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ORIGINAL ARTICLE

CTZ and Calcium Hydroxide Pastes Did Not Cause Hepatic and Renal Alterations in Mice

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ABSTRACT

Objective: To evaluate the possible renal and hepatic alteration by root canal filling pastes in mice. **Material and Methods:** Fifty-four mice were divided into nine groups and received one polyethylene tube implant containing two filling pastes (CTZ or calcium hydroxide pastes). Empty polyethylene tubes were used as a negative control. All tubes were implanted subcutaneously in the back of the mice. After time intervals of 7, 21, and 63 days, 1.5 mL of blood was collected by cardiac puncture, and serum samples were used for serological testing. Urea, creatinine, aspartate transferase (AST), alanine transferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT) were evaluated. Data were analyzed by 2-way ANOVA ($p<0.05$). **Results:** When comparing CTZ and calcium hydroxide pastes and empty tubes and experimental time intervals, no significant differences in the results were found for any of the biochemical parameters analyzed ($p>0.05$). No differences were observed in the interactions (material*experimental time intervals) and the biochemical parameters analyzed ($p>0.05$). **Conclusion:** CTZ and calcium hydroxide pastes did not cause hepatic and renal alterations in mice, demonstrating the pastes' safety.

Keywords: Calcium Hydroxide; Pediatric Dentistry; Root Canal Filling Materials; Subcutaneous Tissue.

Introduction

Most of the techniques recommended for filling root canals of primary teeth with pulp necrosis have proposed instrumentation and chemo-mechanical preparation of the root canal system [1]. Among the most commonly used techniques, calcium hydroxide stands out as one of the root-filling material options, justified by its antibacterial action, biocompatibility, and tissue repair capacity [2].

Due to the difficulties of instrumentation and chemical-mechanical preparation of primary tooth canals, simplified techniques have been developed [3-6]. They recommend the removal of infected tissue within the pulp chamber and the placement of pastes containing antibiotics at the root canal entrances and pulp chamber floor [7,8]. These techniques are based on disinfection principles and help bone tissue repair developed [3-6].

Among the techniques in which chemical-mechanical preparation of root canals is not performed [3,6,9,10], Cappiello [3,9] has suggested the use of a paste composed of chloramphenicol, tetracycline, zinc oxide, and eugenol, known as CTZ paste. This paste has biocompatibility similar to that of calcium hydroxide [11], has antimicrobial action [12,13], and does not promote alterations in alveolar blood cells [14]. Besides, CTZ paste did not cause enamel developmental defects [15] and has shown satisfactory clinical and radiographic results [7,8] and less chair time than that for the ZOE pulpectomy [7,16]. The CTZ technique is more economically viable than using iodoform paste for endodontic treatment of primary teeth, with lower costs [16]. However, the literature on the systemic effects of the paste is scarce. Several biomarkers could help evaluate liver and kidney injuries and toxicity caused by several products [17,18].

Therefore, evaluating the systemic effect of the related antibiotics used in pulp therapy using CTZ paste is essential because bacterial resistance is possible [19]. The present study aimed to evaluate the potential renal and hepatic alteration caused by root canal-filling pastes in mice.

Material and Methods

Ethical Approval

This study was conducted in compliance with the recommendations contained in the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council of The National Academies, Washington, DC, 2011) [20]. The Ethics Committee on Animal Experiments approved all experimental procedures at the Federal University of Piauí (Approval 067/12). The manuscript was written per the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) [18].

Animal Model

The sample size calculation was determined considering a difference of -25% between the means of the control and experimental groups, a coefficient of variation (CV) of 20% to account for biological variability, a 95% confidence interval (CI), and an 80% power analysis. This calculation revealed that a quantity of 6 animals per group was necessary to achieve statistical significance at a p-value < 0.05 level. The study design employed a wholly randomized 3x3 factorial scheme (three materials X three times) with six replications. In the analysis of variance, the degrees of freedom for the residuals, with six replications, were 45, underscoring the representativeness of the sample size. A total of 54 male *Swiss* mice (*Mus musculus*), 6 to 8 weeks old, weighing between 15 and 30g, were used. The animals were housed in individual plastic cages, kept in a vivarium throughout the experiment, and were divided into nine groups. The animals were fed standard rations (Nestlé Purina, Paulinia, SP, Brazil) and provided with access to water *ad libitum*. The mice were examined clinically every two days to diagnose any possible toxic signs and symptoms. The animals were housed in the UFPI vivarium throughout the experimental periods and provided with standard food and water ad libitum. They were periodically observed to monitor the occurrence of local and systemic behavioral abnormalities. Strict control

was maintained over the 12-hour light/dark cycle to ensure that the animals' metabolism remained unaltered. Temperature was maintained within the range of 21°C to 26°C, and humidity was kept between 45% and 55%.

