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ORIGINAL ARTICLE

Clinical and Experimental Study of Autologous Fat Grafting After Processing by Centrifugation and Serum Lavage

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Abstract
Background This clinical and experimental study compared adipose tissue transplant behavior after two different techniques of purifying: centrifugation at 3400 rpm for 3 min and serum lavage without centrifugation.
Methods Clinical evaluation was performed under standardized conditions for lipofilling on a series of 51 female patients, intentionally selected to have similar characteristics and assigned to two groups based on the method of processing. Experimentally, a culture system in diffusion chambers with vitaline membranes was designed to mimic the behavior and to study the morphology of the adipose tissue used for autografting. Survival, structure, and proliferation of the adipose cells in vitro were examined by classical histologic H&E staining and immunohistochemistry for leptin and cyclin D1.
Results The main differences encountered experimentally were the presence of a greater amount of preadipocytes in the noncentrifuged adipose tissue cultures and more distinctly expressed cell proliferation. The postoperative clinical results favored of the serum lavage purifying technique.
Conclusion Our data suggest that with transplantation of noncentrifuged adipose tissue more active preadipocytes are applied which could possibly lead to better potential chances of survival and even de novo development of fat.

Keywords Autologous fat grafting · Adipose tissue cultures · Preadipocytes

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Ever since Neuber's first report on autologous fat grafting [1], its gain in popularity has been restricted by the high resorption rates and poor postoperative results. The introduction of liposuction [2] as a technique for fat harvesting, followed by the invention of lipostructure [3, 4] was supposed to open a new era for the method. Its essential philosophy had changed from free transfer of intact adipose tissue to free fat cell transplantation but the main limitations were still present. This has inspired the creation of many controversial techniques [5] representing a great variety of factors that influence tissue viability and resorption rates [6]. The new concepts were built on attempts to avoid excessive pressure changes, improving the means of purification from potential local inflammation promotors, and application in a fashion that assures sufficient nutritional sources [6–9].

Recent research on lipofilling clearly demonstrated that it is a three-stage procedure consisting of harvesting, purification, and reinjection of the fat [10]. A consensus has even been reached on some stages of its technical implementation such as manual syringe lipoaspiration and three-dimensional reimplantation [8, 11–15]. To date the most controversial part of the procedure is the fat processing. The most frequently used processing methods are washing with saline solution, decantation, and

centrifugation [15]. At one point, choosing between them seemed unnecessary because it had been reported that with the exception of detergents, all other treatments had no statistically significant effect on adipocyte survival [16]. The growing knowledge of the stages of graft viability and its development in the recipient site, however, has put a new emphasis on purifying techniques. Previous histomorphologic studies have documented that adipose tissue consists of two components: lipid inclusion containing adipocytes and stromal cells [17] containing a stem-cell compartment (preadipocytes), which under certain conditions can differentiate into mature fat cells [18]. With this in mind, one wonders whether processing techniques differ by preadipose cell preservation and how this could possibly affect graft survival because it was recently assumed that the primary flaw of previous techniques was selecting the wrong component of adipose tissue [19].

The purpose of the present work was to perform clinical and experimental investigations of the two methods of adipose tissue purification: serum lavage versus centrifugation at 3400 rpm for 3 min. By means of a tissue culture system the histologic characteristics and specific differences of the two graft types were studied and compared with the clinical outcomes after their transplantation.

Materials and Methods

A series of 51 patients were intentionally selected from two different medical centers: the Unit of Plastic and Craniofacial Surgery in Plovdiv, Bulgaria, and the Clinic of Plastic and Reconstructive Surgery in Lille, France. According to the selection criteria, all the participants were females ranging between 16 and 55 years of age, in good health, and under no particular medication. They were admitted over the last 4 years for the initial protocol of lipofilling in the facial region. Their mean age was 32.68 ± 1.54 years. The surgical interventions were indicated for aesthetic reasons, craniofacial malformations or touch-ups after reconstructive operations. Recipient sites were carefully examined to be anatomically undamaged and an improvement in appearance was pursued. The selected donor sites for autologous fat harvesting were the subgluteal regions.

The patients were operated on under general anesthesia with endotracheal intubation and without local anesthetic infiltration. In both centers, fat harvesting was performed using a 2.6-mm-inner-diameter blunt cannula attached to a 10-cc syringe under manual regulation of the negative pressure not exceeding 2 cc. The mean average quantity harvested was 41.23 ± 7.77 cc.

