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Preliminary study of the inflammatory response to subcutaneous implantation of three root canal sealers

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Abstract

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Aim To evaluate the kinetics of the inflammatory tissue response to three root canal sealers using a physicochemical method for quantification of the enhanced vascular permeability and histopathological analysis.

Methodology Twenty-eight male Wistar rats randomly assigned to four groups according to the evaluation periods (1, 3, 7 and 14 days) were used to assess the vascular permeability and histopathological reaction to RoekoSeal, AH Plus and Sealapex (new formulation) sealers, using saline and Chloropercha as negative and positive controls, respectively. Seven rats were sacrificed per period. The biocompatibility of the sealers was evaluated spectrophotometrically and histopathologically.

Results At day 14, Sealapex produced significantly more inflammatory exudate than AH Plus and RoekoSeal ($P < 0.05$); however, there was no significant difference between AH Plus and RoekoSeal ($P > 0.05$). Sealapex (new formulation) was the most irritating sealer, producing severe inflammation with the presence of multinucleated giant cells. RoekoSeal was the most biocompatible sealer, producing the least amount of inflammatory exudate.

Conclusions RoekoSeal root canal sealer was biocompatible when implanted in connective tissue.

Keywords: plasma extravasation, root canal sealers, tissue compatibility.

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Introduction

Root canal sealers are available in different formulations based on epoxy resins, calcium hydroxide [$Ca(OH)_2$], zinc oxide-eugenol (ZOE) and silicone. Most sealers use zinc oxide as the base ingredient with eugenol being the liquid component. Recently introduced sealers are polymers and include epoxy resin-based sealers, such as AH26 and AH Plus. AH26 is toxic when freshly prepared and this toxicity is attributed to the release of a small amount of formaldehyde as a result of the chemical setting process (Leonardo *et al.* 1999b). In a previous study (Huang *et al.* 2002), AH Plus revealed a lower cytotoxicity potential compared to AH26, but both sealers caused a dose-dependent increase in genotoxicity. On the other hand, the manufacturers of sealers such as Sealapex claim the benefits of the biological effects of the added $Ca(OH)_2$ (Silva *et al.* 1997), which stimulates hard tissue deposition and repair (Jaunberzins *et al.* 2000). However, it is still unclear how this process occurs. Silicone-based sealers, such as RoekoSeal, have been introduced recently as a new alternative for root canal filling. Silicone is inert and biocompatible and has been widely

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used in medicine as an implant material (Deva *et al.* 1998).

Previous studies (Figueiredo *et al.* 2001, Huang *et al.* 2002, Kaplan *et al.* 2003, Miletic *et al.* 2005) have reported that the tissue response of different types of root canal sealers varied considerably and concluded that the amount of sealer placed into a root canal, the contact area between the filling material and the adjacent soft and hard tissues, and interactions between dentine and leachable substances influenced significantly the tissue response of an endodontic material. Biocompatibility of root canal sealers has been characterized by several parameters, such as genotoxicity, mutagenicity, carcinogenicity, cytotoxicity, tissue compatibility and microbial effects (Figueiredo *et al.* 2001, Huang *et al.* 2002, Pulgar *et al.* 2002, Schwarze *et al.* 2002, Costa *et al.* 2003, Kaplan *et al.* 2003, Miletic *et al.* 2003, 2005). However, it is impossible to biologically characterize the materials by a single test method and their properties should rather be investigated by different laboratory and *in vivo* assays using a structured approach (Hauman & Love 2003).

The purpose of this study was to evaluate comparatively the kinetics of the inflammatory tissue response to RoekoSeal, AH Plus and Sealapex (new formulation) sealers using a physicochemical method for quantification of the enhanced vascular permeability and histopathological analysis.

Materials and methods

Animal model

Twenty-eight male Wistar rats weighing approximately 275 g were used. The rats were housed in plastic cages under climate-controlled conditions (12 h light/12 h dark; thermostatically regulated room temperature) with free access to food and tap water during the course of the study. The animals were randomly assigned to four groups according to the evaluation periods (1, 3, 7 and 14 days). All experiments were in compliance with the applicable ethical guidelines and regulations of the International Guiding Principles for Biomedical Research Involving Animals (CIOMS, 1985).

