Original Article

Experimental model of oral ulcer in mice: Comparing wound healing in three immunologically distinct animal lines

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Abstract

Background: The oral wound healing is dependent of immune participation and the absence or augment of one specific immune profile can delayed wound healing.

Objectives: So, the objective of this study was t evaluate the wound healing of oral traumatic ulcer (OTU) in cheek mucosa of swiss, balb/c and c57bl6J mice.

Materials and Methods: A total of 144 mice (25-30g) were distributed in three groups: swiss ($n = 48$), balb/c ($n = 48$) and c57bl/6j ($n = 48$). An OTU was performed using a dermatological punch in left cheek mucosa. The animals were euthanized daily ($n = 6$/group/day by 8 days) for evaluation of the ulcer area, weight loss and histological analysis.

Results: There are no differences between ulcer area in three groups; however only swiss group showed total wound healing. Swiss group showed weight loss in 2nd and 3rd days recovering the body mass in 4th day ($P < 0.01$). Balb/c group showed the greater weight loss ($P < 0.05$) and c57bl/6j did not show body mass variation ($P = 0.258$). Histologically swiss group was the only group that showed total reepithelization ($P < 0.001$). Balb/c ($P = 0.022$) and c57bl/6j ($P < 0.001$) showed decrease in histological scores, chronic inflammation on the 8th day. Actinomyces was significantly more observed in surface of OTU of balb/c.

Conclusion: Balb/c mice showed high infection of OTU surface delaying wound healing, and greater weight loss. C57bl/6J mice showed low infection of OTU, but not healing along the eight days. Only the Swiss mice showed wound healing of OTU.

Keywords: Mice, oral ulcer, th1-th2 balance, wound healing

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INTRODUCTION

Oral traumatic ulcers (OTUs) are common pathological entities observed in clinical dentistry, and they do not respond adequate to the usual treatments. Because some patients with systemic disorders have delayed wound healing,[1] a few studies have analyzed the immunological mechanisms involved in these delays.[2,3]

Immunological mechanism studies are an important approach to delineating adequate treatment for OTUs

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because the inflammatory process is the major component of OTU healing.[4] However, these studies, when developed with nonspecific animals, are slow and expensive[3,4] and require some biologic molecular techniques,[5] and when these studies are developed with specific animal lines such as lines without thymus rats, it is very difficult to handle these animals.[6]

In this context, three simple and nonexpensive animal lines have distinct immunological components that can explain the participation of the immune system and inflammatory process in the healing of OTUs. Swiss mice are animals that have no specific immunological response; c57bl/6J mice have a th1 predominant response and Balb/c mice have a th2 predominant response.[7,8]

Among these three animal lines, there can be a response to the real participation of the immune system in OTU wound healing to help further studies choose the most appropriate animal line to examine. Therefore, the objective of this study was to evaluate the wound healing of OTUs in the cheek mucosa of Swiss (no specific immune response), Balb/c (th2 predominantly immune response) and c57bl6J (th1 predominantly immune response) mice.

MATERIALS AND METHODS

Animal care

Approval for the experimental use of laboratory animals was obtained from the local Ethics Committee on Animal Use (CEUA) (protocol 001/2015, May 28, 2015) of University Center Christus (Unichristus) and is in compliance with the Federal Law No. 11794 of October 8, 2008, and the Decree nº 6689, July 15, 2009, available from http: www. planalto.gov.brccivil03Ato200720102008 LeiL11794.htm. The study was designed to minimize the number of animals required for the experiments.

Experimental protocol

A total of 144 male mice (25–30 g) were used in this research. The experimental groups were equally divided into three animal lines: Swiss mice ($n = 48$), c57bl/6J mice ($n = 48$) and Balb/c mice ($n = 48$). The mice were provided by and kept under controlled environmental conditions (24°C relative humidity 40%–60%, 12 h alternate light–dark cycles, food and water *ad libitum*) in the Animal Center of University Center Christus (Unichristus) for this controlled experimental study. After 1 week of acclimatization, the mice that were heavy were randomly distributed into eight groups defined by euthanasia days: day 1–8.

The mice were anesthetized with a fresh-prepared mixture of ketamine 90 mg/kg + xylazine 10 mg/kg injected intraperitoneally and underwent an antisepsis of the oral cavity with 0.12% chlorhexidine with individual and disposable cotton. Then, we performed an oral ulcer in the left cheek mucosa of all animals with a 4 mm diameter and a 1 mm profundity dermatological punch. After ulcer confection, the coating epithelium was excised using a number 15 blades in the Bad Parker scalpel handle. The animals were placed in a thermic bag to avoid hypothermia and were observed during the return of the anesthetic plane.

