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Summary statement: This study shows that the use of local anesthetics in higher concentrations over longer time might impair viability of fibroblasts.
ABSTRACT

Lidocaine, bupivacaine or ropivacaine are routinely used to manage perioperative pain. Sparse data exist focusing on the effects of local anesthetics (LA) on fibroblasts, which are actively involved in wound healing. Therefore, we investigated the effects of the three LA to assess survival, viability and proliferation rate of fibroblasts. Human fibroblasts were exposed to 0.3 mg/ml and 0.6 mg/ml of each LA for 2 days, followed by an incubation with normal medium for another 1, 4 or 7 days (group 1). Alternatively, cells were permanently incubated with LA for 3, 6 or 9 days (group 2). Live cell count was assessed using trypan blue staining. Viability was measured by the tetrazolium bromide assay, proliferation tests were performed with the help of the colorimetric bromodeoxyuridine assay. Production of reactive oxygen species (ROS) was determined, measuring the oxidation of non-fluorescent-2,7′-dichlorofluorescin. Treatment of cells with the three LA showed a concentration-dependent decrease of live cells, mitochondrial activity, and proliferation rate. Group arrangement played a significant role for cell count and proliferation, while exposure time influenced viability. Among the analyzed LA, bupivacaine showed the most severe cytotoxic effects. Increased production of ROS correlated with decreased viability of fibroblasts in lidocaine- and bupivacaine-exposed cells, but not upon stimulation with ropivacaine. This study shows a concentration-
dependent cytotoxic effect of lidocaine, bupivacaine and ropivacaine on fibroblasts *in vitro* with more pronounced effects after continuous incubation. A possible mechanism of cell impairment could be triggered by production of ROS upon stimulation with lidocaine and bupivacaine.
INTRODUCTION

Pain control with local anesthetics is a major issue in perioperative medicine. Local anesthetics (LA) are topically injected (such as intraarticular application) or applied through a perineural or wound catheter for pain management [1-7]. Clinically used concentrations of LA vary from 2 mg/ml to 10 mg/ml, depending on the chosen type and duration of analgesia.

Lidocaine, bupivacaine and ropivacaine are all amide type local anesthetics. Recent publications have suggested potential adverse effects of lidocaine, bupivacaine and ropivacaine on articular chondrocytes in vitro [8-10]. Moreover, studies have also shown toxic effects of local anesthetics on tissues which are involved in postoperative recovery and wound healing, challenging the safe continuous application of local anesthetics in clinical practice [11,12].

Wound healing after surgery is a natural process of regenerating tissue. A set of complex biochemical events takes place in a closely orchestrated cascade to repair tissue. These events overlap in time and may be artificially categorized into 3 steps: inflammatory, proliferative and remodeling phase. Fibroblasts play a crucial role in the proliferative phase. They migrate from normal tissue into the wound area from its margins, where they grow and form a new, provisional extracellular matrix by excreting collagen and fibronectin.
Due to the crucial role of fibroblasts in the wound healing process, we investigated the effects of different concentrations of local anesthetics on viability and proliferation of fibroblasts. Based on previous results in an inflammatory model of acute lung injury [13], we hypothesized that local anesthetics do not have an adverse effect on fibroblasts.
MATERIAL and METHODS

Fibroblasts

In this study, human osteosarcoma cells (LGC Standard GmbH, Wesel, Germany), an osteoblast-like cell type with the morphology of human fibroblasts were used. According to a study from Jukkola et al. in 1993, these cells have the characteristics of proliferative wound fibroblasts [14]. Cells were cultured in α-modified eagle medium (MEM, LGC Standard GmbH, Wesel, Germany) with 10% fetal bovine serum (FBS, LGC Standard GmbH) and 10'000 U/l penicillin/streptomycin (LGC Standard GmbH) at 37ºC and 5% CO₂.

Lidocaine (Lidocain CO₂ 2% Sintetica®) was purchased from Sintetica AG, Mendrisio, Switzerland, bupivacaine (Bucain®) from DeltaSelect GmbH, Munich, Germany, and ropivacaine (Naropin®) from AstraZeneca, Wedel, Germany.

