

Research Article

Subchronic Systemic Toxicity of New Endodontic Material Based on Calcium Hydroxyapatite and Calcium Silicates

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As an alternative to MTA, a new endodontic material based on hydroxyapatite and calcium silicates (ALBO-MPSA) has been synthesized, and its biocompatibility has been studied in many *in vitro* and *in vivo* studies. The current study aims to evaluate a subchronic systemic toxicity of ALBO-MPSA on the rat animal model, as a continuation of the previous studies. Biochemical parameters of blood and histological parameters of the liver, kidneys, and spleen of the rats were analyzed after 120 days of consumption of the aqueous extract of ALBO-MPSA. The results showed no myelotoxic effect or autoimmune effect on peripheral blood cells and no pathological effect on the liver, kidney, and spleen tissues. Besides, no changes in the skin and hair of the rats, neither the change in the consumption of food and water, nor the change in their usual behavior were noticed during the experiment.

1. Introduction

Calcium silicate cements are bioactive materials used in various endodontic indications, such as pulp capping, pulpotomy, apexogenesis, apexification, perforation repair, and root-end filling [1–4]. The mineral trioxide aggregate (MTA) is certainly one of the most important and most widely investigated calcium silicate-based materials due to its clinical reliability and effective bone inductive potential in periapical defects after endodontic surgical treatment [5–8]. Biocompatibility of MTA is attributed primarily to its physicochemical reaction with surrounding tissues and the release of Ca^{2+} ions [9].

However, MTA shows many drawbacks during the clinical application, such as long setting time, difficulty in handling, and potential discoloration of teeth [10–12], and

therefore, researchers have been trying to introduce new materials which would overcome these limitations of MTA (Bioaggregate and Biodentine) [13].

In an attempt to develop appropriate endodontic cement, a new material based on hydroxyapatite and calcium silicates (ALBO-MPSA) has been synthesized a few years ago using the nanotechnology approach [14]. The biocompatibility of ALBO-MPSA has been thoroughly studied and confirmed at *in vitro* and *in vivo* conditions and compared to MTA [14–21].

The aim of this paper was to further examine ALBO-MPSA biocompatibility, over the subchronic toxicity study. The effects of systemic toxicity of ALBO-MPSA were evaluated on the basis of biochemical parameters of blood and histological parameters of the liver, kidneys, and spleen of experimental rats after a 120-day period. The zero

hypothesis was that ALBO-MPSA does not show significant systemic toxic effects.

2. Materials and Methods

2.1. Preparation of the Test Material. The test material ALBO-MPSA is composed of 40% hydroxyapatite, 20% calcium silicates, 20% gypsum, and 20% BaSO₄ as a radiocontrast agent. Hydroxyapatite and calcium silicates were synthesized using the nanotechnology approach, as it was described in detail in our previous references [14–16], while gypsum dihydrate and BaSO₄ were purchased from Whip Mix Corporation, USA, and Merck, Germany, respectively. Detailed physicochemical characterization of HA-CS was given previously [14].

In this study, the aqueous extract of the ALBO-MPSA was used, which was obtained after immersion of ALBO-MPSA in distilled water in concentration of 100 mg/ml and decantation after 5 days (in accordance with the standard ISO 10993-12:2012—Biological evaluation of medical devices—Part 12: Sample preparation and reference materials).

2.2. Subchronic Systemic Toxicity Investigation. The experimental protocol for this investigation was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Belgrade, and Ministry of Agriculture and Environment Protection of the Republic of Serbia (decision number 01-831/2) and realized in accordance with recommendations of European Good Laboratory Practice (86/609 EEC) related to the use and protection of laboratory animals.

2.2.1. Experimental Protocol. This study was performed in accordance with the standard ISO 10993-11:2017 (Biological evaluation of medical devices—Part 11: Tests for systemic toxicity).

