Effects of Levobupivacaine on Wound Healing

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BACKGROUND: Local anesthetic infiltration along the incision may be used to provide surgical anesthesia or postoperative analgesia. However, the effect of local anesthetics on wound healing remains controversial. In this investigation, we evaluated the effects of levobupivacaine on wound healing.

METHODS: Sixty Wistar albino female rats weighing 230 ± 20 g were included, with 10 rats in each group: group early c (early control): 3 mL isotonic saline; group early l2.5 (early levobupivacaine 1.25): 1.25 mg/kg per 3 mL levobupivacaine; group early l1.25 (early levobupivacaine 2.5): 2.5 mg/kg per 3 mL levobupivacaine; group late c (late control): 3 mL isotonic saline; group late l1.25 (late levobupivacaine 1.25): 1.25 mg/kg per 3 mL levobupivacaine; and group late l1.25 (late levobupivacaine 2.5): 2.5 mg/kg per 3 mL levobupivacaine. Rats in groups early c to early l2.5 were euthanized on the 8th day. Rats in groups late c to late l2.5 were euthanized on the 21st day. Wound tension strength, tissue hydroxyproline, and fibrotic index levels of the tissue samples from the early c and early l2.5 and late c and late l2.5 groups, respectively, on the 8th and 21st days were examined.

RESULTS: Levobupivacaine decreased wound tension strength on the 8th day, especially a 2.5 mg/kg dose (P < 0.001), and increased it on the 21st day (P < 0.001). It also increased the inflammatory response (P < 0.001) and collagen synthesis (8th day, P = 0.109; 21st day, P = 0.103) on both the 8th and 21st days.

CONCLUSIONS: While levobupivacaine had a positive effect on wound healing during the early period, negative effects were observed thereafter. Additional studies at the molecular level are necessary to determine the cause of these apparently opposite effects. (Anesth Analg 2013;116:495–9)

Local anesthetic infiltration along the incision may be used to provide surgical anesthesia or postoperative analgesia. However, the effects of local anesthetics on wound healing are not well understood. Normal wound healing consists of 4 phases: hemostasis, inflammation, proliferative, and remodeling. During the proliferative phase, tissue granulation, epithelization, and collagen production occur. An increase in fibroblasts occurs during the proliferative phase in normal wound healing (i.e., the fibrotic index increases). Collagen production and release begin on the 3rd day and continue for 3 weeks. Collagens released from fibroblasts and their cross-linkage enhance wound tension strength. The amount and quality of collagen synthesis determine the wound tension strength, which is the mechanical integrity of the wound. The final phase of wound healing is the remodeling phase, which is characterized by the reorganization of collagen fibrils and gradually increasing wound tension strength.¹²

Several previous studies report that lidocaine delays wound healing and decreases wound tension strength,³⁴ whereas 2 reports suggest that it does not affect wound healing.⁵,⁶ Studies⁷⁻⁸ that discuss reducing the cytotoxic effect of local anesthetics on inflammatory cells/fibroblasts increase concerns regarding the use of local anesthetics. An increase of fibrotic index and collagen amount at the wound site do not always result in improved healing because an irregular sequence of collagen fibers can have a negative effect on wound healing.⁹,¹⁰ Therefore, the evaluations of wound healing based solely on the measurements of cellular response or tissue collagen amount have limited practical clinical importance. Rather, assessment of wound tension strength is essential as it measures mechanical integrity. A decrease in wound tension strength can cause wound dehiscence after a loss of mechanical integrity. The primary goal of this study was to assess the change in wound tension strength. Measurements of tissue hydroxyproline levels and fibrotic index were used to examine the reason(s) for the change in wound tension strength.

METHODS
This study was conducted at the Istanbul University Experimental Medical Research Institution (DETAE), using rats from Istanbul University DETAE from September 20, 2010 to October 25, 2010 after approval by the Istanbul University Animal Experiments Local Ethics Committee. During the operations, the rats were treated humanely according to the Guide for the Care and Use of Laboratory Animals. Sixty Wistar albino female rats (weighing 230 ± 20 g) were used, with 10 rats in each of 6 groups. For anesthesia, 1 dose of 10 mg/kg intraperitoneal ketamine HCl (Ketalar vial, 50 mg/mL, Eczacibasi Medicine and Commerce A.C., Istanbul, Turkey) and 5 mg/kg subcutaneous xylazine (Rompun vial, 23.32 mg/mL, Bayer Turkish

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Accepted for publication August 30, 2012.