Subcutaneous injection of sealers

Each animal received five injections, corresponding to three root canal sealers - RoekoSeal Automix (silicone-based; Roeko Dental Products, Langenau, Germany), AH Plus (epoxy resin-based; Dentsply De Trey, Konstanz, Germany) and Sealapex new formulation (calcium hydroxide-based; Kerr/Sybron, Romulus, MI, USA), a negative control (saline) and a positive control (Chloropercha; Tanrac Ltd., Gävle, Sweden). Seven rats were sacrificed *per* period.

The sealers were mixed according to the manufacturers' instructions under aseptic conditions. The rats were anaesthetized with ketamine hydrochloride (Pisa SA Lab., Mexico City, Mexico), had their backs shaved and cleaned (Isodine, Boehringer Ingelheim Lab., Mexico City, Mexico), and five experimental sites were randomly demarcated. The sealers were placed into sterile hypodermic syringes (Terumo Syringe 5 cc; Medical Co., Tokyo, Japan) using a metal spatula in contact with a vibrator to prevent void formation and approximately 0.2 mL of material were injected subcutaneously into the demarcated sites through a size 5 needle attached to the syringes. The animals had their tails washed and dried, and a catheter (24 g¾''; Punzocat Equipo Médico Vizarra, Mexico City, Mexico) was introduced to facilitate the injection of 2% Evans blue (Sigma-Aldrich Quimica S.A., Toluca, Mexico; 20 mg kg^{-1} body weight), which was administered intravenously in the caudal vein using hypodermic syringes (Terumo Syringe 3 cc, Medical Co, Tokyo, Japan).

Sample recollection and quantification of the enhanced vascular permeability (spectrophotometric analysis)

The rats were sacrificed by anaesthetic overdose at the predetermined periods (1, 3, 7 and 14 days). As described by Udaka *et al.* (1970), the dorsal skin was dissected and the skin lesions were punched out with a 1-cm diameter standard steel punch set. Each piece of skin containing the lesion was placed into a test tube (Corning 9820; Corning Science Mexico, Tamaulipas, Mexico) and the dye was extracted with 10 mL of 99.9% formamide (Productos Quimicos Monterrey, Monterrey, Mexico) at 40 °C for 72 h. After this incubation period, the resulting solutions were centrifuged at 2,500 rpm for 5 min. Optical density was measured in 0.2 mL of the supernatant after glass wool filtration. Initially, the curve of absorption spectrum of Evans blue was determined and 99.9% formamide was used as a negative control. Measurements were made at 620 μm (A^{620}) in a spectrophotometer (Labsystem Multiskan MS, Labsystems, Helsinki, Finland). Each measurement was repeated five times. Transformation

of the mean absorption of each sample into μg/site was done using the following equation: μg=absorption x calculation factor (68) × total formamide volume. A high amount of dye extracted indicates more inflammatory exudate.

Histopathological analysis

Two samples of each group were evaluated in each period. The excised tissue samples were removed in a standardized circumference area around the injected material. These samples were then submitted to routine processing for histopathological analysis. The paraffin-embedded specimens were semi-serially sectioned until exposing the tested materials at a thickness of approximately 5 μm. An approximate number of 30 semi-serial sections were obtained from each specimen. All histological sections were stained with haematoxylin and eosin and examined by a trained, experienced pathologist blinded to the groups and to the specimens with an optical microscope (Motic Model., DMB5-5 Digital Biological Microscope, Micro Optic Industrial Group Co. Ltd, Xiamen, China) and using the Motic Image Advanced 3.0 software (Micro Optic Industrial Group Co. Ltd) to determine the presence of granulation tissue, neutrophils, macrophages, plasma cells and fibroblasts. For the histopathological analysis, the most representative characteristics of the inflammatory infiltrate regarding the type (acute or chronic) and intensity (mild, moderate or severe) in each group were described qualitatively.

