DISCUSSION

The isolation and culture of multi-potent MSCs opened up a new field of stem cell research. However, protocols for isolation and expansion of MSCs vary widely between these trials, which could affect the efficacy of the therapy. It is therefore important to develop ***international standards*** of MSCs production, which should be evidence-based, regulatory authority-compliant, of good medical practice grade, cost-effective, and clinically practical.[100]

Adipose tissue is a rich, ubiquitous and accessible source for multi-potent stromal/stem cells. Stem and precursor cells in freshly stromal vascular fraction (SVF) usually account for up to 3% and this is approximately 2,500 folds more than frequency of MSCs in bone marrow.[101] The adipose tissue SVF consists of a broad and heterogeneous cellular compartments including ; vascular cells (endothelial and perivascular populations), hematopoietic cells (resident and circulating cells), and stromal fibroblast.

The SVF can be isolated by enzymatic digestion of intact fat tissue or lipoaspirate, followed by the depletion of mature adipocytes through centrifugation. In 1976, human AT-MSCs were successfully isolated from the adipose SVF by selective adherence to culture plastics. The adherent fraction of the adipose SVF was later identified as a source of mesenchymogenic progenitors[5], termed adipose-derived stem/stromal cells (ASCs).[102]

Several studies have tried to identify the origin of stromal/ stem cell population within adipose tissue in situ. ***This is a complicated attempt because*** no marker has currently been described which unambiguously identifies native AT-MSCs. In 2013, the International Fat Applied Technology Society (IFATS) published a revised statement to point out the minimal phenotypic criteria to characterize the uncultured SVF and the adherent stromal/stem cell population from adipose tissue.[103] ***In SVF, native AT-MSCs are now characterized as being positive for CD34 and negative for CD45, CD 235a and CD 31 cells***.[103] CD 235a (glycophorin A) to monitor any contaminating erythroid lineage cells, the leukocyte common antigen CD45 is used to identify haematopoietic cells and their progenitors and CD 31 (PECAM-1) to detect endothelial cells and their progenitors.[104]

Isolated and cultured AT-MSCs are a ***non-uniform*** preparation consisting of several subsets of stem and precursor cells. The composition of AT-MSCs or their sub-population seems to vary between different laboratories and preparations. The ***heterogeneity*** of AT-MSCs may result from the lack of a standardized isolation and culture protocol. [104] It has been described, for example, that single –cell derived clonal MSC populations are also highly heterogeneous and contain undifferentiated stem/progenitors and lineage –restricted precursors with varying capacity to proliferate and differentiate.[105, 106]

Patients on regular ***insulin*** therapy due to diabetes mellitus were excluded as insulin may influence differentiation of AT-MSCs to adipocytes.[107] Also regular ***corticosteroids administration*** was excluded as it may influence differentiation of AT-MSCs as; dexamethazone can be used as an inductive factor in media to induce differentiation to adipocytes, osteoblasts, chondrocytes and myocytes. [1] ***Breast cancer*** patients were also

excluded as malignancy may be associated with genetic aberrations which may alter the behavior of isolated AT-MSCs.

Several protocols were tried ; Bochev et al[15] who adopted his protocol from kern et al[14], both were similar to Zuk et al[5] for ***chemical isolation*** of AT-MSCs using collagenase enzyme. Also we tried Zeng et al [98] for ***mechanical isolation*** of AT-MSCs. Then we tried to develop and standardize our own lab protocol of isolation through the modification of these protocols.

Zeng et al[98] protocol *(protocol I, methods, page 18)* for mechanical isolation used the unique property of AT-MSCs ; ***plastic adherence***. Turning T-25 cm^2 culture flasks every 12 hours helps only plastic adherent cells to crawl from pieces of adipose tissue and grow while others die. The cells isolated using this protocol were scanty, disperse and died after 7 days. This disagreed with Dr. Zeng findings who succeeded in isolating and propagating MSCs from adipose tissue using this protocol.

When we used Bochev et al [15] protocol *(protocol IIb , methods, page 21)*, the resulting population was too low and the isolated cells didn't survive more than 5 days. Some modifications were done *(protocol IIa, methods, page 19)* ; ***we prolonged the time to which adipose tissue was exposed to collagenase enzyme from 30 minutes to 1 hour***. ***Also incubation at 37^0 C was done in a shaking water bath instead of an incubator***. This increased the initial SVF pellet, for example; ~ 40 cm lipoaspirate, initial SVF pellet was about 19 $x10^6$ cells/ml with total cell count 38 $x10^6$ cells in 2 ml. The increase may be due to prolonged exposure time of pieces of adipose tissue to collagenase enzyme and this helped the release of more cells from the vascular stroma, also the shaking water bath served as a source of mechanical agitation that caused the release of more cells.

