Evaluation of osteoblast-like cell response to Proroot™ MTA (mineral trioxide aggregate) cement

G. A. PELLICCIONI[1]*, G. CIAPETTI[2], E. CENNI[2], D. GRANCHI[2], M. NANNI[1], S. PAGANI[2], A. GIUNTI[2,3]
[1]*Dipartimento di Scienze Odontostomatologiche, Alma Mater Studiorum/Università di Bologna, via San Vitale 59, 40100 Bologna, Italy*
[2]*Laboratorio di Fisiopatologia degli Impianti Ortopedici, Istituto Ortopedico Rizzoli, Via di Barbiano 1/10, 40136 Bologna, Italy*
E-mail: ciapetti@ior.it
[3]*Dipartimento di Scienze Anatomiche, Umane e dell'Apparato Locomotore, Alma Mater Studiorum/Università di Bologna, Italy*

Some endodontic sealers have been shown to cause local and systemic effects, mainly due to microleakage of chemicals from the sealer. To avoid the risk of toxic effects *in vivo*, the biological compatibility of filling materials has to be assessed. *In vitro* compatibility of Proroot™ MTA cement in comparison with two different fillers used in clinical practice, was examined by testing the adherence, viability, proliferation and secretion of collagen of osteoblast-like cells.

In our experimental system, Saos-2 cells challenged with Proroot™ MTA for 24 and 72 h showed a better behaviour than the cells exposed to the other compounds under assay. We found that the cells attached to the rough surface of Proroot™ MTA cement and spread onto the rough surface. Moreover, the cells on Proroot™ MTA were viable, grew, and released some collagen even at 72 h, while cell metabolism and growth was dramatically reduced onto sEBA and amalgam surfaces. A parallel behaviour was found after the cells were challenged with extracts of the different fillers.

In conclusion, according to our *in vitro* study, Proroot™ MTA showed a good interaction with bone-forming cells: such behaviour may partially account for its satisfying clinical performance.

1. Introduction

The placement of a root end filling material during periradicular surgery is a procedure of a paramount importance to hermetically seal the endodontic space when this goal has not been achieved by a conventional root canal therapy [1]. Several materials have been developed and tested for this use, but to date, no root end filling material is demonstrated to be the ideal one, yet.

Recently a material, named Proroot™ MTA, has been commercialized in the European market and it is quoted to own many of the ideal characteristics [2]. Such cement derives from the original formula of mineral trioxide aggregate (MTA) that was developed at the University of Loma Linda [3, 4].

The MTA cement has been extensively studied and has always showed very good results in all *in vitro* [5] and *in vivo* [6, 7] tests, either alone or when compared with other root end filling materials [8–15]. Particularly regarding the cytotoxicity, MTA was found to be less toxic than (intermediate restorative material) IRM or Super EBA [16–18]. However, the Proroot™ MTA is a modified version of the original formula, on which most of the studies have been conducted [19]. Such changes have been adopted to improve handling characteristics and colour of this material, i.e. features which are relevant for the clinician [20].

It seems indeed interesting to test the biocompatibility of Proroot™ MTA to confirm the good results achieved with the originally patented MTA.

The aim of this study was to further investigate the cytotoxicity of Proroot™ MTA grey as compared with silver amalgam and Super EBA™ cement using an osteoblastic cell model.

Experimental protocol included preparation of the solid specimens, i.e. the cement mixture layered onto the bottom of culture wells, as well as of ''extracts'' from solids, i.e. substances which are eluted from solid into culture medium. Both preparations were tested with osteoblast-like cells. Bone-forming cells have been chosen because of the correlation with *in vivo* situation,

*Author to whom all correspondence should be addressed.

where the ''filler substance'' comes in contact with bone. Since the materials *in vivo* will be in contact with bone, two osteoblast-like cell lines widely used in biocompatibility testing were employed. MG-63 cells were used in preliminary experiments where the goal was the optimisation of the assay. Afterward, Saos-2 cells which are human, mineralise in culture and are well-characterized with respect to expression of osteoblastic traits were used in the experiments [21].

