

Exogenous Factors Capable of Enhancing Mesenchymal-origin Cell Proliferation *in vitro*: Promising Applications in Large Scale Cell-bioprocess Development

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Received November 14, 2013; Revised December 12, 2013; Accepted December 16, 2013

Abstract Musculoskeletal damage is a major health-related problem in working groups as well as in growing age population throughout the world. Due to the inefficiency of the current surgical treatment, Regenerative Medicine has recently grasped the attention of scientists. Tissue-engineering and cell-based therapies, two prominent branches of regenerative medicine, are considered as the most suitable techniques to repair the tissue damage. However, a huge number of autologous cells are required to address these practices. Since only small numbers of cells are obtainable from the patient, there requires expansion of cell number *in vitro*. Nevertheless, these primary cells grow slowly *in vitro* and lose their general characteristics during prolonged culturing period. It has been shown through this review paper that several exogenous factors, which can be classified as physical, chemical, biological and culture-environmental, can modulate the culture parameters efficiently. These can, therefore, support in promoting higher proliferation rate and also in maintaining the desired characteristics after a culturing period.

Keywords: *exogenous factors, clinical graded cell-proliferation, osteoblast proliferation, induced cell proliferation, biosafety of culture medium*

Cite This Article: Partha S. Saha, "Exogenous Factors Capable of Enhancing Mesenchymal-origin Cell Proliferation *in vitro*: Promising Applications in Large Scale Cell-bioprocess Development." *Journal of Biomedical Engineering and Technology* 1, no. 3 (2013): 50-60. doi: 10.12691/jbet-1-3-4.

1. Introduction

Maximum success of regenerative medicine comes from using of primary cells collected from the patient itself. These types of the primary cells include cells of the musculoskeletal tissues, such as osteoblast, osteocytes, chondrocytes, myoblasts, tendon and ligament cells. In other words, both of the streams call for huge number of viable, autologous cells. Besides the *in vitro* tissue regeneration used in tissue-engineering to populate cells on the 3D structure, these cells are being bio-processed into large population with the help of micro-carrier cultivation technique or different types of bioreactors, which are highly efficient, automated, robust and scalable culture configurations used to generate cells that are suitable to use for the clinical or industrial applications [1].

There are several major challenges before the tissue-engineering and cell-based therapy which restrict them to be widely available as a clinical product. Among these hurdles, the scaling-up of the primary cells is prime one. The situation is aggravated when only the autologous cells are needed to be grown. This is because only very low number of primary cells can be harnessed from tissues of a patient. For example, number of mesenchymal stem cells (MSCs) present in the bone marrow is about 0.01- 0.001% of the total number of the mononuclear cells in the bone

marrow. In addition to that, the availability of the MSCs from the bone marrow further decreases with advancing age of the patient [2].

Furthermore, these cells grow very slowly *in vitro*, and in the most of the cases, these cells tend to lose their genotypic and phenotypic entity over the culture period. For example, MSCs cease to grow in around 40-50 doublings [3].

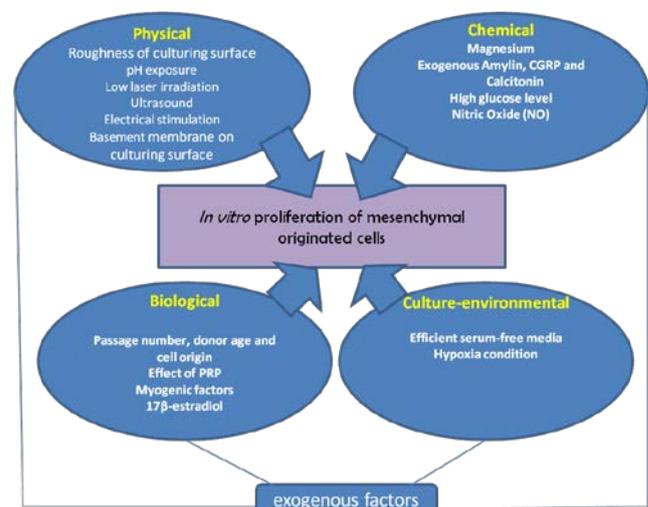


Figure 1. Classification of exogenous factors

Therefore, these limitations indicate the need of a more fast, robust, and reproducible cell-expansion to produce clinically-relevant numbers of cells in shorter periods of time with lower passage numbers. Adoption of a systematic approach can overcome the bottlenecks of the cell-expansion *in vitro*. In addition, the effective method must be well-adoptable, cost-effective, and abundant for a scalable production of clinical grade cell *in vitro*.

The aim of this review article is to explore the most effective exogenous factors, which are found to modulate *in vitro* proliferative activities of different musculoskeletal tissue-related cells without affecting their general features. The exogenous factors are categorized here under the headings of physical, chemical, biological and culture-environmental terms (Figure 1). In this case, proliferation of MSCs and osteoblasts are given emphasized so as to focus on the bone and cartilage defects, the widely explored area of regenerative medicine.

2. Exogenous Factors: Physical

Some of the factors are associated to yield higher cell proliferation when the cells are exposed to them physically. Those external agents are, for example, low laser irradiation, ultrasound, electrical stimuli, basic substances, etc. Roughness of the culturing surface and application of basement membrane have proved to be effective to harness higher proliferation.

2.1. Effect of Roughness of the Culturing Surfaces Can Enhance the Cell Proliferation

The surface roughness can be an important factor which can be controlled to obtain a large cell population of osteoblast cells in a particular culture. Some discrepancies have been observed to demonstrate the relation between these two aspects. For example, no effects have been reported by Mante *et al.* (2003) in their report [4]. However, Nishimoto *et al.* (2008) and Yamashita *et al.* (2009) have reported a positive response of surface roughness, affecting a better osteoblastic cell growth in cell culture [5,6]. These discrepancies may be because of several associated factors such as cell type used, serum concentration, surface fabrication methods, culture conditions, type of substratum, and even sterilization techniques, etc. [7].

According to a most recent report, a positive response of surface roughness of Ti-6Al-7Nb (a dental implant) surface on proliferation of human osteoblast-like cells is shown [7]. In their experiment, SaOS-2 cells (a human osteoblast-like cell line) were cultured in the presence of 10% FCS, being seeded on the surfaces of a Glass slide (as control), Ti1200, Ti400, and Ti180 with increasing surface roughness (measured in Ra or average roughness) [7].

Next, after 24h and 72h the nuclei of the cells were stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) to observe the cell attachment on the surfaces, and higher cell proliferation was observed at 72h than that of 24h. Moreover, it was also observed that except the Ti 1200 surface, the cells proliferated, statistically significantly and more than the control, with the increase of the surface roughness [7].

Therefore, from this experiment, it can be said that roughness of a culture surface, as an exogenous factor, can influence the proliferation of cells of an osteoblastic origin. It is claimed by the author that this growth of cells is because of the change in the microenvironment, which is caused by the surface roughness [7].

2.2. Enhancing Cell Proliferation with pH Exposure

Changes in extracellular pH are one of the conditions which can affect the milieu of the cells in a culture. With the help of a Na^+/K^+ -ATPase dependent Na^+/H^+ antiport, cells maintain their intracellular pH (pHi) or cytoplasmic pH in response to change in extracellular pH condition [8].

It has been observed by Musgrove *et al.* that different stages of the cell cycle are dependent on the pHi. In other words, it was observed that with the increase of the pH, the enrichment at S, G, and M phases occurred, and conversely, with the decrease of the pHi, there was an arresting of cells at G₁ phase [8]. It had been seen that an extracellular acidification below pH 6.5 also decreased the pHi of the cells and that led the cells to synthesis a reduced DNA. Additionally, in 1994, Ramp *et al.* observed the effect of the extracellular pH on the activities of embryonic chick osteoblasts *in vitro*, and found that the decreased pH changed the phenotype of the cells, while keeping the DNA content and synthesis constant [9].

