Brushite cement containing gelatin: evaluation of mechanical strength and *in vitro* degradation

(Cimentos de brushita contendo gelatina: avaliação da resistência mecânica e degradação in vitro)

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Abstract

Calcium phosphate cements (CPCs) are potential materials for repairing bone defects, mainly due to their excellent biocompatibility and osteoconductivity. Nevertheless, their low mechanical properties limit their usage in clinical applications. The gelatin addition may improve the mechanical and biological properties of CPCs, but their solubility in water may increase the porosity of the cement during degradation. Thus, the aim of this work was to investigate the influence of gelatin on the setting time, compressive strength and degradation rate of a brushite cement. CPCs were prepared with the addition of 0, 5, 10 and 20 wt% of gelatin powder in the solid phase of the cement. The results indicated that the setting time increased with gelatin. Furthermore, cement with 20 wt% of gelatin had an initial compressive strength of 14.1±1.8 MPa while cement without gelatin had 4.5±1.2 MPa. The weight loss, morphology and compressive strength were evaluated after degradation in Ringer's solution. According to the weight loss data, gelatin was eliminated of samples during degradation. It was concluded that the presence of gelatin improved CPCs mechanical properties; however, as degradation in Ringer's solution evolved, cement compressive strength decreased due to gelatin dissolution and, consequently, an increase in sample porosity.

Keywords: β-tricalcium phosphate, bone cement, brushite cement, gelatin.

Resumo

Os cimentos de fosfato de cálcio (CPCs) são materiais potenciais para reparar defeitos ósseos, principalmente devido à sua excelente biocompatibilidade e osteocondutividade. No entanto, suas baixas propriedades mecânicas limitam suas aplicações clínicas. A adição de gelatina pode melhorar as propriedades mecânicas e biológicas dos CPCs, mas sua solubilidade em água pode aumentar a porosidade do cimento durante a degradação. Assim, o objetivo deste trabalho foi investigar a influência da gelatina no tempo de pega, na resistência à compressão e na taxa de degradação de um cimento de brushita. Os CPCs foram preparados com a adição de 0, 5, 10 e 20% em massa de gelatina em pó na fase sólida do cimento. Os resultados indicaram que o tempo de pega aumentou com a adição da gelatina. Além disso, o cimento com 20% de gelatina apresentou resistência à compressão inicial de 14,1±1,8 MPa, enquanto o cimento sem gelatina teve 4,5±1,2 MPa. A perda de massa, a morfologia e a resistência à compressão foram avaliadas após a degradação em solução de Ringer. De acordo com os dados da perda de massa, a gelatina adicionada foi eliminada das amostras durante a degradação. Conclui-se que a presença de gelatina melhorou as propriedades mecânicas dos CPCs; no entanto, à medida que a degradação em solução de Ringer evoluiu, a resistência à compressão dos cimentos diminuiu devido à dissolução da gelatina e, consequentemente, um aumento na porosidade das amostras.

Palavras-chave*: β-fosfato tricálcico, cimento ósseo, cimento de brushita, gelatina.*

INTRODUCTION

Calcium phosphate cements (CPCs) are of interest in the biomedical and dental field, mainly due to its excellent biocompatibility, osteoconductivity, resorbability and bone replacement capability [1-5]. The moldability and injectability of most CPCs are key properties to leverage their applicability once the cement paste can be molded into bone defects of complex geometries or be extruded through a needle attached with a syringe, allowing the performance of minimally invasive surgical procedures [1, 4, 6, 7]. CPCs are made from a combination of a powder and a liquid, that form a self-setting paste when mixed [8]. The powder is composed of one or several calcium phosphate compounds and the liquid phase consists of aqueous solutions. Despite several formulations, all CPCs may be divided into two groups according to the end products of the setting reaction: apatite or brushite CPCs [3]. The production of high purity apatite cements is not a simple process since several factors can affect their final properties or even prevent their formation, such as the purity of the starting reagents [9,

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10]. The major disadvantage is associated with its slow *in vivo* degradation [11]. Due to the low solubility of apatite cements, these are reabsorbed by an active mechanism, regulated by living cells [1].

