be found in sites of chronic inflammation of long durations, such as in the case of a bacterial infection or necrotic tissue, and they can also persist if they are continually recruited by chemotactic agents.

Despite the variety of topical agents, antibiotics, local anesthetics, antihistamines, non-steroidal anti-inflammatory agents and enzymatic preparations used for symptom relief, there is no consensus on the efficacy of medications that are currently available because many of these agents have not been properly evaluated and have only been used empirically. Thus, the objective of this study was to establish an experimental traumatic ulcer model that could be used in future alternative therapy studies.

Methods

The Ethical Principles of Animal Experimentation of the Brazilian College of Animal Experimentation (COBEA, institution associated with the International Council for Laboratory Animal Science and Brazilian Legislation on Experimental Animals (Federal Law nº. 6.638 (1979) were adopted.

The research project was submitted and approved by the Ethics Committee of Animal Research (CEPA) of UFC, protocol nº.82/08.

A total of 60 rats were studied (Rattus norvegicus albimus, Rodentia mammalia) that were all: Wistar lineage adult males aged between 2 – 3 months and weighing between 250 and 300g. The rats were acquired from the Central Animal Colony of UFC.

The rats were kept in plastic cages with pine-sawdust covered floors at a temperature of 24°C in a dark-light cycle of 12 hours with free access to water and commercial food (Bio-base).

The animals were randomly distributed into 10 cages with 6 animals each, identified and weighed daily. The date of sacrifice was recorded for each rat.
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Experimental protocol

The animals were anesthetized with 10% chloral hydrate (30ml/0.1g) via intraperitoneal injection, and the surgical process was initiated the eyelid reflex and a constant breathing rhythm ceased. The animals were observed during the post-operative period until the total recovery of reflexes.

After the anesthetic stage was reached, each animal was put on a surgical table in dorsal decubitus and immobilized with adhesive tape. The mucosa was sterilized by using a swab covered in 0.12% chlorhexidine digluconate.

The ulceration was made on the left cheek mucosa by abrasion using a nº 15 scalpel blade. To standardize the lesion area, an 8-mm-diameter demarcator was used. The operation technique was standardized for all the animals and was performed by the same examiner. The ulceration was not performed in the control group, in which the animals were anesthetized, and the variation of body weight was observed.

The animals were observed daily for 10 days. They were weighed and their ulcers were measured with a 0.5-mm precision digital paquimeter (D= biggest diameter and d= smaller diameter) to calculate the area (A= π. r. R)^1. The animals were gradually sacrificed: each group was sacrificed in intervals of 24 hours through the end of the 10-day study period. After the animals were sacrificed, a section of the cheek mucosa containing the induced ulcer was collected.

Histopathological analysis

The collected cheek mucosa fragments were identified and immersed in 10% formol for 24 hours. After fixation in formol, the specimens were macroscopically analyzed, subjected to dehydration in crescent alcoholic series, diaphanized in xylol, impregnated in paraffin and melted at 60%. Then, the fragments were put into paraffin-forming blocks at room temperature. The fragments were sectioned to 5 µm in thickness through the use of a microtome and histology using routine coloration by eosin-hematoxylin was performed. Analysis was performed with an optical microscope from the Oral-dental Pathology Laboratory of the Odontological Clinic Department of UFC, and the histological characteristics of the ulcer along with their corresponding cicatrization phases were described.

The histopathological parameters were determined and scored from 0 to 4 according to the information below (adapted from Cho et al.):

0. No ulcer / remodeled connective tissue
1. No ulcer / fibrosis + slight chronic inflammation
2. With ulcer / fibrosis + moderate chronic inflammation
3. With ulcer / chronic inflammation process (granulation tissue)
4. With ulcer / acute process (dilated vessels, mixed inflammatory infiltrate with neutrophilis)

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 (demo version). Student’s t test was performed to verify the difference between the averages of the weights obtained before and after ulceration in each group. The comparison test of the ulcerated areas among groups was analyzed using ANOVA followed by the Tendency post-test. The histological scores were compared by Kruskal-Wallis non-parametric test followed by the Dunn post-test. Data of the ulcer area and time were adjusted to a dose-response model to determine the time at which the ulcers were 50% cicatrized. A value of p<0.01 was considered a statistically significant response in all cases.

Results

Macroscopic evaluation of injuries

During the first 4 days, the injuries in the cheek mucosa of the animals were clinically visible and were crateriform, bleeding and red (Figure 1).