Therefore, rather than by means of decreasing pH, it could be reviewed that attempts should be taken to promote proliferation by increasing the pH. With this aim, in 1995, Yamaguchi and his co-worker suggested that an increase in extracellular pH increased the formation of the number of gap junctions between osteoblasts, which may be the reason for the increased functionality [10]. The proliferative activity or the synthesis and mitogenesis of the osteoblasts in an increased pH was first observed by Kaysinger and Ramp in 1998. In their experiment, osteoblasts derived from trabecular bone were grown in a medium with a controlled pH, ranging from 7.0 to 7.8. It was observed that the DNA content increased in a range of pH 7.0 to 7.2, leveled off in a range from pH 7.2 to 7.6, and then again increased from pH 7.6 to 7.8 [11].

The author also observed that in the range of pH 7.0 to 7.6, the proliferation, the collagen activity, collagen synthesis and alkaline phosphatase activity increased; however, these parameters remained suppressed at pH 7.8, suggesting this value as a limit of the optimal condition for the viable osteoblasts [11]. Therefore, maintaining a culture pH in a range of 7.0 to 7.6 can be regarded as an exogenous means by which the increased proliferation of the osteoblast can be promoted.

2.3. Cell Proliferation Aided by Low Laser Irradiation

Low-level Laser Therapy (LLLT) uses the far-red to near infrared regions of the electromagnetic spectrum, ranging from 630–1000 nm in wave-length. Several findings in the last two decades have established that LLLT or light-emitting diode arrays helps in increasing cell proliferation along with generating significant biological effects, such as collagen synthesis and release of growth factors [12].

The first effect of the photoactivity of laser irradiation on osteoblastic cells was observed by Yamada in 1991. The cell growth rate and the DNA synthesis were found to be increased when osteoblastic cells (MC3T3-E1) were exposed to low power helium-Neon laser beam [13].

In 2005, Khadra *et al.* irradiated osteoblast-like cells derived from human mandibular alveolar bone, cultured on titanium implant material, with the GaAlAs diode laser at dosages of 1.5 or 3 J/cm². They investigated the cell proliferation after 48, 72 and 96h, and observed highest cell proliferation after 96 h. Moreover, they observed an increased cellular production of ALP, osteocalcin and TGF- β_1 in a dose-dependent manner. The increased level of osteocalcin and TGF- β_1 account for the higher osteoblastic differentiation, and on the other hand, the enhanced level of ALP indicates higher cellular activity [14].

Again in the same year, Abramovitch-Gottlieb *et al.* observed higher cellular density and distribution (or, tissue coverage) of MSCs - seeded on three dimensional coralline biomatrices - in response of low-level laser irradiation in comparison to control samples, indicating the enhanced proliferation [15].

In 2008, Oliveira *et al.* observed a LLLT dependent increase in cell proliferation along with an alteration in morphology of the mitochondria (from a filamentous to a granular appearance). They justified that bio-stimulatory effect of radiation on mitochondrial activity as a cause of proliferation [12]. In accordance with that finding, in 2006, Pinheiro and Gerbi reported that it was possible to biomodulate the non-differentiated mesenchymal cells to become osteoblasts, which could again bring about a faster change to osteocytes [16,17].

According to a most recent finding, it has been suggested that the effect of LLLT can elicit the release of growth factors including, insulin-like growth factor-I (IGF-I), basic fibroblast growth factor (bFGF), and receptor of IGF-I (IGFBP3) from osteoblast cells. This can be concluded as a reason of faster regeneration with simultaneous osteoblast proliferation in response to LLLT [18].

2.4. Cell Proliferation by Treating the Culture with Ultrasound

Ultrasounds are those sound waves which have the frequency above of 16 to 20 kHz. Among different types of ultrasound types, therapeutic ultrasound is of the frequency between 1 and 3 MHz and with intensities of 0.1 to 0.2 W/cm². Thermal and nonthermal mechanisms are the two types of mechanisms by which the therapeutic ultrasound exerts its physical effects on cells and tissues. Non-thermal ultrasounds are known to stimulate the tissue regeneration by inducing angiogenesis [19]. Doan *et al.*, in 1999, carried out an experiment to observe the effect of ultrasound on osteoblastic and fibroblastic cells *in vitro* by exposing both types of cells with two different frequencies (i.e. 1 MHz and 45 kHz) of ultrasound of varying intensities. Cell proliferation was assayed with the DNA assay in terms of incorporation of [³H] Thymidine into the synthesized DNA. The results showed that there was increased cell proliferation in fibroblasts and osteoblasts at higher intensities of both frequencies [19].

Therefore, it can be concluded that ultrasound of higher frequency and intensity can be regarded as an effective exogenous factor to enhance osteoblast proliferation *in vitro*.

2.5. Increased Cell Proliferation with Electrical Stimulation

Electrical stimulations are being used widely by several means in orthopedics with an aim to curing fractures, spinal fusion surgeries, re-growing of bone in failed joint fusion and healing of bone non-unions [reviewed in [20]].

In 1986, Ferrier *et al.* established that in response to a constant electrical field, osteoblasts and osteoclasts migrate towards opposite directions i.e. osteoblasts move towards the negative electrode, while osteoclasts to positive electrode [21,22].

Later, it was observed that upon exposure to 10 μ A of an alternating current for 6 hours a day, a 46% increase in osteoblast proliferation and 307% increase in calcium deposition by osteoblasts was observed after 2 days and 21 days, respectively, in comparison to non-stimulated samples [23].

As mentioned before, Titanium implants are being used mostly as orthopedic biomaterials. The drawbacks of conventional titanium surface features have recently been replaced by modified, nano-structured forms. It can be found that an electrical stimulation (Direct Current) at a frequency of 20 Hz and a varying voltages (of 1V, 5V, 10 V, and 15V) with a pulse duration of 0.4 ms/h/day in an osteoblastic cell culture on anodized titanium surface led to a 72% enhancement after 5 days in comparison to non-stimulated conventional titanium [20].

2.6. Cells Cultured on Basement Membrane Proliferate Faster and Maintain Multi-lineage Differentiation Potential

A vital role is played by extracellular matrix (ECM) in organ morphogenesis as well as in post-injury maintenance and reconstruction. In 2000, Kaur and Li anticipated that since stem cells like all other basal cells are adhered with the basal membrane with the help of integrins, therefore, the adhesive action of the basement membrane can be a determinant of the proliferation and differentiation of the stem cells [24].

In 2004, Matsubara *et al.*, put forward the significance of tissue culture dishes coated with a basement membrane-like extracellular matrix (bm-ECM), which was produced by PYS-2 cells or primary endothelial cells, for an extensive culturing of human mesenchymal stem cells. They also showed the ways to overcome the drawbacks of the commonly used plastic tissue culture dishes for culturing of the mentioned cells [25].

The bmECM is known to be composed of heparan sulfate, entactin, type IV collagen, and laminin [26]. In their experiment, the authors cultured MSCs on bmECM-coated dishes in the presence of 10% human serum. In contrast, the same amount of the MSCs of same source was cultured on plastic culture dishes with or without FGF (1ng/ml) [25]. High growth rate was observed when the primary cells were cultured onto plastic dishes. However, the growth rates slowed down rapidly in secondary and tertiary cultures. Moreover, those MSCs were cultured onto the plastic plate showed morphological alterations as

their appearance changed from spindle-shape to flat-shape with the increase of culture period, indicating the senescence [25].

Those hMSCs which were seeded directly on bmECM showed much higher cumulative cell number and longer proliferative life span than that on plastic. More specifically, on day 50, the cumulative cell number was 105-fold greater in bmECM than that on plastic. Conversely, the cells grown on the bmECM maintained spindlelike appearance until the 5th passage, showing their potentiality of suppressing cell senescence. Most importantly, the authors observed that MSCs on bmECM, even after they have lost their replicative capacity, maintained the multilineage differentiation potential [25].

The author also asserted that the extensive culturing of the cells - in a culture vessel aided with the bmECM - can meet the clinical need of cell expansion from small volumes of aspirate before transplantation [25].

3. Exogenous Factors: Chemical

This category aims to illustrate some of the chemical agents, which, if added externally to a culture medium, may change the culture condition favorable for different cells -involved in the musculoskeletal repair- to proliferate faster.

3.1. Melastatin-like Transient Receptor Potential-7 and Magnesium Stimulated Osteoblast Proliferation by PDGF

Besides the most important bone morphogenetic proteins (BMPs), other growth factors including platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β) and fibroblast growth factor (FGF) play a vital role in osteogenic differentiation. Several *in vitro* and *in vivo* studies on PDGF have shown that it enhances DNA and collagen synthesis as well as promotes bone matrix deposition in osteoblast cultures [27,28].

