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COMPARATIVE CYTOTOXICITY EVALUATION OF MEDICINES USED FOR PULP THERAPY OF PRIMARY TEETH

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SUMMARY:

Introduction: Despite the eight decades of widespread clinical use of formocresol growing evidence from both experimental and clinical studies clearly indicates that formaldehyde is leaking out during pulpotomy and may participate in the development of non-target tissue damage of local and systemic character. Special attention has been paid to pulp-capping materials and especially mineral trioxide aggregate (MTA) as probable alternative of formocresol in vital pulpotomy in primary teeth, in line with its excellent biocompatibility and pro-dentinogenic properties. The overwhelming evidence that MTA is superior in terms of biological compatibilityand clinical success as compared to formocresol has conditioned the dramatic shift to MTA in routine pulpotomies.

Objective: The aim of this study was to assess the biocompatibility of resorcinol/formalin (RF) pulpotomy preparation in comparison to mineral trioxide aggregate (MTA) and calcium hydroxide cement (CHC).

Methods: Cell survival was assessed by the MTT-assay (after 48, 72 or 144 h) in five cell lines, namely: HD-MY-Z, HEK-293, SH-SY-5Y, Neuro-2A, SaOS-2. In addition treatment-induced morphological perturbations and induction of necrosis and apoptosis were assessed in HEK-293.

Results: RF evoked strong, concentration-dependent cytotoxicity, which was evident even at significant dilution of the parent solution. In general the cytotoxicity of RF was not greatly influenced by the exposure period, especially at the higher concentrations under evaluation. In contrast the MTA extracts proved to be generally devoid of cytotoxic effects. MTA treatment actually increased the viability of SaOS-2 osteosarcoma cells, which could be attributed to the presence of calcium ions in the MTA-eluate which in turn stimulates the proliferation of this cell line.The CH cement extracts showed marginal cytotoxicity which was far less pronounced than that of RF and slightly superior compared to MTA.

Conclusions: In contrast to RF that exerted prominent cytotoxicity, MTA and CHC were biocompatible, with no evidence for decreased mitochondrial dehydrogenase activity, morphological changes in monolayer integrity or induction of apoptosis and/or necrosis. This contribution is the first systematic *in vitro* evaluation of the cytotoxicity of resorcinol-formalin *vs*. viable pulpotomy agents. It gives further evidence for the safety advantages of viable pulp therapy products CHC and especially MTA as compared to the RF preparation, routinely used in Bulgaria for decades.

Key words: pulpotomy, formaldehyde, resorcinol, MTA, biocompatibility, calcium hydroxide, cytotoxicity, MTT-assay, apoptosis, necrosis.

INTRODUCTION

Worldwide, formaldehyde-based products continue to be used routinely in dentistry (1). Most dental schools in Europe, North America and elsewhere still widely advocate the dominant clinical use of formocresol (a *m*-cresol/formaldehyde based product) pulpotomies in both carious primary and permanent teeth (2-4). In Eastern Europe, and Bulgaria in particular, primary teeth pulpotomies are performed employing a chemically analogous and therapeutically equivalent protocol based on a saturated resorcinol solution in formalin (RF), instead for formocresol (5-8).

Despite the eight decades of widespread clinical use of formocresol growing evidence from both experimental and clinical studies clearly indicates that formaldehyde is leaking out during pulpotomy and may participate in the development of non-target tissue damage of local and systemic character (1, 9, 10). This has been argued by some authorities who consider formocresol to be devoid of significant toxicity in humans when applied in low doses (i.e. in pulpotomies) (1, 11, 12). Regardless, the immunologic and systemic distribution of ^{14}C labeled formaldehyde has been well documented (13, 14) and the toxic effects of formocresol and

other formaldehyde containing agents have been clearly demonstrated *in vivo* and *in vitro* (9, 10, 15-23). Recent clinical data evidently reinforce these observations (2, 4, 10, 13, 24).

The evidence obtained shows that formocresol even in reduced concentrations has the potential to result in negative immunologic, systemic, toxicological, and overt clinical consequences. More specifically formaldehyde employed during pulpotomy could evoke inflammation of surrounding non-target tissue and exert cytotoxic (16, 17, 21, 23), genotoxic and mutagenic effects (9, 19) leading to tissue damage ranging from vascular insult and inflammation (3, 4, 25) to necrotic (26, 27) and osteolytic changes (13). It is also capable of damaging the enamel and the succedaneum teeth (17). Moreover the pulpal proteins that have been treated by the formocresol or formalin/resorcinol are rendered by the host as an altered entity, recognized as potentially antigenic and immunologically foreign. Thus in addition to the local, non-specific inflammatory events evoked by formocresol the host's immune system could be sensitized to the treatment-modified materials. These could be in turn phagocytized by macrophages, enzymatically processed by neutrophils and stimulate the generation of B and T-cell immune response (13). This is further complicated by the overlying immunologic response to the bacteria and its by-products from the carious lesion that the treatment is supposed to remove and by non-specific stimulation of innate immunity mechanisms and cytokine production (13, 25, 28). Besides, the genotoxic, carcinogenic and mutagenic potential of formaldehyde has been so extensively appreciated to be regarded as proven (9, 13, 19, 20, 22, 29). The International Agency for Research on Cancer classified formaldehyde as definitely carcinogenic for humans in June 2004, leaving the profession to look for alternative regimens (30). On the basis of the information available, an expert working group has concluded that there is now sufficient evidence that formaldehyde causes nasopharyngeal cancer in humans, a rare cancer in developed countries, limited evidence for cancer of the nasal cavity and paranasal sinuses, and "strong but not sufficient evidence" for leukemia (4, 30). Therefore, biologic manifestations of a simple formocresol pulpotomy could well extend beyond the transient local events and can be significant and potentially systemically harmful to a patient (2-4, 24).

