Preventive Functions of Hemp-Derived Biomaterials Against Periodontal Deterioration Caused by Fine Dust

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Article

Preventive Functions of Hemp-Derived Biomaterials Against Periodontal Deterioration Caused by Fine Dust

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Abstract: Although fine dust can cause various disorders, including cardiovascular, neurological, renal, reproductive, motor, systemic, respiratory, and cancerous diseases, there are not enough research for an oral disease. In this study we used various methods, including quantitative PCR, flow cytometry, immunocytochemistry, ELISA, and Alizarin O staining, to reveal the preventive effects of hemp-derived biomaterials in periodontal cells against fine dust. In this study, the two biomaterials (MSE; mature hemp stem extract and IE; induced exosomes derived from gingival cells exposed to MSE) revealed three main biological functions against fine dust. First, two materials suppressed the levels of inflammatory genes and activated anti-inflammatory genes in gingival cells. Second, these materials activated differentiation to osteoblast, periodontal ligament cells (PDLCs) and pulp progenitor cells (PPCs) from periodontal ligament stem cells (PDLSCs). Third, IE and MSE activated upregulation of LL-37 and down-regulation of MCP-1 in gingival cells and PDLSCs, respectively. Consequently, MSE and IE showed potential as a functional biomaterial in various fields, including pharmaceuticals, cosmetics, and foods.

Keywords: cannabis; exosome; periodontal disease; PM10; periodontal ligament stem cell

1. Introduction

Fine dust, also known as particulate matter, is found in the air, and the 2.5-μm (PM2.5) and 10-μm (PM10) particles it contains are very harmful to humans and animals [1]. In particular, elderly people and children, as well as patients with respiratory diseases, are more susceptible to fine dust [2,3]. Fine dust can cause various disorders, including cardiovascular, neurological, renal, reproductive, motor, systemic, respiratory, and cancerous diseases [4,5]. Pathogenic bacteria and viruses in PM10 enhance pathogenic infection in the oral environment [6–8]. Moreover, PM10 causes oral inflammation and cancer, as well as various other cancers in human body [9]. Further, gingival inflammation accelerates bacterial platelet aggregation, elevating C-reactive protein and amyloid A fibrinogen in the liver, as well as atherosclerosis in humans [10]. According to a recent study [11], fine dust suppresses the osteogenic differentiation of adipose-derived stem cells. Moreover, fine dust causes dermal tissue inflammation in humans and pets [11]. Skin inflammation harms dermal immunity, leading to adipose-derived stem cells (ASCs) differentiation in the subcutaneous fatty tissues of the skin [11,12]. When exposed to fine dust, dermal cells upregulate apoptotic proteins, including BAX and CytC, and downregulate antiapoptotic proteins, including AKT, P50, P52, and BCL-2 [11].

Dental stem cells are classified into seven types, including dental pulp stem cells, dental follicle progenitor cells, gingival mesenchymal stem cells, periodontal ligament stem cells (PDLSCs), stem

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cells for apical papilla, stem cells from human exfoliated deciduous teeth, and natal dental pulp stem cells [13]. PDLSCs are differentiate into various cell types including cementoblasts, osteoblasts, fibroblasts, neurons, endothelial cells, cardiomyocytes, periodontal ligament cells (PDLCs), and pulp progenitor cells (PPCs) [14]. Periodontitis suppresses PDLSC differentiation into various types of cells [15]. The downregulation of cathelicidin (LL-37 peptide) in inflamed gingival cells enhances periodontium inflammation in neutrophils and various epithelial cells, including connective cells, fibroblasts and gingival cells [16]. Recent reports have shown that cathelicidin suppresses the expression of monocyte chemoattractant protein-1 (MCP-1) in PDLCs under antimicrobial activity [17–19] . Contrary to MCP-1, cathelicidin activates the expression of secretory leukocyte protease inhibitor in PDLCs [20].

Exosomes of approximately 40–100 nm in size are secreted from almost all cell types into the serum, urine, cerebrospinal fluid, ascites fluid, milk, or saliva [21,22]. Exosomes contain various molecules, including functional proteins, carbohydrates, mRNAs, microRNA, and DNA [23]. In general, cells exposed to stimulants, such as phytoextracts, drugs, and pathogens [24], secrete exosomes with dramatically altered components, compared to unstimulated conditions [24]. Altered exosomes can have adverse or beneficial effects on cells [24]. Furthermore, altered exosomes exert various functions, including immune system modulation, prognostic biomarkers for diseases, and cancerous activity in the human body [22,24]. Based on these characteristics, induced exosomes (IEs) are considered as biomaterials in various fields, including the pharma, cosmetics, and food industries [24].