FIGURE 1 - Clinical aspects of the traumatic ulcers in the left cheek mucosa within one (A), two (B), three (C) and four days (D) after the induction of the trauma with an nº 15 scalpel blade.
From days 5 to 8, the size of the injuries decreased; however, some of them were not totally cicatrized and still had irregular borders and the presence of a whitish halo surrounding the reminiscent injury (Figure 2).

On the ninth and tenth days, the cheek mucosa of the animals where the ulceration was induced was cicatrized, uniform and presented regular borders (Figure 3).

**Body weight and ulcerated area analysis**

The surgical procedures were satisfactory, and all the animals remained alive during the operation without any pre-operative or anesthetic complications.

Concerning the weight (Figure 4), a progressive reduction was observed in the animals subjected to ulceration until the fifth day, which was statistically significant compared to the weight initial the procedure. From this day on, the animals started to gain weight, however, it was more evident from the tenth day when it became statistically significant (p<0.01). In regards to the control group, no significant difference was observed between the final and initial weights (p>0.01).

For the analysis of the ulcerated area (Figure 5A), a tendency for linear regression with time was observed, and the injury in the cheek mucosa of the animals was almost completely cicatrized by the end of the 10-day period. Concerning the contraction behavior of the ulcer in relation to time (Figure 5B), the adjustment of the dose-response curve showed that 50% of the ulcerated area was contracted in about 4.5 days or 108 hours after ulcer induction.
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FIGURE 5 - Area with trauma-induced ulcers in the cheek mucosa of rats. (A) Relation between the area of the ulcer and time was analyzed by ANOVA test with Tendency post-test ($R^2=0.87; p<0.0001$). (B) Relation of the percentage of ulcer contraction and time ($p<0.0001$).

Histopathological analysis

Microscopic analysis of the sections in the cheek mucosa of rats on the first and second days after the induction of the trauma revealed the presence of an acute inflammatory process constituted by extended part of ulceration associated with dilated vessel, hemorrhagic areas and mixed inflammatory infiltrate with the predominance of neutrophils (score 4) (Figure 6A-B). On the third and fourth days after the injury, the histological profile was composed by a lymphoplasmocytic inflammatory infiltrate, proliferation of fibroblasts and some neoformed capillaries (score 3) (Figure 6C).

FIGURE 6 – (A) Photomicrograph of histological sections of the cheek mucosa of rats (1st and 2nd days after ulceration) showing the presence of vessels and ulcer (black arrow) associated with intense mixed leukocytic inflammatory infiltrate (HE, 200X). (B) Photomicrograph of histological sections of the cheek mucosa of rats (1st and 2nd days after ulceration) showing the mixed leukocytic inflammatory infiltrate (HE, 400X). (C) Photomicrograph of histological sections of the cheek mucosa of rats (3rd and 4th days after ulceration) with the presence of lymphoplasmacytic inflammatory infiltrate and some neoformed capillaries (white arrow) (HE, 400X).

Analysis of days 5 and 6 post-injury revealed the persistence of the ulcer associated with a moderate chronic inflammatory process with the presence of fibroblastic proliferation of organized collagen fibers (Score 2) (Figure 7A-B). After day 7, the microscopic alterations were characterized by the absence of the ulcer with remodeled connective tissue sometimes with a discreet inflammatory infiltrate (Score 1) (Figure 7C-D) and sometimes with absence of inflammation (Score 0) (Figure 7E-F).

However, on days 7 and 8, when the ulcers were not cicatrizated, it was observed secondary infection by *Actinomyces* sp.
After the histological analysis and evaluation, scores were tabulated for regression, and statistical significance was observed among the groups between days 1 and 9, 1 and 10, 2 and 9, 2 and 10, 3 and 9, and 3 and 10 (p < 0.01) (Figure 8). We observed that groups at the 1st, 2nd and 3rd days displayed similar results, but the scores decreased after day 4.

**Discussion**

The use of animal models has allowed for analysis of some aspects of the oral cavity and other associated structures that would not be easily studied in humans.

In the studies involving the oral mucosa, rats are commonly used because they present some advantages in relation to other animals. Among these advantages are: the low cost, the easy manipulation, maintenance in controlled environmental and sanitary conditions including special diets. Another advantage is the similarity between the oral mucosa of rats and humans, which is basically made up of epithelial tissues on the surface and subjacent connective tissue, called basal lamina which offers support and nutrition to the epithelium.

