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The effect of bevacizumab, 5-fluorouracil, and triamcinolone on the healing modulation of surgical wounds in rats

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Summary. In this study, we aimed to analyze the effect of 5-fluorouracil, triamcinolone, and bevacizumab on scar modulation in an experimental rat model of surgical lesions. Rats (*Rattus norvegicus albinus*) were divided into four groups: bevacizumab, 5-fluorouracil + triamcinolone, bevacizumab + 5-fluorouracil + triamcinolone, and control (received no medication) groups. A linear, dorsal incision was created and sutured for the first intention wound healing, mimicking the surgical incision of upper blepharoplasty. Treatments were initiated on day 7, and the rats were euthanized on day 14. Only in the 5-fluorouracil + triamcinolone group was there a difference in the number of infiltrated monocytes. There was 56%, 86%, and 85% decrease in the number of neovessels in the bevacizumab, 5-fluorouracil + triamcinolone, and bevacizumab + 5-fluorouracil + triamcinolone groups, respectively, compared with the control. Picrosirius red staining showed higher collagen density and more organized collagen in the treatment groups than in the control group. Scar modulation was observed in all groups, but the 5-fluorouracil + triamcinolone group presented the best results. To our knowledge, this is the first study to evaluate the influence of three medications in combination on healing. When used together, these medications can prevent the development of unsightly scars, and are therefore promising alternatives to corticosteroids.

Key words: BVZ, 5-FU, Triamcinolone, Scar, Scarring modulation

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Introduction

During recent years, surgical procedures, particularly aesthetic surgeries, have increased worldwide. The countries where most cosmetic procedures are performed are Brazil and the United States, with breast implants, liposuction, and blepharoplasty, the focus of the present study, being the commonly performed procedures (ASAPS, 2019). Among women over 65 years of age, upper blepharoplasty surgery remains the most frequently performed aesthetic procedure; however among men and women, it is the third and fifth most frequent aesthetic procedure, respectively (ASAPS, 2019).

Besides visual field improvement, blepharoplasty is used to improve appearance by removing excess skin and fat bags in the eye region. The eyelid has the thinnest skin in the human body and has little tension. These characteristics allow excellent healing following blepharoplasty. However, there are some patients who, despite these favorable characteristics, develop fibroproliferative scarring during the postoperative period (Kalasho et al., 2019). Employing suitable surgical techniques combined with postoperative follow-up is essential for satisfactory healing. Nevertheless, even with adequate technical care in the preoperative and postoperative periods, scar disorders may occur, which include the development of fibroproliferative disorders in 5-15% of patients and may culminate in the formation of keloids and hypertrophic scars (dos Santos et al., 2015).

Besides the apparent aesthetic issues, there may be a few changes in scars that impair palpebral dynamics. Currently, corticosteroids, especially triamcinolone, are used as the first-line treatment, but they are typically prescribed to treat skin hypopigmentation, adjacent tissue atrophy, and subcutaneous whitish deposits; moreover, they are not effective in all patients (Sevilla and Perez, 2018). Research is ongoing to identify

innovative biological modifiers that could serve as therapeutic alternatives to corticosteroids. 5-Fluorouracil (5-FU) and bevacizumab (BVZ) are safe alternatives that are already used in other ophthalmic treatments (How et al., 2010; Arain et al., 2018). In the present study, we evaluated the effect of triamcinolone, BVZ, and 5-FU on scars (both macroscopically and histologically) on the dorsal skin using a surgical-wound-induced rat model. To our knowledge, this is the first study to use the combination of three drugs in scar evaluation.

Materials and methods

Animal procedures

A prospective, randomized, non-blinded rat study was carried out to evaluate the effect of BVZ and 5-FU + triamcinolone, alone or in combination, on *in vivo* scar formation from experimental dorsal lesions. This study was approved by the Ethics Committee on the Use of Animals (CEUA) under the protocol number 9537270217.

In this study, 24 rats (*Rattus norvegicus albinus*) from the Central Bioterium from a public university were randomly distributed (utilizing https://www.randomizer.org) into four groups, containing six rats each. All rats were healthy and aged 2-3 months with a body weight of 200-300 g at the beginning of the experiment.

Three rats were housed in each polypropylene cage lined with autoclaved white pine shavings. The luminosity, temperature, noise intensity, and relative humidity were maintained by the natural environment. Filtered water and Labina feed (Purina, St. Louis, MO, USA), suitable for the species, were offered to the animals *ad libitum*. The cages were sanitized twice a week with glyceraldehyde solution. The bed shavings were changed every 2 days, and then incinerated after removal.

For the incision, the rats were anesthetized via intramuscular injection in the lower limb (20-22-gauge needle) with 10% ketamine and 2% xylazine at 0.01 mL/g body weight. The eyes were lubricated with 0.9% saline to prevent corneal ulcers under ketamine anesthesia, as this drug inhibits eye blinking (Damy et al., 2010). An oval lesion measuring 2×1 cm cm was made into the shaved dorsal skin of each rat using a No. 15 scalpel blade, following an outline made using an acrylic mold developed in-house. The wound was then sutured with four simple nylon 6.0 stitches (Fig. 1).

