


Processing Adipose Tissue to Make it More Stable When Used for Refilling: A Morphologic and Immunohistochemistry Evaluation

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Abstract

Breast reconstruction has gained from lipofilling the possibility to recover the aesthetic outcome of anatomical profile in a more natural appearance. However, until today, the long-term graft survival remains unpredictable, and sometimes it does not guarantee a well-adequate aesthetic result. In the present work, the morphological changes, occurring in fat mass used for refilling, harvested by the Coleman's procedure or through the washing/fragmenting procedure were analysed. Adipocyte size; immunohistochemistry against CD8, CD31, CD68 and M2-type macrophages and catalase enzyme, were analysed *in vitro* on fat mass cultured for 4 weeks. Our observation reveals an increase of connective tissue around the mass and a high number of immune cells occurrence in fat mass harvested by the Coleman's procedure. Instead, the washing/fragmented procedure would reduce the number of immune cells within the fat mass, increase the size of adipocytes, and cause a wider presence of active vessels profile and greater catalase expression. We hypothesize that the fat mass processed by the Coleman's procedure would remain more reactive due to a higher number of immune and macrophages cells, prone to develop cystic formation, leading to a suboptimal integration in the recipient site. On the other hand, the conditions more prone to realize an optimal integration would occur in the fat mass processed by the washing/fragmenting procedure: a reduced number of immune cells, low amount of connective tissue, presence of larger adipocytes. Follow-up monitoring did support our conclusion, as we observed a reduction of re-intervention for refilling procedure in patients treated with the washing/fragmenting procedure.

Keywords

lipofilling, adipose cell, M2 macrophages, breast refilling, fat transplantation

Introduction

Lipofilling consists of autologous fat transplantation that offers a minimal tissue reaction and easy availability. The difficulties of survival and stabilization of transplanted fat mass have initially oriented the lipofilling procedure to small aesthetic adjustments.¹ However, due to the same nature of the anatomical part, and the acquiring of technical amelioration in adipose tissue grafting, lipofilling was applied also to a large reconstructive anatomical profile of the breast.² Actually, breast demolitive surgery has gained from lipofilling the possibility to recover the aesthetic outcome of anatomical profile in a more natural appearance. Thus, it has become an elective technique of choice.^{3,4}

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The lipofilling technique applied to the breast mass is performed according to a standardized procedural protocol, initially indicated by Coleman (Coleman's lipofilling procedure).⁵ However, until today, the long-time outcomes of the Coleman's lipofilling procedure remain affected by the same kind of uncertainty, because the long-term survival of great amount of transplanted fat mass remains unpredictable. It has been observed in long-term follow-up studies that 20–80% of the graft volume is lost. Furthermore, Chung et al.⁶ have reported how the Coleman's procedure for fat injection could be harmful to adipocytes. This implies an increase of the number of lipofilling procedure, and sometimes a not well-adequate aesthetic result.⁷

Fat graft survival and integration in the host tissue environments depends on availability in terms of revascularization, low inflammatory reaction and integration in extracellular environment. In addition to the presence of adipocytes as cells that characterise the adipose tissue, the fat microenvironment is characterised by a plethora of different cellular types, such as preadipocytes, vascular stromal cells, staminal cells, macrophages and other leukocytes.^{8,9} All these cells are able to react with each other in conditions of tissue stress, such as inflammation¹⁰ and environmental stress.⁸ An appropriate revascularization of the grafted tissue has been regarded as the first critical point for a correct integration to avoid cell loss by hypoxia and the following inflammation response.^{11,12} However, it has been observed that the loss of the adipose graft tissue is apoptotic in nature, indicating the occurrence of cell signals leading cells to die rather than to develop hypoxic/necrotic conditions.^{13–15} These evidences confer to revascularization of the fat graft a not real critical importance for the graft integration; otherwise, clinical and experimental studies have reported a continuous volume loss of the grafted adipose cells even after the grafts appeared revascularized.^{16,17}

The washing of the lipoaspirate to remove superfluous fluid, free lipids and debris and a reduction of centrifuge times are effective technical solutions to preserve both large number of mesenchymal stem cells and mature adipocytes and to increase the stability of the fat graft.^{18,19}

Recently, technical facilities, based on washing and fragmenting, have optimized the fat mass harvesting procedure. The washing and filtering/fragmenting the fat mass, allowed to obtain a more fluid mass, more manageable and ready to be inserted into recipient sites.^{20,21}

The aim of the present study is to analyse *in vitro* the structural morphology and evolution of the fat mass harvested by the classical Coleman's procedure in comparison to those observed following washing and fragmenting the fat mass.

Materials and Methods

Patients

The experimental plane was performed on 20 women undergoing breast reconstructive surgery, following surgical

mastectomy performed to remove tumoral mass. All patients enrolled in the present study have ended a wash-out pharmacological period of antitumoral therapy, had no signs of cancer recurrence and they were free from systemic pathologies (such as hypertension or diabetes). The procedures were performed in the Plastic Surgery Division of Hospital: Azienda Ospedaliero-Universitaria Maggiore della Carità in Novara and were in accordance with the World Medical Association Declaration of Helsinki (June 1964) and subsequent amendments. The over-exceeding adipose tissue not used for refilling and allocated to disposal was used in our study. Since the surgical procedures did not differ from those widely adopted in the clinical practise, no ethical committee was required to authorize it. All participants gave written informed consent.

From September 10, 2018 to January 14, 2019, in ten patients aged 42±4 years (Body Mass Index<30 Kg/m²), fat graft was harvested and processed using the Coleman's modified procedure.

From January 28, 2019 to June 28, 2019, in other ten patients aged 43±6 years (Body Mass Index<30 Kg/m²), fat graft was harvested and processed by washing/fragmenting procedure.

