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Preliminary evaluation of the in vitro cytotoxicity of PMMA-co-EHA bone cement

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ABSTRACT

This work reports a preliminary in vitro cytotoxicity assessment of new poly (methyl methacrylate)-co-ethyl hexylacrylate (PMMA-co-EHA) bone cement by evaluating the effect of its leachables on the viability of human osteoblast-like cells (MG63 line) and their progression through the cell cycle. MG63 cells were exposed to 72 h-extract dilutions of PMMA-co-EHA and their viability was tested using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Also, putative changes in the progression of cells through the cell cycle were monitored using flow cytometry. For that the relative nuclear DNA content and the ratio of cells at G_1:S:G_2 stages of the cell cycle were measured after three exposure periods (24, 48 and 72 h). The obtained results revealed a dose-dependent influence of the cement extract in MG63 cell metabolism when compared to cells cultivated in a culture medium only. The MTT assay showed that a moderate number of cells died after exposure to the most concentrated extract. The cell cycle analysis revealed that leachables of PMMA-co-EHA led to significant changes in cellular proliferation, with cells exposed for 48 h to the most concentrated extract being arrested in the S phase of the cell cycle. However, despite the initial period of cytotoxicity, the obtained results suggest that after 72 h of exposure, the surviving cells are able to recover from this arresting condition and continue to proliferate. Therefore, this preliminary study indicates that, at the biological level, PMMA-co-EHA may have potential of being used as a bone cement matrix. However, a more detailed research work is needed to fully understand the factors responsible for the initial cytotoxicity observed.

1. Introduction

Human aging is accompanied by progressive weakening of the skeletal system. Hip and knee are the most common arthroplasties, as a result of the particularly high mechanical stresses that these joints must endure [1]. In the majority of joint replacement procedures, surgeon chooses to use an auto-polymerizing poly (methyl methacrylate) (PMMA)-based bone cement to anchor the implant to contiguous bone [2]. Despite a good success rate, the PMMA-based bone cements that are in current use exhibit several drawbacks [3], such as local cellular death due to exothermic polymerization and toxicity of the unreacted methyl methacrylate (MMA) monomer that leaches out of the material, apart from intrinsic brittleness and poor resistance to fatigue [4]. Thus, the development of new bone cements with improved properties is still an important issue in the biomaterial field and many research efforts have resulted in a large number of alternative formulations [5–9]. Some of the authors have recently developed a new bone cement matrix of a copolymer of PMMA and ethyl hexylacrylate (EHA) (1:1) with the purpose of bringing some improvements to the commonly used formulations. This particular composition was chosen among a series of compositions whose mechanical properties and in vitro bioactivity were already evaluated in a previous study [10], where this copolymer showed to have potential for orthopaedic applications.

However, before the implementation of a biomaterial, it is of upmost importance to evaluate its cytotoxicity [11–13]. Therefore, in the present work we evaluated the in vitro cytotoxicity of the copolymer of PMMA and EHA by the known 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay used to assess the cellular viability after exposure to cement extracts [14]. This biochemical test is based in the activity of the mitochondrial enzyme succinate dehydrogenase, and is a measure of cellular viability, since only living cells are able to metabolize the MTT reagent into a purple formazan product [15]. In parallel, we used flow cytometry (FCM) to determine if any of the cement leachables interfered with the progression of cells through the cell cycle, since bone proliferation is a determinant factor in the biocompatibility of a biomaterial [16]. The applicability and usefulness of FCM in the assessment of biomaterial biocompatibility have already been demonstrated in several studies [17–19]. In the present study, we focused on the capability of this

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technique in the analysis of the cell cycle status of cells in culture, having developed a simple and rapid protocol for the analysis of MG63 cells after exposure to cement extracts.

2. Materials and methods

The cytotoxicity tests were, in general, performed according to ISO 10993-5 standard: "Test for cytotoxicity — in vitro methods" guidelines [20].

