# *In vitro* toxicity of MTA Compared with other Primary Teeth Pulpotomy Agents

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***Objective:*** *The main goal of this work is to compare the In vitro toxicity of MTA with other primary teeth pulpotomy agents.* ***Study Design:*** *The In vitro toxicity of MTA, calcium hydroxide, ferric sulphate solution, diluted formocresol and Buckley's formocresol were tested using MTT and Neutral Red Uptake cell viability assays. The results for MTA were compared to those obtained for the other substances using ANOVA and Tukey statistical tests (p<0,05).* ***Results:*** *MTA had the lower in vitro toxicity and Buckley's formocresol, the higher, with statiscally significant difference.* ***Conclusion:*** *Among the primary teeth pulpotomy agents tested, MTA showed the lower In vitro toxicity, standing as the most promising substitute to formocresol.*
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## INTRODUCTION

Pulp therapy in primary teeth is one of the most controversial areas in pediatric dentistry. Especially the vital pulp treatment, where formocresol has been, for the last 70 years, the most widely used substance.[1] Even with the acceptable clinical and radiographic results, some concerns about formocresol's toxicity and potential mutagenicity from sistemic contamination are raised by some authors.[2,3]

Modern trends in dentistry claim for more biocompatible substances, especially those that are going to be in direct contact with the pulp tissue.[4] Buckley's formocresol, despite its known toxicity, has been widely used as a pulpotomy agent for primary teeth, mainly because of its good clinical results. Alternative substances are being studied and developed in order to substitute formocresol as the medicament of choice for primary teeth pulpotomies, such as: Diluted formocresol, calcium hydroxide, ferric sulfate and mineral trioxide aggregate.[5,6,7]

The biocompatibility of the mineral trioxide aggregate (MTA) has already been shown in many studies[8] supporting its clinical use in endodontic procedures such as root perforations[9] and pulpotomy for both permanent and primary teeth. Actually MTA, when used as a primary teeth pulpotomy agent has been shown excellent compatibility with the pulp tissue,[10] even inducing dentin bridge formation and less pulp inflammation than formocresol and ferric sulfate.[11]

Among many different procedures to evaluate the citotoxicity and biocompatibility of dental materials, the reproducible and standardized protocols of the International Organization for Standardization (ISO)[12] are particularly efficient helping professionals to select and evaluate materials and devices.

The comparison of the *in vitro* toxicity of the MTA with other primary teeth pulpotomy substances has not been studied already. The main goal of this study is to define an *in vitro* toxicity rank for primary teeth pulpotomy agents, based on ISO standards, in order to provide a scientific basis to help pediatric dentists choose a more biocompatible substance for a routine procedure, such as vital pulp treatment.

## MATERIALS AND METHODS

The materials tested in this study were: Buckley's formocresol (FC) (Formula e Ação™, São Paulo, Brasil), 20% Diluted formocresol (DFC) (Formula e Ação™, São Paulo, Brasil), Calcium Hydroxide (CH) (Formula e Ação™, São Paulo, Brasil), 15,5% Ferric Sulfate solution (FS), (Formula e Ação™, São Paulo, Brasil) and Mineral Trioxide Aggregate (MTA Angelus™, Londrina, Brasil).

The crude extracts of each material tested were obtained by incubation of 0.1g of each material in 1mL of Dulbecco's modification of Eagle's medium (DMEM; Gibco BRL, Grand Island Biological Co., Grand Island, NY) without fetal calf serum, for 24 hours, at 37ºC in 5% $CO_2$ atmosphere. Afterwards, the supernatant was carefully removed

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and submitted to serial dilutions in DMEM containing 5% of fetal calf serum ranging from $10^{-1}$ to $10^{-5}$ and used immediately for cytotoxicity assay.

Balb/c 3T3 fibroblasts (CCL 163, clone A31, American Type Culture Collection), were plated at a density of 2 x$10^4$ cells/cm$^2$ and incubated in DMEM supplemented with 100 units/mL of penicillin, 100 mg/mL of streptomycin, 2mM L-glutamine, and 5% fetal calf serum (FCS) for 24 hours at 37ºC, in an atmosphere of 5% $CO_2$, to allow attachment. Afterwards, the medium was removed and the cells were exposed to the different dilutions of the extracts in DMEM + 5% FCS for 24 hours at 37ºC and 5% $CO_2$. Cells that were not exposed to the extracts (DMEM + 5% FCS only) were used as negative control. All experiments were done twice in five replicates.