Surgical Procedure

Coleman's modified procedure²² was performed in patients under general anaesthesia and after local infusion of lidocaine/adrenaline; in those patients, 200 mL of adipose mass was aspirated manually from sub-umbilical abdominal area and centrifuged for 2 min, 1,500 r/min. The upper and lower fractions obtained were manually discharged.¹⁰

The washing/fragmenting procedure was performed in patients under general anaesthesia and after local infusion of lidocaine/adrenaline; in those patients, fat graft aspiration was performed manually in the sub-umbilical abdominal area. About 200 mL fat graft was taken in each patient. The haematic and plasmatic residues were discharged through settling and double washing with saline. Differently from the Colman's procedure, no centrifugation was executed. The total fat mass of each patient was separated and diluted (4 mL fat mass +4 mL PBS) in opportune containers (Rigeneracons device, Rigenera[®], Torino, Italy), in which the mixture was crumbled for 60 s.^{20,21}

The obtained product has been recovered through a syringe without needle.

All patients were monitored for the next 8 months to verify the need to replicate the procedure due to the lipofilling resorption. All replicated lipofilling procedures were performed according to refilling initial procedure.

Experimental Procedure

The primary aim of the present work, was focused on observing morphological change occurring in the same fat mass

available for lipofilling; therefore, no disaggregating manipulation was performed on the fat mass cultured during experimental procedure.

The fat mass (about 2 mL) was leaned on dishes (diameter 35 mm; area= 961 mm²) and cultured in 2 mL of X-VIVO15 medium (Lonza, Milan, Italy) in a CO₂ incubator at 5% CO₂, 37°C. The medium was immediately added and was enough to submerge the fat mass. The mass was initially floating after medium addition, then the more compact mass obtained through the Coleman's procedure settled within 1 h, whereas the mass obtained through the washing/fragmenting procedure within 1.5 h. The medium was changed every 2 days.

To analyse morphological evolution and cell activation, a small part of fat mass was immediately collected to analyse its morphological appearance (T0). Thereafter, a complete portion (inner and outer part) of the fat mass placed on dishes was taken after 1,2,3 and 4 weeks of culture (T1–T4).

Each portion was fixed in buffered formalin and processed for paraffin inclusion. Slides (5 µm thick) were stained by hematoxylin/eosin to analyse the general morphology, the adipocytes size and to analyse the extent of connective tissue. CD31 immunohistochemistry (primary antibody: clone JC70, cod. 760-4378; Hoffmann-LaRoche, DE) was used to identify active vessels and leukocytes immunoreactive cells;²³ CD68 immunohistochemistry (primary antibody: clone KP-1, cod. 790-2931) was used to identify macrophage cells; CD8 immunohistochemistry (primary antibody: clone SP-57, cod. 790-4460) was used to identify the occurrence of cytotoxic lymphocytes in the fat mass; Ki-67 immunohistochemistry (primary antibody: clone 30-9, cod. 790-4296) was used to detect the occurrence of active replicative events; anti M2-macrophage antibody (anti CD-163, ab87099, Abcam, Cambridge, UK) was used to identify the anti-inflammatory/reconstructive M2-type macrophages and anti-Catalase antibody (ab16791, Abcam) was used to identify the occurrence of oxidative stress protection. Immunoreaction product was revealed using biotinylated secondary antibody and avidin/biotinylated enzyme complex (Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA) together with 3,3'-diaminobenzidine as chromogen (DAB chromogen kit, Vector Laboratories).

Morphometric analysis was performed using the image analyser (Leica Qwin) connected to the microscope. Adipocyte size was evaluated by drawing the adipocytes border in the analysed area (10*10⁴ µm²); the higher value was taken as representative value of adipocytes size of the field. The higher value was interpreted as the value corresponding to diametrical section (therefore representative of the real dimension of adipocytes), while the other values were interpreted as secant sections (not passing along cell diameter).

Quantification of immunohistochemical positive cells was performed by counting the number of positive cells occurring or evaluating their size by drawing immunopositive cells edge in the analysed area (14*10⁴ µm²).

Connective matrix was quantified as % of area occupied in respect to the total area; positive vessels were detected as total area (µm²) of longitudinal displayed immunopositive elements, visualized inside the analysed area (10*10⁴ µm²).

Catalase was quantified by detecting the optical density (OD) of different immunopositive elements inside the analysed area (data expressed as %, where 0% equals blank and 100% equals black).

Catalase immunoreaction was detected in 3 different compartments, morphologically identified: adipocytes (identified by their outer edges), immune cells (identified in the roundish, homogeneously staining elements visible along the edge of adipocytes or in the stromal area) and the stromal environment (identified in the dilated spaces between adipocytes edge).

Six different fields were examined for each section, 3 sections for each experimental sample (fat mass from single patient). The data were detected in blinded by 2 independent observers and their results collected.

Data were reported as Mean ± Standard Error of Mean (SEM); Kolmogorov–Smirnov test was performed to check normality distribution of data. Statistical significance was checked by ANOVA followed by Newman–Keuls post-hoc test, using statistical software program Prism 5.0 (GraphPad Software Inc, San Diego, CA, USA). A *P* value <.05 was taken for significance.

Results

The BMI of patients enrolled in the 2 experimental group was for both of them <30 Kg/m², identifying the subjects as not overweight or obese. Therefore, BMI has not gained further attention.

Upon rough examination, the fat mass harvested by the washing/fragmenting procedure was cleaner than that processed by the Coleman's procedure (Figure 1A).

The morphological analysis of the fat mass harvested by the Coleman's procedure (Col-p) showed the presence at T0 of a wider connective net (as visualized by trichromic stain) than that observed in the fat mass harvested using the washing/fragmented procedure (WF-p) (Figure 1B). Using the Col-p, the connective net increased, particularly between the second (T2) and the third (T3) weeks (Figure 1D). Starting from the third week of culture time, in the outer zone of the fat mass, a wide presence of connective net, small adipocytes and large microvessels could be observed. This formation encapsulated an inner part where the connective net was less present and almost absent in the core (Figure 1B). In the fat mass harvested by WF-p, the connective net remained scarcely appreciable and did not increase during the culture time (Figures 1C,D).