Magnesium (Mg^{2+}) is a divalent cation and is mostly abundant intracellularly. Mg^{2+} is involved with numerous biological processes, such as a cofactor for many enzymes, regulator of various transporters and ion channels, modulator of intracellular pH, etc. Therefore, because of all these essential functions, Mg^{2+} is considered to be involved with cell contraction, secretion, motility, and proliferation. Cell proliferation needs an influx of extracellular Ca^{2+} and Mg^{2+} , and this occurs in response to mitogenic stimuli [29].

Additionally, Melastatin-like transient receptor potential (TRPM) is a group of eight members (TRPM 1–8) of voltage independent Ca^{2+} permeable cation channels. Among them, TRPM7 is associated with cell proliferation and survival (Nadler *et al.*, 2001). Most importantly, in osteoblast proliferation, TRPM7 channels are involved with the intracellular Mg^{2+} homeostasis [30].

Therefore, it is clear that osteoblast proliferation is related with the Mg^{2+} and TRPM7 channel. Abed and Moreau first attempted to relate the effect of Mg^{2+} concentration with the osteoblast proliferation in culture in the presence of PDGF [29]. Human osteoblast-like MG-63 cells were cultured with specific concentration of Mg^{2+} in the presence of PDGF-BB for a certain period of time, and

then the cell proliferation was assessed by the MTT assay. The result showed that PDGF was not effective for initiating proliferation in the absence of optimum extracellular concentration of Mg^{2+} (i.e. below 0.01mM). This suggested that influx of extracellular Mg^{2+} is essential for PDGF-induced cell proliferation. The TRPM7 channels are regarded as a key player for Mg^{2+} influx induced by PDGF. However, similar reduction of proliferation was also observed with other hOB cell lines, such as SaOs and U2OS [29].

3.2. Effect of the Exogenous Amylin, CGRP and Calcitonin on Osteoblastic Cell Proliferation

Amylin, Calcitonin gene-related peptide (CGRP), and calcitonin belong to the same family of peptides as they have a common ancestral origin and also share many biological actions, including activities on bone cells [32]. It has been found that their proliferative activity stimulates PKC activity, but not cAMP involved pathways [33].

3.2.1. Amylin-induced Proliferation

Amylin is a peptide composed of 37 amino acids which is co-secreted with insulin from beta cells of the pancreas. Patients who have insulin-dependent diabetes mellitus (IDDM) lose bone mass [34]. Therefore, it is concluded that both insulin and Amylin synergically help in bone formation. Villa *et al.* in 1996 investigated the effect of Amylin on human osteoblastlike cells (hOB cells) in a primary culture [35]. The bone cells, derived from patients having no metabolic or malignant bone disease, were cultured in Iscove's modified Dulbecco's medium (IMDM) in the presence of 20% fetal bovine serum (FBS) before the cells were shifted to a new serumfree culture medium, containing increasing concentrations (10^{-10} – 10^{-6} M) of amylin. After culturing for a stipulated period of time, an enhanced proliferation of the cells was observed in comparison to the control. More precisely, there was an increase in the G2-M fraction in a dose-dependent manner with a maximum effect –which is two folds or 200% - at an amylin concentration of 10^{-6} M [35]. From the results, the authors suggested that amylin stimulates the osteoblast proliferation which may indicate what can occur *in vivo*.

By 1998, it was an established concept that amylin possessed an osteoblastic cell proliferation effect along with the antiosteoclastic effect [35,36]. Being swayed by the potentiality of amylin to stimulate bone formation both *in vitro* and *in vivo*, in 1998, Cornish *et al.*, with an aim to establish it as a potential therapeutic tool against osteoporosis, took a step forward to assess the structure–activity relationship (SAR) activity of the whole peptide and its fragment on the cell proliferation and thymidine incorporation was used to assess the proliferation [37].

They observed that it was not its fragments but the whole molecule of amylin alone that can produce a maximum effect at a concentration of 10^{-9} M compared to the control and other amylin fragments.

3.2.2. CGRP-induced Proliferation

Calcitonin gene-related peptide (CGRP) also has 37 amino acids in its peptide structure, which has considerable homology with amylin [38]. Situated in sensory nerve ending in bone [39], it stimulates the

insulin-like growth factor-I (IGF-I) [40], in turn, having an anabolic effect on bone metabolism [41]. This metabolism is facilitated by the expression of CGRP and CGRP receptors by human osteoblast-like cells. In contrast, *in vitro* CGRP inhibits osteoclast activity [42].

In 1999, Cornish *et al.* showed the proliferative effect of CGRP in rat osteoblasts [43]. However, in 1999, Villa *et al.* examined the proliferative effect of CGRP on the primary culture of human osteoblastic-like cells. In that experiment, Villa *et al.* cultured hOB cells derived from trabecular bone with an increasing concentration (i.e. 10^{-9} – 10^{-7} M) of α CGRP for 24h, before they were checked for the incorporation of the [3 H] thymidine. The results showed that the maximal effect of CGRP on the cell proliferation was achieved at 10^{-8} M [39].

3.2.3. Calcitonin-induced Proliferation

Calcitonin plays an important role to modulate skeletal homeostasis. However, calcitonin receptors and calcitonin-type receptors are expressed in hOB cells. In 2002, Villa *et al.* examined calcitonin-induced hOB cell proliferation *in vitro* [33]. In that experiment, they cultured the hOB cells derived from trabecular bone, in a serum-free medium containing increasing concentration of calcitonin. Cell proliferation was assessed on the basis of [3 H] thymidine incorporation by the DNA of the cells during growth. The results showed that the maximum [3 H] thymidine incorporation happened with the concentration of 10^{-10} M [33].

3.3. Effect of High Glucose in the Extensive Culture of MSCs

Due to their proliferative and differentiation activity, MSCs are a potential therapeutic tool. However, several reports have identified the unsuccessful therapeutic attempts by stem cell therapy [44,45,46]. Hyperglycaemic condition, which is passed by transplanted stem cells *in vivo* or *in vitro*, is regarded as one of the reasons of such a failure [reviewed in [47]].

The effect of the hyperglycaemic condition on the proliferative activity of the stem cells was first shown by one investigator [47]. In their experiment, the stem cells were taken from three different sources: bone marrow (BM), subcutaneous fat (SF) and omentum fat (OF), and those cells were cultured in DMEM-HG solution, containing 25 mmol/L of glucose and 10% FBS as supplement.

The result showed that MSCs remained unaffected without showing any detrimental effect in high glucose concentration until passage 20. Moreover, MSCs from all the sources found to proliferate extensively in higher glucose concentration *in vitro* [47]. Interestingly, the population doubling time (PDT) was found to be more in MSCs derived from SF and OF than that of BM [47].

This indicated that proliferative rate decreased for the bone marrow derived MSCs in hyperglycaemic condition. In other words, MSCs derived from SF and OF, survive better in hyperglycaemic condition than that of bone marrow derived MSCs [47].

3.4. Nitric Oxide (NO) Induced Cell Proliferation

NO is an inter- and intra-cellular messenger which is involved in several physiological and pathological conditions. Nitric acid is important for metabolism and skeletal muscle contraction. Again, the biological role of NO depends on the local concentration of the molecule as well as on the microenvironment. For example, at moderate concentration of the NO generators have a proliferative effect on smooth muscle, but at higher concentration they have inhibitory effects on the same cells [reviewed in [48]].

3.4.1. Sodium Nitroprusside-induced Myoblast Proliferation

It was found that exogenous as well as endogenous NO are involved with the protein synthesis, metabolism, and contraction of skeletal muscle *in vivo*. On the other hand, myoblast mitosis and satellite cell proliferation are the essential features of regeneration. Therefore, the effect of NO on these processes was first addressed by [48].

In their experiment, myoblasts derived from turkey embryo was cultured in the presence of Turkey Growth Medium (TGM) with different concentrations of SNAP (S-nitroso-N-acetylpenicillamine) and SNP (Sodium Nitroprusside), two NO donors, for 72, 96, 120 and 144h and the proliferation was observed [48].

