ORIGINAL SCIENTIFIC ARTICLE

INFLUENCE OF DIRECT PULP CAPPING WITH CALCIUM HYDROXIDE AND MINERAL TRIOXIDE AGGREGATE ON SYSTEMIC OXIDATIVE STRESS IN RATS

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ABSTRACT

Direct pulp capping is a procedure where materials are placed on exposed vital pulp tissue in order to stimulate reparative dentinogenesis and preserve pulp vitality. Carious pulp exposure and direct pulp capping are usually accompanied by pulp inflammation which can affect pro- and anti-oxidative systems locally or systemically. Therefore, this study aimed to investigate the potential influence of direct capping of inflamed rat dental pulp with calcium hydroxide (CH) and mineral trioxide aggregate (MTA) on parameters of systemic oxidative status.

Dental pulps of the first maxillary molars of Albino rats (n=32) were exposed and capped with either CH (n=8), MTA (n=8) or were left untreated (n=8). Animals with healthy pulp were used as a healthy control (n=8). After four weeks, animals were euthanized and blood samples were collected for biochemical analysis of parameters of systemic oxidative stress by spectrophotometric method.

Untreated control had the significantly higher ($p < 0.05$) values of pro-oxidative parameters and lower ($p < 0.05$) values of anti-oxidative parameters (superoxide dismutase and reduced glutathione) compared to healthy control. CH and MTA groups showed reduced values of pro-oxidative parameters compared to untreated control and values of anti-oxidative parameters comparable to healthy control.

Pulp exposure led to disbalance in systemic oxidative parameters while direct pulp capping with calcium hydroxide and mineral trioxide aggregate restored the levels of systemic oxidative parameters to that of animals with healthy dental pulp. These results indicate the importance of direct pulp capping and the potential influence of untreated inflamed pulp on systemic health.

Keywords: *Direct pulp capping, calcium hydroxide, mineral trioxide aggregate, oxidative stress.*

INTRODUCTION

The dental pulp tissue is located within dentinal walls, however pulp exposure can occur as a result of dental caries or trauma or can be induced iatrogenically during caries excavation or tooth preparation (1,2). Following traumatic and iatrogenic pulp exposure, dentin and adjacent odontoblast cells are destroyed causing reversible inflammation that, if left untreated, can cause irreversible damage to pulp tissue. This initial inflammation is even more evident in cases of carious pulp exposure (1,3). Direct pulp capping is defined as covering of exposed vital pulp tissue with bioactive material in order to stimulate formation of reparative dentin and preserve pulp vitality (4). Besides the size of pulp exposure, the presence of microorganisms or the patient's age, the type of material used for direct pulp capping affects the success rate of the capping procedure (5). Over time, different materials have been proposed and developed for direct pulp capping (calcium hydroxide (CH), zinc oxide eugenol cement, mineral trioxide aggregate (MTA), enamel matrix derivative, resin-modified glass-ionomer, hydroxyapatite etc.)(2).

CH has been considered the "gold standard" material in direct pulp capping for a long period mainly due to its good properties that include antimicrobial activity, promotion of mineralization, and the formation of a reparative dentin bridge (6). Successful direct pulp capping with CH has been attributed to local rise in pH that induces bactericidal effects and sterile local necrosis of the exposed pulpal tissue. This necrosis produces a mild inflammation that promotes the differentiation and migration of immune and odontoblast-like cells, stimulating the repair processes in dental pulp (7). However, many limitations in the use of CH were recognized including poor sealing ability, low mechanical properties, high resorption, and induction of dentin bridge formation with tunnel defects which may be a pathway for the spread of microorganisms (8). Several bioactive materials, such as MTA, have been proposed to overcome these disadvantages, These materials demonstrated high biocompatibility, excellent sealing properties, and homogenous dentin bridge formation (8). Placing MTA on tissue leads to hydration of cement, formation of calcium silicate hydrate and release of calcium hydroxide, which further dissociates into OH^- and Ca_2^+ ions (9). Ionic exchange with dental pulp tissue makes MTA a bioactive material that acts antimicrobial, regulates the production of pro-inflammatory cytokines, and stimulates the differentiation and activity of odontoblast-like cells (10). Although MTA has not been shown to cause superficial necrosis as calcium hydroxide, it similarly produces high pH environment which together with ion release stimulates acute and mild inflammation that favors the onset of reparative processes in the pulp (11). Both CH, and MTA showed high success rates in direct pulp capping of healthy pulp tissue at around 90% (8). However, in cases of carious pulp exposure which is often accompanied with reversible pulp inflammation, these rates are significantly lower dropping to below 60%, as shown for CH in previous study (12).