At T0, by using the Col-p, the adipocytes size was smaller than that detected by the WF-p (Figures 1B,C,E), indicating that WF-p is less traumatic on mature adipocytes than Col-p. Using the Col-p, the adipocytes further decreased their

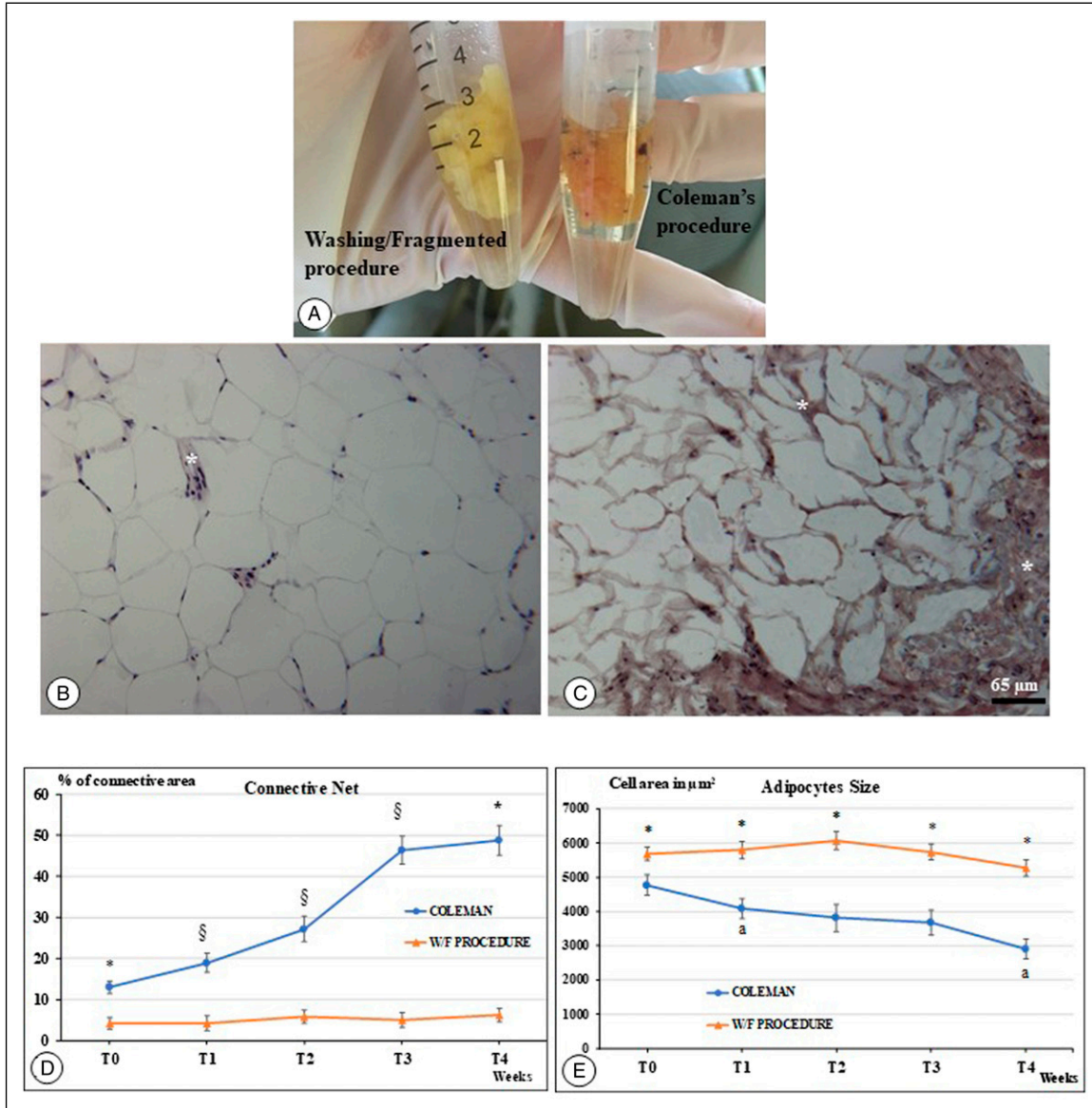


Figure 1. Photograph panel illustrating the appearance of the fat mass harvesting by the 2 lipofilling procedures (A), and the microanatomical appearance of the fat mass following the washing/fragmenting procedure (B) and the fat mass following the Coleman's procedure (C). Note the wider presence of connective matrix and smaller size of adipocytes in the fat mass treated by the Coleman's procedure in comparison to the washing/fragmenting procedure. Culture time: fourth week (T4), Line-graph illustrating morphometric analysis of connective tissue (D) and adipocytes size (E) performed along the different culture time. * = $P < .05$ vs. the other experimental group at the same time; § = $P < .05$ vs. the other experimental group at the same time and previous culture time of the same experimental group; a = $P < .05$ vs. previous culture time of the same experimental group.

dimension from 1 week of culture time (T1) until the fourth week of culture (T4). On the contrary, using the WF-p, the adipocytes size showed no significant changes during the culture time (Figure 1E).

CD31 immunoreactive vessels showed, at all culture times examined, a larger immunoreactive area belonging to immunopositive vascular profile, in the fat mass harvested by WF-p respect to fat mass harvested by Col-p (Figure 2). In the fat mass harvested by WF-p, an increase of vessels

immunoreactive area was observed until the third week of culture, followed by an apparent decrease, with no statistical significance (Figure 2). In the Col-p, an increase of vessels immunoreactive area was observed at the second week of culture. Then a decrease was found at the fourth week of culture (Figure 2).