Experimental groups

Serial dilutions were chosen with lidocaine, bupivacaine and ropivacaine resulting in concentrations of 0.3 mg/ml and 0.6 mg/ml, representing comparable tissue concentrations measured in clinical practice [15].

In group 1, cells were exposed to the LA for 2 days followed by another incubation time of 1, 4 or 7 days with normal medium without LA. In group 2,
cells were permanently exposed to local anesthetics for 3, 6 or 9 days. The LA-containing medium was changed every second day to provide stable and constant drug concentrations.

Control cells were incubated with medium only for the according period of time. All changes of medium performed in the treated group were similarly done in control cells.

**Cell count**

On day 3, 6 and 9, living cells were counted manually in the Neubauer chamber, using trypan blue [16,17].

**Cell viability**

The tetrazolium bromide (MTT) assay is a well-known and recognized method to measure cell viability *in vitro*.[18] The method is based on the reduction of the yellow tetrazoliumsalt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide into purple formazan crystals by mitochondrial dehydrogenases. Dehydrogenases are active in living cells only. Conversion of MTT is therefore directly related to cell viability.

**Cell proliferation**

Proliferation tests were performed with the help of the colorimetric
bromodeoxyuridine (BrdU) assay (Roche, Basel, Switzerland). The test analyzes the proliferation of cells by utilizing BrdU as an analog of the DNA nucleotide thymidine, which is incorporated into the synthesized DNA of actively dividing cells. The amount of BrdU incorporation is reflected in the intensity of absorbance of the final reaction, measured with an ELISA-Reader at 450 nm (reference wavelength 620 nm) [19,20].

**Fluorometric assays for determination of caspase-3 activity**

Caspase-3 activity was determined by measuring proteolytic cleavage of the fluorogenic caspase-3 substrate Ac-Asp-Glu-Val-Asp-AMC (Calbiochem, Laefelfingen, Switzerland). Cells were incubated for 1 hour at 37ºC with 2.5 µM substrate. The fluorescence of the cleaved reporter group was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

**Determination of reactive oxygen species (ROS)**

To quantify possible ROS generation by fibroblasts, experiments were performed measuring the oxidation of non-fluorescent 2,7'-dichlorofluorescin (DCFH) (Sigma, Buchs, Switzerland) substrate to highly fluorescent DCF by ROS. As the experimental setup could only be performed for a short exposure, LA were incubated for 5 hours with fibroblasts. Cells were loaded with DCFH during a 60-min incubation in Hanks Balanced Salt Solution (HBSS, Sigma, Buchs,
Switzerland) supplemented with 30 mM glucose (D-(+)-Glucose (Sigma, Buchs, Switzerland), pH 7.4, and 50 µM DCFH-DA (Sigma, Buchs, Switzerland) at room temperature in the dark. Cells were washed three times with HBSS to remove any extracellular probe from the extracellular environment. Thereafter, cells were exposed to various concentrations of local anesthetics in HBSS. The amount of generated DCF was measured using a fluorescence Synergy HT (Bio-TEK, Winooski, VT). The excitation filter was set at 485 nm, the emission filter was set at 530 nm. At the same time, cell viability and activity of caspase-3 were determined as well.

**Statistical analysis**

Values were expressed as mean ± SD. Results are presented in percentage to control. Cell count and ELISA data regarding viability, proliferation rate, and caspase-3 activity were analyzed using three-way ANOVA. Pearson’s product moment correlation coefficients were computed between ELISA results regarding production of reactive oxygen species and cell viability. OriginPro 8G (OriginLab, Northampton, MA, USA) and SPSS (SPSS, Inc., Chicago, IL) were used for statistical analyses. A probability of $p < 0.05$ was considered as statistically significant.
RESULTS

Cell count

In group 1, no negative effect of lidocaine and ropivacaine regarding cell survival was observed for the concentration of 0.3 mg/ml (Fig. 1A). In the presence of bupivacaine cell death ranged between 20% and 40%. With the concentration of 0.6 mg/ml, cell survival in the lidocaine and ropivacaine group was similar with 50% to 90%, while a prominent effect on cell death rate was observed for bupivacaine with 30% survival after 3 days, 5% after 6 days and no survival after 9 days of incubation (Fig. 1B).

