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Isolation And Chondrogenic Differentiation Of Mesenchymal Stem Cells Derived From Oral Mucosal Tissue And Adipose Tissue.

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ABSTRACT

Stem cell therapy has a prevailing role as a potential treatment option in dentistry. Dental tissue regeneration provides an alternative to the current conventional restoration therapies. The oral cavity is the most affluent stem cell source in the human body. These stem cells are now accepted as being fundamental to different types of dental and non dental tissue regeneration. The aim of this study was to isolate human mesenchymal stem cells (MSCs) from adipose tissue (ASCs) and oral mucosal tissue (OMSCs) and confirm their differentiation potentials, including the chondrogenic lineage. ASCs and OMSCs cultures were analyzed for cell shape, cell cycle, proliferation potential (MTT assay) and stem cell markers (CD90, CD105). The chondrogenic differentiation potential of ASCs and OMSCs induced with chondrogenic induction medium and was estimated by means of Alcian Blue stain as well as quantitative real time RT-PCR using collagen II. Our data discovered that ASCs and OMSCs showed a significant increase in cell viability from day 14 to day 21, representing high cell proliferation rate(80-90%), where ASCs proliferated faster than OMSCs. Moreover, ASCs and OMSCs can efficiently differentiate into cartilage forming cells expressing collagen II. This study provides evidence that ASCs and OMSCs can be used in tissue engineering/regeneration protocols as an accessible stem cell source.

Keywords: stem cells, differentiation, chondrogenic, adipose tissue, oral mucosa tissue.

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INTRODUCTION

Application of regenerative medicine technology may offer new therapies for patients with injuries, end-stage organ collapse, or other clinical troubles. Scientists in the pasture of regenerative medicine and tissue engineering are now applying the ideology of cell transplantation, material science, and bioengineering to create biological substitutes that will restore and maintain normal function in diseased and injured tissues. The stem cell field is a rapidly advancing aspect of regenerative medicine as well, and new discoveries here create new options for this type of therapy ***(Bhateja S, 2012 and Vinicius, 2013).***

Tissue engineering includes three basic mechanisms: Inducting signals, responsive cells, and a matrix/scaffold ***(Titi et al, 2015 and Deepika,et al., 2015).*** Mesenchymal stem cells (MSCs) are multipotent stem cells that can be isolated from many tissues/organs, such as bone marrow and adipose tissue, with the properties of self-renewal for long periods through cell division ***(Indumathi et al, 2015).*** In addition, under certain physiological or trial circumstances, MSCs are promising progenitor cell sources for stem cell transplantation, tissue engineering and regeneration. Exploring suitable sources of stem cells for reparative and regenerative purposes is an important assignment in frontage of researchers ***(Verma et al., 2014; Potdar and Jethmalani, 2015).*** Adipose tissue-derived stem cells (ASCs) were first isolated by ***Zuk et al. (2001)*** as a rich accessible source of MSCs with minimal patient discomfort, less invasive and low risk of side effects. ASCs were found to be able to keep strong proliferative ability, maintain their phenotypes and have stronger multi-differentiation potential ***(Davies et al, 2014).*** ASCs have many clinical rewards over bone marrow mesenchymal stem cells, by its unique expression of antigens CD49d and CD106 ***(Lee et al, 2015).*** In addition, the differentiation potential of ASCs can be maintained with aging ***(Hakan et al, 2012and Mizuno et al, 2012).***

Recent attention has been focused on the exploitation and existence of dental tissue-derived stem cells in tissue engineering, providing potential cell sources for regeneration of tooth structures as well as other tissues/organs ***(Park et al, 2016, Marco et al, 2015 and Ensany et al, 2014).*** The most widely known MSCs of dental origin are dental pulp stem cells (DPSCs) ***(Marcella et al, 2014and Tatullo et al, 2014)*** and periodontal ligament stem cells (PDLSCs) ***(Bright et al, 2014 and Chen et al, 2012).*** Besides that, several other kinds of MSCs of dental origin have also been gradually secluded by the researchers, such as, exfoliated deciduous teeth (SHED) ***(Kerkis et al, 2012)***, apical papilla (SCAP) dental follicle (DFPCs) ***(Torkzaban et al, 2012)*** as well as oral mucosal tissue ***(Bong et al, 2012).***