CD31 and CD68 immunoreactive cells analysis showed a higher number of immunoreactive cells in the fat mass harvested by Col-p compared to the fat mass harvested by

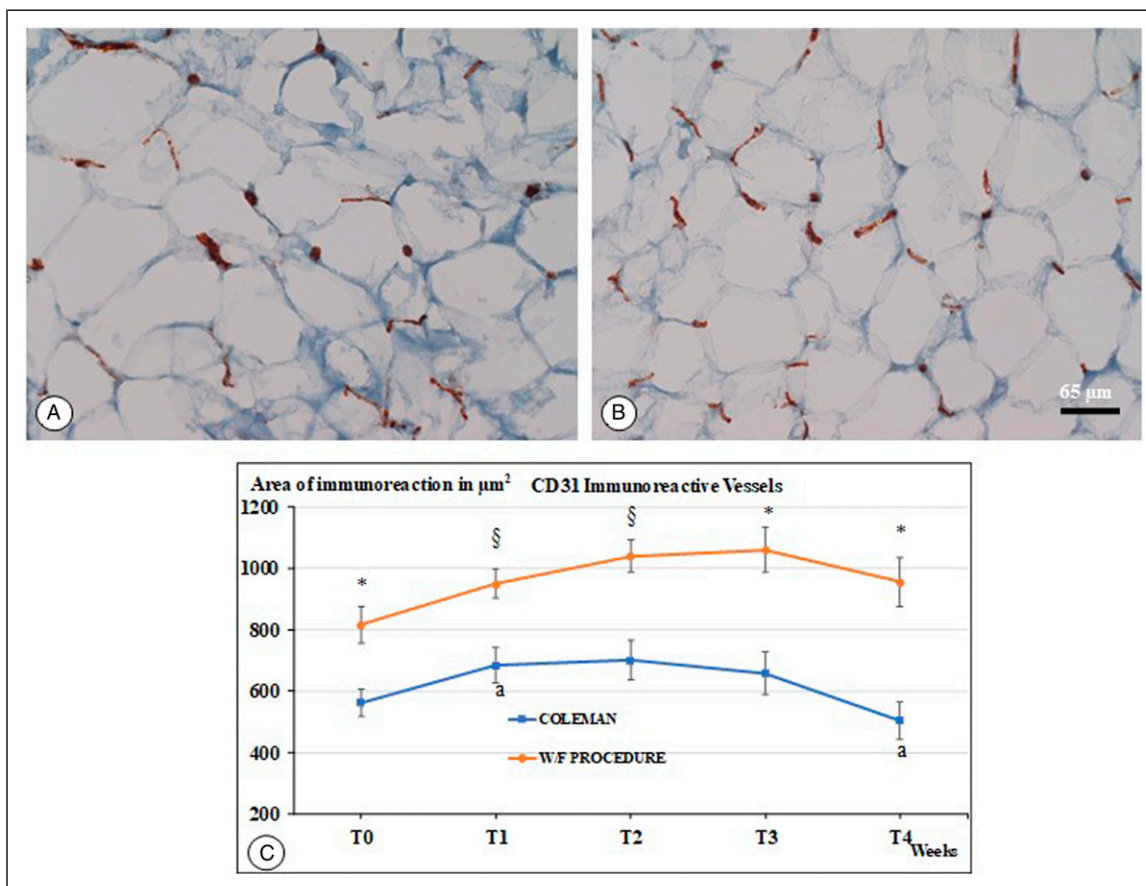


Figure 2. Microphotograph panel illustrating the CD31 immunoreactive vessels profile occurring in the fat mass following the Coleman's procedure (A) and the washing/fragmenting procedure (B). Culture time: third week (T3). Line-graph illustrating morphometric analysis of the CD31 immunoreactive vessels profile occurrence along the different culture time (C). Statistical significance symbols as in Figure 1.

WF-p at T0 and during all following times of culture (Figures 3A–D). During culture time, both in the fat mass harvested by Col-p and by the WF-p, a wide increase of immunopositive cells was detected after 1 week (T1), whereas, the number of immunopositive cells gradually decreased until the fourth week of culture (Figures 3E,F). The size of CD68-positive cells was larger in the fat mass harvested by Col-p ($319.4 \pm 37.6 \mu\text{m}^2$) vs. WF-p ($265.1 \pm 30.3 \mu\text{m}^2$; $P < .05$), independently from the culture time. Only scanty and disperse CD8-positive cells were detected, with no differences between Col-p and WF-p (9–11 immunopositive cells on exploring area $14 \times 10^4 \mu\text{m}^2$), indicating the absence of an active inflammatory status (Figures 4A,B). No Ki-67 immunopositive signals were detected following Col-p or WF-p indicating any active proliferation events occurring (Figures 4C,D).

M2-type positive cells were detected both in the fat mass harvested by Col-p and WF-p, mainly associated to connective profile (Figure 5). It was evident the occurrence of larger dimension of M2 macrophages following Col-p (cell size: $384.4 \pm 43.8 \mu\text{m}^2$) vs. WF-p (cell size: $211.1 \pm 32.6 \mu\text{m}^2$; $P < .05$), regardless of the culture time. Between the initial culture time (T0) and the second week of culture, the number

of immunoreactive cells was statistically higher following Col-p than WF-p. At T3 and T4, the number of M2-type macrophages was comparable in the fat mass harvested by Col-p or WF-p (Figure 5). Following both Col-p and WF-p, the number of M2-type macrophages increased along the culture time (Figure 5).

