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Chapter

Alternative Strategies for Stem Cell Osteogenic Differentiation

Carla Cristina Gomes Pinheiro and Daniela Franco Bueno

Abstract

Discovering strategies that increase the osteogenic differentiation potential of mesenchymal stem cells (MSCs) can lead to new perspectives for bone disease treatments. The possibility to associate the mesenchymal stem cells with scaffolds and to use them in bone regeneration as well as the number of studies to understand the signaling pathway of osteogenesis are increasing. Identifying osteogenic induction factors is extremely important and crucial for the success of bone regeneration. Studies have shown that proteins, such as bone morphogenetic proteins (BMPs), trichostatin A and IGF-1, can be efficiently used for osteogenic regeneration. However, the use of these proteins increases the treatment cost. Fortunately, low-level laser therapy (LLLT) may be a new alternative for adjuvant therapy to treat bone regeneration because it has biostimulatory effects on the conversion of mesenchymal stem cells into osteoblasts and on the induction of ex vivo ossification. The principle of tissue photobiomodulation with LLLT was first described in dermatology for healing wounds; however, other applications have been described, with anti-inflammatory and anti-edema effects and cellular proliferation and differentiation. Following this way, we will discuss some alternative strategies for osteogenic differentiation and suggest that the low-power lasers can be an innovative instrument for cell differentiation.

Keywords: osteogenesis, mesenchymal stem cells, low-level laser therapy, low-power laser, osteogenic differentiation

1. Introduction

Bone transplantation is one of the most common tissue transplants in the world, second only to blood transplant. There are approximately 15 million bone fractures per year worldwide and about 10% of those will experience no tissue regeneration, potentially leading to complications such as infections and pain [1]. Technological advances and increase in life expectancy of the global population have sparked interest in and use of alternative strategies in regenerative medicine.

Tissue bioengineering is an interdisciplinary field where engineering and biological science strategies are applied jointly in order to develop biological substitutes to restore, maintain, and/or increase the function of damaged tissues [2, 3].

In concern to bone tissue engineering different medical areas as well as dentistry areas have developed bone tissue engineering strategies (stem cells (SCs), biomaterials, and growth factors) to rehabilitate congenital malformations and craniofacial syndromes associated with bioengineering [3, 4]. Therefore, the main

goal of bioengineering is to overcome limitations imposed by current conventional treatments, which are based on reconstructive surgery or organ transplant. Above all, it aims at being able to produce substitutes for organs and tissues with immune tolerance, so that transplantation can be achieved without the risk of rejection by the organism [5].

Three elements are necessary for bone tissue bioengineering: osteoconduction, osteoinduction, and osteogenesis; together, these three elements form the basis for obtaining a new, functional bone tissue [6, 7]. Given the increase in regenerative medicine studies and the need to find a biological source to promote tissue formation, that is, osteogenesis, stem cells appear to be a potentially unlimited biological source [8].

Stem cells (SCs) can be defined as cells that are capable of: (1) proliferation and self-renewal and (2) answering to external stimuli and giving rise to different specialized cell lines. Consequently, they are considered important for regenerative medicine [8]. Stem cells are classified based on their source and plasticity; hence, they can be divided into three different groups: embryonic stem cells, induced pluripotent stem cells (iPSCs), and adult stem cells.

Embryonic stem cells are those derived from the inner mass of a blastocyst (4 or 5 days after the egg has been fertilized), that are capable of differentiating in the three germ layers (endoderm, ectoderm, and mesoderm). They are known as being pluripotent. However, the therapeutic use of these cells has been questioned by several studies due to teratoma formation after transplantation in animals, potential immune rejection by the host, and strong association with ethical issues [9].

An increasing number of studies have been published about induced pluripotent stem cells (iPSCs). iPSCs are somatic cells—able to differentiate into the same cell type—but genetically altered, with four genes being inserted into their genome: OCT-4, SOX2, c-Myc, and KLF4. This increases their ability to differentiate and decreases their plasticity, changing them from somatic to pluripotent cells [9].