The fact that two different continuous cell lines gave comparable results, as only Proroot™ MTA surface allowed cell attachment and growth, provides an additional proof of its biocompatibility.

2. Materials and methods

2.1. Material preparation

The composition of the materials, as given by the manufacturer, is reported in Table I.

Each material was mixed according to manufacturer's instructions under the laminar air hood using sterile disposable materials.

Proroot™ MTA § was mixed (powder/liquid ratio 3 : 1 by weight) for 1 min.

Super EBA™ * (powder/liquid ratio 1 : 1 by weight) was mixed for 1 min.

Amalgam # (alloy/mercury ratio 1.3 : 1 by weight) was vibrated, according to manufacturer's instructions, for 9 s with an appropriate amalgamator.

After placing the different materials in the wells of Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL), they were left to set for 1 week under laminar air flow hood in a wet environment. Before seeding with cells the surface of each material was pre-wetted with culture medium for 2 h.

2.2. Preparation of extracts

The cement components were mixed according to the manufacturer's indications as previously described. The dough time of the mixtures was between 1 min (Proroot™ MTA and Super EBA) and 8 s (amalgam). After 4 h of setting, the solidified specimens were extracted in D-MEM culture medium for cells (see under ''Cells'') according to the International Standard for Biological Testing of Medical Devices (1 g/5 ml of medium for 72 h at 37 °C) [22]. The control for the material extracts was provided by D-MEM stored in a polystyrene flask for cell culture.

2.3. Cells

MG-63 and Saos-2 osteoblast-like cells (Istituto Zooprofilattico Brescia, Italy) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U of penicillin/ml, 10 µg of streptomycin/ml, 2 mM glutamine and 0.1 mM nonessential amino-acids. The cells were grown in 75 cm^2 flasks at 37 °C in a humidified 5% CO_2-95% air atmosphere. For the experiments with solid samples 1×10^5 cells were seeded onto material surfaces; with the extracts, first 1×10^5 cells were seeded in the wells, then the extracts were added (1 ml per well) after 24 h. In both cases the cells were re-incubated for 24 and 72 h at these endpoints the supernatants were separately collected and LDH and collagen release were evaluated. The cells were tested using MTT, neutral red or Alamar blue staining for cell viability/activation, Hoechst 33342 dye for cell number, and acridine orange for morphological observation of cells onto surfaces. Cells grown onto tissue culture polystyrene provided the controls (crl).

2.4. Viability

2.4.1. MTT test

The MTT conversion method has been modified in our laboratory for testing the cytotoxicity of materials [23].

At the fixed endpoints 100 µl of MTT (5 mg/ml in PBS, SIGMA) were added to each well. The plates were incubated for 3 h at 37 °C, then the medium was removed and 500 µl of DMSO added to the well. After gentle shaking for dissolving the formazan crystals, the blue solution was transferred to a 96-well plate and the absorbance was read in the microplate-reader, using a test wavelenght of 540 nm.

2.4.2. Neutral red assay

Neutral red is a vital dye actively endocytosed by cells through the intact membrane and stored within lysosomes of viable cells; the assay was performed by the method of Hansen [24].

Briefly, neutral red dye (SIGMA) was dissolved 50 µg/ml in MEM and, after discarding the supernatants, 0.2 ml added to the wells for 2 h at 37 °C. Following removal of the medium, 0.1 ml of lysing solution (50% ethanol in 1% acetic acid) were added to the wells.

After gentle shaking of the microplate for one minute, the colour intensity of each well was read at 540 nm using the Spectra III plate reader (Tecan, Austria) and optical density values (OD) of replicate wells were averaged.

