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Histomorphologic and Volumetric Analysis of Implanted Autologous Preadipocyte Cultures Suspended in Fibrin Glue: A Potential New Source for Tissue Augmentation

Thomas Schoeller, M.D.,[1] Sean Lille, M.D.,[2] Gottfried Wechselberger, M.D.,[1] Angela Otto, M.D.,[1] Arian Mowlawi, M.D.,[2] and Hildegunde Piza-Katzer, M.D.[1]

Innsbruck, Austria and Springfield, Illinois, USA

Abstract. Previous efforts to use adipocyte transplants for tissue augmentation have been limited by high and unpredictable resorption rates. Preadipocytes are precursor cells that are capable of replication and differentiation into mature adipocytes. Furthermore, they are more resilient to ischemia, making them a desirable transplant media. Utilizing fibrin glue as a transport vehicle and a prefabricated intramuscular capsule pouch as the recipient site, we have demonstrated the successful transplantation of cultured preadipocytes without the previously presented resorption sequelae. Histological analysis at 2 weeks has demonstrated establishment of vascular supply and the complete resorption of fibrin glue. Most importantly, using planimetric analysis, volume retention has been demonstrated in implanted areas up to 1 year following implantation. Finally, BrdU labeling has been utilized to demonstrate the lack of increased and uncontrolled replication rate, an index of potentially tumorigenic tissue. In conclusion, we have demonstrated a potentially new and safe source of tissue augmentation in the rat model.

Key words: Fat grafts—Tissue engineering—Fibrin glue—Tissue augmentation—Cell culture

The gain in popularity of fat transplantation in the last 15 years as a method of augmenting selected soft tissue deficiencies has been restricted by high resorption rates [1]. The advent of liposuction allowed for readily available suction-derived donor material [2]. The theoretical benefit to this approach was that graft revascularization could be enhanced as a result of particulating the fat by cannular aspiration [3]. However, graft preparation through cellular fragmentation induced by the aspiration process, in reality, did not enhance adipose cellular nutrition [4]. Cellular fragmentation incurred through mechanical handling resulted in devascularization death of many of the living, intact cells and subsequent replacement by fibrosis [5]. As a result, much attention has focused on developing alternative harvesting, processing, and implanting techniques during fat transfer with the goal of improving ultimate transplant survival. Interest in harvesting techniques has stressed the importance of careful tissue handling [6] and has led to several studies comparing blunt suction with sharp suction and suction techniques to direct excision [4,7]; others have published unique atraumatic techniques such as using a "vented" syringe with a Mercedes tip and syringe suction lipectomy [8,9].

Implanting techniques have also been investigated with goals of minimizing the initial ischemic insult to transplanted tissue [10]. Microfat packet transfers and multifragmented pieces for injection have been used in an attempt to maximize nutrient flow upon transplantation by providing a larger surface area to volume ratio [11,12]. Others have had limited success at decreasing initial resorption by providing a well-vascularized recipient bed such as an intramuscular site [11]. Despite all mentioned efforts, when assessed at 6 to 9 months followup, resorption rates have been unacceptable, with transplanted tissue being replaced with significant fibrosis. Histological cross-sections have yielded only rem-

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Correspondence to Thomas Schoeller, M.D., Univ.-Klinik für Plastische und Wiederherstellungschirurgie, Anichstraße 35, A-6020 Innsbruck, Austria

nant cystic spaces with only a small number of adipocytes surviving [4].

Recent attempts at improving graft survival have involved the development of alternative processing methods. Transfer of fat grafts with basic fibroblast growth factor (bFGF) bound to dextran beads has allowed for fat weight maintenance at 6 months follow-up [13]. Fat grafts enriched with serum-free culture medium supplemented with anabolic nonsteroidal hormones have shown improved retention at 15 weeks [14]. Not so successful has been the use of insulin to supplement fat grafts prior to transfer without any benefit to adipocyte retention *in vivo* [4,15]. Finally, the advent of fat cell culture has provided the impetus for the propagation and implantation of precursor adipocyte cells. When excised fat is digested and separated by centrifugation, three layers are obtained including mature adipocytes—the floating layer, an acellular supernatant, and a stromal pellet-containing preadipocyte cell [16]. This preadipocyte layer can be cultured *in vitro* and later replanted into the host.

