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Original article

Chronic metabolic and induced stress impacts mesenchymal stromal cell differentiation and modulation of dental origin *in-vitro*

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ABSTRACT

The impact of induced (smoking) and metabolic stress (diabetes) on dental stem cells with respect to pre-impact consideration on differentiation and bone formation were investigated. The progenitor stem cells isolated from dental pulp, follicle and gingival tissues were phenotyped and subjected to nicotine and high glucose stress mimicking the smoking and diabetic condition *in-vitro*. The results showed that the cellular viability post treatment with 100 μM nicotine and 10uM glucose was about 86% to 89% respectively in all the three cell types while about 73% in combined nicotine and glucose treatment. No variation in the expression of pro-inflammatory TNF-α, IL-1β and IL-12 in all the three cell types were noticed. The observed viability in nicotine treated cells were due to elevated IL-6, IL-10 while in glucose was due to brain derived neurotropic factor (BDNF). Higher expression of IL-4, IL-6, IL-10, TGF-β and heme oxygenase −1 (HO-1) were found high in both stressors treated cells. Differentiation and mineralization markers Alkaline phosphatase (ALP), Collagenase I (COL1), Osteocalcin, Runt related transcription factor 2 (RUNX2), Osteopontin and Bone sialoprotein were expressed in the dental pulp stem cells (DPSCs) and gingival mesenchymal stem cells (GMSCs) at varying levels post nicotine or glucose treatment while not significantly observed in dental follicular stem cells (DFSCs). Therefore, it is evident that the stem cells of varied dental origin responded to the stress are more or less uniform with physiological delay in differentiation into osteoblast. It is evident from the study that, the metabolic or induced stress subverts the process of regenerative healing by mesenchymal stromal cells with their anatomical niche.
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1. Introduction

Stem cells present in the various anatomical niche and are tissue-specific pools of progenitor cells. The resident stem cells of mesenchymal or stromal origin have varied functions from regenerative to modulatory depending on the signal (Yu et al., 2015) and are susceptible to stress, both metabolic and acquired following chronic exposures (Greco and Rameshwar, 2012). The current understanding is that the differentiation capabilities and regenerative functions largely depend on the paracrine (Morsczeck et al., 2017) and, to an extent, autocrine signals (Takahashi et al., 2019) from the cells. Moreover, the mobilization of these stem cells from the niche to the site of injury is hindered by stressed tissue phenotypes like untreated diabetes mellitus (Mahmoud et al., 2019; Zhen et al., 2017) and acquired stress like long-term continuous smoking (Ng et al., 2013; Ng et al., 2015). However, there is insufficient evidence on the dental stem cells' fate and functionality under external and internal stress and whether these stem cells are exposed to chronic stress within the dental niches. It is also unknown whether the stem cells mobilized to the injury site have lower than usual susceptibility levels than adult cells (Tower, 2012).

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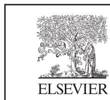

