Osteogenic differentiation of rat bone mesenchymal stem cells cultured on poly (hydroxybutyrate-co-hydroxyvalerate), poly (ε-caprolactone) scaffolds

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Abstract
Bioresorbable biomaterials can fill bone defects and act as temporary scaffold to recruit MSCs to stimulate their differentiation. Among the different bioresorbable polymers studied, this work focuses on poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) and poly(ε-caprolactone) (PCL). Were prepared blends of PHBV and PCL to obtain PHBV based biomaterials with good tenacity, important for bone tissue repair, associated with biocompatible properties of PCL. This study assesses the viability of Vero cells on scaffolds of PHBV, PCL, and their blends and the osteogenic differentiation of mesenchymal stem cells (MSCs). Materials were characterized in surface morphology, DSC and Impact Strength (IS). Vero cells and MSCs were assessed by MTT assay, cytochemical and SEM analysis. MSC osteogenic differentiation was evaluated through alizarin red staining and ALP activity. We found some roughness onto surface materials. DSC showed that the blends presented two distinct melting peaks, characteristic of immiscible blends. IS test confirmed that PHBV-PCL blends is an alternative for increase the tenacity of PHBV. MTT assay showed cells with high metabolic activities on extract toxicity test, but with low activity in the direct contact test. SEM analysis showed spreading cells with irregular and flattened morphology on different substrates. Cytochemical study revealed that MSCs maintained their morphology, although in smaller number for MSCs. The development of nodules of mineralized organic matrix in MSC cultures was identified by alizarin red staining and osteogenic differentiation was confirmed by the quantification of ALP activity. Thus, our scaffolds did not interfere on viability of Vero cells or the osteogenic differentiation of MSCs.
1 Introduction

Huge tissue losses due to traumas, infections, tumors, or degenerative diseases have been treated for decades through the removal of the patient’s tissues and substitution of damaged areas by prostheses. Yet, these methods demand revision surgeries, cause physical limitations, and, consequently, decrease the quality of life of such patients [1]. Thus, methods involving engineering to produce tissues with the same properties as the injured ones, leading to more efficient recovery and rehabilitation, are particularly interesting [2, 3].

Regenerative medicine based on tissue engineering is a research field that may positively contribute to that purpose. This investigation line has used mesenchymal stem cells (MSCs) to optimize the regeneration process and reestablish the lost structure and function [4]. The main known sources of MSCs are bone marrow, adipose tissues, peripheral blood, and umbilical cord blood [4, 5]. Studies have shown that, when stimulated by specific signs, MSCs derived from such sources can differentiate into cells of bone and cartilage tissues [4, 6, 7]. The capacity to induce the differentiation of MSCs into other cell types with specific functions allows recruiting compatible cells to the damaged area, modulating and optimizing the tissue regeneration process [4–7].

Studies have sought to elucidate the efficiency of MSCs associated with different biomaterials in the most different clinical applications. Broadly speaking, they aim at clarifying not only the isolation, maintenance, and differentiation mechanisms of MSCs in vitro, but also and mainly their clinical application and interaction with scaffolds, which are possible applications [2–4, 7].

In orthopedics, such biomaterials can fill bone defects and still act as temporary scaffold to recruit MSCs to the damaged place in order to stimulate their differentiation into osteoblasts [8]. Nevertheless, the most appropriate biomaterials to fill these defects and favor the growth of bone cells still need definition.

Among the bioresorbable polymers used for that purpose are polyhydroxyalkanoates (PHAs), which are produced by microorganisms from renewable carbon sources. Among PHAs, we highlight poly(3-hydroxybutyrate) (PHB), poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), poly(4-hydroxybutyrate) (P4HB), poly(hydroxybutyrate-co-hydroxyhexanoate) (PHBHHx), and poly(3-hydroxyoctanoate) (PHO). Since such compounds can degrade in the presence of body fluids, they are indicated as raw materials to develop orthopedic temporary scaffolds that may substitute the metallic implants conventionally used to fix fractures and substitute bone segments [9]. On the other hand, poly(ε-caprolactone) (PCL) is a bioresorbable material approved by the Food and Drug Administration for use in humans. It has important characteristics, such as biodegradability, moderate undesirable host reactions, compatibility with a wide range of other polymers and good processability. Thus, PCL allows the manufacture of a variety of structures and shapes for tissue engineering [3, 10].

