Evaluation Study

CALCIUM SULFATE ACTS ON MESENCHYMAL STEM CELLS: AN EXPRESSION PROFILE EVALUATION

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ABSTRACT

Calcium sulfate, with the chemical formula $CaSO_4$, exists in several hydrated and anhydrous forms, including gypsum (calcium sulfate dihydrate), hemihydrate (plaster of Paris), and anhydrite. It is insoluble in water and exhibits excellent thermal stability, making it suitable for high-temperature applications. Calcium sulfate is non-toxic and environmentally friendly, contributing to its widespread use in various fields. For this reason, we studied how calcium sulfate can induce osteoblast differentiation in dental pulp-derived stem cells by measuring the expression levels of bone-related and extracellular matrix-related genes using real-time -Polymerase Chain Reaction (real-time -PCR). The obtained results showed an upregulation of the SP7 and MMP7 genes. Calcium sulfate-based materials are used in dental procedures for socket preservation, guided bone regeneration, and sinus augmentation. In periodontal surgery, calcium sulfate barriers prevent soft tissue ingrowth and promote bone regeneration around dental implants. Calcium sulfate can activate several genes of mesenchymal stem cells, stimulating bone regeneration.

KEYWORDS: *calcium sulfate, dental implants, bone regeneration, gene expression*

INTRODUCTION

Calcium sulfate, with the chemical formula $CaSO_4$, exists in several hydrated and anhydrous forms, including gypsum (calcium sulfate dihydrate), hemihydrate (plaster of Paris), and anhydrite. It is insoluble in water and exhibits excellent thermal stability, making it suitable for high-temperature applications. Calcium sulfate is non-toxic and environmentally friendly, contributing to its widespread use in various fields (1, 2).

Best known for its industrial purposes, calcium sulfate has also found valuable applications in the medical field due to its biocompatibility, resorbability, and versatility. Calcium sulfate serves as a bone graft substitute in orthopedic and dental surgery. Its porous structure and resorbability facilitate bone regeneration by providing a scaffold for new bone

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formation. Calcium sulfate implants gradually dissolve, releasing calcium and sulfate ions that promote osteogenesis and angiogenesis and are eventually replaced by new bone tissue (3).

Calcium sulfate-based hemostatic agents are utilized to control bleeding during surgical procedures. When applied to bleeding sites, calcium sulfate forms a gel-like matrix that accelerates clot formation and promotes hemostasis. Its biocompatibility and absorbability minimize tissue irritation and allow safe resorption without adverse effects (4).

Calcium sulfate acts as a carrier for local drug delivery in various medical applications. Pharmaceutical agents, growth factors, antibiotics, and analgesics can be incorporated into calcium sulfate matrices and delivered directly to target tissues, such as bone defects or periodontal pockets. The controlled release of therapeutic agents from calcium sulfate carriers enhances treatment efficacy while minimizing systemic side effects.

Calcium sulfate-based materials are used in dental procedures for socket preservation, guided bone regeneration, and sinus augmentation. In periodontal surgery, calcium sulfate barriers prevent soft tissue ingrowth and promote bone regeneration around dental implants. Calcium sulfate pastes are also utilized in endodontic therapy for root canal disinfection and obturation. As calcium sulfate has a wide range of applications in regenerative dentistry, we investigated the effect of calcium sulfate on mesenchymal stem cells (MSCs) *in vitro*.

MSCs have garnered significant attention in regenerative medicine due to their multipotent differentiation capacity, immunomodulatory properties, and tissue repair and regeneration potential. Initially identified in bone marrow, MSCs can now be isolated from various tissues, including adipose tissue, umbilical cord blood, and dental pulp (5, 6). MSCs possess several key characteristics that make them valuable for regenerative medicine. MSCs can differentiate into multiple cell lineages, including osteocytes (bone cells), chondrocytes (cartilage cells), adipocytes (fat cells), myocytes (muscle cells), and even neurons (nerve cells). MSCs secrete various cytokines and growth factors that modulate the immune response, reducing inflammation and promoting tissue repair. MSCs have a high proliferation rate, allowing for the generation of sufficient cells for therapeutic use. MSCs can migrate to sites of injury or inflammation, contributing to tissue repair and regeneration (7).

MSCs can be isolated from various tissues, each with advantages and limitations. Bone marrow-derived MSCs (BM-MSCs) are the most well-studied and have been widely used in clinical applications (7, 8). However, the isolation process is invasive and yields limited cells. Adipose-derived MSCs (AD-MSCs) can be obtained through less invasive procedures and yield more cells than bone marrow. Umbilical cord-derived MSCs (UC-MSCs) offer a non-invasive source with high proliferative potential and low immunogenicity. Dental pulp stem cells (DPSCs) are another accessible source of MSCs with potential applications in dental and orthopedic regenerative therapies (9).