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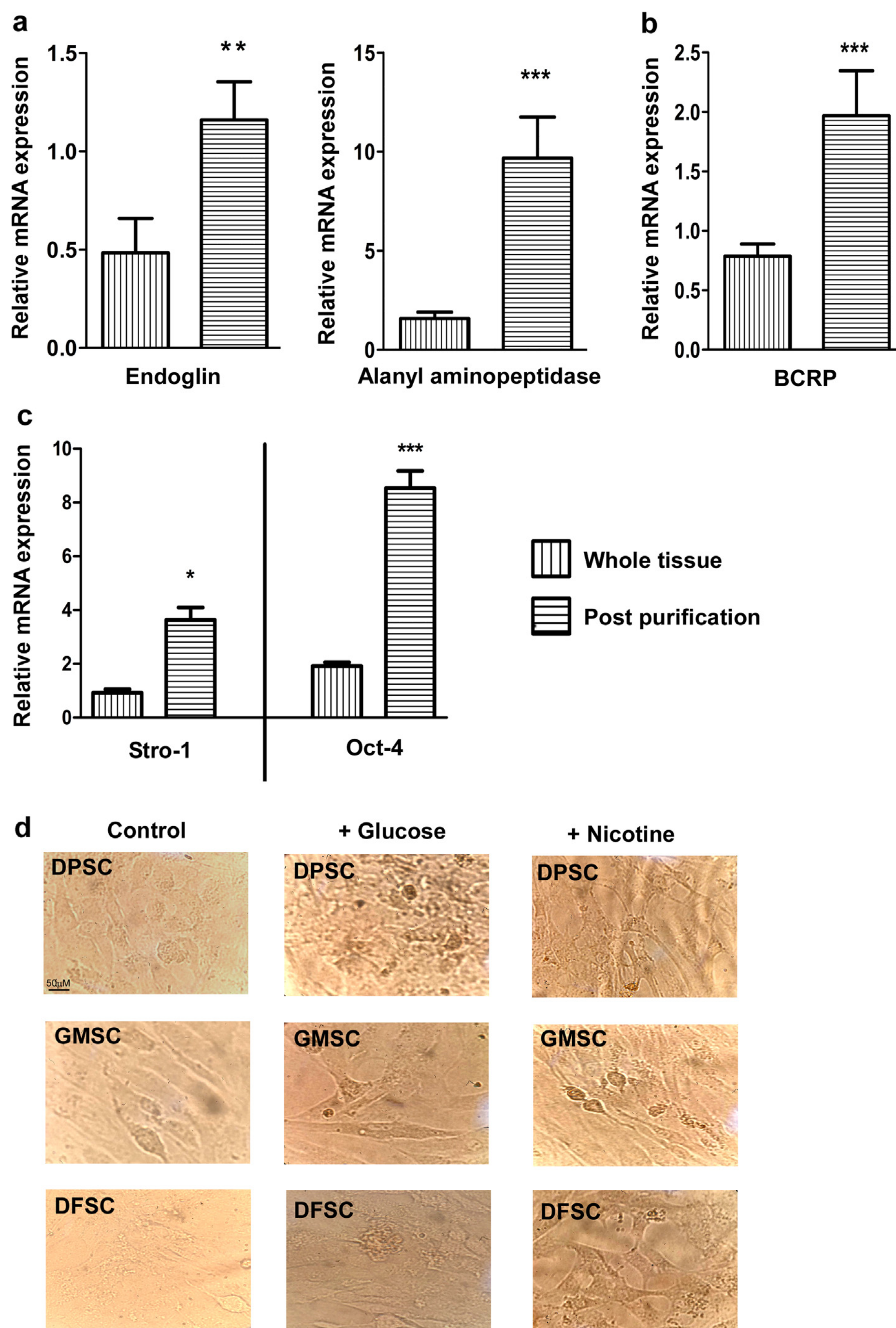


Fig. 1. Characterization of the isolated stem cells pre and post processing of the tissues. a. Endoglin and Alanyl aminopepdidate expression in the DPSC. b. BCRP expression for DFSCs. c. Stro-1 and Oct-4 expression in GMSCs. d. Morphological observation of the various stem cells in culture pre and post stress with nicotine or glucose.

The importance of deducing such functional lacunae is to address clinical issues such as osseointegration of dental implants and alveolar bone healing following regenerative surgical procedures in patients with underlying conditions (Murakami, 2000). With the recent advances in the therapeutic use of stem cells in dentistry, it can be noted that the improved pace of bone regeneration and osseointegration makes it convenient and safe to treat multiple implant sites simultaneously (Paz et al., 2018).

The current literature shows that many stem cells sources, especially of stromal origin, in the oral region have been characterized well. The current knowledge of stem cell sources of dental origin includes dental pulp stem cells (DPSCs), exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), gingival mesenchymal stromal cells (GMSCs) etc have been studied extensively for their regenerative dentistry applications (Bakopoulou and About, 2016). When used as implanted donor cells or coated over dental implants, these stem cells interact with the host's immune cells. This is an important event as the cell-to-cell communication with the adjoining tissues and their microenvironment play a pivotal role in the successful and accelerated bone regeneration or osteointegration of the implant (Li et al., 2019; Yang et al., 2017). Advances in regenerative dentistry show that the stromal stem