TABLE I Composition of the materials, as given by the manufacturers

§Proroot™ MTA cement	Dentsply Tulsa Dental, Tulsa, Ok	*Powder*: Portland cement (Tricalcium silicate, Dicalcium silicate, Tricalcium aluminate, Tetracalcium aluminoferrite) ≈ 75%; Bismuth oxide ≈ 20%; Calcium sulfate dihydrate (Gypsum) ≈ 5%; trace elements (crystalline silica, calcium oxide, potassium and sodium sulfate) up to 0.6% *Liquid*: water
*Super EBA™ cement	Harry J. Bosworth Co, Skokie, Ill.	*Powder*: 60% Zinc oxide, 34% Alumina and 6% Natural resin *Liquid*: Benzoic acid 62.5% and Eugenol 37.5%
#Valiant™ Ph.D™ Amalgam	Dentsply Caulk, Dentsply International Inc, Milford, DE	*Alloy* (400 mg) composition: Silver, Tin, Copper, Palladium *Mercury* (358 mg)

2.4.3. Alamar blue test

The Alamar blue assay measures the products of the redox reactions occurring in mitochondria of viable cells: it is used as an index of both viability and metabolic activity of cells [25]. 100 µl of Alamar Blue™ solution (Biosource, CA) were added to the wells (one for each material and control cells). The wells were incubated for 4 h at 37 °C, then the medium was transferred to a 96-well plate in triplicates and the fluorescence read in the CytoFluor™ 2350 (Millipore Corporation, Bedford, MA, USA) reader for microtiter plates with EX 490 nm–EM 530 nm.

2.5. Number of cells

The number of cells was determined by fluorometric quantification of DNA using an assay adapted from West *et al*. [26]. The bis-benzimidazole dye Hoechst exhibits high fluorescence upon binding to the double-stranded DNA. Hoechst 33342 (SIGMA) 5 µg/ml in PBS was added to the cells for 30 min at 37 °C in the dark: then the fluorescence was evaluated using the CytoFluor™ 2350 plate reader with EX 360 nm–EM 460 nm. The results were expressed as relative fluorescence units (RFU).

2.6. Lactate dehydrogenase assay

Lactate dehydrogenase enzyme released from membrane-damaged cells is used for measuring cell death due to toxic agents: it was quantified using a commercial kit from SIGMA based on pyruvate substrate reduction. The absorbance of the product, which is proportional to cell death in the sample, was read at 490 nm.

2.7. Collagen production

Details of the staining procedure for collagen using picrosirius dye have been reported elsewhere [27]. Briefly, cell supernatants were separately collected and dispensed into wells of a microtiter plate in triplicates (100 µl/well). The plates were incubated at 37 °C for 16 h (humidified) and then 24 h at 37 °C (dry). After rinsing with distilled water, the wells were filled with 0.1% Sirius Red F3BA in saturated picric acid (w/v) (100 µl/well) for 1 h at room temperature. The excess dye was removed with 10 mM HCl and the bound dye eluted using 0.1 M NaOH. The absorbance of the eluted dye was read at 540 nm in the Spectra III reader and the results expressed as optical densities.

2.8. Morphology

The method, originally detailed by Darzynkiewicz *et al*. (1992) for analyzing DNA of cells by flow cytometry, was modified and used for examination of cells onto materials by fluorescence microscopy [28]. Briefly, the cells seeded in chamber slides were permeabilized using 0.1% Triton X-100 in 0.08 N HCl/0.15 N NaCl for 30 s. Following *in situ* staining with acridine orange 6 µg/ml in 1 mM EDTA, 0.15 M NaCl and 0.1 M citrate-phosphate buffer (pH 6) for 10 min the cells were examined using a fluorescent microscope with 515–575 nm EX-EM wavelengths.

TABLE II MTT test with solids at 24 h

	MTA	Amalgam	Super EBA	Control
W/o cells	13	1112	5	8
With cells	433	1394	33	319

2.9. Data presentation

The results from biochemical assays are expressed as Optical Density ($OD \times 10^3$) in the first part, i.e. Tables II–IV, and as percentages of values of cement-exposed cells *vs*. control cells, set = 100, in the experiments with Saos-2 cells. Mean and standard deviation of three separate experiments are given in the graphs.

