Original Article

Infiltration of local anesthetics into the surgical wound: effect on inflammation and fibrous scar in rats

Infiltração de anestésicos locais na ferida cirúrgica: efeito sobre a inflamação e cicatriz fibrosa em ratos

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■ **ABSTRACT**

Introduction: Pain relief after surgery remains one of the most significant medical challenges, mainly in aesthetic surgery. The infiltration of the surgical incision with local anesthetics has been increasingly used to reduce pain and other analgesic use. However, little is known about the effect of this injection on healing. The objective is to evaluate the interference of local anesthetics in the area of inflammatory infiltrate and fibrosis scar in rats. **Methods:** Two linear incisions each were made on the dorsal region of 40 Wistar rats. The left incision was infiltrated with doses of 1.8ml of bupivacaine, levobupivacaine, ropivacaine, or 0,9% saline solution infiltration. The right incision did not receive infiltration, serving as a control group. After seven days, samples of the incisions were collected for histological morphometric evaluation. **Results:** When compared with the control groups, the area of inflammatory infiltrate was found larger in the bupivacaine, ropivacaine, and levobupivacaine groups. The bupivacaine group presented a larger inflammatory infiltrate than the levobupivacaine and ropivacaine. The fibrous scar area was larger in the levobupivacaine and ropivacaine groups. There was no difference between the groups that received anesthetic and saline solution. **Conclusion:** As there was no difference between the anesthetics and saline solution groups, the volume applied, or the trauma may have been the cause of the larger areas of infiltrating and scar associated with local anesthetics application.

Keywords: Inflammation; Bupivacaine; Levobupivacaine; Ropivacaine; Cicatrix.

■ **RESUMO**

Introdução: O alívio da dor após a cirurgia continua sendo um dos desafios médicos mais significativos, principalmente na cirurgia estética. A infiltração da incisão cirúrgica com anestésicos locais tem sido cada vez mais utilizada para reduzir a dor e outros analgésicos. No entanto, pouco se sabe sobre o efeito desta injeção na cicatrização. O objetivo é avaliar a interferência dos anestésicos locais na área de infiltrado inflamatório e cicatriz de fibrose em ratos. **Métodos:** Duas incisões lineares foram feitas cada uma na região dorsal de 40 ratos Wistar. A incisão esquerda foi infiltrada com doses de 1,8ml de bupivacaína, levobupivacaína, ropivacaína ou solução salina 0,9% infiltrada. A incisão direita não recebeu infiltração, servindo como grupo controle. Após sete dias, amostras das incisões foram coletadas para avaliação morfométrica histológica. **Resultados:** Quando comparada com os grupos controle, a área de infiltrado inflamatório foi encontrada maior nos grupos bupivacaína, ropivacaína e levobupivacaína. O grupo da bupivacaína apresentou um infiltrado inflamatório maior do que a levobupivacaína e a ropivacaína. A área da cicatriz fibrosa foi maior nos grupos levobupivacaína e ropivacaína. Não houve diferença entre os grupos que receberam anestésico e solução salina. **Conclusão:** Como não houve diferença entre os grupos de anestésico e soro fisiológico, o volume aplicado ou o trauma podem ter sido a causa das maiores áreas de infiltração e cicatriz associadas à aplicação dos anestésicos locais.

Descritores: Inflamação; Bupivacaína; Levobupivacaína; Ropivacaína; Cicatriz.

INTRODUCTION

Pain relief after surgery remains one of the most significant medical challenges, and inadequate treatment may delay hospital discharge and patient recovery. In addition, postoperative pain is acute and, when improperly conducted, may lead to the development of chronic pain and greater use of analgesics, including opioids, and their consequences[1,2].

The infiltration of surgical incisions with local anesthetics (LA), mainly those long-lasting, has been increasingly used in different types of surgeries, and the results described in studies are encouraging[1,2]. The effects of this application have been tested in several experimental models[3-7]. However, the impact of LA infiltration on surgical incision healing has not yet been fully established.

