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Biomedical Materials

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Reduced interleukin-6 immunoexpression and birefringent collagen formation indicate that MTA Plus and MTA Fillapex are biocompatible

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Abstract

Considering that endodontic sealers release some components which may promote delay in the repair process, our purpose was to evaluate the tissue reaction promoted by MTA Plus and MTA Fillapex in comparison with AH Plus (standard control) and Endofill, which has a long clinical track record. One hundred rats were distributed into five groups: MTA Plus (Avalon Biom Inc., Bradenton, FL, USA), MTA Fillapex (Angelus, Londrina, PR, Brazil), AH Plus (Dentsply DeTrey GmbH, Konstanz, Germany), Endofill (Dentsply, Petrópolis, RJ, Brazil) and CG (control group, empty polyethylene tubes). The polyethylene tubes filled with sealers or empty (CG) were implanted into subcutaneous. After 7, 15, 30 and 60 days, the tubes surrounded by capsules were paraffin-embedded. In HE-stained sections, the volume density of inflammatory cells (VvIC) was estimated in the capsules. The number of interleukin-6-immunolabelled cells (IL-6), a pro-inflammatory cytokine, was also computed in the capsules. The birefringent collagen content was quantified in picrosirius-stained sections. Data were analysed by ANOVA and Tukey tests ($p \leqslant 0.05$). At 7 days, the capsules showed moderate inflammatory reaction. In all groups, VvIC and IL-6-immunostained cells reduced significantly from 7 to 60 days. At 60 days, IL-6 immunoexpression was reduced significantly in MTA Plus and MTA Fillapex in comparison with AH Plus; no difference was found in the VvIC among MTA Plus, MTA Fillapex, AH Plus and CG whereas Endofill exhibited the highest VvIC. The reduction in VvIC was parallel to an increase in the collagen in all the groups, except Endofill. MTA Plus, MTA Fillapex and AH Plus induce a response that culminates in the regression of inflammation and formation of a fibrous capsule over time. The lower IL-6 immunoexpression in the capsules of MTA Plus and MTA Fillapex than AH Plus suggests that the immune response is suppressed more rapidly in the MTA-based sealers.

Introduction

A proper root canal sealer should provide a complete sealing and stimulate the periodontal tissue repair [1–3]. Most endodontic sealers have shown limited capacity to stimulate periapical tissue repair and/or have unsatisfactory physical and chemical properties. Thus, several studies have been performed in attempt to improve the physicochemical and biological properties of the root canal sealers [3–5] and new materials have been developed.

MTA (Mineral Trioxide Aggregate, Angelus, Londrina, PR, Brazil), a tricalcium silicate-based material, has been widely used as reparative cement in cases of root perforation [6, 7], pulpotomies [8] and as a root-end filling material [9] due to its biological properties [4, 10, 11]. The calcium hydroxide, which is produced as a reaction product of the MTA during its hydration [12], may be responsible for bioactivity of this material [3, 10, 13]. However, MTA does not have proper flow, and has no ability to be used as root filling canal sealer [4, 5, 9]. Considering the physicochemical

properties and its biocompatibility, MTA has been altered to provide viscosity necessary to endodontic sealers [4, 5, 14]. For this reason, new calcium silicate-based materials have been developed such as MTA Fillapex (Angelus) and MTA Plus (Avalon).

MTA Fillapex was manufactured from the mineral trioxide aggregate with addition of natural resin, salicylate resin, bismuth oxide and silica nanoparticles [14]. Although MTA Fillapex is a tricalcium silicate-based material, the calcium ions releasing is low compared with MTA and calcium hydroxide has been not detected during its hydration [3, 15]. Some studies have reported that the MTA Fillapex exhibits biocompatibility [16] and bioactivity [13] whereas other studies have demonstrated that this material is highly cytotoxic [17–19] and induces inflammatory reaction for prolonged time [20, 21]. On the other hand, there is evidence that the cytotoxicity of the MTA Fillapex is high in the initial periods, but reduces over time [13, 22]. In addition, the cytotoxicity of MTA Fillapex depends directly on its concentration [19].

According to the manufacturer, MTA Plus (Avalon) contains smaller particles of calcium silicate than MTA and it is provided either with water or an anti-washout gel ampoules for mixing [12, 23]. The mixture with salt-free water-soluble gel allows the use this material as a root filling sealer. There is evidence that MTA Plus releases calcium ions when immersed in water or Hank's saline solution [12]. In addition, the hydration of MTA Plus originates calcium hydroxide [12], suggesting that this material may induce the formation of calcified deposits. MTA Plus in contact with human osteoblastic cells stimulates cell proliferation, migration and the formation of mineralised nodules [5]. Moreover, this sealer stimulates the alkaline phosphatase expression by human osteoblastic cells [5] and human dental pulp cells [24] indicating the potential differentiation of these cells. Although some studies have demonstrated that the MTA Plus exhibits low cytotoxicity and bioactivity, *in vivo* investigation could contribute for the better comprehension of the tissular response to this endodontic sealer.

The host response to the root canal sealers plays an important role in the outcome of the endodontic therapy, since the cells produce growth factors, cytokines and chemokines, which control the tissue healing process. It is expected that a biocompatible material may stimulate the host cells to release cytokines and growth factors, promoting the regression of the inflammatory reaction initially formed [11, 20, 25, 26] and allowing the tissues to recover their structural organisation [7, 26, 27]. Connective tissue repair involves several overlapping cellular and molecular events which culminate in the fibroblast proliferation and collagen formation [26, 28].

Considering that the components released by root filling sealers may induce extensive cellular damage and subsequent delay in the repair process, the aim of the present study was to evaluate the tissue reaction promoted by MTA Plus and MTA Fillapex in the subcutaneous. Moreover, these findings were compared with AH Plus, used as standard control, and Endofill, which has a long clinical track record. To evaluate the tissue reaction, the volume density of inflammatory cells and the interleukin-6 (IL-6) immunoexpression, a pro-inflammatory cytokine, were analysed in the capsules at different time points. Moreover, whether the endodontic sealers allow the formation of a fibrous capsule over time was also evaluated.

Materials and methods

Experimental procedures

One hundred adult male Holtzman rats (*Rattus norvegicus albinus*) weighing 220–250 g were used. The rats were individually housed in stainless steel cages with controlled temperature (23 ± 2 °C) and humidity (55 ± 10%) on a 12 h light/dark cycle with light onset at 7:00 am. The rats received water and food (Guabi rat chow, Paulínia, SP, Brazil) *ad libitum*. The animal care and the experimental procedures were conducted in accordance with Brazilian national law on the animal use. The Ethical Committee for Animal Research of Araraquara Dental School (São Paulo State University—UNESP, Brazil) approved the protocol regarding the animal use and experimental procedures.

The animals were distributed into five groups containing 20 animals each: MTA Plus (Avalon Biom Inc., Bradenton, FL, USA), MTA Fillapex (Angelus, Londrina, PR, Brazil), AH Plus (Dentsply DeTrey GmbH, Konstanz, Germany), Endofill (Dentsply, Petrópolis, RJ, Brazil) and CG (control group, empty polyethylene tubes). The materials were mixed according to manufacturer's recommendations under aseptic conditions. MTA Plus was prepared with vehicle for root canal filling (1 g:350 μl). MTA Fillapex and AH Plus were prepared mixing equal quantity of the pastes whereas the Endofill was prepared by mixing zinc oxide and liquid (1 g:220 μl). The chemical components of each endodontic sealer are described in the table 1.

One hundred polyethylene tubes (Embramed Indústria Comércio, São Paulo, SP, Brazil) with 10.0 mm length and 1.6 mm diameter, previously sterilised with ethylene oxide, were used. After mixing, the tubes were filled with the materials and immediately implanted into the dorsal subcutaneous tissues.

