DOES AN ALTERATION IN NOCICEPTIVE RESPONSE TO MINERAL COMPONENTS OF DENTAL COMPOSITES INVOLVE CHANGES IN OXIDATIVE STATUS? A BRIEF REPORT

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DA LI PROMENE NOCICEPTIVNOG ODGOVORA NA MINERALNE KOMPONENTE DENTALNIH KOMPOZITA PODRAZUMEVAJU PROMENE OKSIDATIVNOG STATUSA? KRATAK PREGLED

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ABSTRACT

Since that use of bioactive mineral components of dental composites have been accompanied with various toxicities, including neurotoxicity, the aim of the study was to examine the effect of chronic application of hydroxyapatite, tricalcium phosphate and amorphous calcium phosphate in nanoparticles (nHA, nTCP, nACP) to parameters of sensitivity to thermal pain stimuli. Although the systemic toxicity of those compounds is frequently attributed to an oxidative damage, we also decided to examine the potential effects of Filipendula ulmaria extract on nociception alterations induced by the nano-sized mineral components of dental composites. Forty-two Wistar albino rats were divided into control and six experimental (equal) groups that orally received either nHA, nTCP, nACP alone, or simultaneously with FU extract for 30 days. Nociceptive alterations were quantified in the hot plate and tail flick test. The chronic administration of nHA and nACP resulted in significant increase in reactivity to thermal stimulus, with no significant change observed in nTCP group when compared to the control in the hot plate test, while simultaneous application of FU extract prevented any significant alteration of time to respond. The reaction time in the tail flick test for all three groups that received only nano calcium phosphates was reduced, with no changes in the groups treated with FU extract. The results of this study confirmed that calcium phosphates of mineral components of dental composites produced hyperalgesic effects, and this side effect were significantly attenuated by antioxidant supplementation.

***Keywords**: nanoparticles of calcium phosphates, Filipendula ulmaria, nociception, oxidative status, rat.*

SAŽETAK

Budući da je upotreba bioaktivnih mineralnih komponenata zubnih kompozita praćena različitim oblicima toksičnosti, uključujući i neurotoksičnost, cilj ove studije je bio ispitivanje efekata hronične primene hidroksiapatita, trikalcijum fosfata i amorfnog kalcijum fosfata u obliku nanočestica (nHA, nTCP i nACP) na parametre osetljivosti na termičke stimuluse. Kako se sistemska toksičnost ovih jedinjenja često pripisuje oksidativnom oštećenju, odlučili smo da ispitamo i potencijalne efekte ekstrakta biljke Filipendula ulmaria na promene nocicepcije izazvane nanočesticama mineralne komponente dentalnih kompozita. Četrdeset i dva pacova Wistar albino soja podeljeno je na kontrolnu i šest eksperimentalnih (jednakih) grupa koje su oralno primale nHA, nTCP ili nACP, samostalno ili u kombinaciji sa ekstraktom FU, tokom 30 dana. Nociceptivne promene su kvantifikovane pomoću testa vruće ploče i testa povlačenja repa. Hronična primena nHA i nACP rezultirala je značajnim povecanjem reaktivnosti na termički stimulus, bez značajnih promena primećenih u nTCP grupi u poređenju sa kontrolnom grupom u testa vruće ploče, dok je istovremena primena ekstrakta FU sprečila bilo kakvu promenu u vremenu odgovora. Vreme reakcije u testa povlačenja repa za sve tri grupe koje su primale samo nano kalcijum fosfate je smanjeno, bez promena u grupma tretiranih eskstraktom FU. Rezultati ove studije potvrdili su da su kalcijum fosfati mineralne komponente dentalnih kompozita proizvele hiperalgezijske efekte, koji su značajno umanjeni korišćenjem antioksidantne suplementacije.

