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Original Article

Impact of different implant materials on osteoblast activity after oral implantation

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Abstract: Objective: The current study aimed to explore the effects of nanohydroxyapatite/polycaprolactone (nHA/PCL) composites in different proportions on osteoblast proliferation, cell cycle, alkaline phosphatase activity, and mRNA expression of osteogenesis-related genes. Methods: Lingual bone lamellae were collected from healthy volunteers. The primary culture of human osteoblast was carried out by enzyme digestion and was identified. Effects of nHA/PCL composites in different proportions on osteoblast proliferation and cell cycle distribution were detected by MTT assay and flow cytometry. Alkaline phosphatase activities and mRNA expression of osteogenesis-related genes were detected by ELISA and real-time polymerase chain reaction (PCR). Results: The primary culture of human osteoblast was successful and identified. There were no significant differences in cell proliferation, alkaline phosphatase activity, and mRNA expression of osteogenesis-related genes between the PCL implant material group and blank control group. Cell proliferation, alkaline phosphatase activity, and mRNA expression of osteogenesis-related genes in nHA/PCL (40:60) and nHA/PCL (60:40) implant materials groups were higher than those in PCL implant material and blank control groups (all $P<0.001$). There were no significant differences in cell proliferation, alkaline phosphatase activity, and mRNA expression of osteogenesis-related genes between nHA/PCL (40:60) and nHA/PCL (60:40) implant materials groups. There were no significant differences in osteoblast cell cycle distribution among these groups. Conclusion: nHA/PCL composite can significantly promote osteoblast proliferation and differentiation, improving intracellular alkaline phosphatase activity.

Keywords: Nanohydroxyapatite/polycaprolactone, osteoblast, cell proliferation, cell cycle, alkaline phosphatase, osteogenesis-related gene expression

Introduction

Oral implantation provides good retention for dentures and has the features of being lifelike, aesthetic, and comfortable. For patients with tooth loss, oral implantation improves mental status and quality of life, providing a new option for patients with conventional dentures [1, 2]. Thus, it is especially important to select suitable implant materials [3]. Suitable implant materials can increase the biomechanical strength and biological integration of the implant-bone interface and promote the formation of osseointegration. It is believed that osseointegration is the standard for judging implant material property and clinical efficacy [4, 5]. Therefore, methods allowing implant materials to effectively combine with the bone and accelerate the healing of bone tissue around implant materials have been research hotspots.

Hydroxyapatite (HA) is the main component of the inorganic phase of human bone tissue, with stable chemical properties, good mechanical properties, and biocompatibility. The chemical formula is $Ca_{10}(PO_4)_6(OH)_2$. However, due to poor strength resistance and difficult degradation, HA has been limited in clinical application [6, 7]. Polycaprolactone (PCL), a kind of linear aliphatic polyester, has high biocompatibility and good biodegradability, but also has the disadvantage of insufficient strength [8]. In recent years, the emergence of nanotechnology has promoted the development of oral implantology. Studies have demonstrated that the size and morphology of pores in nanometer materials can regulate the growth and adhesion of bone cells and expression of bone-related genes [9, 10]. The microstructure of nanohydroxyapatite (nHA) is like that of the natural bone microstructure. This is beneficial to the exchange of nutriment and intervention of cells,

making the material more biocompatible and degradable. Nanohydroxyapatite/polycaprolactone (nHA/PCL) composite highlights the advantages of nHA and PCL and is a clinically promising bone substitute material. nHA/PCL composite microscopically simulates natural bone with a nanoscale crystal and lamellar structure. It has similar porosity to natural bone and has good cell conductivity and biocompatibility. Also, its biodegradation rate is compatible with the speed of osteogenesis [11]. Some studies have reported that mRNA expression of osteogenesis-related genes of bone marrow mesenchymal stem cells has increased after the induction of nHA/PCL [12]. Different proportions of nHA and PCL in nHA/PCL composites affect the biological characteristics of cells [13]. It is unclear whether the effects of physical and chemical property changes are caused by different proportions of nHA and PCL on osteoblast activity after nHA/PCL composite-to-osteoblast contact. Therefore, the current study explored the effects of nHA/PCL composites in different proportions on osteoblast activity, aiming to understand the molecular mechanisms of nHA/PCL composites on osteoblasts, providing an experimental basis for clinical application of oral implantation.