The animals were anaesthetised with intraperitoneal injection of ketamine (80 mg kg^{-1} of body weight) combined with xylazine hydrochloride (8 mg kg^{-1} of body weight). After shaving and disinfection, a 2.0 cm long incision was performed, and one polyethylene tube was placed into the subcutaneous pocket. After 7, 15, 30 and 60 days of implantation (n = 5 per group in each period), the rats were euthanized with overdose of ketamine and the tubes

Table 1. Composition of the root canal sealer.

Root Canal sealer	Components
MTA Plus	Powder: tricalcium silicate, dicalcium silicate, bismuth oxide, calcium sulphate, silica Gel: salt-free water-soluble gel
MTA Fillapex	Salicylate resin; diluting resin; natural resin; bismuth oxide; nanoparticulated silica; mineral trioxide aggregate (MTA) and pigments
AH Plus	Paste A: epoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments, aerosol Paste B: adamantane amine, N,N-dibenzyl-5-oxanonane, TCD-diamine, calcium tungstate, zirconium oxide, aerosol, silicone oil
Endofill	Powder: zinc oxide, resin (Staybelite), bismuth subcarbonate, barium sulphate and sodium borate Liquid: eugenol and almond oil

surrounded by connective tissue were removed and, immediately, fixed for histological procedures.

Histological procedures

After fixation for 48 h in 4% formaldehyde at pH 7.2 buffered with 0.1 M sodium phosphate at room temperature, the specimens were dehydrated and embedded in paraffin. From each implant, 60 longitudinal sections (6 μm thick) were carried out; five non-serial sections were stained with Carazzi's haematoxylin and eosin (HE) for morphological analysis of the capsules adjacent to the opening of the tubes containing the material. In addition, the HE-stained sections were used for obtaining the volume density of the inflammatory cells (VvIC) and fibroblasts (VvFb). Sections were submitted to the picrosirius-red method and the birefringent collagen content was estimated. Sections adhered to silanized slides were submitted to immunohistochemistry for detection of IL-6 and the number of immunolabelled cells in the capsules was also computed.

Volume density of inflammatory cells (VvIC) and fibroblasts (VvFb)

Volume density (%) was undertaken using an Olympus camera (DP-71, Olympus, Tokyo, Japan) attached to a light microscope (BX51, Olympus) and an image analysis system (Image Pro-Express 6.0, Olympus), as previously described [29]. In five implants from each group per time point, three HE-stained non-serial sections were captured at x695 magnification. Using the image analysis system, a standardised reticular grid containing 204 intersections/points was superimposed on each image. The number of points on the inflammatory cells (neutrophils, lymphocytes, plasma cells and macrophages) and fibroblasts was computed; blood vessels, extracellular spaces, collagen fibres and particles of material in the capsules were computed as other structures (VvO). The VvIC and VvFb were performed by one calibrated and blinded examiner.

Measurement of capsule thickness

In each animal, three HE-stained non-serial sections were used, totalling 15 sections per group/period. In each section, the capsule was captured using a camera (DP71, Olympus) attached to a light microscope (BX51, Olympus) at x65 magnification. In the central portion, the thickness of the capsule was estimated using an image analysis system (Image-Pro Express 6.0, Olympus). Thus, the mean value of capsule thickness/animal and per group was obtained. The measurements were performed by one calibrated and blinded examiner.

Immunohistochemical detection of IL-6

Sections adhered to silanized slides were subjected to immunohistochemistry for detection of IL-6. For antigen retrieval, the deparaffinized sections were immersed in 0.001 M sodium citrate buffer pH 6.0, and heated in a microwave oven for 20 min at 90 °C–94 °C. After a cooling-off period, the endogenous peroxidase was inactivated with 5% hydrogen peroxide for 20 min. The sections were washed with 0.1 M saline-phosphate buffer (PBS) at pH 7.2 followed by incubation for 30 min in 2% bovine-serum-albumin (Sigma-Aldrich Chemie, Munich, Germany) at room temperature. Then, the sections were incubated in a humidified chamber at 4 °C for 16–18 h with goat anti-IL-6 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was diluted at 1:100. After washing in PBS, the immunoreactions were detected by the Labelled StreptAvidin-Biotin system (LSAB-plus Kit; Dako, Carpinteria, CA, USA). The sections were incubated for 30 min at room temperature with multi-link solution containing biotinylated mouse/rabbit/goat antibodies, washed in PBS and incubated with streptavidin-peroxidase complex for 30 min at room temperature. The peroxidase activity was revealed by 3,3′-diaminobenzidine (Dako, Carpinteria, CA, USA) for 2–3 min; the sections were counterstained with haematoxylin. As negative control, sections were incubated with non-immune serum (Sigma-Aldrich Chemie, Munich, Germany) in place of primary antibody.

Numerical density of IL-6-immunolabelled cells

The numerical density of immunolabelled cells was estimated in all specimens (implants), as previously

described [10, 11, 25]. In each section, a standardised area (0.09 mm^2) was captured using an Olympus camera (DP-71, Olympus) attached to a light microscope (BX51, Olympus) at x695 magnification. The number of immunolabelled cells (brown-yellow colour) was computed using an image analysis system (Image Pro-Express 6.0, Olympus). For each implanted tube, the immunolabelled cells (IL-6-positive cells) were counted in the standardised area (0.09 mm^2) of the capsule by one blinded and calibrated examiner. The quantification was carried out twice, with intervals of at least 8 weeks. Thus, the number of IL-6-immunolabelled cells per mm^2 of capsule per animal was obtained [10, 11, 25].

Analysis of collagen under polarised light microscopy

For the collagen content estimation in the capsules, the sections were stained with 0.1% picrosirius-red solution and analysed under light microscope (BX51, Olympus, Tokyo, Japan) equipped with filters to provide polarised illumination [26, 30]. In each specimen, three non-serial sections stained with picrosirius-red were used, totalling 15 sections per group in each period. In each section, a standardised area (0.09 mm^2) of the capsule was obtained at x40, and a total area of 0.27 mm^2 was evaluated per implant.

Birefringent collagen content was estimated using ImageJ® image analysis software (http://rsbweb.nih.gov/ij) following the methodology performed by Koshimizu *et al* [30]. The data of birefringent collagen (red, orange, yellow and green colours) was determined and expressed as percentage of the total number of collagen pixels which in turn was expressed as a percentage of the total number of pixels in the image.

Statistical analysis

Differences among the groups in each period and the differences of each group over time were evaluated by two-way ANOVA analysis and Tukey post-test (GraphPad Prism 5.0 software, San Diego, CA, USA) at significance level of $p \leqslant 0.05$. All data were presented as mean and standard deviation.

Results

Morphological and quantitative findings

At 7 days, the implants in all the groups were surrounded by thick capsules containing around 15%–16% of inflammatory cells, mainly lymphocytes, plasma cells and macrophages (figures 1(A)–(J); table 2). Moreover, some fibroblasts, blood vessels and occasional multinucleated giant cells were also observed in the capsules (figures 1(F)–(J)). Inflammatory reaction was not seen in the adjacent tissues to these well-defined capsules (figures 1(A)–(E)).

In the period of 15 days, an evident difference in the distribution and arrangement of the cell types was observed in the capsules of MTA Plus and MTA Fillapex in comparison to the other groups (figures 2(A)–(E)). The frequency of inflammatory cells in the capsules of MTA Plus and MTA Fillapex was 7.2% and 6.0% while in the AH Plus and Endofill was 10.1% and 11.9%, respectively (table 2). Moreover, in the innermost portion of the capsules in contact with MTA Plus and MTA Fillapex contained predominantly fibroblasts (figures 2(A) and (B)). Otherwise, in AH Plus, Endofill and control group, the inflammatory cells and fibroblasts were distributed throughout the capsules (figures 2(C)–(E)).

