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The effect of partially stabilized zirconia on the biological properties of HA/HDPE composites *in vitro*

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Abstract The effect of partially stabilized zirconia (PSZ) on the biological properties of the hyroxyapatite - high density polyethylene (HA/HDPE) composites was studied by investigating the simultaneous effect of hydroxyapatite and PSZ volume fractions on the *in vitro* response of human osteoblast cells. The biocompatibility of composite samples with different volume fraction of HA and PSZ powders was assessed by proliferation, alkaline phosphatase (ALP) and cell attachment assays on the osteoblast cell line (G-292) in different time periods. The effect of composites on the behavior of G-292 cells was compared with those of HDPE and TPS (Tissue Culture Poly Styrene as negative control) samples. Results showed a higher proliferation rate of G-292 cells in the presence of composite samples as compared to the HDPE sample after 7 and 14 days of incubation period. ALP production rate in all composite samples was higher than HDPE and TPS samples. The number of adhered cells on the composite samples was higher than the number adhered on the HDPE and TPS samples after the above mentioned incubation periods. These findings indicates that the addition of PSZ does not have any adverse affect on the biocompatibility of HA/HDPE composites. In fact in some experiments PSZ added HA/HDPE composites performed better in proliferation, differentiation and attachment of osteoblastic cells.

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1. Introduction

The main driving force behind the use of calcium phosphates as bone substitute material is their similarity to the mineral component of bone. In addition to being non-toxic, they are biocompatible and most importantly exhibite bioactive behavior, being integrated into the tissue by the same processes active in remodeling healthy bone [1, 2]. HA is biocompatible and undoubtedly osteoconductive, it suffers from limited bioactivity due to its stability and hence extremely slow degradation rate in biological fluids [3, 4]. Hydroxyapatite-high density polyethylene (HA/HDPE) composites have been developed as a bone replacement material [5]. The mechanical properties of this composite and the biological response to it have been studied extensively by a number of researchers [6–20]. In cell culture studies with human osteoblasts, the cells was shown to grew and spread over the composites, attaching themselves to the hydroxyapatite particles on the sample surface [9, 17–20].

In our previous study, we showed that the partial replacement of hydroxyapatite with partially stabilized zirconia (PSZ) is beneficial in the improvement of both the fracture strength and failure energy values in the composite samples [21]. The primary objective of this study was to investigate whether altering the PSZ volume fraction in the composite samples would have any significant effect on the biological properties of hyroxyapatite-high density polyethylene (HA/HDPE) composite bulk samples. Zirconia ceramics have several advantages over other ceramic materials, due to the transformation toughening mechanisms operating in their microstructure that can give components made out of them very intresting mechanical properties [22]. Zirconia has a significantly lower coefficient of friction against articular cartilage and should be beneficial in applications where the material is employed as the bearing surface for

hemiarthroplasty devices [23]. Biocompatibility of zirconia has been evaluated by both the in vitro and in vivo tests [22]. Results showed adverse reactions relatable to the presence of zirconia [22, 24–26]. In this present study, for the first time, we have investigated the effects of surface structure of PSZ added HA/HDPE composites on the proliferation, differentiation and attachment of osteoblast cell line (G-292) in different time periods.

2. Materials and methods

2.1. Materials and sample preparation

Hydroxyapatite powder (Merck, 102196) was used as the filler material for the preparation of HA-HDPE composites. The matrix polymer was a high density polyethylene(0.944gr/cm3, supplied by Arak petrochemical Co., in Iran). As received hydroxyapatite powder was calcined at 1200°C for 2h prior to use. Partially stabilized zirconia (Table 1) was synthesized by adding 4wt.% CaO into high purity (99.8%) chemical grade monoclinic zirconia powder [21].

HA/HDPE and HA+PSZ/HDPE composite samples with different ceramic powder volume fractions (Table 2) were prepared by first blending and compounding the HDPE and ceramic powders to produce a homogeneous composite mixture.

The composite mixtures were shaped in to block shapes by compression molding. The shaped samples were cut into $2 \times 2 \times 2$ mm and $10 \times 10 \times 2$ mm blocks. The samples were sterilized by gamma irradiation at a dose of 25 KG (Co60 γ cell-220) using standard procedures for medical devices.

