Stem Cells – Regenerative Medicine using non-fetal sources

Introduction:

Menstrual blood, sub-cutaneous fat or adipose tissue, bone marrow, milk and the dental regions of the human body **are other newly explored areas for rich source of stem cells** of different lineage and properties. Some of these sources are even better in terms of stemness and other characteristics (like having a rich cytokine profile) and can be easily grown in vitro as compared to many stem cells obtained from already established sources. Stem cells from these sources are easy to obtain without jeopardizing the patient's health and safety. (1) Secondly they do not require invasive procedures, nor there are any ethical dilemma. These cells also have tremendous potentialities in autologous stem cell therapies. Menstrual blood, for example, is thrown away like cord blood or placenta. (1) However, the presence of rich source of stem cells in the menstrual blood has recently initiated the concept of 'menstrual blood banks' like cord blood bank. It is estimated that 8 to 10 ml of menstrual blood can have 10 to 100 million of mesenchymal stem cells and, therefore, on an average trillions of mesenchymal stem cells can be harvested and collected from 30 to 35 ml of menstrual blood. Throwing away such biological substance amounts to massive wastage of precious source of Stem Cells.(2)

Menstrual Blood Stem cells:

The Endometrium is made of simple columnar epithelial cells and consists of two zones namely the inner functionalis and the deeper basalis overlying the myometrium. The functionalis layer sheds every month with menstruation and is adjacent to the uterine cavity. This layer is further divided into stratum compactum, a superficial layer next to the uterine cavity and stratum spongiosum, the deeper part composed mainly of blood vessels, stromal cells and loosely arranged cells. The lower basalis contains the lymphoid aggregates and blood vessels and normally remains unaltered during the process of menstrual cycle. It serves as the germinal compartment every month for generating new functionalis. It is postulated that this layer contains the niche for adult stem cells or progenitor cells. (3, 4, 5)

During each ovarian cycle the endometrial lining of the uterus undergoes shrinkage, necrosis and is almost entirely expelled and reconstructed periodically. This process is totally under the ovarian hormonal regulation of estrogen and progesterone. Characterization of adult stem cells in the endometirum is extremely tough because these stem cells constitute only a very small part of the endometrial tissues. In 2004, for the first time it was reported that these small proportions of adult stromal cells, obtained from the endometrial tissues or lining after biopsy, exhibited clonogenicity (capacity to differentiate and self renewal properties). (6) Therefore, it was established that endometrium harbours two types of adult stem cells, one clonogenic epithelial and the other stromal cells. (7, 8)

There is still a limited literature and information pertaining to menstrual blood stem cells (MensSC's), collected directly from the endometrial tissues. But recent research and accumulating evidences has substantiated the presence of epithelial and stromal / stem cells in endometrial tissues. (9, 10) This dynamic behaviour of the endometrial tissues during each menstrual cycle has made it one of the most convenient sources in the search for endometrial stem cells. (11, 12) Collection of menstrual blood is extremely easy using a silicone grade cup and large volumes can be collected in 1 to 4 hours without the requirement of any invasive procedures.

Schwab and Gargett demonstrated the presence of endometrial stem cells through characterization studies by expressing the perivascular markers of CD146 and PDGF-R Beta, phenotypes which are

quite similar to MSC's. (13) Dimitrov and hic co-workers (14) observed that these stem cells are positive for CD 105, CD73 and CD 90 and absence of haematopoietic stem cell surface antigens and HLA-DR thereby suggesting a stem cell profile close to mesenchymal stem cells (MSC). (15)

SSEA-4, Oct-4 or octamer 4 are unique pluripotent stem cell markers which normally varies with the age of the stem cell, was also found to be expressed in the endometrium and not in the myometrium, therefore, suggesting the presence of embryonic or pluripotent like stem cells from the endometrial lining of the uterus. (16, 17) Brentz and his colleagues further supported the presence of OCT-4 in the follicular and the luteal phase of the endometrium (18). One word of caution, however, exists among the researchers is that Oct-4, a pluripotent marker is also present among germ cell tumours and embryonic carcinomas, therefore, indicating the need for a complete re-assessment of all marker profiles. (19)