In group 2 with a permanent incubation of fibroblasts with LA at a concentration of 0.3 mg/ml, cell survival of 70%-80% upon time was found for lidocaine and ropivacaine. Cell death was more evident in the bupivacaine group, showing a time-dependent decrease of survival (Fig. 1C). For the concentration of 0.6 mg/ml, cell survival decreased over time with no major differences between the three LA (Fig. 1D).

Concentration of lidocaine, bupivacaine and ropivacaine has a significant effect on cell death (for lidocaine p< 0.001, bupivacaine p< 0.001, and ropivacaine p=0.001). Group arrangement significantly influences cell survival as well: p=0.001 for lidocaine, p=0.029 for bupivacaine, and p=0.01 for ropivacaine.
**Cell viability**

Cell viability determined in fibroblasts from group 1 showed a similar pattern as trypan blue assays: only minor impairment over time was observed for the three LA with the concentration of 0.3 mg/ml (Fig. 2A). While viability was not diminished after incubation with lidocaine and ropivacaine in a concentration of 0.6 mg/ml, MTT decreased time-dependently after incubation with bupivacaine (Fig. 2B).

In group 2, MTT did not change upon incubation with lidocaine and ropivacaine with the lower concentration. However, no cells survived after 9 days of bupivacaine exposure (Fig. 2C). With the higher concentration, fibroblasts experienced serious impairment of viability with increasing time of exposure. The most pronounced effect was observed in the bupivacaine group (Fig. 2D).

Correlation analysis revealed a time- and concentration-dependent effect on cell viability for all three LA with the following values: Lidocaine time p=0.019, concentration p< 0.001; bupivacaine time p=0.05, concentration p< 0.001; ropivacaine time p=0.004, concentration p< 0.001. An effect based on the type of stimulation (group 1 or 2) was not observed.

**Proliferation rate**

Thymidine incorporation over time upon incubation with each of the three LA was not changed after exposure to a low concentration of LA (Fig. 3A). With the
concentration of 0.6 mg/ml, again proliferation rate was only decreased in the bupivacaine group (Fig. 3B).

In group 2 with continued incubation with the low concentration of LA, proliferation rate decreased to 80% in the lidocaine and ropivacaine group (Fig. 3C). This effect was more pronounced with the concentration of 0.6 mg/l. Bupivacaine had with both concentrations a more pronounced effect on thymidine incorporation compared to the two other LA (Fig. 3D).

Concentration of the LA has a statistical significant impact on proliferation rate (lidocaine: p< 0.001, bupivacaine: p< 0.001, ropivacaine p=0.001) as well as had the group constellation (lidocaine: p< 0.001, bupivacaine: p=0.009, ropivacaine p=0.001).

**Apoptosis rate**

Apoptosis of fibroblasts was determined upon exposure to lidocaine, bupivacaine and ropivacaine. In group 1, apoptosis rate was diminished for all 3 LA in a similar way for both concentrations (Fig. 4A, B).

With permanent incubation with LA apoptosis rate decreased in a time- and concentration dependent fashion for lidocaine. An increase of the apoptosis rate was observed at 3 days of incubation with the concentration of 0.3 mg/ml (bupivacaine, ropivacaine) and 0.6 mg/ml (ropivacaine) (Fig. 4C and 4D).
Calculations regarding the influence of incubation time, concentration of LA and group arrangement on apoptosis only reached statistical significance for lidocaine with p< 0.001 for concentration and p=0.006 for group.

