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Original Research

The Effect of an Extract of Sappanwood, Protosappanin A and Protosappanin B on Osteogenesis in Periodontitis

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Abstract

Background: Sappanwood is widely used in the prevention and treatment in diseases due to its ability to seal blood vessels, dissipate stasis, and relieve pain. Important monomer components of sappanwood, Protosappanin A (PA) and Protosappanin B (PB) have anti-tumour and antimicrobial medicinal properties. This study investigated the anti-inflammatory and osteogenic differentiation effects of a crude extract of Sappanwood (ESP), PA and PB against periodontitis in periodontal ligament stem cells (PDLSCs). **Methods**: Oil Red O staining was used to assess the ability of adipocytes to differentiate. Alizarin Red staining was used to assess the capacity to differentiate into osteoblasts. Third-passage PDLSCs were grown in either basic medium alone or basic media with varying doses of ESP (0.0625 mg/mL, 0.03125 mg/mL and 0.125 mg/mL), PA and PB (2.5 µM, 5 µM, 10 µM). The CCK-8 assay was used to measure cell proliferation. Real Time PCR (RT-qPCR) and Enzyme-Linked Immunosorbnent Assay (ELISA) assay were used to measure gene expression. The capacity to differentiate into osteoblasts was evaluated using Alizarin Red staining, and Alkaline Phosphatase (ALP) staining and activity. **Results**: The development of lipid droplets and mineralized nodules was examined using Oil Red O staining and Alizarin Red staining. Flow cytometry revealed that PDLSCs were CD29 (98.23%) and CD44 (98.81%) positive, but CD34 (0.16%) and CD45 (0.09%) negative. CCK-8 assay showed that ESP at three concentrations (0.03125 mg/mL, 0.0625 mg/mL and 0.125 mg/mL) and 2.5 µM, 5 µM and 10 µM PA and PB had no cytotoxicity at 5 and 7 days ($p < 0.05$). qRT-PCR and ELISA assay indicated that ESP, PA and PB downregulated the inflammatory cytokines IL-8, IL-6, IL-1β, IL-10 and IL-4 and elevated the mRNA expression of osteogenesis cytokines RUNX2 , OSX and OCN in PDLSCs ($p < 0.05$). Alizarin red staining, and ALP staining and activity showed that ESP, PA and PB increased mineralized nodules and the ALP content of in PDLSCs ($p < 0.05$). **Conclusions**: ESP, PA and PB can reduce the inflammatory response and amplify the osteogenic differentiation of PDLSCs. Therefore, ESP, PA and PB may have potential pharmacological effects in controlling the progression of periodontitis and promoting periodontal tissue regeneration.

Keywords: periodontitis; periodontal membrane stem cells; sappanwood; Protosappanin A and Protosappanin B; osteogenic differentiation

1. Introduction

Periodontitis is defined by periodontal tissue destruction and may lead to tooth loss. Currently, engineering and life sciences research in tissue engineering have provided methods to replace missing alveolar bone, periodontal ligament, and root cementum [1].

The periodontal ligament is rich in periodontal ligament stem cells (PDLSCs). These cells are capable of multinomial differentiation and self-renewal [2]. Studies have shown that PDLSCs maintain dynamic periodontal balance and regulate periodontium regeneration. The interaction between PDLSCs and the peripheral periodontitis niche is important for periodontal tissue repair. Injured PDLSCs may disrupt the microenvironment by exacerbating the host immune response, resulting in abnormal angiogenesis, and promoting osteoclast activity [2]. The periodontal regenerative capacity of PDLSCs is impaired in the inflammatory microenvironment [3]. Therefore, regulation of the inflammatory response for PDLSCs is crucial for the generation of periodontal tissue. A vital component of Sappanwood, Protosappanin A (PA), may prevent atherosclerosis by inhibiting NF-B signalling, hyperlipidaemia, and inflammation in hyperlipidaemic rabbits.

Sappanwood, a red dye in ancient China, has the advantages of bright colours and can easily be produced. Studies have documented the antitumor effects and regulatory apoptotic effects of Sappanwood [4,5]. Sappanwood also plays a neuroprotective role through multitarget pharmacological mechanisms and prevents brain injury caused by ischemia/reperfusion [6]. Sappanwood has the advantages of low price, convenient extraction and preparation, and a wide range of materials and sources. This dye also has more research value because of its good efficacy and low

cytotoxicity as a natural medicine [7,8]. As a vital component of Sappanwood. Protosappanin A (PA) may prevent atherosclerosis by inhibiting NF-κB signalling, hyperlipidaemia, and inflammation in hyperlipidaemic rabbits [9]. Additionally, PA protects against osteoporosis by partially lowering the formation of reactive oxygen species (ROS) in RAW264.7 cells [10]. Protosappanin B (PB) was found to have anti-inflammatory activity [11]. However, an extract of Sappanwood (ESP), PA, and PB have not been used to prevent or cure periodontal diseases. Thus, in this study, we examined the impact of ESP, PA, and PB on the inflammatory response and osteogenic differentiation of PDLSCs.

2. Materials and Methods

2.1 Materials

ESP was purchased from Yunnan Hongxiang Yitang Pharmaceutical (Yunnan, Kunming, China) in China and dissolved it in pure sterile water for cell treatment. We extracted medicinal materials by heating them at 100 °C and boiling them for 24 hours in water as a solvent. PA and PB were purchased from Yunnan Xili Biotechnology (Yunnan, Kunming, China) and dissolved in DMSO (Solarbio, Beijing, China) for cell treatment. We used 10 µg/mL lipopolysaccharides (LPS) from Porphyromonas gingivalis (Invitrogen, Carlsbad, CA, USA).