Sponsorship was not used in this study. All the funding was obtained by the authors.

The authors declare no conflicts of interest.

Reprints will not be available from the authors.

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DOI: 10.1213/ANE.0b013e318273f48e
Chemistry Industry Ltd. C., Istanbul, Turkey) were administered to rats under sterile conditions. Rats were separated into the following 6 groups:

1. Group early c (early control): 3 mL isotonic saline
2. Group early l1.25 (early levobupivacaine 1.25): 2.5 mg/kg per 3 mL levobupivacaine
3. Group early l2.5 (early levobupivacaine 2.5): 2.5 mg/kg per 3 mL levobupivacaine
4. Group late c (late control): 3 mL isotonic saline
5. Group late l1.25 (late levobupivacaine 1.25): 2.5 mg/kg per 3 mL levobupivacaine
6. Group late l2.5 (late levobupivacaine 2.5): 2.5 mg/kg per 3 mL levobupivacaine

Rats in groups early c to early l2.5 were killed on the 8th day. Rats in groups late c to late l2.5 were killed on the 21st day.

After the dorsal surface hair of all rats was shaved and the surgical site was disinfected, 3 mL isotonic saline, 1.25 mg/kg per 3 mL levobupivacaine (Chirocaine, Abbott Laboratories, Istanbul, Turkey), and 2.5 mg/kg per 3 mL levobupivacaine was administered to groups early c and late c, groups early l1.25 and late l1.25, and, groups early l2.5 and late l2.5, respectively, at the incision site, and a cutaneous–subcutaneous incision was made 2 minutes later. A continuous suture was used at the incision sites with 3-0 silk. A daily dressing at the incision sites was used for 7 days. No prophylactic or therapeutic antibiotic was administered. After a 4 cm × 2 cm patch of skin and subcutaneous tissue was removed under sterile conditions and under anesthesia from the incision sites of rats of groups early c, early l1.25, and early l2.5 on the 8th day, all rats were killed under high-dose anesthesia (ketamine and xylazine) with intracardiac injection. After sutures were removed, the tissue samples were divided into 3 pieces. One sample was added to 10% formaldehyde solution for pathologic examination. One sample was placed into a microcentrifuge tube, minimizing room temperature exposure, and was stored at –30°C for biochemical examination. The last sample was used to measure wound tension strength.

Sutures at the incision sites in the rats in groups late c, late l1.25, and late l2.5 were removed under sterile conditions on the 10th day. Sutures in the rats to be killed on the 21st day were removed so that a “10th day suture reaction” would not develop, because nonabsorbable silk suture material was used. On the 21st day, tissue samples were obtained and evaluated as described above. Levobupivacaine dosages used in this study were similar to dosages used in other studies from the literature.11–13 In our study, milligram per kilogram concentrations and a 3 mL volume of levobupivacaine were used to standardize the doses. A smaller volume was preferred in comparison with other clinical studies in the literature to minimize the effect of the volume. A total volume of 3 mL was administered at the 4-cm incision site in all rats.

The 8th postoperative day is the proliferative phase of wound healing, and during this period, the synthesis of collagen and fibroblast increases.1,2 It is typically during this period (7–10 days) that primary sutures are removed.14 Therefore, the clinical outcome was based on the early-phase variables studied on the 8th day. The 21st day is the remodeling phase of wound healing, and this is when wound tension strength becomes evident.1,2 As a result, late-phase variables in this study were evaluated on the 21st day.

### Measurement of Wound Tension Strength

Wound tension strength was measured on fresh tissue samples with a 10-N single-action tensiometer. Tissues were clamped to the 2 ends of the tensiometer, and a tension of 1 N/min was applied. An increase in wound tension strength positively affects wound healing in primary wounds.1

### Biochemical Method

The amino acid hydroxyproline is abundant in collagen. The basis of this procedure is alkaline hydrolysis of tissue homogenates and the formation of free hydroxyproline. Chloramine T was used for the oxidation of free hydroxyproline to obtain the pyrrole. Then, Ehrlich solution was added and a chromophore was formed at 550 nm. Hydroxyproline is found in large amounts in collagen.15 In this study, tissue hydroxyproline levels were used to determine the collagen amount in the tissue. An increase in collagen in a wound can positively affect wound healing, depending on the sequence of the collagen fibers.1

