Effect of Washes and Centrifugation on the Efficacy of Lipofilling With or Without Local Anesthetic

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Background: Among the different parameters that influence fat graft survival and lipofilling success, the use of local anesthetic and the way to process the fat before injection have often been pointed out. Likewise, we evaluated different techniques for processing adipose tissue before its injection and analyzed the quality of the grafts.
Methods: Adipose tissue from the same patient was gently harvested from one side of the abdomen after infiltration of a tumescent solution without lidocaine and from the other side of the abdomen using a tumescent solution containing lidocaine 2%. Harvested tissue was prepared with different protocols, from simple decantation to advanced protocols including single or multiple washes and centrifugations. Each type of processed adipose tissue was then injected subcutaneously into immunodeficient mice. Adipose grafts were collected after 1 month and analyzed by histology with a detailed scoring method.
Results: After lidocaine use, decantation protocol led to adipose grafts of poor quality with high resorption rate and oil vacuole formation. Larger grafts were obtained after centrifugation, but centrifugation alone resulted in increased fibrosis and necrosis, with or without the use of lidocaine. Finally, multiple washes and centrifugations greatly improved the quality of the lipografts.
Conclusions: Centrifugation alone is not sufficient and must be associated with multiple washes to improve graft quality. This article aims to provide further evidence of lidocaine and washing/centrifugation effects in fat grafting to provide easy tips aimed at ensuring graft efficiency with a long-term clinical outcome. *(Plast Reconstr Surg Glob Open 2015;3:e496; doi: 10.1097/GOX.0000000000000465; Published online 27 August 2015.)*

Autologous fat grafting (AFG) is now a widely used technique for soft-tissue filling, providing a natural outcome and gaining increasing popularity. Unfortunately, this kind of procedure is still not perfect in terms of engraftment efficiency, and no standardized protocol yet exists that enables an efficient prediction of clinical outcome. Techniques differ between surgeons who are still striving for the best clinical outcome and patient satisfaction. Despite ongoing research and a real will to refine the technique, the major disadvantage of lipofilling persists, namely fat graft resorption, leading to variable and unpredictable results.[1,2]

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Numerous parameters of the lipofilling procedure have been described by many teams as being critical to graft survival.[3–6] Some intervene before the procedure, such as infiltration with the influence, in particular, of local anesthetics,[7–9] others during the harvesting phase, such as aspiration pressure[10–13] or the size of the cannula,[14,15] and others after liposuction, such as the washing or centrifugation of adipose tissue.[10,16–20]

Of all the parameters identified, our team has recently shown that two of them could have a major impact on cell survival. Indeed, we initially demonstrated in vitro that conventional clinical concentrations of lidocaine were toxic to adipose-derived stem cells,[21] but we also showed in vivo that the speed and duration of centrifugation carried out to wash and compact the tissue could strongly influence graft survival and the amount of oil present therein.[22] However, no study has so far shown, in vivo, the influence of repeated washings and centrifugations on graft survival, with or without lidocaine. It is this work that has been conducted to compare the different adipose tissue preparation techniques following liposuction. The effects of a simple decantation, single or multiple washes followed by centrifugation, in the presence or absence of lidocaine during harvesting, were analyzed to investigate graft survival in vivo in immunodeficient mice. The effectiveness of the graft was evaluated through a detailed analysis via scoring of several parameters including the size of the grafts, the amount of oil, and the presence of fibrosis.

Finally, according to the results of our study, we propose 2 protocols that use lidocaine or not to obtain the best results during lipofilling.

MATERIALS AND METHODS

Patients

Subcutaneous tissue samples of human white fat were obtained from 2 normal weight or slightly overweight women (age 31 and 57, with body mass index of 26.4 kg/m² and 23.6 kg/m², respectively) undergoing liposuction for cosmetic reasons. Except for oral contraception, the subjects were not receiving prescribed medication at the time of liposuction.

Adipose Tissue Harvesting

Surgery was performed under general anesthesia to allow sampling on one side of the abdomen without local anesthetics. Before aspiration, infiltration was performed with 2 different tumescent solutions: one side of the abdomen was first infiltrated with a solution containing adrenalin 1 mg/L for 1 L of Ringer's lactate (RL), but without lidocaine, and then the other side was infiltrated with 0.8 mg/mL lidocaine (40 mL lidocaine 2% and adrenalin 1 mg/L for 1 L of RL) (Fig. 1).

