



ORIGINAL ARTICLE

Fixation of free gingival grafts with cyanoacrylate glues: A histomorphometric and immunohistochemical study

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KEYWORDS

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Abstract *Aim:* The objective of this study was to evaluate the inflammatory process resulting from the use of two cyanoacrylate-based adhesives to stabilize grafts.

Methodology: A total of 45 male Wistar rats were randomly divided into three groups (n = 15/group) treated with ethyl cyanoacrylate glue (TG1), octyl-2-cyanoacrylate glue (TG2) or suture threads (CG). After de-epithelialization in the anterior gingival region of the mandible, the graft was removed from the donor site (hard palate), taken to the recipient site and stabilized according to the protocol of each group. After 7, 14, and 45 days, the animals were euthanized. The graft area was analysed macroscopically, histologically, histochemically (Masson trichrome), and immunohistochemically positive cell count for TGF- β , α -SMA, RANKL, OPG, FGF, and IL-10. The Kruskal-Wallis/Dunn test (SPSS 20.0, p < 0.05) was used for analysis.

Results: There was no difference in the clinical parameters among the three groups, but TG1 showed the lowest mononuclear inflammatory cell count and the highest amount of total collagen. FGF immunoreaction was significantly higher for the CG group, but the TG2 showed a significant reduction in the RANKL/OPG ratio.

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Conclusion: TG1 had a mild inflammatory response and a higher collagen deposition than other glues, and TG2 had a reduction in the RANKL / OPG ratio.

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1. Introduction

The free gingival graft is a surgical technique characterized by the disinsertion of the gingival tissue, containing the epithelial and connective tissues, from its donor area to its transfer to the recipient area (Deo et al., 2019). One of the existing techniques for graft stabilization is the use of conventional sutures. However, this technique has disadvantages, causing tissue irritation, promoting an inflammatory response and may lead to a hematoma at the puncture site, therefore, adequate stabilization of the gingival tissue is not achieved (Oh et al., 2017; Gümüş and Buduneli, 2014).

Thus, the literature has proposed that the use of tissue adhesives in wound closures has great advantages compared to conventional techniques. Among them, cyanoacrylates are often used as tissue adhesives in the medical field, due to their hemostatic, anti-inflammatory characteristics and their high adhesion capacity in a humid environment (Cortellini et al., 2012).

Thus, one of the main clinical advantages of cyanoacrylates is the possibility of polymerization when they come into contact with water and / or blood due to the excellent bonding strength to the tissues (Davis and Derlet, 2013; Clarke, 2011; Hung et al., 2014; Andreotti Damante et al., 2020). Therefore, the objective of this study was to evaluate the inflammatory process resulting from the use of two cyanoacrylate-based adhesives for graft stabilization.

2. Materials and methods

2.1. Animals and ethical principles

This study was submitted to and approved by the Ethics Committee on the Use of Animals (CEUA) of the University of Fortaleza, filed under CEUA number 3,597,270,218 and under Federal Law number 11.794 (2008) and decree number 6.689 (2009) that regulate animal experimentation in the country.

Male Wistar rats ($n = 45$) (*Rattus norvegicus*), 3 to 5 weeks of age, weighing ± 200 g, from the vivarium of the University of Fortaleza were used. The animals were kept in individual cages in the vivarium during the experimental procedure, remaining in a controlled macroenvironment with access to a water supply and specific feed at will.

The animals were randomized into three experimental groups: a control group (CG) whose grafts were stabilized by employing simple sutures on the graft margins; test group 1 (TG1) whose grafts were stabilized using only ethyl cyanoacrylate glue and group 2 (TG2) whose grafts were stabilized using only octyl-2 glue cyanoacrylate.

2.2. Surgical procedure

To perform the experimental surgical procedure, rats were anaesthetized with 2% xylazine hydrochloride (Syntec®) and 10% ketamine hydrochloride (Syntec®) (10 mg/kg and

90 mg/kg, respectively) intraperitoneally. After anaesthesia, de-epithelialization was performed in the anterior mandible of each animal using a 15c scalpel blade. The de-epithelialized area comprised the entire region of the buccal mucosa in the region of the incisors.

