THE EFFECTS OF BISPHOSPHONATES ON OSTEONECROSIS OF JAW BONE: A STEM CELL PERSPECTIVE

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ABSTRACT

THE EFFECTS OF BISPHOSPHONATES ON OSTEONECROSIS OF JAW BONE: A STEM CELL PERSPECTIVE

Bisphosphonates (BPs) are commonly used drugs for the treatment of osteoporosis in routine clinical practice. Although BPs are successfully used for osteoporosis treatment, they may also cause bisphosphonate-induced osteonecrosis of the jaw (BIONJ). Up to present, the researchers have proposed several factors but the effects of BPs on dental stem cells' proliferation, differentiation or maintenance capacity have not been evaluated yet. In current study, the stem cells isolated from dental pulp, periodontal ligament and tooth germ were characterized by specific cell surface marker expression, differentiation and immunostaining assays. Osteogenic differentiation of the Dental Pulp Stem Cells (DPSCs), Periodontal Stem Cells (PDLSCs) and Human Tooth Germ Stem Cells (hTGSCs) treated with Zoledronate (ZOL), Alendronate (ALE) and Risedronate (RIS) were evaluated by mineral staining assays and gene expression analysis. BPs did not cause any negative effect on the osteogenic differentiation of DPSCs, PDLSCs and hTGSCs. Moreover, scratch assay was performed to detect the effects of ZOL, ALE and RIS on migration capacity of these dental stem cells, and migration related genes, extracellular matrix proteins and cytokines were analyzed. The results revealed that all BPs tested significantly inhibited migration ability of the dental tissue-derived stem cells. To evaluate the effects of ZOL, ALE and RIS on angiogenesis, two experimental models, aortic ring and tube formation assays, were performed with human umbilical vein endothelial cells (HUVECs). The results showed that BPs robustly inhibited angiogenic potential of the endothelial cells. The drastic effects of BPs on migration of DPSCs, PDLSCs and TGSCs, and angiogenesis of endothelial cells were determined. Inhibition of the migration capacities of the stem cells localized in proximity to the jaw bone, and interference with angiogenesis process might be the possible explanation for BIONJ observed after BP treatment. Further *in vivo* studies are highly warranted to investigate dental stem cell conditions in BP treated animals to elucidate the importance of these cells in BIONJ formation.

ÖZET

BIFOSFONATLARIN ÇENE KEMİĞİ OSTEONEKROZU ÜZERİNE ETKİSİ: KÖK HÜCRE PERSPEKTİFİNDEN

Bifosfonatlar (BF), osteoporoz tedavisinde rutin klinik uygulamalarda yaygın olarak kullanılan ilaçlardır. BF'ler osteoporoz tedavisinde başarılı bir şekilde kullanılmalarına rağmen, bifosfonata bağlı çene osteonekrozuna (BON) neden olmaktadırlar. Bu güne kadar, araştırmacılar tarafından bu konuda birçok sebep öne sürülmesine rağmen, BF'nin diş kökenli kök hücrelerin çoğalmasına, farklılaşma kabiliyetine veya onarma kapasitesine etkileri henüz aydınlatılamamıştır. Mevcut çalışmada, diş pulpasından, periodontal bağ dokusundan ve olgunlaşmamış gömülü dişten izole edilen hücreler, özel hücre yüzey belirteç anlatımı, farklılaşma kapasitesi ve immünboyama deneyleriyle ile karakterize edildi. Zoledronate (ZOL), Alendronate (ALE) ve Risedronate (RIS) ile muamele edilen karakterize edilmiş kök hücrelerin osteojenik farklılaşması, mineral boyama ve gen anlatımı analizi ile değerlendirildi. BF'ler, DPSC'ler, PDLSC'ler ve TGSC'lerin osteojenik farklılaşması üzerinde herhangi bir olumsuz etki yaratmadı. Ayrıca, bu diş kök hücrelerinin migrasyon kapasitesi üzerine ZOL, ALE ve RIS'in etkilerini saptamak için çizilme testi gerçekleştirildi ve migrasyonla ilişkili genler, hücre dışı matris proteinleri ve sitokinler analiz edildi. Test edilen tüm BF'lerin, diş doku kökenli kök hücrelerin migrasyon kabiliyetini önemli ölçüde azalttığı ortaya konuldu. ZOL, ALE ve RIS'in anjiyogenez üzerindeki etkilerini değerlendirmek için iki model deney; aortik halka deneyi ve tüp formasyonu deneyi insan umbilikal ven endotel hücreleri (HUVEC'ler) ile gerçekleştirildi. Sonuçlar, BF'lerin endotel hücrelerinin damar oluşturma potansiyelini güçlü bir şekilde engellediğini gösterdi. BF'lerin DPSC, PDLSC ve TGSC'lerin migrasyonu ve endotel hücrelerinin anjiogenezi üzerindeki olumsuz etkileri belirlendi. Çene kemiği yakınında localize olan kök hücrelerin migrasyon kapasitelerinin engellenmesi ve bölgesel anjiyogenezin engellenmesi, BF tedavisinden sonra görülen BON için muhtemel açıklama olabilir. BON oluşmasında, BF'lerin bu hücreler üzerindeki etkilerini aydınlatmak için BF ile tedavi edilen hayvanlardaki diş kök hücrelerini araştırmaya yönelik yapılacak *in vivo* çalışmalar daha açıklayıcı sonuçlar verecektir.

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ADSCs	Adipose Derived Stem Cells
ADT	Androgen Deprivation Therapy
ALE	Alendronate
ALP	Alkaline Phosphatase
ASCs	Adult Stem Cells
BCA	Bicinchoninic Acid Assay
bFGF	Basic Fibroblast Growth Factor
BIONJ	Bisphosphonates Induced Osteonecrosis of Jaw
BMD	Bone Mineral Density
BMP	Bone Morphogenetic Protein
BMSCs	Bone Marrow-Derived Stem Cells
BPs	Bisphosphonates
BSP	Bone sialoprotein
CBFA-1	Core Binding Factor Alpha 1
COL II	Collagen Type II
COL1A1	Collagen Type I Alpha 1
DAPI	4′,6-di-amidino-2-phenyl-indole
DFSCs	Dental Follicle Stem Cells
dH_2O	Distilled Water
DMEM	Dulbecco’s Modified Eagle’s Medium
DPSCs	Dental Pulp Stem Cells
DSCs	Dental Stem Cells
DXM	Dexamethasone
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ESCs	Embryonic Stem Cells
FABP4	Fatty Acid-Binding Protein 4
FBS	Fetal Bovine Serum
FGFs	Fibroblast Growth Factors

FLK-1	Flt Related Receptor Tyrosine Kinase
FLT-1	Fms Related Tyrosine Kinase 1
FPP	Farnesyl Diphosphate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GH	Growth Hormone
HDMECs	Human Dermal Microvascular Endothelial Cells
HH	Hedgehog
HSCs	Hematopoietic Stem Cells
hTGSC	Human Tooth Germ Stem Cells
HUVECs	Human Umbilical Vein Endothelial Cells
IBMX	3-Isobutyl-1-methylxanthine
ICAM-1	Intercellular Adhesion Molecule 1
IGF-I	Insulin-like Growth Factor I
iPSCs	Induced Pluripotent Stem Cells
KLF4	Kruppel Like Factor 4
MCP-1	Monocyte Chemoattractant Protein 1
MMP	Matrix Metalloproteinase
MSCs	Mesenchymal Stem Cells
MSX 1	Msh homeobox 1
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
N-BPs	Aminobisphosphonates
non-N-BPs	Non-aminobisphosphonates
OCN	Osteocalcin
OCT4	Octamer Binding Transcription Factor 4
ONN	Osteonectin
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PDLSCs	Periodontal Ligament Stem Cells
PFX	Paraformaldehyde
PSA	Penicillin/ Streptomycin/ Amphotericin
PSCs	Pluripotent Stem Cells

qPCR	Real Time Polymerase Chain Reaction
RIS	Risedronate
RUNX2	Runt-Related Transcription Factor 2
SCAP	Stem Cells of Apical Papilla
SCs	Stem Cells
SDF-1	Stromal Cell Derived Factor 1
SHED	Stem Cells from Human Exfoliated Deciduous Teeth
SOX10	Sex Determining Region Y Box 10
SOX2	Sex Determining Region Y Box 2
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline and Tween-20 Solution
TGPCs	Tooth Germ Progenitor Cells
TIMP-1	TIMP metallopeptidase inhibitor 1
TIMP-2	TIMP metallopeptidase inhibitor 2
TRIS-HCL	Tris Hydrochloride
VE-CADHERIN	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
ZOL	Zoledronate

1. INTRODUCTION

1.1. STEM CELLS

Stem cells (SCs) are undifferentiated cells, which have self-renewal capacity and differentiation potential into various functional cell types [1]. As SCs divide, two different cells generate by undergoing asymmetric division; (i) one of them keeps stem cell properties just like parent cell, and (ii) the other one is defined as a progenitor cells with specialized function [2]. Stem cells can differentiate into specific cell lineages after receiving proper signals from the surrounding microenvironment, which allows regeneration of damaged organs or tissues in the body.

According to their differentiation capacities, stem cells are divided into four main groups (Figure 1.1). Totipotent stem cells obtained from morula stage are able to generate a whole organism (all tissues and placenta) [3]. Pluripotent stem cells (PSCs) existing in the inner cell mass of blastocyst are referred to as embryonic stem cells (ESCs) [4] and induced pluripotent stem cells (iPSCs) are able to differentiate into about 200 various cell types [5-7]. Multipotent stem cells or adult stem cells (ASCs) are obtained from adult tissues and can differentiate into a limited number of cell lineages [8]. Unipotent stem cells (progenitor cells) have the differentiation potential into one special cell type.

ESCs, derived from early stage of embryo, and iPSCs, reprogrammed somatic cells by transduction with specific transcription factors, can differentiate into ectoderm, mesoderm and endoderm derived cells in the body. Due to these unique properties, they are planned to be used as alternative cell sources for future regenerative medicine. They are more proliferative and have more differentiation capacity compared to multipotent adult stem cells such as bone marrow derived stem cells (BMMSCs) and adipose derived stem cells (ADSCs), etc [9]. However, it is difficult to work with PSCs, especially originating from human, because it is hard to direct them to the desired cell lineages due to their high unstable conditions. In order to direct the differentiation of PSCs, many factors in the cell microenvironment such as cell-cell interactions, physical and chemical factors should strictly be controlled [10]. Ethical problems and tumorigenicity have hindered commonly

using embryonic stem cells in clinical applications [11]. Moreover, while more simple culture methods are used for ASCs, more complicated culture methods are used for PSCs. As an example, PSCs are cultured with feeder cells (to form a feeder layer), on matrigel or other biocompatible biomaterials; however, ASCs can be cultured on traditional culture dishes [12].

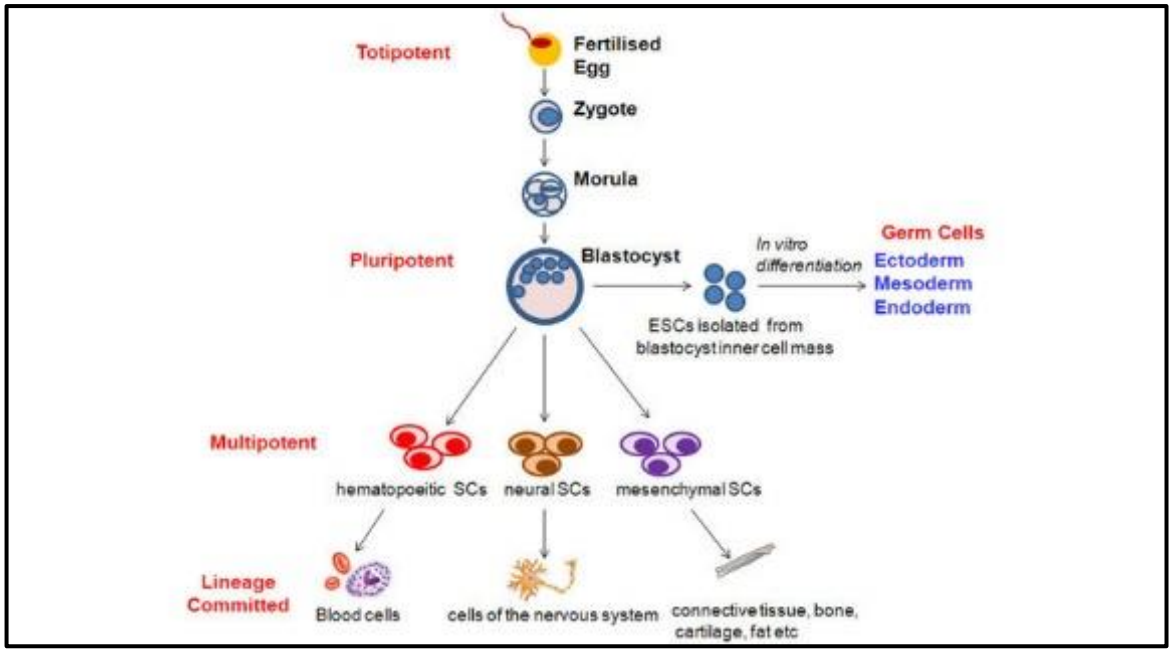

Figure 1.1. Stem cell classifications according to their differentiation potential [13].

Although ASCs isolated from various body parts have limited differentiation capacity they possess major roles in maintaining blood homeostasis, tissue and skin turnover. After the embryonic development, ASCs are located in different parts of the body and play critical roles in tissue homeostasis through replacing cells to maintain tissue integrity in case of disease and injury [14, 15]. ASCs are composed of two sub-classes; hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). Along with producing all blood cells [16], HSCs can also differentiate into neurogenic-like cells [17]. On the other hand; in general, MSCs have differentiation potential into marrow stroma, bone, muscle, cartilage, tendon and fat [18].

First characterized MSC source is bone marrow [19]. These cells can adhere to plastic culture dishes and differentiate into cells in various clonal subpopulations [20, 21]. In the

last decade, several MSC types have been isolated from almost every parts of body including skeletal muscle [22], adipose tissue [23], amniotic fluid [24], placenta [25], umbilical cord blood [26], dental pulp [27] and dental follicle [28]. These cells are able to differentiate into adipogenic, osteogenic, chondrogenic and neurogenic cell lineages [29, 30]. Moreover, other mesodermal-derived tissues such as, muscle and tendon cells could be obtained from MSCs by using appropriate differentiation protocols [31, 32]. In addition to mesodermal lineages, it has been proven that these cells can also generate ectodermal and endodermal originated cells including hepatocytes, retinal pigment epitelium, neural cells, and hepatocytes, lung cells [33-38].

The general characteristic properties of MSCs are that they are plastic adherent in *in vitro* conditions, express CD105, CD73 and CD90, do not express hematopoietic surface markers such as CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II, and they can easily undergo adipogenesis, chondrogenesis and osteogenesis under specific *in vitro* conditions [39].

MSCs manage cell turnover, induce tissue repair and regeneration, and regulate the immune system enabling restoration of damaged tissues. They repair the tissue in two different ways; (i) stem cells either differentiate directly into required cell types, or (ii) they release vital factors that organize the repair process [40]. For bone tissue integrity and regeneration, MSCs are crucial cell types due to their osteogenic potential. They are also privileged against the immune response [41], and they can release immunomodulatory agents and therapeutic factors [42, 43]. MSCs are mesoderm-derived residues from the embryonic development period and they are undifferentiated cells localized in the connective tissues of different organs and bone marrow. Dental tissue also contains several MSCs with different properties. Dental Stem Cells (DSCs) are classified according to their source in the oral cavity and have different properties based on their stem cell characteristics.

1.1.1. Dental Stem Cells (DSCs)

DSCs are obtained from various teeth types and different parts of the tooth such as from the pulp of adult teeth, pulp of exfoliated teeth, ends of extended roots, surrounding tissue

of the unerupted teeth referred to as dental follicle, and periodontal ligament (Figure 1.2). All these cells derive from the neural crest and have general features of MSCs including, having surface markers of stem cells and differentiation capacity into three mesenchymal cell lineages; adipocytes, osteoblasts and chondrocytes [44].

Stem cell isolation from the tooth was first performed by Gronthos *et al*. in 2000 from dental pulp tissue [27]. In 2003, the Miura *et al*. characterized stem cells from human exfoliated deciduous teeth (SHED) [45]. After a year, Seo *et al*. discovered periodontal ligament stem cells (PDLSCs) [46]. Morseckeck *et al.* discovered dental follicle cells in 2005, but these cells were later considered to be dental follicle stem cells (DFSCs) [28]. The last dental stem cell type was isolated from apical papilla in 2006 referred to as stem cells from apical papilla (SCAP) [47].

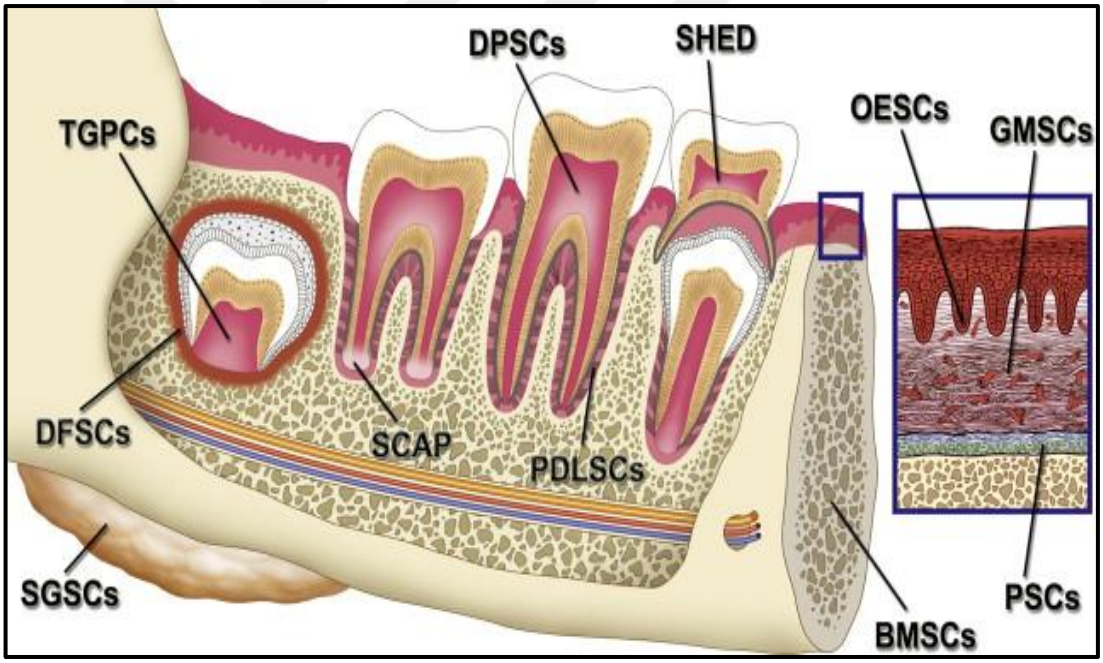

Figure 1.2. Sources of adult stem cells in the oral and maxillofacial region [48].

1.1.1.1. Dental Pulp Stem Cells (DPSCs)

Stem cell isolation from the tooth was first performed by Gronthos et al. in 2000 from dental pulp tissue. DPSCs are spindle-shaped resembling to fibroblasts cells, that are

highly proliferative and clonogenic. DPSCs express Sox-2, Nanog, Oct 4, c-Myc, Vimentin, Nestin which are considered as ESC markers [49-53]. In addition, CD29, CD44, CD73, CD105 and CD106, as MSC cell surface markers, have been used to identify DPSCs [54]. Moreover, DPSCs can generate bone, pulp, and dentine-like tissues when transplanted into experimental animals [55]. DPSCs were also differentiated into odontoblast in *in vitro* [56].

DPSCs have been shown to produce functional dental tissue complex after transplanted into immunocompromised mouse [55]. Researchers have also shown that DPSCs have differentiation ability into dentinogenic, osteogenic, myogenic, neurogenic, adipogenic and chondrogenic cell types [57-62]. As DPSCs have the capacity of differentiation into bone, dentine and pulp-like tissues; they are mostly preferred in bone and periodontal tissue regeneration applications [63, 64]. Interestingly, DPSCs have been proven to display greater osteogenic differentiation capacity compared to BMMSCs and periosteal cells; hence, they are proposed to be more appropriate cell source for bone restoration approaches during dental implantations [65].

In comparative studies, DPSCs exhibited shorter doubling time and higher stem/progenitor cells numbers. In the same study, DPSCs displayed markedly better alkaline phosphatase (ALP) activity in *in vitro* conditions compared to BMMSCs after three week incubation period [66]. These studies revealed that DPSCs could serve a valuable option for the field of orthopedics and maxillofacial remodeling. As a different point of view, DPSCs have also been used for the muscle, heart and brain regeneration and/or repair in animal models [67]. In the first clinical study of DPSCs used for alveolar bone remodeling was successfully carried out, and DPSC/collagen complex was found to be able to repair whole human mandible bone damages [68].

For a successful tissue generation, newly established systemic blood network is required for oxygen and nutrient transfers to regenerating cells, and evacuation of carbon dioxide and waste materials. Therefore, stem cells with angiogenic potential would be more applicable in bone regeneration applications. Other than their osteogenic potential, angiogenic activity of DPSCs has been shown by reporting the expressions of angiogenesis-related markers such as vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-2 (Flk-1), and von willebrand factor (vWF) in

endothelially differentiated DPSCs in *in vitro* conditions [69]. The cells were found to give rise to bone cells as well as vessel complexes in which Flk-1 expression plays a critical role, indicating that DPSCs would be important candidate for future bone therapy approaches [63]. Consistently, DPSCs were reported to undergo endothelial differentiation confirmed by the expression of vWF, VEGF receptors, Flk-1 and vascular endothelial growth factor receptor-1 (Flt-1), and intercellular adhesion molecule-1 (ICAM-l). Furthermore, when DPSCs were cultured with VEGF on matrigel coated wells, they formed tube-like structures, supporting the functional angiogenic capacity of the cells. Microscopic evaluations revealed phenotypic difference between VEGF-treated or non-treated DPSCs. While non-treated groups remained as classic fibroblast morphology, VEGF-treated groups obtained endothelial structure [69].

1.1.1.2. Periodontal Ligament Stem Cells (PDLSCs)

Periodontal ligament tissue has heterogeneous cell populations and ability to remodel itself. This tissue consist of various cell lines such as osteoblast, cementoblast, fibroblast, smooth muscle cells, vascular endothelial cells and nerve cells. Thus, researchers thought that this population could contain valuable stem cell resources. PDLSCs have various differentiation potential according to the isolation area. For instance, cells isolated from the surface of alveolar bone differentiate into alveolar bone better in comparison to cells isolated from the surface of root [59].

PDLSCs have specific markers including Nanog, Sox2, Klf4 and Oct4 as ESC markers and Slug, p75, Nestin and Sox10 as neural crest markers. They are able to differentiate into ectodermal and mesodermal lineages including chondrogenic, osteogenic, neurogenic and cardiomyogenic cells. Moreover, they can also transform into insulin producing endodermal lineage-derived cells, indicating three germ layer differentiation potential of PDLSCs. Their highly proliferative potential was attributed to great expression of telomerases [70].

PDLSCs express scleraxis, a tendons specific transcription factor, as well as STRO-1 and CD146. Besides, scleraxis are expressed more in PDLSCs than BMMSCs and DPSCs. In this context, it is not surprising that tendon and the periodontal ligament are

morphologically similar. In a comparison study for PDLSCs and BMMSCs, it was found out that expression of alkaline phosphatase (ALP) in PDLSCs was detected 7 day before the expression in BMMSCs [46].

PDLSCs display some bone-related cell surface markers as BMMSCs do, indicating the importance of these cells for bone repair and regeneration studies. Moreover, PDLSCs have the potential of differentiation into osteoblast- and cementoblast-like cells. Therefore, PDLSCs might provide homeostasis and periodontal tissue repairmen in the body. In addition to these, PDLSCs exhibit adipogenic, chondrogenic and osteogenic differentiation under the appropriate conditions [46]. PDLSCs have the capacity of angiogenesis specific genes expression, including VE-cadherin, CD31, vWF [71].

1.1.1.3. Stem Cells From Human Exfoliated Deciduous Teeth (SHED)

SHED are isolated with the method similar to that is used for DPSC isolation. As other dental tissue-derived stem cells, they have also colony forming ability and high proliferation capacity. SHED were reported to be more proliferative due to having shorter doubling time compared to DPSCs and BMMSCs [72]. These cells have also differentiation of adipogenic, neurogenic and osteogenic differentiation potential [45, 72]. Also, SHED are able to chondrogenesis and myogenesis [73]. Similar to other dental tissue-derived stem cells, SHED express CD146 and STRO-1 [74].

According to *in vivo* studies, SHED were able to produce dentin-like tissue consisting odontoblast-like cells. Surprisingly, they could not exhibit odontogenic ability as much as DPSCs [45]. Besides, they were not able to differentiate into osteocyte- and osteoblast-like cells. On the other hand, they supported the differentiation of recipient murine cells into osteoblasts [72]. These studies, in general, support the idea that SHED are immature type of DPSCs.

1.1.1.4. Dental Follicle Stem Cells (DFSCs)

The dental follicle is a connective tissue sac which includes periodontal ligament, osteoblast and cementoblast progenitors. Therefore, DFSCs are able to produce bone,

cementum and periodontal ligament from fibrous tissue [75, 76]. CD13, CD44, CD73, CD105, STRO-1, Nestin, Notch-1 and GoPro49, a newly identified golgi protein [77], have been used to characterize DFSCs using flow cytometry analysis [78]. DFSCs reside in surrounding the unerupted tooth of enamel organ. During the periodontium development, these cells turn into osteoblast, periodontal ligament, fibroblast and cementoblast cells. These cells have also potential to get mineralized under specific osteogenic differentiation medium in *in vitro* culture conditions [79]. DFSCs appear like fibroblast cells and have ability of neurogenesis and adipogenesis, similar to DPSCs.

1.1.1.5. Stem Cells of Apical Papilla (SCAP)

SCAP are isolated from the apical papilla which is the soft tissue at the root of the developing tooth. After root development, apical papilla merges to the pulp tissue. Like other stem cells originating from the tooth, SCAP resemble fibroblast cells and have clonogenic properties, express STRO-1 and CD146. Distinctly, their expansion ability is greater than DPSCs and express a unique cell surface marker, CD24. SCAP are able to differentiate into neurogenic and chondrogenic cells under *in vitro* conditions. Besides, they can generate dentine-like tissue that includes odontoblast-like cells in *in vivo*. In addition, researchers showed that SCAP can differentiate into the odontogenic and adipogenic lineages under optimized stimulus. Interestingly, these cells can express neural markers without any neurogenic induction but if they are neurogenically induced, these markers are expressed more. SCAP are claimed to be suitable stem cells for tissue regeneration and better choice in comparison with the cells obtained from mature tissues [47, 80, 81].

1.1.1.6. Human Tooth Germ Stem Cells (hTGSC)

Ecto-mesodermal interactions generating the neural crest cells give rise to dental germ tissue during the embryonic development [58]. The tissue includes progenitor cells with differentiation potential towards various dental components such as dental papilla, dental follicle, dental pulp and dental organ [82]. In the late stages of the tooth development, these cells are still found in some dental components including dental pulp, papilla and

periodontal ligament [27, 45, 46, 83]. Similar to other dental tissue-derived stem cells, hTGSCs have multipotent properties and can differentiate into various cell lineages. In childhood, organogenesis of the tooth germ starts at the age of 6 [58], supporting germ-derived stem cells might have high proliferation and multipotent capacities.

hTGSCs can differentiate into various mesoderm-derived cell types. hTGSCs are obtained from the center tissue of the extracted teeth. Thus, studying with these cells does not create any orthodontic or ethical problems. hTGSCs isolated from young adults provide an alternative and more potential MSC source for the utilization of dental tissue engineering. Osteogenic and odontogenic differentiation capacities of hTGSCs have been proven in *in vitro* conditions. Besides, hTGSCs have differentiation capacity into various cell lineages derived from ectoderm, mesoderm and endoderm germ layers [84]. In the aspect of adipose tissue engineering, hTGSCs derived adipogenic cells may be one promising approach for the therapy of fatty defects or deep burns. hTGSCs expressing CD29, CD44, CD73, CD90, CD105 MSC cell surface markers can successfully differentiate into endothelial and epithelial cell lineages. Consistently, hTGSCs also form tube-like structure when cultured on matrigel in *in vitro* conditions [85].