Previous studies in the field of lipid research have documented that adipose tissue consists of two components: lipid-inclusion containing adipocytes and stromal cells that are devoid of fat goblets [17]. *In vitro* evidence has shown that the stromal cells contain a stem cell compartment which, under certain conditions, can differentiate into mature fat cells [18]. These stem cells, unlike the mature adipocytes, divide and proliferate but do not partake appreciably in lipid storage [19]. This process of adipogenesis, once thought to be confined to the neonatal period, is now recognized to extend throughout life [20]. In fact, successful *in vivo* transfer of immature fat cells (preadipocytes) has resulted in these cells differentiating again into mature fat cells and reaccumulating lipids [21–23]. We proposed that the primary flaw of previous techniques is the result of selecting the wrong component of the adipose tissue. Choosing the preadipocyte cells, which have been shown to be more resistant to ischemia, and including fibrin glue as the delivery vehicle could significantly improve fat graft survival and result in more predictable volume retention [24].

Despite early enthusiasm for the previously published techniques, significant postoperative graft resorption and volume reduction continue to occur, necessitating additional graft placements to maintain satisfactory clinical results. The biological efficacy of the current modalities to enhance the ratio of fat graft take have shown limited success experimentally, and, at best, unpredictable and highly variable outcomes clinically. In this study, we cultured autogenous preadipocytes harvested from the epidydimal fat pad of rats. FIbrin-glue seeded with preadipocytes was then injected into a prefabricated intramuscular capsule. Macroscopic, histological, and volumetric analysis were then conducted during the 1 year time course of the experimental period. The technique of *in vivo* maturation following transplantation of fat cell precursors grown in cell culture may provide potentially new exciting modalities in filling soft tissue defects.

Material and Methods

Animal Model

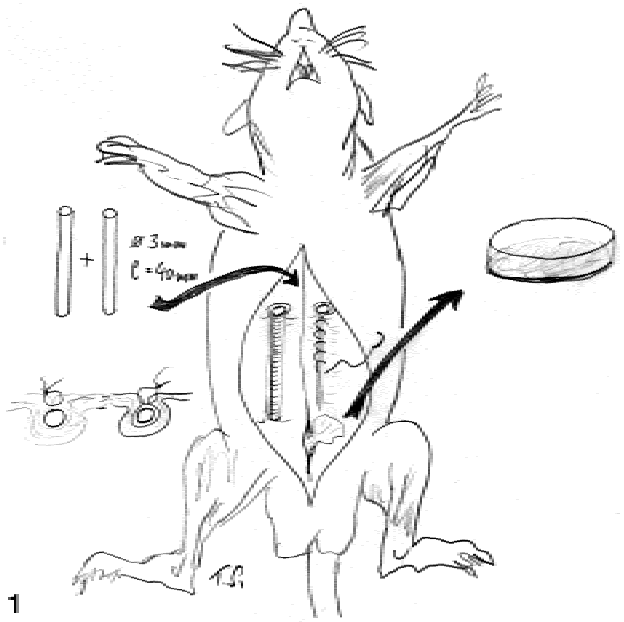
Adult male Wistar rats (total $n = 30$) weighing between 350 g and 450 g were housed in the animal facilities at Southern Illinois University (SIU) School of Medicine. Animals were handled in accordance with the guidelines set forth by the American Association for Accreditation of Laboratory Animal Care, and all protocols were approved by the SIU Animal Care and Use Committee.