MATERIALS AND METHODS

Dental pulp stem cells (DPSCs) isolation

Dental pulp was extracted from the third molars of healthy subjects and digested for 1 h at 37°C in a solution containing 1 mg/ml collagenase type I and 1 mg/ml dispase, dissolved in phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 500 µg/ml clarithromycin. The solution was then filtered using 70 µm Falcon strainers (Merck, Darmstadt, Germany) to separate mesenchymal stem cells from fibroblasts. Stem cells were cultivated in α-MEM culture medium (Merck, Darmstadt, Germany) supplemented with 20% Fetal Bovine Serum (FBS), 100 µM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Merck, Darmstadt, Germany). The flasks were incubated at 37 °C and 5% CO_2, and the medium was changed twice weekly.

DPSCs were characterized by immunofluorescence for the positive mesenchymal stem cell markers CD105, CD90, and CD73 and the negative marker CD34 described in Sollazzo et al. (10).

Cell treatment

DPSCs were seeded at a concentration of 1.0×10^5 cells/ml in 9 cm^2 (3 ml) wells containing DMEM supplemented with 10% serum and antibiotics added with calcium sulfate (Surgiplaster, Classimplant, Roma, Italy) at the concentration of 0.001 mg/ml. Another set of wells containing untreated cells was used as a control. The treatment was carried out at two-time points: 24 hours and 4 days.

The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. At the end of the treatment period, the cells were lysed and processed for total RNA extraction.

RNA isolation, reverse transcription, and quantitative real-time-PCR

According to the manufacturer's instructions, total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Hilden, Germany). Pure RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

cDNA synthesis was performed starting from 500 ng of total RNA using the PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan). The reaction mixture was incubated at 37 °C for 15 min and inactivated by heating at 70 °C for 10 s. cDNA was amplified by real-time quantitative PCR using an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). All real-time-PCR reactions were performed in a volume of 20 µl. Each reaction contained 10 µl of 2X qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, Ltd, London, UK), 400 nM of each primer, and 100 ng cDNA.

Custom primers designed on genes belonging to the "extracellular matrix, adhesion molecule" pathway, "osteoblast differentiation", and "inflammation" pathway were purchased from Merck (Darmstadt, Germany). The selected genes grouped by functional pathway are listed in Table I.

All experiments were performed using non-template controls to exclude reagent contamination. Real time-PCR was performed using two analytical replicates. The amplification profile was initiated by incubation for 10 min at 95°C, followed by a two-step amplification for 15 s at 95°C and 60 s at 60°C for 40 cycles. In the final step, melt curve dissociation analysis was performed.

Table I. *Selected genes tested in real time-PCR grouped by functional pathway.*

Pathway	**Gene**
Osteoblast differentiation	SPP1 (Osteopontin) SPARC (Osteonectin) RUNX2 (Runt-related transcription factor 2) ALP (Alkaline phosphatase) FOSL1 (FOS-like antigen 1) SP7 (Osterix) ENG (Endoglin)
Extracellular matrix, adhesion molecule	COL1A1 (Collagen type I alpha1) COL3A1 (Collagen type III alpha 1) COL4A1 (Collagen type IV alpha 1) MMP7 (Matrix Metallopeptidase 7) MMP12 (Matrix Metallopeptidase 12) MMP14 (Matrix Metallopeptidase 14)
Inflammation	IL1a (Interleukin 1 Alpha) IL1R1 (Interleukin 1 Receptor type 1) IL6 (Interleukin 6) IL6R (Interleukin 6 Receptor)
Reference gene	RPL13 (Ribosomal protein L13)

Statistical analysis

The gene expression levels were normalized to the expression of the reference gene (RPL13) and expressed as fold-changes relative to the expression in untreated cells. Quantification was performed using the delta-delta Ct method.

RESULTS

The DPCSs were phenotypically characterized using immunofluorescence. Fig. 1a shows a cytoskeletal filament stained with vimentin. The cell surfaces were positive for mesenchymal stem cell markers CD90 (Fig. 1b) and CD73 (Fig. 1c) and negative for markers of hematopoietic origin CD34 (Fig. 1d).

