In vitro antimicrobial and cytotoxic effects of Kri 1 paste and zinc oxide-eugenol used in primary tooth pulpectomies

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Abstract

The antimicrobial and cytotoxic effects of Kri 1 paste, an iodoform-based primary tooth filling material, were compared with zinc oxide-eugenol (ZOE), using in vitro techniques. Antimicrobial evaluation involved measuring inhibition zones of Streptococcus faecalis *on brain heart agar. Cytotoxicity evaluation involved direct cell-medicament contact experiments of 4-hr and 24-hr duration using fresh and set medicaments, and indirect cell-medicament contact experiments of 24-hr duration using fresh and set medicaments. ZOE produced a greater zone of bacterial inhibition than Kri 1 paste. Kri 1 paste cytotoxicity remained high regardless of the amount of setting time in the 4-hr direct contact experiment, while ZOE cytotoxicity decreased with setting time. Both Kri 1 paste and ZOE had high cytotoxicity regardless of setting time in the 24-hr direct cell-medicament contact test. ZOE cytotoxicity decreased to control levels after only 1 day of setting in the indirect contact experiments, compared with greater than 7 days for Kri 1 paste. The results suggest ZOE has better antimicrobial activity than Kri 1 paste. ZOE also has lower cytotoxicity, although prolonged cell-medicament contact may result in both medicaments having similarly high cytotoxicity. (Pediatr Dent 16:102–6, 1994)*

Introduction

To maintain function, esthetics, arch length, and arch symmetry, primary teeth should be maintained in the dental arch until their proper exfoliation time.[1, 2] Endodontic treatment of primary teeth has been a successful method of maintaining nonvital primary teeth with and without periapical pathosis. However, success is related to strict tooth selection criteria and to thorough debridement of the canals followed by obturation with a suitable filling material.[2, 3]

Zinc oxide-eugenol (ZOE) has traditionally been used as a root canal filling material in the primary dentition. Recently, an iodoform paste (Kri 1 paste, Pharmachemie AG, Zurich, Switzerland) has been recommended as an alternative medicament to ZOE in root canal therapy for nonvital primary teeth.[4–6] Walkhoff originally described the formula for an iodoform paste in 1928 and used it in permanent teeth. Later, Castagnola and Orlay,[7] Juge,[8] and Barker and Lockett[9] reported using Walkhoff's paste as a filling material in permanent teeth, but long-term treatment success was compromised since the paste eventually resorbed, leaving a deficit in the root canal.

The reported rationales for using Kri 1 paste over ZOE in primary teeth are: ease of use, rapid resorption from periapical tissues, and superior antimicrobial action.[4, 5] Kri 1 paste is easier to use than ZOE because it is supplied as a premixed paste that can be placed directly into the root canal. Kri 1 resorbs rapidly from the periapical tissues, however, Woodhouse, et al.[10] have shown osteolytic changes in the bone surrounding Kri 1 paste implants in cats, persisting past the retention time of the material. No osteolytic changes were found surrounding ZOE implants. Several studies have investigated the antimicrobial action of Kri 1 in vivo[9, 11–13] and in vitro.[14–16] No studies have compared the antibacterial action and attendant cytotoxicity of Kri 1 paste and ZOE in vitro.

The purpose of this study was to examine the claim of superior antimicrobial action for Kri 1 paste by comparing the antimicrobial and cytotoxic effects of Kri 1 paste and ZOE in vitro.

Methods and materials

Antimicrobial evaluation

Kri 1 paste (iodoform 80.8%, camphor 4.86%, p-chlorophenol 2.025%, menthol 1.215%, was used as supplied. ZOE (zinc oxide U.S.P./eugenol U.S.P. — Sultan Chemists, Inc., Englewood, NJ) was mixed to the same consistency as Kri 1 paste immediately before experimentation (2.5 g zinc-oxide to 1 ml eugenol).

Ninety-millimeter-diameter petri dishes were filled with 12 ml of BBL Brain Heart Infusion with PABA (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD) and BBL Purified Agar. Six-millimeter-diameter wells were created in the agar using the end of a sterilized glass pipette. The wells were filled with 0.05 ml of either Kri 1 paste or ZOE dispensed from tuberculin syringes.