Twenty-five Wistar rats (*Rattus norvegicus*) at the age of 8–12 weeks and average body weight of 250 ± 10 g were included in the investigation. The rats were placed in properly marked cages and kept in a room for experimental animals where the room temperature was 23 + 3°C and air humidity was 55 + 5%, with 8–12 air changes/h, with artificial lighting at intervals of 12 h day/night. The animals had free access to food and water, and their health status was monitored daily during the experiment.

After the adaptation period of 7 days, these 25 animals were randomly divided into two groups: experimental (15 animals) and control (10 animals) groups. The animals in the experimental group were given 1 ml of the aqueous extract of the ALBO-MPSA everyday at the same time during 120 days, and the animals in the control group were given 1 ml of distilled water.

Health status of the rats, their behavior, changes in the skin and hair, changes in food and water consumption, and changes in urination and defecation were monitored and recorded daily during the experiment. The body weight of experimental animals was checked at the beginning of the

experiment and then every forty days to the end of the experiment.

2.2.2. Blood Analysis. On the last day of the experiment, blood samples were taken from the lateral tail vein of all animals for complete blood analysis (leukocytes, hemoglobin, and platelets), including analysis of biochemical parameters of the blood: the levels of alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST), to evaluate the function of the liver, and levels of urea, creatinine, and bilirubin, to evaluate the function of kidneys. Also, the values of serum protein, glucose, and alkaline phosphatase (ALP) were determined.

2.3.3. Histopathological Analysis. After 120 days, the rats from both control and experimental groups were sacrificed by intravenous administration of thiopentone sodium solution at a dose of 170 mg/kg, and the tissue samples of the liver, kidneys, and spleen from each animal were taken for the histopathological analysis. The tissue samples were fixed in 10% buffered formalin solution and then processed by the routine procedure. One paraffin block from the liver, kidneys, and spleen was made for each animal, after which 5 μm thick tissue sections were made from paraffin blocks and dyed by hematoxylin-eosin (HE).

Semiquantitative histopathological analysis of liver tissue samples included an evaluation of the following parameters: the presence of inflammatory infiltrates in the portal tract and its graduation 0–3: 0—without infiltrates, 1—mild infiltration, 2—moderate infiltration, and 3—strong infiltration, the dominant cells' type in inflammatory infiltrates (lymphocytes or neutrophils), and other changes (degenerative and similar) in portal spaces. In the lobules of the liver, the following parameters were investigated: the intensity of inflammatory changes, the type of hepatocyte necrosis, the occurrence of steatosis, binuclear hepatocytes, and the accumulation of pigments in hepatocytes. The lobular necroinflammatory intensity is semiquantitatively graduated (0—without lobular activity, 1—light lobular activity with 1–3 necrosis per lobule, 2—moderate with 3–5 necrosis per lobule, and 3—strong with more than 5 necrosis per lobule). As part of the lobular activity, enlargement and hyperplasia of Kupffer's cells in sinusoids were studied (0—no change, 1—light hyperplasia of Kc, 2—moderate hyperplasia of Kc, and 3—severe hyperplasia of Kc).

Analysis of kidney tissue samples included an evaluation of the presence of interstitial haemorrhage and its graduation (0—without haemorrhage, 1—focal and rare haemorrhage in less than 10% of the sample, 2—moderate haemorrhage in 10–30% of the sample, and 3—severe, extensive haemorrhage in more than 40% of the sample) and degenerative changes of the tubular epithelium and tubular dilatation (0—without degenerative changes and dilatation, 1—light degenerative changes and dilatation of up to 10% of tubules in the sample, 2—moderate degenerative changes and dilatation of 10–30% of tubules in the sample, and 3—strong degenerative changes and dilatation of more than 30% of tubules in the sample), as well as semiquantitative

analysis of the presence of inflammatory infiltrates in the kidney interstitium (0—without inflammatory infiltrate, 1—mild inflammation, 2—moderate inflammation, and 3—severe inflammation) and determination of the dominant type of cells in the inflammatory infiltrate (lymphocytes and neutrophils). Also, the presence of the foci of glomerular collapse has also been evaluated.

