Local Anesthetics Have a Major Impact on Viability of Preadipocytes and Their Differentiation into Adipocytes

Maike Keck, M.D.
Maximilian Zeyda, Ph.D.
Karina Gollinger
Sonja Burjak
Lars-Peter Kamolz, Ph.D.
Manfred Frey, Ph.D.
Thomas M. Stulnig, Ph.D.

Vienna, Austria

Background: Autologous fat transplantation is a well-established technique in surgery. Moreover, the use of preadipocytes in soft-tissue engineering is currently being intensely investigated. Current efforts focus on identifying maneuvers that may minimize resorption and provide predictable late results. The aim of this study was to investigate the influence of different local anesthetics frequently used in clinical practice on the viability of preadipocytes and their ability to differentiate into adipocytes.

Methods: Human preadipocytes were isolated from subcutaneous adipose tissue of 15 patients and treated with bupivacaine, mepivacaine, ropivacaine, articaine/epinephrine, and lidocaine for 30 minutes. Viability was determined directly after treatment and during the ensuing cultivation. Differentiation of preadipocytes was determined by expression of the adipocyte marker adiponectin.

Results: Although the immediate effects of mepivacaine and ropivacaine were only moderate, treatment with articaine/epinephrine and lidocaine strongly impaired preadipocyte viability. Cells normally attached to the culture dishes and proliferated irrespective of the previous treatment. During long-term cultivation, articaine/epinephrine-treated cell viability decreased markedly, whereas other local anesthetics had no impact. Despite normal phenotypic appearance of cells treated with bupivacaine, mepivacaine, ropivacaine, and lidocaine, all local anesthetics markedly impaired adipocyte differentiation as determined by adiponectin expression.

Conclusions: The authors' results show that there is a marked influence of local anesthetics not only on the quantity but also on the quality of viable preadipocytes as determined by their ability to differentiate into mature adipocytes. Therefore, these results should be considered in the context of autologous fat transfer and soft-tissue engineering. (*Plast. Reconstr. Surg.* 126: 1500, 2010.)

Autologous fat transplantation with lipoinjection for soft-tissue augmentation is a commonly used surgical technique. Adipose tissue grafts have been used for soft-tissue augmentation in a diverse range of surgical procedures for more than 100 years.[1–4] Current efforts focus on identifying maneuvers that may minimize resorption and provide predictable late results. The most important problem of this method is the yet unpredictable resorption rate of the transplanted tissue. Explantation of adipose tissue as performed during the procedure of autologous fat transfer confers stress to preadipocytes and adipocytes. Disruption of blood supply during fat harvesting may result in hypoxia and apoptosis of the heterogeneous population of cells present in adipose tissue.[5–8] This may explain why a mean resorption rate of only 40 to 50 percent is described, and some studies also report approximately only 10 percent viable cells in the transplant.[9–13]

Preadipocytes play an important role in soft-tissue augmentation,[14] because these adipocyte precursor cells have a higher survival rate under ischemic conditions than mature adipocytes and even have the ability to proliferate and differentiate into

From the Division of Plastic and Reconstructive Surgery, Department of Surgery, and the Clinical Division of Endocrinology and Metabolism, Department of Medicine III, Medical University Vienna.
Received for publication March 1, 2010; accepted May 3, 2010.

DOI: 10.1097/PRS.0b013e3181ef8beb

Disclosure: *The authors have no financial interest to declare in relation to the content of this article.*

mature adipocytes after transplantation.[15–17] Moreover, they are used for in vivo and in vitro tissue engineering.[18] Research in tissue engineering has developed rapidly over the past several years and offers alternative strategies for soft-tissue augmentation, which might be superior to traditional surgical options. The conventional reparative approach is replaced by a regenerative approach.

Because of their key role in autologous fat transfer[19,20] and soft-tissue augmentation, we focused our examinations on preadipocytes to evaluate the impact of local anesthetic treatment on the quantity and quality of preadipocytes yielded from adipose tissue grafts. In a previous work, we have shown that incubation of preadipocytes with lidocaine and articaine plus epinephrine caused a significant reduction of cell viability by immediate necrotic effects.[21] In this study, we chose local anesthetics according to their frequent use in clinical practice and investigated their effects beyond immediate reduction of cell viability. Therefore, we determined the ability of treated preadipocytes to grow and differentiate into adipocytes.

MATERIALS AND METHODS

Subcutaneous adipose tissue was obtained from 15 healthy patients undergoing reductional plastic surgery. This study was approved by the ethics committee of the Medical University of Vienna and the General Hospital Vienna (EK no. 275/2006). All subjects gave written informed consent before taking part in the study.