Following the incubation of Balb/c 3T3 cells in the presence of different dilutions of each extract, the medium was removed, washed in phosphate buffered saline (PBS) and cells were harvested and counted using a Neubauer chamber. The number of cells corresponding to each extract concentration was calculated relative to the control (untreated) group, considered as 100%. The plot of the relative number of cells in function of extract concentration allowed the graphical estimative of dose (mg/mL) which kills 50% ($LD_{50}$) of the cells in relation to the control group, DMEM + FCS in the absence of material extract.

Cell viability was evaluated using the MTT assay and neutral red uptake (NRU) as described previously.[13,14] Briefly, Balb/c 3T3 cells were seeded into 24-well microtiter plates at a density of 2 x $10^4$ cells/cm$^2$. After 24 h the culture medium was replaced with each material extract at $LD_{50}$ in DMEM and incubated for another 24h. Afterwards, the medium was replaced after washing with PBS; 0.5 mg/mL of MTT (Sigma, St. Louis, Mo., USA) in PBS or 50 mg/mL of neutral red (in DMEM free of FCS) were added to each well.

For MTT assay, after an incubation time of 4 h, unreacted dye was removed by aspiration and the dark blue formazan crystals, which were solubilized by the addition of a 0.2% sodium dodecim sulfate were quantitated at a wavelength of 570 nm in a spectrophotometer (Ultrospec 2000™).

For the NRU assay, after an incubation time of 3 hours, medium was removed, cells were washed once with PBS and the incorporated dye was solubilized in 1% acetic acid in 50% ethanol and quantitated at wavelength of 540 nm in a spectrophotometer (Ultrospec 2000™).

Relative cell viability [%] related to control wells containing cell culture medium without extract was calculated by [A] test/[A] control x 100. The MTT and NRU tests were conducted twice in five replicates for each substance tested. The *in vitro* toxicity of the substances was ranked as a percentage of the control group (cells not exposed to the extracts).

The mean value of the five replicates from two independent experiments was submitted to ANOVA and Tukey's test for statistical analysis (InStat™, CA, USA). The significance level was $p<0.05$.

## RESULTS

Figure 1 shows the dose related inhibition of cell proliferation after a 24 hours exposure to all the dilutions of extracts of the primary teeth pulpotomy agents tested. The $LD_{50}$ concentration for every substance tested is defined by the intersection of the plotted lines with the line that represents 50% of the relative cell number. The estimated $LD_{50}$ values for each substance are shown on Table 1. Based on $LD_{50}$ values, the cytotoxicity of the primary teeth pulpotomy agents can be ranked as FC>DFC>FS>CH>MTA. The substance that presented the higher *in vitro* toxicity was Buckley's Formocresol (FC). Calcium hydroxide (CH) revealed to be approximately 10 times more toxic than MTA, according to Table 1 ($p<0.05$). When comparing FC and DFC $LD_{50}$ values with those obtained from MTA, it can be concluded that FC and DFC are, respectively, $10^4$ and 2,6 x $10^4$ times more toxic than MTA. The $LD_{50}$ value for MTA was 79,72 mg/mL, and with $p=0.0023$, could be considered significantly lower than CH, FS, DFC and FC.

Based on the $LD_{50}$ values, the mechanisms of toxicity were analyzed by applying the MTT and NRU cell viability colorimetric assays. These tests show, respectively, the interference of the extracts of the substances tested in mitochondrial metabolism and cell membrane integrity. Figure 2 shows the cytotoxic effects of the primary teeth pulpotomy agents based on MTT and NRU tests. Comparing the cytotoxicity of the substances tested in the MTT and NRU colorimetric assays, FC and DFC showed to be the most toxic

**Table 1.** Estimated LD50 values of the substances tested.

| Pulpotomy agents | LD50 (mg/mL) |
|---|---|
| MTA | 79.72 |
| Calcium Hydroxide (CH) | 7.74 * |
| Ferric Sulfate (FS) | 0.24 * |
| Diluted Formocresol (DFC) | 8.29 . $10^3$ * |
| Buckley's Formocresol (FC) | 3.07 . $10^3$ * |

*p values less than 0,05 when compared with MTA.

