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Biocompatibility of new calcium-silicate based sealer TotalFill BC Sealer HiFlow in subcutaneous tissue of rats

Carolina Mourato Pedro Coelho

Orientador: Prof. Doutor João Miguel Marques dos Santos

Co-orientadora: Mestre Diana Bela da Luz Sequeira

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Coelho C.[1], Sequeira D.B.[2], Santos J.M.[3]

1) 5th year student of Integrated Master in Dentistry, Faculty of Medicine, University of Coimbra, Portugal

2) Lecturer, Institute of Endodontics, Faculty of Medicine, University of Coimbra, Portugal

3) Assistant Professor, Institute of Endodontics, Faculty of Medicine, University of Coimbra, Portugal

Área de Medicina Dentária da Faculdade de Medicina da Universidade de Coimbra

Av. Bissaya Barreto, Bloco de Celas

3000-075 Coimbra

Portugal

Tel.: +351 239 484 183

Fax: +351 239 402 910

E-mail: carolinacoelho96@gmail.com

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1. ABSTRACT

Introduction: Endodontic treatment comprehends a chemical and mechanical preparation of the root canals, followed by a three-dimensional obturation. Endodontic sealers must present adequate chemical and handling features and must be biocompatible. The aim of this study was to evaluate the biocompatibility of 2 new hydraulic calcium silicate-based materials, TotalFill BC Sealer (FKG, La Chaux-des-Fonds, Switzerland) and TotalFill BC Sealer HiFlow (FKG, La Chaux-des-Fonds, Switzerland) through subcutaneous implantation in connective tissue of rats.

Materials and Methods: Subcutaneous implantation was performed in 16 young Wistar rats. The study comprised 2 time periods and 4 experimental groups: control group, AH Plus (Dentsply DeTrey, Konstanz, Germany) group, TotalFill BC Sealer and TotalFill BC Sealer HiFlow. Hematoxylin-eosin coloration was used to score the inflammatory reaction, macrophage infiltrate and to measure the thickness of the fibrous capsule. Von Kossa staining was performed to evaluate the mineralization potential. Kruskal-Wallis test was used to analyze non-parametric data followed by the Dunn's test. To analyze the influence of the time within each material, a Mann-Whitney U test was used. $P \leq 0.05$.

Results: Eight days after implantation, AH Plus provoked an inflammatory reaction more intense when compared with the control and TotalFill BC Sealer ($P \leq 0.05$). TotalFill BC Sealer HiFlow present a higher score for the macrophage infiltrate than TotalFill BC Sealer and control ($P \leq 0.05$). The fibrous capsule thickness in this period was significantly higher for the TotalFill BC Sealer group than for the control and AH Plus ($P \leq 0.05$) and the mineralization potential was higher for the TotalFill BC Sealer HiFlow group when compared with the control and AH Plus ($P \leq 0.05$). At day 30, the score for the inflammatory reaction remained higher for the AH Plus group than for the control and TotalFill BC Sealer ($P \leq 0.05$). The macrophage infiltrate of the TotalFill BC Sealer HiFlow was significantly higher than control and AH Plus groups ($P \leq 0.05$) and the fibrous capsule of the TotalFill BC Sealer and TotalFill BC Sealer HiFlow was thicker than the control ($P \leq 0.05$). Finally, the mineralization potential was significantly higher for TotalFill BC Sealer and TotalFill BC Sealer HiFlow groups ($P \leq 0.05$).

Conclusion: Both TotalFill BC sealer and TotalFill BC HiFlow are biocompatible and present potential bioactivity when implanted in the subcutaneous tissue.

KEY-WORDS: calcium silicate-based sealers, biocompatibility, subcutaneous implantation, animal model, root canal filling material, endodontics.

2. RESUMO

Introdução: O tratamento endodôntico compreende a preparação químico-mecânica dos canais radiculares seguida de uma obturação tridimensional. Os cimentos endodônticos devem possuir características físico-químicas adequadas e devem ser biocompatíveis. O objetivo do nosso estudo foi avaliar a biocompatibilidade de 2 cimentos endodônticos à base de silicato de cálcio, TotalFill BC Sealer (FKG, La Chaux-des-Fonds, Suíça) e TotalFill BC Sealer HiFlow (FKG, La Chaux-des-Fonds, Suíça) através da implantação subcutânea em tecido conjuntivo de ratos.

Materiais e métodos: A implantação subcutânea foi realizada em 16 ratos Wistar. O estudo compreendeu 2 períodos de tempo e 4 grupos experimentais: controlo, AH Plus (Dentsply DeTrey, Konstanz, Alemanha), TotalFill BC Sealer e TotalFill BC Sealer HiFlow. A coloração com hematoxilina-eosina foi realizada para avaliar a reação inflamatória, o infiltrado de macrófagos e para medir a espessura da cápsula fibrosa. A coloração com Von Kossa foi utilizada para avaliar o potencial de mineralização dos materiais. Para a análise estatística dos dados não-paramétricos utilizou-se o teste Kruskal-Wallis, seguido do teste de Dunn. Para analisar a influência do tempo entre os materiais utilizou-se o teste Mann-Whitney U. $P \leq 0.05$.

Resultados: Oito dias após a implantação, o AH Plus provocou uma reação inflamatória mais intensa quando comparada com a do controlo e do TotalFill BC Sealer ($P \leq 0.05$). O grupo do TotalFill BC Sealer HiFlow apresentou um maior infiltrado de macrófagos do que o controlo e o TotalFill BC Sealer ($P \leq 0.05$). Neste período, a espessura da cápsula fibrosa foi significativamente maior para o TotalFill BC Sealer do que para o controlo e AH Plus ($P \leq 0.05$) e o potencial de mineralização foi maior para o grupo do TotalFill BC Sealer HiFlow relativamente ao controlo e AH Plus ($P \leq 0.05$). Aos 30 dias, a reação inflamatória continuou a ser maior para o grupo do AH Plus do que para o controlo e TotalFill BC Sealer ($P \leq 0.05$). O infiltrado de macrófagos do TotalFill BC Sealer HiFlow foi significativamente maior do que o controlo e o AH Plus ($P \leq 0.05$) e as cápsulas fibrosas do TotalFill BC Sealer e do TotalFill BC Sealer HiFlow apresentaram-se mais espessas do que as do controlo ($P \leq 0.05$). Por fim, o potencial de mineralização foi significativamente maior nos grupos do TotalFill BC Sealer e TotalFill BC Sealer HiFlow ($P \leq 0.05$).

Conclusão: Ambos os cimentos TotalFill BC e TotalFill BC HiFlow são biocompatíveis e apresentam potencial bioativo quando implantados em tecido subcutâneo.

PALAVRAS-CHAVE: cimentos silicato de cálcio, biocompatibilidade, implantação subcutânea, modelo animal, material de obturação, endodontia.

3. INTRODUCTION

Endodontic treatment aims to create a cleaned and well-shaped root canal system, with adequate instrumentation techniques and proper irrigation solutions, followed by a three-dimensional obturation (1).

The root canal filling is classically performed using a dense core such as gutta-percha, a solid obturation material with good biological compatibility, in combination with a root canal sealer to provide hermetic sealing, since gutta-percha alone does not seal the root canal (2–4). This represents a challenge during endodontic treatment due to the complex and varying anatomy of the root canal system, characterized by the presence of isthmuses and ramifications (5).

Root canal sealers used in the obturation procedure must provide suitable marginal properties, low solubility, adequate setting time and have to be impervious, biocompatible and non-toxic (1,3,6). These sealers have the ability to interact physically with dentine creating mechanical retentions, penetrating inside dentin tubules and filling the irregularities of the root canal system, like fins, isthmus and lateral canals. Besides they may also interact with dentine in a chemical way through the formation of tag-like structures at the cement-dentin interface (5).

Sealers fill the voids between gutta-percha points and dentin walls, thus presenting an important role in the outcome of root canal treatments, preventing regrowth of microorganisms that persist after chemo-mechanical preparation or intracanal reinfection through coronal microleakage. Therefore, they are relevant to prevent and cure apical periodontitis of microbial origin (1,7).

Endodontic sealers and their components can provide different periapical reactions after root canal treatment due do the risk of extrusion through the apical constriction or the existence of lateral and accessory canals (1,6,8,9). These reactions are influenced by the chemical composition of the endodontic sealer (10). Furthermore, tissue response to those sealers together with aggression caused by the pre-obturation procedures and the damage caused by the pathological process may affect tissue's healing and therefore, the overall outcome of the endodontic treatment (1,4,9).

Several root canal sealers have been introduced in the market and it is mandatory to know the response induced in the periapical tissues prior to their use in humans (1,11,12). Based on their chemical composition, sealers are classified as glass ionomer, zinc-oxide eugenol, resin-based, calcium hydroxide, silicone and bioceramic-based root canal sealers (6,13).

Obturation technique also influence the quality of sealing. The standard procedure is cold lateral compaction, however this technique may induce micro-cracks in the root and is time-consuming. In order to overcome these disadvantages other techniques have emerged namely warm vertical compaction, core-carrier (Thermafill) and single cone techniques (14).

Warm obturation techniques have gained more popularity because of the higher percentage of interface between gutta-percha and the sealer. Besides, the high temperature helps to fill the small and peripheral details of dentin walls by pushing gutta-percha against them. Nevertheless, there have been concerns related to possible negative effects on sealer properties due to the heat to which they are submitted during this procedure step (8,15,16).

Single cone techniques require less treatment time when compared to lateral compaction and provides an improved adaptation to the root canal walls (14). However, the branches and irregularities of the root canal are mainly filled with endodontic sealer, whereas in warm vertical compaction, not only the sealer is pushed against dentin walls but also the plasticized gutta-percha (5,9).

AH Plus (Dentsply DeTrey, Konstanz, Germany) is an epoxy resin-based root canal sealer that as good properties such as low microleakage, ability to bond to dentin, antibacterial activity against *Enterococcus faecalis* and dimensional stability due to low polymerization shrinkage after entering the root canal (3,5). It is composed by 2 pastes, paste A containing iron oxide and epoxy resin and paste B containing silicone oil and amines (17). However, it exhibits toxicity immediately after mixing, gradually reducing upon setting (3).

To overcome these disadvantages, hydraulic calcium silicate-based sealers also known as bioceramic sealers have been developed and they present good physicochemical and biological properties (3,5,13,18,19).