The oral mucosa illustrates the soft tissue lining of the oral cavity, including the buccal mucosa and the gingivae ***(Stephens and Davies, 2015 and Jones and Klein, 2013).*** Although the capability of dental mesenchymal stem cells to give rise to dental tissue as well some other tissues has been reported, regrettably the accessibility and availability of these stem cells are quite restricted ***(Rouabhia, 2015).*** Comparatively, gingival MSCs (GMSCs) compose more tempting alternatives to the other dental originated MSCs in terms of that they are much easier to get as a byproduct from the clinically resected gingival tissues. Hence, it is of great interest to authorize the multiple differentiation potentials of GMSCs for potential tissue engineering applications ***(Jin et al, 2014 and Treves et al, 2013).***

Zhang et al. ***(2009)*** first sheltered a population of oral progenitor cells within gingival tissue, termed GMSCs, which formed clonogenic colonies, expressed a typical MSC surface marker profile (CD90, CD105 positive and, CD45 negative)***(Zhang et al, 2012)*** and wrapped up the ability to differentiate into multiple mesodermal lineages *in vitro* ***(Yu et al, 2013 and Fournier et al, 2013).*** Notably, single colony derived GMSCs verified the capacity for self-renewal and formation of connective tissue-like structures *in vivo*. ***Jin et al (2014)*** and ***Geetanjali et al. (2010)*** confirmed that GMSCs are superior to BMSCs for clinical applications.

Articular cartilage is an especially attractive target for tissue engineering strategies because it has been documented that the injuries of articular cartilage, an avascular tissue without direct access to a significant source of reparative cells, do not spontaneously heal. The vast majority of approaches to repair or regenerate articular cartilage are cell-based, aiming to provide a population of reparative cells to the injured site ***(Bai et al, 2011)***. Cells used to develop these strategies can be either differentiated chondrocytes isolated from unaffected areas of the joint surface ***(Dani et al., 2012)*** or progenitor cells capable of differentiating into chondrocytes and can be isolated from a variety of tissues. As harvesting a tissue biopsy from valuable healthy articular cartilage will result in an additional injury, which ultimately cannot repair itself, this cell source does not seem to be a good choice ***(Fortier et al., 2012).*** Thus, the present study seeks to investigate whether ASCs

and OMSCs are capable of differentiating into chondroblast like cells when exposed to chondrogenic induction medium in vitro.

MATERIAL AND METHODS

Sample Collection:

Adipose tissue and oral mucosal samples were collected from the ten white healthy albino rats from the animal house of the National Research Center. Under general anesthesia, adipose tissue from inguinal region and the oral mucosa from cheek were aseptically collected. Incineration of the deceased rats was done at the incinerator of the National Research Center.

***Isolation and culture of ASCs and OMSCs*:**

The oral mucosa was scraped with a lancet to peel the epithelium from it and both specimens were minced into small 1mm3 pieces. The minced pieces were collected in sterile, labeled 1.5 ml Eppendorf tubes to which a digesting solution was added. Enzyme digestion (3 mg/ml collagenase type I and 4 mg/ ml dispase) was carried out according to Gronthos et al, for 60 minutes at 37°C. The culture medium (DMEM) with L-glutamine supplemented with 10 % fetal bovine serum (Gibco, Invitrogen Life Technologies, USA), antibiotics and finally antimycotic agent was added. The tubes were centrifuged for 20 minutes at room temperature to obtain a cell pellet of isolated cells. The supernatant was discarded and then the cells in the pellet were re-suspended in complete culture medium by successive pipetting. Media were changed every 2-3 days.

Passaging was performed when the primary cell culture of adherent cells reached 70% confluence and was named passage zero (P0). Later passages were named accordingly. And cells were propagated and expanded till passage 3 (P3). Cultures were washed twice with (PBS) and trypsinized with 0.25% trypsin in 1m (EDTA) (GIBCO/BRL) for 5 minutes at 37°C. After centrifugation, cell pellets were re-suspended in 1 ml complete medium and divided into two plates (passaging) both followed by immersion in complete culture medium to increase cell numbers. Thus the primary cell culture was propagated and expanded. The cells were counted under inverted microscope.