3. Results

In preliminary experiments using MG-63 cells and the MTT test, the values recorded by amalgam were very high, with no difference between samples with and without cells (Table II). It was postulated that the value scored by the amalgam w/o cells may be due to reactive groups still present onto material surface: such groups were able to convert MTT salt to formazan product, giving false positive results. The hypothesis was confirmed by the MTT test performed using the extracts of the materials, where the reactive radicals are no more present: the viability of cells grown onto Proroot™ MTA and amalgam were similar and quite close to the value of control cells (Table III).

In order to get reliable results on cell viability onto cements, i.e. unaffected by the reactivity of material surfaces, the method from Hansen was modified. MG-63 cells were stained with neutral red before seeding onto material surface; after 24 h of culture onto materials the neutral red within cells was extracted and measured, and the cells on Proroot™ MTA showed the highest viability (Table IV).

Saos-2 cells, which also are osteoblast-like cells, were used in the second part of the study. After seeding of Saos-2 onto solid surfaces of materials the viability/activity of cells was assessed using Alamar blue: the range was amalgam > Proroot™ MTA > sEBA at 24 and Proroot™ MTA > sEBA ≥ amalgam at 72 h (Figs. 1 and 2).

But after subtraction of the ''blank'' value, i.e. medium onto material but no cells, differences were observed in the results, with Proroot™ MTA substantially ranging from 49.9 to 39.7% of crl, while the activity of cells onto sEBA and amalgam was close to zero (Figs. 1 and 2: ''blank'' values marked with °). The toxic effect of the materials was measured as release of lactate dehydrogenase from the cytoplasm of cells: at 24 h sEBA and amalgam were found to cause cell death more than that induced by Proroot™ MTA (140.6 and 132.72 *vs*. 101.1, respectively) Fig. 3. At 72 h sEBA was the more toxic material (Fig. 2).

TABLE III MTT test with extracts at 24 h

	MTA	Amalgam	Super EBA	Control
With cells	311	337	50	319

TABLE IV Neutral red test with extracts at 24 h

	MTA	Amalgam	Super EBA	Control
With cells	1397	649	1197	1750

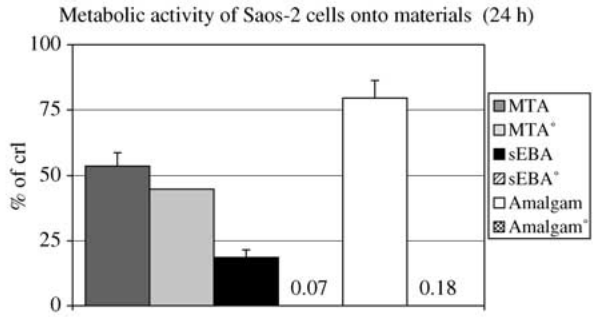

Figure 1 Metabolic activity of Saos-2 cells onto materials at 24 h: results are expressed as percentage of unexposed cells (= crl cells). ° = values after blank subtraction.

The number of cells onto materials has been measured indirectly by quantification of DNA content in the cells. At 24 and 72 h after seeding, the number of cells onto Proroot™ MTA was approaching control values (85.9 and 84.7, respectively) (Fig. 4).

The cells onto sEBA and amalgam were found to have the same behaviour at 24 h, with values approaching control cells, but after 72 h the number of cells was reduced to 50–40% of crl.

When the collagen released from cells was measured, the Proroot™ MTA-challenged cells were able to release a consistent amount of collagen at 24 and 72 h, while cells onto sEBA and amalgam are not stimulated to this activity (Fig. 5).

The behaviour of cells exposed to cement extracts is depicted in Figs. 6 and 7. After exposure of the cells to the extracts from filler polymers for 24 h, the same trend was appreciated. The cells challenged with Proroot™ MTA showed the best viability (with no interference of the material itself with the assay) and proliferation in comparison with the other compounds.

