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# The effect of mineral trioxide aggregate on phagocytic activity and production of reactive oxygen, nitrogen species and arginase activity by M1 and M2 macrophages

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## Abstract

**Rezende TMB, Vieira LQ, Cardoso FP, Oliveira RR, de Oliveira Mendes ST, Jorge MLR, Ribeiro Sobrinho AP.** The effect of mineral trioxide aggregate on phagocytic activity and production of reactive oxygen, nitrogen species and arginase activity by M1 and M2 macrophages. *International Endodontic Journal*, **40**, 603–611, 2007.

**Aim** To assess the influence of co-culture with mineral trioxide aggregate (MTA) on phagocytosis and the production of reactive oxygen intermediates (ROI) and nitrogen (NO) species and the arginase activity by M1 and M2 peritoneal macrophages.

**Methodology** Cellular viability, adherence and phagocytosis of *Saccharomyces boulardii* were assayed in the presence of MTA. Macrophages were stimulated with zymosan for ROI assays and with *Fusobacterium nucleatum* and *Peptostreptococcus anaerobius* and IFN-$\gamma$ for NO production and arginase activity, when in contact with capillaries containing MTA. Data were analysed by T, ANOVA, Kruskall–Wallis and Mann–Whitney tests.

**Results** M2 macrophages displayed greater cellular viability in polypropylene tubes, greater ability to ingest yeast and smaller production of ROI and higher arginase activity when compared with M1 macrophages. Both macrophages, M1 and M2, presented similar cell adherence and NO production. The addition of bacterial preparations to macrophages interfered with NO and arginase productions. MTA did not interfere with any of the parameters measured.

**Conclusions** Phagocytosis and the ability of the two macrophage subtypes to eliminate microbes were not affected by MTA.

**Keywords:** macrophage, mineral trioxide aggregate, phagocytosis, reactive nitrogen species and arginase activity, reactive oxygen species.

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## Introduction

Mineral trioxide aggregate (MTA) consists of hydrophilic tri-calcium silicate particles, tri-calcium aluminates, tri-calcium oxide, silicate oxide and other mineral oxides (Lee *et al.* 1993b, Torabinejad *et al.* 1993, Abedi & Ingle 1995, Torabinejad & Chivian 1999). MTA is sold under the brand name ProRoot® (Dentsply Maillefer, Ballaigues, Switzerland) and, in Brazil, MTA-Angelus® (Odonto-lógika, Londrina, Brazil). As MTA is indicated for the use in inflamed or infected areas, it ought to be biocompatible and not affect cell behaviour.

Macrophages predominate amongst the several cells present in the inflamed pulp and periapical tissues (Stern *et al.* 1981, Kawashima *et al.* 1996). Recently, macrophages have been divided into two subtypes: M1 and M2, according to their ability to produce different types of responses (Mills *et al.* 2000, Bastos *et al.* 2002,

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Mantovani *et al.* 2002, Mosser 2003, Rezende *et al.* 2005). M1 macrophages are activated in the classical way, in the presence of IL-12, by IFN-γ, to produce nitric oxide (NO) and reactive oxygen intermediates (ROI) in response to microbes (Mosser 2003). In the presence of oxygen, NO generates reactive nitrogen intermediates which will cause damage to cells and hence to infectious agents (Nathan & Shiloh 2000). ROI are intermediate reduction products of oxygen, such as superoxide, hydrogen peroxide, and hydroxyl radical, as well as reactive products of these with halides and amines (Nathan & Shiloh 2000). Together, nitrogen and oxygen intermediates mediate resistance to pathogens (Nathan & Shiloh 2000). On the other hand, M2 macrophages preferably convert arginine into urea and ornithine, which results ultimately in collagen production and cellular proliferation (Mills *et al.* 2000, Bastos *et al.* 2002, Mosser 2003). M2 macrophages have been found to pre-dominate in BALB/c mice and in mice genetically deficient in the p40 chain of IL-12/23 by homologous recombination (IL-12p40$^{-/-}$) (Bastos *et al.* 2002).

