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Research Article

Local Anesthetics Impair *In Vitro* Growth of Human Osteoblasts: A Laboratory Investigation

John M. Bonvini[1], Jose A Aguirre[1], Roman Mantel[2], Claudio Camponovo[3], Barbara Rupnik[1], Andrea Saporito[4] and Alain Borgeat[1*]

[1]Department of Anesthesiology, Balgrist University Hospital Zurich, 8008 Zurich, Switzerland

[2]Institute of Radiology and Nuclear Medicine, Triemli Hospital, 8063 Zurich, Switzerland

[3]Department of Anesthesiology, Clinica Ars Medica, 6929 Gravesano, Switzerland

[4]Department of Anesthesiology, Ospedale Regionale di Bellinzona e Valli, 6500 Bellinzona, Switzerland

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Corresponding author:
Alain Borgeat,
Department of Anesthesiology, Balgrist University Hospital Zurich
Forchstrasse 340, 8008 Zürich, Switzerland, Tel: +41 44 386 31 01; Fax: +41 44 386 11 09;
Email: alain.borgeat@balgrist.ch

ABSTRACT

Introduction: Intraarticular infusion of Local Anesthetics (LAs) is widely used, but the direct effect of LAs on osteoblasts has not been extensively investigated. Therefore, we analyzed the effect of LAs on growing osteoblasts in an *in vitro* model.

Methods: Growing fetal human osteoblasts were either exposed 2 or 9 days to lidocaine, bupivacaine or ropivacaine solutions at concentrations of 1.25, 0.62 and 0.3125 mg/mL. Cell number, viability, proliferation and apoptosis were determined at day 3, 6 and 9. Additionally, the opening of the mitochondrial permeability transition pore was visualized by quenching of calcein fluorescence and intracellular calcium was assessed by Fluorescence Activated Cell Sorting.

Results: LAs led to time-, concentration, exposure type and LA-dependent decrease in cell count ($p<0.001$). Cell viability and proliferation were higher with ropivacaine compared to other LAs ($p<0.001$). Apoptosis was pronounced for lidocaine, where bupivacaine was the stronger inhibitor of bone healing ($p<0.001$). Opening of the mitochondrial permeability transition pore was positively correlated with increasing concentration and exposure time to the LAs.

Conclusion: This *in vitro* model suggests that LAs negatively affect osteoblast growth. Apoptosis is not the only mechanism, collapse of the mitochondrial membrane potential following opening may play a role in cellular toxicity. Subpopulation of surviving cells showing increased viability and proliferation might attenuate deleterious LAs side effects.

INTRODUCTION

Local Anesthetics (LAs) play an important role in perioperative analgesia as the main drugs of regional aesthesia, being used for neuraxial and peripheral nerve block, local infiltration and surgical site analgesia [1]. Aside their well-known analgesic and anti-arrhythmic properties they have a broad spectrum of biological effects [2-5]. The negative side effects of LAs are well-known and documented [6-8] while other effects including bone healing are less clear. The articular cartilage and synovial membrane of the knee in rabbits show histopathological changes after intra-articular injection of bupivacaine [9]. Bupivacaine promotes articular chondrolysis in different *in vitro* [10-12] and animal models after continuous intra-articular shoulder infusion in rabbits [13].

Chondrocytes are of mesenchymal origin similar to osteoblasts, fibroblasts, myocytes and tenocytes. Local anesthetics contribute to reduced their cell viability, and inhibit cell growth of fibroblasts, [14] myocytes, [7] tenocytes, [15] and disc [16] and mesenchymal stem cells [17]. However, the direct effect of lidocaine on osteoblasts, on the contrary to chondrocytes, has not been extensively investigated. Therefore, the aims of this investigation were: first, to assess the effect of lidocaine, bupivacaine and ropivacaine on osteoblasts in an *in vitro* model, focusing on cell count, cell viability, proliferation rate and apoptosis of human osteoblasts, and second, to investigate different mechanisms to explain lidocaine toxic effect in this context. We hypothesized a decreased cell growth of osteoblasts in the presence of LAs.

MATERIAL AND METHODS

Cells

Fetal human osteoblasts (hFOB 1.19, and purchased from LGC Standards GmbH, Wesel, Germany). Cells were grown to confluence in DMEM/F12 (1:1) (Dulbecco's Modified Eagle Medium, F-12 Nutrient Mixture (Ham); Gibco®, Life Technologies Corporation, Paisley, UK), supplemented with 10% fetal bovine serum (FBS, Gibco) and 300 mg/L geneticin (G 418 disulfate salt; Sigma-Aldrich, St. Louis, MO, USA) in an incubator with 5% CO2 and 95% air at 33.5 °C.