Aesthetic surgeries sometimes are significant, and consequently, there may be severe pain in the postoperative period. The infiltration of LA could improve this pain; however, it is necessary to assess whether this infiltration influences healing, which could harm the final result.

OBJECTIVE

This study aimed to evaluate the interference of the infiltration of long-lasting LAs in the surgical incision on the inflammatory infiltrate and on the fibrous scar areas in rats.

METHODS

This study was carried out in a public tertiary hospital (*Hospital de Clínicas, Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais,* Brazil) from January 2018 to January 2020 approved by the committee on ethics in animal use under protocol number 314. All the animals were treated following the recommendations of the institutional animal care committee.

We followed the committee's recommendations on ethics in animal use to comply with the principle of 3R (reduction, refinement, and replacement). No sample calculation was performed. Forty Wistar rats, 20 males and 20 females, were divided into four groups of 10, with the same number of females and males in each group. The animals did not present a difference in their weight, ranging from 152 to 378 grams, with an average of 264 grams (±0.06).

The rats were placed in cages with controlled temperature (24±1°C) and in cycles of 12 hours with light-12 hours in the dark. They were fed a standard diet with water *ad libitum* for 12 hours before the experimental protocol.

The surgical procedure was performed under general anesthesia, induced by xylazine hydrochloride 2%, 5mg/kg, associated with ketamine, 70mg/kg, administered intraperitoneally. When anesthesia was confirmed (loss of tail reflex, paws and muscle relaxation), the dorsal area of the animal was trichotomized, cleaned with povidone-iodine and dried with sterile gauze after 2 minutes. Two linear incisions of about 2cm each were made on the dorsal region, symmetrical concerning the midline, reaching the subcutaneous tissue. The left incision was infiltrated with doses of 1.8ml of LA (Figure 1), and the right incision did not receive infiltration (control group). Another group received 1,8ml of 0.9% saline solution (SS) application in the left incision and nothing in the right. Thus, six application points (0.3ml per point) distributed along the 2cm incision were made.
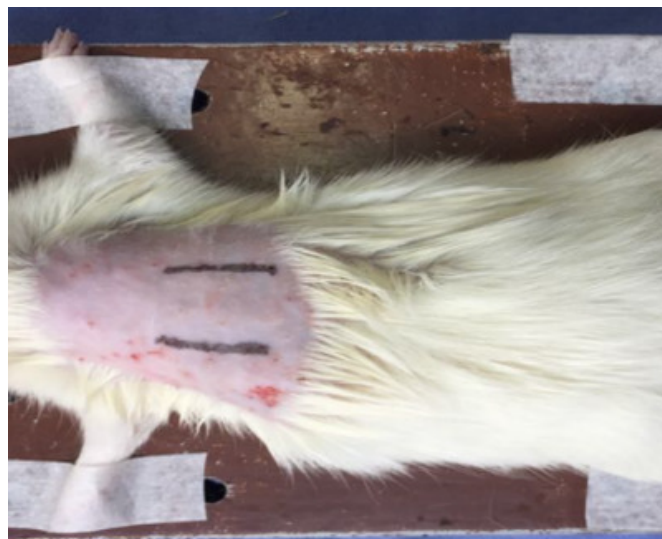

Figure 1. Sutured surgical incisions, symmetrical in relation to midline, on the dorsal area of the test animal.

The groups were named according to the substance applied in the incision:
- Bupivacaine - infiltration of bupivacaine 0.25%;
- Levobupivacaine - infiltration of enantiomeric excess levobupivacaine 0.25%;
- Ropivacaine - infiltration of ropivacaine 0.25%;
- Saline solution - infiltration of saline solution 0.9%.

There was no evaluation of anesthetic equipotency. Therefore, we chose to standardize the volume and number of application points in the groups.

