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Research Article

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GenAdipose Device gives Clean Lipoaspirate for Generation of Nanofat or Microfat for Autologous Reinjections. CD44-Expressing Adipocytes Observed in Some Human Lipoaspirates

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Abstract

GenAdipose device developed by Genzir Technologies provides clean fat from lipoaspirates to prepare Stromal Vascular Fraction (SVF)-rich microfat or nanofat with filters of varying sizes. Nanofat has gained popularity in recent times in the fields of regenerative medical therapeutics and research beyond cosmetology purposes. Adipose tissue serves as a repository for multipotent stem cells that can transform into chondrocytes, adipocytes, neurons, myocytes etc. based on cell differentiation environment. Nanofat is generated from lipoaspirate of a patient for autologous transfer and consists of high concentrations of stem cells, endothelial cells and growth factors. Nanofat extracted by using GenAdipose device and nanofilters is safe and has applications in cosmetology, arthritis, muscle pain, plantar fasciitis and multiple other chronic disorders. Its therapeutic potential in autologous regeneration has been validated by clinicians and found to have tremendous procedural and qualitative advantage compared to manual methods of obtaining clean lipoaspirates.

Keywords: Lipoaspirate, Adipose-derived stem cells (ADSC), Stromal vascular fraction (SVF), Nanofat, GenAdipose (GA) device, Plantar fasciitis, Arthritis

Introduction

Mesenchymal stem cells in adult adipose tissue were discovered recently in 2001 and since then have been tested in various therapeutic applications [1]. Adipose tissue is the largest endocrine tissue in humans that secretes adipokines to regulate metabolism, energy homeostasis and the immune system [2]. Adipose tissue is a connective tissue comprising of adipocytes, pre-adipocytes, stem cells, vascular endothelial cells, fibroblasts and immune cells (together known as stromal vascular fraction) embedded in collagen fibers. Adipose-derived stem cells (ADSCs) exhibit paracrine activity by secreting active biomolecules such as cytokines, antioxidants, chemokines and growth factors [3]. Hence ADSCs are involved in processes that control angiogenesis, apoptosis, immune response to facilitate therapeutic tissue regeneration. ADSCs are known to secrete vascular endothelial growth factor (VEGF), angiogenin (ANG), platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), and other angiogenic cytokines to facilitate tissue regeneration through angiogenesis. ADSC se-

creted growth factors support nutrients and oxygen supply, and waste removal from the newly formed tissues [4]. Simultaneously, they secrete insulin-like growth factor-1 (IGF-1) to exert protective anti-apoptotic effect in the existing and newly regenerated tissues [5]. Tissue regeneration also requires suppression of inflammation to avoid tissue damage. ADSCs regulate the immune system response by suppressing CD8+ and CD4+ T-lymphocytes, NK-cells, dendritic cells proliferation, and promoting M2 macrophage polarization and regulatory T-cell proliferation [6]. ADSCs are typically identified by a combination of positive and negative cell surface markers, for example, CD105, CD73, and CD90 positivity and CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR negativity [7]. Put together, stromal vascular fraction from adipose tissue has therapeutic benefits and can be used for autologous regenerative medicine.

Embryonic stem cells face ethical challenges, bone-marrow derived stem cells are low in number and induced pluripotent stem cells are limited in therapeutic option as yet [8]. Thus, limitations of stem cell access from other sources, potency, ease of isolation, stem cell number, and procedural difficulties have made mesenchymal stem cells from adipose tissue a good choice for regenerative medicine.

In this short communication, we shall present data from a medical device "GenAdipose" we developed at Genzir Technologies to obtain clean lipoaspirate to generate therapeutic quality of nanofat or microfat.

Methodology

Sample collection: Lipoaspirate from five patients was collected in 1X PBS, pH 7.2 (Sigma) with 1X antibiotic-antimycotic solution (Sigma) and processed the same day for various experiments.

GenAdipose Device: A device was developed to clean up lipoaspirates from tumescent fluid and blood. It comprises of (a) bottom polycarbonate component, (b) top polypropylene cap with multiple ports including a luer lock insert and (c) A mesh that holds the lipoaspirate. Once the lipoaspirate was collected in the device through AI (Adipose In) port, it was washed multiple times (usually 5X times) by addition of normal saline or 1X PBS, pH7.2, by connecting the device through the WO (waste out) port to the vacuum/suction pump. After thorough washing, clean fat was collected back through the luer lock syringe (AO; Adipose Out) port. Typically, 50ml lipoaspirate obtained from the patient gives 15ml of clean adipose tissue.

