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Biological response of new activated acrylic bone cements with antiseptic properties. Histomorphometric analysis

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Abstract The biological response to an acrylic bone cement cured with 4,4′-bis-dimethylamino benzydrol (BZN) as activator of reduced cytotoxicity and antiseptic properties, has been carried out and compared with that obtained for CMW 3 cement. Histomorphometrical data (undecalcified trichromic Goldner staining) were obtained by measuring the most significant variables at the bone-cement interface. Quantitative results of tissue response revealed that newly formed bone and connective tissue were maximum at 4 weeks whereas bone marrow increased with time of implantation for both cements. Statistical analysis ($p < 0.05$) showed no significant differences in newly formed bone and bone marrow with time and between both groups, however, connective tissue significantly decreased between 4 weeks and 12 weeks for BZN cement, and between 12 weeks and 24 weeks for CMW3. By comparing both cements at each time, lower significant percentage of connective tissue at the bone-cement interface of the BZN cement, was obtained at 12 and 24 weeks, however, a very low amount of connective tissue was found for both cements. All the results indicate that the new activated system could be applied clinically in a relatively short time, after the corresponding preclinical study.

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Introduction

One of the main advantages of the poly(methyl methacrylate) bone cement is the mechanical support that provides immediately after its implantation. The major biological disadvantage of this cement is its limited biocompatibility, leading to the formation of a fibrous tissue layer at the bone-cement interface [1]. The bone cement induces damage to the tissue during the initial phase and is not sufficiently biocompatible to encourage repair. The prevalence of foreign body giant cells at the contact interface reflects the poor compatibility of the bone cement surface. Both methyl methacrylate monomer, MMA, and its additives can cause irreversible damage to the cells [2] and in vivo studies demonstrated that hot toxic chemicals released from bone cement during polymerisation may inhibit bone blood perfusion and remodelling of bone [3]. In vitro studies [4] on the toxicity produced by the currently used activator *N*,*N*-dimethyl-4-toluidine, DMT, demonstrated that it causes a delay on the cell replication when it is exposed to culture of osteoblasts, it is able to inhibit protein synthesis, causing both qualitative and quantitative changes in chromosomal arrangement [5] and even modifying the process of bone mineralization [6]. It has been suggested that a medium to high concentration of DMT in the liquid phase of the cement induce a remarkable platelet activation [7]. Few commercial bone cement formulations contain an activator different from DMT. One of them is the cement Sulfix-60® which has incorporated a tertiary aromatic amine of reduced toxicity, *N*,*N*-dimethylamino phenethanol [8]. The Sulfix-60® cement extracts assessed in vitro with osteoblast-like cells produced the most positive response among other

commercial formulations [9]. However, with respect to in vivo studies scarce references are found in the literature.

In a previous paper we have analysed the properties and preliminary in vivo response to acrylic bone cements cured with *N*,*N*-dimethylaminobenzyl-alcohol (DMOH) and 4,4′-bis-dimethylamino benzydrol (BZN) [10]. The cement formulated with the activator BZN gave the most promising response, providing a marked cellular activity with a good osseous neoformation. This finding was attributed not only to the reduced cytotoxicity of BZN but also to its antiseptic properties, due to its similarity to crystal violet [11–13]. As a continuation of that work, new in vivo experiments have been conducted in order to analyse both qualitative and quantitative histomorphometric response when the cement is implanted in the femur of rabbits. The histomorphometry was conducted by measuring the evolution of cement, neoformed bone, bone marrow and connective tissue at the bone-cement interface with time of implantation. Parallel experiments were performed by implantation of a commercially available cement, e.g., CMW·3, in the same conditions and its response was compared to that of the experimental cement containing BZN.

Experimental

Materials

Methyl methacrylate monomer (MMA) (Acros) was used as received without further purification. Benzoyl peroxide (BPO) (Merck) was used after crystallisation from ethanol. BZN (Sigma), poly (methyl methacrylate) (PMMA) beads (Industrias Quirúrgicas de Levante, S.A.) and zirconium dioxide (Comercial Riba, S.A.) were used as received. The radiopaque acrylic bone cement CMW 3® (DePuy International Ltd, United Kingdom) was used as the commercial formulation.