Brushite cement with a formulation based on H_3PO_4 and β-tricalcium phosphate (β-TCP) presents some advantages, such as lower cost, faster preparation, better control of chemical composition and reactivity and improved physical and chemical properties [12]. Notwithstanding, their short setting time and low mechanical properties may limit their clinical application [7]. The degradation of brushite cement *in vitro* is associated with physical-chemical processes, such as erosion, fragmentation and dissolution with the release of calcium and phosphate ions into the solution. The chemical equilibrium between the dissolution and reprecipitation of ions determines the rate of degradation of brushite cement [13]. Although the resorption of brushite cements is associated with their high solubility [8], *in vivo* supersaturation of calcium and phosphate ions suggests that the dissolution of brushite cement must occur slowly and therefore cannot fully explain the complete *in vivo* degradation. According to studies, the degradation of brushite cement *in vivo* is aided by macrophages that phagocyte cement particles [14]. Porosity is another important factor in CPC resorption, a very dense structure may lead to slow resorption rate. CPCs have small pores, insufficient to allow osteoblasts and osteoclasts to penetrate into the material for resorption and formation of new bone [15].

A common path to overcome CPCs' undesirable mechanical performance would be by the addition of reinforcements, i.e. polymers or fibers [16-19]. Although, one should take into account that the size of the fibers may alter powder wettability and paste homogeneity, compromising cements' applicability as well as their final properties, mainly degradation rate and bioactive interaction with bone [17, 20]. The incorporation of polymers into cements is an excellent alternative to improve their clinical applicability. The polymers can provide better mechanical performance and also better properties, such as injectability, setting time, cohesion, degradation rate and biological response [18]. Chitosan [21], alginate [22], silk fibroin [23], collagen [24] and gelatin [25, 26] are some examples. The formation of CPC/polymer composite follows the natural bone, that consists of apatite and fibrous collagen [7, 27, 28]. The incorporation of gelatin in powder phase of CPC increased setting time and porosity. According to the amount of gelatin added, mechanical properties of composite initially increased [29], but decreased with degradation, due to increased macroporosity [15, 30, 31].

Gelatin is a natural polymer produced by the hydrolysis of collagen extracted from the skin and bones of animals [32]. It is soluble in water, biocompatible and biodegradable [33, 34]. Gelatin can support initial cell adhesion and proliferation, stimulated alkaline phosphatase (ALP) activity and collagen production [30, 35] and its resorption mechanism of gelatin occurs by enzymatic degradation *in vivo* [36-38]. Thus, gelatin has shown to be an attractive alternative in the formulation of cements. The aim of the current study was to investigate the influence of gelatin on setting time, physicochemical, mechanical and morphological characteristics of brushite cements, as well as their changes during *in vitro* degradation.

MATERIALS AND METHODS

Calcium phosphate cement: CPC solid phase, i.e. β-TCP, was synthesized through a solid-state reaction at 1050 °C of a 1:2 molar ratio mixture of $CaCO_3$ (Sigma-Aldrich, Germany) and $CaHPO_4$ (Sigma-Aldrich, Germany). The β-TCP powder was milled in a horizontal ball mill for 48 h using an alumina grinding media of Ø15 mm and ball to power ration of 20:1 w/w. The particle size of the resulting powder was analyzed by laser diffraction (Mastersizer 2000, Malvern). CPC liquid phase consisted of an aqueous solution of 2.67 $mol.L^{-1}$ phosphoric acid (H_3PO_4, Synth, Brazil) and 0.08 $mol.L^{-1}$ citric acid ($C_6H_8O_7$, Synth, Brazil). Citric acid was added to slow the setting reaction down by promoting a dispersant effect on powder [7, 39]. Liquid-to-powder ratio was 0.80 $mL.g^{-1}$. To evaluate the influence of gelatin (Synth, Brazil) on the properties of the cement, 5, 10 and 20 wt% were mixed with β-TCP powder. Cement pastes were molded into cylindrical Teflon molds (6x12 mm), let to set in 100% humidity for 24 h. After drying, cement samples were polished and demolded for further characterization.

