

New Insights into Lidocaine and Adrenaline Effects on Human Adipose Stem Cells

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Received: 22 June 2012 / Accepted: 20 August 2012 / Published online: 13 December 2012
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Abstract

Background Adipose stem cells have gained great interest in plastic and reconstructive surgery with their ability to improve engraftment after fat transfer for soft tissue filling. It is therefore essential to know the effect of the drugs commonly used during the lipoaspiration procedure, such as lidocaine and adrenaline. Indeed, these drugs are infiltrated at the fat donor site for local anesthesia and for reduction of bleeding. This study analyzed the effects of these drugs on the viability of adipose-derived stem cells and on their inflammatory status.

Methods Adipose-derived stem cells from lipoaspirates were grown in culture before being treated with different clinical doses of lidocaine at different times of exposure (1–24 h), and with adrenaline (1 µg/mL). Cytotoxicity was measured by lactate dehydrogenase assay and by flow cytometry with annexin V/propidium iodide staining. In parallel, the secretion of the proinflammatory cytokines tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) was tested by enzyme-linked immunoassay.

Results Lidocaine affected cell viability after 24 h, even when the cells were exposed for only 1 or 2 h. Apoptosis was not involved in lidocaine cytotoxicity. Regarding inflammation, no TNFα was produced, and lidocaine decreased the levels of IL-6 and MCP-1 in a dose-dependent manner. In contrast, adrenaline did not influence cell viability or cytokine secretions.

Conclusions Adipose tissue should be handled appropriately to remove lidocaine and adrenaline, with such procedures as washing and centrifugation. This study provides new insights into the use of lidocaine and adrenaline for fat transfer or stem cell isolation from lipoaspirates.

Level of Evidence II This journal requires that authors assign a level of evidence to each article. For a full description of these Evidence-Based Medicine ratings, please refer to the Table of Contents or the online Instructions to Authors www.springer.com/00266.

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Keywords Adipose stem cells · Adrenaline · Fat grafting · Lidocaine · Lipoaspiration

Introduction

For many years, adipose tissue has been used in autologous fat grafting for soft tissue filling. Fat grafting represents a

method of choice for various clinical applications such as facial rejuvenation and reconstruction, scar correction, breast augmentation and reconstruction, and treatment after radiation therapy [1]. Moreover, adipose tissue provides a great source of stem cells, which have been shown to have powerful potential for regenerative medicine [2, 3].

In recent years, a growing interest in adipose stem cells has emerged in plastic and reconstructive surgery. Indeed, surgeons attach increasing importance to the presence of these stem cells in fat grafts [4–6], and they even endeavor to enrich lipotransfers with adipose stem cells [7–9]. Adipose stem cells are reported to play an important role in the take and survival of fat grafts, allowing neovascularization and modulating the local inflammatory response [10, 11].

For all these reasons, it is important to focus on the way to harvest adipose tissue, and this report focuses particular attention on the procedure of infiltration before lipoaspiration, which conditions the status of stem cells.

Lidocaine and adrenaline are commonly used during lipoaspiration. They are infiltrated in a tumescent solution at the fat donor site to ensure the patient's comfort. As findings have already shown, lidocaine is a good local anesthetic compared with others [12]. Initially, this drug was known to inhibit sodium voltage-dependent channels in nerve cells [13], thus preventing nervous signal conduction and avoiding peri- and postoperative pain for patients undergoing local anesthesia. On the other hand, adrenaline is used to reduce bleeding during lipoaspiration, producing vasoconstriction in subcutaneous fat, which also contributes to prolonging lidocaine action by decreasing its clearance [14].

The effects of the two aforementioned molecules, alone or combined, are not well known, especially concerning adipose stem cells. It has already been reported that lidocaine can impair cell growth and affect cell viability in various cell types, including human fibroblasts [15], chondrocytes [16, 17] and myoblast cells [12]. Only a few studies have tried to highlight the effect of these drugs on the viability of adipose tissue, and the results are controversial [18–21]. Moreover, apoptosis is suggested to be one of the mechanisms underlying lidocaine-induced cytotoxicity [22], but the results also remain controversial [23].

In addition, it seems that inflammation also is able to influence engraftment [24]. Tissue inflammation could be deleterious for the graft, considering that inflamed cells can secrete proinflammatory cytokines that contribute to macrophage infiltration, which could finally lead to graft damage [24].

It is therefore essential to know the effect of these two drugs, widely used by medical practitioners, on the viability and the inflammatory status of stem cells. Thus, this study analyzed the effect of these two drugs on adipose stem cell viability and on their inflammatory status.