2.1. In vitro cell culture

MG63 human osteoblast-like cells were cultured in α-Minimal Essential Medium (α-MEM) supplemented with 10% of fetal bovine serum (FBS), 10,000 units mL^{-1} of penicillin–streptomycin, 2.5 μg mL^{-1} of fungizone and 0.0085 mg mL^{-1} of gentamicin (GIBCO Invitrogen Corporation). Cells were routinely subcultured every third day in 75 cm^2 flasks and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Preparation of the PMMA-co-EHA bone cement and the cement extracts

The composition of the PMMA-co-EHA cement is shown in Table 1. It was obtained following the classical method, by combining solid and liquid components. The solid component consisted of commercial PMMA beads (Aldrich, average MW 120,000), with a mean particle size of 160 μm. The liquid component was a mixture of the monomers MMA and EHA (both Aldrich) used without prior purification. Benzoyl peroxide (BPO, Merck) was used as a polymerization initiator and N,N dimethyl-p-toluidine (DMPT, Fluka) was used as an activator of the redox initiation. The bone cement was cured at room temperature during 24 h.

PMMA-co-EHA pieces ($5 \times 3 \times 3$ mm^3) were sterilized by autoclaving at 121 °C and 1 atm during 20 min. An appropriate number of PMMA-co-EHA pieces were immersed in conic tubes with complete culture medium and incubated for 72 h at 37 °C with constant shaking (60 rpm). In all tests, a ratio of material outer surface/extraction fluid volume equal to 3 cm^2 mL^{-1} was maintained. The extract was then diluted in growth medium (50 and 25% of the extract in the medium). An extraction control (EC-72 h) consisting of complete α-MEM medium was placed in the same conditions as the testing materials. This control was performed to assure that the extraction conditions by themselves did not alter the properties of the culture medium.

2.3. MTT assay

Cells were re-suspended in the culture medium at a density of 1.5×10^5 cells mL^{-1} and plated (100 μL/well) into 96-well microplates. Plates were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After that, the medium was replaced by the same volume of both diluted (25% and 50%) and undiluted extracts. The MTT solution was prepared in 1x phosphate-buffered saline (PBS; GIBCO Invitrogen) into a final concentration of 1 mg mL^{-1}. After 72 h of incubation, the culture medium was discarded and 50 μL of the MTT solution was added to each sample, followed by incubation for 4 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Afterwards, the supernatant was removed and the formazan crystals were dissolved by the addition of 100 μL/well of dimethyl sulphoxide (DMSO; Sigma). Plates were then slightly stirred at room temperature for an additional 2 h period in the dark. Finally, the optical density was recorded on a multiwell microplate reader (Labsystem Multiskan EX) at 570 nm. As a positive control (C^+), a 24 h-extract of Cu sheet pieces 25% diluted in the growth medium was used. Cells incubated in the medium not subjected to extraction conditions were considered as the negative control (Ctrl). Four replicates were made for all treatments. The obtained results were expressed as percentage of cell viability.

Table 1
Composition of the evaluated PMMA-co-EHA bone cement (wt.%).

Sample[a]	Liquid component		Solid component
	MMA	EHA	PMMA
PMMA-co-EHA	16.7	16.7	66.6

[a] BPO 2 wt.% of the solid component and DMPT 1 wt.% of the liquid component.

2.4. Cell cycle analysis

The influence of the PMMA-co-EHA extract in the movement of cells through three of the main phases of the cell cycle (G_1, S and G_2) was determined by flow cytometry. After re-suspension at 0.5×10^5 cells mL^{-1} in the culture medium, MG63 cells were plated (400 μL/well) in 24-well microplates. Following a 24 h of incubation at 37 °C under a 5% CO_2 humidified atmosphere, the medium was replaced by same volume of diluted (25 and 50%) and undiluted extracts. Cells were exposed to material extracts and controls (EC-72 h and Ctrl) for 24, 48 and 72 h. After each period, cells were harvested by treatment with trypsin-EDTA (GIBCO Invitrogen) and washed once with PBS pH 7.2. The MG63 cell pellet was further re-suspended in 1 mL of WPB buffer [21,22]. After that, 50 μg mL^{-1} of RNase A (Sigma) and 50 μg mL^{-1} of propidium iodide (PI; Fluka) were added to samples. Samples were incubated at room temperature in the dark for a minimum period of 15 min until analysis in the flow cytometer. The relative fluorescence intensity (FL) of PI-stained nuclei was measured on a Beckman-Coulter EPICS-XL (Beckman-Coulter®) flow cytometer equipped with an air-cooled argon-ion laser tuned to 15 mW and emitting at 488 nm. Integral fluorescence from nuclei was collected through a 645 dichroic long-pass filter and a 620 band-pass filter and converted on 1024 ADC channels. The results were acquired using the SYSTEM II software (version 3.0 Beckman-Coulter®), in which at least 5000 nuclei were acquired per sample. PI-signals corresponding to debris and cell aggregates were eliminated by creating a gating region in the FL pulse integral vs. FL pulse height cytogram. Four replicates were performed for each treatment. The percentage of cells in each stage of the cell cycle was analyzed using the Cylchred software (version 1.0.2, Cardiff University).

