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Involvement of growth factors in molecular effects of ibuprofen in dental pulp stem cells

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Abstract
Stem cells represent promising candidates for regenerative therapy of craniomaxillofacial bone defects, where common techniques, such as autogenous bone graft, allografts or others possess shortcomings and limitations in restoring the morphology and function in bone loss. The efficacy of regenerative therapy with mesenchymal stromal cells (MSC) depends on a combination of the interactions between transplanted MSCs and cellular and molecular components of the recipient, and any current pharmacotherapy in the recipient with effects on transplanted MSC and the bone microenvironment. In the present investigation, dental pulp stem cells (DPSC) were isolated from human impacted third molar teeth. DPSC were treated with ibuprofen *in vitro* at clinically relevant concentration and relative expression of selected genes were assessed. Our preliminary data suggest a significant effect of ibuprofen as indicated by upregulation of the relative expression levels of growth factors, vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). While the effects of stem cell therapy in bone regeneration are being investigated in ongoing clinical trials, the effects of commonly used pharmacotherapy should be studied for its potential impact on the paracrine effects of stem cells and consequently bone regenerative processes.

Key words: bone defects, dental pulp stem cells, regenerative therapy

Introduction

Restoration of extensive bone loss and defects in the craniomaxillofacial area is challenging due to complex three-dimensional structural needs and remains an unaddressed challenge in modern medical science. Common techniques, such as autogenous bone grafts, allografts, xenografts or bioactive materials have certain advantages but also possess shortcomings, including limitations in restoring the morphology and function in bone loss [1]. Mesenchymal stromal cells (MSCs) were recently used in clinical studies to facilitate bone healing and present a promising new biomedical technology approach [2–4]. However, the efficacy of treatment using stem cells remains as a major challenge in establishing new approaches to optimize-MSC based bone regeneration. The efficacy of regenerative therapy depends on a combination of the interactions between transplanted MSCs and cellular and molecular components of the recipient, and any current pharmacotherapy in the recipient with effects on transplanted MSC and the bone microenvironment.

Bone healing

Bone healing is typically accompanied by complications arising from complex processes including inflammation, tissue repair and re-modelling, with the engagement of many intracellular pathways. Angiogenesis, the sprouting of new blood vessels from existing ones, is a highly regulated process that is fundamental for successful healing by providing oxygen and nutrients to the injured site. Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are important for angiogene-

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sis and for mediating crosstalk between various signaling pathways. VEGF is a potent endothelial chemokine and mitogen with its significant role in neovascularization. Upon binding to its receptor which is located on endothelial cells, the cells release matrix metalloproteinases that cleave the surrounding extracellular matrix [5]. As a consequence, VEGF interferes with bone formation, resulting in the lengthening and endochondral ossification as evidenced by expression of its receptors by chondrocytes in the epiphyseal growth plate. The putative role of HGF in bone healing is angiogenesis, where the VEGF signaling pathways can be activated through the HGF receptor called c-MET. This activation induces a similar endothelial cell response without competing with the VEGF surface receptors [6]. Although the role of HGF in the osteogenesis is also not fully elucidated, it has been shown to be expressed during bone healing, promoting the osteogenic differentiation of MSC and upregulating receptors for bone morphogenetic proteins (BMP), thereby stimulating BMP signalling [7].

Characteristics of stem cells

Stem cells possess unique potential for self-renewal and multi-lineage differentiation [8]. For this property, dental or nondental mesenchymal stem cells are commonly used in tooth and periodontal regeneration therapy. MSCs also known as mesenchymal stromal cells are adherent, fibroblast-like cells with the ability to differentiate into distinct mesodermal lineages, which can produce bone, cartilage, fat, and fibrous connective tissue. MSCs are characteristic by a specific profile of surface markers (positive for markers CD73, CD90 and CD105 and negative for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR) [9]. Although MSCs possess multiple differentiation abilities, their main therapeutic mechanism is rooted in paracrine effects. In particular, MSCs secrete a variety of biologically active molecules including cytokines and growth factors which, among others, promote angiogenesis, modulate apoptosis, and suppress inflammation [10].

Non-steroidal anti-inflammatory drugs

Moreover, many other variables exist in the complex process of bone healing when MSCs are used in therapy. It has been demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) have no significant cytotoxic effect on bone marrow stem cells from mice, while the proliferation suppressive effects occurred at concentration covering therapeutic doses (nonselective NSAIDs 10^{-5} M and COX-2 inhibitors 10^{-6} M) [11]. Therapeutic management of pain after surgery is an essential factor that can influence the results of stem cell therapy. The most frequently used medication in the management of minor to moderate postoperative pain in dentistry are non-steroidal anti-inflammatory drugs (NSAIDs). They exert antipyretic, analgesic, and anti-inflammatory effects via a decrease in production of prostaglandins (PGs) and inhibition of cyclooxygenases (COXs). Prostaglandin E2 (PGE2) is produced by prostaglandin synthetase and binds to its G-protein coupled receptor EP1-EP4. Activation of G protein coupled receptors triggers many signaling pathways and affect several transcription factors and gene expression levels that are involved in cell growth, apoptosis, proliferation, immune responses, and angiogenesis [12].

Aims

In the present study, we investigated the effects of ibuprofen on DPSC characteristics, especially its effects on the relative expression levels of growth factors, VEGF-A and HGF.