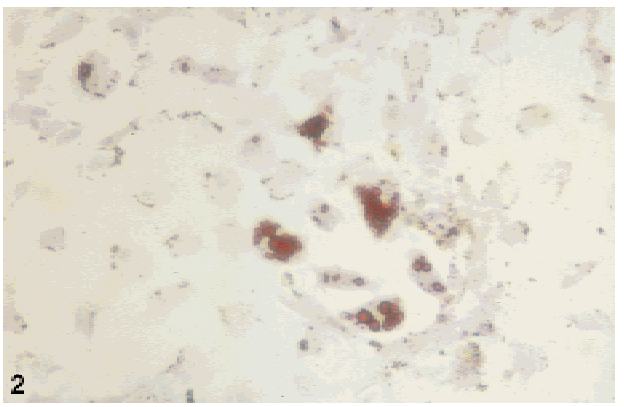
All rats were anesthetized with intraperitoneal pentobarbital (42 mg/kg). All surgical procedures were performed under clean conditions. The abdominal area was shaved and a 5% iodine solution was applied. In each animal, a 6-cm longitudinal midline incision was made over the abdominal skin and the rectus abdominis muscles were exposed. Two sterile, cylindrical silicon tubes (diameter = 5 mm, length = 40 mm) were secured into place by imbricating each rectus abdominis muscle around the implant using a nonabsorbable 4-0 prolene running suture; this resulted in a muscle pouch on both sides and late provoked a fibrous capsule formation around the silicon tube facilitating exact identification of implanted cells. The peritoneal cavity was then opened and the right epidydimal fat pad was harvested (Fig. 1).

From an initial 1 ml of the harvested fat, which was assessed by water displacement, preadipocytes were isolated to a pellet by centrifugation. These cells were resuspended, counted, and plated for culture. After confluence of plates was obtained following 6–9 days of culture (Fig. 2), the cells were resuspended, counted, and combined with fibrin glue as delivery vehicle. Animals were reanesthetized and the abdominal muscular layer was exposed again. A small incision was made at the cephalad end of the muscle-enveloped capsule, and the silicon cylinder was removed (Fig. 3). A purse-string suture using 4-0 prolene was placed at this site and the preadipocyte-fibrin glue suspension was then injected slowly into the cavity. Following injection, the needle was withdrawn around the tightening purse-string suture.

Experimental Groups

Animals were divided into different time course analysis. Group 1 animals ($n = 6$) were sacrificed after 2 weeks, group 2 animals ($n = 6$) after 1 month, group 3 animals ($n = 6$) after 3 months, group 4 ($n = 6$) after 6 months, and group 5 ($n = 6$) after 12 months. The animals were euthanized using a 1 ml intracardiac dose of sodium pentobarbital (160 mg/kg body wt). Both rectus muscles of each animal were harvested for analysis. Histological sectional analysis was performed every 5 mm along the longitudinal capsule axis. The specimens were embedded in paraffin and histological sections were cut at 5 μm for hematoxylin-eosin (H&E) staining and planimetric analysis.