Isolation of Preadipocytes and Local Anesthetic Treatment

Minced adipose tissue was washed in phosphate-buffered saline and digested with 2 mg/ml collagenase type IV in Hanks' buffered salt solution (both obtained from Sigma Chemical Co., St. Louis, Mo.), 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid supplemented with 50 μg/ml streptomycin, 50 U/ml penicillin (both from Invitrogen, Carlsbad, Calif.), and 25 μg/ml amphotericin B (Sigma) for 1 hour at 37°C with constant shaking. Cells were filtered through a 250-μm nylon filter and centrifuged for 10 minutes at 380 *g*. Red blood cells were lysed in hypotonic buffer, cells were centrifuged for 10 minutes at 380 *g*, and cell pellets were resuspended in Dulbecco's Modified Eagle Medium/Ham's F12 (Sigma), supplemented with 14 mM/liter sodium bicarbonate, 16.5 mM/liter biotin, 8.5 mM/liter pantothenate, 15 mM/liter glucose, 13.5 mM/liter 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (all Sigma), 2 mM/liter L-glutamine, 200 μg/ml kanamycin, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen), and 10% fetal calf serum (HyClone; Thermo Scientific, Logan, Utah). After filtration through a 70-μm nylon mesh, cells were dispensed under constant stirring onto six-well plates and incubated at 37°C in a humidified atmosphere with 5% carbon dioxide for 24 to 48 hours. Subsequently, nonadherent cells were removed by washing with phosphate-buffered saline, adherent cells trypsinized and, after washing/centrifugation, resuspended in 1% bupivacaine, 1% mepivacaine, 0.5% ropivacaine, 4% articaine plus epinephrine 1:100,000, and 2% lidocaine or physiologic saline solution as control for 30 minutes at room temperature. After another washing step, viability was quantified by propidium iodide exclusion using a fluorescence-activated cell sorter (FACSCanto; BD Biosciences, San Jose, Calif.). Viability after local anesthetic treatment was compared with that after saline control treatment (88.2 ± 1.9 percent propidium iodide–negative cells), which was taken as 100 percent.

Adipocyte Differentiation

A specific feature of mature adipocytes is expression of adiponectin (*ADIPOQ* gene).[22] To assess local anesthetic on adipocyte maturation, preadipocytes were reincubated at equal viable cell densities (50,000 cells/cm^2) in the same culture medium as before for 4 days for proliferation. To induce adipocyte differentiation, cells were incubated for 12 days in Dulbecco's Modified Eagle Medium/Ham's F12, 33 μM biotin, 17 μM pantothenate, 1 nM triiodothyronine, 100 nM dexamethasone (all from Sigma), 500 nM human insulin (Roche, Basel, Switzerland), 1 μM rosiglitazone (generously provided by Johnson & Johnson, New Brunswick, N.J.), and, for the first 3 days, 250 μM IBMX (Sigma).

Subsequently, total RNA was prepared from five samples by homogenizing cells in Trizol reagent (Invitrogen) followed by RNA extraction according the manufacturer's instructions. RNA was transcribed into cDNA by Superscript II using random hexamer priming (Invitrogen). Quantitative real-time polymerase chain reaction was performed using an adiponectin-specific commercial Assay-on-Demand (Applied Biosystems, Foster City, Calif.) normalized to ubiquitin C expression as endogenous control (Applied Biosystems). Expression of specific mRNA in each sample was quantitated in duplicates on an ABI PRISM 7000 Cycler (Applied Biosystems) using the $\Delta\Delta C_T$ method.