Clinically, the patients were assigned to two groups depending on the method of graft purification. In group I, consisting of 27 participants (22 from Lille and 5 from Plovdiv), the obtained adipose tissue was centrifuged at 3400 rpm for 3 min, resulting in sedimentation and formation of three layers. The serum at the bottom and the oil at the top were removed as previously described [3, 10, 12]. In group II, which consisted of 24 patients from Plovdiv, the fat tissue was transferred from the 10-cc syringes in which it was obtained to 20-cc syringes and washed by additionally drawn 10 cc of physiologic serum. To obtain a fat graft free of oil and blood, the saline solution was changed one or two times and removed. For the transplantation of both graft types the three-dimensional technique of reinjection was applied [11] and in all cases a normocorrection was performed.

The follow-up period was 1 year and the method of postoperative evaluation was standardized for both medical centers. Clinical outcomes were documented by preoperative and postoperative digital photographs taken at baseline and the regular control visits at 3, 6, and 12 months. Positioning, facial expression, focal distance, and camera adjustments were standardized. Results were appraised at the end of the follow-up period by the operating surgeons, the patients themselves, and an independent medical observer who was not involved in the treatment. After the examination, the postoperative effect (correction) was classified as sufficient or insufficient by the operating surgeon according to the extent of soft tissue defect correction. The other two evaluations were performed by the patients and the independent observer without them knowing which handling technique was applied. Each evaluation was independent of the others. Results were appraised using a three-stage assessment scale: satisfactory, nonsatisfactory, and absent. Patients' assessments were obtained via a questionnaire that they filled out at the end of the follow-up period. The evaluation of the independent observer was done by comparing the preoperative and final postoperative digital photographs for every case. The images were presented at PowerPoint slides (Microsoft Corp., Redmond, WA), each for a duration of 30 s.

Statistical analysis was performed by using Fisher's exact test and the Mann-Whitney U test. A p value of ≤ 0.05 was considered significant.

The first 10 cc of adipose tissue samples obtained from five different patients of each group was submitted for experimental study. Part of this material, processed by either method of purification, was cultivated in diffusion chambers with vitaline membranes [20] in a tissue culture incubator at 37°C and at 5% CO_2 atmosphere for 7 days. Culture medium 199 and 5% calf serum were used and replaced every 2 days. At the end of cultivation the

cultures were fixed in 10% formalin solution, routinely processed, and embedded in paraffin. Consequent deparaffinized sections were used for hematoxylin and eosin (H&E) staining and immunohistochemistry. Immunohistochemical demonstration of leptin (as a marker of preadipocytes) was carried out by the avidin-biotin-peroxidase (ABC) method as described by Atanassova and Popova [21]. Primary antibodies—polyclonal rabbit anti-mouse leptin antibody (Alexis Biochemicals) in dilution 1:5000 and antibody of cyclin D1 (DACO Corp.) in dilution 1:200—were used for investigating proliferation of leptin and adipocytes, respectively. The experimental results were semiquantitatively analyzed.

Results

There was no statistically significant difference between the average quantities transplanted in the two groups: 10.73 ± 2.60 cc in group I and 10.40 ± 1.01 cc in group II. No donor or recipient site complications such as necrosis, infections, hematoma, seroma, or cyst formations were noted in either group. Postoperative swelling was not counteracted and it was comparable for all patients.

At the end of the 1-year follow-up period the analysis of the data from the professional medical examinations (surgeons) showed a tendency for better results only in group II ($p = 0.15$) (Table 1). At the same time, a statistically significant difference in favor of the serum lavage purifying technique was found in both other evaluations, i.e., the patient's subjective assessment ($p < 0.0001$) and the independent observer's assessment ($p = 0.001$) (Tables 2 and 3).

Histologic analysis (H&E staining) showed that both types of cultures consisted of typical mature-like adipocytes, revealing no morphologic changes. The cells were unilocular, with one large lipid drop occupying almost the entire cell and pushing the cytoplasm and the nucleus to the periphery. No picnosis of the nuclei was observed. Among these adipocytes were singly scattered multilocular adipose

Table 1 Data from the professional medical examination

Group	Assessment					
	Sufficient correction		Insufficient correction		Total	
	Count	%	Count	%	Count	%
Group I (centrifuged)	16	59.26	11	40.74	27	100
Group II (noncentrifuged)	19	79.17	5	20.83	24	100

Statistical analysis by Fisher's exact test detected no significant difference, $p = 0.15$

Table 2 Data from the patients' subjective evaluation

Group	Assessment							
	Satisfactory result		Nonsatisfactory result		Absence of result		Total	
	Count	%	Count	%	Count	%	Count	%
Group I (centrifuged)	7	25.93	7	25.93	13	48.15	27	100
Group II (noncentrifuged)	21	87.50	2	8.33	1	4.17	24	100