Catalase immunoreaction was detected following both Col-p and WF-p, ubiquitously, in different elements of adipose tissue, such as adipocyte cells, immune cells and stromal environment (where the catalase intensity can be developed by endothelial cells and stromal fibroblasts) (Figure 6). Comparing the immunoreaction intensity of adipocytes following Col-p or WF-p, we have observed higher intensity of immunoreaction in the WF-p group (Figure 6). In particular, in the WF-p group, the catalase expression increased gradually until the second week of culture and then decreased towards initial values observed at T0. No change in catalase intensity was observed in adipocytes following Col-p (Figure 6E). Catalase intensity in immune cells, following both the Col-p and WF-p, did not show any significant change along the culture time. However, the catalase intensity in immune cells in the WF-p group was higher than that measured in

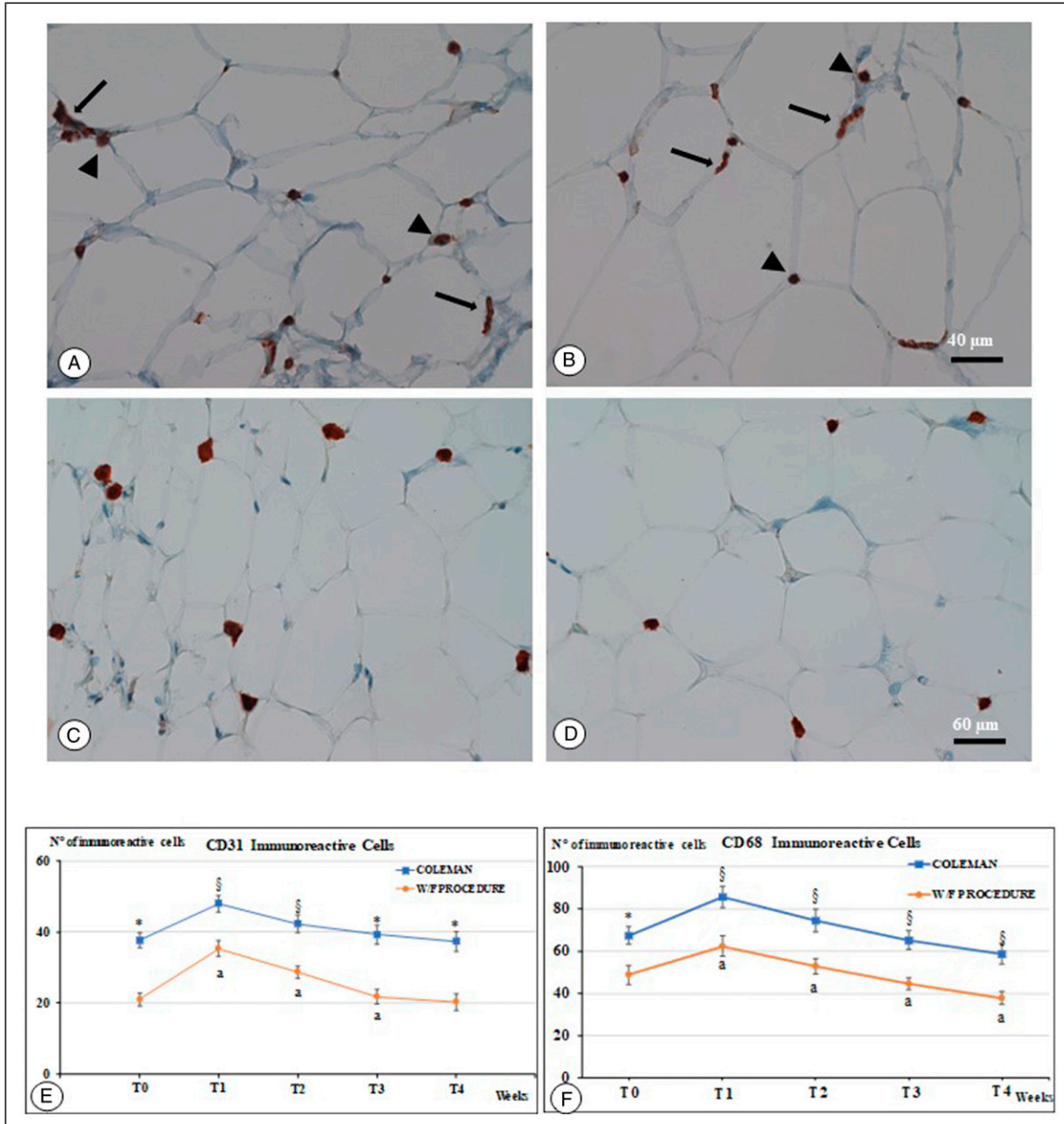


Figure 3. Microphotograph panel illustrating the presence of CD31 immunoreactive leukocytes in the fat mass following the Coleman's procedure (A), and in the washing/fragmenting procedure (B); CD68 immunoreactive macrophages in the fat mass following the Coleman's procedure (C), and in the washing/fragmenting procedure (D). Culture time: first week (T1). Arrows, indicate the immunopositive vessels profile, arrowheads, indicate immunopositive leukocytes. Line-graph illustrating the analysis of the number of CD31 (E) and CD68 (F) immunoreactive leukocytes along the different culture time. Statistical significance symbols as in Figure 1.

immune cells in the Col-p group (Figure 6F). The catalase intensity in stromal environment following WF-p was higher than that found following Col-p. A gradual increase of catalase intensity was observed following Col-p or WF-p along the culture time (Figure 6G).

Analysing the follow-up of patients for 8 months, we have noted that in the group of patients treated through the WF-p, no re-interventions were performed. On the contrary, in the patients treated by Col-p, 1 patient needed a re-intervention

after 1 month and for 2 patients after 2 months. In addition, for one of the latter 2, another re-intervention was again required after 3 months (Table 1).