The animals were dewormed with Basken® suspension (König do Brazil Ltda., Santana de Parnaíba, SP, Brazil), administered orally in a single dose seven days before the experimental procedures began.

Root Canal Pastes

Two pastes were tested: one calcium hydroxide-based and the other contained antibiotics in the paste composition (CTZ paste). As the negative control, an empty polyethylene tube was used. The blood samples collected from each animal were submitted to biochemical analysis at time intervals of 7, 21, and 63 days. These specific experimental timeframes were chosen following the biocompatibility study's design, which represents this research project's primary outcome. Additionally, these timeframes align with the standards established by the International Organization for Standardization (ISO), as outlined in ISO 10993. This ISO series encompasses various standards for evaluating the biocompatibility of medical devices, including ISO 10993-1:2009, ISO 10993-2:2006, ISO 10993-6:2007, ISO 10993-11:2006, and ISO 10993-12:2007.

A total of nine groups of six animals (n=6) were distributed as follows: G1 received one polyethylene tube containing CTZ paste for seven days; G2 received one polyethylene tube containing CTZ paste for 21 days; G3 received one polyethylene tube containing CTZ paste for 63 days; G4 received one polyethylene tube containing calcium hydroxide paste for seven days; G5 received one polyethylene tube containing calcium hydroxide paste for 21 days; G6 received one polyethylene tube containing calcium hydroxide paste for 63 days. Animals in the Control Group received one empty polyethylene tube for 7 (G7), 21 (G8), and 63 (G9) days.

The micronized powder base for the CTZ paste was provided in 250 mg capsules prepared by a compounding pharmacy (Teresina, Piauí, Brazil). The paste contained 62.5 mg chloramphenicol, 62.5 mg tetracycline, 125 mg zinc oxide, and 0.1 mL of eugenol (Biodinâmica Química e Farmacêutica, Ibiporã, PR, Brazil) [11]. Micronization reduced the powder particles' size, ensured homogeneity, and improved the paste's pharmacological properties [17,18].

The Calcium Hydroxide ($Ca(OH)_2$) powder (analytical grade) (Biodinâmica Química e Farmacêutica, Ibiporã, PR, Brazil) was provided in 250 mg capsules and was reconstituted in 0.2 mL of distilled water (Isofarma Industrial Farmacêutica Ltda, Eusébio, CE, Brazil). The powders of each of the two pastes were placed on sterile glass plates and mixed with their respective vehicles using a metal spatula immediately before use.

Experimental Design for Subcutaneous Implantation

Mice were pre-medicated using tramadol at a dose of 2 mg/kg (Hipolabor Farmacêutica Ltda., Sabará, MG, Brazil) by deep intramuscular injection using a 1 mL syringe. After 10 minutes, anesthesia was induced using a combination of xylazine (10 mg/kg) (Xilazin®, Syntec do Brasil Ltda., Cotia, SP, Brazil) and ketamine (80 mg/kg) (Clortamina®, Instituto BioChimico Indústria Farmacêutica Ltda., Itatiaia, RJ, Brazil) prepared in the same syringe, and administered by deep intramuscular injection. The back of the animal was shaved, and the surgical region was disinfected with a 1% chlorhexidine gluconate solution (Rioquímica S/A, São José do Rio Preto, SP, Brazil) [11].

Polyethylene tubes measuring 1 mm in diameter and a peripheral catheter type scalp No. 27 (Solidor®, Shijiazhuang, China) were sterilized in ethylene oxide and closed at one extremity using Allis forceps (Quinelato, Schobell Industrial Ltda, Rio Claro, SP, Brazil). Tube segments measuring 1 cm long were filled with CTZ or calcium hydroxide paste. A linear incision measuring 0.5 cm was made in the caudal lumbar dorsum, and the tube was inserted into the tissue, a suture with 5.0 nylon thread (Shalon Fios Cirúrgicos Indústria, São Luis de Montes Belo, GO, Brazil). In the first 24 hours after implantation, the animals received tramadol (2 mg/kg) administered by deep intramuscular injection every 6 hours [11].