Soon after de-epithelialization and the adequate preparation of the recipient site, the graft from the donor site of the animal's hard palate in the posterior region of the maxilla was removed. The graft area was 2 mm long \times 2 mm wide. Both epithelial and connective tissue were removed, with a thickness of 1 mm. Immediately after removing the tissue from the donor area, the graft was repositioned at the recipient site where the fixation protocol was performed according to each experimental group.

In the technique performed in the control group, graft stabilization was performed with Vicryl 5.0 suture thread (Ethicon®), with two sutures in the lateral regions; both were interrupted simply. In test groups 1 and 2, ethyl cyanoacrylate glue and 2-octyl cyanoacrylate were used, respectively, and were applied to all graft edges, which was fixed at the recipient site and pressed gently.

The animals were euthanized 7 days, 14 days, and 45 days after the surgical procedure using anaesthetic overdose (270 mg/kg ketamine hydrochloride and 30 mg/kg xylazine hydrochloride) when the grafts were analysed macroscopically and removed for histological analysis.

2.3. Clinical assessment of graft stability

The graft recipient sites were evaluated clinically by a single evaluator for the following clinical parameters: oedema, redness, and necrosis. For the inflammatory parameters, the surgical bed was assessed by scores: absent oedema (0), mild oedema (1) moderate oedema (2) severe oedema (3), and absence (0) or presence (1) redness (Zambon et al., 1989; Vastani and Maria, 2013).

Finally, to evaluate the necrosis of the grafted tissue, a UNC15 millimetre probe (Golgran®) was used to measure the graft necrosis area, when present, which was considered necrotic tissue that presented a colour change, different from the normal mucosa. The largest diameter and the smallest diameter were measured to calculate the area based on the formula described by Cavalcante (Cavalcante et al., 2011).

2.4. Histological processing and microscopic analysis

The mucosal tissues related to the recipient area were removed and fixed in 10% buffered formaldehyde, processed histologically, embedded in paraffin, and subjected to 4- μ m cuts for staining by haematoxylin and eosin (HE) and Masson's trichrome (EasyPath®). Five fields were photographed at 400x magnification using a microscope (Leica®) coupled to a camera (Leica MC 170 HD®) and LAS V4.3 software

(Leica®) in the areas of greatest cellularity for polymorphonuclear, mononuclear and giant cells on slides stained with haematoxylin-eosin using the 'Cell Counter' command of ImageJ software (Java®) (Brizeno et al., 2016).

The slides stained by Masson's trichrome were photographed using the same methodology to quantify the total collagen present. The photomicrographs were evaluated by the image analysis software ImageJ® (RSB) after calibration of the images by the command 'Color Deconvolution' (Image > Plugins > Color Functions for the blue colour). After calibration, the images were binarized (Process > Binary > Make Binary), and the percentage of collagen area was marked in black, corresponding to the total collagen (Analyse > Analyse Particles), and measured, obtaining the area fraction as a percentage (Park et al., 2015).

2.5. Preparation of a tissue microarray (TMA) block

After histological and histomorphometric analysis, two representative 2 mm micro-campuses of the graft were selected and marked on the histological slides for subsequent pairing with the block and removal of the sample using the Tissue Micro Arrayer® device (Quick-Ray™ UNITMA, Reference: UT06®). Afterwards, a recipient block containing 70 wells of the same diameter and depth received the samples of paraffin tissue, organized and mapped (Andrade, 2007). After cooling the receiver block at room temperature, 3 µm cuts were made and placed on silanized slides for an immunohistochemical reaction.

2.6. Reactions and immunohistochemistry analysis

Briefly, the silanized slides were dewaxed and submitted to antigenic recovery with 0.1 M citrate solution pH 6.0. Endogenous peroxidase activity was blocked for 30 min with 0.3% hydrogen peroxide followed by 1% protein blocking for 30 min. The slides were incubated overnight with the following antibodies: anti-transformant growth factor beta (TGF-β) (1: 100, ab92486, Abcam®), anti-smooth muscle actin (α-SMA) (1: 200, ab32575, Abcam®), anti-nuclear activator receptor kappa B ligand (RANK-L) (1: 200, ab45039, Abcam®), anti-osteoprotegerin (OPG) (1: 250, ab73400, Abcam®), anti-fibroblast growth factor (FGF) (1: 1250, ab8880, Abcam®) and anti-interleukin-10 (IL-10) (1: 200, ab217941, Abcam®).