The results showed that 5.0 μ M SNP (one of the lowest concentration) promoted maximum cell number, and in contrast, a higher concentration of 50 μ M brought about a lower cell number than the control [48].

3.4.2. Effect of Nitric Oxide on Mouse Clonal Osteogenic Cell (MC3T3-E1) Proliferation *in vitro*

It was found by one author that NOS inhibitor, NG-monomethyl-L-arginine could inhibit the proliferation of several osteoblast-like cell lines in a dose-dependent manner. This clearly indicated that NO had a proliferating effect on bone cells [49].

However, Kanamaru *et al.* in 2001 investigated the proliferative effect of SNAP, a NO donor, on the DNA synthesis of the mouse osteogenic cell-line, MC3T3-E1, *in vitro*. For this purpose, the cells were cultured with SNAP. A few hours before terminating the incubation, the cells were treated with radioactive [3 H] TdR. Later on, the [3 H] thymidine incorporation was analysed in order to examine DNA synthesis. The results showed that the osteogenic cells could incorporate [3 H]-TdR in a dose-dependent manner. Moreover, the authors claimed that that proliferation was induced by SNAP directly as upon application of a specific NO scavenger, the cell proliferation was attenuated [50].

4. Exogenous Factors: Biological

Here are some of the biological factors which directly or indirectly affect the cultural condition or cellular responsiveness. This can be brought about by inherent characteristics of the cells or by adding different concentrations of additives, which are the sources of growth factors or may act as stimuli for the cells to proliferate in medium.

4.1. Effect of Passage Number, Donor Age and Cell Origin on the Cell Proliferation

4.1.1. Effect of Passage Number in ex-vivo Cell Expansion and the role of Dexamethasone

It has been reported that “after the first passage, BMSC had a markedly diminished proliferation rate and gradually lost their multiple differentiation potential. Their bone-forming efficiency *in vivo* was reduced by about 36 times at first confluence as compared to fresh bone marrow” [51].

In 2006, It was again investigated the effect of passage number on the proliferative and differentiation ability of bone marrow MSCs. Their observation was the same as the observation of the earlier author. However, additionally it was concluded by them that culture expansion caused MSCs to lose their stem cell properties. Moreover, the author noted the remarkable potential of MSCs of passage number less than 7 to give higher proliferation [52].

However, Xiao *et al.* in 2010, put forward the significance of using dexamethasone (Dex), which has a role in maintaining osteogenic potential to prevent the loss of proliferative and multi-lineage differential potential. According to the author, Dex can do this by keeping the surface phenotype intact. More specifically, it was found that Dex maintains this stemness during repeated passaging at a very low concentration (i.e. 10^{-8} M) by positively regulating the gene associated with cell proliferation [53].

4.1.2. Effect of the Passage Number and the Donor Age on Cell Proliferation

In 2008, Pradel *et al.* investigated more deeply the significance of the relationship between the passage number and the donor age so as to address the question – how often the osteoblast-like cells can be passaged before they lose the proliferative ability and undergo senescence and growth arrest. For this purpose, they cultured and then subcultured osteoblast cells from the human jaw of two age groups i.e. beyond 40 years and younger than 40 years. In the cell proliferation analysis with EZ4U assay (test basis: reduction of yellow colored tetrazolium salt to intense red color formazan derivative with the reduction of the number of intracellular mitochondria), it was found that at 4th passage, there was highest proliferation rate in either age group. Moreover, over 5th passage, there was a reciprocal relationship between the cell proliferation and differentiation [54].

It is well known that the regenerative potential of different cells in musculoskeletal tissues decline with age. The major cause is the changed microenvironment or alteration in the intrinsic properties of stem and progenitor cells. The effect of donor age on the expansion of MSCs is an important phenomenon.

In several studies, no age-associated effects of serum supplements on proliferation of some cells, such as myoblasts and human lung and skin fibroblasts, could be observed [55,56]. However, in addition to BMMSC-donor age, the medium-donor age can also modulate the proliferation rate of the cells. For example, in 2012, Lohmann *et al.* cultured MSCs derived from bone marrow of the *caput femoris* of a donor in human platelet lysate (HPL) obtained from 31 donors aging 25 years to 57 years. The results showed that HPLs derived from younger donors had a stimulating effect on the expansion rate of MSCs and their osteogenic differentiation [57].

4.1.3. Effect of Cell Origin on Cell Proliferation

The origin of the MSCs may determine their proliferative potential. Avanzini *et al.*, in 2009, showed the influence of the cell source on their own proliferative ability. In their experiment, the authors took the MSC-like clones obtained from two Umbilical Cord Blood (UCB) units along with BM-MSCs and cultured them ex-vivo in the presence of 5% platelet lysate supplemented media. Later, the cumulative cell counts were measured from P0 to P5. It was found that although started the experiment with a lower number of UCB-MSCs than the BM-MSCs, the former cells proliferated with higher proliferation potential, so much so that they could yield similar cell number at P5 as compared to BM-MSCs. More specifically, the cumulative population doublings were found to be 12.9 for UCB3-MSC and 13.3 for UCB6-MSC, whereas BM-MSCs were only with 10.9 [58].

4.2. Effect of PRP on Cell Proliferation

Cell proliferation and the differentiation are involved with the wound healing process at the cellular level, and this process is regulated by different growth factors.

One of the most effective means to deliver the cell growth promoting GFs to the wound site is via the use of platelet-rich plasma (PRP). PRP, an autologous source of various growth factors, is obtained from freshly drawn venous blood by means of sequestering and concentrating [59]. PRP contains seven different GFs secreted by platelets: TGF- β_1 , TGF- β_2 , PDGF-AA, PDGFBB, PDGF-AB, VEGF, and EGF. These factors have strong mitogenic, capillary growth and angiogenic actions which promote bone and tissue healing. Therefore, nowadays they are widely used in oral and maxillofacial reconstruction and periodontal regeneration [reviewed in [59,60]].

However, since 1998, after the remarkable research on clinical effectiveness of a specific form of PRP on the oral and maxillofacial reconstructive surgery [61], many scientific works have been carried out to test the usefulness of PRP in different aspects of regenerative therapies [59]. As part of that investigation Kanno *et al.* evaluated the effect of PRP on the proliferation and differentiation aspects of two hOB cell lines (HOS and SaOS-2 cells). They checked different concentrations of PRP, along with Platelet Poor Plasma (PPP) on cell viability by the MTT assay [59].

The pre-cultured cells were treated with different concentrations of PRP (5% and 10%) and PPP (5% and 10 %) and cultured for 24, 36, 48 and 60 hours before the MTT assay was performed. From the results, they concluded that PRP administration enhanced the cell viability in a dose-dependent manner [59].

Until 2006, it was not clear at what optimum concentration PRP exerts its proliferative effect the most. In the same year, Graziani *et al.* performed an experiment *in vitro* on osteoblasts (OB) and fibroblasts (FB) to examine the PRP (at concentrations : 2.5X, 3.5X and max (4.2 – 5.5X) mediated growth. Later, after 24 and 72h, the proliferation was measured by MTT assay. The result showed that PRP exerts a dose-specific effect on OB and FB proliferation, and the highest proliferation was achieved at the concentration of 2.5X and reduced proliferation at higher concentrations [60].

4.3. Myoblast Proliferation Using Myogenic Factors

As reviewed by Hawke and Garry in their publication that certain growth factors and hormones, which were released from a number of tissues/cells could modulate different satellite cell activity, such as chemotaxis, differentiation and proliferation. Among these types of cells, certain cells which are involved in causing immune response are also involved. Some of the factors which are secreted by them are LIF, IL-6, EGF, TGF- β , IGF-I, IGF-II, FGF, HGF, PDGF, etc. [62].

An inflammatory response is accompanied by macrophages, which are cells involved in the immune responses in the body. However, in response to muscle damage, the satellite cells form new functional myoblasts, which fuse together to form myofibers. On the other hand, being stimulated they secrete a number of cytokines and growth factors and thus activates mitosis and chemotaxis processes, in addition to the removal of the cell debris [reviewed in [63,64]]. Taking the action of macrophages *in vivo* for promoting the cell number in 1994, Cantini *et al.* co-cultured rat satellite cells with exudate macrophages and obtained a huge proliferation of cells compared to the control. The proliferation was assessed by an immunoblotting assay of myosin heavy chain (MHC) [65].

