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Biological evaluation of partially stabilized zirconia added HA/HDPE composites with osteoblast and fibroblast cell lines

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Abstract In the present study, the biocompatibility of partially stabilized zirconia (PSZ) added hydroxyapatite (HA)—high density polyethylene (HDPE) composites was evaluated by proliferation and cell attachment assays on two osteoblast cell lines (G-292, Saos-2) and a type of fibroblast cell isolated from bone tissue namely HBF in different time intervals. Cell-material interactions on the surface of the composites were observed by scanning electron microscopy (SEM). The effect of composites on the behavior of osteoblast and fibroblast cells was compared with those of HDPE and Tissue Culture Poly Styrene (TPS) (as negative control) samples. Results showed that the composite samples supported a higher proliferation rate of osteoblast cells in the presence of composite samples as compared to the HDPE and TPS samples after 3, 7 and 14 days of incubation period. It was showed that an equal or in some cases an even higher proliferation rate of G-292 and Saos-2 osteoblast cells on composite samples in compare to negative controls in culture period ($P < 0.05$). The number of adhered cells on the composite samples was equal and in some cases higher than the number adhered on the HDPE and TPS samples after the above mentioned incubation periods ($P < 0.05$). Adhered cells presented a normal morphology by SEM and many of the cells were seen to be undergoing cell division.

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1 Introduction

Because of its chemical composition and crystallographic structure similar to those of human hard tissues, HA has been extensively studied for use as bone and tooth implants [1]. It is biocompatible and most importantly exhibits bioactive behavior, being integrated into the tissue by the same processes active in remodeling healthy bone [2, 3]. HA is osteoconductive, it suffers from limited bioactivity due to its stability and hence extremely slow degradation rate in biological fluids [4, 5]. However, as HA is intrinsically poor in mechanical properties, bone replacement parts made from HA have been used only in non-load-bearing areas such as the ossicles in the middle ear [1]. Therefore, for full utilization of bioactive HA-based implants, improvements in mechanical properties are necessary. HA/HDPE composites have been developed as a bone replacement material [6]. The mechanical properties of this composite and the biological response to it have been studied extensively by a number of researchers [7–21]. In cell culture studies with human osteoblasts, the cells was shown to grew and spread over the composites, attaching themselves to the HA particles on the sample surface [10, 18–21].

In the previous study, it has been shown that the partial replacement of HA with PSZ is beneficial in the improvement of both the fracture strength and failure energy values in the composite samples [22]. It was also showed that the addition of PSZ into HA/HDPE composites not only does not have negative effect on osteoblasts

behavior but also in some cases increase their proliferation and attachment [23]. The primary objective of this study was to investigate the simultaneous effect of HA and PSZ volume fractions on the in vitro response of on two osteoblast cell lines (G-292, Saos-2) and a type of fibroblast cell isolated from bone tissue namely HBF in different time periods. 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 interesting mechanical properties [24]. 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 [26]. Biocompatibility of zirconia has been evaluated by both the in vitro and in vivo tests [24, 25]. Results showed adverse reactions relatable to the presence of zirconia [27, 27–29]. In this present study the authors have investigated the effects of surface structure of PSZ added HA/HDPE composites on the proliferation and attachment of two osteoblast cell lines (G-292, Saos-2) and a type of fibroblast cell isolated from bone tissue namely HBF in different time intervals.

2 Materials and methods

2.1 Materials and sample preparation

HA powder (Merck, 102196) was used as the filler material for the preparation of HA/HDPE composites. The matrix polymer was a HDPE (0.944 gr/cm3, supplied by Arak petrochemical Co., in Iran). As received HA powder was calcined at 1,200 °C for 2 h prior to use. PSZ (Table 1) was synthesized by adding 4 wt% CaO into high purity (99.8%) chemical grade monoclinic zirconia powder [22].

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 × 2 × 2 mm and 10 × 10 × 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.

Table 2 Composition 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

2.2 In vitro cell culture

The bone cell lines with following characteristics 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).

Three cell lines were selected from bone tissue; G-292 Colon A141B1, Saos-2 (the human primary osteogenic sarcoma cell line) and fibroblast (isolated from human bone tissue). All of these cell lines obtained from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran, Tehran, Iran.