In vitro degradation: for the *in vitro* degradation studies, the specimens were weighed and immersed in 15 mL of Ringer's solution (Oxoid, BR0052G, UK) and incubated at 36.5 °C (Nova Ética, 410-D) for 7, 14, 21 and 28 days. At the end of each period, samples were washed with deionized water and dried at 100 °C for 24 h. Then, samples were weighed again. The immersion solution was refreshed each 48 h, in order to avoid changes in pH during degradation [40]. *In vitro* degradation rate was assessed for each time period by measuring weight loss, compressive strength and microstructure of the samples. The weight loss (W_L) was determined by comparing the initial weight (W_o) of the specimen and the dry specimen weight (W_d) after the degradation process, according to Eq. A [41]. At least 10 samples were used at each degradation time.

$$W_L\,(\%) = \frac{W_o - W_d}{W_o}\,.\,100 \tag{A}$$

Characterization: the initial and final setting times of cement paste were determined according to the ASTM C266-04 standard [42]. Assays were performed in triplicate. The apparent density of the specimens before degradation was determined by Archimedes' method. Assays were performed in triplicate. Crystalline phases composition of samples B_0 and B_20 were qualitatively investigated by X-ray diffraction (XRD, Shimadzu, XRD7000), CuKα, 10° to 40° (2θ), after each degradation time. JCPDS files used for phase identification were 09-0077 for brushite, 09-0080 for monetite and 09-0169 for β-TCP. The compressive strength of the samples before and after the degradation assay was measured in a universal testing machine (Instron, 5500R) equipped with a 30 kN load cell at a crosshead speed of 0.5 mm.min^{-1}. At least ten specimens were used for each compressive strength test. Structural morphology of the cements' surface of fracture was determined by scanning electron microscopy (SEM, Jeol, JSM6360-LV and 5900-LV). The samples were coated with a thin layer of gold (<30 nm; Baltec, MCS010) before the examination.

Statistics: one-way analysis of variances (ANOVA) was used to evaluate the statistical significance of compressive strength and weight loss. In all cases, the results were considered statistically different with $p<0.05$. Tukey's test was used for *post hoc* analysis to determine multiple comparisons. All statistical measurements and computations were made on RStudio environment [43].

RESULTS AND DISCUSSION

After milling, the powder of β-TCP presented a mean particle size of 3.45 μm (D50 value) and a particle size distribution between 1.10 μm (D10 value) and 26.21 μm (D90 value). Brushite cements are produced through acid-base reaction [1]. Among all compositions which result in brushite, the one developed in this study may be considered the most simple and inexpensive, i.e. β-TCP reacting with phosphoric acid to form brushite. The setting reaction of the cement is shown in Eq. B:

$$\beta\text{-Ca}_3(\text{PO}_4)_{2(s)} + \text{H}_3\text{PO}_{4(aq)} + 6\text{H}_2\text{O}_{(l)} \rightarrow 3\text{CaHPO}_4.2\text{H}_2\text{O}_{(s)} \quad \text{(B)}$$

The initial and final setting times of the brushite cements prepared are displayed in Table I. As can be observed, the setting times increased with the weight percentage of gelatin, without compromising the final product of the reaction. Indeed, as reported in [30], the addition of polymers tends to increase setting time of cements due to the increase in paste viscosity, inhibiting the ionic diffusion through the cement paste [30]. As noted for all formulations, gelatin positively interfered with the setting reaction of the cement, because the fast setting of the brushite cements (~2-4 min) compromised the workability of the paste. Yin et al. [44] describe the same behavior in brushite cements prepared with the mixture of β-TCP and monocalcium phosphate monohydrate (MCDM). According to [44], the addition of gelatin powder to the cement inhibits the dissolution and the diffusion of calcium and phosphate ions, retarding the cement setting time.