The medications (BVZ, 5FU, Triancinolona) were applied, after the removal of the stitches on
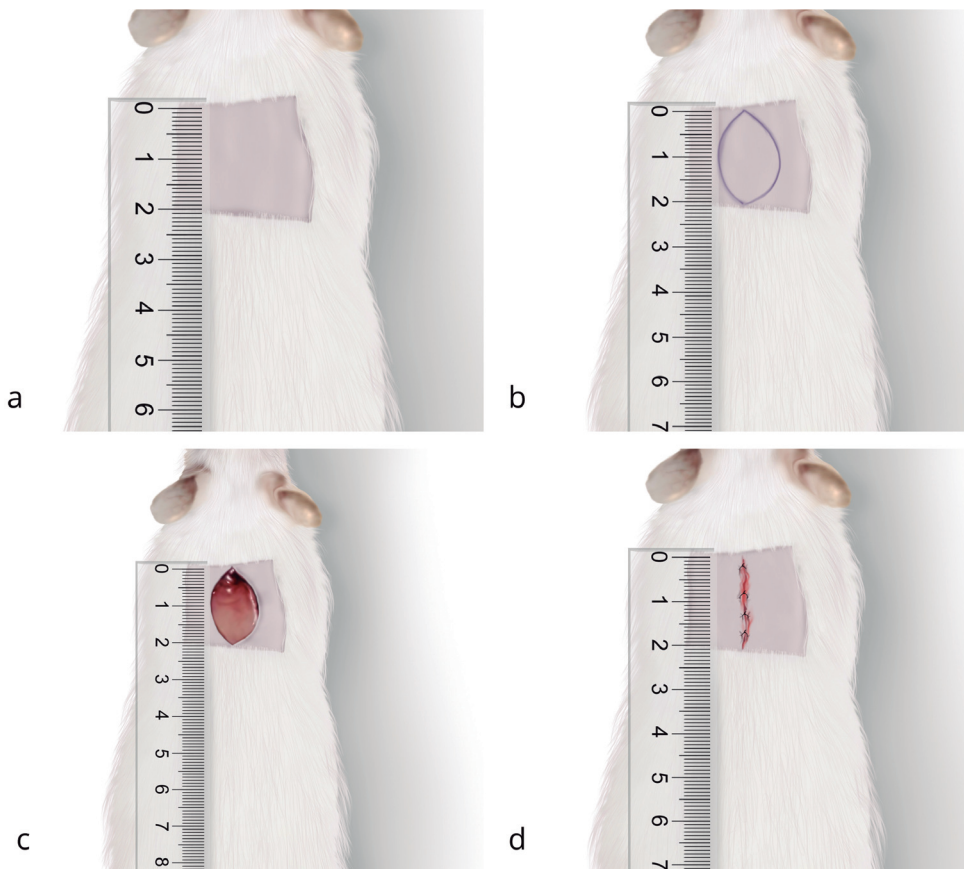

Fig. 1. Demonstration of the incisions made in rats. **a.** A 2.2-cm by 2.2-cm shaved area. **b.** Marking outlined using plastic mold with the border measuring 2 cm in the craniocaudal direction and 1 cm in the latero-lateral direction. **c.** Incision and dissection of the demarcated area. **d.** Border sutured with simple Nylon 6.0 stitches. All rats were healthy and aged 2-3 months with a body weight of 200-300 g at the beginning of the experiment.

postoperative day 7, through subcutaneous injection with the retro injection technique in a continuous and proportional way to the entire extension of the scar. The four treatment groups are listed in Table 1. The rats were administered Tramal 50 mg capsules, diluted in 500 ml in the drinking water for 7 days in the morning, the aim of analgesia. After 24h, all remaining solutions were discarded, and new solutions were prepared for the next 24h.

Fourteen days following lesions, the rats were euthanized following the Euthanasia Practice Guidelines of the National Council for the Control of Animal Experimentation (Concea) (Brazil. Ministry of Science 2018).

Macroscopic evaluation

Each rat was analyzed daily, and the following observations were recorded: (a) wound integrity, (b) suture tension between wound borders, (c) epithelialization of the wound at the border interface, and (d) signs of infection and dehiscence of sutures.

Table 1. Medications administered to each group.