Adipose tissue was harvested by manual syringe liposuction with a 2-mm-diameter 8-hole aspiration cannula (1.2-mm hole diameter; Adipsculpt, France). Aspiration was performed with 10-mL Luer-lock syringes by creating a light negative pressure by slowly withdrawing the plunger in a gradual manner in order not to damage adipose tissue.

Adipose Tissue Processing

The whole procedure (from infiltration and liposuction to manipulation of fat) was conducted to leave adipose tissue in contact with the infiltrated solution for 2 hours (with or without lidocaine). Then, the 10-mL syringes were placed vertically and left to settle for 5 minutes to remove the infiltration mixture. For the decantation protocol: syringes were allowed to settle for a further 5 minutes. For decantation with washing, 25 mL of the harvested fat tissue was transferred into a 50-mL tube and then rinsed with 25 mL of RL. For the centrifugation protocol, tissue was centrifuged for 1 minute at 400*g*. For the washing and soft centrifugation protocol, 25 mL of the harvested fat tissue was transferred into a 50-mL tube and then rinsed and centrifuged with 25 mL of RL (400*g* for 1 minute). For multiple washings and centrifugations, 25 mL of the harvested fat tissue was rinsed and centrifuged with 25 mL of RL (100*g* for 1 second), this step was repeated a second time, followed by a last wash with a soft centrifugation (400*g* for 1 minute). Centrifugation speed and duration were fixed according to previous results obtained by our team.[22] The different protocols used for fat processing are summarized in Table 1.

Fat Grafting in Severe Combined Immunodeficiency Beige Mice

All experiments were conducted at the CYROI animal laboratory, Reunion Island (approval no. 974001 issued by the Veterinary Services of Reunion Island), and approved by the CYROI Ethical Committee for Animal Welfare. Severe combined immunodeficiency Beige mice (Charles River Laboratories, Lyon, France) were used for in vivo fat grafting experiments. For both protocols tested, the subnatant liquid and the supernatant oil were removed; the tissue was then transferred into 1-mL syringes and injected into 8-week-old female severe combined immunodeficiency Beige mice. For each adipose tissue sample, a total of 24 mice (4 per condition) were injected subcutaneously in the dorsolateral region with 1 mL per side (2 mL of tissue per mouse) with a 1.6-mm-diameter cannula, for a total of 48 lipografts (12 conditions tested). The whole experiment was repeated twice, with adipose tissue from 2 different patients.