Bioceramic materials are designed to repair and reconstruct damaged parts of the musculoskeletal system (20) and are classified as bioactive, bioinert and biodegradable (21). Endodontic bioceramic materials are dimensionally stable and not sensitive to moisture and blood contamination. When setting, they become hard and insoluble. Their pH increases until 12 due to the release of calcium ions in the hydration reaction. After this, a precipitation reaction occurs when bioceramic material contact with tissue's fluids forming hydroxyapatite. This latter reaction may explain the hard tissue inductive properties evoked by hydraulic calcium-silicate based sealers (19,21).

TotalFill BC Sealer (FKG, La Chaux-des-Fonds, Switzerland) is part of the novel generation of this group of sealers, a pre-mixed ready-to-use injectable calcium silicate-based sealer whose main components are calcium silicates, calcium hydroxide, calcium phosphate, thickening agents and zirconium oxide as a radiopacifier agent. This sealer exhibits biocompatibility and antimicrobial activity (3,13,18,19) and requires the presence of moisture from dentinal tubules to achieve complete setting (8,13). Due to its high pH and ability to release calcium hydroxide, this sealer also possesses potential antimicrobial effect against *Enterococcus faecalis* (8). Cytotoxicity assays on TotalFill BC Sealer reveal that this sealer demonstrates an excellent biocompatibility with human gingival fibroblasts (22), an enhancement of osteoblastic

differentiation on human periodontal ligament cells and a low expression of inflammatory mediators (8). However, its flow ability and setting time when heat was applied, decreased significantly, which can negatively affect the quality of the obturation when a warm obturation technique is used (15).

Recently available for dental professionals TotalFill BC Sealer HiFlow (FKG, La Chaux-des-Fonds, Switzerland) is a calcium-silicate based sealer developed especially for warm obturation since its physicochemical properties remain stable at temperatures corresponding to those inside the root canal during warm procedures. According to the manufacturer it provides lower viscosity and higher flow ability when heated and is more radiopaque than its predecessor, and this data has been confirmed by an independent study (8). It also revealed a biocompatibility profile similar to BC Sealer (8,16).

To the best of authors' knowledge, there are only 2 studies that assessed cytotoxic potential of this new sealer and none evaluated the biocompatibility of TotalFill BC Sealer HiFlow.

Biocompatibility is one of the most important properties of endodontic sealers and its evaluation comprises initial evaluation of sealers' cytotoxicity, *in vivo* laboratory tests in animals and finally, usage studies in primates or humans (1,6,12).

The purpose of the current study was to evaluate the biocompatibility and bioactive potential of 2 new bioceramic root canal sealers compared to those provided by a commonly used sealer, AH Plus, through subcutaneous implantation in connective tissue of rats.

The null hypothesis is that there would be no difference between the histologic scores observed for AH Plus, TotalFill BC Sealer and TotalFill BC Sealer HiFlow on both observation periods.

4. MATERIALS AND METHODS

In vivo implantation is the experiment following cell culture studies since *in vitro* assays cannot reproduce the physiology and anatomy of human tissues. Wistar rats are mammals and their body temperature is similar to humans as well as their physiology and drug/anesthesia administration, and for this reason they are commonly used worldwide in laboratory research.

Besides, they are homoeothermic and have the ability to regulate their body temperature generating heat through metabolic processes and heat loss control mechanisms. Additionally, they are not expensive and are easy to be taken care of and manipulate.

This study followed the ARRIVE guidelines (23).

16 young Wistar rats were used for the *in vivo* assay (age, 8-10 weeks, body weight, 110-240g). The sample size was established based on previous research (1,2). All procedures were conducted in accordance with the standards of the National Institutes of Health, according to the Guide for the Care and Use of Laboratory Animals. The Institutional Ethics Committee on the Use of Animals of the Faculty of Medicine of the University of Coimbra approved this study (ORBEA 17/2015). The animals were observed at each 8-hours by animal caretakers during the whole period of the experiment.

The animals were anesthetized with ketamine 50mg (Ketalar, Pfizer, United Kingdom) and chlorpromazine 5mg/mL (Largactil, Laboratórios Vitória, Portugal), via intramuscular (i.m.) with a dosage of 0,3 mL/100g in the thigh. Dorsal hair was removed while the animals were positioned in ventral *decubitus* (Figure 1). After this, they were disinfected with a povidone-iodine solution (Egrema, Paracelsia, Porto, Portugal). Incisions were made in each one of the four quadrants of the dorsal region using a nº15 scalpel blade, equidistant from the spine with an orientation from head to tail. Two scapular and two caudal pockets were created. Before receiving the implants, blunt-tipped scissors were used to dissect the subcutaneous tissue, parallel to the spine, to create surgical recesses with approximately 20mm of depth. This procedure was performed under aseptic conditions, with sterile material and using surgical gloves and masks.

Four polyethylene tubes (9mm length and 0,9mm internal diameter) were implanted in the subcutaneous tissue of each animal. One of the tubes in each animal was left empty serving as the negative control, and the other three were filled with AH Plus, TotalFill BC Sealer and TotalFill BC Sealer HiFlow (APPENDIX I – Composition of the sealers). These sealers were prepared under aseptic conditions and mixed according to the manufacturers' instructions immediately before implantation.

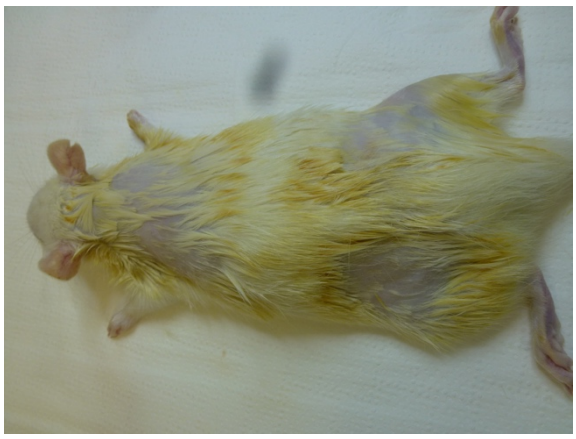


Figure 1 – Dorsal hair removal with the animal positioned in ventral *decubitus*.

After implantation, the incisions were closed with a non-resorbable suture (silk 4/0) and the animals were housed in individual boxes, in light, temperature and air-controlled rooms (Tecniplast, 9ARMI/4120) with proper food and water *ad libitum*. No adverse events were registered during the immediate post-operative period or at follow-up until the end of the experiment.

The study comprised two different time periods, 8 and 30 days (n=8) and four experimental groups. At the end of each experimental period, the animals were sacrificed by anesthetic overdose. The location of the implants was found through tactile sensitivity and surgical removal of the implants was made with adjacent 1cm safety margins of the surrounding tissues (Figure 2 – 3).

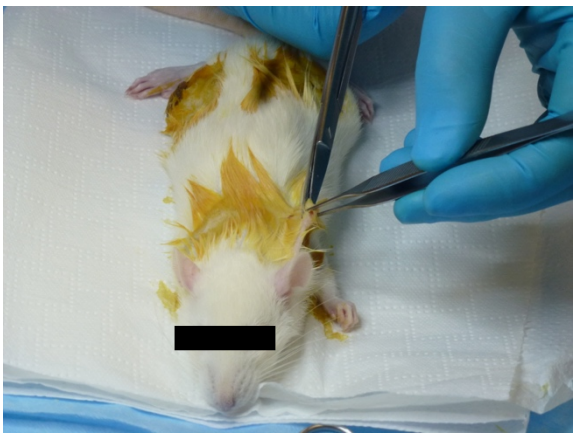


Figure 2 – Surgical removal of the implants after animal's euthanasia.

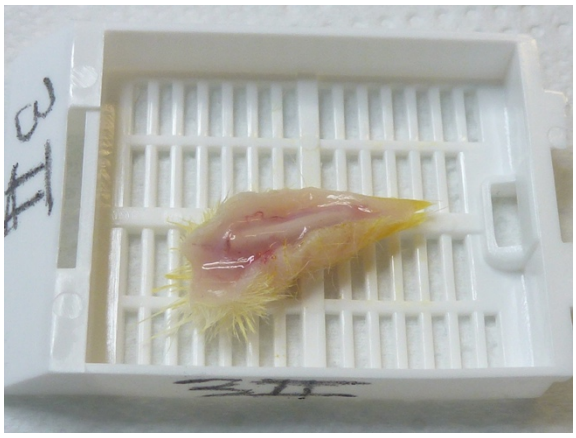


Figure 3 – Biopsy sample with safety margins from the polyethylene tube.

The biopsy samples were then fixed in 10% neutralized formaldehyde solution (Panreac, Barcelona, Spain) and following fixation each sample was placed in an alcohol water solution, in order to dehydrate the tissue samples. After dehydration, tissues were cleared, replacing alcohol by xylene (Panreac, Barcelona, Spain) and the xylene by paraffin (Paraplast Regular, Sigma Aldrich, St Louis, USA) to form blocks. Finally, these tissue blocks were cut into 5 µm sections using a microtome (Leica RM 2155, Leica, Lisbon, Portugal). The thin sections were mounted on microscope slides and stained with a hematoxylin and eosin histologic coloring technique and also with the Von Kossa coloration in order to evaluate the mineralization potential (APPENDIX II – Histology technique).

The stained histological sections were analyzed with a light microscope (Nikon Eclipse Ci-L, Tokyo, Japan) and digital photos were obtained using an accoupled camera to the microscope (Nikon Digital Sight DS-Fi1, Tokyo, Japan) and analyzed by a blind investigator to the implantation timing and sealer.

Tissue reaction was assessed according to a scoring system for: inflammatory reaction (magnification field 100x: 0, absent with few inflammatory cells; 1, mild with less than 25 cells; 2, moderate with 25 to 125 cells; 3, severe with more than 125 cells); macrophage infiltrate (magnification field 100x: 0, less than 10 cells; 1, 10 to 30 cells; 2, more than 30 cells); thickness of the fibrous capsule (magnification field 40x: measured in 3 points (2 measurements near each of the margins of the top opening of the polyethylene tube and a third one in the middle, according to the example on Figure 1A)); and mineralization (0, absent; 1, present in less than half of the length of the tube opening; 2, present in more than half of the length). The evaluation system to score the parameters observed in the samples is explained in Table 1.