The next day, the slices were incubated with anti-rabbit/anti-mouse polymer Dako Envision Dual Link System HRP (K4061, Dako®) for one hour. The slides were incubated with 3,3-diaminobenzidine (DAB) (K3468, Abcam®) for 5 min. The slides were counterstained with Harris 7% haematoxylin for 10 s and assembled with Enthellam®. For the positive control, histological sections of pyogenic granuloma were used, and for the negative control, suppression of the primary antibody.

Five fields were photographed at 400× magnification using a microscope (Leica®) coupled to a camera (Leica MC 170 HD®) and software (LAS V4.3) (Leica®) in the areas of greatest cellularity to count cells with cytoplasmic positivity for the markers mentioned above using the 'Cell Counter' command of ImageJ software (Java®) (Brizeno et al., 2016; Zhang et al., 2015).

2.7. Statistical evaluation

The macroscopy scores were expressed as the median (minimum–maximum), and the other data were expressed as the mean and standard error. The data were submitted to the Shapiro-Wilk normality test and compared by the Kruskal-Wallis/Dunn test and Spearman's correlation (SPSS 20.0 for Windows, $p < 0.05$).

3. Results

3.1. Stabilization of free gingival grafts with cyanoacrylate-based adhesives does not alter the clinical parameters compared to the gold standard.

In all experimental groups, the signs of oedema and redness were mild and present. In the CG, there was no significant variation in the signs of oedema and redness throughout the experimental protocol, but in groups TG1 and TG2, there was a significant reduction in these signs on D14 in relation to D7 (Table 1).

Regarding the area of necrosis, there was no significant difference between groups on days 7, 14 and 45 and all groups showed a significant reduction in the area of necrosis on D14 in relation to D7 (Table 1).

3.2. Fixation of free gingival grafts with cyanoacrylate-based adhesives reduces the inflammatory process and alters the collagen/fibroplasia process

On days 7, 14 and 45, there was no significant difference in the PMN count. (Fig. 1) The CG, TG1 and TG2 showed a significant reduction in this parameter 14 days after the surgical procedure (Table 1).

The CG showed the highest number of inflammatory mononuclear cells in groups TG1 and TG2 on D7, with no significant difference between the groups on D14 and D45. All groups showed a significant reduction in the number of mononuclear cells from day 14. (Table 1) (Fig. 1)

There was no significant difference in the number of multinucleated giant cells in the three experimental groups on any day or throughout the experimental (Table 1).

Regarding the percentage of collagen (Fig. 1) in the graft, there was no significant difference in the three experimental groups on day 7, but TG1 showed an increased level of collagen in relation to the other groups on day 14. On day 45, the groups showed no differences between them. The CG showed a significant increase in the percentage of collagen area only at D45, TG1 demonstrated this increase from D14, and TG2 did not show a significant variation in the percentage of collagen area over time during the study (Table 1).

3.3. Fixation of free gingival grafts with sutures requires greater expression of collagen markers for adequate tissue repair

The expression of α-SMA (Fig. 2) was significantly higher in the CG than in the other groups on days 7 and 14, with no difference on day 45. There was a significant increase in the number of α-SMA-positive cells in the CG and TG1 from day 14,

Table 1 Clinical and histological parameters of free grafts fixed in the gingiva of rats submitted to de-epithelialization in the anterior region of the mandible.