Polymer blends are the physical mixture of two or more polymers, where the properties resulting from the mixture are related to the properties of pure polymers. In this study, we produced blends of PHBV and PCL to obtain biomaterials based PHBV with good tenacity, an important mechanical characteristic for bone tissue substitutes, associated with the plasticizing and biocompatible properties of PCL. The objective was to assess the behavior of such blends in the presence of cell cultures as fibroblasts and MSCs induced by osteogenic differentiation.
2 Materials and methods

2.1 Preparation of scaffolds of poly (hydroxybutyrate-co-hydroxyvalerate), poly (caprolactone), and their blends

Were used poly(hydroxybutyrate-co-valerate) (PHBV) containing 12% valerate and polymer PCL type CAPA 6500 - with molar mass (MW) of 50,000 g/mol provided by BIOCYLE (PHB Industrial S/A) (lot FE133) and Solvay, respectively. Specimens of the polymers and their blends were prepared in the Materials Engineering Department of the Federal University of São Carlos. Blends of PHBV/PCL were prepared in 75:25 and 50:50 proportions. The initial mixtures of polymer granules were produced, extruded, and molded into specimens by injection molding. Polymer blends were extruded using a modular co-rotating twin-screw (L/D = 35) Werner & Pfleiderer ZSK 30 extruder and specimens were injected using a fully automated Arburg Allrounder injection molding machine. The different polymer compositions produced were injected to obtain circa 2 mm thick specimens in the form of plates according to the proportions produced were injected to obtain circa 2 mm thick specimens in the form of plates according to method develop by Casarin et al. [10]. These were cut into appropriate sizes, sterilized by (25 KGy) gamma radiation, and submitted to cytotoxicity tests. This study used the following materials: PHBV, PCL, PHBV/PCL 75:25, and PHBV/PCL 50:50.

2.2 Vero cell culture

Vero cells, a fibroblastic cell line derived from the kidney of the African green monkey (Cercopithecus aethiops), provided by Adolfo Lutz Institute, São Paulo, Brazil, were used. They were cultured in Ham’s F-12 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, provided by Nutricell, Campinas, Brazil), and 1% penicillin/streptomycin (PS, Hyclone) at 37 °C with 5% CO₂. Vero cell lines are internationally recommended as a standard to study cytotoxicity and cell-substratum interactions with biomaterials [11, 12].

2.3 Isolation and culture of bone marrow mesenchymal stem cells of rats

To obtain MSCs, the femurs, tibias and humeri of Wistar-Kyoto rats were removed. The project was approved by the Ethics Committee on Animal Experimentation of Faculty of Medical Sciences, State University of Campinas (Protocol 1958-1). After their epiphyses were cut off, they were introduced into 5 ml blood collection tubes and centrifuged for 10 min at 400 g. The precipitated bone marrow was homogenized using a (2 ml/bone) PBS/EDTA solution, circulated through a (20 µm) filter, placed on 15 ml of Ficoll-Hypaque®, and centrifuged for 25 min at 300 g. The fractions of mononucleated cells were collected and centrifuged for 10 min at 200 g. After the supernatant was discarded, the precipitate was homogenized in 10 ml of PBS/EDTA. The suspension obtained was washed and centrifuged three times at 200 g for 10 min. Cells were then counted in a Neubauer chamber and placed in DMEM medium with low glucose concentration supplemented with 10% FBS and 5% CO₂.

2.4 Surface morphology of biomaterials specimens by optical microscopy

The morphological analysis of specimens surfaces was performed using a Stereo Microscope (Physix, model SZ40). Photographs were taken from all samples with a digital camera coupled to a stereoscope at ×1.0, ×1.5, ×2.0, ×3.0, and ×4.0 magnification.

2.5 Differential scanning calorimetry (DSC)

DSC analysis was realized by using the equipment TA, DSC Q100, in N2 atmosphere. In the case of PHBV and the blends the temperature was scanned from −30° to 200 °C and for PCL the temperature was changed from −80° to 150 °C, with the heating of 10 °C/min.

2.6 Impact strength

The impact strength (IS) was realized with notched specimens in an equipment Izod Impact Ceast code 6545100, with a 2,0 J pendulum according to ASTM D-256-02 [13]. A set of ten specimens/sample were used to obtain the average value and standard deviation of the IS.