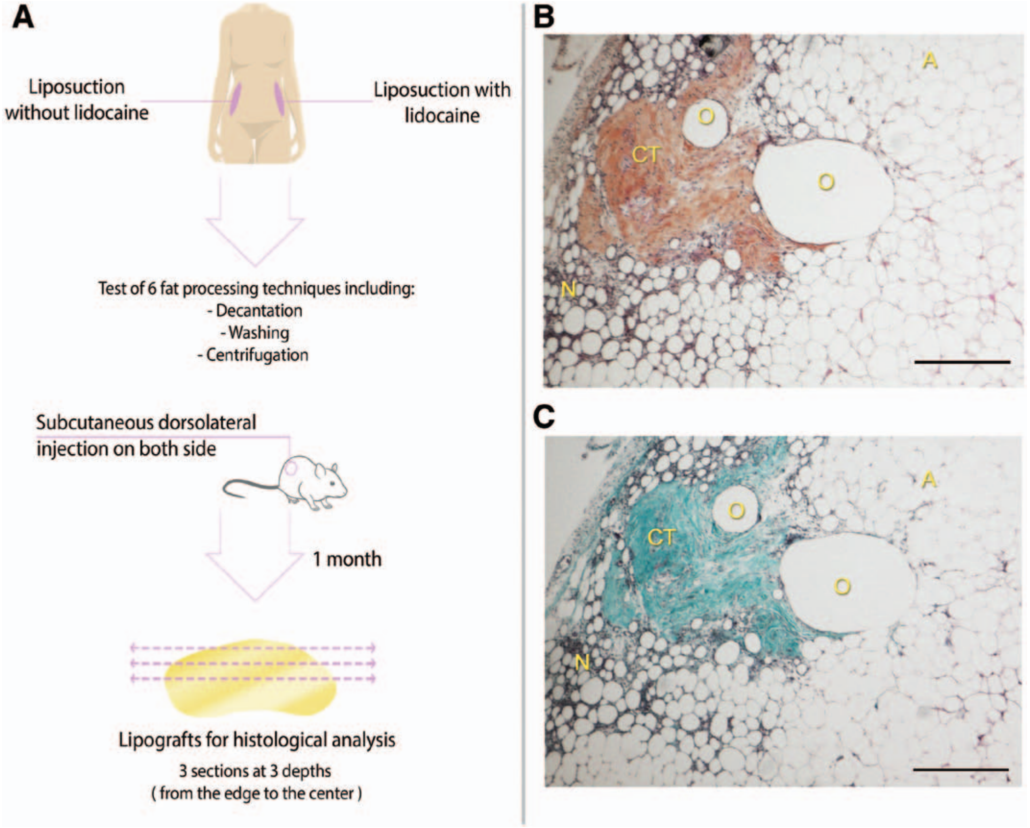

Fig. 1. Methodology, from tissue harvesting to graft analysis. A, Fat grafts were carried out in mice after liposuction with or without lidocaine and following adipose tissue processing by 6 different techniques including decantation, washing, and soft centrifugation. The entire lipograft was removed 1 month later to be analyzed by histology. Longitudinal sections were made at 3 different graft depths (from the edge to the center). Hematoxylin/eosin/saffron (B) or Masson's Trichrome (C) staining was performed on the sections. Different criteria were then evaluated: total graft area, oil vacuoles (O), connective tissue (CT), cell organization and adipocyte shape (A), and necrotic area (N).

Table 1. Protocols of Fat Processing

	Decantation	Washing	Centrifugation
Decantation	5 min	0	—
Washing + decantation	5 min	1	—
1 centrifugation	—	0	1 min at 400*g*
1 washing + centrifugation	—	1	1 min at 400*g*
2 washings + centrifugations	—	2	1 s at 100*g* 1 min at 400*g*
3 washings + centrifugations	—	3	1 s at 100*g* 1 s at 100*g* 1 min at 400*g*

Histological Analysis of Lipografts

Mice were killed and dissected 1 month after injection. The entire lipograft was carefully collected, taking care not to damage them during dissection, before being preserved in formol and paraffin embedded. Five-micrometer tissue sections were then prepared as follows: 6 longitudinal sections were prepared from each complete lipograft, at 3 different depths (2 per depth) to analyze different planes (from the edge of the graft to the center).

Then, sections were stained with hematoxylin/eosin/saffron and Masson's trichrome (Groat's hematoxylin/Ponceau red/Light green). Photographs were taken with an AZ100 microscope (Nikon France S.A., Champigny sur Marne, France), magnification from 10× to 100×. Blind observation of the grafts and sections was made by 2 investigators. From each section, total area measurement, oil vacuoles, necrotic areas, graft organization, cell morphology, and vessel quantification were analyzed with NIS-Elements AR software (Nikon). A scoring method detailed in Table 2 was used to assess global graft efficiency.[23,24]

Statistical Analysis

Statistical analysis was performed using GraphPad PRISM 6 software (GraphPad Software, Inc., La Jolla, Calif.). Data were analyzed by a 2-way analysis of variance followed by a Tukey's posttest for

Table 2. Scoring Method for Histological Analysis of the Lipografts

Scoring Criteria	Evaluation	Score
Total graft area	$>60\times10^5$ pixel^2	5
	40×10^5 pixel^2 < area < 50×10^5 pixel^2	4
	30×10^5 pixel^2 < area < 40×10^5 pixel^2	3
	20×10^5 pixel^2 < area < 30×10^5 pixel^2	2
	10×10^5 pixel^2 < area < 20×10^5 pixel^2	1
	$<10\times10^5$ pixel^2	0
% Vacuoles (mean from sections of 3 different depths)	<5%	5
	5–10%	4
	10–15%	3
	15–20%	2
	20–25%	1
	>25%	0
Fibrosis	Absence	3
	Minimal	2
	Moderate	1
	Extensive	0
Normal connective tissue (no fibrotic)	Well organized	1
	Few or no collagen fibers/absence	0
Cell organization		
Adipocyte size and shape	Homogeneous	1
	Heterogeneous	0
Cellularity (stromal cells)	Normal stromal cells between adipocytes	1
	Few stromal cells	0
Necrotic area	Absence	3
	Minimal	2
	Moderate	1
	Extensive	0
Macroscopic appearance	Good fat take, well vascularized, no visible oil vacuoles, good maintenance	1
	Visible oil vacuoles, reduced size, sign of resorption	0
	Maximal score	20

The higher scores are attributed to healthy criteria, whereas the lower scores are assigned to injured grafts. Combining both healthy and injured criteria allowed to estimate global graft efficiency (score 20 representing 100% graft efficiency). Vascularization was excluded from scoring criteria because of high variability and no significant differences in the results.

multiple comparisons. Statistical significance was set at $P < 0.05$ (*) or $P < 0.01$ (**) or $P < 0.001$ (***) or $P < 0.0001$ (****). Data are expressed as mean ± SEM.

