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Effect of centrifugation and washing on adipose graft viability: A new method to improve graft efficiency

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KEYWORDS
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Summary *Background:* Adipose tissue grafting is a promising method in the field of surgical filling. We studied the effect of centrifugation on fat grafts, and we propose an optimised protocol for the improvement of adipose tissue viability.
Methods: Adipose tissue was subjected to different centrifugations, and the volumes of interstitial liquid and oil released were measured to choose the optimal condition. Tissue from this condition was then compared to tissue obtained from two traditional techniques: strong centrifugation (commonly 3 min at 3000 rpm/900 g), and decantation, by injecting into immunodeficient mice. The cytokine interleukin-6 (IL-6) and chemokine monocyte chemotactic protein-1 (MCP-1) were assayed 24 h post-injection, and after 1 month of grafting the state of the lipografts was evaluated through macroscopic and histological analysis, with oil gap area measurement.
Results: Strong centrifugation (900 g, 1800 g) is deleterious for adipose tissue because it leads to until threefold more adipocyte death compared to low centrifugation (100 g, 400 g). In addition, mice injected with strong centrifuged and non-centrifuged adipose tissue have higher rates of blood IL-6 and MCP-1, compared to those grafted with soft centrifuged fat. Moreover, extensive lipid vacuoles were detectable on histological sections of the non-centrifuged lipografts, whereas lipografts from soft centrifugation contain a higher amount of connective tissue containing collagen fibres.
Conclusion: It is necessary to wash and centrifuge adipose tissue before reinjection in order to remove infiltration liquid and associated toxic molecules, which in the long term are

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deleterious for the graft. However, strong centrifugation is not recommended since it leads very quickly to greater adipocyte death. Thus, soft centrifugation (400 g/1 min), preceded by washings, seems to be the most appropriate protocol for the reinjection of adipose tissue.
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The technique of re-injecting fat obtained by lipoaspiration in order to correct volume deficiencies was first described at the end of the 19th century by F. Neuber.[1] More recently, SR. Coleman revived interest in "lipofilling", by publishing his technique: the "lipostructure."[2] Although this method gives more reproducible and stable results, the disadvantages of the technique persist; namely the reduction in graft size in the months following the operation,[3] necessitating an over-correction of the volumes to be treated, and the pronounced post-operative effects (bruising, oedemas). Surgeons have therefore sought to improve Coleman's technique in order to improve graft success.

Thus, although numerous studies have been carried out, a standard procedure has not yet been adopted by all practitioners and today there is still no agreement on the best method for handling adipose tissue in order to improve graft success. In all of the techniques used, however, there seem to be two parameters that strongly influence the quality of injected adipose tissue: centrifugation and washing, which are used or not depending upon the practitioner.

In this study, we began by determining the effect that speed and centrifugation time has on the volume of interstitial liquid present in adipose tissue, as well as on adipocyte viability. From these results, we put in place a washing and soft compression protocol for the reinjection of the tissue. Lastly, we compared this protocol with two methods usually employed in filler surgery: decantation (without washing or centrifugation) and centrifugation according to the lipostructure protocol (without washing, and with strong centrifugation). After injection of adipose tissue in immunodeficient mice, graft success was evaluated by blood inflammation dosage, and by examining the tissue structure and quantifying the oil lacuna.

Material and methods

Patients

Subcutaneous tissue samples of human white fat were obtained from nine normal weight or slightly overweight human subjects undergoing liposuction for cosmetic reasons. Patients were exclusively female, with body mass index (BMI) $= 23.1 \pm 1.9$ kg/m^2, and age $= 43 \pm 9$ years. Apart from oral contraception, the subjects were not receiving treatment with prescribed medication at the time of liposuction. Surgery was performed under local anaesthesia, and for some patients, also under general anaesthesia. The study was approved by the Ile de la Réunion ethics committee for the protection of persons undergoing biomedical research.

Adipose tissue harvesting protocol

The fat tissue was harvested by a surgeon with a syringe technique (manually), after infiltration of a tumescence solution (40 ml lidocaine 2% + adrenalin 1 mg l^{-1} for 1 L Ringer Lactate) for local anaesthesia. Liposuction procedures were performed with a 2-mm diameter single hole aspiration cannula, hole surface 3.4 mm^2 (ref CF201090 INEX, France). Aspiration was performed with 10-ml Luer-lock syringes by creating a light negative pressure (<2 ml in the syringe) by slowly withdrawing the plunger in a gradual manner.