**Production of ROS**

The possibility of LA-induced expression of ROS was assessed. This could lead to an increased rate of cell death. Production of ROS after short time incubation (5 hours) with lidocaine or bupivacaine was enhanced with increasing concentrations from 0.3 mg/ml, 0.6 mg/ml to 1.3 mg/ml (**Fig. 5A**). For ropivacaine, no production of ROS was observed. After incubation with lidocaine and bupivacaine a decrease of viability was measured, while viability of fibroblasts was not impaired in the presence of ropivacaine (**Fig. 5B**). Viability of fibroblasts negatively correlated with production of ROS (**Fig. 6A, B**). The highest correlation was observed in the bupivacaine group (Pearson correlation of -0.74) (**Tab. 1**), while the correlation value for lidocaine was -0.53. As no ROS were generated in the presence of ropivacaine, correlation coefficient was not relevant for this LA. Caspase-3 activity did not increase upon short term incubation with any of the 3 LA tested (data not shown).
DISCUSSION

This *in vitro* study shows a cytotoxic effect of lidocaine, bupivacaine and ropivacaine on fibroblasts. In group 1 with exposure to local anesthetics for 2 days, followed by an incubation with normal medium, cells were only slightly impaired upon stimulation with lidocaine and ropivacaine. However, bupivacaine had a significant concentration-dependent impact. In group 2, where fibroblasts were permanently exposed to LA, cells were impaired time- and concentration-dependently with all LA. The most negative effect was observed after exposure to bupivacaine. We assume that single injections do not impair the tissue.

Compared to previous investigations the present study is original because:
1. Three different local anesthetics were tested, 2. Experiments were performed in cell culture of human fibroblasts, 3. Different concentrations of LA were evaluated, 4. Different incubation periods were assessed. 5. A possible mechanism of cytotoxicity was tested. This broad and carefully designed approach allows drawing detailed conclusions concerning wound healing in the presence of LA.

Previous experiments have demonstrated a possible impairment of the proliferation rate of cells such as type II pneumocytes or endothelial cells [21,22]. These data reflected rather the impact of local anesthetics in very low concentrations as found in the respiratory or vascular compartment. Our study,
however, focused on concentrations as observed after injection into a wound area. A retrospective analysis of shoulder arthroscopy with intraarticular bolus injection of 0.25% bupivacaine with epinephrine described chondrolysis as a devastating complication [23]. A direct causative correlation between chondrolysis and application of local anesthetics could not be shown, but the authors strongly advised against the use of large doses of intra-articular placement of local anesthetics [23]. The use of topical corneal anesthetics for pain relief after corneal abrasion seems to have limited use as well [24]: Bisla et al. have examined epithelial regeneration in the presence of lidocaine 100 – 1000 µg/ml, and have observed a dose-dependent impairment of epithelial wound healing with concentrations higher than 250 µg/ml, which would also point out the negative effect of LA [24]. All these studies pointed out a potential harm using local anesthetics, but did not specify the exact mechanism of impairment.

Results from our study confirm other experimental findings, demonstrating that bupivacaine has a higher toxicity potential compared to lidocaine and ropivacaine [25,26]. In addition, it also corroborates the results from Sturrock et al., demonstrating a compromised cell survival in hamster lung fibroblasts with an ED50 of 0.06% for bupivacaine compared to 0.09% for lidocaine [27].

The present study suggests that the observed cell death is not mainly due to increased apoptosis rate since activity of caspase-3 was significantly correlated to the amount of living cells. An exception was observed for the short
stimulation period of 3 days for bupivacaine and ropivacaine. Caspase-independent mechanisms of cell death have been described in LA-induced cytotoxicity due to a change in intracellular Ca\(^{2+}\) homeostasis [28-33]. It is postulated in myocytes that LA induce Ca\(^{2+}\) release from the sarcoplasmic reticulum by interaction with ryanodine receptors [34,35]. Other studies suggested an inhibition of Ca\(^{2+}\) reuptake into the sarcoplasmic reticulum, regulated by Ca\(^{2+}\) ATPase activity [35,36]. Beside dysregulated intracellular Ca\(^{2+}\) the involvement of production of ROS is another possible mechanism of LA-induced cell death [37,38]. As described for cocaine, LA and/or its oxidative metabolites might trigger the release of ROS, which have a toxic effect on hepatocytes [37,38]. Other authors claimed a correlation between the dysregulation of mitochondrial Ca\(^{2+}\) and production of ROS, therefore reflecting a possible combination of the two proposed insult pathways [39]. A trigger such as LA or as described by Brookes et al. ischemia/reperfusion might lead to a mitochondrial Ca\(^{2+}\) overload, mitochondrial dysfunction, production of ROS, which exacerbates mitochondrial damage [39].

