Exploring the potential of human adipocytes in periodontal regeneration: A review

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ABSTRACT
Stem cells, initially identified in embryonic tissues and later in numerous adult tissues, tend to possess the potentiality to differentiate into various cell types. Though most flexible of all stem cell lines, ethical issues restrict the use of embryonic cells. Furthermore, induced pluripotent stem cells (iPS) and adult stem cells (e.g: bone marrow stroma) can also be used. However, procurement of autologous bone marrow has its potential limitations. An alternate source of autologous adult stem cells which can be procured in large quantities, under local anesthesia, with minimal discomfort would be of keen interest. In the present context, human adult adipose tissue may be the best appropriate alternative source of mesenchymal stem cells. Studies have shown that adipose stem cells (ASCs) extracted from subcutaneous human adult adipose tissue tend to contain heterogeneous cell population called stromal vascular fraction (SVF). It may be used directly or cultured in for selection and expansion of an adherent population, and hence, they are called ASCs. The adipose tissue, obtained by suction-assisted lipectomy (i.e., liposuction), are processed to obtain a fibroblast-like population of cells, also called processed lipoaspirate (PLA). PLA cells has the potentiality to differentiate in vitro into adipogenic, chondrogenic, myogenic, and osteogenic cells in the presence of lineage-specific induction factors. This attributable feature of ASCs may be of significant importance in future clinical cell-based therapy for periodontal disease as well. This review describes current knowledge & recent advances in ASCs & their application. This review describes current knowledge and recent advances in ASCs and their application in periodontal regeneration.

KEYWORDS: Adipose-derived stem cells, Periodontal disease, Processed lipoaspirate, Stem cells, Stromal vascular fraction, Tissue engineering.

INTRODUCTION
Periodontal disease is a chronic inflammatory condition of the supporting structures characterized by persistent inflammation, connective tissue breakdown, and alveolar bone destruction. If left untreated, it continues with progressive alveolar bone destruction, leading to increased tooth mobility & subsequent tooth loss. One of the major goals of periodontal therapy is to obtain regeneration of the affected tissues to their original architecture & function.

Several procedures have been developed to achieve periodontal regeneration, including root surface conditioning,1-3 bone graft placement,3-5 guided tissue regeneration,1-3,5-7 enamel matrix derivatives8-10 & growth factors11 application. However, these procedures used alone or in combination have limitations in obtaining a predictable outcome, especially in cases of advanced periodontal breakdown. So, recent advances in regenerative medicine & stem cell biology have provided the opportunities for tissue engineering & gene-based approaches in periodontal therapy.

Multilineage stem cells have substantial therapeutic potential...
for applications in tissue engineering & gene therapy. Potent stem cells widely useful for these applications are: embryonic stem cells\(^1\) (ESCs), induced pluripotent stem cells (iPSCs)\(^2\), & autologous stem cells.\(^3\) Although they seem to be appealing due to their pluripotentiality, their practical use is limited due to the potential problems of cell regulation & ethical considerations. The autologous stem cells obtained from bone marrow have proven experimentally promising but its procurement is a major issue. Studies have shown that a population of stem cells can be isolated from human adipose tissue.\(^4\) Subpopulation of fibroblasts present within the fat tissues can be processed to obtain the processed lipoaspirates (PLA). These PLA cells, in vitro, can potentially differentiate into adipogenic\(^5\),\(^6\), chondrogenic,\(^7\) myogenic\(^8\),\(^9\) osteogenic cells\(^10\),\(^11\)\(^12\),\(^13\)\(^14\) in the presence of lineage-specific induction factors. The evidences have suggested that the periodontal microenvironment may induce ADMPC (Adipose-derived multilineage progenitor cells) to differentiate into periodontal tissues and that the ADMPC themselves might secrete various factors that stimulate resident progenitor cells.\(^15\) These unique properties make ADMPC an important cell source for stem cell-based therapeutic approaches in the field of periodontology. The effect of autologous mesenchymal stem cells transplantation has been investigated in clinical trials for periodontal regeneration of healthy patients.\(^16\) This review study discusses the potential use of adipose-derived stem cells in the field of periodontal regeneration.