Analysis by Mann-Whitney U test found a statistically significant difference, $p < 0.0001$

Table 3 Data from the independent observer evaluation

Group	Assessment							
	Satisfactory result		Nonsatisfactory result		Absence of result		Total	
	Count	%	Count	%	Count	%	Count	%
Group I (centrifuged)	9	33.33	8	29.63	10	37.03	27	100
Group II (noncentrifuged	19	79.17	3	12.50	2	8.33	24	100

Analysis by Mann-Whitney U test found a statistically significant difference, $p = 0.001$

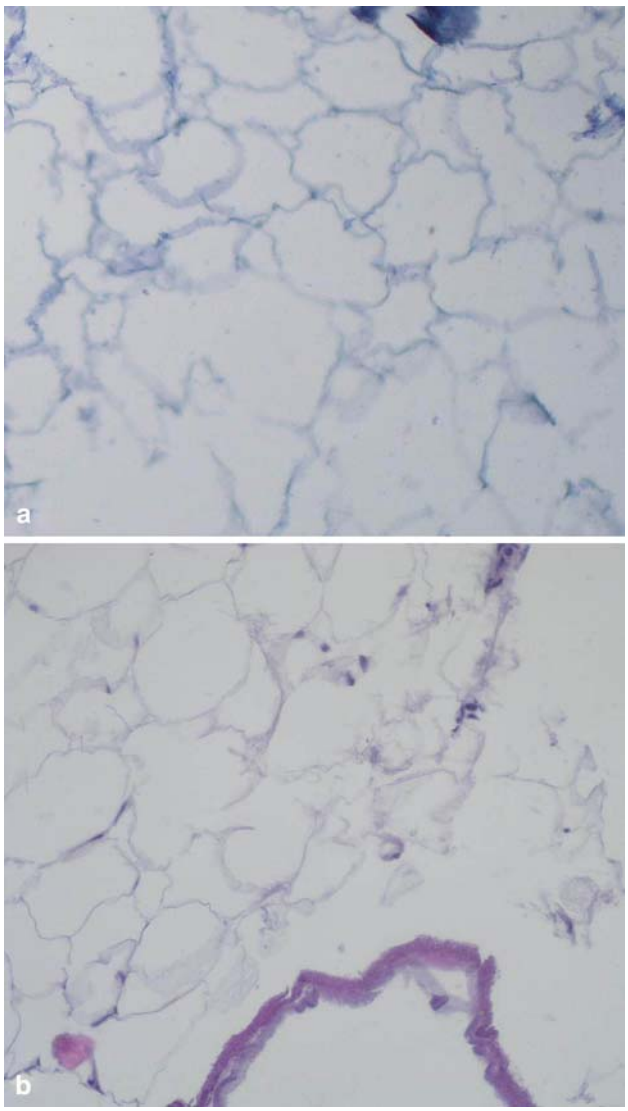

Fig. 1 Hematoxylin-eosin staining of adipose tissue cultures. Magnification 200×. **a** Centrifuged adipose tissue culture. **b** Noncentrifuged adipose tissue culture