Characterizations of ASCs and OMSCs by Flow cytometry:

Adherent cells (at the end of the 4th passage) were trypsinized and adjusted to 1×106 cells/ml. Then 1x105 cells were incubated with 10µl of monoclonal antibodies: CD45 FITC, CD90 PE and CD105 PE, (Beckman coulter, USA) at 4 ◦C in the dark. Same species iso-types served as a negative control. After 20 minute incubation, 2 ml of PBS containing 2% FCS solution were added to each tube of monoclonal treated cells. The mixtures were then centrifuged for 5 minutes at 2500 rpm followed by discarding the supernatant and re-suspending cells in 500µl PBS containing 2% FCS. Cell analysis was performed using CYTOMICS FC 500 Flow Cytometer (Beckman coulter, FL, USA) and analyzed using CXP Software version 2.2.

Assessing Proliferation Capability:

MTT Assay Protocol

The proliferation capacity was judged by close follow up of confluence rate i.e. culture plates reaching 70 % confluence according to culture days Cultures from ACSs and OMSCs monitored using inverted light microscope (Olympus, USA).Cells were cultured in 100 ml of culture medium in a flat-bottomed 96 well plate. The MTT reagent was added (10 ml per well) and the plate was incubated for 2 to 12 hours. Detergent reagent was added to each well and the absorbance of each sample in a microplate reader was measured at 550 - 600 nm, depending upon the filters available.

Chondrogenic differentiation at the 3rd passage: 2.5x105 of cells was centrifuged in 15 ml conical polypropylene tube (BD, USA) at 10,000 rpm for 5 minutes. Pellets were suspended in chondrogenic induction medium containing DMEM, 500 ng/ml bone morphogenetic protein 6, 10 ng/ml transforming growth factor β3, 100 nmol/l dexamethasone, 50 µg/ml ascrobate-2 phosphate, 40 µg/ml lprolene and 100 µg/ml pyruvate

(# A10070-01 kit). Pellets were incubated at 37ºC and 5% CO2. The medium was changed every 3 days for 30 days.

Assessment of differentiation:

Flow cytometric analysis:

Differentiated cells were tested for the expression of stem cell surface markers (CD90, CD105). Cell analysis was performed using CYTOMICS FC 500 Flow Cytometer (Beckman coulter, FL, USA) and analyzed using CXP Software version 2.2 ***(Ekaterina and Vladimir, 2012).***

Staining and RT-PCR:

On day 14 and 30, the cells were stained using Alcian Blue stain to indicate the amount of cartilage proteoglycan. On day 14 and 30, the total RNA was extracted from all dishes and real time RT-PCR was performed to analyze the mRNA level of the chondrogenic differentiation marker collagen II.

Statistical analysis:

Data were coded and entered using the SPSS (version 21). Data was summarized using mean and standard deviation. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test in normally distributed quantitative variables while non-parametrical Kruscal-Wallis test and Mann-Whitney test were used for non-normally distributed quantitative variables by Pearson's correlation. P values < 0.05 were considered as statistically significant.

RESULTS

Isolation and Culture:

Stem cells were successfully isolated from Adipose tissue. After enzymatic digestion and initial seeding, ASCs conformed an elongated shape and some cells started to be spindle shaped (Fig.1). Adipose tissue continued to proliferate and propagate reaching 80-90% confluence by day 10. In addition, stem cells were also successfully isolated from oral mucosal tissue. After enzymatic digestion and initial seeding, OMSCs conformed an elongated shape and some cells started to be spindle shaped than ASCs (Fig.2). OMSCs continued to proliferate and propagate reaching 80-90% confluence by the end of the second week

