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Effect of Root Canal Sealers on Mouse Peritoneal Macrophage Functions

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ABSTRACT. Three root canal filling materials, *viz*. calcium hydroxide-based cement (Apexit®, resin-based cement (AH-plus®) and glass-ionomer based material (Ketac Endo®) were tested for their influence on several functions of peritoneal macrophages from Balb/c mice. Macrophage functions were evaluated by the adherence, phagocytic, candidacidal and Nitro blue tetrazolium-dye assays. Ketac-Endo® enhanced all macrophage functions in the first 2 d ($p \leq 0.05$), when compared to the positive control, but this effect had changed after 7 and 14 d, causing inhibition of these functions. Other materials suppressed substrate adherence capacity and phagocytosis, while significantly stimulating macrophage microbicidal activity ($p \leq 0.05$) in a time-dependent manner.

Abbreviations

AI	adherence index	PI	phagocytic index
MΦ	macrophage(s)	TGC	thioglycollate
NBT	Nitroblue tetrazolium		

Materials used as root canal sealers must be capable of adaptation and adhesion to the root canal dentine surface, be stable in volume, to be unaffected by moisture, be highly water-insoluble and, beyond these characteristics, they must also be well tolerated by the surrounding periapical tissue (Spangberg *et al.* 2002). Biocompatibility of endodontic materials is of primary importance, because of the direct contact, especially when extruded, with the periapical tissue for a prolonged period of time (Hauman *et al.* 2003). However, it has been shown that most commercially available sealers irritate the apical tissues and exhibit toxic potentials of various degree (Miletić *et al.* 2000, 2005). Whenever tissues are exposed to endodontic material at the tooth apex an inflammatory response is initiated, with the presence of polymorphonuclear cells and MΦ. MΦ play an essential role in the pathogenesis of human periapical pathosis and are implicated in bone resorption and processing of foreign material (Liapatas *et al.* 2003). In the early inflammatory reaction they act as both phagocytosing and secretory cells, releasing many biologically very important mediators (Trindade *et al.* 2001). MΦ can modulate the immune response in several ways. Therefore, in the case of biomaterials used for root canal obturation, their interaction with the cells at the tooth apex should encourage the healing process and not irritate and damage the apical tissue.

Our aim was to investigate the effect of various root canal sealers on several MΦ functions (adherence, phagocytosis, intracellular killing and digestion of the material). As good and readily accessible source of MΦ of high yield and viability for studying their reactions we used the peritoneal exudate collected after stimulation with 3 root canal filling materials.

MATERIALS AND METHODS

Root canal sealers. Three root canal sealing materials were used: calcium hydroxide-based cement (Apexit®; *Vivadent*, Liechtenstein), resin-based cement (AH-plus®; *De Trey*, Germany) and glass-ionomer based sealer (Ketac Endo®; *ESPE*, Germany). The materials were mixed according to the manufacturer's instructions.

Animals. Three female BALB/c mice (obtained from the *Medical Faculty, University of Rijeka*) per each group and timepoint, aged 2–3 months, were used. The animals were kept in plastic cages and were

given standard laboratory rodent food and water *ad libidum*. Utmost precautions were taken so that the animals remained free from infection by environmental pathogens.

The experiments were conducted in accordance with the guidelines found in the *International Guiding Principles for Biomedical Research Involving Animals*. The Ethical Committee at the *University of Rijeka* approved all of the animal experiments.

Collection of macrophages. The animals were injected into the peritoneal cavity with the solutions containing freshly mixed sealers (1 g sealer per 3 mL 0.85 % NaCl). 0.85 % NaCl as a negative and TGC (*Becton Dickinson*, USA) as a positive control were injected in the same volume and the same manner. Mice were sacrificed by cervical dislocation 2, 7 and 14 d later. At the appropriate timepoint, the peritoneal cavity was washed with 5 mL of RPMI medium (*Institute of Immunology*, Zagreb, Croatia). After a 2-min massage, the injected medium containing peritoneal cells was slowly aspirated. A quantitative analysis of the cell suspension was made immediately after collection in a Neubauer chamber. Elicited populations (TGC, sealers) were up to 90 % MΦ and >95 % viable as determined by trypan blue exclusion. The suspension (morphologically distinct MΦ/mL) was adjusted with medium to a cell concentration of 1/nL for further analyses.

