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Influence Of Local Anesthetics On Wound Healing

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ABSTRACT

Evaluating the scarring differences between areas previously anesthetized with different local anesthetics is interesting to verify whether the choice of anesthetic helps in surgical healing in relation to inflammation, neovascularization and collagen deposition. To analyze the influence of ropivacaine and levobupivacaine on the inflammatory phase of surgical incision healing. Thirty male Wistar rats were divided into 3 groups to evaluate the effects of these local anesthetics in comparison with each other and with a control group. The animals were subjected to general anesthesia and subsequently scheduled for administration of the solutions and their samples were excised after 5 days. The scar area, neovascularization and collagen deposition were studied. Regarding inflammation and neovascularization, there were no significant differences between the groups, with all of them presenting pronounced levels. As for collagen deposition, this group exhibited significantly higher levels of type i, ii, and iii collagens compared to groups c and r. Regarding collagen deposition, this group demonstrated significantly higher levels of type i, ii and iii collagen when compared to groups c and r. No differences were observed between the groups in relation to the variation in scar measurements, inflammation and neovascularization. However, there was a significant difference in the production of collagens with an intensity of 6 to 12 times greater in collagen types i, ii and iii with the use of levobupivacaine.

Keywords: Local anesthetics. Wound healing. Neovascularization. Collagen. Levobupivacaine. Ropivacaine.

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INTRODUCTION

Postoperative wound healing is a crucial step in the recovery process of patients undergoing surgical procedures. The use of them has been associated with better pain control and faster surgical recovery.[1-5]

Postoperative pain is a common concern among patients and can negatively affect quality of life, mobility, and functional recovery.[6] The use of local anesthetics has shown promising results in the management of this pain, mainly due to their direct action on peripheral nerves and the reduction of local inflammatory response.[7-10] In addition, it may have a positive impact on wound healing.[11,12] However, the molecular mechanisms involved in these processes are not yet fully understood.

The local anesthetics most commonly used in clinical practice include lidocaine, bupivacaine, and ropivacaine.[13] These substances are administered through various techniques, such as local infiltration, peripheral nerve blocks, and topical administration. It is important to consider the pharmacokinetic and pharmacodynamic properties of these agents when selecting the ideal local anesthetic for postoperative wound healing.[14]

In this context, the present study aimed to analyze the role of local anesthetics in the healing of postoperative wounds and their clinical implications by verifying whether there were differences in scar measurements, histological differences in the inflammatory process, neovascularization, and collagen deposition in the healing process of rats.

MATERIALS AND METHOD

The study was conducted at the Medical Research Institute of the Mackenzie Evangelical Faculty of Paraná, Curitiba, PR, Brazil, between 2020 and 2022. It was approved under number 2019-003354, by the Animal Ethics Committee of this faculty (CEUA/FEMPAR) in accordance with Federal law n^{o}. 11.794/2008, in the normative resolutions of the National Council for the Control of Animal Experimentation (CONCEA) and in the Internal Regime of the Commission.

The norms of the Practical Manual of Standardization for Academic Works of the Mackenzie Evangelical Faculty of Paraná (2019) and the Veterinary Anatomical Nomenclature (2012) were applied.

Sampling and experimentation

Thirty male Wistar rats (Rattus Novergicus albinus) were used, aged 90 days and weighing between 250-280 g. They were divided into 3 groups, with 10 animals each: levobupivacaine group (L), ropivacaine group (R), and control group (C).

Preparation for the experiment:

The rats received water and standard feed (Nuvilab CR1® - Nuvital Nutrientes S/A) *ad libitum* and were kept in a controlled room temperature between 18-22 ºC, under proper humidity conditions with a 12-hour light-dark cycle. They were housed in 30 polypropylene cages, one for each animal.