2.2. *In vitro* cell culture

The human primary osteogenic sarcoma cell line G-292 Colon A141B1 (NCBI C-116; National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran) were cultured in Dulbecco's Modified Eagle´s Medium (DMEM) (GIBCO, Scotland) supplemented with 10% fetal calf serum (FCS) (Seromed, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, USA). G-292 cells were harvested with 0.25% trypsin-EDTA solution (Sigma, USA) in phosphate-buffered saline (PBS, pH 7.4) and seeded onto the composites at a density of 2×10^3 cells/well into 96-well microtiter plates for cytotoxicity/proliferation and differentiation assays. These cells were cultured on the surface of the composite samples at 8×10^3 cells/well into 24-well plates for attachment and cell morphology determination assays. The cells were incubated at 37°C in humidified air with 5% CO_2 for a period of 3, 7 and 14 days. The cultured medium was changed at selected time intervals, with care not to cause any disturbance to culture conditions.

2.3. Cell proliferation

Proliferation study was performed to investigate the biofunctionality of the materials. The growth and proliferation of G-292 cells on the composites were measured using dimethylthiazol diphenyl tetrazolium bromide (MTT) assay. Briefly, G-292 cultured cells were resuspended in culture medium at a density of 2×10^3 cells/100μl and were added to each of the wells in the 96-microliter plates (NUNC, Denmark). The plates were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 in air. The composite samples with composition given in Table 2, were placed on the G-292 cells. Four G-292 cultured wells with no sample were used as negative controls [(Tissue Culture Poly Styrene (TPS)]. In addition, three wells containing only HDPE were used as composite control. The plates containing the composites and the cells were incubated at 37°C with 5% CO_2 for 3, 7 and 14 days. The medium was changed every three days. At predetermined intervals, composites were taken out of wells. 10μl of MTT (5mg/ml in medium) was added to each of the wells and incubated for another 5h at 37°C in a humidified atmosphere of 5% of CO_2 in air. At this stage the MTT was removed and 100 μl/well of isopropanol (Sigma, USA) was added in order to dissolve the formazan crystals. The plates were placed in the incubator for 10 min and then in a cold room for 15 min prior to absorbance measurements.

Table 1 Chemical analysis for the partially stabilized zirconia powder

Oxide	Na_2O	MgO	Al_2O_3	SiO_2	P_2O_5	CaO	TiO_2	Fe_2O_3	ZrO_2	HfO_2
Wt %	0.13	0.35	0.12	<100ppm	0.093	4.0	0.20	0.032	95.1	<100ppm

Table 2 Compoistion and calculated bulk density values of the composite samples prepared in this study

Sample code	HA (Wt %)	PSZ (Wt %)	HDPE (Wt %)	Composite bulk density (g/cm^3)
20HA	45.65	0	54.35	1.38
15HA5PSZ	30.88	19.93	49.19	1.53
40HA	69	0	31	1.83
20HA20PSZ	26.03	50.57	23.40	2.42

The optical density (OD) was read on a multiwell microplate reader (ICN, Switzerland) at 570 nm. The experiment was repeated three times. The cells number were calculated based on standared curve prepared from results of cell proliferation in different densities.

2.4. Cell differentiation

Osteoblastic phenotype was determined biochemically by measuring ALP production from the G-292 cells. The cells were seeded into a 96-microliter plates at a density of 2×10^3 cells/100 μl for each well. The composite samples were placed on the G-292 cells. Four G-292 cultured wells with no sample were used as negative controls (TPS). In addition, three wells containing only HDPE were used as composite control. The plates containing the composites and cells were incubated for 3, 7 and 14 days at 37°C in humidified air with 5% CO_2. The culture medium was changed at selected time intervals. G-292 cells were lysed on the 3rd, 7th and 14th days (freeze/thaw). ALP activity was determined using a Cobas-Bio (Roche, UK) centrifugal analyzer; p-nitrophenol phosphate in diethanolamine buffer (Randox, UK) was used as a substrate for ALP. The reaction product, p-nitrophenol, is yellow at alkaline pH=9.8, and can be quantified at a wavelength of 405 nm. The experiment was repeated three times.

2.5. Cell attachment

One milliliter of G-292 cells at a concentration of 8×10^3 cell/well were added to the surface of each composite sample in a 24 well plates (Greiner, Germany). The plates were placed into an incubator at 37°C for 2h to allow for the cells to attach to the surfaces. Negative and composite control samples were also used in this experiment similar to the other tests. After 2h, 1.5 ml of DMEM was added to each well. The plates containing the cells and composite samples were incubated at 37°C with 5% CO_2 for 3, 7 and 14 days. The culture medium was changed every three days. On the 3rd, 7th and 14th days, the plates were removed from the incubator and rinsed with PBS. Adhered cells were lifted from the substrates with a 0.25% trypsin solution. The cells were washed using the medium and a mixture of cell suspension with equal volume of 0.2% of trypan blue solution. Finally the cells were enumerated by using neobar lam and light microscope. The experiment was repeated three times.

