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Structural characterization and stimulating effect on osteoblast differentiation of a purified heteropolysaccharide isolated from Hedysarum polybotrys

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ABSTRACT

Radix Hedysari polysaccharides (HPS) is the principal active fraction of Radix Hedysari (RH). The information about HPS3d, the main fraction of HPS3, and its effect on bone is still unknown. In the present study, the purified HPS3d was obtained by anion-exchange column. It consisted of 94.38% polysaccharide, 3.40% protein and 13.30% uronic acid. The molecular weight was measured to be 84.6 kDa. The backbone consisted of galactopyranose and galacturonopyranose, and the side chains were composed of glucopyranose, rhamnopyranose and arabinofuranose. The FT-IR and elemental analysis showed that HPS3d was the sulfated polysaccharide. HPS3d upregulated alkaline phosphatase (ALP) activity and the expression of other osteogenic marker genes in osteoblast. In addition, HPS3d increased the expression and transcriptional activity of Runt-related transcription factor 2 (Runx-2) and Osterix, the two master genes of osteoblast differentiation. These findings suggest that HPS3d stimulates osteoblast differentiation by activation of Runx-2 and Osterix.

1. Introduction

The skeleton is a dynamic and heterogeneous tissue whose remodeling is largely the result of the coordinated action of osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation, while osteoclasts are in charge of bone resorption (Kawamura et al., 2007). When this balance is broken, osteoporosis occurs. Such as elderly postmenopausal women who experience high rate of osteoporotic fractures caused by hormonal changes (Ferguson, 2004). To prevent or treat osteoporosis, either anti-osteoporotic drugs (anti-resorptives), anabolic drugs (bone formation), or both are used (Brennan et al., 2009; Leong, Zhou, Lim, & Li, 2009). Such as bisphosphonates, hormone replacement therapy (HRT), calcitonin, vitamin D, and calcium have been used to date. However, conventional drug therapies have both pros and cons. For example, long-term use of estrogen therapy increases the possibility of breast and endometrial cancers (Grodstein et al., 1997; Gustafsson & Warner, 2000). It is very important to find and develop new agents which have high recovery efficiency of bone mass without side effects. Natural products have received more and more attentions for the treatment of osteoporosis. Several plant-derived compounds have positive effects on bone with no or fewer undesirable side effects (Jeong et al., 2011).

Radix Astragali (RA), known as Huangqi, which is the root of *Astragalus membranaceus* (Fisch.) has been widely used as an immunostimulant, cardiotonic, hepatoprotective, antidiabetic, and antiviral drug (Shon, Kim, & Nam, 2002; Toda & Shirataki, 1999). Radix Hedysari (RH), the dry root of *Hedysarum polybotrys Hand.-Mazz.* known as Hongqi in China, is a famous-region drug of Gansu province in China used in traditional Chinese medicine (TCM). RA and RH belong to the same family. Although RH has been considered an authentic and specifically different medicine since 1985,

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as recorded in the Chinese Pharmacopoeia, traditionally these two herbs are usually considered to possess the same efficacy in TCM. Today, RH is often used as the substitute of RA, and in some regions RH is esteemed as the uppermost class of RA (Liu et al., 2010, 2012). The comparative chemical analysis of the major constituents of RA and RH by HPLC shows that there are some common compounds in both herbs (Liu et al., 2012). Liu et al. (2010) reported that polysaccharides extract (PE) of these two herbs might be the main bioactive compounds that were responsible for immunoregulatory function and RH could be used instead of RA in the immunoregulatory function aspect in the clinical application. It is reported that RA could stimulate differentiation of osteoblast (Choi et al., 2011). So we hypothesized that RH could also display a stimulating effect on osteoblast differentiation.

RH has been used to treat variety of chronic adult diseases such as strengthening the immune system, diarrhea, diabetes mellitus, chronic nephritic proteinuria and inflammation (Wang et al., 2013). The Radix Hedysari polysaccharides (HPS) is the principal active fraction that is responsible for the various pharmacological activities of RH (Dang et al., 2013). In our previous study, HPS were purified into four homogeneous fractions of different molecular weight range (HPS1, HPS2, HPS3, HPS4) by DEAE-52 and Sephadex G-200 column chromatography and we found HPS3 was an important active fraction of HPS (Hu, Li, Zhao, Feng, & Wang, 2010), this polysaccharide is of special interest to us.

In previous works, several glucans were extracted and purified from RH and the structures of them were identified (Li, Chen, Wang, Tian, & Zhang, 2009; Liu, Li, Meng, & Chen, 1997; Shi et al., 2012). However, to the best of our knowledge, further information about HPS3 such as the structural characteristics and the effect on osteoblast had never been reported. Therefore, aim of the present study was to identify the structural characteristics of HPS3 and explored whether HPS3 could stimulate the differentiation of osteoblast.

2. Methods and materials

2.1. Materials

The roots of Hedysarum polybotrys were collected from Wudu, Gansu Province and identified by Prof. Zhigang Ma, School of Pharmacy, Lanzhou University. The voucher specimen was deposited in the herbarium of School of Pharmacy, Lanzhou University. The root of Hedysarum polybotrys was air-dried in shade and ground into powder with a mill (60-mesh). Monosaccharide standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All the other chemicals used were of analytical grade.