Anesthesia for solution administration:

All animals participating in the experiment remained fasted, prior to the procedure for 6 hours from the standard feed and 2 hours from water, and were subjected to general anesthesia with intramuscular application of ketamine hydrochloride (Ketalar®) at a dose of 50 mg/kg and xylazine hydrochloride (Virbaxyl 2%®) at a dose of 5 mg/kg. The procedures were only initiated after confirming immobility to the nociceptive stimulus, allowing the assumption of unconsciousness and adequate analgesia, and this anesthetic plan remained until the end of the operation. All rats were placed on a surgical table covered with a sterile field, in the prone position, and no fixation was used. After trichotomy of the dorsal region and using a Sharpie® pen, the area to be infiltrated with the corresponding solutions for each group and subsequently excised in circular lesions with an average diameter of 0.8 cm containing epidermis, dermis and the upper cellular tissue generated by a Rhosse® Punch no. 8 and converse scissors was demarcated.

Infiltration of the solutions in groups C, L, and R:

All animals were under general anesthesia at the time of infiltration of the solutions pertinent to each group. The toxic dose of each local anesthetic used was respected. The standard was the application of a solution containing the maximum allowed dose of the drugs (ropivacaine 3mg/kg and levobupivacaine 3mg/kg) and the solution was completed with 0.9% sodium chloride (NaCl), totaling 1 ml of solution. Subcutaneously, with a hypodermic needle, 0.5 ml of the solution was applied in the center of each demarcated area, in the interscapular region. (Figure 1A).

A B C

Figure 1: A) Infiltration of the anesthetic in the incision area; B) Circular incisions in the dorsal area; C) Postoperative recovery

Excision of the areas infiltrated by the NaCl 0.9%, levobupivacaine, and ropivacaine solutions:

Under the same anesthetic plan, still without responses to nociceptive stimuli, the rats were subjected to surgical excision (Figure 1B). The extensions of the surgical wounds were measured with a 150 mm PPV 1506® digital plastic caliper, and the measurements of each initial circular incision were recorded for later comparison.

Recovery after anesthetic-surgical procedure:

The animals were monitored during the recovery from the anesthetic-surgical procedure for 6 hours (Figure 1C). Ambulation and food intake were the criteria considered in the recovery. Afterward, they returned to their cages and continued to receive water and feed *ad libitum*, and were kept in the previously mentioned environmental conditions, remaining in the animal facility for 5 days. On that day, euthanasia was performed with a chamber containing the anesthetic gas isoflurane (Forane® Abbvie) at high concentrations. Next, they were placed in the prone position, and the measurement of the scar areas with the caliper was performed. The skin fragment in the healing process of each circular incision infiltrated on the 1st day of the experiment was carefully removed from each animal using a No. 15 scalpel blade to incise the skin and perform the excision of the material. Subsequently, the specimens were placed in vials containing Bouin's solution (picric acid, formaldehyde, and acetic acid) for 48 hours. After this period, the fragments were transferred to properly identified containers and forwarded to the experimental pathology laboratory. The tissue repair process was evaluated through histological sections stained with hematoxylin & eosin (H&E) and picrosirius.

Histopathological evaluation by H&E

The samples were fixed in Bouin's solution (picric acid, formaldehyde, and acetic acid) and washed in PBS pH 7.4 for 2 h. They were then dehydrated in increasing concentrations of ethanol (70-100%), cleared in 2 baths of xylol for 1 h each, embedded and blocked in paraffin. Subsequently, the blocks were cut on a microtome (Spencer 820) at 3 µm and deposited on positively charged slides (*Imunnoslide, Easypath*®). Next, they were deparaffinized in xylol, rehydrated in decreasing concentrations of ethanol (100-70%) and distilled water, and then processed for H&E panochromatic staining, followed by dehydration in ethanol, clearing with xylol, and mounting with *Entellan*® (Merk) and a coverslip. The slides were analyzed in 3 fields for angiogenesis with the counting of vessels. Using the *ImageJ software*, the thickness of the epidermis was measured, which will be the average width between the dermis and the keratin layer of the epidermis.