In the tissue samples of the spleen, the presence of congestion in red pulp and the existence of white pulp hypertrophy (0—no hypertrophy, 1—light hypertrophy, 2—moderate hypertrophy, and 3—severe hypertrophy) were estimated.

Statistical analysis was performed using the SPSS software (version 2010; Chicago, IL, USA).

3. Results

3.1. Clinical Symptoms and Observations. By daily monitoring of the tested animals, no adverse effects related to their behavior, no changes in skin and hair, no changes in food and water consumption, and no changes in urination and defecation have been observed. Body weight of all animals mildly and consistently increased during the observation period (Figure 1).

3.2. Results of Blood Analysis. Blood analysis showed that there was no statistically significant difference between the hemoglobin and thrombocyte values in the experimental and control groups, but the leukocyte values were significantly higher in the control group than in the experimental group ($p < 0.001$; Table 1).

Analysis of the biochemical parameters (Table 2) showed that the ALT level was similar in the experimental and control groups, while the AST level was higher in the control group than in the experimental group, but without significant differences. Urea, creatinine, bilirubin, glucose, and ALP values were similar in experimental and control groups and without statistically significant differences.

3.3. Results of Semiquantitative Histopathological Analysis. Histopathological analysis of the liver tissue pointed out the absence or eventually the presence of a mild inflammatory infiltrate in the portal liver areas. A mild inflammatory reaction with eosinophils infiltration was more common in the experimental group of rats but not statistically significantly higher in comparison to the control group (Figure 2).

No degenerative changes in the biliary ductal epithelium of the liver were observed in the experimental group nor in the control group. Necrosis of individual hepatocytes in lobules, marked by a slight lymphocyte inflammatory infiltrate, was registered in more than 50% of samples in the experimental and control groups. Moderate lobular activity was registered only in two cases in the experimental group (13.3%) (Figure 3).

Fatty changes in hepatocytes were not observed in any case, while binuclear hepatocytes were observed only in 20% of cases in the experimental group (Figure 4).

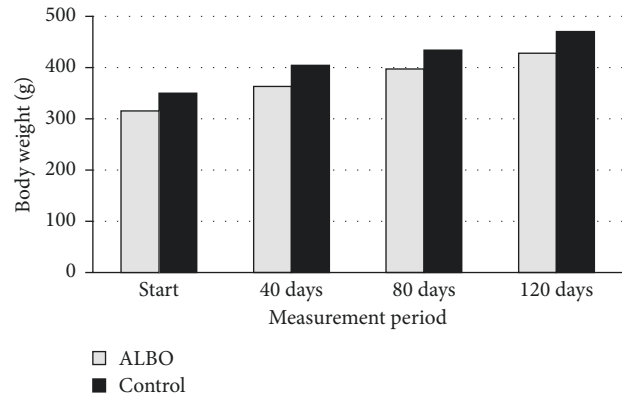


FIGURE 1: Average body weights of experimental and control animals during the study of subchronic systemic toxicity of the dental material ALBO-MPSA.

TABLE 1: Results of blood analysis.

	ALBO-MPSA	Control
Leukocytes	3.00 ± 1.63	6.83 ± 0.77
Hemoglobin	132.38 ± 46.1	140.8 ± 6.76
Thrombocytes	431.67 ± 257.28	713.20 ± 181.58

TABLE 2: Results of analysis of biochemical parameters.