Apparently the above stated evidence-based dentistry has provided overwhelming scientific and clinical data to support the removal of formocresol from human use especially in the pediatric population (10, 13). Thus during the last several years the clinical role of formocresol has been subject to significant clinical debate and reevaluation (1, 10). It has been shown that many clinical studies supporting the use of formocresol have been based on old, imprecise, short-term data (2-4, 10, 24). On these grounds many clinicians in Western Europe and North America tend to be increasingly reluctant regarding the usefulness of formocresol. Thus in a survey in Great Britain it has been shown that 54% of the pediatric dentists stated they were concerned about the safety profile of formocresol. Conversely, in an USA study 37% of endodontists and 18% of the pediatric dentists were concerned about the carcinogenicity of formocresol (13).

As a result of this compelling evidence, there has been a rigorous and diligent effort to design and elaborate alternative materials and procedures for use in pulpotomies (4, 31-33). Ideally such agents would guarantee the radicular pulp preservation in primary teeth until the time of their physiological exfoliation, while at the same time avoiding the side effects and long term toxicities from formaldehyde-containing medicaments. The most important medicaments and procedures studied in primary teeth pulpotomy include electrosurgery, laser, glutaraldehyde, ferric sulphate, freeze-dried bone, bone morphogenic protein and osteogenic protein, among others (2-4, 15, 24). Recently, advances in biomedical research opened avenues for the design of new methods of dental treatment, aiming at regeneration of the dentin-pulp complex. Such approaches have been based on the understanding of the molecular and cellular mechanisms regulating the dentinogenesis processes during dental tissue repair and their potential for clinical exploitation (2, 4, 10, 31). Accordingly, the research focus has been re-targeted from preservation and conservation to regeneration of the remaining pulp tissue (3, 4, 31, 32, 34-36). In this context special attention has been paid to pulp-capping materials and especially mineral trioxide aggregate (MTA) as probable alternative of formocresol in vital pulpotomy in primary teeth, in line with its excellent biocompatibility and pro-dentinogenic properties (32, 34, 37-49). The overwhelming evidence that MTA is superior in terms of biological compatibility (18, 28, 39, 41, 47, 50-53) and clinical success as compared to formocresol (2, 3, 24, 33-36, 45, 53-64) has conditioned the dramatic shift to MTA in routine pulpotomies (2-4, 31-36, 55, 61). Nevertheless, one must continue to ascertain the *in vitro* properties, long-term efficacy, biological and clinical use of MTA. Moreover there are alternative capping agents (e.g. Portland cement, calcium hydroxide and calcium silicate based cements etc.), which could be regarded as possible cheaper alternatives for MTA (4, 45, 58), requiring for further experimental and clinical research and evaluation. Despite the cytotoxicity of formocresol has been subject to numerous investigations the *in vitro* biosafety and compatibility profile of resorcinol/formalin being the dominant pulpotomy preparation in Eastern Europe is generally unexplored. On these grounds this study is aimed at thorough comparative evaluation of biocompatibility profiles of the three regimens most often employed in primary teeth pulpotomy in Bulgaria, namely resorcinol/formalin solution for mortal pulp management, or calcium hydroxide and MTA cements for vital pulp treatment.

MATERIALS AND METHODS

Pulpotomy agents and solutions. Formulations of the different agents were prepared in the laboratory according to the composition of the different materials as used clinically. The used chemicals were of reagent-grade quality, obtained from licensed dental material supply companies.

The RF solution was prepared according to the Bulgarian clinical protocol, as a saturated solution of resorcinol into 40% aqueous formaldehyde (5, 6). It was then diluted 1:1 with PBS to yield formaldehyde concentration of ca. 20%, thus mimicking the HCHO content in the most widely used Buckley's formulation of formocresol. Thereafter, for the bioassays the cells were exposed to serial dilutions of the RF solution, namely 1:25, 1:100, 1:200 and 1: 1,000 in PBS. The selected concentrations were either higher or lower as compared to the clinically relevant level of exposure of non-target tissues, corresponding to 1:100 formocresol (Buckley's formulation) (26, 65).