At 30 days (figures 2(F)–(J)), the frequency of inflammatory cells was 4.5%, 6.6%, 5.2%, 6.3% and 4.0% in the MTA Plus, MTA Fillapex, AH Plus, Endofill and Control groups respectively (table 2). After 60 days of implantation (figures 3(A)–(J)), the thin capsules were formed by fibrous connective tissue, except in the Endofill (figures 3(D) and (I)). The capsules showed fibroblasts/fibrocytes between bundles of collagen fibres; few inflammatory cells were found in the capsules of these groups (figures 3(F))–(H) and (J)). On the other hand, a moderate inflammatory reaction exhibiting macrophages and plasma cells was still observed in the capsules adjacent to Endofill (figure 3(I)). The quantitative analysis revealed that the frequency of inflammatory cells in the capsules of MTA Plus, MTA Fillapex, AH Plus and Control groups varied from 3.0% to 4.3% whereas in the Endofill was 8.8% (table 2).

Volume density (%) of inflammatory cells (VvIC) and fibroblasts (VvFb)

According to table 2, the highest VvIC was verified in the capsules of all the groups at 7 days. In this period, significant differences in the VvIC among the groups were not detected ($p \geqslant 0.05$). In the capsules of all groups, a significant reduction in the VvIC was observed from 7 to 15 days. After 15 days, the lowest values of VvIC were observed in the MTA Plus, MTA Fillapex and control group. Significant differences were not detected between AH Plus and Endofill ($p = 0.5558$), which exhibited the highest values of VvIC. However, the capsules of AH Plus and Endofill showed significant reduction in the VvIC from the 15 to 30 days ($p = 0.0006$; $p < 0.0001$, respectively). Significant differences among the groups were not found after 30 days. At 60 days, significant differences were not detected in the VvIC between the capsules of MTA Plus ($p > 0.9999$), MTA Fillapex ($p = 0.9445$), AH Plus ($p = 0.9975$) with the control group. In contraposition, the VvIC was significantly higher in the capsules of Endofill than control group ($p = 0.0007$); in the Endofill, the VvIC increased two-fold in comparison with the other groups.

Regarding the VvFb (table 2), significant differences between MTA Plus ($p = 0.9922$), MTA Fillapex ($p = 0.6023$), AH Plus ($p = 0.8527$) with control group were not detected at 7 days. In these groups, the capsules exhibited the lowest number of fibroblasts. At 15 and 30

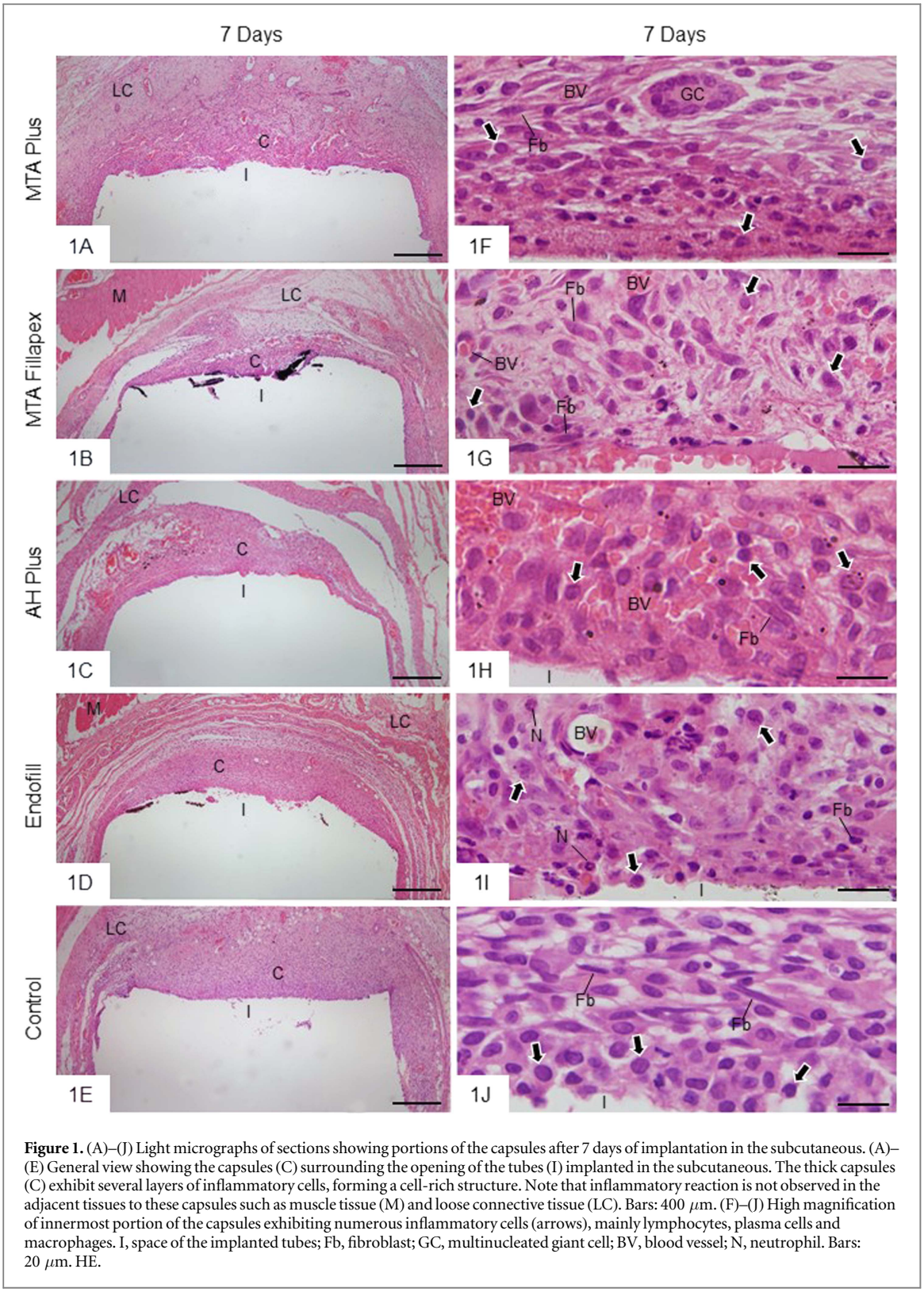

Figure 1. (A)–(J) Light micrographs of sections showing portions of the capsules after 7 days of implantation in the subcutaneous. (A)–(E) General view showing the capsules (C) surrounding the opening of the tubes (I) implanted in the subcutaneous. The thick capsules (C) exhibit several layers of inflammatory cells, forming a cell-rich structure. Note that inflammatory reaction is not observed in the adjacent tissues to these capsules such as muscle tissue (M) and loose connective tissue (LC). Bars: 400 μm. (F)–(J) High magnification of innermost portion of the capsules exhibiting numerous inflammatory cells (arrows), mainly lymphocytes, plasma cells and macrophages. I, space of the implanted tubes; Fb, fibroblast; GC, multinucleated giant cell; BV, blood vessel; N, neutrophil. Bars: 20 μm. HE.

days, statistical differences in the VvFb were not observed among the groups ($p > 0.05$). However, at 60 days, the VvFb was significantly lower in the capsules of MTA Fillapex than MTA Plus ($p = 0.0032$), Endofill ($p = 0.0216$) and control group ($p = 0.0271$). According to table 2, none statistical difference ($p > 0.05$) in the VvFb was detected in the capsules of MTA Plus and control group over time. In the capsules of MTA Fillapex, a significant increase in the incidence of fibroblasts was observed from 7 to 15 days ($p = 0.0014$) whereas in the AH Plus and Endofill the highest VvFb was found at 30 and 15 days, respectively.