Enzymatic digestion method to obtain single-cell population from lipoaspirate. Clean lipoaspirate from GenAdipose tissue was collected and subjected to enzyme digestion at 370C for 1hr. Centrifuged at 2000rpm for 5min to collect single cell population at the bottom of the tube and washed with PBS one more time. Top layer of undigested fat and oil was discarded. Cell pellet was resuspended in PBS and counted by trypan blue method. Enzyme Cocktail (Collagenase type I (@ 0.5% + 0.36mM CaCl2 + 1X Antibiotic in 10mM PBS, pH 7.2) added to 5ml clean lipoaspirate for tissue dissociation.

Histochemistry: Three Lipoaspirate samples per patient were collected in 10% formalin. (1) Direct aspirate from patient. (2) After washing with the GenAdipose device. (3) After passing the lipoaspirate collected from GenAdipose device through nanofilter (0.8mm) multiple times (10 in this case). Different sized nanofilters are available to generate nanofat. After fixation for 24hours, the samples were embedded in wax and 6µ sections were cut and placed on slides for hematoxylin and eosin staining [9].

Isolation of stromal Vascular Fraction (SVF) using mechanical shearing with brass filter: This protocol is followed to assess the total number of stromal vascular cells present in a certain amount of lipoaspirate. Actual procedure contains SVF-enriched nanofat with some vasculature and intact adipocytes before re-injections. 40ml lipoaspirate tissue was collected in the GenAdipose device. Lipoaspirate was washed with DNS 4X times. 5ml of clean washed tissue was passed through a 0.8mm brass filter for 40 times (1ml at a time) and collected in a falcon tube. 2ml fresh PBS was added to the nanofat and centrifuged the tube at 2000 rpm for 6min in a Remi-8C centrifuge to collect first batch of SVF. The upper fat layer was collected, passed through the nanofilter again 40 times and centrifuged to collect SVF (Batch 2). Discarded the top oil layers and combined the pellets from two batches in 500µl PBS. Re-suspended cells were counted using the Trypan blue method.

Fluorescence Microscopy: Once SVF was isolated, cells were fixed with 70% ethanol for 15min at RT. Centrifuged at 2000rpm in Remi-8C centrifuge for 7min. The pellet was re-suspended in 1X PBS, pH 7.2 for microscopy. Cells were either directly stained with antibodies or permeabilized with 0.1% Triton-X-100 in PBS for 5min prior to staining and counter-stained with DAPI. 1 million cells each were stained with antibody combination (1) CD44 (FITC-conjugate) and CD34 (R-PE conjugate) or (2) CD105 (Alexa Fluor 488) and CD45 (PE-Cy 5.5 conjugate). CD44 and CD105 are positive markers for Adipose-Derived Stem Cells (ADSC) whereas CD34 and CD45 are negative markers for ADSC. Antibody staining was carried out in the dark for 1hr in cold (4^0C) following manufacturer's instructions. Antibodies were purchased from ICON Biosystems. The stained cells were washed and re-suspended in 50µl PBS and 50µl glycerol, mixed and spotted on slide with a cover slip to capture images under 10X magnification using Leica Fluorescent microscope [9].

Statistical Analysis: p-value was calculated using standard T.TEST for cell counts from five samples in duplicate. $P<0.5$ was considered significant since these are different patient samples and

procedural variation was taken into account.

Safety and sterility studies of the sterile packed devices were carried out in cells and animal models according to ISO standards (ISO 10993-5; ISO 10993-10; ISO 10993-11; ISO 11737-1) by Bioneeds (I) Private Limited, Bangalore (Data not shown).

Results

Quick and clean lipoaspirate using the GenAdipose device

Procedural details on using the GenAdipose device are demonstrated in the video submitted (**Supplementary data**) (**Exhibit Video demonstrating the use of GenAdipose device**). The device facilitates generation of clean adipose tissue in 5-10 minutes by washing with normal saline or PBS that is 3X-5X the volume of lipoaspirate, by connecting the device to the vacuum/suction pump. Manual methods take multiple centrifugation steps or multiple changes of wash buffer using syringes and the wait time is longer than 10 min for each step. The device can be kept upright while collecting the clean fat through the luer lock syringe port.

Architecture of clean lipoaspirate and nanofat

Histochemical analysis done by HnE staining reveals that lipoaspirate after wash and collection from the GenAdipose device had well-spread adipocytes with nuclei and other vasculature. Nanofat derived from passing the clean lipoaspirate had some adipocytes and many single nucleated cells. Most blood cells were washed out, especially red-blood cells (Figure 1).