2.2 Isolation and Culture of PDLSCs

Permission to conduct this study was obtained from the Kunming Medical University School's ethical committee (no. KYKQ2021MEC025). PDLSCs were removed from premolars without caries and periodontitis. These samples were obtained from healthy individuals who voluntarily agreed to be part of this study (donors' age: 14–18 years of age). The extracted teeth were submerged in a phosphate-buffered saline (PBS) solution consisting of 10% antibiotics (Solarbio, Beijing, China). After three PBS washes, the periodontal membrane was scraped off one-third of the root under aseptic conditions. The small pieces of periodontal tissue were digested with collagenase I (3 mg/mL, Gibco, Grand Island, NE, USA) and Dispase II (4 mg/mL, Sigma Chemical Co. St. Louis, MO, USA) for 30 min. The cells were maintained in basic medium with 89% alpha minimal essential medium (αMEM, Biological Industries, Kibbutz Beit Haemek, Israel), 10% foetal bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel) and 1% antibiotics solution (Solarbio, Beijing, China). All investigations involved cells from the third passage. We established the experimental groups to determine the effects of ESP, PA and PB on the inflammatory response and osteogenic differentiation of PDLSCs. Blank control groups (basal medium, DMSO $\leq$0.1% V/V), ESP groups (0.03125 mg/mL, 0.0625 mg/mL and 0.125 mg/mL), and PA and PB groups (2.5 µM, 5 µM, 10 µM) were used.

2.3 Cell Surface Marker Measurement by Flow Cytometry

Markers on the cellular surfaces of the PDLSCs were examined utilizing the flow cytometry approach. We followed the guidelines provided by the manufacturer (Agilent NovoCyte, Santa Clara, CA, USA). We used PBS to wash the trypsinised cells and cultured them with the markers CD44, CD105, CD34 and CD45 (Abcam, Wales, UK) for 20 min at 4 °C in complete darkness. The outcomes were examined using the NovoExpress program (version1.4.1, Santa Clara, CA, USA).

2.4 Multipotent Differentiation Assays

We changed the medium to adipogenic or osteogenic medium from the wells after 2–3 weeks to assess adipogenic or osteogenic differentiation. Cells were treated in osteogenic medium with vitamin C (50 µg/mL, Sigma, Chemical Co. St. Louis, MO, USA) and β-sodium glycerophosphate (10 mmol/L, Sigma Chemical Co. St. Louis, MO, USA). The media were replaced every three days. To test for adipogenic differentiation, cells were cultured in adipogenic medium with dexamethasone (0.4 µg/mL, Sigma, Germany), insulin (5 µg/mL, Sigma, Chemical Co. St. Louis, MO, USA), indomethacin (72 µg/mL, Sigma, Chemical Co. St. Louis, MO, USA) and IBMX (111 µg/mL) (Sigma Chemical Co. St. Louis, MO, USA). At the end of four weeks, the cells were washed with PBS before being fixed for 30 minutes in 2 mL of 4% formaldehyde solution. We instilled 1 mL of Oil Red O working solution (Cyagen, Guangzhou, China) or 1 mL of alizarin red dye solution (Cyagen, Guangzhou, China) into one well for 3–5 min after three times with PBS. Oil Red O staining and Alizarin Red staining were performed to detect lipid droplets and mineralized nodules which were examined under an inverted microscope (Leica, Weztlar, Germany).

2.5 Cell Counting Kit-8

We determined the cytotoxicity of ESP, PA and PB to PDLSCs. According to the standard of 2000 cells per well, we transferred cells into a 96-well plate containing various concentrations of ESP, PA and PB with refreshment every other day. Consistent with the guidelines provided by the manufacturer, 90 µL of αMEM and 10 µL of CCK-8 solution (Dojindo Laboratory) were added to each well after changing the medium. The cells were incubated for 2 hours at 37 °C with 5% CO_2. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.6 ALP Activity and ALP Staining

The Alkaline Phosphatase (ALP) level in PDLSCs was assessed by an ALP activity kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and an ALP staining kit (Beyotime Institute of Biotechnology, Shanghai,China). Osteogenic medium was used to cultivate third passage cells in six-well plates for one week. Using ALP

Table 1. Primer for real-time polymerase chain reaction (PCR).

Gene	Primer	
Interleukin 8 (IL-8)	Forward	5′- AACTGAGAGTGATTGAGAGTGG-3′
	Reverse	5′- ATGAATTCTCAGCCCTCTTCAA-3′
Interleukin 6 (IL-6)	Forward	5′- CACTGGTCTTTTGGAGTTTGAG-3′
	Reverse	5′- GGACTTTTGTACTCATCTGCAC-3′
Interleukin 10 (IL-10)	Forward	5′- CTTGCTGGAGGACTTTAAGGGTTAC-3′
	Reverse	5′- CTTGATGTCTGGGTCTTGGTTCTC-3′
Interleukin 4 (IL-4)	Forward	5′- ACAGCAGTTCCACAGGCACAAG-3′
	Reverse	5′- CGTACTCTGGTTGGCTTCCTTCAC-3′
Interleukin β (IL-β)	Forward	5′- GCCAGTGAAATGATGGCTTATT-3′
	Reverse	5′- AGGAGCACTTCATCTGTTTAGG-3′
Osteocalcin (OCN)	Forward	5′-CTACCTGTATCAATGGCTGGG-3′
	Reverse	5′-GGATTGAGCTCACACACACCT-3′
Osterix (OSX)	Forward	5′-CGGCAAGAGGTTCACTCGTTCG-3′
	Reverse	5′-TGGAGCAGAGCAGGCAGGTG-3′
Runt-related transcription factor 2 (RUNX2)	Forward	5′-AGGCAGTTCCCAAGCATTTCATCC-3′
	Reverse	5′-TGGCAGGTAGGTGTGGTAGTGAG-3′
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward	5′-CTTTGGTATCGTGGAAGGACTC-3′
	Reverse	5′-GTAGAGGCAGGGATGATGTTCT-3′

staining, we conducted PBS staining three times and fixed the sample in 4% paraformaldehyde. Then, we viewed the sample under an inverted microscope (Leica, Weztlar, Germany). Similarly, after osteogenic differentiation, we lysed the cells using RIPA buffer and three PBS washes. We introduced a liquid phase containing ALP into a 96-well plate for 15 min at 37 °C and measured the sample's optical densities using a spectrophotometer set to 520 nm.