In 2007, Meng and his co workers named a sub set of endometrial mesenchymal stem cells as endometrial regenerative cells or ERC's which in comparison to bone marrow derived mesenchymal stem cells and adipose tissue derived stem cells has a more rapid proliferation and growth kinetics including higher angiogenic properties and growth factors. (20,21) Allickson et.al has shown that Menstrual Stem cells or MenSC's can be cultured upto 47 times before these went senescence. (22)

Hida et.al in 2008 through their pioneering animal study involving myocardial infarction showed the therapeutic efficacy of MenSC. The research group reported the transdifferentiation of MenSC's in nude myocardial infracted rats by injecting MenSC' s into centres and margins of the infarcted rats. When compared with BM-MSC, the GFP labelled MenSC's showed better recovery and proved the presence of cardiac troponin-1 and sarcomeric alpha actinin after immunostaining studies. (23)

Menstrual blood have immunomodulatory properties and based on this concept, Phuc et.al has shown that mononuclear cells collected from menstrual blood stem cells can also help in the generation of dendritic cells from menstrual blood derived monocytes like umbilical cord blood or peripheral blood or haematopoietic stem cells.(24)

Murphy et.al in 2008 has reported the therapeutic efficacy of menstrual blood derived stem cells injected intra muscularly in mice model for the treatment of critical limb ischemia after ligation of the femoral artery. (70)After 14 days, intact limbs were observed in case of all the mice with signs of normal walking. To test this efficacy the author has registered for a clinical trial. (25)

Cui et.al in 2007 through a pioneering work showed the therapeutic use of MenSC's in mice suffering from Duchenne Muscular Dystrophy. Immunohistochemistry studies using DAPI revealed the presence of both animal and human nuclei in the dystrophin positive myocytes as compared to control or dystrophin negative myocytes. (26)

In murine model using Streptozotocin induced diabetes, it has been shown that human endometrial mesenchymal stem cells when differentiated and then transplanted showed better recovery compared to undifferentiated human menstrual endometrial mesenchymal stem cells (hEMSC) and control thereby suggesting the fact that human endometrial mesenchymal stem cells can be differentiated into insulin producing cells. (27) Human endometrial derived mesenchymal stem cells has the capacity to develop and differentiate into dopamine producing neuronal cells after their intra cranial transplantation in animal models of Parkinson's disease. These animals showed significant improvements in striatal dopamine and dihydroxyphenylacetic acid concentrations. (28, 29)

However, in diseases like endometriosis it is proposed by the scientists that endometrial stem cell/progenitor cells, apart from bone marrow derived mesenchymal stem cells, might play an important role by reaching and adhering to ectopic areas like the walls of the peritoneal cavity because of inappropriate shedding resulting in an extreme pain in the peritoneal cavity of the patient. (30) This is one of the theories apart from the classical retrograde menstruation theory as proposed by Sampson. (15) Although no such direct evidence suggests the role of endometrial stem cells in the pathogenesis of endometriosis different studies have suggested the evidence for ectopic endometrial growth in many animal studies. (31, 32)

Breast milk stem cells:

Breast milk contains a lot of important bioactive and other unknown nutritional components that are beneficial in imparting immunity to the neonate as studies have shown that the cells in early lactating mothers are predominantly leukocytes unlike the mature human milk which are mainly epithelial in nature. (33) Also the neonate for the first 6 months survives only on breast milk. Further research has revealed the presence of a profound cellular hierarchy in breast milk starting from the early stage stem cells which might be pluripotent in nature to progenitors and more adult or differentiated stem cells. (33)