Measurement of capsule thickness

According to table 3, a significant reduction in the thickness of the capsules was observed in all the groups

Table 2. Volume density (%) of inflammatory cells (VvIC), fibroblasts (VvFb) and other (VvO) in the capsules from the MTA Plus, MTA Fillapex, AH Plus, Endofill and control (empty tubes) groups after 7, 15, 30 and 60 days.

		MTA Plus	MTA Fillapex	AH Plus	Endofill	Control
7 days	VvIC	15.4 (1.0)^{a,1}	16.2 (3.1)^{a,1}	15.2 (2.7)^{a,1}	16.0 (2.7)^{a,1}	15.6 (3.6)^{a,1}
	VvFb	8.9 (1.5)^{a,1}	6.8 (1.1)^{a,1,3}	7.7 (1.8)^{a,1}	12.4 (0.9)^{b,1,2}	9.5 (2.1)^{a,b,1}
	VvO	75.7 (2.4)	76.9 (1.2)	77.1 (1.8)	71.5 (2.1)	74.8 (2.1)
15 days	VvIC	7.2 (1.2)^{a,b,2}	6.0 (2.0)^{a,2}	10.1 (2.9)^{b,c,2}	11.9 (1.5)^{c,2}	5.2 (0.9)^{a,2}
	VvFb	11.8 (2.2)^{a,1}	14.6 (4.4)^{a,2}	11.9 (5.0)^{a,1,2}	15.8 (4.4)^{a,1}	12.7 (3.1)^{a,1}
	VvO	81.0 (2.7)	79.3 (2.4)	77.9 (3.9)	72.2 (3.8)	82.0 (2.9)
30 days	VvIC	4.5 (1.1)^{a,2,3}	6.6 (2.1)^{a,2}	5.2 (0.8)^{a,3}	6.3 (1.6)^{a,3}	4.0 (1.1)^{a,2}
	VvFb	13.0 (4.4)^{a,1}	10.7 (1.6)^{a,1,2}	14.1 (3.7)^{a,2}	10.2 (1.6)^{a,2}	13.6 (5.0)^{a,1}
	VvO	82.4 (2.5)	82.6 (2.7)	80.6 (2.5)	83.3 (1.2)	82.3 (2.0)
60 days	VvIC	3.8 (0.9)^{a,3}	3.0 (0.5)^{a,3}	4.3 (1.2)^{a,3}	8.8 (0.6)^{b,3}	3.9 (0.6)^{a,2}
	VvFb	9.2 (0.7)^{a,1}	5.2 (0.9)^{b,3}	8.1 (2.3)^{a,b,1}	8.8 (2.3)^{a,2}	9.1 (2.3)^{a,1}
	VvO	87.0 (1.0)	91.8 (0.9)	87.5 (1.8)	82.3 (1.4)	86.9 (1.2)

Note. Mean (standard deviation). The comparison between groups in the same period is indicated by superscript letters in the lines, same letters = no statistically significant difference. The comparison between periods in the same group is indicated by superscript numbers in the columns; same numbers = no statistically significant difference. Tukey test ($p \leqslant 0.05$).

over time. In all periods, the capsules of the control group were thinner than the other groups ($p \leqslant 0.024$). At 7 days, the capsules of MTA Plus were thinner than MTA Fillapex ($p = 0.011$), AH Plus ($p < 0.0001$) and Endofill ($p < 0001$) while significant differences were not found between AH Plus and Endofill ($p = 0.265$). Significant differences were not observed between the capsules of MTA Plus and AH Plus ($p = 0.312$) as well as MTA Fillapex and Endofill ($p = 0.798$) at 15 days. After 30 days, significant differences were not detected among MTA Plus, MTA Fillapex and AH Plus ($p \geqslant 0.516$) while the thickness of the capsules of the Endofill was higher than the other groups ($p \leqslant 0.0007$). At 60 days, the thickness of the capsules of MTA Plus was significantly reduced in comparison with Endofill ($p = 0.017$) while significant differences were not observed among MTA Fillapex, AH Plus and Endofill ($p \geqslant 0.250$).

Numerical density of IL-6-immunolabelled cells

In the sections submitted to immunohistochemistry for IL-6 detection, immunolabelled cells (brown-yellow colour) were observed in the capsules of all the groups (figures 4(A)–(J)). At 7 days, several inflammatory cells (lymphocytes, plasma cells and macrophages), fibroblasts and endothelial cells of the blood vessels exhibited strong cytoplasmic immunolabelling (figures 4(A)–(E)). On the other hand, in all the groups, occasional inflammatory cells and fibroblasts exhibiting weak immunolabelling were observed at 60 days (figures 4(F)–(J)), except in Endofill (figure 4(I)). In this group, evident immunolabelling was observed in several inflammatory cells and fibroblasts. Immunolabelled cells were not found in the negative controls (data not shown).

The quantitative analysis (table 3) revealed that, in the period of 7 days, the number of immunolabelled cells was significantly lower in the capsules of MTA Plus than in MTA Fillapex ($p = 0.0004$), AH Plus ($p < 0.0001$), Endofill ($p < 0.0001$) and control group ($p < 0.0001$). Otherwise, the highest number of immunolabelled cells was observed in the capsules of AH Plus and Endofill. At 15 and 30 days, significant differences were not detected among MTA Plus, MTA Fillapex and AH Plus ($p > 0.05$). From 30 to 60 days, significant reduction in the number of immunostained cells was observed in the capsules of MTA Plus ($p < 0.0001$), MTA Fillapex ($p = 0.0269$) and Endofill ($p = 0.0302$). After 60 days, a significant reduction in the immunoexpression was found in the capsules of MTA Plus and MTA Fillapex in comparison with AH Plus ($p = 0.0035$; $p = 0.0192$, respectively) and Endofill ($p = 0.0005$; $p = 0.0015$, respectively). At 15, 30 and 60 days the lowest values of immunolabelled cells were observed in the capsules of control group whereas the highest values were detected in the capsules of Endofill.

Birefringent collagen content

At 7 days, the capsules contained few birefringent collagen content (figures 5(A)–(E)) whereas, in the period of 60 days, an enhanced birefringent collagen content was observed in all the groups (figures 5(F)–(J)). According to table 3, the lowest birefringent collagen content was detected in the initial period. From 7 to 15 days, a significant increase ($p < 0.0001$) in the collagen content was detected in the capsules of all groups. At 15 and 30 days, the highest birefringent content was observed in the capsules of MTA Plus and AH Plus. At 15 days, significant differences were not detected between MTA Plus and control group ($p = 0.9095$); significant differences were not observed between AH Plus and control group in the time point of 30 days ($p = 0.4122$). From 30 to 60