Methods

Acrylic bone cements

The experimental cement was formulated by adding the liquid component containing the monomer MMA and the activator BZN to the solid component consisting of PMMA beads, zirconium dioxide as radiopaque agent, and BPO as radical initiator, in a typical solid:liquid ratio of 2:1. The radiopaque CMW 3 was used as a control and was prepared according to the manufactures instructions. The composition of both experimental and commercially available formulations are shown in Table 1. The experimental cement will be named as BZN cement for abbreviation purposes.

Table 1 Chemical composition of the acrylic bone cements analysed in this work

	Commercial cement, CMW 3		Experimental cement, BZN	
Solid component (wt%)	PMMA	88.00	PMMA	88.5
	BPO	2.00	BPO	1.5
	$BaSO_4$	10.0	ZrO_2	10.0
Liquid component (wt%)	MMA	96.54	MMA	99.00
	DMT	2.487	BZN	1.00
	Ethanol	0.44		
	Ascorbic acid	0.022		
	Hidroquinone	0.0022		

Surgical procedure

Adult female New Zealand rabbits of average weight 3.820 Kg (3.450–4.260 Kg) were used for in vivo experiments. The rabbits were pre-medicated with atropine sulphate (0.3 mg/Kg, IM) and chlorpromacine (10 mg/Kg, IM). General anaesthesia was given by intramuscular injection of ketamine hydrochloride (50 mg/Kg, IM) and fentanile (0.17 mg/Kg, IM). After shaving the skin, the surgical field was cleaned with iodine and a longitudinal incision was made. Two bone defects were created manually in the femur, one of them in the femoral condyle, epiphysis, in order to study the effects of cement on cancellous bone, since in clinical practice, mainly this type of bone is exposed to the acrylic cement, and the other one was created in diaphysis. The defects were 5 mm in diameter and 5–8 mm depth. At that moment, the corresponding acrylic bone cement was prepared by mixing both liquid and solid components and the mixture was stirred for a while to obtain a paste or dough of cement with suitable viscosity to be injected in the femoral cavity. Then, the corresponding paste or dough was injected with a syringe and the cement was allowed to cure inside, so that the exothermic polymerisation reaction occurred inside the femoral cavity after implantation. Once the cement was set, the muscle was sewn with a suture of vicryl and then the skin with discontinuous silk. After surgery, the animals were allowed to move freely in their cages without joint immobilization and they were sacrificed by an intravenous injection of pentotal®. A total of 18 rabbits (36 legs) were operated on. CMW 3 cement was implanted in the right leg and the experimental cement

in the left one. A batch of 6 animals were killed at 4, 12 and 24 weeks respectively, after operation. The periods of time analysed were established based on findings of previous studies [10].

Samples extraction and histopathological analysis

After the sacrifice, a skin incision was made to reach the femoral bone and it was disarticulated from the knee to the hip. The extracted femur and the surrounding tissue removed were fixed in a saline solution of formaldehyde (10 v%). The femoral condyle was cut transversally and then was embedded in methacrylate resin. This method has the advantage to allow bone processing without decalcification [14]. 5 μm sections were obtained with a microtome (Microm-HM 350 S). The sections were stained with Goldner technique to visualize new bone formation and examined under a light microscope (Nikon Microphot FXA) connected to a digital colour camera (COOLPIX 995). The macroscopic photographs were taken with a digital camera (COOLPIX 4500).

Histomorphometric analysis

The histomorphometric analysis was conducted on explanted samples of sections containing both control and experimental cements, separately. For each case, a zone of 30 mm^2 area of histological section (6 μm thickness) at the interface bone-cement was selected, where the cement was always located in the centre, and surrounded by newly formed bone, connective tissue and bone marrow. The areas of the four variables (cement, new bone, bone marrow and connective tissue) were measured in μm^2, normalized and expressed in percentages. Histomorphometric parameters were measured with an image analysis system consisted of a camera (Digital Nikon Camera DXM 1200) connected to a microscope (Nikon Microphot-FXA) and to a computer (Intel Pentium IV). The programs Nikon ACT-1 and Eclipse Net were used for taking images and for the measurement of the parameters.

Statistical analysis

The values of the percentages of cement, newly formed bone, connective tissue and bone marrow, obtained by histomorphometry for each cement and each time of implantation, were evaluated with the use of a one-way ANOVA test, at a significance level of $p < 0.05$. A Tukey's multiple comparison procedure was used to compare both groups for each time post implantation, and the three times with each other for each group.