Liquid and oil measurement after centrifugation

After harvesting, 50 ml of adipose tissue was centrifuged (centrifuge LMC-3000, Biosan, Latvia) at different speeds and times: no centrifugation, 100 g/1 s, 100 g/1 min, 400 g/1 min, 900 g/1 min, 900 g/3 min, 1800 g/10 min. The remaining subnatant volume of liquid and supernatant volume of oil were measured after centrifugation and reported as a percentage of the initial tissue volume.

Adipose tissue manipulation protocols

The 10 mL syringes were placed vertically and left to settle for 2 min to remove the infiltration mixture. Decantation protocol: syringes were allowed to settle for a further 2 min. High-speed centrifugation protocol: syringes were spun at 900 g for 3 min (centrifuge IEC, Medispin). Soft centrifugation protocol: 25 ml of the harvested fat tissue was transferred into a 50 ml centrifuge tube, and rinsed/centrifuged twice with 25 ml of Ringer Lactate (100 g for 1 s; centrifuge LMC-3000, Biosan, Latvia), followed by a last wash with a soft centrifugation (400 g for 1 min).

Injection of prepared adipose tissue in severe combined immunodeficiency Beige mice

For the three protocols, after removing the subnatant liquid, and the supernatant oil, the tissue was then transferred into 1-ml syringes, and injected into 8 weeks old (±1 week) female severe combined immunodeficiency (SCID) Beige mice (Charles River Laboratories, Lyon, France). The protocol was carried out at the animal laboratory of CYROI, Reunion Island (approval n°974001 issued by the Veterinary Services of Reunion Island) and approved by the CYROI Ethics Committee for Animal Welfare. For each adipose tissue sample, 18 mice (six per condition) were injected dorsolaterally with 1 ml per side (2 ml of tissue per mouse) while withdrawing the 1.6-mm diameter cannula (ref.

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CF151090 INEX, France). Adipose tissue taken from two patients was used for the injection of 36 mice. Blood and lipografts were taken from these 36 mice.

Histological sections and staining of lipografts

Mice were sacrificed 1 month after injection, dissected and photographs of human lipografts were taken before their removal. Lipografts were preserved in formol to be paraffin-embedded. Five micrometre tissue sections were then prepared, followed by haematoxylin/erythrosin/saffron (HES) and Masson trichrome (Groat's haematoxylin/Ponceau red/light green) stainings and photographed with an inverted microscope, magnification ×40 and ×100. From sections, area measurement was done with the NIS-Elements software, magnification ×1.2. All grafts and sections analysed were done by blinded observers.

Blood sampling

Retro-orbital blood sampling in ethylene diaminetetraacetic acid (EDTA) was performed at 24 h after tissue grafting. Samples were immediately centrifuged at 9000 rpm for 5 min to isolate plasma, and frozen at −20 °C for subsequent enzyme-linked immunosorbent assay (ELISA).

Quantification of serum interleukin-6 and monocyte chemotactic protein-1 by ELISA

Plasma samples were assayed for mouse interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) contents with Ready-SET-Go ELISA kits (Cliniscience, Montrouge, France), according to the manufacturer's instructions. ELISA sensitivity: 4 pg ml^{-1} for IL-6; 15 pg ml^{-1} for MCP-1.

Statistical analysis

All values were measured as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5 software. Differences were tested for significance, compared to the soft centrifugation condition, by the one-way analysis of variance (ANOVA) and Dunnett post-test; $P < 0.05$ (*) or $P < 0.01$ (**) or $P < 0.001$ (***).