Local anesthetics

Lidocaine (Lidocain CO_2 2% Sintetica®, Sintetica AG, Mendrisio, Switzerland), bupivacaine (Bucain®, DeltaSelect GmbH, Munich, Germany), and ropivacaine (Naropin®, Sintetica AG, Mendrisio, Switzerland) were used in these experiments. All used drugs were preservative free.

Quantitative assays

After growing to confluence cells were incubated with different LAs from 1.250 mg/mL (high concentration), to 0.625 mg/mL and 0.3125 mg/mL (low concentration). In group 1, cells were permanently (continuous) exposed to LAs. In group 2, cells were exposed to the LA for 2 days (transient) followed by incubation with normal growth medium. According to each time point control cells were incubated with growth medium. In all three groups medium was changed on days 2,4,6,8. Assays were performed after 3, 6, and 9 days. All experiments were performed in triplicate in the same cell lines and the mean value was considered.

Cell count: Osteoblasts were counted by using fluorescent DNA quantitation assay [18]. Briefly after lysing the cells, Hoechst 33258 (Bisbenzimide Hoechst 33258 Solution: Sigma-Aldrich, St. Louis, MO, USA) which specifically binds to adjacent AT base pairs of DNAs was added. The resulting fluorescence changes was measured efficiently at an excitation wavelength of 360 nm and an emission of 465 nm. All experiments were performed in triplicate in the same cell lines and the mean value was considered.

Cell viability: The tetrazolium bromide (MTT) assay (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, St. Louis, MO, USA) is a well-known and recognized method to measure cell viability *in vitro* [19]. Briefly, in living cells, mitochondrial dehydrogenases catalyze tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide into formazan crystals. This reaction can be quantified by the ELISA-system (test wavelength 570 nm, reference wavelength 620 nm). All experiments were performed in triplicate in the same cell lines and the mean value was considered.

Cell proliferation: Proliferation tests were performed with the help of the colorimetric bromodeoxyuridine (BrdU) assay (Invitrogen Ltd, Paisley, UK) [20,21]. The test analyses the proliferation of cells by utilizing BrdU as an analogue of the DNA nucleotide thymidine, which is incorporated into the synthesized DNA of actively dividing cells. The amount of BrdU incorporation is then quantified with an ELISA-reader at 450 nm (reference wavelength 620 nm). All experiments were performed in triplicate in the same cell lines and the mean value was considered.

Fluometric assays for determination of caspase-3activity: Caspase-3 activity was determined by measuring proteolytic cleavage of the fluorogenic caspase-3 substrate Ac-Asp-Glu-Val-Asp-AMC (Calbiochem, San Diego, CA, United States) [14]. Cells were incubated for 1 h at 37 °C with 2.5 µM substrate. The fluorescence of the cleaved reporter group was assessed at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. Camptothecin was used as positive control (4 µM). All experiments were performed in triplicate in the same cell lines and the mean value was considered.

Semi-Quantitative assays

Wound healing assay: Osteoblasts grown to confluence in Ibidi® Culture Insert 24, TC-treated plates (Ibidi GmbH,

Planegg / Martinsried, Germany) for two days. Local anesthetics where then added at low (0.3125 mg/ml) or high (1.250 mg/ml) concentrations. After 48 h of incubation the well spacer was removed leaving a standardized 500 µm wide gap open. Plates were further incubated with medium and LAs for another 15 h, followed by a staining step using the Diff-Quik staining Set (Cruinn Diagnostics Limited, Dublin, Ireland) to achieve a better contrast. Residual gap opening was then determined by direct microscopy and image analysis using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA). All experiments were performed in triplicate in the same cell lines and the mean value was considered.

Mitochondrial transition pore assay: Osteoblasts were seeded (80,000 cells/ml) on µ-Slide 8 well plates (ibiTreat, Ibidi® GmbH, Planegg / Martinsried, Germany) and cultured for 24 h. Thereafter culture medium was changed to medium containing LAs in a concentration of 0.3125, 0.625 and 1.250 mg/ml. After 6, 24, 48 h cells were labelled using the Image-iT ™ LIVE Mitochondrial Transition Pore Assay Kit (I35103) (Invitrogen Ltd., Paisley, United Kingdom). Cells were then washed twice with modified hank's balanced salt solution (HBSS: HEPES [10 mM], L-glutamine [2 mM], succinate [100 µM]), followed by incubation with 150 µl/well of labeling-solution (Calcein AM [1.25 µM], MitoTracker Red CMXRos [250 nM], Hoechst 33342 dye [1.25 µM], CoCl2 [1.25 mM]) at 33.5 °C for 20 min in the dark. Thereafter, cells were again washed once with modified HBSS. Ionomycin 5 µM was used as positive control and cells in plain HBSS as negative control.