	BVZ 25 mg/mL	5-FU 50 mg/mL	Triancinolone 20 mg/mL
Group 1	0.1 mL	-	-
Group 2	-	0.9 mL	0.1 mL
Group 3	0.05 mL	0.45 mL	0.05 mL
Control Group	-	-	-

BVZ: bevacizumab; 5-FU: 5-fluorouracil

Microscopic evaluation

After euthanasia, segments of the central region and area adjacent to the lesion border were removed using a No. 15 scalpel blade for histological evaluation. The tissue pieces were fixed in 10% buffered formaldehyde, processed, and stained. Two stains were used. The first stain was hematoxylin and eosin to evaluate monocytes, fibroblasts, hemorrhage, hemosiderin, neovessels, and collagen. The second stain was picrosirius red, which is more specific for collagen. The slides were evaluated by optical microscopy. The semi-quantitative scoring system used to evaluate the hematoxylin and eosin-stained tissues was based on a previous study (Cardoso et al., 2016); collagen, neovessels, monocytes, fibroblasts, hemosiderin and re-epithelization were graded as absent (score 0), discrete (up to 25% of the committed area, score 1), moderate (from 26% to 50% of the committed area, score 2), and accentuated (more than 50% of the committed area, score 3). The identification of monocytes was performed according to their morphology, kidney-like shape of the nucleus which is big and centralized, ranging from 25 to 50um, Neovessels were considered when small vessels were observed with one or two erythrocytes in their interior (Table 2). Immunohistochemical tests were not performed.

Collagen density and scar maturation were evaluated using sections stained with picrosirius red and visualized by polarized light microscopy. For the analysis, thick and strong birefringent collagen fibers fluoresced yellow and red, representing collagen type I, whereas thin, scattered, and weak birefringent fibers fluoresced green, representing collagen type III. The images were analyzed using Image J software, using the "Threshold

Table 2. Semi-quantitative scoring system used to evaluate the hematoxylin and eosin-stained tissues was based on a previous study (Cardoso et al., 2016).

	Collagen	Neovessels	Monocytes	Fibroblasts	Hemosiderin	Re-epithelialization
Absent score 0	No collagen fibers observed	No neovessels observed	No monocytes observed	No fibroblasts observed	No hemosiderin deposit observed	No re-epithelialization
Discrete Score 1	Presence of scarce collagen fibers, occupying up to 25% of the tissular area observed	Presence of scarce neovessels, occupying up to 25% of the tissular area observed	Presence of scarce monocytes, occupying up to 25% of the tissular area observed	Presence of scarce fibroblasts, occupying up to 25% of the tissular area observed	Presence of scarce hemosiderin deposit, occupying up to 25% of the tissular area observed	Re-epithelialization up to 25% of the wound
Moderate Score 2	Presence of collagen fibers, occupying around 26 to 50% of the tissular area observed	Presence of neovessels, occupying around 26 to 50% of the tissular area observed	Presence of monocytes, occupying around 26 to 50% of the tissular area observed	Presence of fibroblasts, occupying around 26 to 50% of the tissular area observed	Presence of hemosiderin deposit, occupying around 26 to 50% of the tissular area observed	Re-epithelialization around 26 to 50% of the wound
Accentuated Score 3	Presence of several collagen fibers, occupying more than 50% of the tissular area observed	Presence of several neovessels, occupying more than 50% of the tissular area observed	Presence of several monocytes, occupying more than 50% of the tissular area observed	Presence of several fibroblasts, occupying more than 50% of the tissular area observed	Presence of several hemosiderin deposit, occupying more than 50% of the tissular area observed	Complete re-epithelialization or closed wound

Colour" plug-in. The collagen percentage was determined using the automated particle analysis according to the selection and measurement of areas based on color. The values for each collagen type in "Threshold Colour" were standardized for all images as follows: hue 0-40 for red (collagen type I) and 45-120 for green (collagen type III), and saturation 0-255 and brightness 5-225 for both types of collagen (Biondo-Simões et al., 2006).

Microscopic analyses were performed by two different pathologists, and any discrepancies were resolved by the group.

Statistical analysis

All variables were tested for normal distribution and homogeneous variance. When the distribution was deemed normal with homogeneous variance, parametric tests were used. When the distribution was not normal, or the variance was not homogeneous, non-parametric tests were used. For histological analyses, Mann-Whitney and Kruskal-Wallis tests, followed by Dunn's multiple comparison tests were utilized. For the comparison of epithelial thicknesses, ANOVA, followed by Holm-Šidák test was used. Tukey's test was used for

collagen density analysis. Proportions and correlations were also evaluated. Sigma Stat 2.3® was utilized to perform the statistical tests. Results with a p-value of <0.05 were considered statistically significant.

Results

In the macroscopic evaluation, performed daily, all rats showed re-epithelialization at the border interface (Fig. 2); delay in healing, dehiscence of sutures, or sign of infection were not observed. For the histological evaluation, the tissues were stained with hematoxylin and eosin (Table 3, Figs. 3, 4) and picrosirius red (Table 4, Fig. 6). In the morphometric analysis among groups, there was a difference in the number of monocytes only in Group 2 (*p*=0.03) and a reduction in the number of neovessels by 56%, 86%, and 85% in Groups 1, 2, and 3, respectively (*p*=0.002) (Tables 3, 5, Fig. 6). For Figure 6, the bleeding and hemosiderin parameters were not presented, as they did not present statistical differences and the values were equal to zero. There was no significant difference in the number of fibroblasts, bleeding, or hemosiderin. Analysis of picrosirius red-stained sections by fluorescence microscopy revealed higher collagen density (Group 1: 16%, Group 2: 87%,

Table 3. Parameters evaluated in the histological analysis (hematoxylin and eosin staining) and the results obtained for each group.