Results

Adipose tissue viability after centrifugation

In order to directly determine the extent of adipocyte death caused by centrifugation, we measured the volume of oil released from adipose tissue after various centrifugations. Figure 1 shows the percentage of oil released from adipose tissue for each of the centrifugation conditions: the more strongly the tissue was centrifuged (from 100 g to 1800 g), the more oil was released (from 2% to 10%). The oil released came mainly from adipocyte death, demonstrating that strong centrifugation, from 900 g, was deleterious for adipose tissue (conditions 5/6/7). Soft centrifugation, up to 400 g, did not cause adipocyte death (conditions 2/3/4),

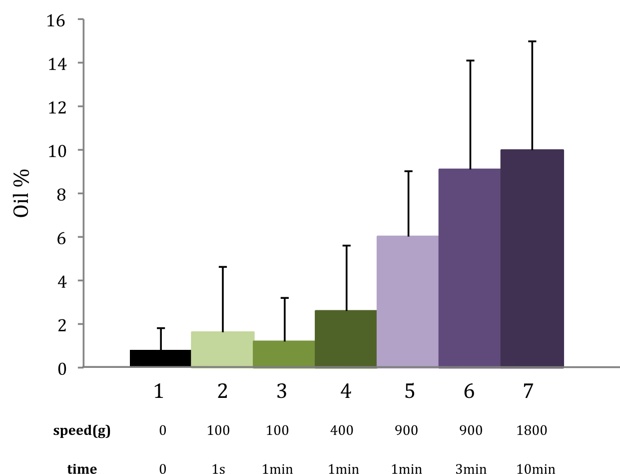

Figure 1 Oil released from adipose tissue after centrifugation. After harvesting, tissue was centrifuged according to the following protocols: 1. Decantation/2 min, 2. 100 g/1 sec, 3. 100 g/1 min, 4. 400 g/1 min, 5. 900 g/1 min, 6. 900 g/3 min, 7. 1800 g/10 min. The initial volume of tissue was measured before centrifugation, and the volume of oil obtained after centrifugation was also measured. The graph shows the mean percentage ± SD of oil volume obtained for an initial volume of adipose tissue, in 3 different patients.

since the percentage of oil was equivalent to that of non-centrifuged adipose tissue (<3%, condition 1).

Liquid released from adipose tissue after centrifugation

Figure 2 shows the percentage of interstitial liquid extracted from adipose tissue after various centrifugations. This graph demonstrates that the more the tissue was centrifuged at a higher speed, the more it was possible to extract liquid. This was true for soft centrifugation between 100 g and 400 g, where it was possible respectively to extract 12% and 17% liquid after centrifugation for 1 min (conditions 3 and 4). However, compared to 400 g (17%), centrifugation at 900 g (condition 5, 1 min), or even longer (condition 6, 3 min) resulted in the extraction of more interstitial liquid from the adipose tissue, but the difference was not significant (20% and 22%, respectively). The volume of liquid extracted from adipose tissue reached a maximum of about 25% with condition 7 (1800 g/10 min), which probably represents the total volume of interstitial liquid inside adipose tissue.

Macroscopic appearance of the grafts

One month after injection, the grafts were well integrated, irrespective of the protocol used (Figure 2: left for soft centrifugation − 400 g/1 min, middle for decantation, and right for high centrifugation − 900 g/3 min). Grafts were surrounded with mouse connective tissue and were vascularised (Figure 3). However, the grafts derived from the decantation protocol were distinctly different from the other conditions in that they displayed a much greater quantity of macroscopically visible oil. It was not possible

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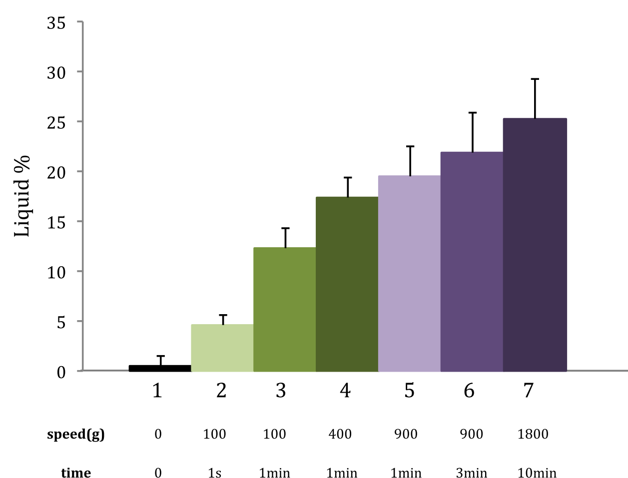

Figure 2 Liquid released from adipose tissue after centrifugation. After harvesting, tissue was centrifuged according to the following protocols: 1. Decantation/2 min, 2. 100 g/1 sec, 3. 100 g/1 min, 4. 400 g/1 min, 5. 900 g/1 min, 6. 900 g/3 min, 7. 1800 g/10 min. The initial volume of tissue was measured before centrifugation, and the volume of liquid released from tissue after centrifugation was also measured. The graph shows the mean percentage ± SD of liquid volume obtained for an initial volume of adipose tissue, in 3 different patients.

to distinguish the two other methods upon their macroscopic appearance.