During infection, macrophages play a crucial role in eliminating pathogens and in the healing process. Hence, this study endeavors to determine phagocytic activity, production of free radicals and arginase activity in the presence of MTA from two sources (MTA-Angelus® and ProRoot®). M1 macrophages were isolated from C57BL/6 mice, and M2 macrophages from IL-12p40$^{-/-}$ in the C57BL/6 background. Cellular viability, cellular adherence and phagocytosis were assayed, as well as the production of ROI and NO and arginase activity.

## Materials and methods

### Mice

Male and female 4- to 8-week-old wild-type (wt) C57BL/6 (CEBIO – UFMG, Belo Horizonte, Brazil) and C57BL/6 deficient in the p40 chain for IL-12/IL-23 (IL-12p40$^{-/-}$; Gnotobiology and Immunology Laboratory, UFMG, Belo Horizonte, Brazil) by targeted mutation (Magram *et al.* 1996) were kept in a conventional animal house with barriers, temperature and light control. Food and water were offered *ad libitum*.

### Isolation of macrophages

Cells were isolated from the peritoneal cavity of wt and IL-12p40$^{-/-}$ C57BL/6 mice 5 days after injection of 2 mL of 3% thioglycolate medium (Biobrás S.A., Montes Claros, MG, Brazil) in the peritoneum. Cells were resuspended in complete medium: RPMI 1640 without phenol red (Sigma Chemicals Co., St Louis, MO, USA), supplemented with 10% of fetal calf serum (Nutricell, Campinas, SP, Brazil), 0.1% of 0.05 mol L$^{-1}$ β-mercaptoethanol (Sigma Chemicals Co.), 0.2% of penicillin (100 U mL$^{-1}$)/streptomycin (0.1 mg mL$^{-1}$) and 200 mmol L$^{-1}$ L-glutamine (Oliveira Mendes *et al.* 2003).

### Cell cultures

Cells ($1 \times 10^5$ cells mL$^{-1}$) were cultured in 1 mL of medium, in 24-well culture plates (Nunclon; Nalge Nunc International, Naperville, IL, USA), for the assessment of viability; $5 \times 10^5$ cells mL$^{-1}$ for the phagocytosis assay; $1 \times 10^6$ cells mL$^{-1}$ for the verification of cellular adherence, cellular viability, ROI production and arginase activity; and $2 \times 10^6$ cells mL$^{-1}$ for NO detection. All incubations were performed in an incubator with humidified atmosphere containing 5% $CO_2$, at 37 °C.

### MTA manipulation

Both MTA brands were prepared in accordance with manufacturers' instructions in sterile conditions. Soon after preparation, MTA was inserted into the tips of previously sectioned sterilized capillary tubes (test group), so that their contact with the cell suspension could be standardized (Oliveira Mendes *et al.* 2003). Empty capillary tubes were used in control cultures. Capillaries were sterilized by exposure to 25 kGray Gamma ray irradiation (CDTN, Belo Horizonte, MG, Brazil).

### Cell viability

Cells were cultured for 24 h, in polypropylene tubes. Cellular viability was determined by the trypan blue exclusion assay (Oliveira Mendes *et al.* 2003).

### Cell adherence

Polypropylene tubes containing macrophages were incubated for 2 h with capillaries (test and control groups) in an incubator with humidified atmosphere containing 5% $CO_2$, at 37 °C. Tubes were agitated in a vortex agitator for 5 s, at low speed. Twenty microlitres of the cellular suspension were removed placed into a Newbauer chamber and incubated for 1 h at 37 °C as

above. The percentage of adherent and nonadherent macrophages was then established by counting under an optical microscope (Lee *et al.* 1993a).

