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Lipoplasty-Derived Stem Cells Lipogenesis "An Experimental Study"

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ABSTRACT

While the fate of fat grafting remains unpredictable, the search for the ideal soft tissue filler continues. Engineered adipose tissue substitutes are not yet available despite their clinical need for both aesthetic soft tissue augmentation and reconstruction following trauma and tumor excision. This experimental study elucidate the potential of using human adipose-derived stem cells harvested through suction assisted lipoplasty and suspended in fibrin glue to produce an engineered adipose tissue substitute.

INTRODUCTION

With the ideal goal being the "replacement of like with like", autologous fat transfer represents a logical approach for soft tissue augmentation and reconstruction. Excluding a minority of reports in which long term survival has been documented, issues of predictability, reproducibility, and long-term efficacy remain significant obstacles to the use of autologous fat transplantation **[1,2]**. Many innovation have been reported to overcome these problems **[3-7]** and the search for the ideal soft tissue filler continues.

Clinical stem cell therapies comprise an attractive alternative. Embryonic stem cells are appealing because of their pluripotential nature that would provide an ideal source of cells with regenerative potential and capability of self-renewal. However, their practical use is limited because of ethical consideration and concerns over cell regulation and control of their growth and differentiation **[8,9]**. Furthermore, they could be rejected by an immunocompetent host **[10]**. On the other hand, adult stem cells, when used autologously, are immunocompatible and have no ethical issues related to their use. Adult stem cells have been isolated from several tissue sources, notably bone marrow **[11]**, but also periosteum **[12]**, skeletal muscle **[13]**, dental pulp and periodontal ligament **[14]** from peripheral **[15]**, placental and umbilical cord **[16]** blood. Recently, an abundant source of adult stem cells that can differentiate into multiple lineages has been isolated from human adipose tissue **[10,17-18]**.

Subcutaneous adipose tissue is a particularly attractive reservoir of progenitor cells, because it is easily accessible, abundant and self-replenishing **[19]**. It is derived from the mesodermal germ layer and contains a supportive stromal vascular fraction (SVF) that can be readily isolated **[10,20]**. This SVF from adipose tissue consists of a heterogeneous mixture of cells, including endothelial cells, smooth muscle cells, pericytes, leukocytes, mast cells and pre-adipocytes **[21-23]**. In addition to these cells, the SVF contains an abundant population of multipotent adipose-tissue-derived stem cells (ASCs). Not only these cells possess the capacity to differentiate in vitro into cells of mesodermal origin such as adipocytes, chondrocytes, osteoblasts and myocytes **[10,24-29]**, but also into neural cells of ectodermal origin **[30]**.

Aim of work:

This study sought to evaluate whether adipose-derived stem cells harvested through suction assisted lipoplasty could be used to engineer adipose tissue in vivo.

MATERIAL AND METHODS

Human adipose tissue was harvested by suction assisted lipectomy from five healthy female donors between the ages of 20 and 35 years. Primary cultures of adipose-derived stem cells were established as described by Zuk et al. **[10]**. Briefly, the raw lipoaspirate was extensively washed with equal volumes of phospho-buffered saline (PBS) to remove contaminating red blood cells and debris. The extracellular matrix was subsequently digested with 0.075 collagenase for 30 minutes at 37ºC. Enzymatic activity was neutralized with Delbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) followed by centrifugation at 1200 x g for 5 minutes. The resulting pellet was

resuspended and cultured overnight in non–induction control medium containing DMEM, 10% FBS and 1% antibiotic/antimycotic solution. The plates were then extensively washed with PBS to remove residual non-adherent red blood cells. Cultures were maintained at 37ºC and the medium was changed twice/week.

Once confluent, the cells were suspended in fibrinogen (10^7 cells/ml) and aliquoted to a 48-well culture plate (200µL/well). A solution of bovine thrombin and CaCl2 was added to form disc-shaped gels or constructs. These fibrin constructs were either cultured in adipogenic media for an additional week to induce differentiation of stem cells, or kept in non-induction medium to act as controls. The adipogenic **[10,31,32]** media consisted of non-induction control medium supplemented by 0.5mM isobutylmethylxanthine (IBMX), 1µM dexamethasone, 10µM insulin and 200µM indomethacin.