Cells were directly visualized using Leica Widefield DMI 6000 LX microscope (Leica Microsystems AG, Heerbrugg, Switzerland) with appropriate specific software (Leica LAS AF 2.6). A 63x magnification objective (Leica, HCX PLAPO lambda blue) was used and photos were taken at 33.5°C with a 44.1x magnification camera (Hamamatsu-C9100-13-340204, Solothurn, Switzerland). For different stainings, following filters were used: A4 for Hoechst 33342 (ExcitationFilter BP 360/40; DichromaticMirror: 400; SuppressionFilter: BP 470/40); L5 for Calcein (ExcitationFilter BP 480/40; DichromaticMirror: 505; SuppressionFilter: BP 527/30); TX2 for Mitotracker Red CMXRos (ExcitationFilter BP 560/40; DichromaticMirror: 595; SuppressionFilter: BP 645/75). The following camera-settings were used: Hoechst 33342 (Exposure: 7 ms, Gain: 1, EM-Gain: 823), Calcein (Exposure: 15 ms, Gain: 1, EM-Gain: 770) and Mitotracker Red CMXRos (Exposure: 6.1 ms, Gain: 1, EM-Gain: 370). Imaging processing was achieved using Adobe Photoshop. All experiments were performed in triplicate in the same cell lines and the mean value was considered.

Intracellular calcium

Cell alive imaging: Osteoblasts were seeded on µ-Slide 8 well plates (150,000 cells/ml) and cultured for 48 h. Then, the medium was removed and cells were stained with 5 µM Fluo-8am (Fluo-8am, ab142773: Abcam, Cambridge, UK) for 60 min. Cells were then washed three times with phosphate buffered saline (PBS, Gibco) and left in 200 µl culture media completed with 2 mM Ca^{++}.

Cells were recorded at 33.5 °C with 63x magnification and an L5 filter as described above with the following settings: Exposure: 15 ms, Gain: 1, EM-Gain: 324. To achieve a better sensitivity 2x2 binning was used. Images were taken every 0.5 seconds. Baseline fluorescence was determined, then 66.7 µl bupivacaine 0.5%, 66.7 µl ionomycin 20µM (positive control) and 66.7 µl PBS (negative control) were added with an automated sample applicator to 200 µl medium to achieve end concentrations of 1.250 mg/ml (bupivacaine) and 5 µM (ionomycin). Fluorescence measurement was then continued for 5 min. Data analysis was performed using the Leica Application Suite Advanced Fluorescence software (LAS AF Light, version 4.3) (Leica Microsystems AG, Heerbrugg, Switzerland). All experiments were performed in triplicate in the same cell lines and the mean value was considered.

Clinical flow cytometry: Osteoblasts were grown to confluence as described, trypsinized and stained for 90 min with the calcium indicator Fluo-3am (Fluo-3am, 73881-1mg: Sigma-Aldrich, St. Louis, MO, USA) in a concentration of 5 µM at 33.5 °C. After staining, cells were maintained in culture medium completed with 2 mM Ca^{++}. The fluorescence was determined using the blue 488 nm laser and 503 LP mirror with 530/30 filter on a clinical *flow cytometry system* (FACS Canto II™ BD Biosciences: Mississauga, Ontario, Canada).

Baseline fluorescence was obtained for 60 seconds. Immediately afterwards LAs were added to a final concentration of 1.25 mg/ml. PBS served as negative, Ionomycin [5 µm] as positive controls. The measurement was

continued for 30 min, fluorescence medians were visualized using Flowjo 7 (Flowjo LLC, Ashland, OR, USA) All experiments were performed in triplicate in the same cell lines and the mean value was considered.

Statistical analysis

All statistical analyses were performed using R (R Core Team, 2015) with "lme4"-, "pkbrtest", and "multcomp" packages [22,23]. Linear mixed model analyses were used to assess the influence of LAs on the amount of DNA, NADPH-related metabolic activity, proliferation rate, and caspase activity levels. The different LA concentrations, duration of incubation, and type of exposure (continuous vs. transient) were introduced as random intercepts. Osteoblasts without exposure to LAs (control=100%) were used as reference. Post-hoc comparisons on the mixed models were performed using the "glht"-function of the "multcomp" package with the Tukey method. Values are shown as mean ± SD. Considering that the amount of DNA decreased in the culture wells during our experiments, we showed residuals of NADPH-related metabolic activity (Figure 2), proliferation rate (Figure 3), and caspase activity (Figure 4) corrected for the amount of DNA. P<0.05 was considered significant. Residual gap analysis was performed with one-way ANOVA and posthoc Bonferroni correction for multiple comparisons.