	Control Group Median (Min-Max)	Group 1 Median (Min-Max)	Group 2 Median (Min-Max)	Group 3 Median (Min-Max)	p-value¹	Dunn's Post-test
Monocytes	2 (1-2)	2 (1-2)	1 (0-1)	2 (1-2)	0.030*	CG > G2, G1 > G2, G3 > G2
Fibroblasts	2 (1-2)	1 (1-2)	1 (0-1)	1 (0-2)	0.060	-
Bleeding	0 (0-0)	0 (0-1)	0 (0-0)	0 (0-1)	0.200	-
Hemosiderin	0 (0-2)	0 (0-1)	0 (0-0)	0 (0-0)	0.090	-
Neovessels/ Angiogenesis	78 (17-100)	34 (19-52)	11 (5-26)	11.5 (6-21)	0.002*	CG > G2, CG > G3
Collagen	1 (1-2)	1 (1-2)	3 (1-3)	3 (1-3)	0.003*	CG < G2, CG < G3, G2 > G1, G3 > G1

CG, control group (No treatment); G1, group treated only with bevacizumab (Group 1); G2, group treated with 5-fluorouracil and triamcinolone (Group 2); G3, group treated with bevacizumab, 5-fluorouracil, and triamcinolone (Group 3), 1Kruskal-Wallis test.
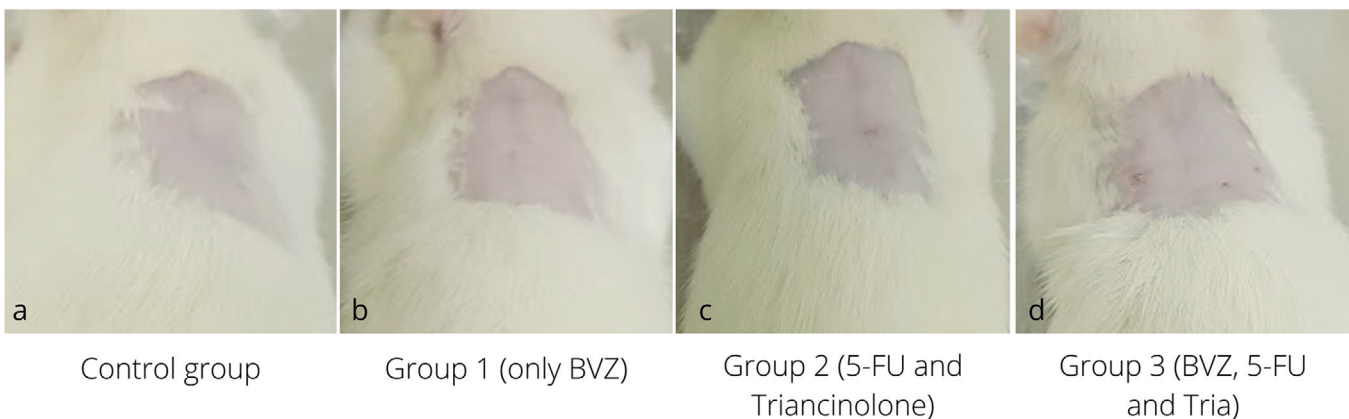

| Control group | Group 1 (only BVZ) | Group 2 (5-FU and Triancinolone) | Group 3 (BVZ, 5-FU and Tria) |

Fig. 2. Macroscopic appearance of scars 14 days after incision and 7 days after treatment with BVZ, 5-FU and Triancinolone. **a.** Control group. **b.** Group 1 (only BVZ). **c.** Group 2 (5-FU and Triancinolone). **d.** Group 3 (BVZ, 5-FU and Triancinolone). BVZ: bevacizumab; 5-FU: 5-fluorouracil.

and Group 3: 59%) and more organized fibers in the treatment groups than in the control group (CG) ($p<0.001$).

Discussion

Scars are unavoidable in medical practice, and surgeons should be prepared for the early diagnosis and treatment of hypertrophic scars and keloids. Scarring can affect the daily functioning and psychological well-being of patients (Trace et al., 2016).