Presence of cells in the mammary glands during lactation was first identified by Anton Van Leeuwenhoek, but it was Alexander Donne in 1837 who confirmed granular bodies and granules in colostrum. (34) Presence of mesenchymal stem cells was first proposed by Bizzozero and Vassale but Czemy and Gruber considered them as leucocytes whereas Cregoire and Engel considered these cells as epithelial cells. (34) Later on different scientists found different cells in the breast milk using sophisticated technique and concluded the presence of foam cells, leucocytes, histiocytes and epithelial cells. Like the endometrial system, the mammary gland can undergo maturation only during pregnancy and lactation after child birth with different repeated cycles of proliferation, differentiation and apoptosis with respect to hormonal signals. (35)This repeated ability of the breast to undergo remodelling has formed the basis of presence of stem or progenitor stem cells in the breast. (36)

Scientist observed the presence of epithelial colonies when breast milk was cultured in vitro through multiple passages and this was further proved and confirmed by Cregan in 2007 who revealed the presence of stem cells and progenitor stem cells in the breast milk. (37) These cells under in vitro culture, expressed CK5 positive, Nestin, CK18 positive and CK14 positive cells. (38) Presence of bi potent stem cells in the breast milk has been also confirmed and expresses the p63 gene, CD49f and self renewal properties in spheroid culture.(39) Further the high rate of proliferation or self renewal is maintained due to the presence of OCT4, SOX2 and NANOG pluripotent transcriptional markers which resembles these breast milk stem cells more like embryonic in nature. (40) Further these breast milk derived stem cells formed colonies which were pretty similar to hESC's when cultured on mouse feeder layers. (41) Scientists believe that these extensive remodelling of the mammary glands is due to the high proliferation rate and extensive pluripotent like stem cell characteristics. Further, human breast milk stem cells showed differentiation capabilities outside their normal mammary alveolar cell lineage into osteoblasts, chondrocytes, neural stem cells, adipocytes, heaptocytes, cardiomyocytes and pancreatic beta cells (41). They do not form tumours when injected subcutaneously inside SCID mice, suggesting an attractive property for regenerative medicine therapies unlike embryonic stem cells.

Scientists have now confirmed that a great majority of the breast milk stem cells are viable (around 70 to 100%) during the time of lactation, popular to contrary belief that they are not. (42, 43) Therefore, infants ingest live cells during the time of breast feeding and some of these cells are stem cells which are pluripotent in nature giving rise to the concept of microchimerism or passage of

maternal breast milk stem cells into the baby through breast feeding. These cells are quite well tolerated inside the infant's system due to the not so matured immune system of the infant. (44) Future prospects of regenerative medicine includes breast milk stem cell banking in treatment therapies.

Bauls:

In rural Bengal (Indian province) there is a sacred sect called Bauls, who follow their own religion, disregard caste system, does not believe in having any permanent shelter and is always on the move. The word Baul comes from Sanskrit word "vatul" meaning mad affected by wind. (45)The Bauls are an unique sect because in their system of thinking they speak of the four moons which are actually menstrual blood, semen, stool and urine in medical terminologies. (45) Swallowing the menstrual blood is a part of the Baul rituals. Further rituals include a practice where the oldest Baul in the group drink the milk of the youngest post natal lactating mother, after the birth of the baby. (46) A further introspection by sociologists and scientists has revealed that none of the Bauls are fat or obese; most of them are agile, lean and thin in their physical appearance. This is related to the high degree of well being and good health of the Bauls, which some sociologists and scientists believe can be actually be related to the feeding of the menstrual blood or breast milk, which are rich sources for stem cells. (45) However, the science behind such justification is yet to consider this behaviour of Bauls and their apparent youth and if there is any role of stem cells in such cases can be a subject for further health research. (45)

Dental stem cells:

Other attractive sources for non-fetal stem cells are from dental pulp, human exfoliated deciduous teeth or SHED, periodontal ligament stem cells or PDLSC's & progenitor stem cells from the dental follicle or DFPC's. These different populations of stem cells have a good content of mesenchymal stem cells and their plasticity, capacity for self renewal & proliferation and differentiation are proved already. (47)