Statistical analysis

Data were analysed statistically by a nonparametric Tukey's multiple-comparison post hoc test to assess differences among the groups. Statistical significance was defined at $P < 0.05$ in a two-tailed test, using a JMP 4.0 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Spectrophotometric analysis

The results of the spectrophotometric analysis are presented in Table 1. There was no statistically significant difference ($P > 0.05$) among the tested sealers at 24 h. At the 3-, 7- and 14-day periods, RoekoSeal had the lowest absorbance values, followed by AH Plus and Sealapex. These results indicate that RoekoSeal produced the least amount of inflammatory exudate, differing significantly from the other materials and controls ($P < 0.05$).

Histopathological analysis

The findings of the histopathological analysis revealed a correlation with the results of the spectrophotometric analysis. For the negative control, a mild irritation at the site of injection of the saline solution was evident both at 24 h and at 3 days. No evidence of irritation or inflammatory response was found at 7 and 14 days (Fig. 1). For the positive control, an immediate local irritation was noted 24 h after injection of Chloropercha.

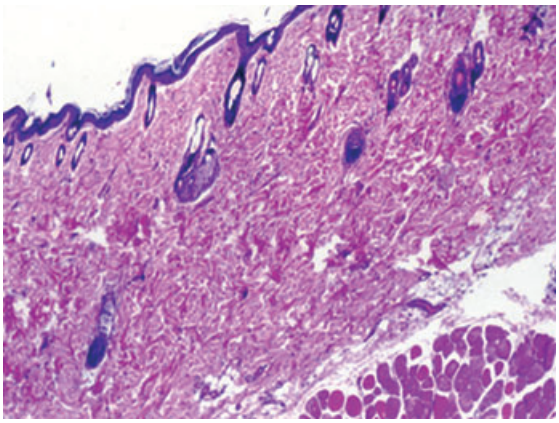

Figure 1 Negative control (saline). Absence of inflammatory cells after 14 days (×40).

Table 1 Amount of dye extracted (μg/site) for all experimental groups

Endodontic Sealer	1 day	3 days	7 days	14 days
RoekoSeal	20.128 ± 7.61^{a}	23.930 ± 8.43^{c}	14.960 ± 4.35^{d}	11.424 ± 7.34^{f}
AH Plus	23.120 ± 13.87^{a}	34.272 ± 5.98^{bc}	25.840 ± 14.14^{de}	22.576 ± 6.52^{f}
Sealapex	18.768 ± 5.71^{a}	43.520 ± 5.44^{b}	31.280 ± 7.88^{e}	23.120 ± 2.72^{g}
Negative control	19.856 ± 6.25^{a}	23.936 ± 14.41^{c}	14.416 ± 8.70^{d}	10.336 ± 2.72^{f}
Positive control	29.920 ± 3.26^{a}	46.240 ± 13.05^{b}	40.256 ± 5.98^{e}	35.904 ± 9.79^{g}

Different letters indicate statistically significant difference at 5%.

At 3 days, an acute inflammation and great infiltration of leucocytes and macrophages was found. At 7 days, a chronic inflammation with presence of lymphocyte and plasma cell was evident. Multinuclear cells were found at 14 days (Fig. 2). Sealapex had similar findings to those of the positive control. A severe acute inflammation was noted followed by chronic inflammation at 14 days (Fig. 3). AH Plus produced a severe acute inflammation at 24 h and 3 days. Polymorphonuclear (PMN) cells and macrophages were found surrounding the material. At 7 days, mononuclear cells were observed with the presence of macrophages and plasma cells until the 14th day of evaluation, mediating a chronic inflammatory response (Fig. 4). RoekoSeal sealer presented different characteristics from those of the other root canal sealers. At 24 h, there was an infiltrate of neutrophils indicating a mild acute inflammation. At 3 days, lymphocytes and fibroblasts were observed surrounding the material and capsule formation was noted at the seventh day of evaluation. At 14 days, a fibrous scar tissue was observed with total absence of inflammatory cells (Fig. 5), which suggests tissue compatibility of this sealer.