The animals were weighed and euthanatized by cervical dislocation daily in six animals per day of each line ($n = 18$/day).

Clinical parameters

On the day of euthanasia, the OTUs were measured using a digital tachymeter (precision of 0.05 mm) on the left cheek to avoid the oral area ulcer (minor diameter × major diameter × $\pi/4$), as previously described (Cavalcante *et al.,* 2011 and Brizeno *et al.,* 2016). Thus, the left cheek was surgically removed and conditioned in buffered formalin 10% to histologic process.

The body mass variation was evaluated by the weight proportional variation as previously described ([final weight − initial weight]/initial weight × 100).[4]

Histologic process and analysis

The left ulcerated cheeks were macroscopically analyzed, dehydrated in alcoholic series, diaphanized in xilol and impregnated in paraffin (65°C). Slides of 4 μm were performed in a semiautomatic microtome and were stained with hematoxylin and eosin. The histologic slides were analyzed qualitatively and by scores as previously described:[9] 0 = no ulcer and connective tissue totally healed; 1 = no ulcer and fibrosis and slight chronic inflammation in connective tissue; 2 = with ulcer and fibrosis and moderate chronic inflammation in connective tissue; 3 = with ulcer and chronic inflammation process (granulation tissue) in connective tissue and 4 = with ulcer and acute process (dilated vessels, mixed inflammatory infiltrate with neutrophils).

The presence/absence of colonization signs of *Actinomyces* in the ulcer surface or in connective tissue was evaluated dichotomically.

Statistical analysis

Quantitative data (oral ulcer area and body mass variation) were analyzed by the Shapiro–Wilk test, expressed as the mean ± standard error of the mean and analyzed by

1- or 2-way ANOVA test and the Bonferroni *post hoc* test (parametric data). The histologic scores were expressed as median (minimum and maximum) and analyzed by the Kruskal–Wallis test and the Dunn *post hoc* test. The prevalence of colonization signs of *Actinomyces* was expressed as absolute and percental frequencies and analyzed by Chi-square test.

GraphPad Prism 5.0 (California corporation, San Diego, California, USA) for windows was used for statistical analysis, and a probability value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Oral ulcer area

In relation to the 1st day (4.9 ± 0.3 mm²) Swiss group, there was a significant decrease in ulcer area from the 4th day (day 4: 1.4 ± 1.0 mm²; day 5: 1.1 ± 0.3 mm² and day 6: 0.7 ± 0.0.6 mm²) healing completely on the 7th day (day 7 and day 8: 0.0 ± 0.0 mm²) ($P < 0.001$). In relation to the 1st day (4.9 ± 0.4 mm²) Balb/c group, there was a significant decrease in ulcer area from the 5th day (day 5: 1.4 ± 0.7 mm²; day 6: 1.5 ± 0.9 mm² and day 7: 0.9 ± 0.6 mm²) that did not heal completely on the 8th day (0.2 ± 0.1 mm²) ($P < 0.001$). In relation to the 1st day (4.6 ± 0.3 mm²), the c57bl/6j group showed a significant decrease in ulcer area from the 5th day (day 5:2.1 ± 0.2 mm²; day 6: 2.3 ± 0.2 mm² and day 7: 1.9 ± 0.2 mm²) but did not heal completely on the 8th day (0.7 ± 0.1 mm²) ($P < 0.001$). There were no differences in wound healing between the Balb/c, c57bl/6J and Swiss groups behind the evaluated time ($P = 0.348$) [Figure 1].

Body mass variation

In the Swiss group, there was a significant decrease in weight variation on the 2nd (−9.0% ± 1.7%) and 3rd (−10.1% ± 2.0%) days, and the animals returned to baseline on the 4th day (−1.4% ± 1.2%) and did not modify their body variation on the 8th day (0.6% ± 0.6%) ($P < 0.001$). In the Balb/c group, there was a significant decrease in weight gain on the 2nd (−15.2% ± 4.2%) and 3rd (−16.1% ± 2.1%) experimental days, and the animals returned to baseline on the 4th day (−3.7% ± 1.1%) and did not modify their body variation on the 8th day (−1.8% ± 0.5%) ($P < 0.001$). In c57bl/6J, there were no differences in body weight variation in eight experimental days ($P = 0.258$). The Balb/c group showed a significant weight reduction on the 3rd day compared to the Swiss group, and c57bl/6J showed a significant weight gain on the 3rd and 4th days ($P < 0.05$) [Figure 1].