Being interested by this research, a few years later, the same authors carried out another experiment in which rat myoblasts were cultured in macrophage conditioned medium (MCM). The result showed that proliferation only happened in case of Myo-D positive (MyoD+) cells, but not in case of Myo-D negative (MyoD-) cells. In the proliferation assay, about 77% more proliferation was obtained in the case of myoblasts treated with MCM [64].

4.4. 17 β -estradiol induced Cell Proliferation

Estrogen has a significant role in influencing metabolism, growth and differentiation in many tissues. Multiple types of cells, including osteoblasts and MSCs, contain estrogen receptors (ER- α and ER- β) through which estrogen exerts its regulatory functions [66].

It was known before the year 1988 that deficiency of estrogen plays an important role in causing postmenopausal osteoporosis which showed a relation of estrogen with bone formation. To know about the mechanism of action on the bone-forming cells, Ernst *et al.* took hOB cells derived from calvarins of newborn rats with different concentrations of 17 β -estradiol (0.1 nM and 1 nM) and the results showed that there was an increase in cell proliferation by 41% and 68% (in terms of number of CFU-F), respectively in comparison to vehicle controlled counterparts.

However, 17 β -estradiol, a stereoisomer had no such effect [67]. The result showed that 17 β -estradiol caused maximum cell proliferation at concentration of 0.1–1nM. The effect was also inhibited by anti-estrogen tamoxifen in a dose dependent manner, confirming the specific action of 17 β -estradiol in causing cell proliferation [67]. As mentioned earlier estrogen receptors are found on MSCs. Since improvement of MSCs proliferation is essential for the clinical application of MSC-based tissue engineering, therefore, with an aim to improve this, the effect of 17 β -estradiol supplement was first applied to enhance hMSCs proliferation [68]. In order to do so, Hong

et al. cultured MSCs obtained from male and female donors (human) in the presence of 17 β -estradiol supplement of concentrations of (10^{-12} M - 10^{-6} M) in both conventional cell culture media and steroid free culture media for 4 days and/or 6 days. The proliferation was measured, using a MTS-based colorimetric method. The results showed that in presence of 17 β -estradiol enhanced cell proliferation in both of the conditions. Though with the progress of passaging, the MSCs lose their responsiveness, yet the presence of 17 β -estradiol helped to maintain high proliferative rates in passage 8, indicating the effectiveness of the 17 β -estradiol in preventing cell senescence [68]. The result supports the concept of estrogen's potentiality to up-regulate the telomerase activity and to prevent shortening of telomerase of MSCs via ER- α [69].

5. Exogenous Factors: Culture-environmental

Cells, being a dynamic structure, are highly responsive to their environment. *In vitro* environment should be such that they offer the cells a reproducible, recognizable and regular functioning condition. Therefore, in a cell culture several factors should be kept in such a state of balance that the cells get maximal stability. Therefore, this chapter aims at comparing different media (especially, serum-free media) in terms of their cell proliferation efficiencies.

5.1. Background

Cells, being a dynamic structure, are highly responsiveness to their environment. *In vitro* environment should be such that it offers the cells a reproducible, recognizable and regular functioning condition. Therefore, in a cell culture several factors should be kept in such a state of balance such that the cells get maximal stability.

In 1974, Waymouth first highlighted the importance to design a culture milieu which can support the cell-stability during its growth and proliferation *in vitro*. He also put forward the significance of some factors that affect the cell stability *in vitro*, such as serum proteins, cations, enzymes, pH and ionic balance, microorganism, O₂ tension, macromolecular growth factors and hormones [70].

Stability *in vitro* can be in terms of genetic stability and phenotypic stability. An ideal media, on the other hand, should be free from any unknown variable components and must fulfill the requirements for the particular cells in a culture. It is true that failure to devise a suitable media can lead to unexpected results [70].

5.2. Choice of Suitable Culture Medium to Attain Most Cell Proliferation with Biosafety

The aim of serum in a cell culture is to promote growth without altering the desired phenotype and genotype of cells and leading to achieve a sustained growth.

In cell culture media, the most used essential supplement is FBS. But the harvest, production and use of this are related with the various scientific and ethical concerns. The cells cultured in FCS or FBS carry the risk of transmitting the xenogenic, zoonoses, prions, viral or

bacterial infections. Moreover, it may cause local inflammatory or immune response against the animal proteins which may lead to antibody production and rejection of the transplanted cell or non-engraftment. This problem cannot be overcome even by washing after the expansion. On the other hand, in many countries, the use of bovine serum is restricted [58,71].

Therefore, for few decades, considerable efforts are being made to find serum free alternatives to expand the hMSC clinically. As a substitute of FCS/FBS, the autologous and heterologous serum or plasma are assessed for their efficiency of proliferation and other aspects.

Convenience, availability and the expense determine the use of serum from species to species. Besides the serum from other animals, human serum (autologous or pooled) are available as growth supplement. Their uses are limited because of some disadvantages, such as probable chance of containing the toxic components, batch to batch variability and lack of defined composition. In addition to the naturally obtained serum, synthetic serum substitutes are also available, for example Ultoser™ G, which has batch-dependent, constant hormonal and chemical composition [72].

Most GMP-grade expansion protocol still uses FBS as supplement. Due to their risk at bio-safety, regulatory guidelines are aiming to minimize the uses of FBS. Therefore, the search for the alternatives is on the way. The alternatives include the use of autologous and allogenic human serum, cord blood serum, human plasma, human platelet derivatives, including the platelet lysate and platelet released factors [reviewed in [73]].

Besides ensuring the bio-safety, rapid cell proliferation is also desired in a manufacturing setting. Therefore, cell proliferation efficiencies of different media (especially, serum-free media) are compared, keeping different useful aspects of media in consideration.

5.2. a. In search of finding the most suitable medium or to optimize culture conditions for the proliferation of osteoblast-like cells *in vitro*, Pradel *et al.*, in 2008 carried out a cell culture, where the osteoblast-like cells from human jaw were grown for 8 days in three different culture media: Optiminimal essential medium (Opti-MEM), DMEM (Dulbecco's modified Eagle Medium) and RPMI (Roswell Park Memorial Institute) with 5 or 10% FCS with 4% HEPES. The AZ4U assay was used to check the proliferation of hOB cells which showed that Opti-MEM with 10% FCS produced the best proliferation in a culture period of 4 to 8 days [54].

5.2. b. Again, for expanding adult human bone marrow MSCs, the most suitable culture medium was sought for by [74]. BM-MSCs were cultured in DMEM/12 with 10% UCB serum, 10% FCS, human blood serum and MesenCult culture medium. The result on proliferation in 10% UCB serum was similar to MesenCult culture medium. Moreover, the proliferation rates in these were greater than the yield in 10% FCS and in human blood serum [74].

5.2.c. Hankey *et al.* compared the proliferation efficiency of 8 different human serums on osteoblast cells. With this aim, they cultured hOB cells from 16 patients with different serums with given concentrations. After 10 days of culture, cell numbers were counted on the basis of their [³H]-Thymidine incorporation into their DNA. The result showed that AHS (heat treated) showed the higher

proliferation than FBS, and Ultoser™ G showed lowest proliferative response. Cumulative Thymidine was 2.4 – 6.7 folds more in AHS than FBS. In other words, AHS (heat-treated) could grow the total cell number of 7300 – 24200 cells, whereas FBS (heat treated) with only 5500 – 18600 cells [72].

The results also showed that although FBS contained high levels of growth promoter and hormones, AHS, on the other hand, contains more specific factors for hOB cells to proliferate increasingly while maintaining the osteoblast phenotype [72].

5.2.d. An alternative media to FBS is autologous serum (AS) and concentration of 1%, 3% and 10% were evaluated for their efficiency in comparison to FCS (10%) by [71]. They isolated MSCs from 9 donors and seeded equal numbers of cells (20 MSC/cm²) in 3 different media containing 1%, 3% and 10% AS and another media with 10% FCS. The proliferation was found to be greater in 10% AS in both primary and passage 1, and the MSCs were found to possess osteogenic potential [71].