Material and methods

Materials

Primary culture of human osteoblast: This study was approved by the Ethics Committee of the Second Hospital of Shanxi Medical University. All participants providing specimens provided informed consent. The specimens were taken from healthy people that received physical examinations in the Department of Stomatology, Second Hospital of Shanxi Medical University. These people did not suffer from chronic diseases like hypertension and diabetes or oral diseases. Lingual bone lamellae were taken out when pulled out of the third molar. Blood stains in lingual bone lamellae were washed sufficiently under aseptic conditions. Bone tissue was cut into pieces and moved to the centrifuge tube. Trypsin (0.25%) was added to completely digest the bone tissue, then blowing was performed slowly. Bone tissue was centrifuged at 1,500 r/min for 10 minutes. The supernatant was discarded. DMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 ug/mL streptomycin was added to the sediment to prepare cell suspension. The cell suspension was placed in a culture flask and the culture flask was placed in a 5% CO_2 incubator (Model 3111, Thermo Fisher Scientific Inc., USA) at 37°C to carry out the primary culture of human osteoblast. Subsequent experiments were performed on cells in the logarithmic phase. Osteoblasts were identified by immunohistochemical staining collagen I. The operation was carried out in strict accordance with manufacturer instructions of the human collagen I immunohistochemistry kit (Boster Biological Technology Co. Ltd., Wuhan, China).

nHA/PCL composite: Three composites were prepared, according to different proportions of nHA and PCL, including PCL (group A), nHA/PCL (40:60) (group B), and nHA/PCL (60:40) (group C). These composites were formed into sheet shapes under pressure of 15 MPa for 3 minutes. The surfaces were polished, cleaned, and sterilized by high temperatures and high pressure for subsequent experiments. The material without composite was in the blank control group.

Main reagents: DMEM medium, fetal bovine serum (FBS), trypsin, and EDTA were purchased from Gibco, USA. Alkaline phosphatase (ALP) kit and MTT were purchased from Sigma-Aldrich, USA. Polymerase chain reaction (PCR) kit was purchased from BD Biosciences, USA. PCR primer synthesis was purchased from Shanghai GenePharma Co. Ltd., China. Mouse anti-human collagen I antibody (1:300) was purchased from Santa Cruz Biotechnology, Inc., USA.

Detection of the effects of implant materials on osteoblast proliferation by MTT assay

Human osteoblasts in the logarithmic phase were conventionally digested, centrifuged, and re-suspended to prepare a single cell suspension. The cell suspension was put in 96-well cell culture plates at a density of $3 * 10^4$ cells/well, then incubated in a 5% CO_2 incubator at 37°C. After cell adherence, the medium in wells was discarded and different proportions of composites and 100 μL medium were added. After incubating for 72 hours, 20 μL of 5 mg/mL dMTT was added to each well. After incubating for 4 hours, the supernatant was discarded and 150 μL DMSO solution was added, shaking

for 10 minutes in a dark place. The 96-well culture plate was placed in a microplate reader (Thermo Fisher Scientific Inc., USA) to detect the optical density (OD value) of each well at a wavelength of 490 nm. Five wells in each group were repeatedly detected and the average value was calculated.

Cell cycle ratios of osteoblasts

In each group, osteoblasts were put in 6-well cell culture plates at a density of 5 * 10^5 cells/mL. After 72 hours of incubation, osteoblasts were washed with phosphate buffered saline (PBS) and collected in a flow tube. Osteoblasts were fixed with 75% ethanol overnight at 4°C. Before detection, osteoblasts were digested with 0.25% trypsin - 0.02% EDTA and centrifuged. The supernatant was discarded to collect cells. One milliliter DNA staining solution (1% RNA enzyme, 2 mmol/mL EDTA, and 5 mg/L propidium iodide) was added to the cells, incubating for 30 minutes at room temperature in a dark place. Cell cycle was measured by a flow cytometer (Thermo Fisher Scientific Inc., USA). Proliferation index (PI) = (S-phase cell percentage + G2/M-phase cell percentage)/(S-phase cell percentage + G2/M-phase cell percentage + G0/G1-phase cell percentage) * 100%. Five samples were set up in each group and the average value was calculated.