The cells were harvested with 0.25% trypsin-EDTA solution (Sigma, USA) in phosphate-buffered saline (PBS) (pH 7.4) and seeded at a density of 2 × 10^3 cells/well in 96-well microtiter plates (Nunc, Denmark) and the composite samples added into the plates for cytotoxicity/proliferation assay. These cells were cultured on the surface of the composite samples at 8 × 10^3 cells /well in 24-well plates (Greiner, Germany) for attachment and cell morphology assays. The cultures were incubated at 37 °C in a humidified air with 5% CO_2 for 14 days with observations made at 3, 7 and 14 day time points. The cultured medium was changed every three days.

2.3 Cell proliferation

Proliferation rate of osteoblast and fibroblast cells over the composite samples was measured using dimethylthiazol diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were plated into a 96-well microtiter plate (NUNC, Denmark) at a density of 2 × 10^3 cells/well. The plates were incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2 in air. The composite samples with

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	<100 ppm	0.093	4.0	0.20	0.032	95.1	<100 ppm

composition given in Table 2 were placed on the cells. Four 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 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. Ten microlitre of MTT (5 mg/mL in medium) was added to each of the wells and incubated for another 5 h at 37 °C in a humidified atmosphere of 5% of CO_2 in air. Formed formazan crystals were next dissolved by addition of 100 µl/well of isopropanol (Sigma, USA). Then the plates were incubated at 37 °C for 10 min and transferred to 4 °C for 15 min prior to absorbance measurements. The optical density (OD) was recorded on a multiwell microplate reader (ICN, Switzerland) at 570 nm. The experiment was repeated three times.

2.4 Cell attachment

Osteoblast and fibroblast cells were seeded onto composite samples placed in a 24-well plate at a concentration of 8×10^3 cells/well and they were allowed to attach to the surface of the composites. Appropriate negative and composite control wells were also prepared as described earlier. The plates were incubated for a period of 2 weeks with media changed every three days. At various time intervals (3, 7 and 14 days) the composites were removed from the plates and adhered cells were removed by trypsinization and live cells were enumerated on a hemocytometer using 0.2% trypan blue dye exclusion method. The experiment was repeated three times.

2.5 Cell morphology

Twenty four-well plates were prepared as described for attachment assay. The plates were incubated at 37 °C for 72 h. Next the cells were fixed using 2.5% glutaraldehyde in 0.2 M PBS and they were dehydrated through a series of alcohol concentrations (70, 80, 90 and two times 100%). Dehydration was followed by air drying (overnight). The samples were next sputter-coated before examination by a Jeol SEM at an accelerating voltage of 15 keV.

2.6 Statistical analysis

Statistical analysis was performed on SPSS statistical package and values were considered significant at $P < 0.05$. The student's *t*-test was used to compare the data for the composite and control samples.

3 Results

3.1 Sample preparation

The ceramic particle distribution in the composite sample containing 20 vol% HA 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 polyethylene matrix.

3.2 Cell proliferation

An increase with time in OD of viable cells was observed over the 14 days of incubation period for all samples include: composites, HDPE and TPS (Fig. 2a, b, c). The rate of osteoblastic cell proliferation was observed to peak on 14th day with the composite samples giving an equal or in some cases an even higher proliferation rate in compare to negative controls in culture period. In the case of G-292 cells, the significant difference between 15HA5PSZ composite and 20HA, HDPE and TPS was observed on the 7th and 14th days ($P < 0.05$) as shown in Fig. 2a. 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. Approximately same results were observed for Saos-2 and HBF cells. Interestingly, the highest growth rate was obtained for 40HA composite in all time intervals (Fig. 2b, c). The difference