1.2. BONE DEVELOPMENT AND MAINTENANCE

The skeleton of the mammalian body has three various embryological origins: (i) the lateral plate mesoderm, which engenders the appendicular skeleton; (ii) paraxial mesoderm, which generates the axial skeleton; and (iii) ectodermal neural crest, which creates the facial skeleton. Bones are generated by osteoblasts secreting a specific bone matrix [86, 87].

Skeletal development occurs through two main mechanisms: (i) in intramembranous ossification, the MSCs (osteochondral progenitors) differentiate into osteoblasts to form membranous bone, and (ii) in endochondral ossification, the MSCs differentiate into chondrocytes to form a cartilage template of the future bones [88]. Each skeletal element decides its genesis mechanism and anatomic features (i.e. shape and size) according to its place. This flexibility is gained during pattern formation in early embryonic development. Cell-cell interactions or communications have crucial functions in the bone maintenance

and development, which is mediated by various signaling factors including hedgehog (Hh), Wnt, bone morphogenetic protein (Bmp), Notch/Delta and fibroblast growth factor (Fgf). These mediators direct the cell fate, proliferation, maturation, and polarity later in the bone development [89]. Androgens, estrogens and vitamin D are known as steroid hormones have also crucial roles on the bone development. In addition, each one of them has specific roles on skeletogenesis and balance of bone mineral density (BMD) [90].

The jaw is a special mammalian part which provides mastication. It is derived from neural crest cells including dental stem cells. The development of the jaw starts with localization of the neural crest MSCs and differentiation into the jaw blastema and chondrocytes. Then, chondrocytes generate Meckel's cartilage (i.e. two bilateral cartilaginous rods). Neural crest cells differentiate into osteoblast forming the intramembranous bone. Meckel's cartilage serves as a scaffold for the ossification. During development of long bones through endochondral ossification, development of the jaw takes places with an intramembranous process. While neural crest MSCs do not generate the bone itself, they from the alveolar gap required for the development of tooth [90].

The maintenance of bone tissue integrity is regulated by bone cells during osteogenesis and skeletal growth through modeling (uncoupled) and remodeling (coupled) mechanisms. Modeling mechanism is necessary for the bone development, shape and size preservation to achieve normal and stable growth as well as providing resistance to mechanical stress. Osteoblasts and osteoclasts play crucial roles in this mechanism at different time and places.

Remodeling mechanism is required for endurance of bone regeneration, maintenance of undamaged bone and adjustment of optimum calcium level (Figure 1.3). In case of an imbalance between these the two mechanisms, osteopetrosis (bone resorption < bone formation) or osteoporosis (bone resorption > bone formation) take place [91, 92].

1.3. OSTEOPOROSIS

Osteoporosis, characterized by reduction in the bone mass and BMD, is a skeletal disease, which is commonly experienced by women in postmenopausal stage and elderly people [93]. About 75 million people in the USA, Europe and Japan have osteoporosis. As the

bone fracture is the most prominent result of osteoporosis, the risk for bone fracture in postmenopausal women is almost three times more than the risk for healthy men at the same age [94].

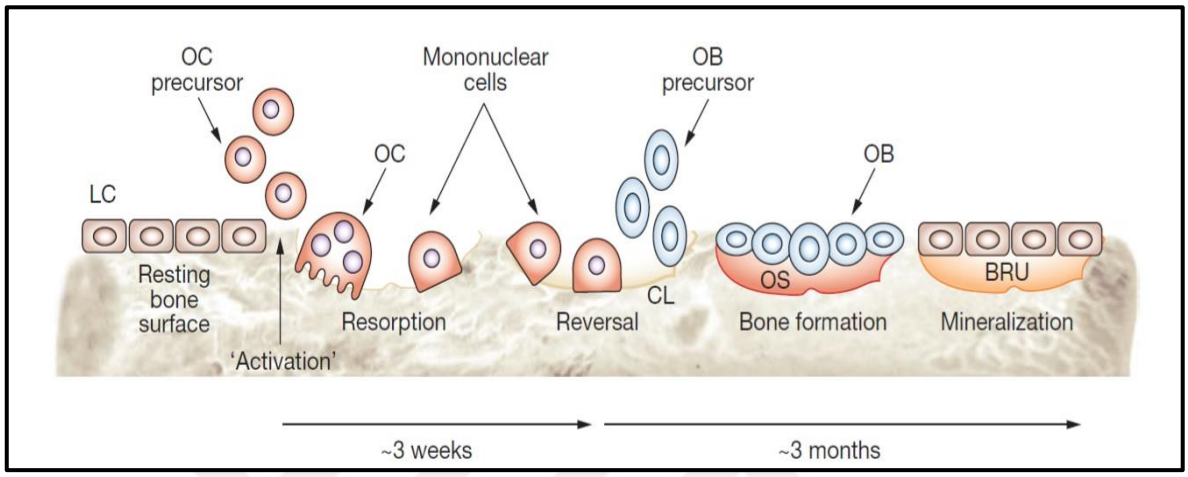

Figure 1.3. Bone remodeling cycle. Abbreviations: BRU, bone remodeling unit; CL, cement line; LC, lining cells; OB, osteoblast; OC, osteoclast; OS, osteoid [95].

Normally, bone mass and density continuously increase in childhood and puberty, and reach a maximum point until the age of twenty. After skeletal maturation, developing bone tissue quantity is named as peak bone mass. Although men generally have bigger bones and thicker cortical layer than women, the intensity of bone is nearly the same in men and women. After reaching peak bone mass, it remains constant. Both genders go into a short consolidation stage at the age of 30, and their bone mass and density begin to decrease. Age-related reduction in the bone mass takes place at a constant rate for men during their whole lifetime, whereas the maximum decrease in bone mass for women occurs around 5-10 years after their menopausal stage [96].

Age-related bone mass reduction arises from imbalance between the osteoclasts' resorptive activity and the production activity of osteoblast. Due to detrimental loss of trabecular bone structure and connection, inner layer of the bone becomes less intense. Peak bone mass is a main indicator for the osteoporosis risk and affected by several parameters including heritage, gender, diet, hormonal level, physical activity and environmental factors, and of those, heritage is the most crucial factor [96].

Estrogen has key roles in the bone development and remodeling, and it is necessary for the development of normal bone and mineral accumulation to reach peak bone mass. As the main characteristic of osteoporosis, estrogen deficiency results in severe decrease in the bone mass and impairment in the bone microarchitecture. Estrogen is able to regulate proliferation and apoptosis of osteoclast cells [94]. Estrogen deficiency increases osteoclasts' lifetime and decreases osteoblasts' and osteocytes' lifetime. The sum of all these indications propose that estrogen deficiency can cause disequilibrium between the bone formation and resorption resulting in severe bone loss [97].

Anti-resorptive agents including hormones and chemical drugs have been used in treatment of osteoporosis to decrease bone resorption rate, providing balance between osteoblast and osteoclast activity, and adjusting proper levels of calcium and phosphorus ions [98]. These anti-osteoporosis drugs have generally been used along with calcium and vitamin D supplements to prevent possible bone fracture. For each patient with osteoporosis, it may be necessary to prescribe different drug combinations, due to their different personal lifestyle, clinical and familial histories.

Bisphosphonates (BPs) are commonly used for the treatment of osteoporosis in the clinical practice. They are the most preferred drugs to treat osteoporosis, particularly postmenopausal osteoporosis, as they are strong anti-resorptive agents. Some of these drugs are also used for male patients with glucocorticoid-induced osteoporosis [96].

1.4. BISPHOSPHONATES (BPs)

BPs, analogues of inorganic pyrophosphate, decrease bone resorption. They have high affinity for hydroxyapatite crystals and phosphorous–carbon–phosphorous structures in the bone architecture. The efficacy of BPs is highly associated with the structure and length of the side chain of BPs [99]. The BPs include a carbon atom, two phosphate groups and two side chains (R1and R2). The side chains determine the characteristic of the BPs. Modifying the phosphate groups or side chains in the chemical formula may lead to development of novel BPs with altered functions. R1 groups are responsible for the affinity capacity of BPs to hydroxyapatite crystals. R2 groups, on the other hand, identify the BPs' potential and action of mechanism. BPs show significant toxicity against osteoclast cells and interfere

with some metabolic pathways in those cells reducing the activity. The most prominent role of BPs is to prevent bone resorption arisen from high osteoclast activity [100, 101].

There are mainly two classes of bisphosphonates; (i) the first group is without any amino group (non-aminobisphosphonates, non-N-BP) such as etidronate, tiludronate and clodronate, and (ii) the second group is aminobisphosphonates (N-BPs) such as zoledronate, alendronate, risedronate, pamidronate and ibandronate (Figure 1. 4) [101]. The non-N-BP is metabolized and converted into cytotoxic ATP analogues, which accumulates in the cytosol of osteoclast and lead to cell death by apoptosis [102]. On the other hand, N-BPs binds to key enzymes and block HMG-CoA reductase pathway (mevalonate pathway) and inhibits farnesyl diphosphate (FPP) synthase, that obstructs the prenylation of small GTPases including Ras, Rho, Rab. Thus, N-BPs provide blockage of osteoclast cells' function and recovery (Figure 1. 5). BPs can be administered both orally and intravenously. Absorption rate of BPs is very low in the body (0.7% - 2.5%) [96]. As oral uptake of the BPs have limited bioavailability and absorption, intravenous way is generally preferred. The absorption rate of BPs by each bone is not the same. BPs are firstly received by bone tissues with high turnover ratio. Then, they diffuse into some soft tissue including spleen, kidney etc [103].

Although the effects of BPs on bone tissue is widely investigated and elucidated, their effects on the tooth development have not been adequately addressed. There are only a few studies about this topic in the literature. In one of these few studies, BPs have been reported to effect odontogenesis and dental structure in pregnant women or children during deciduous and permanent teeth development [103]. One possible mechanism for this positive effect was given as that free calcium and phosphate ions due to the bone resorption may contribute to the tooth eruption and development via forming hydroxyapatite crystals. Tooth development mechanism is very complex event and identified by cell-cell and cell-extracellular matrix communications. These interactions result in transforming of odontoblasts into the dentin and ameloblasts. Bone resorption via osteoclasts is necessary for the tooth eruption, but some of the BPs have also critical side effects negatively influencing the eruption, development and mineralization of tooth. BP treatments can cause osteoclast apoptosis leading to inefficient tooth eruption [103].

Positive effects of the BPs have been identified in children. BPs, particularly zoledronic acid (ZOL), are used for the treatment of metastatic bone disease in children. They are also used for the therapy of acute hypercalcemia occurred after increased calcification. In addition to these, they are reported to have beneficial outcomes for some osteonecrotic conditions such as chemotherapy-related osteonecrosis [104].

ZOL is newly presented drug to prevent osteoporosis derived from fracture by intravenous infusion in postmenopausal women. Alendronate (ALE) is introduced to treat and block postmenopausal osteoporosis. Risedronate (RIS) is used to treat and inhibit postmenopausal osteoporosis and glucocorticoid-induced osteoporosis. BPs, in general, decrease the fracture risks occurred in the osteoporosis by 70% and 40% for men and postmenopausal women, respectively. Cell proliferation, differentiation and gene expression of differentiated osteoblasts are regulated by BPs. Besides, BPs orchestrate pre-osteoblast formation in the bone marrow, providing early osteoblastogenesis, and stimulate vital growth factors and cytokines synthesis. Although BPs are commonly used for many diseases associated with bone, their usage is not suitable for some patients who have gastrointestinal disease or reflux (50).

1.4.1. Zoledronate (ZOL)

ZOL is a new generation (nitrogen containing) BPs and has higher potential than the other BP family drugs. Recent studies shown that ZOL is more appropriate than the other orally taken BPs as it induces more BMD. In a preclinical study, ZOL is reported to be more beneficial and safe than pamidronate. Moreover, ZOL resulted in reduction skeletal-related events (SRE) in the breast cancer patients who are cancer cells metastases to bone tissue [105].

In another study, progression of the breast cancer bone metastasis was reduced when ZOL was accompanied with the endocrine therapy and enhanced survival rate compared to only endocrine therapy. ZOL has anti-tumor and anti-metastatic properties via suppression of angiogenic activity of endothelial cells and adhesion of cancer cells to bone, triggering apoptosis and inhibiting tumor-cell invasion. ZOL is a unique BP in the aspect of

providing long-term effective treatment in the prostate cancer patients with metastases to bone tissue [106].

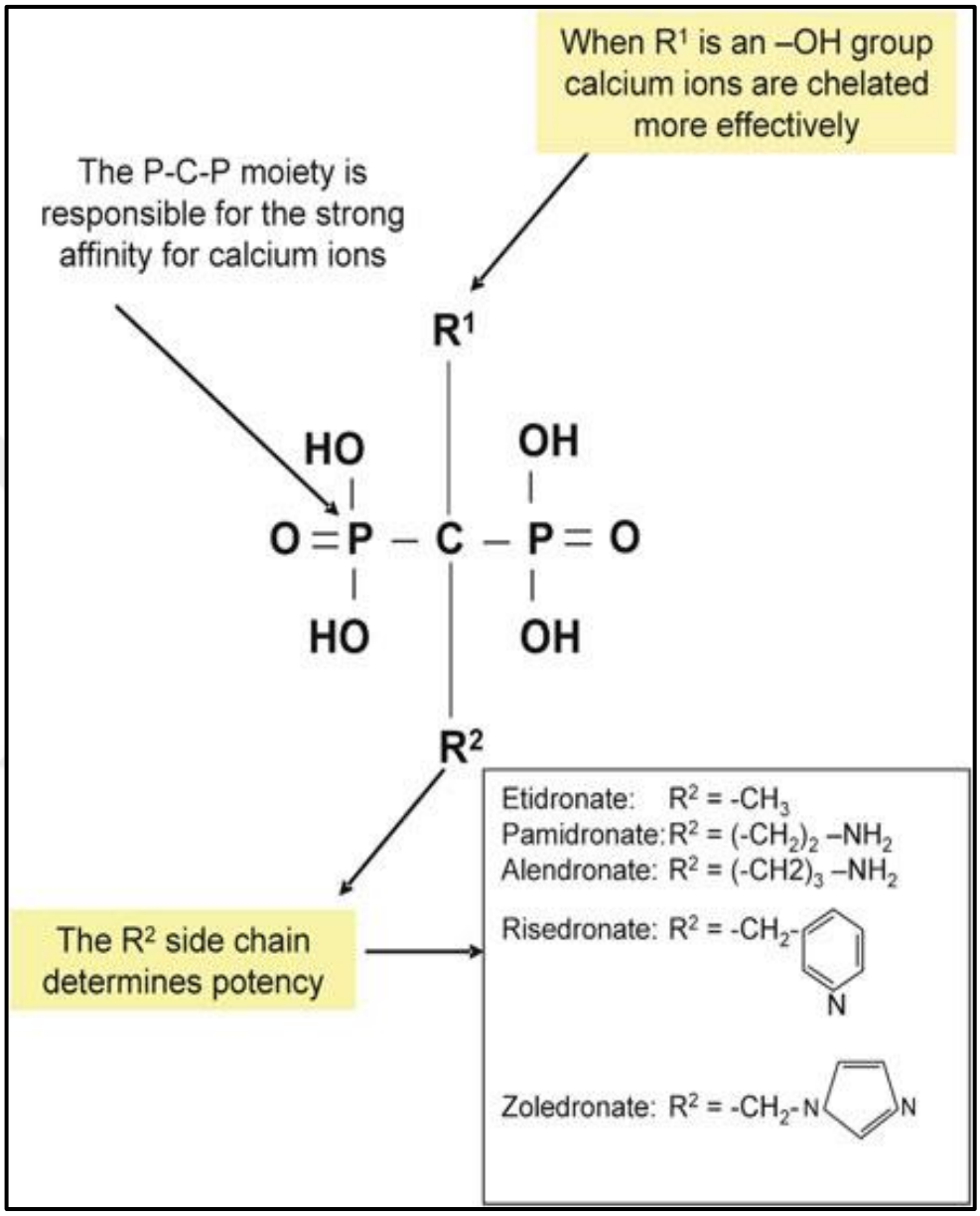

Figure 1.4. Basic chemical structure of bisphosphonates [107]

1.4.2. Alendronate (ALE)

ALE is included in the nitrogen-containing BPs group used for osteoporosis treatment. A well-known effect of the ALE is inhibiting bone loss and decreasing the risk of spondyle and hip bone fracture at a rate of approximately 50% [96].

In a clinical trial with androgen deprivation therapy (ADT) for non-metastatic prostate patients, ALE therapy was reported to inhibit bone mass reduction compared to non-treated group. There are two challenges for the ALE treatment; (i) bioavailability of ALE is very low, and (ii) ALE cause serious side effects, including oesophageal irritation, and erosion-related with bleeding. Thus, the patients should be examined before the prescription as 60% of the patients stopped ALE therapy in the first year of use [103].

1.4.3. Risedronate (RIS)

RIS is used for treatment of postmenopausal and glucocorticoid-induced osteoporosis. Risedronate is an orally taken medicine, and its reductive effects on osteoporosis related bone loss is well established. In postmenopausal individuals, RIS uptake have been reported to reduce the levels of markers related with bone degeneration at a ratio of 50% through increasing the BMD. On the other hand, RIS is also associated with several adverse events, such as oesophageal irritation and long term uptake of RIS may cause bisphosphonate-induced osteonecrosis of the jaw (BIONJ) [103].

Although BPs are common agents used to treat osteoporosis and related side effects, they also have several adverse effects. The oral administration of RIS, ALE and ibandronate might lead to oesophagus irritation, painful swallowing, headache, intestinal obstruction, catharsis, rash and pain in stomach.

Intravenous administrations of BPs have resulted in short-term flu, arthalgia, transient fever and myalgia (e.g. acute phase reaction) with rate of 25%. Moreover, heartburn, joint and bone pain, muscle, episcleritis, atrial fibrillation, iritis, conjunctivitis and orbital inflammation with rate of up to 1% have been recorded in BP administered patients. More importantly, long term administrations of BPs have caused BIONJ with rate of 0.028-4.3% when applied intravenously. In addition, oral administration of BPs increases the risk of BIONJ depending on the dosage and the time of the treatment [98].

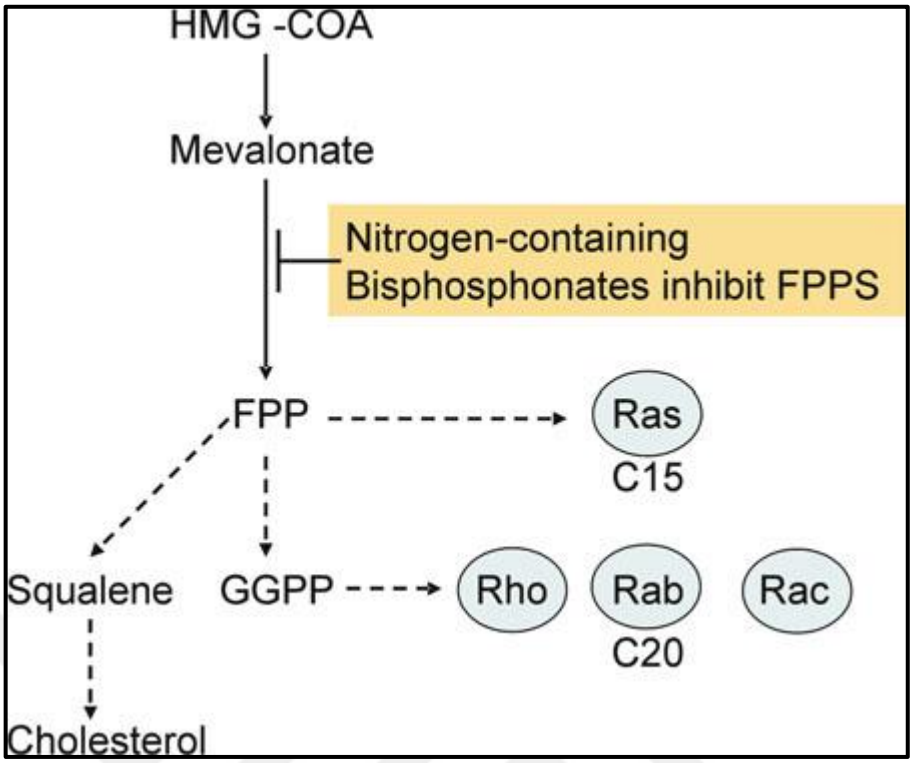

Figure 1.5. Mechanism of action of nitrogen-containing bisphosphonates [103]

1.5. BISPHOSPHONATES-INDUCED OSTEONECROSIS OF JAW (BIONJ)

BIONJ is highly related to intravenous administration of high dose BPs. As it was mentioned before, the main effect of the BPs is to inhibit the osteoclast activity; hence, BPs influence the bone turnover [101, 108, 109]. Normally in bone remodeling, osteoclastic activity is closely associated with bone deposition of osteoblast. Both mechanisms are essential for maintain of physiologic micro damage. When the bone turnover is excessive or in case of injuries including dental surgery and trauma, the bone integrity cannot be maintained due to local micro damages, resulting in jaw bone necrosis in patients receiving BPs [110].

The occurrence of jaw bone necrosis is not only associated with cumulative amount of BPs, it is also related to types and functions of the drugs. Besides, BPs also exert anti-angiogenic function. It has been shown that BPs induce avascular necrosis in the jaw by destroying local blood vessels. BPs, especially the ones with amino groups, exhibit side-

effects on the regional blood supplement. Other than *in vitro* studies, BPs have been shown to obstruct endothelial function *in vivo* as well [111, 112].

BPs, especially N-BPs, may cause serious tissue necrosis by inhibition of blood vessel formation and inhibits regional blood supply. This effect can be partially explained by the complex relationship of BPs with the insulin-like growth factor I and growth hormone which are related with bloodstream in bones. Moreover, expression of specific angiogenic marker, VEGF, was reported to be lower, and significant endothelial cell inhibition have been proven for BP treated groups in *in vivo* and *in vitro* conditions [113, 114].

The maxilla and mandible bones are more susceptible to infection with respect to other bones. The jaw is an open part in the oral cavity, which tends to be infected due to having large microbiologic diversity especially when there are dental problems. Besides, treatment processes of dental issues may also cause infection in the jaw bone. In these conditions, the usage BPs might also increase risk for fracture and tendency to BIONJ [110].

BPs bind to bones at physiological conditions; however, at acidic pH, they separate from hydroxyapatite structure. Thereby, a decrease at pH results in high levels of active BPs and a subsequent cytotoxic effect to various cell types. A recent investigation has shown the effects of the N-BPs, ibandronate and ZOL, and the non-N-BP, clodronate, on MSCs at various pH values (6.3, 6.7, 7.0, and 7.4) *in vitro*. N-BPs' high doses led to considerable reduction in survival and activity of the stem cells through dose- and pH value-dependent manner [115-117].

Renal failure, diabetes, age, immunosuppressant therapy, alcohol and cigarette consumption are other co-factors for osteonecrosis of the jaw [118]. In addition, the genetic factors affect susceptibility or resistance to the disease. A recent study have shown that patients with multiple myeloma treated with BPs intravenously have high BIONJ risk because of having single-nucleotide polymorphism in the cytochrome P450-2C gene [118]. To sum up, the various conditions effect the occurrence of BIONJ, but intravenous administration of BPs and dental operations are two main risks for BIONJ. Decrease in the MSC pool in the necrotic tissues was reported along with reduced osteogenic and adipogenic differentiation capacity compared to MSCs derived from healthy tissues [119]. However, the fate of the stem cells has not been investigated in case of BIONJ.

Up to present, the researchers have proposed several hypotheses for BIONJ including inhibition of local macrophage function, occurrence of micro-fracture on the jaw bone, abnormalities in vascular systems of the jaw, infectious inflammatory response, local necrosis due to anti-angiogenic activity of the BPs or side effects of adjuvant therapies such as chemotherapy. However, BIONJ have not been investigated in terms of dental derived stem cells proliferation, differentiation and maintenance so far. Effects of BPs on DPSC, PDLSC and hTGSC proliferation, survival, differentiation or maintenance capacities have not been evaluated yet. In this study, effects of BPs on osteogenesis and migration of DPSCs, PDLSCs and hTGSCs, and angiogenesis of endothelial cells were determined.

2. MATERIALS & METHODS

2.1. STEM CELL ISOLATION & CHARACTERIZATION

DSCs were isolated from 8 patients (10-18 years-old) following a routine dental treatment. Written informed consents of the patient were obtained after receiving approval from the Institutional Ethics Committee of Yeditepe University, Turkey. Pulp tissue was removed from center of the adult tooth, periodontal tissue was procured by scraping the adult tooth root with the scalpel and germ tissue was collected from third molars for DPSCs, PDLSCs and TGSCs isolation subsequently. Separated tissues were minced into small pieces by scalpel and incubated with Dulbecco's Modified Eagle's Medium (DMEM, #41966-029, Invitrogen, Gibco, UK) containing 10% Fetal Bovine Serum (FBS, #10500-064, Invitrogen, Gibco, UK) and 1% Penicillin/ Streptomycin/ Amphotericin (PSA, Invitrogen, Gibco, UK). Cells were incubated at 37 °C in a humidified air atmosphere with 5% CO2 and cells started to spread from the minced tissue after 2-3 weeks. Then, the cells were trypsinized with 0.25% trypsin/EDTA (#25200-056, Invitrogen, Gibco, UK) passaged and propagated.

Surface Marker Analysis

Isolated cells were used for surface marker analysis when they reached enough confluency at passage 3. Briefly, the cells were fixed 4% paraformaldehyde (PFX) (w/v), washed with Phosphate Buffer Saline (PBS) and treated with specific surface markers of MSCs such as CD29, CD44, CD73, CD90, CD105 (Abcam, Cambridge, UK) and HSCs, including CD14, CD31, CD34 and CD45 (Abcam, Cambridge, UK).

Cells were incubated with these conjugated antibodies for overnight at 4 °C and washed two times with PBS. Cells were suspended in PBS and analyzed with BD FACSCalibur™ (BD Biosciences, Singapore) flow cytometry device.

Staining Assays

DPSCs, PDLSCs and hTGSCs were seeded on six well plates (#3516, Corning Plasticware, Corning, NY) at a cell concentration of 10^5 cells/well. Osteogenic, chondrogenic and adipogenic induction medium (Table 2.1) were applied to the cells for 10 days and media were changed every other day. At the end of the differentiation period, the cells were fixed with using 4% Paraformaldehyde PFX (w/v) for 30 min. Osteo-, chondro- and adipo-genic differentiation processes were checked by von Kossa, Alcian Blue and oil red O staining, respectively.

von Kossa was used to identify the minerals deposition of stem cells. Fixed cells were stained with the 3% Silver Nitrate Solution and the wells were exposed with UV light for 30 min and washed three times with dH_2O. 5% Sodium Thiosulfate was applied on the wells for 2 min. and washed three times with dH_2O. Finally Nuclear Fast Red was added onto the wells and visualized by Zeiss PrimoVert light microscope with an AxioCam ERc5s camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). The fixed cells were exposed to Alcian Blue for 30 min. Then, the wells were washed three times with dH_2O and visualized by light microscope. Adipogenic differentiation was confirmed by oil red O staining which stains lipid droplets. Briefly, fixed cells were incubated with oil red stain for 15 min and washed three times with dH_2O. Stained cells were visualized by light microscope.