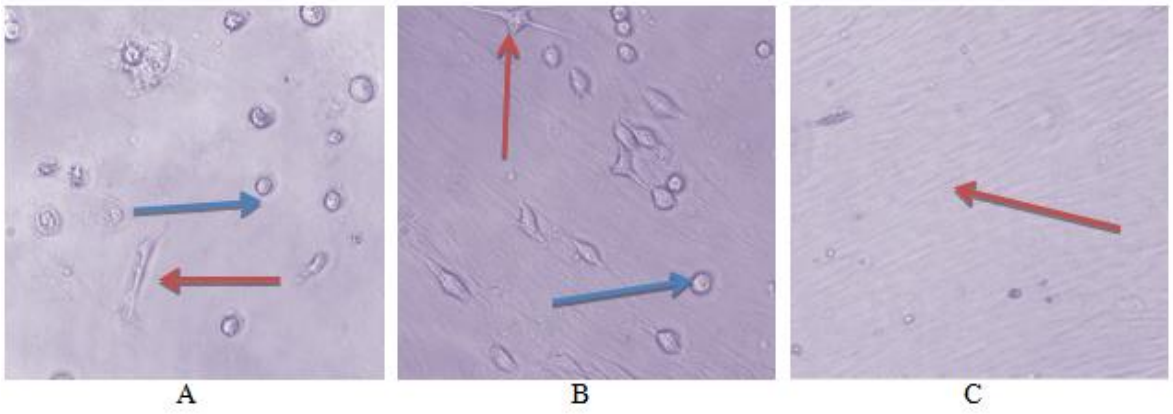

***Fig (1)*: Showing ASCs after isolation a) one week; show a stellate cells, b) two weeks: cells increase in number and attain more spindle appearance, c) three weeks: cells increase in number and become compressed (blue arrows showing hematopoietic cells while red arrows show different morphology of stem cells)**

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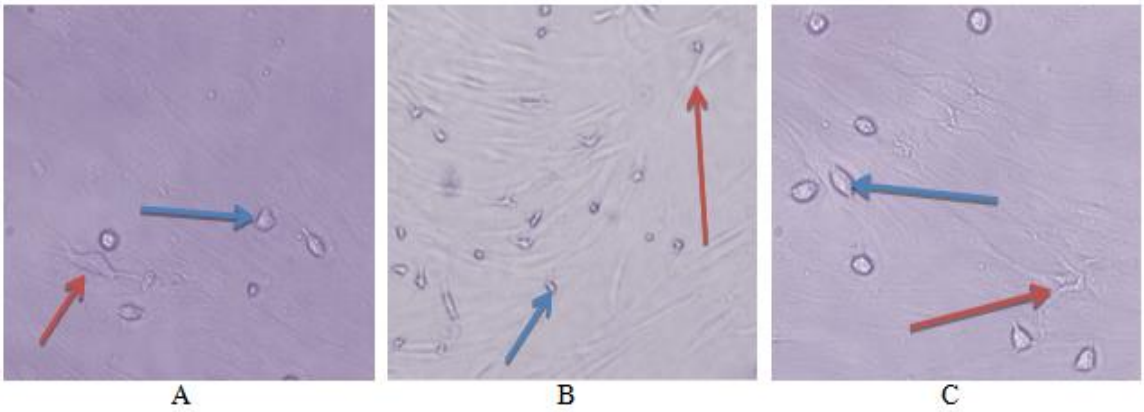

***Fig (2*): Showing OMSCs after isolation a) one week; show morphological diversity, b) two weeks: cells increase in number and attain more spindle appearance, c) three weeks: cells become more compressed and showing confluence 80- 90%.**

Characterization of stem cells by flow cytometry:

The expression of CD90, CD105 and CD45 were assessed using flow cytometric analysis which revealed that ASCs and OMSCs were positive for CD90 (ASCs: 82 ±2%, OMSCs: 97 ±1%)(Fig.3). as well as for CD105 (ASCs: 98.58% and OMSCs: 99.75%)(Fig.4). On the other hand, the cells were negative for the leucocyte precursor marker CD45 (ASCs: 0.04 and OMSCs: 0.2%).

Fig (3): Flow cytometric analysis of ASCs(A) and OMSCs(B) for CD90

Fig (4):Flow cytometric analysis of ASCs(A) and OMSCs(B) for CD105

MTT Assay:

On day 14, the number of viable cells in ASCs cultures was found to be significantly higher than that of OMSCs and this was also demonstrated on day 21. It was also demonstrated that the proliferation of ASCs and OMSCs increased significantly (P value ≤ 0.05) from day 14 to day 21 (Fig.5).