RESULTS

General Features of the Lipografts

After 1 month, all grafts were well integrated into the murine tissue and lipografts were removed in their entirety, without being cut, so as to perform histological analysis of the whole grafts. After graft collection, macroscopic observations revealed that the grafts were smaller with the decantation conditions compared with the centrifugation conditions (data not shown).

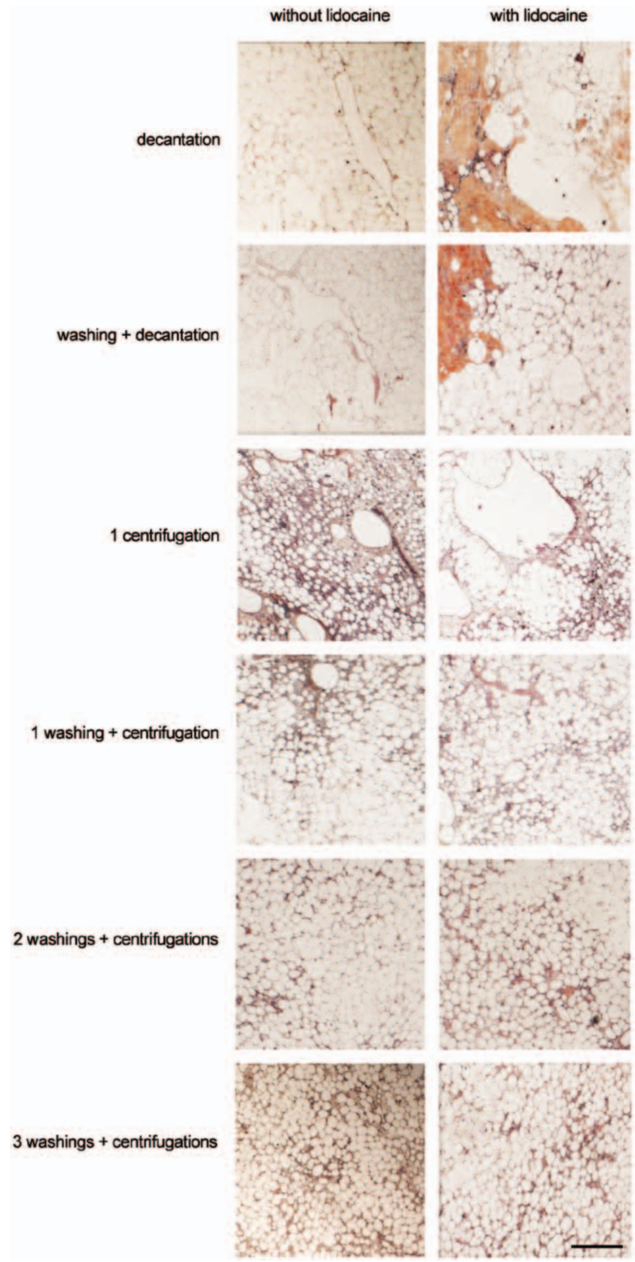

Fig. 2. Histological structure of the lipografts. Representative images of deep sections for each condition, with (B) or without (A) lidocaine, after hematoxylin/eosin/saffron staining. Images are representative of 4 lipografts taken from 4 different mice, in 2 different experiments. Scale bar represents 500 μm.

Gross observation of the histological sections also revealed that the decantation protocol led to the formation of oil vacuoles inside the grafts, especially when using lidocaine for tumescent anesthesia (Fig. 2). Decantation also led to heterogeneous (in size and shape) and loosely packed adipocytes. Conversely, all grafts from centrifuged tissue (with or without washing(s)) showed better compaction of adipocytes and less cell heterogeneity. An increase in the number of washings with centrifugations increases graft quality. However, simple centrifugation without washing led to large oil vacuoles and obvious fibrosis or necrotic areas (Fig. 2). To confirm these observations, quantitative analyses are described hereafter.

Adipose Graft Size

For histology, lipografts were cut longitudinally, in a homogeneous manner, so as to estimate the size of each graft and properly compare the different conditions. This allowed quantification of total area of each graft section, with a total of at least 12 sections analyzed per condition (Fig. 3).