Cannabis sativa, hemp, has various applications in foods, architectural materials, natural drugs, animal bedding and feed, textiles, clothing, bioplastics, and biofuel [25,26]. Recent reports [27] have associated the pharmacological potentials of cannabis with antimicrobial activities, neuroprotection, management of gastrointestinal disorders, anti-seizure, and anti-cancer activities [27]. Matured hemp stem chemicals comprise fibers (23%), proteins (23%), ash (7%), CBD (0.001%), and THC (0.0064%), but few reports have shown their biological activity [28,29]. Phytochemical profiling of ethanol extracts [28,29] shows that tannin, saponin, and phenolic compounds (quercetin, apigenin, and rutin) are significantly higher than those in the stem. Among all hemp components, the stem and roots are regarded as low-value pharmaceutic and cosmetic byproducts. Thus, further research is needed to demonstrate the stem as a high value product.

This research documented the bioactivities of mature hemp stem extract (MSE) and IEs derived from gingival cells exposed to MSE against fine dust in gingival cells and PDLSCs.

2. Results

2.1. Protection of Gingival Cells Against Fine Dust

To evaluate the effects of the two materials dervied from hemp, the cytotoxic concentration (CC_{50}) of gingival cells and PSDLSCs were established for PM16 (PM10 of 16 μg/mL), MSE of 1,600 μg/mL, and IE of 60 ng/mL (Figure 1). Compared with the control, PM10 suppressed the levels of anti-apoptotic genes, including AKT, NFκB-P50, NFκB-P52 and BCL2, whereas MSE increased their levels. Additionally, MSE intensely activated these genes in gingival cells, even under fine dust treatment (Figure 2). The approximate levels of these genes under MSE treatment were 3.8 times higher than those under fine dust treatment (Figure 2). Contrary to these results, MSE suppressed apoptotic genes in gingival cells, and their levels were 2.84 times lower than those fine dust treatment (Figure 2). Notably, compared with MSE, IE was more effective; under fine dust, IE activated anti-apoptotic genes 2.5 times more than MSE (Figure 3). Moreover, apoptotic gene levels were 8.8 times higher than those under MSE treatment (Figure 3).

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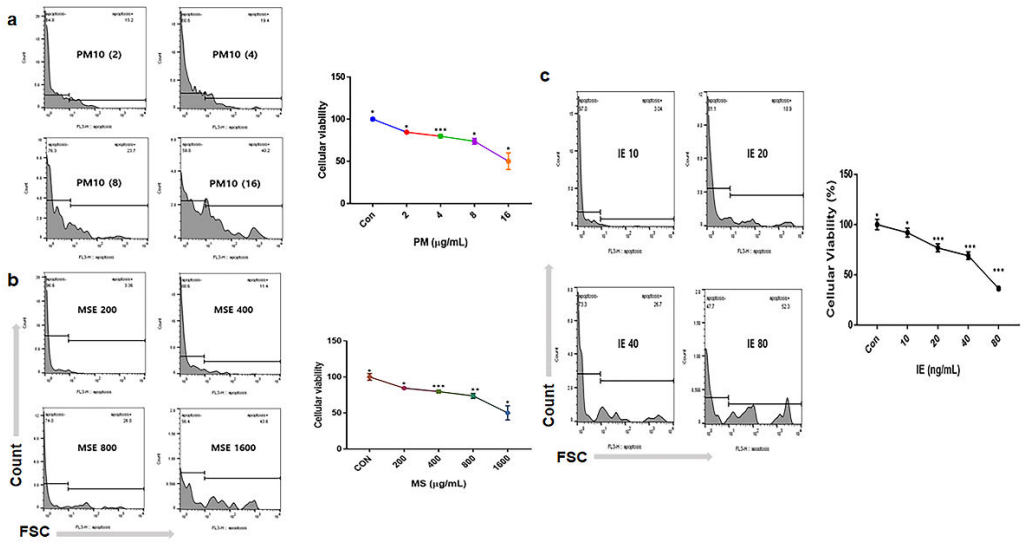

Figure 1. Treatment dosages of fine dust, mature stem extract (MSE), and induced exosomes (IEs). (a, b) Cytotoxicity concentration (CC_{50}) dose of fine dust (PM10) and MSE in gingival cells. (c) IE treatment dose in periodontal ligament stem cells (PDLSCs). Con; control, (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