2.5. Statistical analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) (SigmaStat for Windows Version 3.1, SPSS Inc.) employing the Dunnet's test to compare results of test cultures with

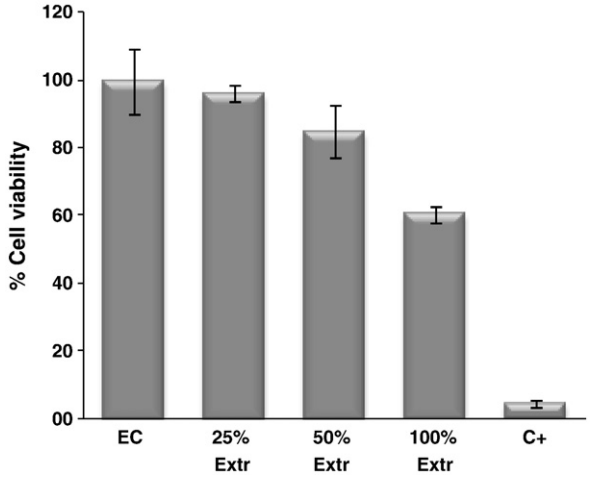

Fig. 1. Percentage of MG63 viable cells after 72 h-exposure to the diluted (25% Extr, 50% Extr) and undiluted (100% Extr) extracts of PMMA-co-EHA as determined by the MTT assay. Percentage of cellular viability was calculated considering that negative control cultures had 100% viability. Results corresponding to the extraction (EC) and positive (C^+) controls are also presented.

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those of the negative control. In all cases the level of statistical significance was set at $p \leq 0.05$. The results are expressed as mean ± standard deviation of the mean ($n = 4$).

3. Results

3.1. MTT assay

The cytotoxic effects of possible substances that leach out of PMMA-co-EHA bone cement during extraction were evaluated by quantifying the cellular viability using the MTT assay (Fig. 1). The obtained results evidenced a negative correlation between extract concentration and cell viability. Indeed, after 72 h of exposure, the undiluted extract induced a moderate reduction (about 40%) of viable MG63 cells, while this toxic effect was reduced with the dilutions, reaching values near 100% of viability when cells were exposed to the most diluted extract (25% Extr). The positive and extraction controls resulted according with our expectations, with the positive control with copper having a large effect in culture's viability, reducing it to values around 5%, and with the extraction control having no effect on culture's viability, showing that the extraction conditions had no influence in the observed cytotoxicity.