**Isolation of adipocytes-derived stem cells:** Rodbell & colleagues in the 1960s pioneered the methods to isolate cells from adipose tissue using a rat.\(^17\)\(^18\)\(^19\)\(^20\)\(^21\) A study was done by Tobita et al, 2007 in Wistar rat in which control group was passed platelet rich plasma (PRP) only & cases group were passed with adipocyte stem cells & PRP.\(^22\) This study showed that after two to four weeks of implantation, a small amount of alveolar bone regeneration was observed & eight weeks after implantation, periodontal ligament like structure was observed along with the alveolar bone. Human studies were also done by several groups.\(^23\)\(^24\) Different progenitor cells were successfully isolated and characterized from human orbital adipose tissue.\(^25\)

**Obtaining the adipose tissues:** Adipose tissue is harvested by liposuction procedure. Under local anesthesia, around 1cm incision is given, through which a hollow blunted cannula is inserted into subcutaneous space. The cannula is connected externally to a gentle suction which mechanically disrupts fat tissue & draws them out from the adipose compartment. In order to minimize the blood loss & contamination of tissue by peripheral blood cells, a solution of saline & epinephrine is infused into adipose compartment.\(^26\) In this way, raw lipoaspirate (~300 cc) is obtained.\(^27\)

**Processing of raw lipoaspirate:** When preserved at room temperature, the aspirated fat should be processed as quickly as possible. It can be stored or transported overnight without adipose-derived stem cell yield loss or changes in biological properties if it is preserved at 4°C.\(^28\) The obtained raw lipoaspirate is extensively washed with equal volume of phosphate buffered saline (PBS). The extracellular matrix is then enzymatically digested at 37°C for 30 min with 0.0075% collagenase\(^29\) or 40 min with 0.1% collagenase.\(^30\) This enzymatic activity is neutralized with Dulbecco’s modified Eagle’s medium (DMEM), containing 10% FBS(Fetal Bovine Serum) & the cell suspension is centrifuged at 1200 xg for 10 minutes to isolate stromal vascular cells (SVC)\(^31\),\(^32\) pellets from primary adipocytes.

**Obtaining processed lipoaspirates:** SVF pellet is resuspended in basal medium containing 10% fetal bovine serum\(^33\) or in 160 mM NH\(_4\)Cl & incubated at room temperature for 10 minutes to lyse the contaminating red blood cells.\(^34\) The cell suspension is then filtered through 100µm cell strainer / nylon mesh to remove cellular debris. The cells are plated & incubated at 37°C / 5% CO\(_2\) in control medium. The plates are then washed extensively with PBS to remove residual non-adherent RBCs. The medium should be changed in every second day till the cells reach 80-90% confluence. The cells were maintained at subconfluent levels in order to prevent their spontaneous differentiation. In this way, a large number of ASCs can be harvested approximately 2,50,000 cells per gram of tissue.\(^35\)\(^36\) Furthermore, the use of PRP augments the proliferation of human ASCs.\(^37\) ASCs can be successfully stored for more than six months which ensures the availability of autologous banked ASCs for clinical applications in the future.\(^38\)\(^39\)

**Characterisation of adipocytes stem cells:**

1. **Indirect immunofluorecence of PLA cells:** It uses two antibodies; the unlabeled first (primary) antibody specifically binds with the target molecule, and the secondary antibody, that carries the fluorophore, recognizes the primary antibody and binds to it. PLA cells are then processed by using monoclonal antibodies to specific CD markers and lineage-specific protein. The cells are plated onto glass chamber slides, fixed in 4% paraformaldehyde in 100 mM NaPO\(_4\) buffer (pH 7.0) for 15 min.\(^40\) The cells are then extensively washed for 10 min in 100 mM glycine in PBS (PBS/glycine) & blocked for one hour in

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immunofluorescent blocking buffer (IBB) that contains 5% bovine serum albumin (BSA), 10% FBS, 1× PBS, 0.1% Triton X-100) followed by incubation for one hour in IBB containing the following cell-specific monoclonal antibodies:

1) Anti-smooth muscle actin: -To identify smooth muscle cells & pericytes.
2) Anti-Factor VIII: -To identify endothelial cells.
3) ASO2: -To identify fibroblasts & cells of mesenchymal origin.