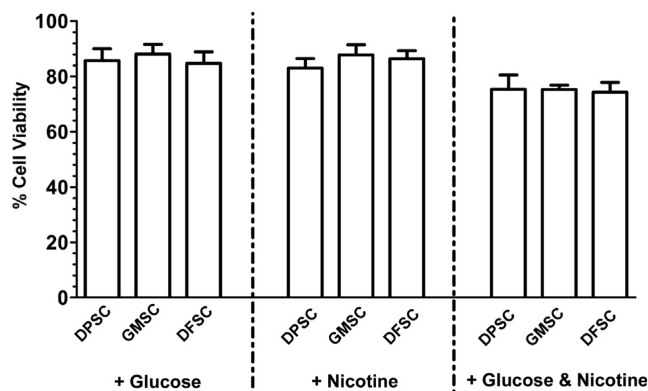


Fig. 2. Viability of the DPSCs, GMSCs and DFSCs during the induction of the stress to check whether the cells are able to be viable for downstream experiments.

cells' regenerative capabilities have inbuilt factors that could propel them to be biologically active in signaling bone regeneration or osteointegration (Li et al., 2019; Chalisserry et al., 2017).

From a clinical perspective, bone regeneration and osseointegration are the two desirable outcomes of periodontal reconstructive surgery and dental implant site development. The presence of uncontrolled diabetes mellitus and chronic smoking are the two major risk factors in the occurrence of periodontal disease (Ganesan et al., 2017). Both types of diabetes mellitus enhance the inflammatory events within the periodontal tissues, adversely affect the bone formation in the new bone, and upregulate the expression of Receptor activator of nuclear factor kappa-β ligand (RANKL) in the presence of dental plaque. Activation of nuclear factor-kappa β and pro-inflammatory cytokines such as tumor necrosis factor and interleukin-1 in diabetes mellitus is due to increased glucose levels, reactive oxygen species, and advanced glycation end-products in the periodontal tissues (Graves et al., 2020). Similarly, several studies show that cigarette smoking alters the periodontium's oxidative stress mechanisms, causes constriction of the microvasculature, and delays wound healing (Chang et al., 2018; Sanz and Teughels, 2008).

This led us to hypothesize that the stressors, namely, glucose and nicotine, primarily affect the homeostasis or stemness of the stromal cells at the anatomical nice while stromal cells of the different site have a dissimilar response to these conditions. Hence, we decided to experimentally investigate the response of DPSCs (Hata et al., 2015), GMSCs (Zhao et al., 2019), and dental follicular stem cells (DFSCs) (Zhou et al., 2019) towards high glucose akin to diabetes and nicotine as observed with chronic smokers *in-vitro*.

2. Materials and methods

2.1. Ethics statement

The study protocol involved the collection of extracted teeth from medically healthy patients between the age of 18–25 years who consented to participate in the study. All procedures were carried out by approved and relevant guidelines for handling human subjects. Only cases that required removal of third molar teeth for therapeutic purposes and presented with follicle around the root when examined radiographically were considered for the study. Informed consent was obtained from each of these patients before the collection of extracted teeth and subsequent removal of the pulp tissue, gingival tissue, and follicular tissue from those teeth as per the protocol approved by the scientific research committee and Institutional Review Board (IRB) at the College of Dentistry, King Khalid University (SRC/ERC/2018-19/114). A total of 26 samples, each with dental pulp, gingival tissue, and follicular tissues, were isolated. Since we lost dental follicle and gingival tissue from 2 specimens, $24 \times 3 = 72$ samples (24 samples from each tissue group) were included in this study.

2.2. Tissue processing

The tissue samples were immediately transferred to the stem cell unit in a 1.5 ml Eppendorf tube containing plain DMEM with 100 U/ml penicillin, and 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B transport medium at 4 °C. The processing of the samples was carried out in cold conditions as per the protocol described in detail elsewhere (Fawzy El-Sayed and Dörfer, 2016). Briefly, the tissues were roughly torn to small bits and then treated with collagenase IV for 20 min at 37 °C and were strained through a 70 µm strainer. The cells were washed once with ice-cold Phosphate-buffered saline (PBS) for 10 min at 1200 rpm at 4oC. The cells were then enumerated for cell viability and phenotyping. The stem cells' pure population was achieved by positive selection with CD73, CD13, and Stro-1 magnetic labelled beads after removing the floating cells after 4 h' sedimentation in a 100 mm tissue culture dish. The rest of the cells were cryopreserved till the day of experimentation. Confirmation of the DPSCs, DFSCs, and GMSCs was done checking the expression of endoglin and alanyl aminopeptidase, breast cancer resistance protein (BCRP), stro-1, and Oct-4, respectively, by RT-PCR in the isolated cells.