The current treatment options of fibroproliferative scars involve non-invasive and invasive techniques, with the primary treatment being intralesional injection of
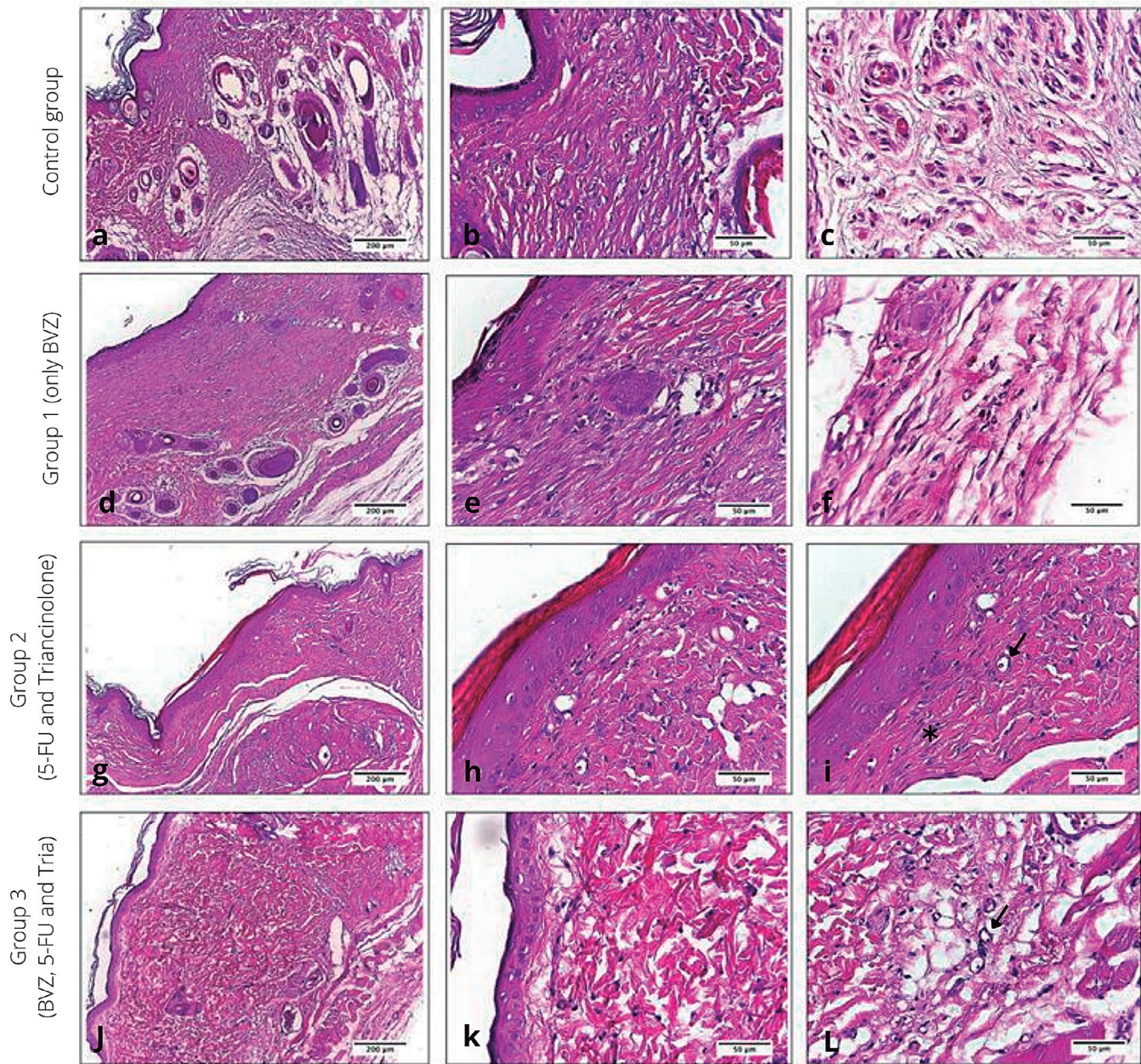
Fig. 3. Microscopic appearance of sutured incisional wounds in Wistar rats after 14 days of healing and 7 days of treatment. Control group: **a.** 5x magnification. **b.** 10x magnification. **c.** 40x magnification. Group 1 (only BVZ): **d.** 5x magnification. **e.** 10x magnification. **f.** 40x magnification. Group 2 (5-FU and Triancinolone): **g.** 5x magnification. **h.** 10x magnification. **i.** 40x magnification. Group 3 (BVZ, 5-FU and Triancinolone): **j.** 5x magnification. **k.** 10x magnification. **l.** 40x magnification. Scale in µm. Mononuclear cells were fewer (p<0.05) in group 2 (*) than in group 1, group 3, and control. Groups 2 and 3 (black arrow) presented less (p<0.05) angiogenesis than the control group. The sections were stained with hematoxylin and eosin.

corticosteroids. These treatments often do not meet the expectations of surgeons, as they may lack efficacy and cause adverse effects in some patients. New drugs that can act alone or in combination with corticosteroids are currently being studied. Antimetabolite drugs such as 5-FU (Chen et al., 2019) and anti-angiogenic drugs such as BVZ (Kwak et al., 2016) have already shown promising results for healing and in other areas of medicine (Artzi et al., 2019). To the best of our knowledge, the present study is the first to use 5-FU, BVZ, and triamcinolone in an *in vivo* model of skin healing.

In our macroscopic evaluation, the surgical wounds in all three groups presented a similar appearance, and the lesion closed completely. Although we did not perform the assessment of wound closure kinetics, macroscopically, we did not observe any delay in healing or dehiscence. In the morphometric study, the number of monocytes was lower in Group 2 than in the other groups. This observation could be explained by the inhibition of proliferative response by triamcinolone (Artzi et al., 2019). Scar modulation in Group 3, which received only 50% of the triamcinolone dose, was similar to that in Group 2, thus reducing the use of corticosteroids.