### Phagocytosis assay

Cells ($1 \times 10^6$ in 1 mL) were incubated for 2 h in 24-well culture plates (Nunclon, Nalge Nunc International, Miami, FL, USA), onto round glass coverslips (Glasstécnica, São Paulo, SP, Brazil) in an incubator as above. Nonadherent cells were removed by washing with warm complete medium, afterwards $10^7$ CFU of *Saccharomyces boulardii* (Floratil; Merck S.A., Rio de Janeiro, RJ, Brazil) and capillaries with or without MTA were added to the medium and plates were incubated for 1 h. Unbound yeast cells were removed by washing with complete medium and the coverslips were covered for 1 min with 1 mL of tannic acid at 1% (Merk), so that the distinction could be made between extracellular and intracellular yeast cells. One drop of fetal calf serum was applied onto each coverslip. The dried coverslips were stained with Panótico Rápido (Laborclin Ltd, Pinhais, PR, Brazil) and glued to microscope glass slides with Entellan (Merk) for observation under optical microscope at 1000× magnification in oil immersion (Giaimis *et al.* 1992). To determine the percentages of macrophages with phagocyted yeast, the percentages of macrophages with adhered yeast, as well as the number of phagocyted yeast/cells, a minimum of 200 cells were counted.

### Reactive oxygen intermediates assay

Cells were cultured in polypropylene tubes in an incubator as above. After 24 h, $1 \times 10^6$ cells were transferred to a C96 White Maxisorp (Nalgene, Rochester, New York, USA) plate in 100 μL; $10^7$ zymosan particles (Sigma Chemical Co.) and 0.05 mmol $L^{-1}$ luminol in 1640 RPMI without phenol red were added to each well. Plates were read every 2 min for 118 min in a luminometer (LumiCount®; Packard Instrument Company Inc., Downers Grove, IL, USA) (Trusk *et al.* 1978). This procedure will measure the luminol-amplified chemiluminescence due to the production of superoxide, hydroxyl radical and oxygen peroxide in response to ingestion of zymosan particles by phagocytes (Trusk *et al.* 1978). Results were expressed as the area under each of the curves obtained in the 118-min period, calculated as follows:

$$I = h[f_2 + f_3 + \cdots + f_{(n-1)}] + \frac{(f_1 + f_n)h}{2}$$

where I is the area underneath the curve, h the time interval, $f_1$, $f_2$,…,$f_n$ indicate light units at times 1, 2,…, n (n measurements).

### NO production

Cells were cultured with capillaries (test and control groups) in 24-well culture plates (Nunclon) in an incubator (as above) with $10^7$ CFU of heat-killed *Fusobacterium nucleatum* (ATCC 10953) or *Peptostreptococcus anaerobius* (ATCC 27337). Some cultures were activated with 10 U $mL^{-1}$ of recombinant IFN-γ (Pharmingen, San Diego, CA, USA). Cells were cultured for 72 h and NO production was assayed as nitrites in the supernatants. Sodium nitrite (0.977–125 μmol $L^{-1}$) was used as standard (Green *et al.* 1982). Sensibility was 0.5 mmol $L^{-1}$.

### Arginase activity

Cells were cultured with capillaries (test and control groups) in polypropylene tubes in an incubator as above with $10^7$ CFU of heat-killed *F. nucleatum* (ATCC 10953) or *P. anaerobius* (ATCC 27337), for 48 h. Arginase activity was measured in cell lysates as described by Corraliza *et al.* (1994). Briefly, cells were lysed with 50 μL of 0.1% Triton X-100 (Sigma Chemicals Co.). After 30 min on a shaker, 50 μL of 10 mmol $L^{-1}$ $MnCl_2$ (Merck), 50 μL of mmol $L^{-1}$ Tris-HCl (Merck) were added, and the enzyme was activated by heating for 10 min at 55 °C. Arginine hydrolysis was conducted by incubating 25 μL of the activated lysate with 25 μL of 0.5 mol $L^{-1}$ L-arginine (Merck; pH 9.7) at 37 °C for 60 min. The reaction was stopped with 400 μL of $H_2SO_4$ (96%)/$H_3PO_4$ (85%)/$H_2O$ (1/3/7, v/v/v). As a degree of arginase activity, the urea concentration was measured at 540 nm after addition of 25 μL of α-isonitrosopropiophenone (Sigma Chemical Co.) dissolved in 100% ethanol, for 45 min at 95 °C. One unit of arginase activity was defined as the amount of enzyme that catalysed the formation of 1 μmol urea $min^{-1}$. The standard curve was prepared with increasing amounts of urea between 1.625 and 100 μg.