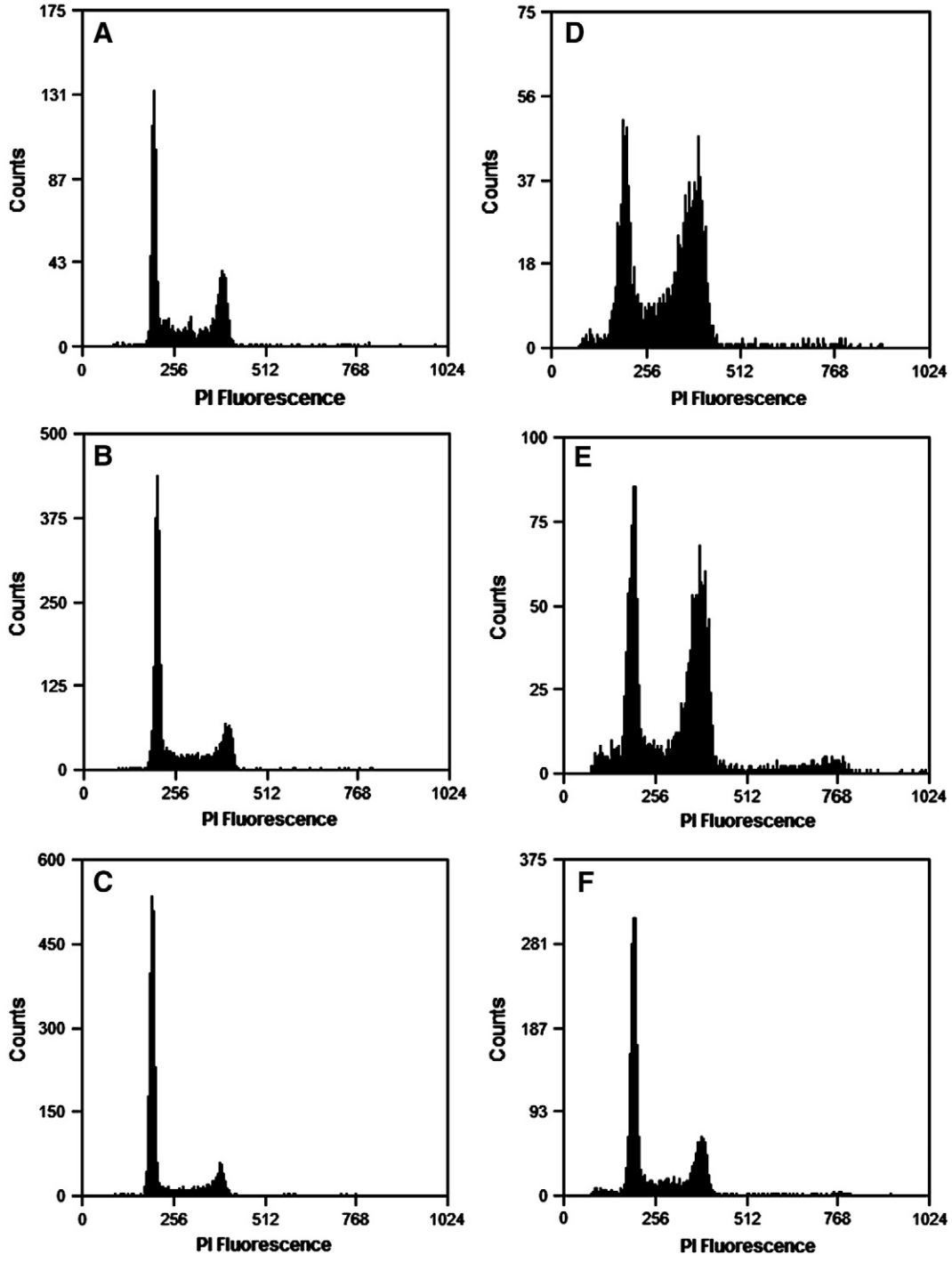

Fig. 2. Fluorescence light intensity histograms of propidium iodide (PI)-stained nuclei isolated from MG63 cells grown in: (A–C) culture medium for 24 h (A), 48 h (B) and 72 h (C) and (D–F) after treatment with the undiluted PMMA-co-EHA extract at 24 h (D), 48 h (E) and 72 h (F).