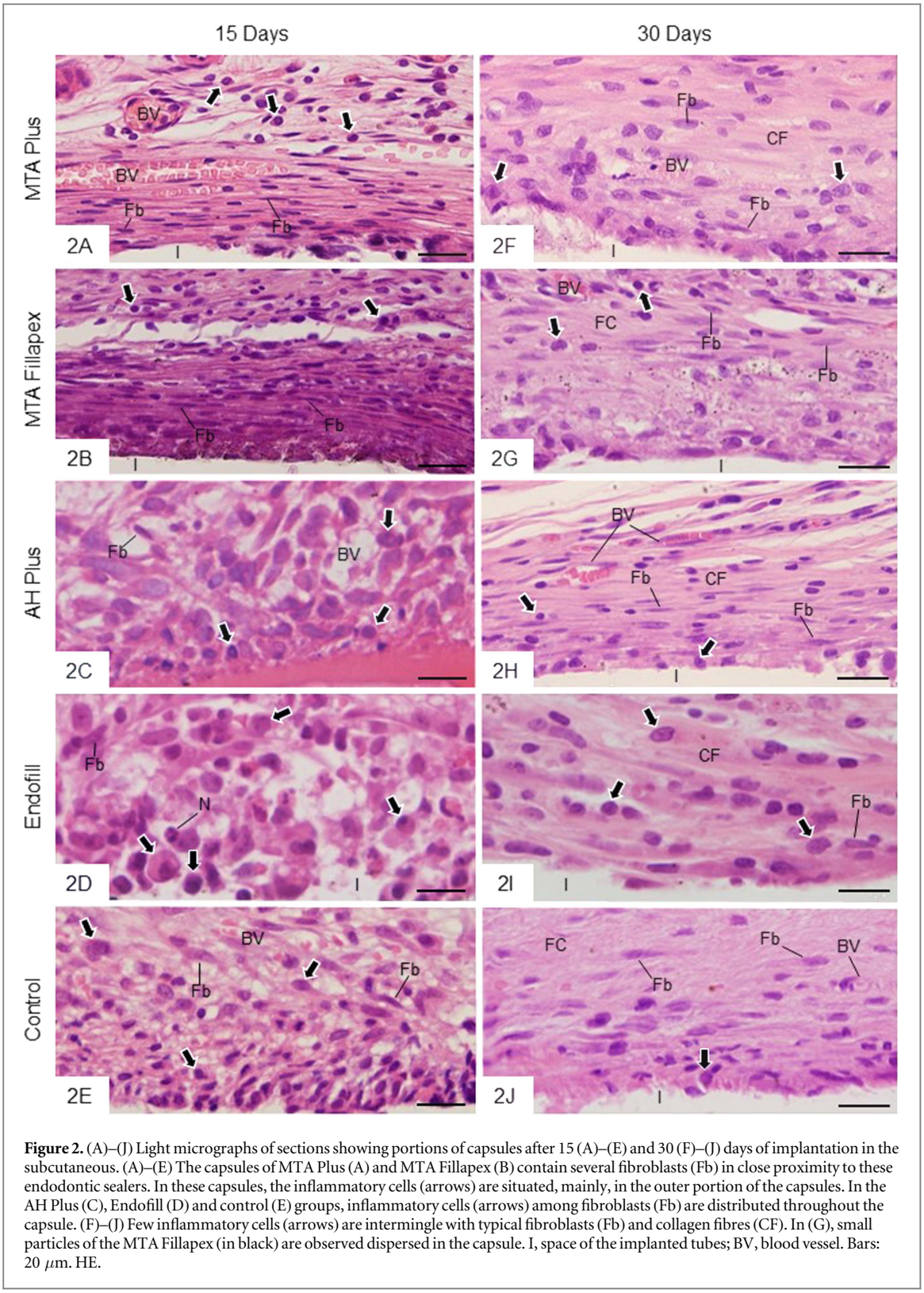

Figure 2. (A)–(J) Light micrographs of sections showing portions of capsules after 15 (A)–(E) and 30 (F)–(J) days of implantation in the subcutaneous. (A)–(E) The capsules of MTA Plus (A) and MTA Fillapex (B) contain several fibroblasts (Fb) in close proximity to these endodontic sealers. In these capsules, the inflammatory cells (arrows) are situated, mainly, in the outer portion of the capsules. In the AH Plus (C), Endofill (D) and control (E) groups, inflammatory cells (arrows) among fibroblasts (Fb) are distributed throughout the capsule. (F)–(J) Few inflammatory cells (arrows) are intermingle with typical fibroblasts (Fb) and collagen fibres (CF). In (G), small particles of the MTA Fillapex (in black) are observed dispersed in the capsule. I, space of the implanted tubes; BV, blood vessel. Bars: 20 μm. HE.

days, a significant increase in the collagen content was detected in the capsules of MTA Plus ($p = 0.0002$) and MTA Fillapex ($p < 0.0001$). In the period of 60 days, the collagen content was similar in MTA Plus ($p = 0.3664$), MTA Fillapex ($p = 0.9987$) and AH Plus ($p = 0.5268$) when compared to control group. Otherwise, the collagen content was significantly lower in the capsules of Endofill than in MTA Plus ($p < 0.0001$), MTA Fillapex ($p = 0.0001$), AH Plus ($p < 0.0001$) and control group ($p = 0.0003$).

Discussion

Implantation into subcutaneous is a recommended method by ISO 10993 [31] to evaluate the biocompatibility