Discussion

In this study, the method used for the spectrophotometric analysis was originally proposed by Udaka *et al.* (1970), and has often been employed to quantify the irritating potential of several substances injected intradermally as well to evaluate the effectiveness of antiinflammatory drugs. This method analyses the plasmatic exudate produced after an increase in vascular permeability that can be assessed by spectrophotometric measurement of the amount of extracted Evans blue dye (Rutberg *et al.* 1977, Nagem-Filho *et al.* 2003, da Silva *et al.* 2004).

The methodology used in this study has been shown to be effective (Nagem-Filho *et al.* 2003, Silva *et al.* 2004, Tokita & Yamamoto 2004). Vascular permeability alterations can be demonstrated by means of intravenous injection of vital dyes. The vital dye binds to plasma albumin to form a protein bound dye complex, which is suitable as a plasma marker for detection of protein leakage in an area of oedema. Microbial, mechanical or chemical irritation of pulp and periradicular tissues leads to inflammation.

The analysis of the plasmatic exudation occurred in the negative control group showed that the reaction to saline was significantly reduced at day 14, indicating that the operatory trauma caused by the injection itself could have influenced the results within the first 7 days. Therefore, the 14-day period can be considered as the most relevant of the different time intervals, as its results permitted the actual determination of the irritating potential of the cements, employing the present methodology.

This study evaluated three formulations of endodontic sealers using a physicochemical method for quantification of the enhanced vascular permeability and histopathological analysis, using saline and Chloropercha as negative and positive controls, respectively.

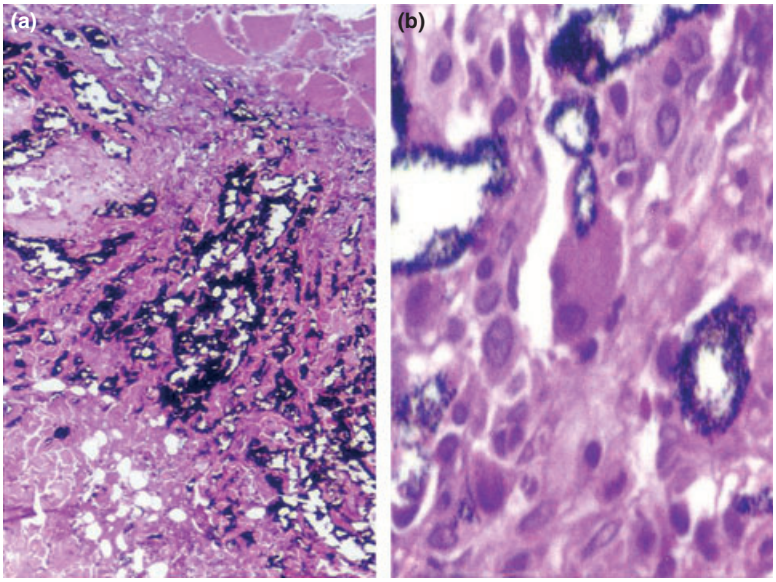

Figure 2 Positive control (Chloropercha). (a, b) Presence of PMNs, macrophages and multinucleated giant cells 14 days after subcutaneous injection of the material (×100).