It was found that AT-MSCs were isolated easier from ***adipose tissue near fascias and blood vessels than those far from fascias and blood vessels***. This may be because adipose tissue near fascia and blood vessels contain more stem cells and these locations act as a reservoir of stem cells. This was similar to the findings of Zeng et al[98] and Traktuev et al[108] who demonstrated that AT-MSCs are rarely distributed among adipocytes, but are predominantly associated with vascular structures; in the walls of adipose microvasculature.

Basal culture media consists of amino acids, glucose and ions including calcium, magnesium, potassium, sodium and phosphate. There is no doubt that the types of culture medium used affect proliferation and differentiation of MSCs. Another modification was the ***type of medium*** used. Dr. Bochev used low glucose (1g/L) Dulbecco's modified Eagle's medium without L-glutamine (LG-DMEM), we tried this medium first in our trials, our isolated cells couldn't proliferate and died within 5-7 days even with the addition of 10% FBS. We thought about using another alternative, we used ***high glucose (4.5 g/L) Dulbecco's modified Eagle's medium with L-glutamine***, a tremendous shift in our results occurred. An obvious increase in proliferation was noticed; For example; the initial plating density was 6.4 x 10^5 cells/ml, at first passage the number of cells were 3 $x10^7$ cells/ml, and cells could survive and were propagated in vitro for almost 40 days. This may be attributed to ; **L-glutamine** which is an essential nutrient for energy production as well as protein and nucleic acid synthesis and cell culture.[100] Also, **glucose** appeared to have the most critical effect on cell growth, viability and metabolic state.[100] It has been already shown that human AT-MSCs increase their lactate production, proliferation and viability

when exposed to higher glucose environment. [109] Whether this astonishing shift in proliferation is due to high glucose content alone or L-glutamine alone or both remains to be investigated. The same medium ; ***high glucose (4.5 g/L) Dulbecco's modified Eagle's medium with L-glutamine*** was used for MSCs isolation and expansion.

It is known that growth factor supplement to culture medium enhances proliferation with maintenance of important properties of MSCs. In particular, fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-beta (TGF-ß) and insulin-like growth factor (IGF) play a role in MSCs proliferation. FBS has been most frequently used to supply growth factors to MSCs as it contains all these factors and readily available.[100] However, it should be noted that FBS shows considerable variation in growth factor activity from batch to batch, also some safety issues may be associated with FBS as; viral transmission, anaphylatoxic reactions and production of anti-FBS antibodies. [100] To avoid such risk related to the use of animal materials, the use of human products including ; platelet products has been proposed.

PRP is a concentration of autologous human platelets in a small volume of plasma, containing at least ***seven*** major growth factors including ; platelet-derived growth factors (PDGFs), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1) and transforming growth factor-beta (TGF-ß).[1] These growth factors are released by actively de-granulated platelets and known to facilitate the wound-healing process. Thus, PRP can be used in this field for its ability to stimulate tissue regeneration.[1] The use of PRP as a source of growth factors and cytokines help to create a ***FBS-free culture*** that leads to decrease the risks of allergic reaction that faced researches during the use MSCs in regenerative medicine after using a FBS culture.[50] In our study, we used PRP *(protocol IIIa, methods,page 22)* in concentrations (80%, 50% and 10%) and platelet lysate *(protocol IIIb, methods, page 22)* in 10%, 50% and 80% concentrations ; the isolated cells couldn't survive and proliferate and died within 5-7 days. This disagreed with Hatim Hemeda et al [99] who found that platelets are good replacement for FBS as a source of growth factors in MSCs' culture. Our results could be explained by; the small volume of blood used, the unavailability of continous supply of autologous PRP during the culture period as our subjects performed their operations and were discharged within two days and the unsteady behavior of PRP as; sometimes when autologous PRP was placed with the culture media in T-25 cm^2 Nunc culture flasks a ***coagulum*** retaining all the cells was formed. Therefore, the use of platelet products as a source of growth factors and also the use of other sources for growth factors, like recombinant cytokines instead of FBS should be further investigated.

Cell seeding density for MSCs isolation is another important factor to determine the efficiency of MSCs yield as; it affects the adherence of MSCs, contamination by other cell types and initial growth of adhered MSCs. [100] After several trials, we found that initial plating density ranging from 6 $x10^4$ cells to 6 $x10^5$ cells in T-25 cm^2 Nunc culture flasks achieved higher yield which agreed with Sotiropoulou et al [110] results when culturing MSCs isolated from bone marrow.

Morphologically, our isolated AT-MSCs were fibroblast-like cells that usually appeared on 2^{nd} day of initial culture and were characterized by being adherent to plastic of culture flasks. By 7^{th} day of culture numerous colonies of fibroblast-like adherent cells

were seen that reached 100% confluence by 18th day. This agreed with the findings of Bochev et al [15], kern et al [14] and Zuk et al[5].