Grafts from decanted conditions (with and without lidocaine) appear smaller than those from all of the centrifugation conditions, and washing does not significantly increase graft size when decantation is used (Fig. 3). A single soft centrifugation results in a 1.2- to 1.8-fold increase in graft size, and further centrifugations including washing steps could result in up to a 2.5-fold increase in graft size, either with or without lidocaine.

Thus, measurement of total area confirmed the macroscopic findings: the grafts that have undergone at least one centrifugation are more likely to be larger compared with adipose tissue grafted after simple decantation, even after washing. Moreover, prior exposure of adipose tissue to lidocaine does not seem to greatly affect lipograft size as no significant differences were found.

Influence of Fat Processing on Adipose Cell Survival

When dying, adipocytes release oil contained in their cytoplasmic lipid droplet. Thus, adipose graft survival was assessed by measurement of oil vacuoles within sections from different depths of the lipografts, from the edge to the center of each graft.

Regarding decantation conditions, only 9.8% ± 1.4% to 14.6% ± 3% of oil vacuoles could be detected when not using lidocaine (Fig. 4A, hatched bars), whereas a mean 31.3% ± 8.3% of oil vacuoles was generated after lidocaine exposure without washing of adipose tissue (Fig. 4B, hatched bars). However, after lidocaine exposure, additional washing in the decantation protocol induces a highly significant 3-fold decrease in oil vacuoles (Fig. 4B, hatched bars).

Interestingly, although washings and centrifugations decrease oil vacuole formation, thus improving graft quality (Fig. 4B), for all centrifuged conditions (with or without washing(s)), no significant differences were observed between lidocaine-exposed grafts and nonexposed grafts.

Either with or without lidocaine, single centrifugation led to a mean 23.1% ± 2.8% of oil vacuoles. This high percentage of oil vacuoles was significantly attenuated by increasing number of washings with centrifugations, with the lowest percentage of oil vacuoles obtained with 3 washings with centrifugations (8.4% ± 1.8%).

Comparison of the washing with decantation protocol and the washings with centrifugation protocols demonstrates that equivalent oil ratios were found, with no significant differences.

Adipose Graft Efficiency

Based on the scoring method detailed in Table 2 and the previous results, global graft efficiency was assessed to find the optimal fat processing protocol.

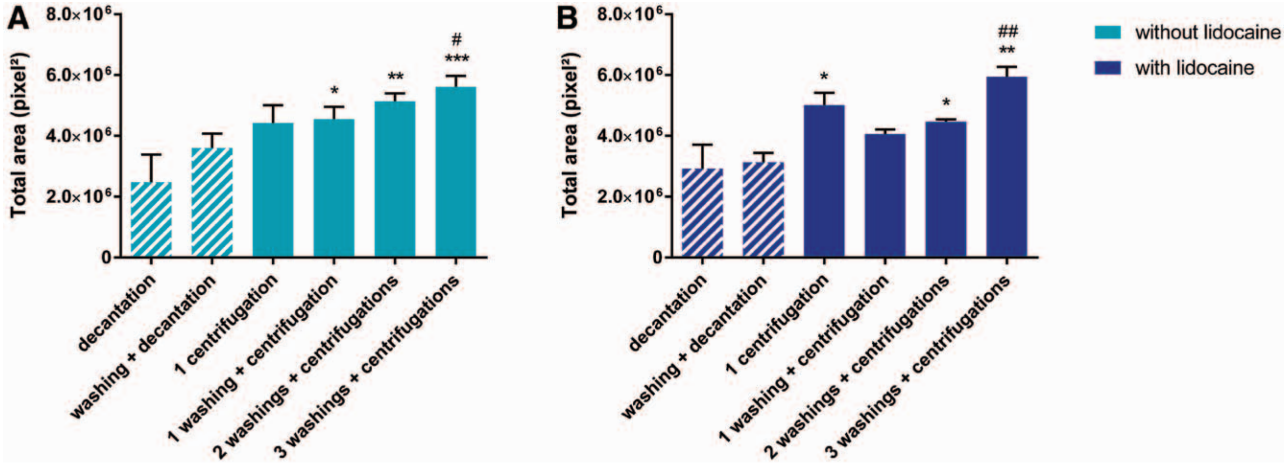

Fig. 3. Lipograft size, without lidocaine use (A) and with lidocaine use (B). Total area of each graft was measured on longitudinal histological sections. Only human tissue was considered and not murine tissue, to determine the true remaining human tissue 1 month after injection. Graphs represent the mean areas of 4 grafts per condition, with 3 (to 6) sections analyzed per graft. Data are represented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs decantation condition. #P < 0.05, ##P < 0.01 vs washing + decantation condition.