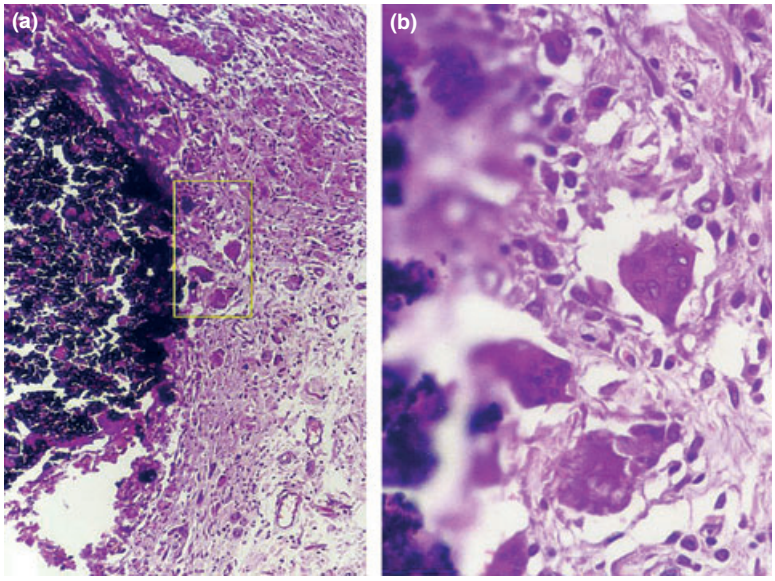

Figure 3 Sealapex. (a, b) Presence of multinucleated giant cells with a foreign body reaction after 14 days (a: ×40; b: ×100).

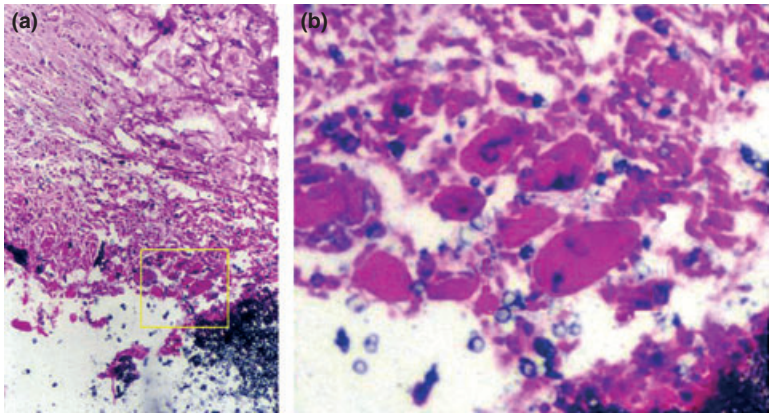

Figure 4 AH Plus. (a, b) Presence of inflammatory cells, macrophages and lymphocytes after 14 days (a: ×40; b: ×100).

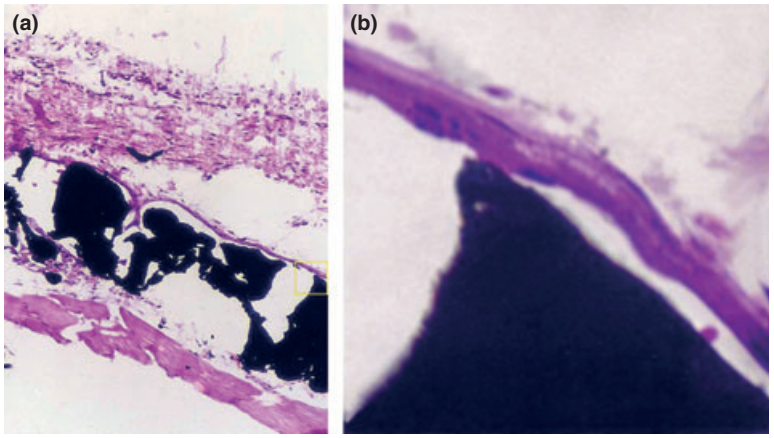

Figure 5 Roeko Seal. (a, b) Presence of fibroplasias 14 days after the subcutaneous injection of the material (a: ×40; b: ×100).

Nelson Filho *et al.* (1999) have demonstrated the innocuous effect of saline when implanted in rat subcutaneous tissue, in the same way as observed in this experiment. Accordingly, in this study, saline had the greatest tissue compatibility in all evaluation periods, while Chloropercha was the most irritating material, producing a severe inflammatory response with the presence of multinucleated giant cells.

