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DENTISTRY SECTION

BIOCOMPATIBILITY OF ROOT CANAL FILLING MATERIALS: DIFFERENCES BETWEEN VITALITY AND FUNCTIONALITY TESTS

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Biocompatibility of root canal filling materials is of great interest because they can come into permanent contact with the living periapical tissue, and induce mild or severe inflammatory responses. Usually biocompatibility tests only determine non-cytotoxic effects of dental materials, even if their functional interactions with cells also play a role in the host responses. The purpose of this study is to evaluate peripheral blood monocyte (PBM) vitality and functionality after contact with 5 different root canal filling materials: Thermafil (gutta-percha), Real Seal and Real Seal 1 (methacrylic resins), AureoSeal (MTA) and SuperSeal (EBA). Cellular vitality was determined by MTT test and cellular functionality by Chemiluminescence (CL) technique. Dishes of the materials were covered with cell culture medium (0.5 cm^2/mL) and incubated for 24 h.The extracts were added to PBMs and the latter, after 2 h of incubation, were analysed by MTT and by Chemiluminescence (CL). All results are expressed as mean ± SEM. The group means were compared by analysis of variance. Results showed that SuperSeal and AuroSeal exhibited a moderate cytotoxic effect, while the toxicity induced by RealSeal, RealSeal 1 and Thermafil was lower. SuperSeal and AuroSeal induced a significant decrease of both oxidative burst and basal reactive oxygen species (ROS) production. RealSeal 1 caused a doubling of basal ROS production in respect to control. The results demonstrate that a low cytotoxic effect does not guarantee a total integrity of cellular functionality and more differences among biocompatibility of root canal materials can be detected when a functionality test is used.

An excellent tissue compatibility of endodontic filling materials is required for clinical use, since these materials can come into direct contact with the living connective tissues of the periapex (1). Ideally, a good endodontic filling material should be biologically compatible and well tolerated, avoiding any possible interference and/or delay of the healing process (2). Usually the most toxic products are the endodontic sealers, especially when freshly prepared, which can elicitate periapical inflammation at varying degrees; their toxicity tends, however, to decrease over time and does not appear to prevent tissue healing (3). On the other hand, a large overfilling/overextension with endodontic sealers and other filling materials can cause severe tissue reaction and permanent damage (4).

Key words: biocompatibility, oxidative burst, peripheral blood monocytes, root canal filling materials

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A large variety of materials has been used to fill root canals. Gutta-percha is the most widely used, because it is well tolerated by host tissues (5); however, other toxic compounds present in the gutta-percha cones can produce irritation or even degeneration of the living tissues.

Biocompatibility studies of endodontic filling material have usually been performed mainly on fibroblasts by assessing cell vitality after tissue/ material contact. A lower number of studies - using other cells and different tests, including functionality tests, - have been published. The role played by peripheral blood monocytes (PBMs) and macrophages both in the interactions between biomaterials and host and in the inflammation reactions is of particular interest; the behaviour of such cells in presence of the different kinds of root canal filling materials should be therefore examined. PBMs and macrophages, in fact, are highly involved in the wound healing process, coordinating tissue repair through the production of a broad spectrum of factors influencing angiogenesis and extracellular matrix synthesis. PBMs furthermore contribute to the immune defensive system with the phagocytosis of microbial intruders. Previous studies suggested a possible interaction between the uncured methacrylic monomers and the monocyte–macrophage system (6-9), assessed by the analysis of interleukin-1β and tumour necrosis factor-α (inflammatory mediators produced by macrophages) (10); moreover, a recent paper (11) shows that different endodontic sealers (based on MTA or zinc oxide- eugenol, etc.) may alter the secretion of some cytokines from PBMs. Other studies (11, 12) indicate that methacrylates are able to alter the oxidative burst of PBMs and granulocyte polymorphonucleates. During phagocytosis-associated respiratory burst PBMs produce - by the plasmatic enzymatic complex of NADPH oxidase - toxic oxidants such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (13). The latter can act as a second messenger in the activation of the genes producing interleukins (14, 15), thus the analysis of oxidative burst helps to evaluate the functionality of the monocyte-macrophage system. These findings support the importance of also evaluating the effect of dental materials on phagocytes, determining a possible activating or stabilizing action on oxidative burst. The purpose of this study is therefore to assess PBM vitality and functionality after contact with the eluates of five different root canal filling materials: Thermafil (a gutta-percha based material), Real Seal and Real Seal 1 (both of them containing methacrylic resins), AuroSeal (containing MTA) and SuperSeal (containing EBA). Cellular vitality was determined by MTT test; cellular functionality was analysed by Chemiluminescence (CL), a technique able to transform the reactive oxygen species (ROS) produced by PBMs into photons through the reaction with a probe such as luminol or lucigenin.