Upon inflammation, immune cells, such as neutrophils, are drawn to the site of pulp exposure in response to injury and microbial insults (13). By propagating the inflammatory process in the pulp, neutrophils have been shown to secrete reactive oxygen species (ROS) and reactive nitrogen species (RNS) (14). The formation of ROS and RNS is a normal part of cellular metabolism, however, if they are present in high concentrations or due to insufficient activity of anti-oxidative systems, they cause oxidative stress and can be an important mediator in cellular damage (15). This oxidative disbalance can be reflected systemically and can affect other tissues and organs (16). Dental materials are shown to affect oxidative status having both pro- and anti-oxidative effects depending on the material (17), despite that, effects of direct pulp capping on oxidative parameters in inflamed exposed pulp has not been studied to date.

Since carious pulp exposure and direct pulp capping are accompanied by pulp inflammation and given the complexity of reparative dentinogenesis, we postulated that pro- and anti-oxidative systems can be affected by direct pulp caping procedure. Therefore, we aimed to investigate the potential influence of direct pulp capping in inflamed rat dental pulp with CH and MTA on parameters of systemic oxidative status.

MATERIAL AND METHODS

Animals and ethical considerations

All experiments were approved by the Animal Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia (approval number 01-16176/3). The experimental procedures were performed in accordance with the European Union Directive for the Welfare of Laboratory Animals (No. 2010/63/EU), the Directive of the Council of the European Communities (86/609 / EEC), the principles of good laboratory practice and guidelines for animal research.

Eight-week- old male Wistar rats, average weight 250 ± 50 g, maintained in the vivarium of the Faculty of Medical Sciences, University of Kragujevac were used in study. Animals were exposed to a standard diet with unrestricted access to food and water (*ad libitum*) at normal room temperature (22-25 °C) and day/night cycles of 12 hours.

The sample size (n= 8) for each group was calculated based on the study of a similar design (18) using G-Power software (v3.1.9.7; Faculty of Mathematics and Natural Sciences, Dusseldorf, Germany) considering the power of 0.80 and probability of alpha error 0.05 for the T-test conditions for two independent samples.

Experimental pulp exposure and direct pulp capping

Prior to experimental procedure, animals (n=24) were anesthetized by intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). The cavities were prepared on occlusal surfaces of the left first maxillary molars using sterile round carbide burs (FG.TC 1/2; Micro

Diamond Technologies, Afula, Israel). After reaching a preparation depth at around 1 mm, the dental pulps were exposed using a sterile endodontic K-file size ISO 30/02 (KFS03021; Dentsply Sirona, New York, USA). The cavities were left exposed next 48 hours to induce reversible pulp inflammation as previously described (19).

After 48 hours, the animals were re-anesthetized as previously described. The cavities were washed with sterile saline solution, dried with sterile paper points and disinfected with a paper point soaked in 2% sodium hypochlorite solution. The appropriate capping material was placed in each cavity directly on exposed pulp tissue. The cavities were restored with light-curing glass-ionomer cement (Fuji II LC; GC Europe, Leuven, Belgium) (19).

After the capping procedure, rats were separated in cages depending on the applied material, and divided into four groups as follows:

1. CH (Dycal; Dentsply Sirona, New York, USA) (n=8): calcium hydroxide was prepared according to the manufacturer's instructions and placed on exposed pulps of the left maxillary molars;
2. MTA (MTA Biorep; ITENA Clinical, Paris, France) (n=8): MTA was prepared according to the manufacturer's instructions and placed on exposed pulps of the left maxillary molars;
3. Untreated control (UC) (n=8): the exposed pulp of the left maxillary molars was covered with a collagen membrane (BioMend; Zimmer Biomet Dental, FL, USA) soaked with a drop of sterile distilled water.
4. Healthy control (HC) (n=8): Experimental animals whose pulps were not exposed or capped.