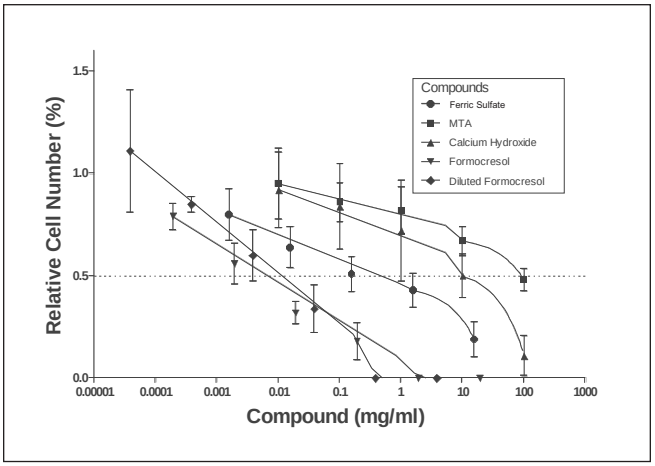


**Figure 1.** Dose-related inhibition of cell proliferation by the primary teeth pulpotomy agents tested. See Table 1 for estimated LD50 values.

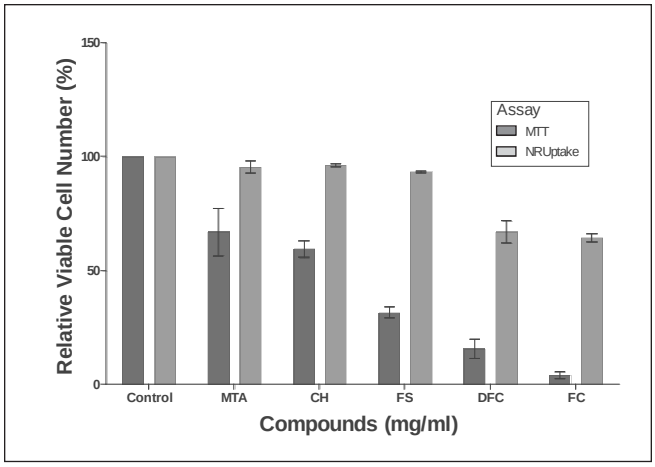


**Figure 2.** Relative number of viable cell at the LD50 for each material tested using MTT or NRUptake as an endpoint. Bars represent the mean (± SD) of two independent experiments in five replicates.

materials, followed by FS, CH and MTA, with a statistical significant difference (p=0.0013). All substances seem to affect more the mitochondrial activity than the cell membrane integrity, specially for FC and DFC that promoted the death of more than 80% of cells comparing with the control group ($p<0.05$). On the other hand, cell membrane integrity seem to be affected only by FC and DFC that killed more than 30% of the cells in comparison to the control group ($p<0.05$).

## DISCUSSION

The $LD_{50}$ value (lethal dose to 50% of the cells when compared to a control group) is one of the most reliable indicators of toxicity, because it defines the toxic concentration that is exactly in the middle point between the first signs of toxicity and the complete absence of metabolism or cell death.[12,15] In the present study, in addition to be applied as a parameter to define a toxicity scale, $LD_{50}$ was also applied to each one of the substances as a reference for the accomplishment of the MTT and NRU assays. $LD_{50}$ was also defined as a parameter for MTT and NRU colorimetric assays to estimate the toxicity of dental bleaching agents and root canal irrigants[16,17,18] and to establish a toxicity rank for dentin primers and monomers for composite resins.[15,19]

Buckley's formocresol has already been compared with other agents utilized in primary teeth pulpotomies, always presenting a higher degree of toxic reaction,[20] and being considered as the main responsible for pulpal degenerative inflammatory process than calcium hydroxide, eugenol and glutaraldehyde at 2.5%.

Table 1 shows that the MTA was considered, among the evaluated materials, the least cytotoxic. This low cytotoxicity degree for the MTA is supported by authors,[21] that also used the ISO protocols for cytotoxicity assays, such as in this research, compared this material with others with endodontic indication, concluding that the MTA does not have toxic effect in fibroblasts from the L-929 lineage. MTA has already been approved as a material for direct pulp application in a study using dog pulp tissue.[22] This research showed that dental pulps, when in contact with this material, are induced to form adjacent dental tissue and do not present inflammatory degenerative alterations. The low *In vitro* toxicity of MTA was also shown in a study that compared this substance with resin modified glass ionomer,[8] and on another study, evaluating whether regular and white MTA are able to induce genetic damage in primary human cells.[23] The biocompatibility of MTA was compared with an hydroxide containing cement when exposed to cultured human dental pulp cells.[24] The results of this study showed that MTA stimulated cell proliferation whereas the hydroxide containing cement had no such effect. The biocompatibility of MTA and glass ionomer cement using a cell culture technique was also compared.[9] These authors recommended the use of MTA as a perforation repair material because of its lower cytotoxicity.