2.7 Alizarin Red Staining

Cells were incubated in osteogenic medium for 14 days with the medium replaced every three days. The cells were fixed for 30 min in a 2 mL solution of 4% formaldehyde after being rinsed with PBS. After three washes with PBS, 1 mL of Alizarin Red dye solution (Cyagen, Guangzhou, China) was instilled into one well for 3–5 min to stain the cells. We used Alizarin Red staining to detect lipid droplets examined by an inverted microscope (Leica, Weztlar, Germany).

2.8 Quantitative Real-Time PCR

After the stimulation of 10 µg/mL lipopolysaccharides (LPS) from the *Porphyromonas gingivalis* (Invitrogen, Carlsbad, California, USA) for 24 h. PDLSCs were cultured at a density of 5×10^4 cells/well in 6-well plates for 1 day in the groups with LPS described in Section 2.2. For detection of the osteogenic genes by RT-qPCR, PDLSCs were cultured for 7 and 14 days under conditions of osteogenic induction. PBS buffer was used to wash the cells three times. Total RNA was isolated from PDLSCs using an Eastep® Total RNA Extraction Kit (Promega, Beijing, China). The reverse transcription of each sample into cDNA was performed according to the manufacturer's instructions using a commercial kit (TaKaRa, Otsu, Japan). The mRNA levels of these targets were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which served as a reference gene internally and was calculated by the $2^{-\triangle\triangle Ct}$ method. The outcomes are presented as fold variations compared to the control. Table 1 lists the primers that were employed in this study.

2.9 Western Blot (WB) Analysis

We used RIPA buffer (Solarbio, Beijing, China) containing protease inhibitors to extract proteins from cells in six-well plates and centrifuged the samples at 12,000 rpm for 30 minutes. The protein concentration was measured by a BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The extracted proteins were denatured at 100 °C for 5 minutes, separated in 5× sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer, and transferred to PVDF membranes. The PVDF membranes were blocked and incubated with primary antibodies against Osteocalcin OCN (Abcam, Cambridge, UK), and GAPDH (Abcam, Cambridge, UK). Secondary antibodies were chosen based on the species of origin of the primary antibodies. After incubation with secondary antibodies, the membranes were placed in developer solution (Beyotime Institute of Biotechnology, Shanghai, China), and the blots were visualized by a chemical imaging system. The expression levels of the target proteins were normalized to that of GAPDH.

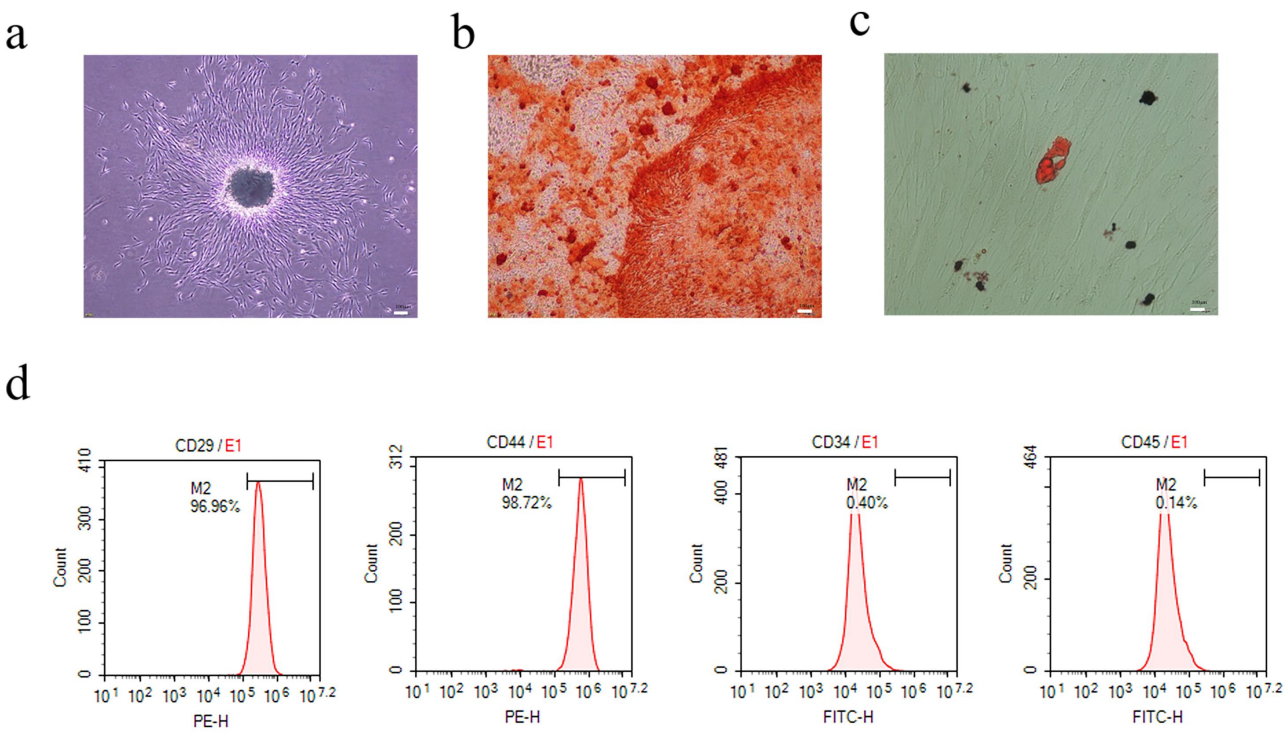

Fig. 1. Morphology and identification of periodontal ligament stem cells (PDLSCs). (a) PDLSCs (P3) formed a spiral arrangement in a fusiform shape (scale 200 µm). (b) PDLSCs were stained with Alizarin Red to assess osteogenic differentiation, showing red mineralized nodules (scale bar 50 µm). (c) Lipid differentiation of PDLSCs showed red oil droplets (scale bar 20 µm) by Oil-Red O staining. (d) PDLSCs expressed positive markers (CD29, CD44) but not negative markers expresse (CD45, CD34).

2.10 ELISA Assay

The supernatants of PDLSCs were collected and centrifuged at 4 °C (300 ×g) for 10 min. The concentration of IL-1β, IL-10 and IL-4 was tested by ELISA kits (Lianke-Bio, Hangzhou, China) according to the manufacturer's instructions. We measured the sample's optical densities using a spectrophotometer set to 450 nm and 630 nm.