Aliquots of 0.1 ml of 10^6 CFU/ml *Streptococcus faecalis* (ATCC 19433) in BBL thioglycollate medium were then spread over the surface of the agar plates. The plates were inverted and incubated at 37°C for 24 hr. Two measurements of the diameter of bacterial growth inhi-

bition around each well were made using a millimeter ruler, and the values were averaged. These values were then used to calculate area of bacterial inhibition (area $=\pi\ d^2/4$), after subtracting the area of the wells. Agar plates containing wells, but no medicaments, were spread with *S. faecalis* and incubated as positive controls. Agar plates containing wells, but no medicaments or *S. faecalis*, were also incubated to ensure maintenance of sterile conditions (negative controls). All experimental procedures were carried out under sterile conditions using a SterilGARD hood® (The Baker Company, Sanford, ME).

Cytotoxicity evaluation

Two in vitro cytotoxicity test methods were used — the direct cell-material contact test of Spångberg[17] and the indirect cell-material contact test of Safavi, et al.[18]

Five- to 7-day cultures of L929 mouse fibroblast cells were used for the cytotoxicity tests. The culture medium used was minimal essential medium (MEM) with Eagle's salts (Flow Laboratories, McLean, VA) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, and 2.2 mg sodium bicarbonate per ml. Streptomycin (50 µg/ml) and penicillin (100 IU/ml) were also added to the medium. The medium was changed every other day and on the day before the experiments.

Radiochromium (^{51}Cr) was supplied as sodium chromate in sterile isotonic saline (New England Nuclear,™ Du Pont Company, Wilmington, DE). Cell monolayer cultures were labeled by incubation with 1 µCi per 10^5 cells 20–24 hr before the experiments. The labeled cells were harvested using 0.125% trypsin and washed three times in Ca^{2+}-free and Mg^{2+}-free phosphate buffered saline (PBS) before use. The cells were resuspended in MEM at a concentration of $4x10^4$ cells/ml.

For direct cytotoxicity testing, 0.3 ml of ZOE or Kri 1 paste was placed in the bottom of 16-mm-diameter cell culture wells (Transwell; Costar,® Cambridge, MA). Materials were tested immediately after mixing (fresh), or after 1-day or 7-day setting times. Two ml of ^{51}Cr-labeled L929 cells were placed in the culture wells and incubated in contact with the medicaments for 4 or 24 hr at 37°C.

After incubation, 1 ml of the fluid in the test chamber was transferred to a test tube and centrifuged (500 x g) for 10 min. One-half ml of the supernatant was transferred to a test tube for counting in a gamma particle counter for 1 min. Negative controls of the L929 suspension were used. During cell dispensing, 0.5 ml aliquots of cell suspension were randomly distributed into six test tubes and used as reference samples. The percentage of ^{51}Cr released from the experimental and control samples was calculated according to the formula:

$$^{51}Cr\ release\ (\%) = \frac{(T\text{-}b)}{(R\text{-}b)} \times 100$$

where T = ^{51}Cr released in test samples, b = background radiation and R = ^{51}Cr released in reference samples.

For the indirect cytotoxicity evaluation, 0.1 ml of either Kri 1 paste or ZOE was placed in the 6.5-mm-diameter chamber insert of a cluster well cell culture plate. The porous bottoms of the cell culture inserts (uniformly spread 0.4 µm holes) were 1 mm from the bottom of the well. This allowed independent access from the chamber insert into the well through the medium. One milliliter of ^{51}Cr-labeled L929 cells was dispensed into each cell culture well. The chamber inserts containing the test medicaments were then placed into the wells. Empty chamber inserts were placed in the cell culture wells for control. Randomly withdrawn 0.5-ml cell samples were transferred to test tubes to be used as reference samples for calculating chromium release. The plates containing the cells were incubated at 37°C for 4 hr immediately after mixing (fresh) and after 1, 7, 14, and 28 days.

The chamber inserts were stored in PBS and incubated at 37°C and 100% humidity between experiments. The percentage of ^{51}Cr release in test and control samples was calculated by the same formula used in the direct toxicity test.

The antimicrobial data were analyzed using Student's *t*-test while the cytotoxicity data were analyzed using ANOVA.