2.7 MTT assay

This assay used the modified Mosmann method [14]. Extracts of the tested materials (PHBV, PCL, and their blends) were obtained by incubating them in Ham’s F-12 medium containing 10% FCS at a proportion of 0.2 g/ml medium for 24 h at 5% CO2 and 37 °C. This method is in agreement with ASTM F813-83 for evaluation of biomedical devices [15]. For the extract toxicity assay, Vera cell suspensions (3 × 10^6 cells/ml) were inoculated into a 96-well cell culture plate (n = 5) and incubated at 37 °C for 24 h. After this, the culture medium was replaced by the extract obtained from the tested materials, and the cells were maintained under these conditions for 24 h. For the direct cytotoxicity assay, a suspension of 3 × 10^6 cells/ml (n = 5) was directly cultivated on the materials for 24 h. Ham’s F-12 medium with phenol 0.5% was used as the positive control toxicity and polystyrene extract as the negative control toxicity in
both tests. After incubation, the medium was removed and
the wells were washed with 200 µl PBS. Next, 200 µl of
Ham’s F12 medium with 10 mM of Heps buffer and 50 µl
of thiazolyl blue tetrazolium bromide solution (MTT, Sigma)
were added, and the plate was incubated in the dark for 4 h at
37 °C. After that, the medium with MTT was removed, and
200 µl of dimethyl sulphoxide was added. The absorbance
curve was determined in a microplate reader (Bio-Rad 550
microplate spectrophotometer) at λ = 540 nm. Commercial
software (Microcal® Origin version 6.0) was used for
statistical calculation. Student’s t test was employed for
assessing statistical differences between each sample and
controls, while one-way analysis of variance (ANOVA) was
employed for assessing statistical differences between all
samples. P < 0.05 was considered statistically significant.

2.8 Scanning electron microscopy (SEM)

For this assay, 3 × 10⁶ cell/mL of Vero cells and MSCs were
inoculated on materials in 100 µL of DEMEM medium
supplemented with 10% FBS into a 96-well plate (Corning)
containing the three different tested materials and incubated
at 37°C in 5% of CO₂ for 24 h. After fixation in para-
formaldehyde (Sigma) 2.5% and glutaraldehyde (Sigma)
2.5%, the samples were post-fixed in 1% solution of osmium
tetroxide (OsO₄, Sigma) for 1 h at room temperature in the
dark, washed in distilled water, dehydrated in ethanol, critical
point dried in CO₂ (Balzers, CDT 030), coated with gold in a
sputter coater (Balzers CTD 050), viewed and photographed
on an electron microscope (JEOL 5800).

2.9 Cytochemical analysis

Vero and MSCs cells were cultivated in contact with PCL,
PCL/PHBV 50:50, PCL/PHBV 75:25 and PHBV. To do so,
3 × 10⁶ cells/ml (n = 4) suspended in low glucose DMEM
medium with 10% FBS were inoculated on the materials
and the plate was cultivated for 24 h at 37 °C. The cells
were then fixed in formaldehyde 10% for 24 h and stained
with toluidine blue (TB) at pH 4.0 to detect the presence
of nucleic acids and glycosaminoglycan acids [16, 17]. The
images were obtained with a Leica inverted microscope
(model DM IL, Leica Microsystems, Wetzlar, Germany)
with 10× and 20× objective lens.

2.10 Induction of osteogenic differentiation

MSCs were inoculated in 24-well plates and osteogenic
differentiation was induced according to Neuhuber et al. [18].
After 48 h, material samples were added in the wells and the
culture medium was replaced with osteogenic induction
medium consisting of low-glucose DMEM with 15% FBS,
1% penicillin/streptomycin, 100 nM dexamethasone, 50 µM
ascorbate-2-phosphate, and 10 mM glycerol-phosphate. As a
control, MSCs were cultured without any material. The
medium was changed every 3 days during 21 days. After this
period, the cells were tested for alkaline phosphatase activity
and development of mineralized bone matrix.

2.11 Alkaline phosphatase assay

Alkaline phosphatase enzyme activity (ALP) was assessed
with a specific kit (Sigma Fast p-nitrophenyl phosphatase
Tablets N1891) according to the manufacturer’s recommenda-
tions. After 21 days of osteogenic differentiation, 50 µl of
the differentiation medium contained in the 24-well plate
were transferred into a 96-well plate and incubated for 2 h at
37 °C. Next, 200 µl of the ALP substrate solution were added
and the plate was maintained in the dark for 30 min at room
temperature. Absorbance was read at 405 nm. Statistical
significance (P < 0.05) was assessed using one-way analysis
of variance (ANOVA).