Results

Within the development of more biocompatible acrylic bone cements, the use of low toxicity activators substituting the traditional currently used *N*,*N*-dimethyl-4-toluidine (DMT) has been attempted. Among the different less toxic tertiary aromatic amines employed, the efficiency of BZN as an activator in the redox system amine/BPO to initiate the free radical polymerisation of methyl methacrylate, has been demonstrated [10]. Residual monomer content, indic-

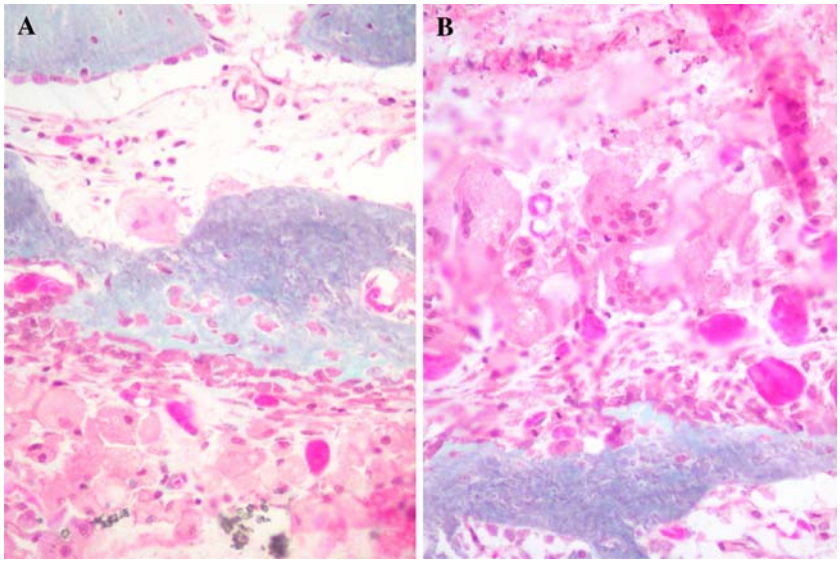

Fig. 1 Histological reaction to BZN cement 4 weeks post implantation. (**A**) A micrograph of the trabeculae with osteoblasts synthesizing osteoid actively and also osteoblasts remodelling the newly formed bone. Goldner's stain, ×20. (**B**) Micrograph showing at the bottom a neoformed trabeculae adjacent to connective tissue, and above it some inflammatory cells, and in the centre some multinucleated cells. Goldner's stain, ×20

ative of the polymerisation conversion, of an experimental cement containing BZN/BPO as the initiator system, was quantify as 2.5%, that is, in the range of that remaining in commercially available cements. The curing parameters of the as-obtained cement measured at 25 °C, were within those accepted by the international standard ISO 5833 [15], 75 °C peak temperature and 10.5 min setting time, giving a maximum temperature lower than the currently used formulations. Mechanical properties were not impaired with respect to values given in the literature for commercial brands [16, 17] and present a compressive strength of 79 MPa, and a maximum tensile strength of 52 MPa. Based on those promising results, an examination of the biological response of this experimental cement has been performed in vivo, simulating the surgical use of the acrylic bone cements, that is, the in vivo implantation of the cement dough has been conducted and the histological and histomorphometric response analysed with time of implantation. The implantation of the commercially available CMW 3 has been carried out under the same conditions and studied for comparison purposes.

Histological analysis

The biological response of the bone tissue after the implantation of dough of cement in the femur of rabbits was studied in a period from 4 weeks to 24 weeks. The response for the cement containing BZN after 4 weeks of implantation revealed the existence of necrotic cellular residues in the central portion as well as an important inflammatory