Materials and Methods

Origin of Adipose Tissue Samples

Subcutaneous (abdominal, buttocks, hips, and thighs) tissue samples of human white fat were obtained from normal weight or slightly overweight human subjects (females ages 34–59 years, mean age 45 years, with a mean body mass index of 23.6 kg/m²) treated with liposuction for cosmetic reasons while under general anesthesia. Except for oral contraception, the subjects were not receiving treatment with prescribed medication at the time of liposuction.

All the experiments were performed with samples obtained from at least three different patients, and each sample was tested in six replicates. The study was approved by the Ile de la Réunion ethics committee for the protection of individuals participating in biomedical research.

Purification of the Stromal Vascular Fraction and Cell Culture

The total procedure has already been described [25]. Briefly, tissue samples obtained by liposuction were digested for 1 h at 37 °C with collagenase (NB4; Serva, Heidelberg, Germany). Digested tissue then was centrifuged at 900 g for 4 min, and the cell pellet (stromal vascular fraction) was washed three times, then filtered through Steriflip 100 µm (Millipore, Molsheim, France).

After centrifugation, cells were resuspended in M199 medium (PAN Biotech, Aidenbach, Germany). Cell number and viability were assessed by Trypan blue dye exclusion (Trypan solution 0.4 %; Sigma-Aldrich, Saint-Quentin Fallavier, France). The isolated cells had already been analyzed by flow cytometry to assess the phenotypic characteristics of the adipose stem cells [26].

Approximately 300,000 cells/cm² were plated in 24-well plates with M199 containing L-glutamine, glucose (2 g/L), amphotericin B (10 mg/mL), streptomycin (0.4 mg/mL), and penicillin (400 U/mL) (PAN Biotech) with 20 % fetal bovine serum (FBS) (PAN Biotech). The cells then were cultured for proliferation in medium with 10 % FBS at 37 °C in 5 % carbon dioxide before the experiments.

Drug Treatment

Adipose-derived stem cells (ADSCs) were kept only in primary culture without any passage, and when they reached 80 % confluence (after 4 days of culture), the cells were treated with different clinical concentrations of lidocaine together with the same culture medium [1.7 mmol

(0.4 mg/mL), 3.4 mmol (0.8 mg/mL), 6.8 mmol (1.6 mg/mL)] and for different times of exposure (1, 2, 4, 12, and 24 h). The same conditions were used in combination with adrenaline at 1 µg/mL (1:1,000,000), and adrenaline alone also was tested. Nontreated cells were used as the control cells for each time of exposure.

For experiments involving drug washout (after 1 and 2 h of treatment), the medium containing lidocaine, adrenaline, or both was removed, and the cells were rinsed twice, then incubated with fresh culture medium for 24 h. The control cells also were rinsed and incubated with fresh medium. The pH (~8) of each treatment medium had been verified to ensure that the only parameters capable of variation were the concentration of the drugs and the time of treatment. No significant difference in terms of pH could be noted.

Microscopic Observations

The ADSCs were observed under phase contrast microscopy (Nikon Eclipse TS100), and photomicrographs were taken using a digital camera (Nikon Coolpix P5100) to highlight cell density changes (Nikon, Champigny sur Marne, France).

Detection of Cytotoxicity by Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme released in culture media when the cell membrane is damaged and thus is a good indicator of cytotoxicity. The release of LDH in the media was measured using the LDH Cytotoxicity Assay Kit from ScienCell (Cliniscience, Montrouge, France) according to the manufacturer's instructions. Enzymatic measurements of LDH release were spectrophotometrically performed at 490 nm. As a positive control for death, 1 % TritonX-100 (Sigma-Aldrich, Saint-Quentin Fallavier, France) was used. Absorbance values then were correlated with the number of viable cells to quantify cytotoxicity.

Flow Cytometry Analysis with Annexin V and Propidium Iodide Staining

After treatment, cells were collected ($\sim 5 \times 10^5$ cells) and incubated for 15 min with 10 µL of annexin V-FITC in 100 µL of binding buffer from the Annexin V-FITC Apoptosis Detection Kit Plus (BioVision, Cliniscience, Montrouge, France) and for 5 min with 2.5 µg/mL of propidium iodide (Santa Cruz; Cliniscience). Stained cells were analyzed by flow cytometry using FC-500 and MXP software (Beckman Coulter, Villepinte, France). The collected data then were analyzed with FlowJo software, version 7.6.5, for Microsoft (TreeStar, San Carlos, CA).