Immunocytochemistry (ICC) Assay

Immunostaining experiments for Osteocalcin (#sc-30044, Santa Cruz, USA), Collagen type II (Col II) (#sc-59772, Santa Cruz, USA) and Fatty acid binding protein 4 (Fabp4) (#2120, Cell Signaling, USA) were performed to confirm osteo-, condro- and adipo-genic differentiation, respectively. The fixed cells were incubated with Osteocalcin, Col II and Fabp4 primary antibodies overnight at 4°C. Cells were washed with PBS three times for 10 min and were incubated with AlexaFluor-488 goat anti-rabbit immunoglobulin G (Invitrogen, Carlsbad, USA) secondary antibody for 1 h at 4 °C. 4′,6-di-amidino-2-phenyl-

indole (DAPI) (Applichem, Darmstadt, Germany) was used to stain nuclei. The results were visualized using a confocal microscope (LSM 700, Zeiss, Heidelberg, Germany).

Table 2.1. Contents of Differentiation Medium

Osteogenic Medium	100 nM Dexamethasone, 10 mM β-Glycerophosphate, 0.2 mM Ascorbic acid
Chondrogenic Medium	1× Insulin-Transferrin-Selenium (ITS −G), 100 nM Dexamethasone, 100 ng/ml TGF-β 14 µg/ml Ascorbic acid 1 mg/ml BSA
Adipogenic Medium	100 nM Dexamethasone, 5 µg/ml Insulin 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) 60 µM Indomethacin

2.2. PREPARATION OF BPs

In this study, three kinds different of second generation bisphosphonates were used in the current study. Zoledronic acid monohydrate (ZOL) was purchased from Sigma-Aldrich (#SML0223, St. Louis, MO, USA). Main stock solution was prepared in the dH_2O at a concentration of 6 mM. Alendronate Sodium Salt (ALE) was purchased from Calbiochem (#121268-17-5, Germany). Main stock solution was prepared in the dH_2O at a concentration of 30 mM. Risedronate sodium (RIS) was purchased from Sigma-Aldrich (#SML0650, St. Louis, MO, USA). Main stock was prepared in the dH2O at a concentration of 16 mM. The main stock solutions were filtered through a 0.22-µm filter (Sartorius AG, Göttingen, Germany) and kept at -20°C. For further studies, BPs were diluted to lower doses in DMEM for cell culture studies.

2.3. CELL VIABILITY ASSAY

Cell viability analysis were completed in order to check the cytotoxic effects of BPs (ZOL, ALE and RIS) on DPSCs, PDLSCs and hTGSCs. Cell viability was measured by the 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS)-assay (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) according to the manufacturer's instructions. Cell viability analysis were performed for stem cells under regular culture conditions and differentiation culture conditions and for HUVECs (Human Umbilical Vein Endothelial Cells) for further angiogenesis analysis.

First, DPSCs, PDLSCs and hTGSCs were seeded onto 96-well plate (#CLS6509, Corning Plasticware, Corning, NY) at a concentration 5000 cells/well. Next day, 5 μM, 1 μM, 0.5 μM, 0.1 μM, 0.05 μM, 0.01 μM, 0.005 μM concentrations of ZOL, ALE and RIS were applied to the cells with osteogenic differentiation medium during 21 day and medium was changed every other day. At the end of the differentiation period, 110 μl MTS solution mix (10 μL MTS reagent + 100 μL DMEM) was added to each well and incubated for 2 h at 37 °C. Absorbance nm was measured at 495 with ELISA plate reader (Biotek, Winooski, VT). 0.01 ZOL, 0.1 μM ALE and 0.05 μM RIS for DPSC, 0.05 ZOL, 0.1 μM ALE and 0.1 μM RIS for PDLSC, 0.005 ZOL, 1 μM ALE and 0.5 μM RIS for hTGSC were determined as sub-lethal doses for osteogenic differentiation studies.

Secondly, DPSCs, PDLSCs and hTGSCs were seeded onto 96-well plate at a concentration 5000 cells/well. Next day, 100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM and 0.1 μM concentration of ZOL, ALE and RIS were applied to the cells in complete growth medium (DMEM low glucose + 10% FBS + 1% PSA). After 24h, MTS assay was performed as described above. 100 μM were determined as sub-lethal doses for migration studies. Thirdly, HUVECs were used for cell viability analysis HUVECs were obtained from ATCC (CRL-1730) and were cultured in DMEM including 10% FBS and 1% PSA. HUVECs were seeded onto 96-well plates at a concentration of 5000 cells/well. Next day, 100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM and 0.1 μM concentration of ZOL, ALN and RIS were administered to the cells in complete growth medium (DMEM high glucose

+ 10% FBS + 1% PSA). After 24h, MTS assay was performed as described above. 100 μM were determined as sub-lethal doses for angiogenesis analysis.

2.4. OSTEOGENIC DIFFERENTIATION

In order to determine the effects of BPs on osteogenic differentiation potential of DPSCs, PDLSCs and hTGSCs, the cells were seeded onto 6-well plate at a concentration of 150.000 cells/well. Next day, cells were treated with osteogenic differentiation medium containing 10% FBS, 1% PSA, 100 nM Dexamethasone (DXM), 10 mM β-Glycerophosphate and 0.2 mM Ascorbic acid. In addition to control group BPs added differentiation media were applied to the cells. For treated group, the differentiation medium was mixed with previously identified highest non-toxic doses of BPs by cell viability assay at day 21. Differentiation media were changed every other day during 21 days and cells were incubated in a humidified incubator at 37 °C and 5 % CO2 conditions during differentiation. Differentiated cells were characterized by von Kossa and Alizarine Red staining and q-PCR analysis.

2.5. VON KOSSA STAINING

The cells were fixed with 4% PFX (w/v) for 30 min after osteogenic differentiation. To show relative calcium levels, von Kossa Method staining for Calcium Kit (#24633) was used according to the manufacturer's instructions. Mineral depositions were identified using Zeiss PrimoVert light microscope with an AxioCam ERc5s camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) and quantitatively calculated by using Image J software.

2.6. ALIZARINE RED STAINING

The cells were fixed with absolute ethanol for 30 min after osteogenic differentiation. To show relative calcium levels, 2% Alizarine Red Stain (#CM-0058) was applied for 15 min at RT. The wells were washed with PBS three times. Mineral depositions were identified using Zeiss PrimoVert light microscope with an AxioCam ERc5s camera (Carl Zeiss

Microscopy, LLC, Thornwood, NY, USA) and quantitatively calculated by using Image J software.

2.7. REAL TIME PCR (qPCR) ANALYSIS

qPCR was used to determine mRNA expression levels of the target genes quantitatively. In the current study, qPCR analysis were conducted to identify expression levels of osteogenic differentiation and migration related genes. *bone morphogenetic protein 2* (BMP2), *msh homeobox 1* (MSX1), *osteocalcin* (OCN), *osteonectin* (ONN) and *runt-related transcription factor 2* (RUNX2) were selected for osteogenic differentiation analysis. C*ollagen type 1 alpha 1* (COL1A1), FIBRONECTIN and LAMININ were selected for migration analysis. *Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) was selected as a housekeeping gene for all experiments (Table 2.2). Primers were synthesized by Macrogen (Seoul, Korea).

Table 2.2. Primers List Using For RT-PCR Assay

Gene	Species	Sequence	Ref
BMP2	Human	F 5’ TCATAAAACCTGCAACAGCCAACTCG 3’ R 5’ GCTGTACTAGCGACACCCAC 3’	[120]
MSX1	Human	F 5’ AAGTTCCGCCAGAAGCAGTA 3’ R 5’ ACATCTGTGTTTTCCCTGCC 3’	[121]
OSTEO-CALCIN	Human	F 5’ GTGCAGAGTCCAGCAAAGGT 3’ R 5’ CAGCCAACTCGTCACAGTC 3’	[122]
OSTEO-NECTIN	Human	F 5’ ATGAGGGCCTGGATCTTCTT 3’ R 5’ CTGCTTCTCAGTCAGAAGGT 3’	[29]
RUNX2	Human	F 5’ TCCACACCATTAGGGACCATC3’ R 5’ TGCTAATGCTTCGTGTTTCCA3’	[123]
COL1A1	Human	F 5’ CCACGCATGAGCGGACGCTAA 3’ R 5’ ATTGGTGGGATGTCTTCGTCTTGG 3’	[124]

FIBRO-NECTIN	Human	F 5’ AGCCTGGGAGCTCTATTCCA 3’ R 5’ CTTGGTCGTACACCCAGCTT 3’	[125]
LAMININ	Human	F 5’ CACATGTCCGTCACAGTGGA 3’ R 5’ TAGAGGCTGACCACCTCCTC 3’	[125]
GAPDH	Human	F 5’ TGGTATCGTGGAAGGACTCA 3’ R 5’ GCAGGGATGATGTTCTGGA 3’	[126]

Total RNAs were isolated using High Pure RNA Isolation Kit (#11 828 665 001, Roche, USA) according to the manufacturer’s instructions. cDNA synthesis was conducted using High Fidelity cDNA synthesis kit (#05081955001, Roche, USA) according to the manufacturer’s instructions. SYBR Green master mix was used for qPCR experiments. Experiments were carried out in a 10 µl mixture of synthesized cDNAs, SYBR Green master mix, primers and PCR grade distilled water (#SH30538.02, Hyclone, Utah, USA). Contents of qPCR mix and reaction conditions are given in (Table 2. 3 and Table 2. 4). qPCR experiments were performed by CFX96 RT-PCR system (Bio-Rad, Hercules, CA). All data were normalized to GAPDH.

Table 2.3. Contents of RT-PCR mix

Reagents	**Amount**
Applied Biosystems™ SYBR™ Green Master Mix	5 µl
Primer Forward (10 pmol)	0.5 µl
Primer Reverse (10 pmol)	0.5 µl
PCR Grade Distilled Water	1.5 µl
cDNA (100 ng/ml)	2.5 µl

Table 2.4. Reaction conditions of RT-PCR analysis

Cycle	Repeats	Step	Dwell time	Set point
Initial Denaturation	1	1	3 min	95 °C
Denaturation	39	1	30 sec	95 °C
Annealing		2	40 sec	60 °C
Elongation		3	45 sec	72 °C
Final extension	1	1	10 min	72 °C
Melt curve	110	1	12 sec	-0.5 °C/cycle

2.8. SCRATCH ASSAY

Scratch assay (wound healing assay) was performed to determine the effects of BPs on migratory characteristics of DPSCs, PDLSCs and hTGSCs. The cells were seeded onto 6-well plate at a concentration of 150.000 cells/well for four different experimental groups including ZOL, ALN, RIS treatment and non-treated Control group (growth medium), Cells were scratched with a sterile 1000 µl pipette tip in each well when they reached 100% confluency. Then, the wells were washed with 1xPBS to remove unattached cells. 100 µM of each BPs determined by cell viability assay were applied to the cells. Pictures of wound areas were taken by using Zeiss PrimoVert light microscope with an AxioCam ERc5s camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) at 0h and 24h. Wound closures were measured and calculations were done by seven randomly selected areas with Zen 2 (blue edition) software. Besides, the cells were collected for total RNA

and protein isolation at the end of scratch assay. COL1A1, FIBRONECTIN and LAMININ genes were selected for migration analysis and GAPDH was used as a housekeeping gene.

2.9. WESTERN BLOT ANALYSIS

Protein isolation and western blot analysis were carried out to determine expression levels of migration related proteins. Buffers were prepared as shown in Table 2.5 and reagents were purchased from Biorad Laboratories (Richmond, CA). Briefly, total protein was isolated from cells using RIPA Buffer (#sc-24948, Santa Cruz, USA) and protein concentrations were estimated using BCA assay (#23227, Pierce, Rockford, USA). Primer antibodies against Col1a1 (#ab34710), Fibronectin (#ab2314) and Laminin (#ab11575) were used for protein expression analysis (Abcam, Cambridge, MA, USA). GAPDH was used to normalize protein expression (#8884, Cell Signaling, Beverly, MA, USA).

20 µl mix of protein and 4X Leammli Buffer (#1610742, Biorad, USA) was prepared for each sample by incubation at 95 ºC for 5 min. 20 µg protein samples were loaded onto each well of Any kD™ Mini-PROTEAN® TGX™ precast gels (#456-9033, Biorad, USA) and electrophoresed by applying 90V for 10 min and 120V for 60 min. proteins were transferred to nitrocellulose membranes (122-0115, Biorad, Germany) with the 0.45 µm pore size by applying 175 mA for 90 min. Then, membranes were incubated in a blocking solution including 5% non-fat dry milk powder (36-6404, Biorad, USA) prepared in tris-buffered saline and Tween-20 solution (TBS-T) for 1h at room temperature. The membranes were incubated with primary antibodies in blocking solution at 4 °C overnight. The membranes were washed with 1xTBS-T three times for 10 min and treated with anti-mouse (#7076) or anti-rabbit (#7074) IgG secondary antibodies prepared in TBS-T at room temperature for 1h. Then, membranes were washed with 1xTBS-T three times for 10 min and visualized after Clarity™ ECL Western Blotting Substrate (# 1705060, BioRad, USA) administration. Results were photographed with ChemiDoc MP imaging system (BioRad, USA). Band intensities were calculated with Image Lab software program and normalized to GAPDH.

Table 2.5. Western Blotting Solutions

10X Running Buffer	25 mM Tris base 190 mM Glycine 0.1% SDS, pH 8.3
10X Transfer Buffer	25 mM Tris base 190 mM Glycine 20% Methanol
TBS-T	20 mM Tris-HCl 150 mM NaCl 0.1% Tween 20, pH 7.6
Blocking Buffer	5% Non-fat dry milk prepared in TBS-T

2.10. HUMAN CYTOKINE ARRAY

Human cytokine array was carried out to detect migration related cytokine levels in ZOL, ALE and RIS treated DPSCs, PDLSCs and hTGSCs protein samples. Briefly, cells were seeded onto 6-well plate at a concentration of 150.000 cells/well for four different experimental groups including 100 μM ZOL, ALE, RIS treatment and non-treated Control group (growth medium). The effects of BPs on the cytokine array profile of DPSCs, PDLSCs and hTGSCs were evaluated by human cytokine array C5 (#AAH-CYT-5-8, RayBiotech) according to the manufacturer's instructions. Briefly, membranes were incubated with blocking solution for 30 min. Samples (100 μg protein) are added on the membranes for 3h at RT. Membranes were washed three times with wash buffer I and three times with wash buffer II. Biotinylated AB cocktail was added on the membranes for overnight at +4 ºC. Then, membranes were washed three times with wash buffer I and three times with wash buffer II. HRP-Streptavidin was applied on the membranes for 2h at RT. Membranes were washed three times with wash buffer I and three times with wash

buffer II and visualized after chemiluminescence detection buffer administration. Results were photographed with ChemiDoc MP imaging system. Blots intensities were calculated with Image J software program and analyzed with graphpad software program.

2.11. AORTIC RING ASSAY

Aortic ring assay was done to evaluate effects of ZOL, ALE and RIS on angiogenic activity of endothelial cells. Thoracic aortas from Sprague Dawley rats were removed. Pre-chilled 48-well tissue culture plates were coated with 75 µl of Matrigel Matrix (#354234, BD Biosciences, Bedford, MA). Thoracic aortas were carefully separated from fibroadipose tissues and cut into 1-mm-long cross-sections. Aorting rings were placed on Matrigel-coated wells, and covered with an additional 75 µl of Matrigel.

100 µM concentrations of BPs, negative control (NC: growth medium) and positive control (PC: growth medium + 50 ng/ml VEGF) were arranged as experimental groups. BPs were applied for 24h and then media were exchanged with complete growth medium. Angiogenic sprouting was monitored by using Zeiss PrimoVert light microscope with an AxioCam ERc5s camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) after 5-days.

2.12. TUBE FORMATIN ASSAY

Tube formation assay was done to determine effects of ZOL, ALE and RIS on tube forming capacities of HUVECs. Briefly, pre-chilled 24 well plates were coated with 150 µl matrigel and incubated for 30 min at 37℃ to provide polymerization. HUVECs were seeded onto matrigel-coated wells at 10^5 cells/well. For treated groups 100µM ZOL, ALE and RIS were added into the wells.

The tube-like structures appeared after 7h and pictures were taken by using Zeiss PrimoVert light microscope with an AxioCam ERc5s camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). The tube-like structure numbers were calculated in randomly selected five areas by using Image J software.

2.13. STATISTICAL ANALYSIS

One-way analysis of variance and Tukey post hoc tests were used for the statistical analysis. The values of *P<0.05 were accepted as significant.

3. RESULTS

3.1. CHARACTERIZATION

The isolated stem cells exerted fibroblast cell like morphology (Figure 3.1a, Figure 3.2a and Figure 3.3a). The surface marker expression profile showed that DPSCs, PDLSCs and hTGSCs expressed CD29, CD44, CD73, CD90, CD105 which are specific surface markers of MSCs and did not express CD14, CD31, CD34 and CD45 which are specific surface markers of HSCs (Figure 3.1b, Figure 3.2b and Figure 3.3b). Isolated DPSCs, PDLSCs and hTGSCs were successfully differentiated into osteo-, chondro- and adipo-genic lineages. Calcium deposition and osteocalcin expression were demonstrated with von Kossa staining and osteocalcin immunostaining, respectively, after osteogenic transformation of cells. Alcian Blue staining and Col II immunostaining were used to prove chondrogenic transformation of cells lipids droplets were stained with oil red O after adipogenic differentiation. Fabp 4 immunostaining was conducted to confirm adipogenesis (Figure 3.1c, Figure 3.2c and Figure 3.3c). Results demonstrated that DPSCs, PDLSCs and hTGSCs have MSC properties including specific surface marker expression and differentiation ability to osteogenic, chondrogenic and adipogenic lineages.

3.2. CELL VIABILITY ASSAY

Cell viability analyses were performed to decide the maximum tolerated non-toxic concentration of BPs for DPSCs, PDLSCs and hTGSCs. Maximum tolerated non-toxic concentration of BPs that resulted in 90% or higher cell viability, were selected. 5 μM, 1 μM, 0.5 μM, 0.1 μM and 0.05 μM ZOL treatments significantly decreased cell viability compared to control group for DPSCs in osteogenic differentiation culture as approximately 15%, 15%, 20%, %60 and %80, respectively. 0.01 μM ZOL was determined as the highest non-toxic concentration for DPSCs. 5 μM (17%), 1 μM (20%) and 0.5 μM ALE (74%) treatments significantly reduced cell viability compared to control group for DPSCs in osteogenic differentiation culture. 0.1 μM ALE was determined as highest non-toxic concentration for DPSCs. 5 μM (15%) , 1 μM (16%) and 0.5 μM RIS (

16%) and 0.1 μM RIS (86.6%) treatments significantly decreased cell viability compared to control group for DPSCs in osteogenic differentiation culture. 0.05 μM RIS was detected as higher non-toxic dose (Figure 3.4).

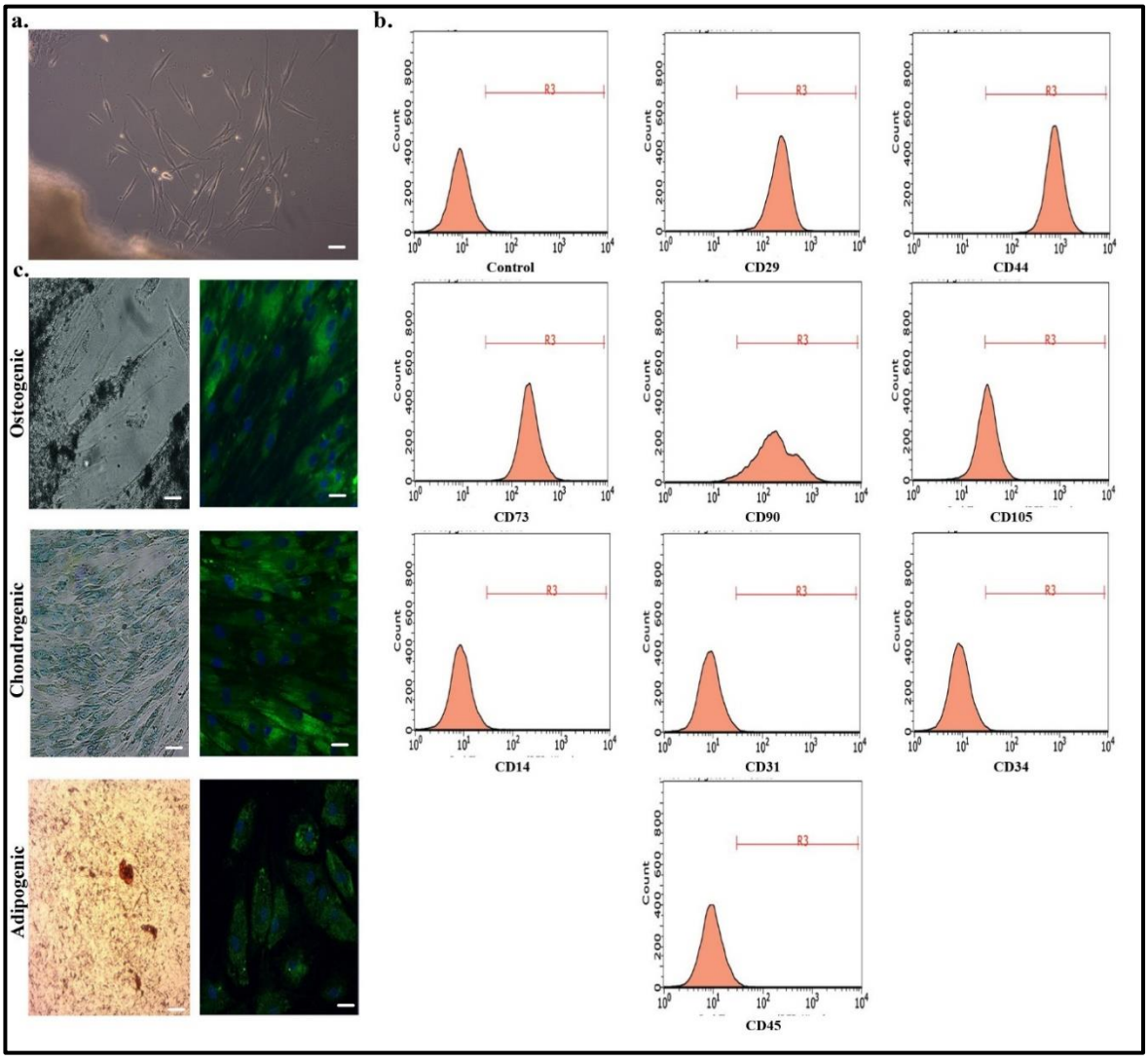

Figure 3.1. Characterization of Dental Pulp Stem Cells (DPSCs). a. Fibroblast-like cell morphology of DPSCs, (*scale bar* 200 μm). b. DPSCs expressed CD29, CD44, CD73, CD90, CD105 which are specific surface markers of MSCs and did not express CD14, CD31, CD34 and CD45 which are specific surface markers of HSCs. c. von Kossa, Alcian Blue and oil red O staining indicating successful differentiation of DPSCs into osteo-, chondro- and adipo- genic cell lineages. Immunostaining of Osteocalcin, Col II and Fabp4 confirming osteo-, chondro- and adipo- genic differentiation (*scale bar* 100 μm).

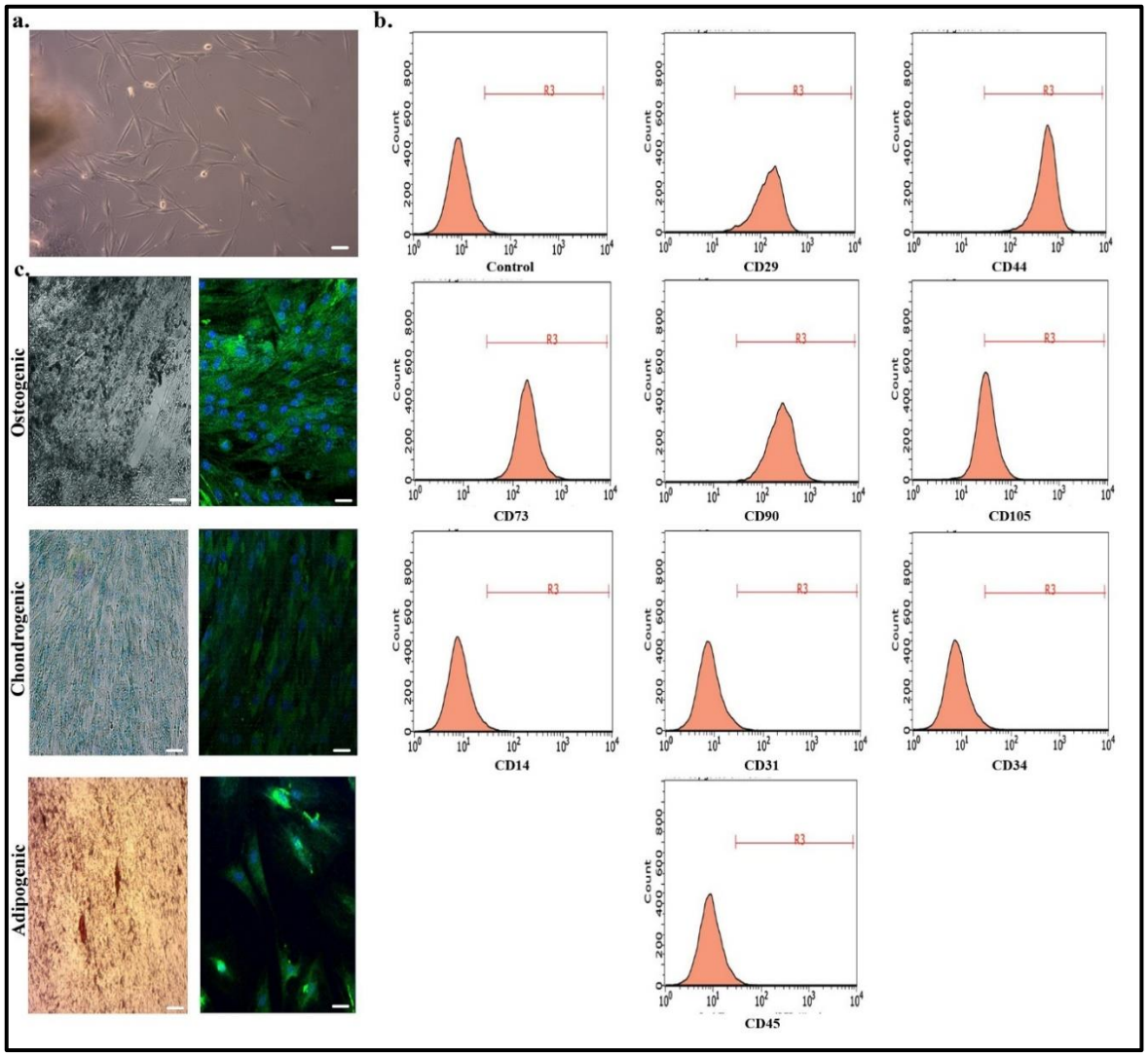

Figure 3.2. Characterization of Periodontal Ligament Stem Cells (PDLSCs). a. Fibroblast-like cell morphology of PDLSCs, (*scale bar* 200 μm). b. PDLSCs expressed CD29, CD44, CD73, CD90, CD105 which are specific surface markers of MSCs and did not express CD14, CD31, CD34 and CD45 which are specific surface markers of HSCs. c. von Kossa, Alcian Blue and oil red O staining indicating successful differentiation of PDLSCs into osteo-, chondro- and adipo- genic cell lineages. Immunostaining of Osteocalcin, Col II and Fabp4 confirming osteo-, chondro- and adipo- genic differentiation (*scale bar* 100 μm).