Another type of stem cell is the multipotent stem cell, which includes adult stem cells. They have lower plasticity than pluripotent cells and, although they can differentiate into some types of cells of adult tissues, they are unable to differentiate into germ layers. Adult stem cells are found in the body and are responsible for tissue maintenance and repair [5].

The first adult SCs described in the literature were those found in bone marrow, which have been used in the treatment of several diseases affecting the hematopoietic SCs since the 1950s. Hematopoietic SCs found in bone marrow can give rise to all types of blood cells (lymphocytes, red blood cells, platelets, etc.). In addition, studies about bone marrow transplant have led to the discovery of another important cell type—larger and adherent—that support regeneration of other tissues: the mesenchymal stem cells. Since then, several studies have begun using particularly these stem cells [10, 11].

2. Mesenchymal stem cells (MSCs)

Even after birth and growth, we can still find stem cell niches in different tissues—bone marrow, adipose tissue, skeletal muscle, dental pulp, placenta and umbilical cord, and fallopian tube—usually involved in tissue maintenance and repair [12–17].

Those are known as adult mesenchymal stem cells (MSCs). Their own characteristics are preserved, that is, they remain multipotent and undifferentiated, capable of self-renewal and differentiation into multiple cell lines—under specific in vitro conditions—including osteogenic, chondrogenic, adipogenic, and myogenic lineages [18].

The first three sources are considered key differentiation lineages in determining MSCs' multipotentiality [19]. In 1976, Friedenstein et al. isolated cells with morphological features that were described as colony-forming unit-fibroblasts (CFU-Fs). Bone marrow stromal cells were first described as bone progenitor cells present in its stromal fraction [12]. In 1991, Caplan named those stromal cells as mesenchymal stem cells with potential for cell expansion while remaining undifferentiated, the cells being a great option in cell therapy for tissue regeneration [11]. Subsequent studies have shown that these cells are able to remain undifferentiated when cultured for prolonged periods of time. Moreover, they have the ability to differentiate into mesodermal cell lineages, including chondrocytes, osteoblasts, adipocytes, and myoblasts [5].

Currently, the definition of MSCs includes several morphological and immunophenotypic factors as well as functional features. According to the International Society for Cellular Therapy (ISCT), MSCs: (i) are plastic-adherent when maintained in in vitro conditions; (ii) show positive expression of the CD13, CD29, CD44, CD54, CD73, CD90, CD105, CD166, and Stro-1 cell surface markers and negative expression of the CD14, CD19, CD34, CD45, and HLA-DR markers; and (iii) are a group of clonogenic cells, capable of differentiating into several mesodermal cell lineages [19].

A range of studies have shown that multipotent MSCs can also differentiate into unrelated germline cells in a process known as transdifferentiation. Thus, in addition to differentiating into mesodermal cells—such as bone, fat, and cartilage—MSCs also have the potential for endodermal and neuroectodermal differentiation [20]. Even though adult MSCs are generally considered to originate from mesoderm, some authors describe embryonic MSCs derived from neuroepithelium and the neural crest, such as MSCs from deciduous dental pulp [20, 21].

Adult MSCs can be isolated from several tissues, with similar membrane receptor functions and expressions. However, none of those membrane receptors is considered a MSC-specific cell surface marker; rather, MSCs show a profile of cell surface markers, with positive and negative expression, varying according to source and cell heterogeneity [22, 23].

Furthermore, important features of MSCs for clinical use are their non-immunogenicity, as described in the literature, and immunomodulatory properties, which can be observed from two different perspectives, namely: (i) immunosuppressive effects of allogeneic MSCs, inducing immune tolerance; and (ii) effect of inflammatory cytokines in MSCs' activity and differentiation, in cell-to-cell interactions [8, 24–27].

Bone marrow is considered one of the main sources of MSCs, both in experimental studies and clinical use [26]. Yet, bone marrow MSCs are obtained through a painful surgical incision that produces a low number of harvested cells [28], with only about 0.001–0.01% of the total population of nucleated cells being identified as MSCs [5, 29].