2.2. General methods

Total carbohydrate content was determined by phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), with glucose and galactose as the standards at 490 nm. Protein content was assayed by Bradford method with bovine serum albumin as reference protein (Bradford, 1976). Uronic acid content was determined according to GC method (Lehrfeld, 1987), and *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973) by measuring the absorbance at 525 nm and using glucuronic acid as standards.

2.3. Extraction, isolation and purification of HPS3

Polysaccharides were extracted by hot water following the method of the earlier report (Shi et al., 2012). Sevage method (Staub, 1965) was used for the deproteination of HPS3. And then, the deproteined polysaccharide was dialyzed and freeze-dried. HPS3 (300 mg) was dissolved in 5 ml distilled water, and then applied to a DEAE-52 cellulose column, which was (2.6 cm × 70 cm) equilibrated with 0.05 M NaAc-buffer (pH 5.2). The column was eluted with 0.05 M NaAc-buffer (pH 5.2) at a flow rate of 25 ml/h and then 0.1 M and 0.3 M NaCl in 0.05 M NaAc-buffer (pH 5.2). Test tubes were collected using an automated step-by-step fraction collector. Total carbohydrate content of each tube was measured at 490 nm by the phenol sulphuric acid colorimetric method (Dubois et al., 1956), and the main fraction (designated HPS3d) was collected.

2.4. Measurement of molecular weight

The molecular weight of HPS3d was determined by size exclusion chromatography-multi angle laser light scattering (SEC-MALLS) method. SEC-MALLS measurements were carried out on a DAWN HELEOS-II laser photometer (DAWN HELEOS-II, Wyatt Technology Co., Santa Barbara, CA, USA), combined with an Ultrahydrogel™ 1000 and an Ultrahydrogel™ 500 column and equipped with a Model 600 pump HPLC system (Waters, USA), differential refractive index detector (Model 2414) and UV detector (Model 2998).

The HELEOS-II laser photometer was calibrated by NaCl aqueous solution. The average value of refractive index increment (dn/dc) was determined to be 0.135 ml/g at 632.8 nm. HPS3d solution (20 μl, 5 mg/ml, dissolved by mobile phase, filtrated by 0.22 μm microfiltration membrane) was injected and eluted at 0.8 ml/min with 0.1 M $NaNO_3$ containing 0.02% (w/w) NaN_3 as the mobile phase. Data collection and processing were under the control of a personal computer driven by the Wyatt Technology ASTRA program. The light scattering signal was detected simultaneously at 18 scattering angles (θ) ranging from 15 to 165°.

2.5. Elemental analysis

Elemental analysis was carried out using an Elementar Vario EL instrument (Elementar, Germany) to analyze carbon (C), hydrogen (H), oxygen (O), nitrogen (N) and sulfur (S). Sulfate content ($-SO_2O^-Na^+$, sodium salt) was calculated from sulfur analysis result according to the following relation: Sulfate group = 3.22 × S%.

2.6. Partial acid hydrolysis

Partial degradation of polysaccharide by acid hydrolysis is based on the fact that some glycoside linkages are more labile to acid than others. HPS3d (100 mg) was hydrolyzed with 0.3 M trifluoroacetic acid (TFA) (100 ml), kept at 100 °C for 1 h. After TFA was removed by evaporation, the remains were dialyzed with distilled water for 48 h. The solution inside of the dialysis bag (MWCO 3500) was concentrated and lyophilized, and then applied on Sephadex G-200 column (2.6 cm × 90 cm). The main peak fraction was collected and lyophilized to obtain sub-fraction, designated as HPS3di, which was the backbone of HPS3d.

2.7. Analysis of monosaccharide compositions

Conversion of aldoses and aldonic acids to peracetylated alditols according to earlier report by Lehrfeld (1985). Briefly, 0.5 M sodium carbonate solution (0.156 ml) was added to a mixture containing approximately 4 mg each of L-arabinose, D-xylose, D-mannose, D-glucose, D-galactose, myo-inositol, D-glucuronic acid, D-galacturonic acid and HPS3d, HPS3di (5 mg) in a culture tube, respectively. The solution was maintained at 30 °C for 45 min and then treated with sodium borohydride (1 ml of a 4% solution) for 1.5 h at room temperature. Excess sodium borohydride was decomposed by the dropwise addition of acetic acid (25%) until bubbling

stopped (6–8 drops). To remove sodium ions, the solution (2 ml) was applied onto a cation-exchange resin (732,H^+ form) column and eluted with 6 ml of water. The elution was evaporated to dryness by evaporator (45 °C in vacuo) and repeated twice after add methanol (3 ml) to remove Borate from the residue. Then heating at 85 °C in vacuo for 2 h converted the aldonic acids into aldonolactones. The residue was dissolved by pyridine (2 ml) in Teflon tube, and added I-propylamine (2 ml), and then the tube was capped and heated at 55 °C for 30 min. The solution was cooled (less than 45 °C), the cap was removed, and nitrogen was bubbled through the reheated solution (55 °C) until dry. The residue was dissolved in pyridine (1 ml) and acetic anhydride (1 ml) and heated at 95 °C for 1 h.