***Ključne reči:** nanočestice kalcijum fosfata, Filipendula ulmaria, nocicepcija, oksidativni status, pacov.*

ABBREVIATIONS

HA - hydroxyapatite
TCP - tricalcium phosphate
ACP - amorphous calcium phosphate
FU - Filipendula ulmaria

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INTRODUCTION

Although the use of dental composite has began in the 1960s, today they represent the gold standard in tooth restoration (1). Their complex structure, composed of organic resin and mineral filler, provides good aesthetic and physical properties, very similar to dental tissues, but without any influence on the remaining enamel and dentin (2). The application of calcium phosphate, such as hydroxyapatite (HA), tricalcium phosphate (TCP) and amorphous calcium phosphate (ACP) in the bone tissue replacement and confirmation of its bioactivity is very well confirmed (3). In order to achieve bioactive effect in the physiological environment, the development of dental composites has been moved to integration of HA, and other more soluble forms of calcium phosphates to mineral component (4, 5). The basic mechanism of composite bioactivity is the partial dissolution of calcium phosphate molecules in a thin layer of saliva, located between solid dental tissues and fillings, which lead to increase in local concentrations of Ca^{2+} and PO_4^{3-} ions with subsequent deposition of HA layer on the surface of dental tissues and remineralization of solid tissue (6). With the expansive development of nanotechnology and its application in medicine, during the time, nanoparticles were included in prophylactic and therapeutic procedures in dentistry (7). Novel dental composites contain nano-sized calcium phosphates with large specific surface area, which provide a high degree of dissolution, while on the other hand, the solubility is determined by the chemical structure of compound (8). To achieve adequate bioactive effect, it is necessary to ensure that calcium phosphate release and dissolute for a long time period (9). However, the prolonged effect of dissolved compounds, carried by the physiological path of saliva, sets the importance of toxic effects to other oral tissues and the organism in general as a high priority issue.

Calcium phosphates have been considered as biocompatible materials for decades, while the recent investigation of these compounds toxicity, when applied as nano-sized particles, still offer contradictory results. Systemic toxicity of calcium phosphates has been demonstrated through several *in vivo* experiments using nanoparticles of hydroxyapatite (nHA). Thus Liu et al. showed a toxic effect of nHA parenteral administration confirmed hepatotoxicity by means of an increase in AST, ALT and alkaline phosphatase (10). Wang et al. reported that low doses of nHA, after intraperitoneal administration in rats, causes apoptosis in liver and kidney cells, but still without necrosis (11). Although the numerous results of other metallic nanoparticles, such as ZnO, MgO, CuO and CeO, include the neurotoxicity of those compounds manifested by alterations in nociception (12-17), to our knowledge this behavioral pattern that may also be affected by calcium phosphate nanoparticles has not been evaluated yet.

Evaluating the mechanisms of metallic nanoparticles toxicities, Mosu et al. suggested that systemic administration of nHA induces generation of reactive oxygen species (ROS) that leads to an increase in p53 transmitters and activation of apoptosis in renal cells (18). Also, the study on C6 cell culture showed that cell damage was mediated by an oxidative mechanism with consequent apoptosis (19). As the recent investigations have shown that the oxidative state disturbance may be the basic mechanism of cell damage, it seems reasonable that treatment with antioxidants may be beneficial in reducing side effects following the application of dental composites with nano-sized calcium phosphates, as the active substance. Also, the natural products with high antioxidant potential have a wider application, and become more and more interesting to the scientific community. Among the natural supplements with the confirmed antioxidant potential is the extract of *Filipendula ulmaria* – FU (20).

Filipendula ulmaria (L.) Maxim., better known as meadowsweet, is a perennial herbaceous plant that is widespread in Europe and Asia with a long history of medicinal use (21). In addition to flowers, leaves and roots are also used for healing purposes, especially in the therapy of various inflammatory processes (22). The extract of this plant is rich in phenolic compounds and shows a wide range of pharmacological activities (23). Numerous studies have shown the mechanisms of action of FU, whereby Katanic and coworkers showing that FU have a strong antioxidant and antimicrobial potential (23, 24). In addition to those described, it also shows antihypertensive, neuroprotective and many other pharmacological activities, so there are numerous indications for its use (24).