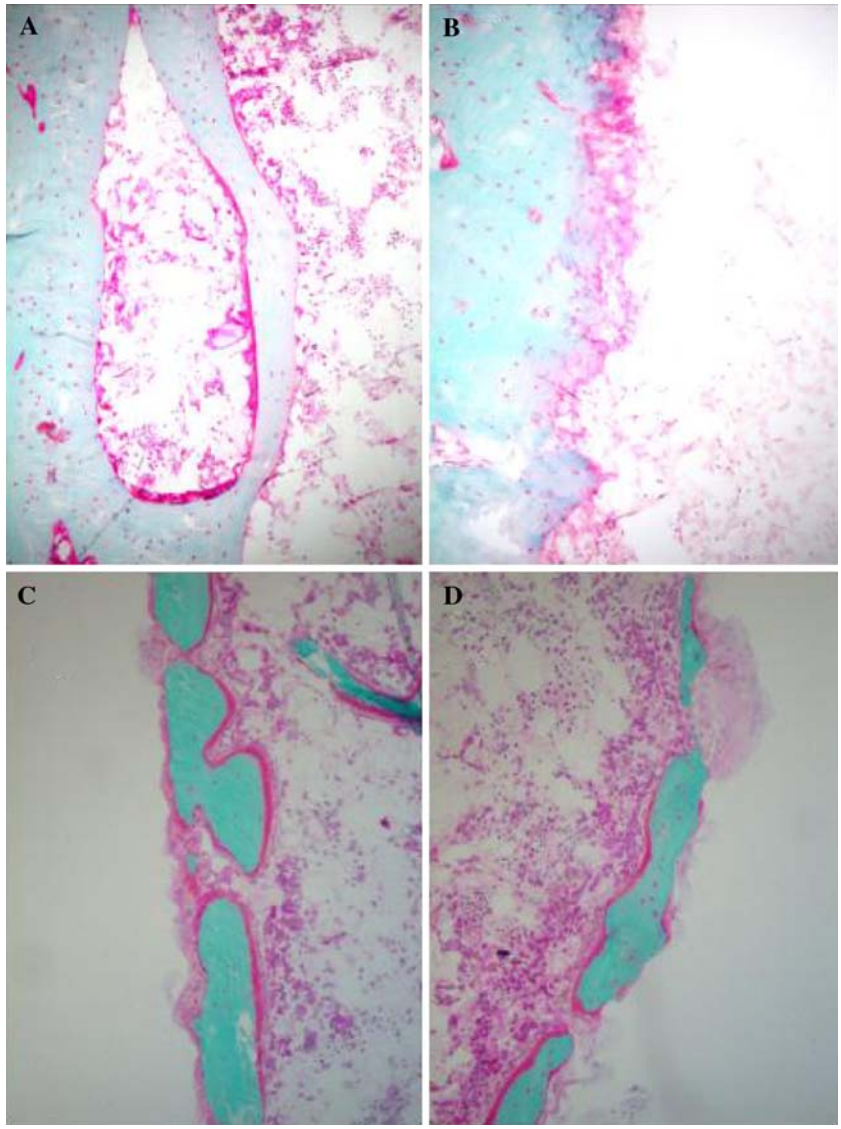

Fig. 2 Histological reaction 12 weeks post implantation. (**A**) Micrograph of BZN cement response showing a detail of endostium with a new cavity having haematopoietic bone marrow. Goldner's stain, ×10. (**B**) A detail of the diaphysis cortical bone—BZN cement interface in connexion with bone marrow. The interface is formed by a thin layer of connective cells with osteoformed capacity. Goldner's stain, ×20. (**C**) Micrograph showing CMW 3 cement in contact with neoformed bone, and osteoid in contact with old bone opposite to the cement. Goldner's stain, ×10. (**D**) Micrograph showing the CMW 3 cement, multinucleated giant cells, and in the centre neoformed bone and osteoid containing precursor cells of hematopoietic bone marrow. Goldner's stain, ×10

response characterised by agglomerates of foreign body giant cells isolated within empty spaces and shaped according to the voids between cement beads. At this period the histological analysis also showed an increase of the cortical bone, mainly at the endosteal surface where it interfaces with bone marrow. This proliferation was at the expense of osseous trabeculae anastomosed which contained osteoblasts within the bone in small lacunae. Osteoblasts were found in clusters of cuboidal cells along the bone surface, forming a layer of bone matrix that they were producing before calcification (Fig. 1). This implies that during the phase of repair, new bone architecture can be formed where it has previously existed. The response produced after implantation of CMW 3 for this period was comparable with that of BZN cement regarding necrosis and osseous neoformation. Figure 2 shows the biological reaction to BZN and CMW 3 cements at 12 weeks post implantation. In both cases, the newly formed bone presented normal characteristics, it was mature regarding the cells disposition and extracellular matrix, and also with respect to haematopoietic marrow. The histological response 24 weeks after implantation was characterized by few necrotic residues, less abundant for the BZN cement what indirectly indicates a higher osseous neoformation for this formulation compared to CMW 3, at this time. Figure 3A and B shows the response for the BZN cement. The haematopoietic marrow presenting normal cytology is shown in Fig. 3A, and Fig. 4B shows the cortical diaphysis, the neoformed bone and bone marrow close to the cement, showing normal cytological characteristics. For CMW 3, the cement presented a complex and