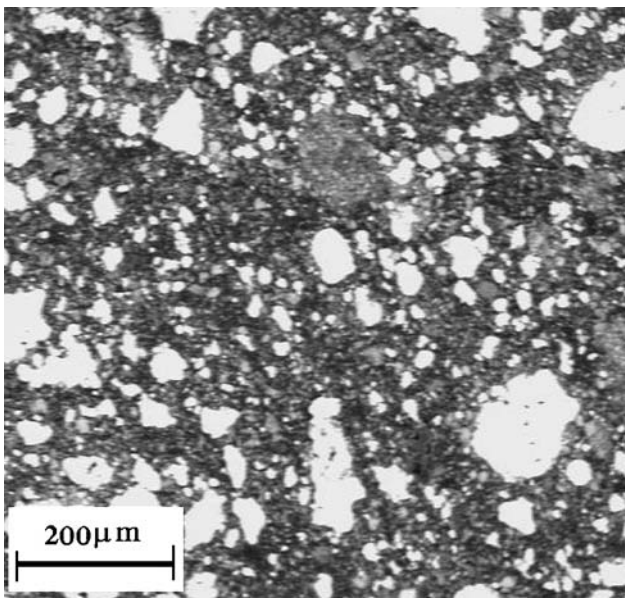

Fig. 1 SEM micrograph showing the distribution of the filler particles in the polymer matrix: Sample containing 2o vol% HA + 20 vol% PSZ

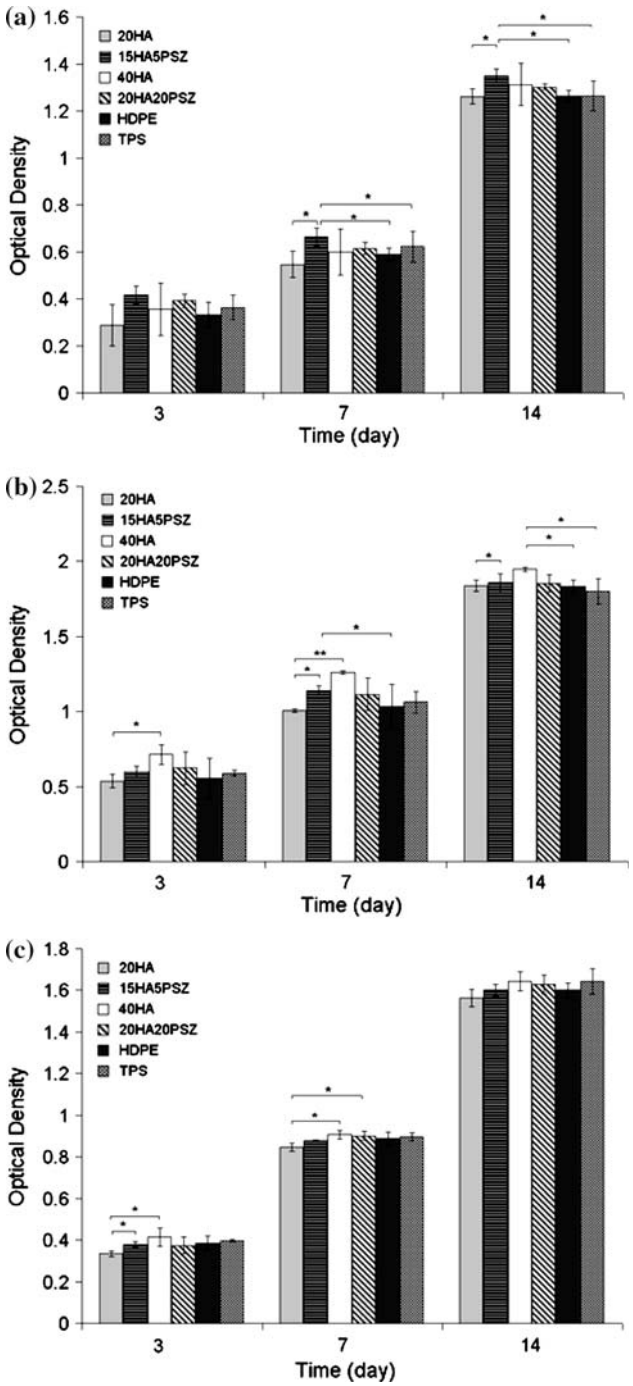

Fig. 2 Cell proliferation of different cells grown on composite samples after different time periods, **a** osteoblastic G292 cells, **b** osteoblastic Saos-2 cells, and **c** fibroblastic HBF cells (*$P < 0.05$, **$P < 0.01$)