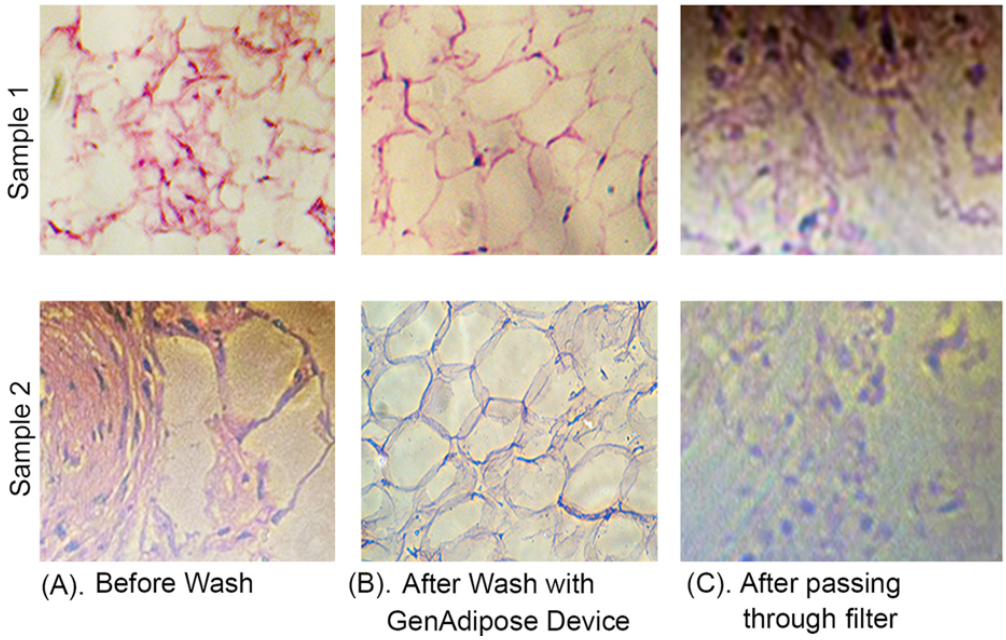

Figure 1: Histochemical analysis of lipoaspirate with hematoxylin and eosin staining. Representative samples demonstrate the architecture of adipose tissue with nuclei, adipocytes, vascular structure and lipid. (A). Immediately after collecting the lipoaspirate (B). Lipoaspirate after washing with GenAdipose device using normal saline (C). Stromal Vascular Fraction (cells) and partial intact adipose tissue after passing through the brass filter multiple times.

Cell count and analysis of SVF with fluorescence microscopy of samples obtained from GenAdipose device

Clean adipose tissue was passed through 0.8 mm nanofilter multiple times to obtain single cell population known as stromal vascular fraction (SVF). Cells were counted by trypan blue method. Cell numbers were typically between 6-8 ± 2 million per ml ($p<0.3$) of clean lipoaspirate. Manual method using enzymatic digestion resulted in less number of nucleated cells and large number of RBCs, confirmed by fluorescent microscopy. Passing the lipoaspirate 10-15 times through the brass filter resulted in SVF and partial intact tissue, whereas passing for 30-40 times through the filter gave higher number of single-cell population (SVF) and lipid layer. The tissue dissociated completely.

Microscopic analysis by immunofluorescence method showed expression of positive CD44 and CD105 cells. CD45 and CD34 expression was absent in freshly isolated SVF. Hematopoietic stem cells are rich in CD34; we did not find them in ADSC derived from GenAdipose device combined with nanofiltration. Expression of positive stem cell markers was robust in SVF isolated with this method. Both nucleated and non-nucleated cells expressed CD44, as seen when stained with DAPI (data not shown). This was expected as CD44 expression is observed on many cell types (Figure 2,3).