Traumatic ulcers are the most common inflammatory ulcerative conditions in the oral cavity, and the cheek mucosa is the most traumatized site. Such traumas are caused by poorly adapted prosthesis or occlusal disharmony, tooth crowns or fractured restorations and even by accidental bites while chewing or habits. Although it is the focus of intense research, there is no consensus in the literature about a definite therapeutic protocol, and many times inadequate treatments, such as the use of synthetic substances, phytotherapy and herbs are used by some of the population. Therefore, there is a need to establish experimental models able to test such substances as well as the ones available on the pharmaceutical market.
In the present study, the evolution phases of the macroscopic aspect of the ulcers followed the same patterns observed by Marcondes in 2002 who evaluated the tissue repair of surgical lesions in the oral mucosa of rats using a 2-mm “punch” for dermatological biopsy. Their study revealed that 20 days after the surgical procedure, there was a complete repair of the lesion in all the animals. These data corroborate our clinical findings in which the cheek mucosa of all the animals were completely cicatrized 10 days after beginning the experiment.

In regard to body weight, there was a decrease in the body mass in all groups during the first five days. This initial loss was caused by the process of ulcer induction in the mucosa, impairing feeding. As the cicatrization proceeded, the feeding process was reestablished, and consequently weight gain, which explains the increase in body mass after the fifth day. This result was confirmed after the analysis of the dose-response curve once the ulcerated area was already 50% contracted in this period. These results are similar to the findings by Alves et al. who inoculated Walker Carcinoma 256 tumor cells into open alveoli of the first and second inferior molars after extraction. The animals lost weight in the first two days of the experiment but gained weight after the fifth day. This demonstrates that open injuries in the oral cavity impair the feeding process, causing the animal to lose weight.

Regarding the analysis of the ulcerative area, no significant decrease was observed during the first two days. However, after the third day, the area of the injury started to recede at a constant rate and progressively declined until the final day. These data confirm the study by Cross et al. where a model for the evaluation of the cicatrization dynamics in injuries made in the dorsal area of rats and guinea pigs was established. According to the authors, the correlation coefficient (R), which was similar to what was found in our graph on the reduction of the ulcerated area (Figure 5A), approximates 1, suggesting a high reproducibility of the studied results.

Through microscopic evaluation, after day 7, a positive significant effect of cicatrization on reepithelialization (the time for epithelial reconstruction) was observed. This was similar to the findings by Amorim et al. who evaluated the cicatrization process of 1-cm incisions in rat bladders. During the first two days after induction of the ulcer, a slight decrease in tissue repair was observed due to the extended amount of ulceration and the predominance of polymorphonuclear neutrophils. However, Okabe and Amagase reported that after the first three post-surgical days, the rate of repair recovers once the acute inflammatory process reaches its peak and other activities start to modify the environment, which leads to tissue repair. In this phase, a change in the histological content can be observed, including the presence of a mononuclear cell infiltrate, mainly lymphocytes and macrophages. These macrophages increase their metabolism when activated and begin to release products that act as intermediates of inflammatory response events and growth factors, promoting the repair of tissues damaged during the inflammatory process. However, on days 7 and 8, one animal of each group still displayed persistent acute inflammation. This may be due to a secondary infection by bacteria from the oral microbiota during surgical injury, which slows down the repair process of the cheek mucosa due to maintenance of a local inflammatory reaction.

To complete the repair process, abundant granulation tissue, characteristic of chronic inflammation, grows form the border in a centripetal pattern at the thickness of the surrounding skin/mucosa, which reduces the size of the injury. This is performed by activated fibroblasts, here myofibroblasts, which have similar properties to the smooth contractible muscle cells that allow for the necessary contraction and tension to repair the damage.

The cicatrization process, similar to what happens in any inflammatory process, consists of steps with a sequence of alterations that can be observed throughout the period of tissue repair. The role of growth factors in the migration, proliferation and differentiation of epithelial cells, in the formation of connective tissue and growth of new vessel is of great importance.

Thus, the proposed model is similar to human traumatic ulcers regarding both the pathological characteristics and the cicatrization mechanism. These ulcerations represent inflammatory lesions that are extremely symptomatic that also impair feeding and the patient’s quality of life.

Conclusions

The cicatrization process of the mucosa in rats is similar to cicatrization in human beings. The proposed cheek mucosa ulcer model in rats can be considered an efficient process that offers reliable, reproducibility, and a low cost. The permanence time of the ulcer allows for testing of alternative therapies in vivo that have so far only been used empirically.

References

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