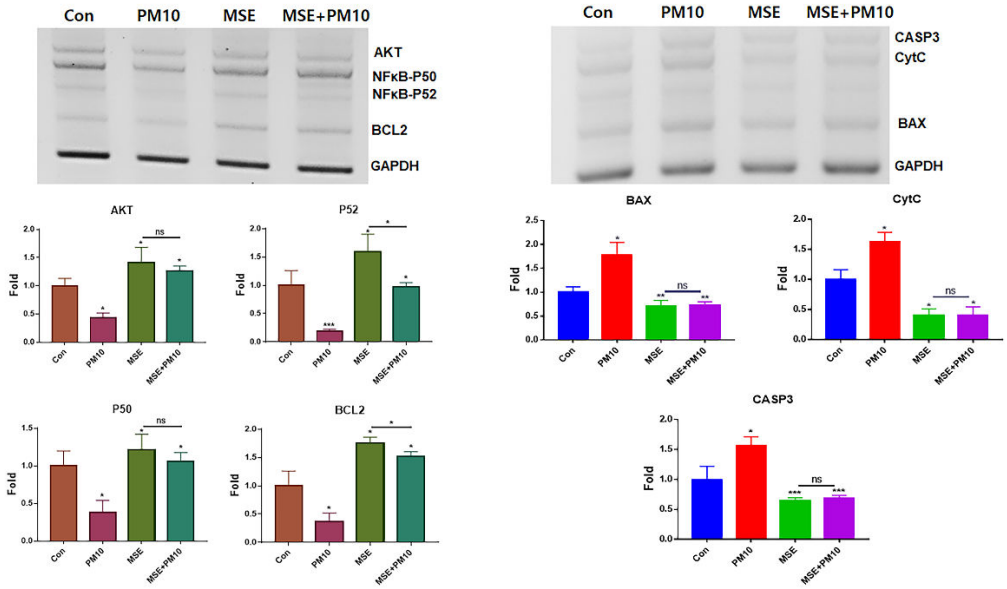

Figure 2. Levels of anti-apoptotic and apoptotic genes in gingival cells under MSE treatment. Levels of apoptotic (BAX, CytC, CASP3) and anti-apoptotic (AKT, NFκB-P50, NFκB-P52, BCL2) genes in gingival cells under MSE and fine dust treatment. MSE+PM10, PM10 treatment after MSE exposure, ns; not significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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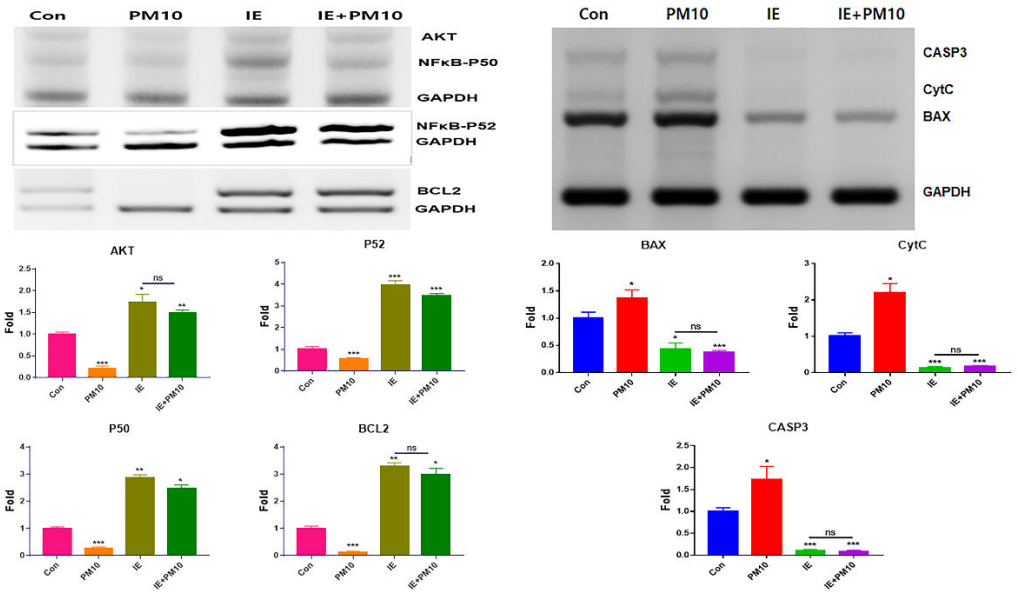

Figure 3. Levels of anti-apoptotic and apoptotic genes in gingival cells under IE treatment.Levels of apoptotic (BAX, CytC, CASP3) and anti-apoptotic (AKT, NFκB-P50, NFκB-P52, BCL2) genes in gingival cells under IE treatment (*p < 0.05, **p < 0.01, ***p < 0.001).

2.2. Activation of Osteogenic Differentiation By The Two Materials

The two biomaterials activated PDLSC differentiation into osteoblasts, PDLCs, and PPCs despite the exposure to fine dust (Figures 4 and 5).