RESULTS

Cell count

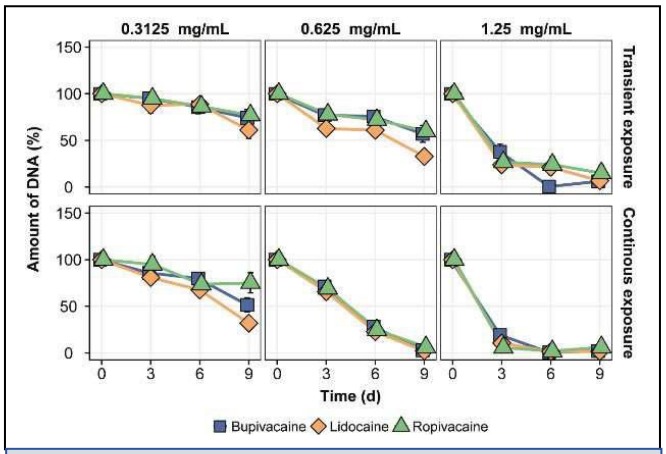

Figure 1a: Amount of DNA in osteoblasts. Osteoblasts without exposure to local anesthetics have been defined as the control group (=100%). Data are expressed as mean ± SD. The concentration of the local anesthetics during exposure is indicated in the titles of the subplots.

Exposure of osteoblasts to LAs significantly reduced the amount of DNA, a surrogate for the cell count (all p<0.001) (Figure 1a). This effect was most pronounced for lidocaine and least for ropivacaine. Higher LA concentrations led to a higher DNA decrease (Figure 1a). Transient compared to continuous exposure to LAs decreased DNA to a lesser extent (p<0.001).

Cell viability

NADPH-related metabolic activity of the surviving cells increased by a mean of 18% to 33% after exposure to all LAs (all p<0.001). This was especially pronounced in cells exposed to ropivacaine. NADPH-related metabolic activity was significantly influenced by LA concentration (p<0.001), as well as by the mode of exposure (transient vs. continuous) (p<0.001). The relationship between NADPH-related metabolic activity and these factors was, however, not linear (Figure 1b).

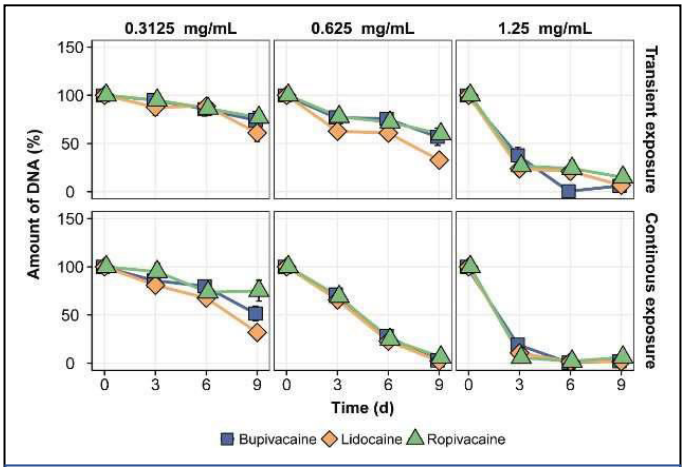

Figure 1b: NADPH-oxidase related metabolic activity in osteoblasts was measured using MTT assays. Considering that the amount of DNA decreased in the culture wells during experiments, residuals corrected for the amount of DNA are shown. Osteoblasts without exposure to local anesthetics have been defined as the control group (=100%). Data are expressed as mean ± SD. The concentration of the local anesthetics during exposure is indicated in the titles of the subplots.

Cell proliferation

The proliferation rate of surviving osteoblasts (assessed by BrdU) was increased in response to LA exposure (all p<0.01). Proliferation rate was most stimulated by ropivacaine with a mean increase of about 40%. This effect was particularly pronounced in cells transiently exposed to high LA concentrations (Figure 1c).

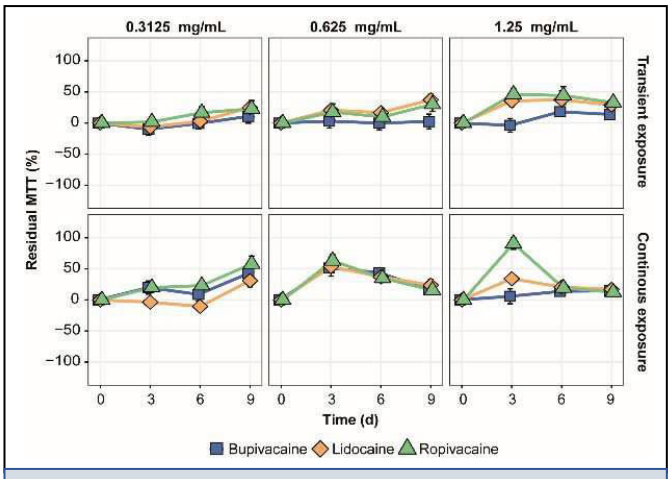

Figure 1c: Proliferation in osteoblasts was measured using BrdU assays. Considering that the amount of DNA decreased in the culture wells during experiments, residuals corrected for the amount of DNA are shown. Osteoblasts without exposure to local anesthetics have been defined as the control group (=100%). Data are expressed as mean ± SD. The concentration of the local anesthetics during exposure is indicated in the titles of the subplots.