The collected data were recorded for each group. The evaluation of the microscopic results was adapted from the scheme established by Myers et al.15, an arbitrary system for

classifying wound healing was established, based on the inflammatory reaction, fibroblastic and capillary proliferation, and collagen maturation, as observed in sections stained with H&E. The cellular elements of the wounds were counted or estimated based on the number per high-power field, to obtain a value of 0 to 4+. The amount of edema, muscle necrosis, and similar changes were estimated. Each histological element received a negative or positive factor, the latter for characteristics that indicated wound healing. The 0 to 4+ value of each element in each section was multiplied by the negative or positive factor; the sum of these values was considered as the wound classification. By this method, completely healed wounds should have a classification of 100.

The information obtained from microscopic observation was classified according to the intensity in which they were found and transformed into quantitative variables for each histological finding, being classified according to their occurrence/intensity:

Vascular proliferation

It was considered absent when no vessels were evident in the histological section; discrete when few vessels were visible, scattered in isolation in the context; moderate when they appeared more frequently and dispersed in the optical field; and marked when they were evident with high frequency, arranged throughout the context.

Mononuclear cells:

They were classified as absent when they were not visualized in the optical field; discrete when evidenced in isolation, allowing the distinction of areas free of infiltrate; moderate when they appeared more frequently, constituting dense aggregates, but allowing the visualization of areas free of infiltrate; and marked when they were evidenced with high frequency, constituting dense and juxtaposed aggregates, without areas free of infiltrates.

Polymorphonuclear cells:

They were classified as absent when they were not visualized in the optical field; discrete when they were visible sparsely, in isolation, with many areas free of infiltrates; moderate when they were visible forming aggregates, but with adjacent areas free of infiltrates; marked when they appeared with high frequency, forming dense aggregates, without areas free of infiltrates.

Fibroblastic proliferation:

They were classified as absent when no proliferation of fibroblasts was evident; discrete when there were scattered proliferated fibroblasts in the loose connective tissue; moderate when in moderate quantity, constituting small multidirectional cell bundles; and marked when there was a large number of proliferated fibroblasts constituting compact aggregates of multi directionally arranged cells.

Collagen synthesis:

It was classified as absent when no collagen fibers were observed deposited; discrete when the deposition occurred in small quantity, characterized by fibers deposited among the proliferated fibroblasts; moderate when the collagen deposition formed eosinophilic, thick fiber bundles, interspersed with areas of loose connective tissue and proliferated fibroblasts; and marked when there was a large deposition of collagen fibers, constituting thick eosinophilic fiber bundles, compactly arranged among the proliferated fibroblasts and without areas of loose connective tissue.

Re-epithelialization:

It was classified as absent when no epithelium was visible in the optical field; discrete or moderate when it appeared incompletely or partially; marked when it was visible in a total or complete manner on the connective tissue.

Histopathological evaluation by picrosirius staining

Concomitantly, sequential sections were processed and subjected to staining for collagen I and III. Briefly, the sections were placed in 0.1% *sirius red* in a saturated solution of picric acid for 30 min, then placed in 1% acetic acid for 10 min, washed in distilled water and stained for 1 min with Harris hematoxylin. They were then differentiated in 1% hydrochloric acid in 70% ethanol, washed in running water for alkalinization for 10 min, dehydrated in ethanol, cleared with xylol and mounted with *Entellan*® (Merk) and coverslip.

The stained slides were analyzed under a polarized light microscope (Olympus BX40) to show the birefringence of the collagen fibers, where collagen I is observed in yellow and red, and collagen III in green. The amount of each collagen was evaluated using *ImageJ software*, where the HSB "*Hue-Saturation-Balance*" threshold was applied to the images of the 3 fields, allowing the counting of the red, yellow and green areas, enabling the observation of the amount of each of these collagens in the samples in pixels (unit of measurement of a digital image). The data obtained were analyzed using *GraphPad Prism 6.0 software*.