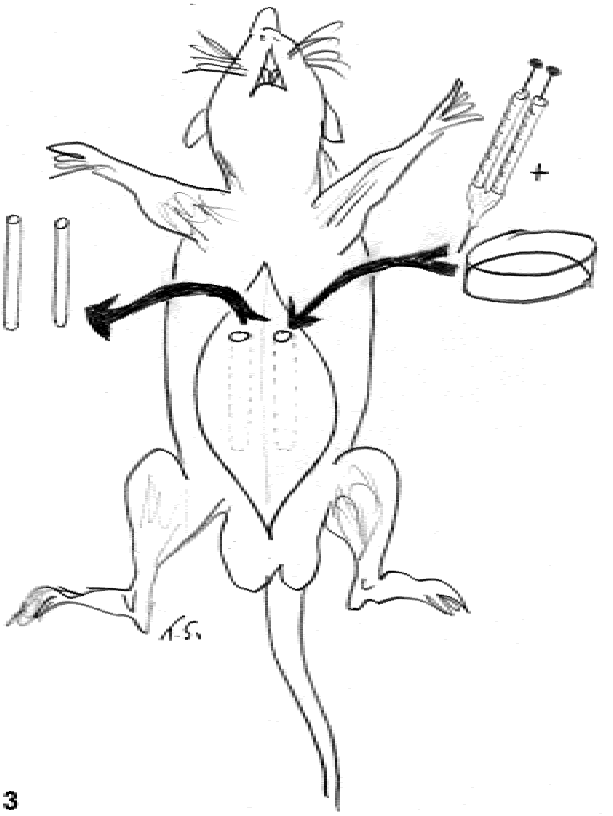


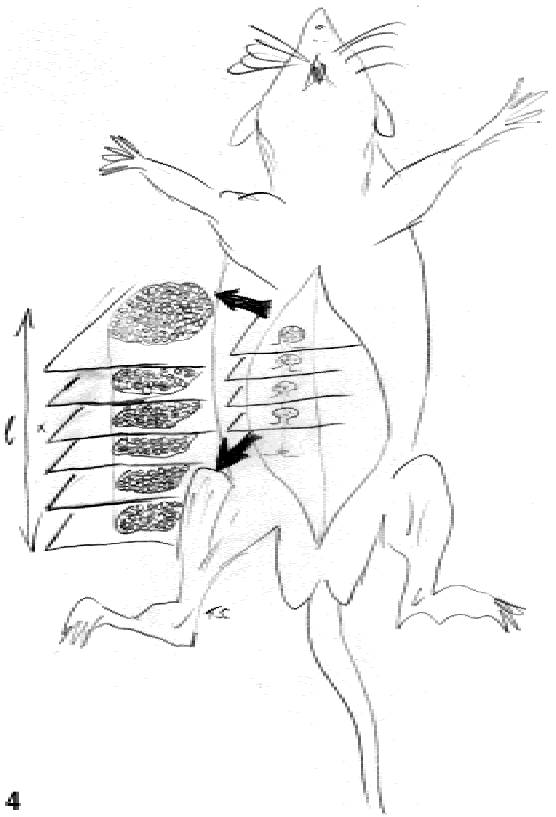
Fig. 1. First stage of the experiment: implantation of a silicon tube into the rectus muscle and harvest of epididymal fat pat for preadipocyte culture.
Fig. 2. Mature culture for preadipocytes before reimplantation suspended in fibrin glue.
Fig. 3. Second stage of the experiment: explantation of the silicon tube and implantation of the matured preadipocyte cultures suspended in fibrin glue.

Cell Preparation

From the harvested fat, 1 ml was washed twice in a buffer of 0.135 M NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.25 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 1.25 mM Na_2HPO_4, 10 mM HEPES, and 1% (w/v) bovine serum albumin (BSA) (Mitchell SE). The tissue was finely minced with scissors and digested 1–2 h in a solution of buffer containing 3.5% BSA, 1.0 mg/ml collagenase, and 0.5 mg/ml glucose at 37°C using a shaking water bath. The cell suspension was centrifuged for 10 min at 250 *g*. Red blood cells were lysed using a lysing buffer of 10% 0.17 M Tris in 0.16 M NH_4Cl for 10 min at room temperature. The suspension was centrifuged, washed with buffer, and a specific volume of cell suspension was counted using a hemacytometer. After another centrifugation step, the cell pellet was resuspended in Hamís F-12 medium/Dulbecco's Modified Eagle Medium (DMEM) in a 1:1 ratio supplemented with 10% fetal calf serum (FCS), 15 mM NaHCO3, 15 mM HEPES, 33 μM biotin, 17 μM pantothenate, 100 U/ml penicillin, 0.1 μg/ml streptomycin, and 10 μg/ml transferrin (Wabitsch), and the cells in 75 sq. cm tissue culture flasks. The flasks were incubated in a humid atmosphere at 37°C and 5% CO_2. After 3 or 4 days, the FCS was removed from the medium and 66 ng/ml insulin and 1 nM triiodo-thyronine (T3) was added to induce adipose differentiation. The medium was changed three times during the incubation period. After reaching confluence, between 6–9 days, the cells were detached using a trypsin/EDTA solution. The cells were resuspended on 0.4 ml of the thrombin/$CaCl_2$ component of fibrin glue prior to reimplantation.

Agents

DMEM, HamísF-12, FCS, HEPES, Trypsin/EDTA, penicillin, and streptomycin were used along with type 4 collagenase, BSA, glucose, biotin, pantothenate, transferrin, insulin, and T3 all resuspended in 1 ml of the