Histologic analysis: Stages of wound healing

All mice in the Swiss (4, 4-4), Balb/c (4, 4-4) and c57bl/6J (4, 4-4) groups showed connective tissue exposed to the oral cavity and intense inflammatory infiltrate composed predominantly of polymorphonuclear neutrophils on the 1st days of the experimental protocol ($P = 1.000$) [Table 1 and Figure 2].

In the Swiss group, the median of the 3rd experimental day (4, 3-4) still showed a predominance of acute inflammatory cells evolving to granulation tissue on the 4th day (3, 2-4). From the 7thseventh (1, 0-3) and 8th days (1, 0-1), there was a significant reduction in histologic scores in the Swiss group, showing total wound reepithelization and a slight residual of mononuclear inflammatory cells in connective tissue ($P < 0.001$) [Table 1 and Figure 2].

In the Balb/c group on the 3rd day, granulation tissue was predominant in exposed connective tissue (3, 3-3). This situation lasted until the 5th day (3, 2-3) when there was a significant reduction in histologic scores in relation to the first experimental day ($P = 0.022$). However, the histologic scores have a discreet elevation from the 6th day, and the ulcer remained open to the oral cavity with prolonged granulation tissue in connective tissue, without significant differences until the 8th day (3, 3-4) [Table 1 and Figure 2].

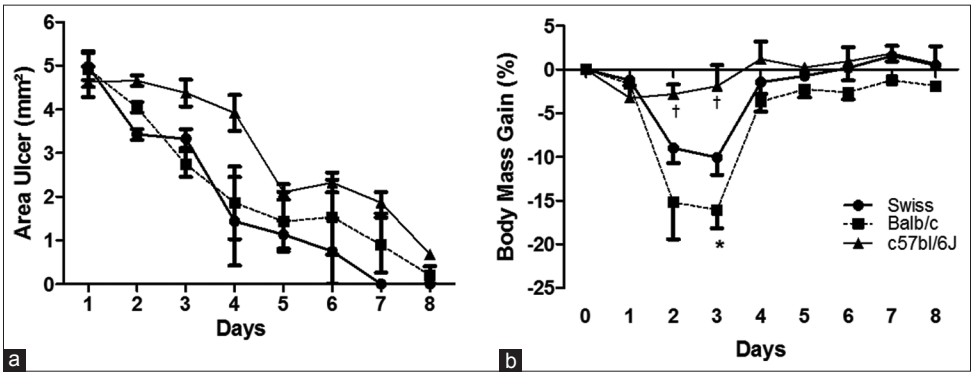

Figure 1: Clinical evaluation of area ulcer (a) and body mass gain (b) of Swiss, Balb/c and c57bl/6J mice with oral ulcers in the left cheek mucosa. *$P < 0.05$ versus Swiss group; †$P < 0.05$ versus Swiss group (mean ± standard error of the mean, 2-way-ANOVA/Bonferroni)

Table 1: Histologic evaluation of wound healing stages and signs of infection of Swiss, Balb/c and c57bl/6j mice with oral ulcers in cheek mucosa

	Experimental day								*P*
	1	2	3	4	5	6	7	8	
Histologic scores									
Swiss	4 (4-4)	4 (4-4)	4 (3-4)	3 (2-4)	3 (3-3)	3 (2-3)	1 (0-3)†	1 (0-1)†	<0.001[a]
Balb/c	4 (4-4)	4 (4-4)	3 (3-3)	3 (3-4)	3 (2-3)†	3 (3-3)	3 (1-4)	3 (3-4)*	0.022[a]
C57bl/6j	4 (4-4)	4 (4-4)	4 (3-4)	3 (3-3)	3 (3-3)	3 (1-3)†	3 (1-3)†	2 (1-3)†	<0.001[a]
P	1.000[b]	1.000[b]	0.058[b]	0.839[b]	0.168[b]	0.740[b]	0.185[b]	0.015[b]	
Signs of infection									
Swiss (%)	5 (83)	3 (50)	4 (67)	1 (20)†	0 (0)†	1 (20)†	1 (20)†	1 (20)†	0.027[c]
Balb/c (%)	3 (75)	5 (87)	3 (50)	1 (20)	1 (17)	3 (50)	3 (60)	3 (60)	0.085[c]
C57bl/6j (%)	1 (20)	0 (0)**	1 (17)	2 (40)	0 (0)	0 (0)	2 (33)	2 (33)	0.524[c]
P	0.078[c]	0.022[c]	0.207[c]	0.711[c]	0.411[c]	0.155[c]	0.411[c]	0.411[c]	

[a]Kruskal–Wallis/Dunn; [b]Mann–Whitney; [c]χ^2; data shown as median (minimum–maximum) or frequency (%). *$P<0.05$, versus Swiss; **$P<0.05$ versus Balb/c group on the same day; †$P<0.05$, versus day 1 of same group

The c57bl/6J group, similar to the Swiss group, showed a modification of the inflammatory profile in the 4th day with a reduction of acute inflammation and the presence of predominant granulation tissue in exposed connective tissue (3, 3-3). This profile has a significant reduction in histologic scores on the 6th day (3, 1-3) compared with the 1st experimental day ($P < 0.001$). There was no significant repair from the 6th day, and on the 8 experimental days, the ulcer remained open, with a median of 2 (1-3) (with ulcer and fibrosis and moderate chronic inflammation in connective tissue) [Table 1 and Figure 2].