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3.2. Cell cycle analysis

Using flow cytometry we analyzed the changes in cell cycle phases at different culture endpoints after incubation with the PMMA-co-EHA extracts. Fig. 2 shows representative histograms of relative fluorescence light intensity of PI-stained nuclei isolated from control and bone cement extract-grown cells collected in each culture point. In any of the histograms, a first peak corresponding to cells that are in the G_1 phase of the cell cycle and a second peak with the double fluorescence of the first one, which corresponds to cells in the G_2 phase of the cell cycle, are evident. Cells that are actively synthesizing DNA, i.e., in the S phase, are scored between those peaks, showing an intermediate nuclear DNA content. Proportions of cells at different stages of the cell cycle are represented in Fig. 3. After 24 h in contact with the undiluted PMMA-co-EHA extract (100% Extr) there was a statistically significant reduction of cells in the G_1 phase ($p<0.001$) when compared with control culture (27.0% and 44.1%, respectively) and this trend was maintained in the subsequent exposure periods. However, as it was observed in control and diluted treatments, the percentage of cells in this phase raised with time, as the cultures became confluent. On the contrary, the 100% PMMA-co-EHA extract increased the number of cells in the S phase, relative to those cells growing in the culture medium, reaching its maximum in the first 24 h (62.4%) and suffering a decrease after that period in all cultures. Nevertheless, at 72 h of exposure, the cultures growing in both 50 and 100% extracts (50 and 100% Extr) showed a higher and significant percentage of cells in the S phase ($p<0.001$) when compared to control (22.2 and 22.7%, respectively vs. 17.2%). Moreover, during the first 48 h of culture, the percentage of G_2 phase cells exposed to different extracts, including the diluted and undiluted, did not show significant differences when compared with the control. However after 72 h of exposure, the cells exposed to the most concentrated extract showed a statistically significant higher proportion of G_2 cells ($p<0.001$) than in the control (19.6% and 9.9%, respectively), while the diluted extracts (50 and 25% Extr) did not induce significant changes in the number of cells in the G_2 phase.

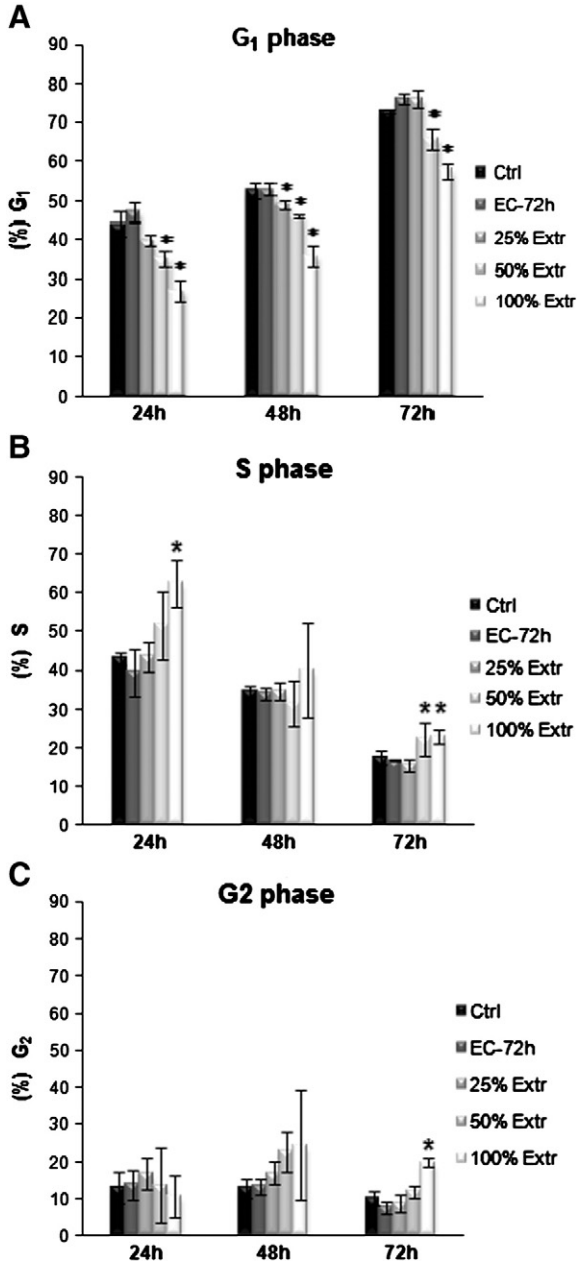

Fig. 3. Proportion of MG63 cells in each cell cycle phases after 24 h, 48 h and 72 h of culture exposure to diluted (25% Extr and 50 % Extr) and undiluted (100 % Extr) PMMA-co-EHA extract: (A) G_1, (B) S, and (C) G_2. The results obtained for the negative and extraction controls (Ctrl and EC-72 h, respectively) are also reported. Percentages represent mean ± standard deviation of the mean of four replicates. The symbol * indicates significant differences between Ctrl and treated samples ($p<0.001$) at each exposure time.