Alkaline phosphatase activity of osteoblasts

Human osteoblasts in the logarithmic phase were put in 96-well cell culture plates at a density of 1.0 * 10^5 cells/mL. Cell suspensions (100 μL) were added to each well. The cell suspension was discarded after 24 hours of serum-free culturing. Different proportions of nHA/PCL composites and DMEM medium containing 10% FBS were added. After 3 days of incubation, the medium was discarded. Detection was carried out according to ALP kit instructions. A color-developing agent was added and blended. The absorbance (A value) of each well was measured with a microplate reader at a wavelength of 405 nm. The relative activity of ALP was determined by the percentage of ALP activity in each well/the activity in the blank control group. Five wells in each group were repeatedly detected and the average value was calculated. Protein quantification detection in each well was strictly performed according to operation procedures of the BCA (Bicinchoninic Acid) protein assay kit (Tiangen Biotech (Beijing) Co., Ltd., China). ALP activity = (A value in detecting tube/A value in standard tube * 0.003 mg)/the amount of protein in detecting tube (mg).

Detection of mRNA expression of osteogenesis-related genes via real-time PCR

Human osteoblasts in the logarithmic phase were put in 6-well cell culture plates at a density of 4.0 * 10^5 cells/well. The cell suspension was discarded after 24 hours of serum-free culturing. Different proportions of nHA/PCL composites and DMEM medium containing 10% FBS were added. After 3 days of incubation, the medium was discarded. Total RNA in each group was extracted with TRIzol Reagent (Invitrogen Corporation, USA). Total RNA was synthesized into cDNA according to instructions of the reverse transcription kit. GAPDH served as the internal reference. GAPDH upstream primer: 5'-CAAGGTCATCCATG ACAACTTTG-3', downstream primer: 5'-GTCCACCACCCTGTTTCTGTAG-3'. Osteopontin upstream primer: 5'-CTTTACAGCCTGCACCCAGAT-3', downstream primer: 5'-CCTTTCCGTTGTTGTCCTGAT-3'. Osteonectin upstream primer: 5'-GCCAGAACCATCATTGCAAA-3', downstream primer: 5'-TTCTCAAAGTCTCGGGCCAA-3'. Osteocalcin upstream primer: 5'-GACACCATGAGGACCATCTTT-3', downstream primer: 5'-TTTTGGAGCTGCTGTGACAT-3'. There was 20 uL reaction system, including 2.0 uL cDNA, 1.0 uL upper primer, 1.0 uL lower primer, 10.0 μL SYBR Green PCR Master Mix, and 6.0 uL ddH_2O. After centrifugation, amplification was performed by Applied Biosystems 7,500 quantitative PCR. Reaction conditions: pre-denaturation for 3 minutes at 94°C, denaturation for 30 seconds at 94°C, renaturation for 30 seconds at 60°C, and extension for 90 seconds at 68°C, for 30 cycles. With CAPDH as the reference gene, mRNA expression of osteopontin, osteonectin, and osteocalcin, in each group, was calculated using the $2^{-\Delta\Delta Ct}$ method. Five samples were set up in each group and the average value was calculated.

Statistical analysis

SPSS 21.0 statistical software was used to analyze all experimental data. Measurement data are expressed as mean ± standard deviation ($\bar{x}$ ± sd). Multi-group comparisons were performed with one-way analysis of variance.