GC analysis was taken on a Shimadzu GC-2010 (Shimadzu, Japan) with flame ionization detector (FID), using a fused-silica capillary column OV-101 (50 m × 0.25 mm, i.d × 0.33 μm film thickness). The GC operating conditions was performed as follows: injector temperature: 210 °C; injection mode: split; split ratio: 50; injection volume: 1 μl; carrier gas: nitrogen; detector temperature: 280 °C. Temperature program: 160 °C, held for 4.0 min, to 190 °C at rate of 5 °C/min, held for 4 min, to 210 °C at rate of 3 °C/min, held for 15 min, then to a final temperature of 260 °C at rate of 10 °C/min, held for 5 min.

2.8. Infrared spectral analysis

The Fourier transform IR spectra (FT-IR) were recorded with a Nicolet Nexus spectrometer (Nicolet, USA) between 400 cm^{-1} and 4000 cm^{-1}. The sample was analyzed as KBr pellets.

2.9. Cell culture

The MC3T3-E1 osteoblastic cell line was obtained from the Chinese Academy of Medical Sciences. It was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 U/ml streptomycin, and it was maintained at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air throughout the experiment.

2.10. Cell viability assay

Cells were seeded in 96-well culture plates and treated with different concentrations of HPS3d (0.5 to 32 mg/ml). HPS3d was dissolved in DMEM/F-12 with FBS and sterilized via a 0.2 μm micropore filter. After 72 h, cell viability was measured by MTT assay. This assay is based on the ability of viable cells to convert soluble MTT into an insoluble dark blue formazan reaction product. Cells were treated with 0.5 mg/ml MTT solution for 2 h. DMSO was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After all the crystals were dissolved, the plates were read on a microplate reader at a wavelength of 570 nm.

2.11. Alkaline phosphatase (ALP) activity

After treated with HPS3d (0.5 to 8 mg/ml), cells were washed with phosphate buffered saline (PBS) and frozen (−70 °C) in 300 μl of Tris-Triton (0.1 M Tris base, 0.2% Triton X-100). After thawing, the cells were centrifuged (12,000 rpm, 5 min) and the supernatant was used for analysis. ALP activity was normalized to total protein content.

2.12. RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

The total cellular RNA was isolated using TRIzol reagent and RTPCR was performed using RevertAid™First Strand cDNA Synthesis Kit and Go Taq® Flexi DNA Polymerase according to the manufacturer's instructions. PCR was performed with mouse-specific primers: sense strand osteopontin (OPN), 5′- CTCCAATCGTCCCTACAGTCG-3′, antisense strand OPN,5′-CCAAGCTATCACCTCGGCC-3′; sense strand osteocalcin (OCN), 5′-ACCATCTTTCTGCTCACTCTGCT-3′, antisense strand OCN,5′-CCTTATTGCCCTCCTGCTTG-3′; sense strand bone sialoprotein (BSP), 5′-GGCTATTGATCAAGCAGCACACA-3′, antisense strand BSP, 5-CGCAGTTAGCAATAGCACAAACAC-3′; sense strand Runt-related transcription factor 2 (Runx-2), 5-CACTGGCGGTGCAACAAGA-3′, antisense strand Runx-2, 5′-ATGACGGTAACCACAGTCCCATC-3′; sense strand Osterix, 5′-AAGTTATGACGGGTCAGGTACA-3′, antisense strand Osterix, 5′-AGAAATCTACGAGCAAGGTCTCCAC-3′; sense strand β-actin, 5-ATGGAGCCACCGATCCACA-3′, antisense strand β-actin, 5′-CATCCGTAAAGACCTCTATGCCAAC-3′.

2.13. Western blot analysis

After various treatments, cell lysates were subjected to 12% SDS-PAGE. The proteins were then transferred to PVDF membranes (Millipore). The membrane was incubated with the appropriate primary antibody, followed by incubation with secondary antibody. Immunoreactive proteins were detected by the enhanced chemiluminescence (ECL; Millipore, USA) method. The densities of obtained Western blotting bands were analyzed using Image J (v. 1.37) software (NIH, Bethesda, MD).

2.14. Statistical analysis

All experiments were performed with triplicate independent samples and were repeated at least thrice, giving qualitatively identical results. All data were presented as the mean ± SD of each group. Statistical analyses were performed using one-way ANOVA. $P < 0.05$ was considered to be significant.

3. Results

3.1. Isolation, purification of polysaccharides

The crude polysaccharide HPS was isolated from the dried roots of Hedysarum polybotrys by hot-water extract in 45% yield. Then the concentrated supernatant was precipitated with 95% ethanol (to give 30%, 50%, 70% v/v final ethanol concentrations) to obtain three crude polysaccharides accordingly, then they were dried and given them namely HPS1, HPS2, and HPS3,which all presented in yellowish form. The total sugar content of HPS3 was found to be 45.4% as determined by the phenol-sulfuric acid method, which was the highest in three crude polysaccharides.