Statistical analysis was performed using PRISM8 software (version 8.4.2, GraphPad Software, LLC). To analyze non-parametric data Kruskal-Wallis test was used followed by the Dunn's test. To analyze the influence of the time within the material, a Mann-Whitney U test was used. The *P*-value for significance was set at 0.05.

Table 1 – Score system used to evaluate histopathologic features of the specimens.

Scores	0	1	2	3
Inflammatory reaction	Absent with few inflammatory cells	Mild with less than 25 cells	Moderate with 25 to 125 cells	Severe with more than 125 cells
Macrophage infiltrate	Less than 10 cells	10 to 30 cells	More than 30 cells	-
Mineralization	Absent	Less than half the mineralized area	More than half the mineralized area	-

5. RESULTS

As previously mentioned, our study was composed by two different assessment periods, 8 and 30 days, each with 8 rats and four experimental groups: control group, AH Plus, TotalFill BC Sealer and TotalFill BC Sealer HiFlow. After both observation periods, macroscopic clinical evaluation showed a satisfactory wound healing and all implants remained *in situ*.

5.1. CONTROL GROUP

At day 8, a mild inflammatory reaction was observed in 75% of the samples (Table 2) and a thin immature fibrous capsule was present (Figure 4A-C). Some granulation tissue emerged inside the tube. Very few macrophages were observed in both time periods.

After 30 days the capsule remained thin but surrounded by mature connective tissue and the results showed a resolution of the inflammatory reaction (Figure 5A-C). Mineralization was considered absent in both periods (Figure 6A-B, 7A-B).

Table 2 – Absolute and relative frequencies for histologic evaluation of the samples according to groups and periods.

Scores	Control group		AH Plus		TotalFill BC Sealer		TotalFill BC Sealer HiFlow	
	8 days	30 days	8 days	30 days	8 days	30 days	8 days	30 days
Inflammatory reaction								
0	2 (25)	6 (75)	0 (0)	0 (0)	1 (12,5)	7 (87,5)	0 (0)	4 (50)
1	6 (75)	2 (25)	1 (12,5)	4 (50)	7 (87,5)	1 (12,5)	5 (62,5)	4 (50)
2	0 (0)	0 (0)	6 (75)	4 (50)	0 (0)	0 (0)	3 (37,5)	0 (0)
3	0 (0)	0 (0)	1 (12,5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Macrophage infiltrate								
0	8 (100)	8 (100)	4 (50)	6 (75)	7 (87,5)	3 (37,5)	2 (25)	0 (0)
1	0 (0)	0 (0)	4 (50)	2 (25)	1 (12,5)	5 (62,5)	2 (25)	6 (75)
2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (50)	2 (25)
Mineralization								
0	8 (100)	8 (100)	8 (100)	8 (100)	3 (37,5)	2 (25)	0 (0)	0 (0)
1	0 (0)	0 (0)	0 (0)	0 (0)	4 (50)	5 (62,5)	6 (75)	5 (62,5)
2	0 (0)	0 (0)	0 (0)	0 (0)	1 (12,5)	1 (12,5)	2 (25)	3 (37,5)

5.2. AH PLUS

Images from day 8 show a moderate inflammatory reaction in 75% of the samples (Figure 4D-F) and a reduced layer of fibrous tissue, forming a thin fibrous capsule in the tissue-implant interface. The macrophage infiltrate was scored 1 in half of the samples. No mineralization occurred in both time periods (Figure 6C-D, 7C-D).

At day 30, a thin fibrocelular capsule (Figure 5D-F) was present and a mild to moderate inflammatory reaction was observed with the presence of lymphocytes and plasma cells.

5.3. TOTALFILL BC SEALER

At day 8, the fibrous capsule for this group was thicker than the other groups (Graphic 2). A mild inflammatory reaction was observed, and the number of macrophages was reduced (Figure 4G-I). The majority of the samples presented less than half of the length of the tube opening mineralized (Figure 6E-F).

Thirty days after implantation, this groups continued to present the thickest fibrous capsule (Figure 5G-I) and the macrophage infiltrate was scored 1. In the majority of the specimens, mineralization was observed in less than half of the tube opening (Figure 7E-F).

5.4. TOTALFILL BC SEALER HIFLOW

In the first period, a mild to moderate inflammatory reaction was present associated with a mild to severe macrophage infiltration (Figure 4J-L). Most of the specimens present a thin fibrous capsule and mineralization occurred in less than half of the area for the majority of samples (Figure 6G-H), even if some presented more than half of the length of the tube opening mineralized (2 out of 8).

At day 30, chronic inflammation reaction was absent or mild (Figure 5J-L) and most of the samples showed a moderate macrophage infiltrate. The thickness of the fibrous capsule remained thin. Mineralization was present in all samples with less than half of the area adjacent to the tube opening mineralized in 5 specimens and more than half in 3 specimens (Figure 7G-H).

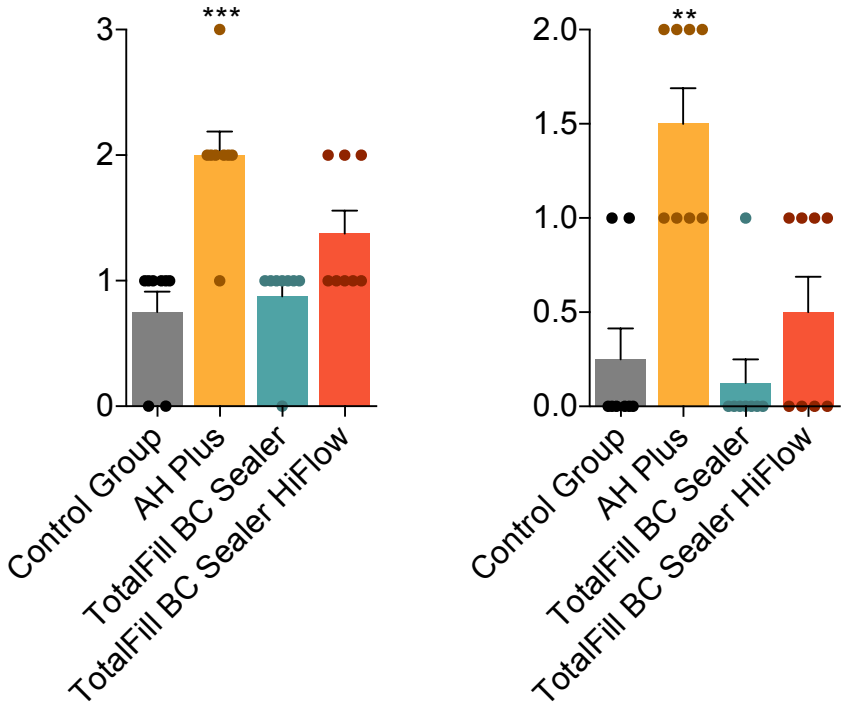
5.5. GROUP COMPARISON

After eight days, we found significant effects on the inflammatory response ($H_{(3)} = 18,20$, $p = 0,0004$). AH Plus showed the highest mean score for this parameter. There were statistically significant differences when comparing AH Plus with the control group ($2,000 \pm 0,1890$, $n = 8$;

0,7500 ± 0,1637, n = 8; p = 0,0009) and AH Plus with the TotalFill BC Sealer group (2,000 ± 0,1890, n = 8; 0,8750 ± 0,1250, n = 8; p = 0,0032).

At day 30, the effects on the inflammatory response remained significant ($H_{(3)}$ = 17,36 , p = 0,0006) and AH Plus remained with the highest score of all groups. There were significant effects in the same groups as after 8 days: AH Plus with control (1,500 ± 0,1890, n = 8; 0,2500 ± 0,1637, n = 8; p = 0,0044) and between AH Plus and TotalFill BC Sealer (1,500 ± 0,1890, n = 8; 0,1250 ± 0,0,1250 , n = 8, p = 0,0009). There were no statistically significant differences between other sealers and control and in between sealers (p > 0,05).

The results for the inflammatory reaction are represented in Graphic 1 and the adjusted *P*-values for this parameter in Table 3.



Graphic 1 – Scores for the inflammatory reaction (8 and 30 days).

Table 3 – Adjusted *P*-values for the inflammatory reaction of the four experimental groups (8 and 30 days).

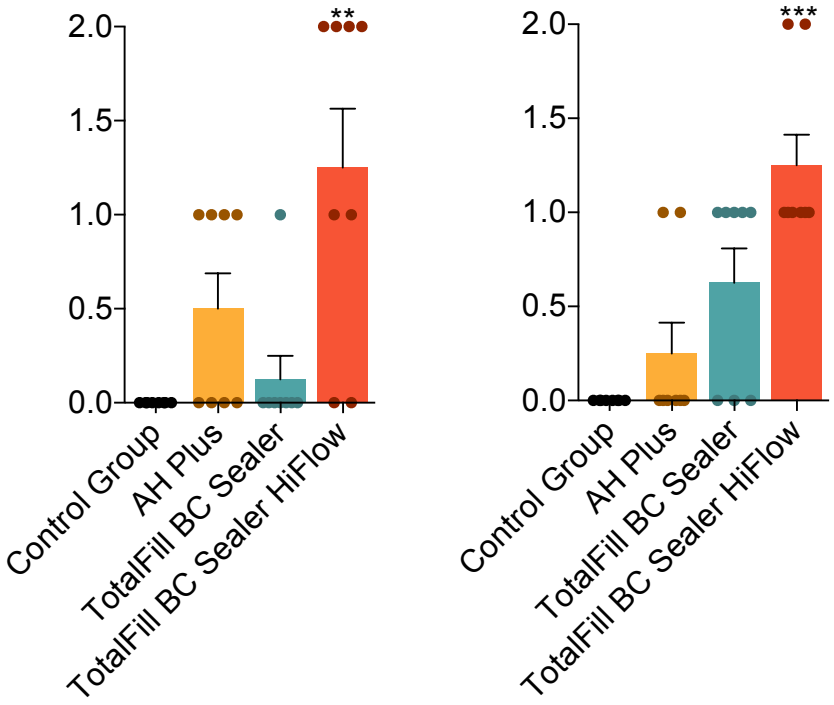
	Inflammatory reaction (8 days)				Inflammatory reaction (30 days)			
	Control	**AH Plus**	**TotalFill BC Sealer**	**TotalFill BC Sealer HiFlow**	**Control**	**AH Plus**	**TotalFill BC Sealer**	**TotalFill BC Sealer HiFlow**
Control	-	0,0009*	>0,9999	0,3101	-	0,0044*	>0,9999	>0,9999
AH Plus	0,0009*	-	0,0032*	0,3819	0,0044*	-	0,0009*	0,0648
TotalFill BC Sealer	>0,9999	0,0032*	-	0,6426	>0,9999	0,0009*	-	>0,9999
TotalFill BC Sealer HiFlow	0,3101	0,3819	0,6426	-	>0,9999	0,0648	>0,9999	-

*Statistically significant

Regarding macrophage infiltrate, we also found significant effects at the first observation period ($H_{(3)}$ = 13,74 , p = 0,0033). TotalFill BC Sealer HiFlow had the highest mean score for the macrophage infiltrate. The groups that had statistically significant differences were TotalFill BC Sealer HiFlow and control (1,2500 ± 0,3134, n = 8; 0,0000 ± 0,0000, n = 8; p = 0,0046) and TotalFill BC Sealer HiFlow and TotalFill BC Sealer (1,2500 ± 0,3134, n = 8; 0,1250 ± 0,1250, n = 8; p = 0,0211).