Two vital pulpotomy capping agents namely mineral trioxide aggregate (MTA; white, Angelus Solu3xes Odontolygicas, Londrina, PR, Brazil) and $Ca(OH)_2$ (CHC; Dycal®-Dentsplay) were used. They were prepared according to the manufacturer's instructions and after mixing, the cements were stored in an incubator at 100% relative humidity and 37°C for 1 day of hydration. The cements were then sterilized in ultraviolet light for 1 hour and stored in serum-free RPMI-1640 medium in a polypropylene tube for 3 days. The concentrations of the cement elution solutions were adjusted to 1, 10, 30, and 100 mg/mL.

Cell lines and culture conditions. In this study the following cell lines were used:, HD-MY-Z (Hodgkin lymphoma, human), HEK-293 (human embryonal kidney epithelium), SAOS-2 (human osteogenic sarcoma), SH-SY-5Y (human neuroblastoma) and Neuro2A (murine neuroblastoma). Except for HEK-293 and SH-SY-5Y, which originated from American Type Cell Culture (ATCC), all cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). The cells were grown in controlled environment – cell culture flasks at 37°C in an incubator 'BB 16-Function Line' Heraeus (Kendro, Hanau, Germany) with humidified atmosphere and 5% CO_2. HD-MY-Z were cultured as semi-adherent, and the other cell lines as monolayer cultures. SAOS-2 cells were grown in 85% McCoy's 5A supplemented with 15% fetal bovine serum (FBS), whereas for the other cells the growth medium was RPMI-1640 supplemented with 10% (FBS) and 2 mM L-glutamine. The cell lines were reset by trypsinization two times per week.

MTT-dye reduction assay. Cell survival was assessed by the MTT-dye reduction assay, which is based on the ability of mitochondrial succinate dehydrogenases in viable cells to reduce a yellow tetrazolium salt to violet formazan product which is detected spectrophotometrically. The procedure was carried out as previously described (66). Exponentially growing cells were plated in 96-well sterile plates at a density of 10^4 cells/well in 100 *m*L of medium and were incubated for 24 h. Thereafter the cells were exposed to serial dilutions of the resorcinol/formalin solution or the cement extracts for 48, 72 or 144 h. For each concentration a set of 8 wells was used. After the exposure period 10 *m*L aliquots from a 5 mg/ml MTT solution were added to each well and the plates were further incubated for 4 h at 37°C in a humidified 5 % CO_2 atmosphere. The formazan crystals were solubilized by addition of HCOOH (5 %) acidified 2-propanol. The MTT-formazan absorbance was read on a microprocessor controlled micro-plate reader (Labexim LMR-1). Results were normalized as percentage of the untreated control (set as 100% viability).

Apoptosis/necrosis assay. The DNA fragmentation as a quantitative merit of the ability of tested compounds to induce cell death was detected using a commercially available 'Cell Death Detection ELISA^{PLUS}'™ kit (Roche Applied Science), after 12 or 24 h treatment of HEK-293 cells, according to the manufacturer's instructions. The levels of DNA-fragments in the cytosolic fraction of treated cells (apoptotic fragmentation) and those in the culture supernatants (necrotic) were presented as enrichment factors (EF). These were calculated by dividing the 405 nm absorption of treated samples by the corresponding absorption of the untreated control. Each test was run in triplicate.

Morphological assay. The 48 h MTT-assay treatment protocol was mimicked in established confluent HEK-293 cell monolayers (cultured in sterile Petri dishes) in order to register defects in the monolayer integrity and cellular density, undetectable by the MTT-dye reduction assay. The exposed cultures where examined by a phase-contrast light microscope and photographed with a digital camera 48 h post treatment. The results were interpreted using the grade scale, described in USP 28 (2005) (grades 0-4) for assessment of the cytotoxic potential of tested materials, as follows: grade 0 – none reactivity (discrete intracytoplasmic granules, no cell lysis); grade 1 – slight reactivity (Not more than 20% of the cells are round, loosely attached and without intracytoplasmic granules; occasional lysed cells are present); grade 2 – mild reactivity (not more than 50 % of the cells are round and devoid of intracytoplasmic granules, no extensive cell lysis and empty areas between cells); grade 3 – moderate (Not more than 70% of cell layers contain rounded cells or are lysed); grade 4 -severe (nearly complete destruction of the cell layers).