Table 4. Analysis of collagen density using tissues stained with picrosirius red.

Variables	Control Group mean (±SD)	Group 1 mean (±SD)	Group 2 mean (±SD)	Group 3 mean (±SD)	p-value	Tukey's Post-Test
Collagen density	15.94 (5.08)	18.53 (2.53)	29.73 (7.03)	25.36 (8.83)	<0.001*	G2>CG, G3>CG, G2>G1
Increase	-	16%	87%	59%		

CG, control group (No treatment); G1, group treated only with bevacizumab (Group 1); G2, group treated with 5-fluorouracil and triamcinolone (Group 2); G3, group treated with bevacizumab, 5-fluorouracil, and triamicinolone (Group 3); SD, standard deviation.

Table 5. Analysis of reduction in neovessels in the evaluated groups.

Variables	Control Group median (min-max)	Group 1 median (min-max)	Group 2 median (min-max)	Group 3 median (min-max)	p-value	Dunn's Post-test
Number of Neovessels	78 (26-100)	34 (19-52)	11 (5-26)	11.5 (6-24)	0.002*	CG>G2, CG>G3
Reduction	-	56%	86%	85%		

CG, control group (No treatment); G1, group treated only with bevacizumab (Group 1); G2, group treated with 5-fluorouracil and triamcinolone (Group 2); G3, group treated with bevacizumab, 5-fluorouracil, and triamicinolone (Group 3). p-value Kruskal Wallis.
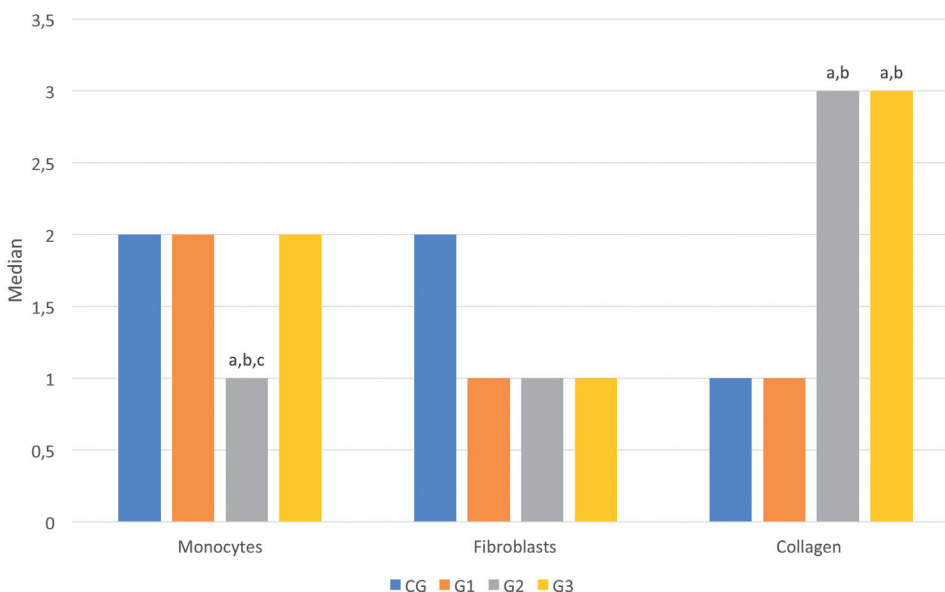

Fig. 4. Evaluated parameters in histopathologic analysis. CG: control group (no treatment); G1: group treated only with bevacizumab; G2: group treated with 5-fluorouracil and triamicinolone; G3: group treated with bevacizumab, 5-fluorouracil and triamicinolone. **a.** statistical difference in comparison to control group. **b.** statistical difference in comparison to G1. **c.** statistical difference in comparison to G3.

In the present histological evaluation, the mean number of fibroblasts was lower in the treatment groups than in the CG, and it was more evident in Group 2. Activated fibroblasts and excess collagen production play important roles in the formation of hypertrophic and keloid scars (Singer and Clark, 1999). The neutralization of vascular endothelial growth factor (VEGF) by BVZ (Kwak et al., 2016) and inhibition of fibroblast proliferation by 5-FU (Trace et al., 2016) explain the inhibition of neovessel formation, reduction in fibroblasts, and reduction in mature collagen observed in this study.

In the evaluation of collagen density by picrosirius red staining, Groups 2 and 3 demonstrated a higher percentage of type I collagen, which fluoresces reddish yellow with this stain. Type I collagen is considered more mature and is associated with better healing. The use of BVZ alone did not produce satisfactory results, with no significant difference compared with the use of CG.