Interleukin-6, Tumor Necrosis Factor- α , and Monocyte Chemotactic Protein Secretion by Enzyme-Linked Immunoassay

After lidocaine and adrenaline treatments, media from ADSCs were assayed for tumor necrosis factor-alpha (TNF α), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1) content with Ready-SET-Go human enzyme-linked immunoassay (ELISA) kits (eBioscience; Cliniscience) according to the manufacturer's instructions. The ELISA sensitivity was 4 pg/mL for TNF α , 2 pg/mL for IL-6, and 7 pg/mL for MCP-1. The ratio of cytokine secretion was determined by the quantity of cytokine from each condition related to that from negative control subjects.

Statistical Analysis

Data were analyzed via Student's *t* test for two-group comparisons (ELISA) or by an analysis of variance (ANOVA) followed by a Dunnett's *t* test for comparisons of multiple doses or times (JMP; SAS Institute Inc., Cary, NC, USA). Statistical significance was set at a *p* value lower than 0.05. Data are expressed as mean \pm standard error of the mean.

Results

Effect of Lidocaine on Adipose Stem Cell Viability

Lidocaine treatment was followed by LDH assays to measure cytotoxicity. After 1 and 2 h of treatment, lidocaine did not affect cell viability (Fig. 1a). Cytotoxicity started to increase after 4 h of treatment, but the results were not significant (Fig. 1a). At 12 and 24 h of treatment, cytotoxicity increased with the increasing dose of lidocaine, up to 70 % for the highest dose (1.6 mg/mL) at 24 h. Thus, lidocaine induced cytotoxicity in a dose- and time-dependent manner but not for short exposures (<2 h).

This is consistent with microscopic observations showing that cell density changes depend on the dose of lidocaine and the time of exposure. Indeed, whereas control cells grew vigorously, ADSCs rounded with the highest concentration of lidocaine (1.6 mg/mL) and lost their adherence to the plastic plates after an increased time of exposure. Finally, cell density was markedly lower at 24 h of treatment (Fig. 1b).

Effect of Adrenaline on Adipose Stem Cell Viability

At 24 h of lidocaine treatment, cells were co-treated with adrenaline at 1 µg/mL. Figure 2 shows that adrenaline alone was not deleterious for the cells after 24 h of treatment because the level of cytotoxicity was the same as the