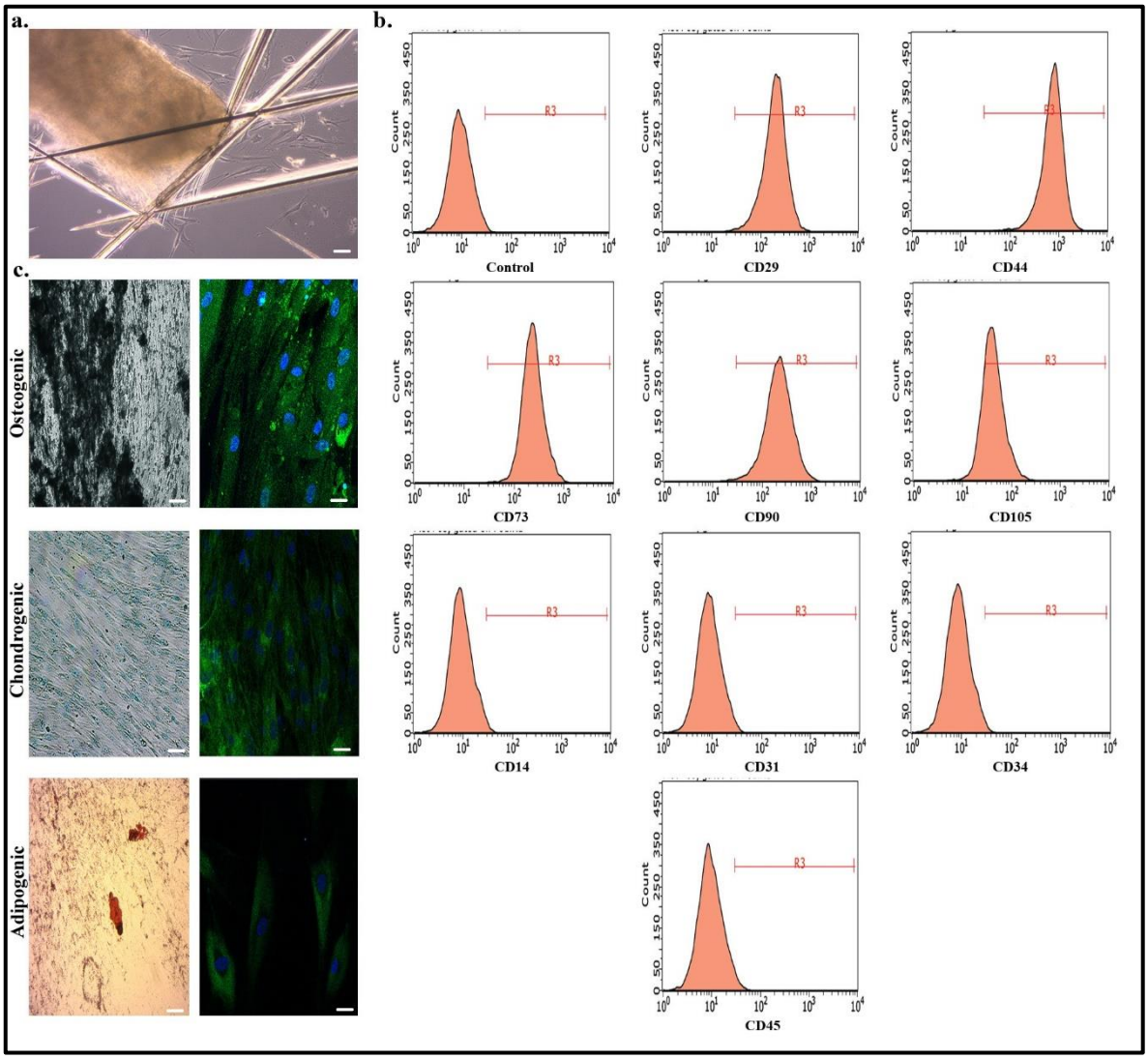

Figure 3.3. Characterization of human Tooth Germ Stem Cells (hTGSCs). a. Fibroblast-like cell morphology of hTGSCs, (scale bar 200 μm). b. hTGSCs expressed CD29, CD44, CD73, CD90, CD105 which are specific surface markers of MSCs and did not express CD14, CD31, CD34 and CD45 which are specific surface markers of HSCs. c. von Kossa, Alcian Blue and oil red O staining indicating successful differentiation of hTGSCs into osteo-, chondro- and adipo- genic cell lineages. Immunostaining of Osteocalcin, Col II and Fabp4 confirming osteo-, chondro- and adipo- genic differentiation (scale bar 100 μm).

5 μM, 1 μM and 0.5 μM and 0.1 μM ZOL treatments significantly reduced cell viability 15%, 15%, 17% and 41%, respectively, compared to control group for PDLSCs in osteogenic differentiation culture 0.05 μM ZOL was determined as highest non-toxic concentration for PDLSCs. In addition, 5 μM, 1 μM and 0.5 μM ALE and RIS treatments significantly decreased cell viability compared to control group. 0.1 μM ALE and 0.1 μM RIS were determined as highest non-toxic concentration for PDLSCs. (Figure 3.5). 5 μM, 1 μM, 0.5 μM, 0.1 μM, 0.05 μM and 0.01 μM ZOL treatments significantly decreased cell viability compared to control group for hTGSCs in osteogenic differentiation culture as 21%, 22%, 22%, 23%, 34% and 66%, respectively. 0.005 μM ZOL was determined as highest non-toxic concentration for hTGSCs. 5 μM ALE treatment caused significant reduction in cell viability as 18% when compared to control group. 1 μM ALE was determined as highest non-toxic concentration for hTGSCs. 5 μM (20%) and 1 μM (80%) RIS treatments significantly decreased cell viability compared to control group. 0.5 μM RIS was detected as highest non-toxic dose hTGSCs (Figure 3.6).

In addition to osteogenic culture system, various concentrations of BPs including 100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM and 0.1 μM were applied in complete growth medium to DPSCs, PDLSCs and hTGSCs. All three kinds of BPs did not exert significant differences in cell viability compared to control group (growth medium application). 100 μM ZOL, 100 μM ALE and 100 μM RIS were determined as highest non-toxic concentrations, for DPSCs (Figure 3.7), PDLSCs (Figure 3.8) and hTGSCs (Figure 3.9). Selected doses were used for the migration experiments.100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM and 0.1 μM BPs were applied in complete growth medium to HUVECs. Selected doses of BPs did not cause significant difference in cell viability. 100 μM ZOL, 100 μM ALE and 100 μM RIS were determined as highest non-toxic concentrations for HUVEC cells (Figure 3.10).

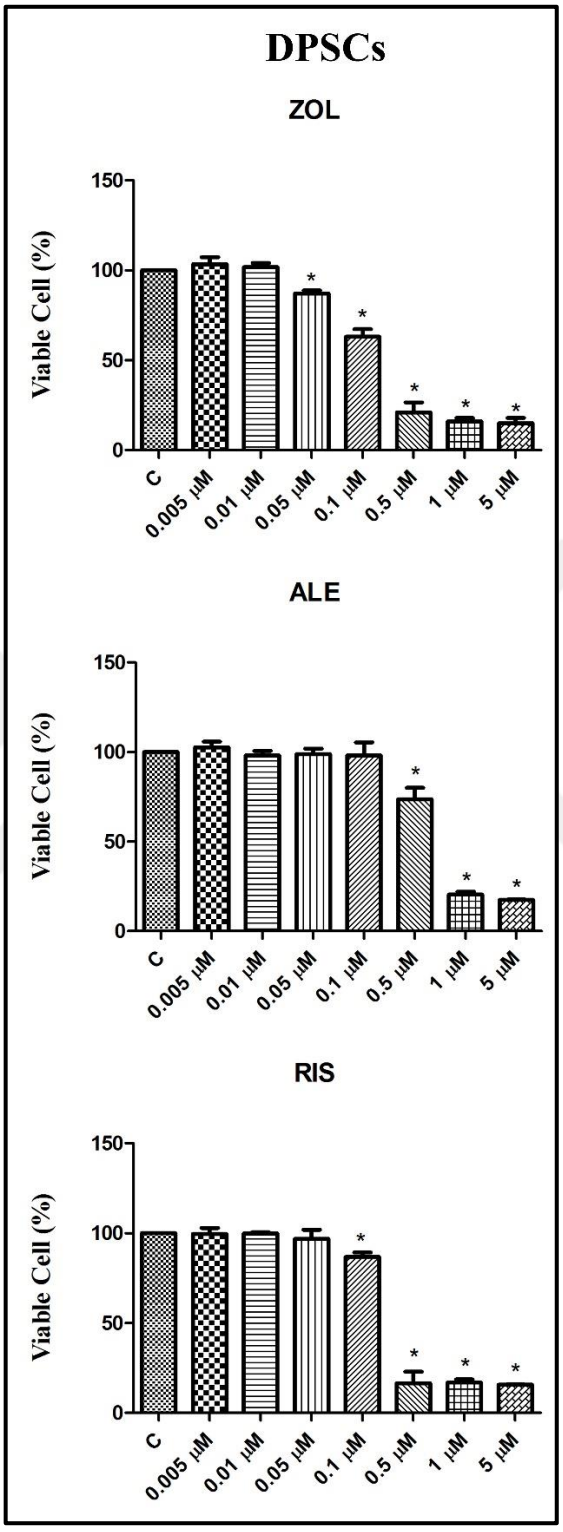

Figure 3.4. The effects of various concentrations of ZOL (Zoledronate), ALE (Alendronate) and RIS (Risedronate) on cell viability of DPSCs at day 21 in the osteogenic differentiation culture. Abbreviations: C: Control group (stand-alone osteogenic differentiation medium treatment), *P<0.05.

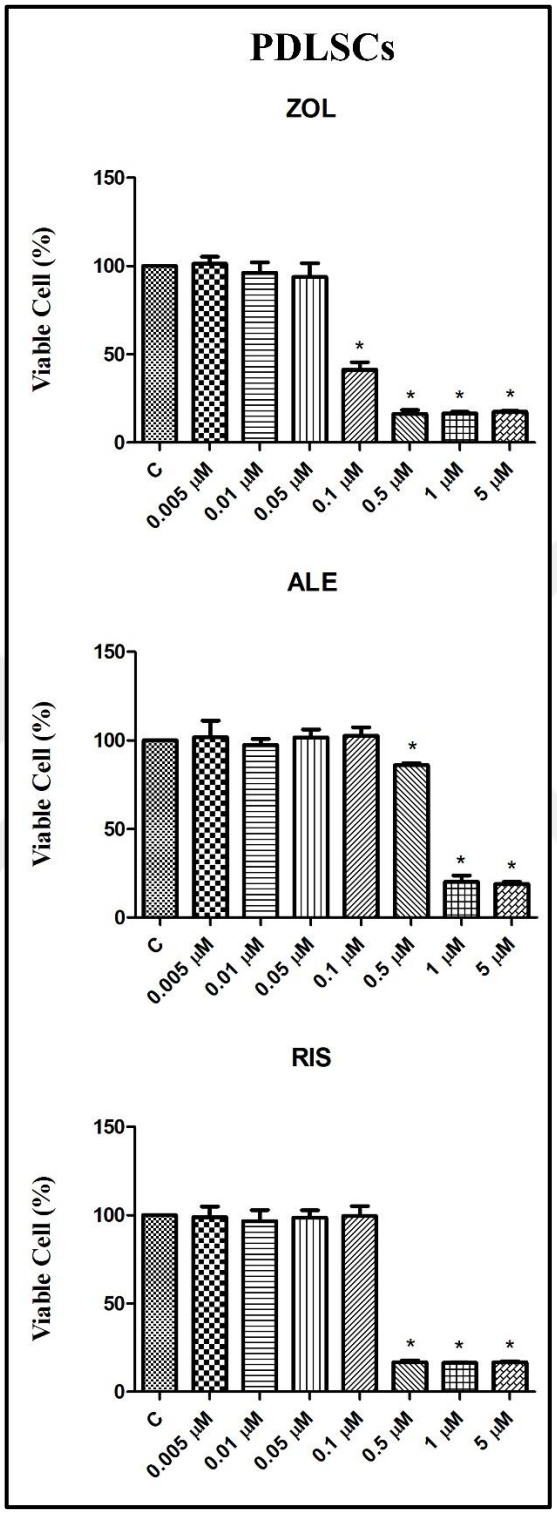

Figure 3.5. The effects of various concentrations of ZOL (Zoledronate), ALE (Alendronate) and RIS (Risedronate) on cell viability of PDLSCs at day 21 in the osteogenic differentiation culture. Abbreviations: C: Control group (stand-alone osteogenic differentiation medium treatment), *P<0.05.

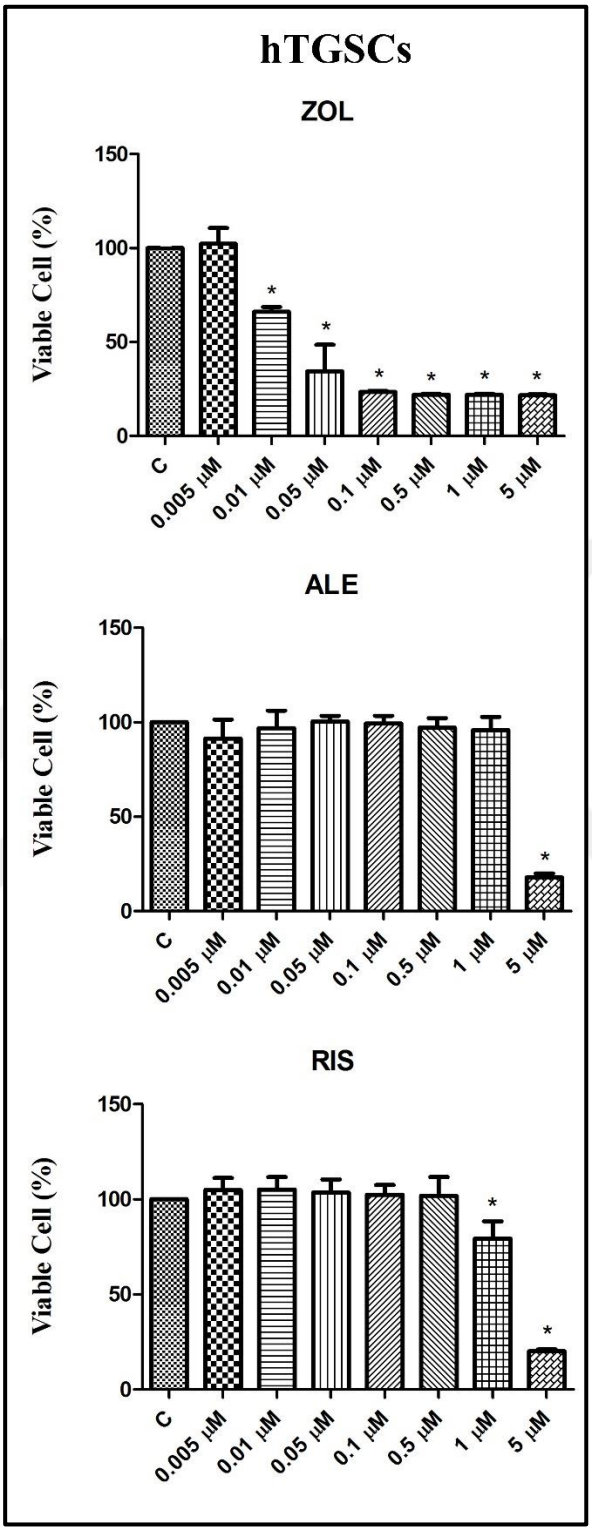

Figure 3.6. The effects of various concentrations of ZOL (Zoledronate), ALE (Alendronate) and RIS (Risedronate) on cell viability of hTGSCs at day 21 in the osteogenic differentiation culture. Abbreviations: C: Control group (stand-alone osteogenic differentiation medium treatment), *P<0.05.

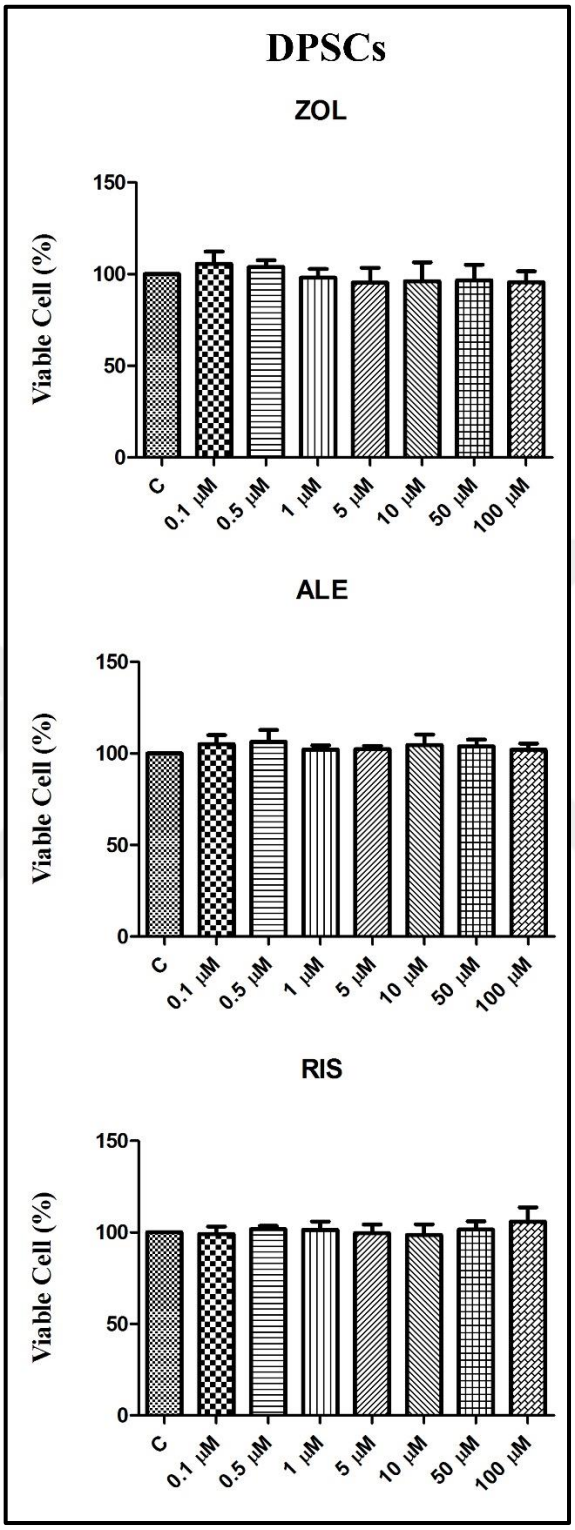

Figure 3.7. The effects of various concentrations of ZOL (Zoledronate), ALE (Alendronate) and RIS (Risedronate) on cell viability of DPSCs at day 1 in the growth medium. Abbreviations: C: Control group (stand-alone treated with only growth medium medium treatment), *P<0.05.

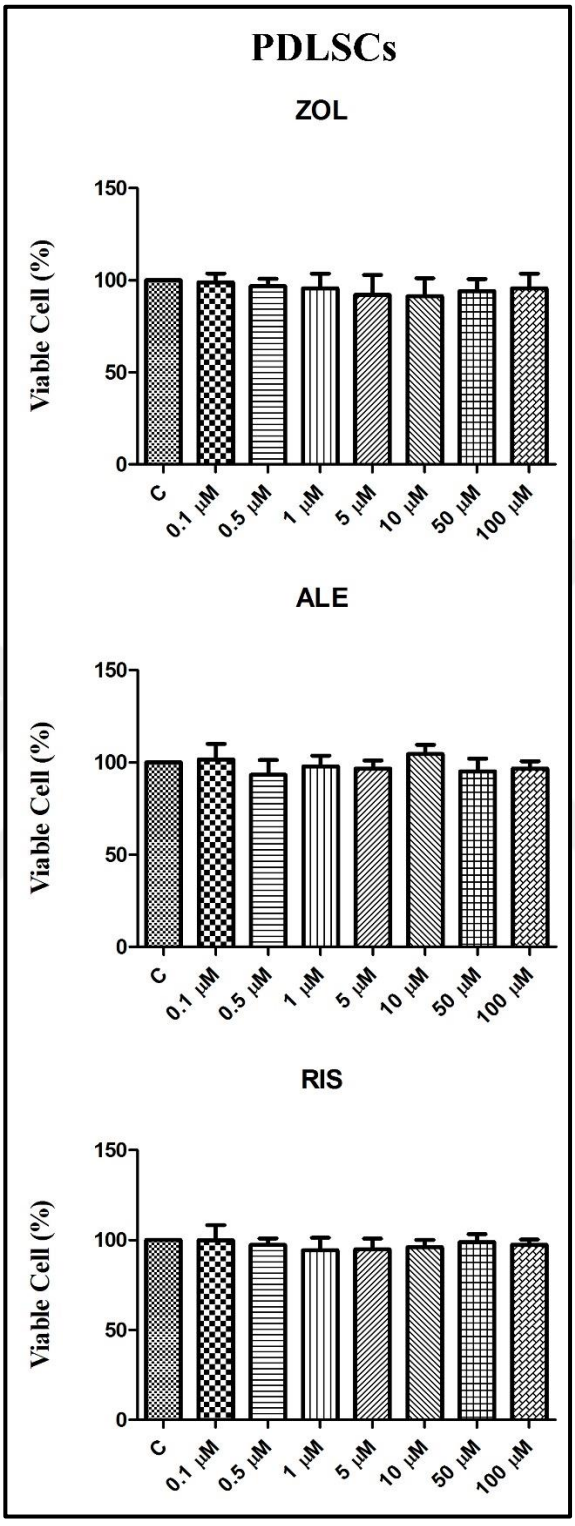

Figure 3.8. The effects of various concentrations of ZOL (Zoledronate), ALE (Alendronate) and RIS (Risedronate) on cell viability of PDLSCs at day 1 in the growth medium. Abbreviations: C: Control group (stand-alone growth medium treatment), *P<0.05.

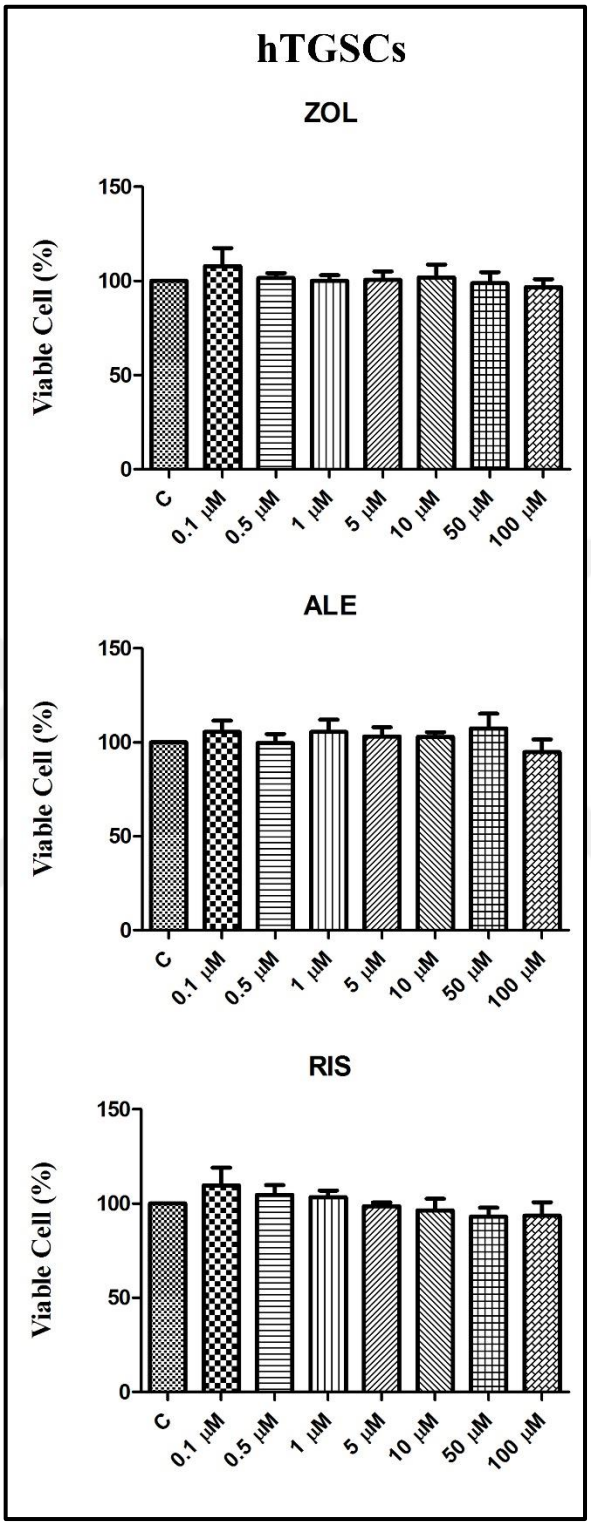

Figure 3.9. The effects of various concentrations of ZOL (Zoledronate), ALE (Alendronate) and RIS (Risedronate) on cell viability of hTGSCs at day 1 in the growth medium. Abbreviations: C: Control group (stand-alone growth medium treatment), *P<0.05.

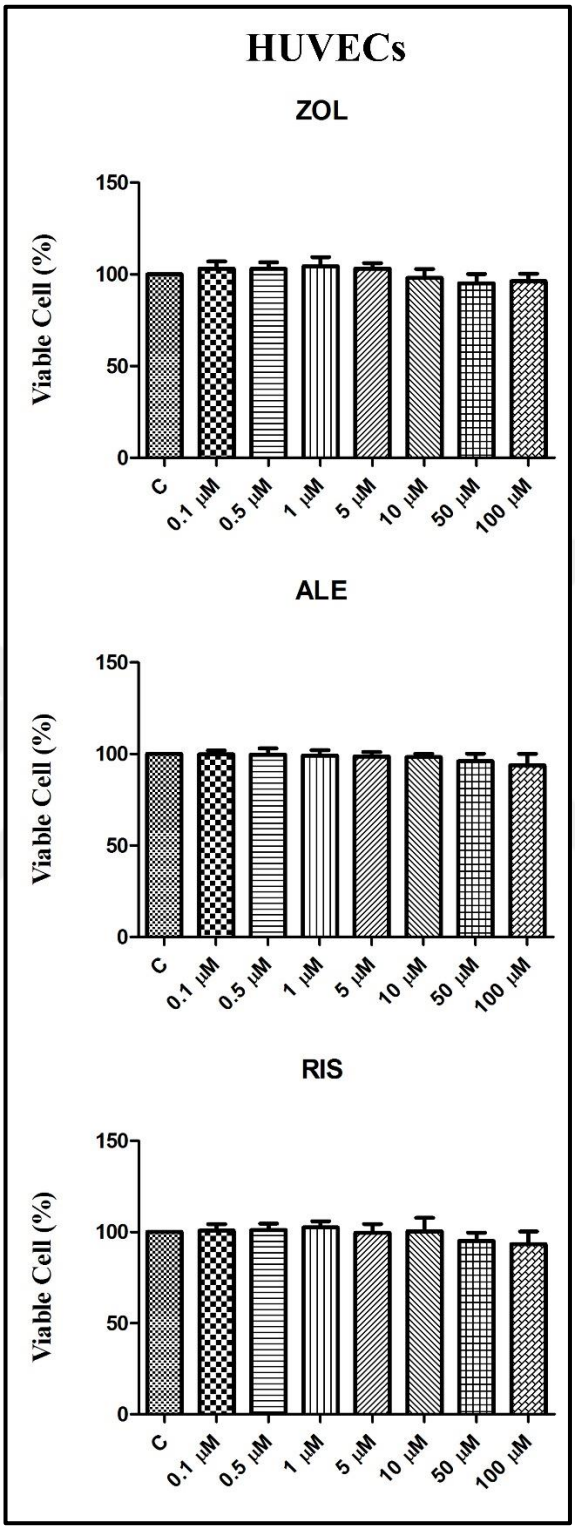

Figure 3.10. The effects of various concentrations of ZOL (Zoledronate), ALE (Alendronate) and RIS (Risedronate) on cell viability of HUVECs at day 1 in the growth medium. Abbreviations: C: Control group (stand-alone growth medium treatment), *P<0.05.

3.3. OSTEOGENIC DIFFERENTIATION

DPSCs, PDLSCs and hTGSCs were differentiated into osteogenic cell types in the presence of BPs and analyzed by von Kossa staining, Alizarin Red staining and q-PCR analysis.