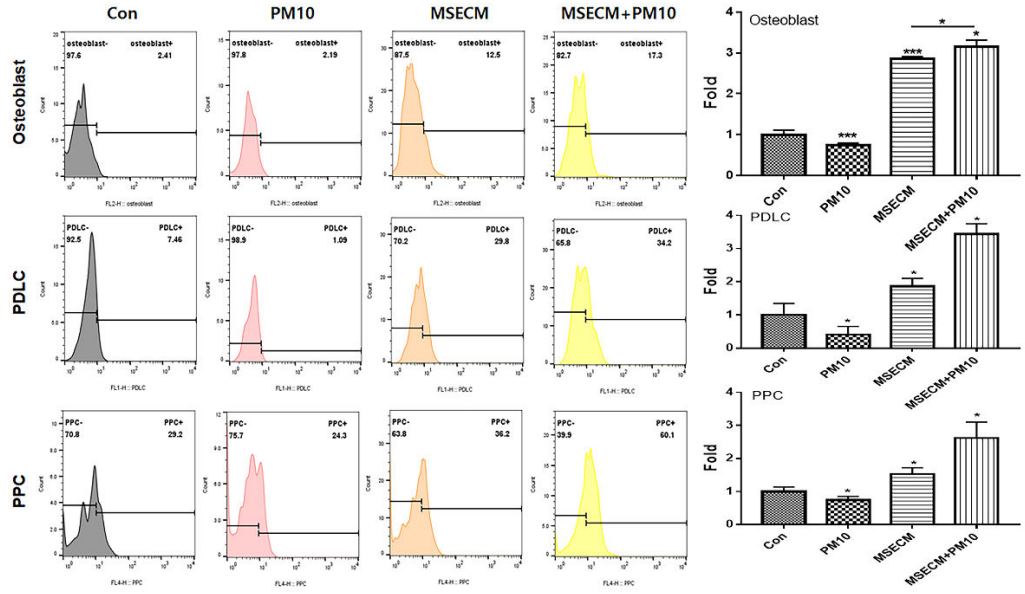

Figure 4. Differentiating patterns of PDLSCs under the MSE-conditioned medium.

Differentiation to osteoblasts, periodontal ligament cells (PDLCs), and pulp progenitor cells (PPC) from PDLSCs. MSECM, matured hemp stem extract conditioned medium; PM10, fine dust (*p < 0.05, **p < 0.01, ***p < 0.001).

The conditioned medium from gingival cells treated with MSE (MSECM) activated differentiation to osteoblasts in PDLSCs. Unlike the effect of fine dust, osteotic differentiation was 3.9 times higher under MSE (Figure 4). Notably, the differentiation was 5.8 times higher under IE despite the exposure to fine dust (Figure 5). Based on the results of alizarin O staining (Figure 6a), IE strongly activated the formation of osteocytic colonies, whose activity was 7.2 times higher than that under

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fine dust (Figure 6a). Compared with MSECM, the values under IE were approximately 1.4 times higher (Figure 6a). These results corresponded with those of flow cytometry (Figure 4 and 5).

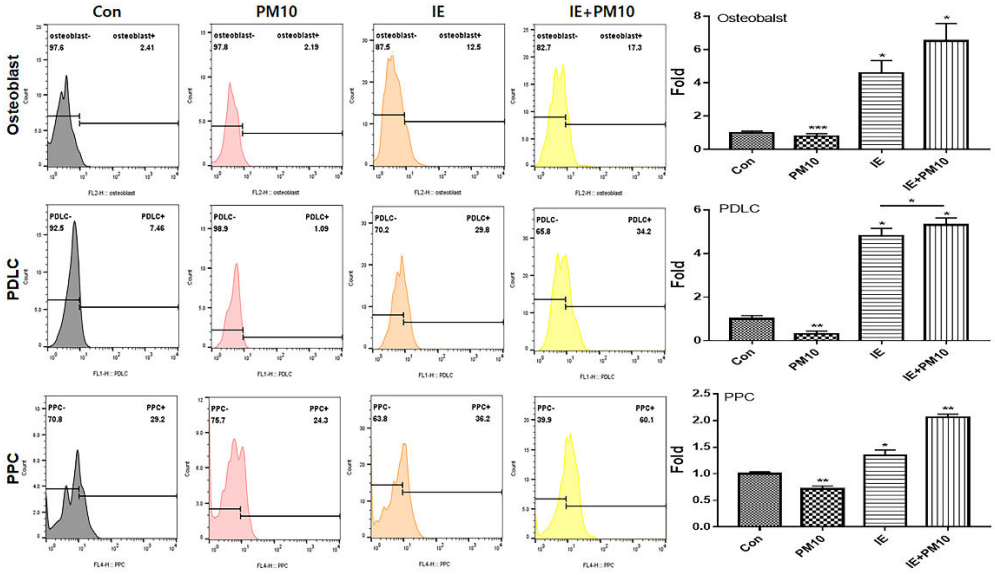

Figure 5. Differentiation patterns of PDLSCs under MSECM and IEs.