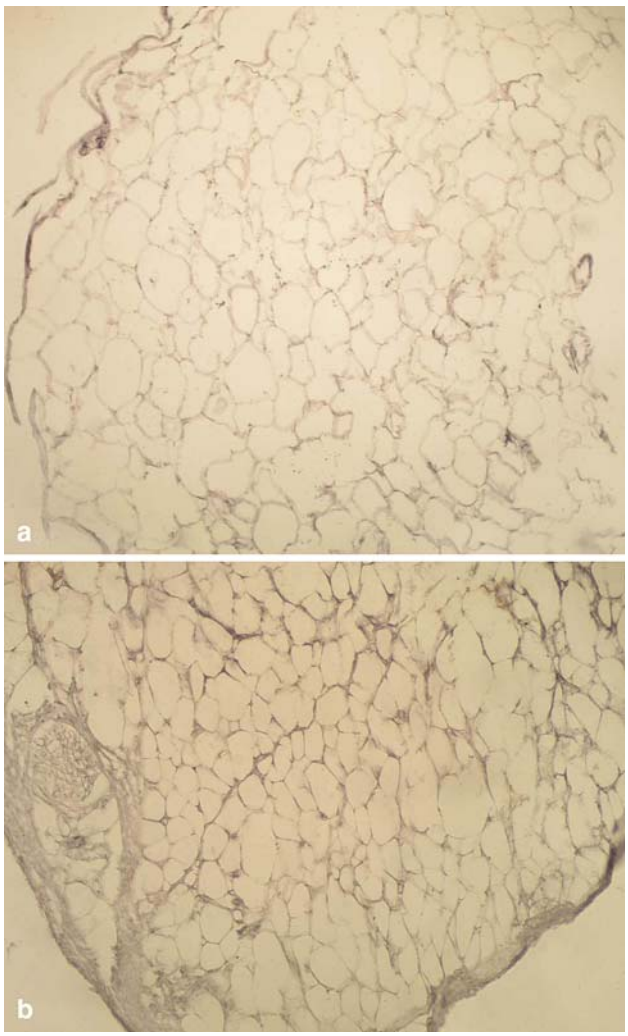

Fig. 2 Leptin immunohistochemical expression in adipocytes and preadipocytes. Magnification 200×. **a** Centrifuged adipose tissue culture. **b** Noncentrifuged adipose tissue culture

cells containing tiny lipid droplets and a roundish, centrally located nucleus. Because of their histologic appearance they were referred to as preadipocytes (adipose cells not yet having finished their differentiation). In the cultures of the noncentrifuged adipose tissue (group II), together with the two types of adipose cells, small fragments of connective tissue were seen. Some capillary vessels and preadipocytes were observed within the connective tissue (Fig. 1).

The immunohistochemical reactions for leptin demonstrated positive activity in the uni- and multilocular adipose cells in both types of cultures. This fact confirmed the presence of preadipocytes in the cultures in view of the fact that leptin is considered a significant marker not only for mature adipocytes but also for preadipocytes [21, 22]. In the cultures of centrifuged adipose tissue (group I) there were single preadipocytes with a positive reaction for leptin. In contrast, in the cultures of noncentrifuged adipose tissue (group II) many leptin-positive multilocular fat cells were observed among the unilocular ones, as well as in the preserved fragments of connective tissue. The other cell types present in the fragments were leptin-negative (Fig. 2).

In both types of cultures there was positive immunohistochemical expression of cyclin D1. This was seen in the preadipose cells located among the mature adipocytes as well as in those present in the connective tissue fragments (Fig. 3).

Semiquantitative analysis showed that as a whole there were more preadipocytes in the noncentrifuged adipose tissue cultures (group II) than in the centrifuged ones (group I) (Table 4).

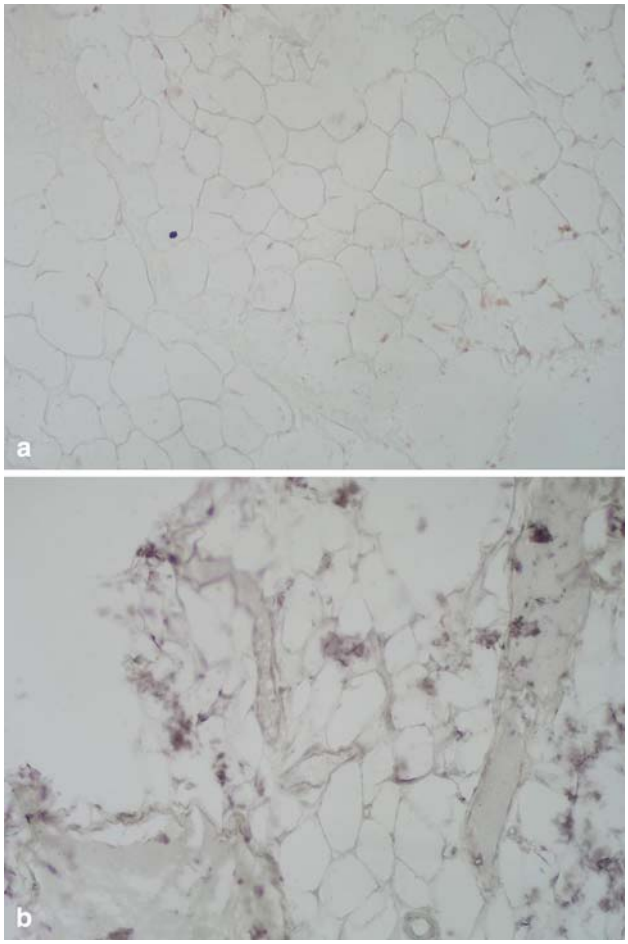

Fig. 3 Cyclin D1 immunohistochemical expression in preadipocytes. Magnification 200×. **a** Centrifuged adipose tissue culture. **b** Noncentrifuged adipose tissue culture

Table 4 Semiquantitative analysis of the experimentally estimated characteristics of the centrifuged and noncentrifuged adipose tissue cultures