Statistical analysis

Statistical analysis of the difference in the measurements of the scar areas, the variations in the diameter of the circular incision in millimeters were studied. For this, the differences between the measurements of the initial and final diameters of the circular incisions were calculated. In no case was an increase in the diameter of the circular incision observed. Data preparation was performed in MS Excel. The statistical analysis graphs were made using *JMP version 17 software* (SAS). The T-test was performed to evaluate possible variation between the means of the left and right incisions. The test of variance equivalence (TOST) and the Levene test were performed to evaluate the homogeneity of the variances. The

ANOVA test checked if any group had different means. The Dunnett test was used to verify if the groups had a difference from the control group. The analyses were performed for each type of collagen. In this way, 3 groups were investigated: collagen I, collagen II and collagen III. The Welch test was applied to verify if any group had a significant difference. The significance level adopted was $p<0.05$.

RESULTS AND DISCUSSION

All animals survived the experiment and no signs of infection were found in the surgical wounds.

Analysis of the diameter of the circular incisions

It was noted that all groups showed some type of evolution between the initial and final diameter. Group C had a median of 0.2 mm, a result similar to group R. Group L had a median of 0.3 mm, a slightly higher measure than the other groups (Figure 2).

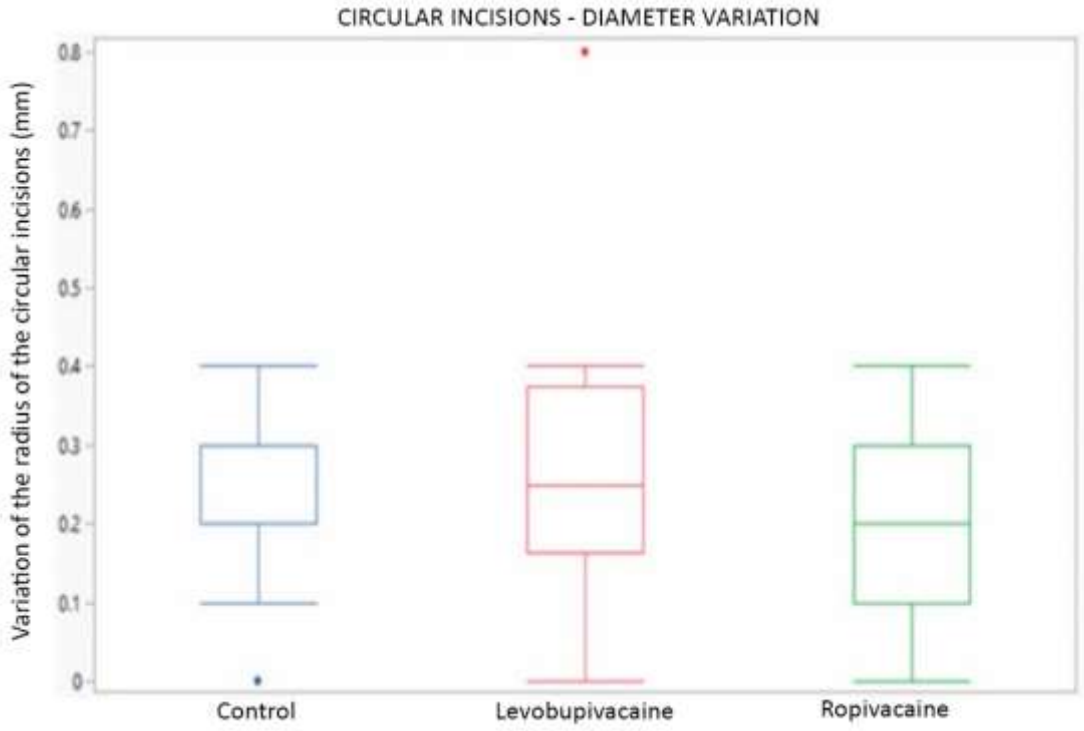

Figure 2: Boxplot of the variation in the diameter of the circular incisions.

Therefore, no statistically significant differences were found between the means of re-epithelialization for groups L and R in relation to group C.

Analysis of the inflammatory process by H&E

The visualized data were applied to the Meyers et al. table (Table 1) in which each column has weight within the histological evaluation, with regard to the characteristics of tissue healing.