After thirty days, we verified significant effects on the macrophage infiltrate response ($H_{(3)}$ = 18,76, p = 0,0003) and TotalFill BC Sealer HiFlow continued to show the highest macrophage infiltrate. There were statistically significant differences between TotalFill BC Sealer HiFlow and control (1,2500 ± 0,1637, n = 8; 0,0000 ± 0,0000, n = 8; p = 0,0003) and TotalFill BC Sealer HiFlow and AH Plus (1,2500 ± 0,1637, n = 8; 0,2500 ± 0,1637, n = 8; p = 0,0094).

The results for the macrophage infiltrate after 8 and 30 days are represented in Graphic 2 and Table 4.



Graphic 2 – Macrophage infiltrate scores (8 and 30 days).

Table 4 – Adjusted *P*-values for the macrophage infiltrate of the four experimental groups (8 and 30 days).

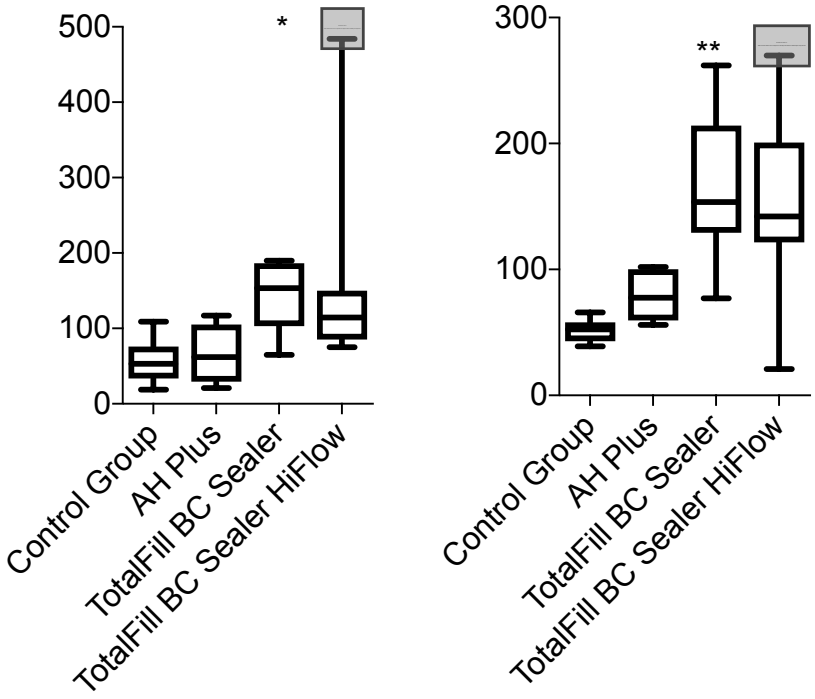
	Macrophage infiltrate (8 days)				Macrophage infiltrate (30 days)			
	Control	**AH Plus**	**TotalFill BC Sealer**	**TotalFill BC Sealer HiFlow**	**Control**	**AH Plus**	**TotalFill BC Sealer**	**TotalFill BC Sealer HiFlow**
Control	-	0,4538	>0,9999	0,0046*	-	>0,9999	0,1437	0,0003*
AH Plus	0,4538	-	>0,9999	0,6761	>0,9999	-	>0,9999	0,0094*
TotalFill BC Sealer	>0,9999	>0,9999	-	0,0211	0,1437	>0,9999	-	0,4251
TotalFill BC Sealer HiFlow	0,0046*	0,6761	0,0211	-	0,0003*	0,0094*	0,4251	-

*Statistically significant

For the thickness of the fibrous capsule parameter, at day 8, significant effects were found ($H_{(3)}$ = 16,28 , p = 0,0010). TotalFill BC Sealer HiFlow presented the thickest fibrous capsule of all groups. We found significant differences between TotalFill BC Sealer HiFlow and control (158,5 $\pm$ 47,47 , n = 8; 56,38 $\pm$ 10,39, n = 8; p = 0,0376), between TotalFill BC Sealer and control (144,8 $\pm$ 16,33, n = 8; 56,38 $\pm$ 10,39, n = 8; p = 0,0049), TotalFill BC Sealer and AH Plus (144,8 $\pm$ 16,33, n = 8; 66,63 $\pm$ 18,39, n = 8; p = 0,0249).

Thirty days after implantation, the effect of the sealers in the fibrous capsule's thickness continued to be statistically significant ($H_{(3)}$ = 19,19 , p = 0,0002) and TotalFill BC Sealer had the thickest fibrous capsule. There were observed significant differences between TotalFill BC Sealer and control (164,3 $\pm$ 28,58, n = 8; 52,00 $\pm$ 3,218, n = 8; p = 0,0006) and TotalFill BC Sealer HiFlow and control (152,5 $\pm$ 25,82, n = 8; 52,00 $\pm$ 3,218, n = 8; p = 0,0039).

The mean results obtained measuring the thickness of the fibrous capsule are presented in Graphic 3 and the adjusted *P*-values for this parameter is represented in Table 5.



Graphic 3 – Fibrous capsule thickness in μm (8 days and 30 days).

Table 5 – Adjusted *P*-values for the fibrous capsule thickness of the four experimental groups (8 and 30 days).

	Thickness of the fibrous capsule (8 days)				Thickness of the fibrous capsule (30 days)			
	Control	**AH Plus**	**TotalFill BC Sealer**	**TotalFill BC Sealer HiFlow**	**Control**	**AH Plus**	**TotalFill BC Sealer**	**TotalFill BC Sealer HiFlow**
Control	-	>0,9999	0,0049*	0,0376*	-	0,7328	0,0006*	0,0039*
AH Plus	>0,9999	-	0,0249*	0,1455	0,7328	-	0,1140	0,3725
TotalFill BC Sealer	0,0049*	0,0249*	-	>0,9999	0,0006*	0,1140	-	>0,9999
TotalFill BC Sealer HiFlow	0,0376*	0,1455	>0,9999	-	0,0039*	0,3725	>0,9999	-

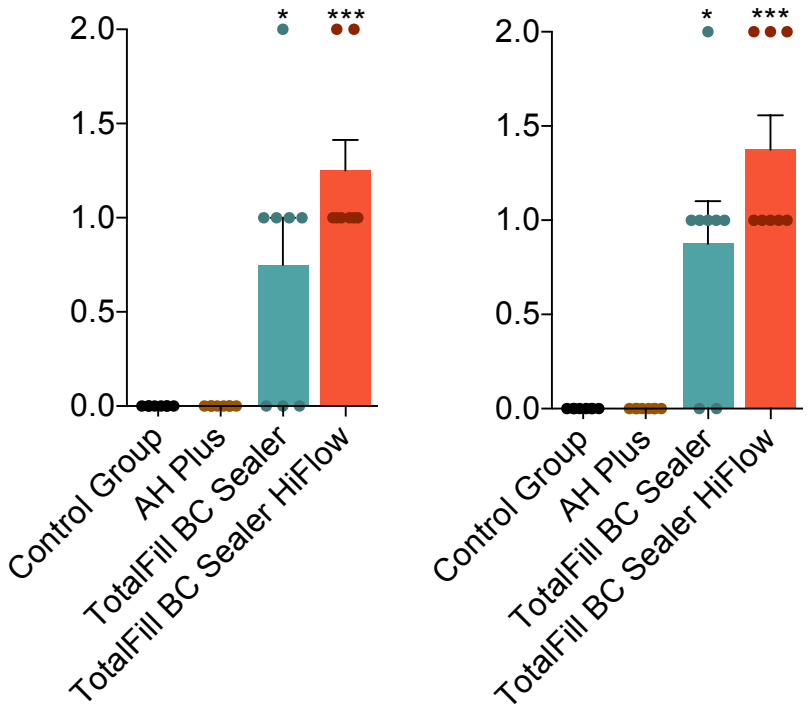
*Statistically significant

The last parameter analyzed was the mineralization potential and we found significant effects in the first period ($H_{(3)}$ = 22,55, p < 0,0001). TotalFill BC Sealer HiFlow had the greatest area of mineralization. TotalFill BC Sealer HiFlow and control (1,250 ± 0,1637, n = 8; 0,000 ± 0,000, n = 8; p = 0,0005) showed statistically significant differences as well as TotalFill BC Sealer HiFlow and AH Plus (1,250 ± 0,1637, n = 8; 0,000 ± 0,000, n = 8; p = 0,0005).

At day 30, the effects on the mineralization remained significant ($H_{(3)}$ = 24,13, p < 0,0001) and TotalFill BC Sealer HiFlow continued to show the greatest mineralization potential. The groups

that presented significant differences were: TotalFill BC Sealer and control (0,8750 ± 0,2266, n = 8; 0,000 ± 0,000, n = 8; p = 0,0382), TotalFill BC Sealer HiFlow and control (1,3750 ± 0,1830, n = 8; 0,000 ± 0,000, n = 8; p = 0,0004), TotalFill BC Sealer and AH Plus (0,8750 ± 0,2266, n = 8; 0,000 ± 0,000, n = 8; p = 0,0382) and TotalFill BC Sealer HiFlow and AH Plus (1,3750 ± 0,1830, n = 8; 0,000 ± 0,000, n = 8; p = 0,0004).