After time intervals of 7, 21, or 63 days, animals from each group were pre-medicated with tramadol (2 mg/kg) by deep intramuscular injection and euthanized with a combination of xylazine (10 mg/kg) and ketamine (80 mg/kg) anesthetic prepared in the same syringe, and administered by deep intramuscular injection. Soon after confirming the animal's death, 1.5 mL blood was collected by cardiac puncture (vacuum tubes with activator, Vacuplast®, Cotia, Brazil) to evaluate the hepatic and renal biomarkers.

Biochemical Analysis

The blood from each mouse (1.5 mL) was collected by cardiac puncture, and separated by centrifugation (TDL model 80-2B), and preserved at -20ºC until further analysis. Biomarkers were studied by measuring serum dosages of aspartate transferase (AST), alanine transferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), urea, and creatinine, with the intention of analyzing the pastes' possible nephrotoxicity and/or hepatotoxicity.

To carry out the biochemical analysis, the reagents were prepared according to the manufacturer's recommendations (Doles Reagents and Equipment Laboratories Ltd., Goiânia, GO, Brazil). The quantities of serum used according to the enzyme reagent were as follows: AST 50 µL, ALT 50 µL, ALP 10 µL, GGT 50 µL, Urea 10 µL, and creatinine 50 µL, and analyzed.

The enzyme activity was determined using Doles® Commercial Kits (Doles Reagents and Equipment Laboratories Ltd., Goiânia, GO, Brazil) and read by using a semi-automatic spectrophotometer (Doles®, D 250, version 3616B, Goiânia, GO, Brazil).

Statistical Analysis

Data were tabulated and analyzed using the statistical program SPSS® 20.0 version (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test was applied, and the data were found to be parametric ($p>0.05$). Descriptive analysis (means and standard deviation) was performed. Two-Way Analysis of Variance (2-way ANOVA) test was conducted to assess differences in the means of biochemical parameters among the materials (CTZ, calcium hydroxide, and control), various experimental time intervals, and interaction (material ✻ experimental time intervals). The level of significance was $p < 0.05$.

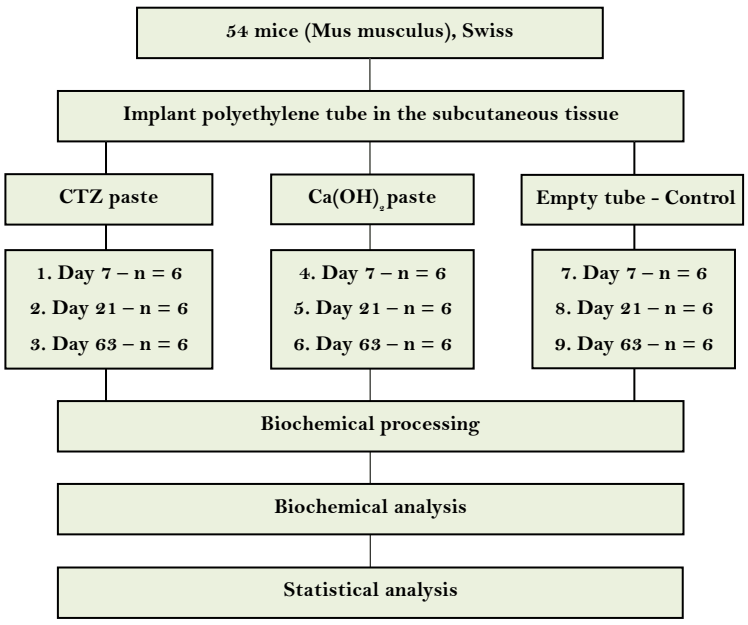

Figure 1. Study flowchart.

Results

Table 1 presents the mean and standard deviation of the biochemical parameters. Therefore, serum levels of urea, creatinine, AST, ALT, ALP, and GGT in the animals with subcutaneous implants with CTZ and calcium hydroxide pastes were similar to those found in the empty tube group (Table 1).

Table 1. Biochemical parameters of pastes at the specified periods.