Table 1: Scores for characterization of the healing process (Myers et al. 1961)

CLORETO DE SÓDIO 0,9%							
Variáveis	**Ausente (0)**	**Discreto (+1)**	**Moderado (+2)**	**Espessa (+3)**	**Acentuada (+4)**	**Fator**	**Total**
Neutrófilos				x		-10	-7
Eosinófilos	x					-4	-4
Inflamação aguda		x				-4	-3
Inflamação crônica			x			2	4
Reepitelização		X				5	6
Proliferação de fibroblastos				x		5	8
Proliferação vascular				x		5	8
Tecido de granulação					X	5	9
ESCORE TOTAL							**21**
LEVOBUPICAÍNA							
Variáveis	**Ausente (0)**	**Discreto (+1)**	**Moderado (+2)**	**Espessa (+3)**	**Acentuada (+4)**	**Fator**	**Total**
Neutrófilos				x		-10	-7
Eosinófilos	x					-4	-4
Inflamação aguda		x				-4	-3
Inflamação crônica		x				2	3
Reepitelização			x			5	7
Proliferação de fibroblastos					X	5	9
Proliferação vascular					X	5	9
Tecido de granulação				x		5	8
ESCORE TOTAL							**22**
ROPIVACAÍNA							
Variáveis	**Ausente (0)**	**Discreto (+1)**	**Moderado (+2)**	**Espessa (+3)**	**Acentuada (+4)**	**Fator**	**Total**
Neutrófilos				x		-10	-7
Eosinófilos	x					-4	-4
Inflamação aguda		x				-4	-3
Inflamação crônica	x					2	2
Reepitelização			x			5	7
Proliferação de fibroblastos				x		5	8
Proliferação vascular				x		5	8
Tecido de granulação				x		5	8
ESCORE TOTAL							**19**

The results obtained from the comparison of the effects of levobupivacaine (group L), ropivacaine (group R) and the control group (group C) did not show significant differences in relation to re-epithelialization, fibroblast proliferation and vascular proliferation (Figure 3).