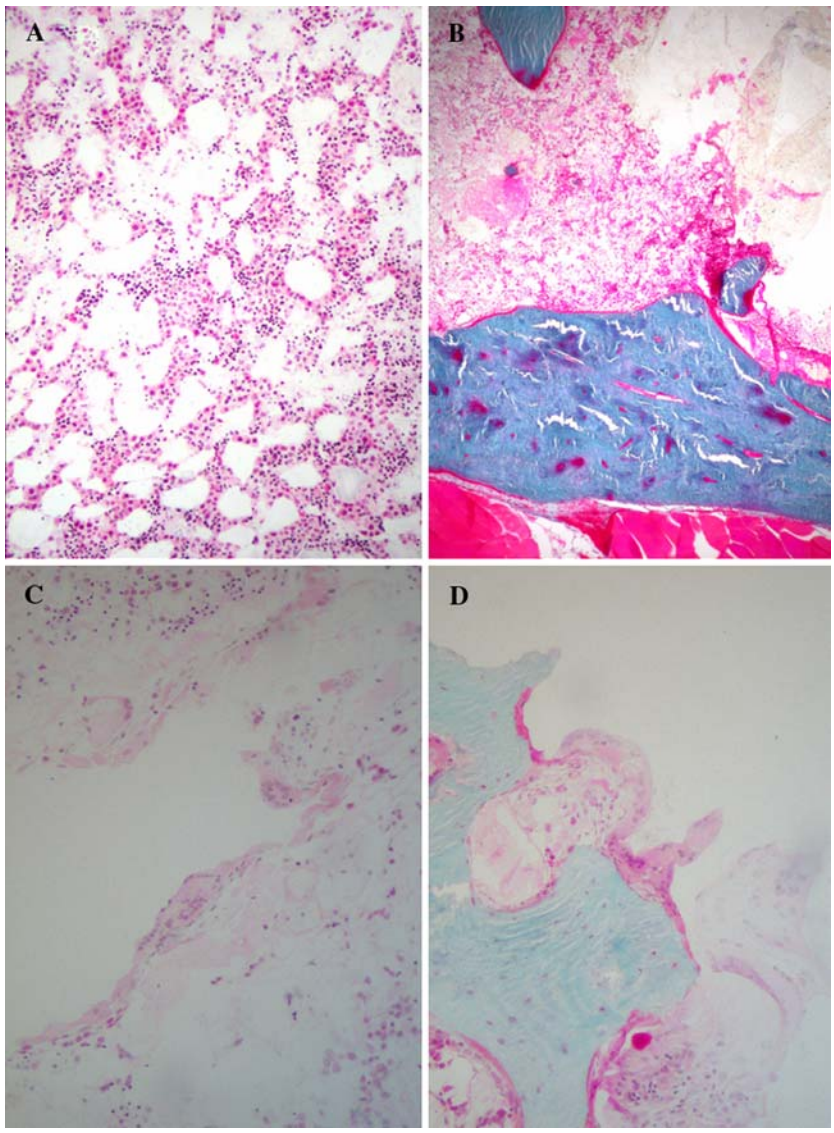

Fig. 3 Histological reaction 24 weeks post implantation. (**A**) Response to BZN cement showing haematopoietic bone marrow of normal characteristics. Goldner's stain, ×10. (**B**) A panoramic view showing at the bottom the cortical dyaphysis, on the right hand, the cement and on the left hand, neoformed bone and bone marrow close to the BZN cement, with normal cytology. Goldner's stain, ×4. (**C**) Micrograph showing the CMW 3 cement adjacent to bone and delimited by connective tissue with penetration of papilla into the cement. Goldner's stain, ×10. (**D**) Photograph showing the CMW 3 cement in contact with mature bone, multinucleated giant cells and a papillar structure inside the cement. Goldner's stain, ×10