On the 8th experimental day, the Balb/c group (3, 3-4) showed histologic scores significantly higher than the Swiss (1, 0-1) and c57bl/6J groups (2, 1-3) ($P = 0.015$) [Table 1 and Figure 2].

Histologic analysis: Signs of infections

The presence of signs of infection showed surface ulcer area and connective exposed tissue in most animals. *Actinomyces* were observed in 83% of Swiss mice on the 1st day, with a significant reduction of this prevalence from the 4th day (20%) until the last day of protocol (20%) ($P = 0.027$) [Table 1 and Figure 2].

The Balb/c group showed a high prevalence of *Actinomyces* during the 8 days of evaluation. On the 1st day, the prevalence of signs of infection was 75%, and there was no significant variation during the experimental protocol ($P = 0.085$) [Table 1 and Figure 2].

The c57bl/6J mice showed a low frequency of surface and profundity infection. On the 1st day, only 20% of animals showed *Actinomyces* on the oral ulcer surface, and this prevalence was equally low along the 8 experimental days ($P = 0.524$) [Table 1 and Figure 2].

On the 2nd day, the Balb/c group showed a higher significant infection rate than the c57bl/6J group (0%) ($P = 0.022$).

DISCUSSION

OTUs are complex pathological entities, and their healing depends heavily on the inflammatory process and immunological system. Some studies have shown that the control of pro-inflammatory processes,[10] the reduction of pro-oxidant molecules,[11] the modulation of an anti-inflammatory response by the use of growth factors[12] and the infusion of undifferentiated mesenchymal cells[13] can accelerate the wound healing of OTUs.

These processes normally occur within 10 days in rats without a specific immune response[9] and are dependent on a balance in pro (th1) and anti-inflammatory (th2) cytokines.[2] Overexpression of pro-inflammatory (th1) cytokines is important to delay wound healing in OTUs, and its blockage with compounds that augment the th2 response accelerates the processes.[10,13,14] Therefore, the th2 response seems to be the most appropriate process for wound healing of OTUs.

Paradoxically, the Balb/c group showed an unappropriated inflammatory process and delayed OTU healing. The Th2 response is associated with a greater production of cytokines such as interleukin (IL)-4, -5, -6, -9, -13 and-10, which are important for stimulus of antibody production and control of infections.[15] Some of these cytokines, such as IL-6 and IL-10, are indispensable to wound healing of epithelial injuries lesions.[16,17] However, the predominance of a th2 response is harmful to wound healing.

Mice lacking the interferon (IFN)-gamma receptor (th1 response) have an impaired ability to resolve inflammatory processes associated with a prolonged capacity of T cells to exhibit a th2 cytokine profile.[18] Despite stimulating wound repair, th2 cytokines are antagonists of the pro-inflammatory profile (th1), inhibiting the classical