Based on the lack of data for the action of calcium phosphate nanoparticles to the central nervous system, the aim of the study was to examine the effect of chronic application of HA, TCP and ACP in the form of nanoparticles (nHA, nTCP, nACP) on parameters of sensitivity to thermal pain stimuli. Following the trend and the extent of research of the antioxidant effects of natural products, and according to the suggested mechanism of cell damage induced by nano-sized calcium phosphates, we also chose to examine the potential effects of FU extract on nociception alterations induced by the nano-sized mineral component of dental composites.

MATERIALS AND METHODS

Animals and treatment

Two-month-old male Wistar rats (180-220 g, n=42) were purchased from the Military Medical Academy, Serbia. The animals were kept in transparent cages (three animals per cage) in controlled standard environmental conditions of temperature 23±1 °C, humidity 50±5 %, with light/dark cycle 12/12h, and were provided standard chow and water ad libitum for the duration of the study. All protocols lasted for 30 days.

The animals were randomly divided into seven equal groups (6 animals per group) as follows:

1. Control group;
2. HA group, orally received nHA (17.8 mg/kg b.w.);

3. HA+FU group, orally received nHA (17.8 mg/kg b.w.) and FU extract (100 mg/kg b.w.);
4. TCP group, orally received nTCP (11 mg/kg b.w.);
5. TCP+FU group, orally received nTCP (11 mg/kg b.w.) and FU (100 mg/kg b.w.);
6. ACP group, orally received nACP (9.65 mg/kg b.w.); and
7. ACP+FU group, orally received nACP (9.65 mg/kg b.w.) and FU (100 mg/kg b.w.).

The mineral components of dental composites in nanoparticles were purchased from the Sigma-Aldrich, Germany: Hydroxyapatite nanopowder, <200 nm particle size (BET), ≥97%, synthetic; Tricalcium phosphate hydrate nanopowder, <200 nm particle size (BET); Calcium phosphate, amorphous nanopowder, <150 nm particle size (BET). The FU extract preparation was performed according to previously established procedure (23). The doses of mineral components used in this study were selected to equimolary equalize the lowest dose of nHA that showed the toxic effects in the previous report for the experiments performed in vivo with nHA (11). It should be noted that the doses administered are similar to the concentrations of mineral components released from dental composites in vitro (25), while the oral administration was chosen in order to mimic the authentic route of application in humans. The dose of FU extract was selected based on our previous study which confirmed the biological efficacy of this natural product (22), and the final concentration of all applied substances was calculated based on the average water intake in the previous 24 hours, dissolved in tap water.

All research procedures were carried out in accordance with the European Directive for the welfare of laboratory animals No 86/609/EEC and the principles of Good Laboratory Practice, and in accordance with the ARRIVE guidelines. All experiments were approved by the Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Behavioral testing

The behavioral testing was performed 24 h after completing the protocols. Approximately at 8 a.m., rats were placed in the testing room, and allowed to accommodate for 1 h before behavioural testing. The nociception assessment was performed in hot plate test and tail flick test under appropriate conditions (23). In order to remove potential interfering odours, the apparatus for both tests were cleaned with water and ethanol (70%) for each animal.

Hot plate test

The hot plate test was conducted according to algorithm previously defined in our lab (26). The appliance consisted of a square metal plate measuring (43 x 43 cm) and glass walls (30 cm) (Fig. 1). Each animal was placed in the central part of the plate and the temperature was maintained at 51.5 ± 0.5°C. The duration of the test was individual and defined by the appearance of a specific reaction to a thermal stimulus – in the form of licking the hind paw, shaking the hind paw or bouncing off the ground with all 4 limbs at the same time. To prevent burns, the test time was limited to 180 seconds. The parameter monitored in this test is the reaction time expressed in seconds.

Tail flick test

The tail flick test is the nociception test in which a high-intensity heat stimulus is directed at the rat's tail according to the procedure described by Bannon et al. (27). The animals were placed on a raised grid and covered with an appropriately sized tube to disable movement (Fig. 2). After achieving a temperature of 75°C, a heat stimulus was placed in the middle of the tail and the reaction of the experimental animal was monitored. A strong enough stimulus was necessary to provoke the expected reaction of the animal – the tail flick. By measuring the time from the initiation of the painful stimulus to the manifested form of the expected reaction, the results of this test were quantified and expressed in seconds.