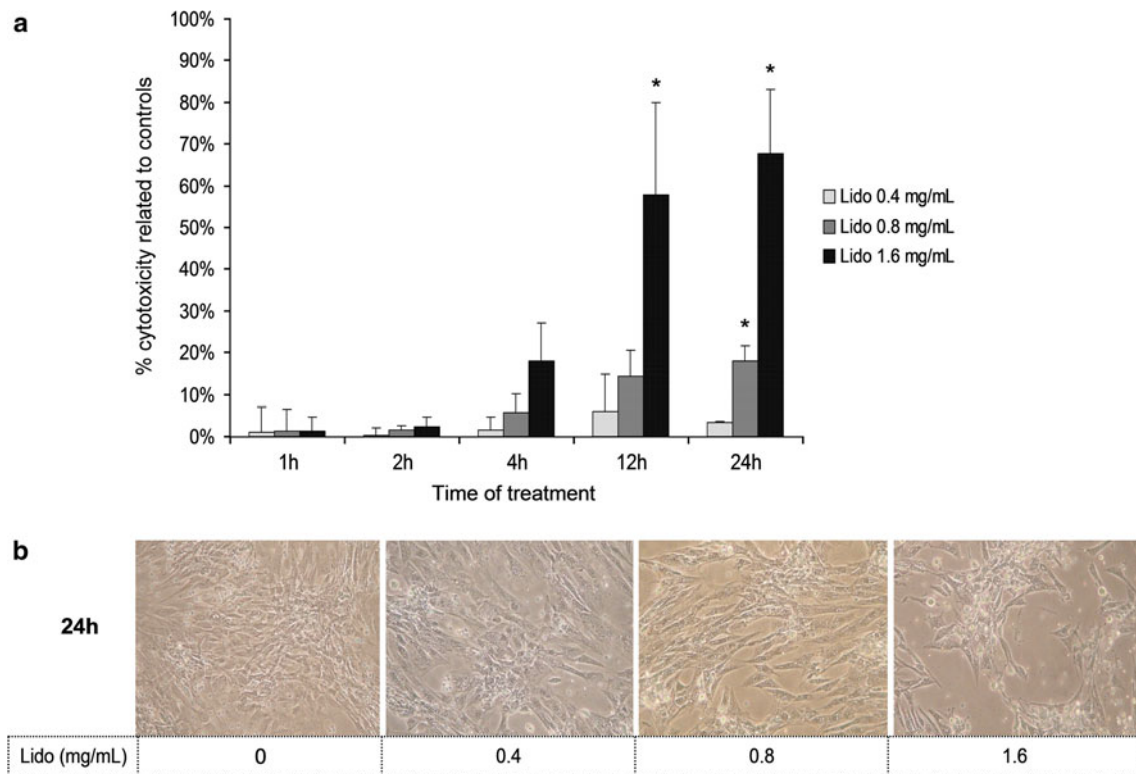


Fig. 1 Cytotoxicity measurement of adipose-derived stem cells (ADSCs) after lidocaine treatment. The ADSCs were treated with different concentrations of lidocaine (Lido) up to 24 h after treatment. **a** Cytotoxicity determined by lactate dehydrogenase (LDH) assay. A blank (no treatment control) and a positive control of death (treatment with 1 % Triton X-100) were included in all the assays. Cell death is

shown relative to that of the untreated sample (0 %) and the positive control condition (set to 100 %). The graph shows the mean \pm standard error of the mean (SEM) of the results from four patients ($n = 6$ for each condition for each patient). * $P < 0.05$ versus control cells. **b** Representative photos of nontreated ADSCs versus ADSCs treated with lidocaine after 24 h (magnification $\times 100$)

basal level related to nontreated cells (data not shown). Moreover, when combined with lidocaine, adrenaline also did not affect cell viability, whatever the lidocaine dose used (Fig. 2). Indeed, the results did not differ from those obtained with lidocaine alone (Fig. 2). Thus, we can conclude that only lidocaine had a harmful effect on ADSCs after extended exposure.

Apoptosis Analysis with Annexin V and Propidium Iodide Staining

We next investigated whether lidocaine could induce apoptosis. Flow cytometry analysis showed very low levels of annexin V positive staining corresponding with basal apoptosis (~ 1 %) (Fig. 3a). No significant differences were shown between lidocaine-treated cells and nontreated cells, even after longer exposure (24 h). Moreover, we could not detect any increase in propidium iodide staining between treated and nontreated cells for the first 2 h after treatment, which confirmed the LDH results.

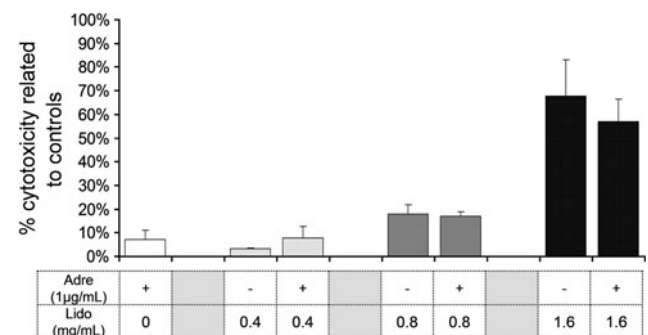


Fig. 2 Cytotoxicity after 24 h of treatments with or without adrenaline. ADSCs were treated for 24 h with adrenaline 1 μ g/mL alone (Adre) or in association with different concentrations of lidocaine (Lido). The previous results of cytotoxicity induced by lidocaine alone after 24 h are reported to compare the conditions Lido and Adre + Lido. A blank (no treatment control) and a positive control of death (treatment with 1 % Triton X-100) were included in all LDH assays. Cell death is shown relative to that of the untreated sample (0 %) and the positive control condition (set to 100 %). The graph shows the mean \pm standard error of the mean (SEM) of the results for three patients ($n = 6$ for each condition for each patient)