Half of the constructs were kept in vitro while the other half of constructs were implanted into the dorsal subcutaneous space of immunocompromised mice for another four weeks. The constructs were harvested, processed and stained with Oil-Red-O and osmium tetraoxide to detect the presence of lipid containing vacuoles within the cells histologically **[17,33,34]**. Briefly, the cells were fixed for 1 hour in a 4% formaldehyde/1% calcium solution, washed with PBS and overlaid for 5 minutes with an Oil-Red-O staining solution that is composed of 2% Oil-Red-O reagent in 70% ethanol:acetone (1:1). Cells were subsequently washed with 70% ethanol and distilled water and counterstained with Mayer's Hematoxylin. In addition, total cellular RNA was isolated and reverse transcribed using conventional protocols as described elsewhere **[10,23]**. The expression of an adipose-specific transcription factor, peroxisome proliferator-activated receptor gamma (PPAR-gamma), using conventional reverse transcription-polymerase chain reaction (RT-PCR) was performed. Non-induced PLA cells were examined as a negative control. Lineage-specific cell lines (3T3-L1) were analyzed as positive controls for the adipogenic lineage.

RESULTS

Following a total of five week period of adipogenic differentiation in vitro, the constructs were processed and stained with the lipogenic stains Oil-Red-O and osmium tetraoxide. Cells developed lipid-filled Intracellular vacuoles which stained prominently red with Oil-Red-O (Fig. 1) and black with osmium tetraoxide (Fig. 2). The controls stained negatively.

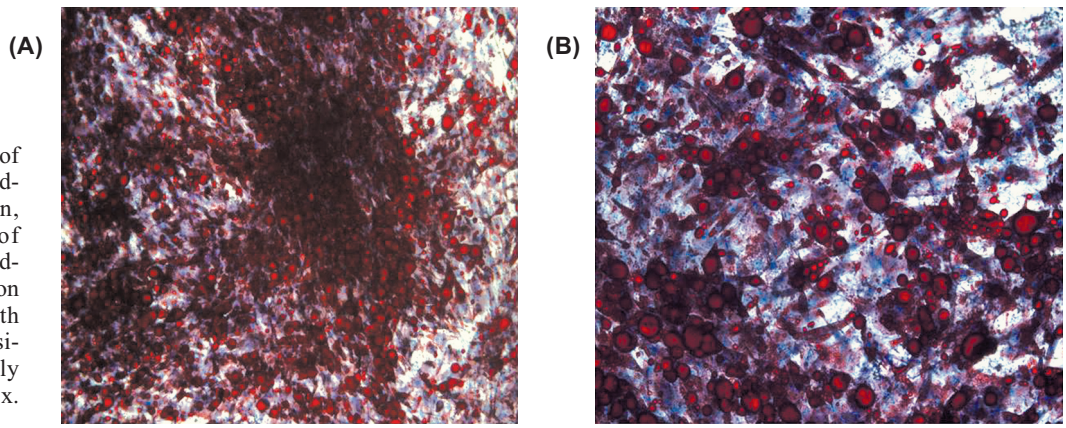

Fig. (1): Staining of costructs with Oil-Red-O, a lipogenic stain, following a total of five- week period of adipogenic differentiation in vitro. (a) Cells with intracellular lipid vesicles stain prominently red at 100x (b) at 200x.