2.3. In-vitro diabetic and smoking condition model

The isolated and transiently cryostored cells were retrieved in 6 well plates. The plates were prepared with sterile coverslips previously for differentiation staining. Once the cells attained a colony of 60% or more by day 7 or 9, the cells were challenged with 100 µM Nicotine treatment and 10 µM glucose individually for 24 h. Post-exposure, the cells were moved from stressor medium to osteoblast differentiation medium for 9–13 days. The coverslips were retrieved, fixed in ice-cold methanol for 15 min, and proceeded to alkaline phosphatase and alizarin red staining for phenotypic detection of osteoblast differentiation and mineralization (Caplan, 2007; Inada et al., 2017). The cells from the rest of the plate are scraped with sterile cell scrapers, and the collected cells are centrifuged for further assessment of survival and differentiation markers by the genomic array.

3. Results

The cells isolated and purified by magnetic cell sorter confirmed the stem cell/progenitor linage via the expression of specific markers through mRNA expression. The expression of endoglin and alanyl aminopeptidase confirmed the dental pulp's stromal cells (Fig. 1a), while BCRP expression was specific to follicular stem cells (Fig. 1b). Expression of stro-1 and Oct-4 confirmed the gingival stromal cells (Fig. 1c). Since we had used phenotypic markers for magnetic sorting and in our experience, the cells using magnetic sorter give more than 97% pure cells, we did not perform flow cytometry. The normal morphology of the DPSCs, DFSCs, and GMSCs showed fibroblast-like morphology, and we checked any changes associated with the cells after stress (Fig. 1d).

The results of the *in-vitro* stressors like nicotine and glucose have varied effects on the types of stem cells tested. The viability of the cells was initially checked to ensure that the concentration of the nicotine or glucose used for experiment does not immediately kill the cells. We observed more than 85% viability post 24 h in all three types of cells with minute variations (Fig. 2). The combination of nicotine and glucose at our concentrations