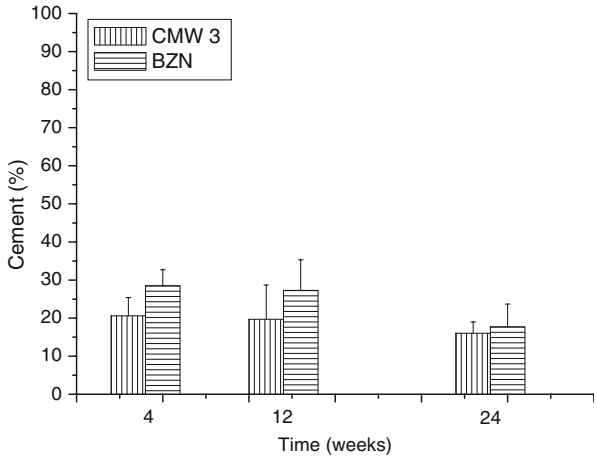

Fig. 4 Histomorphometric analysis of the variable cement with time for both types of cements

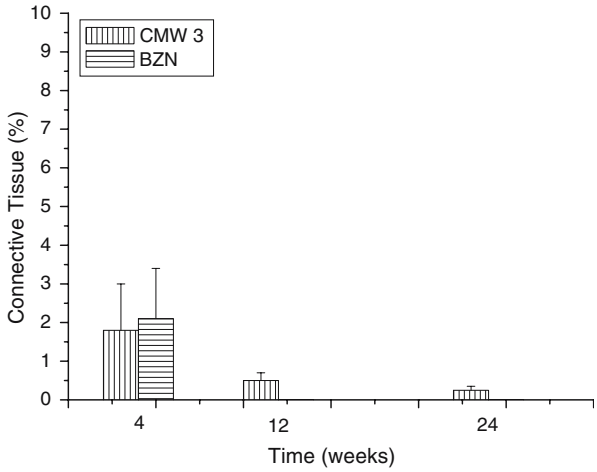

Fig. 6 Histomorphometric evolution of the connective tissue at the bone-cement interface with time for both types of cements

irregular interface, containing connective tissue with multinucleated giant cells and precursors of bone marrow haematopoietic cells (Fig. 3C and D).

Histomorphometric and statistical analysis

The histomorphometry of the biological reaction was carried out by quantifying the amount of cement, newly formed tissue, connective tissue and bone marrow at the bone-cement interface, and the evolution of each variable with time are represented separately in Figs. 4–7. The variable cement did not significantly change between 4 weeks and 24 weeks for both formulations, as expected due to the in vivo stability of the poly(methyl methacrylate) cement (Fig. 4). The newly formed bone (Fig. 5) presented a maximum amount at 4 weeks for both cements (33.9 ± 7.9% and 30.2 ± 9% for CMW 3 and BZN, respectively) and then it decreased slightly for CMW 3 up to 24 weeks (22 ± 3%), but for BZN cement it decreased at 12 weeks (22.7 ± 5%) and then recovered at 24 weeks (28 ± 16%). That is, there was a higher amount of neoformed bone for the BZN cement at 24 weeks, but no significant differences were observed when the periods of time for each cement and both groups were compared at any time. The histomorphometric results regarding the connective tissue at the bone-cement interface are shown in Fig. 6. The maximum amount of connective tissue was measured at 4 weeks (1.8 ± 1.8% for CMW 3 and 2.1 ± 1.4% for BZN cement) and showed a trend to decrease with time of implantation. The decrease was significant for BZN between 4 weeks and 12 weeks, and between 4 weeks and 24 weeks, and for CMW 3 cement

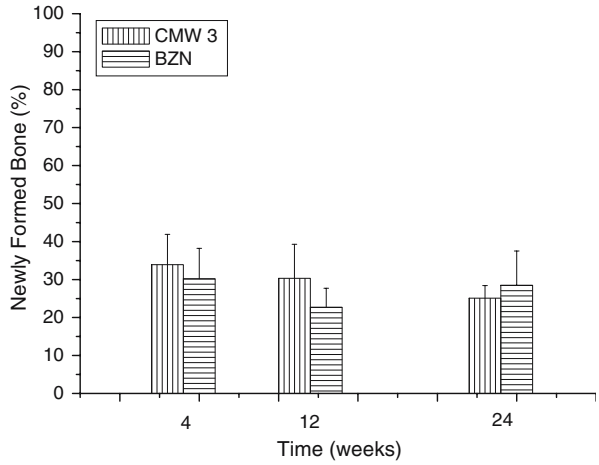

Fig. 5 Histomorphometric evolution of the newly formed bone at the bone-cement interface with time for both types of cements