Apoptosis

Caspase activity was increased in osteoblasts exposed to lidocaine ($p=0.009$), but not after exposure to bupivacaine or ropivacaine. The duration of incubation had a significant influence on caspase levels ($p=0.001$), however without linear relationship or a clear dose-effect response (Figure 1d). Transient exposure to LA lead to higher caspase activity levels than continuous exposure ($p<0.001$).

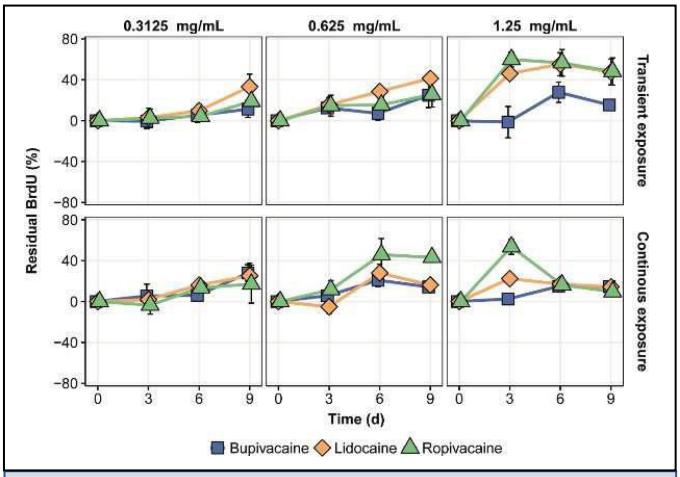

Figure 1d: Apoptosis rate was determined by measuring caspase activity in osteoblasts. Considering that the amount of DNA decreased in the culture wells during experiments, residuals corrected for the amount of DNA are shown. Osteoblasts without exposure to local anesthetics have been defined as the control group (=100%). Data are expressed as mean ± SD. The concentration of the local anesthetics during exposure is indicated in the titles of the subplots.

Wound healing assay

At low concentrations (0.3125 mg/ml) the gap closure assay was uninfluenced by LAs compared to control cells. At a higher concentration such as 1.250 mg/mL gap closure was significantly impaired by all three LAs (all $p<0.001$) with bupivacaine as a stronger inhibitor than ropivacaine ($p<0.05$) and lidocaine ($p<0.001$) (Figure 2a,b).

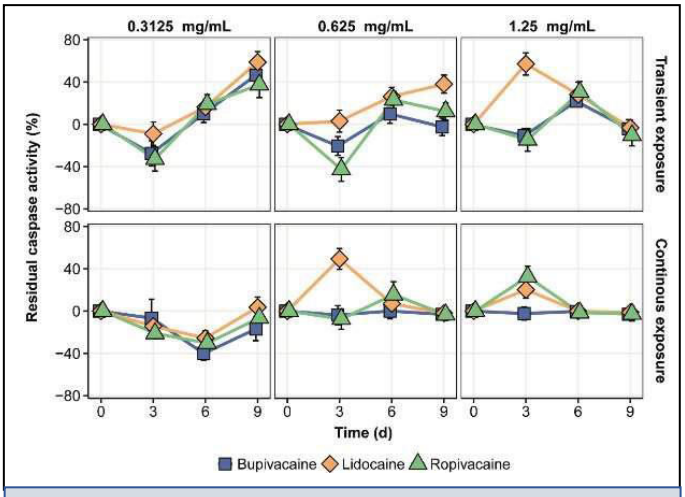

Figure 2a: Wound healing assay: direct microscopy.

Residual gap opening was measured after 15h of incubation with low and high concentrations of LAs

Mitochondrial permeability transition pore (MPTP) activity

Quenching of mitochondrial calcein fluorescence following opening of the MPTP showed a concentration and time dependent pattern (Figure 3 a-c). Increasing the concentration or the exposure time to the different LAs reduced mitochondrial fluorescence, signaling opening of the MPTP and subsequent mitochondrial membrane potential collapse inevitably leading to cell death. Different LAs show different patterns with bupivacaine showing the most deleterious effect even at low concentrations and short exposure times (Figure 3c).