Two minutes after infiltration, the incisions were sutured using a 4.0 prolene suture thread. There was an observation period of 2 hours after the application of the local anesthetic. The test animals received an

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intramuscular tramadol injection (2mg/kg) during the postoperative period every 12 hours for three days. The surgical wound was treated once a day, and the animals were sacrificed at the end of the seventh day.

Immediately after sacrifice, tissue samples from the two incisions with an average diameter of 2cm² were removed and fixed in 4% buffered formaldehyde for approximately 4 hours. Posteriorly, they were cleaved, and samples of the scar were processed and embedded in paraffin to make histological sections of about 5 micrometers (μm) thick. Next, histological sections were stained using the hematoxylin-eosin (HE) techniques to evaluate the inflammatory infiltrate and the Masson's trichrome (MT) staining for the evaluation of the fibrous scar. The slides were analyzed under a standard light microscope (OLYMPUS® BX40) by a single previously trained observer, who was unaware of the group to which each animal belonged (blind).

To perform the morphometric analysis of the area of the inflammatory infiltrate (HE) and the fibrous scar (MT), we selected the areas with the largest inflammatory infiltrate around the surgical scar (HE) and the focus with the largest scar diameter (MT), respectively. To determine the area of the inflammatory infiltrate and the scar, Image J® 1.32j semiautomatic image analyzer system (National Institutes of Health, U.S.) was used, using μm as reference unit and the 100X magnification field of light microscope Olympus BX41 (Olympus®, São Paulo, Brazil).

Biostat® program, version 5.0, was used for statistical analysis. Comparisons among the different groups were made using a one-way ANOVA test with Bonferroni post-test and Kruskal-Wallis test with Student-Newman-Keuls post-test. The results were considered significant when the probability of rejection of the null hypothesis was less than 5% ($p<0.05$).

RESULTS

Morphologically evaluating, the inflammatory infiltrate was predominantly mononuclear, permeated by occasional polymorphonuclear cells, especially neutrophils. Table 1 shows the results of the inflammatory infiltrate area in the different groups.

The inflammatory infiltrate area was significantly larger in the bupivacaine, ropivacaine, and levobupivacaine groups compared with their controls (incisions that did not receive any infiltration). When comparing the groups with each other, the bupivacaine group had a significantly larger inflammatory infiltrate than the levobupivacaine and ropivacaine groups. In addition, the SS 0.9% group presented a substantially larger area of infiltrating than its control group. However, no difference was observed between the SS 0.9% group and the LA groups tested.

Figure 2 illustrates the inflammatory infiltrate area in four different animals. Table 2 shows evaluation data of the fibrous scar area.

When evaluating the fibrous scar area, a significant difference was observed between the levobupivacaine and ropivacaine groups and their controls. Moreover, the fibrous scar area was larger when these two anesthetics were applied. However, when comparing the LA groups with the SS 0.9% group, no difference was found.

Figure 3 shows the fibrous scar in four different animals.

DISCUSSION

LA infiltration into the skin and subcutaneous tissue is widely used for analgesic purposes after surgical correction in different types of surgery[1,2]. Long-lasting LAs, such as bupivacaine, ropivacaine, and the enantiomeric excess levobupivacaine, corresponds to 75% of the levorotatory isomer and 25% of the dextrorotatory isomer are regularly used. Theoretically, the infiltration of these anesthetics is the most rational method of analgesia since they block the nociceptive afferents and, therefore, pain and secondary stress[8-10].

There has been controversy over the interference of LA in the healing of surgical wounds. The effects of injection or topical application of LA have been tested in several experimental models, using healing time, tensile strength measures, inflammatory process area, regeneration, granulation tissue, and angiogenesis[3-7]. These studies used rodents whose skin heals rapidly, less susceptible to inhibitory agents and systemic factors that may limit healing[3].