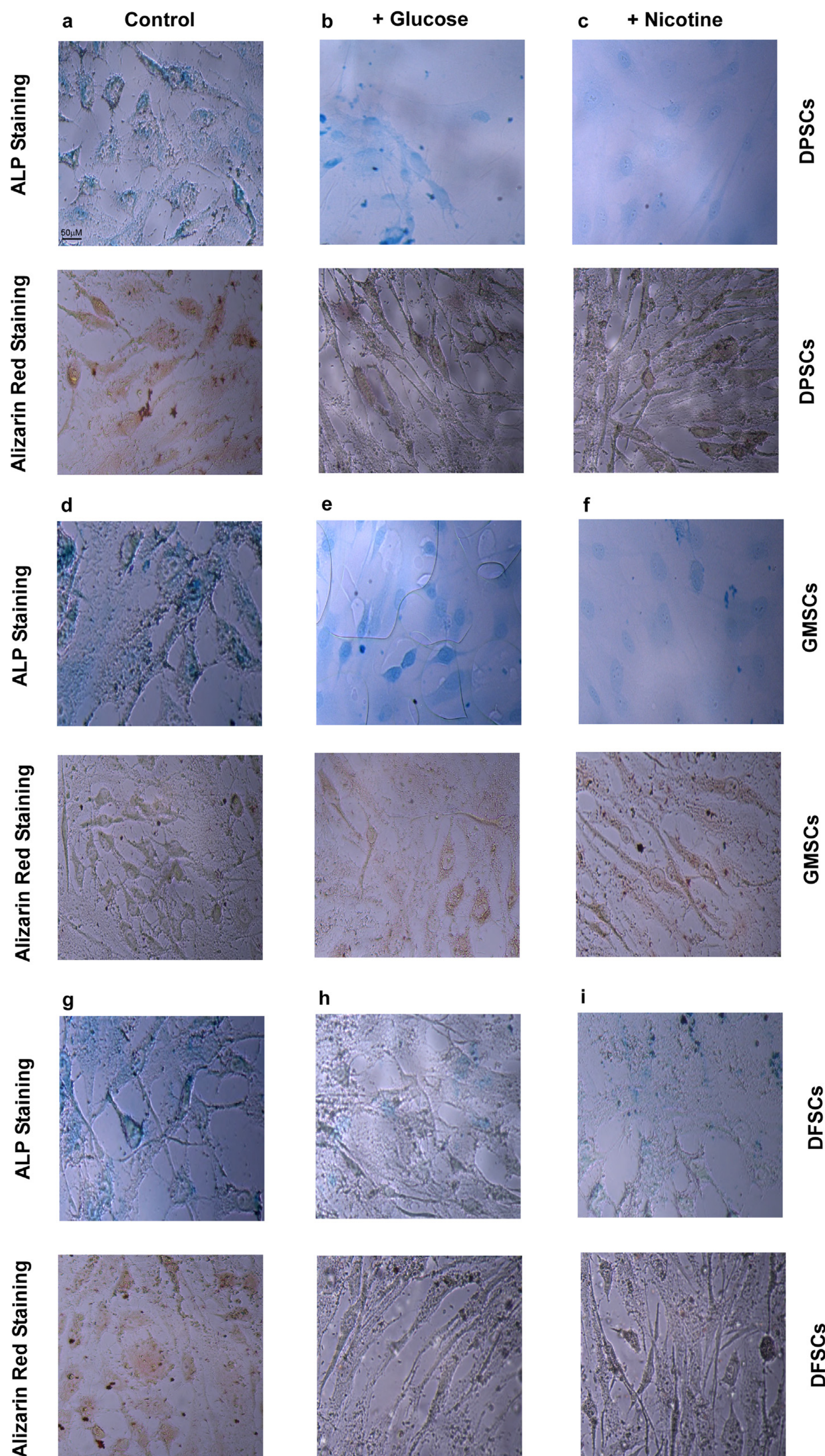


Fig. 3. The ALP and Alizarin Red staining for showing differentiation and mineralization. The a-c shows ALP & Alizarin Red staining of DPSCs post nicotine and glucose treatments. d-g and g-I shows the staining patterns for GMSCs and DFSCs respectively.

resulted in ~75% viability showing that our concentrations are sufficient to exert stress rather than immediate cell death (Fig. 2). From our staining results, it is evident that the DPSCs and GMSCs were more vulnerable to both nicotine and glucose stress deeming the cells differentiated to osteoblast with good mineralization as seen in Fig. 3 a – c & d - f. On the other hand, the DFSCs were very