Assay of macrophage functions. Adherence capacity. Two plastic test tubes (16 × 160 mm) were filled with 0.2 mL of the cell suspension and incubated 2 h in a horizontal position at 37 °C in a humidified atmosphere of 5 % CO_2 in air. After gentle removal, the number of nonadherent MΦ was counted in a Neubauer chamber. The results were expressed as the AI:

$$AI = 100 - [(M\Phi_{non}/mL)/(M\Phi_{ini}/mL)] \times 100,$$

where $M\Phi_{non}$ are nonadherent MΦ and $M\Phi_{ini}$ initial MΦ.

Ingestion of heat-killed *Candida albicans*. Equal volumes (200 μL) of MΦ and heat-killed *C. albicans* (10/nL) suspensions were placed inside plastic rings (2 mm high, 16 mm in diameter) stucked onto microscopic slides, covered with coverslips and incubated (30 min, 37 °C) in a humidified atmosphere with 5 % CO_2. After incubation the slides were washed with RPMI medium (to remove nonphagocytosed *C. albicans*), dried overnight, stained with Wright's stain (*Merck*, Germany) and examined under the microscope. At least 100 MΦ were counted per slide. The result was expressed as PI meaning by multiplying the percentage of adherent cells that had completely ingested ≥1 *C. albicans* by the average number of *C. albicans* per positive MΦ.

C. albicans killing assay. The microbicidal ability of MΦ was determined by coculturing MΦ with viable *C. albicans* (10/nL) suspensions. Peritoneal MΦ and the yeast were rotated end-over-end (1 h, 37 °C, 5 % CO_2). The contents were then diluted in cold distilled water to lyse the MΦ and release intracellular yeast. Liberated *C. albicans* were then stained with 0.1 % methylene blue. Light microscope examination allows to distinguish between dead (blue) and living (transparent) yeast cells. At least 300 yeast cells were examined to determine the percentage of killed *C. albicans* per 100 blastoconidia.

Nitroblue tetrazolium reduction assay (NBT test). An indirect measurement of the respiratory burst by the MΦ is based on the fact that the superoxide anions produced by MΦ have the ability to reduce NBT to dark blue formazan. Two hundred μL of MΦ suspension was incubated (15 min, 37 °C) with an equal volume of 0.1 % NBT solution. Smears were prepared, air-dried and stained with Wright's stain. A total of 100 MΦ were examined under a microscope for the presence or absence of dark blue formazan in cells.

Statistical analysis. Each experiment was made in duplicate and performed 3×. The results were evaluated by 2-way ANOVA analysis and shown as mean ± SD. Post-hoc LSD test was performed for analyzing the differences between the root canal sealers and the control in each incubation interval. Pearson's correlation coefficient was used to assess the correlation among indexes.

RESULTS

Positive control (TGC) served as a comparison with the examined materials. The AI of TGC elicited MΦ increased progressively from 79.4 ± 3.45 % on 2nd d to 93.7 ± 3.25 % on 14th d as well as after injection of Apexit (76.8 ± 7.86 % on 2nd d; 93.1 ± 1.72 % on 14th d). Following the treatment with AH-plus, the AI showed a similar progressive character, but the values were lower (74.9 ± 9.35 % on 2nd d; 87.3 ± 4.30 % on 14th d). Only Ketac Endo significantlly increased the AI (94.7 ± 1.50 %, $p \leq 0.05$) on 2nd d. However, on 7th d the same material reduced the adherence capacity (74.5 ± 7.30 %) with a decreasing tendency to 14th d (71.9 ± 3.56 %, $p \leq 0.05$). The differences were statistically significant ($p \leq 0.05$) when compared to the positive control as well as to Apexit or AH-plus (Table I *upper left*).