After a four-week follow-up period, the animals were sacrificed followed by dissection of left maxillae and blood sample collection. The experimental animals were exposed to short-term ether anesthesia before sacrifice. The sacrifice was performed by cervical dislocation. Blood samples were collected from jugular vein and placed into test tubes containing sodium citrate anticoagulant. Blood samples were processed and stored immediately. Blood was centrifuged to separate plasma and red blood cells (RBCs).

Pathohistological evaluation

After dissection, the left maxillae were fixed in 4% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA, USA) for 24h. The samples were decalcified in 10% EDTA/phosphate-buffered saline solution (Sigma-Aldrich, St Louis, MO, USA) for three weeks and embedded in paraffin. Serial 5µm sections were cut using a rotary microtome system. Haematoxylin-eosin (H&E) stained sections were examined under DM4000B light microscope with DMC295 camera and Qwin V3 software (Leica Microsystems, Wetzlar, Germany). Histological scores of pulp inflammation and mineralization were evaluated using grading system that was previously reported (19) and presented in Table 1.

Evaluation of Systemic Oxidative Status

The concentration of pro-oxidative markers (nitrites (NO_2^-), hydrogen peroxide (H_2O_2), and superoxide anion (O_2^-)) and index of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) were evaluated in plasma samples. The parameters of the anti-oxidative system including the level of reduced glutathione (GSH), and the activities of catalase (CAT) and superoxide dismutase (SOD) were measured in the lysate of erythrocytes. All oxidative parameters were evaluated spectrophotometrically using methods previously reported and described briefly.

Determination of the Index of Lipid Peroxidation

Index of lipid peroxidation was assessed by measuring TBARS using 1% thiobarbituric acid (Sigma-Aldrich) in 0.05 NaOH (Sigma-Aldrich), incubated with plasma at 100°C for 15 minute and determined spectrophotometrically (λ=530 nm) (20)

Nitrite Determination

The level of NO_2^- was determined as an index of nitric oxide (NO) production with Griess's reagent (20). A total of 200 µl of plasma was precipitated with 100 µl of 3 N perchloric acid (Thermo Fisher Scientific) and 400 µl 20mM ethylenediaminetetraacetic acid (Sigma-Aldrich), left on ice for 15 min, and centrifuged at 6000 rpm for 15 min. After pouring of the supernatant, 220 µl of potassium carbonate (Thermo Fisher Scientific) was added. To the 200 µl of final mixture, 250 µl Griess's reagent (Abcam, Cambridge, UK) and 125 µl of previously prepared buffer for NO were added followed by incubation at room temperature for 15 min before measuring. The levels were measured spectrophotometrically (λ= 550nm).

Superoxide Anion Radical Determination

The 50 µl of plasma sample and 950 µl of assay mixture was mixed. The assay mixture contained 50 mM TRIS-HCl (tris (hydroxymethyl) aminomethane-hydrochloric acid) buffer (pH = 8.6) (Sigma-Aldrich), 0.1 mM ethylenediaminetetraacetic acid (Sigma-Aldrich), 0.1 mg/ml gelatin (Sigma-Aldrich) and 0.1 mM nitro blue tetrazolium (Sigma-Aldrich). The level of O_2^- was measured three times every 60 seconds at a wavelength of λ=530 nm (20).

Hydrogen Peroxide Determination

H_2O_2 was measured as a level of Phenol red oxidation by hydrogen peroxide catalyzed by horseradish peroxidase (20). A total of 800 µl of Phenol red solution (MP Biomedicals, Solon, OH, USA) and 10 µl of rhubarb peroxidase (Sigma-Aldrich) were added to 200 µl of plasma sample. After incubation at room temperature for 10 minutes, absorbance was measured at λ=610 nm.