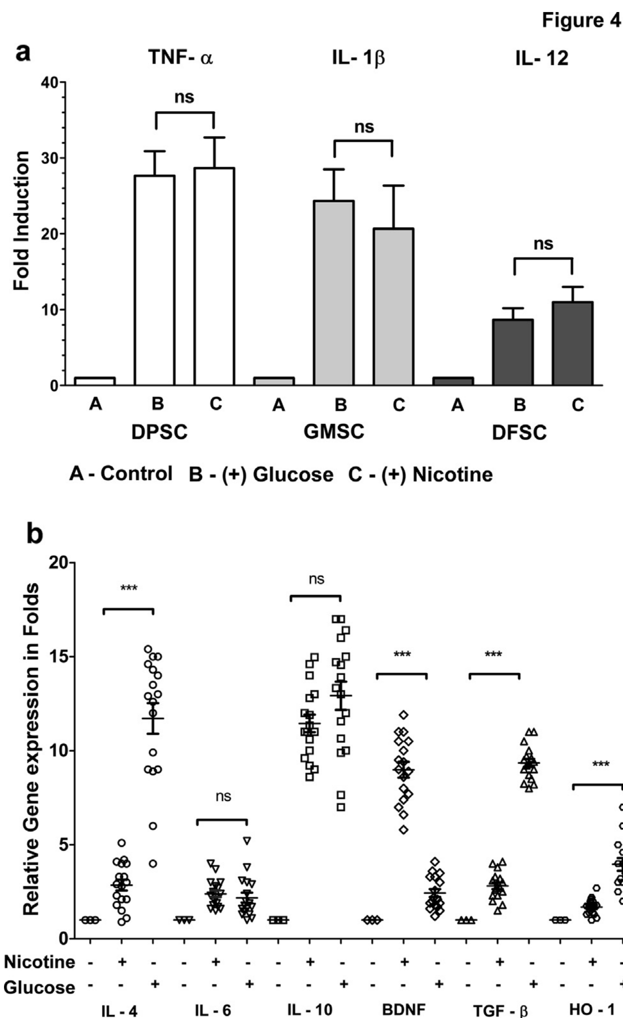


Fig. 4. a Pro-inflammatory maker's expression substantiating requirement for the cell viability and differentiation patterns. b. Paracrine array of cytokines and survival factors required for survival of the stem cells under stress.

much susceptible to the stressors, and we could not observe differentiation and mineralization (Fig. 3 g – i) similar to what was observed with DPSCs and GMSCs.

To substantiate our results of differentiation, we assessed the expression of cell survival, osteoblast differentiating markers. The results showed the expression of some pro-inflammatory markers like TNF-α, IL-1β, and IL-12 (Fig. 4 a) that were less equal in all the three cell types. The array of the paracrine markers and their expressions were similar to all the three cell types, and it is depicted here unless there is a gross and significant change or omission of expression of any markers. Hence, the markers are maximum of three hits in all three samples. The markers that keep the cell viable post nicotine stress (Fig. 4 b) were IL-6, IL-10, and brain-derived neurotropic factor (BDNF), while the high glucose treated cells (Fig. 4 b) showed expression of IL-4, IL-6, IL-10, TGF-β, and heme oxygenase -1 (HO-1) compared to the control cells which were either treated with nicotine or glucose. Further, the differentiation markers like Alkaline phosphatase (ALP), Collagenase I (COL 1), Osteocalcin, Runt related transcription factor 2 (RUNX2), Osteopontin and, Bone sialoprotein was expressed in the DPSCs and GMSCs at varying levels post nicotine or glucose treatment; however, we could not observe much expression of these markers in DFSCs (Fig. 5).

4. Discussion

4.1. Response of the stromal cells from various anatomical niche

We investigated the homeostasis and the response of the stromal cells from various anatomical niche namely, DPSCs, GMSCs, and DFSCs towards metabolic and included stressors *in-vitro*. The initial results of the mesenchymal stromal cells characterization were well in agreement with published results (Avinash et al., 2017; Pilbauerová et al., 2019; Tomasello et al., 2017). The specific markers' expression was useful in coming to an agreement about the various sources or niches where the stromal stem cells are found.

The exposure of stem cells to the chronic stressor of the privileged dental anatomical niches reveals that, the cells are not entirely skewed towards apoptosis or necrosis. However, the stem cells' regenerative capacity irrespective of the dental origin is hindered due to stresses cellular phenotypes that require a real-time investigation. Though our observations did not suggest a gross difference in the survival markers' expression pattern, it is not surprising as some of these markers are also known to be pro-inflammatory with specialized functions of cell proliferation (Yang et al., 2013).