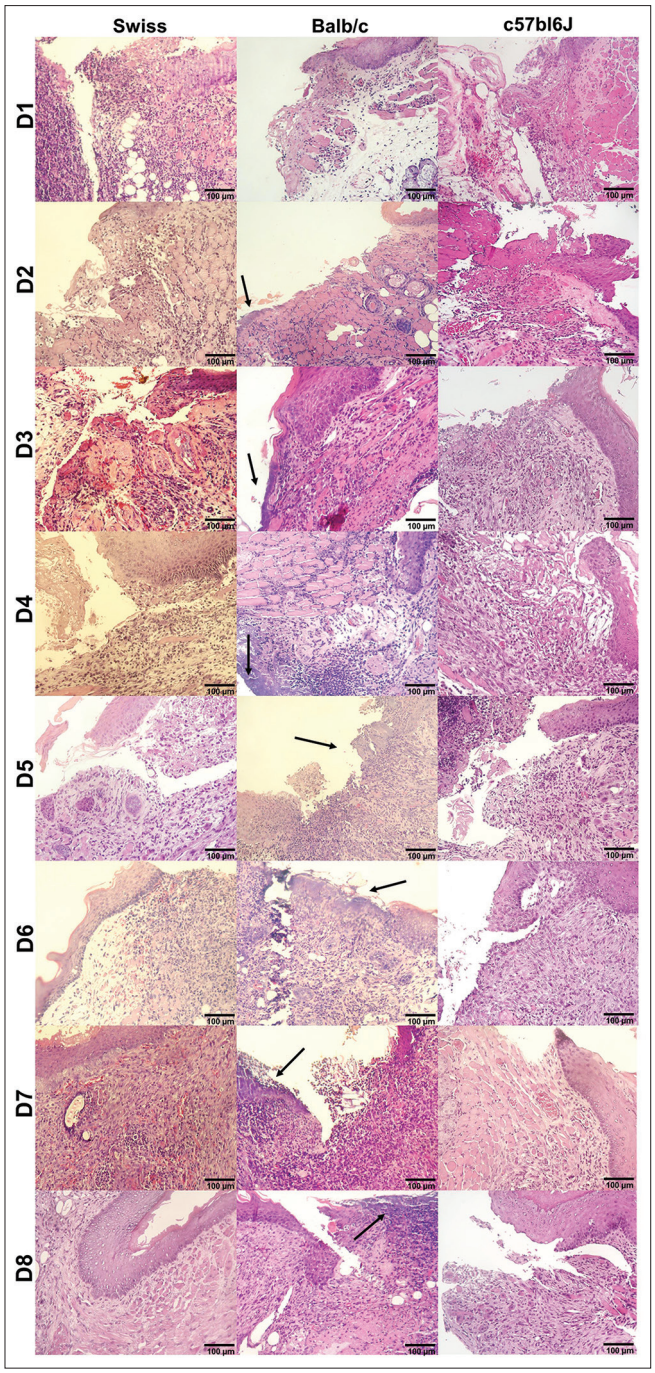

Figure 2: Microscopic characteristics of OTU in Swiss, Balb/c and c57bl/6J mice showing intense acute inflammation in days of experimental course and granulation tissue formation in the 4th day in both groups (×200). Black arrows demonstrate microscopic signs of infection by *Actinomyces* spp

activation of macrophages that play a major role in combating oral microbiota and preventing infection.[16,17,19]

In this context, the c57bl/6J mice (th1 predominant immunological response) showed a great antimicrobial capacity. The infection rate of OTU was low throughout the protocol experiment. The Th1 response is extremely important to control the microbiota of the gastrointestinal tract,[20] and cytokines such as tumor necrosis factor-alpha, IFN-gamma, and IL-1 beta activate macrophages and monocytes, augmenting their phagocytic ability,[21] which is important in the control of superficial infection of OTUs. In addition, th1/th2 polarization is responsible for the development of autoimmunity and long infection processes in the gastrointestinal tract.[22]

Although the th1 response shows potent antimicrobial effects preventing the infection of OTUs and loss of weight (Oliveira *et al.,* 2016), the OTUs of c57bl/6J mice (th1 predominant response) did not heal completely along the 8 experimental days. Thus, the balance in th1 and th2 immunological profiles seems to be the most appropriate way to wound healing.

The Swiss mice showed the best results in course of OTUs healing. Despite the Swiss group losing more weight, probably due to high infection rates in the 1st days of experimental protocols that lead to overproduction of pro-inflammatory and pro-nociceptive cytokines,[4,23] this overproduction quickly diminished the infection frequency. On the 4th day, in the absence of infection, the unspecific immune response directed to wound healing with a natural anti-inflammatory stimulus.[9]

The control of pro-inflammatory processes is indispensable to accelerate the healing of OUT,[8-11] but the suppression of this response leads to augmented infection rates, apoptosis and delayed wound healing.[4]

The inability of the immune system to respond to response to infection in surface and connective tissue (th2 predominantly immunity) and the inability of the immune system to convert the pro-inflammatory response in an anti-inflammatory response (th1 predominant immunity) play the major role in delayed wound healing in this study. Thus, the combination of antimicrobial approaches with anti-inflammatory drugs can be a promising investment in future perspectives of pharmacological formulations to accelerate wound healing.[24]

CONCLUSION

The Balb/c mice (th2 predominant immunity) showed a greater infection rate for OTUs, delayed wound healing and greater weight loss. The c57bl/6J mice (th1 predominant immunity) showed a greater antimicrobial ability but without completely healing their OTUs. Thus, this study shows that the balance in initial th1 (antimicrobial) and later th2 (anti-inflammatory and prohealing) responses

is the best way to accelerate wound healing of OTUs (Swiss mice, nonspecific immunity).

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Conflicts of interest

There are no conflicts of interest.

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