Data processing and statistics. *The cytotoxicity MTT-assay was carried out in eight separate experiments, whereas the morphological, apoptosis/necrosis assays were run in triplicate. Statistical processing exploited Student's t-test with pd≤0.05 set as significance level.*

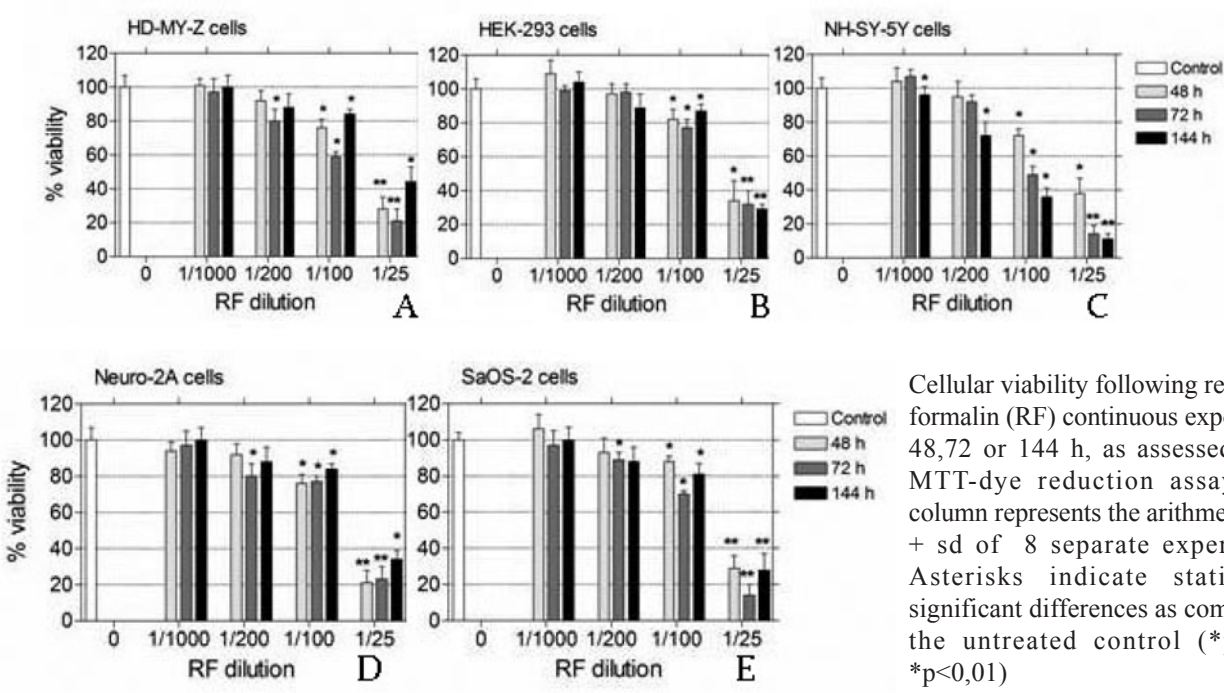
RESULTS

The cytotoxicity of the agents was assessed by the MTT-dye reduction assay in a panel of cell lines, representative for cellular populations that would have been exposed to the chemical entities in pulpotomy medications within a clinical situation, namely: HD-MY-Z (histiocytes/ fibroblasts), HEK-293 (epithelium), SH-SY-5Y and Neuro-2A (neurons) and SaOS-2 (calcified tissue). The cell survival after exposure to resorcinol/formalin (RF) solutions, or eluate extracts from MTA or $Ca(OH)_2$ cement (CHC) is presented in figures 1,2 and 3 respectively.

RF evoked strong, concentration-dependent cytotoxicity, which was evident even at significant dilution of the parent solution (Fig. 1). At the highest concentration tested (1:25 dilution) the cellular viability was greatly decreased in all cell lines. In general the cytotoxicity of RF was not greatly influenced by the exposure period, especially at the higher concentrations under evaluation.