	Time			<i>p-Value</i>
	D7	D14	D45	
Edema*				
Suture	1 (0–1) ^{Aa}	0 (0–0) ^{Aa}	0 (0–0) ^{Aa}	0.056
Superbonder	1 (1–2) ^{Aa}	0 (0–0) ^{Ab}	0 (0–0) ^{Ab}	0.001
Dermabond	1 (1–2) ^{Aa}	0 (0–0) ^{Ab}	0 (0–0) ^{Ab}	0.008
<i>p-Value</i>	0.131	1.000	1.000	
Redness *				
Suture	1 (0–1) ^{Aa}	0 (0–0) ^{Aa}	0 (0–0) ^{Aa}	0.056
Superbonder	1 (1–1) ^{Aa}	0 (0–1) ^{Ab}	0 (0–0) ^{Ab}	0.009
Dermabond	1 (1–1) ^{Aa}	0 (0–0) ^{Ab}	0 (0–0) ^{Ab}	0.007
<i>p-Value</i>	0.142	1.000	1.000	
Area of necrosis †				
Suture	32.00 ± 11.14 ^{Aa}	7.50 ± 2.50 ^{Ab}	0.00 ± 0.00 ^{Ab}	0.042
Superbonder	26.00 ± 11.22 ^{Aa}	2.00 ± 2.00 ^{Ab}	0.00 ± 0.00 ^{Ab}	0.038
Dermabond	37.50 ± 13.77 ^{Aa}	3.33 ± 3.33 ^{Ab}	0.00 ± 0.00 ^{Ab}	0.017
<i>p-Value</i>	0.679	0.267	1.000	
PMN†				
Suture	96.60 ± 19.66 ^{Aa}	8.00 ± 6.70 ^{Ab}	1.75 ± 1.75 ^{Ab}	0.010
Superbonder	53.00 ± 24.77 ^{Aa}	2.40 ± 0.75 ^{Ab}	0.00 ± 0.00 ^{Ab}	0.002
Dermabond	53.75 ± 16.87 ^{Aa}	32.00 ± 18.58 ^{Ab}	0.75 ± 0.75 ^{Ab}	0.033
<i>p-Value</i>	0.272	0.282	0.503	
MN†				
Suture	44.60 ± 6.62 ^{Aa}	10.75 ± 3.20 ^{Ab}	13.75 ± 4.71 ^{Ab}	0.014
Superbonder	25.00 ± 4.12 ^{Ba}	20.80 ± 3.35 ^{Ab}	9.60 ± 1.96 ^{Ab}	0.039
Dermabond	37.50 ± 4.70 ^{Aa}	16.33 ± 8.17 ^{Ab}	4.50 ± 1.32 ^{Ab}	0.027
<i>p-Value</i>	0.037	0.201	0.181	
CG†				
Suture	2.40 ± 1.12 ^{Aa}	1.75 ± 1.75 ^{Aa}	2.00 ± 1.35 ^{Aa}	0.764
Superbonder	0.80 ± 0.80 ^{Aa}	2.40 ± 1.50 ^{Aa}	0.60 ± 0.60 ^{Aa}	0.379
Dermabond	0.00 ± 0.00 ^{Aa}	2.00 ± 2.00 ^{Aa}	1.00 ± 1.00 ^{Aa}	0.484
<i>p-Value</i>	0.171	0.714	0.315	
Collagen (%) †				
Suture	30.57 ± 9.91 ^{Aa}	23.33 ± 10.48 ^{Aa}	43.75 ± 5.62 ^{Ab}	0.028
Superbonder	27.91 ± 11.49 ^{Aa}	41.25 ± 8.94 ^{Bb}	43.72 ± 4.58 ^{Ab}	0.035
Dermabond	35.51 ± 9.64 ^{Aa}	33.34 ± 1.25 ^{Aa}	42.02 ± 6.32 ^{Aa}	0.299
<i>p-Value</i>	0.650	0.020	0.892	

p < 0.05, median (minimum - maximum); † p < 0.05 (mean ± SEM); Kruskal-Wallis test (Different capital letters = difference between groups; different lower letters = difference between the evaluation moments. Edema scores: 0 = Absent; 1 = Mild; 2 = Moderate; 3 = Severe. Redness scores: 0 = Absent; 1 = Present[9], Vastani et al, 2013).

but in TG2, there was no significant variation in the number of α -SMA-positive cells (Table 2).

The results were similar for the immunoexpression of FGF (Fig. 2), which showed a greater number of positive cells in the CG than in the TG1 and TG2 on day 7, with no difference on days 14 and 45. The CG and TG1 showed a significant increase in FGF expression on day 14, although TG2 did not show significant variation in the cellular expression of this marker (Table 2).

Regarding IL-10 (Fig. 2), there was no significant difference in the number of positive cells among the three groups on day 7, but on day 14, the immunoexpression for IL-10 was significantly higher in the CG than in the other groups. The CG was the only group that showed a significant increase in IL-10 over the time course of the study (Table 2).