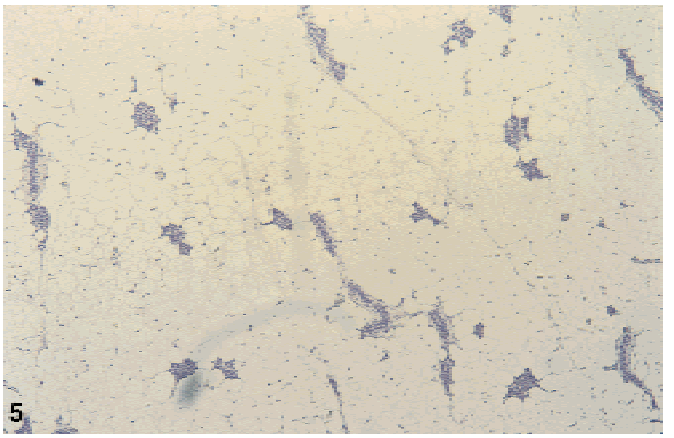


Fig. 4. Method of volume assessment of the harvested specimens: planiometric analysis of 10 consecutive histological cross-sections × length of the fat cylinder (l = 4 cm; length of silicone tube).
Fig. 5. BrdU-labeled cells from the implanted preadipocyte cultures after 2 weeks without uncontrolled cell proliferation.

thrombin/$CaCl_2$ component of fibrin glue and further mixed with 1 ml of the fibrinogen solution at the moment of implantation into the capsule-pouch. The (human) freeze-dried sealer protein concentration contained 100 to 130 mg/ml total protein (70 to 110 mg/ml fibrinogen, 2 to 9 mg/ml plasmafibronectin (CIG), 10 to 50 U/ml Factor XIII, 0.04 to 0.12 mg/ml plasminogen) and was dissolved with a bovine aprotinin solution (3,000 KIU/ml). The 500 IU/ml thrombin component of fibrin glue was mixed with a 40 mmol/ml calcium chloride solution.

Staining

Fat samples from each animal were fixed for 2 h in 10% formalin and slide sections were made. The fat cells in the slides were stained following the methods described by Sorisky et al. [25]. A 0.35% Oil Red O isopropanol solution was diluted with an equal volume of water, filtered, and added to the fixed cells for 2 h. Next, the slide cells were washed and incubated overnight at 4°C with Giemsa stain. Finally, the cells were washed and photographed. Histomorphological slides included those obtained after initial harvest to quantify percentage of preadipocytes in cell population and following various reimplantation periods, as described in experimental groups.

Volumetric Analysis

Volumetric analysis was used to verify the volume retention for each of the experimental groups. Initial volume before culture processing was defined constant as 1 ml. For determination of final volume retention, nine histological cross-sections every 5 mm along the 40 mm cylindrical axis of each animal were obtained and stained following euthanasia. Cross-sections were photographed with a magnification scale and these pictures were then scanned into a standard PC. The area within the capsule containing the fatty tissue was marked manually and a software program was used to calculate the marked area considering the magnification factor. The mean value of these nine areas was used as the basis area to further calculate the volume along the 4 cm longitudinal capsule extension (Fig. 4).

BrdU Labeling

Evaluation of potentially proliferating differentiated and/or undifferentiated preadipocytes from 3-month-old animals was made using the thymidine analog 5-bromo-2′-deoxyuridine (BrdU). BrdU tablets were dissolved and infused in lactated ringers solution to provide for a total dose of 5 mg BrdU/kg weight of animals. Following 1 h of infusion, the animals were euthanized with an overdose of sodium pentobarbital (160 mg/kg body wt). The fat implants as well as control intestinal tissue were then fixed in 4% paraformaldehyde in PBS by perfusion, embedded in paraffin, and cross-sections were cut. Proliferating cells were identified using a monoclonal antibody against BrdU. Stained fat cell nuclei were counted and

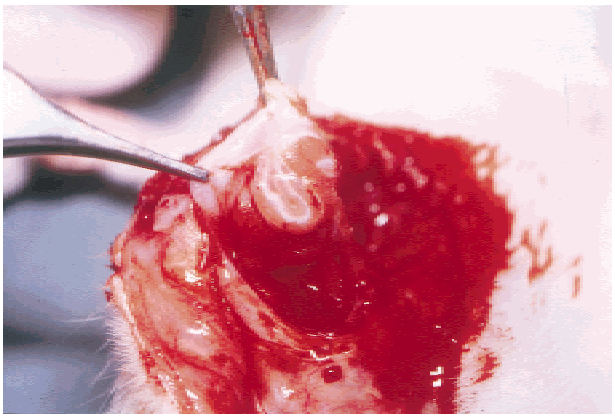


Fig. 6. Macroscopic view after rectus muscle harvest; the fat pad from the implanted preadipocyte cultures is clearly distinguishable from the capsule.