Using flow cytometery, Initial stromal vascular fraction (SVF) pellet i.e. day 0 was ***negative*** for CD45, CD34, CD90, CD73, CD105, CD49d, CD 19, CD3, CD 14 and HLA-DR. This disagreed with what we previously mentioned in *(discussion, page 33)* which stated that ; IFATS has published a revised statement to point out the minimal phenotypic criteria to characterize the uncultured SVF and the adherent stromal/stem cell population from adipose tissue and that native AT-MSCs are now characterized by being ***CD34 positive*** cells.[103] Our result may be attributed to; the use of CD34-PE antibodies, class III clone BIRMA-K3 for verification of CD34 expression. It was reported that there are some technical difficulties concerning the verification of CD34 expression[104] as there are multiple classes of CD34 antibodies recognizing unique immunogens and influencing the signal.[111] Therefore, Bourin et al [103] recommended the use of class III CD34 antibodies (i.e., clone 581 or 4H11) for SVF characterization[111] .

Our isolated cells were **positive** for CD90, CD73 and CD105 and **negative** for CD45, CD 19, CD3, CD 14 and HLA-DR on day 7, 14, 21 and 28. This agrees with which was reported by Zuk et al[5], Kern et al [14], Bochev et al[15], and Zeng et al[98]. ***CD90***, also known as Thy1, is a glycosylphosphatidylinositol-linked protein involved in cell-cell and cell-matrix interactions, ***CD73*** is an ecto-5'-nucleotidase that converts extracellular adenosine monophosphate to adenosine and ***CD105***, also known as endoglin, is a type I membrane glycoprotein that functions as an accessory receptor for transforming growth factor-beta (TGF-ß) superfamily ligands. The isolated cells were also **positive** for CD 49d on day 7,14,21 and 28 similar to Zuk et al[5] findings who demonstrated that the stem cell population isolated from adipose tissue, which he named processed lipoaspirate (PLA), expressed CD 49d. ***CD49d*** is an integrin alpha subunit. It makes up half of the α4 β1 lymphocyte homing receptor.

Isolated cells were **positive** for CD34 on day 7, 14, 21 and 28. This finding disagreed with Zuk et al[5], Kern et al [14], Bochev et al[15], and Zeng et al[98] . Their studies showed that MSCs were **negative** for CD34. ***CD 34*** also known as mucosialin, was used to characterize haematopoietic progenitor cells. Recently, it was found that it may be a potential marker to identify AT-MSCs. [104, 112] However, it has been reported that long-cultured AT-MSCs don't express CD34, perhaps it is lost due to artificial environment. Therefore, the possible explanation for the studies that showed the absence of CD34 expression in cultured plastic adherent fibroblast-like cells in higher passages is that they didn't investigate the expression of CD34 earlier in their cultures.

Based on our findings we noticed the following; cells isolated and cultured from lipoaspirate material showed better proliferation rate than those isolated and cultured from adipose tissue obtained from abdominoplasty and breast reduction. This agrees with the fact that the preparations of AT-MSCs are heterogeneous. This heterogeneity may be attributed to different reasons; some of them are related to the donors from which AT-MSCs are isolated. These donors differ in age, body mass index and ethnicity. [113] It is also important to consider ; *operation procedure*, which may differ between different clinics, the *time* lapse until cell isolation procedure starts and the *temperature* at which adipose tissue is stored until cell isolation.[104]

By observing the behavior of isolated adherent fibroblast-like cell ; ***proliferation*** of cells was found to increase from ***day 0*** to reach maximum by ***day 14*** and then starts to decrease and becomes plateau as the cells senesce and die. Concerning ***CD expression*** of isolated cells, we found that CD34, CD90, CD73, CD105 and CD49d were expressed by cells starting from ***day 7*** till ***day 28*** and mean percentage (%) of gated cells out of control ***positive*** for these CDs was ***nearly*** the ***same*** at all days except for ***CD 105*** whose mean percentage of gated positive cells out of control started to decrease by ***day 21***, whether this decrease is significant or not needs further investigation. Therefore, we believe the optimum time for using AT-MSCs in clinical applications to get the maximum benefit out of them is between ***day 10*** and **day 18**.

SUMMARY

Cell-based therapy and regenerative medicine offer a new hope for the treatment of various diseases in which there is loss of substance, volume, tissue or organ damage. Recently, scientists have been interested in MSCs due to their capacity to differentiate into many cell lineages. MSCs are multi-potent adult somatic stem cells that have fibroblast-like morphology and can be guided in vivo and in vitro to terminally differentiate into osteoblasts, chondrocytes and adipocytes. In addition to their multipotency and their potential use in stem cell therapy, it was also discovered that MSCs also posses immune-regulatory properties. They either don't express or negligibly express low levels of HLA class II antigens and co-stimulatory molecules such as CD80 and CD86. MSCs don't activate alloactive T-cells, which indicates that these cells are not inherently immunogenic.