Immunoexpression for TGF- β (Fig. 2) did not vary significantly between groups or between assessment periods (Table 2).

3.4. Fixation of free gingival grafts with ethyl-2-cyanoacrylate reduces the RANKL/OPG ratio in free gingival grafts

Immunoexpression for RANKL and OPG (Fig. 3) did not vary significantly between the experimental groups. However, the RANKL/OPG ratio decreased significantly in the group treated with ethyl-2-cyanoacrylate on the 45th postoperative day compared to that present on the 7th and 14th days after periodontal surgery; nevertheless, there were no differences between groups (Table 3).

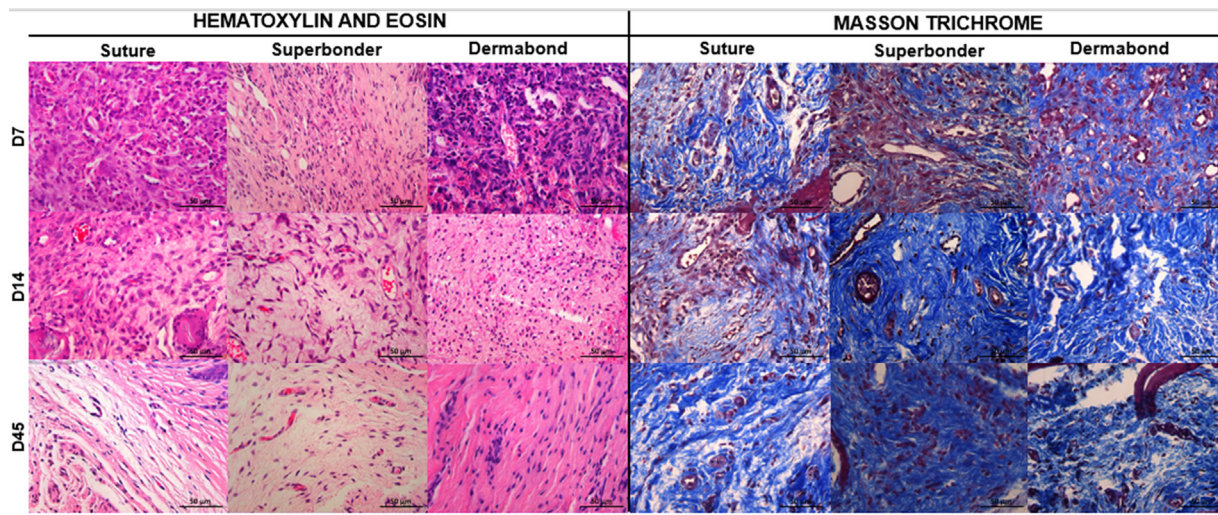


Fig. 1 Cell and tissue profile of free grafts fixed in the gingiva of rats submitted to de-epithelialization in the anterior region of the mandible (400x, Hematoxylin-eosin, and Masson's Trichomium).