Relative mineral levels determined by von Kossa staining, were significantly increased by 0.01 μM ZOL and 0.1 μM ALE treatment in DPSCs (Figure 3.11).

0.05 μM RIS treated group did not cause significant effect on relative mineral levels compared to control group (Figure 3.11).

Relative mineral levels were not significantly different for 0.05 μM ZOL, 0.1 μM ALE and 0.1 μM RIS treated groups compared to control group in PDLSCs (Figure 3.12).

1 μM ALE treated group significantly increased relative mineral levels compared to control group (Figure 3.13).

0.005 μM ZOL, and 0.5 μM RIS treatment did not cause any effect on relative mineral levels compared to baseline in hTGSCs (Figure 3.13).

Alizarin Red staining results showed that BPs (0.01μM ZOL, 0.1 μM ALE, 0.05 μM RIS for DPSCs and 0.05 μM ZOL, 0.1 μM ALE, 0.1 μM RIS for PDLSCs) treated groups did not cause significant effect on relative calcium deposition levels compared to control groups for DPSCs (Figure 3.14).

Alizarin Red staining results showed that BPs (0.01μM ZOL, 0.1 μM ALE, 0.05 μM RIS for DPSCs and 0.05 μM ZOL, 0.1 μM ALE, 0.1 μM RIS for PDLSCs) treated groups did not cause significant effect on relative calcium deposition levels compared to control groups for PDLSCs (Figure 3.15).

1 μM ALE treated group significantly increased relative calcium deposition levels compared to control group (Figure 3.16).

0.005 μM ZOL and 0.5 μM RIS treated groups did not cause significant effect on relative calcium deposition levels for hTGSCs, (Figure 3.16).

Gene expression analysis indicated that 0.01 μM ZOL treatment significantly increased BMP2 (~3.5 fold), MSX1 (~1.25 fold) and OCN (~3.1 fold) expression but did not change ONN and RUNX2 expression compared to control group for DPSCs (Figure 3.17).

0.1 μM ALE treatment significantly increased gene expression levels of BMP2 (~1.5 fold) and MSX1(~1.3 fold) but did not change OCN, ONN and RUNX2 expression levels compared to control group for DPSCs (Figure 3.17)

0.05 μM RIS treatment significantly increased BMP2 (~2.5 fold), MSX1(~1.5 fold), OCN (~1.5 fold), ONN (~1.6 fold) and RUNX2 (~1.4 fold) gene expression levels compared to control group for DPSCs (Figure 3.17).

All three kinds of BPs treatments elevated BMP2 gene expression level compared to control group for PDLSCs. 1 μM ALE and 0.5 μM RIS treatment upregulated MSX1 expression (~1.4 fold) compared to control group for PDLSCs (Figure 3.18).

0.005 μM ZOL treatment significantly increased BMP2 (~1.8 fold), MSX1 (~2 fold) and OCN (~2.5 fold) expression levels compared to control for hTGSCs (Figure 3.19).

1 μM ALE treatment significantly increased BMP2 (~2 fold), OCN (~1.2 fold) and ONN (~2 fold) genes expression levels. Besides 0.5 μM RIS treatment significantly upregulated BMP2 (~1.5 fold) and ONN (~1.7 fold) expression compared to baseline (Figure 3.19).

ZOL, ALE and RIS applications did not reduce osteogenic gene expression levels of DPSCs, PDLSCs and hTGSCs. BMP2, MSX1, OCN, ONN and RUNX2 levels were significantly low in negative control groups (undifferentiated) as expected.

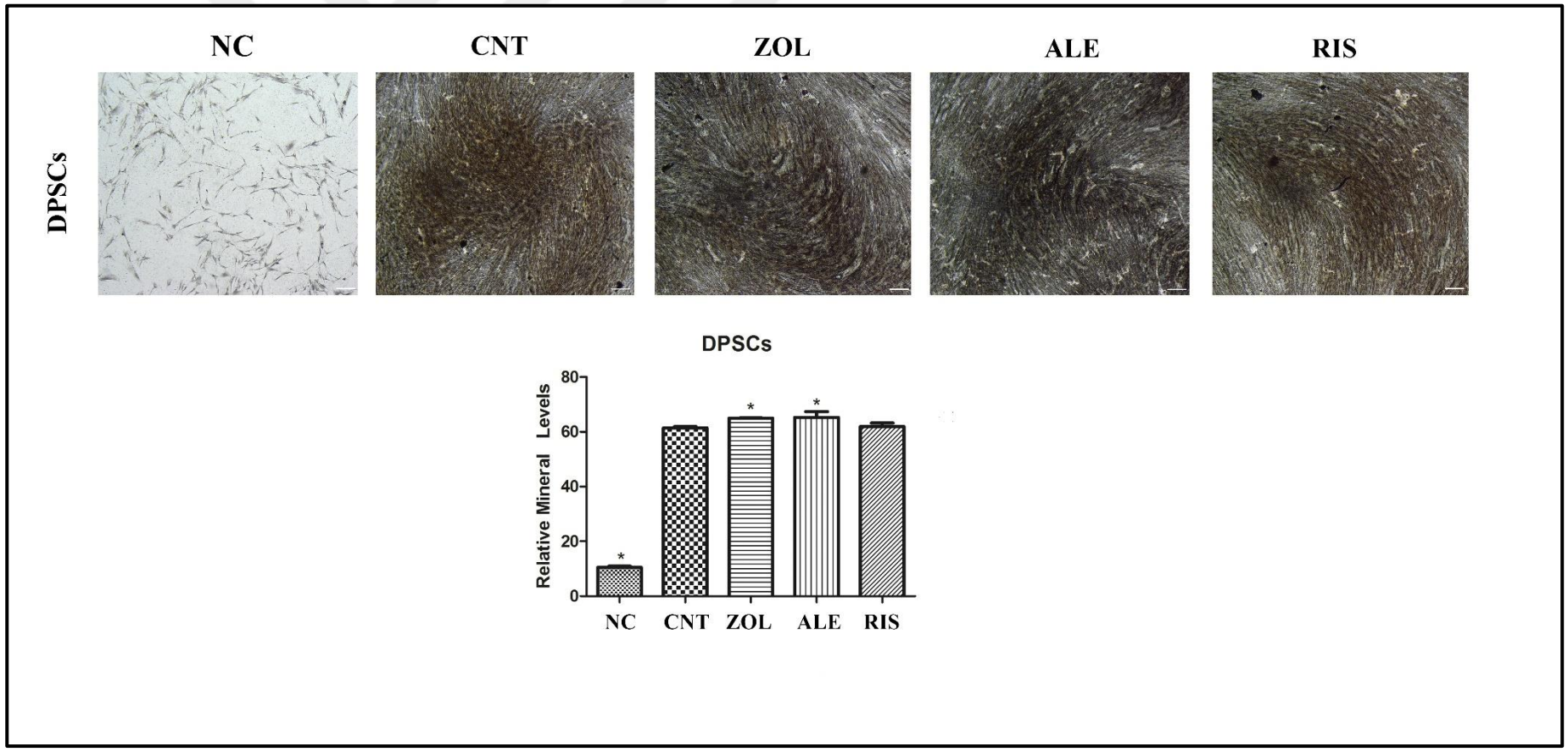

Figure 3.11. von Kossa staining of DPSCs (*scale bar 200 μm*) and relative mineral levels of the differentiated cells. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone osteogenic differentiation medium treatment), NC: Negative Control group (stand-alone growth medium treatment), *: $P<0.05$.

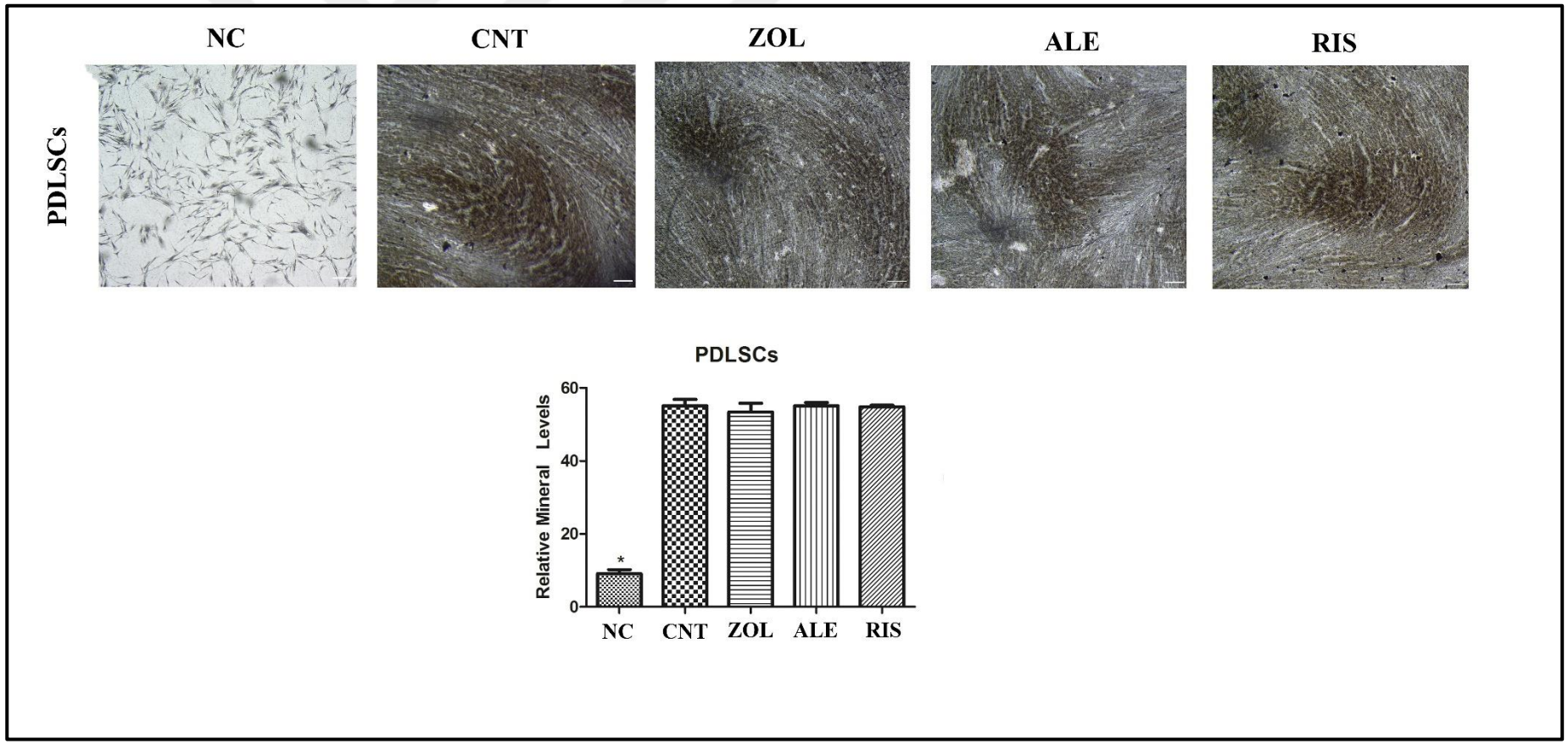

Figure 3.12. von Kossa staining of PDLSCs (*scale bar 200 µm*) and relative mineral levels of the differentiated cells. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone osteogenic differentiation medium treatment), NC: Negative Control group (stand-alone growth medium treatment), *: $P<0.05$.

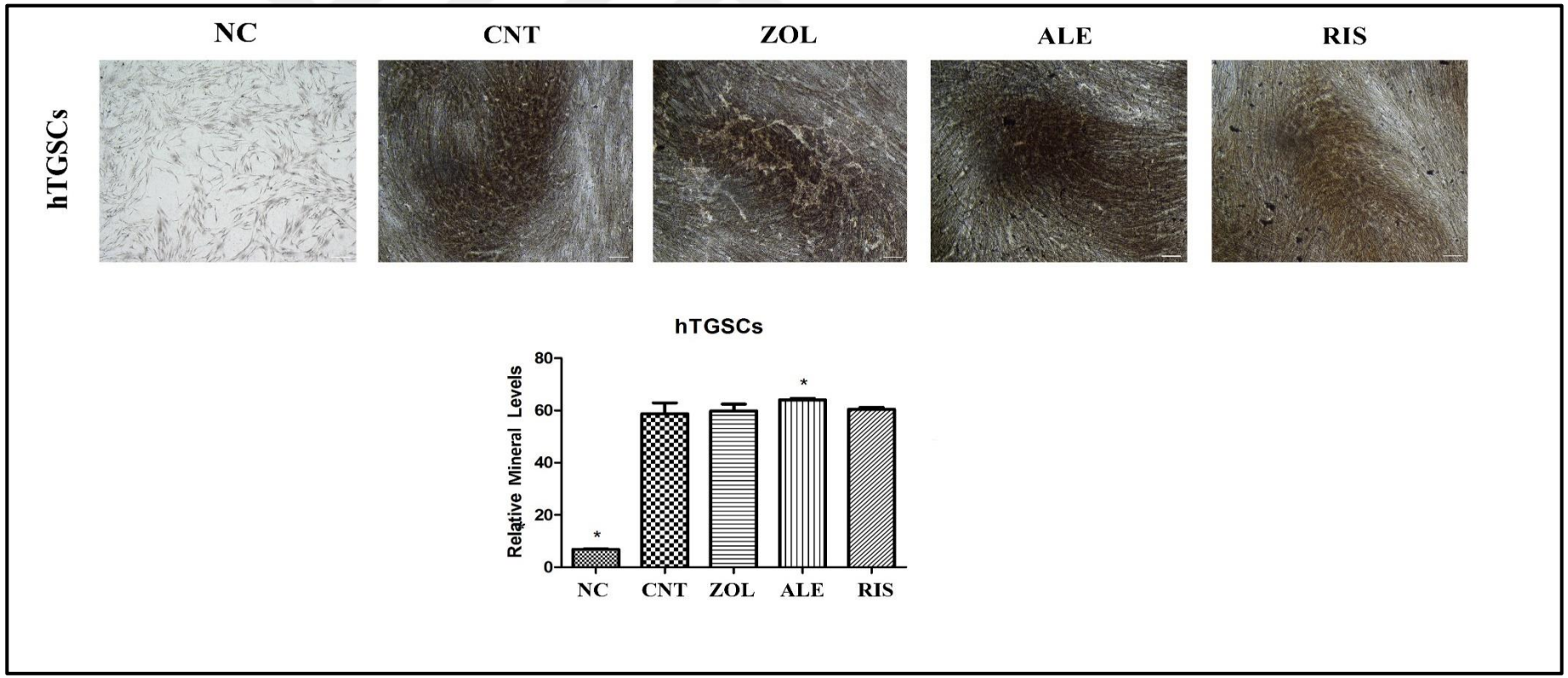

Figure 3.13. von Kossa staining of hTGSCs (*scale bar 200 µm*) and relative mineral levels of the differentiated cells. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone osteogenic differentiation medium treatment), NC: Negative Control group (stand-alone growth medium treatment), *: $P<0.05$.

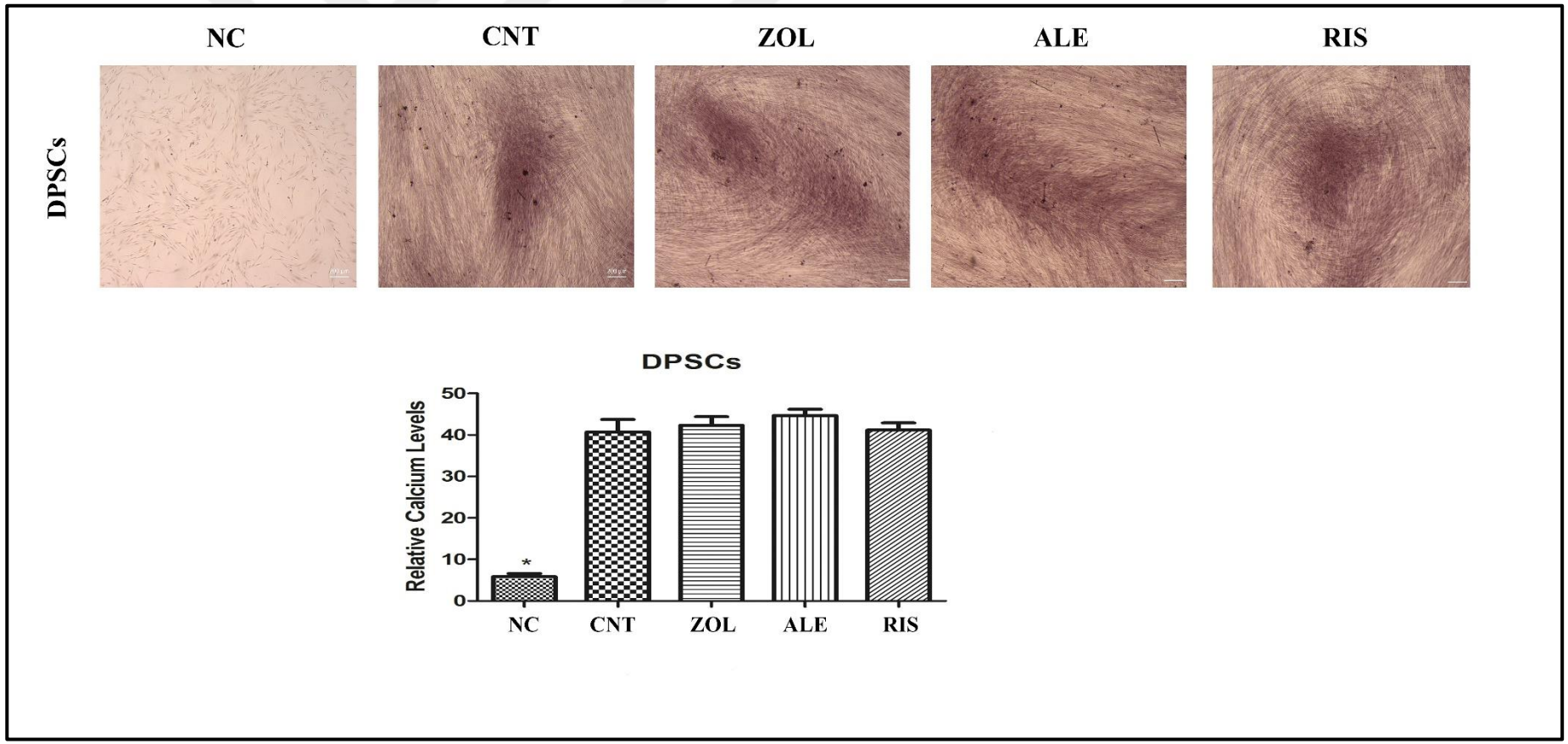

Figure 3.14. Alizarin Red staining of DPSCs (*scale bar 200 μm*) and relative calcium levels of the differentiated cells. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone osteogenic differentiation medium treatment), NC: Negative Control group (stand-alone growth medium treatment), *: $P<0.05$.

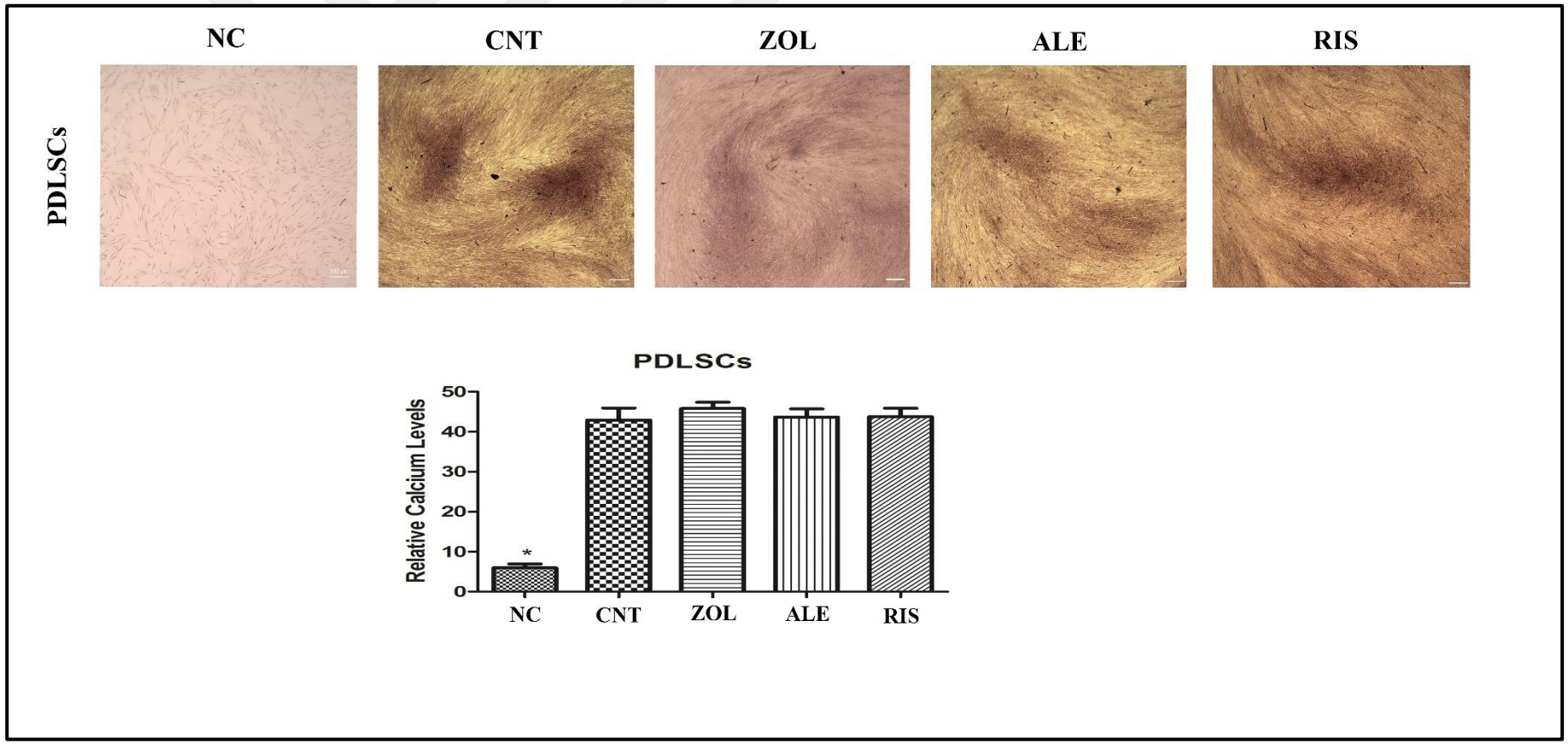

Figure 3.15. Alizarin Red staining of PDLSCs (*scale bar 200 µm*) and relative calcium levels of the differentiated cells. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone osteogenic differentiation medium treatment), NC: Negative Control group (stand-alone growth medium treatment), *: $P<0.05$.

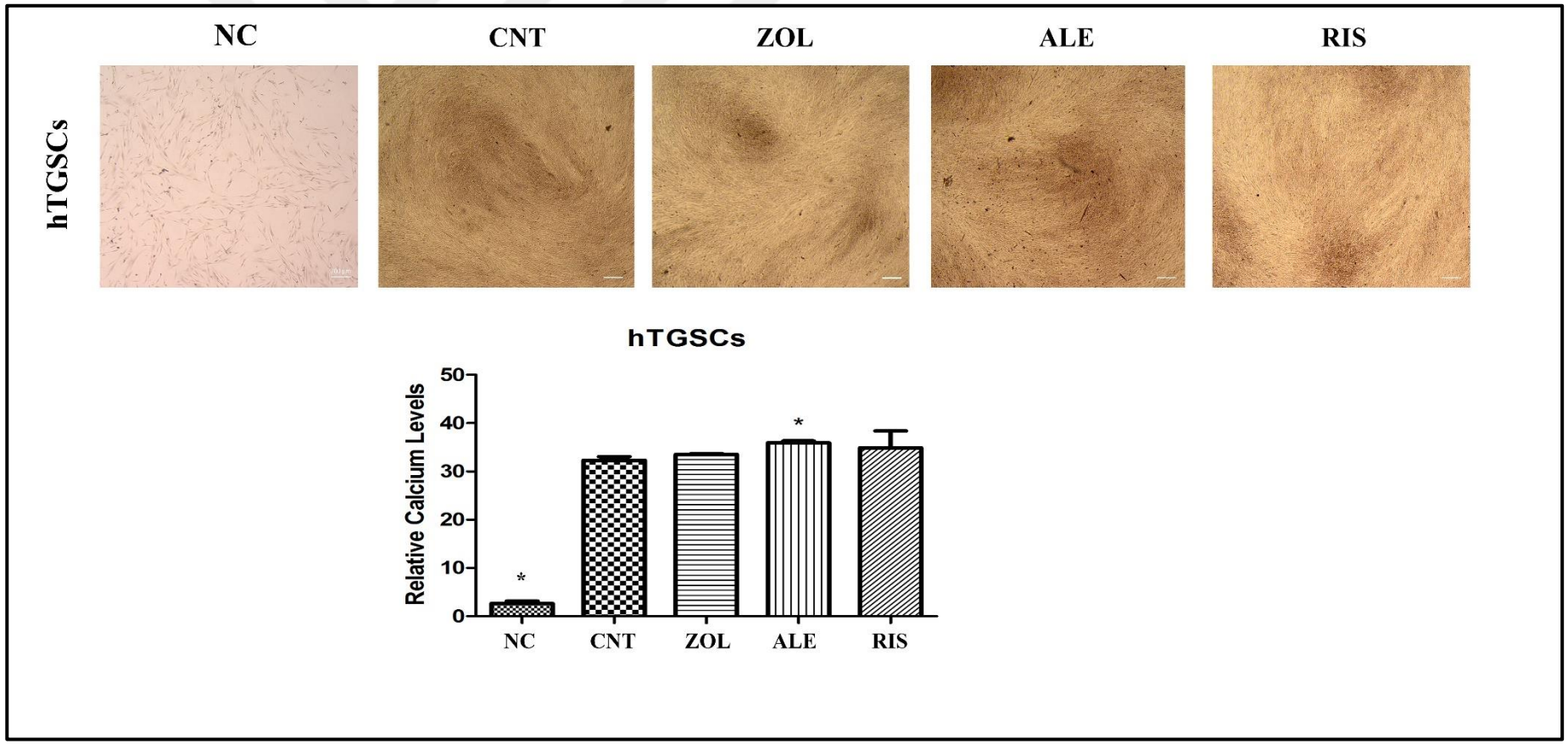

Figure 3.16. Alizarin Red staining of hTGSCs (*scale bar 200 µm*) and relative calcium levels of the differentiated cells. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone osteogenic differentiation medium treatment), NC: Negative Control group (stand-alone growth medium treatment), *: $P<0.05$.