Table 1. Evaluation criteria for rat pulp tissue response based on inflammation and mineralized tissue formation

	Score	Definition
Pulp inflammation		
	0	Absent or very few inflammatory cells
	1	Mild: inflammatory cells only next to dentin bridge or area of pulp exposure
	2	Moderate: inflammatory cells are observed in the part of coronal pulp tissue
	3	Severe: all coronal pulp tissue is infiltrated or necrotic
Pulp mineralization		
	0	No trace of mineralization in the pulp
	1	Increased deposition of hard tissue along the surface of the exposed pulp tissue
	2	Extensive mineral deposition in the coronal pulp tissue

Determination of Reduced Glutathione

The level of GSH was determined on the basis of GSH oxidation with 5.5-dithio-bis- 6.2-nitrobenzoic acid in erythrocyte lysate (20). A mixture was prepared containing 50 µl of sample, 200 µl of 0.1% ethylenediaminetetraacetic acid solution (Sigma-Aldrich) and 385 µl of previously prepared precipitation buffer. This mixture was incubated on ice for 15 minutes and then centrifuged at 4000 rpm for 10 minutes till extract was formed. Afterwards, 300 µl of extract, 750 µl of disodium hydrogen phosphate (Sigma-Aldrich) and 100 µl of 5,5-dithio-bis-6,2-nitrobenzoic acid (Sigma-Aldrich) were added to the new tubes and incubated at room temperature for 10 minutes. The absorbance was measured at λ= 412 nm.

Determination of Catalase activity

CAT activity was determined in a previously diluted erythrocyte lysate (20). A total of 100 µl of erythrocyte lysate was diluted in 10 ml of distilled water and 100 µl of 70% ethanol alcohol (Reahem, Novi Sad, Serbia). A 100 µl of previously prepared dilution was added to 50 µl of previously prepared CAT buffer with 1 ml of 10mM H_2O_2 (Sigma-Aldrich). Measurements were repeated 6 times for each sample. Absorbance was measured in quartz cuvettes at λ=230 nm.

Determination of Superoxide Dismutase

SOD was measured in a mixture containing 100 µl of erythrocyte lysate and 1 ml of carbonate buffer (Thermo Fisher Scientific). After vortexing the mixture, a 100 µl of adrenaline (Sigma-Aldrich) was added. Absorption measurements were performed in duplicate at λ=470 nm (20).

Statistical analysis

Statistical data processing was performed in the statistical software IMB SPSS Statistics for Windows v23.0 (IBM Corp., Armonk, NY, USA). The Shapiro – Wilk normality test was used to assess the normality of the data distribution. Depending on the normality of the distribution, the Nonparametric Kruskal-Wallis H and Mann-Whitney U tests or the parametric One-Way ANOVA and Independent Samples T-test were used. Results are presented as mean ± standard deviation (SD). A value of $p < 0.05$ was set as an indicator of statistical significance.

RESULTS

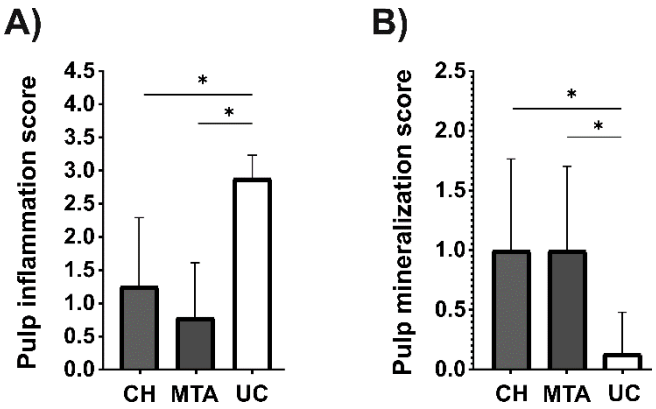
Pulp inflammation and mineralization

Contrary to the sound dental pulp tissue on H&E sections of HC group, UC group presented high level of tissue disorganization and necrosis. H&E sections of UC group presented severe inflammation accompanied by loss of odontoblast layer, inflammatory cell infiltrate and cellular necrosis with absence of pulp mineralization in exposed coronary pulp. CH and MTA groups mostly presented no or mild inflammation with restored odontoblast-like cell layer, mineralized bridge formation and absence of inflammatory cell infiltrate in coronary pulp under the site of pulp exposure. Evaluation of pathohistological scores is presented on Figure 1.

The highest value of pulp inflammation score (associated with the greatest inflammation) was observed in UC group which was significantly higher ($p < 0.05$) compared to CH and MTA groups (Figure 1A). Pulp mineralization score values showed no significant difference ($p > 0.05$) between CH and MTA groups, however, both groups presented signi-

ficantly higher values ($p < 0.05$) (indicating high level of mineralization) compared to UC group (Figure 1B).