### Statistical analysis

Data were analysed using parametric (ANOVA and *t*-test) and nonparametric (Mann–Whitney and Kruskal–Wallis) tests ($P < 0.05$). Analyses were made using the SPSS 8.0 Inc. (Statistical Package for Social Sciences, Chicago, IL, USA) software.

### Animal ethical committee

These experiments were authorized by CETEA – UFMG (protocol no. 35/2002).

## Results

### Cell viability

Cellular viability was greater than 80% in polypropylene tubes (Fig. 1), for both macrophage subtypes. Analyzing macrophages subtypes, M1 macrophages survived better than M2 ($P < 0.05$). MTA did not affect viability when compared with controls in all conditions tested.

### Cell adherence

Cell adherence was similar between M1 and M2 macrophages. MTA did not affect the capacity of either macrophage subtype to adhere to glass (Fig. 2).

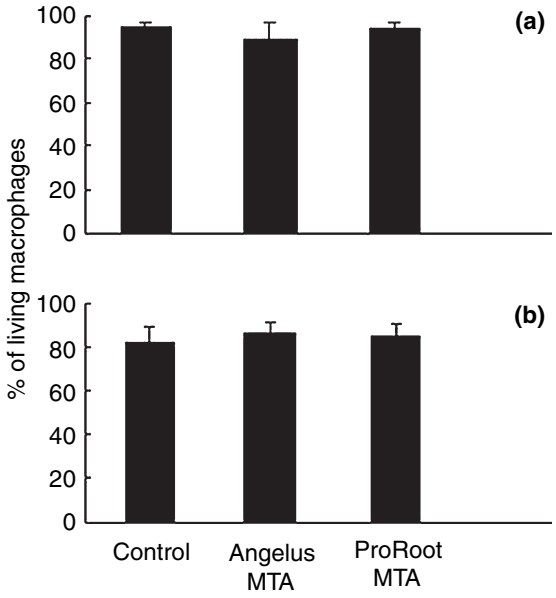


**Figure 1** Percentage of living M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice, after incubation in polypropylene tubes with capillaries containing MTA from both commercial sources. Controls were cultured with empty capillaries. Cultures were maintained for 24 h as described in materials and methods. Bars represent the mean of two experiments, lines stand for the standard error of the means.

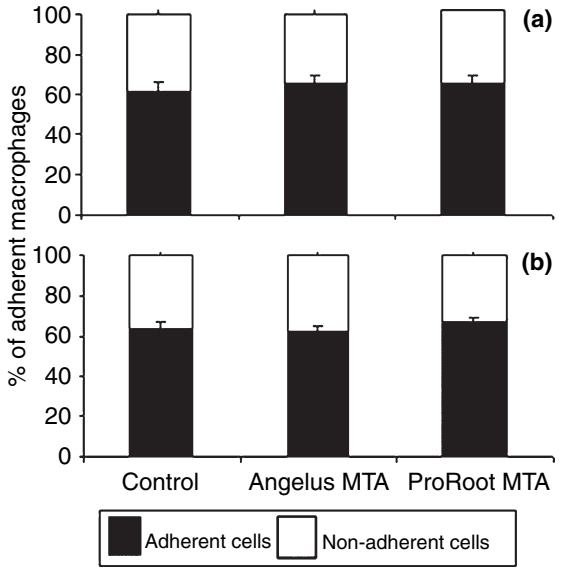


**Figure 2** Percentage of adherent M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice, after incubation in culture plates with capillaries containing MTA from both commercial sources. Controls were cultured with empty capillaries. Cultures performed as described in materials and methods. Bars stand for the mean results of five experiments made in duplicates. Lines indicate standard error of the means.

### Phagocytic activity

The percentage of macrophages with adhered yeast to their surface yeast was similar for M1 and M2 subtypes (Fig. 3a,b). However, M2 macrophages presented larger numbers of ingested yeast cells in all conditions examined ($P < 0.05$) (compare Fig. 3c,d). MTA did not affect either parameter. Macrophages in contact with MTA presented bi-refractive structures inside, suggesting that MTA was ingested (Fig. 7).