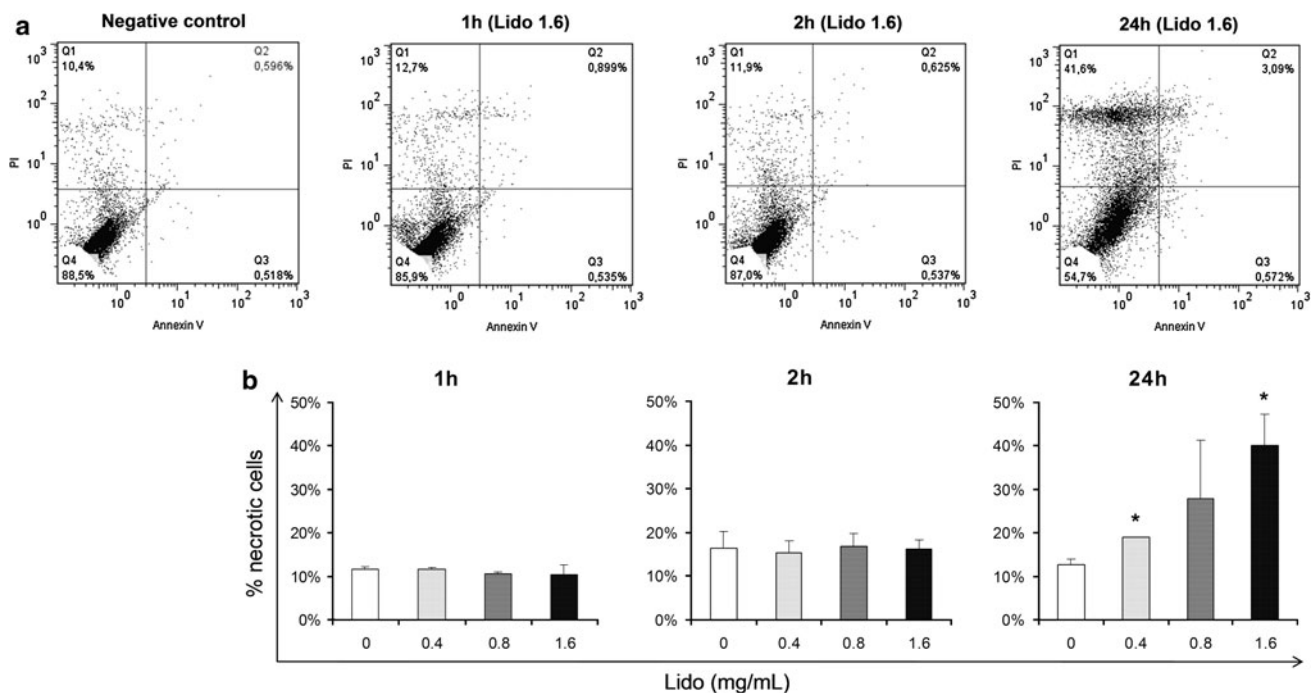


Fig. 3 Annexin V/propidium iodide (PI) staining after treatment. ADSCs were treated with different concentrations of lidocaine (Lido) and adrenaline for 1, 2, and 24 h before being subjected to annexinV/PI staining. **a** Typical dot plots of one in three independent experiments (from 3 patients) showing lidocaine treatment (1.6 mg/mL). Quadrants Q1, Q2, Q3, and Q4 correspond to necrotic cells, late apoptotic cells, early apoptotic cells, and viable cells,

respectively. No statistical significance was detected in the comparison of lidocaine alone and lidocaine with adrenaline. **b** Summary of annexin V-negative and PI-positive staining (necrotic cells) corresponding to each period of lidocaine treatment. The graph shows the mean \pm standard error of the mean (SEM) of the results for three patients. * $P < 0.05$ versus control cells

However, propidium iodide staining increased significantly at 24 h of treatment and with increasing doses of lidocaine (Fig. 3). Thus, lidocaine could induce necrosis but no early or late apoptosis. Treatments with adrenaline alone or combined with lidocaine led to the same results: Adrenaline did not influence annexin V or propidium iodide staining (data not shown).

Time of Cytotoxicity Induction after Treatments

Figures 1 and 3 showed no cytotoxic effect of lidocaine with short exposure (1–2 h). Nevertheless, cytotoxicity might be delayed by complex mechanisms that occur within the cells, which could be shown a long time after drug exposure. To be sure that lidocaine or adrenaline did not induce a delayed cytotoxic effect, cells were treated for 1 or 2 h, then washed and cultured for an additional period of 24 h before being tested for cytotoxicity.

As shown in Fig. 4a, cell death did not exceed 3 %, except at the highest lidocaine concentration, which led to about 10 % cytotoxicity. These data were much lower compared with 24-h treatment results but were higher than the results obtained directly after 1–2 h of exposure.

In the same way, we could not detect any cytotoxic effect of adrenaline (<5 %), even when it was combined

with the highest dose of lidocaine. Indeed, we could reach the same rate of cell death (8–10 %) as with lidocaine alone (Fig. 4b). Finally, washout experiments demonstrated that lidocaine could affect ADSCs even after 1- or 2-h treatments, especially at a high concentration.

Study of Inflammation

To determine whether lidocaine and adrenaline could promote inflammation, the proinflammatory cytokines (IL-6, TNF α , and MCP-1) were quantified in the media by ELISA after 1–2 h of treatment, times for which no cytotoxicity were measured. Regarding TNF α , whatever the dose of lidocaine, whether combined with adrenaline or not, and whatever the time of exposure, we could not detect any secretion in either treated or nontreated cells (data not shown).