MSCs were first identified by ***Friedenstein et al*** year 1976 by culturing of bone marrow on plastic culture dishes, they were able to discard non-adherent hematopoietic stem cells and identify plastic adherent cells or colony-forming unit-fibroblasts (CFU-F). These plastic adherent cells were spindle shaped and formed foci of two to four cells, which remained inactive for 2–4 days and then multiplied rapidly. After passaging several times in culture, they became more homogeneously fibroblastic in appearance. They also were found to have the ability to make small colonies that resemble cartilage or bone.

MSCs were more recently identified by the international society of cellular therapy (ISCT) based on ***three cellular*** properties; adherence to plastic, ***positive*** expression of CD90, CD 73, CD 105 and ***negative*** expression of CD45, CD34, CD14 or CD11b, CD79 a or CD19 and HLA -DR, and differentiation into multiple cell types including adipocytes, chondrocytes and osteoblasts.

Bone marrow represents the main source for the isolation of multi-potent MSCs, however there are some features that could restrict or make the use of bone marrow-derived mesenchymal stem cells (BM-MSCs) unacceptable, such as; highly invasive harvesting procedure and the significant decline in number, differentiation potential and maximal life span of these cells with increasing age. Therefore, it became essential to search for other sources of MSCs. Another favourable source from which isolated mesenchymal stem cells display similar properties to BM-MSCs is adipose tissue. ***Adipose tissue*** appear to be a very encouraging alternative source of MSCs and regenerative cell therapy, as it could serve as a more convenient rich source of MSCs. Adipose tissue can be obtained in a less traumatic manner and in larger quantities than bone marrow. In addition, adipose-derived mesenchymal stem cells (AT-MSCs) show lower morbidity during the harvesting procedures.

Although many scientists have successfully isolated MSCs from adipose tissue, there is still no standard method for isolating and culturing highly homogenous population of MSCs. ***Our aim*** was to develop an efficient method for isolation and identification of MSCs.

We tried several protocols until we succeeded in developing our own protocol ***(protocol IIa, methods, page 19)*** which is; adipose tissue obtained from 5 subjects undergoing abdominoplasty, breast reduction or liposuction was ; washed 3-4 times with sterile PBS to clean its surface from blood clots, RBCs and local anaesthetics. Adipose

tissue was then digested by adding equal volume of 0.075% working collagenase solutions to adipose tissue material in T-25 cm^2 Nunc culture flasks for one hour at 37^0C in a water bath with shaking 500 RPM. The collagenase is de-activated by adding equal volume of high glucose DMEM media with L-glutamine, then pour and distribute all the contents of flasks in 15 ml falcon tubes. The mixture was then centrifuged at 3000 rpm for 10 min at 4 ^{0} C. The supernatant was then discarded and the pellet, was re-suspended in 5 ml sterile PBS and centrifuged at 2000 rpm for 5 minutes. Red cell lysis buffer was then added for 5 minutes at room temperature to lyse RBCs, then mixture was centrifuged at 2000 rpm for 5 minutes. Obtained pellet was re-suspended in 5 ml sterile PBS and centrifuged at 2000 rpm for 5 minutes. The resulted pellet represents the initial SVF. The initial SVF pellet was then re-suspended in 2ml high glucose DMEM with L-glutamine and 10% FBS, counted and cultured in T-25 cm^2Nunc culture flasks containing high glucose DMEM (4.5g/l) with L-glutamine and 10% FBS. Flasks were then incubated in 37^0C at humidified atmosphere containing 5% CO_2. After overnight incubation, culture flasks were then washed with sterile PBS to remove non-adherent cells and new media was added (high glucose DMEM with L-glutamine+10%FBS+8%P/S). First adherent fibroblast-like cells usually appeared at ***day 2***, 15-20 colonies of fibroblast-like adherent cells appeared by ***day 7***. Fibroblast-like adherent cells reached 70% confluence by ***day 15*** and 100% confluence by ***day 18***. Culture flasks were maintained until fibroblast-like adherent cells reached 80-90% confluence with media being changed every 48 hours. The cells were harvested using 0.05% trypsin /1.0mM EDTA at 37 °C for 10 minutes. The cell cultures were expanded for 2-8 passages. We used same culture medium for isolation and expansion of AT-MSCs.

By immunophenotyping; cells of initial SVF pellet ***didn't*** express CD90, CD73, CD105, CD49d, CD34, CD45, CD14, CD19, CD3 and HLA-DR. By day he 7^{th} day ; immunophenotyping of our isolated fibroblast-like adherent cells showed that they were ***positive*** for CD34,CD90,CD73,CD105 and CD49d and ***negative*** for CD45,CD14,CD19,CD3 and HLA-DR. Same results was obtained on day 14,21,28.