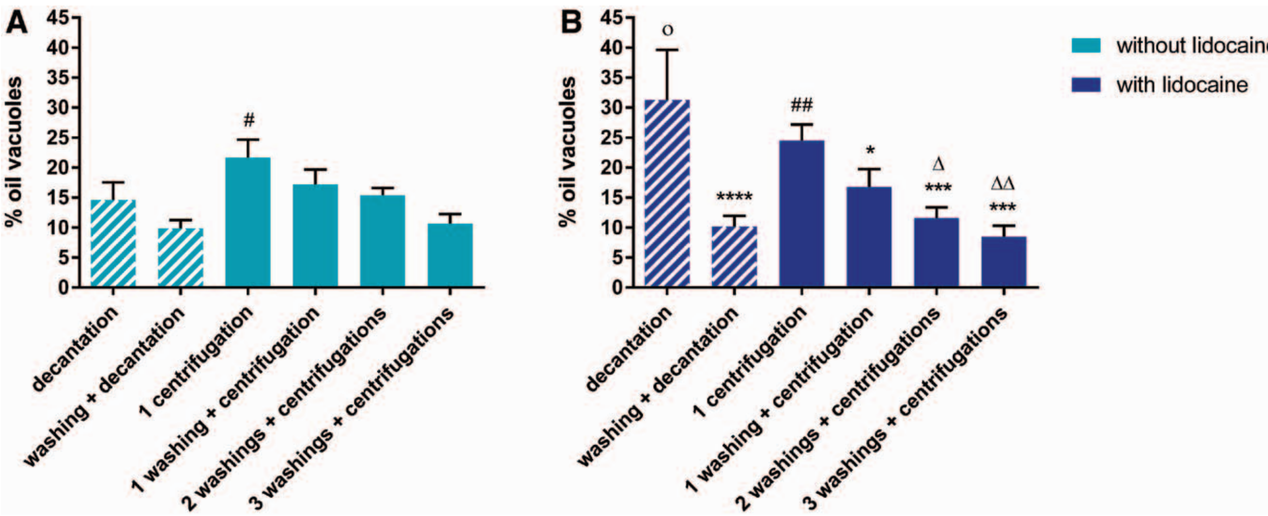

Fig. 4. Measurement of oil lacuna within the lipografts, without (A) and with (B) lidocaine use. Ratio of oil vacuoles was measured relative to total graft size of each section. The graph shows the mean ± SEM of the results from 4 grafts per condition, with 3 sections analyzed per graft. Significance is set to $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs decantation protocol; $^{\circ}P < 0.05$ vs decantation without lidocaine; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs washing + decantation; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs centrifugation.