The cells are again washed with PBS/glycine & incubated for one hour in IBB containing a fluoroisothiocyanate (FITC)-conjugated secondary antibody mounted with a solution containing DAPI(diamidino-2-phenylindole) to detect the nuclei.

2) Spectrophotometric assays:

a) Alkaline Phosphatase (AP): Samples of PLA cells are placed in osteogenic medium (OM) for around six weeks followed by washing with PBS. AP enzyme activity is then assayed using a commercial AP enzyme kit according to the method of Beresford et al(1986). AP activity is expressed as nanomoles of p-nitrophenol produced per minute per microgram of protein.

b) Total Calcium: PLA cells are placed in osteogenic medium for six weeks, then washed with PBS (no Ca++ or Mg++), harvested & extracted in 0.1 N HCl at 4°C for around four hours & lastly centrifuged for five minutes at 10,000 x g. A commercial kit is used to determine total calcium (millimolar Ca++) per microgram of protein.

c) Dimethylsulfoxide: High-density micromass protocol is employed to differentiate PLA cells in chondrogenic medium (CM) for three weeks. PLA nodules are harvested & assayed for sulfated proteoglycans.

3) Cell Senescence Assay: The existence of senescence-associated β-galactosidase (SA-β-gal or SABG) was proposed in 1995 by Dimri et al following the observation that when β-galactosidase assays are carried out at pH 6.0, only cells in senescence state develop staining & not the proliferating cells. It is a hypothetical hydrolase enzyme which helps in catalyzing the hydrolysis of β-galactosides into monosaccharides in senescent cells. Cells from each culture passage (passage 1 to passage 15) are fixed for five minutes in 2% formaldehyde/glutaraldehyde and incubated in a β-Gal reaction buffer (containing 1 mg/ml X-Gal, 40 mM citric acid/sodium phosphate buffer (pH 6.0), 5 mM each of potassium ferrocyanide and potassium ferricyanide, 150 mM NaCl, and 2 mM (MgCl2). Senescent cells (blue) are identified by light microscopy.

4) Flow Cytometry: Flow cytometry helps in analyzing light scatter which distinguishes different cells based on their size, shape, & internal complexity. The antibodies or ligands conjugated with fluorescent probes are used to measure the presence and amount of specific intracellular and cell surface molecules. Similarly, fluorescent indicators help in measuring the transport of ions across the cellular membranes, as well as the mitochondrial activity & other metabolic parameters. PLA samples are cultured in control medium 72 hours before analysis & then flow cytometry with a FAC scan argon laser cytometer is performed. Briefly, cells are harvested in 0.25% trypsin/EDTA & are fixed in ic-cold 2% formaldehyde for 30 min. The fixed cells are then washed in the buffer solution (PBS, 2% FBS, 0.2% Tween-20) & then incubated for 30 min in flow cytometry buffer containing fluorescein isothiocyanate-conjugated monoclonal antibodies to SH3, STRO-1, and the CD antigens: 13, 14, 16, 31, 34, 44, 45, 49d, 56, 62e, 71, 90, 104, 105, and 106. PLA cells are stained with a phycoerythrin-conjugated nonspecific IgG to assess background fluorescence.

Multilineage differentiation potential of PLA cells:
PLA cells possess adipogenic, chondrogenic, osteogenic, myogenic & neurogenic lineages depending upon their cultured in specific induction media (Table 1). DMEM is used for differentiation into all the cell types.