### Production of reactive oxygen intermediates

Analysis of the area under each ROI production's curves showed that in general, M1 macrophages produced more ROI than M2, but when the test and control groups were compared between two macrophages' types there was no statistical differences. Addition of zymosan induced significantly higher levels of ROI production by M1 macrophage ($P < 0.05$). MTA did not affect ROI production in any of the conditions examined (Fig. 4).

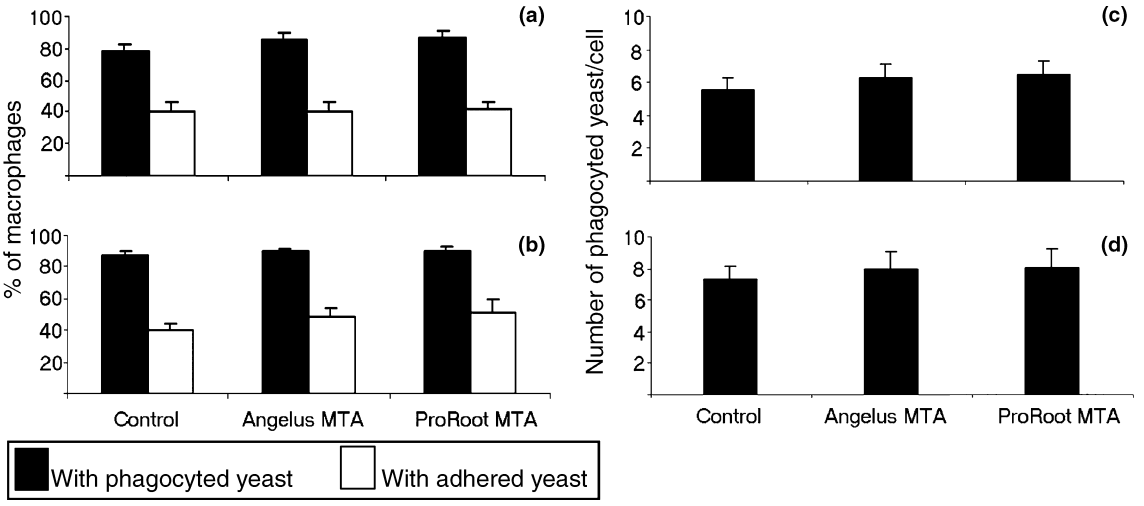


**Figure 3** Percentage of M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice displaying phagocyted or adherent yeast cells, after incubation in culture plates with capillaries containing MTA from both commercial sources. Numbers of yeast cells per M1 (c) and M2 (d) macrophages are also shown. Controls were cultured with empty capillaries. Cultures performed as described in materials and methods. Bars stand for the mean results of five experiments made in duplicates. Lines indicate standard error of the means. All M1 data were statistically different from M2, under the same conditions.