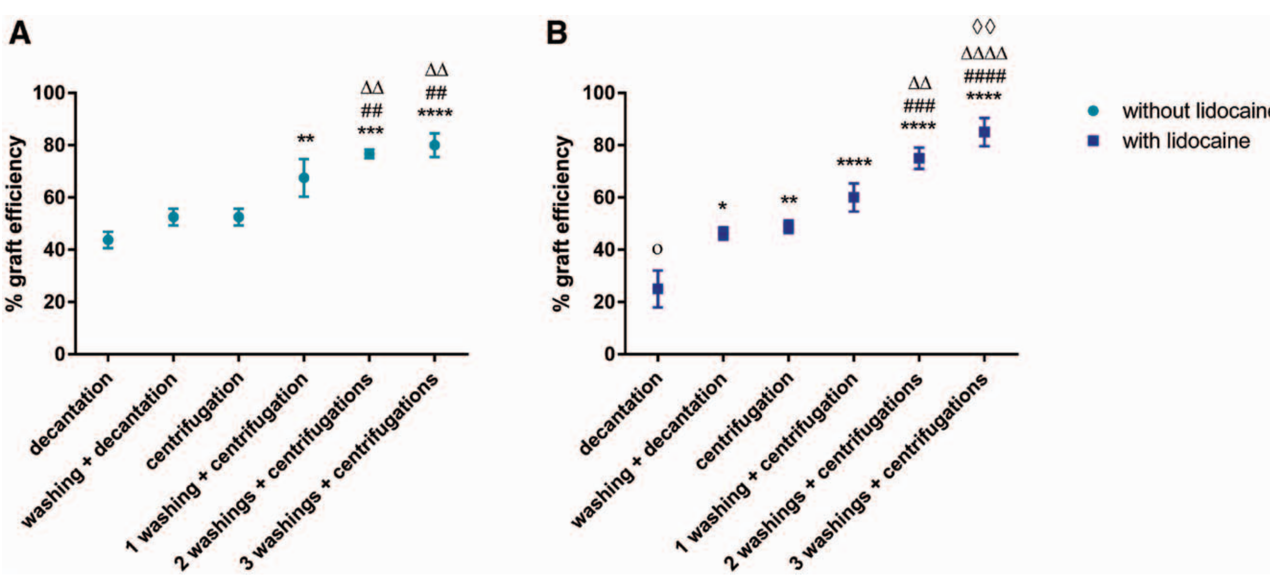

Fig. 5. Adipose graft efficiency, without (A) and with (B) lidocaine use. Global graft efficiency was established thanks to different scoring criteria (see Materials and Methods). Representative graphs of the mean percentage efficiency ± SEM resulting from 4 grafts per condition, with 3 sections analyzed per graft. Significance is set to $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ vs decantation protocol; $^{\circ}P<0.05$ vs decantation without lidocaine; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.0001$ vs washing + decantation; $^{\Delta\Delta}P<0.01$, $^{\Delta\Delta\Delta\Delta}P < 0.0001$ vs centrifugation; $^{\Diamond\Diamond}P < 0.01$ vs 1 washing + centrifugation.

Briefly, the scores were obtained by analyzing cell organization, connective tissue, fibrosis, and necrosis and taking into account the previous quantifications for graft size and oil vacuoles. These scores do not provide absolute quantification of graft efficiency but represent a reliable relative efficiency index based on the comparison of the fat processing techniques.

Without previous lidocaine exposure, decantation, decantation + washing, and centrifugation protocols alone gave moderate results, with only 43.70% ± 3.15%, 52.00% ± 3.21%, and 52.50% ± 3.23%, respectively, of graft efficiency. It is noteworthy that centrifugation alone was not significantly better than the washing + decantation condition (Fig. 5A). Moreover, one washing step with centrifugation could slightly increase graft efficiency but optimal protocols were attributed to multiple washings and centrifugations (2 or 3), reaching around 80% graft efficiency (Fig. 5A). Indeed, we can observe a fairly marked trend in graft efficiency for these last 2 conditions, and no significant differences could be noted between 2 and 3 washings.

With previous lidocaine exposure, the same range of results was obtained except for the decantation protocol, which resulted in even worse graft efficiency, with only 25.00% ± 7.07% (Fig. 5B). Additional washing steps resulted in significantly improved graft quality (mean 1.85-fold increase) but were still insufficient to attain a high quality level (only 46.25% ± 2.39%). Also, single soft centrifugation hardly reached 50% efficiency. Finally, increasing the number of washings and centrifugations correlated with an increase in graft efficiency and fat processing. Three washings with centrifugations were found to be the optimal protocol leading to around 80% graft efficiency (Fig. 5B), which is similar to the results obtained without lidocaine (Fig. 5A). In the case of previous lidocaine infiltration, the third wash provided real added value with a marked trend in graft improvement. It was also significantly different from the situation with 1 washing step, which was not the case without lidocaine (Fig. 5).

DISCUSSION

The abundance of literature on the effectiveness of the different protocols and techniques used in the transfer of autologous grafts shows that the subject is still a hot topic and has been so for many years. However, it is necessary to point out that numerous important advances have been made in recent years, with consensus on a number of key elements that significantly improve graft efficiency. Of these, 2 main factors have been identified during the harvesting phase: aspiration pressure[11–13] and cannula size.[14,15,25] Two other factors before and after the liposuction phase seem to be fundamental but nonetheless controversial: the presence or absence of local anesthetics[7–9] and treatment of fat by washing and/or centrifugation.[10,16–20,26]

However, the work carried out, including that by our team, to objectify the influence of these 2 parameters often use an in vitro approach, which does not give any indication of potential clinical success.[7,20,21,27] Publications concerned with clinical cases of lipofilling often lack in situ objectivity of graft size and quality and, in particular, the presence or absence of oil cysts. Thus, the in vivo xenograft model in immunodeficient mice is a good model to study because it enables a precise histological quantification of engraftment success and graft quality.[23,28]

Also, it is very important in this type of study to consider the maximum number of factors for an objective global analysis of graft effectiveness (graft size, quantity of oil cysts, fibrosis, necrosis, etc.). The analysis of a single objectification criterion often leads to erroneous conclusions about the actual quality of a graft (eg, when only oil percentage is analyzed, one might conclude that a simple decantation + wash is as effective as 3 washes + centrifugation, whereas histological analysis using several criteria demonstrates that this is not the case: see “Results” section).