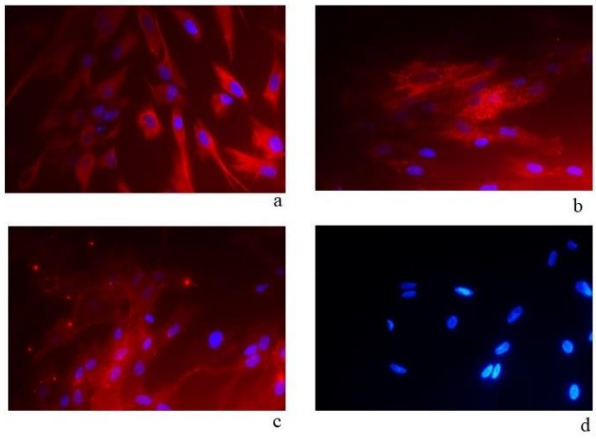

Fig. 1. *DPCSs by indirect immunofluorescence (Rodamine). Immunofluorescence staining of vimentin (a), mesenchymal stem cell marker CD73 (b), CD90 (c), and hematopoietic markers CD34 (d). Nuclei were stained with DAPI. Original magnification x40.*

The effect of calcium sulfate treatment on DPSCs was analyzed in quantitative real-time-PCR after 24 h and 4 days of treatment, respectively, and the expression levels of osteoblast-related genes, extracellular matrix, and inflammation pathways were measured. Table II reports the fold change obtained after 24 h and 4 days.

The analyzed genes are significantly upregulated if they show ≥ 2-fold change in expression. In contrast, significantly downregulated genes show ≤ 0.5-fold change in expression compared to the levels found in untreated cells. In DPSCs, after 24 h of treatment, SP7 was strongly up-regulated, as were MMP7 and IL6. Although with a less pronounced fold change, MMP12, MMP14, and IL1a were also upregulated. Of note, the expression level of FOSL1 increased the most after 4 days (Table II).

Table II. *Expression profile analysis in DPSCs after 24 h and 4 days of treatment with calcium sulfate. Numbers express the fold-changes with respect to untreated cells. In bold, significant gene expression levels. Nd – not determined.*

	24 h	**4 days**
SPP1	nd	nd
SPARC	0.49	0.27
RUNX2	0.56	0.84
ALP	0.22	0.03
FOSL1	1.65	**4.75**
SP7	**20.91**	0.24
ENG	0.83	0.73
COL1A1	0.34	0.25
COL3A1	0.6	0.2
COL4A1	0.4	0.5
MMP7	**5**	0.2
MMP12	**2.7**	0.3
MMP14	**2.7**	2
IL1a	**2.2**	0.2
IL1R1	0.8	1.7
IL6	**3.4**	1.5
IL6R	1.2	0.9

DISCUSSION

Calcium sulfate serves as a bone graft substitute in orthopedic and dental surgery. Its porous structure and resorbable nature facilitate bone regeneration by providing a scaffold for new bone formation. Calcium sulfate implants gradually dissolve, releasing calcium and sulfate ions that promote osteogenesis and angiogenesis, ultimately replaced by new bone tissue. In dentistry, calcium sulfate-based materials are used in dental procedures for socket preservation, guided bone regeneration, and sinus augmentation. In periodontal surgery, calcium sulfate barriers prevent soft tissue ingrowth and promote bone regeneration around dental implants. Calcium sulfate pastes are also utilized in endodontic therapy for root canal disinfection and obturation.

Since bone regenerative activity is driven by mesenchymal stem cells, in this study, we wanted to evaluate the effect of calcium sulfate on DPSC after 24 hours and 4 days of treatment. Therefore, we analyzed the expression profile of genes involved in pathways related to osteoblast differentiation, extracellular matrix, and inflammation. Our results demonstrated that calcium sulfate acts on DPSCs, activating SP7 and MMP7, among other genes.

SP7, also known as Osterix, is an osteoblast-specific transcription factor, a downstream target of RUNX2. It is considered an up-regulator of late osteogenic markers and is required for bone formation and mineralization *in vivo* (11). Indeed, SP7 plays a master role in the maturation of osteoblasts from mesenchymal progenitors and their differentiation into osteocytes (12, 13). Interestingly, in our study, after 24 hours of treatment of DPSCs with calcium sulfate, almost 21-fold changes in SP7 overexpression were achieved.

Collectively, matrix metalloproteinases (MMPs) are capable of degradation of the entire extracellular matrix (ECM). Remodeling of bone tissue is ensured by osteoclasts, which actively degrade the bone matrix using a powerful array of proteinases, including MMPs. We found that three MMPs in our model were significantly overexpressed. Specifically, MMP7, also known as matrilysin-1, is a secreted zinc- and calcium-dependent endopeptidase, considered the smallest MMP enzyme; MMP12, also known as macrophage metalloelastase (MME); MMP14, also known as membrane-type 1 matrix metalloproteinase (MT1-MMP), a transmembrane protein.

MMP7 is considered to be a potent proteoglycanase. Its increased expression observed in osteocytes and collagen bundles of haversian canals during osseointegration has been suggested to reflect its role in maintaining matrix content and turnover and in solubilizing osteoid matrix (14). In our study, it was the second most upregulated gene among those analyzed.