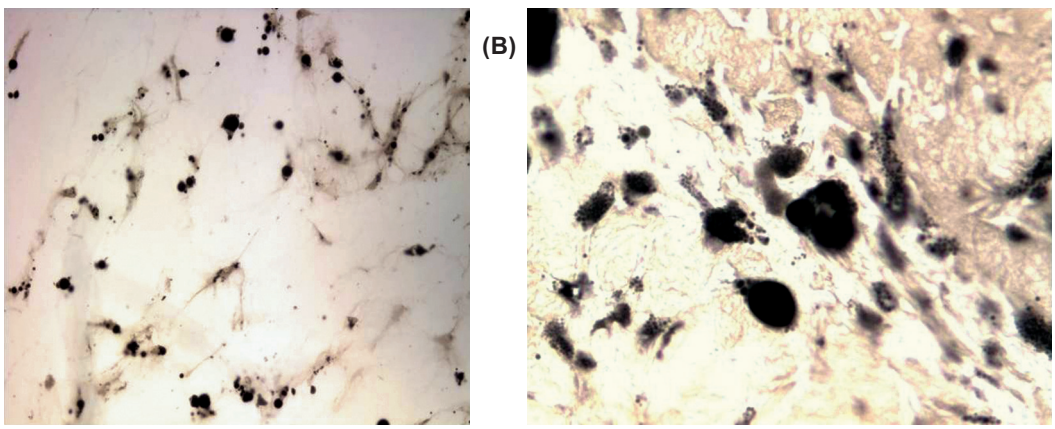

Fig. (2): Staining of costructs with Osmium tetraoxide, a lipogenic stain, following a total of five-week period of adipogenic differentiation in vitro. (a) Lipid containing cells stain black at 100x (b) at 400x.

A minor reduction in construct size was noted and neovascularization was observed in and around the scaffold four weeks after implantation in vivo (Fig. 3). Histological analysis revealed scattered lipid-bearing cells within a surrounding fibrin matrix with no evidence of microcalcification (Fig. 4). The presence of lipid-containing cells was further confirmed by osmium tetraxoide staining (Fig. 5). Additionally, the expression of an adipose-specific transcription factor, PPAR-gamma, using RT-PCR could be demonstrated (Fig. 6). Constructs that remained in the non-induction media were negative, indicating that only constructs cultured in adipogenic medium could be induced and differentiated towards the adipogenic lineage.

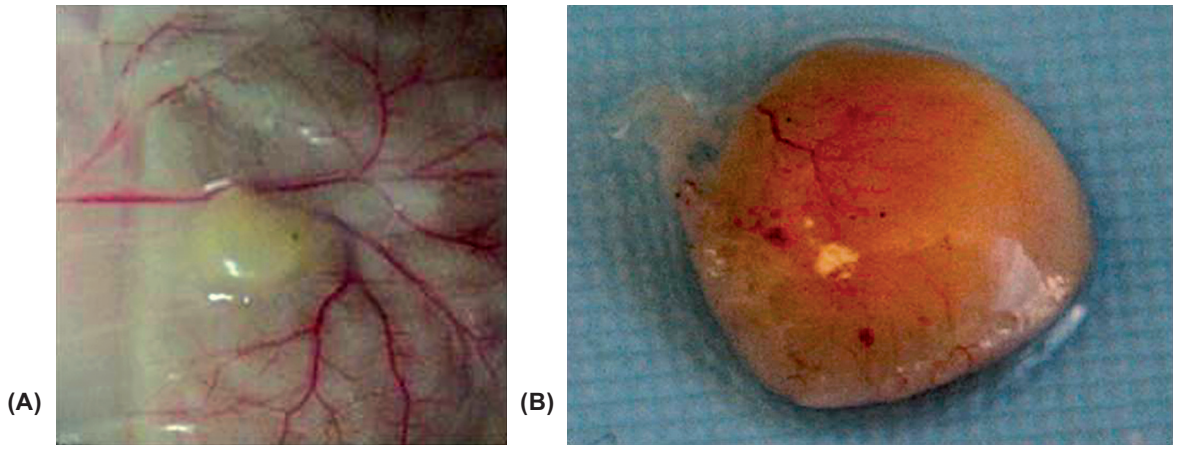

Fig. (3): Four weeks following in vivo implantation, neovascularization was observed around (a) and in the scaffold (b).