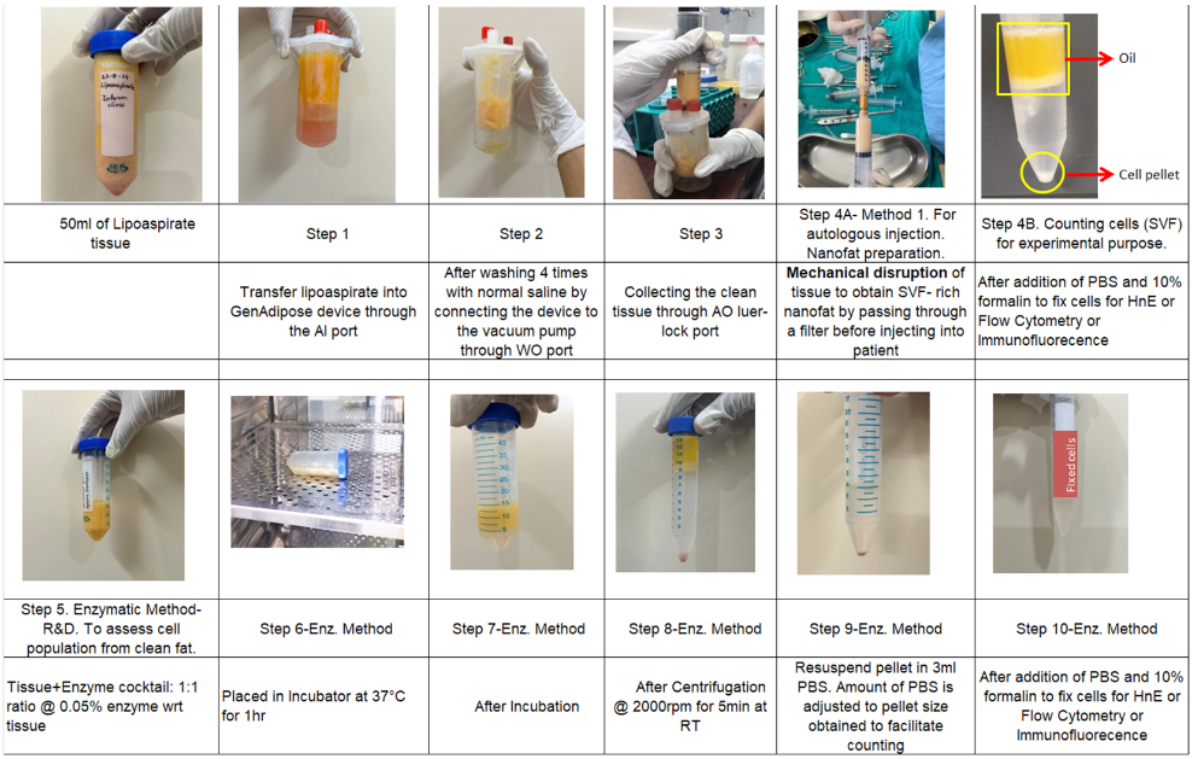

Figure 2: Illustration and step-wise description of GenAdipose device use. Clean lipoaspirates from GenAdipose device can be used for generation of nano/microfat with the help of a filter for re-injections OR can be digested with enzyme to obtain stromal vascular fraction for research use.

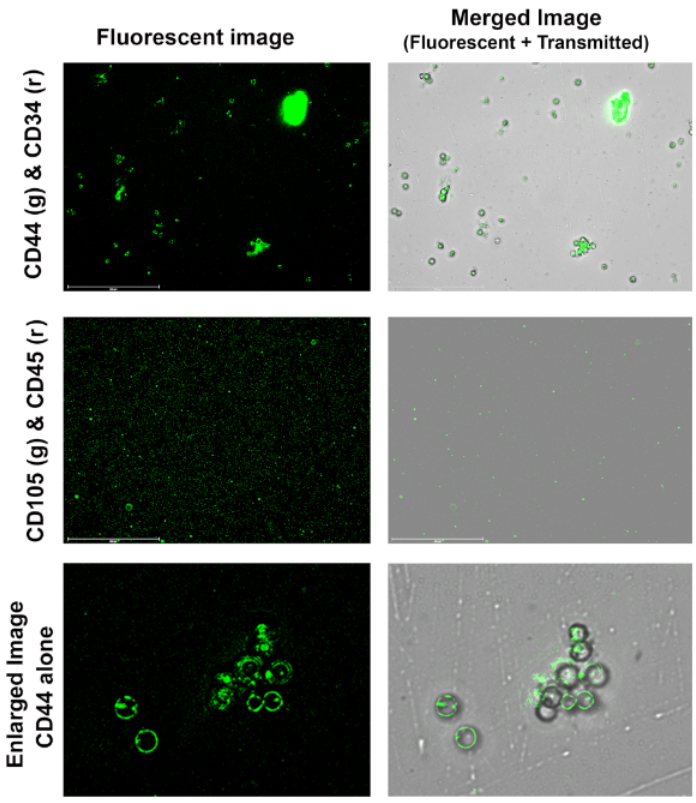

Figure 3: Fluorescent microscopy of freshly isolated stromal vascular fraction (single cells) with GenAdipose device and brass filter reveal positive CD44 and CD105 cells. CD34 and CD45 were negative. Enlarged image of CD44 staining shows expression of CD44 on adipocytes as well along with adipose-derived stem cells. Scale-200 µm. g=green fluorescence. r=red fluorescence.