Table I - Cements formulations and setting time: T_i - initial setting time; and T_f - final setting time.

[Tabela I - Formulações dos cimentos e tempos de pega: T_i - tempo de pega inicial; e T_f - tempo de pega final.]

Sample	Gelatin (wt%)	T_i (min)	T_f (min)
B_0	0	2.58±0.22	4.62±0.33
B_5	5	3.33±0.10	6.37±0.13
B_10	10	3.72±0.05	6.80±0.05
B_20	20	4.12±0.12	7.67±0.17

Figs. 1a and 1b show the XRD patterns of the formulations B_0 and B_20, respectively, after different degradation periods (0, 7, 14, 21 and 28 days). The presence of XRD lines related to β-TCP indicated that the setting reaction of the cements was not complete, i.e. not all β-TCP was transformed into brushite. The presence of gelatin did not affect appreciably XRD pattern of the cement powder. Further, the XRD patterns revealed diffraction lines related

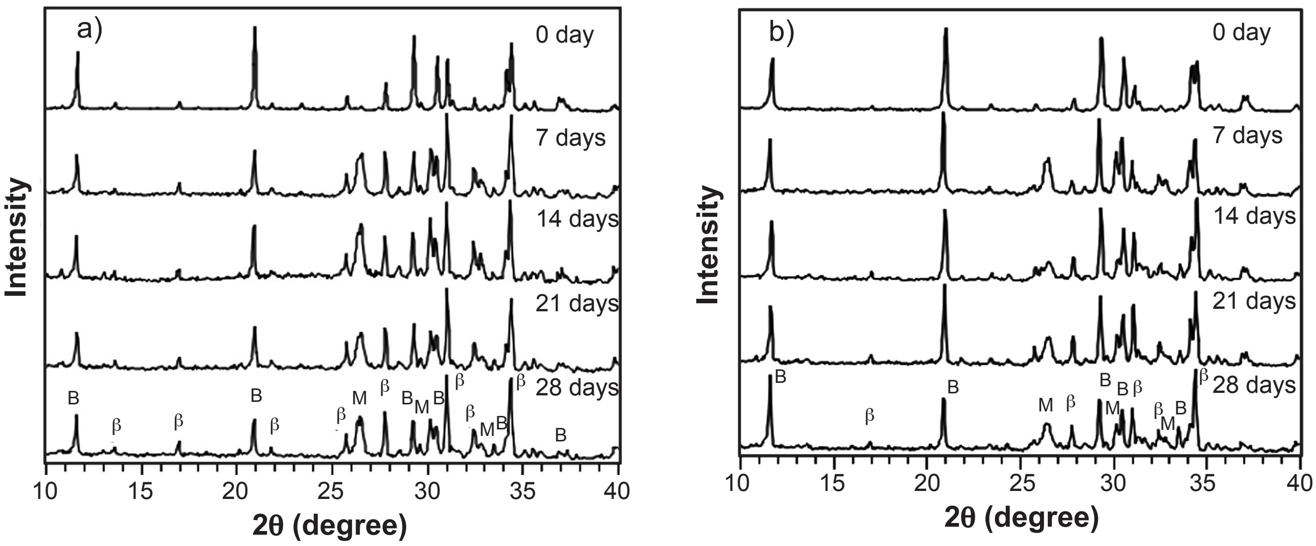

Figure 1: XRD patterns of the cements with 0 (a) and (b) 20 wt% gelatin after different degradation periods. β: β-TCP, B: brushite, M: monetite.