5.2.e. Pooled human serum or AB serum has a high clinical significance because it is available in large quantities. In 2007, Kocaoemer *et al.* compared first the proliferative effect of 10% concentration of pooled human AB serum (AB-HS), thrombin activated platelet-rich plasma (tPRP) and FCS on adipose tissue-derived MSCs (AT-MSCs). The flow cytometric result demonstrated that AB-HS and tPRP provided higher proliferative effect than FCS [75]. In terms of the population doubling, the expansion together caused by AB-HS and PRP is 2.8 times higher than that of FCS.

5.2.f. Pooled human Platelet Lysate (pHPL) is another human derived alternative. In 2009, Bieback *et al.* compared the proliferative action of 10% pHPL on the BM-MSCs, with the 10% FBS, tPRP and AB serum. It was found that pHPL not only favoured rapid cell expansion, but it was found to be supportive for the long term expansion without compromising differentiation, immunomodulatory and immune phenotype of the cells [73].

5.2.g. In 2006, Mauleman *et al.* compared the cell expansion between commercially available serum-free media and Ultoser® (UC), which is supplemented with serum substitute in comparison to classical medium i.e. α MEM with 15% FBS. After primoculture (P0) and P1 more MSCs were found in UC in comparison to α MEM, indicating more effectiveness of the UC in expansion [76].

5.2.h. Again in 2007, human Platelet lysate was compared against FCS supplemented culture conditions. The result showed a significant rise of cumulative cell number [77]. MSCs show less immunogenic potential. So, replacement of FCS can be done by Platelet Lysate (PL), a liquid rich in huge quantity of growth factors and derived from blood platelets after freeze/thaw process. Similar, experiments in 2008 by the same authors again showed the same effectiveness of PL for rapid generation of hMSCs in a short time [78].

5.3. Creating Hypoxia condition *in vitro* to Induce Cell Proliferation

It is a challenge to obtain the MSC expansion while maintaining their self-renewal capacity as it can be lost over the culture period. Understanding the *in vitro*

parameters well is a means to achieve the target effectively. Oxygen is regarded as a potent signaling molecule which has the ability to affect the fundamental characteristics of several progenitor cells [79]. Again, the oxygen has roles in proliferation, metabolism, apoptosis, expression of GFs, ECM secretion and differentiation pattern [reviewed in [80]].

In vivo, hematopoietic stem cells (HSCs) coexist with hMSCs in bone marrow, which is a severely hypoxic microenvironment; however, they maintain excellent self-renewal and proliferative capabilities [81]. Therefore, it is clear that hypoxia conditions support the growth and proliferation of MSCs. It was found that murine MSCs were affected by oxygen tension and exhibited higher colony formation with high proliferative rate when grown in 5% O₂ [82].

Taking all these into account, Garyson *et al.* in 2006, assessed the effect of hypoxia (2% O₂) conditions on MSC expansion in a 3D construct. In a culture period of 1 month, hMSCs showed an extended lag phase, but they proliferated continuously and showed a higher proliferation rate in comparison to a normoxic (20% O₂) condition. Moreover, it was found that the hypoxic cells maintained more osteoblastic and adipocyte differentiation markers than the cells under normoxic conditions [80].

Later, in 2007, the authors assessed the effect of hypoxia in a monolayer or 2D culture. In that experiment, they cultured hMSCs at 2% O₂ condition in contrast with 20% O₂. The cells were found to grow extensively even after reaching confluence by forming multiple cell layers. The growth rate was found more in every passage than the normoxic condition. Furthermore, they found those to achieve 30 fold higher hMSCs over 6 weeks of culture without losing the multilineage differentiation capabilities [80].

6. Conclusion

It can be seen that several investigations have been carried out by different investigators to improve the culture system to obtain higher proliferation. Maximizing proliferation by using exogenous factors can also be economical as they may limit the use of expensive materials, having partial roles in cell culture. Maintaining the natural characteristic is also essential during the period of the proliferation and in most of the aspects of proliferation this has been highlighted, proving that application of exogenous factors may not alter the usual behaviour of the cells. Therefore, reviewed procedures to obtain higher cell expansion give direction to use them in bioprocess development for the cells which have mesenchymal origin or others. In addition, rapid proliferation without modulating behaviour of the cells will make the cells to be readily available for the emergency patients. Overall, this article can also be used as a reference for further studies to be carried out by combining different exogenous factors together to formulate out suitable cell bio-processing platforms, like bioreactors.

Acknowledgement

I am thankful to Professor Iain R. Gibson, Institute Medical Science, University of Aberdeen, Aberdeen, Scotland, United Kingdom for his cordial assistance for designing this article.

References

- [1] Serra M, Brito C and Alves PM. "Bioengineering strategies for stem cell expansion and differentiation." *Canal Bioquímica*; 7: 30-38. 2010.
- [2] Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, McKenzie S, Broxmeyer HE, Moore MA. "Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny." *Blood*; 56(2): 289-301. 1980.
- [3] Fehrer C and Lepperdinger G. "Mesenchymal stem cell aging". *Experimental Gerontology*; 40:926-930. 2005.
- [4] Mante M, Daniels B, Golden E, Diefenderfer D, Reilly G, Leboy PS. "Attachment of human marrow stromal cells to titanium surfaces." *J Oral Implantol*; 29: 66-72. 2003.
- [5] Nishimoto SK, Nishimoto M, Park SW, Lee KM, Kim HS, Koh JT, Ong JL, Liu Y, Yang Y. "The effect of titanium surface roughening on protein absorption, cell attachment, and cell spreading." *Int J Oral Maxillofac Implants*. 23, 675-680. 2008.
- [6] Yamashita D, Machigashira M, Miyamoto M, Takeuchi H, Noguchi K, Izumi Y, Ban S. "Effect of surface roughness on initial responses of osteoblast-like cells on two types of zirconia." *Dent Mater J.*; 28, 461-470. 2009.
- [7] Osathanon T, Bessinyowong K, Arksornnukit M, Takahashi H, Pavasant P. "Human osteoblast-like cell spreading and proliferation on Ti-6Al-7Nb surfaces of varying roughness." *J Oral Sci*. 53(1):23-30. 2011.
- [8] Musgrove E, Seaman M, Hedley D. "Relationship between cytoplasmic pH and proliferation during exponential growth and cellular quiescence." *Exp Cell Res.*; 172(1):65-75. 1987.
- [9] Ramp WK, Lenz LG, Kaysinger KK. "Medium pH modulates matrix, mineral, and energy metabolism in cultured chick bones and osteoblast-like cells." *Bone Miner* 24:59-73. 1994.
- [10] Yamaguchi DT, Huang JT, Ma D. "Regulation of gap junction intracellular communication by pH in MC3T3-E1 osteoblastic cells." *J Bone Miner Res*. 10:1891-1899. 1995.
- [11] Kaysinger KK, Ramp WK. "Extracellular pH modulates the activity of cultured human osteoblasts." *J Cell Biochem.*; 68 (1):83-9. 1998.
- [12] Oliveira P DA, de Oliveira RF, Zangaro RA, Soares CP. "Evaluation of low-level laser therapy of osteoblastic cells." *Photomed Laser Surg*. 26 (4):401-4. 2008.
- [13] Yamada K. "Biological effects of low power laser irradiation on clonal osteoblastic cells (MC3T3-E1)." *Nihon Seikeigeka Gakkai Zasshi.*; 65(9):787-99. 1991.
- [14] Khadra M, Lyngstadaas SP, Haanaes HR, Mustafa K. "Effect of laser therapy on attachment, proliferation and differentiation of human osteoblast-like cells cultured on titanium implant material." *Biomaterials*; 26(17):3503-9. 2005.
- [15] Abramovitch-Gottlieb L, Gross T, Naveh D, Geresh S, Rosenwaks S, Bar I, Vago R. "Low level laser irradiation stimulates osteogenic phenotype of mesenchymal stem cells seeded on a three-dimensional biomatrix." *Lasers Med Sci.*; 20(3-4):138-46. 2005.
- [16] Pinheiro, A.L.B., and Gerbi, M.E.M.M. "Photo engineering of bone repair processes." *Photomed. Laser Surg*. 24, 169-179. 2006.
- [17] Boulton M, Marshall J "He-Ne laser stimulation of human fibroblast proliferation and attachment *in vitro*." *Lasers Life Sci.*; 1:123-134. 1986.
- [18] Saygun I, Nizam N, Ural AU, Serdar MA, Avcu F, Tözüm TF. "Low-level laser irradiation affects the release of basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), and receptor of IGF-I (IGFBP3) from osteoblasts." *Photomed Laser Surg.*; 30(3):149-54. Jan., 2012.
- [19] Doan N, Reher P, Meghji S, Harris M. "In vitro effects of therapeutic ultrasound on cell proliferation, protein synthesis, and cytokine production by human fibroblasts, osteoblasts, and monocytes." *J Oral Maxillofac Surg.*; 57(4):409-19. Apr., 1999.