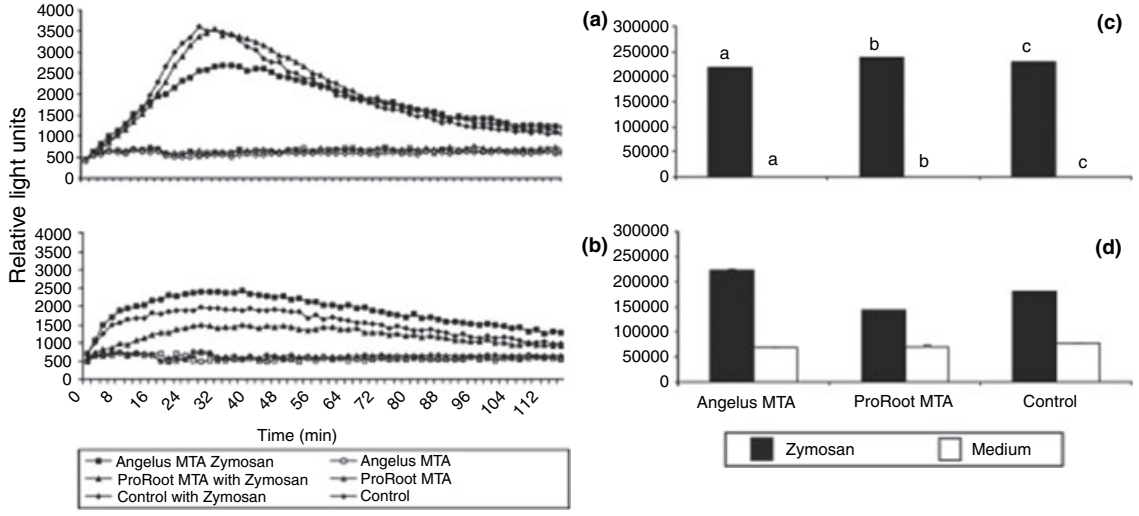


**Figure 4** Kinetic of reactive oxygen intermediates (ROI) production by M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice. ROI total production by M1 macrophages (c) and M2 macrophages (d). Cells were cultured with capillaries containing MTA and stimulated with zymosan as described in materials and methods. Bars stand for the mean results of five experiments made in duplicates. Lines indicate standard error of the means. Different letters indicate statistical difference for ROI production in response to zymosan when compared with macrophages in medium alone, by Mann–Whitney test.

## NO production

M1 and M2 macrophages produced similar levels of NO in all conditions examined (Fig. 5). Addition of bacterial preparations to macrophages induced NO production, and activation by IFN-γ increased this production, especially in the cultures stimulated with *F. nucleatum* ($P < 0.05$). MTA had no significant effect on NO production.

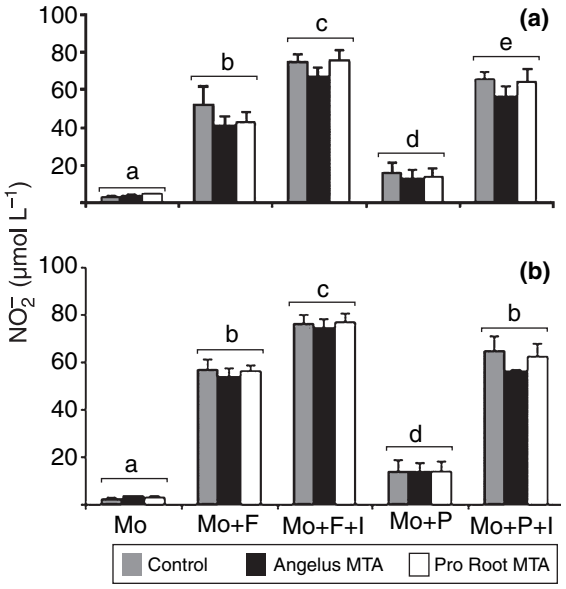


**Figure 5** Nitric oxide (NO2-) production by M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40$^{-/-}$ mice. Cells were cultured with capillaries containing MTA and stimulated with *F. nucleatum* (F) or *P. anaerobius* (P), and IFN-gamma I) as indicated. Bars stand for the mean results of five experiments made in duplicates. Lines indicate standard error of the means. Different letters indicate statistical difference in the same macrophage subtype (M1 or M2), $P < 0.05$. No differences were found between M1 and M2 macrophages in the same conditions.