Concerning IL-6, levels of secretion decreased in cells treated with lidocaine (Fig. 5a), from 20 % with the lowest dose of lidocaine (0.4 mg/mL) to as much as 30 % with the highest dose (1.6 mg/mL) after 1 h, and even more after 2 h, with the decrease reaching 50 % compared with control cells. When combined with adrenaline, the lidocaine had the same effect. This decrease did not seem to persist after longer exposure (24 h, data not shown).

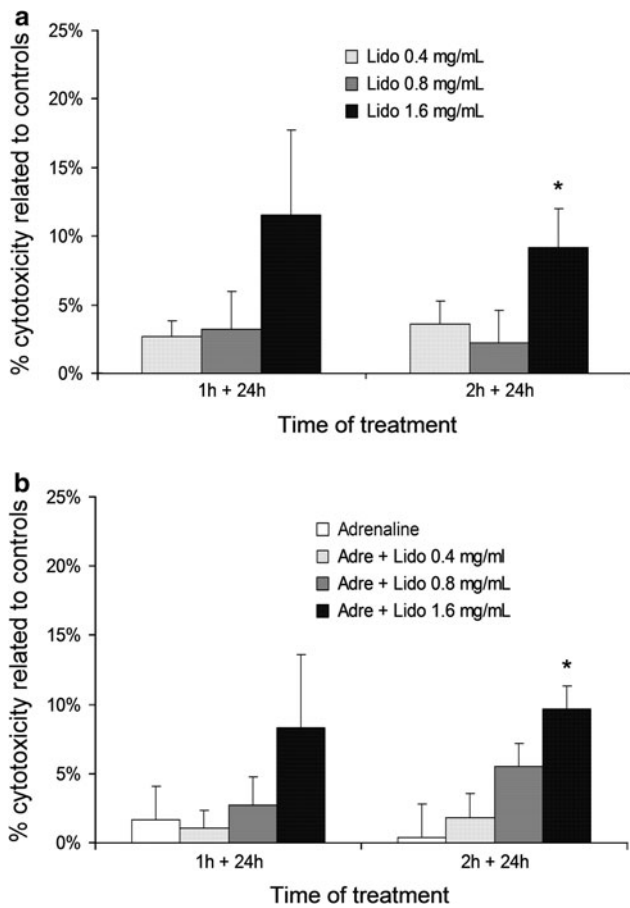


Fig. 4 Washout experiments after treatments. Cytotoxicity was determined by LDH assay. **a** Washout experiment after lidocaine treatment. The cells were washed after treatment for 1 and 2 h with different concentrations of lidocaine (0.4, 0.8, and 1.6 mg/mL). **b** Washout experiment after adrenaline treatments. The cells were washed after treatment with adrenaline 1 µg/mL alone or in association with the same different concentrations of lidocaine. The times 1 + 24 h and 2 + 24 h represent the washout experiments after 1- and 2-h treatments. The graph shows the mean \pm standard error of the mean (SEM) of the results for three patients ($n = 6$ for each condition for each patient). * $P < 0.05$ versus control cells

We obtained the same pattern with MCP-1 levels, as shown in Fig. 5b, with significantly decreased secretion in lidocaine-treated cells (from 20 % for the lowest dose to 40 % for the highest dose, for both 1 and 2 h of treatment), whereas adrenaline still did not have any influence on the secretion of this cytokine. From this result, we conclude that lidocaine was able to decrease IL-6 and MCP-1 secretion in a dose-dependent manner, whereas adrenaline did not affect this effect.

Discussion

Only a few studies exist on the conditions of adipose tissue collection, and it is important to know the best way to proceed when adipose tissue is aimed at reconstructive or

regenerative medicine to preserve adipose stem cells and thus to improve the viability and the quality of the grafts. The tumescent technique for lipoaspiration is widely used, but the concentration of the drugs infiltrated at the fat donor site may vary between surgeons.

This study confirmed that lidocaine is an attractive local anesthetic for the lipoaspiration procedure before autologous fat grafting or stem cell therapy [27]. Indeed, consistent with our previous results [27], lidocaine caused cytotoxicity but not apoptosis in a time- and dose-dependent manner but did not directly affect ADSC viability at low concentrations or for short exposures (<2 h), even when combined with 1 µg/mL of adrenaline.