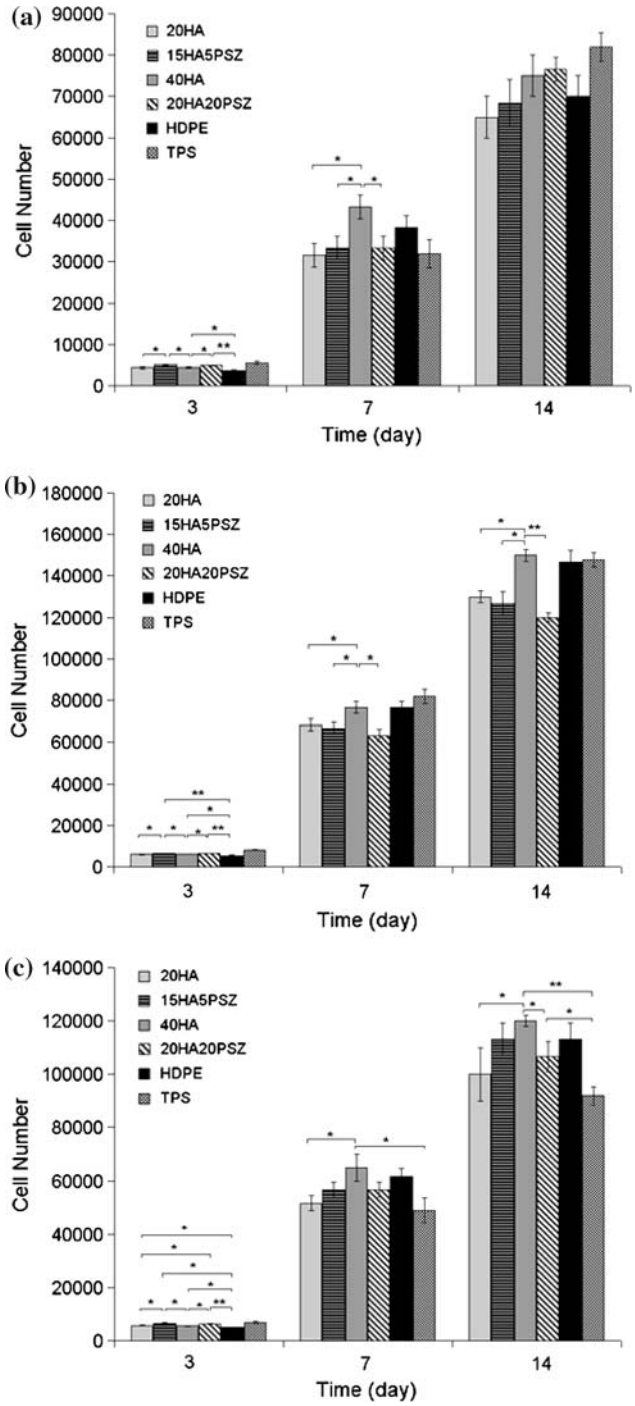

Fig. 3 Outcome of different cells attachment to the surface of composite samples after different time periods, **a** osteoblastic G292 cells, **b** osteoblastic Saos-2 cells, and **c** fibroblastic HBF cells (*$P < 0.05$, **$P < 0.01$)

between the proliferation rates of 40HA and control samples (HDPE and TPS), in Saos-2 culture, was significant on day 14 ($P < 0.05$) as shown in Fig. 2b. Also the significant difference between 40HA composite and other samples was observed on the 3rd and 7th days ($P < 0.05$) as shown in Fig. 2b, c.

3.3 Cell attachment

The number of osteoblast and fibroblast cells adhered on the composite, HDPE and TPS samples are shown in Fig. 3a, b, c. A continuous increase in the number of

adhered cells can be seen all over the 14 days of incubation period. On 3rd day of incubation, the number of osteoblast or fibroblast adhered cells on all of the composite samples was significantly higher than those on the HDPE sample ($P < 0.05$ and $P < 0.01$) (Fig. 3a, b, c). In comparison 40HA composite showed higher cell attachment than 20HA composite and the difference was significant on the 7th day ($P < 0.05$) for all cell types. About G292 cells, 40HA composite had higher cell number adhered than other composite, HDPE and TPS samples on 7th day of incubation. The difference between 40HA and 20HA20PSZ composites and TPS sample was significant ($P < 0.05$ and $P < 0.01$ respectively) on the 3rd day of incubation for all cell types. In comparison with HDPE and TPS samples, in case of HBF cells, the number of adhered cells to the surface of 40HA composite sample was higher than those to the surface of HDPE and TPS, in days 7th and 14th. The difference between 40HA and TPS sample was significant