Table 1. Lineage-specific differentiation

| Medium | Serum | Supplementation |
|--------|-------|-----------------|
| Control | 10% FBS | None |
| Adipogenic (AM) | 10% FBS | 0.5 mM isobutyl-methylxanthine (IBMX), 1 µM dexamethasone, 10 µM insulin, 200 µM indomethacin, 1% antibiotic/antimycotic |
| Myogenic (MM) | 10% FBS, 5% HS | 0.1 µM dexamethasone, 50 µM hydrocortisone, 1% antibiotic/antimycotic |
| Chondrogenic (CM) | 1% FBS | 6.25 mg/ml insulin, 10 mg/ml TGF-β, 50 nM ascorbate-2-phosphate, 1% antibiotic/antimycotic |
| Osteogenic (OM) | 10% FBS | 0.1 µM dexamethasone, 50 µM ascorbate-2-phosphate, 10 mM ß-glycerophosphate, 1% antibiotic/antimycotic |
Table 2. Markers and assays of lineage-specific differentiation

| Lineage   | Serum       | Lineage-specific determinant                          |
|-----------|-------------|-------------------------------------------------------|
| Adipogenic| 10% FBS     | Lipid accumulation                                     |
|           |             |                                                       |
| Myogenic  |             |                                                       |
|           | 1. Phase contrast microscopy | 1. Multinucleation                           |
|           | 2. Myosin- and MyoD1-specific and MyoD1 expression monoclonal antibodies | 2. Skeletal muscle myosin heavy-chain |
| Chondrogenic | 1. Alcian Blue (pH 1.0) stain | 1. Sulfated proteoglycan-rich matrix |
|           | 2. Collagen II-specific monoclonal antibody | 2. Collagen II synthesis                        |
| Osteogenic | 1. AP stain | 1. AP activity                                         |
|           | 2. Von Kossa stain | 2. Calcified matrix production                        |

Adipogenesis:

Adipogenic inductive compounds (Table 1):
- a) Glucocorticoid receptor ligands (dexamethasone),
- b) Insulin,
- c) Biotin,
- d) d-pantothenate,
- e) Cyclic AMP agonist (forskolin) &
- f) Equivalent Peroxisome proliferator activated receptor gamma agonist (PPARγ)

In response to these adipogenic induction medium, ASCs tend to differentiate into adipocytes.16,50-52 Within two weeks after placing in adipogenic medium, the ASC contain vacuoles filled with neutral lipid cells. The cells are fixed at room temperature in 4% formaldehyde/1% calcium for 60 minutes, washed with 70% ethanol, incubated in 2% (wt/vol) Oil Red O reagent for five minutes at room temperature. Oil Red O stain (Table 2) is used to stain intracellular lipid droplets accumulation.53 Excessive stain are removed by washing with 70% ethanol & counterstaining is done with hematoxylin for two minutes. ASCs tend to undergo morphological changes & reduce their proliferation rate during the differentiation. During differentiation, parameters including leptin, adipogenic mRNA like fatty acid binding protein, aP2 mRNA levels are increased by several hundred-fold.51,54,55

Clinical implication:
- i. Envisioning the therapies that facilitate cartilage repair
- ii. Substantial cartilage remodeling and repair

Osteogenesis: Osteogenic induction medium (Table 1):
- a) Ascorbate, b) β-glycerophosphate, c) Dexamethasone d) Vitamin D3.

ASCs cells are incubated for three weeks in DMEM containing 1% FBS, 40mg/ml proline, 100 mg/ml pyruvate, 50 mM L-ascorbic acid-2-phosphate, 10 ng/ml transforming growth factor-β.57 The cells are then fixed for one hour with 4% paraformaldehyde & rinsed with PBS. After induction, cell condensation occurs followed by ridge spheroid/nodule formation by two days. Chondrogenesis is confirmed using the histologic stain Alcian Blue (Table 2) at acidic pH (one & below) (pH 2.5, Wako) which stains sulfated proteoglycans present in cartilagenous matrices. Quantitation of sulfated proteoglycan levels revealed a time-dependent increase in cartilage-induced PLA cells up to two weeks of induction, followed by a slight decrease at three weeks. Expression of aggrecan, chondroitin sulphate, cartilagenous collagen type II & IV isoforms are suggestive of chondrogenesis. Standard hematoxylin and eosin staining are used to assess cellular morphological features of the processed lipoaspirate cell nodules (paraffin sections), & the presence of collagens was confirmed with Goldner’s trichrome stain.