2.11 Statistical Analysis

For each experiment, all results from at least three replicates were reported as the mean ± SD. To determine whether there was a difference between the experimental group and the control group, we used one-way analysis of variance by GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1 Isolation and Identification of PDLSCs

PDLSCs were isolated from the periodontal tissue of healthy individuals. They were plastic adherent with a fibroblast-like morphology when incubated (Fig. 1a). When stimulated in an osteogenic and lipid-forming inductive medium, these cells could be converted into adipocytes and osteoblasts (Fig. 1b,c). Flow cytometry revealed that PDLSCs were CD29 (98.23%) and CD44 (98.81%) positive, and negative for the markers CD34 (0.16%) and CD45 (0.09%) (Fig. 1d) (**Supplementary Fig. 1**). These results confirmed that the isolated PDLSCs were stem cells with powerful multipotency.

3.2 Cytotoxicity of ESP, PA and PB in PDLSCs

CCK-8 assay were used to determine the cytotoxicity of ESP, PA and PB to PDLSCs. Cells treated with 0.0625 mg/mL, 0.03125 mg/mL and 0.125 mg/mL ESP showed no cytotoxicity at 1, 3, 5 and 7 days (Fig. 2a). We established a concentration gradient of PA and PB and found that 2.5 µM, 5 µM and 10 µM PA and PB had no cytotoxicity at 5 and 7 days (Fig. 2b,c). Finally, we chose 0.03125 mg/mL, 0.0625 mg/mL and 0.125 mg/mL ESP, and 2.5 µM, 5 µM and 10 µM PA and PB for the follow-up experiments.

3.3 Effects of ESP, PA and PB on the Inflammatory Response of PDLSCs

LPS (10 µg/mL) significantly increased IL-8, IL-6, IL-1β expressions and decreased IL-4, IL-10 expressions by PDLSCs (Fig. 3a–f) (**Supplementary Fig. 2**). ESP reduced IL-8 and IL-6 mRNA expression (Fig. 3a,b) at three concentrations and 0.0625 mg/mL ESP increased the expression of IL-4, IL-10 at the RNA level in PDLSCs (**Supplementary Fig. 2a**). 5 µM PA reduced the RNA expression of IL-8 and 2.5 µM PA increased the expression of IL-10 at the RNA level (Fig. 3c) (**Supplementary Fig. 2b**).

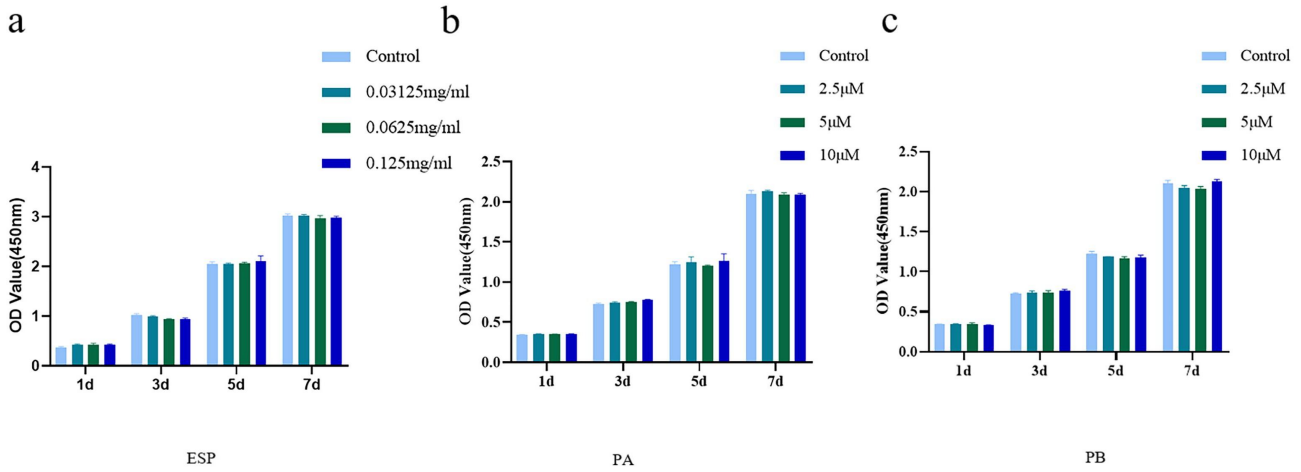

Fig. 2. Cytotoxicity of extract of Sappanwood (ESP), Protosappanin A (PA) and Protosappanin B (PB) to PDLSCs. (a) The cytotoxicity of of ESP at 0.03125 mg/mL, 0.0625 mg/mL and 0.125 mg/mL to PDLSCs of the control groups on days 1, 3, 5 and 7. (b,c) The cytotoxicity of PA and PB at 2.5 μM, 5 μM and 10 μM to PDLSCs contrasted with that of control groups on days 1, 3, 5 and 7. Each bar represents the mean ± standard deviation (n = 3).

Three concentrations of PB reduced the RNA expression of IL-8 and IL-1β RNA expression and PB decreased IL-6 RNA expression at 2.5 μM (Fig. 3e,f) (**Supplementary Fig. 2c**). 2.5 μM PB increased IL-10 RNA expression (**Supplementary Fig. 2c**). The ELISA assay showed that the secretions of pro-inflammatory cytokines including IL-1β were elevated and anti-inflammatory cytokine IL-4 and IL-10 were decreased when PDLSCs were exposed to 10 μg/mL LPS (**Supplementary Fig. 3a,b**). 5 μM PA and 2.5 μM, 5 μM, 10 μM PB reduced the secretion of IL-1β (**Supplementary Fig. 3b**). However, ESP could not reduce the secretion of IL-1β (**Supplementary Fig. 3a**). 0.03125 mg/mL and 0.125 mg/mL ESP and 2.5 μM, 5 μM, 10 μM PA increased the secretion of IL-4 in PDLSCs. PB could not increase the secretion of IL-4. ESP, PA and PB did not increase the secretion of IL-10 (**Supplementary Fig. 3a,b**). These results suggested that ESP, PA, and PB could attenuate the inflammatory response.