- [20] Ercan and Webster, "Greater osteoblast proliferation on anodized nanotubular titanium upon electrical stimulation." *Int J Nanomedicine*; 3(4): 477-485. December, 2008.
- [21] Ferrier J, Ross SM, Kanehisa J, Aubin JE. "Osteoclasts and osteoblasts migrate in opposite directions in response to a constant electrical field." *J Cell Physiol*; 129: 283-288. 1986.
- [22] Dubey AK, Gupta SD, Basu B. "Optimization of electrical stimulation parameters for enhanced cell proliferation on biomaterial surfaces." *J Biomed Mater Res B Appl Biomater*; 98(1):18-29. Jul., 2011.
- [23] Supronowicz PR, Ajayan PM, Ullmann KR, et al. "Novel currentconducting composite substrates for exposing osteoblasts to alternating current stimulation." *J Biomed Mater Res*, 59:499-506. 2001.
- [24] Kaur P., Li A. "Adhesive properties of human basal epidermal cells: an analysis of keratinocyte stem cells, transit amplifying cells, and postmitotic differentiating cells." *J. Invest. Dermatol.*; 114: 413-420. 2000.
- [25] Matsubara T, Tsutsumi S, Pan H, Hiraoka H, Oda R, Nishimura M, Kawaguchi H, Nakamura K, Kato Y. "A new technique to expand human mesenchymal stem cells using basement membrane extracellular matrix." *Biochem Biophys Res Commun*. 16; 313(3):503-8. 2004.
- [26] Martinez-Hernandez A., Chung A.E. "The ultrastructural localization of two basement membrane components: entactin and laminin in rat tissues." *J. Histochem. Cytochem.*; 32: 289-298. 1984.
- [27] Centrella M, McCarthy TL, Canalis E. "Platelet-derived growth factor enhances deoxyribonucleic acid and collagen synthesis in osteoblast enriched cultures from fetal rat parietal bone." *Endocrinology*; 125: 13-19. 1989.
- [28] Pfeilschifter J, Oechsner M, Naumann A, Gronwald RG, Minne HW, Ziegler R.. "Stimulation of bone matrix apposition *in vitro* by local growth factors: a comparison between insulin-like growth factor I, platelet-derived growth factor, and transforming growth factor beta." *Endocrinology* 127: 69-75. 1989.
- [29] Abed E, Moreau R. "Importance of melastatin-like transient receptor potential 7 and magnesium in the stimulation of osteoblast proliferation and migration by platelet-derived growth factor." *Am J Physiol Cell Physiol.*; 297(2):C360-8. 2009.
- [30] Abed E, Moreau R. "Importance of melastatin-like transient receptor potential 7 and cations (magnesium, calcium) in human osteoblast-like cell proliferation." *Cell Prolif*; 40: 849-865. 2007.
- [31] Nadler MJ, Hermosura MC, Inabe K, Perraud AL, Zhu Q, Stokes AJ, Kurosaki T, Kinet JP, Penner R, Scharenberg AM, Fleig A. "LTRPC7 is a Mg²⁺ ATP-regulated divalent cation channel required for cell viability." *Nature*; 411: 590-595. 2001.
- [32] Reid IR and Cornish J. Amylin and calcitonin gene-related peptide. In: Principles of Bone Biology (2nd ed.), edited by Bilezikian JS, Raisz LG, and Rodan GA. San Diego, CA: Academic, 2002, 641-654.
- [33] Villa I, Dal Fiume C, Maestroni A, Rubinacci A, Ravasi F, Guidobono F. "Human osteoblast-like cell proliferation induced by calcitonin-related peptides involves PKC activity." *Am J Physiol Endocrinol Metab.*; 284(3):627-33. 2002.
- [34] Bouillon R. "Diabetic bone disease." *Calcif. Tissue Int*; 49: 155-160. 1991.
- [35] Villa I, Rubinacci A, Ravasi F, Ferrara AF, Guidobono F. "Effects of amylin on human osteoblast-like cells." *Peptides*. ;18 (4):537-40. 1997.
- [36] Alam, A. S., B. S. Moonga, P. J. Bevis, C. L. Huang, and M. Zaidi. "Amylin inhibits bone resorption by a direct effect on the motility of rat osteoclasts." *Exp. Physiol*. 78: 183-196. 1993.
- [37] Cornish J, Callon KE, Lin CQ, Xiao CL, Mulvey TB, Coy DH, Cooper GJ, Reid IR. "Dissociation of the effects of amylin on osteoblast proliferation and bone resorption." *Am J Physiol.*; 274:827-33. May, 1998.
- [38] Van Rossum, D., Hanisch, U.K., Quirion, R. "Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors." *Neurosci. Behav. Rev*. 21, 649-678. 1997.
- [39] Villa I, Melzi R, Pagani F, Ravasi F, Rubinacci A, Guidobono F. "Effects of calcitonin gene-related peptide and amylin on human osteoblast-like cells proliferation." *Eur J Pharmacol.*; 409(3):273-8. 2000.
- [40] Vignery, A., McCarthy, T.L. "The neuropeptide calcitonin gene-related peptide stimulates insulin-like growth factor I production by primary fetal rat osteoblasts." *Bone*; 18, 331-335. 1996.
- [41] Marie, P.J. "Cellular and molecular alterations of osteoblasts in human disorders of bone formation." *Histol. Histopathol.*; 14, 525-538. 1999.
- [42] Zaidi, M., Chamber, T.J., Gaines, R.E., Morris, H.R., McIntyre, I. "A direct action of human calcitonin gene related peptide on isolated osteoclasts." *J. Endocrinol*. 115, 511-518. 1987.
- [43] Aiyar, N., Rand, K., Elshourbagy, N.A., Zeng, Z., Adamou, J.E., Bergsma, D.J., Li, Y. "A cDNA encoding the calcitonin gene-related type I receptor." *J. Biol. Chem.*; 19, 11325-11329. 1996.
- [44] Andreas L, Young GY, Robyn AB, Lan W, Anna LN, Joel FH. "No evidence for significant transdifferentiation of bone marrow into pancreatic β -cells *in vivo*." *Diabetes*; 53: 616-623. 2004.
- [45] Castro RF, Jackson KA, Goodell MA, Robertson CS, Liu H, Shine HD. "Failure of bone marrow cells to transdifferentiate into neural cells *in vivo*." *Science*; 297: 1299. 2002.
- [46] Meza-Zepeda LA, Noer A, Dahl JA, Micci F, Myklebost O, Collas P. High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. *J Cell Mol Med*; 12: 553-563. 2008.
- [47] Dhanasekaran M, Indumathi S, Rajkumar JS, Sudarsanam D. "Effect of high glucose on extensive culturing of mesenchymal stem cells derived from subcutaneous fat, omentum fat and bone marrow." *Cell Biochem Funct*. doi: 10.1002/cbf.2851. 2012.
- [48] Ulibarri JA, Mozdziaik PE, Schultz E, Cook C, Best TM. "Nitric oxide donors, sodium nitroprusside and S-nitroso-N-acetylpencilamine, stimulate myoblast proliferation *in vitro*." *In vitro Cell Dev Biol Anim.*; 35(4):215-8. 1999.
- [49] Riancho, J.A., Zarrabeitia, M.T., Salas, E., and Gonzalez-Macias, J. "Impairment of osteoblast growth by nitric oxide synthase inhibitors: an effect independent of nitric oxide and arginine transport inhibition." *Methods Find Exp. Clin. Pharmacol*. 18: 663-667. 1996.
- [50] Kanamaru Y, Takada T, Saura R, Mizuno K. "Effect of nitric oxide on mouse clonal osteogenic cell, MC3T3-E1, proliferation *in vitro*." *Kobe J Med Sci.*; 47(1):1-11. 2001.
- [51] Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R. "Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy." *Exp Hematol.* ; 28(6):707-15. 2000.
- [52] Hu JB, Zhou Y, Jiang DD, Tan WS. "Proliferation and differentiation characteristics of human bone marrow mesenchymal stem cells during ex-vivo expansion." [Article in Chinese] (abstract) *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi.* ; 22 (1):7-10. 2006.
- [53] Xiao Y, Peperzak V, van Rijn L, Borst J, de Bruijn JD. "Dexamethasone treatment during the expansion phase maintains stemness of bone marrow mesenchymal stem cells." *J Tissue Eng Regen Med.*; 4(5): 374-86. 2010.
- [54] Pradel W, Mai R, Gedrange T, Lauer G. "Cell passage and composition of culture medium effects proliferation and differentiation of human osteoblast-like cells from facial bone." *J Physiol Pharmacol.*; 59 (5):47-58. 2008.
- [55] Kondo H, Nomaguchi TA, Sakurai Y, Yonezawa Y, Kaji K, et al. "Effects of serum from human subjects of various ages on proliferation of human lung and skin fibroblasts." *Exp Cell Res*; 178: 287-295. 1988.
- [56] George T, Velloso CP, Alsharidah M, Lazarus NR, Harridge SD. "Sera from young and older humans equally sustain proliferation and differentiation of human myoblasts." *Exp Gerontol* 45: 875-881. 2010.
- [57] Lohmann M, Walenda G, Hemeda H, Jousen S, Drescher W, Jockenhoevel S Hutschenreuter G, Zenke M, Wagner W. "Donor Age of Human Platelet Lysate Affects Proliferation and Differentiation of Mesenchymal Stem Cells." *PLoS One*; 7 (5): 1-11. 2012.
- [58] Avanzini MA, Bernardo ME, Cometa AM, Perotti C, Zaffaroni N, et al. "Generation of mesenchymal stromal cells in the presence of platelet lysate: a phenotypic and functional comparison of umbilical cord blood- and bone marrow-derived progenitors." *Haematologica.* ; 94(12):1649-60. 2009.
- [59] Kanno T, Takahashi T, Tsujisawa T, Ariyoshi W, Nishihara T. "Platelet-rich plasma enhances human osteoblast-like cell proliferation and differentiation." *J Oral Maxillofac Surg.*; 63(3):362-9. 2005.
- [60] Graziani F, Ivanovski S, Cei S, Ducci F, Tonetti M, Gabriele M. "The *in vitro* effect of different PRP concentrations on osteoblasts and fibroblasts." *Clin Oral Implants Res.*; 17(2):212-9. 2006.