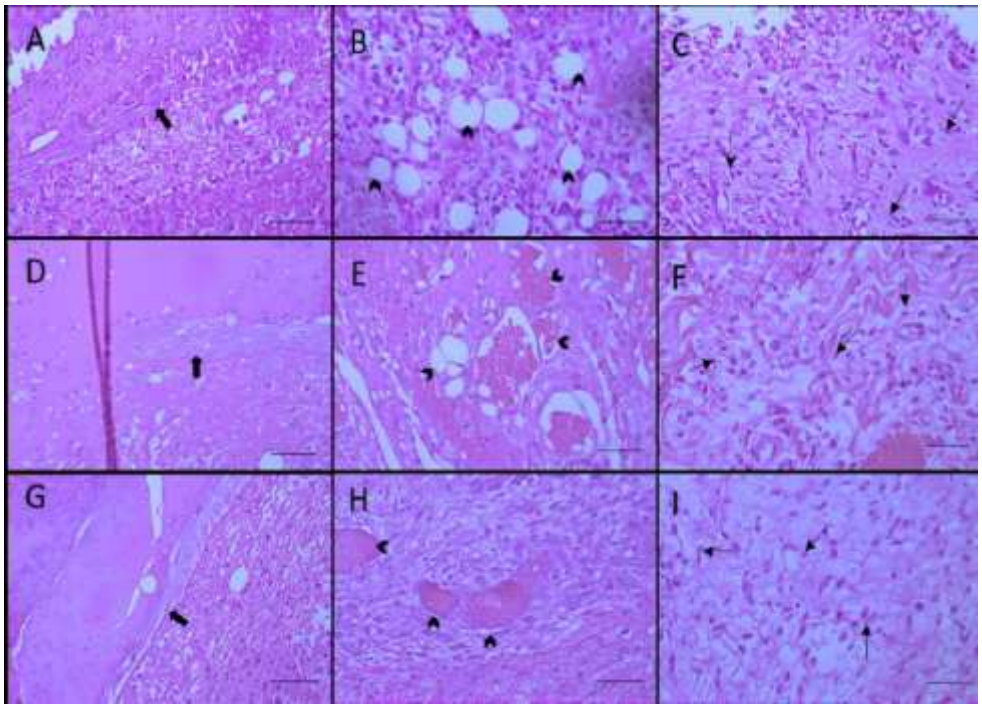

Figure 3: Re-epithelialization, fibroblast proliferation and vascular proliferation: A, B, C) Controls; D, F) levobupivacaine; G, I) ropivacaine, re-epithelialization (wide arrow), neovascularization (arrowhead), fibroblasts (thin arrow) (50 µm, H&E).

Collagen deposition analysis by picrosirius

Samples from group L showed substantially greater deposition of type I, II and III collagens compared to groups C and R (Figure 4).

Group L had a mean type I production six times higher than groups C and R ($p<0.0002$, Figure 5); type II, 12 times higher than groups C and R ($p<0.0001$, Figure 6); type III, twelve times higher compared to groups C and R ($p<0.0002$, Figure 7).

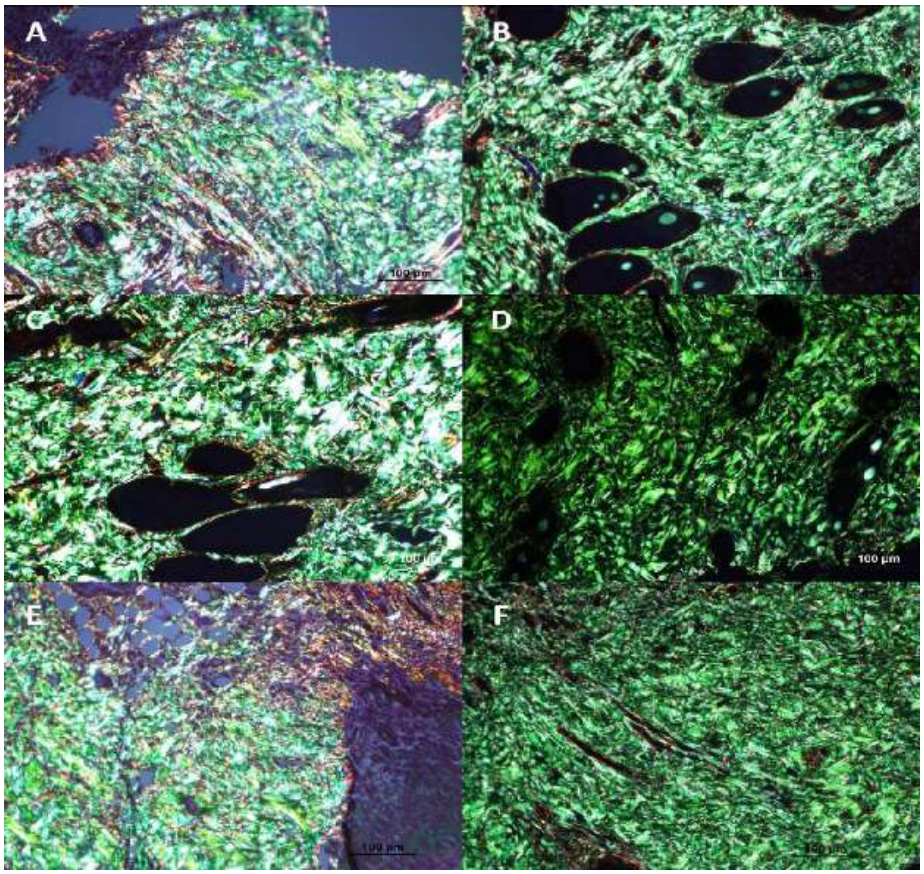

Figure 4: Collagen deposition: A, B) Controls; C, D) levobupivacaine; E, F) ropivacaine, type I collagen (yellow and red), type III collagen (green) (picrosirius, 100 µm).