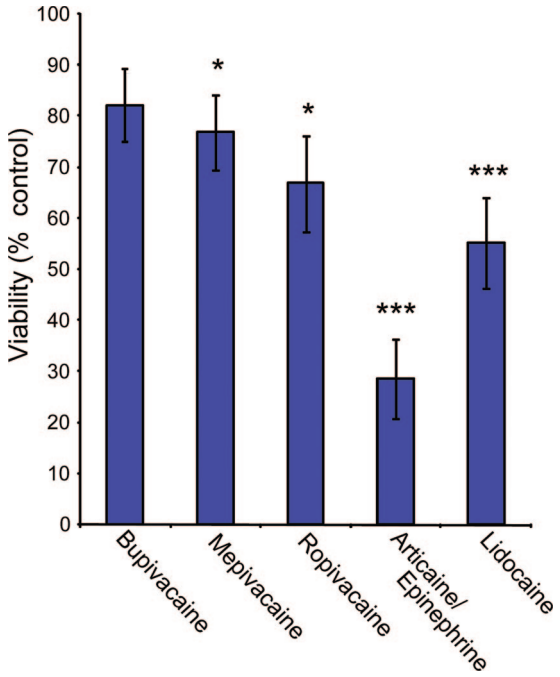

Fig. 1. Viability of preadipocytes after treatment with local anesthetics. Viability was assessed by flow cytometry immediately after treatment with indicated local anesthetics. The diagram shows cell viability in mean ± SEM, with the saline control treatment taken as 100 percent ($n = 15$). Significant differences from control are indicated by *asterisks*: *$p < 0.05$; ***$p < 0.001$.

Statistical Analysis

Data are expressed as means ± SEM. Univariate analysis of variance followed by Dunnett post hoc testing was calculated.

RESULTS

Effects of Local Anesthetic on Preadipocyte Viability

With the exception of bupivacaine, all local anesthetics significantly reduced cell viability, but to differing extents, as determined directly after treatment and compared with saline control treatment (Fig. 1). Although the effects of mepivacaine and ropivacaine were relatively moderate, treatment with articaine/epinephrine and lidocaine left only 32 ± 9 percent and 61 ± 6 percent of preadipocytes viable, respectively. As demonstrated in Figure 2, *above*, showing similar cell densities on day 5 of induced cell differentiation, cells normally attached to the culture dishes and proliferated irrespective of the previous treatment when seeded at identical viable cell numbers per well. Interestingly, during long-term cultivation, articaine/epinephrine-treated cell viability markedly decreased as determined by reduced cell density, small size, and round shape indicating apoptosis (Fig. 2, *below*), whereas bupivacaine, ropivacaine (not shown), mepivacaine, and lidocaine (Fig. 2, *below*) had no impact on viability also

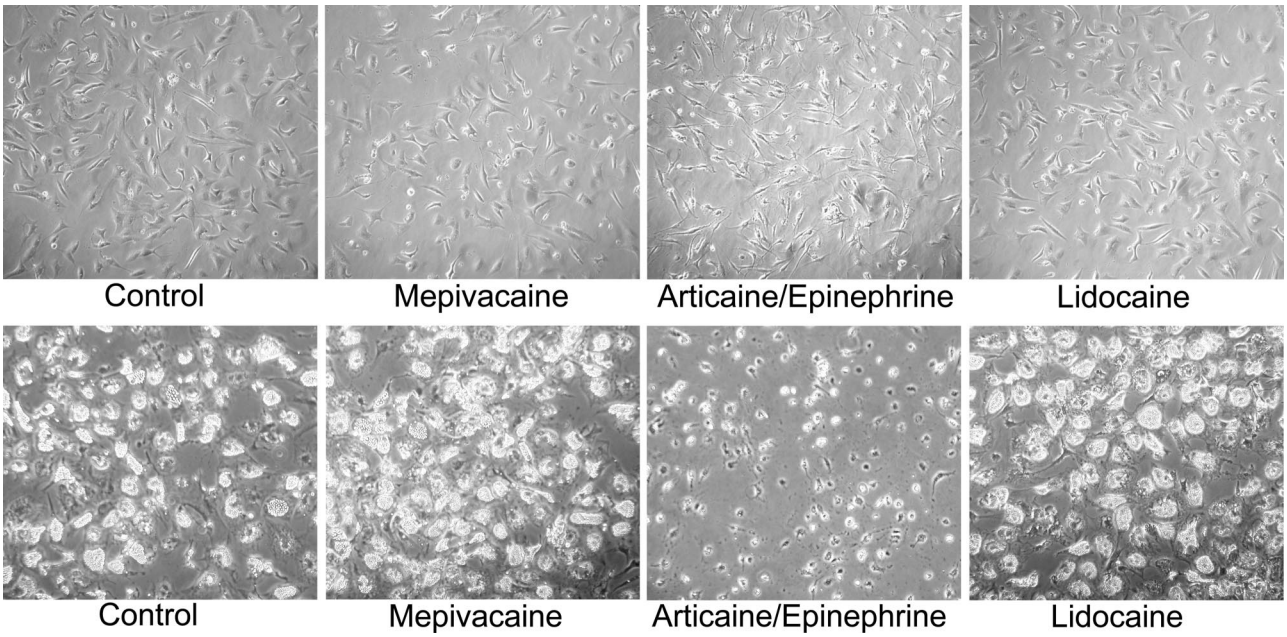

Fig. 2. Effects of local anesthetics on preadipocyte culture and differentiation. After local anesthetic and control (physiologic saline solution) treatment, cells were seeded at the same viable cell densities, cultivated, and induced to differentiate into adipocytes as described in the Materials and Methods section. Images show typical examples of cells treated as indicated on days 5 (*above*) and 12 (*below*) after induction of differentiation are shown.

on day 12 of the differentiation process compared with control treatment.