MATERIALS AND METHODS

Isolation of peripheral blood monocytes

Venous blood (10 mL) obtained from healthy volunteers was diluted with physiological solution (10 mL); dextran in physiological solution (6%, 4 mL) was then added to enhance the sedimentation rate of erythrocytes at 1 × *g*. After 30 min, the white blood cell suspension was centrifuged on a Lymphoprep (Pharmacia, Sweden) according to the manufacturer's instructions; lymphomonocytes, suspended in DMEM (Dulbecco's modified Eagle medium) with HEPES (10mmol/L), glucose (1.0 g/L), $NaHCO_3$ (3.7 g/L), penicillin (100 Units/mL), streptomycin (100μg/mL) and 10% Fetal Calf Serum (1×10^6 cells/mL), were put into both luminometer vials and into 96-well plates to allow the adhesion (37°C, 5% CO_2 humidified atmosphere, 1 h). Non-adhered cells were then gently removed by three washes with modified Krebbs–Ringer phosphate (KRP) buffer and counted by Nucleocounter (Sartorius Stedim S.p.a, Florence, Italy) to calculate, by subtraction from the whole, the number of adhered cells (16): the latter were mainly monocytes (> 90%), as determined using the aspecific esterase test, a typical monocyte enzyme (17).

Canal filling materials

- Thermafil (Tulsa Dental, Tulsa, OK, USA) consists of a flexible central carrier coated with a layer of a-phase gutta-percha.
- AUROSEAL (Giovanni OGNA S.p.A., Milan, Italy) is composed by 30% Portland cement (PC, containing SiO_2, Al_2O_3, Fe_2O_3, CaO, MgO, Na_2O, K_2O)
- SUPERSEAL (Giovanni OGNA S.p.A., Milan, Italy), composed by zinc oxide (ZnO, 30%), calcium tungstate ($CaWO_4$, 30%), aluminium oxide (Al_2O_3, 34%); liquid 2-etoxy benzoic acid (62.5%) and eugenol (34%).
- REALSEAL 1™ (SybronEndo, Orange, CA, USA) is composed by a polysulfone based carrier with

difunctional methacrylate resin, bioactive glass, radiopaque filler and colouring agents.

- REALSEAL (SybronEndo, Orange, CA, USA) is a mixture of UDMA, PEGDMA and Bis-GMA resins, silane-treated barium borosilicate glasses, barium sulphate, silica, calcium hydroxide, bismuth oxychloride with amines, peroxides, photo initiator, stabilizers and pigments.

Treatment of peripheral blood monocytes with materials

Dishes of the materials (5 mm Æ, 2 mm depth) were covered with cell culture medium and incubated in the dark for 24 h at 37°C under sterile conditions. The ratio between the sample surface and volume medium (0.5 cm^2/mL) was selected according to the International Organization for Standardization (ISO) (18). After the incubation, the extracts were added to PBMs (≈100.000) by medium change (150 µL) and similar volumes of DMEM were added also to the control wells. After 2 h of incubation at r.t., the PBMs were washed three times with PBS, and analysed by MTT to evaluate the cytotoxic effects and by CL to measure cell functionality.

MTT test

MTT test was performed according to Wataha et al. (19): a solution (20 mL) of MTTin PBS (phosphate buffer, 5mg/mL) was added to the medium (200 mL) and, after incubation (4 h, 37°C) the intracellular Formosan crystals produced were dissolved in a solution of HCl in isopropanol (4×10^{-2}N, 200 mL). The optical density (OD) of the solution contained in each well was determined using an automatic microplate photometer (Packard Spectracount™, Packard BioScience Company, Meriden, USA) at a wavelength of 570 nm. Each experiment was performed in sextuplicate.