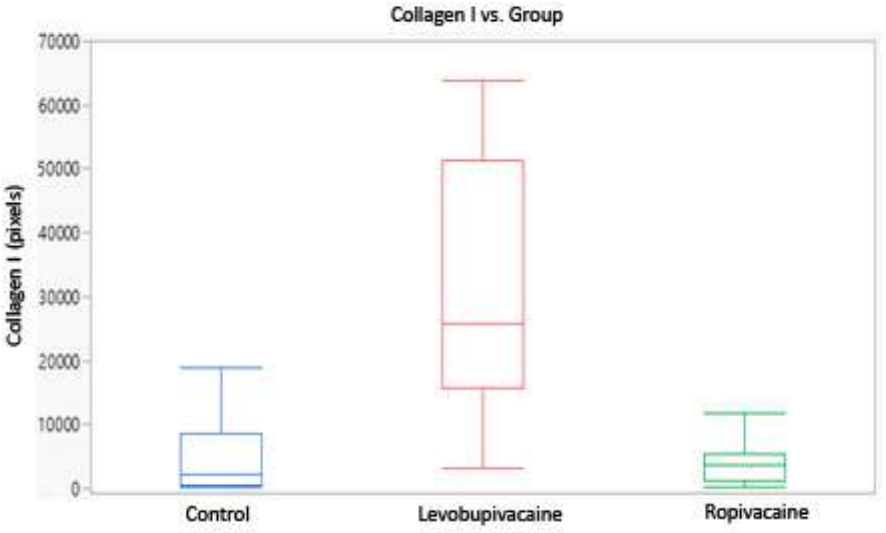

Figure 5: Collagen type I in pixels.

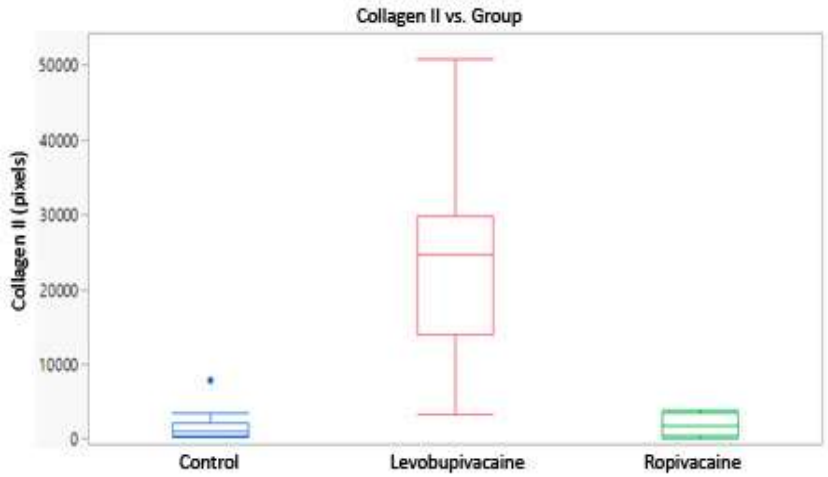

Figure 6: Collagen type II in pixels.

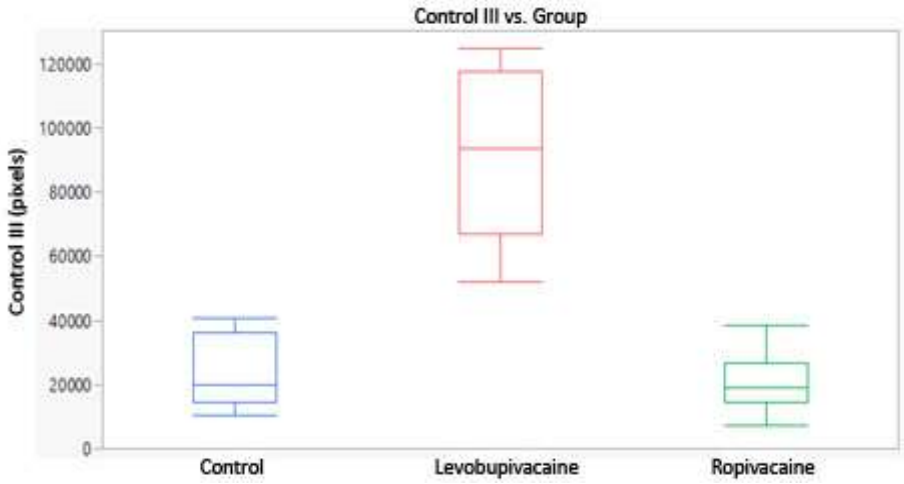

Figure 7: Collagen type III in pixels.