Effects of Local Anesthetics on Adipocyte Differentiation

In addition to these quantitative analyses, we investigated the effects of local anesthetics on the quality of preadipocytes as assessed by their ability to differentiate into adipocytes. Irrespective of pretreatment, cells started to form lipid droplets on day 5 after induction of differentiation (Fig. 2, *above*). Also, at the end of the differentiation process, no differences in cell size or lipid droplet formation were detectable between control and local anesthetic–treated cells (Fig. 2, *below*; data not shown), with the exception of articaine/epinephrine-treated cells that were markedly smaller while a similar percentage of these cells were loaded with lipid droplets (Fig. 2, *below*). Despite this normal phenotypical appearance of cells treated with bupivacaine, mepivacaine, ropivacaine, and lidocaine, all local anesthetics markedly affected adipocyte differentiation as determined by their expression of the differentiation marker adiponectin (Fig. 3). The strongest impairment of adiponectin expression was observed after lidocaine treatment (Fig. 3).

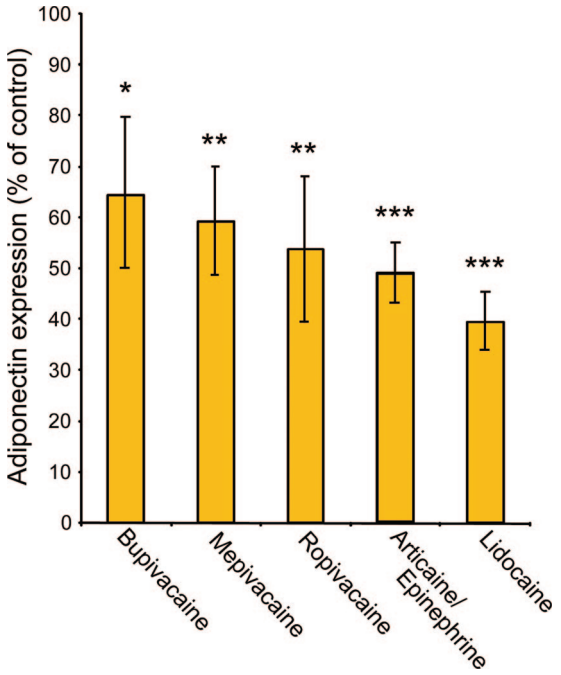

Fig. 3. Adiponectin expression of preadipocytes after treatment with local anesthetics. After treatment with indicated local anesthetics, preadipocytes were differentiated into adipocytes for 12 days. Adiponectin mRNA expression was assessed by quantitative real-time reverse-transcriptase polymerase chain reaction and normalized to ubiquitin C expression. The diagram shows mean ± SEM of adiponectin expression with the saline control treatment taken as 100 percent ($n = 5$). Significant differences from control are indicated by *asterisks*: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

DISCUSSION

A high number of patients suffer from disfiguring loss of soft tissue because of posttraumatic defects, tumor defects, or congenital deformities. Thus, successful long-term treatment of soft-tissue defects remains a desirable goal. One possibility for correcting some of these defects is autologous fat transplantation. A major challenge of this method is predicting the resorption rate of the transplanted tissue to determine the exact amount of fat that needs to be transplanted. In clinical practice, not much is known about the survival rates of the transplanted fat cells. Several studies have been conducted to demonstrate that specimen handling, type of canula, body region aspirated, and anatomical characteristics of patients all affect the degree of resorption of the transplanted fat graft from liposuction aspirate.[5,6,8,23,24] Overall, the fewer traumas that occurred during harvesting, handling, and transplantation, the more consistent the volume that was maintained in the long term.[9] Our results implicate that the use and the choice of local anesthetics has to be considered as well.