Therefore, due to the aforementioned difficulties, alternative sources of MSCs—such as lipoaspirated adipose tissue, dental pulp, umbilical cord tissue, and skeletal muscle among others—have been studied, as they are often discarded and can be easily procured and manipulated in order to obtain MSCs [16, 22, 30, 31]. Cells obtained from sources other than the bone marrow contribute greatly to the development of cell therapies and consequently to the choice of the best cellular source for clinical uses and better response to target tissue regeneration [6, 16, 17, 25].

The possibility to use a non-invasive source of MSCs in bone tissue engineering has been increased by researches, because of the ease of obtaining the tissue, since they are discarded and do not involve ethical controversy. Since the year 2000, described by Gronthos, mesenchymal stem cells derived from dental pulp (DPSCs) have been studied by other researchers, and the use of DPSCs in vitro and in vivo

has generated a great expectation for the translational use in tissue bioengineering, especially for bone neoformation [8, 30–32]. The profile of DPSCs when compared to stem cells derived from human adipose tissue (hASCs), the DPSCs present an increase in the extracellular matrix formation capacity and presented expression profile for osteogenic genes (RUNX2, BGLAP and ALP) [33]. These comparative results between alternative sources for translational use may help us find the best source of stem cells for each type of tissue to being repaired.

Recently re-emerged as an attractive source of osteogenic progenitor cells (OCPs), the periosteum can be isolated from several locations in the body, such as the anterior tibia, and the spinous process [34]. Periosteal OCPs were involved in bone repair and may also differentiate in response to paracrine signals from mechanically stimulated osteocytes. However, the interconnection of load stimulation with the molecular mechanisms is still unclear. On the other hand, another group of researchers recently described the presence of an immature cell with clonal multipotency and self-renewal characteristics in the long bones and calvarium of mice denominated with periosteal stem cells (PSCs) that are also involved in the support of the bone tissue repair [35]. With the advancement of technology, a new cellular and molecular markers can be innovative therapeutic target to open the best possibilities for promising therapies.

3. Strategies for osteogenic differentiation

A basic premise for a cell to be characterized as MSC is its ability to differentiate into a range of mesenchymal tissues—as mentioned above. Thus, stimulus for osteogenic differentiation must be efficient, resulting in viable and functional cells that produce bone extracellular matrix. This functionality is highly important for cellular characterization and applications in regenerative medicine [36].

In accordance with the basic requirements for carrying out tissue bioengineering, selection and strategy of signs of differentiation (osteoinduction) are other key aspects that should be explored. These are external inducers that promote cell proliferation and differentiation to regenerate the new tissue [36–38].

The biomaterial is not only involved as a structural support but can also be used as an inducer of osteogenic factors depending on its composition. The biomaterial classes most cited in the literature are the active ceramics, biodegradable polymers, and biodegradable metals. The mechanisms of the interaction between the cell and the biomaterial as well as of the osteogenic stimuli have not been clarified yet [39].

Another growing trend in bioengineering is the use of three-dimensional (3D) culture system, this possibility of cell culture is innovative and being explored by researchers, one of the factors that draws attention to this technique is the release of bioincomparable or non-absorbable compounds and the possible customization of the area to be regenerated [40].

Osteogenic induction and differentiation are often achieved via growth factors, which—through molecular mechanisms involving pathways, such as Wnt, BMP, FGF, and PTH, and genes that are essential for osteogenesis [41], such as RUNX 2, COL, ALP, OCN, OP, BGLAP, and SSP1—play a key role in osteogenesis and osteogenic differentiation, as shown in **Figure 1** [42–44]. In this context, identifying those factors is crucial for successful tissue regeneration.