2.6. Statistical analysis

Each of the experimental points reported in this study is an average value taken out of three measurements. The standard deviations are indicated as error bars in the figures. Statistical analysis was performed using Sigmaplot v.5 software (SPSS Inc). Data are reported as mean ± SD at a significance level of $p < 0.05$. The student's t test was used to compare the in vitro data for the composite and control samples. Differences were considered statistically significant when the p value was < 0.05.

3. Results and discussion

The ceramic particle distribution in the composite sample containing 20 vol % hydroxyapatite and 20 vol % PSZ (20HA20PSZ) is shown in Fig. 1. Except for the few areas with agglomerated particles, there is a rather uniform distribution of filler particles in HDPE matrix.

In the development of bioactive material both mechanical and biological characteristics must be considered [17, 27]. In an earlier study we showed some improvement in the mechanical properties of HA/HDPE composites with partial replacement of HA with PSZ [21]. When evaluating biological response, it is necessory to select the appropriate cell type, as the biocompatibility of a material is determined by its ability to elicit a specific host response in a specific application [17]. Osteoblastic cells have been reported to probe the substrate surface with filopodia presented on the cell lamellipodia and produce the organic matrix of bone in a highly organized maner [27–32].

An increase with time in total number of viable cells was observed over the 14 days of incubation period for all samples include: composites, HDPE and TPS (Fig. 2).

The rate of osteoblastic cell proliferation was seen to peak on 14th day with the composite samples giving an equal or

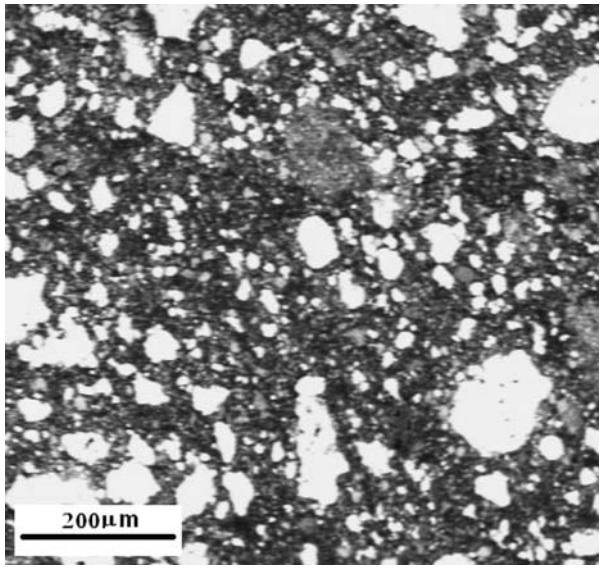

Fig. 1 SEM micrograph showing the distribution of the filler particles in the polymer matrix: Sample containing 2o vol % HA + 20 vol % PSZ.