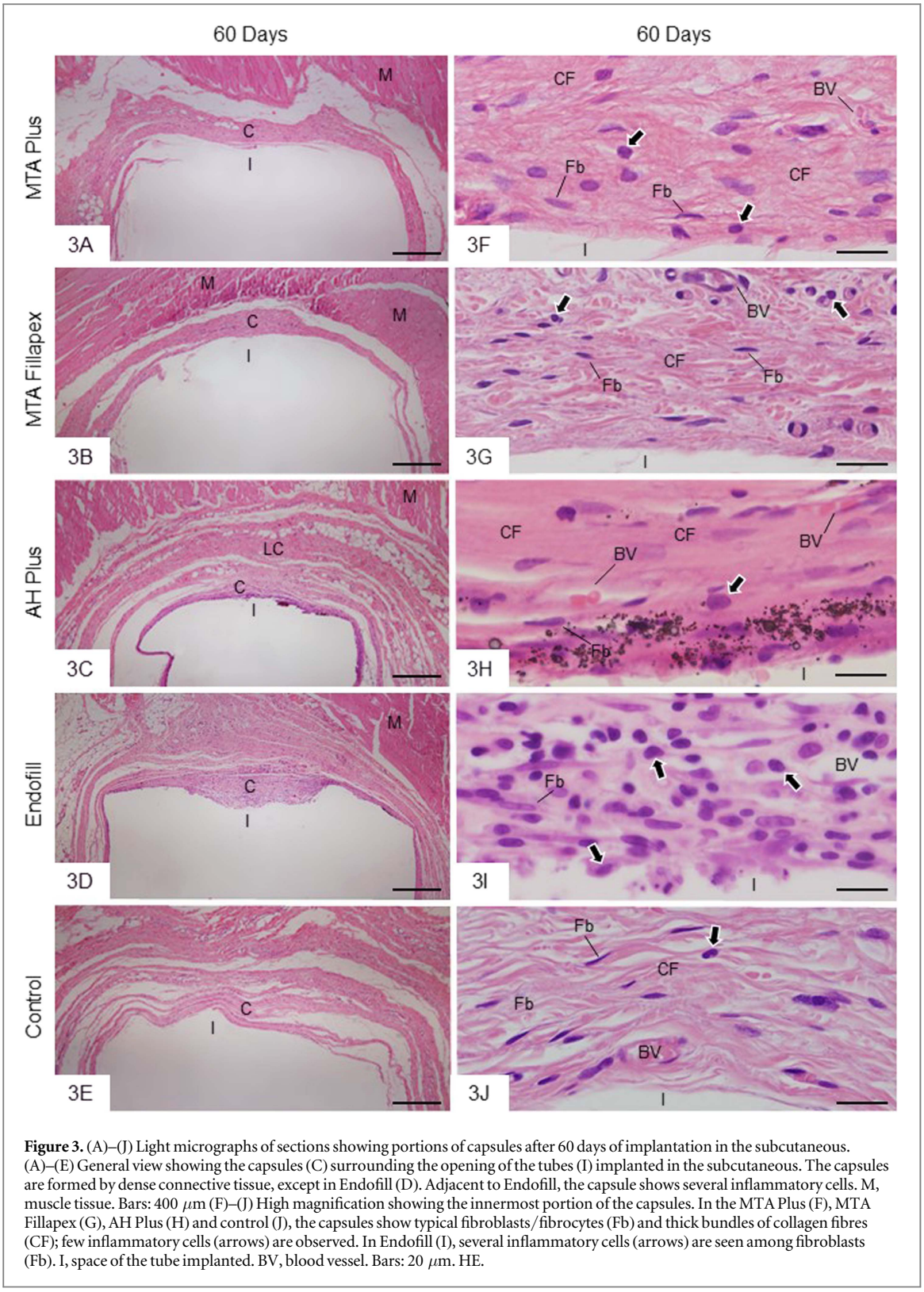

Figure 3. (A)–(J) Light micrographs of sections showing portions of capsules after 60 days of implantation in the subcutaneous. (A)–(E) General view showing the capsules (C) surrounding the opening of the tubes (I) implanted in the subcutaneous. The capsules are formed by dense connective tissue, except in Endofill (D). Adjacent to Endofill, the capsule shows several inflammatory cells. M, muscle tissue. Bars: 400 μm (F)–(J) High magnification showing the innermost portion of the capsules. In the MTA Plus (F), MTA Fillapex (G), AH Plus (H) and control (J), the capsules show typical fibroblasts/fibrocytes (Fb) and thick bundles of collagen fibres (CF); few inflammatory cells (arrows) are observed. In Endofill (I), several inflammatory cells (arrows) are seen among fibroblasts (Fb). I, space of the tube implanted. BV, blood vessel. Bars: 20 μm. HE.

of dental materials and, therefore, it has been widely used [4, 10, 11, 20, 25, 32, 33]. The use of polyethylene tubes prevents the diffusion of the material, besides simulating the situation in the filling root canal [32, 33]. In the present study, although the highest number of inflammatory cells was observed in the capsules adjacent to all materials at 7 days, the root canal sealers exhibited low irritant effect since no difference was detected between the capsules adjacent to the endodontic materials and the control group (empty tube) at 7 days. At 60 days, the morphological analysis revealed that MTA-based and AH Plus root filling sealers were surrounded by capsules exhibiting fibroblasts and scarce inflammatory cells. The quantitative results clearly showed that MTA Plus, MTA Fillapex and AH Plus exhibit favourable biological response since low number of inflammatory cells was verified in the capsules

Table 3. Capsule thickness (μm), number of IL-6-immunostained cells per mm^2 and birefringent collagen (Col) content (%) in the capsules from the MTA Plus, MTA Fillapex, AH Plus, Endofill and control (empty tubes) groups after 7, 15, 30 and 60 days.

		MTA Plus	MTA Fillapex	AH Plus	Endofill	Control
7 days	Capsule	371 (74)^{a,1}	477 (39)^{b,1}	597 (61)^{c,1}	661 (83)^{c,1}	274 (41)^{d,1}
	IL-6	562 (27)^{a,1}	769 (102)^{b,1}	1013 (106)^{c,1}	931 (111)^{c,d,1}	840 (167)^{b,d,1}
	Col	5.8 (1.4)^{a,1}	4.6 (1.5)^{a,1}	5.3 (1.6)^{a,1}	3.6 (1.9)$^{a,.1}$	6.1 (1.5)^{a,1}
15 days	Capsule	302 (57)^{a,1}	455 (44)^{b,1}	363 (49)^{a,2}	490 (63)^{b,2}	129 (21)^{c,2}
	IL-6	607 (57)^{a,1}	662 (71)^{a,1}	600 (54)^{a,2}	800 (100)^{b,2}	342 (52)^{c,2}
	Col	19.9 (1.6)^{a,2}	9.7 (2.2)^{b,2}	17.0 (1.2)^{c,2}	9.1 (2.1)^{b,2}	19.2 (0.5)^{a,c,2}
30 days	Capsule	210 (19)^{a,2}	257 (69)^{a,2}	260 (47)^{a,3}	392 (85)^{b,3}	108 (15)^{c,3}
	IL-6	569 (104)^{a,1}	520 (42)^{a,2}	598 (68)^{a,2}	744 (57)^{b,2}	182 (54)^{c,3}
	Col	21.2 (1.4)^{a,2}	17.1 (0.5)^{b,3}	22.8 (1.7)^{a,c,3}	20.4 (0.7)^{c,3}	24.4 (1.3)^{c,3}
60 days	Capsule	158 (17)^{a,2}	193 (15)^{a,b,2}	221 (39)^{a,b,3}	258 (29)^{b,4}	62 (8)^{c,3}
	IL-6	302 (31)^{a,2}	378 (44)^{a,3}	489 (18)^{b,2}	604 (26)^{c,3}	171 (32)^{d,3}
	Col	25.1 (0.9)^{a,3}	23.7 (0.8)^{a,4}	24.9 (1.0)^{a,3}	19.5 (1.1)^{b,3}	23.4 (1.7)^{a,3}

Note. Mean (standard deviation). The comparison between groups in the same period is indicated by superscript letters in the lines, same letters = no statistically significant difference. The comparison between periods in the same group is indicated by superscript numbers in the columns; same numbers = no statistically significant difference.Tukey test ($p \leqslant 0.05$).

at 60 days. Moreover, at 60 days, the inflammatory reaction was similar in the capsules adjacent to MTA Plus, MTA Fillapex and AH Plus sealers when compared with control group. Thus, the host initial immuno-inflammatory response caused by these endodontic sealers induced the regression of the inflammatory reaction and the formation of collagen-rich capsules. In contrast, the presence of the chronic inflammatory process in the capsules of Endofill after 60 days of implantation reinforces the concept that this material presents irritant potential to the tissues for long time.

AH Plus, an epoxy-based root canal filling sealer, was used in the present study as a control since this cement is considered a gold standard [19, 21]. The moderate inflammatory reaction observed initially was diminished over time concomitantly with the presence of birefringent collagen fibres, indicating that this material may induce a biological response that culminates in tissue repair. With regard to the MTA Plus, no evidence of cytotoxic effect was observed in primary culture of human dental pulp cells [24, 34]. Moreover, the increase in the alkaline phosphatase enzyme activity expressed by dental pulp cells [24, 34] and by human osteoblast-like cells [5] suggests that this endodontic sealer exhibits bioactivity [5, 24, 34]. In rat molars, the MTA Plus used as pulp capping promoted a mild inflammatory reaction and induced the reparative dentine formation after 30 days [35]. However, *in vivo* studies evaluating the tissue response of the MTA Plus as root canal filling sealer have not been found. In the present study, although MTA Plus induced a moderate inflammatory reaction on the 7th day, this sealer exhibited a significant reduction in the number of inflammatory cells in parallel to the significant increase in the collagen content in the capsules over time. In addition, these changes were accompanied by a significant reduction in the capsule thickness indicating that this MTA-based sealer is biocompatible.

It is known that the inflammatory reaction induced by endodontic materials in the tissues involves a complex cascade of cellular and molecular events with participation of several cytokines and growth factors. The interaction of these molecules with the host cells (resident and inflammatory cells) define the response outcome, which can result in the regression of the inflammatory process or a chronic inflammatory response [36]. The IL-6 plays an important role in the recruitment and survival of neutrophils and macrophages, B-lymphocyte differentiation and regulation of T cell functions [36–38]. Thus, the highest number of IL-6-immunolabelled cells in the capsules on the 7th day indicates that this interleukin is involved in the beginning of the inflammatory reaction in the capsules surrounding the root filling sealers. In fact, the surgical trauma may be at least in part responsible for the high number of immunostained cells in all the groups at 7 days since enhanced IL-6 immunoexpression was also verified in the control group (empty tubes) in this time point either in this or previous studies [11, 25]. However, the number of immunolabelled cells in the control group reduced significantly after 15 days of implantation in the subcutaneous.