Discussion

The use in breast reconstruction of fat mass for refilling requires the occurrence of cells which are stable over time and are able to occupy a large volume.^{4,7}

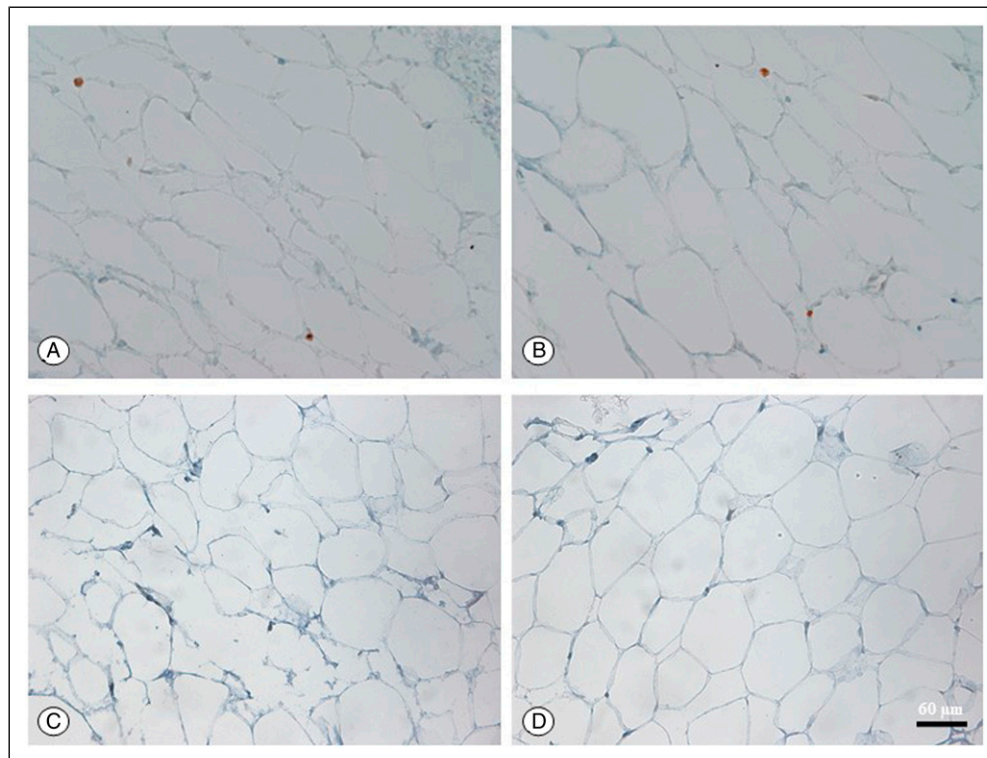


Figure 4. Microphotograph panel illustrating the presence of CD8 immunoreactive leukocytes in the fat mass following the Coleman's procedure (A), and in the washing/fragmenting procedure (B); Ki-67 immunoreactivity in the fat mass following the Coleman's procedure (C), and in the washing/fragmenting procedure (D). Culture time: second week (T2). No differences have been observed between the 2 harvesting procedures.

Since it is not possible to follow the changes encountered by fat mass in the recipient site during the first period after transplantation, we have used *in vitro* observations.

Our morphological analysis has shown that the mass harvested and processed by the Coleman's procedure cannot be considered a stable mass, because over time it evolved towards a hyper-connective mass where the connective capsule surrounded a cellular core composed by adipocytes. Furthermore, the size of adipocytes decreased over time, as a probable consequence of the loss of larger and mature adipocytes. Although this evidence was obtained *in vitro*, the hypothesis that a similar reaction could somehow take place in the recipient site could also be considered and would represent a matter of debate.

On the contrary, in the fat mass harvested and treated by the washing/fragmented procedure, no development of connective tissue has been observed over the 4 weeks of culture, and the adipocytes size was larger than that observed with the Coleman's liposuction. These observations indicate the occurrence of a stressful condition in the Coleman's fat mass, that would alter the architectural structure of the mass, increasing connective tissue and reducing the viability of the largest and mature adipose cells.

The microvascular profile of the fat mass was well conserved and endothelial cells remained active, as evidenced by

the positivity of CD31 surface marker following both the Coleman and the washing/fragmenting procedure, but the fat mass treated by latter procedure showed a more conservative display of active vessels profile, which can be supposed to represent an improvement for the integration of the mass in the recipient site.

Previous studies have been focused on preadipocytes and stem cells from stromal vascular fraction as main elements responsible for tissue recovery and integration in a recipient site.^{22,24} Preadipocytes and stem cells represent part of a heterogeneous population of cells generally named adipose-derived stromal cells (ASC). These cells possess regenerative properties, immunomodulatory properties and are able to promote angiogenesis, furthermore the pre-adipocytes are more stable and resistant than mature adipocytes, and have been considered the cells real responsible of fat graft integration and stabilization.^{22,24,25} Several protocols were studied to ameliorate the initial Coleman's procedure and achieve optimal preservation of preadipocytes and stem cells.^{20,25}

However, immune cells population has not been adequately considered in the analysis of the adipose mass responses. In particular, immune cells could induce adverse reaction during fat graft implantation and contribute to reduction of fat graft survival and integration in the host tissue