The morphology of cells grown onto materials was examined after staining of cells *in situ* using acridine orange: compared to control cells, which are well spread and elongated, the cells onto Proroot MTA were less in number but quite well spread (Figs. 8 and 9).

Very scarce cells were observed onto amalgam, while the examination of the cells (if any) onto sEBA surface

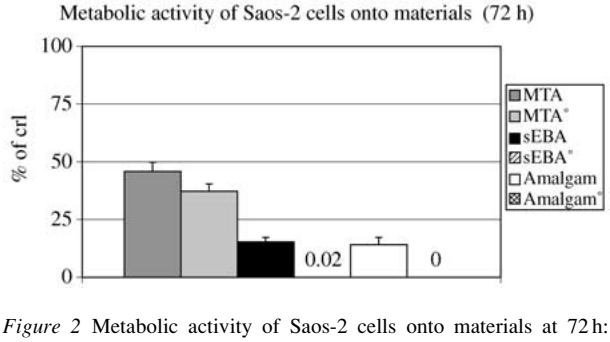

Figure 2 Metabolic activity of Saos-2 cells onto materials at 72 h: results are expressed as percentage of unexposed cells (= crl cells). ° = values after blank subtraction.

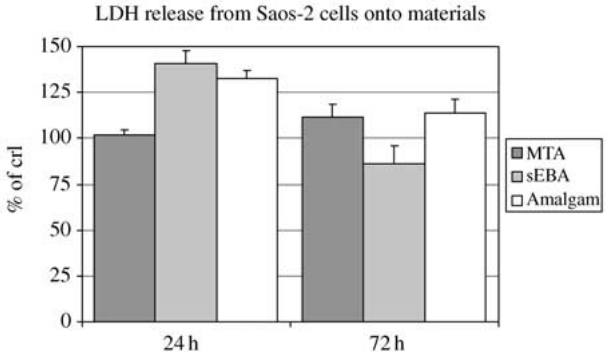

Figure 3 Release of lactate dehydrogenase from Saos-2 cells onto materials at 24 and 72 h: results are expressed as percentage of unexposed cells (= crl cells).

was completely hampered by the fluorochrome-adsorbing surface (Figs. 10 and 11).

4. Discussion

Some endodontic sealers have been shown to cause local and systemic effects, mainly due to microleakage of chemicals from the sealer. To avoid the risk of toxic effects *in vivo*, the biological compatibility of filling materials has to be assessed preliminarily. Furthermore root end filling materials work in intimate contact with bone cells, and the growth of these cells on support materials is needed for good integration of the implanted device. Recent literature has indicated the key-role of osteoblasts in the response of bone tissue to materials. Not only their specific bone-forming activity, i.e. extracellular matrix (ECM) formation, HA deposition, etc., has to be maintained throughout the implant-life, but they play a key role in bone remodeling, as they have been shown to be finely tuning osteoclast activity through intercellular signals and cytokines [29].

To determine whether osteoblast-like cells proliferate and perform their functions on Proroot™ MTA, the adherence, viability, proliferation and secretion of these cells on MTA, as well on super EBA and amalgam for comparison, were assessed.

MG-63 and Saos-2 cells are osteoblast-like cells widely used in the field of biomaterial testing [21, 30]; among osteoblastic cell lines, Saos-2 cells are considered a ''mature'' type of cell, as many traits of human osteoblasts, including high ALP and mineralization ability, are retained by this cell line [31].

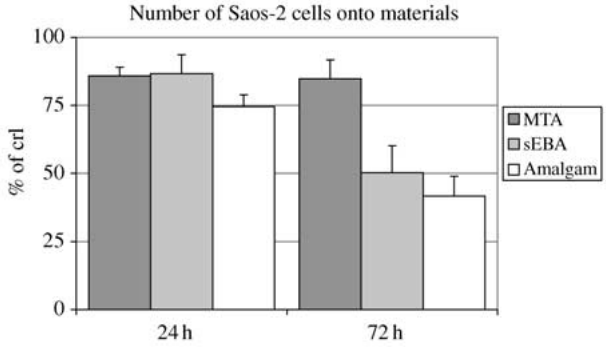

Figure 4 Number of Saos-2 cells onto materials at 24 and 72 h: results are expressed as percentage of unexposed cells (= crl cells).