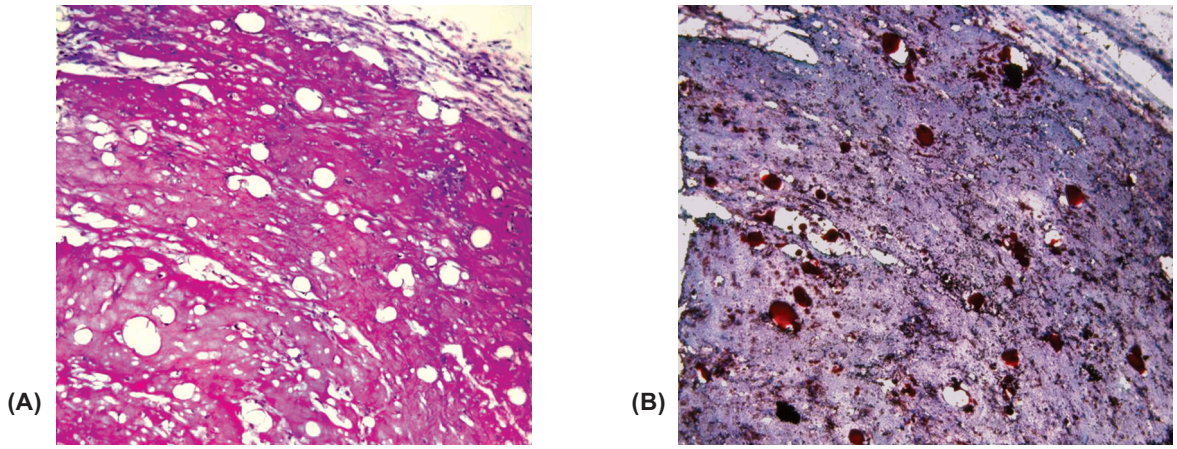

Fig. (4): Scattered lipid-bearing cells within a surrounding fibrin matrix with no evidence of microcalcification as seen following four weeks of in vivo implantation with H&E staining at 100x (a) and Oil-Red-O stain at 100x.

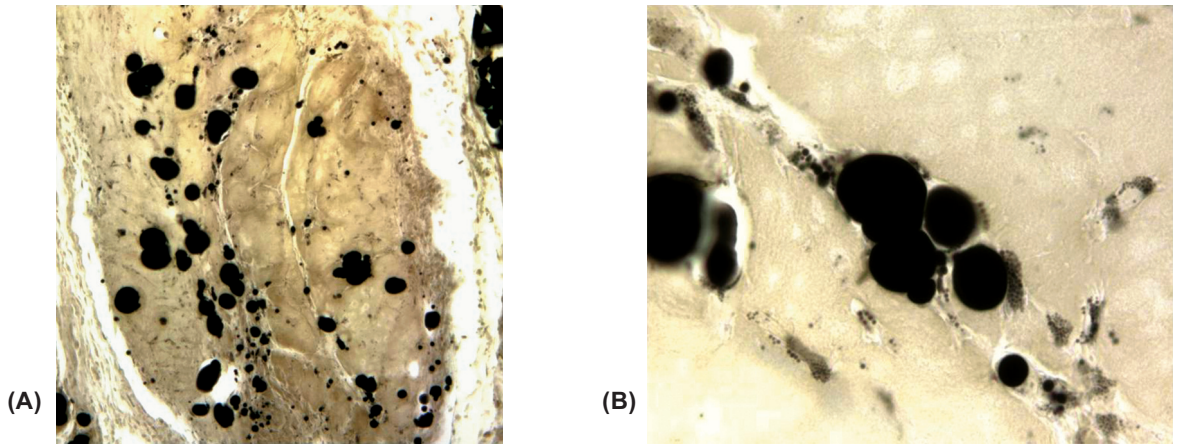

Fig. (5): Staining of costructs with Osmium tetraoxide, a lipogenic stain, confirmed the presence of lipid-containing cells following a four week period of implantation in vivo at 200x (a) and at 400x (b).