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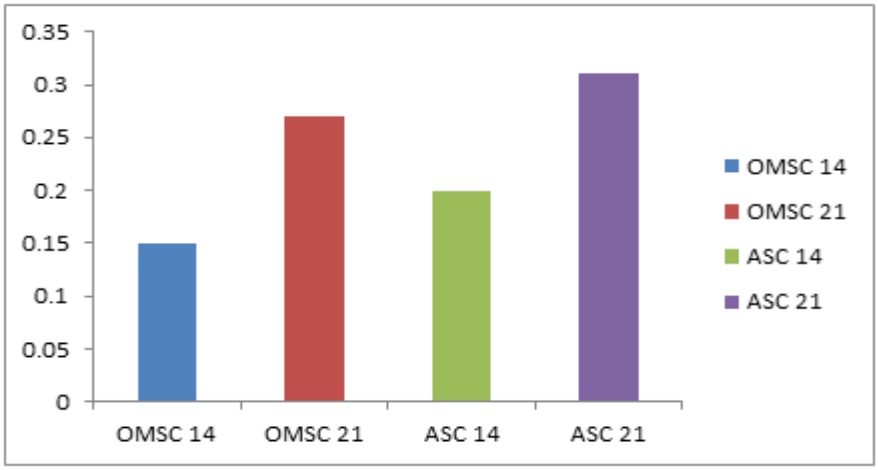

Fig .(5): Bar chart for MTT assay for ASCs and OMSCs on day 14 and day 21

Assessment of Stemness after Differentiation by Flow Cytometry:

CD90 was negatively expressed in the cells (ASCs: 4±1% and OMSCs: 2±1%)(Figs.8) as well as CD105 was also negatively expressed in the cells (ASCs: 0.04% and OMSCs: 3.14%)(Figs.6,7)

Fig (6): Flow cytometric analysis of ASCs (A) and OMSCs (B) for CD90 after differentiation showing negative expression

Fig (7): Flow cytometric analysis of ASCs (A) and OMSCs (B) for CD105 after differentiation showing negative expression.

Assessment of Differentiation

Alcian Blue staining

By the 14th day, staining with Alcian Blue revealed that the differentiated cells had a round morphology, an obvious cartilage like lacunae and dense extracellular matrix that stained strongly for Alcian Blue. By the 30th day, moderate chondrogenesis which display varying degrees of hyaline-like cartilage morphology with the extracellular matrix being Alcian Blue positive and dense intracellular staining (Fig.8, 9).

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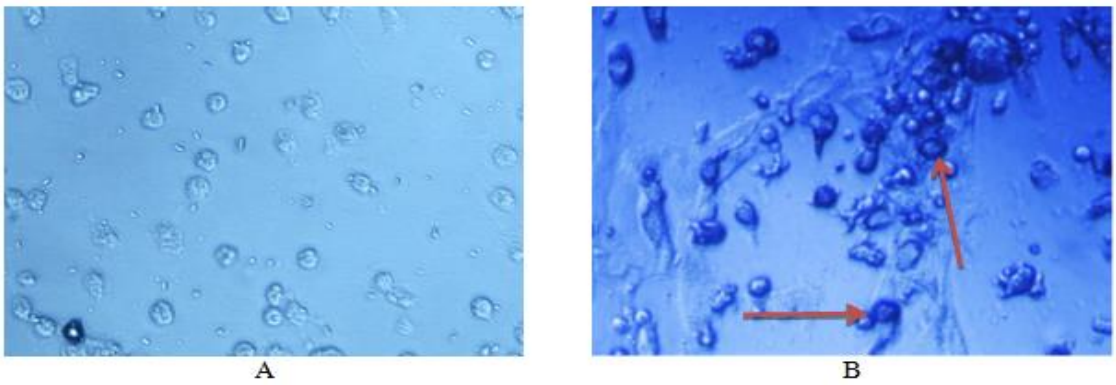

Fig (8): day 14 showing ASCs (A) stained with Alcian Blue stain which appeared homogenous indicating positive extracellular staining and low intensity intracellular staining and OMSCs (B) appeared homogenous indicating positive extracellular staining and high intensity intracellular staining than ASCs (arrows)