The $LD_{50}$ value for the Ferric Sulfate (FS) showed that this substance is about 332 and 32 times more toxic than MTA and CH, respectively. When compared with the FC and the DFC, it is about 78 and 29 times less cytotoxic, respectively. FS, differently from MTA and CH, does not induce regeneration of the adjacent pulp tissue, when applied directly to the pulp. This substance operates by means of hemostasis, and does not stimulate dentinal bridge formation.[25] Histological research, comparing the effect of the FS and the FC in rat pulps,[26] showed that the FS toxicity mechanisms do not differ from those found with FC and DFC, with a remarkable presence of cells from inflammatory infiltrate.

To understand the mechanisms of toxicity of the analyzed substances was one of the main goals of the MTT and Neutral Red Uptake (NRU) colorimetric assays. These experiments help to identify if the involvement of the cell viability was in terms of reducing the mitochondrial activity of the cells (MTT) or affecting the lysosomal function (NRU).[14,27]

The NRU assay demonstrated that the MTA, CH and FS extracts did not affect the cells, when compared with the control group. FC and the DFC decreased the cell viability about 65% in relation to the control group (Figure 2). According to the results of these assays, the toxicity rank of the materials can be established as shown in the $LD_{50}$ test, all of them being significantly different from the control group (p=0.0067). This difference in sensitivity among the colorimetric assays is a common finding. The specificity of the MTT, NRU and cell proliferation assays was already tested[27] in order to establish the cytotoxicity of glass ionomer cements, zinc phosphate and a resin composite. According to these authors, the difference in sensitivity among the colorimetric experiments may differ, depending on the material that is being evaluated. Different degrees of sensitivity between the MTT and NRU assays were noticed in other studies.[28,29,30] These authors agreed that the same material, in the same experimental conditions, can be set into different degrees of cytotoxicity in each one of these experiments. These studies may indicate that liquid substances affect the cell metabolism in a more enhanced way at a mitochondrial level. When the cytotoxicity of different liquids and

powders, which are components of dental materials, was evaluated,[29] it was shown that the MTT and the NRU assays had similar patterns when the powder was analyzed. The liquids, however, seemed to affect more the cells at a mitochondrial level. The present study presented similar results, because FC and DFC (liquids) inhibit the cell viability, when compared to the control group, in a range higher than 70%. It is possible that the formaldehyde, that is a component of both substances, has a more adverse effect on the mitochondrial metabolism than in the lysosomal activity. However, in the NRU assay, these substances affected the cell viability in a range between 11% and 40%. FS, which is also a liquid, presented a different behavior for the two assays. The MTA and the CH, which are powder substances, was positioned in a same range in both assays.

It also can be possible that the liquid substances, due to their easiness to dissolve in an aqueous environment while the extracts are being obtained, seem to be more sensitive to the cytotoxicity tests than the solid substances and the ones, which are only powder.

The *in vitro* toxicity of MTA was analyzed and compared to other perforation repair materials using the total nucleic acid content (NAC), NRU and MTT tests.[30] The authors concluded that MTA was the least cytotoxic material and that the NRU test showed a lower sensitivity to MTA, when compared to MTT and NAC tests.

The results obtained by the FC, DFC and FS in this research, showed that these materials are extremely cytotoxic and should be used as a foundation to search for more biocompatible alternatives for pulpotomies in primary teeth.

The extremely acceptable biocompatibility of MTA has already been shown in many *in vitro*[8,9,24,31,32,33] and *in vivo* studies,[6,10,34,35,36] supporting the clinical use of MTA as a vital pulp therapy agent for both primary and permanent teeth.

## CONCLUSIONS

Based on these results it can be concluded that:

- MTA has the lower *In vitro* toxicity among the most widely used primary teeth pulpotomy agents tested.
- MTA can be considered the vital pulp therapy substance that stands out as the most promising substance, either for permanent or primary teeth, to replace formocresol.

Regarding the mechanisms of toxicity, all substances tested seemed to affect mitochondrial function, especially Formocresol (FC) and diluted formocresol (DFC). These two substances were the only ones that produced a severe response on cell membrane integrity.

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