Intracellular calcium

Neither staining with Fluo-8 (Figure 4a) nor fluorescence measured by activated cell sorting (Figure 4b) was influenced by 1.25 mg/ml bupivacaine.

DISCUSSION

This study showed that LAs are in-vitro cytotoxic for growing osteoblast *in vitro* in a concentration, time and exposure dependent manner. Collapse of the mitochondrial potential seemed to be important in this process. The lowest cytotoxicity was observed for ropivacaine. Bone regeneration is a complex

dynamic process, involving recruitment of mesenchymal stem cells and their differentiation into osteoblasts [24]. Infiltration or instillation of LAs after bone surgery improves postoperative analgesia and reduces opioid requirement after several surgical procedures [25-29]. Local anesthetics concentrations were chosen based on previous works [14,30].

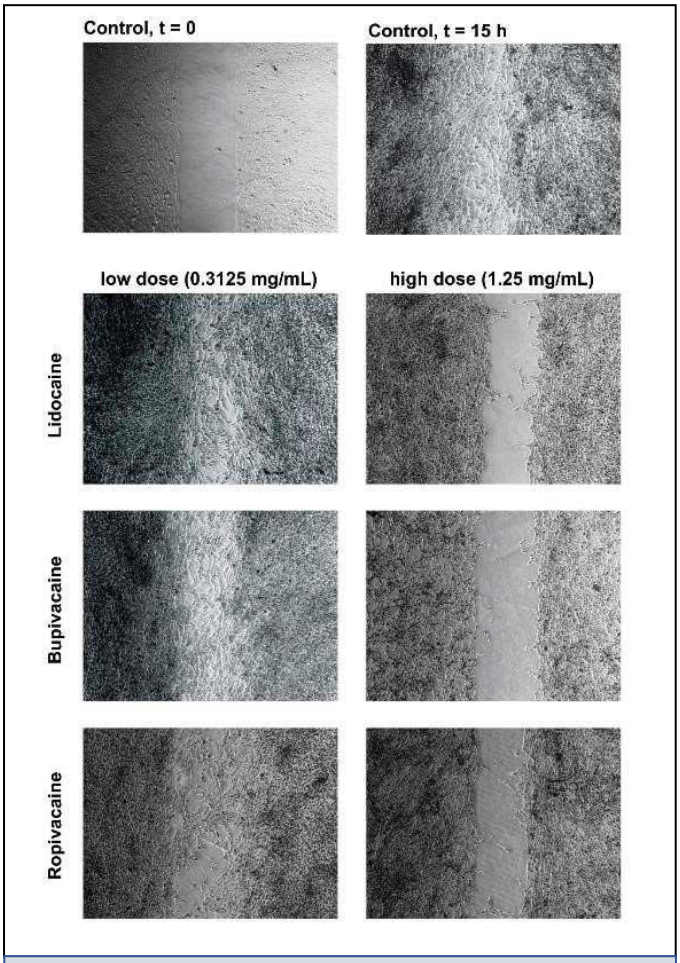

Figure 2b: Wound healing assay: measurements.

Residual gap area after 15 h of exposure to low and high concentrations of LAs.
Results are expressed as mean ± SD
*=p<0.05, **=p<0.001, ns= not significant

We found a significant concentration and exposure dependent decrease in osteoblast DNA after exposure to lidocaine, ropivacaine and bupivacaine. This is in accordance with the results of Pentyala et al., who observed that lidocaine at low concentration (2 mM) enhanced proliferation of osteoblasts, while at high concentration cells did not survive [31]. These results are in line with several other studies on other cell types such as myocytes, chondrocytes, fibroblasts, tenocytes, endothelial cells, keratinocytes, lung fibroblasts [7,9,10,13-15,32] and on mesenchymal stem cells in general [33].

Our results indicated increased cell viability of surviving cells after exposure to all LAs (Figure 1b). This is in contrast with the results of Breu et al. [34] and might be explained by a longer continuous exposure in the present investigation. Cell proliferation of surviving cells was also significantly increased after exposure to LAs ($p<0.01$), especially when using high LA concentrations (1.25 mg/ml) and transient exposure (group 2), suggesting that weaker osteoblasts were first eliminated (Figure 1c). This is the first assay assessing proliferation rate in the surviving cells. Contrasting results, even from our own group [14], might be explained by the fact that the overall decrease in cell count inevitably decreases the overall incorporation of Bromodeoxyuridine (BrdU). Accounting for the quantity of DNA (cell count) renders a more accurate reading of proliferation in surviving cells. Considering the time/dosage/exposure pattern we speculated that cells surviving the first hit (LA exposure) reacted with increased proliferation and higher viability. This positive reaction was blunted by a dose and time dependent LA exposure (Figure 1b,c).