3.1 Bone morphogenetic protein (BMP)

Bone morphogenetic proteins (BMPs) are cytokines from the beta family and are used in clinical applications to stimulate bone regeneration [45]. These proteins are involved in the development of the embryo and in skeletal formation.
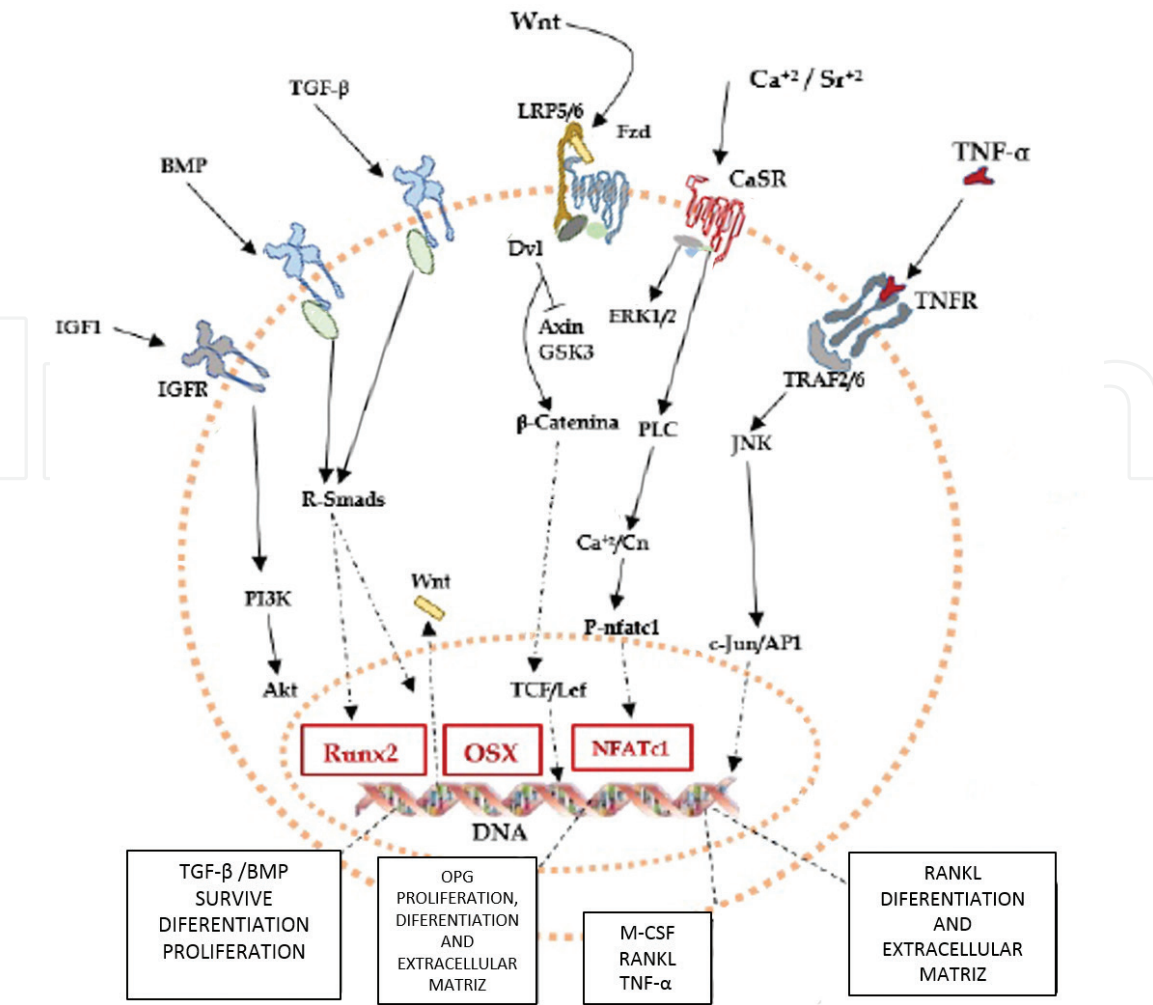
Figure 1.
Representative illustration of osteogenic signaling pathways. These pathways can activate several transcription factors, among them, RUNT (Runx 2), osterix (OSX), nuclear factor of activated T-cells 1 (NFATc1), and transcription factors of the Wnt pathway. Continuous arrows indicate interactions and signaling; dashed arrows indicate the actions described in boxes; and t-bar indicates block.