### Pathological Method

After tissue samples obtained by biopsy were fixed in 10% formaldehyde solution for 24 hours, they were processed and embedded in paraffin blocks, and 5 µm sections were cut with a microtome. These sections were deparaffinized and prepared for staining. Staining was conducted with hematoxylin and eosin (H&E) and Masson trichrome stains. Cell nuclei and cytoplasm were stained blue-violet and pink, respectively, with H&E. Polymorphonuclear cells, edema, blood vessels in the extracellular matrix, and inflammation were determined with this stain. Connective tissue, muscle, and nuclei were seen in green, red-violet, and black-dark brown with Masson trichrome. The histopathological examination was conducted by a pathologist experienced in this field who was blinded to the animal’s group, using light microscopy at 10× and 40× magnifications by randomly sampling the preparation. During the pathological evaluation, the amount and aggregation of polymorphic nuclei cells in the preparation, new vessel formation, existence of fibroblasts, and intensity were graded from 0.5 to 4.5, considering the accumulation of connective tissue matrix, fibrosis formation, and intensity (fibrotic index). An increase in fibrotic index generally causes an increase in wound tension strength, depending on the increase in collagen level and the sequence of the collagen fibers. That is, it positively affects wound healing.

### Statistical Analysis

Median (interquartile range) was used to describe the study data. Data were compared between 2 groups using the Mann-Whitney U test. Data were compared among more than 2 groups using the Kruskal-Wallis test followed by post hoc comparisons pairwise using the Mann-Whitney U test reported after Bonferroni correction. The 99% confidence intervals for median differences between groups were calculated using the Hodges-Lehman method. Correlations were calculated using Spearman rank analysis.
RESULTS

One rat in group late c was found dead in its cage on the 1st postoperative day; therefore, wound tension strength, tissue hydroxyproline, and fibrotic index level could not be examined.

The median wound tension strength of all groups on the 8th day was significantly lower than the wound tension strength on the 21st day (Table 1). The median wound tension strength in group early l1.25 on the 8th day was significantly lower than the values in group early l1.25 (P < 0.001) and group early c (P < 0.001). There was no significant difference between group early c and group early l1.25. The median wound tension strength in group late c on the 21st day was significantly lower than the median wound tension strengths of group late l1.25 (P < 0.001) and group late l2.5 (P < 0.001). The wound tension strength on the 8th day was in the order: group early c > early l1.25 > early l2.5. The wound tension strength on the 21st day was in the order: group late l2.5 > late l1.25 > late c.

A significant increase in median wound tension strength was seen in groups early c and late c (P < 0.001), groups early l1.25 and late l1.25 (P < 0.001), and groups early l2.5 and late l2.5 (P < 0.001; Table 1).

The difference between the median fibrotic index values of the groups on the 8th and 21st days was statistically significant (P < 0.001; Table 2). The median fibrotic index of group early l1.25 on the 8th day was significantly higher than group early c (P < 0.001) or group early l2.5 (P < 0.001). The median fibrotic index of group late l1.25 on the 21st day was significantly higher than the median of group late c (P < 0.001); Table 2).

DISCUSSION

Previous studies reported that local anesthetic drugs used for infiltration anesthesia exhibit neutral,\(^5,6\) negative,\(^16\) and positive effects\(^13\) on wound healing. A critical factor determining the effect on wound healing is wound tension strength, since a decrease in wound tension strength can result in wound dehiscence and may prevent the removal of sutures during an early phase. Increases in fibrotic index and the amount and quality of tissue hydroxyproline are the factors that determine wound tension strength. Individually, these factors are not clinically important; however,

\[ r = 0.447; \]
\[ r = 0.015; \]
\[ r = –0.740; \]
\[ r = 0.472; \]
\[ r = 0.662; \]

**Table 1. Comparison of Median Wound Tension Strengths (Newtons)**

<table>
<thead>
<tr>
<th></th>
<th>c Median</th>
<th>IQR</th>
<th>l1.25 Median</th>
<th>IQR</th>
<th>l2.5 Median</th>
<th>IQR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>8th day (early)</td>
<td>84.62</td>
<td>7.81</td>
<td>78.19</td>
<td>7.81</td>
<td>175.00</td>
<td>12.50</td>
<td>0.0001</td>
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<tr>
<td>21st day (late)</td>
<td>97.50</td>
<td>8.63</td>
<td>163.75</td>
<td>17.63</td>
<td>68.62</td>
<td>1.06</td>
<td>0.0001</td>
</tr>
<tr>
<td>P</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99% CI</td>
<td>6.2–20</td>
<td></td>
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</tr>
</tbody>
</table>