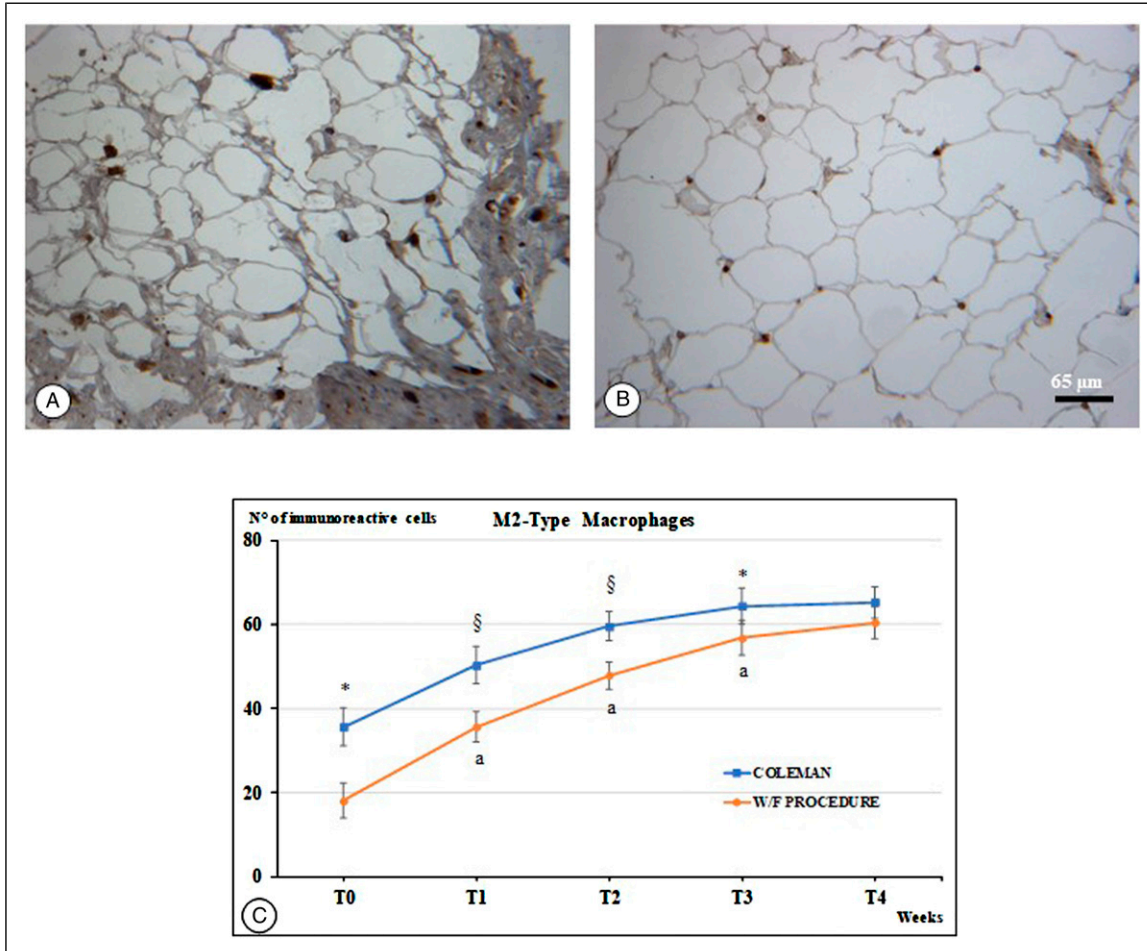


Figure 5. Microphotograph panel illustrating M2-type macrophages (immunohistochemically detected) occurring in the fat mass following the Coleman's procedure (A) and the washing/fragmenting procedure (B). Culture time: first week (T1). Line-graph illustrating the analysis along the different culture time of the number of M2-type macrophages (C). Statistical significance symbols as in Figure 1.

environment, despite the presence of preadipocytes and stem cells.

Immune cells in the adipose tissue have been recognized as important in playing a role in health and disease. A high BMI indicating obesity would be associated with a high presence of immune cells resident in adipose tissue, which may induce patterns of chronic inflammation responsible for the onset or worsening of diseases such as metabolic syndrome and diabetes.^{25,26}

In our study, BMI was not an exclusion parameter, but it has not gained particular attention because the patients enrolled in the study have revealed a BMI < 30 Kg/m², indicating that those subjects were not in overweight. Indeed, the observation of the variability in the number of immune cells detected for each patient has supported the hypothesis that patients were not ascribing to different groups.

Immune cells are constitutive inside the adipose tissue and are mainly represented by macrophages, neutrophils and lymphocytes. We have observed an appreciable number of immune cells in the fat mass harvested by the Coleman's

procedure, represented mainly by activated macrophages (CD68⁺ cells), and by neutrophils and leukocytes (CD31⁺ cells).²³ Those cells showed a widespread activation profile, as documented by an increased number of immunopositive elements during the first weeks after the mass harvesting, indicating a reactive stress response of the fat mass.

Otherwise, the absence of proliferation signals, analysed through Ki-67 and the absence of immune reactions by exogen antigen or altered cells, as evidenced by the scanty presence of CD8⁺ cytotoxic T-lymphocytes,²⁷ would confirm the hypothesis that the cell activation was a phenomenon connected to a state of environmental stress. It is noteworthy that the condition of the *in vitro* culture *per se* did not represent an improper environment for cells; in fact, capillary endothelial cells, even if not functional in terms of blood flow transport, remained reactive for CD31 marker for several weeks.

A part of macrophage cells observed in the fat mass was constituted by M2-type macrophages, which represent the recovering elements inside a tissue phlogistic process. M2-type macrophages appear in the presence of recovering of