Cytotoxic effects of LA have been described in several studies, however, without elucidation of the underlying mechanism [40-42]. Park et al. have shown increased ROS concentration correlating with cell death of Schwann cells after incubation with bupivacaine [43]. The authors thereby suggested a ROS-triggered caspase-3-activated apoptosis in neuronal cells. These conclusions
were supported by results from Perez-Castro, which showed a caspase-3/-7 activation in human neuroblastoma cells after 10-minutes incubation with lidocaine, ropivacaine and bupivacaine [44]. In our study, ROS concentrations increased upon exposure to lidocaine and bupivacaine, while at the same time caspase-3 activity was not influenced. Therefore, the pathway of ROS-induced apoptosis in our model with lidocaine and bupivacaine is rather unlikely. Regarding ropivacaine cytotoxicity, the mechanism of ropivacaine–induced cell impairment still remains unclear and needs further evaluation.

If the cytotoxic effect is related to Na channel blocking is rather questionable. LA are well known to interact not only with Na-, but also with K- and Ca channels [45]. In addition, they interfere with Ca uptake and release from the endoplasmic reticulum [46]. Data are also indicating that LA modify N-methyl-D-aspartate (NMDA) receptor function [47]. All these and probably many more unknown interactions lead to a variety of properties of LA such as myotoxicity [46], anti-inflammatory [13], anti-microbial [48], and anti-cancerogenic effects [49], which cannot be attributed to their well-known action on Na channels.

These in vitro data could lead to the assumption that certain local anesthetics might have similar effects in vivo, especially by using perineural continuous application of local anesthetic or wound instillation leading to tissue LA concentrations over several days, a factor which seems to be crucial for cytotoxicity according to our results. Though, we must bear in mind that the in
vitro model is a limitation of this study, using a cell line. Despite the toxic effects observed with these concentrations, further clinical studies are needed to support the present findings in vivo. Furthermore, perineural catheters for regional anesthesia and pain therapy are worldwide used. Prospective studies with large numbers of patient did not report significant clinical neurotoxic-related complications [50-52]. However, wound healing was not assessed in detail. If neuronal cytotoxicity of LA and cytotoxicity of LA on fibroblasts is comparable remains questionable. Neuronal cells do not proliferate, while fibroblasts are highly active during the phase of wound healing. Therefore, no direct conclusions can be drawn from all theses prospective analysis. Additionally, the average duration of the catheter was in these studies shorter, 56 hours and 3.0 to 4.7 days, respectively [50,52].

The real clinical impact of this study warrants further investigations. However, it seems advisable to limit continuous application of LA for no more than 72-92 hours, to use the lowest effective concentration and to choose the least cytotoxic LA. Application of these techniques in patients with reduced tissue healing (e.g. diabetics, smokers) need to be carefully investigated.
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FIGURE LEGEND

Figure 1
A: Change of cell count after an incubation of 2 days with lidocaine, bupivacaine and ropivacaine with a concentration of 0.3 mg/ml (group 1), assessed after 3, 6 and 9 days. Values are mean ± SD.
B: Change of cell count after an incubation of 2 days with lidocaine, bupivacaine and ropivacaine with a concentration of 0.6 mg/ml (group 1), assessed after 3, 6 and 9 days. Values are mean ± SD.
C: Change of cell count after an incubation with lidocaine, bupivacaine and ropivacaine for 3, 6 and 9 days (group 2) with a concentration of 0.3 mg/ml, assessed after 3, 6 and 9 days. Values are mean ± SD.
D: Change of cell count after an incubation with lidocaine, bupivacaine and ropivacaine for 3, 6 and 9 days (group 2) with a concentration of 0.6 mg/ml, assessed after 3, 6 and 9 days. Values are mean ± SD.