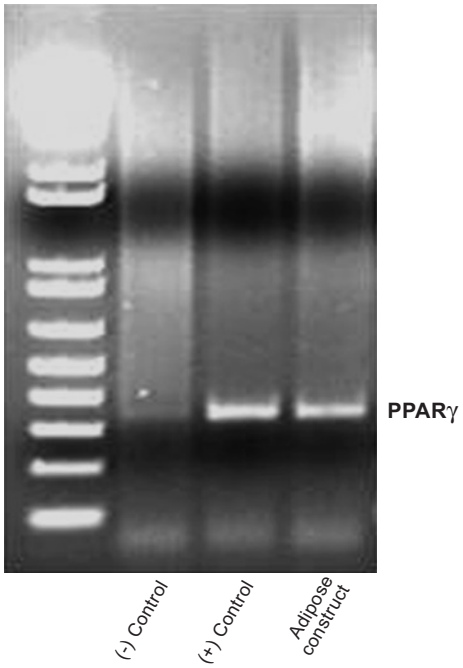

Fig. (6): The expression of an adipose-specific transcription factor, PPAR-gamma, using RT-PCR. Non-induced PLA cells were used as a negative control. Lineage-specific cell lines (3T3-L1) were analyzed as positive controls for the adipogenic lineage.

DISCUSSION

There has been an increased interest in adipose-derived stem cells (ASCs) for tissue engineering applications **[35]**. Zuk et al. **[10]** are credited for first describing adipose tissue as an abundant, accessible and rich source of adipose-derived stem cells with multipotent properties suitable for tissue engineering and the emerging field of regenerative medicine. These adult stem adherent cells were isolated from the fatty portion of liposuction aspirates and were initially termed processed lipoaspirate (PLA) cells. Yoshimura et al. **[36]** further defined cells isolated from the fluid portion of liposuction aspirates (LAF) and verified distinct cell surface marker profiles for them. Yet, they also confirmed quite similar characteristics with PLA cells in regard to growth kinetics, morphology and capacity for differentiation.

The translation of these findings attracted rapidly the plastic surgeons not only because they deal with subcutaneous adipose tissue on regular basis, but also since it could be the answer for some of the problems they face when dealing with soft tissue augmentation and reconstruction. Lipoinjection has long been used to treat facial changes associated with aging and to correct various types of depressed deformities such as hemifacial microsomia and pectus excavatum. It has also been used in breast augmentation by a limited number of plastic surgeons **[6,37,38]**, although the use of autologous fat for breast augmentation has been controversial for safety issues. Microcalcifications resulting from autologus fat transfer might cause confusion in the evaluation of mammograms. Regardless of these controversial issues, it would certainly be an advantage to have adipogenesis without calcification. This study hoped to open a new frontier in soft tissue augmentation and provide a new valuable source of adipogenesis without calcification detected at the site of implantation.

Matsumoto et al. **[39]** compared histologic features and yield of adipose-derived stem cells (ASCs) between human aspirated fat and excised whole fat. Aspirated fat contained fewer large vascular structures, and lower ASC yield. Aspirated fat was transplanted subcutaneously into athymic mice with (cell-assisted lipotransfer; CAL) or without vascular stromal fractions containing ASCs isolated from adipose tissue. The CAL fat survived better (35% larger on average) than non-CAL fat and microvasculature was detected more prominently in CAL fat, especially in the outer layers. They suggested that ASCs differentiated into vascular endothelial cells and contributed to neoangiogenesis in the acute phase of transplantation. These findings may partly explain why transplanted aspirated fat does not survive well and suggest clinical potential of the CAL method for soft tissue augmentation.