2.12 Alizarin red staining

After 21 days of osteogenic differentiation, the cells were
fixed in paraformaldehyde 4% for 10 min, washed in distilled
water, stained with Alizarin Red S (ARS) for 2 min, washed
again in distilled water, differentiated in ethanol 95% and
hydrochloric acid 100% for 15 s and placed in distilled water.
Pictures were shot with an inverted Leica microscope (model
DM IL) with 10× and 20× objective lens. ARS activity was
quantified using the colorimetric method described by Greg-
ory et al [19]. Water was drained and the plate was kept at
room temperature until complete dryness was achieved.
Next, 280 µl of acetic acid 10% were added and the plate
was shaken for 30 min. Due to the resolution of our equipment,
the contents of each experimental sample were pooled and
then transferred to Eppendorf tubes, which were heated to
85 °C for 10 min and then cooled down in ice for 5 min.
Therefore, the assay performed were semiquantitative. After
the tubes were centrifuged at 16.000 g for 20 min, 100 µl of
the supernatant were placed in a tube with 40 µl of amno-
nium hydroxide 10%. The final solution was transferred into
a 96-well plate. Absorbance was read at 405 nm.

3 Results

3.1 Surface morphology of studied biomaterials

Figure 1 shows the images of the specimen’s surface that
allowed verifying the presence of risks characteristics of the
injection process, which resulted in some roughness onto
surface materials. In general, the surface is flat, resembling
the surface of the culture plates.
3.2 Thermal behavior of studied biomaterials by DSC

The Table 1 shows the values of melting temperature (Tm) and transition glass temperature (Tg), beyond of melting enthalpy and crystallinity of the studied samples. The crystallinity percentage of PHBV and PCL were calculated by using the data of theoretical value of fusion enthalpy of PHB hypothetically 100% crystalline ($\Delta H_{f100\%} = 146$ J/g) [20] and of PCL hypothetically 100% crystalline ($\Delta H_{f100\%} = 136$ J/g) [21], as well as the actual values of pure PHBV and PCL. The Fig. 2 illustrates the DSC curve of the studied materials. Considering the obtained data is possible to note that the blends presented two distinct melting peaks, one near around 64 °C indicating the PHBV phase and the other near around 55 °C, characteristic of the do PCL.

3.3 Impact strength of studied biomaterials

The Table 2 describes the values obtained in the impact resistance test. According to these results, it was observed that the resistance value of the impact of the pure PHBV is lower than the value showed by the pure PCL. For blends studied was noted that the blend PHBV/PCL (75:25) showed a value of impact resistance higher than the value presented by the pure PHBV, with an increase of approximately 30% while the blend PHBV/PCL (50:50) showed a value similar to the arithmetic mean of the values presented by the PHBV and PCL.

3.4 MTT assay

The MTT assay showed that in contact with polymer extracts, the number of adherent viable cell was similar to negative control (Fig. 3A). According to the Student’s t test, there are no statistically significant differences between any sample and negative control (no toxic) ($p < 0.05$), except positive control. On the other hand, in direct contact, the quantity of viable cells on different polymers was similar to positive control (Fig. 3B).

3.5 Scanning electron microscopy (SEM)

For Vero cells analysis, we could see on glass coverslip flattened and elongated cells forming a semi confluent layer (Fig. 4A e 4B). On PCL we found cells flattened with irregular morphology (Fig. 4C). On PCL/PHBV (50:50) blends we could see spreading and elongated cell, some
times with some thin cell extensions, on substrates. Vesicles and microvilli were also found in some cells (Fig. 4D). The cells cultured on PCL/PHBV (75:25) blends showed irregular morphology, flattened and retracted with cell prolongations on materials (Fig. 4E). On PHBV, cells with a very flattened morphological pattern were found (Fig. 4F). For Vero cells, we did not found fibrilar material around cells in any sample studied.

It was noticed by MTT assay a small amount of MSCs adhered on the surface of the studied materials. With the use of SEM, few cells were found with a great spaced around them (data not shown). Thus, the SEM objective was to evaluate the cell morphology on polymers. On the glass coverlip, it was noticed the presence of spread cells with varied morphology and star-shaped, oblong and almost bipolar aspect (Figs. 5A & 2B). On PCL, cells with irregular morphology were noticed, mainly with elongated cells (Fig. 2C). On PHBV/PCL (50:50) we found much-flattened