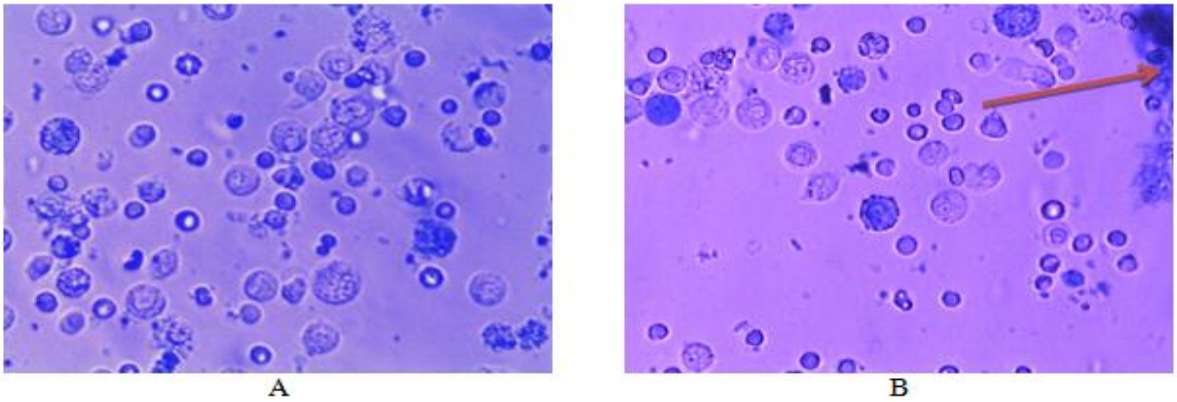

Fig (9): day 30 showing ASCs (A) densely stained intra and extracellularly with Alcian Blue stain and OMSCs (B) positively stained intracellularly with Alcian Blue stain; arrows show extracellular dense stain

PCR:

The chondrogenic differentiation potential was assessed by the expression of collagen II day 14 to day 30 was detected in ASCs and OMSCs. It was recorded that the amount of collagen II expressed was significantly higher in OMSCs cultures than in ASCs at the period of 14 days while on the 30 days ASCs cultures expressed more collagen II than OMSCs but this increase was insignificant (Fig.10).

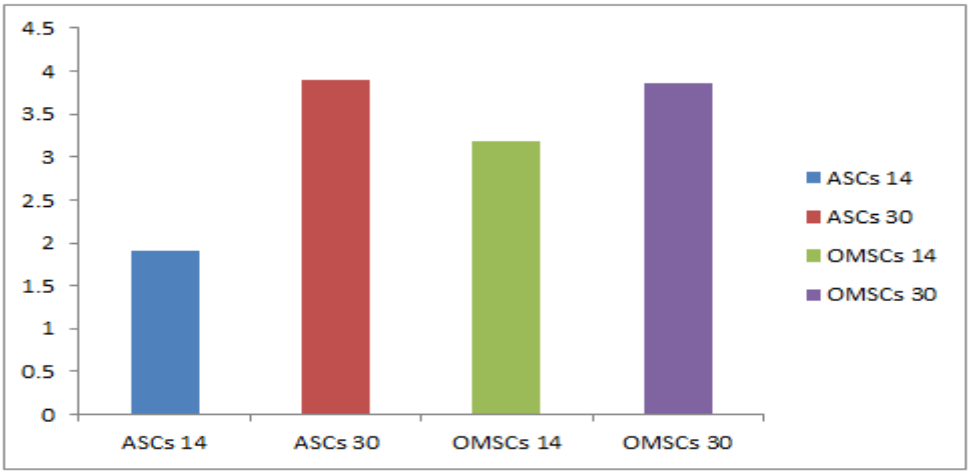

Fig (10): Bar chart showing expression of collagen II on day 14. and day 30 in ASCs and OMSc.

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DISCUSSION

The ambition of tissue engineering and regenerative medicine is to reinstate the functions of diseased tissues and organs. Tissue engineering approaches have necessitated main rudiments such as stem cells, scaffold or matrix, and growth factors ***(Titi et al, 2015 and Kabir et al., 2014).*** Most recent studies of tissues regeneration designed to use MSCs taken from sites that are even more reachable and rich in stem cells ***(Nguyen et al, 2013).*** Adipose tissue has been proven to be an optional source of MSCs as it is characterized by stable proliferation doubling kinetics in vitro, good accessibility and tissue abundance ***(Deepika et al, 2015).***