Figure 1. Pulp inflammation and pulp mineralization evaluation scores



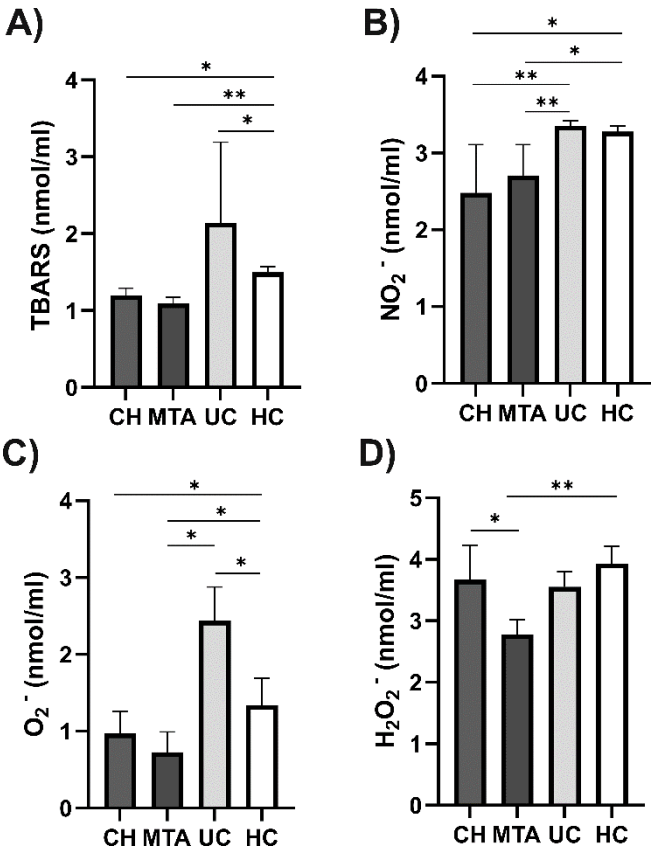
(A) Pulp inflammation scores between CH, MTA and UC groups (B) Pulp mineralization scores between CH, MTA and UC groups. The results are presented as mean ± SD of 8 animals per group. * statistically significant difference ($p < 0.05$) (Kruskal-Wallis post-hoc test);

Effects of direct pulp capping on pro-oxidative markers

The measured values of pro-oxidative markers (TBARS, NO_2^-, O_2^-, and H_2O_2) were compared between the experimental groups (CH, MTA, UC and HC) and presented in Figure 2.

The value of TBARS was the highest in the UC group, which was significantly higher compared to HC group ($p < 0.05$). Although, CH and MTA groups had mean values of TBARS similar to healthy control (HC) group, statistical analysis showed significant difference between CH and HC groups ($p < 0.05$), as well as MTA and HC groups ($p < 0.001$) (Figure 2A). The NO_2^- value was also the highest in UC group. NO_2^- values in CH and MTA groups were significantly lower compared to the UC group ($p < 0.001$), as well as to HC group ($p < 0.05$) (Figure 2B). UC group presented the highest mean value of O_2^- which was significantly higher ($p < 0.05$) compared to CH, MTA, and HC groups (Figure 2C). Both CH and MTA groups showed significantly lower ($p < 0.05$) mean O_2^- values compared to HC group. MTA group also presented the lowest mean value of H_2O_2 which was statistically significant compared to CH ($p < 0.05$) and HC groups ($p < 0.001$). No statistically significant difference ($p > 0.05$) was found between UC and other groups (Figure 2D).