It is known that the chemical nature of endodontic sealers is an important factor that influences the apical healing as a result of mineralized tissue deposition. However, the formation of this tissue can be affected by the inflammatory response elicited by the sealer. In other words, if the tissue irritation caused by the sealer persists, no biological repair is expected.

In this study, the severity of the inflammation produced by the $Ca(OH)_2$ sealer, Sealapex (new formulation), increased at the third day of evaluation, with no resolution until the 14th day. Inflammatory cells were observed at the seventh day and foreign-body multinucleated giant cell reaction occurred after 14 days. The spectrophotometric data revealed a correlation with the histopathological findings. In a recent study in dogs' using the new formulation of Sealapex, Leonardo *et al.* (2007) also observed an intense inflammatory infiltrate, suggesting that the alterations in the original formulation might have affected negatively the tissue compatibility of this material.

Most epoxy resin-based sealers, such as AH Plus, have been shown to have mild-to-moderate irritating effects (Leonardo *et al.* 1999a). Owing to its complex chemical composition, numerous substances may be released from AH Plus to the adjacent tissues and might induce local and/or systemic adverse effects, including genotoxicity or cytotoxicity (Leyhausen *et al.* 1999). AH Plus is an improved formulation of AH 26, and does not release formaldehyde, which could be related to its claimed toxicity (Leonardo *et al.* 1999b). However, amines, which accelerate the polymerization in AH Plus composition, could be responsible for the strong initial tissue response close to this sealer. In this study, AH Plus exhibited a severe tissue response. The peak of irritation occurred in the earlier evaluation period and decreased over time. However, it had a severe tissue response when compared to RoekoSeal and may be considered as a moderately irritating material in the period immediately after mixing.

Few reports are available in the literature about biological testing of silicone-based root canal filling materials (Miletic *et al.* 2003, Öztan *et al.* 2003). Silicone is inert and RoekoSeal's manufacturer claims in the instructions for use that this sealer is biocompatible and insoluble. In this study, subcutaneous implantation of RoekoSeal showed tissue compatibility.

A relevant finding of this study was the existence of correlation between the spectrophotometric and the histopathological findings. However, the decrease in the absorbance values after the third day of evaluation is due to the fact that the spectrophotometric analysis depends on the release of plasma protein inflammatory exudate, which is typically found in acute inflammation and decreases as soon as a chronic inflammation develops. The increase of the absorbance values at the third day and the decrease at 7 and 14 days are indicative of a chronic process. Further studies are necessary to evaluate long term tissue response to the tested endodontic sealers.

Conclusion

Under the tested conditions, AH Plus and Sealapex (new formulation) did not show tissue compatibility, while RoekoSeal was compatible when implanted in subcutaneous connective tissue.

References

Costa CA, Oliveira MF, Giro EM, Hebling J (2003) Biocompatibility of resin-based materials used as pulp-capping agents. *International Endodontic Journal* **36**, 831–9.

Deva AK, Merten S, Chang L (1998) Silicone in nasal augmentation rhinoplasty: a decade of clinical experience. *Plastic Reconstructive Surgery* **102**, 1230–7.

Figueiredo JA, Pesce HF, Gioso MA, Figueiredo MA (2001) The histological effects of four endodontic sealers implanted in the oral mucosa: submucous injection versus implant in polyethylene tubes. *International Endodontic Journal* **34**, 377–85.

Hauman CHJ, Love RM (2003) Biocompatibility of dental materials used in contemporary endodontic therapy. A review. Part 1. Intracanal drugs and substances. *International Endodontic Journal* **36**, 75–85.

Huang FM, Tai KW, Chou MY, Chang YC (2002) Cytotoxicity of resin-, zinc oxide-eugenol-, and calcium hydroxide-based root canal sealers on human periodontal ligament cells and permanent V79 cells. *International Endodontic Journal* **35**, 153–8.

International Guiding Principles for Biomedical Research Involving Animals. Geneva, Switzerland: CIOMS (Council for International Organizations of Medical Sciences); (1985). Available at http://www.cioms.ch/frame_1985_texts_of_guidelines.htm. Accessed October 17, 2008.