| Polymers                | PHBV       | PCL       |
|-------------------------|------------|-----------|
| **T<sub>m</sub> (°C)**  | 164.1      | 164.1     |
| **ΔH<sub>m</sub> (J/g)**| 46.8       | 37.1      |
| **%C<sup>a</sup>**      | 32.4       | 33.9      |
| **T<sub>g</sub> (°C)**  | -0.2       | -0.6      |
| **ΔH<sub>m</sub> (J/g)**| -          | 56.2      |
| **%C<sup>a</sup>**      | -          | 18.2      |
| **PHBV/PCL (75/25)**    | 163.6      | 55.3      |
| **ΔH<sub>m</sub> (J/g)**| 20.3       | 35.7      |
| **%C<sup>a</sup>**      | 28.2       | 52.8      |
| **PHBV/PCL (50/50)**    | -          | 55.3      |
| **ΔH<sub>m</sub> (J/g)**| -          | 68.3      |
| **%C<sup>a</sup>**      | -          | 51.1      |

<sup>a</sup>Percentage of crystallinity of the pure polymers or polymers in the blend (standardized by the experimental levels of the PHBV and PCL in the blends)
cells (Fig. 5D). In PHBV/PCL (75:25) we could observe some star-shaped spread cells on material surface (Fig. 5E). Finally, on the PHBV surface, flattened cells were noticed. No cellular extensions could be observed (Fig. 5F).

3.6 Cytochemical analysis

For Vero cells, TB staining showed confluent spread and many cells adhered to the plate. They showed the same growth pattern as negative control. Regardless of the type of biomaterial, strongly basophilic cells with slightly metachromatic nuclei and evident nucleoli, which characterize cellular activity, were detected. For MSCs, after 24 h culture, TB staining showed typical behavior of these cells. At the semi-confluence stage, oblong, occasionally elongated or star-shaped morphology was noticed and, in agglomeration regions, rather contracted cells with a polygonal aspect were seen. The same pattern of cytoplasmic

Fig. 4 Scanning electronic microscopy (SEM) of Vero Cells cultured by 24hs on experimental conditions: A and B Control on glass coverslip; C PHBV; D PHBV/PCL (75:25); E PHBV/PCL (50:50) and F PCL. Scale bar = 10 or 50 µm
basophilia with a slight nucleolus metachromasia was observed. No influence of the biomaterial types on the activity of the cells in contact with them was noticed. No variations on cell cytochemical results induced by biomaterials were found (Figs. 6A–3F).

3.7 Alkaline phosphatase

Figure 7 shows the semi-quantitative analysis of ALP enzyme activity, a commonly used marker for bone differentiation. In all the different experimental conditions investigated, the production of this enzyme was observed. We did not find significant differences among samples. Thus, the different polymers did not interfere (stimulating or repressing) the production of ALP.

3.8 Alizarin red staining

The incorporation of ARS after 21 days of osteogenic differentiation indicated the development of mineralized bone

Fig. 5 Scanning electronic microscopy (SEM) of MSCs cultured by 24hs on experimental conditions: A and B Control on glass coverslip; C PCL; D PHBV/PCL (50:50); E PHBV/PCL (75:25) and F PHBV
matrix. Figure 8 shows the development of mineralization nodules submitted to osteogenic differentiation under contact culture conditions on all the polymers used as substrates. The quantification of the produced matrix is presented in Fig. 8F, where one can see that, in all the studied conditions, the amount of produced material was similar to that of the control. We did not find differences among samples.

4 Discussion

Cultured bone marrow MSCs hold a potential to transform into bone and cartilage cells. This transformation occurs in well-defined conditions [2–7]. When cultured in the presence of some biomaterials these cells show capacity to form bone tissue and even ectopic bone formation [22–25]. Thus, the search for new materials with
such properties in the presence of MSCs has a significant clinical importance.

For material characterization, however, studied materials had no pore structure we could observe an irregular surface of samples for maintain cell culture. The DSC curves showed of two distinct melting peaks indicating the immiscibility of the PHBV/PCL blends. It was also verified a small decrease of the Tm as result of the increase in the amount of PCL in the blend. Otherwise, with respect to the crystallinity of the material, was observed that a significant reduction occurs only for high percentages of PCL in the blend.

According to IS test, it was observed that the resistance value of the impact of the pure PHBV is lower than the value showed by the pure PCL. Considering that the Tg of PCL is ~73 °C below the ambient temperature, its amorphous phase is totally rubbery, making it a very flexible material with high impact resistance. For blends studied was noted that the blend PHBV/PCL presented intermediate values of IS, between the pure polymers, confirming that the mixture of PCL with PHBV is an alternative to make feasible the use of these biomaterial in orthopedics. Taken together, the characterization data indicates that substrates were shown to be stable for the culture conditions planned in those assays. Because of its biocompatibility with human tissues and because it is of natural origin, PHBV is shown with a promising polymer for tissue engineering and a viable alternative in the repair of bone lesions [26, 27]. Although PHBV is considered a polymer with high potential for biomedical applications, its high crystallinity restricts these applications. The use of PHBV blends with PCL is a proposal to increase the tenacity of PHBV and the biological evaluation of this compound may be important and of great relevance in the context of tissue engineering applied to the treatment of osteochondral defects.