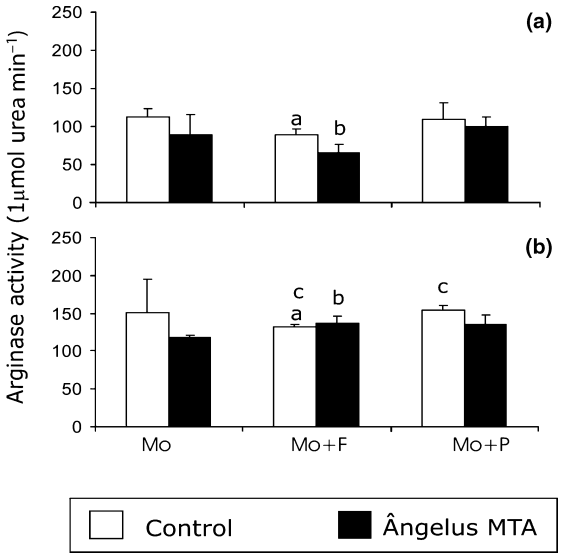


**Figure 6** Arginase activity by M1 macrophages (a) from wt C57BL/6 mice and M2 macrophages (b) from C57BL/6 IL-12p40-/- mice. Cells were cultured with capillaries containing MTA and stimulated with *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P), as indicated. Bars stand for the mean results of three experiments made in duplicates. Lines indicate standard error of the means. a, b indicate $P < 0.05$ for arginase activity when compared two. c indicates $P < 0.05$ when compared to kinds of stimuli in M2 macrophages.

### Arginase activity

Mineral trioxide aggregate did not affect the arginase activity of either macrophage subtype (Fig. 6). M2 macrophage presented higher arginase activity than M1 macrophage when it was stimulated with *F. nucleatum*, in MTA and control groups ($P < 0.05$). In M2 macrophages, control group stimulated with *P. anaerobius* presented higher activity than control group stimulated with *F. nucleatum* ($P < 0.05$).

## Discussion

Mineral trioxide aggregate is indicated for pulp-capping, pulpotomy, repair of root perforations, fractures and apical barriers in root with incomplete apex formation (Adamo *et al.* 1999, Torabinejad & Chivian 1999, Saidon *et al.* 2003). However, little information is available on MTA interaction with the immune responses to infection, which is relevant since in every

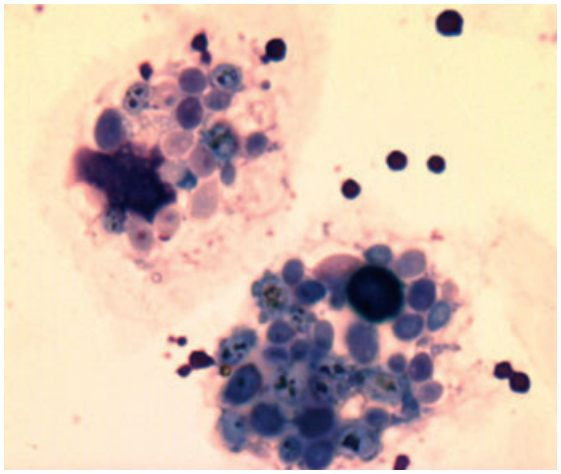

**Figure 7** M1 macrophages containing ingested yeast cells (pink, arrows) and numerous adhered yeast cells (purple, stars). Bi-refractive structures are found on and apparently in macrophages, suggesting that MTA might be ingested by macrophages (arrow heads) 1000× under immersion objective.

clinical condition for which this material is indicated there will be contact of MTA with pulpal, periapical, or periodontal tissues.

As soon as the inflammatory process starts, immune-competent cells are attracted to the site, in an attempt to eliminate the aggressors. Of these cells, infiltrating professional phagocytes, neutrophils and macrophages are amongst the first cells to make contact with the foreign bodies, playing therefore the main role in the pathogenesis of the inflammatory process (van Furth *et al.* 1972, Unanue 1978, Stashenko *et al.* 1998, Metzger 2000, Oliveira Mendes *et al.* 2003).

Recent studies propose that two subtypes of macrophages exist, with different response profiles: M1 and M2 (Metzger 2000, Mills *et al.* 2000). IL-12 would drive macrophages to the M1 subtype (Metzger 2000, Mills *et al.* 2000), whilst the absence or low production of this cytokine would lead macrophages to the M2 subtype (Mills *et al.* 2000, Bastos *et al.* 2002). Hence, IL-12p40$^{-/-}$ mice are a good source of M2 macrophages, although this model does not exclude the production of other cytokines that, along with IL-12, induce a Th1 response, such as IL-18 (Joosten *et al.* 2000).