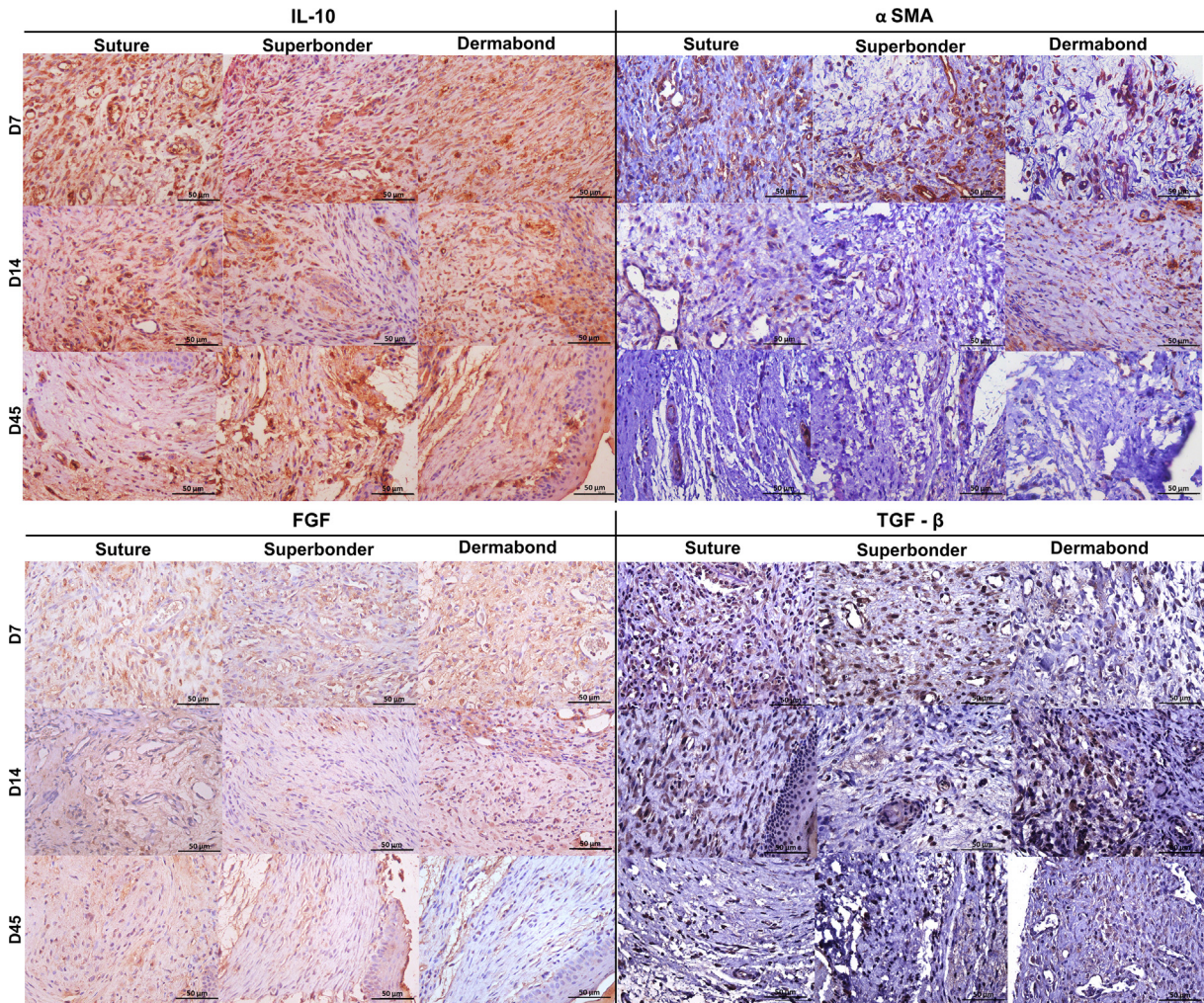


Fig. 2 Immunoreaction profile for IL-10, α -SMA, FGF and TGF- β in free grafts fixed on the gingiva of rats submitted to de-epithelialization in the anterior region of the mandible (400x, IHC).

Table 2 Immunohistochemical profile of free grafts fixed on the gingiva of rats submitted to de-epithelialization in the anterior region of the mandible.