Yoshimura et al. **[40]** took it a step further and applied the concept clinically. They freshly isolated stromal vascular fraction containing ASCs from half of an aspirated fat sample and attached to the other half of aspirated fat sample (CAL) with the fat acting as a scaffold. They hoped that this process would convert relatively ASC-poor aspirated fat into ASC-rich fat and performed conventional lipoinjection (non-CAL; n=3) or CAL (n=3) on six patients with facial lipoatrophy due to lupus profundus or Parry-Romberg syndrome. They found that all patients obtained improvement in facial contour, but the CAL group had a better clinical improvement score than did the non-CAL patients, although the difference did not reach statistical significance.

Kimura et al. **[41]** studied adipose tissue engineering from human preadipocytes in vivo. They isolated preadipocytes from human fat tissue and incorporated them into collagen sponge scaffolds followed by subcutaneous implantation into the back of nude mice. In addition, they suspended

the isolated preadipocytes with gelatin microspheres that contained basic fibroblast growth factor (bFGF) first and prior to their incorporation into the collagen sponge scaffold in another group. Adipose tissue formation at the implanted site of collagen sponge was found within 6 weeks postoperatively only in the presence of gelatin microspheres that contained bFGF. They concluded that the combination of gelatin microspheres containing bFGF and preadipocytes with the collagen sponge is essential to achieve tissue engineering of fat tissue. Cho et al. **[42]** delineated that the implantation of adipogenic-differentiated preadipocytes enhances adipose tissue regeneration in vivo, as compared with the implantation of undifferentiated preadipocytes and that cell transplantation-mediated adipogenesis can be further enhanced by the delivery of bFGF. Hiraoka et al43 argued the need for preadipocytes for adipogenesis in their model. They demonstrated that in situ regeneration of adipose tissue in a defect of rat fat pad could be achieved only by combining gelatin microspheres releasing of bFGF to scaffold collagen without the addition of syngeneic rat preadipocytes. In this study, neither growth factors were utilized to induce adipogenesis, nor were collagen sponge carriers used. Adipogenesis was formed by adult human adipose-derived stem cells. Fibrin glue was used as a carrier similar to Mizono et al. **[44]**.

In an attempt to improve delivery vehicles, Flynn et al. **[45]** used only placental decellular matrix (PDM) and PDM combined with cross-linked hyaluronan (XLHA) scaffolds. They seeded them with primary human adipose-derived stem cells and implanted them in a subcutaneous athymic mouse model and assessed them at 3 and 8 weeks. They identified unilocular and multilocular adipocytes by intracellular lipid accumulation histologically and assessed implant vascularization by staining for murine CD31. Their results demonstrated that both scaffolds maintained their three-dimensional volume macroscopically and supported the mature adipocyte populations in vivo. They concluded that incorporating the XLHA had a positive effect in terms of angiogenesis and adipogenesis. Similarly, this study detected adipogenesis from human adipose-derived stem cells not only histologically but also using the reverse transcription-polymerase chain reaction (RT-PCR). Moreover, a non-sophisticated easily assembled carrier was used instead in this study.

The presently demonstrated adipogenic potential of adult human adipose-derived stem cells is consistent with previous studies designed to explore mesenchymal stem cells multipotentiality in vitro **[10,17-19,46-48]**. In addition, it concurs with other studies designed to investigate the ability of adipose-derived stem cells to generate engineered adipose tissue in vivo **[41-45]**. The main distinguishing features of this study are that growth factors were not utilized to induce adipogenesis and that only simple carriers were employed as delivery vehicles. Adult mesenchymal stem cells can be obtained autologously from the same patient for whom adipose tissue is to be engineered, thus eliminating immunologic rejection issues **[46,49]**. It is envisioned that the present approach or a close approximation can lead to therapeutic applications that would serve the plastic surgeons whom are constantly burdened with the clinical needs for soft-tissue augmentations and reconstructions.

Conclusion:

The results of this experimental study demonstrate that the human adipose-derived stem cells harvested through lipoplasty can be utilized to generate engineered adipose tissue in vivo without calcification at the recipient site. An easily assembled carrier was used, however, additional studies will be required to optimize delivery carriers, evaluate the long term viability of tissue engineered constructs and their clinical utility in both cosmetic and reconstructive surgery.

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