Stem cells obtained from SHED and DPSC's also show a better proliferation, transdifferentiation and osteo-induction capacity compared to BMSC's. (49, 50) The main advantages of using stem cells, collected from the different dental regions are (a) can be an autologus process and thereby requiring no immunosuppressive drugs (b) can be easily expanded and used in the regeneration of bone to repair even critical size defects, mandibular defects post 6 months of surgical reconstruction as seen by researchers and (c) it is a less invasive procedure. (50) Stem cells from the different dental sources also have rich haematopoietic content as they express CD34 positive markers and posses the ability to differentiate into osteoblasts and form calcium deposits and mineralized nodules in vitro. Further Hara et.al showed the presence of Bone Morphogenetic protein-4 or BMP-4 signalling pathway thereby indicating the facts that these might be used as effective tools in cell therapy for bone tissue engineering including pulp and dentin. (51) De Mendoca Costa and his research group has observed that human dental pulp stem cells can be effectively used to reconstruct large sized cranial defects in non-immunosuppressed rats.(52)

Further, presence of a number of growth factors at the dentine ECM like TGF-Beta superfamily, BMP, FGF, VEGF, IGF, Sonic Hedgehog and Wnt, which all are secreted in minute amounts and are able to maintain the self proliferation of these stem cells including cellular response during any stress or injury has been observed. (53) Similar such functions are observed in cases of odontoblasts associated with the pulp tissue which are normally activated during any superficial injury. (54)

In bone regeneration, according to Bakopoulou et al., DPSC's and SCAP, both are of MSC types predominantly which have shown osteocyte differentiation markers including cell migration,

organization, mineralization and can be used for bone tissue engineering. (55) Gomes had shown that these human undifferentiated immature dental pulp stem cells or hIDPSC's can be a valid alternative for ocular surface reconstitution in cases of bilateral total limbal deficiency. (56)

Differentiation of DPSC's from deciduous part of the teeth into pancreatic cell lineage can be also an alternative autologus stem cell treatment for diabetic patients. Similarly Ishkitiev showed the differentiation of dental pulp stem cells into hepatocyte like cells for liver regeneration. (57, 58) Further studies in nude rat suffering from myocardial infarction showed an improvement with increase in the number of blood vessels, pro-angiogenic and anti-apoptopic factors with similar degree of regeneration like that of MSC's. (59)

Various preclinical studies have shown that hIDPSC's are hypoantigenic, can have immunomodulatory properties, similar to MSC's and lacks the expression of HLA class I and II molecules including other co-stimulatory molecules.(60, 61) Also it has been shown that these cells can inhibit the proliferation of T-cells, NK cells and impair dendritic cell maturation. (62)

Dental pulp stem cells normally reside within the perivascular niche of dental pulp and, therefore, are thought to be originating from the cranial neural crest cells or CNC. Gronthos et al has observed that under appropriate neural cell culture in vitro, these dental pulp stem cells can be trans differentiated into neuronal like lineages. (63) Glial cell differentiation of these cells has been demonstrated by Vollner. (64) Taghipour and colleagues showed that induced neural SHED cells can be used for functional recovery in rat models of acute spinal cord injuries. (65) Karaoz et al also showed the transdifferentiation of these SHED cells into neural and epithelial cell lineages apart from adipocytes, osteocytes and chondrocytes. (66) Sakai has further shown that SHED and DPSC cells can be also applied successfully for treating spinal cord injuries. (67)

However, as this is a new field of stem cell therapy, there should be an approach with caution as safety evaluation studies like tumour formation, risk of infection during in vitro maintenance and improper homing or migration of these cells into ectopic sites still needs proper verification and assessment before translating them into clinics. (68)

Stem cells derived from Adipose Tissue and Bone marrow:

Stem cells can be also derived from fat or adipose tissue. Adipose tissues are of several types and can be derived mainly from the subcutaneous fat and bone marrow. In bone marrow it mainly functions in occupying the empty spaces in adults and supports the niche with an array of cytokines and contact regulatory signals for hematopoietic stem and progenitor stem cells. In mammary glands these cells support mainly during lactation. Main role of adipose tissue is to provide endocrine support in the form of producing adipokines, leptin, adiponectin, resistin, lopicalin, osteoponin and other angiogenic like factors apart from structural and energy support.(69, 70, 71)