Figure 2. Systemic pro-oxidative parameters



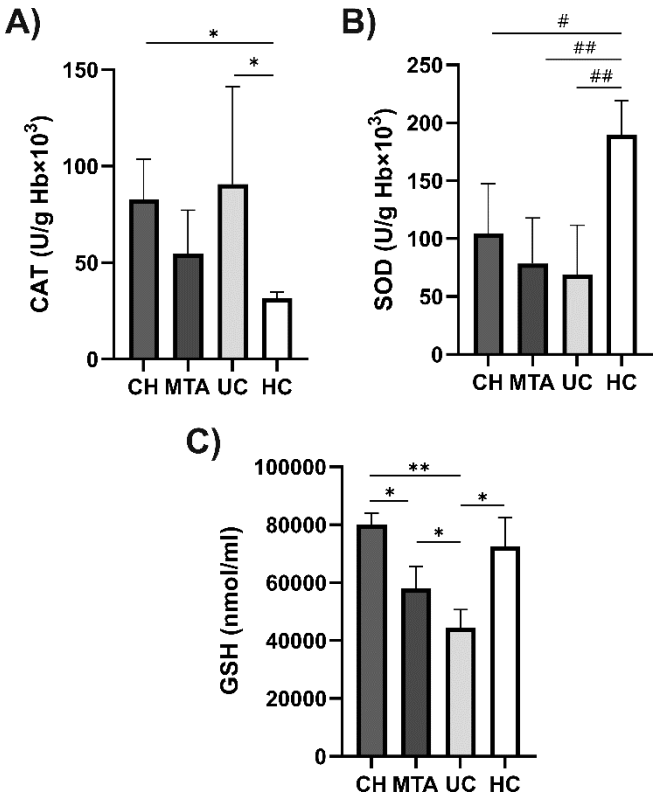
(A) Level of TBARS in systemic circulation of CH, MTA, UC and HC groups (B) Level of NO_2^- in systemic circulation CH, MTA, UC and HC group (C) Level of O_2^- in systemic circulation CH, MTA, UC and HC groups (D) Level of $H_2O_2^-$ in systemic circulation CH, MTA, UC and HC groups. The results are presented as mean ± SD of 8 animals per group. * statistically significant difference ($p < 0.05$) (Kruskal-Wallis post-hoc test); ** statistically significant difference ($p < 0.001$) (Kruskal-Wallis post-hoc test)

Effects of direct pulp capping on anti-oxidative markers

The measured values of anti-oxidative parameters (CAT, SOD, and GSH) were compared between the experimental groups (CH, MTA, UC and HC) and presented in Figure 3.

The mean values of CAT were higher in all groups compared to the HC group. However, a statistically significant difference ($p < 0.05$) was presented between CH and HC and UC and HC groups (Figure 3A). The values for CAT were not statistically significantly different between MTA and HC groups ($p > 0.05$). UC group showed significantly lower ($p < 0.001$) SOD mean value compared to HC group, as well as MTA and CH groups ($p < 0.05$) (Figure 3B). The UC group also showed significantly lower ($p < 0.05$) level of GSH compared to the HC group (Figure 3C). CH and MTA groups showed no statistical difference to HC group ($p > 0.05$), while having significantly higher values ($p < 0.05$) compared to UC group (Figure 3C).

Figure 3. Systemic anti-oxidative parameters



(A) Level of CAT in systemic circulation of CH, MTA, UC and HC groups (B) Level of SOD in systemic circulation CH, MTA, UC and HC groups (C) Level of GDH in systemic circulation CH, MTA, UC and HC groups; * statistically significant difference (p <0.05) (Kruskal-Wallis post-hoc test); # statistically significant difference (p <0.05) (Oneway ANOVA/Turkey post-hoc test); ## statistically significant difference (p <0.001) (Oneway ANOVA/Turkey post-hoc test)

DISCUSSION

In this study for the first time, we analyzed the influence of direct pulp capping of inflamed pulp on systemic parameters of oxidative stress. The goal of direct pulp capping is the healing of exposed dental pulp by the formation of dentin bridge. As shown in our study, direct pulp capping with CH and MTA resulted in mineralized bridge formation presented with higher pulp mineralization scores to UC group (Figure 1B). As in any other wound healing, healing of exposed dental pulp passes through hemostasis, inflammation, cell proliferation, angiogenesis, and eventually tissue remodeling (21,22). Conditions associated with oxidative stress such as inflammation, hypoxia, or the effects of systemic diseases often occur in dental pulp (23). Carious lesion as well as pulp exposure during caries excavation often led to reversible pulp inflammation. During inflammation, immune cells including macrophages and neutrophils produce cytokines as well as ROS in order to destroy pathogens (24,25). In order to cope with ROS production, the cells of the organism possess anti-oxidant systems that include enzymes such as catalase, superoxide dismutase, hydrogen peroxidase and the system of glutathione peroxidase and reductase, among others (17,23,25,26). However, inability of anti-oxidant systems to cope with overproduction of ROS can led to disorders of local oxidative status that can negatively affect surrounding healthy cells causing oxidative stress and leading to irreversible pulp disorders. This can also be reflected systemically, causing the effects of oxidative stress in other organs as well (16,27). One of the properties of direct pulp capping materials is reducing inflammation and preventing the occurrence of irreversible damage to the pulp (28). Thus, we hypothesized that direct pulp capping could affect systemic oxidative parameters in rats with inflamed dental pulp.