3.4 Effects of ESP, PA and PB on Osteogenesis of PDLSCs

Under the condition of osteogenic induction, the mRNA levels of RUNX2 and OSX in PDLSCs were increased compared with those under normal conditions. The expression of RUNX2 was also increased in the PDLSCS treated with three concentrations of ESP (Fig. 4a). Treatment with 0.0625 mg/mL and 0.125 mg/mL ESP increased the expression of OSX at the RNA level in PDLSCs in comparison to that in the control groups over 14 days (Fig. 4b). 0.0625 mg/mL and 0.125 mg/mL ESP increased the expression of OCN at the RNA level in PDLSCs and 0.125 mg/mL ESP increased the expression of OCN at the protein level (**Supplementary Fig. 4a–c**) in comparison to that in the control groups over 14 days. Similarly, PB at the three concentrations upregulated the mRNA expression of OSX (Fig. 4f). The expression of OSX was also increased in the PDLSCS treated with 10 μM PA (Fig. 4d). Treatment with 2.5 μM and 10 μM PA and PB increased the expression of RUNX2 at the mRNA level in PDLSCs compared with that in the control groups for 14 days (Fig. 4c,e).

5 μM and 10 μM PB increased the expression of OCN at the mRNA level in PDLSCs compared with that in the control groups for 14 days. 0.125 mg/mL ESP and 5 μM PB increased the expression of OCN at the protein level (**Supplementary Fig. 4a–c**). To further determine the osteogenic effect on ESP, PA and PB, we performed Alizarin Red staining, ALP staining and activity assay. The content of ALP and mineralized nodules of PDLSCs were increased in a dose-dependent manner at three concentrations of ESP (Fig. 5a,c). The activity and content of ALP in the PDLSCs treated with 5 μM PA and 2.5 μM and 5 μM PB were significantly higher than those in the control group (Fig. 5b,d,e). In addition, Alizarin Red staining demonstrated that 2.5 μM PA and 2.5 μM and 5 μM PB generated a significant increase in mineralization (Fig. 5b). The quantity of ALP staining in the PDLSCs treated with 0.0625 mg/mL and 0.125 mg/mL ESP and 2.5 μM and 5 μM PB were significantly higher than those in the control group (**Supplementary Fig. 5b**). The quantity of Alizarin Red staining demonstrated that 0.0625 mg/mL ESP, 2.5 μM PA and 2.5 μM PB generated a considerable increase in mineralization (**Supplementary Fig. 5a**). These assays showed that ESP, PA and PB can promote the osteogenesis of PDLSCs.

4. Discussion

The treatment of periodontitis is mainly nonsurgical by mechanical subgingival instruments, but this method is palliative, unable to repair periodontal tissue, and cannot

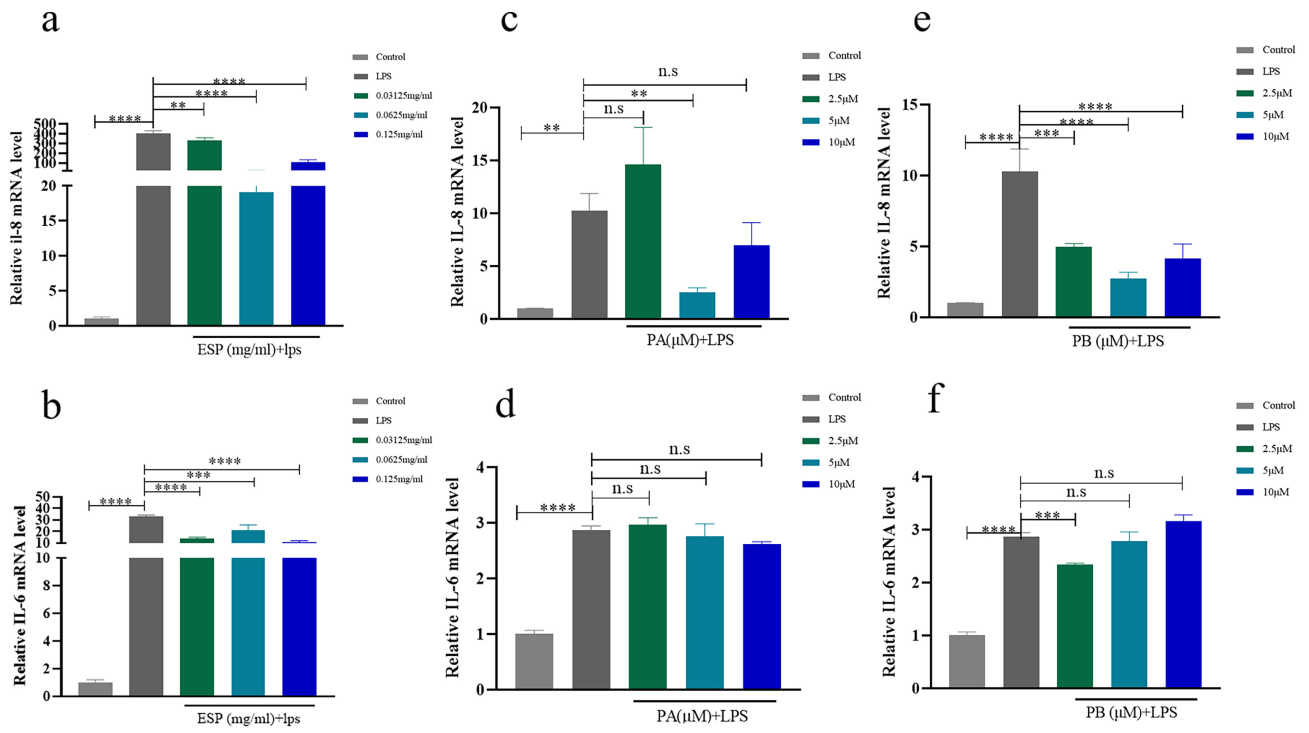

Fig. 3. Effects of ESP, PA and PB on the inflammatory response of PDLSCs. (a,b) The RNA expression of IL-8 and IL-6 with ESP at 0.03125 mg/mL, 0.0625 mg/mL and 0.125 mg/mL in PDLSCs contrasted with that of the control groups. (c–f) The mRNA expression of IL-8 and IL-6 with PA and PB at 2.5 μM, 5 μM and 10 μM in PDLSCs compared to the controls. Each bar represents the mean ± standard deviation (n = 3). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.001$. LPS, lipopolysaccharides.