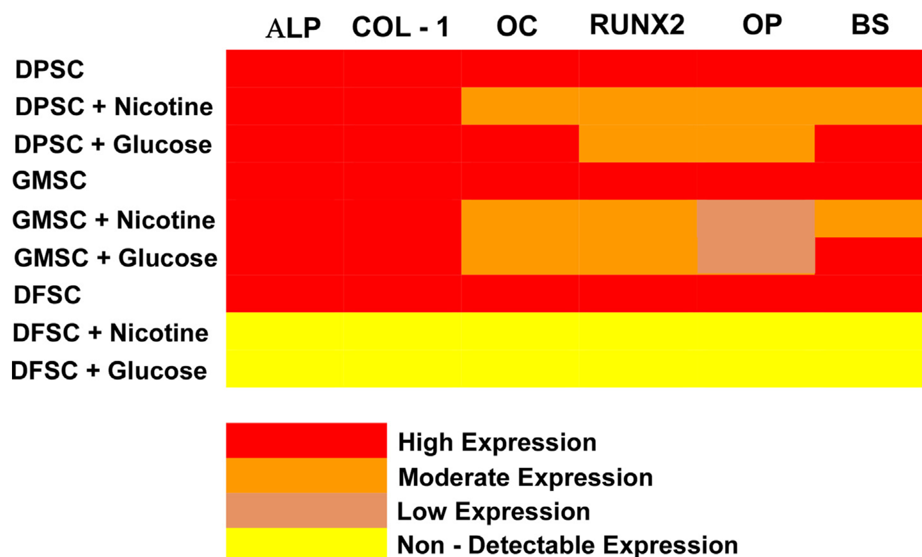
A more interesting observation was regarding the paracrine factors that are necessary for survival. However, the stromal cells of all three origins survived, the expression of the markers slightly varied with the origin of the stem cells to the stressors. This could be attributed to the fact that cell death mechanisms are functions of several exogenous parameters and the ability of the cell to handle specific types of stress (Fulda et al., 2010). The cell response towards metabolic stress or acquired stress varied with markers or expression patterns at this stage, while real implications were observed with negative differentiation in DFSCs. Similar observations were not observed in the literature to substantiate our claim. Therefore, it becomes a novel observation that cellular niche and proximity of stressors are major factors resonating the stem cell differentiation and repair.

We further observed that DFSCs could not effectively differentiate in both the conditions of nicotine and high glucose while the DPSCs and GMSCs readily differentiated though with little variations, which could be obviously observed in the level of mineral deposition or expression of the early and late differentiation markers. These observations of DPSCs and GMSCs were well in agreement with earlier published research (Tomasello et al., 2017). Another observation that was noted was that some of the cells did not show mark of differentiation in the DPSCs and GMSCs, and we could not isolate these negatively skewed cells to check the marker expression and compare with DFSCs, which had entirely not shown any sign of differentiation or expression of the differentiation markers. The expression of survival makers in DFSCs might be indicative of modulatory functions, which warrant further investigation for isolating the cells of differentiation resistant cells from DPSCs and GMSCs that are not succumbed to the selection/differentiation pressure after encountering the stress. This will enable us to know the difference and the transcriptional regulation that positively or negatively skews the cells to differentiate post-stress and the functional aspects which affect the tooth restoration or regenerative adaptation (Fan et al., 2020).

4.2. Clinical implications

The results of this study improve our understanding of the effect of metabolic stress and injury on the differentiation of stem cells. This is of great clinical significance as statistics show that diabetes mellitus and smoking are among the most significant risk

Figure 5



ALP - Alkaline Phosphatase; COL - 1 - Collagenase 1; OC - Osteocalcin;

RUNX2 - Runt Related Transcription Factor - 2; OP - Osteopontin;

BS - Bone Sialoprotein

Fig. 5. The differential expression of the osteoblast and mineralization markers of control (Nil stress) and post treatment with nicotine and glucose. The expression of these markers is depicted as high/moderate/low/not detectable levels.

factors for periodontal disease progression (Ganesan et al., 2017). These act as stressors and adversely affect the treatment outcomes by impeding periodontal tissue regeneration through various mechanisms while attempting procedures for improving the alveolar bone dimensions prior to implant placements (Bazli et al., 2020).

These stressors also result in oxidative stress characterized by disruption of the balance between the production and the inactivation of reactive oxygen species (ROS) and leads to cellular dysfunction and damage. Studies have shown that oxidative stress or damage can increase the levels of pro-inflammatory cytokines (Marchetti et al., 2012) which could grossly affect the differentiation of the mesenchymal stromal cells. In the current study there was not variation in the expression of the pro-inflammatory TNF-α, IL-1b and IL-12 in all the three cell types.