Studies by Keck et al. [20, 21] drew the same conclusion regarding lidocaine-induced cytotoxicity, and we also reinforce their idea because lidocaine does not convincingly seem to induce apoptosis in adipose stem cells [23]. However, these authors found that lidocaine influenced cell death in preadipocytes after only 30 min of treatment. The difference with our results regarding the time of exposure may reside in the protocol of the treatment, which included much higher drug concentrations. Indeed, compared with those we used in our study, they used doses 6-fold higher (comparing their lidocaine 1 % with our 1.6 mg/mL) to 50-fold higher (comparing their lidocaine 2 % with our 0.4 mg/mL).

Another major issue raised in this study concerns the steps of washing adipose tissue before its use. Washing seems to be good for improving cell survival, but even with just 1 h of lidocaine exposure, we could see deleterious effects after 24 h, which is not insignificant. The washing step greatly reduced the rate of death, but we cannot exclude the delayed effect with just 1 h of exposure. It is therefore better to minimize the contact of the cells with drugs. This result supports earlier findings, including the study of Moore et al. [28], in which lidocaine could alter adipocyte metabolism and growth in isolated adipocytes, whereas the cells finally were able to regain their function after being washed. Moreover, knowing that the grafts can be injected into a recipient site that also is under local anesthesia, it should be better to remove most of the residual drugs present in lipoaspirates to avoid drug accumulation inside the grafts as well as deleterious effects.

Thus, in the context of autologous fat grafting, one simple method for rescuing adipose stem cells from cell death could be washing. This step also could include steps of centrifugation to improve drug removal from the tissue. On the contrary, we have already demonstrated in an other study that decantation leads to the worst results in terms of grafting because tissue decantation may increase the time of exposure to drugs present in lipoaspirates [29].

Shoshani et al. [19] showed similar results when they centrifuged tissue before injection in nude mice. Indeed,