Flow cytometric analyses were complemented with microscopic visualizations, which confirmed a reduction in the cellular density soon after a 24 h contact with the undiluted extract in comparison with control cultures. This reduction was also accompanied by an increase of dead cells. After 72 h-exposure, treated cultures showed a recovery in proliferation, reaching confluence levels closer to those of the control (data not shown).

4. Discussion

When a new biomaterial is developed, its biocompatibility must be demonstrated and one of the first approaches should be the in vitro cytotoxicity evaluation. In the present work we report the preliminary data obtained for the new PMMA-co-EHA bone cement whose extracts were tested on MG63 osteoblast-like cells. According to the MTT assay it was possible to verify that extracts of this cement led to a reduction of cells capable of metabolizing formazan, with a subsequent death of a moderate number of cells after a 72 h-exposure period. Thus, we can deduce that leachables of PMMA-co-EHA interfered somehow with the mitochondrial cell function. However, this effect was largely reduced with extract dilution, indicating that this is a dose-dependent response. In accordance with these results, microscopic observations showed that the confluence level was lower after exposure to cement extracts. By other way, FCM results revealed an increment of cells in the S phase when compared to control, which suggests that this extract may have promoted cellular proliferation. A detailed analysis along the incubation period may shed light on the reasons behind these results. Indeed, in the first 48 h there was a significant number of cells, in cultures exposed to cement extracts, whose viability was affected, probably due to cellular arrest in the S-phase (the cells were not able to progress into the G_2 phase and originate new cells), with a consequent decrease of the confluence level because of the death of some cells. However, after 72 h of exposure, some cells seemed to be able to slowly recover from this condition, as evidenced by the significantly higher percentage of cells in the G_2 phase and by the increase in the confluence level in the undiluted extract when compared with the other treatments. Thus, despite an apparent adverse effect of PMMA-co-EHA extracts during the first days, our results suggest that there is a subpopulation of MG63 cells that after an arresting period is able to progress through the cell cycle and thus maintain its proliferation ability. The delay effect on the cell cycle, induced by either the contact with the biomaterial itself or its extract, was already reported in other studies [17,19]. For example, Lopes et al. [17] observed that when MG63 cells

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were grown on P_2O_5 glass-reinforced hydroxyapatite composite discs a delay on the progression of the cells from the G_1 phase into the S phase of the cell cycle was observed causing a low level of cell growth inhibition. Moreover, in the study of Granchi et al. [19] several commercial bone cements were analyzed and the results showed that MG63 cells exposed to the most toxic cement extract (Simplex P RO™ and CMW3™) had more difficulties in starting their replication cycle, but as in our study, this effect was reversible and the surviving cells continued to proliferate. However, we must point that in our work all the samples were steam sterilized by autoclaving (at 121 °C during 20 min) and this procedure itself could be responsible for a reduction of the effect of some cytotoxic leachables.

It is well known that some components (such as nonreactive MMA and DMPT) of typical PMMA-based bone cements are cytotoxic [23–25]. Considering that our PMMA-co-EHA formulation also has these components in its chemical composition we were already expecting some negative influence of PMMA-co-EHA extracts on their cytotoxic results. On the other hand, EHA based materials are considered biocompatible and have recently been proposed as suitable to be used as intra-ocular lenses–drug systems [26,27]. After this study had been accomplished, further in vitro tests were performed with other PMMA-co-EHA formulations [28, unpublished data]. Thus, considering all the results we believe that the observed initial cytotoxicity may be related with the in vitro behavior of PMMA-co-EHA bone cement, especially with its water uptake capability.

5. Conclusion

In conclusion, the results showed that the tested PMMA-co-EHA cement presents some cytotoxicity on cell viability and cell cycle progression, mostly during the first two days of exposure, but after this period the surviving cells are able to recover and maintain their proliferation ability. These data, together with the already obtained data on the mechanical properties of the cement [10], support that the new formulation could have potential for future applications. However, as this is the first study regarding the biological properties of PMMA-co-EHA, further in vitro and in vivo studies with this co-polymer are necessary to confirm the suitability of using this biomaterial in joint replacement procedures.

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