Figure 2
A: Change of viability after an incubation of 2 days with lidocaine, bupivacaine and ropivacaine with a concentration of 0.3 mg/ml (group 1), assessed after 3, 6 and 9 days. Values are mean ± SD.
B: Change of viability after an incubation of 2 days with lidocaine, bupivacaine and ropivacaine with a concentration of 0.6 mg/ml (group 1), assessed after 3, 6 and 9 days. Values are mean ± SD.

C: Change of viability after an incubation with lidocaine, bupivacaine and ropivacaine for 3, 6 and 9 days (group 2) with a concentration of 0.3 mg/ml, assessed after 3, 6 and 9 days. Values are mean ± SD.

D: Change of viability after an incubation with lidocaine, bupivacaine and ropivacaine for 3, 6 and 9 days (group 2) with a concentration of 0.6 mg/ml, assessed after 3, 6 and 9 days. Values are mean ± SD.

Figure 3

A: Change of proliferation rate after an incubation of 2 days with lidocaine, bupivacaine and ropivacaine with a concentration of 0.3 mg/ml (group 1), assessed after 3, 6 and 9 days. Values are mean ± SD.

B: Change of proliferation rate after an incubation of 2 days with lidocaine, bupivacaine and ropivacaine with a concentration of 0.6 mg/ml (group 1), assessed after 3, 6 and 9 days. Values are mean ± SD.

C: Change of proliferation rate after an incubation with lidocaine, bupivacaine and ropivacaine for 3, 6 and 9 days (group 2) with a concentration of 0.3 mg/ml, assessed after 3, 6 and 9 days. Values are mean ± SD.
D: Change of proliferation rate after an incubation with lidocaine, bupivacaine and ropivacaine for 3, 6 and 9 days (group 2) with a concentration of 0.6 mg/ml, assessed after 3, 6 and 9 days. Values are mean ± SD.

Figure 4
A: Change of apoptosis rate after an incubation of 2 days with lidocaine, bupivacaine and ropivacaine with a concentration of 0.3 mg/ml (group 1), assessed after 3, 6 and 9 days. Values are mean ± SD.
B: Change of apoptosis rate after an incubation of 2 days with lidocaine, bupivacaine and ropivacaine with a concentration of 0.6 mg/ml (group 1), assessed after 3, 6 and 9 days. Values are mean ± SD.
C: Change of apoptosis rate after an incubation with lidocaine, bupivacaine and ropivacaine for 3, 6 and 9 days (group 2) with a concentration of 0.3 mg/ml, assessed after 3, 6 and 9 days. Values are mean ± SD.
D: Change of apoptosis rate after an incubation with lidocaine, bupivacaine and ropivacaine for 3, 6 and 9 days (group 2) with a concentration of 0.6 mg/ml, assessed after 3, 6 and 9 days. Values are mean ± SD.

Figure 5
A: Production of reactive oxygen species. Fibroblasts were incubated for 5 hours with lidocaine, bupivacaine and ropivacaine in concentrations of 0.3 mg/ml, 0.6
mg/ml and 1.25 mg/ml. Reactive oxygen species were determined. Values are mean ± SD.

B: Determination of viability (control = 100%). Fibroblasts were incubated for 5 hours with lidocaine, bupivacaine and ropivacaine in concentrations of 0.3 mg/ml, 0.6 mg/ml and 1.25 mg/ml. Cell viability was determined. Values are mean ± SD.

**Figure 6**

Correlation analysis. Production of reactive oxygen species was assessed and correlated to cell viability after incubation with lidocaine (A) and bupivacaine (B).