Graphic 4 shows the mean results regarding the mineralization potential and Table 6 the obtained adjusted *P*-values.



Graphic 4 – Results of the mineralization potential (8 and 30 days).

Table 6 – Adjusted *P*-values for the mineralization potential of the four experimental groups (8 and 30 days).

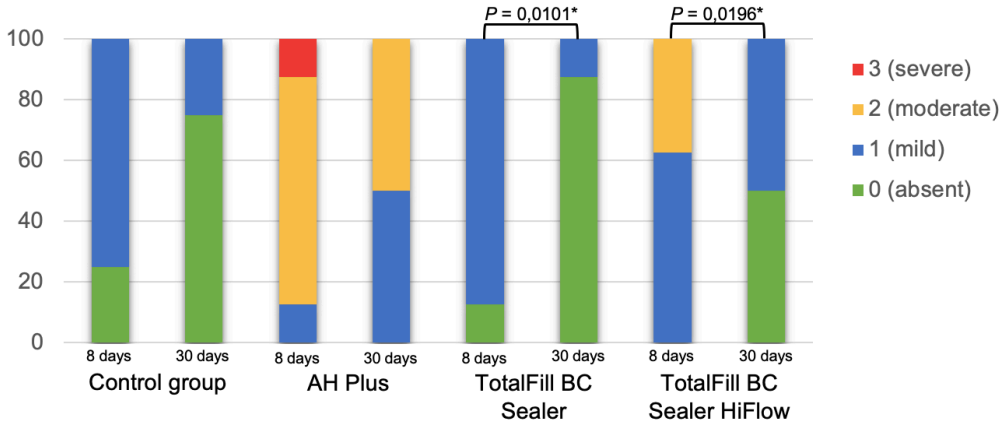
	Mineralization (8 days)				Mineralization (30 days)			
	Control	**AH Plus**	**TotalFill BC Sealer**	**TotalFill BC Sealer HiFlow**	**Control**	**AH Plus**	**TotalFill BC Sealer**	**TotalFill BC Sealer HiFlow**
Control	-	>0,9999	0,0944	0,0005*	-	>0,9999	0,0382*	0,0004*
AH Plus	>0,9999	-	0,0944	0,0005*	>0,9999	-	0,0382*	0,0004*
TotalFill BC Sealer	0,0944	0,0944	-	0,7585	0,0382*	0,0382*	-	>0,9999
TotalFill BC Sealer HiFlow	0,0005*	0,0005*	0,7585	-	0,0004*	0,0004*	>0,9999	-

*Statistically significant

5.6. TIME COMPARISON

We found significant effect on the inflammatory reaction between day 8 and day 30 (Graphic 5) only for the TotalFill BC Sealer (0,8750 ± 0,1250, n = 8; 0,1250 ± 0,1250, n = 8; p = 0,0101) and TotalFill BC Sealer HiFlow (1,375 ± 0,1830, n = 8; 0,500 ± 0,1890, n = 8; p = 0,0196). There were no statistically significant differences in the remaining groups for the inflammatory reaction when comparing the results after eight and thirty days. Nevertheless, the scores decreased in all experimental groups from 8 to 30 days.

Finally, there were no statistically significant differences between time periods for the macrophage infiltrate, fibrous capsule thickness and mineralization parameters, for each experimental group.



Graphic 5 – Inflammatory reaction scores at 8 and 30 days.

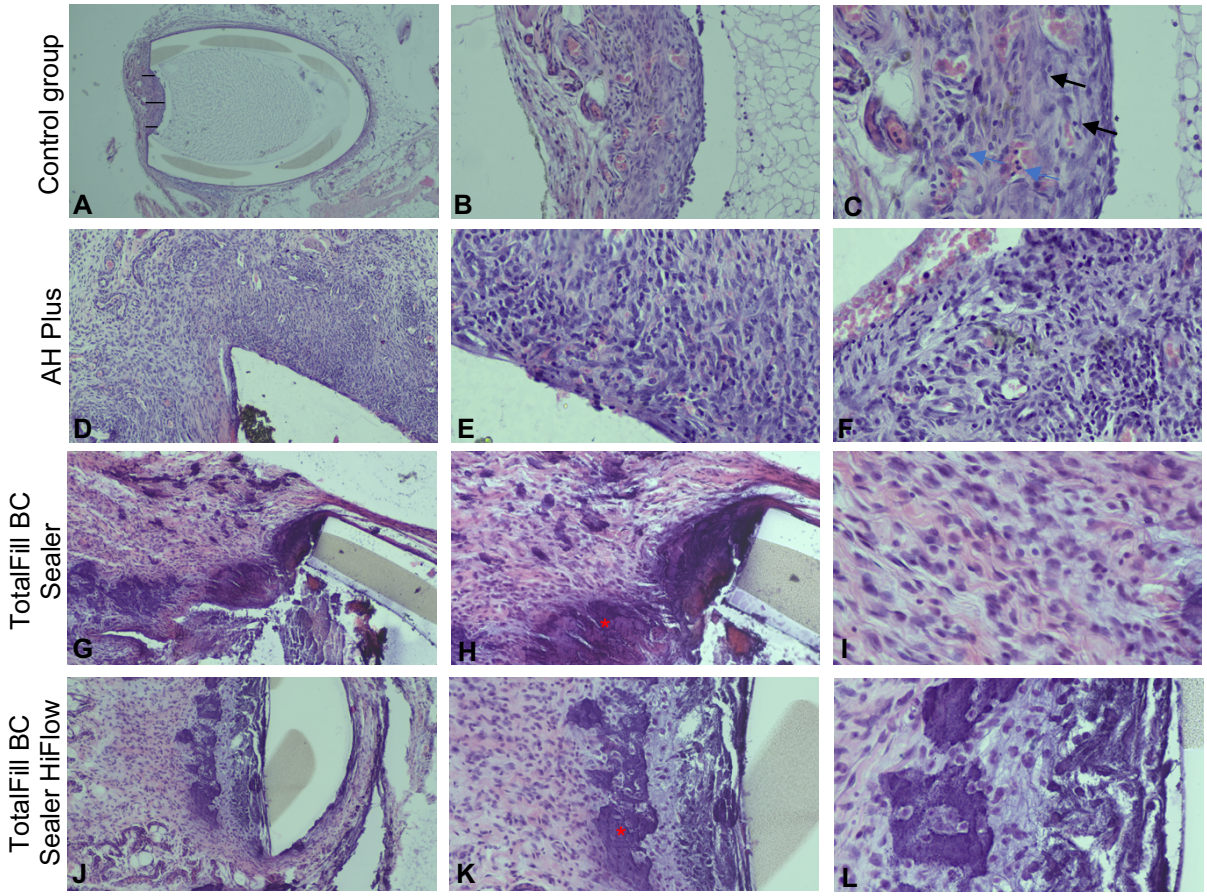


Figure 4 – Histological sections of the interface tissue-implant 8 days after subcutaneous implantation (hematoxylin and eosin staining). A) Control group, 40x magnification evidencing the thin fibrous capsule at the interface between the host tissue and the polyethylene tube (measured in 3 points represented by the black lines). B) 200x magnification the fibrous capsule and mild inflammatory reaction (score 1) at the interface tissue-implant. C) 400x magnification showing in detail the cellular population consisting of PMN (blue arrows) and fibroblasts (black arrows). D) AH Plus group, 100x magnification showing granulation tissue surrounding the polyethylene tube. E) 200x magnification evidencing small congested neo capillaries, fibroblasts and moderate inflammatory reaction (score 2). F) 400x magnification showing the inflammation with mainly lymphocytes and neutrophils. G) TotalFill BC Sealer group, 100x magnification showing a fibrous capsule with calcification (bluish deposits). H) 200x amplification where we can see the fibroblasts and some inflammatory cells (score 1) and the calcified area in more detail (red asterisk). I) 400x magnification showing the fibroblasts (fusiform cells) in a stroma with some collagen fibrils and some lymphocytes, plasma cells and rare neutrophils. J) TotalFill BC Sealer HiFlow, 100x magnification revealing a fibrous capsule with calcification next to the polyethylene tube. K) 200x magnification where we can observe fibroblasts, and inflammatory cells (score 1) next to the calcified area. L) 400x magnification detailing the calcified area (red asterisk). Fibroblasts (fusiform cells) are seen in an edematous and low collagenous stroma. Some lymphocytes are also observed.