	ALBO-MPSA	Control
ALT	57.54 ± 17.83	71.50 ± 31.11
AST	412.62 ± 303.70	844.32 ± 838.57
Urea	5.13 ± 1.05	5.21 ± 0.36
Creatinine	38.23 ± 4.44	40.00 ± 4.84
Bilirubin	1.35 ± 0.41	1.01 ± 0.32
Glucose	5.59 ± 1.36	5.48 ± 0.99
ALP	69.62 ± 8.87	63.00 ± 9.80

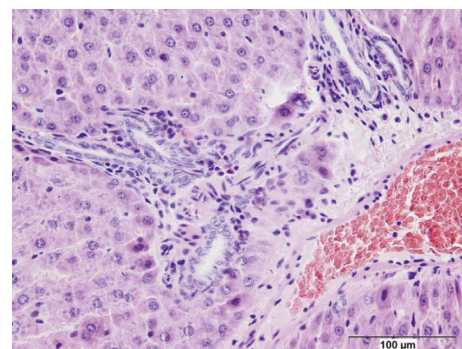


FIGURE 2: A normal-sized portal tract with a slight inflammatory infiltrate with eosinophils included (HE, x400).

An analysis of reactive changes in the liver indicated mainly a slight increase in Kupffer's cells, while a moderate increase was recorded in 26.7% of cases in the experimental group.

Histopathological analysis of the kidney tissue showed no interstitial bleeding in any case and a slight collapse of rare glomeromas in 6.7% of cases in the experimental group.

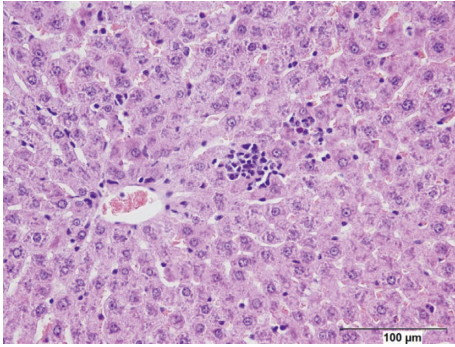


FIGURE 3: Centrilobular area of the liver tissue with 2 focal hepatocyte necrosis (HE, x400).

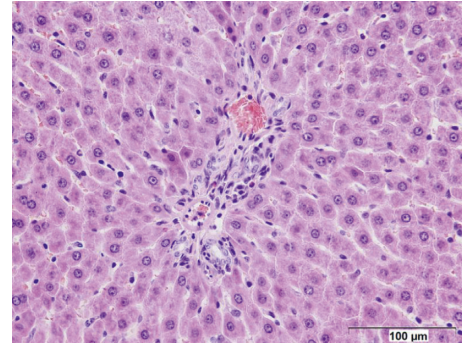


FIGURE 5: Inflammatory infiltrate inside the kidney interstitium (HE, x400).

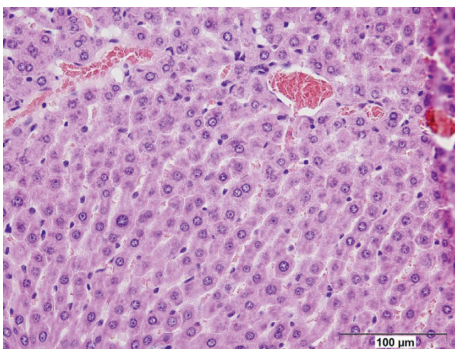


FIGURE 4: Binocular hepatocytes with anisocytosis (HE, x400).

Degenerative changes in the tubular kidney epithelium were not observed in any rat. Inflammatory infiltrate in the interstitium was observed in 6.7% of cases in the experimental group consisting of lymphocytes (100%) and in 55.6% of cases in the control group where lymphocytes were present in 60% of cases (Figure 5).

Histopathological analysis of the spleen tissue showed mildly atrophic or hypertrophic changes in the white pulp (present in 20% of cases in the experimental group and 11.1% of cases in the control group), while in red pulp, mostly mild congestion was registered (in 100% of cases in the experimental group and 77.8% of cases in the control group).

4. Discussion

During the 120-day period, experimental rats were daily given the aqueous extract of ALBO-MPSA, and during that period, no changes in their skin, hair, and usual behavior were noticed.