Chemiluminescence analysis of PBMs

ROS metabolism of leukocytes was studied by the CL technique according to a modification of the method of De Sole et al. (20): the system was made up of luminol (5-amino-2,3-dihydro-1,4-phthalazindione, 100nmol/L) and cells (1×10^5) in the presence or absence of stimulus constituted by opsonized zymosan (0.5 mg) or 150nmol/L of phorbol 12-myristate 13-acetate (PMA) (20). The final volume (1.0 mL) was obtained using modified KRP buffer. CL activity was measured at 25°C for 2 h, using a LB 953 luminometer (Berthold, EG&G Co, Germany). All the experiments were performed in triplicate. The chemiluminescence parameter considered for analysis was photons signal (area) produced by cells during two hours indicated as Area Under Curves (AUC).

Statistical analysis

All the results are expressed as mean ± SEM. The group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Student-Newman-Keuls; if necessary, comparison of means by Student's *t*-test was used. $P< 0.05$ was considered significant. Specimens were rated as severely, moderately or slightly cytotoxic, where the enzymatic activity relative to controls was lower than 30%, between 30% and 60%, or greater than 60%, respectively (21).

RESULTS

Cytotoxicity (MTT) tests: SuperSeal and AuroSeal exhibited a moderate cytotoxic effect, while the toxicity induced by RealSeal, RealSeal 1 and Thermafil was slight. In spite of this, there are no statistically significant differences between the effects induced by specimens (Fig. 1).

Analysis of ROS production. SuperSeal and AuroSeal induced a significant decrease of both oxidative burst and basal ROS production; in particular, the first one induced a decrease of oxidative burst higher than 80% and a reduction of ROS production in resting state of about 70%. The effect of AuroSeal was even more impressive, and in all condition ROS production disappeared almost completely. On the contrary RealSeal 1 caused doubling of basal ROS production in respect to control (Fig. 2).

DISCUSSION

The aim of this work is to analyse the interactions between some root canal filling materials and PBMs. The effect of endodontic sealers on the activity of such cells - involved both in innate and acquired immune defenses, in inflammation and in wound healing process (7) - is remarkable. More specifically, we evaluated the cellular vitality by MTT test and the cellular functionality by CL technique, through the measure of NADPH membrane oxidase activity. The latter enzyme (formed by 6 subunits, some present in plasmatic membrane and others in cytoplasm) determines the ROS production of cells stimulated by antigenic signals. Enzymatic activation is due to a bond between the receptor of immunoglobulin constant fragment (RFc) present on phagocytes and antigen opsonized by immunoglobulins; this reaction induces a translocation of the cytoplasmatic

subunits to plasmatic membrane followed by their assembling.

In vitro it is possible to activate the enzymatic chain directly stimulating, through PMA, the Protein Kinase C and bypassing the receptorial bond. The differences in the oxidative burst stimulation allow to discriminate the action mechanisms of some xenobiotics: as a matter of fact, if a compound inhibits the activation stimulated by Zymosan but not by PMA, an alteration of linking molecules between RFc and kinases responsible for enzyme assembling is predictable (11, 22).

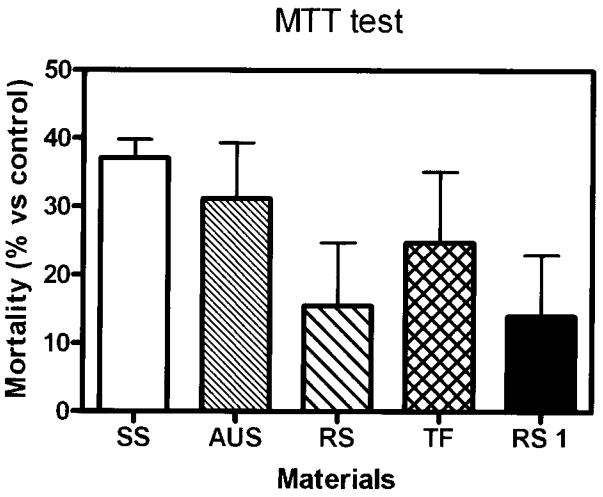

Fig. 1. *Cytotoxicity (MTT) test : the higher the value the more toxic the material.*

In oxidative burst the ROS production is physiologically regulated and directed to a non-specific host immune defense. Low levels of ROS, normally produced by PBMs, also act as second messengers inside cells.