In this study, the combination of BVZ, 5-FU, and triamcinolone (Group 3), even at half the dose of the
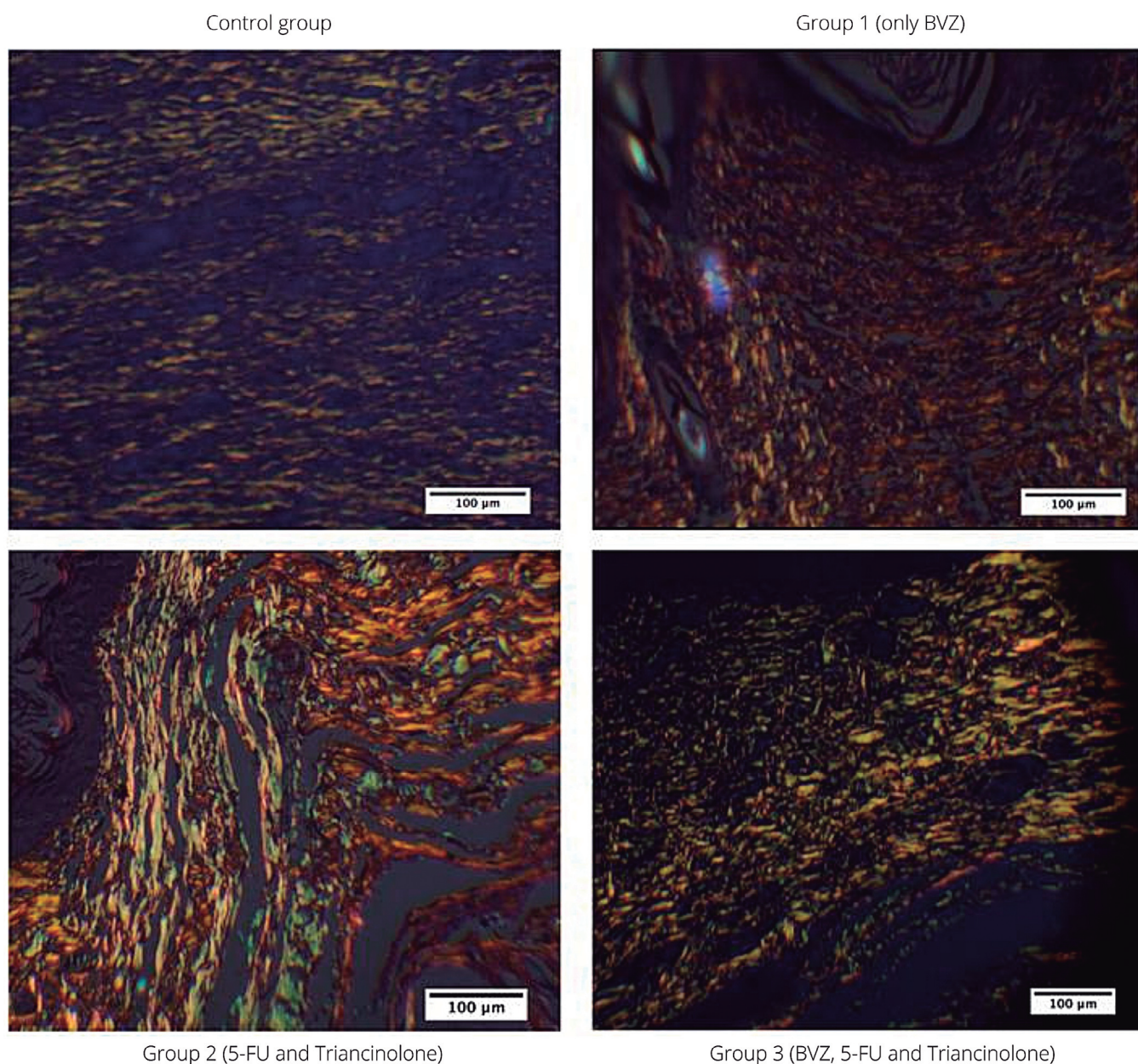
Control group | Group 1 (only BVZ)

Group 2 (5-FU and Triancinolone) | Group 3 (BVZ, 5-FU and Triancinolone)

Fig. 5. Histological images showing collagen after picrosirius red staining. **a.** Control group. **b.** Group 1 (only BVZ). **c.** Group 2 (5-FU and Triancinolone). **d.** Group 3 (BVZ, 5-FU and Triancinolone).

individual drugs, presented the same efficacy as 5-FU + triamcinolone (Group 2), which produced denser type I collagen. It can be inferred that a synergistic action possibly occurred in Group 3, related to the modulating effect on fibroblasts and on collagen and vascular tissue formation, consistent with the findings of Ghoz et al. (2019). They compared the efficacy of 5-FU and Avastin injection (bevacizumab) with that of the control in the management of primary pterygium. High levels of VEGF induce scar formation in skin wounds, thereby increasing vascularization, stimulating fibroblast proliferation, and enhancing collagen deposition, whereas VEGF neutralization reduces angiogenesis and skin fibrosis (Wilgus et al., 2008; Li et al., 2009).