Manochantr et al. showed that after in vitro stimulation of bone marrow-derived MSCs with 100 ng/ml BMP-2, there was upregulation of the level of expression of genes associated with osteogenic differentiation (RUNX2 and OCN) and increase in alkaline phosphatase (ALP) production [46].

During a regular bone remodeling process, typical of an organism maintaining physiological stability, both BMPs and their antagonists are needed since BMPs induce osteo-precursor cells to proliferate and differentiate, thereby leading to formation of bone tissue. Members of the BMP family have different functions and are primarily related to the formation of bone and cartilage [47].

Upon BMP-receptor activation, receptor-regulated SMADs (R-SMADs) are translocated to the nucleus, where they regulate gene transcription by binding to DNA and interacting with DNA-binding proteins. Additionally, SMADs interact with transcription factors, transcriptional coactivators, and corepressors. The transcription factor associated with Runt-Runx is one of the most studied transcription factors for BMP signaling, responsible for regulating processes such as bone formation and hematopoiesis [46, 47].

Runx2 transcription factors cooperatively regulate gene transcription that lead to differentiation of mesenchymal progenitor cells into osteoblasts [48]. Hence, it is widely regarded as a marker for cells committed to the osteochondral lineage and osteoblast differentiation. Runx2 expression is low in mesenchymal cells and is induced by BMP signaling [49].

Osterix (OSX) is another example of a transcription factor mediated by BMP/ SMAD signaling and likely by MAPK signaling and other pathways [50]. Taken together, Runx2 and Osterix are the most studied transcription factors for BMP signaling involved in the differentiation of MSCs into osteoblasts.

Moreover, recombinant BMP-2 (rhBMP-2) has been used for bone induction in humans being treated for long bone fractures and spinal arthrodesis [45]. A clinical study showed improved bone density and quantity formed when compared to the gold standard surgery (anterior iliac crest bone graft), used in maxilla reconstruction in cleft lip and palate patients.

3.2 Insulin-like growth factor type I

Insulin-like growth factor type I (IGF-1) is yet another factor currently being studied as an osteoinducer. IGF-1 is the most abundant growth factor found in the bone matrix and it plays an important role in development and maintenance of skeletal tissue [51]. It has been shown, under in vitro conditions, that IGF-1 is a stimulant for osteogenic differentiation through the increase in expression of ALP, Runx2, and OCN genes in MSCs from molar dental pulp [51].

Previous studies have demonstrated that the stimulant effect of IGF-1 on bone matrix synthesis in cell cultures derived from rat calvaria is a result of at least two distinct regulatory signals: first, the effect on cellular differentiation—osteoprogenitor cells and pre-osteoblasts—in osteoblasts (increased production of bone collagen); and second, the stimulation of osteoprogenitor cells' proliferation, thereby resulting in an increase in the number of functional osteoblasts. Despite working together to increase production of extracellular matrix, those signals differ in origin and can act synergistically with other factors, such as, for example, BMP-9 [37] and OSX, to promote osteogenic differentiation [50].

Insulin-like growth factors are known for mediating skeletal growth and bone formation [37, 52, 53]. Different studies have shown that IGF-1, in particular, promotes differentiation of bone cells in autocrine and paracrine pathways [52, 53]. Previous in vitro and in vivo studies have used IGF-1 to promote osteogenesis while treating dental pulp-derived osteoblastic cells [53, 54] and in an aged rat model, respectively. On the other hand, studies using rat fracture models show that the use of IGF-1 or PDGF alone does not stimulate OCN expression [55]. Nevertheless, using IGF-1 along with MSCs can cause expression of both factors to increase, as well as a significant upregulation of OCN by ODM in comparison to ODM alone.