The results from the present study showed a significant decrease of ROS production induced by the materials containing MTA and EBA (i.e. SuperSeal and AuroSeal). This decrease is not explainable through the cytotoxic effect alone, but also by a direct action of the substance, present in the eluate, on the cells functionality.

RealSeal 1™ eluate caused a significant ROS production growth in the cellular basal state, probably because it acts as an antigenic stimulus; this phenomenon is very interesting because it is well known that ROS, besides their killing effect on bacteria, can play an important role in the damage of cell structures, mainly membrane lipids.

Since the monocyte-macrophage system plays a key function on tissue remodelling and the inflammatory process, every effect of root canal filling materials on the functionality of such cells has to be considered (23). Therefore, the possible clinical effects of the alteration of basal ROS production in monocyte–macrophage function need further investigations by both clinicians and researchers. Some zinc oxide–eugenol cements are reinforced with 2-ethoxy benzoic acid (EBA) to eliminate the problem of absorbability that affected early materials using zinc oxide–eugenol as a retrograde filler

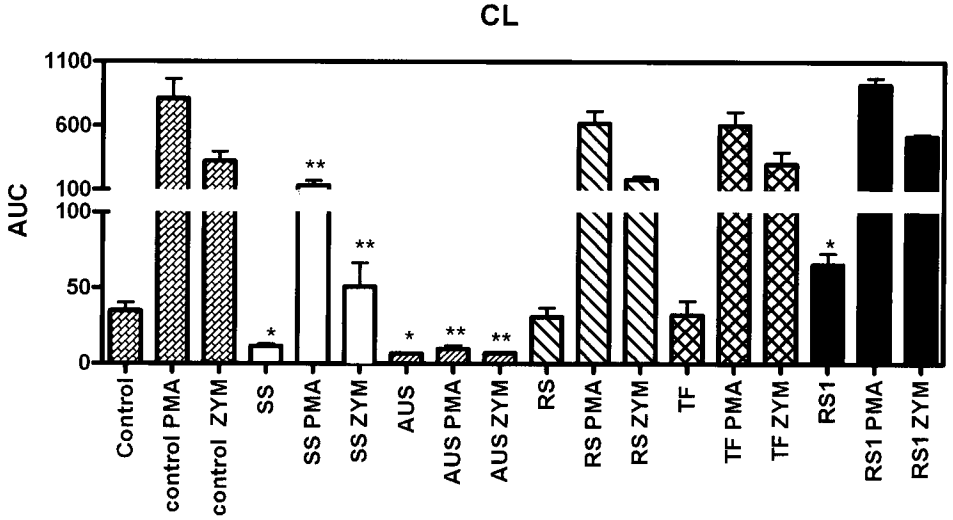

Fig. 2. *Functionality test (analysis of ROS production): the higher the value the higher the ROS production.*

(24). Super-EBA cements contain only one-third of the eugenol present in intermediate restorative materials (IRM) and adhere to the tooth structure in moist conditions. Concern has been expressed about the possible harmful effects on the periapical tissues caused by Super-EBA materials in spite of the reduced amount of eugenol contained. In the late 1990s, a new endodontic sealer containing a mineral trioxide aggregate (MTA), and basically composed by Portland cement (PC), was developed at Loma Linda University (25); more recently, some studies have shown that MTA fulfills the basic requirements of an endodontic material, and for this reason it has been regarded as an attractive product for root-end fillings (26), although expensive and characterized by hard handling (27, 28). MTA has also been successfully used to repair root perforations and as a pulp capping agent (29). However, extended setting time, inadequate compressive strength and poor workability generally limited the use of MTA (30) and research was carried out to develop PC-based cements and root canal sealers, cheaper and with better performances.

In the resin-based root canal sealers, the bi-functional methacrylic molecules act as a bridge between dentine and material (31). However, several studies showed that methacrylic monomers, released from materials, can reach the surrounding tissues through dentinal tubules, accessory and lateral canals, and apical foramina (2) and produce irritation or even degeneration in the surrounding tissues (32).

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