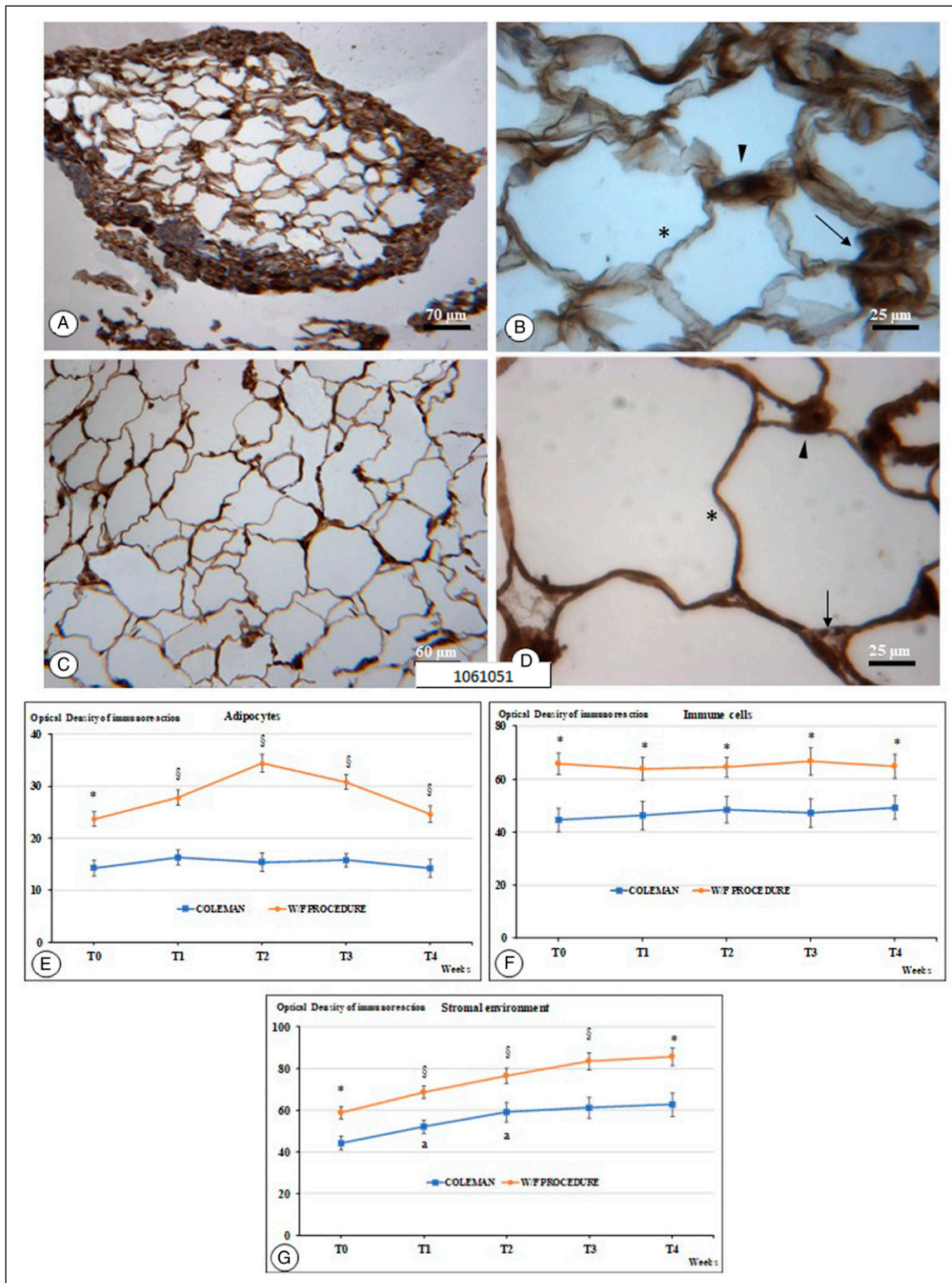


Figure 6. Microphotograph panel illustrating catalase detection and distribution in the fat mass following the Coleman’s procedure (A, B) and the washing/fragmenting procedure (C, D). Culture time: second week (T2). Line-graphs illustrating densitometric analysis of catalase reaction in adipocytes (E), immune cells (F), stroma environment (G), along the different culture time. Asterisk= adipocytes border; arrow= stromal environment; arrowhead= immune cells. Statistical significance symbols as in Figure 1.

Table I. Patients undergone to re-intervention by the Coleman's or the washing/fragmenting procedures.

Patients follow-up (months)	Coleman's procedure		Washing/fragmenting procedure	
1	A first re-intervention	//	//	//
2	//	B first re-intervention	C first re-intervention	//
3	//	B second re-intervention	//	//
4	//	//	//	//
5	//	//	//	//
6	//	//	//	//
7	//	//	//	//
8	//	//	//	//

The ten patient's operated by Coleman's procedure was named (A,B,C... etc.).

the native tissue tri-dimensional scaffold and, probably, they are also responsible to build the connective tissue which characterizes the long-time culture of mass harvested by the Coleman's procedure.²⁸

In our work, we have also explored cell metabolic activation through catalase detection. Catalase is an enzyme presents in all cells, known to protect them from superoxide production. Indeed, in the last years, a great deal of information has been acquired on a broader function of catalase related to gene expression and cell metabolism.²⁹ Results have evidenced a protective role of catalase against apoptosis induced by oxidative insults³⁰ also of ischaemic origin.³¹ Following this evidence, we hypothesize that the higher catalase expression observed in the fat mass following washing/fragmented procedure may indicate a better ability in terms of integration into the recipient site.

Furthermore, even if the analysis of ASC population has not been a purpose of the present work, we can point out that a better preservation of ASC population by disaggregation and filtration procedure has been highlighted by some authors.²⁰ Therefore, an improvement of the preservation of ASC population in the washing/fragmenting procedure adopted in the present work could be hypothesized.

All together, these findings suggest that the adipose tissue harvested by the Coleman's procedure is a reactive mass, having in local immune cells the main source of reactive response, which may, in turn, be responsible for cysts formation, adipose mass dissolution and occasional development of inflammatory events, such as those that can be observed following breast refilling.³²

On the contrary, in the fat mass treated by the washing/fragmenting procedure, the number of immune reactive cells was lower. Following this observation, we hypothesize that the fat mass is less reactive, owing to a lower number of immune cells, and can only respond slightly to potential stressful stimuli that may occur inside any recipient site.

Our work has its main limitation in the *in vitro* experimental protocol adopted, being it a condition quite far from the one that the fat mass would encounter in the living tissue. However, the changes observed during culture time points may well indicate the reactive status of the harvested fat mass.

The follow-up monitoring of the patients in our study would support our hypothesis about the importance of immune/phlogistic cells population inside the fat mass as it is mainly responsible for the instability or unpredictability of the grafting procedure. In fact, we have detected a decrease of re-intervention of refilling procedures in patients receiving fat mass treated by the washing/fragmenting procedure, probably due to greater stability of the adipose tissue.

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Declaration of Conflicting Interests

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