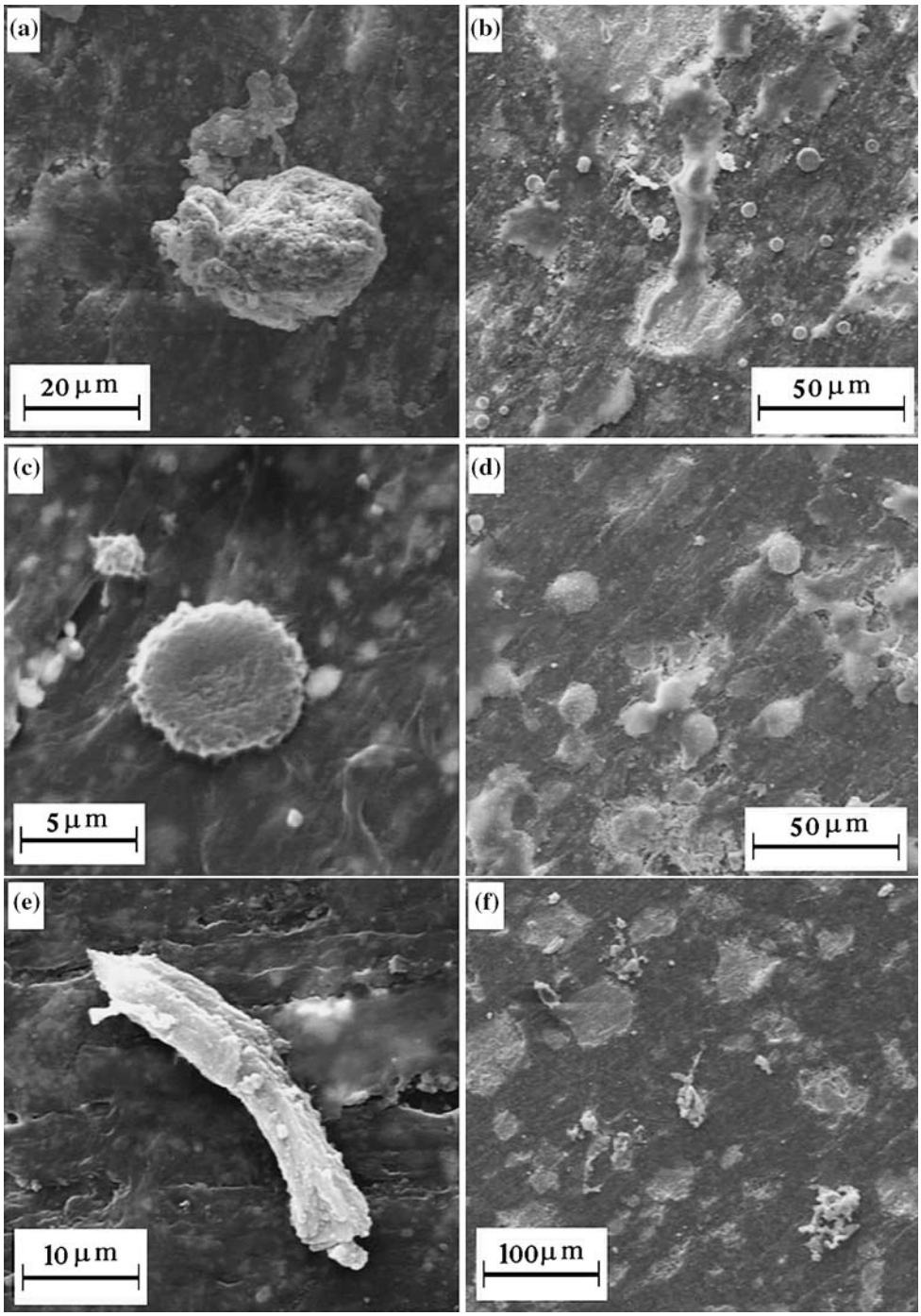

Fig. 4 SEM micrographs showing the cell morphologies in different states of growth on 20HA20PSZ composite including division and flattening, (**a**) and (**b**) G292, (**c**) and (**d**) Saos-2, (**e**) and (**f**) HBF

on 7th and 14th days of culture time ($P < 0.05$ and $P < 0.01$ respectively) (Fig. 3c).

3.4 Cell morphology

The SEM observation of cell morphology showed cell with normal osteoblast and fibroblast morphology, flattened cells with small processes attaching to the surrounding composite in osteoblast cells including G-292 and Saos-2 (Fig. 4a–d) and spindle-like morphology in fibroblast cells (Fig. 4e, f). The process of cell division can be seen in SEM micrographs (Fig. 4d).