\(x – y\) (\(Wu/2\))

\(IQR = \text{interquartile range; CI = confidence interval.}\)

**Table 2. Comparison of Median Values of Fibrotic Index**

<table>
<thead>
<tr>
<th></th>
<th>c Median</th>
<th>IQR</th>
<th>l1.25 Median</th>
<th>IQR</th>
<th>l2.5 Median</th>
<th>IQR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>8th day (early)</td>
<td>1.00</td>
<td>0.25</td>
<td>2.00</td>
<td>2.75</td>
<td>1.00</td>
<td>3.00</td>
<td>0.63</td>
</tr>
<tr>
<td>21st day (late)</td>
<td>2.00</td>
<td>1.00</td>
<td>0.5–1.50</td>
<td>0.001</td>
<td>4.00</td>
<td>0.63</td>
<td>0.0001</td>
</tr>
<tr>
<td>P</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99% CI</td>
<td>1.00–2.00</td>
<td></td>
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</tbody>
</table>

\(x – y\) (\(Wu/2\))

\(IQR = \text{interquartile range; CI = confidence interval.}\)
Effects of Levobupivacaine

In a previous study evaluating the effects of levobupivacaine on wound healing, Dere et al. reported that all groups that received levobupivacaine at various doses exhibited significantly increased wound tension strength relative to the control group. Conversely, we observed that levobupivacaine infiltration, especially at a dose of 2.5 mg/kg, significantly decreased wound tension strength on the 8th day. Therefore, in contrast to the report by Dere et al., levobupivacaine negatively affected wound healing during the early period, whereas positive effects were observed in the late period. We cannot account for the differences in results. Our fibrotic index results were consistent with those of Dere et al. and demonstrated a dose dependence.

Increases in wound tension strength depend on both collagen quantity and its fiber arrangement. Several studies have evaluated the effects of various local anesthetics on collagen production and/or the inflammatory response associated with healing. Vasseur et al. reported that lidocaine infiltration at an incision site inhibits collagen synthesis and causes tissue necrosis. However, Niesgen et al. suggested that lidocaine and bupivacaine inhibited smooth muscle and fibroblast growth by negatively affecting lysophosphatidate. Rodrigues et al. showed that lidocaine decreased the number of mast cells in the wound region. Feder et al. showed that lidocaine, bupivacaine, and ropivacaine had a concentration-dependent cytotoxic effect on fibroblasts. That is, lidocaine suppressed the inflammatory response. However, in contrast to these reports, the fibrotic index increased significantly in our study, and our histopathological examination showed that the local anesthetic used had no cytotoxic effect on fibroblasts.

Collagen fibers are arranged irregularly during the early period of wound healing. This may explain the reduced wound strength, despite the high amount of collagen in the early period of healing. However, hydroxyproline levels and wound tension strength may not always be positively correlated. For example, Nagler et al. reported that halofuginone decreased collagen α-1 gene expression but that it did not significantly decrease wound tension strength. We did not report a correlation between hydroxyproline levels and wound tension strength on either the 8th or 21st day. Conversely, a statistically significant and 74% negative relation between fibrotic index and wound tension strength was detected on the 8th day. We propose that this is due to the negative effect of levobupivacaine on collagen structure/bonding of collagen fibers and a relative decrease in collagen production. This effect may cause a delay in clinical wound healing. A significant and 66.2% positive relationship between fibrotic index and wound tension strength was determined on the 21st day. This may have been due to the removal of the negative effect of levobupivacaine on collagen structure/bonding of collagen fibers, and this result was considered to be consistent with the normal healing process.