Our results revealed that the number of IL-6-immunolabelled cells was significantly higher in the AH Plus sealer than MTA Plus and MTA Fillapex. It is possible that this finding may be due to small quantity of formaldehyde released by AH Plus [39], and to the fact that this material is an epoxy resin-based sealer. The conversion of monomers into polymers from the mixing of the two-component paste of AH Plus occurs slowly and, consequently, this sealer presents a high

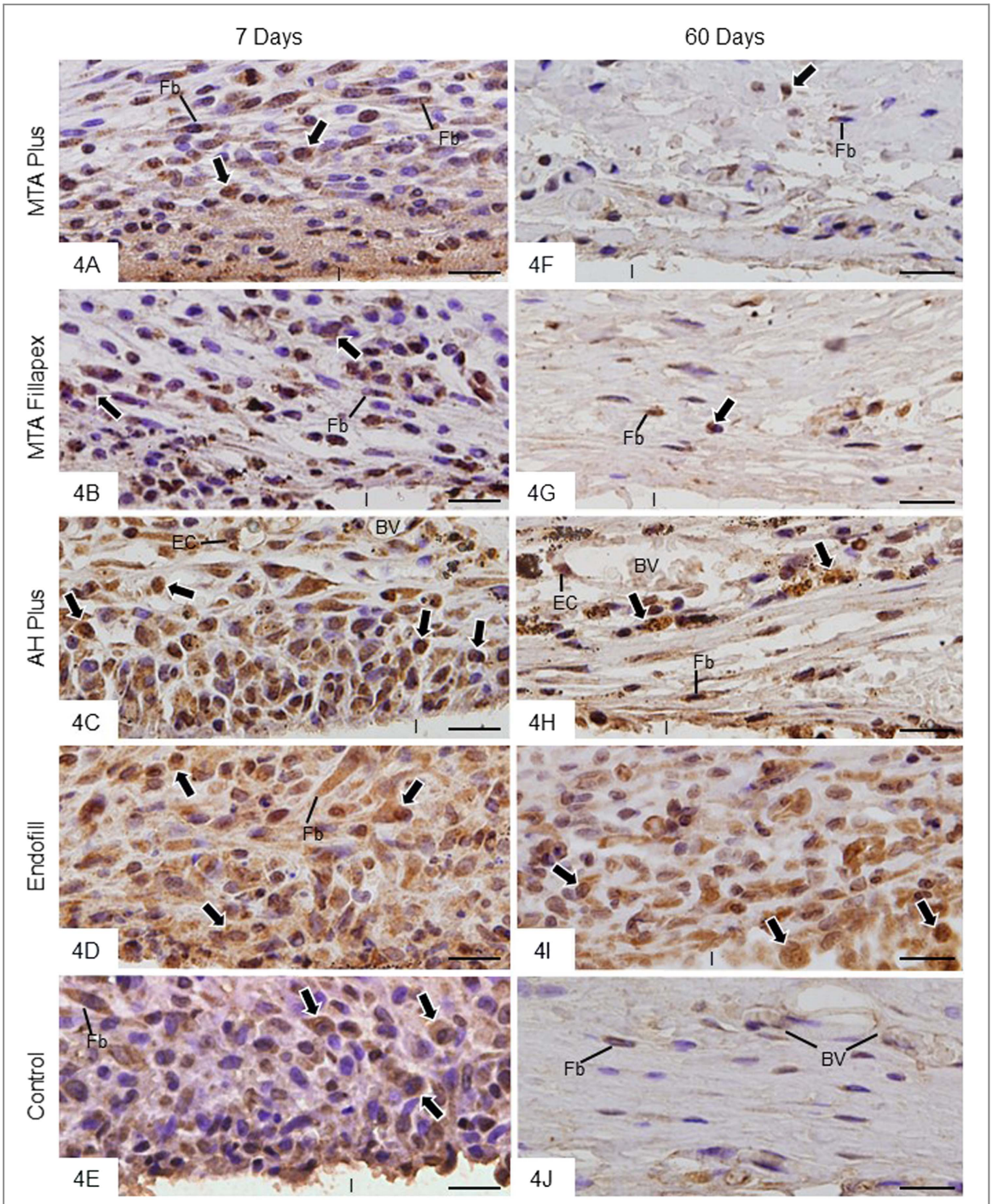

Figure 4. (A)–(J) Light micrographs showing portions of capsules adjacent to the opening of the tubes implanted in the subcutaneous after 7 (A)–(E) and 60 (F)–(J) days of implantation. Sections were subjected to immunohistochemistry for detection of IL-6 (brown-yellow colour) and counterstained with haematoxylin. (A)–(E) (7 days)—several inflammatory cells (arrows), mainly lymphocytes, plasma cells and macrophages, exhibit strong immunolabelling in their cytoplasm. Some immunolabelled fibroblasts (Fb) are also present. (F)–(J) (60 days)—some inflammatory cells (arrows) and fibroblasts (Fb) exhibiting immunostained cytoplasm are sparsely distributed between collagen fibres in all the groups, except in Endofill (I). In this group, several immunolabelled inflammatory cells (arrows) are observed in the capsule. In the (H), particles of AH Plus are observed in the innermost portion of the capsule; some of them are apparently inside the cells (arrows). EC, immunolabelled endothelial cell; BV, blood vessel. Bars: 20 μm.

setting time [40]. Studies have reported that AH Plus presents mutagenic and toxic potential, mainly immediately after mixing [41, 42]. The higher solubility exhibited by AH Plus in comparison with MTA Fillapex [43] may also contribute for high immunoreactivity observed in the capsules of AH Plus mainly at 7 days. In regards the reduced immunoexpression promoted by MTA Plus compared with MTA Fillapex, it is conceivable to suggest that this difference in the tissue response may be due to the composition of these MTA-based sealers. Although both endodontic sealers contain mineral trioxide aggregate, the MTA Plus used in the present study was mixed with water soluble salt-free gel while the MTA Fillapex contains resins (such as salicylate resin, diluting resin and natural resin). It has been demonstrated that these resins exhibit,