Cytotoxicity of local anesthetics on different cell types including neurons has been he subject of several studies.[25–29] Recently, toxic actions of local anesthetics have been shown to correlate with their lipophilic properties.[30,31] However, our results suggest the effects on preadipocyte viability and differentiation to be independent of lipophilic properties, because ropivacaine is less lipophilic than bupivacaine but affects preadipocytes more than bupivacaine. All local anesthetics used in the present study are members of the amide family. Articaine, which in combination with epinephrine shows particularly strong adverse effects in our study, is exceptional because it contains an additional ester group that is metabolized by esterases in blood and tissue,[32] but whether this chemical property is related to the effects shown here remains unclear. Thus, it needs to be elucidated in future studies which of several molecular and cellular mechanisms that have been described for local anesthetics, such as induction of apoptosis,[33,34] induction of calcium flux,[35] activation of signaling kinases, and inhibition of the energy production in the mitochondria,[36–41] are responsible for the effects described here.

Local anesthetic effects on adipose tissue have been the subject of previous studies as well. Shoshani and colleagues investigated the influence of lidocaine and adrenaline on the viability of injected adipose tissue in nude mice. No influence of lidocaine and adrenaline could be detected.[42] The reasons for the apparent difference of the results of this murine study from our in vitro results have to be investigated. However, the present study corroborates earlier data showing that lidocaine and articaine plus epinephrine causes a significant reduction of preadipocyte viability by immediate necrotic effects,[21] extending these observations not only to different local anesthetics but, importantly, to preadipocyte growth and development, putatively important issues that have been neglected so far.

For successful autologous fat transfer, differentiation of the transplanted preadipocytes into adipocytes may be of particular importance.[19,20,43,44] Moreover, preadipocytes have become the focus of research on soft-tissue engineering. Preadipocytes can be expanded in vitro and implanted directly[45] or onto three-dimensional scaffolds.[16–18,46] Therefore, both the quantity of adipocytes surviving the transplantation or isolation and also the ability of preadipocytes to differentiate are of importance when evaluating the procedure of choice to gain adipose tissue with high resorption rate or viable and functional preadipocytes, including the use of local anesthetics.

In a previous study, we showed that 1% lidocaine, 0.5% ropivacaine, and 4% articaine plus epinephrine impaired preadipocyte viability. Here, we extended the study to bupivacaine, mepivacaine, and 2% lidocaine. Furthermore, we investigated the influence on the ability of preadipocytes to differentiate as determined by their ability to express adiponectin, an adipokine with multiple beneficial local and systemic functions.[47]

Most surgeons wait for approximately 30 minutes after tumescence for the infiltration fluid to percolate properly and for its full pharmacologic effects to occur.[48] In our in vitro setting, cells were incubated with the substances for 30 minutes, corresponding approximately to the time of exposure during fat transplantation. The immediate harvest of fat after injection of local anesthetics as practiced by some surgeons could be favorable for limiting exposure time and avoiding the adverse effects as described here. The substances were applied in the same concentrations as those used for infiltration in clinical practice, but resulting in vivo concentrations may be different because of dilution effects which, however, are difficult to estimate. Therefore, confirmation of our results and assessment of their in vivo relevance in clinical studies are topics of great interest.

The results of our in vitro research presented here are intended to improve the overall reliability of the harvest of fat grafts by allowing the surgeon to avoid adverse effects of distinct local anesthetics. Our data indicate that 4% articaine with epinephrine and 2% lidocaine may be inappropriate for these purposes. Furthermore, although the effects of bupivacaine, mepivacaine, and ropivacaine on viability are relatively moderate, the impairment of adipocyte differentiation by treatment with these substances provokes a reconsideration of the use of local anesthesia in general. Reduced levels of adiponectin alone could have serious consequences on the function of reconstituted adipose tissue.[49] However, whether and how reduced expression of adiponectin in normally appearing adipocytes affects autologous fat transfer and soft-tissue engineering remains to be elucidated in conjunction with functional defects of adipocytes derived from anesthetic-treated preadipocytes. In any case, alternatives such as the use of tumescent solution or other anesthetic procedures should be considered.

Altogether, our results show that distinct local anesthetics affect preadipocyte viability to greatly varying degrees, although all local anesthetics investigated here significantly impaired the ability of preadipocytes to differentiate into adipocytes. This could be of major importance in the context of autologous fat transfer and soft-tissue engineering, because the choice of the right local anesthesia for infiltrating the donor site could influence the clinical outcome. We urge that further studies be conducted to evaluate the clinical relevance of our findings.

Maike Keck, M.D.
Department of Plastic and Reconstructive Surgery
Medical University Vienna
Währingergürtel 18–22
1070 Vienna, Austria
maike.keck@meduniwien.ac.at

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