HPS3 was further dissolved in distilled water and deproteined by Savage method. Then, the deproteined HPS3 centrifuged, exhaustively dialyzed, and freeze-dried. On a DEAE-52 cellulose column, four fractions (HPS3a, HPS3b, HPS3c and HPS3d) were obtained from HPS3 by elution with 0, 0.1 M and 0.3 M NaCl in 0.05 M NaAc-buffer (pH 5.2), respectively. The main fraction (HPS3d) were pooled and lyophilized to obtain dry polysaccharide. HPS3d appeared as a white fluffy material. The absorptivity 280 nm in the UV spectrum showed that there were protein in HPS3d. HPS3d consisting mainly of polysaccharide, protein and uronic acid as identified by the phenol sulphuric acid reation, the Bradford method and sulfuric acid–carbazole method. The percentages

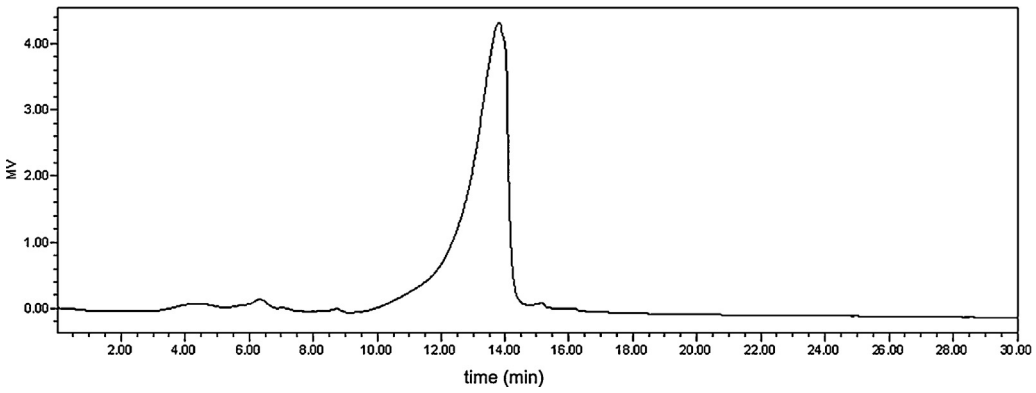

Fig. 1. HPGPC elution profile of HPS3d using a refractive index detector.

of polysaccharide, protein and uronic acid was 94.38%, 3.40% and 13.30%, respectively.

3.2. Molecular weight

In HPGPC profile (Fig. 1), HPS3d dispalyed a single and symmetrically peak, indicating it was a homogeneous polysaccharide.

As an absolute method, SEC-MALLS is used to determine the molecular mass and shape of polymers without standard samples, which is extremely sensitive to macromolecular aggregation. The SEC-MALLS chromatogram patterns of HPS3d in 0.1 M aqueous $NaNO_3$ were shown in Fig. 2A. The molecular weight and chain conformation of HPS3d were determined with SEC-MALLS. The weight average molecular weight, polydispersity index and radius of gyration value, were measured to be 84.6 kDa, 2.54 and 13.3 nm (Rz) in 0.1 M $NaNO_3$, respectively.

The chain conformation of HPS3d described by Mw and the radius of gyration was calculated by the software. Commonly, the chain conformation of a polymer in solution can be judged by the slope of a conformation curve (rms radiums of gyration vs Mw. The slope rate of conformation curve (Fig. 2B) for HPS3d was 0.33 ± 0.01, suggesting that it was a globular shape in 0.1 M $NaNO_3$ and might be a highly branched polymer (Podzimek, 2011).