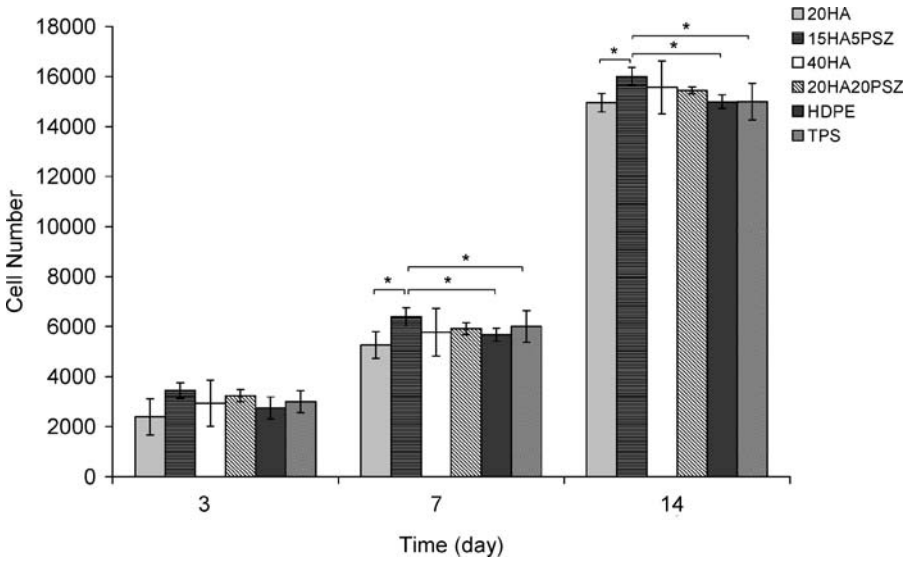

Fig. 2 Cell proliferation of G-292 grown on composite samples after different time periods. 20HA: 20 Vol.% HA, 15HA5PSZ: 15 Vol.% HA and 5 Vol.% PSZ, 40HA: 40 Vol.% HA, 20HA20PSZ: 20 Vol.% HA and 20 Vol.% PSZ, HDPE: High Density Polyethylene, TPS: Tissue Culture Polystyrene (negative control), (*: $p < 0.05$).

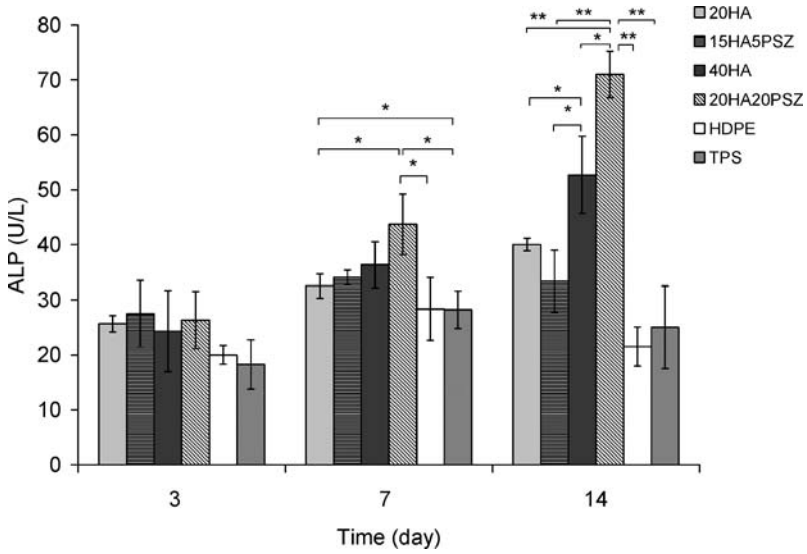

Fig. 3 ALP activity for G-292 cells on composite samples after different time periods. 20HA: 20 Vol.% HA, 15HA5PSZ: 15 Vol.% HA and 5 Vol.% PSZ, 40HA: 40 Vol.% HA, 20HA20PSZ: 20 Vol.% HA and 20 Vol.% PSZ, HDPE: High Density Polyethylene, TPS: Tissue Culture Polystyrene (negative control), (*: $p < 0.05$, ** : $p < 0.005$).

in some cases an even higher proliferation rate in compare to HDPE and TPS samples in culture period. The significant difference between 15HA5PSZ composite and 20HA, HDPE and TPS was observed on the 7th and 14th days ($p < 0.05$).

In all time intervals the 40HA composite sample showed higher proliferation rate than 20HA composite. On the whole the PSZ containing composites showed higher proliferation rate than HA/HDPE composites. The highest growth rate was obtained for 15HA5PSZ composite in all time intervals.

The increase in ALP activity indicated that the cells had ceased to proliferate and had begun to differentiate [9]. ALP is expressed by osteoblastic cells soon after a decrease in proliferation, and it is believed to have roles in hydrolysis of pyrophosphate, and ATP to orthophosphate, which is used to form the nascent $CaPO_4$ mineral. Thus an increase in ALP production and activity in vitro corresponds to bone formation in vivo [27].

In this study an increase with time in ALP production can be seen for composite samples except 15HA5PSZ composite over the 14 day (Fig. 3). Significanty higher increase in ALP activity was noted for G-292 cells cultured on HA/HDPE and PSZ containing composites compared to HDPE and TPS samples.