Using both types of macrophages exposed to two different sources of MTA, this study assessed the cellular viability in polypropylene tubes, and showed that M2 macrophages presented lower levels of cellular viability than M1 macrophages. However, differences in cell viability were small amongst culture conditions, and viability was consistently high. Similar values for the test groups (with MTA) and the control groups (without MTA) were observed. In the literature, similar results were reached using fibroblasts (Keiser *et al.* 2000, Saidon *et al.* 2003), osteoblasts (Koh *et al.* 1997, Mitchell *et al.* 1999) and macrophages (Haglund *et al.* 2003, Rezende *et al.* 2005).

Several studies that evaluate biocompatibility of endodontic materials assayed macrophage adherence and spreading (Zhu *et al.* 2000, Oliveira Mendes *et al.* 2003). MTA did not affect either macrophage subtype in their capacity to adhere to glass, which is consistent with results found for osteoblasts and an osteosarcoma cell line exposed to MTA (Koh *et al.* 1997, 1998, Zhu *et al.* 2000).

Mineral trioxide aggregate is applied onto areas that might be inflamed and/or infected, and it is fundamental that it does not interfere with the host's phagocytosis process. In this study, the capability of both macrophage subtypes to ingest *Saccharomyces boulardii* was evaluated in the presence of MTA, and compared with controls. This yeast was selected because of its size, which makes counting easier, and therefore allows for greater data precision. Neither brand of MTA interfered with the percentage of adhered yeast cells to macrophages or with phagocytosis of yeast cells. However, the number of ingested yeast cells was greater in the M2 macrophages. Conversely, M2 macrophages in contact with zinc oxide eugenol based material had their phagocytosis ability inhibited (Oliveira Mendes *et al.* 2003). This phenomenon was also observed by Bastos *et al.* (2002), who found a greater number of *Trypanosoma cruzi* amastigotes in M2 than in M1 macrophages.

Reactive oxygen intermediates and NO are free radicals that are released by phagocytes exposed to microbes (Marton & Kiss 2000). Both macrophages subtypes produced significant amounts of ROI in response to zymosan. Moreover, in accordance to previous report (Mosser 2003), M1 macrophages produced greater level of ROI than M2. Exposure to MTA did not interfere with the peak of ROI production. This is, to our knowledge, the first study in the literature that assayed the effect of MTA on ROI production.

Considering that *F. nucleatum* and the *P. anaerobius* are bacteria usually found in endodontic infections (Sundqvist 1992, Lana *et al.* 2001), they were chosen as stimuli for the production of NO. These heat-killed bacteria were used with or without IFN-$\gamma$. There was no difference in NO production between control and MTA and between M1 and M2 macrophages. These results are not supported by the current literature (Mills *et al.* 2000, Bastos *et al.* 2002, Mosser 2003). As we have recently shown that M2 macrophages produce higher levels of IL-10 than M1 (Rezende *et al.* 2005), this production of IL-10 might have been sufficient to suppress ROI production, but could be insufficient to inhibit NO production (Bogdan *et al.* 1991). Alternatively, the higher phagocytic capacity of M2 might have provided a greater stimulation to these cells that to M1 macrophages.

Using the same heat-killed bacteria, arginase activity was measured in cell lysates of M1 and M2 macrophage cultures. It is known that M2 macrophages convert L-arginine to L-ornitine and urea and this reaction results in cell growth and differentiation and collagen production. In this study, MTA did not affect arginase activity. M2 macrophage presented higher arginase activity than M1 macrophages. This result agrees with current literature (Mills *et al.* 2000, Bastos *et al.* 2002, Mosser 2003). The higher levels of IL-10, produced by M2 macrophage previously reported (Rezende *et al.* 2005), as a Th2 cytokine, inhibits the activity of type 2

enzyme nitric-oxide synthase (NOS 2) and induces arginase synthesis (Gordon 2003).

## Conclusion

It has recently been reported that MTA does not interfere in the cytokine response by M1 or M2 macrophages (Rezende *et al.* 2005). The present study suggests that MTA did not inhibit phagocytosis, reactive species production and arginase activity, which are protective responses against infections. It is concluded that MTA does not affect effector macrophage activities.

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