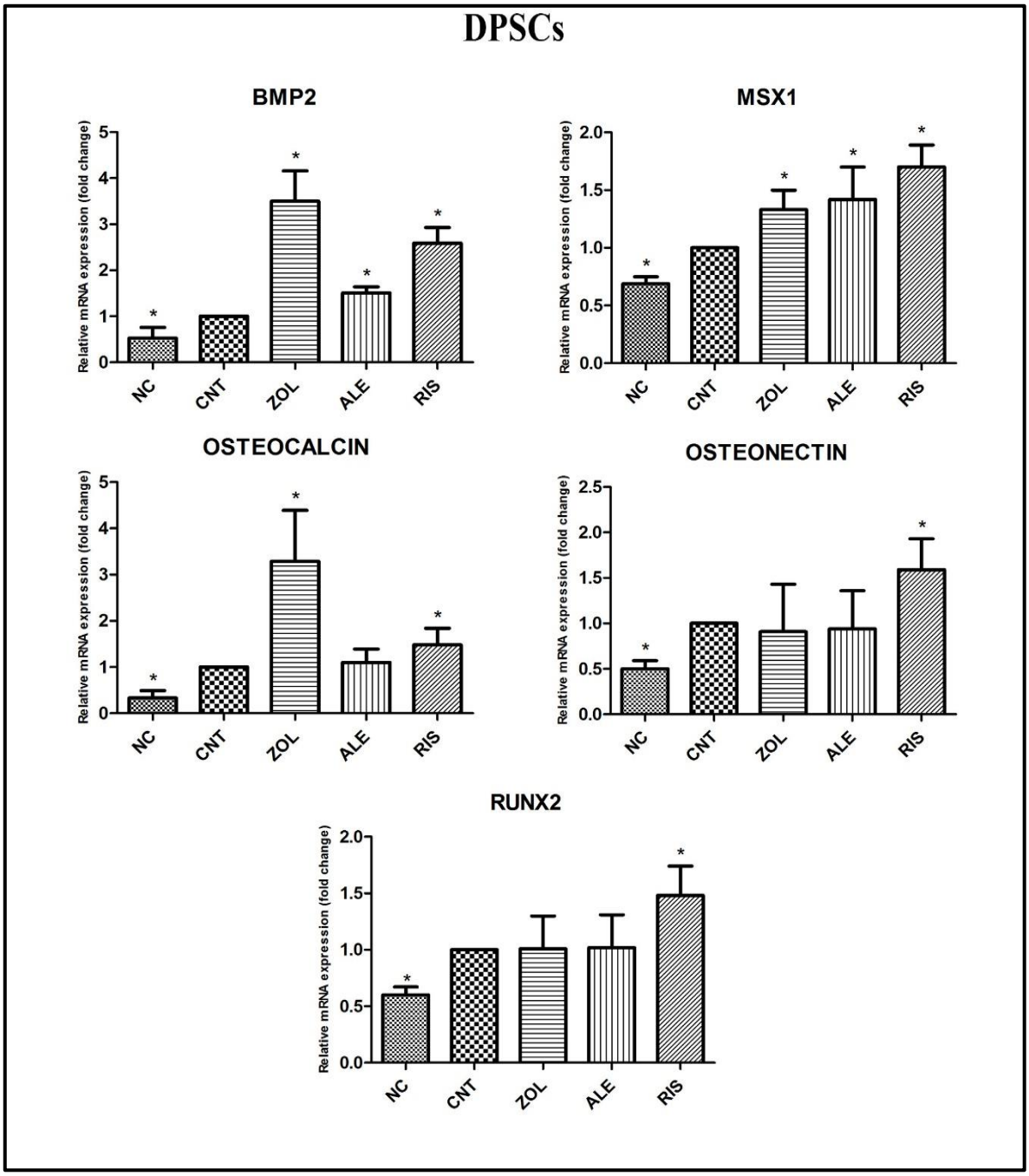

Figure 3.17. The effects of BPs treatment on gene expression levels of osteogenic differentiation marker genes in DPSCs. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone osteogenic differentiation medium treatment), NC: Negative Control group (treated with only growth medium), *: $P<0.05$.

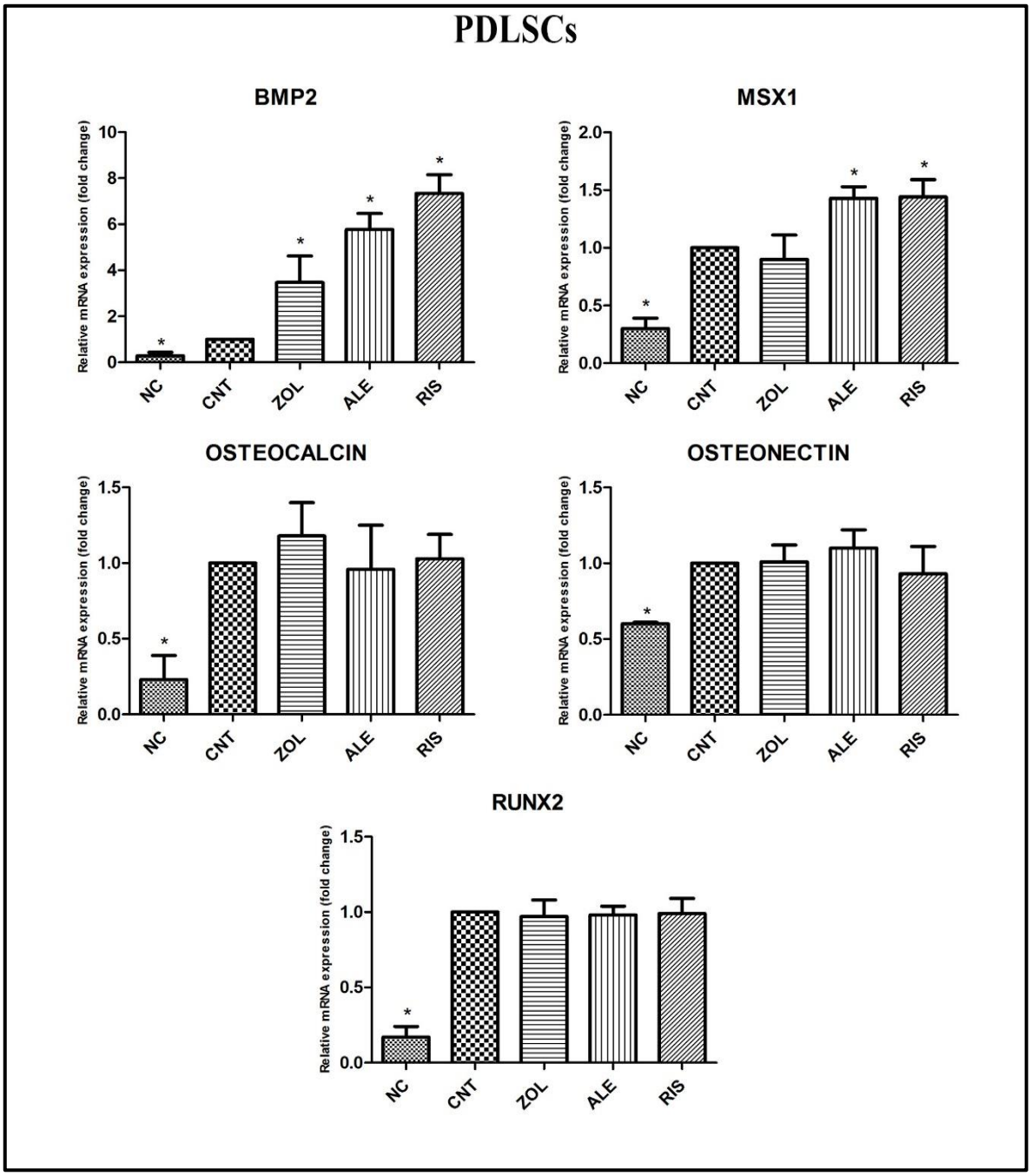

Figure 3.18. The effects of BPs treatment on gene expression levels of osteogenic differentiation marker genes in PDLSCs. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone osteogenic differentiation medium treatment), NC: Negative Control group (treated with only growth medium), *: $P<0.05$.

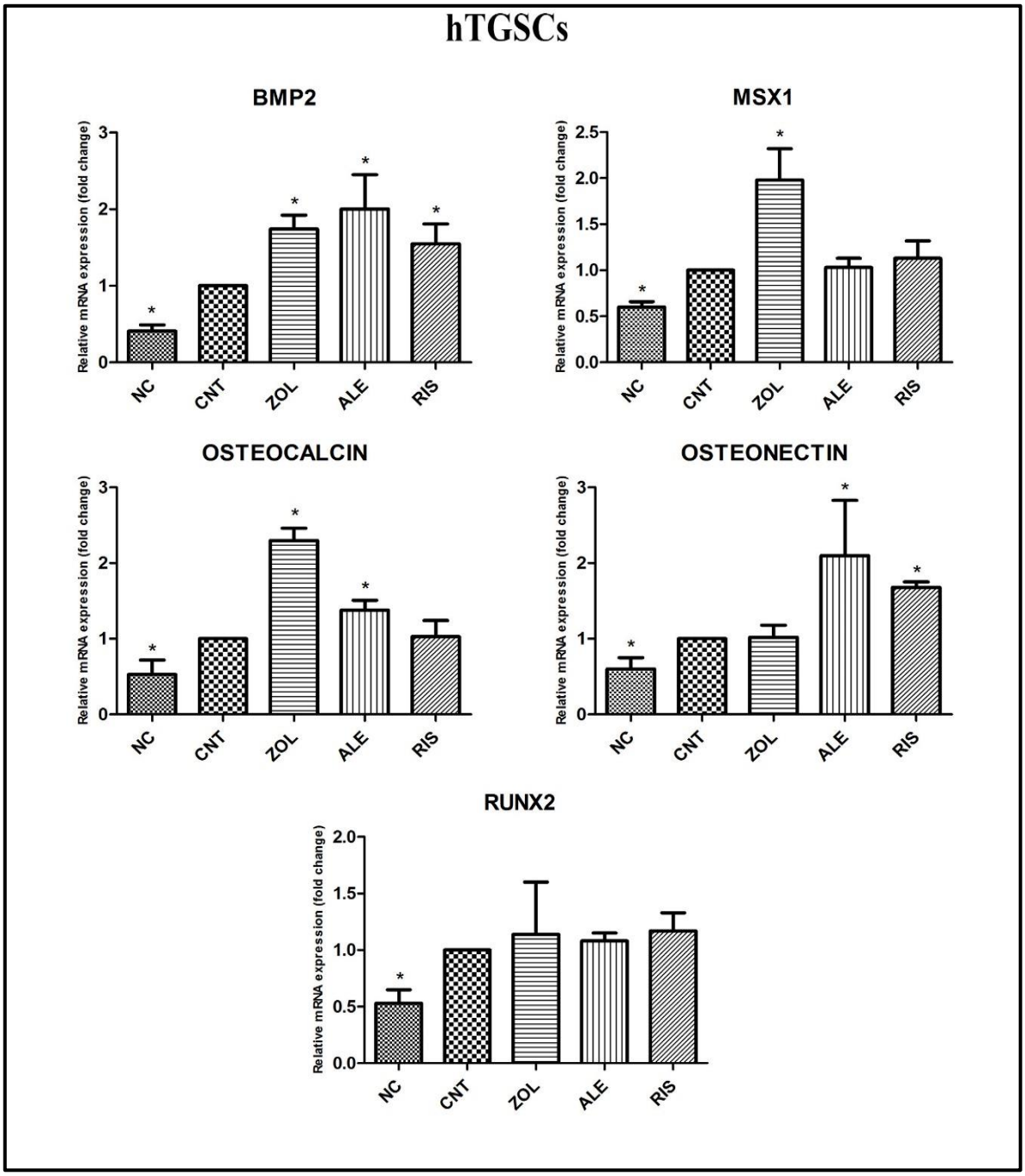

Figure 3.19. The effects of BPs treatment on gene expression levels of osteogenic differentiation marker genes in hTGSCs. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, C: Control group (stand-alone osteogenic differentiation medium treatment), NC: Negative Control group (treated with only growth medium), *: $P<0.05$.

3.4. MIGRATION ANALYSIS

Scratch Assay

In vitro scratch assay was performed to determine the effects of BPs on migration of DPSCs, PDLSCs and hTGSCs. Scratch closure rates were determined in comparison with control group. *In vitro* scratch assay results showed that; 100 μM ZOL, 100 μM ALE and 100 μM RIS treatment significantly reduced wound closure area of DPSCs, PDLSCs and hTGSCs compared to control groups. While closure rate of control group was approximately 68.8% for DPSCs, scratch closure of 100 μM ZOL, 100 μM ALE and 100 μM RIS treated groups were 59.9%, 62.4% and 57.9%, respectively (Figure 3.20). Although closure rate of control group was approximately 83.8% for PDLSCs, scratch closure of 100 μM ZOL, 100 μM ALE and 100 μM RIS applications were 69.9%, 67.9% and 69.2%, respectively (Figure 3.21). Although closure rate of control group was approximately 80.2% for hTGSCs, scratch closure of 100 μM ZOL, 100 μM ALE and 100 μM RIS administrations were 39%, 33.12% and 46.8%, respectively (Figure 3.22).

qPCR Analysis

Gene expression analysis indicated that ZOL, ALE and RIS treatment significantly reduced gene expression levels of COL1A1 as 0.5, 0.81 and 0.59 folds, respectively and LAMININ as 0.56, 0.89 and 0.23 folds, respectively, however, ALE and RIS treatment downregulated FIBRONECTIN expression levels as 0.11 and 0.33 folds, respectively, for DPSCs, respectively (Figure 3.23). ZOL, ALE and RIS treatment significantly reduced gene expression levels of LAMININ (0.6, 0.4 and 0.2 folds, respectively) and FIBRONECTIN (0.5, 0.4 and 0.1 folds, respectively) for PDLSCs, besides ZOL and ALE treatment caused significantly decrease in COL1A1 expression levels as 0.55 and 0.5 folds, respectively (Figure 3.24). ZOL, ALE and RIS treatments significantly downregulated gene expression levels of COL1A1 (0.45, 0.9 and 0.35, respectively) LAMININ (0.65, 0.9 and 0.3, respectively) and FIBRONECTIN (0.3, 0.8 and 0.25, respectively) for hTGSCs (Figure 3.25).

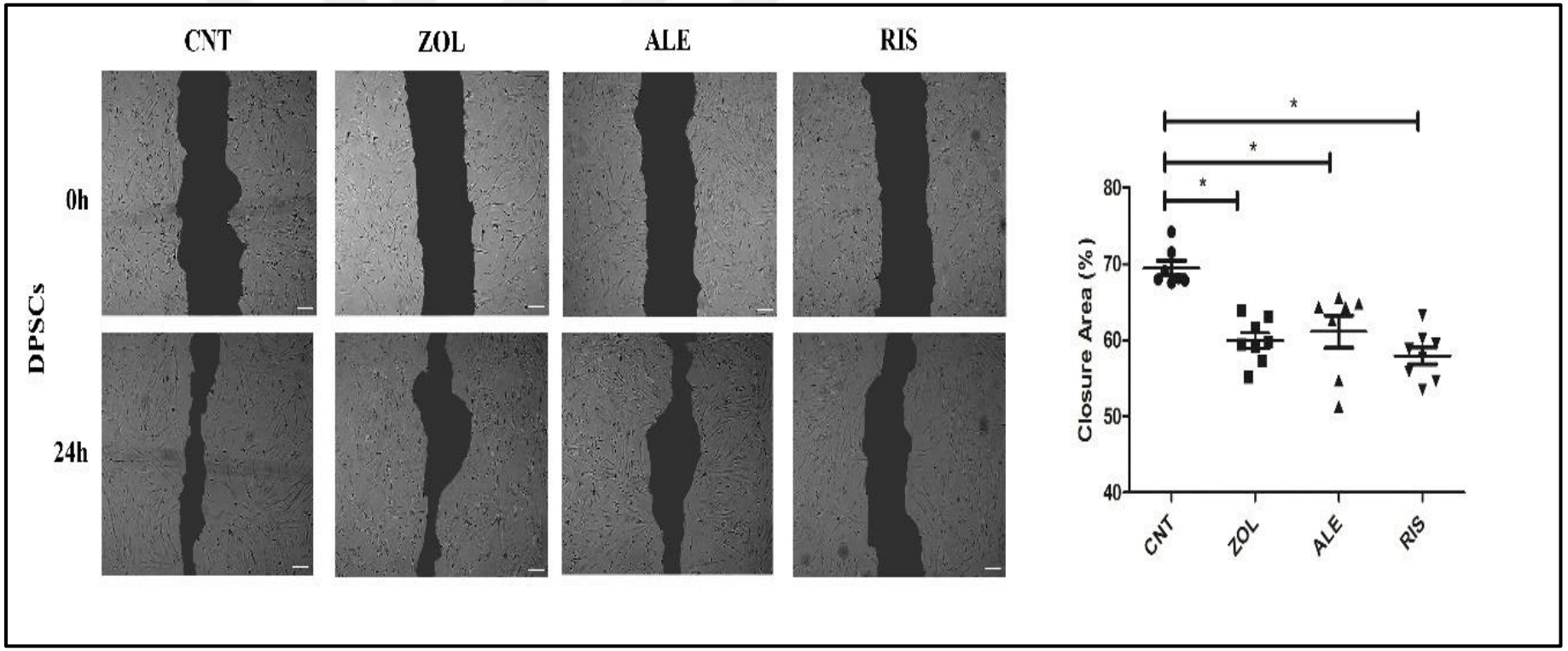

Figure 3.20. Scratch assay of DPSCs after 24h ZOL, ALE and RIS treatment and closure rates of indicated experimental groups (*scale bar 200 μm)*. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone growth medium treatment), *: $P<0.05$.

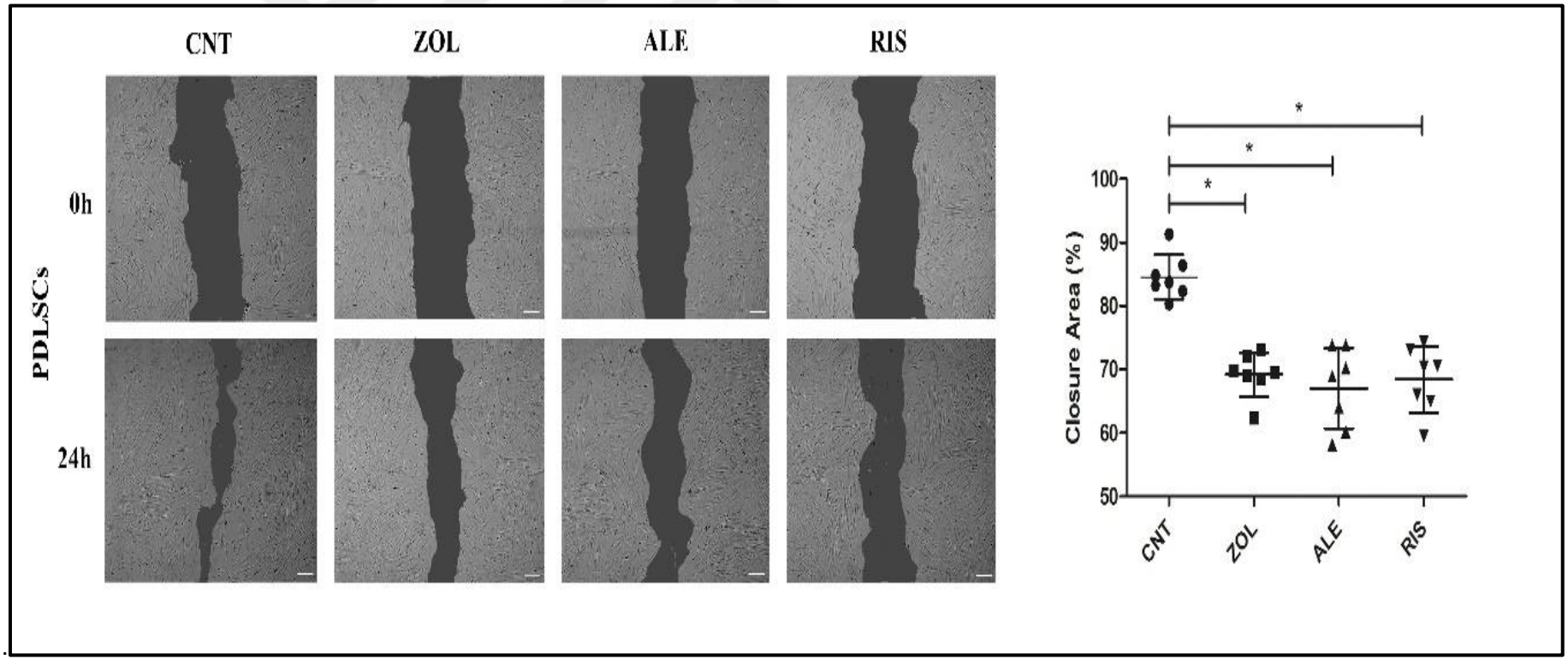

Figure 3.21. Scratch assay of PDLSCs after 24h ZOL, ALE and RIS treatment and closure rates of indicated experimental groups (*scale bar 200 μm)*. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone growth medium treatment), *: P<0.05.

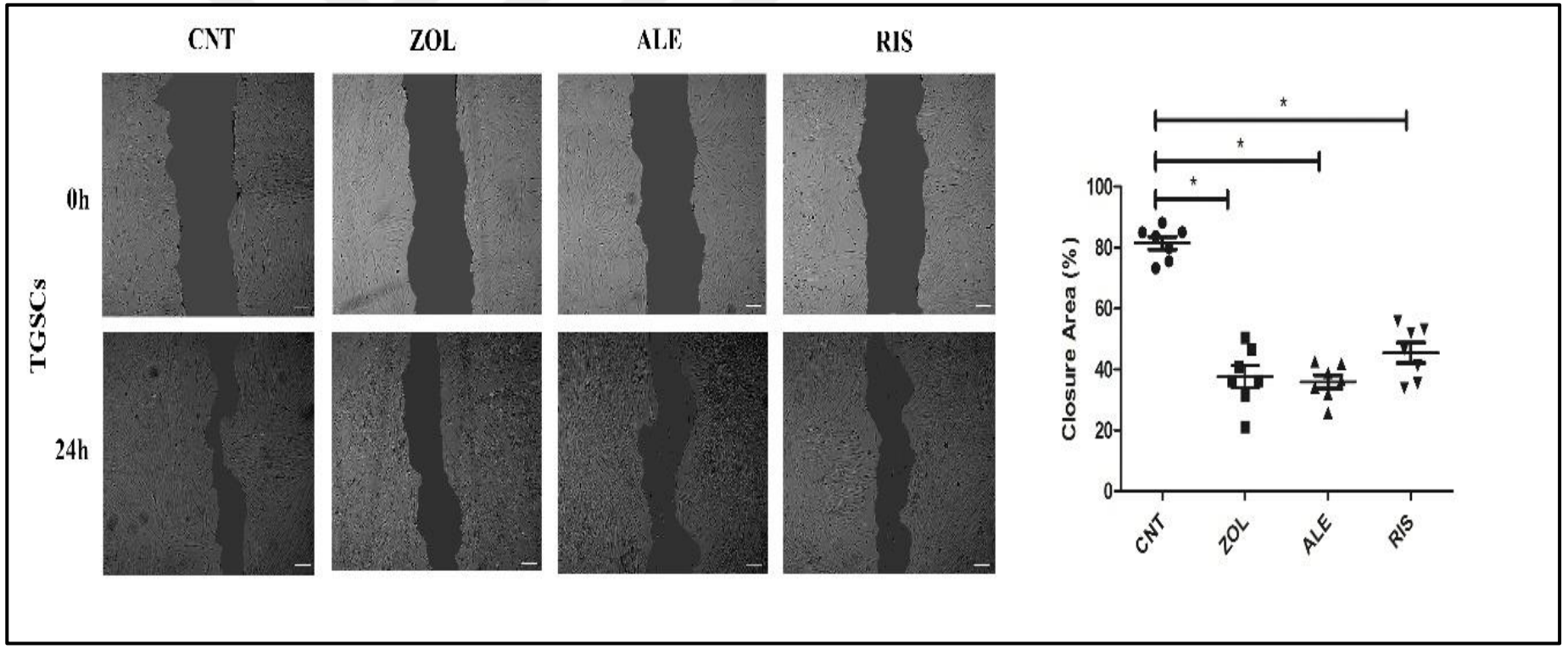

Figure 3.22. Scratch assay of hTGSCs after 24h ZOL, ALE and RIS treatment and closure rates of indicated experimental groups (*scale bar 200 μm)*. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (stand-alone growth medium treatment), *: $P<0.05$.

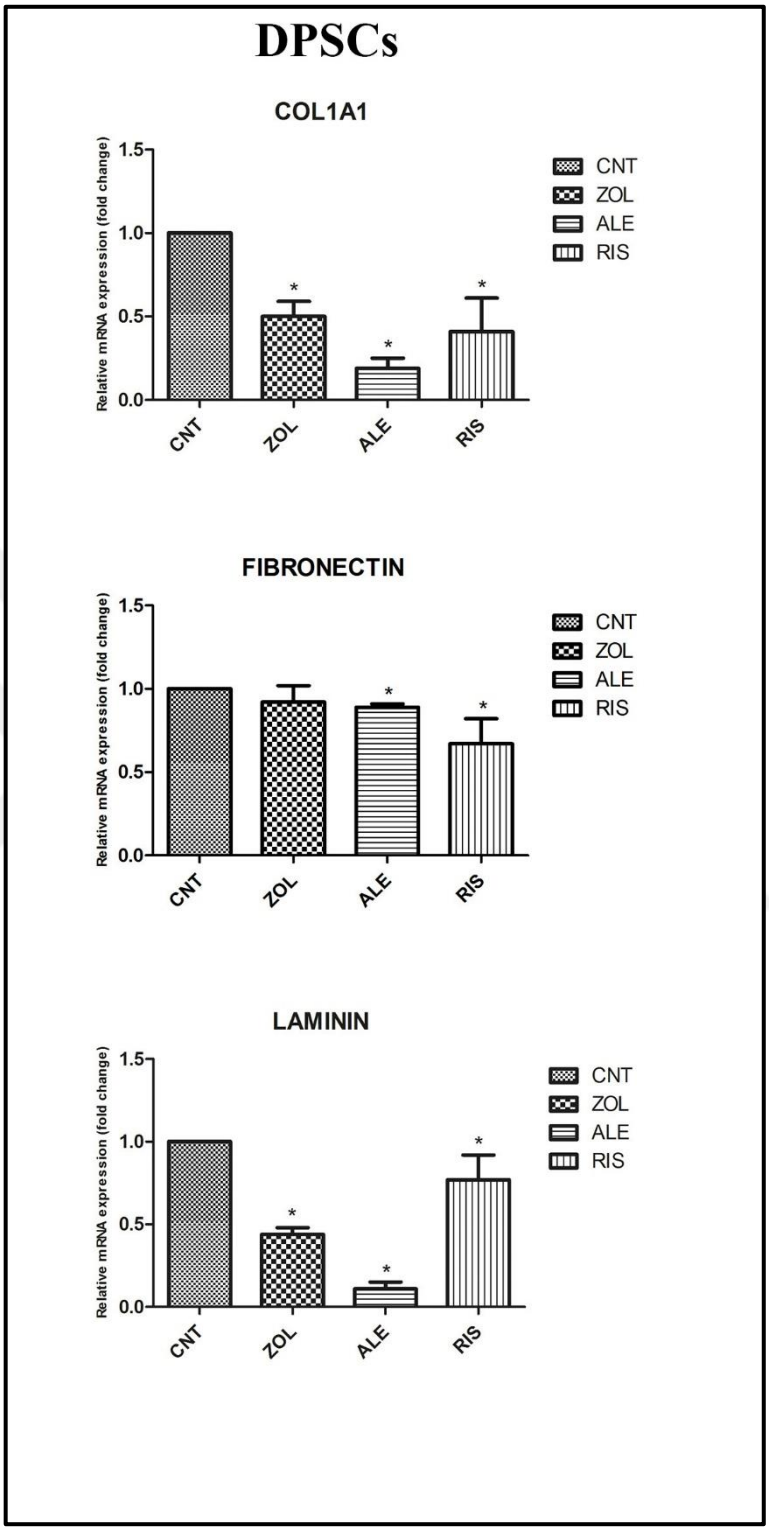

Figure 3.23. The effects of BPs treatment on migration related gene expression levels in DPSCs. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (treated with only growth medium), *: $P<0.05$.