Apoptotic cell death, assessed by caspase 3 activity, seemed to be dependent on the LA used, its concentration, exposure time and pattern (Figure 1d). Our results, especially transient exposure, are in accordance with those of Nakamura et al. [35] demonstrating that prilocaine induced apoptosis in osteoblastic cells in a dose dependent manner. Breu et al [34] also described more necrotic cells upon increasing concentration and time exposure to LA.

The wound healing assay was significantly impaired in the presence of high concentrations of LAs (Figure 2a). This is in accordance with the findings of Lucchinetti et al [33] who could show that cell proliferation impairment already occurred after 3 h of exposure. A better bone healing for all three LAs in the low concentration compared to high dosages was observed (Figure 2b). Abrão et al [36] described a temporary reduction of inflammatory parameters as well as elastin and collagen on day 3 upon a single-shot bupivacaine injection in an animal bone healing model, but this difference disappeared on day 14. Continuous infiltration did influence local inflammatory mediator production after caesarean delivery [37]. The significance of this finding remains unclear.

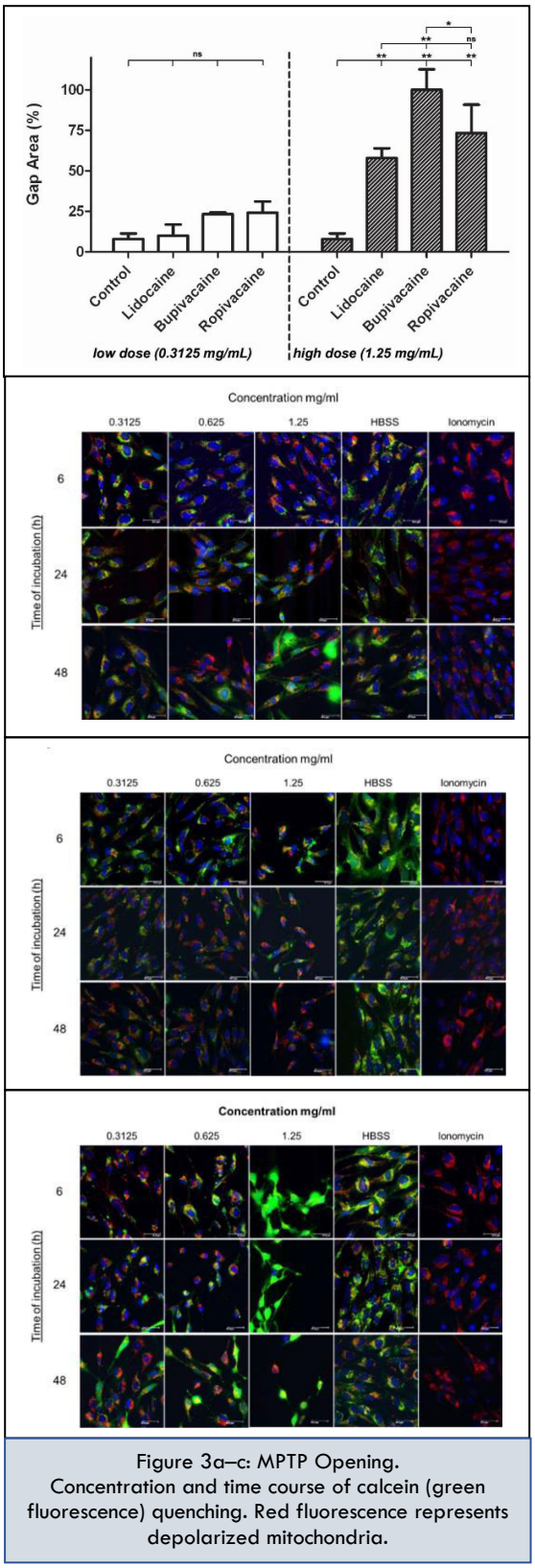

Figure 3a–c: MPTP Opening.
Concentration and time course of calcein (green fluorescence) quenching. Red fluorescence represents depolarized mitochondria.

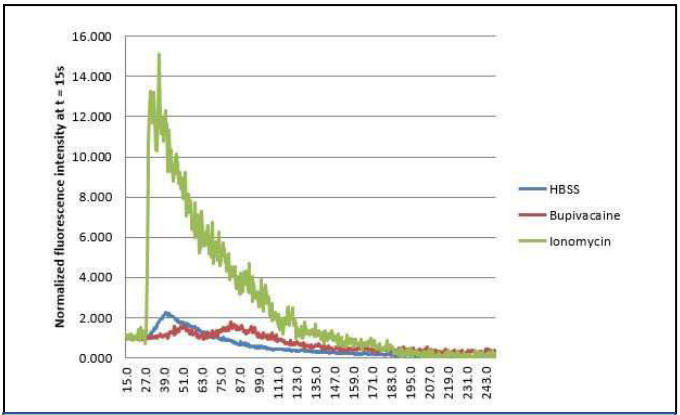

Figure 4a: Fluo-8 (intracellular calcium) fluorescence *in vivo*. Normalized fluorescence after bupivacaine (1.25 mg/ml), HBSS and ionomycin (5 µM).