[Figura 1: Padrões de DRX dos cimentos com 0 (a) e (b) 20% (m/m) de gelatina após diferentes períodos de degradação. β: β-TCP, B: brushita, M: monetita.]

to the presence of monetite ($CaHPO_4$). These peaks became more intense with the increasing immersion period, while brushite peak intensities decreased. This fact can be assigned to the conversion of the brushite into monetite due to the drying treatment of the samples (100 °C during 24 h) [7, 39, 45] after the degradation path, according to the following equation:

$$CaHPO_4.2H_2O_{(s)} \rightarrow CaHPO_{4(s)} + 2H_2O_{(l)} \qquad (C)$$

The SEM micrographs of the surface of fracture of all cements specimens before degradation are shown in Fig. 2. Gelatin provided the formation of a thin layer coating on the formed crystals, maintaining a more homogeneous particle distribution and a more uniform microstructure, improving the fracture toughness of the samples [5], as in the bone matrix, in which the collagen fibers provide ductility and ability to absorb energy [46].

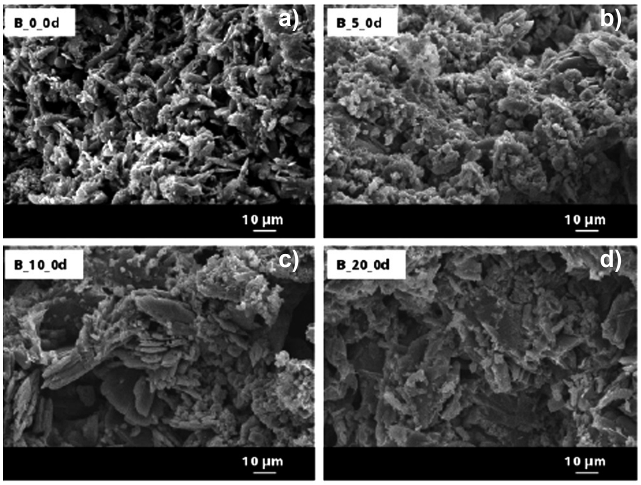

Figure 2: Surface SEM micrographs of the cement samples before degradation.
[Figura 2: Micrografias de MEV das superfícies das amostras de cimento antes da degradação.]

The compressive strength of cement samples as a function of the different periods of degradation is shown in Fig. 3. The compressive strengths before degradation for the specimens were 4.5±1.2 MPa without gelatin and 6.9±1.3, 7.7±1.4, and 14.1±1.8 MPa for B_5, B_10 and B_20, respectively, and were within the range of compressive strength of spongy bone tissue (2-12 MPa) [47]. ANOVA results revealed a $p<0.05$ ($p=0.0000$). Tukey's comparison test yielded a significant difference between the compressive strength of samples. These data suggested that the compressive strength increased with the amount of gelatin added; it was observed compressive strength presented an increase of 213% when 20 wt% of gelatin was added into cement. This was already expected since the addition of gelatin to the CPCs improves the mechanical properties of the cement. Therefore, the plasticizing effect of gelatin improved compressive strength of brushite cements, behavior similar to that of collagen in the bone matrix. Bigi et al. [35] describe the improvement in compressive strength of apatite cements. According to the authors, the addition of 15 wt% of gelatin provided a significant increase in the mechanical strength of the wet cement, even after 7 days immersed in PBS (phosphate buffered saline). Cement enriched with gelatin also exhibited enhanced osteoblast activation and extracellular matrix mineralization processes when compared with a traditional cement *in vitro* biological studies.