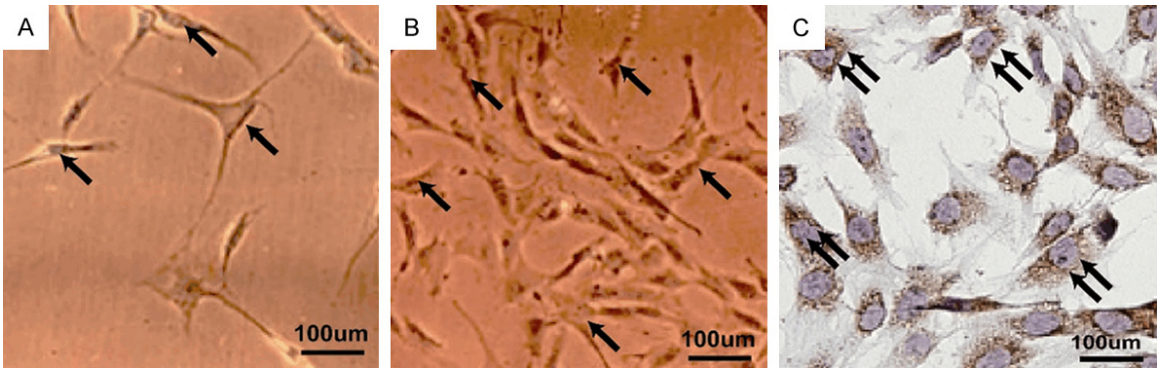

Figure 1. Culture and identification of human osteoblasts (100X). A: 1-day primary culture of osteoblasts. Single arrows indicate osteoblasts; B: 1-week primary culture of osteoblasts. Single arrows indicate osteoblasts; C: Collagen I immunohistochemical staining. Double arrows indicate collagen I positive cells.

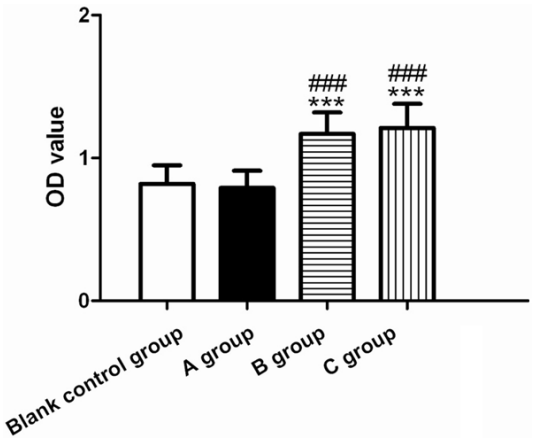

Figure 2. Detection of osteoblast activities in different composites by MTT assay. Compared with the blank control group, ***P<0.001; compared with group A, ###P<0.001.

Comparisons between groups were performed using Bonferroni's method. Enumeration data are expressed as n/%. Comparisons between groups were performed using Chi-squared test. P<0.05 indicates statistical significance.

Results

Culture and identification of osteoblasts

After 24 hours of primary culturing, some osteoblasts began to adhere to the wall and developed many pseudopodia (**Figure 1A**). After culturing for about 1 week, many osteoblasts adhered to the wall and showed in the shape of a triangle, polygon, and fusiform (**Figure 1B**). Osteoblasts were identified by collagen I immunohistochemical staining. Brown staining showed positive expression (**Figure 1C**).

Effects of different composites on osteoblast proliferation

There were no significant differences in OD values between group A and the blank control group. OD values in groups B and C were significantly higher than those in group A and the blank control group (both P<0.001). There were no significant differences in OD values between groups B and C (**Figure 2**).

Comparison of osteoblast cell cycles

There were no significant differences in osteoblast percentages in G0/G1, S, and G2/M phases among these groups. There were no significant difference in PI among these groups (**Table 1**).

Comparison of ALP activities of osteoblasts

Results of analysis of variance showed significant differences in ALP activities of osteoblasts among these groups (P<0.001). ALP activities of osteoblasts in group A were lower than those in the blank control group, but differences were not significant. ALP activities of osteoblasts in groups B and C were significantly higher than those in group A and the blank control group (both P<0.001). There were no significant differences in ALP activities between groups B and C (**Table 2** and **Figure 3**).

mRNA expression of osteogenesis-related genes

Results of analysis of variance showed significant differences in mRNA expression of osteo-

Table 1. Osteoblast percentages in cell cycles phases (%)

Group	G0/G1 phase	S phase	G2/M phase	PI
Blank control	82.73±4.78	14.48±3.65	2.70±0.75	17.20±1.25
A	83.65±4.81	13.19±2.92	3.16±0.83	16.35±1.09
B	84.92±2.37	11.87±2.47	3.21±0.67	15.08±1.04
C	85.13±3.16	11.79±1.95	3.08±0.55	14.87±1.00
F value	0.413	1.019	0.534	2.979
P value	0.746	0.410	0.665	0.063

Note: PI, proliferation index.