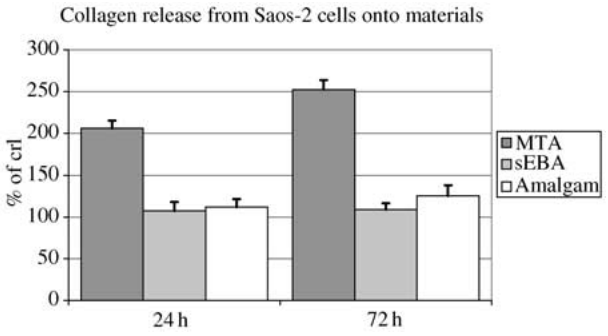

Figure 5 Release of collagen from Saos-2 cells onto materials at 24 and 72 h: results are expressed as percentage of unexposed cells (= crl cells).

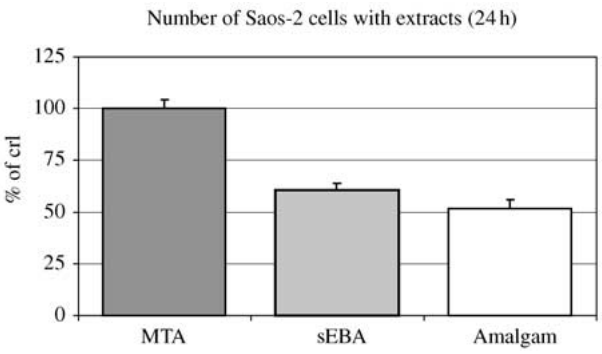

Figure 7 Number of Saos-2 cells treated with material extracts at 24 and 72 h: results are expressed as percentage of unexposed cells (= crl cells).

In preliminary experiments using MG-63 cells the MTT test was found to give false positive results: this was confirmed by measuring MTT conversion in the absence of cells. It has to be underlined that when materials resulting from a polymerization process are tested *in vitro* particular attention has to be paid to the chemical interaction of the material itself with the biochemical detection reagents. In our experience reactive groups of slowly polymerizing compounds may be responsible for unexpected reactions during *in vitro* testing [32]; moreover the reliability of colourimetric methods, and in particular of the MTT dye, has been debated by other Authors [33, 34].

To overcome these problems, biochemical methods have to be validated within each experimental system (''blank'' well, i.e. culture w/o cells, has to be included in designing the protocol), and a variety of cellular endpoints have to be measured within the same assay.

Alamar Blue method is now spreading in the assessment of biomaterial compatibility [35, 36]: the reduction of this dye is proportional to the amount of oxido-reductive reactions in the cells, i.e. it is a marker of cell metabolism. In our hands it turned to be sensitive and easily corrected by ''blank'' subtraction.

Bone formation onto material surface requires the adhesion of viable cells and proliferation, as first step, to be followed by production of extracellular matrix components and mineralization at the interface.

In our experimental system the cells challenged with Proroot™ MTA seem to fulfill these parameters better than sEBA and amalgam, often used for the same clinical application. We found that Saos-2 cells attach to the rough surface of Proroot™ MTA cement, as shown by fluorescence microscopy, and spread wherever possible, adapting to the uneven microtopography of the sample. This behaviour is not observed using the other two materials, which suggests that surface roughness by itself is not sufficient to promote cell adhesion, but the chemistry of the material is governing the interaction with cells, too. Moreover, the cells onto Proroot™ MTA are viable and able to proliferate, even at 72 h, while cell metabolism and growth is dramatically reduced onto sEBA and amalgam surfaces.