The oral cavity is the most wealthy stem cell source in the human body ***(Rouabhia, 2015).*** These oral stem cell populations are allied with a specialized micro-environment that provides key signals to steer stem cell function ***(Fawzy et al, 2012). Zhang et al. (2012)*** first characterized human gingiva-derived MSCs (GMSCs), which exhibited unique immunomodulatory functions, clonogenicity, self-renewal and multi-potent differentiation capacities similar to that of BMMSCs. GMSCs proliferate faster than BMMSCs, display a stable morphology and do not lose their MSC characteristics ***(Fournier et al, 2013)***

The present study focused on the ASCs and OMSCs regarding their characterization, proliferation and capability to differentiate into chondrogenic lineages. After the isolation procedure, diverse morphologies such as spindle and stellate-shaped cells were demonstrated in the cell culture. The cells were able to endure after several passages due to the use of mesenchymal cell promoting culture media as reported by ***Jin et al. (2014).*** In our research, the cells positively expressed the stem cell markers as CD90 and CD105 and negatively expressed hematopoietic cell marker as CD45. These results were in agreement with ***Angelova-Volponi et al (2013)*** and ***Karim M. et al. (2012).***

In the present study, during cell morphology analysis, all cell cultures showed diversity in morphology ranging from spindle to stellate appearance that was steady throughout several passages. Our results were generally in agreement with ***Ge et al. (2012)*** and ***Geetanjali et al. (2010).*** To assess the proliferation capability of the ASCs and OMSCs, MTT assay were elected Our results verified that ASCs and OMSCs showed a significant increase in cell viability from day 14 to day 21, representing high cell proliferation rate(80-90%), where ASCs proliferated faster than OMSCs. Both tissue cultured cells proliferated faster in the first two weeks than they did in the third week. Our results were generally in agreement with ***Davies et al. (2014)*** *and* ***Mohamadreza et al. (2013).***

Flow cytometry was used to evaluate the loss of stemness of the cultured stem cells after induction of chondrogenic differentiatioin ***(Alfonso and Al-Rubeai, 2011).*** The cultured cells were examined for expression of stem cell markers CD90 and CD105. Flow cytometric analysis exposed very much reduced levels of CD90 and CD105 and this suggested that the differentiated cells contain negligible proportion of stem cells indicating loss of stemness. Our results were in accordance with ***Seo M et al. (2012)*** who found that stemness of dental pulp stem cells was almost lost by measuring the expression of stem cell markers (Stro-1 and CD146) which were expressed in a very much reduced levels indicating that cultured dental pulp cells had been already differentiated.

In our study, we cultured the cells in chondrogenic media which was able to induce the cells to differentiate into chondrogenic lineage. Alcian Blue stain was used to assess the chondrogenic differentiation which stains the cartilage proteoglycan. After chondrogenic induction, cells within confluent dishes started to change their spindle-shape into rounded shaped cells (chondrocyte- like appearance). By the 14th day, staining with Alcian Blue discovered that the differentiated cells had rounder morphologies, an obvious cartilage like lacunae and dense extracellular matrix that stained strongly for Alcian Blue. By day 30, the cells attained typical chondrocyte- like appearance. The positive staining indicates presence of sulfated glycosaminoglycans, which proves subsequently the successful chondrogenic differentiation.

This result is in accordance with ***Dani et al., (2012) and Bat et al, (2011)*** who stated that number of growth factors, including fibroblast growth factor, insulin-like growth factor, transforming growth factor (TGF)-β and bone morphogenetic proteins have been implicated in this chondrogenic differentiation process. In the present study, to confirm chondrogenesis, the chondroblast phenotype was also examined by RT-PCR for the expression of collagen II which can be used as an evidence of chondrogenesis. The results of RT-PCR further confirmed the findings established by Alcian Blue staining where Collagen II gene was detected in BMSCs on day 14 and day 21. These findings are in accordance with ***Luis A. et al., (2011),*** induced chondrogenic

differentiation using the desired culture conditions and found that MSCs experience chondrogenic differentiation within 2–3 weeks, producing abundant extracellular matrix composed primarily of cartilage specific molecules such as type II collagen and aggrecan. From the present study, it can be concluded that: ASCs acquire a higher proliferative capacity than OMSCs as evidenced by MTT results, while OMSCs demonstrated a slightly higher capability of chondrogenic differentiation than ASCs as evidenced by collagen II expression.

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