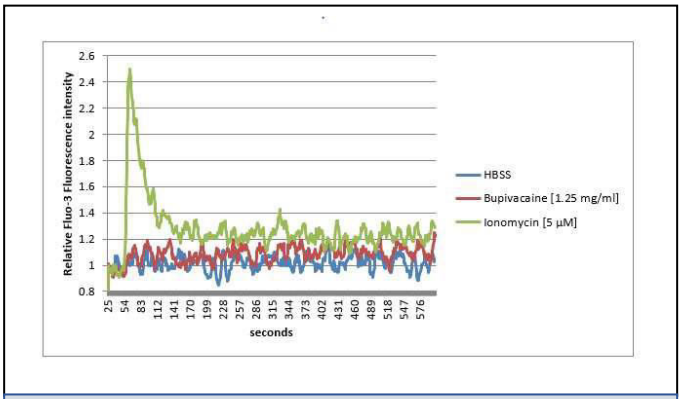

Figure 4b: FACS Fluo-3 (intracellular calcium) fluorescence over 10 min. Normalized fluorescence after bupivacaine (1.25 mg/ml), HBSS and ionomycin (5 µM).

Opening of the MPTP was dose and time dependent, showing the strongest effect for bupivacaine (Figure 3). Our data on intracellular calcium assessed by *in vivo* staining (Figure 4a) or fluorescence activated cell sorting (Figure 4b) did not show an increase in intracellular Ca^{++} concentration. Zadorozhnyi et al. [38] found that the application of richlocaine (1mg/mL) increased the concentration of cytosolic calcium by 105 % in a model of new-born albino rats. The same group reported an increased number of osteoblasts at lower concentration and in presence of hypoxia induced by subtoxic dose of sodium cyanide [38]. This discrepancy could be explained by the drug and model used. Our results also are in some contrast with the findings of Zink et al that could show that bupivacaine and ropivacaine increased cytoplasmic Ca^{++} concentration by enhancing its release from the sarcoplasmic reticulum and inhibiting its re-uptake [39,40]. This difference is most likely explained by different experimental settings using skinned murine myocytes instead of cultivated osteoblasts with unclear Ca^{++} homeostasis. The mitochondrial membrane potential is an essential component in the process of energy storage during oxidative phosphorylation. Together with the proton gradient, it is responsible for the transmembrane potential of hydrogen ions which is necessary for the formation of the ATP [41]. Sustained changes of this mechanism, as observed in this study, may be deleterious and can compromise the cell viability through mitochondrial dysfunction [42]. Accumulation of calcium has been shown to play a major role in the inhibition of mitochondrial ATP production through this effect on the membrane potential [43]. In contrast to this, our study showed that other factors than calcium are involved in this process.

Some limitations have to be addressed in this work. First, we were able to show the detrimental effects of LAs on the cellular level in an *in vitro* model. Whether human osteoblasts *in vivo* are equally susceptible to LA exposure and whether these functional changes result in an inhibited process of bone healing needs to be addressed in further studies. Second, the LAs used were all at the same concentration not considering the different potency of the individual LA. However, the aim of this investigation was primarily not to compare the LAs toxicity, but to show that all the amide-type LA are cytotoxic in a dose- and time dependent manner. Third, all were amide type. Ester type LAs, like chloroprocaine, might present a different profile. Fourth, the majority of the cytotoxicity studies have focused either on different cell types or on mesenchymal stem cells. Fifth, the LAs concentrations were based on plasma blood levels, which may not be similar to those found in clinical practice following local infiltration techniques. Osteoblasts are differentiated cells which might alter specific cellular behavior in the presence of LAs making the comparison with other cell types difficult. Finally, we focused only on Ca^{++} as a trigger for opening the MPTP. Other triggering factors such as oxidative stress remain to be investigated.

CONCLUSIONS

Amide-type LAs showed dose-, time- and exposure pattern-dependent cytotoxic effects on osteoblasts. MPTP opening leading to cell death in this model was not linked to either Ca^{++} or apoptosis. The mechanism responsible for this occurrence needs to be investigated in future studies. Accordingly, limiting exposure time and concentration might

improve cell survival. The clinical use of a continuous infusion and direct application to the wound resulting in high amide-type LA concentrations should be used with caution until the clinical relevance of these findings has been clarified, knowing that in-vitro study does not necessarily translate similarly into clinical practice.

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