The healing of surgical wounds is when the tissue at the lesion site is replaced by vascularized connective tissue. The first healing step is the onset of an inflammatory reaction that will reabsorb extravasated blood and tissue degradation products. Subsequently, there is a proliferation of capillaries and fibroblasts that will form the cicatricial connective tissue. The final volume of the scar depends on the stimuli that regulate the activity of the cells that produce the extracellular matrix and on balance between the matrix synthesis and degradation[11]. There is evidence that the intensity and type of the inflammatory infiltrate will define the extent of the scar[12]. In the present study, the evaluation was carried out seven days after the incision. At this time, healing is probably in the granulation/proliferation stage, which is characterized by fibroblast migration and collagen production in rats[13]. However, even in this stage, significant inflammatory infiltration around the scar was observed in all groups.

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Table 1. Inflammatory infiltrate area in the surgical wound after applying local anesthetics and saline solution in the different animal groups.

Animal Group	Median area (μm²)	Minimum area (μm²)	Maximum area (μm²)
Bupivacaine	1595646.75[a]	353178.5	3785935.5
Bupivacaine control	10301715	181919	2865686.5
Levobupivacaine	975600[b,e]	661763	1829388
Levobupivacaine control	762221.8	97105	971325
Ropivacaine	1278801.3[c,f]	379043.5	4122721.5
Ropivacaine control	340497	340497	565769
SS 0.9%	1440765.3[d]	832288	1959811
SS 0.9% control	749901.3	74383	1054869

Control group: incision that did not receive infiltration. [a]Bupivacaine x Bupivacaine control p<0,05; [b]Levobupivacaine x Levobupivacaine control p<0,05; [c]Ropivacaine x Ropivacaine control p<0,05; [d]SS x SS control p<0,05; [e]Bupivacaine x Levobupivacaine p<0,05; [f]Bupivacaine x Ropivacaine p<0,05; One-way ANOVA. SS: Saline Solution.
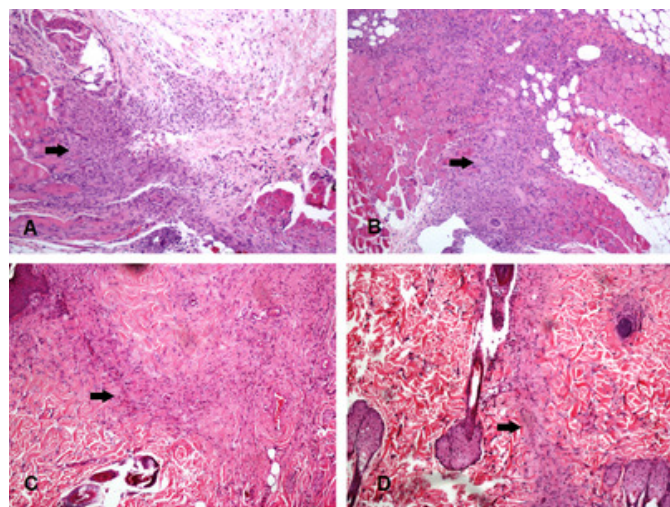

Figure 2. Area of inflammatory infiltrate (arrows) in four different animals. Note that in A (bupivacaine) and B (ropivacaine) the area is larger than in C (levobupivacaine), and D (SS 0,9%) (hematoxylin-eosin, 100X). SS: Saline Solution.
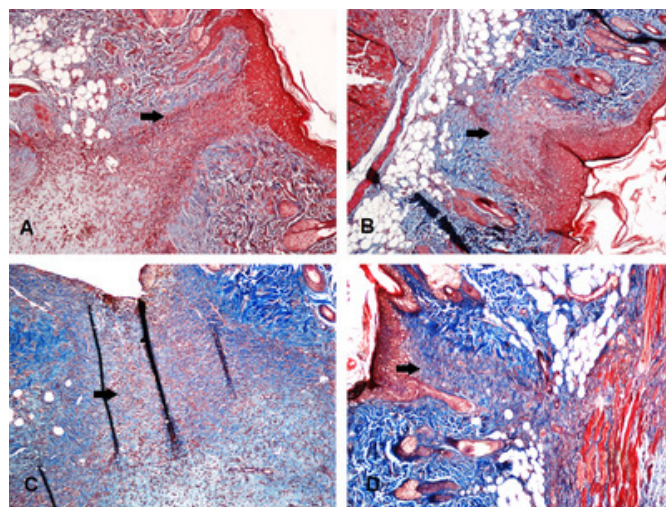