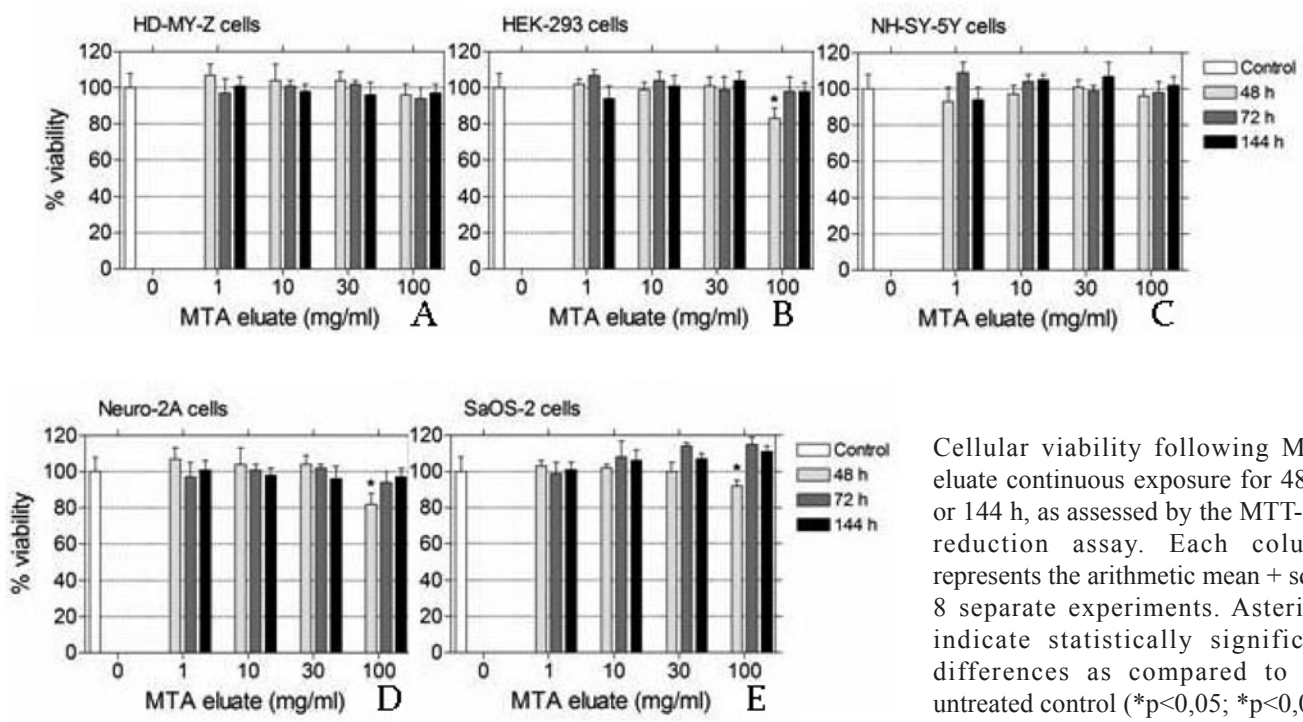
In contrast the MTA extracts proved to be generally devoid of cytotoxic effects. Only slight decreases in cellular viability were encountered at the highest concentration tested after 48 h exposure in HEK-293, SaOS-2, and Neuro-2A cells (Fig. 2). The same treatment intensity however, after longer exposure was associated with less pronounced decrease in cell viability. Noteworthy MTA treatment actually increased the viability of SaOS-2 osteosarcoma cells, which could be attributed to the presence of calcium ions in the MTA-eluate which in turn stimulates the proliferation of this cell line.

The CHC extracts showed marginal cytotoxicity which was far less pronounced than that of RF and slightly superior compared to MTA (Fig. 3). This could be due to release of hydroxide ions during the extraction process and pH-modulation in the culture environment. Noteworthy, the effects of CHC were evident after 48 h exposure, but invariably at longer exposure periods the cell survival fractions tended to increase to values non-significantly different from control group.



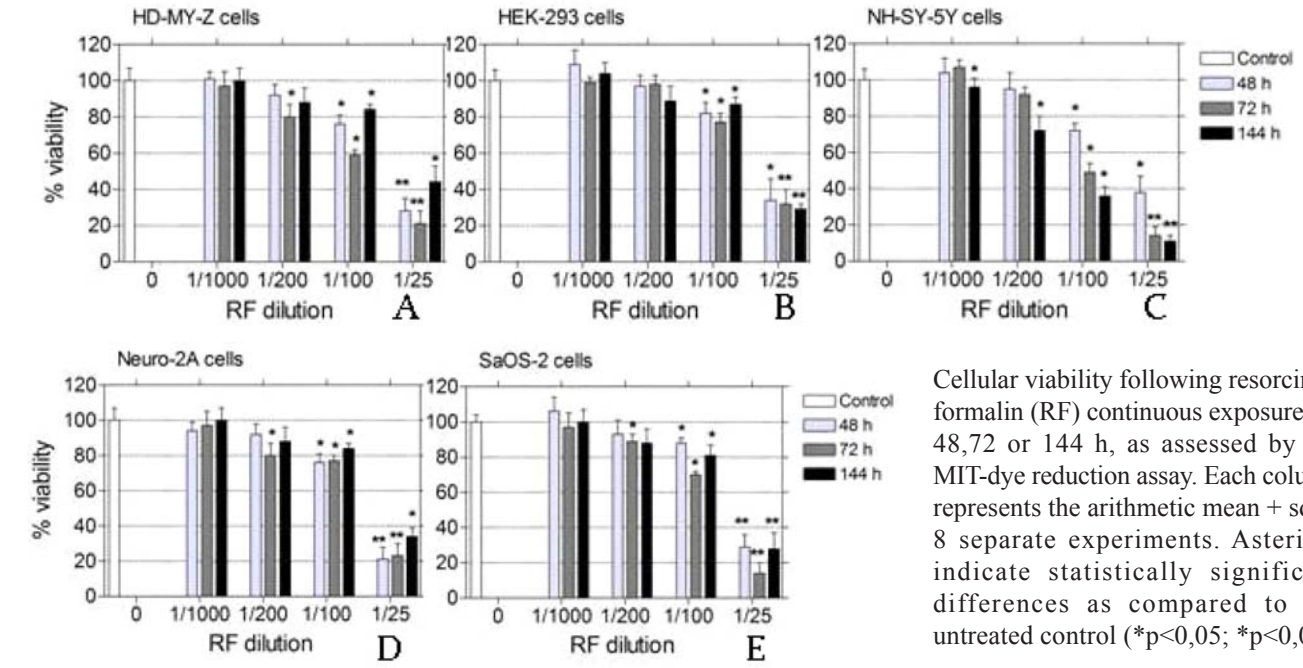
Cellular viability following resorcinol/ formalin (RF) continuous exposure for 48,72 or 144 h, as assessed by the MTT-dye reduction assay. Each column represents the arithmetic mean + sd of 8 separate experiments. Asterisks indicate statistically significant differences as compared to the untreated control (*p<0,05; *p<0,01)