An interesting finding in one of the human patient samples was the expression of CD44 on intact adipocytes, as seen in image 3C. CD44 is the main hyaluronan receptor, present in most tissues including adipose tissue, muscle, liver, pancreas, and endothelium [10,11]. It has been reported that CD44 has a role in cell aggregation, angiogenesis, endothelial cell proliferation, and immune response [12]. Studies revealed that cd44 gene has a role in the molecular pathogenesis of type 2 diabetes [11] and a positive correlation has been established between CD44 expression in adipose tissue/liver and obesity-related metabolic disorders [11,13-15]. Reports have shown that deleting CD44 gene or inhibiting its function by using anti-CD44 monoclonal antibody reduced obesity-mediated tolerance to glucose and insulin in HF-fed mice [14,16]. Irrespective of obesity status, CD44 antagonism displayed anti-diabetic effects in mice [17]. Depletion of CD44 resulted in suppression of adipose tissue inflammation [18]. This finding opens avenues in developing novel therapies using CD44 antagonists and nanofat grafting to treat ailments in obese diabetic patients.

Discussion

Adipose-derived MSCs possess self-renewal capacity; differentiate into multiple lineages such as chondrocytes, osteocytes, adipocytes etc. (observed in our lab experiments too); exhibit paracrine signalling and immunoregulatory properties [19]. Microfat grafting and derivatives are increasingly becoming a popular line of treatment for chronic ailments. Not just for skin regeneration and aesthetics but ailments like arthritis, joint stiffness, plantar fasciitis, alopecia, hair regeneration, sports injuries etc. have found a place for regenerative approach using SVF-rich fat over traditional line of medication.

In 2007, Rigotti et al. demonstrated for the first-time improved tissue hydration and neo-angiogenesis in severe radiation lesions when injected with adipose tissue (MSCs). Magalon et al. took advantage of this information and used adipose-derived MSCs to cure scleroderma. At the same time, Yoshimura et al. got good results with using fat enriched with SVF for breast augmentation. However, there is a caveat in using this method in obese patients or cancer patients and caution must be practiced. It is believed that ADSCs affect tumor promotion and progression [20-22], but their effects on tumor initiation are not confirmed. Nanofat grafting took an aesthetic turn from 2013 onwards when Tonnard et al. used mechanical digestion of adipose tissue to obtain autologous nanofat for lipofilling [23] (reviewed in JCM 2023). Facial restructuring with autologous fat in healthy patients has been in practice; and cartilage 3D bioprinting using ADSC as seed cells for Rhinoplasty or Arthritis or other cartilage growth requirements are giving promising results [24]. There is tremendous potential in tissue re-modelling using scaffolds and ADSC/SVF. GenAdipose device can be used to obtain SVF for such procedures too.

Plantar fascia is a band of tissue that connects the heal to the toes and often in injuries, it gets inflamed and cause a condition Plantar Fasciitis (PF). 15% medical foot injuries result in heel pain. Autologous fat grafting in the heel has shown good results in PF by Kevin Phan and Mathew Linn [25]; and from patients treated in Hyderabad using GenAdipose device. Injected fat may provide a cushioning effect on the heel and alleviate pain by reducing plantar pressure. However, potential minor risks have been reported by Gussenoff, et al. [26] such as scar tissue formation and that the fat graft may promote fascial remodelling. Some other limitations include variable resorption of fat leading to reduced fat retention, especially in long-term therapy monitoring [27].

Lipogems Technology (2013) produces nanofat for autologous fat transfer and has applications in the treatment of wrinkles, skin scars, alopecia treatment and other related issues [28]. The potential benefits of using GenAdipose device is in obtaining clean

fat to prepare microfat with partial intact vasculature or nanofat preparation with rich SVF. Adipocytes were not completely lost with passing the lipoaspirates through the filters, hence, providing lubrication to the joints and at point of injections. We believe this adds additional benefit to the therapeutic potential of microfat or nanofat. These findings are corroborated by studies conducted by Trentani et. al. using micro-fragmented adipose tissue for Osteoarthritis [29]. Multiple chronic disease indications can be targeted for therapy using autologous fat grating method. Osteoarthritis [30], muscle pain, plantar fasciitis, skin problems, hair regeneration, wound healing have all shown improvement with this therapy by itself or in combination with platelet-rich plasma or other traditional medications.

Ethical Guidelines

Ethical permit to collect lipoaspirates from patients undergoing liposuction at any three different clinical sites was obtained from the institutional ethical committee of Krishna Institute of Medical Sciences, Hyderabad.

Conflict of Interest

There is no conflict of interest to disclose.

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