Material	Experimental Time Intervals (Day)	Biochemical Parameters					
		Aspartate Transferase (U/L) μ(±SD)	Alanine Transferase (U/L) μ(±SD)	Alkaline Phosphatase (U/L) μ(±SD)	Gamma-Glutamyl Transferase (U/L) μ(±SD)	Urea (mg/dL) μ(±SD)	Creatinine (mg/dL) μ(±SD)
CTZ	7	113.0 (±21.0)	76.5 (±14.4)	153.0 (±20.2)	6.19 (±1.2)	51.9 (±7.0)	0.543 (±0.06)
	21	89.3 (±20.9)	60.6 (±16.6)	149.0 (±18.8)	6.08 (±1.2)	51.5 (±10.2)	0.524 (±0.10)
	63	96.8 (±32.9)	72.8 (±18.8)	147.0 (±21.4)	5.77 (±1.6)	50.9 (±5.0)	0.488 (±0.11)
Calcium Hydroxide	7	117.0 (±39.9)	72.6 (±21.8)	151.0 (±25.0)	6.22 (±1.4)	51.9 (±5.9)	0.552 (±0.05)
	21	96.8 (±36.7)	61.4 (±18.1)	150.0 (±25.7)	6.05 (±1.6)	51.4 (±10.5)	0.530 (±0.10)
	63	99.8 (±39.0)	73.3 (±17.0)	147.0 (±26.1)	5.75 (±2.3)	51.2 (±9.3)	0.498 (±0.09)
Control	7	111.0 (±17.0)	78.7 (±14.8)	153.0 (±17.1)	6.40 (±1.3)	52.0 (±6.3)	0.561 (±0.07)
	21	92.7 (±32.1)	60.5 (±17.8)	151.0 (±22.5)	6.37 (±1.0)	51.6 (±7.8)	0.537 (±0.09)
	63	109.0 (±45.8)	72.2 (±13.0)	148.0 (±24.0)	5.79 (±1.3)	51.0 (±8.9)	0.509 (±0.06)

μ: Mean; SD: Standard Deviation.

When comparing CTZ and calcium hydroxide pastes, empty tubes, and experimental time intervals, no significant differences in the results were found for any of the biochemical parameters analyzed ($p>0.05$) (Table 2). No differences were observed in interactions (material*experimental time intervals) and the biochemical parameters analyzed ($p>0.05$) (Table 2).

Table 2. Association between materials, experimental time intervals, and interaction and biochemical parameters.

Variables	Biochemical Parameters					
	Aspartate Transferase (U/L) F Statistics (p-value)	Alanine Transferase (U/L) F Statistics (p-value)	Alkaline Phosphatase (U/L) F Statistics (p-value)	Gamma-Glutamyl Transferase (U/L) F Statistics (p-value)	Urea (mg/dL) F Statistics (p-value)	Creatinine (mg/dL) F Statistics (p-value)
Materials	0.126 (0.882)	0.029 (0.971)	0.017 (0.983)	0.089 (0.915)	0.001 (0.999)	0.189 (0.828)
Experimental Time Intervals	1.798 (0.177)	0.908 (0.927)	0.211 (0.811)	0.569 (0.570)	0.052 (0.950)	1.867 (0.166)
Interaction	0.104 (0.980)	0.089 (0.986)	0.004 (1.000)	0.018 (0.999)	0.001 (1.000)	0.004 (1.000)

Interaction: Materials ✻ Experimental Time Intervals; F statistics and p-value: Two-Way Analysis of Variance (2-way ANOVA).

Discussion

To the best of the authors' knowledge, this is the first study demonstrating that CTZ and calcium hydroxide pastes did not cause hepatotoxic and nephrotoxic effects and were, therefore, safe for use in therapies of teeth with endodontic infections. It is relevant to know about materials and drugs used in dentistry and the risks to patient's health [21]. Although there is no scientific evidence regarding possible systemic manifestations of using antibiotic pastes in pulp therapy of primary molars with necrosis, these have been recurrent questions in article submissions and presentations at scientific events. Therefore, the literature needs to provide data on the subject.

This study evaluated several parameters, such as ALT, AST, ALP, GGT, urea, and creatinine levels in serum. Potential drugs for systemic dissemination are submitted to biochemical evaluation by assessing the serum levels of enzymes that indicate the metabolic functions performed by organs such as the liver and kidneys [22]. Renal alterations can be assessed by serum levels of urea and creatinine [23], and alterations in the liver can be determined by measuring biomarkers such as ALT, AST, ALP, and GGT [24–26].