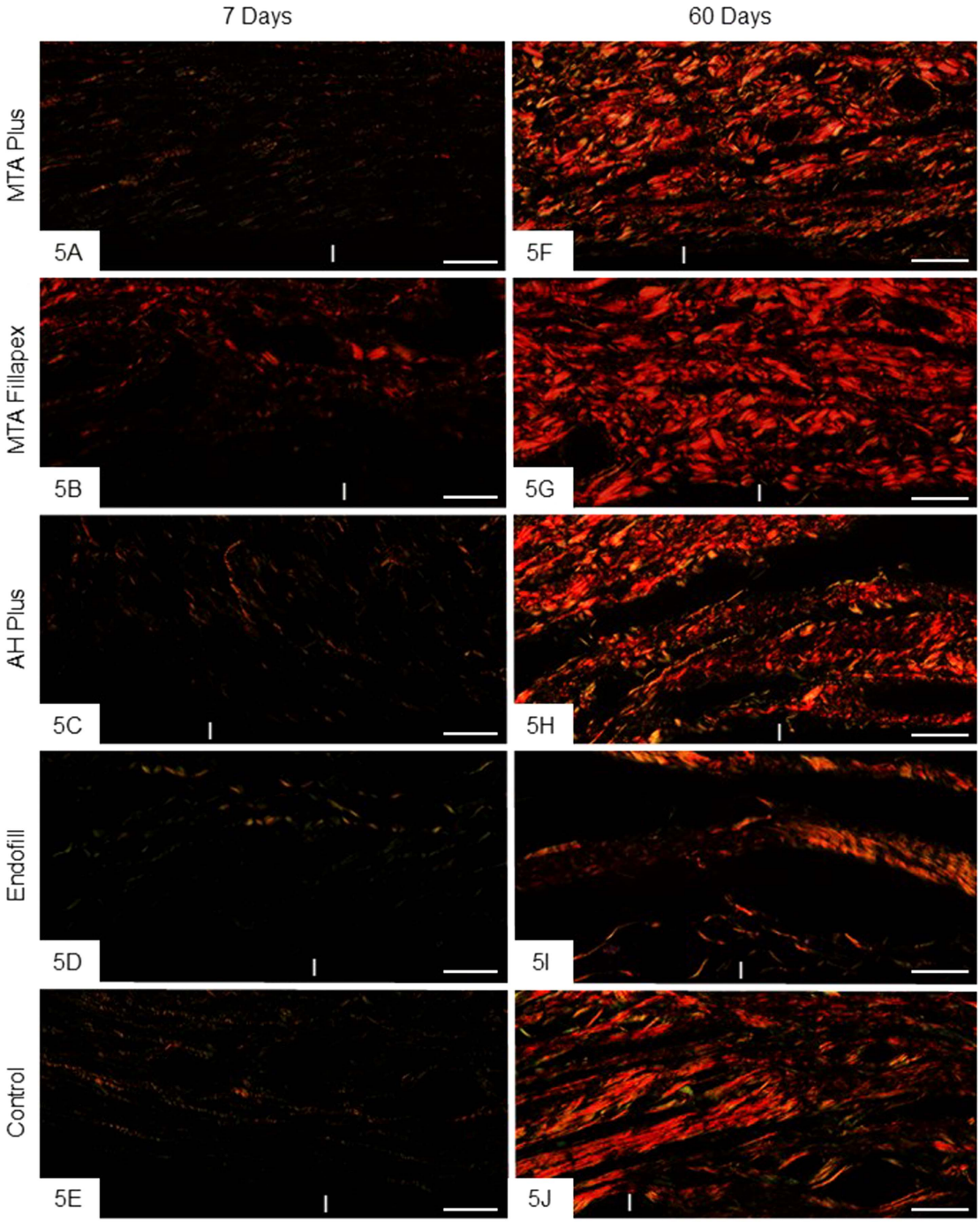

Figure 5. (A)–(J) Light micrographs of sections showing portions of capsules adjacent to the opening of the tubes implanted in the subcutaneous after 7 (A)–(E) and 60 (F)–(J) days of implantation. Sections were stained by picrosirius-red method and analysed under polarised light. (A)–(E) (7 days)—the capsules exhibit few birefringent collagen content (red, yellow and green colours). (F)–(J) (60 days)—evident birefringent collagen is observed in the capsules of all groups, except in Endofill (I). In this group, the capsule exhibits reduced birefringent collagen content in comparison with other groups. Bars: 20 μm.

mainly in the initial periods, irritant potential [19, 20, 34] and, therefore, may have stimulated the production of this cytokine by cells in the MTA Fillapex capsules. Moreover, the MTA Plus induced the formation of thinner capsules than other root filling sealers reinforcing the idea that this endodontic sealer exhibits low irritant potential. Thus, our results strongly suggest that MTA Plus promotes initially lower tissue damage as evidenced by capsule thickness, a parameter indicative of the inflammatory reaction, and by immune response.

The IL-6 level has also been associated with release of matrix metalloproteinases and subsequent degradation of extracellular matrix components [44, 45]. Therefore, the reduced number of immunolabelled cells in the control group after 30 and 60 days of implantation may be responsible for the regulation of the connective tissue remodelling in these capsules. A

significant reduction in the immunoexpression was observed in the capsules juxtaposed to all endodontic sealers from 7 to 60 days. However, MTA-based sealers showed better biological properties than AH Plus since, at 60 days, the capsules of MTA Plus and MTA Fillapex exhibited lower IL-6 immunoexpression than AH Plus. High IL-6 level has been found in the periapical lesions [46] and dental granulomas [47]. Besides modulating the inflammatory process, IL-6 also stimulates the osteoclast formation and consequently induces bone resorption [45, 48–50]. Considering the reduced number of IL-6-immunolabelled cells in MTA Plus and MTA Fillapex, it is conceivable to suggest that these MTA-based sealers may suppress more rapidly the alveolar bone resorption than AH Plus when used in the sealing of teeth with periapical lesions. Moreover, significant differences in the number of inflammatory cells were not detected between MTA-based sealers and control group at 60 days, indicating that the initial inflammatory reaction was replaced by connective tissue containing fibroblasts and collagen fibres, similarly to what is observed during periodontal repair. In addition, the significant reduction in the capsule thickness over time indicates that these endodontic sealers induced the formation of thin capsules. Therefore, our findings indicate that MTA Plus and MTA Fillapex are biocompatible when implanted in the subcutaneous. Some studies have reported that MTA Fillapex causes severe inflammatory reaction [21] and higher cytotoxicity than AH Plus [13, 18]. On the other hand, it has been reported that after initial period of cytotoxicity, the MTA Fillapex stimulates the alkaline phosphatase activity and the deposition of mineralised nodules suggesting that this endodontic sealer exhibits bioactivity after setting time [13, 22]. It is possible that the different results obtained from these studies may be due to some differences in the experimental procedures.

In the period of 60 days, the number of inflammatory cells, mainly macrophages and plasma cells, in the capsules adjacent to Endofill was two times higher than the other groups. Moreover, a high IL-6 immunoexpression was also observed in these capsules, indicating a chronic inflammatory process. These findings are supported by the presence of low collagen content in the capsules of Endofill when compared to other groups. Endofill is a zinc oxide-eugenol based sealer that presents higher solubility than AH Plus [51], and prolonged release of eugenol which exerts a toxic effect to cells [20, 52, 53]. Eugenol activates nuclear factor kappa B and induces cyclooxygenase-2 expression leading to cytoplasmic vacuolisation and toxicity in human osteosarcoma cells *in vitro* [53]. The zinc oxide-eugenol based materials are known to cause chronic inflammation in the periodontal ligament and to promote the release of signalling molecules which lead to bone resorption [1, 7]. Although the mixture zinc oxide/eugenol, used in the present study, contained a minimum of eugenol and provided a putty-like consistency to the Endofill, the irritant effect of this cement was still evident after 60 days of implantation in the subcutaneous. There is evidence that direct contact of zinc oxide and eugenol-based sealers with the tissue fluids promotes the release of large amount of eugenol, which provoke cytotoxic effects [7, 20, 54]. Therefore, our findings support the idea that zinc oxide and eugenol-based sealers induce an irritant chronic effect on the cells, disturbing the tissues structural integrity and hindering the tissue repair.

Conclusions

Although the MTA-based sealers and AH Plus initially induce an inflammatory reaction, these endodontic sealers allow to host cells a response which culminates with the regression of this reaction and formation of thin capsules containing collagen fibres. In addition, MTA Plus and MTA Fillapex promoted a marked reduction in the number of IL-6-immunolabelled cells in the capsules compared with AH Plus over time indicating, therefore, that MTA-containing materials may suppress the immune response more rapidly.

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Conflict of interest

The authors deny any conflict to interests to this study.

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