Figure 3. Fibrous scar area stained in red (arrows) in four different animals. Note that the area is larger in A (levobupivacaine) and B (ropivacaine) than in C (SS 0,9%), and D (Bupivacaine) (Masson's trichrome, 100X). SS: Saline Solution.

Table 2. Fibrous scar area on the surgical wound after applying local anesthetics and saline solution in the different groups of animals.

Animal Group	Median area (μm²)	Minimum area (μm²)	Maximum area (μm²)
Bupivacaine	449402	146280	2016103.5
Bupivacaine control	605389.5	47813.5	1251420.5
Levobupivacaine	818835.5[a,c]	346804	4741842
Levobupivacaine control	450434.0	116934	779886
Ropivacaine	885409[b,d]	295817	2599696
Ropivacaine control	321682	207731	725589
SS 0.9%	530607.8	235281.5	1997949
SS 0.9% control	501198.5	140366	1089956

Control group: incision that did not receive infiltration. [a]Levobupivacaine x Levobupivacaine control p<0,05; [b]Ropivacaine x Ropivacaine control p<0,05; [c]Bupivacaine x Levobupivacaine p<0,05; [d]Bubivacaine x Ropivacaine p>0,05. Kruskal-Wallis.

Levobupivacaine was associated with the smallest area of inflammatory infiltrate and one of the largest areas of a fibrous scar. This observation contradicts the conception that the intensity of the inflammatory infiltrate defines the extent of scar formation[12]. It is possible that the type of inflammatory infiltrate, rather than its intensity, may be associated with a larger or smaller fibrous scar area, which was not evaluated in the current study. The presence of inflammatory infiltrate interferes directly in the scar

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formation; however, different types of inflammatory cells may be present, and these cells may, or not, stimulate the production of extracellular matrix. Another possibility would be that the volume or trauma of the application, not the LA itself, would interfere with the inflammatory infiltrate and the fibrous scar[11], since there was no difference between the LA group and the group that received SS, an apparently inert solution.

The application of long-lasting LA in the incision for postoperative analgesia has been associated with reduced cytokine-induced alterations, and in addition, it minimizes hyperalgesia[14]. Although some studies suggest that these drugs also have anti-inflammatory properties[15], our results demonstrate that bupivacaine and ropivacaine, particularly the latter, are associated with larger inflammatory infiltrate. Furthermore, bupivacaine was associated with an area of infiltrating significantly larger than its levorotatory isomer, levobupivacaine.

A study using rats to evaluate inflammatory and cicatricial processes of the wound after long-lasting LA infiltration, assessing histology and tensile strength on the third and the fourteenth days, observed that, on the third day, there was a significant increase of macrophages in the group receiving bupivacaine. It was also observed that the collagen concentration was increased in the animals infiltrated with bupivacaine compared with ropivacaine and SS. There was no difference in the scar inflammatory response, presence of collagen, and tensile strength on the fourteenth day. The authors concluded that the alterations caused by LA infiltration do not extend beyond the third day, and therefore, do not impair the wound healing process in rats[5]. Our results contradict this supposition.

Another study on the effects of LA infiltration on the eighth day of wound healing in rats demonstrated that both bupivacaine and lidocaine reduced collagen production and resistance to scar rupture, causing significant edema, vascularization, and inflammation when compared with the controls[4]. Similarly, in the present study, we observed that bupivacaine was associated with the largest area of inflammatory infiltrate. Concerning the fibrous scar, bupivacaine seems not to interfere in the fibrous scar. On the other hand, its levorotatory isomer, levobupivacaine, and ropivacaine were associated with larger fibrous scar areas.