The implant failure can be attributed to an imbalance in the interconnected physiological factors such as adipocytokines, increased caloric intake, ROS, metabolic activity, insulin resistance, hyperinsulinemia, altered neutrophil chemotaxis, hyperglycemia, and advanced glycation end-products which are mediated by oxidative stress (Marchetti et al., 2012) induced by diabetes. Similar mechanisms may also be responsible for the degeneration and reduced biomineralization in human osteoblasts (Siddiqui et al., 2020) in smokers too, thus leading to delayed wound healing, increased rate of implant failure, and compromised clinical outcomes (Decker and Wang, 2020; Shang and Gao, 2021; Takamiya et al., 2014).

Smoking is associated with an increased risk of marginal bone loss around implants (Qian et al., 2012) and risk of early implant loss which in most instances are not related to the duration of smoking exposure (Nagao et al., 2021; Naseri et al., 2020). The delayed wound healing and implant failure is linked to noxious chemicals of smoking such as nicotine, carbon monoxide, nitrosamines, aldehydes, and hydrogen cyanide (Levin and Schwartz-Arad, 2005). Studies show that there is a reduction in the oxygen-carrying capacity of red blood cells (Takamiya et al., 2014). hypoxia and deficiency in the recruitment of preosteoblasts, and differentiation into osteoblasts (Balatsouka et al., 2005) that interfere with various stages of wound healing and osseointegration (Casado et al., 2019) in smokers. Therefore, it is imperative to identify the smoking habits and diabetic status of the patients before they are taken up for implant placement procedures.

4.3. Limitations of the current study

The presence of mesenchymal stromal and other progenitor cells at various anatomical niches are naturally protected to mechanical and physiological stress to a great extant. Current study is a comparison of responses to the chronic stress, which has variation on the dose, time frame and reach of the stressors to these niches. Mere exposure to smoke diffused in the media or addition of nicotine does not deem to mimic the physiological conditions provided the later at least satisfies the residual nicotine in various tissues. Further experiments with non-human primates could be one of the suggestions to study *in-vivo* model in detail.

4.4. Future perspective

The microenvironment of stem cells plays a pivotal role in determining the therapeutic outcome of clinical situations, including osseointegration and bone healing. Therefore, newer therapeutic strategies in dental regenerative medicine should focus on optimizing the influence of physical and pathological parameters on the microenvironment for favorable clinical results. Improve-

ment in periodontitis and osteoporosis has been documented following the restoration of mesenchymal stem cells function by protecting them from oxidative damage. There is also a need to further explore the therapeutic role of epigenetic regulation mechanisms such as histone modification regulators and microRNA expression modulation for improvement in bone healing and regeneration of periodontal tissues.

Furthermore, there is also scope for research in regenerative medicine using different modes of administration of exogenous mesenchymal stem cells through various routes as an intravenous infusion and intraperitoneal delivery. These methods have been shown promising results in the management of osteoporosis and osteoarthritis through mechanisms such as endogenous osteoblastogenesis and modulation of osteoclast-mediated bone resorption. A better understanding of the signaling pathways involved will help in developing novel therapeutic modalities to improve the scope of stem cell-based dental regeneration medicine. Similarly, the potential of nanotechnology in mimicking a bionic microenvironment also remains to be explored.

In conclusion, it is evidenced from our observation that the niche of the stem cells plays a key role in regenerative functions. Further, it has varied responses to the long-term metabolic or acquired stress, affecting the implant homing or prosthetic procedures in dentistry. It is, therefore, necessary that these stressors be controlled, which results in the natural mobilization of the stem cells to the site of repair or transplantation of stem cells embedded in the matrix for faster and favorable better clinical outcomes.

CRediT authorship contribution statement

Faris M. Binhomran: Conceptualization, Formal analysis, Methodology. **Ahmed A. Alaskari:** Conceptualization, Formal analysis, Methodology. **Anantharam Devaraj:** Funding acquistion, Methodology. **Samuel Ebele Udeabor:** Methodology. **Ahmed Al-Hakami:** Project administration, Resources. **Betsy Joseph:** Writing – review & editing. **Satheesh B. Haralur:** Data curation, Project administration, Resources. **Harish C. Chandramoorthy:** Conceptualization, Data curation, Formal analysis, Funding acquistion, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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