Our results suggest that levobupivacaine may not be an ideal drug for infiltration anesthesia in regions where sutures are removed early, because levobupivacaine negatively affected wound healing during the early period. Since levobupivacaine positively affects wound healing during the later periods of wound healing, it may be an appropriate local anesthetic agent for infiltration anesthesia in body areas from where sutures are removed later (e.g., back, lower extremity), and in tissues such as the fascia, where wound healing is delayed. Although preincisional levobupivacaine infiltration at a 2.5 mg/kg dose significantly decreased wound tension strength on the 8th day, the inhibitory effects were not as great with a 1.25 mg/kg dose. Thus, this dose may be more suitable in clinical applications. Additional studies at the molecular level of the effects of levobupivacaine on collagen structure are needed to determine the reason(s) for the apparent negative effect on the 8th day and apparent positive effect on wound tension strength of levobupivacaine on the 21st day.

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### Table 3. Comparison of Median Tissue Hydroxyproline Levels (milligrams)

<table>
<thead>
<tr>
<th></th>
<th>c Median</th>
<th>IQR</th>
<th>I25s Median</th>
<th>IQR</th>
<th>I25s Median</th>
<th>IQR</th>
<th>P</th>
<th>99% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>8th day (early)</td>
<td>1.38</td>
<td>0.72</td>
<td>2.13</td>
<td>0.86</td>
<td>2.70</td>
<td>0.87</td>
<td>1.07</td>
<td>1.78</td>
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<tr>
<td>21st day (late)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.50</td>
<td>1.68</td>
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<table>
<thead>
<tr>
<th></th>
<th>c</th>
<th>IQR</th>
<th>I25s</th>
<th>IQR</th>
<th>I25s</th>
<th>IQR</th>
<th>P</th>
<th>99% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>8th day (early)</td>
<td>0.014</td>
<td>0.059 to 1.438</td>
<td></td>
<td>0.028</td>
<td></td>
<td></td>
<td>0.070</td>
<td></td>
</tr>
<tr>
<td>21st day (late)</td>
<td>-0.059</td>
<td>0.014 to 1.334</td>
<td></td>
<td>-0.108</td>
<td></td>
<td></td>
<td>-0.330</td>
<td></td>
</tr>
</tbody>
</table>

IQR = interquartile range; CI = confidence interval.

### Table 4. Relationship Between Fibrotic Index, Tissue Hydroxyproline Level, and Wound Tension Strength

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyproline (8th day)</td>
<td>30</td>
<td>-0.187</td>
<td>0.322</td>
</tr>
<tr>
<td>Fibrotic index (8th day)</td>
<td>30</td>
<td>-0.740</td>
<td>0.0000029</td>
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<tr>
<td>Hydroxyproline (21st day)</td>
<td>30</td>
<td>0.191</td>
<td>0.312</td>
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<tr>
<td>Fibrotic index (21st day)</td>
<td>29</td>
<td>0.252</td>
<td>0.187</td>
</tr>
<tr>
<td>Fibrotic index (8th day)</td>
<td>29</td>
<td>0.662</td>
<td>0.000093</td>
</tr>
<tr>
<td>Hydroxyproline (21st day)</td>
<td>29</td>
<td>0.447</td>
<td>0.015</td>
</tr>
</tbody>
</table>
**DISCLOSURES**

**Name:** Sezgin Zeren, MD.
**Contribution:** This author helped design the study and analyze the data.
**Attestation:** Sezgin Zeren has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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**Contribution:** This author helped design and conduct the study, analyze the data, write the manuscript, and helped with all experimental examinations.
**Attestation:** Sevgi Kesici has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

**Name:** Ugur Kesici, MD.
**Contribution:** This author helped design and conduct the study, analyze the data, write the manuscript, and helped with all experimental examinations.
**Attestation:** Ugur Kesici has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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**Contribution:** This author helped analyze the data and helped with the biochemical examination.
**Attestation:** Salim Isbilir has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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**Contribution:** This author helped design the study and analyze the data.
**Attestation:** Ulku Aygen Turkmen has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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**Contribution:** This author helped analyze the data.
**Attestation:** Hulya Ulusoy has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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**Contribution:** This author helped with all histopathological examinations.
**Attestation:** Vildan Karpuz has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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**Contribution:** This author helped with the biochemical examination.
**Attestation:** Omer Ozcan has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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**Attestation:** Erdal Polat has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

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**Attestation:** Osman Metin Ipcioglu has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

**Name:** Musa Kazim Sari, MD.
**Contribution:** This author helped design the study.
**Attestation:** Musa Kazim Sari has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

This manuscript was handled by: Terese T. Horlocker, MD.

**REFERENCES**