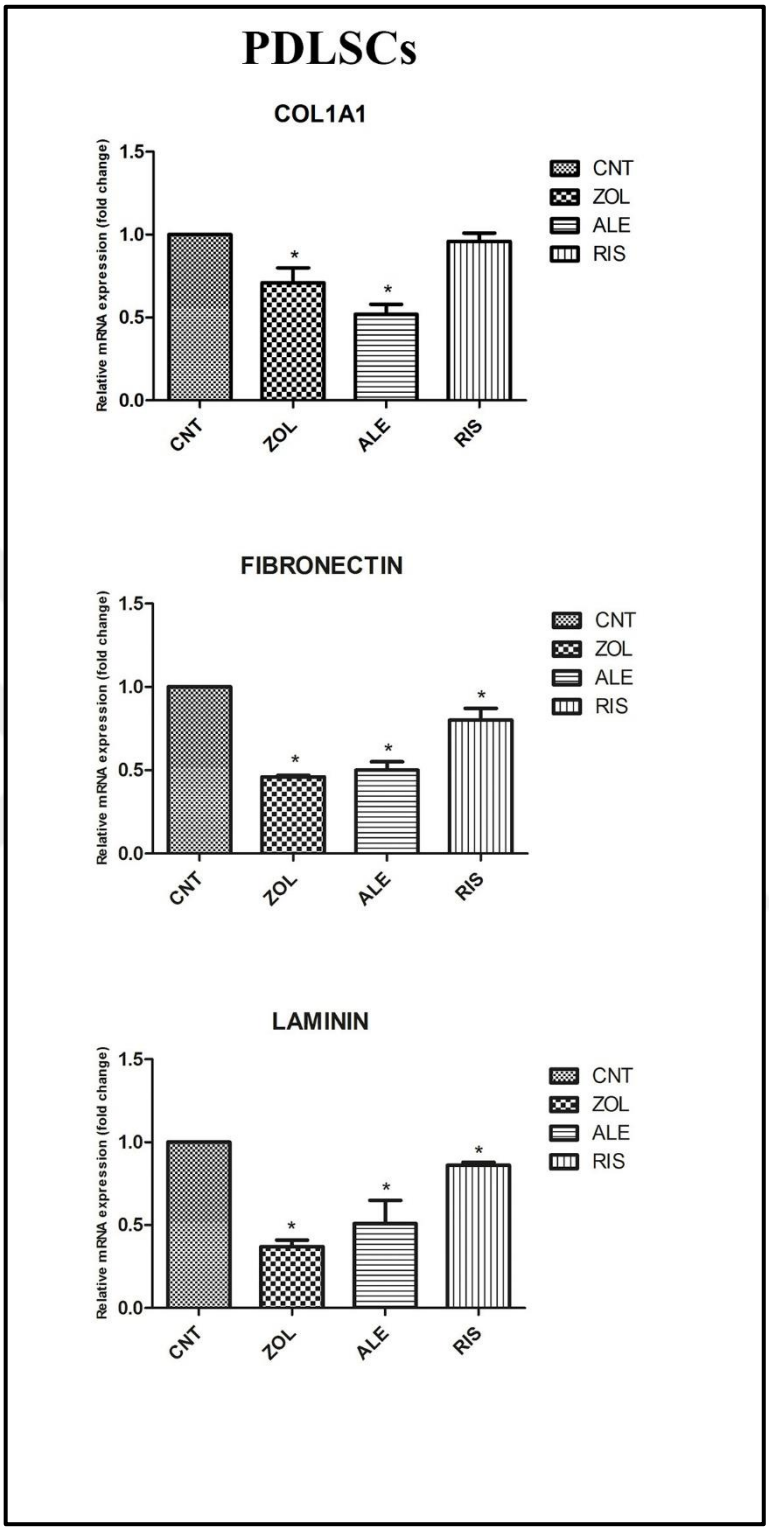

Figure 3.24. The effects of BPs treatment on migration related gene expression levels in PDLSCs. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (treated with only growth medium), *: $P<0.05$.

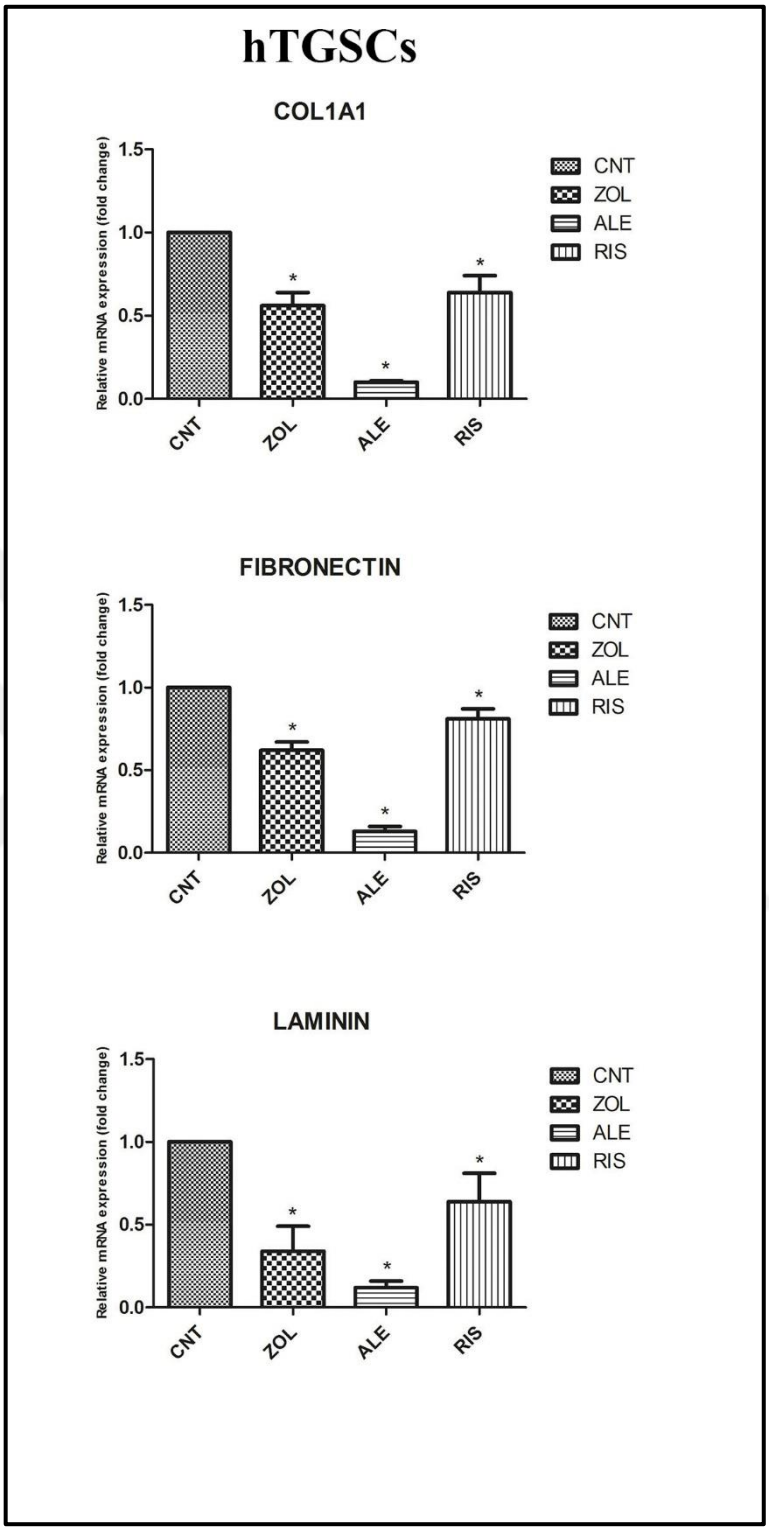

Figure 3.25. The effects of BPs treatment on migration related gene expression levels in hTGSCs. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (treated with only growth medium), *: $P<0.05$.

Western Blot Analysis

Western blot analysis were conducted to analyze the effects of BPs on migration related protein expressions of DPSCs, PDLSCs and hTGSCs. The results showed that ZOL, ALE and RIS treatment significantly decrease protein expression levels of Col1A1 (0.47, 0.78 and 0.48 folds, respectively) and Laminin (~ 0.65 fold) for DPSC as however, Fibronectin protein expression was significantly reduced only by RIS administration as 0.33 fold (Figure 3.26).

ZOL, ALE and RIS applications negatively affected expression level of Fibronectin as approximately 0.25 fold for PDLSCs, besides the BPs caused significantly decrease protein expression level of Laminin as 0.47, 0.18 and 0.23 folds, respectively. ZOL and ALE treatments caused significantly reduction as 0.19 and 0.28 folds in Col1A1 expression, respectively for PDLSCs (Figure 3.27).

ZOL and ALE treatments caused significantly decrease in expression profile of Col1A1 (0.1 and 0.3 folds, respectively) Fibronectin (0.4 and 0.2 folds, respectively) and Laminin (0.5 and 0.4 folds, respectively) expression for hTGSC. While RIS treated cells did not exhibit different expression profile of change Laminin, caused significantly decrease in expression profile of Col1A1 and Fibronectin as 0.18 and 0.21 folds, respectively, in hTGSCs (Figure 3.28).

Scratch Assay, q-PCR and Western Blot analysis were in consistent each other indicating the negative effects of BPs on migratory characteristics of DSCs.

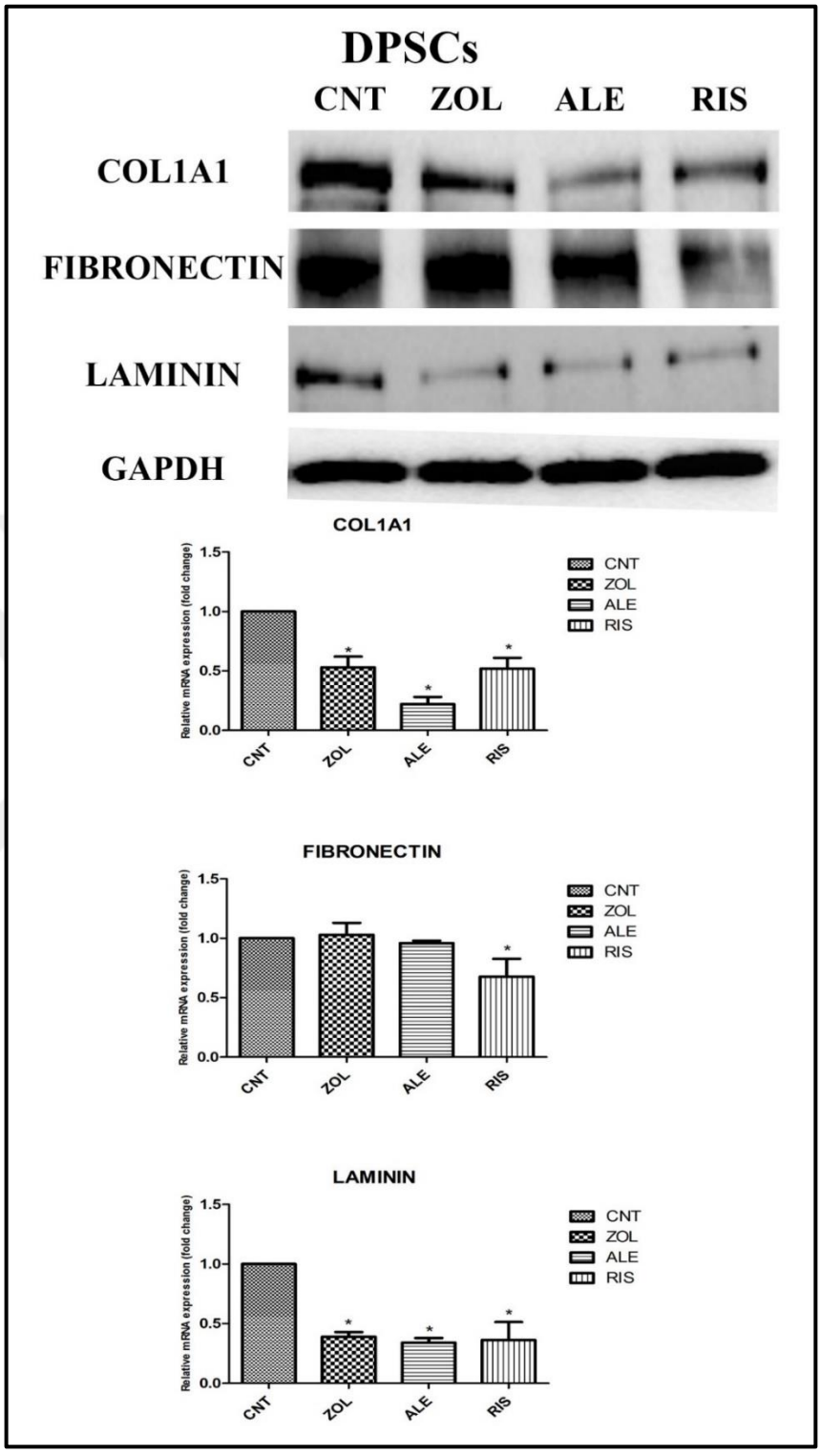

Figure 3.26. Western blot analysis of migration related proteins in DPSCs after different BPs treatment. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (treated with only growth medium), *: P<0.05.

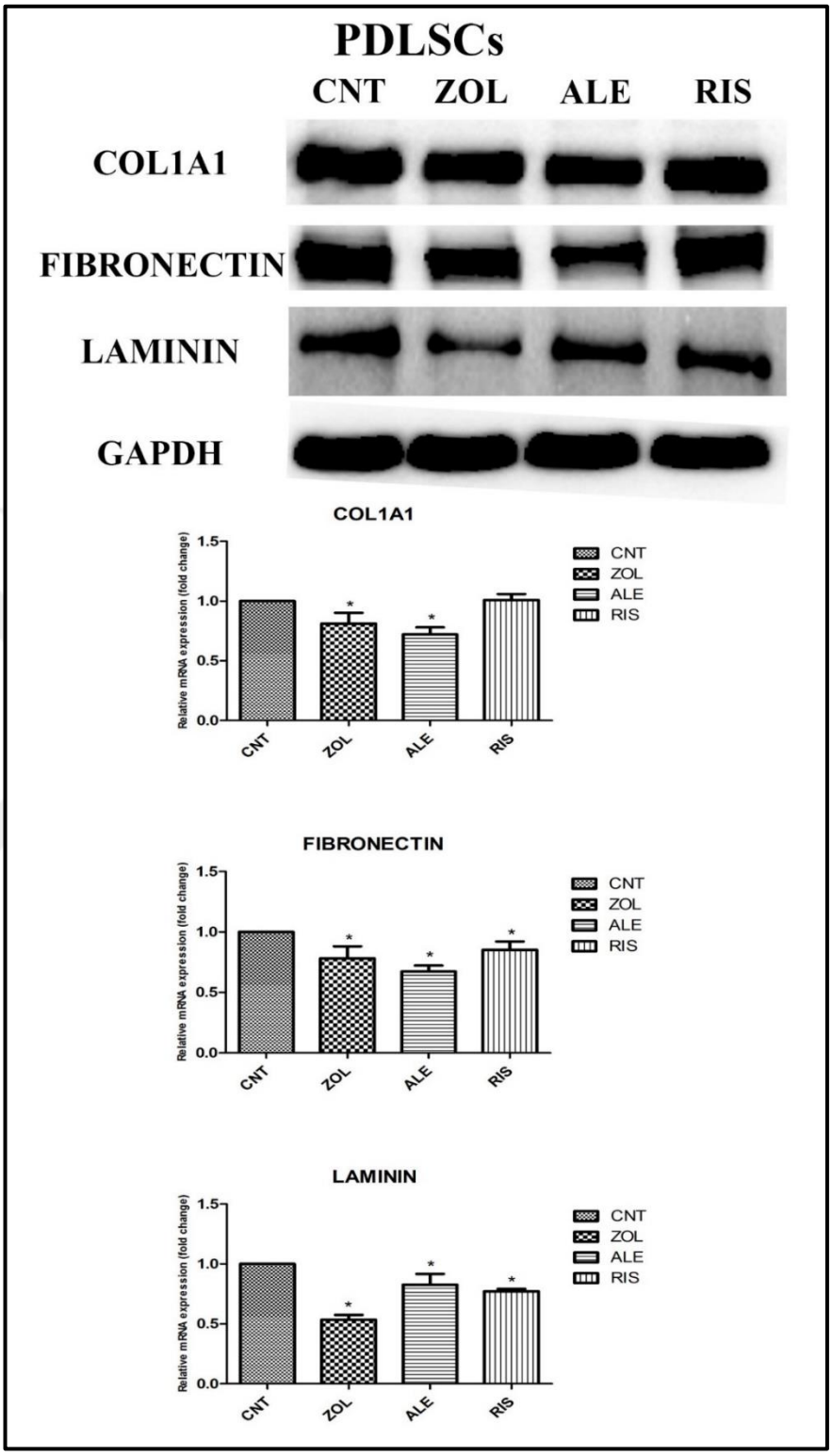

Figure 3.27. Western blot analysis of migration related proteins in PDLSCs after different BPs treatment. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (treated with only growth medium), *: $P<0.05$.

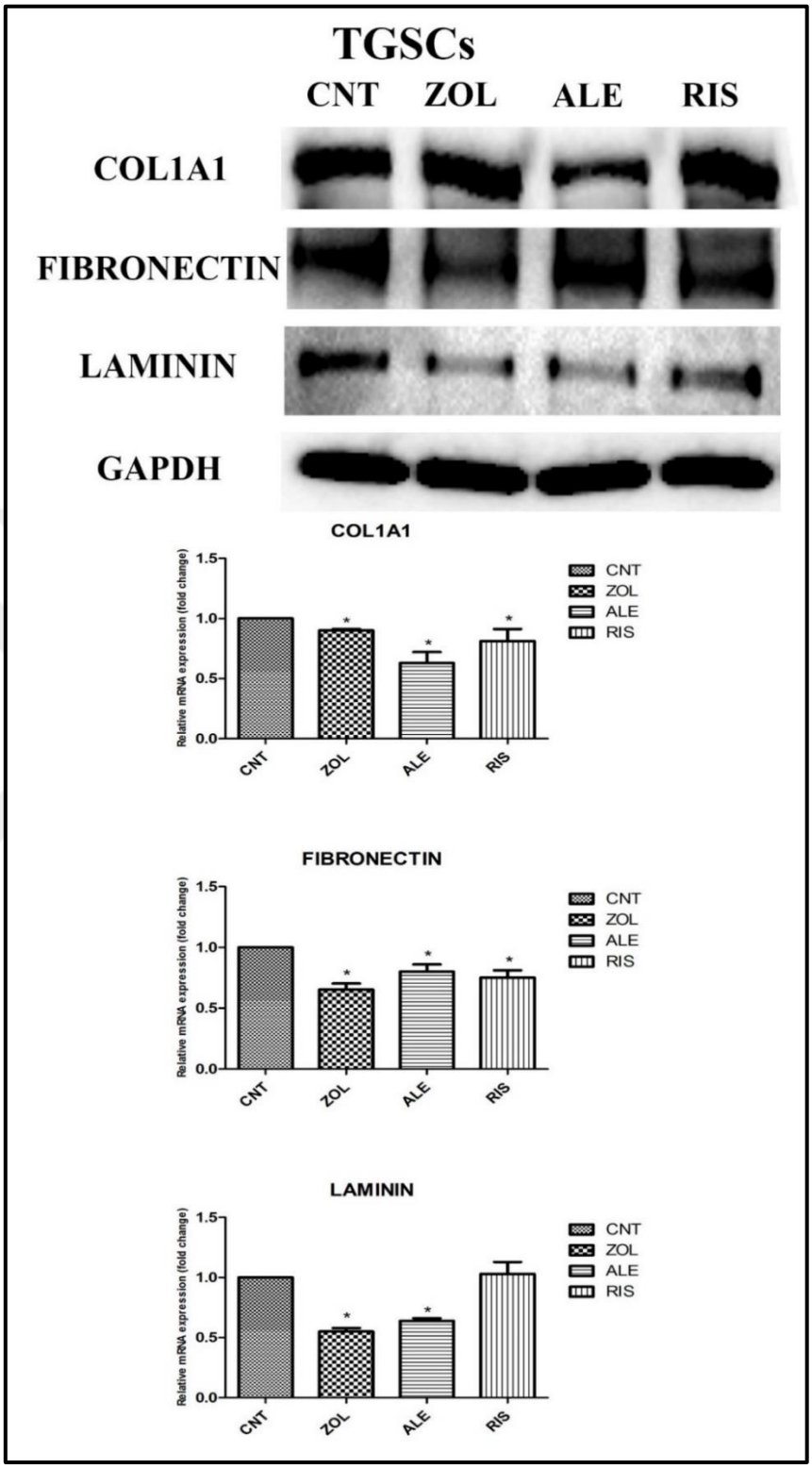

Figure 3.28. Western blot analysis of migration related proteins in hTGSCs after different BPs treatment. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (treated with only growth medium), *: $P<0.05$.

Cytokine Release Analysis

Human cytokine array was carried out to determine migration related cytokine levels in DPSCs, PDLSCs and hTGSCs after ZOL, ALE and RIS treatment. RANTES, SDF-1, MCP-1, EGF, IGF-1 and Eotaxin levels were analyzed as pro-migration factors. TIMP-1 and TIMP-2 levels were analyzed as anti-migratory factors.

The results indicated that, Although RANTES, SDF-1, MCP-1 and Eotaxin levels significantly decreased for DPSCs after ZOL treatment, EGF, IGF-1, TIMP-1 and TIMP-2 levels were significantly increased compared to control group. While ALE treatment downregulated RANTES, SDF-1, MCP-1 and Eotaxin levels, TIMP-1 and TIMP-2 levels significantly increased. EGF and IGF-1 levels did not change after ALE treatment. Although RANTES, SDF-1, MCP-1, EGF and Eotaxin levels significantly decreased, IGF-1 and TIMP-1 upregulated in RIS administration. TIMP-2 level did not change compared to control group (Figure 3.29).

While RANTES significantly decreased, EGF and TIMP-2 levels upregulated in ZOL treatment compared to control, in PDLSCs. RANTES and IGF-1 levels significantly decreased, while TIMP-1 and TIMP-2 levels significantly increased after ALE treatment. RANTES, IGF-1 and EGF levels significantly decreased however, TIMP-1 level upregulated in RIS treatment compared to control group (Figure 3.30).

RANTES, SDF-1, MCP-1, EGF, IGF-1 and Eotaxin levels significantly decreased, TIMP-2 level significantly increased in ZOL treatment, in hTGSCs. TIMP-1 level did not change compared to control group. Although RANTES and MCP-1 levels significantly decreased, TIMP-1 and TIMP-2 levels upregulated after ALE treatment. On the other hand, SDF-1, EGF, IGF-1 and Eotaxin levels did not change significantly in ALE treatment compared to control group. Although RANTES, SDF-1, EGF and IGF-1 levels significantly decreased, TIMP-2 level significantly increased. MCP-1, Eotaxin and TIMP-1 levels remained same in RIS treated cells compared to control group (Figure 3.31).

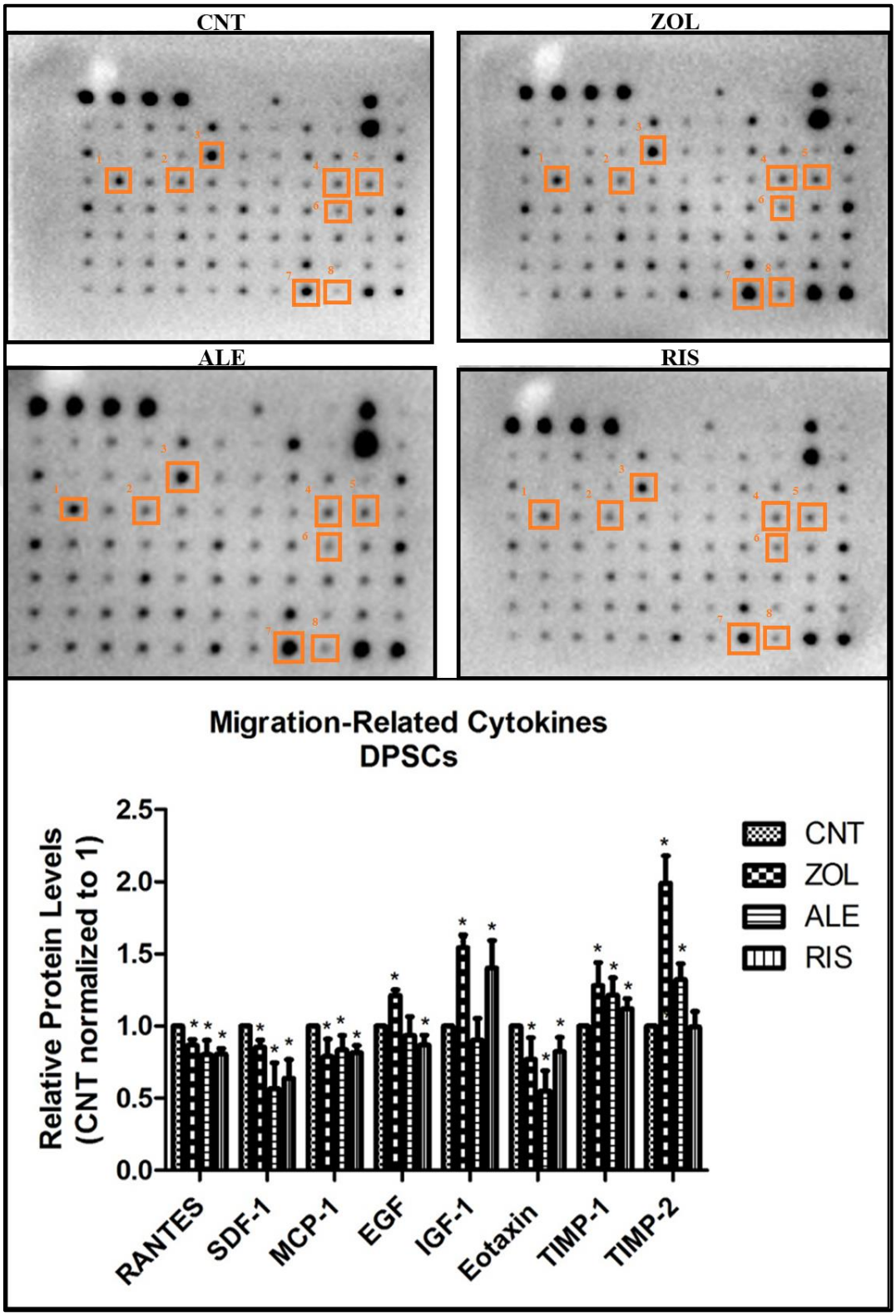

Figure 3.29. The effects of BPs on migration related cytokine levels in DPSCs. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (treated with only growth medium), *: P<0.05.