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& proteins associated with osteoblasts phenotypes such as osteopontin, osteonectin, osteocalcin, collagen type I, BMP-2 and BMP-4, CBFA-1 are suggestive of osteogenic potential of adipocytes stem cells.\textsuperscript{16,54} The first case of autologous ASC use for osseous repair has been reported in the treatment of a calvarial defect in a seven-year-old girl.\textsuperscript{59} A study involved treatment of four patients possessing large cranial defects with a combination of ADSCs and b-tricalcium phosphate granules. Computed tomography scanning subsequently revealed improved ossification in all of the cases.\textsuperscript{60}

**Myogenesis:**

A. Skeletal muscle: Skeletal muscle myogenic medium (MM) (Table 1):-
   a) 0.1 mM Dexamethasone,
   b) 50 mM Hydrocortisone,
   c) 10% FBS
   d) 5% Horse serum

PLA cells, when placed in myogenic medium (MM) for up to six weeks, result in the expression of the multiple myogenic transcription factor myod1, myosin, myf6, myf5 & myogenin\textsuperscript{16,18} followed by fusion & the formation of multinucleated cells that expressed the myosin heavy chain.\textsuperscript{61} The expression of myogenic transcription factors can be confirmed by immunohistochemical staining & real-time-PCR. Thus, skeletal myogenesis is peculiarized by a period of myoblast proliferation, followed by the expression of muscle-specific proteins and fusion to form multinucleated myotubules.

B. Smooth muscles: Smooth muscle inductive medium (Table 1):
   a) MCDB131 supplemented with 1% FBS &
   b) 100u/ml of heparin (for up to six weeks at 37°C with 5% CO\textsubscript{2})

The expression of smooth muscle specific proteins like: smooth muscle actin (SMA), calponin, SM22, smoothelin, h-caldesmon & smooth muscle myosin heavy chain (SMMHC)\textsuperscript{62,63} are suggestive of myogenic potential of adipocytes stem cells. The smooth muscle differentiation has effect on cell size, shape, membrane potential, metabolic activity & responsiveness to external signals.

Differentiated SMCs exhibit two specific phenotypes\textsuperscript{64} namely,

i. Synthetic & proliferative
ii. Contractile and quiescent phenotype

Contractility is an important characteristic of SMCs which plays role in angiogenesis, blood vessel maintenance, & mechanical regulation of hollow organs such as bladder. This property can be of value in the repair of smooth muscle defects in the gastrointestinal and urinary tracts.

**Neurogenesis:** Human ASCs also exhibit neuronal and/or oligodendrocytic markers. The neurogenic induction medium include (Table 1):- DMEM enriched with 500 mM IBMX, 200 mM INDO, & 5 mg/ml insulin for 1 hour. PLAs in neurogenic medium, when express neuronal markers like:- S100, NF70, NSE, NeuN, MAP-2, nestin in addition to GFAP and GalC as markers of astrocytes and oligodendrocytes respectively, are suggestive of neurogenic potential of ASCs. It is still unclear that whether the transplanted cells replace the lost neurons or provide a supporting scaffold for existing stem cells and injured neurons. Specifically, these PLA cells undergo retraction, forming compact cell bodies with multiple extensions. Western blotting test confirms an increase in NSE protein upon induction while real-time-PCR analysis confirms the expression of nestin. This sums up that the expression of nestin, NSE, & NeuN, along with absence of choline acetyltransferase, myelin-basic protein, or GFAP expression, suggests that PLA cells may be capable of assuming an early neuronal or neural precursor phenotype.