Initially cells were isolated from adipocytes and their studies were restricted only to adipocytic lineage. In 1966, Rodbell and Jones (72, 73, 74) showed that these cells can be isolated from stromal vascular fraction or SVF which is a predominantly heterogenous cell population with a mixture of fibroblasts, pericytes and endothelial blood cells. (75, 76) Mitotically isolated fibroblasts from SVF are source for adipocyte precursor cells and forms adipose tissue in vitro.

Both adipose derived stem cells and Bone marrow derived mesenchymal stem cells express markers for CD29 (important for angiogenesis) (77), CD44 (important for ECM formation) (78), CD54 or ICAM-1 (important for immunoglobin supergene family) (79), HLA-DR negative and mostly MHC class I positive and therefore low in immune reactive, has very good immune modulatory properties and

does not elicit any GvHd or allo rejection even after randomized allo-transplantation. (69)The other important markers present are CD71 positive, CD 90 positive, CD105 positive, SH3 positive and absence of haematopoietic markers like CD45 negative, CD34 negative, CD31 negative in both ADSC's and BM-MSC's. (80) Also a section of the cells were found to be CD31 positive or endothelial in nature but have very low expansion rate in vitro. Later on Zuk et al in 2001 first demonstrated that adipose tissue is a rich source for mesenchymal stromal cells and can differentiate into adipocytes, chondrocytes, osteocytes, and myocytes .(81)Further these adipose derived stem cells, meet the guidelines and requirements as stated by the International Stem cell society where they have defined MSC's to have good plastic adherence properties, can be differentiated into adipocytes, chondrocytes and osteocytes in vitro including lack of expression specific haematopoietic markers such as CD45 positive, CD34, CD11b, CD79a and CD14. (82, 83) Further there are also differences between ADSC and BMMSC. ADSC's express CD49d positive and CD106 negative pattern whereas BM-MSC's are CD49d negative and CD106 positive. (84,85)

However, many groups have reported the presence of CD34 positive markers in ADSC's, collected from stromal vascular fractions or SVF, in 95% of their in vitro cell line cultures throughout the course of their culture period. (86) This can be due to several factors like collection of some endothelial cells during the SVF isolation resulting in a highly heterogeneous population, in vitro culture methods, resulting in the expression of various transient and transitional cells. Also low percentage of CD34 positive cells may reflect presence of sub population of EPC's and therefore, supporting the finding that adipose derived CD34 positive and CD133 positive cells are able to form endothelial colonies in vitro and can induce angiogenesis in vivo. (87, 88, 89)Both ADSC's and BM-MSC's through proteome and transcriptome analysis has shown to express stemness markers such as Sox-2, Oct-4 and Rex-1. (90) Normally in cases of ADSC's and BM-MSC's the telomerase activity is not consistent as a result of which the cell doubling time in the initial 2 to 4 days is longest. (91)

Large amounts of adipose tissues can be easily derived through non invasive procedures such as liposuction or lipoectomy and does not involve any ethical issues. This process is becoming attractive as these cells can be harvested from the patient's own subcutaneous fat and can be re utilized for any disease condition. In other words, these cells can be used as autologus stem cell transplantation therapy without any issue. The adipose tissue can be further obtained by tumescent lipoaspiration, ultrasound assisted lipoaspiration, laser assisted or water assisted liposuction or via surgical resection. (92, 93, 94)

International Fat Applied Technological Society in 2004 named these fat derived stem cells as Adipose derived mesenchymal stem or stromal cells. (95) Often there is a debate between naming the cells as Adipose derived stromal cells and Adipose derived stem cells by many. As the stemness of the Adipose derived like cells are still not proven formally by many laboratories, therefore, many scientists prefer the nomenclature stromal over stem cells. This is still a matter of debate though. Through Colony forming unit assay method, investigators have found that the occurrence of non-haematopoietic stem cells in the bone marrow are 1 in 25,000-100,000 in range, whereas in case of isolated nucleated fat/adipose tissue it is only 1 in 50.(96, 97, 98) These were further confirmed by plating ADSC's and BM-MSC's along with characterization studies.