	Time			<i>p-Value</i>
	D7	D14	D45	
α-SMA				
Suture	25.80 \pm 3.71 ^{Aa}	37.00 \pm 2.89 ^{Ab}	16.50 \pm 6.08 ^{Aa}	0.036
Superbonder	3.25 \pm 0.85 ^{Ba}	12.75 \pm 2.29 ^{Bb}	27.00 \pm 4.14 ^{Ac}	0.010
Dermabond	13.25 \pm 4.68 ^{Ba}	6.00 \pm 1.53 ^{Ba}	7.00 \pm 1.15 ^{Aa}	0.221
<i>p-Value</i>	0.009	0.017	0.115	
FGF				
Suture	19.80 \pm 1.07 ^{Aa}	9.75 \pm 1.11 ^{Ab}	12.25 \pm 3.88 ^{Ab}	0.030
Superbonder	13.60 \pm 1.72 ^{Ba}	6.80 \pm 1.28 ^{Ab}	17.00 \pm 4.20 ^{Aa}	0.044
Dermabond	12.00 \pm 0.91 ^{Ba}	7.33 \pm 1.67 ^{Aa}	9.50 \pm 1.50 ^{Aa}	0.112
<i>p-Value</i>	0.016	0.292	0.295	
IL-10				
Suture	26.40 \pm 2.54 ^{Aa}	44.50 \pm 2.36 ^{Ab}	37.50 \pm 0.50 ^{Ab}	0.013
Superbonder	22.40 \pm 4.21 ^{Aa}	21.00 \pm 2.89 ^{Ba}	32.25 \pm 4.97 ^{Aa}	0.379
Dermabond	18.75 \pm 6.55 ^{Aa}	16.00 \pm 0.58 ^{Ba}	18.67 \pm 3.38 ^{Aa}	0.676
<i>p-Value</i>	0.394	0.026	0.137	
TGF-β				
Suture	29.40 \pm 4.13 ^{Aa}	47.50 \pm 8.59 ^{Aa}	31.75 \pm 13.42 ^{Aa}	0.294
Superbonder	20.60 \pm 6.38 ^{Aa}	33.75 \pm 4.66 ^{Aa}	36.75 \pm 4.64 ^{Aa}	0.222
Dermabond	18.50 \pm 7.64 ^{Aa}	36.00 \pm 9.00 ^{Aa}	26.75 \pm 8.60 ^{Aa}	0.331
<i>p-Value</i>	0.478	0.424	0.760	

* $p < 0.05$, Kruskal-Wallis test (mean \pm EPM). Different capital letters = difference between groups; different lowercase letters = difference between the evaluation moments.

4. Discussion

It was observed in this study that the glues based on ethyl cyanoacrylate and octyl-2-cyanoacrylate demonstrated safe fixation of free gingival grafts in rats, inducing a predominantly mononuclear inflammatory reaction, higher collagen deposition and a diminished relationship between RANKL/OPG immunexpression.

The necrotic areas did not differ significantly between the groups, revealing the ability of the three methods to maintain an adequate blood supply. This stabilization is essential for the healing process (Gümüş and Buduneli, 2014).

The clinical findings suggestive of an inflammatory process were low in all groups and were reduced significantly only in the test groups. Part of these results may be related to the prolongation of these parameters in the CG, suggested by the

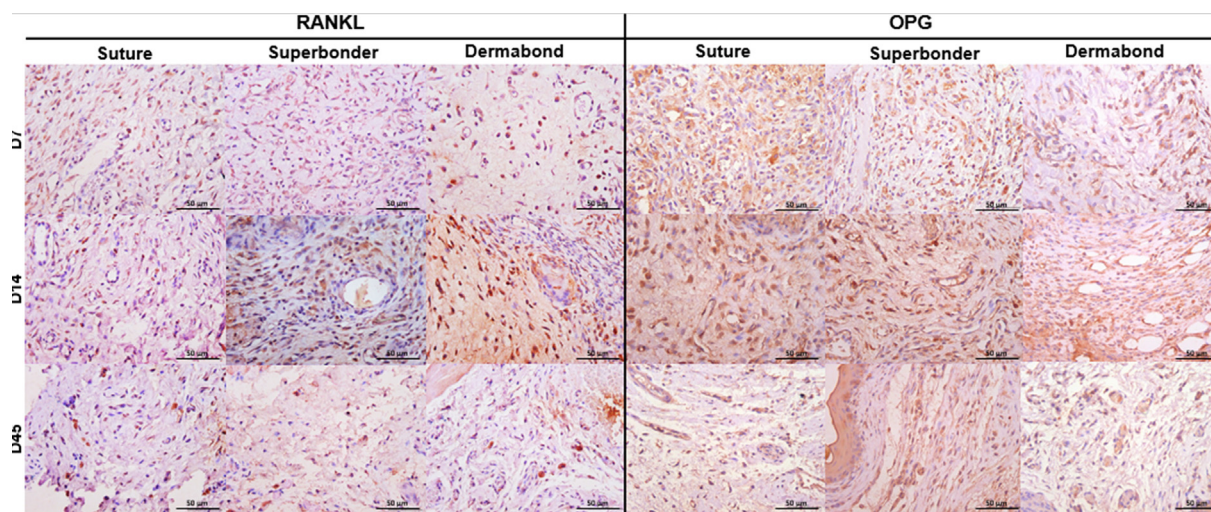


Fig. 3 Immunexpression profile for RANKL and OPG in free grafts fixed on the gingiva of rats submitted to de-epithelialization in the anterior region of the mandible (400x, IHC).