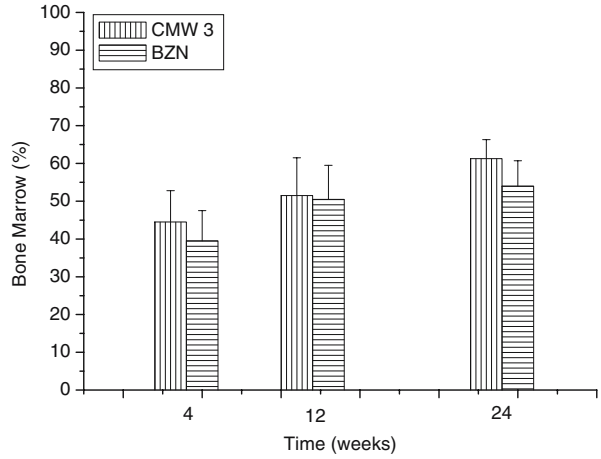

Fig. 7 Histomorphometric evolution of the bone marrow with time for both types of cements

between 12 weeks and 24 weeks, comparing the different periods of time within each formulation. Comparing both types of cements for each time, after 12 and 24 weeks of implantation, CMW 3 gave specimens with connective tissue values significantly greater (0.5 ± 0.2% and 0.3 ± 0.1%, respectively) than the specimens of the BZN group (0.01 ± 0% for both periods of time). The bone marrow presented a trend to increase during the whole period for both cements, filling the space left by decreases in the extend of the other measured variables (Fig. 7). The amount of bone marrow for CMW 3 ranged between 44.5 ± 8.3% (4 weeks) and 61.3 ± 6.8 (24 weeks), and for BZN cement between 39.5 ± 9.8% (4 weeks) and 54.0 ± 6.7 % (24 weeks). However, significant differences were not observed comparing the different periods among themselves for each formulation, and comparing both groups at any time. Figure 8 summarizes the evolution of the corresponding interface with time for both CMW 3 and BZN cements, respectively.

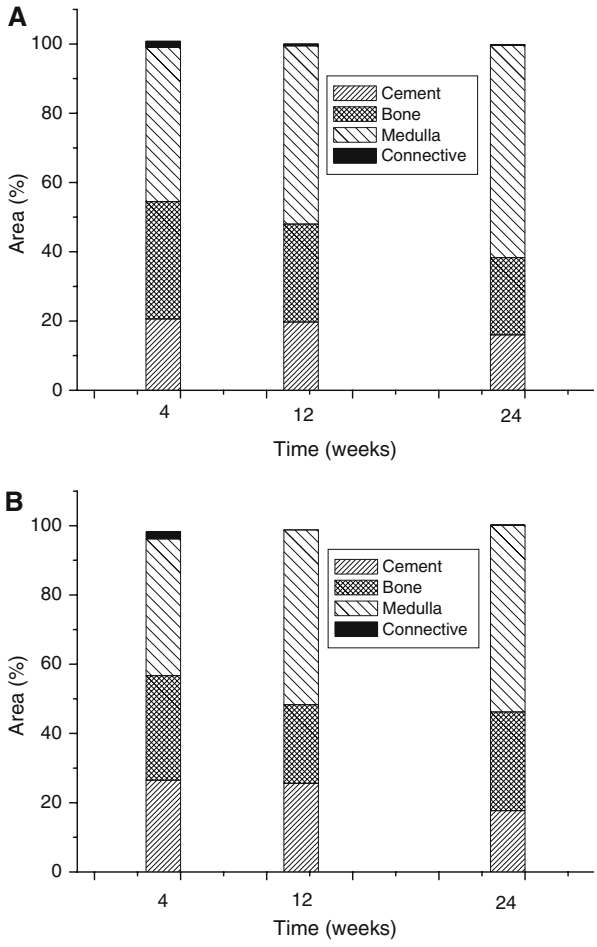

Fig. 8 Summary of the histomorphometric analysis of the tissues at the bone cement interface after different times of implantation. (**A**) CMW 3. (**B**) BZN cement