At 7 and 14 days 40HA composite showed significantly higher ALP production than the 20HA composite. Thus an increase in the hydroxyapatite content, led to the higher ALP production in HA/HDPE composites. Comparison of HA/HDPE and PSZ added HA/HDPE composites showed that the addition of PSZ into the HA/HDPE composites result in improving differentiation and higher ALP production of osteoblastic cells ($p < 0.05$, $p < 0.005$). Overall, the 20HA20PSZ composite showed significantly higher ALP

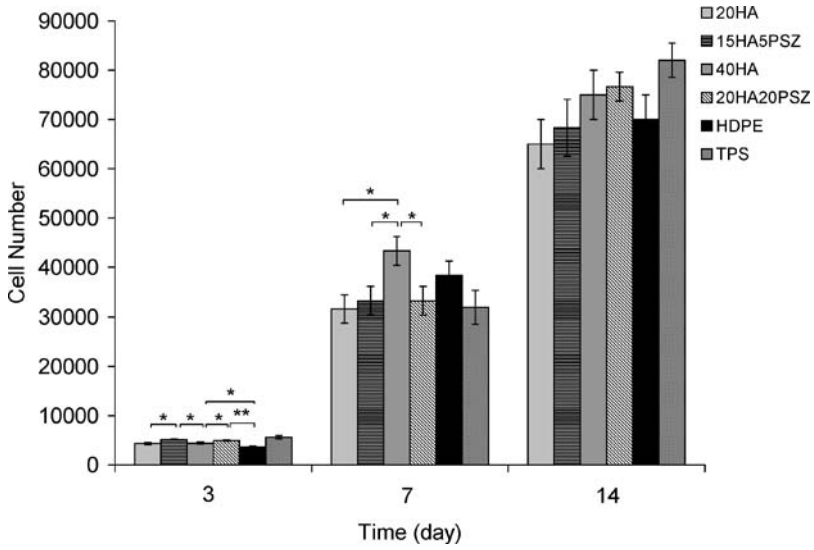

Fig. 4 Outcome of osteoblastic cells attachment to the surface of composite samples after different time periods. 20HA: 20 Vol.% HA, 15HA5PSZ: 15 Vol.% HA and 5 Vol.% PSZ, 40HA: 40 Vol.% HA, 20HA20PSZ: 20 Vol.% HA and 20 Vol.% PSZ, HDPE: High Density Polyethylene, TPS: Tissue Culture Polystyrene (negative control), ($^{*}: p < 0.05$, $^{**}: p < 0.005$).

production over 14 days of incubation($p < 0.05$ and $p < 0.005$). The general trend observed in this study is consistent with the previous studies done on the HA/HDPE composites [17–20, 27]. Osteoblast cells cultured on both composite types showed an initial increase in proliferation rate, followed by an increase in expression of ALP, an osteoblast marker. Similar behaviour on these composites has been observed. Thus indicating the effect of the bioactive component in the composite material [18]. In comparison to the HDPE sample, the biological performance of composite samples was better with increased ALP production and proliferation rate of G-292 cells.

The number of G292 cells adhered on the composite, HDPE and TPS samples are shown in Fig. 4. A continuous increase in the number of adhered cells can be seen over the 14 days of incubation period.

In comparsion 40HA composite showed higher cell attachment than 20HA composite and the difference was significant on the 7 th day ($p < 0.05$). On 7th day 40HA composite had higher cell number adhered than other composite, HDPE and TPS samples. The difference between 40HA and 20HA20PSZ composites and HDPE sample was significant ($p < 0.05$ and $p < 0.005$ respectively) at the 3rd day of incubation. The adhesion of G-292 cells on the composites was as high as that on TPS and in some cases even higher (Fig. 4). These results showed that the surface of composites was sufficiently suitable to support the adhesion and growth of osteoblastic cells. Comparison of PSZ containing composites with HA/HDPE composites showed that the addition of PSZ into the HA/HDPE composite result in higher cell numbers adhered to the composites (Fig. 4).

In this study the biocompatibility of PSZ containing HA/HDPE composites was demonstrated by the ability of the cells to proliferate on the materials. The cell attachment was followed by proliferation and differentiation of the cells when considering osteoblast expression and hence ALP activity, a greater activity was observed in G292 cells on PSZ containing composites as compared to HA/HDPE, HDPE and TPS samples.

Conclusions

The results show that the volume fraction of hydroxyapatite has a significant effect on the bioactivity of the composites. The composites provide a favourable site for cell attachment, with cell processes frequently observed anchoring to the HA particles. Also results show that the addition of PSZ into the HA/HDPE composites does not adversely affect the biological properties of these composites. In fact in some cases, composites with PSZ showed better biological results than HA/HDPE composites. Thus we can increase the mechanical properties of these composites without any decrease in biological properties of the composites.

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