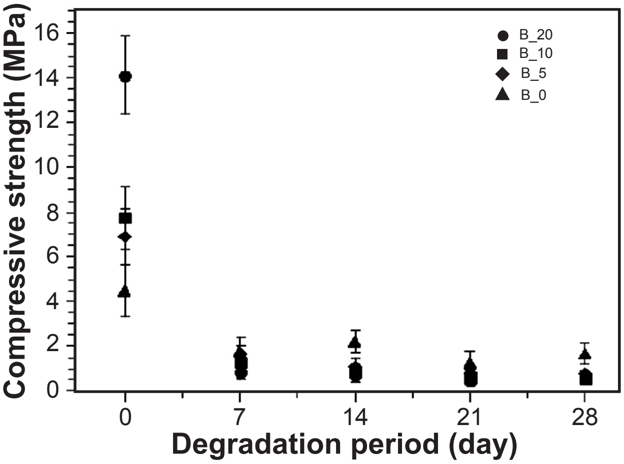

Figure 3: Compressive strength of the cement samples during different degradation periods.
[Figure 3: Resistência à compressão das amostras de cimento em diferentes períodos de degradação.]

The results revealed a reduction in the apparent density of the samples with increasing gelatin addition, before degradation. There was a reduction of 10.9%±6.5%, 10.5%±1.4% and 12.4%±5.6% for the samples B_5, B_10 and B_20, respectively, when compared to sample without gelatin. Indeed, as observed in Fig. 4, which shows SEM micrographs of low magnification, there is an increase in pore size of the samples with increasing fraction of gelatin. Gelatin is also used as a porogenic agent when added to the solid phase of a CPC as powder or microspheres. The formation of macropores is observed with the *in-situ* dissolution of the gelatin [4, 44]. In this study, larger pores might be formed after the addition of the liquid phase to the cement. With the addition of the liquid phase, the gelatin was solubilized forming pores in the cement matrix, and the gel produced in the dissolution of the gelatin-coated cement particles, which provided a better distribution of the mechanical load and contributed to the improvement of the mechanical properties. Therefore, the increase in porosity did not affect the compressive strength of the cements before degradation.

As can be seen in Fig. 3, during degradation, all cement samples exhibited a decrease in compressive strength. After 7 days of degradation, cement without gelatin showed higher compressive strength than the other samples: 1.76±0.70 MPa for without gelatin and 1.68±0.33, 1.28±0.40 and 0.86±0.37 MPa for B_5, B_10 and B_20, respectively. This behavior was maintained throughout the degradation period (28 days). ANOVA results revealed a $p<0.05$ for all degradation

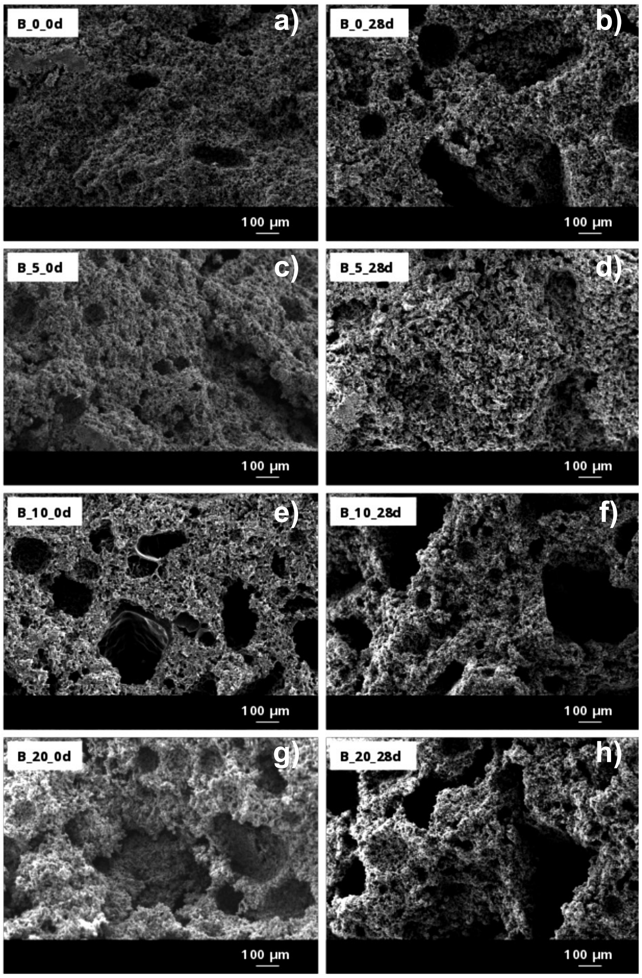

Figure 4: SEM micrographs of cements samples before and after degradation: porosity analysis.
[Figure 4: Micrografias MEV das amostras de cimento antes e após degradação: análise da porosidade.]