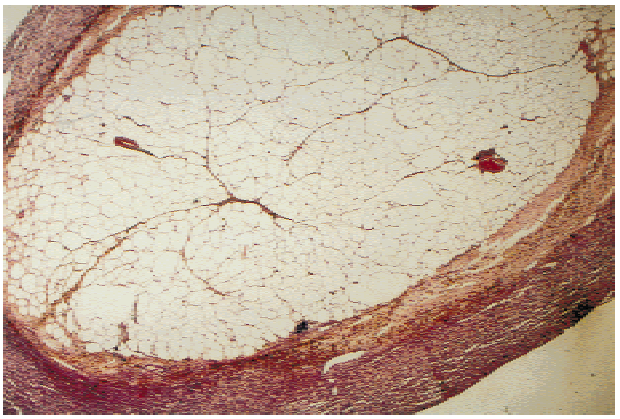


Fig 7. H&E histology of the capsule-surrounded fat cylinder after 1 year showing nearly normal adipose tissue.

the BrdU labeling index (percentage) in the medium was calculated [(stained nuclei/total nuclei) × 100].

Results

After an initial harvest of epididymal fat, 1 ml of fat was further processed to obtain a mean of 5.6×10^4 (range 4.9 to $7.8x \times 10^4$) preadipocyte cells. In fact, the number of preadipocytes represented approximately 20% of the total cell population harvested from histological counts at 40× power. These cells were cultured for 6 to 9 days to achieve confluence and a mean total of 1.3×10^5 cells (range 0.9 to 2.1×10^5). This preadipocyte population was recombined with fibrin glue and implanted into the capsule pouch.

Evaluation of the harvested fat cylinders in the rectus muscle at 1, 3, 6, and 12 months revealed a robust, healthy-appearing lipid structure without evidence of abscess formation and fibrosis. Furthermore, the initial cultured preadipocyte transplant volume (1 ml) was retained in the final volume determinations after 1 year by planimetric analysis, mean volume, 1,12 ml (range 1.02 to 1.20 ml). The statistical results of the volumetric analysis of all harvested fat cylinders at different time points are summarized in Table 1.

Histological analysis of transplanted preadipocytes demonstrated vessel ingrowth and fibrin glue resorption by 2 weeks (Fig. 5). Cell counting at 15 different sites under magnification at a high powered field demonstrated a differentiation of approximately 70% within 4 weeks and 80% of cells and retention at 3 months, and this relationship did not change in the specimens harvested after 6 months and 1 year. At 6 and 12 months, specimens revealed well-organized fatty tissue with only thin, fibrous septa (Fig. 6). No inflammatory cell infiltrate induced by host reaction was present. No evidence of focal cell necrosis, cystic spaces, nor fibrosis was present (Fig. 7). The BrdU labeling index of all specimens showed regular values compared with the controls from intestinal biopsies (Fig. 5).

Discussion

Interest in preadipocyte characterization has been spurred by its potential use as a fat graft substitute, and studying preadipocytes has been aided by their ease of culture [17,26,27]. Preadipocyte counts and physiology have been studied with regard to aging, growth dynamics, influencing growths factors, and anatomic location [28]. In addition, it has been demonstrated that aged preadipocytes may decrease their capacity to both accumulate lipids and to replicate [29]. This has been possible because of the differentiation of mature adipocytes from preadipocytes by the former's ability to express lipid metabolism enzymes of lipoprotein lipase and glycerol-3-phosphate dehydrogenase preferentially [29]. In addition, it has been shown that preadipocytes isolated from genetically obese rats, although greater in number, maintained a decreased ability to differentiate and to replicate [30].

Preadipocytes have been postulated to be more resilient toward ischemia, a desired attribute during the initial period of transplant ischemia from plasma imbibition [24]. In fact, it is likely that surviving maturing preadipocyte transplants undergo transformation by dedifferentiating to the immature preadipocyte stage transiently and then differentiating back into mature cells with the establishment of vascularization. This may obviate the need to place transplants into increased vascularized tissue such as intramuscular or in fat depots.[4].