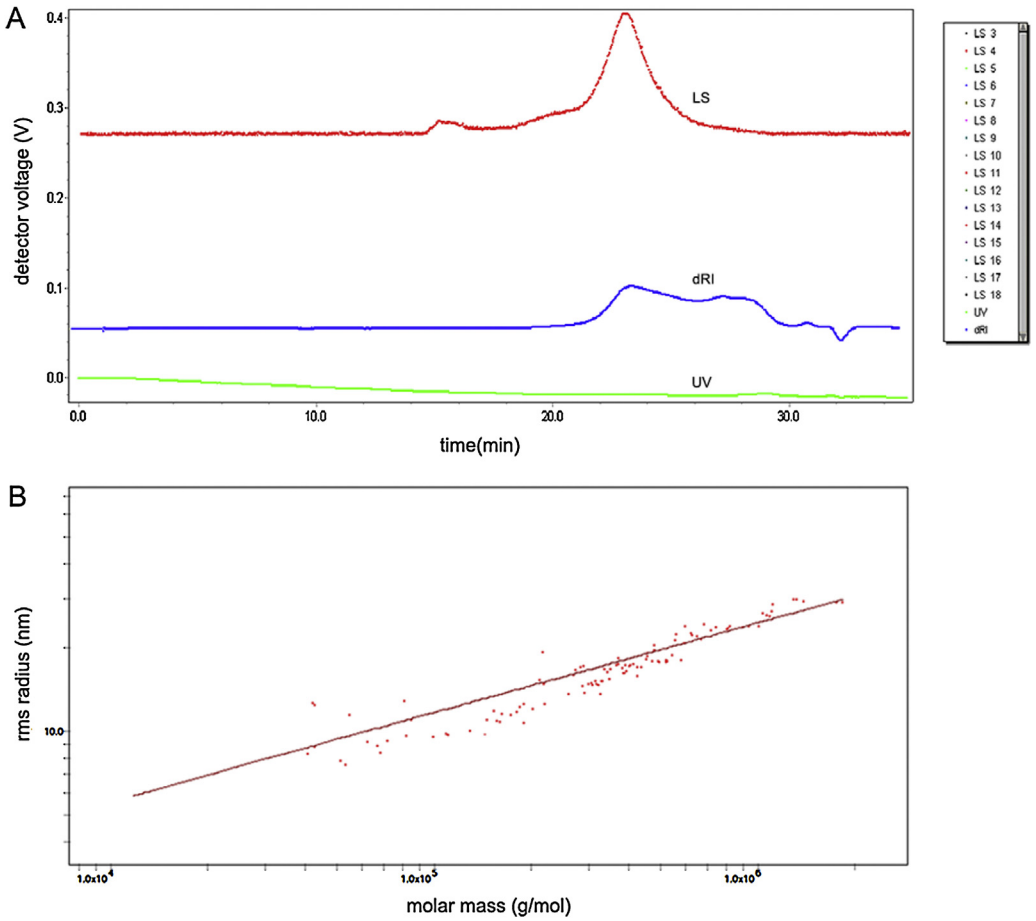

Fig. 2. (A) Profile of SEC-MALLS assay of HPS3d. Light scattering (LS) signal at 90°, UV signal at 280 nm, dRI signal. (B) Conformation plot of rms radiums vs Mw for HPS3d in 0.1 M aqueous $NaNO_3$.

Table 1
GC analysis results.

Samples	Molar ratios				
	Rhamaose	Arabinose	Glucose	Galactose	Galacturonic acid
HPS3d	5.93	39.95	6.76	39.31	8.05
HPS3di	12.36	8.63	9.61	40.75	28.66

3.3. Elemental analysis

The result of elemental analysis of HPS3d revealed that there were mainly C (38.18%, in mass), H (5.66%), O (53.91%), S (1.73%), and the molar ratios of C/H/O were around 1:2:1. 0.52% N in HPS3d demonstrated the presence of protein. The total sulfate content ($-SO_2O^-$) was 5.57%. The results of Kjeldahl method (Williams, 1984) demonstrate that the content of total protein was 3.25% in HPS3d, which in agree with the result of Bradford method.

3.4. Sugar analysis

HPS3d and HPS3di were carried on the further analysis of GC. The results were shown on (Table 1). Compared to HPS3d, much lower content of arabinose in HPS3di, suggesting that arabinose should be present at branch chains in HPS3d. HPS3di mainly contained galactose and galacturonic acid, indicating that they were the components of backbone structure of HPS3d.

3.5. FT-IR spectra analysis

From the FT-IR spectrum of HPS3d (Fig. 3), a broad stretching intense characteristic peak was shown at around 3413.43 cm^{-1} for the hydroxyl groups, and a weak C—H stretching band was observed at 2929.87 cm^{-1} (Santhiya, Subramanian, & Natarajan, 2002). Weak band occurring at 1741.03 cm^{-1} and a strong one at 1616.79 cm^{-1} were derived from the ester carbonyl (—COOH) groups and carboxylate ion stretching band ($-COO^-$), respectively. The absorption at 1075.38 cm^{-1} and 1044.73 cm^{-1} indicated the C—O—C and C—O—H linkage, respectively. The strong absorption at 810.17 cm^{-1} revealed the presence of α-anomeric configuration. In addition, the band at 891.03 cm^{-1} was ascribed to β-type glycosidic linkages (Zhang, 1999).

The absorb band at 1234.58 cm^{-1} describing an asymmetrical S=O stretching vibration and the 820.14 cm^{-1} indicating a symmetrical C—O—S vibration associated to a C—O—SO_3 group.

3.6. HPS3d induced the differentiation of osteoblasts

In the present study, the osteoblasts was treated with different concentrations of HPS3d (control, 0.5 to 32 mg/ml) at 72 h of culture. The results showed the viability of osteoblasts was not affected by HPS3d (0.5 to 8.0 mg/ml). Interestingly, higher concentrations (16 and 32 mg/ml) resulted in a decrease in cell viability (Fig. 4A). The ALP activity (Fig. 4B) and the mRNA expression of OPN, OCN and BSP (Fig. 4C–E) were not affected when the concentration was 0.5 mg/ml. HPS3d (1.0 to 4.0 mg/ml) concentration-dependently enhanced the ALP activity and the mRNA expression of OPN, OCN and BSP and not continuously enhance when the concentration was 8.0 mg/ml ($P > 0.05$).