periods. Tukey's comparison test showed a significant difference between the compressive strength of the sample without gelatin and samples B_5, B_10 and B_20. Starting at 21 days of degradation, compressive strength between the samples B_5, B_10 and B_20 were not significantly different. As shown in Fig. 5, the weight loss of samples may explain the results obtained. As a water-soluble protein, gelatin added to the cements disintegrated in aqueous media, which increased the porosity of the samples during the immersion period. Accordingly, it was observed a decrease in compressive strength of samples; i.e. the higher the amount of gelatin in the cement, the lower the compressive strength after degradation. The cement without gelatin also showed weight loss and increased porosity due to brushite dissolution in Ringer's solution. Renó et al. [48] showed that *in vitro* degradation of brushite cements in Ringer's solution for up to 28 days also led to an increase in porosity and a decrease in compressive strength and concluded that the degradation occurred due to the dissolution of brushite.

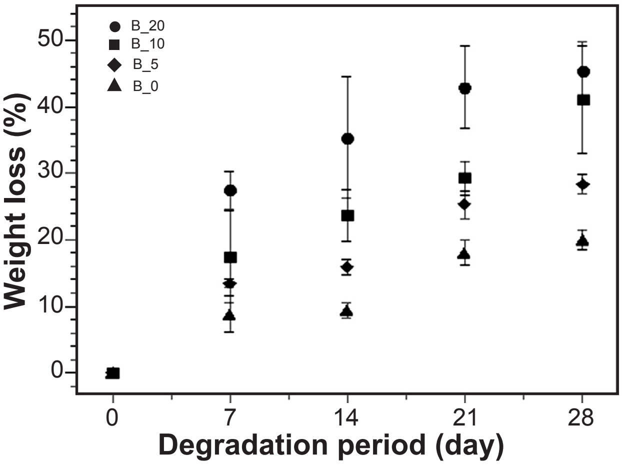

Figure 5: Weight loss ratios of the cement samples during different degradation periods.
[Figure 5: Perdas de massa das amostras de cimento durante diferentes períodos de degradação.]

The microstructure of biomedical implants is a very important property in the *in vivo* bioresorption process, bone ingrowth and vascularization [3]. High degradation rates can promote bone growth into the cement and consequently compensate mechanical resistance loss. The weight loss values suggest that after 7 days of degradation most of the gelatin present in the cement samples were solubilized and removed. Subsequently, in the other periods of degradation, most of the mass loss presented by the samples was due to the dissolution of brushite. After 7 days of immersion, sample B_0 showed 8.8%±2.7% of weight loss, and samples B_5, B_10 and B_20 presented 13.4%±0.7%, 17.4%±6.8% and 27.5%±2.8%, respectively. As can be seen, samples with the addition of gelatin presented higher weight loss. ANOVA results revealed a $p<0.05$ for weight loss during all degradation periods. Tukey's comparison test showed a significant difference between weight loss of sample without gelatin and samples B_5, B_10 and B_20.

CONCLUSIONS

Brushite cements were obtained by adding gelatin into the solid phase, resulting in an increase the compressive strength as higher the content gelatin added. Gelatin also provided a higher degradation rate due to its water solubility. As expected, after *in vitro* degradation, the compressive strength decreased as a function of porosity increase and elimination of gelatin. The incorporation of gelatin in brushite cements should be considered as promising in bone replacement material.

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