The TGC control PI rose from 2nd to 14th d (12.6 ± 7.64 % – 13.7 ± 2.59 % – 23.3 ± 0.88 %). Apexit and AH-plus sensitized MΦ did not demonstrate any major alteration of PI during the first week.

However, opposite to the control, a significant decrease ($p \leq 0.05$) in phagocytotic capacity was manifested on 14th d in the case of both Apexit (7.3 ± 2.23 %) and AH-plus (7.7 ± 1.84 %). A decline of ingestion capability was also noticed in MΦ obtained from Ketac Endo-treated mice; it was significantly higher ($p \leq 0.05$) at all time periods in comparison to Apexit and AH-plus (Table I *upper right*).

Table I. Adherence index of peritoneal macrophages treated with endodontic materials (% of adherent cells), phagocytic index of mouse peritoneal macrophages after exposure to endodontic materials (% of ingested *C. albicans*), *C. albicans* killing by peritoneal macrophages after exposure to endodontic materials (% of killed cells) and NBT dye reduction by peritoneal macrophages after exposure to endodontic materials (% of NBT positive cells) after 2, 7, and 14 d[a]

Material	2	7	14	2	7	14
	Adherence index			**Phagocytic index**		
Positive control	79.4 ± 3.45	84.7 ± 4.21	93.7 ± 3.25	12.6 ± 7.64	13.7 ± 2.59	23.3 ± 0.88
Apexit	76.8 ± 7.86	82.7 ± 2.83	93.1 ± 1.72	12.6 ± 7.64	13.7 ± 2.59	7.3 ± 2.23
Ketac Endo	94.7 ± 1.50*	74.5 ± 7.30	71.9 ± 3.56*	25.9 ± 3.08*	17.5 ± 1.33	13.7 ± 7.22
AH-plus	74.9 ± 9.35	85.9 ± 5.60	87.3 ± 4.30	16.6 ± 4.28	12.3 ± 8.01	7.7 ± 1.84*
	***C. albicans* killing**			**NBT dye reduction**		
Positive control	8.6 ± 1.70	11.0 ± 2.95	21.0 ± 3.00	17.5 ± 0.58	35.3 ± 1.89	41.6 ± 3.82
Apexit	32.0 ± 0.38*	29.7 ± 4.82*	25.1 ± 4.82	39.3 ± 2.15*	38.0 ± 2.82	29.3 ± 4.78
Ketac Endo	33.9 ± 5.02*	30.2 ± 2.28*	14.1 ± 3.22	51.7 ± 0.86*	17.5 ± 3.13	34.3 ± 5.18
AH-plus	18.4 ± 1.53*	27.5 ± 0.82*	32.6 ± 0.28	39.8 ± 1.83*	33.4 ± 2.15	31.0 ± 0.91

[a]Each data point is the mean ± SD of experiments performed in duplicate and repeated 3×; * – statistically significant ($p \leq 0.05$).

The *C. albicans* killing assay of positive control increased during the whole experiment, but the percentage of killed *C. albicans* was generally low (8.6 ± 1.70 % – 11.0 ± 2.95 % – 21.0 ± 3.00 %). *C. albicans* killing assay of peritoneal MΦ elicited by all materials tested was significantly higher during the 1st week ($p \leq 0.05$) (Table I *bottom left*).

As indicated by NBT test, respiratory burst of TGC elicited MΦ correlated with their ingestion capacity. On the 2nd d, all materials increased significantly the intensity of the NBT intracellular reduction in comparison to the control ($p \leq 0.05$) (Table I *bottom right*). On 7th and 14th d in the control group, as well as after treatment with Apexit and AH-plus, the percentage of NBT positive cells was constant (≈40 %). Only in the case of Ketac Endo a strong decrease (in comparison to all other groups) was noticed on 7th d (17.5 ± 3.13 %).