4 Discussion

The ceramic particle distribution in the composite sample 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 [18, 30]. In an earlier study some improvement was resulted in the mechanical properties of HA/HDPE composites with partial replacement of HA with PSZ [22]. When evaluating biological response, it is necessary 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]. It was also showed that the addition of PSZ into HA/HDPE composites not only does not have negative effect on osteoblasts behavior but also in some cases increase their proliferation and division [23]. Osteoblasts produce the organic matrix of bone in a highly organized manner. Fibroblastic and osteoblastic cells have been reported to probe the substrate surface with filopodia presented on the cell lamellipodia [30–35]. In the present study, PSZ added HA/HDPE composites provide a favorable site for osteoblast and fibroblast cells proliferation and attachment. Also in some cases the addition of PSZ into the HA/HDPE composites was appeared with increase in proliferation of osteoblastic and fibroblastic cells in comparison to HA/HDPE composites. The general trend observed in this study is consistent with the previous studies done on the HA/HDPE composites [18–21, 30]. In overall, obtained results showed a good biocompatibility of PSZ added HA/HDPE composites. Even with the occupancy of a part of the well space by composites, the results showed that the cell density (all cells including G-292, Saos-2, and HBF) were enhanced during the incubation with composites as compared to HDPE and TPS.

The adhesion of osteoblast and fibroblast cells on the composites was as high as that on TPS and in some cases even higher (Fig. 3a, b, c). These results showed that the surface of composites was sufficiently suitable to support the adhesion and growth of osteoblast and fibroblast cells. Comparison of PSZ containing composites with HA/HDPE composites showed that the addition of PSZ into the HA/HDPE composite in some cases results in higher cell numbers attached to the composites (Fig. 3). The biocompatibility of PSZ containing HA/HDPE composites was demonstrated by the ability of the cells to proliferate on the materials.

As shown in the results of Sect. 3.2 and 3.3, with comparison between the biological activity of used cells in this study, in proliferation and attachment assay, according to obtained results it can be deduced a better proliferation and attachment in Saos-2 and HBF, respectively. G292 cells showed lower activity in comparison with tow other cells. These behavior may be related to the frequency of growth factor receptors, difference in expression of transcription factors, cell cycle system, cell morphology (size and spindle-like microvillis) and frequency of attachment proteins. HBF with its larger size, special morphology (spindle-like microvillis) and high expression in attachment proteins can attach easer to the surface of the composite samples. Because of its higher proliferation activity, attachment rate of Saos-2 to the surface of composite samples is higher.

The results (Fig. 3c) showed that, in overall, the biological activity and performance of fibroblasts was enhanced by composite samples. The difference was significant in comparison with TPS (days 7 and 14). Probably, chemical and physical structure of composites can influence the adhesion and growth of human bone fibroblast cells. Also, the adhesion of HBF cells to HDPE was better than TPS. This phenomena may be related to the physical feature of composite samples surface. The rough surface of composites could encourage HBF cells to adhere to composites surface more than TPS.

In general, all of obtained results showed that the surface of composites was sufficiently stable to support the adhesion and growth of all tested cells whether osteoblastic or fibroblastic cell.

It is important to understand how materials, in particular those with bioactive components, regulate the process of cell attachment as this determines the success or otherwise of the implant material and subsequent osseointegration [30]. SEM results show that composites were able to support normal osteoblast and fibroblast cell growth on the 20HA20PSZ composite sample (Fig. 4).

5 Conclusions

The results show that the volume fraction of HA has a significant effect on the bioactivity of the composites. The composites provide a favourable site for osteoblast and

human fibroblast cells 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 composite. In fact in some cases composites with PSZ have showed better biological results than HA/HDPE composites. The addition of PSZ into HA/HDPE composites not only does not have negative effect on osteoblasts and fibroblast cells behavior but also in some cases increase their proliferation and attachment. Therefore, an increase in the mechanical properties of these composites without any decrease in biological properties of the composites can be obtained. This study demonstrates that replacing part of HA with PSZ in HA/HDPE composites results in an enhanced biological interaction, which when coupled with enhanced mechanical performance may produce a suitable load bearing bone analogue.

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