Fig. 1 A, B, C, D, E. Cytotoxicity of resorcinol/formalin (RF) in a panel of cell lines after continuous exposure as assessed by the MTT-dye reduction assay.



Cellular viability following MTA eluate continuous exposure for 48,72 or 144 h, as assessed by the MTT-dye reduction assay. Each column represents the arithmetic mean + sd of 8 separate experiments. Asterisks indicate statistically significant differences as compared to the untreated control (*p<0,05; *p<0,01)

Fig. 2 A, B, C, D, E. Cytotoxicity of MTA extracts in a panel of cell lines after continuous exposure as assessed by the MTT-dye reduction assay.



Cellular viability following resorcinol/ formalin (RF) continuous exposure for 48,72 or 144 h, as assessed by the MIT-dye reduction assay. Each column represents the arithmetic mean + sd of 8 separate experiments. Asterisks indicate statistically significant differences as compared to the untreated control (*p<0,05; *p<0,01)

Fig. 3 A, B, C, D, E. Cytotoxicity of calcium hydroxide cement extracts in a panel of cell lines after continuous exposure as assessed by the MTT-dye reduction assay.

The MTT-data where corroborated by a morphological evaluation of established HEK-293 monolayers after 48 h treatment. Evident from the results summarized in Table 1 and Fig. 4 MTA or CHC extracts failed to induce any detectable changes relative to the control group, throughout the evaluated concentration range, while cytotoxic RF proved to greatly affect the monolayer integrity, lower the cell density, and at the highest concentration applied evoked prominent signs of cytotoxicity (rounded cells, detached cells and signs for disruption of membrane integrity).

Table 1. Morphological assessment of the cytotoxic effects of the tested pulpotomy agents against established HEK-293 monolayers after 48 h exposure.

Treatment series	Cytotoxicity grades[1]		
Resorcinol/formalin	**1:25**	**1:100**	**1:200**
	Grade 4	Grade 3	Grade 1
Mineral trioxide aggregate	**100 mg/ml**	**30 mg/ml**	**10 mg/ml**
	Grade 0	Grade 0	Grade 0
Calcium hydroxyde cement	**100 mg/ml**	**30 mg/ml**	**10 mg/ml**
	Grade 0	Grade 0	Grade 0

[1]According to USP28: Grade 0 – None reactivity; Grade 1 slight reactivity; Grade 2 – Mild reactivity: Grade 3 – Moderate reactivity; Grade 4- Severe reactivity (Described in detail in the Materials and methods section).

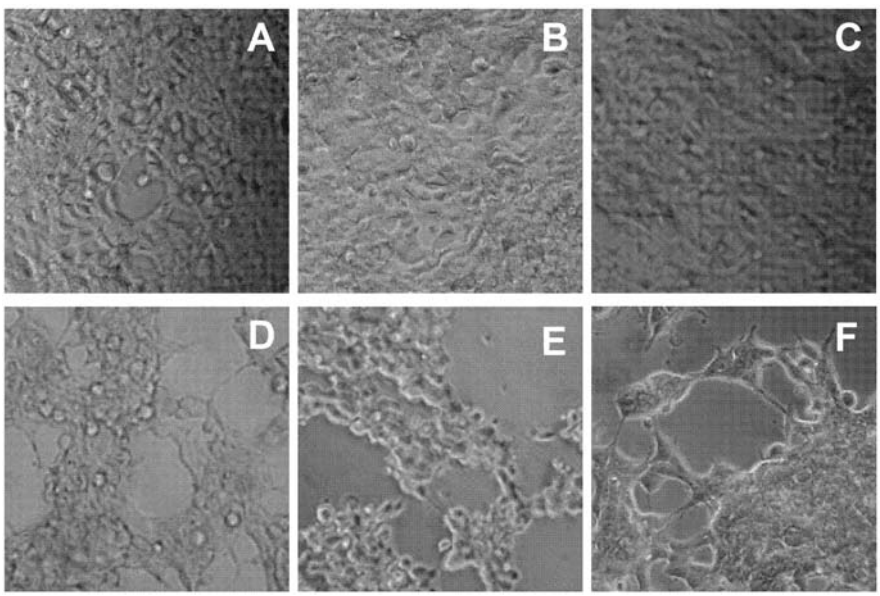


Fig. 4. Representative phase contrast microscopic imaging of HEK-293 monolayers: (A) untreated control; (B) 48 h exposure to 100 mg/ml MTA extract; (C) 48 h exposure to 100 mg/ml CHC extract; (D) 48 h exposure to RF 1:100 dilution; (E) 48 h exposure to RF 1:25 dilution; (F) 48 h exposure to RF 1:25 dilution (higher magnification).