Table 3 Immunoexpression of the RANKL / OPG axis in free grafts fixed on the gingiva of rats submitted to de-epithelialization in the anterior mandible region.

	Time			<i>p-Value</i>
	D7	D14	D45	
RANKL				
Suture	14.40 ± 1.91 ^{Aa}	19.50 ± 3.30 ^{Aa}	15.25 ± 4.91 ^{Aa}	0.520
Superbonder	13.20 ± 2.18 ^{Aa}	19.67 ± 3.38 ^{Aa}	17.25 ± 7.64 ^{Aa}	0.538
Dermabond	15.00 ± 1.08 ^{Aa}	7.33 ± 0.67 ^{Aa}	5.50 ± 1.55 ^{Aa}	0.023
<i>p-Value</i>	0.554	0.054	0.345	
OPG				
Suture	27.20 ± 1.20 ^{Aa}	39.00 ± 3.83 ^{Aa}	32.00 ± 9.26 ^{Aa}	0.239
Superbonder	40.60 ± 5.51 ^{Aa}	23.40 ± 4.80 ^{Aa}	36.00 ± 2.98 ^{Aa}	0.061
Dermabond	20.50 ± 2.72 ^{Aa}	17.50 ± 0.50 ^{Aa}	18.00 ± 3.00 ^{Aa}	0.561
<i>p-Value</i>	0.019	0.070	0.091	
RANKL/OPG				
Suture	0.53 ± 0.07 ^{Aa}	0.49 ± 0.05 ^{Aa}	0.50 ± 0.15 ^{Aa}	0.975
Superbonder	0.34 ± 0.06 ^{Aa}	0.69 ± 0.18 ^{Aa}	0.45 ± 0.19 ^{Aa}	0.214
Dermabond	0.78 ± 0.13 ^{Aa}	0.40 ± 0.05 ^{Aa}	0.30 ± 0.06 ^{Ab}	0.030
<i>p-Value</i>	0.478	0.424	0.760	

* $p < 0.05$, Kruskal-Wallis test (mean ± EPM). Different capital letters = difference between groups; different lowercase letters = difference between the evaluation moments.

presence of suture threads in the gingival graft, which is responsible for maintaining biofilm for a prolonged time. On the other hand, when biological glues were used to fix free gingival grafts, there was less biofilm retention (Kulkarni and Chava, 2003).

The GC showed an increase in the synthesis of IL-10, which is a cytokine that blocks the translocation of cells, attenuating inflammation and interfering with collagen synthesis (Reitamo et al., 1994; Yamamoto et al., 2001). The reduction of inflammatory cells is also related to the decrease in the expression of cytokines of collagen / fibroplasia, such as FGF and TGF- β (Balbino et al., 2005). The increased expression of FGF and α -SMA observed in the GC is necessary to induce collagenesis in the presence of inflammatory cells (Zittermann and Issekutz, 2006), justifying the greater immunoexpression of FGF in the GC. FGF is able to stimulate fibroblasts and osteoblasts, consequently assisting acute and chronic wound healing (Koike et al., 2020).

Interestingly, the RANKL/OPG ratio in the test group 2 showed a significant reduction throughout the experimental protocol. This reveals a substantial clinical impact since the reduction in this axis attenuates the loss bone architecture (Ferreira et al., 2017). From this perspective, its use seems to be feasible and advantageous, since one of the indications of the free gingival graft is root coverage in cases of significant vestibular bone loss.

5. Conclusion

The use of sutures to stabilize free gingival grafts is still an excellent tool, since it showed greater expression of FGF and α -SMA, which are essential in the healing process. The use of biological cyanoacrylate adhesives induced a mild inflammatory response in free gingival grafts with greater collagen deposition. The low cost of glues and the ease of application of these methods seem to have a clinical benefit in stabilizing free gingival grafts than conventional sutures.

Declaration of Competing Interest

The author declare that there is no conflict of interest.

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