Table 2. Comparison of ALP activities of osteoblasts

Group	ALP activity (OD)
Blank control	1.000
A	0.895±0.025
B	1.524±0.031***,###
C	1.589±0.056***,###
F value	534.400
P value	<0.001

Note: Compared with the blank control group, ***P<0.001; compared with group A, ###P<0.001. ALP, alkaline phosphatase; OD, optical density.

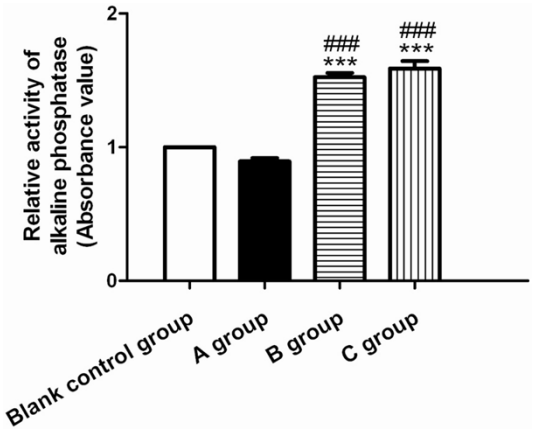

Figure 3. Comparison of ALP activities of osteoblasts. Compared with the blank control group, ***P<0.001; compared with group A, ###P<0.001.

pontin, osteocalcin, and osteonectin among these groups (P<0.001). There were no significant differences in mRNA expression of osteopontin, osteocalcin, and osteonectin between group A and the blank control group. mRNA expression of osteogenesis-related genes in groups B and C was significantly higher than that in group A and the blank control group (both P<0.001). There were no statistical differences in mRNA expression of osteogenesis-related genes between groups B and C (**Table 3** and **Figure 4**).

Discussion

It is currently believed that osseointegration is the key to successful oral implantation with good long-term effects. Osteoblasts are important functional cells involved in bone remodeling and bone formation. The generation of osteoblasts is critical for good implants [14]. Different implant materials can affect the biological integration of the implant-bone interface to varying degrees. An ideal implant material can induce osteoblasts to adhere to the surface of the implant early, proliferating and differentiating. It further induces osteoblasts to synthesize and secrete osteogenesis-related proteins mineralizing into the bone [15]. In recent years, studies on implant materials have focused on nanocomposites. Due to good mechanical properties and biocompatibility, nanocomposites can replace conventional materials [16, 17]. In this study, nHA/PCL composites were selected as research objects. Different proportions of nHA/PCL composites have different physicochemical properties. Therefore, the current study explored the effects of different proportions of nHA/PCL composites on osteoblast activity.

Osteoblasts were isolated and cultured by enzyme digestion. Results showed that cells began to adhere to the wall after 24 hours of culturing. Many cells adhered to the wall after 1 week of culturing. Moreover, collagen I immunohistochemical staining showed that a large amount of collagen I protein was expressed in the cytoplasm. This was consistent with the study results of Ferraz et al. [18]. Gapski et al. evaluated osteoblasts activities by the detection of collagen I protein in a study concerning the effects of titanium material surface and zirconia material surface on osteoblast activity. Their results were in accord with present results [19]. Osteoblast proliferation on the surface of implant material can reflect osteoblast activity of the implant material. MTT assay can be used to detect high sensitivity of cell proliferation. Exogenous MTT can be reduced to a blue-violet crystal formazan by succinate dehydrogenase in living cell mitochondria and deposited in

Table 3. mRNA expression of osteogenesis-related gene

Group	Osteocalcin	Osteonectin	Osteopontin
Blank control	0.976±0.082	1.015±0.075	1.005±0.063
A	0.965±0.074	0.984±0.061	0.955±0.067
B	1.821±0.125***,###	1.573±0.095***,###	1.921±0.145***,###
C	1.952±0.131***,###	1.691±0.121***,###	2.109±0.156***,###
F value	125.600	82.290	135.000
P value	<0.001	<0.001	<0.001

Note: Compared with the blank control group, ***P<0.001; compared with group A, ###P<0.001.