A study evaluating the effect of lidocaine and bupivacaine on wound healing in rats suggested that although these anesthetics influenced local inflammation and proteolytic factors, no effect on wound healing was observed[16]. These findings partially agree with ours.

Another study, using a methodology similar to ours, compared the amount of collagen and the number of mast cells through morphometry in rats after injection of lidocaine with epinephrine or with buffer.

The authors concluded that lidocaine interferes with collagen and reduces the initial amount of mast cells in the surgical wound[17].

Two experimental studies in rats associating levobupivacaine infiltration with ibuprofen and norepinephrine showed greater regeneration of the dermis and epidermis, granulation tissue and angiogenesis than in the control group, suggesting an increase in the regenerative/healing process[6] and increased angiogenesis and tensile strength of the scar[7]. However, unlike our study, levobupivacaine was associated with a non-hormonal anti-inflammatory and a vasopressor, which could interfere with healing.

In rabbits with infiltration of lidocaine and bupivacaine, other authors found no histopathological difference when these LAs were compared with saline solution and suggested that these LAs do not affect healing[18].

LAs act by directly inhibiting the nociceptive fibers of the skin. These fibers, together with the melanocytes, neuropeptides, and interleukins, are part of the cutaneous neuroendocrine system that, among other functions, modulate surgical healing, especially inflammation. LAs block neural impulses temporarily by inhibiting the neuroendocrine response to wound healing stimuli and related signaling, interfering negatively in wound healing. One of the neuropeptides that plays an essential role in the neuroendocrine system is the substance P, which controls mast cells' degranulation and the release of inflammatory proteins in the surgical site. These cells act on wound healing by promoting inflammatory response, angiogenesis, and resorption of the extracellular matrix. In addition, they regulate growth factors and interleukins, essential for inflammation and the proliferative phase of wound healing[19,20].

Amongst the different wound healing proteins, collagen correlates more closely with scar tissue strength. The amount of collagen in the scar site depends on the cicatricial process and may undergo LA interference due to inhibition during the neurogenic inflammatory phase[21].

How SS 0,9% infiltration was associated with a larger area of inflammatory infiltrate and fibrous scar, we question whether, in addition to the action of LA or the SS 0.9% in the scar site, the mechanical effect (distension) caused by the infiltration interferes with the wound healing, possibility already described[11].

When comparing bupivacaine with its isomer, levobupivacaine, the former was associated with the largest infiltrate area, and the last was associated with the largest fibrous scar areas and smallest area of infiltrate. Thus, this modification in the bupivacaine molecule could be responsible for the lower inflammation, and larger fibrous scar observed in the wound infiltrated with levobupivacaine.

CONCLUSION

The present study results indicate that the volume applied, or the infiltration trauma may have been responsible for the larger area de infiltrate and fibrous scar, not the LA itself. Complementary studies, including studies in humans, evaluating the equipotency between the LA and variation in the volume applied, are necessary to better understand the associated mechanisms, particularly in the evaluation of the area of inflammatory infiltrate and fibrosis in different stages (days) of healing, the type of inflammatory infiltrate and the cytokines involved.

COLLABORATIONS

MSC Analysis and/or data interpretation, Conceptualization, Final manuscript approval.

ECSA Analysis and/or data interpretation, Final manuscript approval, Methodology, Writing - Original Draft Preparation.

LAMS Conceptualization, Investigation, Methodology, Supervision.

GCO Analysis and/or data interpretation, Methodology, Writing - Original Draft Preparation.

ACG Conceptualization, Methodology, Writing - Original Draft Preparation.

BJM Conceptualization, Methodology, Writing - Original Draft Preparation.

RME Analysis and/or data interpretation, Conception and design study, Conceptualization, Final manuscript approval, Methodology, Supervision.

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