Differentiation to osteoblasts, PDLCs, and PPCs from PDLSCs. IE from gingival cells under MSE, IE+PM10; PM10 treatment after exposrue to IE (*p < 0.05, **p < 0.01, ***p < 0.001).

2.3. Activation of PDLC Differentiation by the Two Materials

Among the three cell types, the two materials mostly activated the differentiation to PDLCs under fine dust. Compared with the control, the differentiation induced by MSECM and IE was 1.8 and 4.8 times higher, respectively (Figure 4, 5). Additionally, compared with fine dust, differentiated PDLCs were 4.53 times higher under MSECM (Figure 4). Suprisingly, the values under IE were 3.53 times higher than those under MSECM (Figure 5). In the immunocytochemistry results (Figure 6b), corresponding with the flow cytometry results, IE dramatically activated PDLC differentiation from PDLSCs despite the exposure to fine dust (Figure 6b).

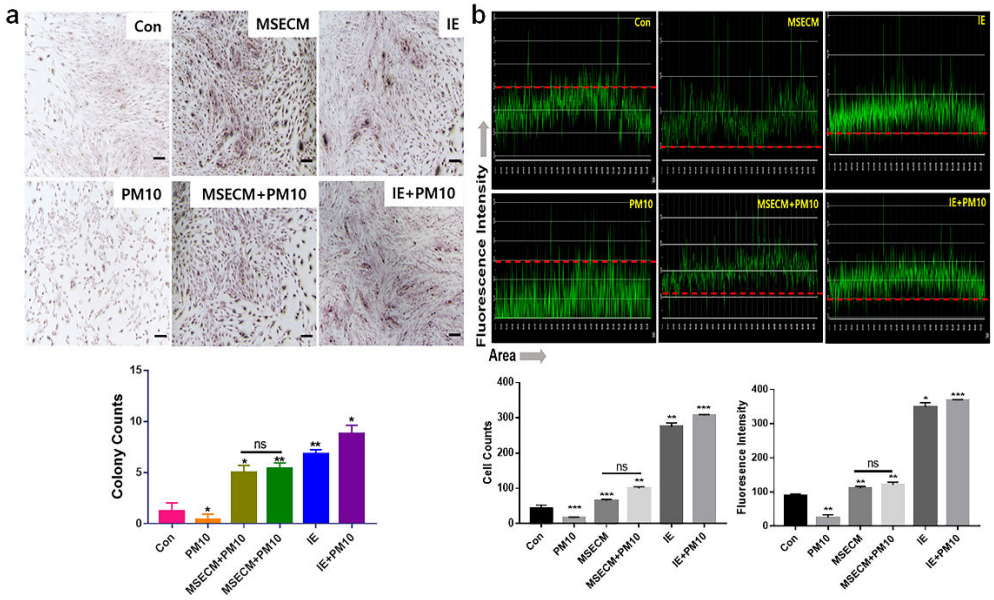

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Figure 6. Immunocytochemistry results for differentiation to osteoblasts and PDLCs under the two biomaterials. (a) Images of osteoblast cells and their colony formation using Alizarin staining. (b) Immunocytochemistry results with the PDLC marker anti-asporin conjugated with green fluorescence. The red dashed lines show the baseline of intensity. The stained cells and colonies are counted using NIS-elements V5.11 software (*p < 0.05, **p < 0.01, ***p < 0.001) (scale bar = 20 μm).

2.4. Activation of PPC Differentiation by the Two Materials

Compared with results of PDLC and PPC, although the differentiation activity to PPCs was the most attenuated under MSECM and IE, the two materials significantly activated differentiation under fine dust. Moreover, contrary to the osteoblasts and PDLC results, PPC differention under MSECM was 1.4 times stronger than that under IE after fine dust treatment (Figures 4 and 5).

2.5. Modulation of Homeostatic Proteins By Two Bio-Materials

Unlike the inactivated expression of MCP-1 in PDLSCs, the two materials activated the expression of cathelicidin antimicrobial peptide (LL-37), a key protein maintaining the dental environment in gingival cells. The levels of LL-37 were 2.2 times higher in gingival cells under MSE (Figure 7a), and MSECM attenuated the levels of MCP-1 approximately 2.7 times compared to those under PM10 (Figure 7b). Interestingly, compared with MSE and MSECM, IE increased the expression of LL-37 approximately 8.2 times in gingival cells and attenuated MCP-1 in PDLSCs approximately 4.2 times (Figure 7a, b).