Discussion

After implantation of an acrylic bone cement the bone-cement interface contained debris particles, connective tissue, osteolysis and/or osteonecrosis, and bone trabeculae fractures which have been attributed to monomer toxicity, the high exothermicity during cement polymerisation and surgical trauma at less than 1 year of implantation [18]. Some studies in the rabbit tibia using the bone harvest chamber seem to indicate that other factors than monomer toxicity and heat trauma, such as the activator DMT, would result in a lasting poor biocompatibility of the acrylic bone cement [19]. In preliminary studies the use of BZN as activator of reduced toxicity in acrylic bone cements has been attempted providing successful results in terms of curing parameters and mechanical and biological properties [10]. The in vivo biocompatibility revealed a better osseous neoformation, more pronounced after 4 weeks post implantation, compared to CMW 3. Based on those preliminary results, the present work focused on the application of histomorphometric analysis to study the quantitative evaluation of tissue reaction at the bone-cement interface. This study was conducted avoiding the traditional acidic demineralisation of samples, which were embedded directly in methacrylate to have more reliable results.

The evolution of the bone-cement interface in both commercial and experimental cements followed the observations stated by Willert and Buchhorn [20], who classified the process in three overlapping stages, initial phase, repair and stabilisation. After 4 weeks of implantation, we can say that the system has entered in the phase of repair, in which fibroblasts grow into the damage areas and begin to replace the necrotic marrow, newly formed bone grows directly onto the surface of the implant, and between the areas of direct bone contact the implants are bordered by soft tissue [20]. In our study, at this time the bone-cement interface for both cements presented the highest amounts of neoformed bone and connective tissue. Histologically, some giant cells were still covering the bone cement in both cases. In the next period, at 12 weeks, for both cements, the neoformed bone suffered from a resorption, the amount of bone marrow increased with respect to the previous period, and the connective tissue decreased, but the latter variable was significantly lower for the BZN formulation with respect to the commercial one. At 24 weeks we can say that both systems are in the phase of stabilization, where the healing process finished with the establishment of the permanent implant bed. In general terms at this stage, the tissues at the interface

to the implant consist of bone trabeculae and membranes of connective tissue of varying thickness, and healthy haematopoietic and fatty bone marrow will be found in the marrow spaces close to cemented implants [20]. At this time, CMW 3 and BZN cements presented the highest amount of bone marrow with respect to previous periods, but the BZN formulation presented a slightly higher amount of neoformed bone and a relatively lower amount of connective tissue than the CMW 3 formulation. Histological findings revealed that the cements were bordered by multinucleated giant cells and macrophages describing exactly the contours of the implanted materials.

The reactions observed for the commercial formulation are consistent with those already described by several researchers. An intervening fibrous tissue layer has been reported between bone and CMW 3 cement at 12 weeks after the operation [21, 22]. Revell et al. [23] reported some CMW 3 results by comparison with those obtained from a novel cement formulated with high molecular weight monomers (PEMBMA) [24–26]. CMW 3 was separated from the surrounding marrow and osseous tissues by a fibrous membrane with a higher thickness than those observed in the PEMBMA cement, indicating that a lower exotherm and monomer release for PEMBMA cement do influence the final biocompatibility. Other in vivo studies performed with the cement called Boneloc®, that contains a combination of less toxic monomers (n-decyl methacrylate and isobornyl methacrylate), and activators (0.5 wt% DMT and 1.0 wt% of dihydroxypropyl-4-toluidine) showed an early bone remodelling activity [27]. The histomorphometric analysis carried out in this work on the substitution of only the activator by one presenting reduced toxicity and antiseptic properties indicated that this change has a slight but positive effect on the final biocompatibility of the system, giving rise to a relatively lower fibrosis at the interface in the long term. The way this finding can affect the survival rate of cemented arthroplasties should be investigated. Clinical studies will be the next step forward and will be carried out in the near future.

Conclusion

The histomorphometric analysis of an acrylic bone cement cured with *N*,*N*-dimethylamino benzydrol, an activator of reduced toxicity and antiseptic properties, provided a rather similar biological response compared to that of the commercial CMW 3, but with a relatively lower amount of connective tissue at the bone-cement interface in the long term. Clinical studies should be carried out in order to determine the influence of this finding on aseptic loosening of cemented prosthesis in service life.

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