The biocompatibility of tested pulpotomy agents was further explored using a commercially available 'Cell death detection ELISA^{PLUS}' kit allowing simultaneous quantification of apoptotic and necrotic cell death. As shown by the results obtained (fig. 5) RF was a potent inducer of both types of cell death, in relation to concentration and treatment duration. Thus at lower levels and shorter exposure apoptosis was the dominant type of cell death in tested samples, whereas the intensified exposure in terms of both concentration and duration was consistent with a gradual increase in the proportion of necrotic cells. In a dissimilar fashion both MTA and CHC failed to induce significant increase of the DNA-fragmentation in either cytosolic fraction or supernatants, relative to control samples (data not shown), indicating that the observed decreased survival fractions in the MTT-assay could be solely attributed to antiproliferative rather to direct cytotoxic properties.

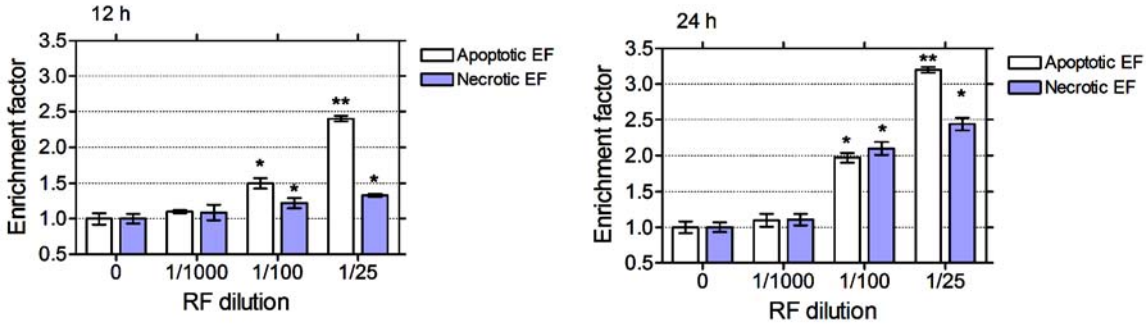


Fig. 5. Induction of apoptotic and necrotic cell death following 12 h or 24 h exposure to different dilutions of resorcinol/formalin (RF) as assessed by 'Cell death detection ELISA^{PLUS}'™ kit, according to the manufacturer's instructions. Each test was run in triplicate. Asterisks indicate statistical significance vs. the untreated control (* pd≤ 0.05; ** pd≤ 0.01). The results are presented as enrichment factor (EF) calculated as the 405 nm absorption of treated samples relative to that of untreated controls, set as 1 (Described in detail in the Materials and methods section).

DISCUSSION

The present study showed that in contrast to RF, that proved to be cytotoxic throughout a range of concentrations and exposure periods, the MTA and CHC were biocompatible, with no evidence for decreased mitochondrial dehydrogenase activity, morphological changes in monolayer integrity or induction of either apoptotic or necrotic cell death.

RF proved to be highly cytotoxic causing time- and dose-related perturbation in cellular viability of all tested cell lines. Noteworthy cytotoxicity was detected even at the 1:100 dilution which is considered clinically relevant (65). These data well correlate to the well-established toxicity of the similar mortal pulpotomy agent formocresol (12, 15-19, 21-24) formaldehyde being the major constituent of both. The second ingredient resorcinol could at least partly contribute to the observed effects, as its intrinsic antiproliferative/cytotoxic properties have been well documented (67-70). The relative role of the phenolic component however, is at best moderate, as it has been well documented that formaldehyde is 20 times as cytotoxic as *m*-cresol (17).

MTA and CHC had only marginal effects on mitochondrial succinate dehydrogenase, detected only at 48 h. With increased exposure the viability of tested samples was no significantly different as compared to the untreated control. This particular response can be attributed to the presence of a non-responsive pool of cell population whose proliferative activity remained unaffected allowing the cell culture to repair. These observations for reversible inhibitory effects of MTA also support those described previously by others (71-73).

An issue of special interest is the stimulatory activity of MTA regarding SaOS-2 neuroblastoma, which could be attributed to the ions released form MTA, during the extraction process providing an optimum amount of calcium for cell proliferation. These data corroborate the well-established osteoinductive and dentinogenic properties of MTA (45, 47, 53, 62, 74).