DISCUSSION

Endodontic materials are capable of inducing changes of MΦ functions. As it is not uncommon to find excess sealer in the periapical tissue it should be biologically inert, with low cytotoxicity and no or minimal influence on local immune and inflammatory reactions. The MΦ are important immunological cells in the periapical tissue responsible for its healing as well as damage (Tomazić-Jezić *et al.* 2001). Therefore, if any of the sealers affect the MΦ functions, they can modulate the local immunological defense mechanisms as well.

Of the three materials studied, glass-ionomer cement (Ketac Endo), which has been accepted in clinical practice because of its good adherence to the canal wall and minimal dimensional changes after hardening (Pommel *et al.* 2003), had the most extreme effect on peritoneal MΦ. Two d post inoculation, this material induced pronounced stimulation of all the analyzed functions. Early stimulation of phagocytotic activity by glass-ionomer cement may be associated with the consistency and physico-chemical properties of the material itself, because the size of the particles appeared to be critical in the process of phagocytosis and therefore in the healing process (Perassi *et al.* 2004; Tomazić-Jezić *et al.* 2001). Kaplan *et al.* (1997) has reported evident disintegration of glass-ionomer-based material in water, which is in accordance with our observation that, after inoculation, Ketac Endo dispersed into fine particles throughout the murine peritoneal cavity. In contrast, the other two materials, like tiny stones, remained hardened at the site of injection. An early stimulating activity of glass-ionomer cement may be positive from the clinical standpoint if it leads to destruction of microorganisms and foreign particles. Apart from phagocytosis, MΦ in this early phase also

secrete biologically powerful mediators that also contribute to the healing of the periapical tissues. However, it is of utmost importance that such stimulation is not prolonged, because persistent activation usually ends with chronic inflammation, accelerating the formation of chronic granulation (Trindade *et al.* 2001) or incomplete tissue healing.

The other two examined materials, calcium-hydroxide-based cement (Apexit) and resin-based cement (AH-plus), slightly decreased AI of murine peritoneal MΦ. The same reduction of AI has also been described by Sedeghein *et al*. (2001) in the reaction of AH 26 on peritoneal MΦ. Consistent with reduced AI and ingestion capacity, a positive relationship between the NBT test and the PI, in the case of both sealers, was also confirmed. Similar data of altered phagocytotic response and nitric-oxide production capability of MΦ after a contact with endodontic sealers has been found by de Oliveira Mendes *et al.* (2003).

The *in vitro* inhibitory effect of other calcium hydroxide materials on the adherence capacity has also been described (Segura *et al*. 1997). Since the presence of calcium ions in the medium is necessary for adherence of MΦ, it is considered that the inhibitory effect of calcium hydroxide-based materials could be due to the chelating effect of their components (Jimenez-Rubio *et al.* 1998). Although calcium hydroxide-based cement exhibited a weak antifungal and moderate bacteriostatic effect (Pezelj-Ribarić *et al.* 2002), adequate sealing ability (Miletić *et al.* 1999), low cytotoxicity (Huang *et al.* 2002*a*; Miletić *et al.* 2005) as well as negligible influence on MΦ functions, would still support its use in our endodontic practice.

A potential advantage of AH-plus (an epoxy-resin-based sealer) could be a sealing ability tighter than that of calcium-hydroxide-based cement (Miletić *et al.* 1999), cytotoxicity that is confined to the early period after mixing (Miletić *et al.* 2005), tissue compatibility with a mild inflammatory reaction (Huang *et al.* 2002*b*) as well as better antimicrobial characteristics (*data not shown*). However, it exhibited a more pronounced influence on MΦ functions, which must be taken into account, particularly in clinical cases with apical pathological processes, where the use of a material which does not satisfy the requirements of biocompatibility, can interfere with the immunological response and healing process.

Our findings demonstrate that endodontic materials influence various aspects of *in vitro* MΦ function. Clinically, slightly activating effect of sealers on MΦ functions may be of clinical benefit, but if the activating stimulus persists, delayed or defective tissue healing may result.

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