**DISCUSSION**

The ultimate goal of the treatment of periodontal disease is the reorganization of functional tissues, predictable regeneration of the lost periodontal tissues and eventually achieving the periodontal health. The regeneration of periodontal tissue requires the restoration of cementum, periodontal ligament, and alveolar bone.\textsuperscript{65} A number of surgical regenerative techniques including osseous grafts,\textsuperscript{3-5} alloplast grafts,\textsuperscript{3} guided tissue regeneration (GTR) technique,\textsuperscript{1,3,5} enamel matrix proteins,\textsuperscript{8-10} chemical mediators,\textsuperscript{11} have been developed to regenerate the lost periodontal tissues. Although these treatment modalities have been widely accepted, the amount of tissue regenerated could not be well-predicted in cases of advanced periodontal defects. So, periodontal tissue regeneration with autologous stem cells (embryonic stem cells, iPSc, bone marrow mesenchyme stem cells) was introduced for cell-based therapy in periodontal diseases. However, the procurement of such stem cells has its own potential limitations.

Till date, human adult adipose tissue may be the best suitable alternative source of mesenchyme stem cells which is obtainable in large quantities, under local anesthesia, with minimal site morbidity & patient discomfort. This alternative source of autologous stem
cells contain multilineage potential to differentiate towards the adipogenic, osteogenic, chondrogenic, neurogenic & myogenic lineages with appropriate medium supplementation. Following induction, the differentiation to specific lineage can be assessed using histology & immunohistochemistry.

Tissue-specific scaffolds (stable supporting structures), signalling systems & vascularization are the major pre-requisites for the differentiation of stem cells into the desired cells & use them effectively to construct the three-dimensional (3D) tissues. Various scaffolds that can be used are: - collagen I (excellent cellular compatibility), porous collagen bead (injectable cell delivery vehicles), placental dermal matrix, XLHA (hyaluronan sulphate) incorporated in placental dermal matrix, hyaluronic acid gel, HFIP silk fibroin chitosan scaffold, gelatin sponges, monofilament polypropylene, polyglycolic acid meshes etc. The autologous transplantation of MSCs in combination with tissue engineering, such as cell sheet technology, has been shown to be effective for regeneration of the periodontium.\(^{66,67}\)

A histological analysis after injection of ASCs+PRP admixture into periodontal defect showed a small amount of alveolar bone regeneration at 2\(^{nd}\)& 4\(^{th}\) weeks of implantation and a periodontal ligament-like structure at 8\(^{th}\) weeks.\(^{32}\) The effect of autologous MSC transplantation has been investigated in clinical trials for periodontal regeneration of healthy patients.\(^{20,68}\) Some studies have shown that the periodontal microenvironment induces ADMPC to secrete various factors that stimulate resident progenitor cells to differentiate into periodontal tissues.\(^{27}\)

Although many clinical trials have demonstrated the efficacy of autologous MSC transplantation, their clinical application is limited by age restrictions, tissue quality and systemic diseases (such as diabetes mellitus, rheumatoid arthritis, systemic lupus erythematosus), which alters the intrinsic properties of MSCs and also increase the difficulty of isolating MSCs.\(^{69}\) Efficient use of allogeneic MSCs may be an alternative strategy that overcomes the limitations of autologous MSC transplantation procedures for the regeneration of large periodontal defects.

In recent years, evidence has supported periodontal regeneration by allogeneic MSC transplantation in periodontal defects using animal models.\(^{70}\) Bone-morphometric analysis following the allogeneic transplantation of ADMPC in a micro-mini pig periodontal defect model showed a significant amount of bone regeneration ability. The histologic analysis showed a comparable regeneration potentiality of ADMPC by allogeneic transplantation as compared to those of autologous transplantation. These results indicate that ADMPC have immune-modulation capability that can induce periodontal tissue regeneration by allogeneic transplantation.\(^{65}\)

**CONCLUSION**

The future of engineering the mesodermally derived tissues from stem cells is very promising & it definitely requires a readily available source of donor cells. Although further characterization of the PLA cells within adipose tissue is necessary, the results obtained from large number of studies suggest that adipose tissue may be another potent source of pluripotent stem cells with multiple germline potential.

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