The use of those factors for cell proliferation and differentiation is still being tested and is correlated with high treatment costs. On the other hand, low-level laser therapy (LLLT) could be a new alternative adjunct therapy for bone regeneration.

3.3 Low-level laser therapy

In the last 30 years, low-level laser therapy (LLLT) has been used mainly for the treatment of wounds; however, its applicability in pathological conditions such as tissue regeneration, pain relief, and inflammation has increased in different branches of regenerative medicine and dentistry [56, 57].

LLLT consists of exposing cells or tissues to low-level red and infrared lasers at wavelengths of 600–1100 nm and energy output of 1–500 mW and is called "low-level" due to its use of low-density light when compared to other forms of laser therapy. This type of irradiation may be a continuous or pulsed wave comprised of a constant, low-density energy beam (0.04–50 J/cm^2). The laser is directed at the target tissue or a monolayer of cells, with power in milliwatts (mW) [36, 58].

LLLT transmits energy at low levels; hence, there is no heat or sound emission nor vibrations. There are no thermal reactions because there is no immediate increase in temperature in the tissue being irradiated by laser. Experiments after low-level laser have shown negligible, immediate heat increase in tissue (±1ºC) [36, 59].

Studies with LLLT have proven effective in biostimulation, increasing the rate of cell proliferation, migration, and adhesion. Several different lasers with varying sources of light—including helium-neon (HeNe), ruby, and gallium-aluminum-arsenide (GaAlA)—have been used in a range of LLLT treatments and protocols [36, 60–63].

As mentioned above, LLLT can promote a range of biological processes, including cell proliferation [59, 64, 65] and differentiation [36, 66]. The effects of LLLT on cell proliferation have been studied in vitro in several types of cells, namely: fibroblasts, endothelium, keratinocytes, myoblasts, and mesenchymal stem cells, among others [36, 66–71]. Nevertheless, the molecular mechanism associated with the stimulatory effects remains unclear.

One possible theory is the ability of LLLT to influence photoreceptors in cells. This mechanism is called photobiology or biostimulation. It has been stated that biostimulation occurs through the electron transport chain in mitochondrial enzymes, inducing high levels of cell respiration by endogenous porphyrin or cytochrome c during tissue stress (lesioned) [62], which increases cell metabolism and function [66]. The response to LLLT's biostimulation effects is an increase in microcirculation, leading to higher ATP production and subsequent increase in DNA and RNA synthesis, thereby improving cellular oxygenation, nutrition, and regeneration [59, 65].

Similar to any drug treatment, LLLT has its own "active ingredient," that is, its irradiation parameters, such as wavelength, power, power density, and energy density. Regarding interaction of the laser with matter, the effects of LLLT have been described by Karu [72] as: primary, acting as modulators of cell function, and secondary, relieving pain or inducing healing. Indeed, those effects depend on appropriate irradiation parameters [72].

Several mechanisms that aim at explaining the mitogenic effects of low-level laser therapy have been proposed, including: light absorption by mitochondrial enzymes; photon absorption by flavins and cytochromes in the mitochondrial respiratory chain, affecting electron transfer; singlet oxygen production through photoexcitation of endogenous porphyrins; and photoactivation of calcium channels, resulting in higher intracellular calcium concentrations and cell proliferation [73, 74].

Furthermore, laser therapy alters cell membrane permeability, causing subsequent physiological changes in the target cells. The magnitude of the biostimulation effect will depend on the wavelength used as well as the physiology of cell at the time [69].