An analysis of hematological parameters of blood after 120 days revealed no significant differences between the experimental and control groups. There was no myelotoxic effect or autoimmune effect on peripheral blood cells which may be due to the fact that the tested material was not in contact with blood [13, 22] but also due to the chemical composition of the material whose biocompatibility has already been confirmed in many studies [14–21].

The morphometric analysis of the liver tissue pointed out the absence of a mild inflammatory reaction in the portal areas of liver in the control and experimental groups, which indicates that there was no significant damage to the liver [1, 13, 23, 24]. Also, the absence of degenerative changes in the biliary ductal epithelium and the rare, mild, and moderate necrosis confirmed that ALBO-MPSA had very low and insignificant impact on the liver tissue [25–27]. Fatty changes in hepatocytes have not been observed. A slight and moderate increase in Kupffer's cells was noticed indicating an increased mitotic activity of hepatocytes in both the control and experimental rats [28–31]. Increased activity of the liver was also indicated by an increase in the number and density of the cells and a moderate increase in the liver sinusoids and hepatocyte surface [25, 32]. In addition, the levels of AST, ALT, and ALP in the blood, which indicate liver damage, were not significantly different in the control and experimental groups, also indicating that there was no hepatocellular liver injury [24, 26].

Histological analysis of the kidney tissue indicated no bleeding in any case, while a mild glomerular collapse was registered in a small number of cases. The values of biochemical parameters referring to kidneys (urea, creatinine, and bilirubin) were also almost identical in the experimental and control groups, pointing out that there was no kidney damage. No degenerative changes in the tubular kidney epithelium and a minority of cases with inflammatory infiltration in the interstitium also indicated a low potential toxic effect of ALBO-MPSA [13, 33].

An analysis of the spleen tissue revealed the presence of light hypertrophic changes in white pulp and a mild congestion in red pulp, but these changes did not affect the function of the spleen and its immune response, which also testifies the nontoxicity of ALBO-MPSA [1, 13].

All these results of the systemic toxicity investigation of ALBO-MPSA showed that there was no negative effect on the liver, kidneys, and spleen, which confirmed the zero hypothesis.

However, there are opposite findings about the biocompatibility of calcium silicate-based materials (MTA, Bioaggregate) which indicate that these materials adversely affect the liver and kidneys of the rat, causing significant inflammatory reactions in comparison to the control group

[13, 34, 35]. Harmful effects are explained by the presence of small amounts of arsenic in the MTA material [12] which exceed the level recommended by the International Organization for Standardization [36–39]. This makes the present study, together with the previous biocompatibility studies of the new endodontic material ALBO-MPSA [14–21], very important as they give evidence about safety of the new material which would be used in the future in dental practice as an alternative to more toxic currently available materials.

5. Conclusions

The present study of subchronic systemic toxicity revealed that the new endodontic material ALBO-MPSA did not cause any pathological effect on the liver, kidney, and spleen tissues of the animal rat model, after oral application during 4 months. Also, it did not lead to changes in the skin and hair and did not affect the consumption of food and water, urination and defecation, and usual behavior of the experimental animals. The results of this study together with the results of previous biocompatibility studies prove that ALBO-MPSA is safe for application in dental medicine.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

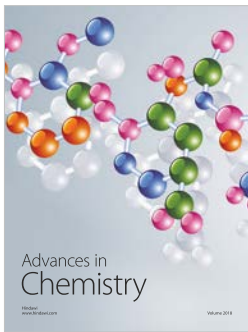
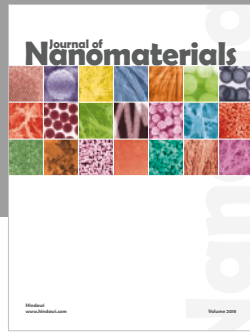
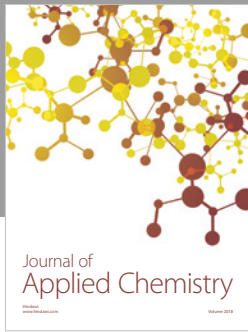
Acknowledgments

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