Histological structure of the lipografts

In the sections from the strong centrifugation protocol (Figure 4, right panel), adipocytes were highly packed, compared to the normal shape found with the soft centrifugation protocol (Figure 4, left panel). Furthermore, grafts from the soft centrifugation protocol contained more connective tissue between and around adipocytes (Figure 4, left panel) than grafts resulting from other methods. This connective tissue contained many cells, identified by haematoxylin staining with several nuclei apparent on each section that was examined. These cells were inside a network partially composed of collagen fibres, as demonstrated by the green colour from the Masson trichrome staining (Figure 6).

From those sections (Figure 5), the area measurement revealed that decantation sections contained more than 20% oil, compared to 10% for the soft and high centrifugation protocols. This result confirmed the oily macroscopic appearance of the graft and the several large oil lacunas observed in sections from the decantation protocol (Figure 4, middle panel).

Serum IL-6 and MCP-1 after adipose tissue grafting in SCID Beige mice

Twenty-four hours after injection, mice with high centrifugated lipografts showed more inflammation than those with soft centrifugated lipografts: fivefold more for serum IL-6 (Figure 7, left panel) and around twofold more for serum MCP-1 (Figure 7, right panel). Mice with decanted lipografts showed no more IL-6 (around 600 pg ml^{-1}), while the rate of MCP-1 was twofold higher than mice with soft centrifugated lipografts. One week after grafting, levels of IL-6 and MCP-1 were the same between the three conditions, which corresponds to the levels of control mice (data not shown).

Discussion

Grafting of autologous adipose tissue is potentially a promising future method for cosmetic reconstruction and body volume augmentation. Indeed, this method remains simple and atraumatic and only leads very seldom to postoperative complications, unlike fillers (injectable collagen, hyaluronic acid, etc.).[4–6] However, numerous publications have demonstrated that techniques used for aspiration, handling and injection of adipose tissue clearly influence, graft success.[7–9]

The aim of this study was to optimise adipose tissue-handling conditions in order to increase post-injection tissular survival. In fact, in order not to damage adipose tissue, samples were collected with a fine-bore cannula[10] (2 mm in diameter) and with minimal depression of the

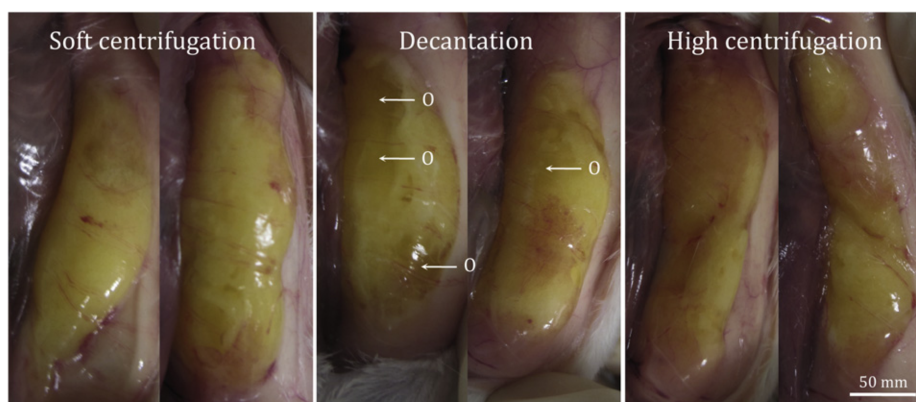

Figure 3 Macroscopic appearance of the lipografts. One month after injection of 1 mL of prepared adipose tissue per side, mice were killed and dissected. Pictures show lipografts from soft centrifugation (400 g/1 min, left panel), decantation (middle panel) and high centrifugation (900 g/3 min, right panel) protocols. O: oil lacuna. Pictures are representative of 6 different mice, in 2 different experiments.