Both ADSC's and BM-MSC's can show multi lineage differentiation also like neuronal cells, cardiac muscle cells, pancreatic cells and hepatocytes. Subcutaneous fat is the most widely collected fat so for isolating adipose derived stem cells. The omentum fat is another attractive source. (99) In 2005, Seo et al and his colleagues reported the differentiation of hASC's into hepatocyte like cells secreting albumin and when transplanted in vivo in mice model, grafted donor cells were observed in the liver resembling a more hepatocyte like morphology and secretion of albumin. (100) Similar observations were made in a SCID mice model where hASC's were injected in case of acute liver failure caused by carbon tetrachloride. They all showed signs and symptoms of revival. (101) In case of diabetes,

transplantation of Beta cells is regarded as the gold technique but can be offered to only a short number of patients due to its low availability and high chances of immune rejection. (102) Regeneration and transplantation of beta cells from ADSC's can be a very attractive alternative. In Crohn's disease, which affects the young (age group between 18 and 40 years) and is often accompanied by bloody stools, diarrhoea, weight loss and auto immune related symptoms has shown to respond well after treatment with adipose derived stem cells. In a phase I clinical trial, adipose stem cells were directly injected into fistulas and showed improvements in 75% of the patients and same result were obtained in case of another Phase IIb trial when compared to fibrin glue. (86, 103)In cases of wound management Rigotti and his colleagues have shown possible successful results after adipose derived stem cell injection in patients who were suffering from wounds due to post mastectomy irradiation. (104) Adipose derived stem cells can also work effectively in graft versus host disease due to its immunomodulatory and immune suppressive properties. Yanez (105) reported unique immunosuppressive properties of ADSC in both in vitro and in vivo studies and, therefore, suggesting that these cells can be used in place of immunosuppressive drugs during organ transplantation. Further studies by the same researcher investigated that allogeneic or third party adipocyte derived stem cell donors did not elucidate any Gvhd and were well tolerated. (86) This very immunomodulatory property of MSC's has been also used in cases of Systemic Lupus Erythrematosus (SLE) and was partially achieved due to suppression of the Th17 lympocytes. In neuronal studies, G.Terenghi and his colleagues have been working on peripheral nerve engineering using Adipose derived stem cells and have found encouraging results where adipose derived cells are being differentiated into glial like cells on engineered scaffolds and then transplanted in animal models. (106, 107) Yoshimura and his colleagues published their clinical trial of a 307 patients who had undergone CAL fat grafting in the breast mainly and showed a 40% to 80% of graft intake, normally which is between 10 to 100% with long term survival of the graft. (108,109, 110, 111, 112, 113, 114) However, absence of another trial arm undergoing standard fat injection procedures was the major limitation of the study. The above clinical trial was supported by Dr. Sterodimas and his colleagues from Brazil where they showed that patients required only one treatment with a form of CAL as opposed to several number of operations in the control arm in case of reconstructing soft tissue defects. (108)

Cytori Therapeutic Includings., reported their own findings where they showed 75% of patient satisfaction after 12 months of follow up who were treated with CAL (115). A total of 71 patients were included and 47 had one procedure and 24 needed only twice. Further follow up results with MRI showed improvement in the breast contours in 54 of the 65 patients. (116)

Tissue Engineering:

Adipocyte derived stem cells are an important and extremely attractive tool in tissue engineering partly because they can be easily isolated and are easy to grow in vitro and in vivo. They are also extremely biocompatible and have greater immune suppressive roles. There have been several established protocols in bone tissue, cartilage tissue and nerve tissue engineering using adipose derived stem cells (117, 118, 119, 120) for treating bone and cartilage defects and spinal cord defects.

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