	Preadipocytes	Leptin expression[a]	Cyclin D1 expression[b]
Centrifuged adipose tissue cultures	+	++	+
Noncentrifuged adipose tissue cultures	++	+++	++

The number of pluses stands for the amount of cells: + = single preadiopocytes located among the adipocytes, ++ = preadipocytes located in the connective tissue fragments and among the adipocytes, +++ = preadipocytes and adipocytes (adipocytes amount in both samples is marked with one +)

[a] Leptin is expressed in adipocytes and preadipocytes

[b] Cyclin D1 is expressed only in preadipocytes

Discussion

Given the great potential versus the disadvantages of autologous fat grafting, scientific studies have been conducted to investigate the influence of all technical factors on graft survival in order to optimize every parameter of the surgical protocol. The objective of our study was to evaluate two methods of graft purification: centrifugation at 3400 rpm for 3 min and serum lavage (without centrifugation). Using clinical and experimental investigations, our work provides scientific information about each method's influence on graft viability and morphology, as well as on the postoperative outcome.

Patients and surgical techniques were carefully selected as it was previously shown that graft survival depends on atraumatic handling and the creation of optimal conditions in the recipient site [5, 6]. Participants in both groups had no pathology that could cause modifications of the adipose tissue structures. Selected recipient and donor sites were those of utmost quality according to the literature, i.e., the facial region because its naturally higher range of blood supply, and the lateral subgluteal zone because it is an optimal source of histomorphologically and functionally stable adipose tissue [6, 8, 23]. The graft was obtained and reinjected under low pressure. The investigated groups differed only by the process of purification. Thus, by setting not only equal but optimal conditions for lipofilling, we were able to study and compare the postoperative effects only in relation to the two particular types of adipose tissue processing.

According to the professional medical examinations at the end of the follow-up period, positive results (sufficient correction) outnumbered negative results (insufficient correction) after application of both types of grafts. The other two assessments, however, demonstrated a statistically significant difference in favor of the results from the serum lavage purification technique. However, a possibility for misapprehension due to the subjectivity of all chosen assessment approaches must always be taken into consideration. Therefore, attention was given to the biology of the graft.

Data in the literature show that the survival of transplanted cells depends on the oxygen and nutrient supply. Tolerance to ischemia differs between various cell types and between a differentiated and an undifferentiated state [24]. Previous histologic studies found that adipocytes tolerate an ischemic period of 4 days maximum after adipose tissue transplantation [25]. Subsequently, they inevitably undergo necrosis if not sufficiently vascularized. At the same time, experiments showed that immature preadipocytes have a higher tolerance to ischemia than mature adipocytes, proven particularly by measurements of their oxygen consumption [24]. Furthermore, compared with mature adipose cells, immature preadipocytes have a considerably smaller volume which allows quicker revascularization after transplantation and future successful reestablishment in the new location. The adipose tissue

stem-cell compartment [17] was previously studied with regard to aging, growth dynamics, and anatomic locations [26]. It was proven that unlike the mature adipocytes, these stem cells (preadipocytes) have the ability to divide and proliferate and thus are realizing the process of adipogenesis, now recognized to extend throughout life [27, 28]. Their successful in vivo transfer had been shown to result in differentiation into mature fat cells and reaccumulation of lipids [18, 25].

Our experimental results are important for further interpretations and better understanding of these facts. The culture model designed by us tried to mimic the processes in vivo and give some information about the morphologic characteristics of the adipose cells in the recipient site after their transplantation. It allowed us to follow their viability, proliferation, and differentiation. The results showed that neither the method of harvesting nor the two purifying techniques harmed the integrity of adipose tissue cells. Both types of cultivated adipose tissue consisted of typical viable, mature adipose cells and preadipocytes, with preserved original morphology. However, the amount of preadipocytes in the noncentrifuged samples outnumbered considerably that in the centrifuged samples, in which they were exceptionally isolated. These results confirm that the technique of serum lavage is more successful as it preserves better the immature fat cells and thus ensures the development of transplants with significantly higher resistance to ischemia. Furthermore, cyclin D1 expression suggested stronger proliferative activity of the adipose cells in the noncentrifuged samples compared with that in the centrifuged samples. According to the previously mentioned data [18, 25–27], the latter spurs the notion of greater possibility of de novo fat formation in the recipient site after transplantation of adipose tissue processed by serum lavage.

Conclusion

Our data suggest that with transplantation of noncentrifuged (washed by serum) adipose tissue more active preadipocytes are applied that could possibly lead to better survival and even the chance for de novo development of fat. The imperfection and subjectivity of the clinical assessment approaches, however, underlie the need for further investigations.

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