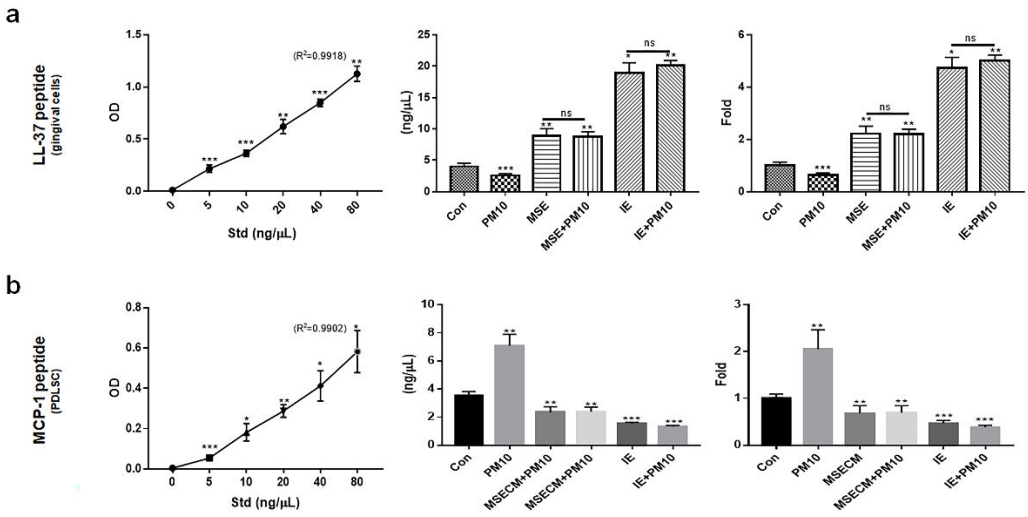

Figure 7. Expression of homeostatic proteins under the two biomaterials. (a) Levels of LL-37 peptide in gingival cells under various conditions. (b) Levels of MCP-1 protein in PDLSCs under various conditions (*p < 0.05, **p < 0.01, ***p < 0.001)

3. Discussion

Recently, the amount of fine dust has been rapidly increasing due to environmental pollution. Fine dust causes various diseases, including respiratory, dermal, and ophthalmic disorders. However, research on dental disorders caused by fine dust is insufficient. In this study, two biomaterials derived from hemp revealed their biological functions against fine dust. The three main biological functions included anti-inflammatory, activation of differentiation, and modulation of proteins associated with dental environment maintenance.

For their anti-inflammatory activity, the two materials suppressed the levels of inflammatory genes and activated anti-inflammatory genes in gingival cells despite the exposure to fine dust (Figure 2, 3). Compared with MSE, IE strongly suppressed apoptotic genes and activated anti-apoptotic genes (Figure 2, 3). Chronic inflammation by gingivitis leads to various disorders, including cardiovascular, respiratory, diabetes, Alzheimer's, cancer, multiple myeloma, and microbial

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carcinoma diseases [30]. According to recent reports [31,32], every 5 μg/m^3 increase in PM10 accelerates periodontitis occurrence and heavy metals in fine dust, causing degradation of the collagen matrix, inhibition of mineralization, and calcium resorption in the dental environment. In addition to outdoor fine dust pollution, indoor air pollution threatens the health of humans and pets [33,34]. Our results (Figures 2, 3) suggest that the two biomaterials are suitable for prevention of periodontitis

Second, the two materials protected and activated the differentiation of PDLSCs despite the exposure to fine dust. These materials activated differentiation to osteoblast, PDLCs and PPCs from PDLSCs (Figures 4-6). PDLSCs are promising cells for periodontal regeneration, osteogenesis, neural damage repair, and periodontal therapy [35,36]. PDLCs are a key player in periodontium regeneration therapies, which use these cells for safe and fast recovery after treatment [37]. In implant therapy, suppression of peri-implantitis is crucial for the therapy success rate [38], and bone loss by inflammation increases its rate of failure. Additionally, osseointegration by the activation of osteogenesis enhances the anchoring and integration of implant fixtures [39]. For these reasons, the reported bioactivities of MSE and IE are crucial for preventing periodontitis and increasing implant success rate. PPCs differentiate into various types of cells including adipocytes, myocytes, osteocytes, chondrocytes, odontoblast-like cells, and neural cells [40]. Our results (Figures 4, 5) showed that differentiation to PPCs from PDLSCs was the most attenuated under treatment with the two materials. This may be attributed to the fact that that IE and MSE activate differentiation to PDLCs and osteoblasts from differentiated PPCs. Dental pulp plays various roles, including blood supply, maintenance of hemostasis under various stimuli, dentin formation, and providing nourishment and moisture to the teeth [41]. The functions of the two materials helps to maintain a healthy pulp and protect the pulp against fine dust.