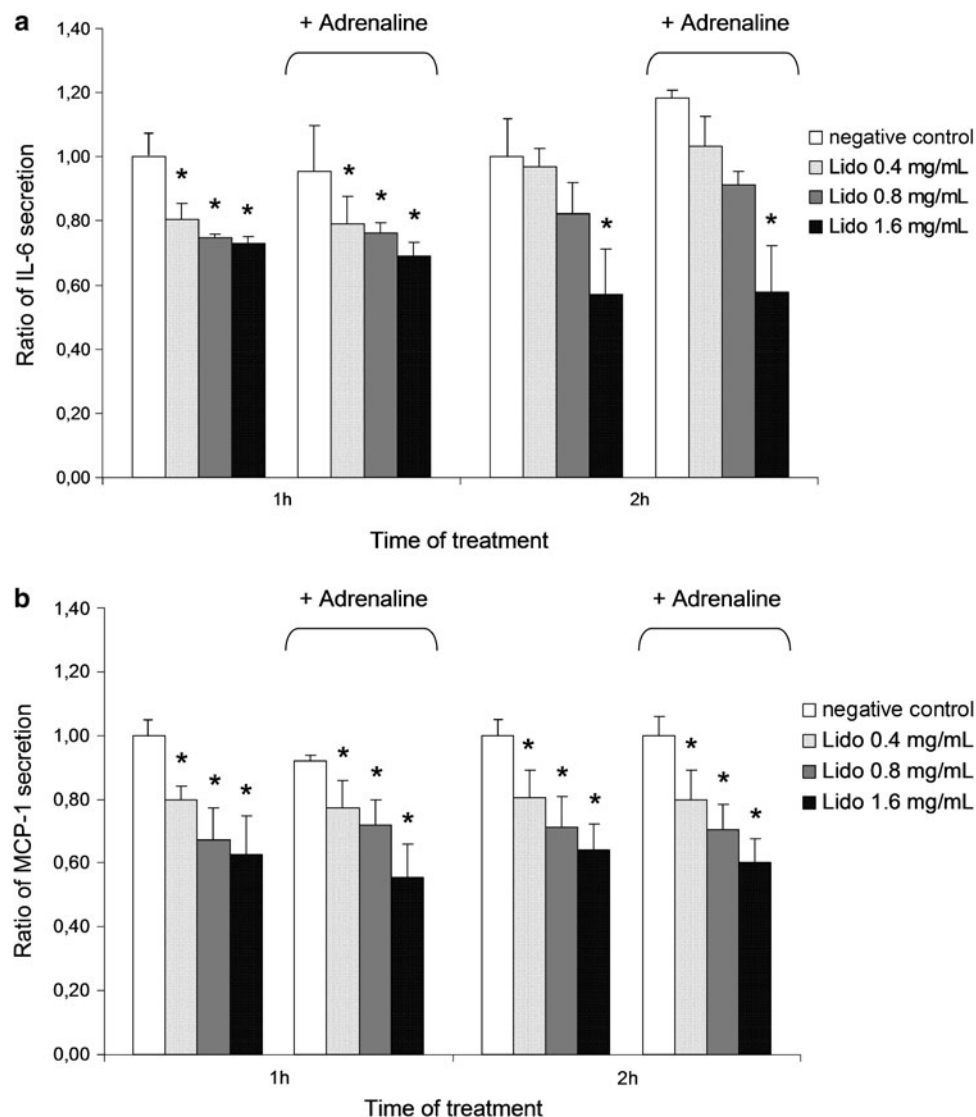


Fig. 5 Ratio of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) secretion by enzyme-linked assay (ELISA). The ELISA assays were performed to quantify **a** IL-6 and **b** MCP-1 in culture media after 1 and 2 h of lidocaine and adrenaline treatment. A blank (no treatment control) was included in all the assays. The ratio

of IL-6 or MCP-1 secretion was determined by the quantity of IL-6 or MCP-1 from each condition related to that from negative control conditions. The graphs show the mean \pm standard error of the mean (SEM) of the results for three patients ($n = 6$ for each condition for each patient). * $P < 0.05$ versus control cells

they concluded that lidocaine and adrenaline did not have any influence on the take of fat grafts or adipocyte viability after lipotransfer in nude mice.

In accordance with our study, this centrifugation step may be essential to preserve the cells. Moreover, it already has been reported that appropriate centrifugation could enhance graft take by concentrating adipose stem cells in the adipose portion [30], and the widely used Coleman technique agrees with this idea [31].

Regarding inflammation, as previously described [32–34], lidocaine seems to have antiinflammatory properties. Indeed, we did not observe any secretion of $\text{TNF}\alpha$, so lidocaine and adrenaline did not induce inflammation with the concentrations tested.

Our data also demonstrated that lidocaine could significantly decrease IL-6 and MCP-1 secretion in the first 2 h of treatment. Because we did not detect any cytotoxic effect during these 2 h, it seems that lidocaine has a beneficial effect on the inflammatory process in ADSCs. Moreover, MCP-1 is involved in the recruitment and activation of peripheral blood monocytes, a key event during inflammation. This decrease of MCP-1 production may prevent the attraction of inflammatory cells to the inflamed area and thus may prevent tissue damage.

These findings should be considered. They are very interesting both for the patient, who can have fewer post-operative constraints such as edema, and for adipose tissue itself, which is in a way “maltreated” during lipoaspiration,

being easily inflamed. However, caution in the use of lidocaine is required because its antiinflammatory property is susceptible to increased infection in the case of gross bacterial contamination [32].

Concerning adrenaline, we have clearly shown that adrenaline has no effect at the concentration of 1 µg/mL. Only lidocaine is involved in cytotoxicity and in inflammatory cytokine levels.

Finally, we must take into account all the aspects of this study to find a good compromise for the use of these drugs during infiltration performed before lipoaspiration. On one hand, several studies, including ours, show that lidocaine seems to exert antiinflammatory action on ADSCs with increasing doses, but on the other hand, cytotoxicity also increased gradually in a dose- and time-dependent manner. We therefore recommend that the lidocaine dose of 0.8 mg/mL not be exceeded. It also might be possible to use a lower dose of lidocaine for tumescent lipoaspiration because it is reported that the use of a high dose is unnecessary [35]. Moreover, to ensure the best efficiency, the time between tissue sampling and tissue or cell preparation should be minimized because we noted a delayed deleterious effect even after 1 h of exposure.

In conclusion, according to our results and our previous findings [29], it seems interesting to wash and centrifuge adipose tissue before fat injection or stem cell extraction. Obviously, these findings have implications for adipose stem cell harvest and also should be applied in regenerative medicine when adipose stem cells from lipoaspirates are used for tissue repair.

Acknowledgments This work has been financially supported by the Ministère de l'Enseignement Supérieur et de la Recherche, by the Association Nationale de la Recherche et de la Technologie, and by the University of Reunion Island. We are grateful to plastic surgeons Delarue P. and Hulard O. who took part in this study and allowed the collection of subcutaneous adipose tissue samples, to the group Clinifutur, to the entire team of the Biochemistry Department of the Félix Guyon Hospital, Reunion island, and to the French Ministry of National Education and Research and the Association Nationale de la Recherche et de la Technologie (ANRT) for their financial support. Finally, we thank all the patients who consented to the collection of tissue samples and thus made this study possible.

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