Previous study reported that pulp tissue had high level of inflammation 28 days after direct capping of reversibly inflamed rat pulp with only collagen carrier, unlike capping with bioactive materials (29). This coincides with the results of our study, where UC group capped with collagen membrane presented high level of pulp inflammation (Figure 1A). As expected, UC group showed a significant increase in systemic pro-oxidative parameters, NO_2^- and O_2^- (Figure 2B and C) compared to groups capped with bioactive materials (CH and MTA). Initially increased ROS production is considered beneficial for wound healing, due to its antimicrobial effect and stimulation of angiogenesis. On the other hand, prolonged and increased ROS production is associated with delayed healing and chronic wounds (22). In the current study, direct pulp capping with CH and MTA reduced the systemic values of the prooxidative parameters opposed to untreated control (Figure 2). These result are in agreement with reduced inflammation in rat pulp capped with different formulations of CH and MTA shown previously and in our study (30).

To avoid oxidative damage, antioxidant system composed mainly of a group of enzymes responsible for the control of ROS is triggered in state of inflammation (31). However, previous studies showed contradictory results on levels of anti-oxidant enzymes in healthy and inflamed dental pulp (32–34). Grossi et al. (32) reported that low levels of SOD could indicate high susceptibility to inflammation, which is in accordance to our results with significantly low levels of SOD in UC compared to HC group (Figure 3B). On the contrary, Topcu et al. (33) reported that inflamed dental pulp tissue have had significantly higher activity of SOD compered to healthy pulp (33). Regarding CAT, previous study showed very low anti-oxidative activity in healthy human pulp tissue (34), similar to our results with lowest level of CAT in healthy control (Figure 3A). Dental materials with proven cytotoxicity, such as dental resins, were shown to produce depletion of GSH in dental pulp cells inducing tissue inflammation and cellular damage (35). Unlike them, CH and MTA were shown to have anti-inflammatory effect and low cytotoxicity when used in direct pulp capping (10,36) In line with that, use of these materials for direct pulp capping showed higher levels of antioxidative parameter GSH compared to UC group in present study (Figure 3C).

Interestingly, some pro-oxidative parameters in CH and MTA groups were presented at lower levels even when compared to healthy control (Figure 2). This divergence in results, as well as others, can be explained as the consequence of different stages of inflammation in pulp tissue that undergoes dentine reparation. Increased levels of ROS in a sample should not be deem as a certain sign of inflammation, because it has been proven that restoring the redox balance in organism takes time (37). One of the limitations of this study was that the pulp inflammation and parameters of oxidative stress were assessed only once at 28 days after pulp capping procedure. Further studies should evaluate and compare these parameters in different time intervals. The disbalances in several parameters could also be a result of disturbances in other tissues and organs. Although all animals were subjected to same procedures and conditions, health of other organs and tissues was not assessed in our study, which could be considered as another limitation. Importantly, prolonged disbalance between the production and elimination of ROS could increase the risk of developing different diseases. It has been shown that an increased production of ROS linked to oral diseases such as chronic periapical lesions could have consequences on systemic health (24,38).

CONCLUSION

In conclusion, our results indicate that pulp exposure leads to disbalance of systemic oxidative parameters reflected in rise of pro-oxidative and fall of anti-oxidative markers. Additionally, direct pulp capping with calcium hydroxide and mineral trioxide aggregate restored the levels of systemic oxidative parameters to that of animals with healthy dental pulp thus revealing the importance of direct pulp capping and the potential influence of untreated inflamed pulp on systemic health. Therefore, we can conclude from these findings that through adequate pulp therapy, oxidative balance can be restored, thereby avoiding the risk of dentally induced consequences on systemic health.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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