Regarding cell viability, the results of the MTT assay demonstrated there are no toxicity by extracts. On the other hand, on direct toxicity assay, we found a similar cell behavior to toxic control. On the other hand, it was observed spreading cells (Vero and MSCs) on SEM analysis forming a monolayer on polymers in the 24 h. We also found a MSCs monolayer on cytochemistry and differentiation assays. This indicates that the materials are non-toxic. If there were toxicity, cell growth would not be observed. Thus, the data were interpreted as an initial difficulty of interaction between cells and polymers. The MTT assay does not indicate toxicity per se. It is an indicator of cellular mitochondrial activity [14]. For this reason, it is commonly and used as an indirect measure of cytotoxicity, but also for cell adhesion and proliferation. Therefore, the MTT trial needs to be analyzed in a broad context. In anchorage-dependent cells, this difficulty may be reflected in cellular activity measured by MTT and, therefore, a low initial interaction with substrata.

Cell adhesion and spreading represent the beginning of the interaction between biomaterial and cell [28]. In this study, the morphology of Vero cells cultured in contact with PHBV, PCL, PHBV/PCL 75:25, and PHBV/PCL 50:50 and assessed by SEM and cytochemistry showed they maintained their morphology, allowing normal cellular adhesion and spreading. In addition, bone marrow MSCs presented typical morphological patterns with scattered, stellate cells although in lesser number when compared to Vero cells. This result was expected, to a certain extent, since MSCs slower adhesion and division kinetics than Vero cells. Thus, despite the slow initial interaction detected by the MTT, once adhered, the cells were able to spread over the studied polymers, presenting a morphological pattern compatible with that expected for anchorage-dependent cells.

For cytochemistry, TB (basic stain that forms electrostatic links with the acid radicals present in the tissues) revealed cells with marked basophilia, slightly metachromatic nuclei, and evident nucleoli, indicating the presence of cell activity. Such activity has been identified in the DNA, RNA, rRNA, and in glycosaminoglycans, since TB combines with the PO4−, SO4−, COO− groups, which are present in these structures at pH 4.0 [14, 15]. These results agree with previous publications reporting that Vero cells on different bioresorbable polymers, among them the PHBV [29]. More recently, extensive cytochemical evaluation has been conducted with fibroblastic cells grown in PHBV/PCL blends. The results presented herein are consistent with those previously described [30].

Besides MSCs identification based on morphological and phenotypic characteristics, the ability to form expanded cultures of different cell types is one of the criteria available
for identifying and distinguishing MSCs cells already committed to a particular path of differentiation. After 21 days inducing the osteogenic differentiation, ALP concentration was dosed and the formation of inorganic nodules was revealed by the impregnation of ARS, which fixes wherever mineralization takes place. ALP is used as an indicator of osteogenic differentiation, while ARS staining identifies the product of this osteogenic differentiation, a mineralized matrix, and quantifies the deposit of these inorganic nodules through colorimetric extraction [19, 31–34]. Our results indicate ALP activity and the presence of mineralized nodules, thus confirming osteogenic differentiation in presence of PCL/PHBV 50:50, PCL/PHBV 75:25, PCL, and PHBV. Such findings agree with previous reports indicate that biodegradable materials, such as poly lactic acid (PLA), poly glycolic acid, PCL and PHBV are being developed as scaffolds to induce bone formation [9, 26, 32, 35, 36].

5 Conclusions

In conclusion, the polymers showed characteristics of immiscible blends and the addition of PCL is an alternative to increase PHBV tenacity. The PCL/PHBV 50:50, PCL/PHBV 75:25, PCL and PHBV not only did not interfere in the normal biology of the cells tested. We did not observe changes in the cytochemical and growth pattern of Vero
cells or MSCs, although the initial adhesion was slower in the tested polymers. The studied materials also did not interfere negatively in the differentiation pattern of MSCs, allowing them to behave as bone cells, producing ALP and mineralized matrix. Such findings open new use perspectives for these materials in the treatment of bone defects or flaws in humans.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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