Third, the two materials enhanced the expression of modulating proteins for a healthy dental environment. Despite the exposure to fine dust, IE and MSE activated upregulation of LL-37 and down-regulation of MCP-1 in gingival cells and PDLSCs, respectively (Figure 7a, b). Although the two materials significantly suppressed MCP-1 protein in PDLSCs, LL-37 was dramatically upregulated by IE (Figure 7a). The LL-37 peptide plays various roles, including oral microbiota control, cancer pro-apoptotic activity, immunomodulation, antimicrobial activity, and promotion of wound healing [42]. This protein maintains oral cavity homeostasis through its biological functions. Compared with healthy condition, concentration of LL37 is 2 times higher in blood during a pro-apoptotic state and the concentration is 8 times during anti-proliferative state in chronic apical periodontitis [42,43]. MSE and IE increased the concentrations of LL-37 approximately 4 and 10 times, respectively, compared with the controls (Figure 7a). Interestingly, these results suggest MSE and IE control the pro-apoptotic and anti-proliferative states, respectively, besides maintaining homeostasis in the oral cavity. The MCP-1 protein is involved in various diseases, including Alzheimer's, Parkinson's, multiple sclerosis, diabetes, tuberculosis, COVID-19, and rheumatoid arthritis [44]. In the oral environment, LL37 secreted from gingival cells suppresses the expression of MCP-1 in PDLCs [45]. Remarkably, compared with the PM10 conditions, IE and MSE dramatically suppressed the expression of MCP-1 (Figure 7b). These results suggest that two materials maintain the homeostatic environment by modulating these two proteins in oral cavity.

4. Materials and Methods

4.1. Cell Culture and Cytotoxicity Test to Establish the Treatment Dosage

After human normal gingival cells (PCS-201-018, ATCC, VA, USA) were cultured in their complete growth media kits (PCS-201-030 and PCS-201-041, ATCC) at 37 °C and 5% CO_2, the cultured cells were treated with MSE, fine dust, and ERM-CZ100 (ERM, Belgium) for 1 day at 37 °C and 5% CO_2. The MSE was prepared via evaporation from 50% ethanol and steamed extraction of matured hemp stem from Cheongsam cultured in Andong City, Korea. IEs were isolated from gingival cells under the established concentration (1600 μg/mL) of MSE. The cultured PDLSCs (SKU: 36085-01and M36085-01S, Celprogen, Torrance, CA, USA) were exposed to IEs to establish the treatment dosage.

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The cytotoxicity was evaluated using a flow cytometer (BD FACScalibur, BD Biosciences, CA, USA) and FlowJo 10.6.1 (BD Biosciences) to establish a cytotoxic concentration (CC_{50}).

4.2. Anti-Apoptotic Activity of MSE

Total RNA in the cultured gingival cells under various conditions (Con, MSE, PM10, MSE+PM10) was isolated from the treated cells using RiboEx reagent (GeneAll, Seoul,Korea), and cDNA was synthesized from the isolated RNA using Maxime RT PreMix (iNtRON, Seongnam, Korea). The cDNA was amplified with primers (Table 1) at the following cycling parameters: 1 min at 95 °C, followed by 35 cycles of 35 s at 59 °C, and 1 min at 72 °C. The amplified DNA was estimated using iBright FL1000 and iBright Analysis Software 4.0.0 (Invitrogen).

4.3. Anti-Apoptotic Activity of IE

Total RNA in the cultured gingival cells under various conditions (Con, IE, PM10, IE+PM10) was isolated from the treated cells using RiboEx reagent (GeneAll, Seoul,Korea), and cDNA was synthesized from the isolated RNA using Maxime RT PreMix (iNtRON, Seongnam, Korea). The cDNA was amplified with primers (Table 1) at the following cycling parameters: 1 min at 95 °C, followed by 35 cycles of 35 s at 59 °C, and 1 min at 72 °C. The amplified DNA was estimated using iBright FL1000 and iBright Analysis Software 4.0.0 (Invitrogen).

4.4. PDLSC Differentiation Patterns Under MSECM

After PDLSCs (Celprogen, Torrance, CA, USA) were cultured in specific media (SKU: M36085-01S) under various conditions (Con, PM10, MSECM, and MSECM+PM10) for a day, the cultured cells were fixed in 2% paraformaldehyde for 4 h and treated with 0.02% Tween 20 for 5 min. The treated cells were incubated with three fluorescence-conjugated immunoglobulins, FITC-anti-asporin (Abbexa, Cambridge, UK), PE-anti-osteopontin (R&D Systems, MN, USA), and APC-anti-cytokeratin 4 (biorbyt, Cambridge, UK) at 37 °C for 2 days. The stained cells were evaluated using a flow cytometer (BD FACScalibur), FlowJo 10.6.1 (BD science) and Prism 7 (GraphPad, CA, USA).