meet the needs of all patients. Although MSC transplantation can effectively restore periodontal tissue, it is still expensive and has disadvantages such as immune rejection [12,13]. Therefore, improving the inflammatory microenvironment of MSCs and promoting the proliferation and differentiation of MSCs in the human body has clinical value for the repair and regeneration of the periodontium

PDLSCs are ideal for tissue engineering research because of their strong self-differentiation and renewal ability [14]. In this study, we identified PDLSCs by osteogenesis and lipid differentiation and surface markers. Our experimental results were consistent with previous studies [15]. We successfully isolated PDLSCs with a typical fibroblastic appearance and detected the expression of stem cell surface markers on PDLSCs using flow cytometry. The findings demonstrated that PDLSCs could be used in the ensuing experiments.

Due to the effectiveness and relative safety of natural plant ingredients, many plant ingredients combined with PDLSCs have become a new strategy for promoting periodontal tissue regeneration and prevention and treatment of periodontitis [15,16]. The anti-inflammatory effect of the extract of sappanwood has been shown previously [17,18]. PA affects cell anti-inflammatory activity and bone metabolism [9,10]. However, no studies have shown that ESP, PA and PB have anti-inflammatory activity and osteogenic effects on PDLSCs. Here we studied the anti-inflammatory and osteogenic differentiation effects on these three components in PDLSCs. In our previous study, we found that ESP had strong inhibitory effects on *Porphyromonas gingivalis*, *gingivalis*, *Clostridium nucleus*, *Prevotella intermedia*, and other core pathogenic microorganisms of periodontitis (MIC $\leq$0.0625 mg·mL^{-1}). Therefore, we tested and selected concentrations of 0.03125 mg/mL, 0.0625 mg/mL and 0.125 mg/mL ESP. Then, we established a concentration gradient of PA and PB for cytotoxicity and selected 2.5 μM, 5 μM, and 10 μM PA and PB for the follow-up experiments.

Inflammation has been implicated in the etiology of periodontal disease [19]. *Porphyromonas gingivalis* is a major etiological agent in the onset and progression of severe forms of periodontal disease [20]. It can initiate the production of various cytokines, such as interleukin-8 (IL-8) [21,22]. Periodontitis patients have higher salivary IL-8 levels, and IL-8 is an important parameter in gingival crevicular fluid reflecting the resting and active stages of periodontitis [23,24]. Moreover, interleukin 6 (IL-6) has recently been associated with worsening periodontal disease and enhancing a cascade of tissue destruction [25,26]. Most cross-sectional studies have shown that serum IL-1β expression levels in patients with periodontitis is higher than that in healthy people, and is positively corre-

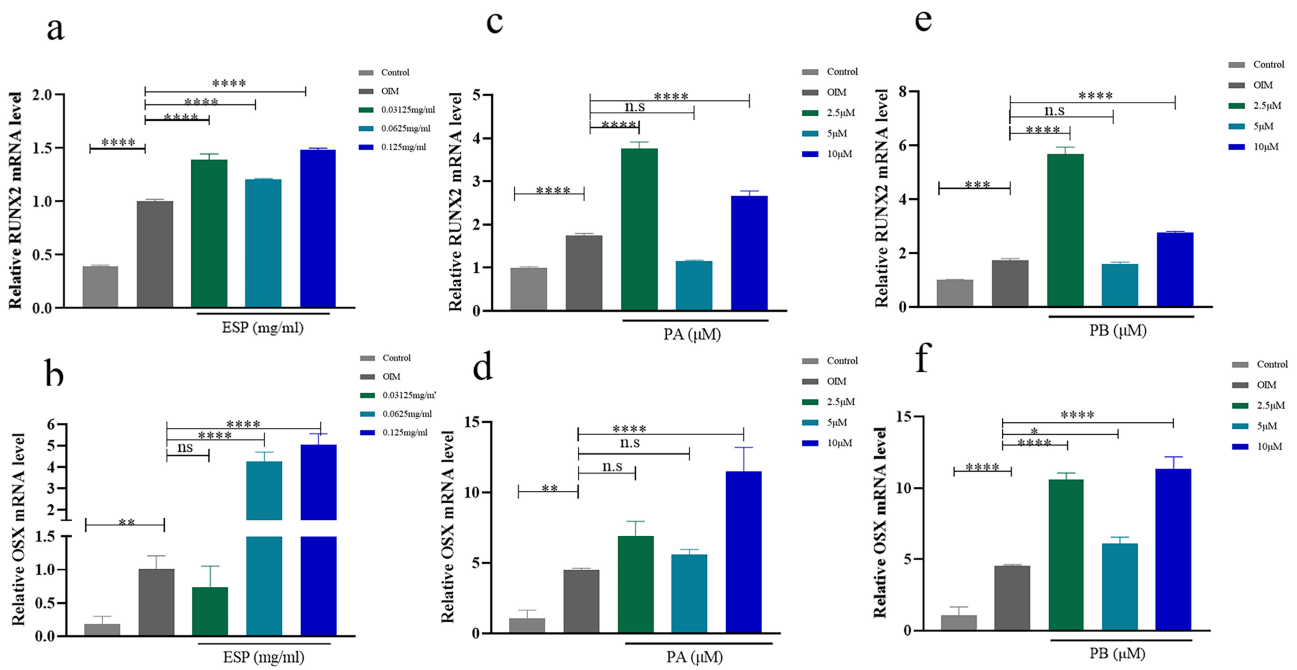

Fig. 4. Effects of ESP, PA and PB on osteogenesis of PDLSCs. (a,b) The mRNA expression of RUNX2 and OSX with ESP at 0.03125 mg/mL, 0.0625 mg/mL and 0.125 mg/mL in PDLSCs *vs*. the control cells after osteogenic induction for 14 days. (c–f) The mRNA expression of RUNX2 and OSX with PA and PB at 2.5 μM, 5 μM and 10 μM in PDLSCs compared to the controls after osteogenic induction for 14 days. Each bar represents the mean ± standard deviation (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.001$. OIM, Osteogenic induction medium; n.s, not significant.