Statistical analysis

The results were expressed as the means±SEM. Parameters obtained in hot plate and tail flick test and oxidative stress markers were initially submitted to Levene's test for homogeneity of variance and to Shapiro–Wilk test of normality. Comparisons between groups were performed using One-way ANOVA, followed by Bonferroni test. The significance was determined at $p<0.05$ for all tests.

RESULTS

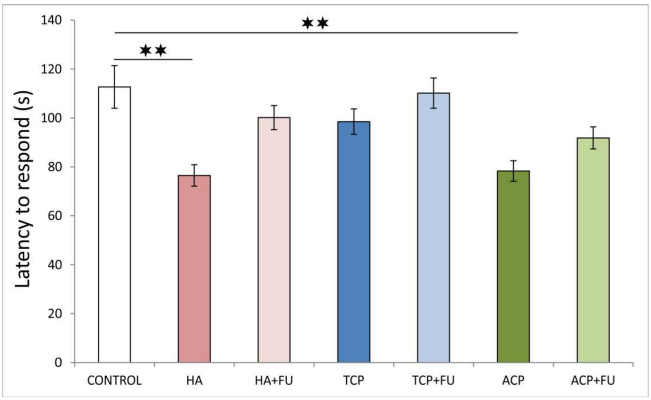
All applied protocols significantly affected the reaction time in the hot plate test (Fig. 3, dF=6, F=6.321). The chronic administration of nHA and nACP resulted in significant increase in reactivity to thermal stimulus ($p<0.01$), with no significant change of observed in nTCP group when compared to the control. At the same time, simultaneous application of FU extract prevented any significant alteration of time to respond in the hot plate test.

As represented in Fig. 4, the results of the tail flick test showed the significant alterations in time to respond following the applied protocols (dF=6, F=10.863). The reaction time for all three groups that received only nano calcium phosphates was reduced ($p<0.01$ for nHA and nACP; $p<0.05$ for nTCP), while in the groups that received nano calcium phosphate along with FU extract no changes in the reaction time were observed when compared to the control. Furthermore, antioxidant supplementation with FU extract resulted in significant elongation of reaction time when compared to groups with single nano-sized calcium phosphate administration ($p<0.01$).

Figure 1. Apparatus used for the hot plate test.

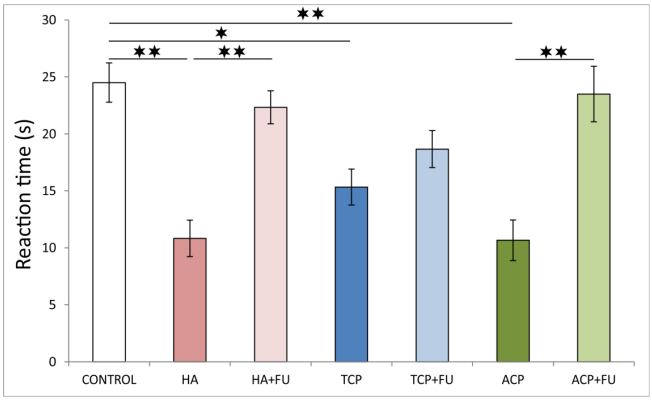
Figure 2. Apparatus used for the tail flick test.

Figure 3. Hot plate test.



The values are mean ± standard error of the mean (SEM), **denotes a significant difference p<0.01.

Figure 4. Tail flick test.



The values are mean ± standard error of the mean (SEM), *denotes a significant difference p<0.05, **denotes a significant difference p<0.01.