The animal model used, Wistar rats, proved to be effective for evaluating the influence of local anesthetics on the wound healing process. The absence of infections in the surgical wounds and the survival of all animals reinforce the validity of the model. However, as pointed out by previous analysis, the physiological differences between humans and rodents should be considered when trying to extrapolate the results to clinical applications.1[6]

The main stages of the wound healing process, such as hemostasis, inflammation and angiogenesis, are influenced by the extracellular matrix (ECM), collagen and its compounds. In response to injury, collagen induces the activation and aggregation of platelets, resulting in the deposition of a fibrin clot at the site of the injury. In the inflammatory phase of wound healing, the activation of immune cells stimulates the secretion of pro-inflammatory cytokines that influence the migration of fibroblasts, epithelial and endothelial cells. Fibroblasts contribute to the deposition of collagen. Simultaneously, the degradation of collagen releases fragments that promote the proliferation of fibroblasts and the synthesis of growth factors that lead to angiogenesis and re-epithelialization.[17]

The results indicated that the infiltration of local anesthetics into the surgical wound may play

a role in the tissue repair process. The group treated with levobupivacaine (group L) showed more pronounced deposition of collagens compared to the other groups. These findings corroborate previous investigations that observed the stimulation of fibroblasts and the formation of granulation tissue with the use of local anesthetics.[18]

The generated data indicate that the local anesthetic appears to exert a significant influence on wound healing, as evidenced by the increased deposition of collagens in the animals in group L. This group showed intense deposition of type I, II and III collagens, indicative of a robust healing and tissue repair response. However, group L also showed greater variability, suggesting a heterogeneous response to the local anesthetic, which deserves to be explored in future research. These findings reveal potential therapeutic applications of levobupivacaine in the control of the inflammatory and healing response with the use of local anesthetics.

A previous study provides a comprehensive view of the repair and regeneration process with the effects of local anesthetics on wound healing. As already recognized cellular and molecular mechanisms involved in wound healing.[19]

In addition, another investigation addresses the anti-inflammatory properties of local anesthetics and their potential to modulate the inflammatory response during wound healing. This research offers relevant insights into the influence of these drugs on this healing, and which strengthens the effects observed with the use of levobupivacaine.[20]

Group L showed significantly higher levels of type I, II and III collagens, suggesting more robust healing. The role of different types of collagens in wound healing has been documented in previous research, which showed that type I and III collagens are crucial for wound strength and structural integrity.[21] In addition, it is worth noting that group L showed greater variability in the results, which suggests that the response to the local anesthetic may be influenced by factors not yet identified, an area that deserves future investigation.

CONCLUSION

In the analysis of the influence of ropivacaine and levobupivacaine on the inflammatory phase of healing, no differences were observed between them in relation to scar areas, inflammation and neovascularization. However, a statistically significant difference was observed in the production of collagens, with an intensity 6 to 12 times higher of type I, II and III collagens in the levobupivacaine group, leading to the conclusion that it may play an important role in the healing process. On the other hand, the ropivacaine group presented results similar to the 0.9% sodium chloride group. Therefore, this drug does not appear to interfere with the healing of surgical wounds.

ABBREVIATIONS

- CEUA/FEMPAR: Ethics Committee on the Use of Animals of Mackenzie

Evangelical Faculty of Paraná.

- CONCEA: National Council for the Control of Animal Experimentation
- H&E: hematoxylin & eosin.
- PPV: plastic precision vernier
- PBS: phosphate buffered saline.
- ECM: extracellular matrix.

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