lated with the clinical parameters of periodontitis [27,28]. Interleukin-10 (IL-10), a cytokine with anti-inflammatory properties, has a central role in infection by limiting the immune response to pathogens and thereby preventing damage to the host [29]. Interleukin-4 (IL-4) induced the repolarization of anti-inflammatory macrophages and the formation of regulatory T cells *in vitro* and the expression of anti-inflammatory factors IL-4 was decreased in patients with periodontitis [30,31]. As a result, treating periodontal disease requires reducing inflammation [32]. LPS (10 μg/mL) has been demonstrated to increase the production of inflammatory mediators and inhibit the osteogenic differentiation of PDLSCs [33]. In our study, 10 μg/mL LPS significantly increased IL-8, IL-6 and IL-1β secretion and declined IL-10, IL-4 by PDLSCs. Our results also showed a moderate effect of ESP on IL-6 and IL-8 reduction. Similarly, we also found that the anti-inflammatory effect of PA agrees with a previous study that found that PA suppressed the expression of inflammatory factors [34]. However, our findings on the anti-inflammatory effects of PB contradict previous research that demonstrated no PB activity in J774.1 macrophage cells [34]. Notably, the anti-inflammatory effect of several protosappanins has not been consistently described in the literature. Previous research found a negligible anti-inflammatory effect of protosappanin B in J774.1 cells, which was related to the suppression of inducible nitric oxide synthase (iNOS) and NO production [11]. The unstable chemicals extracted from sappanwood may be a factor in the inconsistent findings in the literature [35]. Moreover, our investigation discovered that the transcription and protein levels of anti-inflammatory factors IL-10, IL-4, and pro-inflammatory factors IL-1β were not the same, which may be attributed to the complicated gene regulation and protein regulation of living organisms in a dynamic state [36]. Protein ubiquitination and the transcription level feedback control mechanism, as well as the effect of detection time, will influence alterations in the level of expression of these factors [37,38]. IL-1β promotes the expression of collagenolytic enzyme and matrix metalloproteinase, which leads to the destruction of extracellular matrix [39,40]. We found that 5 μM PB had better inhibition of IL-1β. The anti-inflammatory effect may be similar to other natural products by activating the classical PI3K/Akt/NF-κB signalling pathway [41]. 0.0625 mg/mL ESP increased the expression of IL-4, IL-10 and 2.5 μM PA, PB had a better effect on the increase of IL-10 at the RNA level. The ELISA assay showed that 5 μM PA and 10 μM PB had better inhibition of IL-1β. Studies have shown that IL-4 can be increased after periodontal therapy and decreased in patients with chronic periodontitis [42,43]. It can also cause macrophages to change from M1 to M2, which promotes tissue regeneration [44,45], and it can control inflammation by differentiating naive T cells into inducible regulatory T cells in the presence of transforming growth