- [61] Marx RE, Carlson ER, Eichstaedt RM, *et al.* "Platelet-rich plasma: Growth factor enhancement for bone grafts." *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*; 85(6):638-46. 1998.
- [62] Hawke TJ, Garry DJ. "Myogenic satellite cells: physiology to molecular biology." *J Appl. Physiol*; 91: 534-551. 2001.
- [63] Vitello L., Radu C., Malerba A, Segat D, Cantini M, Carraro U and Baroni M D. "Enhancing Myoblast Proliferation by Using Myogenic Factors: A Promising Approach for Improving Fiber Regeneration in Sport Medicine and Skeletal Muscle Diseases." *Basic Appl Myol* 14(1): 45-51. 2004.
- [64] Cantini M, Giurisato E, Radu C, Tiozzo S, Pampinella F, Senigaglia D, Zaniolo G, Mazzoleni F, Vitiello L. "Macrophage-secreted myogenic factors: a promising tool for greatly enhancing the proliferative capacity of myoblasts *in vitro* and *in vivo*." *Neurol Sci.*; 23(4):189-94. 2002.
- [65] Cantini M, Massimino M L, Bruson A, Catani C, Dalla Libera L, Carraro U. "Macrophages regulate proliferation and differentiation of satellite cells." *Biochem Biophys Res Commun*; 202: 1688-1696. 1994.
- [66] Wang Q, Yu JH, Zhai HH, Zhao QT, Chen JW, Shu L, Li DQ, Liu DY, Dong C, Ding Y. "Temporal expression of estrogen receptor alpha in rat bone marrow mesenchymal stem cells." *Biochem Biophys Res Commun*. 18; 347(1):117-23. 2006.
- [67] Ernst M, Schmid C, Froesch ER. "Enhanced osteoblast proliferation and collagen gene expression by estradiol." *Proc Natl Acad Sci USA.*; 85(7):2307-10. 1988.
- [68] Hong L, Zhang G, Sultana H, Yu Y, Wei Z. "The effects of 17- β estradiol on enhancing proliferation of human bone marrow mesenchymal stromal cells *in vitro*." *Stem Cells Dev.*; 20 (5):925-31. 2011.
- [69] Sato R, Maesawa C, Fujisawa K, Wada K, Oikawa K, Takikawa Y, Suzuki K, Oikawa H, Ishikawa K, Masuda T. "Prevention of critical telomere shortening by oestradiol in human normal hepatic cultured cells and carbon tetrachloride induced rat liver fibrosis." *Gut.*; 53(7):1001-9. 2004.
- [70] Waymouth C. "Feeding the Baby"-Designing the Culture Milieu to Enhance Cell Stability." *J Natl Cancer Inst* ; 53: 1443-1448. 1974.
- [71] Stute N, Holtz K, Bubenheim M, Lange C, Blake F, Zander AR. "Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use." *Exp Hematol.* ; 32 (12):1212-25. 2004.
- [72] Hankey DP, McCabe RE, Doherty MJ, Nolan PC, McAlinden MG, Nelson J, Wilson DJ. "Enhancement of human osteoblast proliferation and phenotypic expression when cultured in human serum." *Acta Orthop Scand.*; 72 (4):395-403. 2001.
- [73] Bieback K, Hecker A, Kocaömer A, Lannert H, Schallmoser K, Strunk D, Klüter H. "Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow." *Stem Cells.*; 27 (9):2331-41. 2009.
- [74] Kuang WY, Zhou XF, Zhang GS, Liu LH, Chen SF, Li RJ, Xiao L. "In vitro expansion of the adult human bone marrow mesenchymal stem cells for clinic application in HSCT". [Article in Chinese] (abstract) *Zhongguo Shi Yan Xue Ye Xue Za Zhi.* ; 16 (3):633-8. 2008.
- [75] Kocaoemer A, Kern S, Klüter H, Bieback K. "Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue." *Stem Cells.*; 25(5):1270-8. 2007.
- [76] Meuleman N, Tondreau T, Delforge A, Dejefeffe M, Massy M, Libertalis M, Bron D, Lagneaux L. "Human marrow mesenchymal stem cell culture: serum-free medium allows better expansion than classical alpha-MEM medium." *Eur J Haematol.* ; 76 (4):309-16. 2006.
- [77] Lange C, Cakiroglu F, Spiess AN, Cappallo-Obermann H, Dierlamm J, Zander AR. "Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine." *J Cell Physiol*. Oct; 213(1):18-26. 2007.
- [78] MacPherson H, Noble BS, Ralston SH. "Expression and functional role of nitric oxide synthase isoforms in human osteoblast-like cells." *Bone.* ; 24 (3):179-85. 1999.
- [79] Zhu L.L., Wu L.Y., Yew D.T., Fan M. "Effects of hypoxia on the proliferation and differentiation of NSCs." *Mol. Neurobiol.*; 31:231-242. 2005.
- [80] Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T. "Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs." *J Cell Physiol.*; 207(2):331-9. 2006.
- [81] Ivanovic Z., Bartolozzi B., Bernabei P.A., Cipolleschi M.G., *et al.* "Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors." *Br. J. Haematol.*; 108: 424-429. 2000.
- [82] Lennon DP, Edmison JM, Caplan AI. "Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: Effects on *in vitro* and *in vivo* osteochondrogenesis." *J Cell Phys.*; 187:345-355. 2001.