It has been suggested that porphyrins and cytochromes, which are part of the mitochondrial respiratory chain, are the first photoreceptors in the visible wavelength range. When energy (photons) is absorbed by the photoreceptors' cell membrane, a cascade of cellular response occurs, provoking production of reactive oxygen species (ROS), ATP synthesis, changes in cell membrane permeability, and release of nitric oxide. Those effects in turn lead to an increase in cell proliferation; changes in extracellular matrix synthesis; and local effects in components of the immune, vascular, and nervous system. Besides, intracellular pH levels are altered—a change associated with activation of ATPase. Changes in oxidation-reduction status cause higher levels of intracellular Ca2 and stimulate cell metabolism. High levels of intracellular Ca2 promote several biological processes, such as RNA and DNA synthesis, cell mitosis, and secretion of proteins. It

has been observed that Ca uptake by mammal cells can be induced by monochromatic red light (laser), depending on the dosage applied. Most cellular responses to LLLT derive from changes in mitochondrial and membrane activity, including mitochondrial membrane potential, as shown in **Figure 2**. Despite the positive results that argue for this type of treatment, the underlying action mechanism is yet to be understood [75].

In addition, studies show that ATP can activate P13K signaling pathway (phosphoinositide 3-kinase) through the ERK1/ERK2 genes, a pathway that regulates proliferation of certain types of cells [76]. Studies have also shown that LLLT promotes wound healing, collagen synthesis, nerve regeneration, bone remodeling and repair, and pain relief [57, 59, 77–80].

There are several studies in the literature that state the relationship between osteogenic differentiation, mesenchymal stem cells and LLLT, showing stimulation of matrix production, DNA synthesis, and formation of bone nodules in cultures of osteoblast-lineage cells after LLLT [36, 81, 82]. In 2005, Abramovitch-Gottlieb and colleagues used bone marrow MSCs cultured in 3D coralline (*Porites lutea*) biomaterial and He-Ne red laser irradiation (wavelength of 632.8 nm) to promote osteogenic differentiation [66]. Samples of biomaterial containing irradiated bone marrow MSCs showed an increase in neoformed bone tissue when compared to
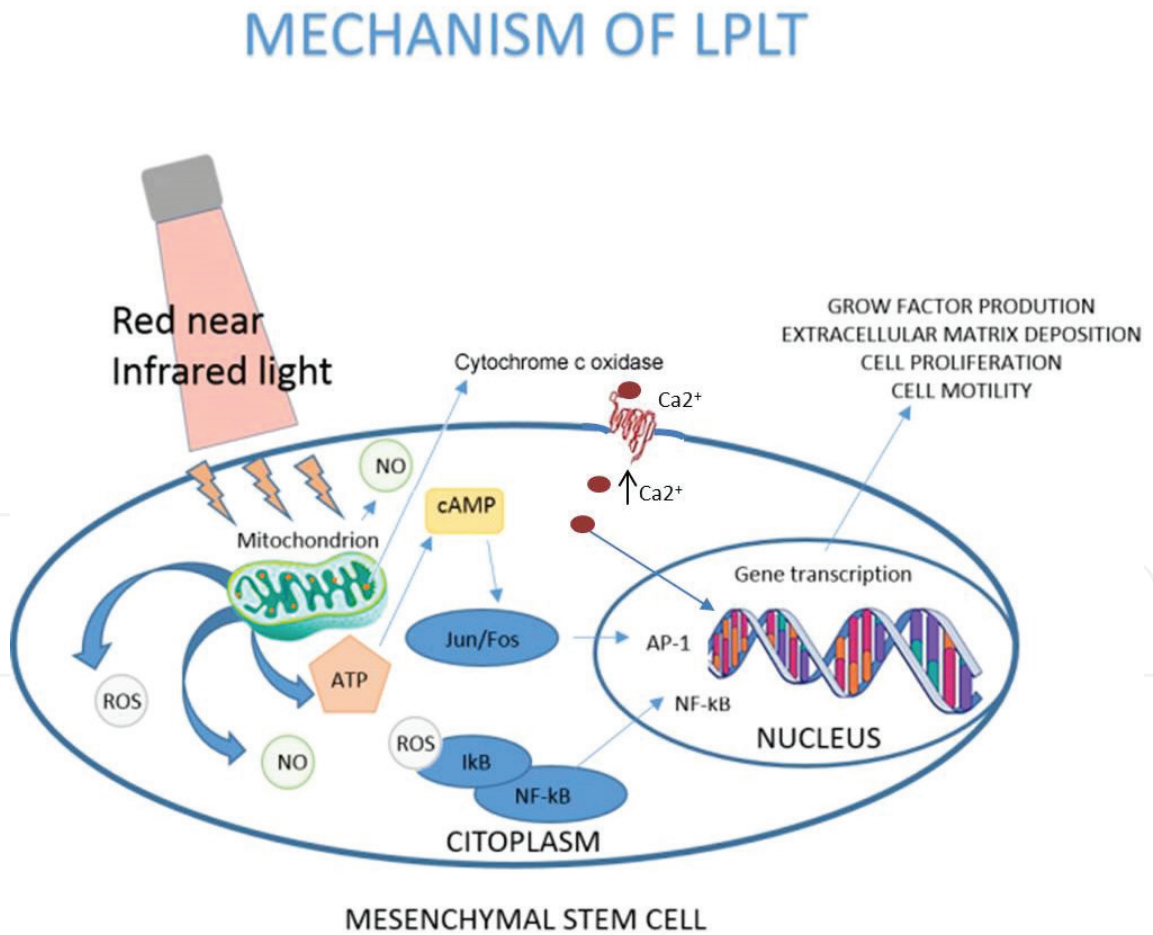