Results from LDH release show that at 24 h less cells are lost onto Proroot™ MTA in comparison to the other fillers; after 72 h the values for sEBA and amalgam are apparently lower than Proroot™ MTA, but they have to be referred to reduced cell populations (as shown by Alamar Blue and Hoechst data), which still undergo a certain death rate.

The good biological compatibility of Proroot™ MTA is demonstrated by the release of collagen from Saos-2 cells: this is very high for cells lying onto MTA at both time-endpoints. Collagen is a major component of the extracellular matrix (ECM) and its deposition onto surfaces represents a critical step in the bone-forming process.

When the cells are challenged with the extracts of the different fillers, the behaviour is very close to that recorded with the corresponding solid samples: the cells are allowed to function and to grow better when

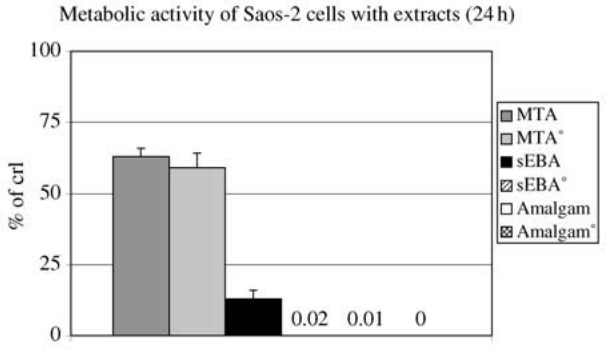

Figure 6 Metabolic activity of Saos-2 cells treated with material extracts at 24 and 72 h: results are expressed as percentage of unexposed cells (= crl cells). ° = values after blank subtraction.

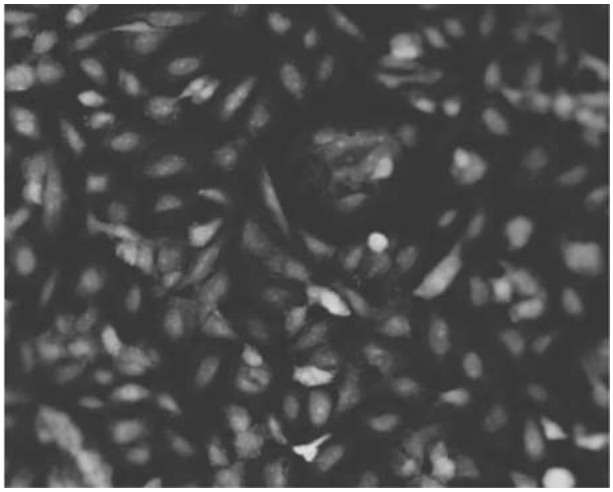

Figure 8 Control cells at 72 h (acridine orange, ×10).

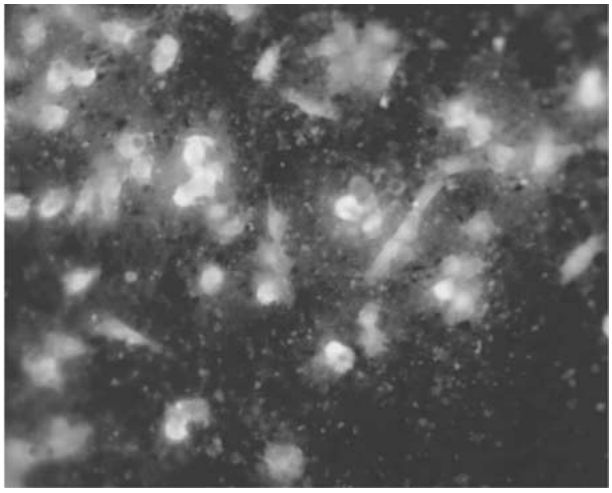

Figure 9 Proroot™ MTA: many cells spread over rough surface (72 h, acridine orange, ×10).

Proroot™ MTA extract is present, compared to sEBA and amalgam extracts.