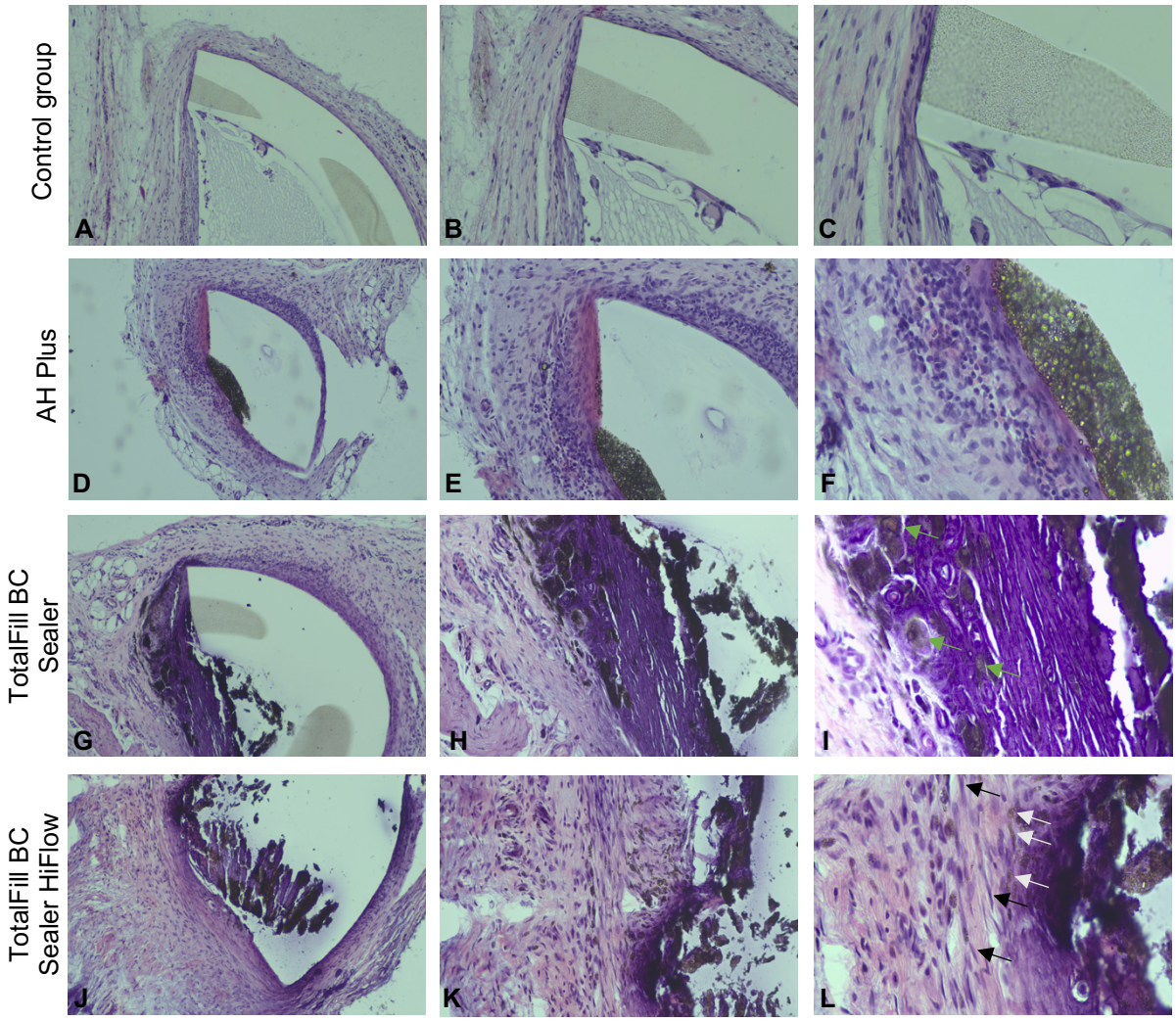


Figure 5 – Histological sections of the interface tissue-implant 30 days after subcutaneous implantation (hematoxylin and eosin staining). A) Negative control, 100x magnification showing the polyethylene tube and content surrounded by a thin fibrous capsule. B) 200x magnification detailing the fibrous capsule with fibroblasts and some inflammatory cells (score 1). C) 400x magnification showing fibroblasts in collagenous stroma and some lymphocytes adjacent to the polyethylene tube. D) AH Plus, 100x magnification showing fibro-inflammatory reaction around the polyethylene space and the material. E) 200x magnification demonstrating a thin fibrous capsules and inflammatory cells (score 2) next to the polyethylene tube and the material. F) 400x magnification where we can observe a thin bundle of fibroblasts surrounded and permeated by lymphocytes and rare neutrophils. G) TotalFill BC Sealer group, 100x magnification revealing a thick fibrous capsule with extensive calcification. H) 200x magnification showing intense calcification (bluish aspect) in the fibrous capsule. Note the material immediately adjacent to the calcified capsule (red asterisk). I) 400x magnification demonstrating birefringent material deposited in the fibrous and calcified capsule (orange arrows) J) TotalFill BC Sealer HiFlow, 100x magnification revealing the material inside the polyethylene tube, surrounded by a fibrous capsule and calcification of almost the entire capsule thickness. K) 200x magnification with similar aspects. Note the fragmentation of the calcified area/tissue. M) 400x magnification revealing in detail

the fibroblasts (fusiform cells – black arrow), collagen deposits and birefringent material in the fibrous capsule thickness (white arrow).

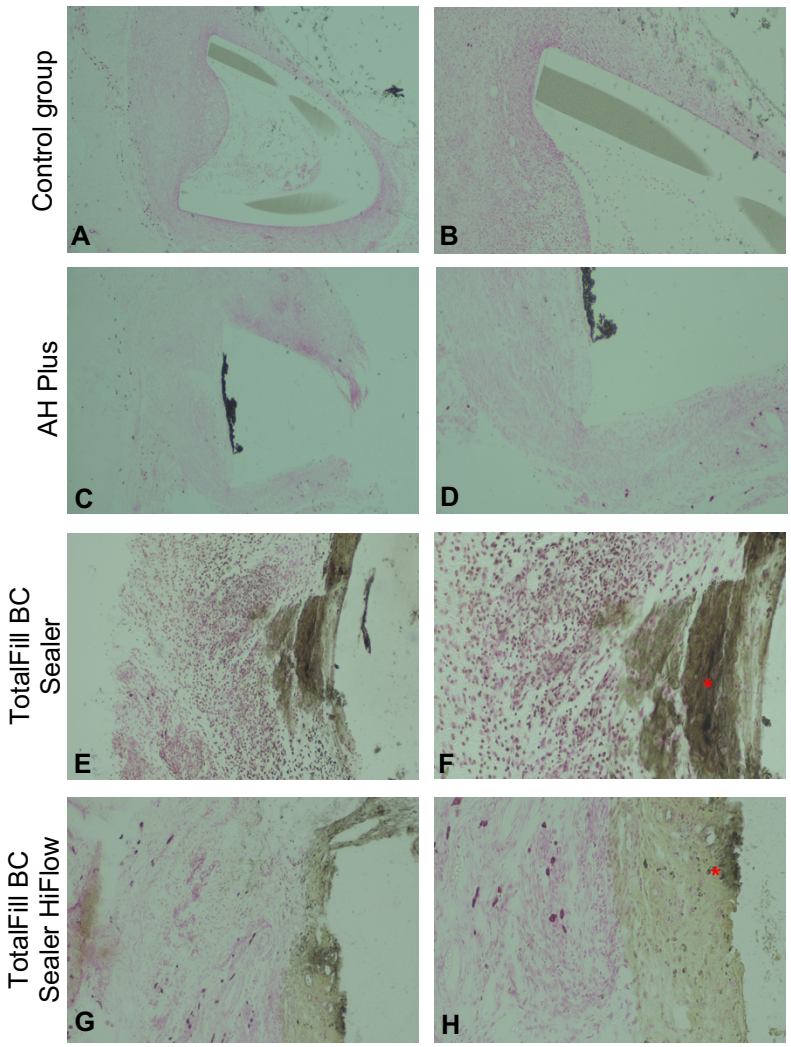


Figure 6 – Histological sections of the interface tissue-implant 8 days after subcutaneous implantation (Von Kossa staining). A) Control group, absence of mineralization. 40x magnification. B) 200x magnification showing a fibro-inflammatory reaction with no mineralization. C) AH Plus group, absence of mineralization, 40x magnification. D) 200x magnification showing the material next to the fibro-inflammatory capsule with no mineralization. E) 200x magnification showing area of mineralization in the capsule (brownish area). F) 400x magnification detailing Von Kossa positive structures (red asterisk) in the capsule and surrounded by lymphocytes and some neutrophils. G) TotalFill BC Sealer HiFlow, 200x magnification showing fibrous area with mild mineralization (right brownish area). H) 400x magnification revealing Von Kossa positive structures (red asterisk) in the fibro-inflammatory capsule.

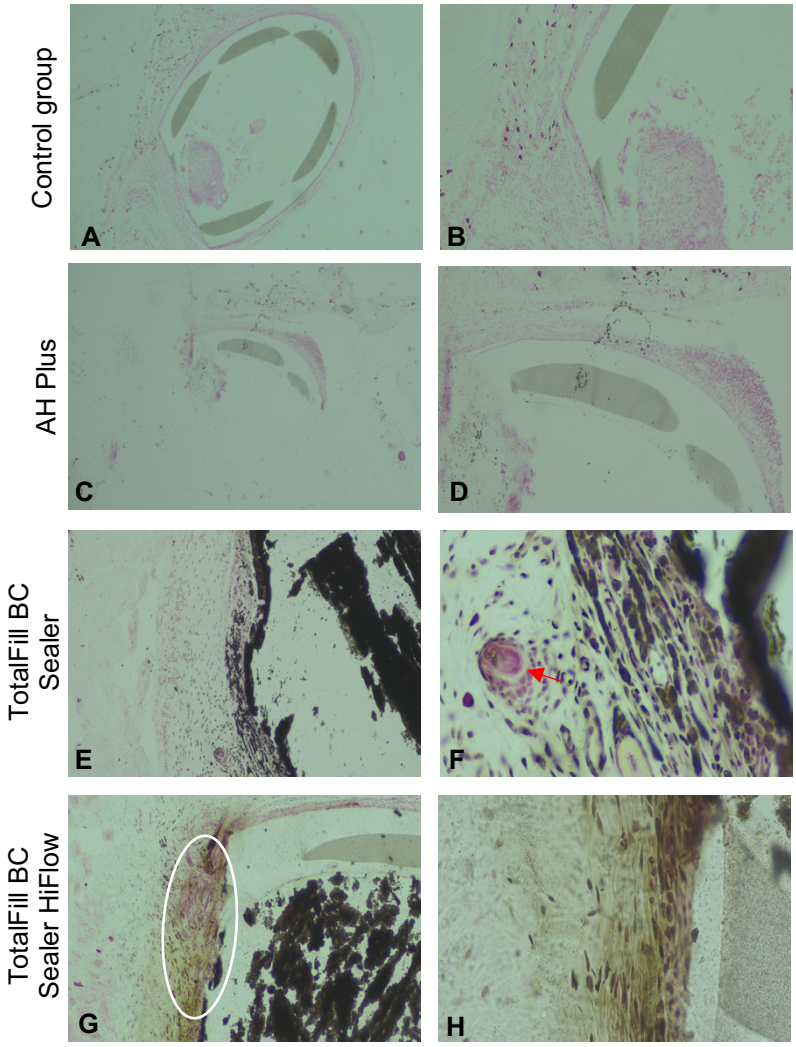


Figure 7 – Histological sections of the interface tissue-implant 30 days after subcutaneous implantation (Von Kossa staining observed with polarized light). A) Control group, absent mineralization, 40x magnification. B) 200x magnification. No mineralization observed in the fibro-inflammatory thin capsule. C) AH Plus group, absent mineralization, 40x magnification. D) 200x magnification. The polyethylene tube is surrounded by thin fibrous capsule with inflammatory cells. No mineralization was observed with Von Kossa staining. E) Total Fill BC Sealer, 200x magnification showing moderate mineralization (blackish area). F) 400x magnification showing birefringent material and mild mineralization in between fibroblasts and surrounded by some inflammatory cells like lymphocytes, plasma cells and rear neutrophils. We can also see edema (red arrow). G) TotalFill BC HiFlow 200x magnification demonstrating moderate mineralization (brownish aspect) in the thin fibrous capsule (white area) and the material (black) inside the polyethylene tube. H) 400x magnification. Mineralization is observed in the fibrous capsule next to the polyethylene tube. Fibroblasts are easily recognized as some lymphocytes and rare neutrophils.