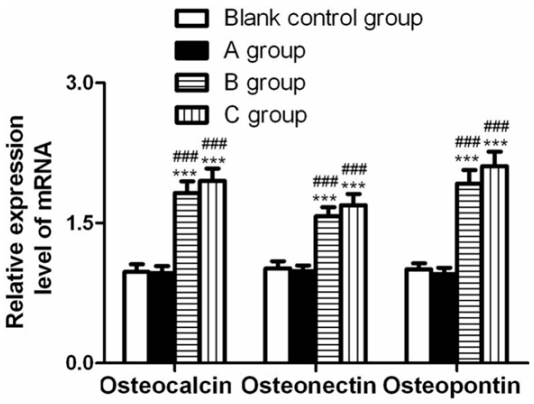

Figure 4. mRNA expression of osteogenesis-related genes (osteopontin, osteocalcin and osteonectin). Compared with the blank control group, ***P<0.001; compared with group A, ###P<0.001.

cells, whereas dead cells are without this feature [20]. In this study, MTT assay was used to detect the effects of different proportions of nHA/PCL on human osteoblast proliferation. Present results showed that cell proliferation activities of nHA/PCL (40:60) and nHA/PCL (60:40) composites were significantly higher than those of PCL and the blank control group, with significant differences. Cell proliferation activities of nHA/PCL (60:40) composites were higher than those of nHA/PCL (40:60) composites, without significant differences. Results indicate that the addition of nHA to the PCL matrix could induce osteoblast proliferation and nHA could promote cell proliferation. Zhang et al. showed that PHBV/PAA (poly-3-hydroxybutyrate-co-3-hydroxyvalerate/polyaspartic acid)-nHA composite could significantly improve osteoblast proliferation, adhesion, and mineralization, compared with PHBV/PAA. This is consistent with present results [21]. Some studies have shown that high contents of calcium and phosphorus in nHA can form a microenvironment suitable for bone formation, which is conducive to osteoblast proliferation [22]. Flow cytometry results of this study showed no significant differences in cell cycle distribution of osteoblasts among these groups, indicating that nHA/PCL composites did not significantly change the cell cycles of osteoblasts. Combined with MTT assay results, nHA/PCL composites do not regulate cell proliferation by affecting the cell cycle. Thus, the way in which nHA/PCL composites promote osteoblast proliferation is worth exploration.

ALP activity, as an indicator of osteoblast function, is a specific marker for early osteoblast differentiation [23]. One study showed that alkaline phosphatase plays an important role in the maturity of the bone matrix. In the absence of alkaline phosphatase, calcification cannot be formed [24]. In addition, osteogenesis-related proteins play an important role in the development of osteoblast differentiation, maturation, and bone matrix calcification. Among proteins, osteopontin is a non-collagen bone matrix glycoprotein. Osteopontin can regulate proliferation, differentiation, migration, and adhesion of cells [25]. Osteocalcin, which is mainly distributed in the teeth and bone tissues, is a marker of bone mineralization. It can bind to calcium ions, promoting bone resorption [26]. Osteonectin also plays an important role in the mineralization of bone matrix. It regulates the interaction between extracellular matrix proteins [27]. Results of this study showed that ALP activities of osteoblasts in nHA/PCL (40:60) and nHA/PCL (60:40) composites, as well as mRNA expression of osteogenesis-related genes (osteopontin, osteonectin, and osteocalcin), were significantly higher than those of PCL and blank control groups, with significant differences. However, ALP activities and mRNA expression of osteogenesis-related genes in nHA/PCL (60:40) composites were higher than those in nHA/PCL (40:60) composites, without significant differences. Results indicate that the addition of nHA to the PCL matrix could promote ALP activity and osteogenesis-related gene expression. nHA could improve the biological activity of implant materials. nHA/PCL could improve osteoblast activity. Mohamadyar-

Toupkanlou et al. indicated that nHA/PCL composites coated with fibronectin could promote differentiation of bone marrow mesenchymal cells into osteoblasts, promoting ALP activity and osteogenesis-related gene (osteocalcin, osteopontin, and Runx2 (Runt-related transcription factor 2) protein expression [28]. However, the mechanisms of nHA/PCL composites affecting osteoblast activity require further study.

In summary, nHA/PCL composites can significantly promote osteoblast proliferation, increase ALP activity, and induce osteoblast differentiation. This is an implant material with good prospects for clinical application.

Disclosure of conflict of interest

None.

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