Materials and methods

Human impacted third molar teeth were collected from healthy donors after obtaining an informed consent in accordance with the Helsinki Declaration and approval of the local ethics committee. The explant technique was used to initialize cell culture and further expansion *in vitro* was varied out in complete culture medium with passaging at 80 % confluence. Expression of surface antigens was quantified in DPSCs using the MSC Phenotyping kit (Miltenyi Biotec, Germany). Ibuprofen (Sigma, Germany) was dissolved in ethanol at 240mM concentration. Cells were cultivated for 24h, 48h and 72h and treated with final concentration of 300μM ibuprofen. Control cells were treated with cultivation medium or 0,1 % ethanol as vehicle control. Total RNA was extracted from DPSCs in passage 9 using Tri reagent (Sigma-Aldrich) and phenol-chloroform extraction, with the verification of total RNA quantity with Qubit RNA XR Assay Kit (Thermo Fisher Scientific, USA). First-strand cDNA was prepared from total RNA using High-Capacity cDNA Reverse kit with RNase inhibitors (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Resulting cDNA was used as a template for quantitative real-time PCR (QuantStudio Real-Time PCR System) to determine

Table 1 | Primers and probes used in quantitative real-time polymerase chain reaction

target gene	accession number of Taqman gene expression assay
VEGF-A	Hs00900055_m1
HGF	Hs00300159_m1
GAPDH	Hs99999905_m1
GUSB	Hs00939627_m1

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the expression level of the selected genes. Glyceraldehyd-3-phosphate dehydrogenase (GAPDH) and β-glucuronidase (GUSB) were used as housekeeping genes. All primers used in the study are listed in Table 1. All statistical analyses were performed using Microsoft Office Excel (2007) or GraphPad Prism version 5.0 (GraphPad Software, San Diego, California USA). Statistical comparisons were performed using the Student t test and the significance threshold was set at $p < 0.05$.

Preliminary results

Considering the evidence suggesting the involvement of NSAIDs in MSC-facilitated bone healing, we investigated the effects of 300μM ibuprofen on gene expression of VEGF-A and HGF in dental pulp stem cells (DPSCs). Our preliminary data suggest a significant influence of ibuprofen on the relative expression levels of VEGF-A and HGF.

Discussion and summary

Research and clinical application of stem cells in the craniomaxillofacial area reached several breakthroughs, however there are multiple issues that require investigation for successful clinical applications and effective bone regeneration. These issues include understanding the impact of commonly used anti-inflammatory drugs on transplanted stem cell characteristics, function and paracrine effects that are important for the regenerative process. Stem cells such as DPSCs can be readily isolated from patient teeth and applied in stem cell therapy. However, given the circumstances of dental treatment using stem cells including pain complications, it is necessary to study the effects of pharmacotherapy for its potential influence on the paracrine effects of stem cells and consequently bone regenerative processes. In the study of Chang et al. showed that anti-inflammatory drugs suppressed proliferation and arrest cell cycle of bone marrow mesenchymal stem cells from mice, but no cytotoxic effect was found. Moreover, no effect was found in the terms of osteogenic differentiation in these cells [11]. Results of Almawi et al. revealed regulation of osteogenic and chondrogenic marker genes in MSC cells by paracetamol and NSAIDs, except diclofenac where no effect was observed [13]. These results demonstrate the urgent need to further investigate the effects of NSAIDs on stem cell properties for successful clinical applications and effective bone regeneration.

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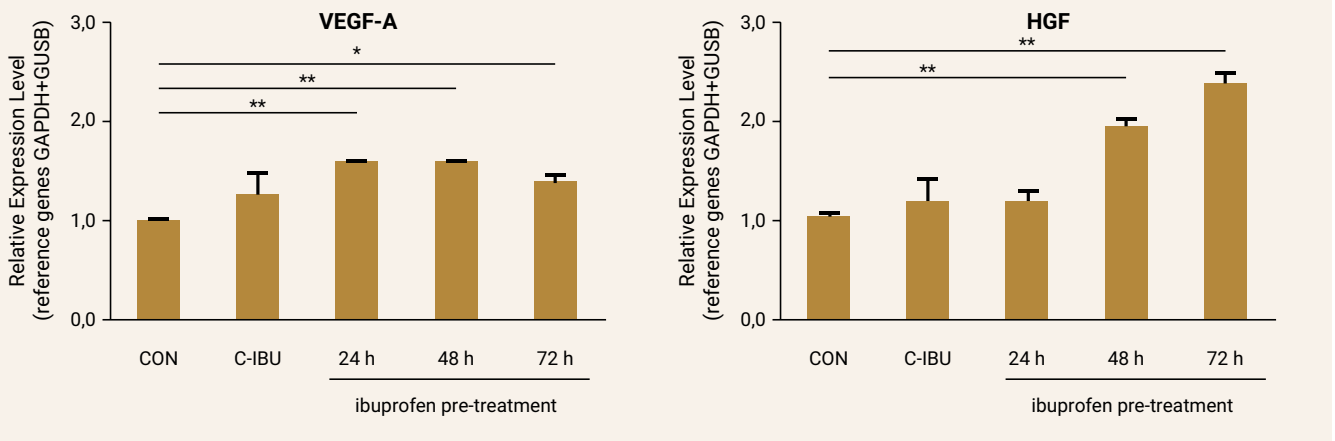
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Fig. 1 | Relative mRNA expression level of VEGF-A and HGF in DPSCs after pre-treatment with ibuprofen. Control cells were treated with cultivation medium (CON) or 0,1 % ethanol as vehicle control (C-IBU). Glyceraldehyd-3-phosphate dehydrogenase (GAPDH) and β-glucuronidase (GUSB) were used as housekeeping genes.



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