Figure 2.
The cellular effect of low-level laser therapy (LLLT) on cellular metabolism. LLLT is proposed to act via mitochondria (cytochrome c oxidase) displacing nitric oxide (NO) from the respiratory chain and increasing levels of adenosine triphosphate (ATP) and reactive oxygen species (ROS). These changes act via intermediaries cyclic adenosine monophosphate (cAMP)-activated transcription factors AP-1. The interaction of the ROS and IkB further transcription factor NF-κB. The LLLT can be photoactive of calcium channels, resulting in higher intracellular calcium concentrations. All stimuli resulting in changes in gene expression and subsequent downstream production of chemical messengers implicated in the cellular changes increase cell proliferation, cell differentiation, cell motility, and growth factors production.

non-irradiated samples. This suggests that tissue bioengineering (biomaterial containing mesenchymal stem cell) together with LLLT have biostimulation effects on osteogenic induction.

Osteogenic differentiation in MSCs has also been reinforced by another study using red laser at 647 nm. MSCs were irradiated with LLLT at differing periods of time and energy levels. Non-irradiated cells (control) were kept under the same conditions as irradiated cells. Samples of cells receiving LLLT showed a significant increase in production of extracellular matrix after 4–5 days compared to non-irradiated cells, indicating that red laser promotes osteoblast differentiation. This increase in extracellular production was maintained with daily irradiation (5, 10, and 20 J) for 21 days, which corresponds to the period of differentiation and maturation of MSCs in osteoblasts [36].

Moreover, in a study using a blue laser, MSCs were irradiated (wavelength of 405 nm) for 180 s through a fiber connected to the bottom of the culture plate. The results showed that irradiation with blue laser can promote extracellular calcification produced by MSCs differentiated into osteoblasts, in addition to inducing translocation of CRY1 protein (cryptochrome 1) from the cytoplasm to the nucleus. CRY1 is a regulator for circadian rhythm and extracellular calcification in MSCs [70]. Based on hypotheses described in previous studies, LLLT can act as adjunct treatment in tissue bioengineering, representing a new strategy in bone rehabilitation.

4. Final considerations

The creation of biobanks of mesenchymal stem cells due to the possibility of isolating and manipulating MSCs from a range of tissues as well as storing them in ultralow temperatures for future use as a bioengineering strategy for bone or other tissues' rehabilitation is of great economic and scientific interest. Yet, strategies and quality management of these biocomponents must still be developed.

The ability of MSCs for osteogenic differentiation has been well established in the literature; however, the analysis of the potential for differentiation between in vitro and in vivo sources of MSCs may direct their use in future therapies.

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