6. DISCUSSION

Endodontic sealers must provide a reduced cell toxicity and low inflammatory reaction or at least insignificant or mild, in order to be considered biocompatible (24). Recent materials are different in their chemical composition and also in physical properties, and for this reason their effects on human tissues need to be tested before its clinical use (25).

In vitro cytotoxicity assays have some limitations and therefore *in vivo* studies are required to investigate the complex cellular and molecular events involved in the immunoinflammatory response induced by endodontic sealers, which may help tissue repair or sustain chronic inflammatory reaction (24,26). Subcutaneous implantation in connective tissue of rats is one of the most adequate tests for determining the type and development of local reactions induced by experimental materials (1,2,27). The outcome of the tested material in studies performed in animals, such as subcutaneous implantation, should be similar to the results of the control group of the study in order to be considered not toxic and biocompatible (25).

The present study shows that TotalFill BC Sealer and TotalFill BC Sealer HiFlow induced lower inflammatory response when compared to AH Plus, rejecting the null hypothesis and confirming that calcium-silicate based sealers present proper biological properties (8,16,22). This results from the basic composition of the sealers and may also be related to the presence of zirconium oxide in TotalFill BC which has been associated with lower inflammatory reaction than sealers with barium oxide in their composition (19,28).

To the best of our knowledge, this is the first *in vivo* study evaluating TotalFill BC Sealer HiFlow biocompatibility in subcutaneous tissue. This material was developed with the aim of having a chemical composition similar to its predecessor, TotalFill BC Sealer, and being efficient with warm compaction techniques, according to its manufacturer. One previous study reported that the setting time of a sealer (ie iRoot; Innovative BioCeramix Inc., Vancouver, Canada) with a composition similar to TotalFill BC Sealer, decreased from 4 hours to only 14 minutes, when heated at 140º (15). The flow ability of this sealer was also assessed in the same study and at high temperatures TotalFill BC Sealer showed a significant flowability reduction. The flow ability and adequate setting time are important features for endodontic sealers in order to seal irregularities in dentinal walls and the apical foramen (15). Atmeh *et al.* concluded that TotalFill BC sealer showed reversible changes when heated at temperatures above 125º, however higher temperatures are not recommended for calcium silicate-based sealers due to the risk of affecting the integrity of the root canal filling materials (29). Regarding the new sealer formulation, Chen *et al.* reported that physicochemical properties of TotalFill BC Sealer HiFlow remained stable when exposed to temperatures present in the root canal during warm vertical compaction (8). Under the referred conditions and according to our results TotalFill BC Sealer HiFlow presented low inflammatory reaction scores after 8 days implantation, and the

inflammatory reaction decreased at 30 days, showing a transient nature and reflecting and appropriate biocompatibility profile.

The inflammatory reaction present in the first time period may be caused by surgical trauma during the placement of the polyethylene tube and also due to eventual toxic effects of the implanted sealers (1,26). Besides, when using calcium-silicate based cements, it is normal to occur an initial inflammatory reaction due to the high alkalinity of these materials, negatively influencing cellular metabolism (24,26,27). During setting, pH value increase and heat is generated by the release of calcium ions from these materials. These phenomena can explain the inflammatory cell recruitment and production and liberation of proinflammatory cytokines (24).

The results of the inflammatory reaction in the AH Plus group were significantly higher than other groups, which might be due the toxicity exhibited when freshly mixed which reduces after setting (3). Release of mutagenic components of AH Plus, minute amounts of formaldehyde and amines may explain this initial cytotoxicity (1,3,30). These findings are in accordance with previous studies with epoxy resin-based sealers that stated that these sealers provide a more intense and longer inflammatory reaction (1,31). Nevertheless, the severity of the inflammatory reaction decreased after 30 days, not only in the calcium silicate-based sealers but also for AH Plus.

These results cannot be directly extrapolated to humans (1), however, in this experimental context, tubes were filled with freshly mixed sealers and extrusion of the materials to the surrounding tissues was promoted and the contact area corresponded to a circle with a minimum of 0.9mm, the inner diameter of the tubes. This is a worst-case scenario than the usual clinical situation where the size of the *foramen* of a prepared canal is smaller (around 0.3 to 0.6mm). As a consequence, sealer particles directly interact with tissues surrounding the open ends of the tubes inducing reactions that might be superior to those found in clinical practice. Also, there has been demonstrated that after setting, AH Plus was no longer cytotoxic to fibroblasts (22). Therefore, we may assume that under regular clinical conditions, all sealers tested in this study showed good biocompatibility.

Calcium silicate-based sealers showed, *in vitro*, potential to stimulate osteoblastic differentiation and promote overexpression of osteo/cementogenic genes (16). Histologically, this capacity can be evaluated by Von Kossa histochemical staining technique which allows the detection of calcium precipitates, in order to assess the bioactivity of these materials (27,32). Both TotalFill BC Sealer and TotalFill BC Sealer HiFlow demonstrate ability to induce mineral deposition shortly after implantation. This may be explained by the alkalinity of the medium provoked by the calcium ions release, therefore stimulating the formation of hydroxyl apatite and the release of bone morphogenic protein 2 and alkaline phosphatase, thus

contributing for the mineralization process (24,27). In order to identify amorphos calcite deposits, the Von Kossa birefringence technique was performed and both calcium silicate sealers presented irregular structures with calcium deposits in the adjacent capsule at 8 days. AH Plus did not show Von Kossa positive structures. The calcium silicate-based sealers showed birefringent mineralized structures in both periods.

Studies with calcium-silicate based sealers suggests that these materials produce calcified tissue through the release of calcium when in contact with tissues' fluids (19,21,32). When they set their pH becomes superior to 12 due to the hydration reaction in which calcium hydroxide is formed followed by dissociation into hydroxyl and calcium ions (hydration reaction) (33). When in contact with tissue fluids, hydraulic calcium silicate-based sealers release calcium hydroxide which interact with phosphates present in tissue fluids to form hydroxyapatite. This reaction may explain the tissue inductive properties of calcium silicate-based materials (precipitation reaction) (21).

$$2(2CaO \cdot SiO_2) + 4H_2O \rightarrow 3CaO \cdot 2SiO_2 \cdot 3H_2O + Ca(OH)_2$$

$$2(3CaO \cdot SiO_2) + 6H_2O \rightarrow 3CaO \cdot 2SiO_2 \cdot 3H_2O + 3Ca(OH)_2$$

(Dicalcium and tricalcium silicates hydration reactions in contact with water)

$$7Ca(OH)_2 + 3Ca(H_2PO_4)_2 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 12H_2O$$

(Precipitation reaction of bioceramics)

Seo *et al.* and Candeiro *et al.* assessed, respectively, the calcium nodule formation and the release of calcium ions of AH Plus and EndoSequence BC Sealer, a sealer with a similar composition to TotalFill BC Sealer. Results from the *in vitro* study of Seo *et al.* show that EndoSequence BC had a significant increase in calcium nodule formation when compared to AH Plus. Candeiro *et al.* evaluated the calcium ion release by using an atomic absorption spectrophotometer and stated that the amount of Ca^{2+} released from AH Plus was far lower than from EndoSequence BC Sealer (3,34). Calcium ions released during setting interact with carbon dioxide in the tissues and originates deposits that are birefringent under polarized illumination (28). Therefore, the results obtained in our study are in accordance with these previous studies regarding mineralization potential as both calcium silicate-based sealers evidence Von Kossa positive structures at both observation periods.

The presence of a fibrous tissue capsule around the tube indicates that the material is being well tolerated by the surrounding tissues (2,24,27,35). Besides, Scarparo *et al.* stated that the existence of an organized fibrous capsule prevents the inflammatory reaction from extending to regions distant from the area in contact with the material, since it limits the area of inflammation (36). Our results are in accordance with these authors since TotalFill BC Sealer showed a significantly thicker capsule than the control group and AH Plus and TotalFill BC

Sealer HiFlow had a thicker capsule than the control group, and therefore, a good adaptation from the rats' connective tissue to these materials. In contrast, Mussel *et al.* suggested that the amount of fibrosis around the material is inversely related to its biocompatibility (25,37).

The presence of macrophages demonstrates the organism's attempt to eliminate the foreign material through phagocytosis (24) and to clear necrotic tissues formed due to previous tissue injury (25). AH Plus showed a decrease in the number of macrophages between both time periods and the TotalFill BC Sealer HiFlow group was associated with higher macrophage infiltrate in the longer observation period. Macrophages were found in the areas of capsules, indicating that the cellular debris and remnants of extracellular matrix may be internalized by phagocytic cells. Besides, the maintenance of a high number of macrophages in BC sealer and BC sealer HiFlow at the longer period may be associated with higher solubility (27), promoting the release of substances and the formation of calcific precipitates (19). Moreover, Xia *et al.* stated that macrophages play an important role in tissue regeneration and when macrophage infiltration is prevented, then healing is impaired. Macrophages have the ability to phagocyte the debris of damaged tissue, necrotic and apoptotic cells and also to produce a spectrum of enzymes for tissue reorganization. Besides, they secrete growth factors for fibroblast, keratinocyte proliferation and bone formation. Finally, macrophages are a resource for the release of angiogenic molecules, which are necessary for the angiogenesis, a fundamental process to wound healing and tissue regeneration (38). Hereupon, the observed increase in the macrophage infiltrate after 30 days for the TotalFill BC Sealer HiFlow group may indicate an attempt of regeneration of the connective tissue.

The similar results obtained with TotalFill BC Sealer and TotalFill BC Sealer HiFlow indicate that this new formulation maintained the good biocompatibility levels and also the ability to form mineralized tissue. TotalFill BC Sealer presented the best performance of all tested sealers and TotalFill BC Sealer HiFlow provided the greatest induction of mineralized tissues. Nevertheless, all sealers showed an adequate biocompatibility profile in the end of the study.