DISCUSSION

The results of this study indicate that calcium phosphate nanoparticles significantly affect nociceptive mechanism, thus altering the response to thermal pain stimuli. Although there is no data of nano calcium phosphates effects of central nervous system, previous studies indicate that systemic application of other metals nanoparticles could significantly affect both peripheral and central nervous system, also including the nociception control (28). Kesmati and co-workers showed that ZnO nanoparticles act analgesic through the central mechanisms of pain regulation, without dose dependence at high concentrations (12). It was later explained by the high rate of receptor saturation (13). Further research concluded that both conventional ZnO and ZnO in nanoparticles (with more pronounced effect of its nanoparticle form), may be effective in reducing acute pain in animal experimental models, with the analgesic mechanism by ZnO applied in nanoparticles could influence antinociceptive action by activation of opioidergic system (14). Also, it was shown that MgO and ZnO nanoparticles directly affect pain perception through changes in glutamate levels, while changes in ion levels, after injection of these nanoparticles, may be effective in altering gene expression in the hippocampus, with overall hypoalgesic effect (15). There is also evidence in the literature that CuO produced antinociceptive effects (16). In contrast, it was reported that CeO reduced the latency time in the hot plate test and that hyperalgesic effect was accompanied with increased oxidative stress expressed by means of cyclooxygenase-2 (17), which almost resembles the results obtained in this study.

Beside the known distribution of calcium phosphate nanoparticles after the systemic application (29), it was already shown that calcium phosphate nanoparticles pass through the blood-brain barrier and enter numerous parts of the brain (30). The results of previous studies indicate that parenteral administration of nHA induced changes in liver function demonstrated through changes in liver enzymes levels (10),

while oral administration of the same compound induced nephrotoxicity of various levels, including renal tissue damage, changes in biochemical parameters, as well as enhancement of proinflammatory cytokine production (18).

Some authors have shown that basic mechanism of cell damage induced by nHA is through oxidative imbalance as the result of the increased production of hydroxyl radicals (31), as well as impaired antioxidant capacity via down-regulation of SOD activity *in vitro* (19). The particle characteristics (size and chemical structure) of calcium phosphate nanoparticles have decisive impact on the inflammatory and apoptotic mechanisms underlying calcium phosphate toxicities in various tissues. The reduction of nHA particle size has been reported to increase the release of NO and pro-inflammatory factors, such as tumor necrosis factor alfa, by the activated microglia in cell culture (31). Analyzing the proapoptotic action (as a mechanism that result in toxic effect), calcium phosphate in form of nHA have shown increase of p53 and decrease of bcl-2 activation, which lead to DNA damage in rat kidney cells (18), while nACP caused apoptosis by selective action on the G1 phase in the cell cycle in leukemia p388 cells (32). Also, other mechanisms of apoptosis induction have been suggested, such as an increase in intracellular Ca^{2+}, probably originating from nanoparticles (33), a modulation of mitochondrial membrane potential (34) and an increase in intracellular PO_4^{3-} (35).

The dose of FU extract used in this study was selected on the basis of previously confirmed antioxidant effect in the same species (23). Therefore, the neuroprotective action of FU extract (by means of the enhanced latency to respond in both tests) could be attributed to the attenuation of previously discussed oxidative damage following the application of calcium phosphate nanoparticles.

Slightly more pronounced response to thermal pain stimuli observed in the tail flick test, when compared to hot plate test, may be considered as confirmation that neurotoxic effect of nano calcium phosphates was more convincing on nociceptive elements that include predominantly peripheral nerves. In addition, according to the results of our study, it seems that antioxidative supplementation with FU extract has shown more beneficial antioxidative effects at the level of nociception control up to the level of spinal cord. At the same time, although it prevented hyperalgesic effect of nano calcium phosphates in hot plate test (when compared to control), FU extract administration did not include significant influence on nociception when compared to the groups that received calcium phosphate nanoparticles solely. The possible explanation for the observed phenomenon may be found in the fact that central mechanisms for pain control (that could be evaluated only in hot plate test) remained less affected to oxidative damage induced by calcium phosphate nanoparticles.

CONCLUSION

In summary, the results of our study may be considered as an experimental confirmation that mineral components of calcium phosphates (commonly used in dentistry), may produce hyperalgesic effects themselves, and this side effect of therapy that includes calcium phosphates may be significantly attenuated by antioxidant supplementation.

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