Like other MMPs, MMP12 can degrade a wide range of ECM components, including type I/IV collagen, aggrecan, decorin, gelatin, elastin, fibronectin, laminin, vitronectin, entactin (14). Interestingly, intracellular MMP12 in macrophages has been observed to have antimicrobial activity (15). In addition to its antibacterial and antiviral properties, MMP12 has been implicated in regulating embryonic development, reproduction, tissue remodeling, wound healing, and repair (16-18). It is generally accepted that MMP12 is undetectable in adult tissues, which normally secrete negligible amounts. However, MMP12 expression has been documented in tissues undergoing rapid remodeling, such as the placenta at term during human fetal development, and in several tumor tissues (19, 20). The upregulation of MMP12 found in our treated DPSCs seems consistent with its activation during tissue remodeling, plausibly induced by calcium sulfate.

Emerging evidence suggests that MMP14 is critical in bone formation, influencing osteoblast differentiation, bone matrix remodeling, and skeletal development (21). MMP14 influences osteoblast function and bone formation through its effects on ECM remodeling, cell signaling, and cell-matrix interactions. During osteoblast differentiation, MMP14 facilitates the turnover of the bone matrix by cleaving collagen and other ECM components, promoting the release of bioactive factors that regulate osteoblast activity and mineralization. Additionally, MMP14 regulates osteoblast migration and invasion, facilitating their recruitment to bone formation and remodeling sites.

Moreover, MMP14 indirectly modulates osteoclast activity and bone resorption through its effects on other cell types and ECM remodeling. MMP14 expressed by osteoblasts and osteocytes promotes the release of soluble factors such as RANKL (Receptor Activator of Nuclear Factor Kappa-B Ligand) and osteopontin, which stimulate osteoclast formation and activity (22). MMP14 also interacts with cell signaling pathways involved in osteoclastogenesis and bone resorption, influencing the balance between bone formation and resorption (23).

IL-6 is a major pro-inflammatory cytokine. In bone tissue, at high levels, IL-6 can bind to receptors on osteoclasts, promoting catabolic bone metabolism and increasing the rate of bone resorption. However, an opposite effect of IL-6 on osteoclast differentiation has been described, leading to a reduction in osteoclastogenesis. This would, therefore, lead to less erosion of bone tissue (24).

It should be noted that among the genes analyzed in our study, the expression of FOSL1 reached a 4.75-fold overexpression after 4 days of treatment, not 24 hours. FOSL1, a member of the Fos family of transcription factors, plays

a significant role in various cellular processes, including proliferation, differentiation, and apoptosis. In recent years, FOSL1 has garnered attention for its involvement in bone biology, particularly in osteogenesis, the process of bone formation (25). FOSL1 belongs to the AP-1 (Activator Protein-1) family of transcription factors, characterized by a basic leucine zipper (bZIP) domain that mediates dimerization and DNA binding. It forms heterodimers with members of the Jun family, constituting the AP-1 transcription complex. FOSL1 activity is modulated by various signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway, the Wnt/β-catenin pathway, and the BMP-Smad pathway, which regulate FOSL1 expression and activity during osteogenesis (26). FOSL1 is critical in osteogenesis, influencing osteoblast differentiation, bone matrix mineralization, and bone homeostasis. During the early stages of osteoblast differentiation, FOSL1 cooperates with other transcription factors such as RUNX2 and Osterix to activate the expression of osteogenic genes, including alkaline phosphatase (ALP), osteocalcin (OCN), and collagen type I (COL1A1). FOSL1 promotes osteoblast proliferation and survival, facilitating the expansion of the osteoblast progenitor pool and the formation of mature osteoblasts. Moreover, FOSL1 regulates bone matrix mineralization by modulating gene expression in extracellular matrix (ECM) synthesis and remodeling. FOSL1 promotes the expression of matrix metalloproteinases (MMPs) and other proteases that degrade ECM components, facilitating mineralized matrix deposition. Additionally, FOSL1 interacts with signaling pathways involved in calcium homeostasis and phosphate metabolism, influencing bone mineralization processes (27).

CONCLUSIONS

Calcium sulfate-based materials are used in dental procedures for socket preservation, guided bone regeneration, and sinus augmentation. In periodontal surgery, calcium sulfate barriers prevent soft tissue ingrowth and promote bone regeneration around dental implants. Calcium sulfate pastes are also utilized in endodontic therapy for root canal disinfection and obturation. Calcium sulfate can activate several genes in mesenchymal stem cells, inducing osteoblast differentiation. Specifically, we have shown that 24-hour treatment of DPSCs with calcium sulfate induces more than 20-fold overexpression of SP7, a transcription factor with a master role in osteoblast differentiation and tissue mineralization. It is our knowledge, however, that more research is needed to elucidate the role of calcium sulfate on MSCs fully.

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