7. CONCLUSION

This study indicates that both TotalFill BC sealer and TotalFill BC HiFlow are biocompatible and present potential bioactivity as they favor calcium precipitation when implanted in the subcutaneous tissue.

At the longer evaluation period, the inflammatory reaction decreased, and all testes sealers presented an adequate biocompatibility profile.

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10. APPENDIX I – Composition of the sealers

Table 1 – Composition of the sealers according to manufacturers.

SEALER	MANUFACTURER	COMPOSITION	PREPARATION MODE
AH Plus	Dentsply DeTrey, Konstanz, Germany	Epoxide paste: diepoxide, calcium tungstate, zirconium oxide, aerosil, pigment; Amine paste: 1-adamantane amine, N, N'-dibenzyl-5-oxa-nonandiamin-1,9, TCD-diamine, calcium tungstate, zirconium oxide, aerosil and silicon oil	The components are mixed in equal proportions of the base and the catalyst paste
TotalFill BC Sealer	FKG, La Chaux-des-Fonds, Switzerland	Zirconium oxide, calcium silicates, calcium phosphate monobasic, calcium hydroxide, filler and thickening agents	Single syringe
TotalFill BC Sealer HiFlow	FKG, La Chaux-des-Fonds, Switzerland	Zirconium oxide, tricalcium silicate, dicalcium silicate, calcium hydroxide and fillers	Single syringe

11. APPENDIX II – Histology technique

1. Indispensable equipment

- Cool plaque – TES 99410 Medite, Germany
- Hot plaque – OTS 403040
- Drying oven – Trade Raypa
- Incubator – Gallenkamp – Economy Incubator with fan, size 1. Garal, Maia, Portugal.
- Hatt Super Chemo Work Station Pbinternacional;
- Water-bath – FALC Italy;
- Microtome – Leica RM 2155, Leica, Portugal
- Paraffine dispenser – TES 99200 Medite, Germany
- Slide boxes – Kartell, Spa, Italy

2. Required supplies

- Formaldehyde solution 10% neutralized, stabilized with methanol, Panreac, Spain
- Ethanol absolute – AGA, Portugal
- Xylene, mixture of isomers, Panreac, Spain
- Mounting medium – DPX, FLUKA, Germany
- Paraffin - Paraplast Regular, Sigma Aldrich, USA
- Microscope slides – Menzel, glaser with 90º matt strip, Germany
- Cover slips – Menzel, Glaser 22 diameter – Germany
- Microtome blades – S35 Feather, Japan
- Tissue embedding cassettes – Kartell Spa, Italy

3. Methodology

a) Fixation

10% formaldehyde - formaldehyde solution 10% neutralized, stabilized with methanol, Panreac, Spain

b) Dehydration

As in the calcified technique, dehydration is essential for processing these samples. Also, it is important to notice that time periods vary depending on the thickness and size of the samples.

Dehydration protocol:

- The biological material is subjected to ascending ethanol series:
- 1st step: 60% ethanol hours to days
- 2nd step: 80% ethanol hours to days
- 3rd step: 90% ethanol hours to days
- 4th step: 96% ethanol from 2 hours to days with intercalary changings
- 5th step: 100% ethanol from 2 hours to days with intercalary changings

c) Pre-impregnation

Before impregnation, sometimes a pre-impregnation procedure is performed using pre-impregnation agents (in case of paraffin, some of these agents may be isoparaffin H, toluol, xylene or chloroform). This step is also known as sample diaphanization.

The purpose of this procedure is to prepare the samples in order to receive the final product for impregnation and inclusion.

This can be done through increasing series:

- 1st step – 2/3 solution of 100% ethanol + 1/3 isoparaffin H during a 6h period to a few days
- 2nd step - 1/2 solution of 100% ethanol + 1/2 isoparaffin H during a 6h period to a few days
- 3rd step - 1/3 solution of 100% ethanol + 2/3 isoparaffin H during a 6h period to a few days
- 4th step – pure isoparaffin H during a 6h period to a few days
- 5th step – then the biological material is directly impregnated as follows:
- 6th step – the biological material is placed in a saturated solution of a combination of isoparaffin H and paraffin for a 2 to 12 hours period and then goes to the drying oven between 12 to 24 hours in pure paraffin.

Or we can do a direct pre-impregnation with 100% xylene and mechanical agitation continues for a period from 6 to 48 hours with changings in between the respective times. In our study, direct pre-impregnation was carried out.

d) Impregnation

In this procedure, sample impregnation is performed in an embedding medium (usually paraffin) in order to obtain thin cuts (microtomy).

Impregnation is made with liquid paraffin. This is achieved by placing the paraffin in the drying oven at a temperature that makes the paraffin liquid, during a period between 6 to 48 hours. It is important to keep two containers with liquid paraffin, so that the samples move from one container to another, after half programmed time.

e) Inclusion

A basic inclusion procedure must include the following steps:

- 1st step: place the sample in a Leuckart mold or bar
- 2nd step: fill the mold or bar with liquid (molten) paraffin
- 3rd step: wait for the paraffin to solidify
- 4th step: remove the bar from the mold, thus obtaining the block with the sample
- 5th step: paraffine heating until it is in a liquid state or using a paraffine dispenser (TES 99200 Medite, Germany, represented by Reagente 5 Química e Eletrónica Lda.). A little amount of liquid paraffine is poured to fill the bottom of the commercial metallic form and the biological material is placed in the bottom with the aid of forceps. The sample must be placed parallel to the sides of the form. Then, the entire form is filled with liquid paraffin. The commercial plastic cassette is placed on the surface of the mold so that when the paraffin solidifies, it remains attached to the surface of the paraffin block that contains the samples. After this, they are taken to the cold (refrigerator) or cool plate (TES 99410 Medite, Germany) until it solidifies and unmolds. In the end, the final touches are carried out with the aid of a scalpel.

f) Microtomy

The sections are obtained through the use of specific devices, the microtomes, for the optical microscope, and the ultramicrotomes, for transmission electron microscopy. The microtomes allow us to obtain thin sections (between 5 to 20μm) while the ultramicrotomes allow to obtain much thinner sections (between 100μm or less).

The microtome of the figure X1 consists of a crank that allows the advance or retreat of the arm that supports the cassette with the sample, vertical movements and constant advances are performed, forcing the sample to pass through a steel knife to obtain the sections.

Figure 1- Microtome – Leica RM 2155, Leica, Portugal

- Microtomy protocol:

The paraffin block containing the sample is thinned out until it appears on the surface of the block. After this, consecutive sections are made with 5 to 10 sections and placed in a tray containing 30% ethanol solution, in a certain order. This step is repeated until the entire sample is finished. Thus, several sequences of several sections are arranged on the board with approximately 5 to 8 μm. Without losing their orientation, we separate the samples one by one and place them in a water-bath (FALC, Italy), according to a certain orientation. Then we move the sample to the slides. The process is repeated until collecting the last sample for the last cut.

The slides are placed on a hot plaque (OTS 403040) in order to allow a good adhesion of the samples to the slides (previously impregnated in glue).

They are placed in a drying oven until coloration procedure. A 4hour period in the drying oven must be respected.

Figure 2 – Water bath (FALC; Italy)

Figure 3 - Hot plaque (OTS 403040)

- Gelatin-coated slides:

Gelatin	3gr / 1,5gr
Distilled water	1000mL / 500mL

Dissolve until 60°C (more or less)

Add:

Chromium alum	0,5gr / 0,25 gr

Thymol / Crystals – shake until dissolve

Filter in hot temperature

- Slides treatment:

1st step – place the blades on the metal supports

2nd step – dip the slides in ether / ethanol during 10minutes

3rd step – put the slides through distilled water 2 times

4th step – put the slides through 96º ethanol

5th step – dry in the drying oven – it is faster and does not collect dust

6th step – dip the slides in the already filtered glue – drain well

7th step – dry in the drying oven or at room temperature

g) Staining technique

In optical microscopy, stain procedure is made with chemicals called dyes. Most cytological dyes are aromatic organic substances, which can be natural from animal origin (carmine), natural from plants origin (hematoxylin) or artificial (acid fuchsin).

Dyes have two very important components: the chromophore, which is responsible for the color of the dye, and the auxochrome or auxochromium, responsible for the electrolytic dissociation and for the binding of the dye to the cellular components. A chromophore associated with an aromatic hydrocarbon molecule is called chromogen, and a chromogen associated with an auxochrome is called a dye. Dyes can be acidic if they have an anionic chromophore group and an auxochromic group (which are negatively charged), or basic if they have a cationic chromophore group and an auxochromic group (which are positively charged). Structures that have an affinity for acid dyes are called acidophiles (positive charge) and structures that have an affinity for basic dyes are called basophils (negative charge).

The staining of cell structures depends on several factors, such as the type of dye used (whether acidic or basic), the pH value and the isoelectric point (where the molecule has a zero total charge, because there is a balance between the positive and negative charges of the dissociated acid and basic groups).

Each histological study requires a certain type of staining, depending on what we want to observe, and the application mode varies depending on the dye that was chose. However, the protocol for the initial treatment of the slides (hydration / dehydration) remains the same, regardless on the dye we intend to use.

In our study, we used Hematoxylin-eosin staining.

h) Hematoxylin-eosin staining

Dewax

1st step – xylene I – 10/15 minutes

2nd step – xylene II – 10 minutes

Hydration process initiates:

3rd step – 100% ethanol I – 10 minutes

4th step – 96% ethanol II – 10 minutes

5th step – 70% ethanol I – 10 minutes

6th step – distilled water – wash

7th step – running water – 10 minutes

8th step – distilled water – wash / drain well

Hydration process ends (dye is applied):

9th step – hematoxylin – 20 minutes

10th step – distilled water – wash 2 times

11th step – running water – 10minutes

12th step – distilled water – wash / drain well

13th step – eosin – 5 minutes

Dehydration process initiates:

14th step – 96% ethanol – splash

15th step – 96% ethanol – dip for a few seconds

16th step – 96% ethanol I – 1 minute and drain off

17th step – 100% ethanol II – 1 minute and drain off

18th step – 100% ethanol I – 2 minutes and drain off

19th step – xylene II – 5 minutes

20th step – clean xylene – 10 to 15 minutes

Immediate placement of the mounting medium (DPX, FLUKA).

Goes to the drying oven for a period of 24 to 48 hours