The results we obtain in this study enable us to confirm our previous results,[22] which were also reported by other teams, on the superiority of centrifugation compared with simple decantation, even without the use of lidocaine (Fig. 5).[26,29–32] Indeed, centrifugation allows the adipose tissue to be compacted and removes interstitial fluid, leading to rapid graft resorption in the case of a simple decantation (Table 3).

When lidocaine is used, the results obtained with a simple decantation are even worse, but easily improved simply by washing or centrifugation. This confirms that in vivo, the deleterious effect of lidocaine on adipose tissue stem cells significantly affects the survival of the entire tissue once grafted.[7,8,21] This also explains the contradictory results obtained by Shoshani et al[23] on the in vivo impact of lidocaine, since 2 centrifugations of the tissue before reinjection (2 centrifugations for 5 minutes at 377*g*) eliminates the cytotoxic substances.

Moreover, and more surprisingly, we also show here that simply washing the adipose tissue once and using decantation without centrifugation, it is possible to obtain a graft efficiency equivalent to that of centrifugation alone (Fig. 5). This is the case

Table 3. Summary of Global Effects of Fat Processing Protocols

Fat Processing Protocol	Impact on the Lipograft	Mechanism/Explanation/Concept
Decantation	Smaller grafts, with heterogeneous and loosely packed adipocytes; large oil cysts when lidocaine is used	Injection of adipose tissue with interstitial fluid leads to rapid resorption
Washing	Improves graft survival	Eliminates lidocaine effect when decantation is used; gets rid of deleterious molecules like proinflammatory cytokines, danger and death-associated molecules
Centrifugation	Larger grafts, with homogeneous and compacted adipocytes	Compaction of adipose tissue and injection of a true volume of fat

whether or not lidocaine is used, although the efficacy remains limited (about 50%). This result agrees with the finding that, during lipoaspiration, a large quantity of inflammatory molecules and cell death factors are released, which probably limit engraftment during reinjection.[3,5] Thus, the simple act of washing the tissue removes some of these factors and leads to improved graft survival (Table 3).[22,32,33]

This theory of inflammatory factors and cell death also seems entirely confirmed by other results that we have obtained. Indeed, we show for the first time that increasing the number of adipose tissue washes before reinjection leads to larger-sized grafts without oil cysts. This result was actually expected when using lidocaine, but this study also demonstrates that the washes are effective for the conditions where lidocaine is not used.

CONCLUSIONS

Our work confirms the deleterious effects of local anesthetics in vivo (previously demonstrated in vitro) and the results of other studies demonstrating that centrifugation (at moderate speed) is superior to the decantation technique for the preparation of adipose tissue for lipofilling. Moreover, and this is probably the most interesting and most innovative side of this work, we show that successive adipose tissue washes can improve significantly the effectiveness of the graft (larger graft size with less oil cysts), both with and without lidocaine. Integration of the results of this study with the findings of our previous work enables us to compile the following recommendations for the clinical lipofilling technique (Table 4):

– without lidocaine infiltration, fat processing with 2 washes and centrifugations (first at 100*g* for 1 second and second at 400*g* for 1 minute) enables good graft maintenance;
– when using lidocaine at the fat donor site, 3 washes with centrifugations (two at 100*g* for 1 second and the last at 400*g* for 1 minute) are preferable.

Obviously, efficient fat grafting also requires that previous recommendations by other research teams are considered, such as the type of cannula (thin) and the light negative aspiration pressure used to harvest fat in order not to damage the tissue.

Following this kind of protocol allows better graft survival and maintenance. It may also prevent the development of oil cysts and steatonecrosis, which are major issues that surgeons must face, especially in AFG to the breast, where megavolumes of fat are required.

Table 4. Clinical Recommendations Arising from This Study

Condition of Fat Harvest	Suggested Protocol
Without lidocaine	Two washings with soft centrifugations
With lidocaine	Three washings with soft centrifugations

See Materials and Methods for detailed protocols.

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