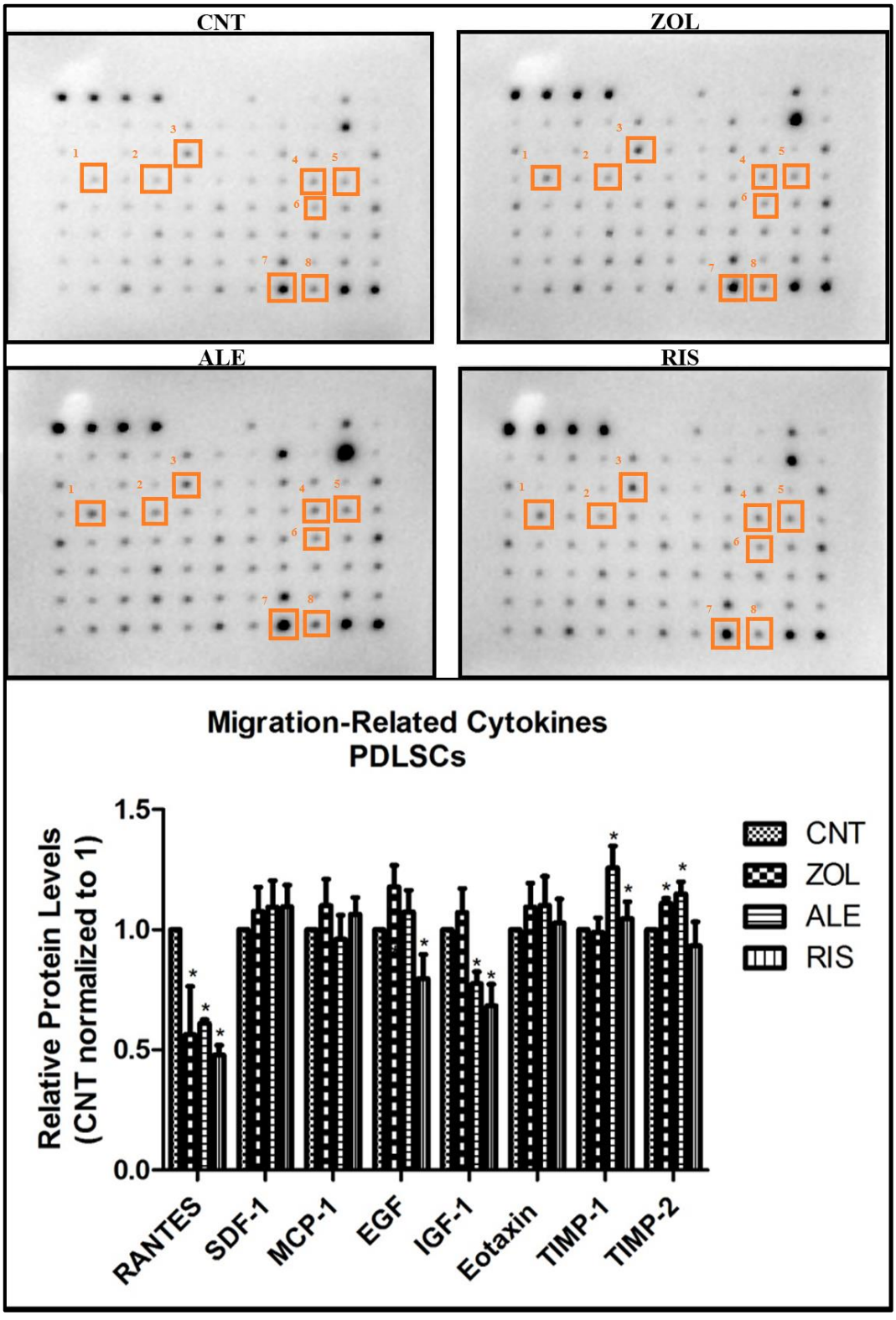

Figure 3.30. The effects of BPs on migration related cytokine levels in PDLSCs. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (treated with only growth medium), *: P<0.05.

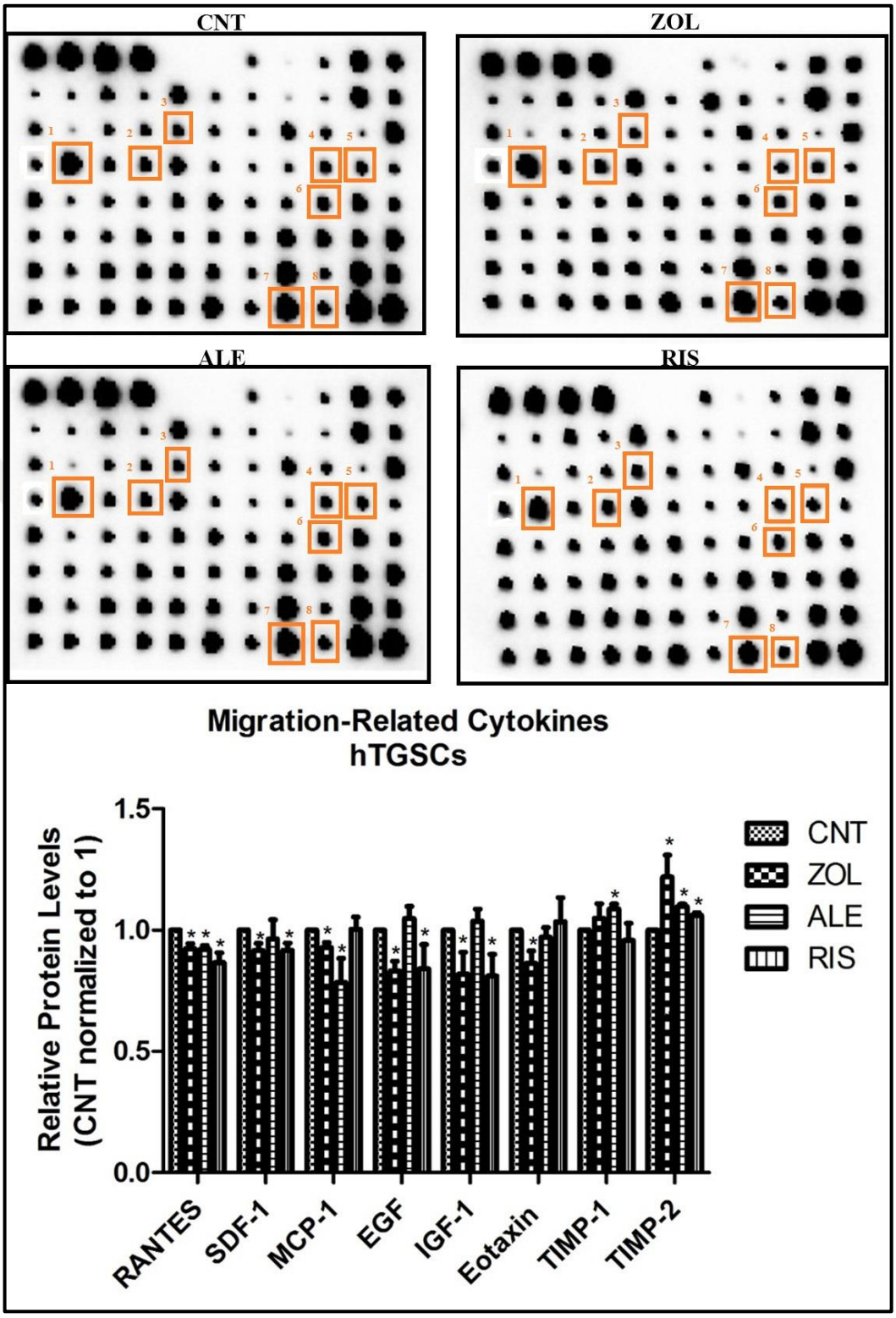

Figure 3.31. The effects of BPs on migration related cytokine levels in hTGSCs. Abbreviations: ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, CNT: Control group (treated with only growth medium), *: $P<0.05$.

3.5. AORTIC RING ASSAY

Non-toxic BPs concentrations (100 μM ZOL, 100 μM ALE and 100 μM RIS) were selected according to cell viability results of HUVECs at 24h. Micro vessel growth in aortic ring assay was reduced after ZOL, ALE and RIS compared to NC and PC groups. In addition, mild micro vessel growth was observed in RIS treated rings compared to ZOL and ALE treatment (Figure 3.32).

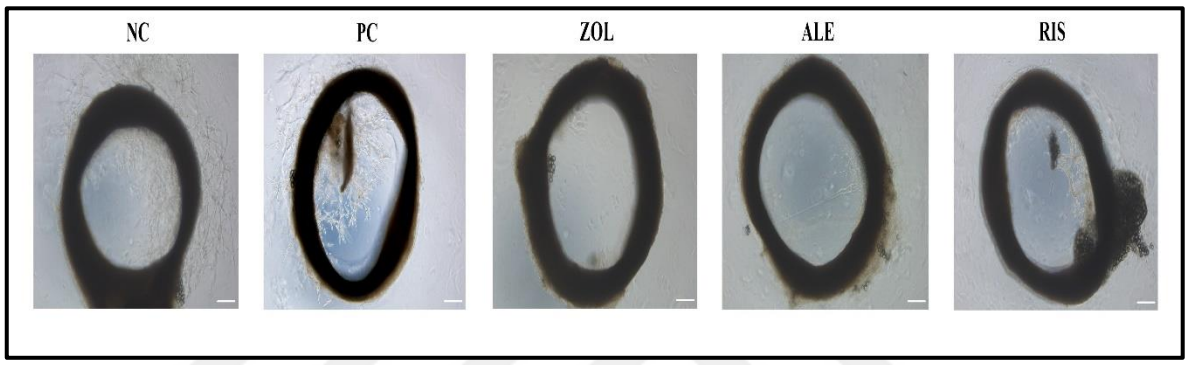

Figure 3.32. The effects of ZOL, ALE and RIS on micro vessel growth in aortic ring assay (*scale bar 400μm*). Abbreviations: NC: Negative Control (Growth medium), PC: Positive Control (Growth medium containing 50 ng/ml VEGF), ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate.

3.6. TUBE FORMATION ASSAY

Tube formation assay was performed to detect the effects of ZOL, ALE and RIS on tube like structure formation capacities of HUVECs which is an indicator of angiogenesis. The average number of tube like structures for ZOL, ALE and RIS treatment and control group were approximately 25.6, 23.6, 27.8 and 55 respectively (Figure 3.33). All three kinds of BPs reduced tube like structures significantly compared to control.

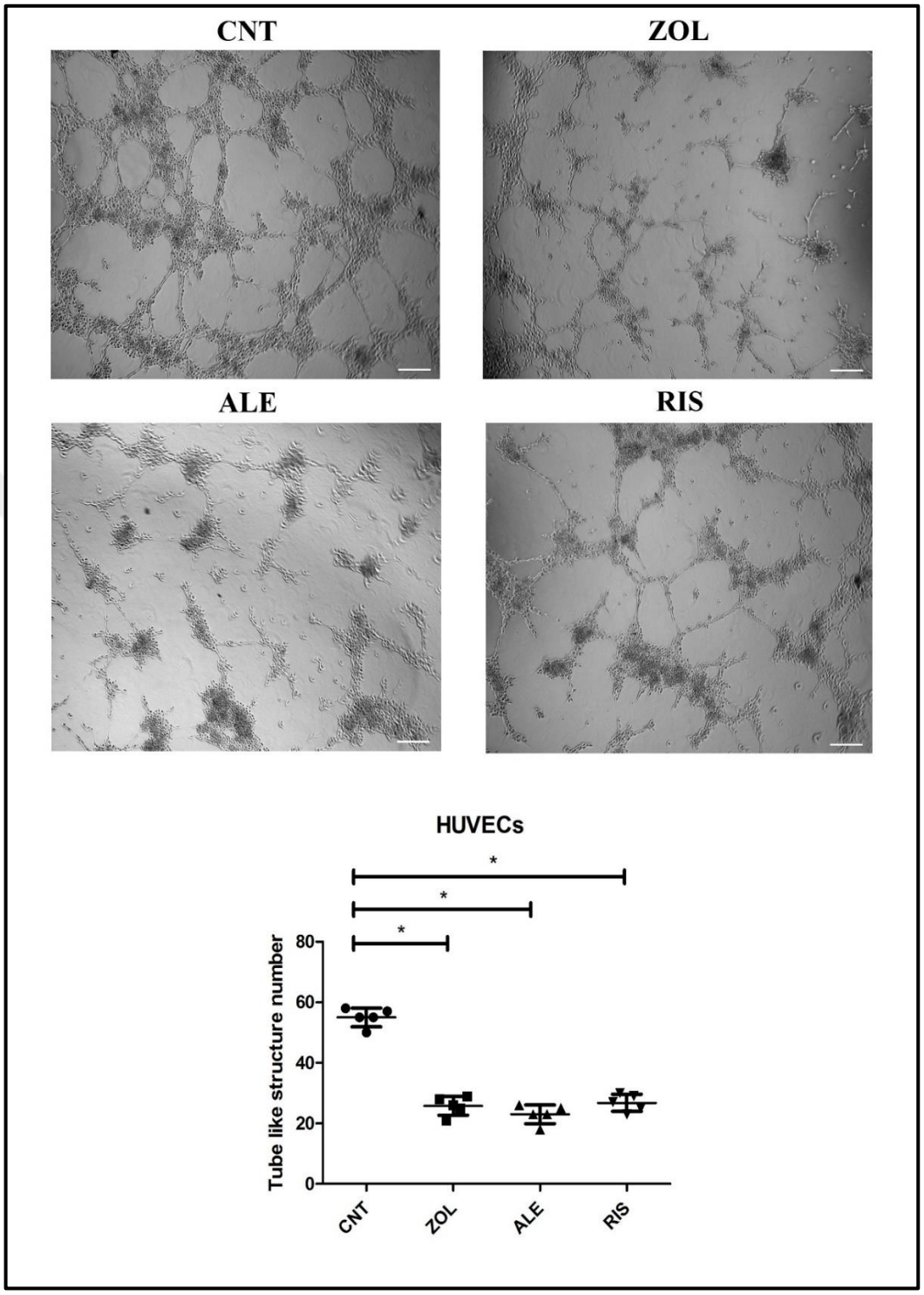

Figure 3.33. The effects of ZOL, ALE and RIS on tube forming capacity of HUVECs and number of tube-like structure by BPs-treated and non-treated HUVECs (*scale bar* 200µm). Abbreviations: CNT: Control group (only growth medium), ZOL: Zoledronate, ALE: Alendronate, RIS: Risedronate, *: P<0.05.

4. DISCUSSION

Osteoporosis, a bone related disease, is diagnosed by BMD and reduced bone mass in which bone fracture risk increases [127]. For the treatment of the osteoporosis, various anti-resorptive agents such as synthetic hormones and drugs are used. BPs, non-N-BPs (non-aminobisphosphonates) and N-BPs (aminobisphosphonates), are mostly preferred anti-resorptive chemicals that block the osteoclast cell activity since they have high affinity for hydroxyapatite crystals and phosphorous–carbon–phosphorous structures in bone architecture [103]. Although BPs are successfully used for the osteoporosis therapy in routine clinical applications, they may have various side effects including oesophagus irritation, intestinal obstruction, headache, painful swallowing, flu, arthralgia, and BIONJ. Literature suggests that BPs induced osteonecrosis of the jaw bone is more probable when there is an excessive bone turnover due to dental surgery or damage, and infection of the jaw bone [128]. While several hypotheses including inhibition of local macrophage function, occurrence of micro-fracture on jaw bone, abnormalities in vascular systems of jaw, infectious inflammatory response, and local necrosis due to anti-angiogenic activity of BPs or side effects of adjuvant therapies such as chemotherapy [110-112] have been presented for BIONJ formation, there is still not a consensus among scientific researchers. However, it is obvious that normal bone regeneration process in the jaw bone is drastically effected by BP treatment so that the structure cannot renew itself after severe toxicity arisen from BPs. The aim of the present study was to evaluate the effects of BPs on characterized DPSCs, PDLSCs and hTGSCs in the aspects of osteogenesis and migration capacity. In addition, anti-angiogenesis properties of BPs were determined using different model study systems.

Bone regeneration in the defected area is mainly driven by unipotent cells, but unipotent cells are derived from more potent progenitor cells such as MSC and their behavior is regulated by vital growth factor and cytokine secretion from these stem cells [129]. In the jaw area, there are various dental tissue-derived stem cells including DPSCs, DFSCs, SCAP, SHED, and PLDSCs. While they, especially PDLSCs and DFSCs, are expected to contribute bone and tooth deficiencies on the jaw, they remain ineffective against toxicity of BP treatment. The effects of BPs on various stem cells have been presented in the literature, but their impacts on dental tissue derived stem cells have not been addressed yet.

Therefore, it is sought to investigate the effects of BPs on osteogenesis and migration capacity of dental stem cells. DPSCs, PDLSCs and hTGSCs, and potential angiogenic or anti-angiogenic activities of the chemicals using different experimental models.

Stem cells are able to reproduce themselves and differentiate into various cell lineages. Based on their differentiation capacities, they can be divided into four main groups: totipotent stem cells, PSCs, multipotent stem cells and unipotent stem cells. Progenitor cells found in various parts of the adult body including bone marrow, adipose tissue, skeletal muscle, dental tissue etc., referred to as ASCs, have multipotent stem cell characteristics with the roles of repairing damaged tissues and providing normal tissue regeneration. According to statement of the international society for cellular therapy, progenitor cells should display 3 criteria to be considered as MSCs: (i) being adherent to the plastic surface, (ii) expressing MSC markers including CD73, CD90 and CD105, and not having HSC markers such as CD14, CD19, CD34, CD45 and HLA-DR, and (iii) display *in vitro* differentiation capacity towards osteoblasts, adipocytes, chondroblasts [39]. In consistent with these criteria, dental tissue-derived stem cells used in the current study were first confirmed for their MSC phenotypes by performing cell surface marker analysis and multipotent differentiation ability.

MSCs are involved in bone development, repair or regeneration. The integrity of bone tissues is provided through remodeling mechanism. Osteoblasts and osteoclasts exhibit critical roles for maintaining and regeneration of bone tissues at different time and places. If an imbalance occurs between osteoblastic and osteoclastic activity, osteopetrosis (bone resorption < bone formation) or osteoporosis (bone resorption > bone formation) take places [96]. Comparison studies of dental stem cells with BMMSCs have presented that they have better osteogenic and odontogenic differentiation capacities [66, 130], showing the superiority of dental stem cells in the jaw regeneration. In addition, as various dental-tissue derived stem cells have been presented for their angiogenic activity through both direct conversion to endothelial-like cells [131] and secretion of angiogenic growth factors [132], they should further support the regeneration by promoting vessel network development in the newly established tissue supplying required nutrients and waste disposal. Therefore, it was hypothesized that the effects of BPs on dental stem cells should be detrimental so that the body cannot overcome necrotic effects of the drugs.

First, various concentrations of each BPs tested, ALE, RIS and ZOL, were applied to each stem cells types to determine the highest sub-lethal concentrations. The toxicity of high dose BPs against various organs and cell types is well-recognized [133]. As administration of toxic dose to osteogenically differentiating stem cells could mislead the outcomes, highest non-toxic dose for each BP was determined for all stem cell types used. Optimized concentrations for BPs were found to be non-correlative and distinct for each stem cell type, supporting different action of mechanisms on each cell type. Similar sub-lethal doses for BPs have been reported in the literature. ZOL treatment at concentrations lower than 1 μM did not significantly decrease the cell viability of stem cells derived from periosteum from the jaw bone (mandible), bone marrow from the jaw bone, and periosteum from long bone (tibia) for a 7-day application period [134]. In another study, treatment of DPSCs and PDLSCs with 0.2-0.8 μM ZOL showed no significant difference on cell growth and survival levels. However, 1 μM ZOL treated cells died after 10 days and 15 days of incubation periods for DPSCs and PDLSCs, respectively [135], suggesting dose- and time-dependent toxicity. As these drugs are beneficial for bone regeneration, lower optimized dose could be expected to have positive results on stem cell activity. *In vitro* study conducted by Still *et al.* supported this idea by presenting augmented colony formation ability of rat BMMSCs by ALE and RIS treatments at concentrations lower than 0.1 μM [136]. As the present study aimed to evaluate cumulative dose effects of BPs on dental tissue-derived stem cells, concentrations lower than the highest sub-lethal concentrations for each experimental models were not evaluated within the context of the thesis.

While the sub-lethal concentrations of BPs tested in this study did not significantly affect osteogenic capacity, enhanced osteogenesis of stem cells by BP administrations have been reported earlier. Sub-lethal doses of BPs were presented to enhance osteogenic differentiation of DPSCs and PDLSCs through increasing Osterix, ALP, osteopontin (OP) and OCN expressions [135]. Other than MSCs, BPs also augmented cell proliferation, osteoblastic differentiation and related growth factor/cytokine synthesis of unipotent cells [137, 138].

Consistently, von Knoch *et al.* reported that ZOL, ALE and RIS treated BMMSCs provided higher proliferation rate and encouraged osteoblast differentiation of the stem cells by increasing expression levels of BMP2, COL1A1, core binding factor alpha subunit

1 (CBFA-1) and bone sialoprotein (BSP) [139]. BMP2 upregulation during osteoblast production after BP application was also shown in another study [140]. While the results of the current study seem contradictory to the previously published data, the concentrations used in those studies are not the highest non-toxic doses and most of the positive effects have been shown on BMMSCs. Although stem cells used in this study are all dental tissue-derived they responded differently to the BP treatments due to the probable cell-specific action of mechanisms. Therefore, it would be normal to expect different results for BMMSCs in comparison with dental-tissue derived stem cells. To clarify this confusion, BMMSCs should also be tested in further studies to elucidate potential source-specific activity of BPs. Another explanation for this issue would be donor dependent characteristics of the stem cells. Therefore, additional work with extended donor lists should be conducted to answer this question.

DSCs respond to injury in the oral cavity by migrating and differentiating into dentin producing odontoblasts [141]. Conditions leading to tissue loss in the local environment enable growth factor and cytokine release and these chemoattractant molecules provide signal for DSC proliferation, migration and differentiation. Although BPs have beneficial activities to treat osteoporosis, osteonecrosis of the jaw bone has emerged as an important side effect [142].

Therefore, the effects of BPs on migratory characteristics of DPSCs, PDLSCs and hTGSCs were evaluated by scratch assay to identify whether BPs impair migration of DSCs. All three kinds of BPs hindered migration capacity of DPSCs, PDLSCs and hTGSCs *in vitro*. This is the first study in the literature demonstrating the inhibitory activity of BPs on dental derived stem cells. Previous research has already identified BPs as deterrent factors for MSC migration which might explain our results. In a previous study, non-toxic concentration of BPs had a negative effect on human placental MSCs migration indicating the pathogenic activity of BPs for stem cell migration that may impair tissue regeneration [143]. Our study defined BPs as anti-migratory components for DSC cell motility which is in consistent with the literature. In addition to MSCs, proliferation and migration of human myogenic cells which are involved in muscle tissue regeneration, was reduced by ALE via blocking of mevalonate pathway [144]. These findings could be explained by the existence of alternative pathways that are affected by BPs in different progenitor populations.

Further extensive research should be conducted to explore the regulatory pathways of BPs related impaired cell migration.

The elements and structure of the extracellular matrix (ECM) is defined by the secretion of protein components that are important for mediating cell migration [145]. Collagen, fibronectin and laminin are three main ECM proteins [146]. ECM proteins are not only involved in general cell migration [145] and wound healing process ,but also provides crucial support to the dental tissues. The role of fibronectin on ameloblast cell proliferation and differentiation have already published [147]. Laminin is important for odontoblast differentiation [148]. Collagen is required for cell proliferation and migration [149]. Because inhibitory activity of BPs in cell migration analyses by scratch assay was observed in DSCs cultures, COL1A1, FIBRONECTIN and LAMININ levels were investigated by gene and protein expression experiments to identify the role of BPs on ECM proteins. The results of gene and protein expression analyses substantially overlapped and significant reduction was observed for COL1A1, FIBRONECTIN and LAMININ. Our findings indicate that BPs decreased the production of important ECM components by DSCs that might impair the cell migration and tissue regeneration in the jaw bone resulting in osteonecrosis.

The role of cytokines on cell migration and tissue remodeling is a complex issue that controls the recruitment of different cell types, cell proliferation and differentiation [150]. The role of pro-inflammatory cytokines on DPSCs odontogenic differentiation has been evaluated in a previous study [151]. Moreover, odontoblast related cytokine secretion from DPSCs has been profiled and paracrine signals provided by DPSCs regulated dentin-pulp complex regeneration pointing the importance of cytokines in DSC differentiation and tissue restoration. Therefore we evaluated the cytokine expression profile of DSCs after BPs treatment to explore the potential regulatory role of BPs on paracrine signaling between DSCs and jaw bone. MCP-1 as a pro survival cytokine secreted by MSCs have been linked to the PI-3 kinase mediated proliferation pathways [152]. In the current study, BPs reduced the expression of MCP-1 suggesting the blockage of survival pathways.

Similar inhibitory activity of BPs was observed for IGF-1 which has been shown to enhance proliferation migration capacity of osteoblast [153]. In another published research IGF-1 expressing MSCs promoted fracture healing and enhanced bone formation through

paracrine and autocrine effects [154]. BPs reduced the expression of IGF-1 in the current study that might explain the negative effect of BPs on DSC supported bone regeneration Previously reported that, RANTES, SDF-1, EGF and Eotaxin induce migration of MSCs [155]. Reduction of the expression of these cytokines by BP treatment could explain both decreased cell migration and potential negative activity in bone regeneration in the oral cavity.

The appropriate organization of ECM is important for many processes including development, tissue repair and cellular functions. Matrix metalloproteinases (MMPs) regulates ECM turn-over and maintains tissue homeostasis. TIMPs are endogenous inhibitors of metalloproteinases which controls ECM catabolism and tissue integrity. TIMPs cause cell growth arrest and induces apoptosis for many cell types including neurons and cancer cells [156]. Furthermore, TIMP-1 and TIMP-2 have inhibitory effect on migration activity of BMSCs [157]. BPs increased the expression of these two metalloproteinase inhibitor. Upregulated expression of TIMP-1 and TIMP-2 could block migration of DSCs to the injury site.

For a complete tissue regeneration, along with having growth factor driven differentiation and migration of progenitor cells to the restoration area, appropriate vascular bed should be established to support and maintain the metabolic needs of the newly forming tissue mass [158]. Thus, efficient angiogenesis should be concomitant with the cell proliferation and differentiation to cope with bone degeneration or damage. As in case of a possible angiogenesis interruption activity of the BPs, jaw osteonecrosis would be further advanced. To test this hypothesis, the impact of non-toxic concentrations of ZOL, ALE and RIS on angiogenesis was investigated using aortic ring and tube formation assays. The data showed a strong inhibition of vessel sprouting and tube formation of endothelial cells. In line with these results, BP treatment hindered angiogenic activity of HUVECs [152, 153] and human dermal microvascular endothelial cells (HDMECs) in *in vitro* conditions [154]. Angiogenesis inhibitory activity of BPs was attributed to the blocking of endothelial function and survival-related pathways such as ERK1/2, JNK, Rock, FAK and PI-3K/AKT/mTOR signaling pathways [159, 160]. Moreover, cancer patients treated with BPs displayed lower angiogenic activity and reduced levels of angiogenesis related proteins in their serum including VEGF, interleukin-17, and insulin growth factor-1 (IGF-

1) [161-164]. Other than direct interference with endothelial cells, progenitor cells which secret paracrine factors regulating blood vessel formation were also claimed to be affected by BP administration. A previous study indicated that ZOL suppressed angiogenesis and osteogenesis by obstructing osteoclasts formation and secretion of platelet-derived growth factor (PDGF)-BB [165].

Similarly, ZOL and ALE treatments were noted to reduce VEGF and angiopoietin-1 expression levels in osteoblast progenitor cells [166]. As a different point of view, growth factor array conducted with all three dental-tissue derived stem cells used in the current study displayed lower levels of TIMP-1 and TIMP-2 protein levels which might be possible partial explanation for anti-angiogenic activity in the jaw bone marrow, as anti-angiogenic action of these inhibitors has been well-established in the literature [156]. However, neither this study nor the literature do not present the exact mechanism of action for inhibitory effects of BPs on endothelial function. Animal models should be accompanied with more detailed *in vitro* studies are highly warranted to clarify the anti-angiogenic activity.

5. CONCLUSION

The current study evaluates the negative effects of BPs on different types of DSCs as a possible reason for BIONJ. Up to now, the researchers have proposed several circumstances for BIONJ including inhibition of local macrophage function, occurrence of micro-fracture on jaw bone, abnormalities in vascular systems of jaw, infectious inflammatory response, local necrosis due to anti-angiogenic activity of BPs or side effects of adjuvant therapies such as chemotherapy and blocking of physiological bone development. However the activity of BPs on DSCs which contribute to the regeneration of dental tissues and bone, has not been studied in detail yet. We used three different types of DSCs such as DPSCs, PDLSCs and hTGSC to determine the BPs induced behaviors in terms of differentiation, migration and cytokine expression.

The current study showed that Although BPs did not exert negative effects on osteogenic differentiation of DPSCs, PDLSCs and hTGSC, they inhibited migration capacity resulting in poor regeneration in the bone tissue. As jaw bone and dental tissues have close proximity, inhibition of DSC migration to the necrotic area may deteriorate the BIONJ. Additionally, local angiogenesis in the necrotic region is important to provide blood supply which is required for complete healing and regeneration. Anti-angiogenic activity of BPs is mediated by their direct activity on endothelial cells.

Overall, the current work is the first study explaining the role of BPs on BIONJ in terms of DSC activity in different aspects including cell migration, differentiation and cytokine mediated signaling. Further studies are required to explore the potential mechanism of BPs on cell migration and differentiation of DSCs *in vitro* and *in vivo*.

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