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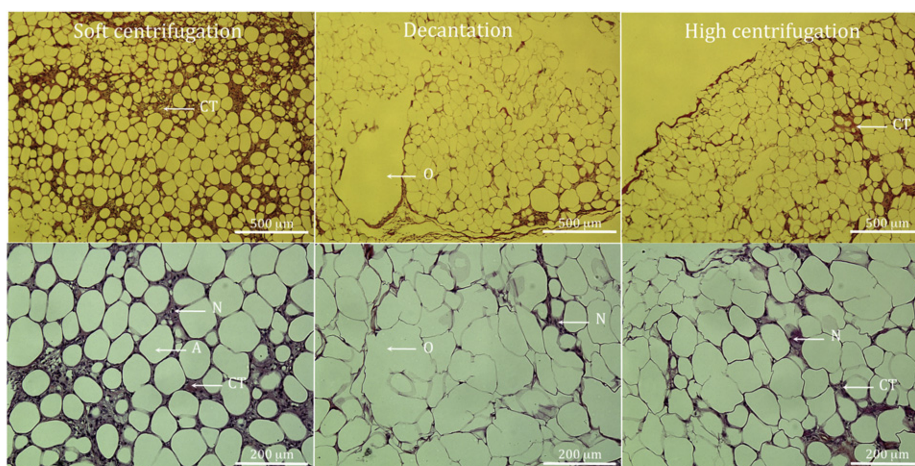

Figure 4 Histological structure of the lipografts. Soft centrifugation (400 g/1 min, left panel), decantation (middle panel) and hard centrifugation (900 g/3 min, right panel) 1 month-lipografts were paraffin-embedded and 5 μm sections were prepared and stained with HES. Pictures were taken with an inverted microscope, ×40 magnification (upper line) and ×100 (lower line). CT: connective tissue; A: adipocyte; O: oil lacuna; N: nucleus. Pictures are representative from 5 sections taken from 6 different mice, in 2 different experiments.

aspiration syringe plunger,[11] which corresponds to around 108 mmHg (2 ml in a 10-ml syringe).[12] According to some studies, this was the best way to prevent adipose tissue trauma, compared to liposuction machines.[13]

We initially sought to determine the parameters required for the optimum centrifugation of adipose tissue for lipofilling. Certain studies do not recommend strong centrifugation since this causes adipocyte and even mesenchymal stem cell death,[14,15] whereas other studies do not observe any impact of centrifugation on adipose tissue viability[16] and even recommend centrifugation at low speeds to increase adipocytes' survival.[17] These studies are however not easily comparable, because the speeds and times of centrifugation differ.

We centrifuged adipose tissue for variable times and speeds in order to determine the condition that was most adapted for two crucial factors: tissue viability (by quantification of the volume of oil, resulting from adipocyte death, Figure 1) and tissue compactness (by quantification of the volume of extracted interstitial liquid, Figure 2). Thus, low-speed centrifugation (up to 400 g) had no effect on oil release (<3%, as the control), whereas at speeds above 400 g, centrifugation irremediably led to adipocyte death, as demonstrated by the significant increase in oil release (threefold more at 900 g). However, centrifugation is recommended insofar as it allows a better volume adjustment of injected adipose tissue, because of the interstitial liquid removal. Compression of adipose tissue sample prevents graft resorption and therefore avoids overcorrection or successive reinjections.[18] Thus, we confirmed that most of the residual liquid was removed at 400 g,[19] even for 1 min, and we arrived at the same conclusion as Lack, that 400 g for 1 min seemed to be the best protocol specifically recommended for facial lipoaugmentation.[13] This centrifugation represents the best compromise between tissue viability and volume of liquid remaining within injected tissue. Moreover, at 400 g, the number and the viability of mesenchymal cells are preserved.[20]

In the literature, results from graft-washing studies are also contradictory. Indeed, a certain work demonstrates that washings lead to better graft quality,[21] in particular as a result of the increase in the viability of mesenchymal stem cells within washed adipose tissue.[14] Other studies demonstrate that washing has no effect upon cell viability, as measured by weight and histological analysis of the grafts.[7,16]