This is assumed as an additional proof that the reaction of cells observed onto solid MTA is not driven by a particular microtopography of the sample in our system, but by a lower chemical toxicity of Proroot™ MTA compared to the other fillers. It is known that free radicals may be released by cements for long time after polymerisation, and these are toxic to cells [37]. Actually, the method of preparation of the extracts is conceived to obtain the ''leakage'' of any water-soluble substance in the medium, which thereafter affects cell behaviour during the assay. This makes the ''elution method'' a valuable tool for biocompatibility assessment of materials.

In conclusion, in our cell culture study Proroot™ MTA shows a good *in vitro* compatibility. This finding is in agreement with other studies concerning similar compounds [38, 19].

We are aware of the limits of the *in vitro* assays, including relevance of the type of cell and short-term contact between cells and materials. But we also believe that *in vitro* data are essential in the pre- clinical testing, as well as for strengthening the clinical results.

During 24 h- and 72 h-challenge of cells with solid

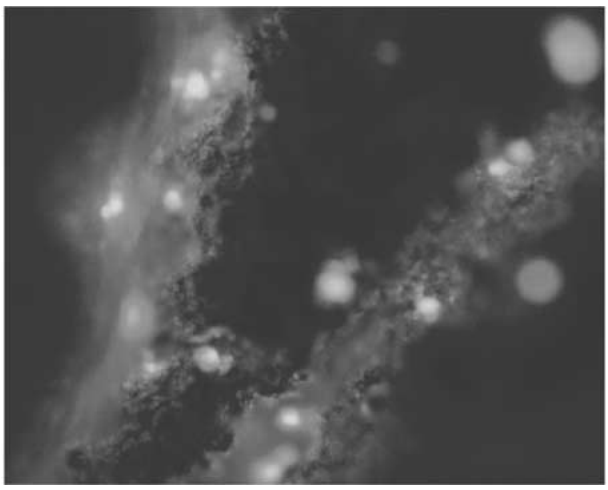

Figure 10 Amalgam: very few cells are seen onto rough surface (72 h, acridine orange, ×10).

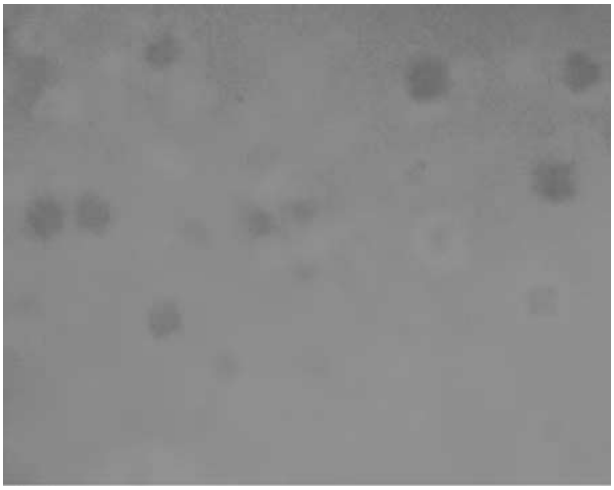

Figure 11 sEBA is totally fluorescent when stained with acridine orange (72 h, acridine orange, ×10).

Proroot™ MTA, as well as with its extract, osteoblast like cells (MG-63 and Saos-2) were able to adhere, spread, remain viable and grow better than cells treated with sEBA and amalgam. Though some ''chemical residue'' is still released from the composite *in vitro*, as shown by a limited reduction of cell viability and growth, the overall toxicity to cells is less severe than that shown by sEBA and amalgam in the same conditions. This would suggest that *in vivo* Proroot™ MTA would not destroy the surrounding cell population, and bone cells could be able to start bone deposition close to the filler material.

Acknowledgments

The work has been partially supported by grant ''Young Research Fund'', Alma Mater Studiorum/University of Bologna, Italy.

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Received 12 December 2002
and accepted 3 June 2003