3.7. HPS3d increased the expression of Runx-2 and Osterix in osteoblasts

Runx-2 and its downsteam factor Osterix are key transcription factors of osteoblastic differentiation (Chiu et al., 2010; Franceschi et al., 2003). We therefore hypothesized that HPS3d stimulated osteoblasts differentiation by modifying the expression and transcriptional activity of these two factors. As shown in Fig. 5A and B, a significant increase in Runx-2 and Osterix mRNA expression were observed starting at 1.0 mg/ml ($P < 0.05$) and reached 5.2 and 6.6-times increase, respectively, at 4.0 mg/ml when compared with control after 72 h incubation by HPS3d. The Western blot analysis also showed that the protein expression of Runx-2 and Osterix were significantly unregulated by HPS3d at 2.0 and 4.0 mg/ml (Fig. 5C–E).

4. Discussion

In this study, we identified the structural characteristics of HPS3d and found that HPS3d induced the expression of various osteogenic genes such as ALP, OPN, BSP, and OCN, as well as the

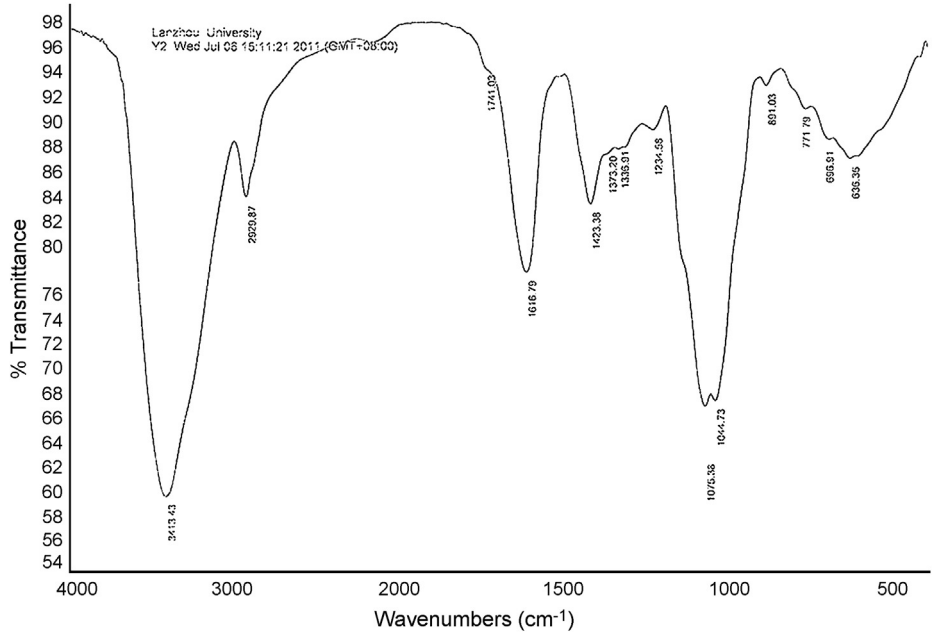

Fig. 3. Infrared spectra of HPS3d.