However, it is obvious that the volume of the interstitial liquid is higher when the adipose tissue is not washed or centrifuged. This residual liquid contains lidocaine, adrenalin (depending on infiltration procedures) and inflammatory molecules (from blood components), recognised to be toxic for adipose tissue, but also and mainly for mesenchymal stem cells,[22,23] which increase graft survival and integration by taking part in neovascularisation.[24] Thus, in

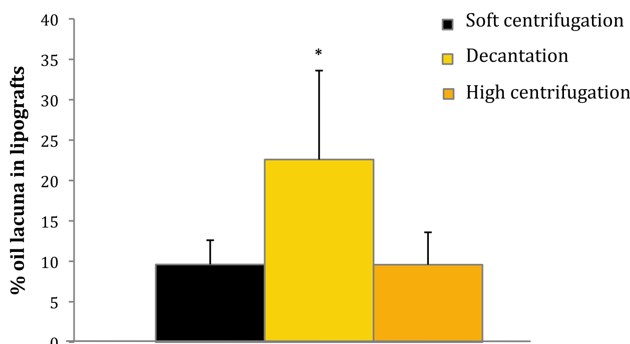

Figure 5 Oil lacunas area in lipograft sections. From one experiment and for each condition, 2 sections per lipograft were done. A total of 6 subsections of 12,4 ± 1,9 mm^2 were analysed per condition. The graph shows the mean percentage ± SD of oil lacuna area obtained per subsection for soft centrifugation, decantation and high centrifugation protocol.

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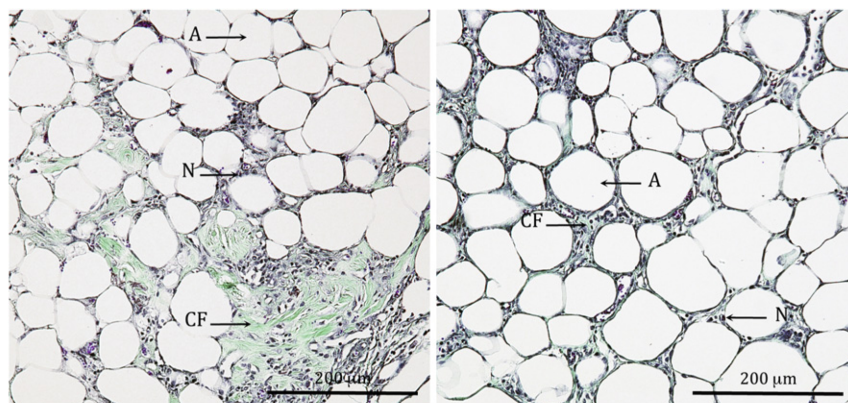

Figure 6 Connective tissue identification. 1 month after injection, lipografts from soft centrifugation (400 g/1 min, left panel) were paraffin-embedded and 5 μm sections were prepared and stained with Masson trichrome. Pictures were taken with an inverted microscope, ×100 magnification. CF: collagen fibres, A: adipocyte; N: nucleus. Pictures are representative from 5 sections taken from 6 different mice, in 1 experiment.

order to eliminate most of these deleterious elements, we decided to carry out two rapid washings (100 g/1 s, completely atraumatic for tissue, Figure 1) before a last washing and centrifugation at 400 g/1 min.

In addition, after having determined the most appropriate protocol for adipose tissue handling, we went on to compare this protocol with two techniques that are currently mostly used: the lipostructure (900 g/3 min) and the decantation, as 25% of the experts do not resort to centrifugation.[25] Thus, we objectivated graft success by injecting adipose tissue obtained with the three techniques into SCID Beige mice.

We observed that injection of strongly centrifuged adipose tissue is deleterious to the host, since it led to widespread inflammation in the model used: IL-6 and MCP-1 plasma levels are higher 24 h post-injection (Figure 7). These molecules are known to start, maintain and increase inflammation.[26] By transposing these results in humans, it is more likely that this will result in localised inflammation at the injection site, which would not promote engraftment.[27] This inflammation is probably most problematic because of the increased levels of MCP-1, which could lead to macrophage infiltration inside the graft and in the long term lead to tissue necrosis. However, 1 week after injection, rates return to normal (data not shown), indicating that this inflammation is only transient. In terms of decantation technique, it also led to higher levels of MCP-1, whereas levels of IL-6 were similar to those of control mice (data not shown). It is likely, since no centrifugation was performed for this condition, that inflammation was due to the persistence of toxic molecules (from infiltration and blood) that could not be removed without centrifugation or washing.