Wendling et al. (2003) reported that the effects of 5-FU on type I collagen expression and the subsequent collagen deposition was able to involve the inhibition of type I collagen transcription controlled by transforming growth factor-β, offering clinical benefits in the treatment of keloids. Here, the number of neovessels was reduced by 56%, 85%, and 86% in Groups 1, 2, and 3, respectively, compared with the CG. However, during post-test evaluation, only Groups 2 and 3 showed a difference compared with the CG. In addition, a reduction in the number of neovessels in Group 3 was similar to that in Group 2, despite using half of the dose of drugs in the former group. Our results corroborate those of How et al. (2010) who reported an anti-angiogenic action of BVZ when used in conjunction with 5-FU + triamcinolone in scar management after glaucoma surgery.

In the present study, there were no significant differences in hemorrhage and amount of hemosiderin among the groups. In addition, the analysis of epithelial thickening of the wound area and its surrounding tissues using Image J revealed no differences in thickening among the groups. Although we attempted to calculate the Scar Elevation Index, an index used in rabbits for scarring evaluation, (Kwak et al., 2016) its use was unlikely as there was no increase in the scar height.

In this study, the role of BVZ and 5-FU + triamcinolone, alone and in combination, as modulators of the *in vivo* scarring process was evaluated by assessing macroscopic and histological parameters after injection into rat skin wounds. The results have implications for the clinical application of these injections to reduce vascularization and stimulate mature collagen development, thus minimizing the development of aesthetically unacceptable scars. In addition, the combined and additive effects of both treatments may be utilized to reduce corticosteroid use in the future.

One limitation of this study is the standardization of BVZ dosage. There are a few studies on the effect of BVZ on scarring, and it has been reported that its use in high dosages may slow the healing process (Gordon et al., 2009). Theoretically, this effect can be potentiated by co-treatment with 5-FU + triamcinolone. Therefore, the drug doses in Group 3 (1.25 mg BVZ + 22.5 mg 5-FU and 1 mg triamcinolone) were half of those used in Groups 1 (2.5 mg BVZ) and 2 (45 mg of 5-FU and 2 mg triamcinolone). After analyzing the results, we observed an improvement in the quality of the collagen produced and there was no vascular compromise, so we suggest carrying out tests with higher dosages that can bring even more satisfactory results, without affecting therapeutic safety. Further studies are warranted to investigate the effect of higher doses of the drugs, especially BVZ. Moreover, we did not include a group treated with triamcinolone alone. Hypothetically, the proposed group may show the same effects as the combination groups in our study. Alternatively, this probability may be low as we observed dissimilar histological results among the groups of our study; thus, the inclusion of the triamcinolone alone group in future studies is strongly recommended.

The effect of 5-FU on fibroblast proliferation and collagen formation and maturation along with the effect of BVZ in reducing angiogenesis resulted in the
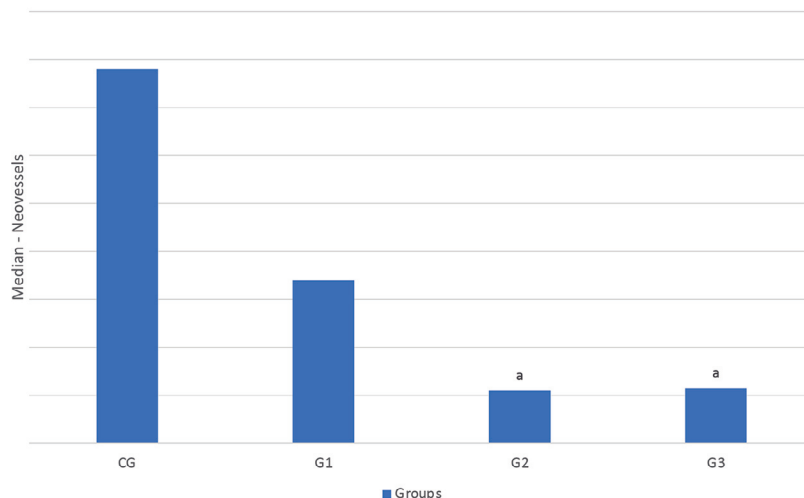

Fig. 6. Neovessels/angiogenesis count in histopathologic analysis. CG: control group (no treatment); G1: group treated only with bevacizumab; G2: group treated with 5-fluorouracil and triamicinolone; G3: group treated with bevacizumab, 5-fluorouracil and triamicinolone. a. statistical difference in comparison to control group.

beneficial effects of the combination therapy in scarring modulation. Here, in the macroscopic evaluation, all groups showed good lesion closure, similar healing of the surgical wound, and no delayed healing or dehiscence of the sutures. Moreover, the lesions demonstrated organized and early maturation of collagen and a decrease in the number of neovessels, supporting the promising effects of these medications. The BVZ-treated group showed similar results to those of the untreated CG. The combination of the three medications is better for scarring modulation, with a greater extent of collagen maturation and angiogenesis inhibition, thus requiring lower corticosteroid doses, which in turn may result in fewer adverse effects.

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