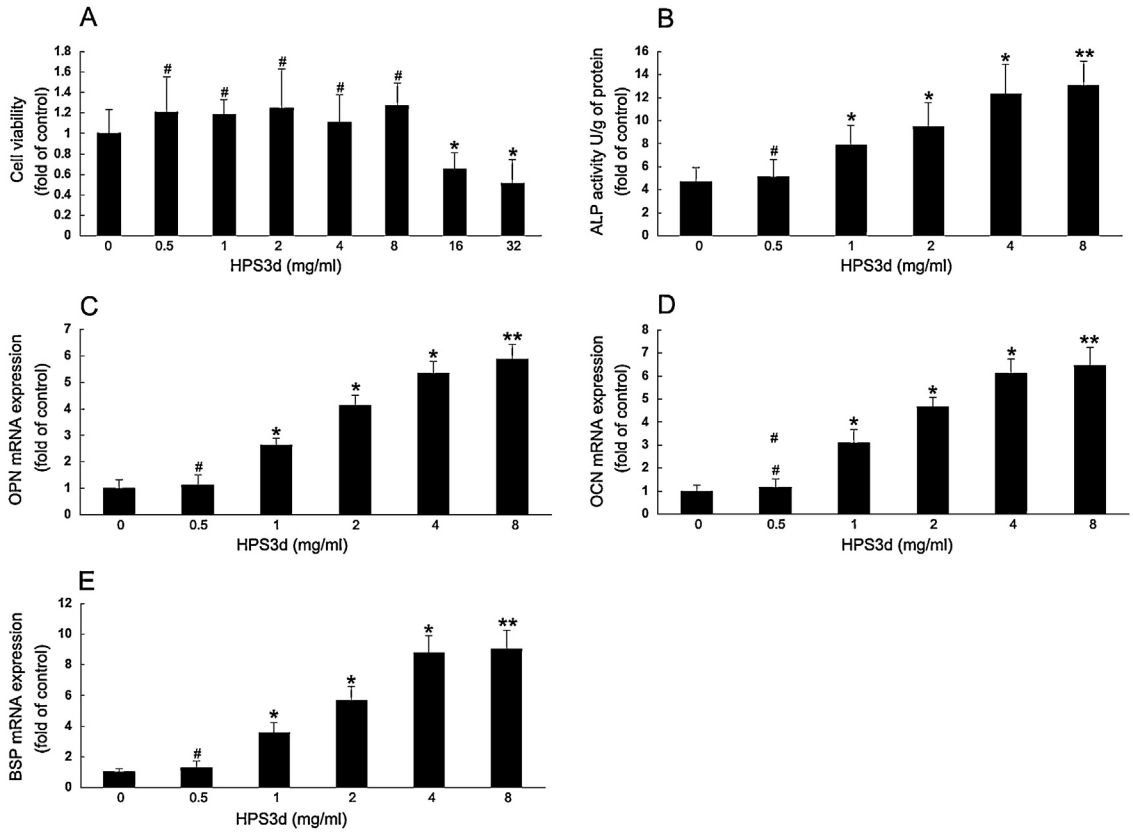

Fig. 4. Effects of HPS3d on cell viability and osteoblast differentiation markers, ALP activity and the mRNA expression of OPN, OCN and BSP in osteoblast. (A) Osteoblasts were treated with HPS3d (0 to 32 mg/ml) for 72 h and cell viability was evaluated by MTT assay. Osteoblasts were treated with HPS3d (0 to 8 mg/ml) for 72 h and (B) ALP activity was assessed by the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol. (C–E) The mRNA expression of OPN, OCN and BSP were determined by RT-PCR. The relative expression of OPN, OCN and BSP was analyzed by GeneTools from SynGene and was normalized to corresponding untreated HPS3d group. The data are presented as the mean ± SD from at least three independent experiments. $^{\#}$ $P > 0.05$, * $P < 0.05$ compared with the control group. ** $P > 0.05$ compared with the 4 mg/ml group.

expression and transcriptional activity of Runx-2 and Osterix, the master genes of osteogenic differentiation, in MC3T3-E1 cells.

The ALP activity and OPN expression are used as early phenotypic markers for osteoblast differentiation (Bilezikian, Raisz, & Martin, 2008; Wennberg et al., 2000), while OCN is a late osteoblastic marker (Higuchi et al., 2002). BSP can promote the nucleation of hydroxyapatite mineralization in vitro and increases calcium incorporation and nodule formation (Gordon et al., 2007; Ogata, 2008). The present results showed that HPS3d not only significantly increased ALP activity but also increased the expression of OPN, OCN and BSP mRNA in a dose-dependent. And the viability of osteoblasts was not affected at the same time.

Runx-2 is an important osteogenic transcription factor that can bind to the osteoblast-specific *cis*-acting element (OSE) 2 in the promoter region of osteogenic genes to initiate the expression of ALP, BSP, OCN and OPN (Franceschi & Xiao, 2003). It is also involved in tumorigenesis (Blyth, Cameron, & James, 2005) and chondrocyte differentiation (Enomoto et al., 2000). The importance of Runx-2 in osteoblast differentiation has been highlighted by the evidence that disruption of one copy of the Runx-2 gene can result in cleidocranial dysplasia (Lee et al., 2006) and that Runx-2 gene knockout in mice prevents osteoblast development (Ducy, Zhang, Geoffroy, Ridall, & Karsenty, 1997). Osterix (Osx or Sp7) is another osteoblast-specific transcription factor. Genetic studies have shown that cortical bone and bone trabeculae formation is abolished in Osterix knock-out mice. Also, in Osx-null mice, expression of type I collagen and osteoblast marker genes is reduced in mesenchymal cells although Runx-2 was expressed. However, Runx-2 expression in Osx-null mice is unaffected, whereas no Osterix transcripts are detected in skeletal elements of Runx2-null mice, indicating that Osterix acts as a downstream gene of Runx-2 in the osteoblast differentiation signaling pathway (Nakashima et al., 2002). Runx-2 and Osterix regulate the expressions of various bone marker genes, including ALP, BSP, OCN and OPN (Gordon et al., 2007; Hinoi et al., 2006). Here, we showed that HPS3d greatly increased the expression and transcriptional activity of Runx-2 and Osterix. Recently, many studies discussed the relationship between mitogen-activated protein kinase (MAPK) family and Runx-2/Osterix. For instance, phosphorylation of p38 MAPK induced the activation of Runx-2 via TAK1 and MEK3 signal pathway during early osteoblastic differentiation (Greenblatt et al., 2010). Furthermore, ERK1/2 mediated Runx-2 phosphorylation and transcriptional activity in bone (Ge, Xiao, Jiang, & Franceschi, 2007). Previous studies also reported that p38 MAPK regulated osteoblastic differentiation through Osterix (Ulsamer et al., 2008; Wang, Goh, & Li, 2007). Thus, whether HPS3d induced osteoblast differentiation and expressions of Runx-2/Osterix were mediated by ERK1/2 or p38 MAPK signaling pathways is a question requiring further research.