In the same way, after 1 month, lipografts from strong or soft centrifugation protocols were large in volume with little resorption, good integration and very little oil, whereas lipografts from the decantation technique appeared smaller, with a much greater quantity of oil (Figure 3). In histological sections, numerous gaps were visible which correspond to oil resulting from adipose cell death (Figure 4), which was twofold higher for this condition (Figure 5). We believe that this very poor graft success was first of all due to the 25% interstitial liquid (Figure 2), which spread and caused resorption, and second to the

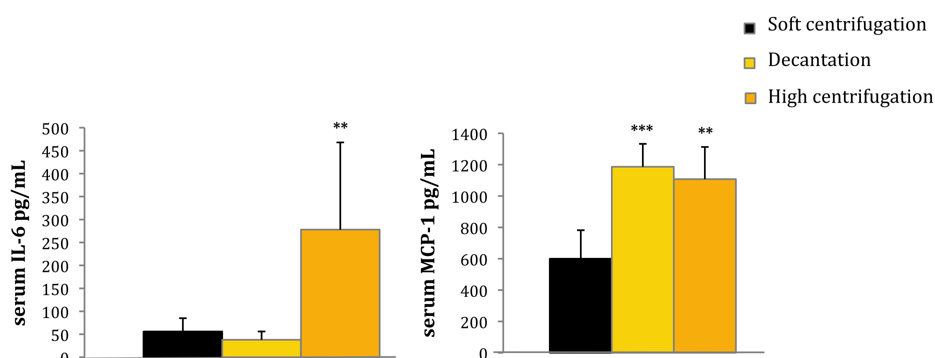

Figure 7 Serum IL-6 and MCP-1 24 h after adipose tissue grafting in SCID Beige mice. 24 h after grafting 2 mL/mice of soft centrifugation-, decantation- and high centrifugation-prepared adipose tissue, mice blood was collected and mouse IL-6 (left panel) and MCP-1 (right panel) were assayed from plasma by ELISA. The graphs show the mean ± SD of the results from 2 patients, $n = 6$ for each condition, for each patient.

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same toxic molecules contained in this persistent liquid. Anaesthetics (as lidocaine) could act directly over time on adipocytes, and/or indirectly through their noxious action on adipose tissue stromal cells.[22,23]

Certain authors affirm however that washing can have harmful effects. In particular, Chajchir et al. have stated that washing removes fibrin content in lipoaspirate, which is necessary for fat graft adherence.[18] We cannot confirm this hypothesis with our results; however, we can assume that the presence of collagen fibres is correlated to lipograft success (Figure 6). On the one hand, collagens might structure the graft, and they could also mediate the migration of other vascular stem cells.[28] Cells from connective tissue probably synthesise these fibres, as type 1 and type 3 collagens[29,30] (and personal data), and they are able to reorganise themselves in order to promote neovascularisation.[31] As there was much connective tissue and many stromal cells in lipografts from soft centrifugation protocol, it looks like there were more collagen fibres. The probability of blood vessels being present within this network might be therefore higher.

Our results agree with those of Conde-Green et al., who show that brief washing and low-speed centrifugation leads to the elimination of contaminants resulting in a more effective graft, whilst preserving the integrity of adipocytes and mesenchymal stem cells.[14] In fact, our results showed that centrifugation at 400 g was sufficient to extract the most of the liquid found in harvested adipose tissue. Moreover, a centrifugation at 900 g was not justified insofar as it did not result in greater interstitial liquid extraction, but it led to adipocyte death.

Thus, the soft centrifugation protocol (with three washings) that we propose represents an optimisation of the techniques that are usually employed: the strong centrifugation used in lipostructure protocols and the decantation method. The reduction in adipose cell death leads to improved graft success, which makes this the method of choice for facial filling and rejuvenation.

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Conflict of interest

There is no potential conflict of interest.

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