4. 5. PDLSC Differentiation Patterns Under IE

After PDLSCs (Celprogen, Torrance, CA, USA) were cultured in specific media (SKU: M36085-01S) under various conditions (Con, PM10, IE, and IE+PM10) for a day, the cultured cells were fixed in 2% paraformaldehyde for 4 h and treated with 0.02% Tween 20 for 5 min. The treated cells were incubated with three fluorescence-conjugated immunoglobulins, FITC-anti-asporin (Abbexa, Cambridge, UK), PE-anti-osteopontin (R&D Systems, MN, USA), and APC-anti-cytokeratin 4 (biorbyt, Cambridge, UK) at 37 °C for 2 days. The stained cells were evaluated using a flow cytometer (BD FACScalibur), FlowJo 10.6.1 (BD science) and Prism 7 (GraphPad, CA, USA).

4.6. Immunocytochemistry for Osteoblasts

After PDLSCs were cultured under various conditions (PM10, MSECM, MSECM+PM10, IE, or IE+PM10), the cultured cells were fixed with 2% paraformaldehyde for 12 h and stained using Alizarin O reagent (Sigma, St. Louis, MO, USA) for 40 min. The stained cells were evaluated using a fluorescence microscope (Eclipse Ts-2, Nikon, Shinagawa, Japan) and imaging software, NIS-elements V5.11 (Nikon)

4.7. Localization of PDLSC Markers Using Immunocytochemistry

After PDLSCs were cultured under various conditions (Con, PM10, MSECM, MSECM +PM10, IE, or IE+PM10), the cultured cells were fixed in 2% paraformaldehyde for 12 h and treated with 0.02% Tween 20 for 10 min. The treated cells were incubated with three fluorescence-conjugated immunoglobulins and FITC-anti-asporin (Abbexa, Cambridge, UK). The stained cells were evaluated

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using a fluorescence microscope (Eclipse Ts-2, Nikon, Shinagawa, Japan) and imaging software, NIS-elements V5.11 (Nikon)

4.8. Homeostatic Modulator Concentrations

After the cultured macrophages were exposed to the four types of IEs (Con, PM10, MSECM, MSECM +PM10, IE, and IE+PM10) for one day, their culture media was isolated. Cytokines in the isolated media were evaluated using LL-37 and MCP-1 ELISA kits (Novus Biologicals, USA and Thermo Fisher Scienfitic) and a microplate reader (AMR-100; Allsheng, Hangzhou, China).

4.9 . Statistical Analysis

All experiments were analyzed using one-way analysis of variance (ANOVA) with post hoc (Scheffe's method) and *t*- tests using Prism 7 software (GraphPad, San Diego, CA, USA).

Table 1. Sequences for PCR primers.

Gene	Seq (5′→ 3′)
AKT	F: GGCTGCCAAGTGTCAAATCC R: AGTGCTCCCCCACTTACTTG
NFκB-P50	F: CGGAGCCCTCTTTCACAGTT R: TTCAGCTTAGGAGCGAAGGC
NFκB-P52	F: AGGTGCTGTAGCGGGATTTC R: AGAGGCACTGTATAGGGCAG
Bcl2	F: CTGCTGACATGCTTGGAAAA R: ATTGGGCTACCCCAGCAATG
BAX	F: AGCGCTCCCCCACTTACTTG R: GACAGGGACATCAGTCGCTT
CytC	F: ATGAATGACCACTCTAGCCA R: ATAGAAACAGCCAGGACCGC
CASP3	F:TCCCTGGGAAGAAAGAGTTGTGG R:TGAACATGGCACCTCTGCAAC
GAPDH	F: GTGGTCTCCTCTGACTTCAACA R: CTCTTCCTCTTGTGCTCTTGCT

5. Conclusions

Based on our *in vitro* research, MSE and purified IEs from gingival cells under the extract displayed their biological functions for dental health against fine dust. Although the biological functions of the two materials were excellent regarding anti-inflammation, activation of differentiation, and modulation of dental environment, the functions of IE against fine dust were more notable.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: full gels.

Author Contributions: Conceptualization and methodology: Eunhee Kim, Yoon jin Park; Writing—original draft preparation, writing—review and editing, supervision: Mihae Yun and Boyong Kim. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable. This study did not involve humans or vertebral animals.

Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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