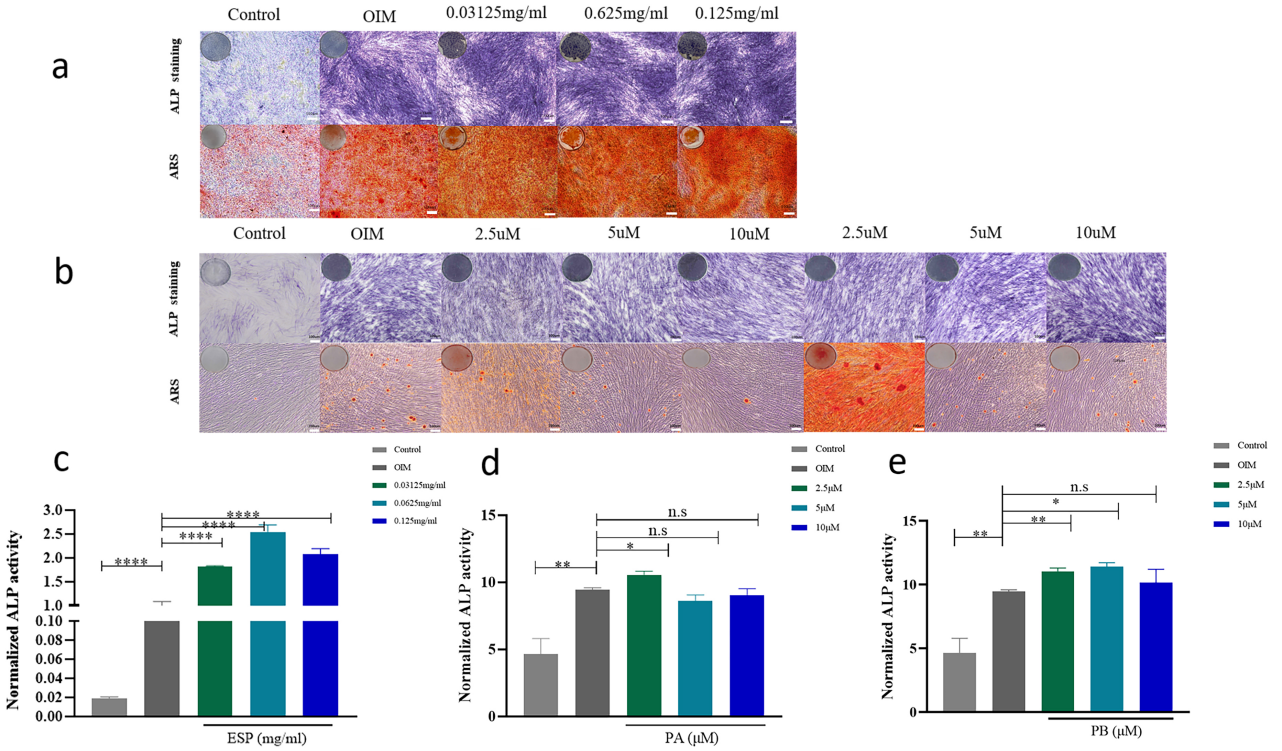

Fig. 5. Effects of ESP, PA and PB to on PDLSCs osteogenesis. The result of the Alkaline Phosphatase (ALP) activity assays after osteogenic induction for 7 days. Representative images of ARS (Alizarin Red staining) after osteogenic induction for 14 days. ALP staining and Alizarin Red staining with ESP at 0.03125 mg/mL, 0.0625 mg/mL and 0.125 mg/mL compared to that of the control groups (scale bar 200 μM). (a) ALP staining and Alizarin Red staining of the PDLSCs treated with PA and PB at the concentrations of 2.5 μM, 5 μM and 10 μM compared to that of the control groups (scale bar 200 μM). (b) The ALP content of PDLSCs with ESP at 0.03125 mg/mL, 0.0625 mg/mL and 0.125 mg/mL compared to that of the control groups. (c) The ALP content of PDLSCs with PA and PB at 2.5 μM, 5 μM and 10 μM of PDLSCs compared to that of the control groups (d,e). Each bar represents the mean ± standard deviation (n = 3). *$p < 0.05$; **$p < 0.01$; ****$p < 0.001$.

factor [46]. IL-10 polymorphisms are a valuable diagnostic method for identifying patients at high risk for periodontitis and have great clinical relevance across ethnic groups, since it is highly expressed in reparative M2-like macrophages and also inhibits proinflammatory cytokine generation [47]. IL-10 has also been recognized as a significant regulator of inflammation and bone homeostasis [48]. 0.03125 mg/mL and 0.125 mg/mL ESP and 2.5 μM, 5 μM, 10 μM PA may improve the periodontal inflammatory microenvironment and regulate bone homeostasis by increasing the secretion of IL-4.

Reconstruction of periodontal tissue defects is a serious challenge. When cultured *in vitro* under inductive circumstances, PDLSCs display osteogenic, adipogenic, and chondrogenic traits [49]. In injured periodontal tissues in animal models, PDLSCs transplantation may increase the formation of new bone and new cement [50–53]. Accumulating evidence suggests that natural products can support the osteogenic differentiation of PDLSCs. Previous studies found that rutin fosters osteogenic differentiation of PDLSCs [15], and ipriflavone stimulates PDLSC proliferation and osteogenic differentiation [54]. Sappanwood mainly includes protohaematoxylin, braziloxylin, high isoflavone, haematoxylin, and other components. Previous studies have shown that it inhibits osteoclastogenesis and bone resorption [55,56]. However, there are few studies on osteogenic differentiation. Therefore, we investigated the effect of ESP, PA and PB on periodontal regeneration from osteogenic differentiation. Osteoblast transcription factor 2 (RUNX2) plays a vital role in both osteogenesis and chondrogenesis [57–59]. RUNX2 requires initiation of prechondroblast mesenchymal separation into a precursor osteoblast lineage, whereas OSX subsequently involves the completion of the osteoblast differentiation pathway [60]. The important role of OSX is attributed to its regulation of osteoblast markers, such as Dkk1, an important antagonist of WNT/β-catenin signalling [61], RUNX2 promotes differentiation of mesenchymal progenitor cells to initiate osteogenesis during osteogenic lineage specification, and OSX supports the maturation of functional osteoblasts [62]. OCN, which is specifically produced by osteoblasts, and is the most abundant non-collagenous protein

in bone, was demonstrated to inhibit bone formation and function [63]. Several studies have confirmed that OCN is one of the mature specific osteogenic markers [64,65]. In the studies involving osteogenic differentiation, ESP, PA and PB increased the expression of RUNX2 and OSX at the RNA level and increased the expression of OCN at the RNA and protein level in PDLSCs in comparison to controls at 14 days. Similarly, we found that the content of ALP and the formation of mineralized nodules of PDLSCs were increased after Alizarin Red staining and measured ALP activity, and that the quantitative data for AR staining and ALP staining were consistent with the staining results. The quantitative ALP staining in the PDLSCs treated with 0.0625 mg/mL and 0.125 mg/mL ESP and 2.5 μM and 5 μM PB were significantly higher than those in the control group. 2.5 μM PA and PB were optimal for promoting RUNX2 and 10 μM PA and PB were optimal for promoting OSX at mRNA level. For OCN expression of downstream genes, 10 μM PB had a better effect on the increase of it and 5 μM PB were optimal for promoting OCN at protein level. In addition, the quantitative Alizarin Red staining demonstrated that 0.0625 mg/mL ESP, 2.5 μM PA and 2.5 μM PB generated a considerable increase in mineralization. Similar to the PCR results, not all concentrations were effective. Given the difference in action between different concentrations, we concluded that osteogenesis at a given stage is regulated by a complex network of genes and proteins. Sometimes the gene or protein expression of osteogenic markers may be time-dependent [66]. We found that the osteogenic effects of ESP were the most effective. The reason for this phenomenon may be that ESP has many components, and their combined effects results in superior outcomes. It was not possible to determine the best PA/PB concentration in our study, nor was it possible to clearly determine the mechanism of action of PA/PB. We will continue to explore this in depth in future experiments.

5. Conclusions

Our study demonstrated that ESP, PA and PB could reduce the inflammatory response and facilitate osteogenic differentiation of PDLSCs. The data indicated that ESP, PA and PB have anti-inflammatory and osteogenic effects. Thus, ESP, PA and PB may be used for bone regeneration and periodontal tissue engineering.

Availability of Data and Materials

Datasets used and/or analyzed for this study are available from the corresponding author upon appropriate request.

Author Contributions

XZ, JC, JZ and YL conceived the study, directed the project, designed the experiments; JZ, YL, XZ and JC interpreted the results and wrote the manuscript; NR, CY, JL provide experimental help; JZ, YL, XZ and JC analyzed the data. JZ and YL revised the manuscript. NR substantial contributions to the conception and design of the work. CY and JL acquisition, analysis, and interpretation of data for the work. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Permission to conduct this study was obtained from the Kunming Medical University School's ethical committee (no. KYKQ2021MEC025).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2808172.

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