Fat transplantation has had variable success in the clinical setting. The inability to sample transplanted fat to assess for resorption rates remains a problem. One investigator published successful maintenance of 400 facial infiltrations over a 6-year period by utilizing careful atraumatic aspirated injected fat parcels [31]. However, the resorption success was measured by the cast shadow of the crease as compared with the controlled contralateral side. This is an inadequate assessment, since, as others have criticized, fat survival is impeded by a host reaction and resulting fibrosis, the bulk of which may be inappropriately judged as success of fat retention [32,36].

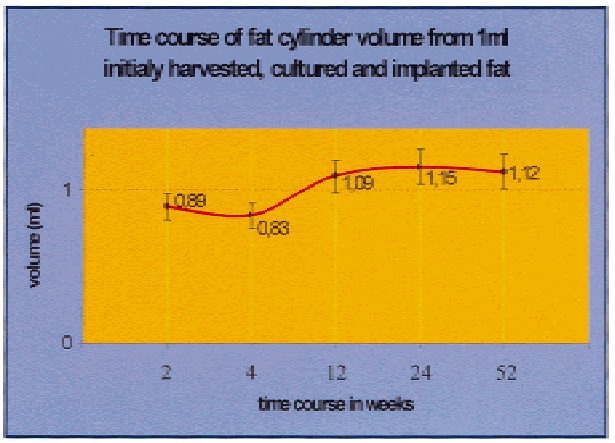


Fig. 8. Graph summarizing the volumetric results after cell culture implantation at different time points.

Our new introduced animal model using the capsule pouch is the first in which a clear identification of the graft, even in the long-term follow-up, and an exact volumetric analysis is possible. A study of nine cases of cheek fat transplants that were later biopsied up to 3 years out revealed pronounced fibrosis between fat lobuli and observation of 40 to 50% resorption. Yet, this investigator found reasonable aesthetic results by utilizing low-power suction and delicate transfer techniques as well as slight overcorrection at time of initial operation [33].

The improved success of transplanting only minimal volumes of fat and limiting transfer into a well-vascularized recipient site has been established [10]. Transplanted fat is revascularized from its outer periphery preferentially down to the center. In an attempt to maximize nutrient diffusability, tissue implants should not exceed 3 mm in diameter [10]. To gain increased bulk, multilayered placement of narrow fat packs can be used in an effort to improve revascularization [33]. However, over expansion of recipient sites must be avoided in order to limit degree of fibrosis [34,35].

Fibrin glue was found to be a suitable delivery with the goal of providing for temporary nourishment as well as a scaffolding for the preadipocytes while waiting for establishment of angiogenesis. It was presumed that the fibrin glue served just this purpose prior to being completely reabsorbed by 2 weeks following transplantation. BrdU labeling of the implanted cultures was necessary to clearly distinguish between a possible invasion of adipose tissue into the capsule cavity and to follow up the fate of the implanted cells in terms of differentiation or malignant transformation. In all specimens over all investigated time courses we found no malignancies and were able to show that the implanted 100% of preadipocytes differentiated into 70% and 80%, respectively, of mature lipid (and therefore volume) storing adipocytes. That means over the course of time slow increase of volume can be expected by reaching the steady state of adipocytes to preadipocytes, which is influenced by several factors such as diet, hormones, activity, anatomical location, genetical predisposition, and individual aging processes.

By providing for both a more resilient preadipocyte cell line as well as a fibrin glue transport medium, we were able to maintain transplanted volume and avoid previously reported foreign body reactions. We were able to retrieve a mean of 1.12 ml of adipose tissue from an initial implantation volume of 1 ml. This volume was comprised of strictly adipose tissue since the fibrin glue infused was completely resorbed by 2 weeks and no evidence of focal cell necrosis, cystic spaces, or fibrosis was identified in histological studies even at 12 months out, the endpoint of our study. Interestingly, net percentage of preadipocyte cells (30%) at 1 month did resemble the initial percentage of preadipocyte cells (20%). The nonreabsorption attained in this study is a definite improvement over previous reported resorption rates of 40 to 60% when followed over 6 to 12 months [32].

This new technique may have he potential to treat wrinkles by injection of preadipocyte cultures or if several passages of those cultured preadipocytes can be stabilized to maintain its characteristics without undergoing dedifferention we are convinced that autologous breast augmentation can be a further indication for this procedure.

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