Jaunberzins A, Gutmann JL, Witherspoon DE, Harper RP (2000) TGF-beta 1 alone and in combination with calcium

hydroxide is synergistic to TGF-beta 1 production by osteoblasts *in vitro*. *International Endodontic Journal* **33**, 421–6.

Kaplan AE, Ormaechea MF, Picca M, Canzobre MC, Ubios AM (2003) Rheological properties and biocompatibility of endodontic sealers. *International Endodontic Journal* **36**, 527–32.

Leonardo MR, Silva LAB, Almeida WA, Utrilla LS (1999a) Tissue response to an epoxy resin-based root canal sealer. *Endodontic Dental Traumatology* **15**, 28–32.

Leonardo MR, Silva LAB, Filho MT, da Silva RS (1999b) Release of formaldehyde by 4 endodontic sealers. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics* **88**, 221–5.

Leonardo MR, Barnett F, Debelian GJ, de Pontes Lima RK, Bezerra da Silva LA (2007) Root canal adhesive filling in dogs' teeth with or without coronal restoration: a histopathological evaluation. *Journal of Endodontics* **33**, 1299–303.

Leyhausen G, Heil J, Reifferscheid G, Waldmann P, Geurtsen W (1999) Genotoxicity and cytotoxicity of the epoxy resin-based root canal sealer AH plus. *Journal of Endodontics* **25**, 109–13.

Miletic I, Jukic S, Anic I, Zeljezic D, Garaj-Vrhovac V, Osmak M (2003) Examination of cytotoxicity and mutagenicity of AH26 and AH Plus sealers. *International Endodontic Journal* **36**, 330–5.

Miletic I, Devcic N, Anic I, Borcic J, Karlovic Z, Osmak M (2005) The cytotoxicity of RoekoSeal and AH Plus compared during different setting periods. *Journal of Endodontics* **31**, 307–9.

Nagem-Filho H, Monteiro CR, Nagem HD, Lage-Marques JL (2003) Effect of dental adhesives on the exudative phase of the inflammatory process in subcutaneous tissue of rats. *Brazilian Oral Research International Endodontic Journal* **17**, 109–12.

Nelson Filho P, Silva LA, Leonardo MR, Utrilla LS, Figueiredo F (1999) Connective tissue responses to calcium hydroxide-based root canal medicaments. *International Endodontic Journal* **32**, 303–11.

Öztan MD, Yilmaz S, Kalayci A, Zaimoglu L (2003) A comparison of the *in vitro* cytotoxicity of two root canal sealers. *Journal of Oral Rehabilitation* **30**, 426–9.

Pulgar R, Segura-Egea JJ, Fernandez MF, Serna A, Olea N (2002) The effect of AH 26 and AH Plus on MCF-7 breast cancer cell proliferation *in vitro*. *International Endodontic Journal* **35**, 551–6.

Rutberg M, Spangberg E, Spangberg L (1977) Evaluation of enhanced vascular permeability of endodontic medicaments in vivo. *Journal of Endodontics* **3**, 347–51.

Schwarze T, Fiedler I, Leyhausen G, Geurtsen W (2002) The cellular compatibility of five endodontic sealers during the setting period. *Journal of Endodontics* **28**, 784–6.

Silva LAB, Leonardo MR, Faccioli LH, Figueiredo F (1997) Inflammatory response to calcium hydroxide based root canal sealers. *Journal of Endodontics* **23**, 86–90.

Silva FB, Almeida JM, Sousa SM (2004) Natural medicaments in endodontics – a comparative study of the anti-inflammatory action. *Brazilian Oral Research* **18**, 174–9.

Tokita K, Yamamoto T (2004) Differential role of neutrophils and monocytes during subcutaneous plasma extravasation. *Laboratory Investigation* **84**, 1174–84.

Udaka K, Takeuchi Y, Movat HZ (1970) Simple method for quantitaion of enhanced vascular permeability. *Proccedings Society of Experimental Biology Medicine* **133**, 1384–7.