When we assessed the proliferation/viability of human cells exposed to MTA extract solution, we found that the MTA was associated with less prominent inhibitory effects than calcium hydroxide. It is well appreciated that toxic compounds within dental materials can initiate adverse reactions in surrounding periodontal tissue. Although MTA and CH cement are biocompatible, they are still foreign to the tissue. The discrepancy between the extracts activity could be due to the composition of cements – MTA is composed of hydrophilic particles and contains tricalcium silicate, tricalcium aluminate, tricalcium oxide, silicate oxide, and other mineral oxides, which set in the presence of moisture (32), whereas the other cement is composed of calcium hydroxide solely (4). The latter has prominent alkaline properties and despite its very low water solubility is still capable of significant release of hydroxide ions (75). The latter could have an adverse effect on the pH microenvironment of cultured cells contributing to the observed effects in the MTT-bioassay. In contrast MTA is composed of insoluble silicates and oxides and is less prone to releasing ions other than calcium, the latter being obviously non-toxic within the chosen experimental conditions. These data generally corroborate the superior biosafety profile of MTA vs. calcium hydroxide (18, 76).

Overall, the findings from the MTT-assay corroborate preceding reports which have shown that MTA and CHC are biocompatible with many cell lines (23, 32, 34, 37-42, 46, 50). In contrast some studies have shown some albeit low-grade cytotoxicity of MTA (71, 77) but this discrepancy with our results could be attributed to the experimental design and the cell lines evaluated. In a biocompatibility study, the material tested can be in either direct or indirect contact with the target cellular population. In the present study we analyzed the bioactivities of the MTA or CHC in an indirect contact with the cells for 2, 3 or 6 days. A proliferation study involving direct contact between the cultured cells and the

freshly mixed cements would have been less meaningful, because cultured cells lack the restorative mechanisms of living tissues (78). Thus most probably the lower survival rate at various concentrations with both the MTA and CHC encountered in preceding studies is due to the employment of direct contact experimental set-up.

The morphological study conducted according to USP XXVIII further supported the significant cytotoxicity of FC and the lack of bio-reactivity of the cement-extracted eluates. These findings further evidence that the marginal inhibitory effects of CHC or MTA documented in the MTT-assay are due to slight inhibition of proliferation without direct cytotoxicity.

The *in vitro* cyto-compatibility testing with cell culture systems, commonly uses cell death based end-point, although they do not differentiate between the mechanistic aspects involved. Cell death is executed by two major mechanisms and correspondingly distinct signaling pathways (apoptosis and necrosis). Apoptosis is a major form of cell death, and it is an important process in a variety of different biological systems, embryonic development and in chemically induced cell death, while generally necrosis is initiated by significant suppra-physiological chemical and/or physical stimuli. Besides the mechanistic differences, at morphological level, necrosis is quite different from apoptosis, as well. During the necrotic process, the cells first swell, then the plasma membrane collapses, and subsequently, the cells are rapidly lysed, whereas in apoptosis cells are transformed into apoptotic bodies, without perturbation of the membrane integrity (79). An issue of concern regarding the relative importance of the cell death mechanisms is that while the interaction between apoptotic and phagocytic cells induces an anti-inflammatory response, necrosis appears to be critical for the initiation of inflammatory, innate- and specific immune responses (79, 80).

Taking into account the above considerations we performed an apoptosis/necrosis discriminating assay in HEK-293 cells using an array of concentrations, higher or lower relative to that found in adjacent non-target tissue in endodontic or pulpotomy procedures tissue (equivalent to 1:100 formocresol dilution). Our observations showed simultaneous activation of necrotic and apoptotic pathways leading to cell death within the same target cellular population after 12 to 24 h exposure, whereby the prevalence of apoptotic and especially necrotic mechanisms was dependent on dose and exposure duration. The MTA and CHC extracts proved to be devoid of apoptosis- or necrosis-inductive properties which further evidences for their superior safety profile as compared to RF. These findings are in agreement with previously published data for the ability of formocresol to trigger apoptotic and necrotic cell death in cultured cells (26), and for the lack of pro-apoptotic activity of mineral trioxide aggregate (37, 51, 52).

CONCLUSIONS

Advances in evidence-based dentistry and dental material science have gained enough evidence to support the notion that the application of formaldehyde-based products in pediatric dentistry albeit widespread is unwarranted because of safety concerns, and consequently, their use in pediatric pulp therapy is obsolete. As a result, numerous investigations for alternatives to formaldehyde/phenol devitalization medicines, some of which have shown efficacy equivalent or even superior to formocresol, have been completed. This contribution is to the best of our knowledge the first systematic, multiple end-point, *in vitro* evaluation of the cytotoxicity of resorcinol-formalin vs. vital pulpectomy capping agents. It gives further evidence for the biocompatibility and safety advantages of viable pulp therapy products calcium hydroxide cement and especially MTA as compared to the resorcinol/formalin preparation, routinely used in Bulgaria for decades.

There can be no doubt that from a 21st century perspective a reparative, biologic, and patient-friendly approach to pediatric pulp therapy is preferable to the absolutist, devitalization approach of resorcinol-formalin primary tooth pulpectomy, and the replacement of the latter by clinically superior and safer alternatives is not only welcome but absolutely indispensable.

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