Elevated plasma levels of liver enzymes are associated with an increased metabolic rate of the liver, indicating liver toxicity, especially when exposed to a drug, virus, or bacterial agent. A drug does not cause liver damage without altering the levels of these enzymes [27]. In this study, measurements of urea and creatine levels and liver enzyme levels were within the normal standards observed in the control group.

Mice may exhibit alterations in biochemical parameters related to sex, strain, and genotype and may be influenced by age, diet, handling during the experiment, and the environment, among other factors [28,29]. Therefore, knowledge of the values of different physiological parameters is essential for the evaluation of homeostasis, alterations induced by pathological processes, and the impact of experimental procedures [30]. It is recommended that each laboratory establishes its range of reference values since using these reagents is standardized for humans [28]. In the present study, the biochemical parameters of the group in which the empty polyethylene tubes were used (control) were considered the reference intervals because they reflected the population in which the tests were applied.

Serum ALT levels increase when alterations occur in vascular permeability or hepatocyte injury. The AST is a mitochondrial isozyme and is not released as soon as ALT, which is primarily cytoplasmic. ALT is a more sensitive indicator of acute hepatotoxicity than AST because, while the former is essentially found only in the liver, the latter can also be found in high concentrations in other organs, such as the kidneys, lungs, and heart [25,26,31]. The levels of GGT can be seen as a field test of the excretory capacity of the liver [25]. The present study observed no alterations in these biomarkers, suggesting that the CTZ and calcium hydroxide pastes were not hepatotoxic. Drawing reliable comparisons between experimental study data and clinical actions in the antibiotic field is challenging, given that clinical and laboratory scenarios are distinct and specific for each study design. ALP is a phosphohydrolase enzyme found in many tissues, with the highest concentrations occurring in the liver, biliary tract epithelium, and bone. ALP is an important biomarker of the plasma membrane and endoplasmic reticulum activity and is often used to assess membrane integrity during pharmacological treatments and in some pathological conditions [32]. As noted in this study, there was no significant difference in the mean ALP values throughout the experimental period for all the materials. This indicated that the CTZ and calcium hydroxide pastes could be safely used because they did not induce biochemical alterations indicative of liver disease in the serum of the mice tested.

Renal tubules reabsorb 40% of the urea excreted by the kidneys; therefore, the blood levels of this parameter indicate renal function and can indicate the glomerular filtration rate [33]. Creatinine clearance

occurs at a relatively constant speed through glomerular filtration and active tubular excretion from the kidneys. The creatinine concentration is independent of the protein level in food and is not affected by the urine volume; it is also more sensitive and specific than the urea concentration for assessing renal function. Usually, the serum creatinine level is proportional to the severity of the disease [34,35]. In this study, no significant differences in the levels of urea and creatinine were found between the study groups and the control group.

In the literature, the normal mean values of AST, ALT, and ALP in mouse serum were lower, and the urea and creatinine levels were higher than those observed in a similar study [29,36]. The difference in our study compared with that of other authors was probably due to differences between strains, the physical and climatic conditions, and the materials and methodology used.

To provide an effective antimicrobial action, CTZ paste is placed at the root canal entrances and on the pulp chamber floor of primary molars, where pulp-periodontal communications are observed due to its lower dentin thickness and the presence of accessory canals [37,38]. Therefore, polyethylene tube implants are used experimentally to simulate the relationship between the tooth apex and periapical tissues because the material within the tubes diffuses within a restricted area [11,19,39,40], similar to the situation occurring in endodontic infections.

The present study's results are essential to elucidate the security of the topical use of antibiotics in endodontic infections. As this was a laboratory study, the results should be interpreted with caution. However, it is important to note that the quantities of CTZ and calcium hydroxide pastes used in the polyethylene tubes in the present study were similar to those used in pulp therapies of primary teeth with pulp necrosis [11].

We were not able to find similar studies published in the literature, so we could not compare these results with previously published findings. The biochemical parameters found in the sera of mice that received polyethylene tube implants containing CTZ and calcium hydroxide pastes were within the range of the control group's reference values. The results showed no differences between time intervals for the biochemical variables when the same treatment was compared throughout the experimental period or in the same experimental interval between study groups and the control.

Conclusion

The CTZ and calcium hydroxide pastes did not cause hepatic and renal alterations in mice.

Authors' Contributions

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LFADM	https://orcid.org/0000-0002-4112-1533	Conceptualization, Methodology, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review and Editing, Supervision and Project Administration.

All authors declare that they contributed to a critical review of intellectual content and approval of the final version to be published.

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None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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