In conclusion, hot water was used to prepare crude polysaccharides from the dried root of *Hedysarum polybotrys Hand.-Mazz.* In this study. The purified HPS3d was obtained by anion-exchange column. HPS3d consisted of 94.38% polysaccharide, 3.40% protein and 13.30% uronic acid. The molecular weight of HPS3d was measured to be 84.6 kDa by SEC-MALLS. HPS3d took globular shape conformation in 0.1 M NaNO3. Results of chemical and spectroscopic analyses showed the backbone of HPS3d mainly consisted of galactopyranose and galacturonopyranose, and that the side chains were composed of glucopyranose, rhamnopyranose and arabinofuranose. The FT-IR and elemental analysis showed that HPS3d was the sulfated polysaccharide. HPS3d induced osteoblast

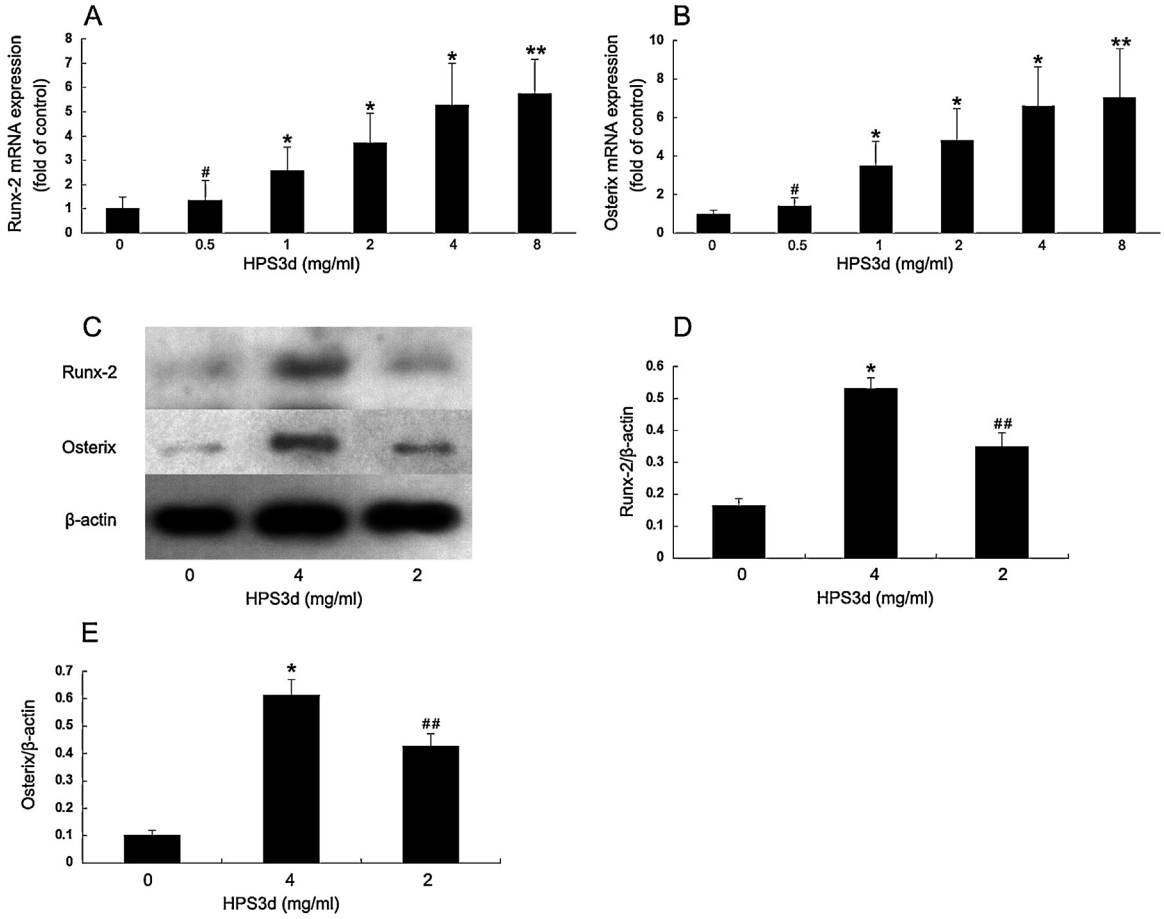

Fig. 5. Effects of HPS3d on mRNA levels and expression of Runx2 and Osterix in osteoblast. (A and B) Cells were treated with different concentrations (0 to 8 mg/ml) of HPS3d for 72 h and the analysis of Runx2 and Osterix mRNA expressionswas by RT-PCR. (C) Cells were treated with 2 and 4 mg/ml HPS3d, respectively, for 72 h and lysed and subjected to Western blot analysis. (D) Quantification of Runx2 and Osterix, respectively, versus β-actin. The data are presented as the mean ± SD from at least three independent experiments. $^{\#}$ $P > 0.05$, * $P < 0.05$ compared with the control group. ** $P > 0.05$ compared with the 4 mg/ml group.

differentiation by activating Runx-2 and Osterix. These findings suggest that HPS3d may be useful in inducing osteogenesis and in facilitating bone mineralization.

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