Results

On average, ZOE-filled wells produced a significantly greater area of inhibition ($P < 0.01$) than did the Kri 1-filled wells (Fig 1). Positive controls showed confluent *S. faecalis* growth over the agar with no areas of inhibition around the wells. Negative controls confirmed the maintenance of sterile conditions (absence of bacterial growth). Serial dilution and plating of the

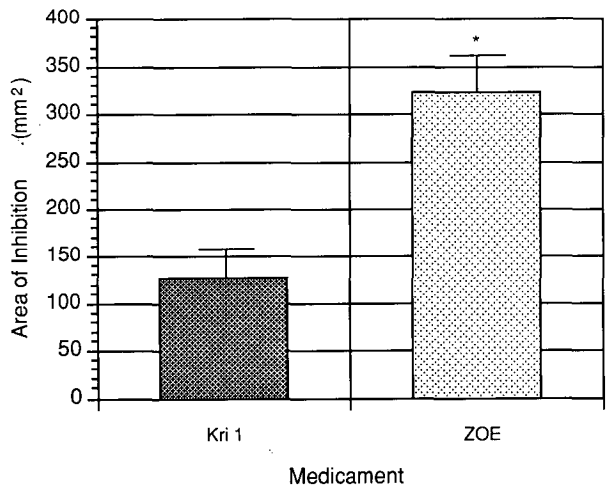

Fig 1. Antibacterial action of Kri 1 paste (N = 15) and ZOE (N = 13) based on *Streptococcus faecalis* inhibition on agar (mean ± SD). * = significantly greater than Kri 1 paste ($P < 0.01$).

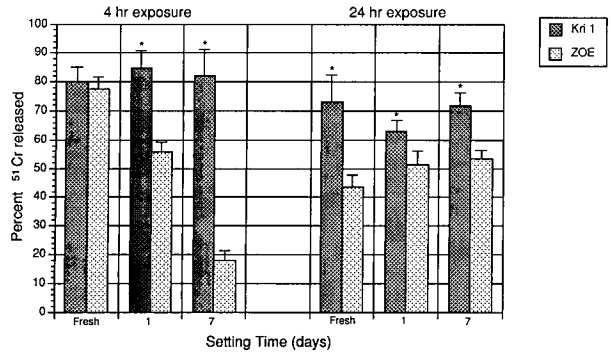

Fig 2. Relative toxicity of Kri 1 paste and ZOE based on percent ^{51}Cr released in the direct contact experiments (mean ± SD of six experiments). Control values have been subtracted from experimental values. * = significantly greater than ZOE ($P < 0.01$).

bacterial suspension confirmed a bacterial concentration of $1x10^6$/ml.

Four-hour exposure of the mouse fibroblast cells to Kri 1 paste resulted in a high release of ^{51}Cr, whether fresh, at 1 day, or at 7 days (Fig 2). This ^{51}Cr release was significantly greater than the controls ($P < 0.01$). Four-hour exposure of the cells to fresh, 1-, and 7-day ZOE resulted in ^{51}Cr release that was significantly greater than the controls ($P< 0.01$). Although fresh ZOE resulted in a high release of ^{51}Cr (approximately equal to that of Kri 1 paste), the release rates after 1 and 7 days decreased to levels that were significantly lower than Kri 1 paste ($P < 0.01$).

Twenty-four-hour exposure of the cells to Kri 1 paste and ZOE also resulted in a high release of ^{51}Cr, independent of the setting time. The release of ^{51}Cr for both Kri 1 paste and ZOE was significantly greater than the controls ($P < 0.01$). The cytotoxicity of Kri 1 paste was significantly greater than ZOE at all setting times in the 24-hr exposure experiments ($P < 0.01$).

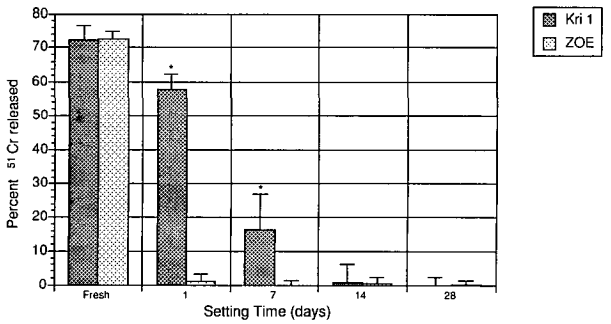

Fig 3. Relative toxicity of Kri 1 paste and ZOE based on percent ^{51}Cr released in the indirect contact experiments (mean ± SD of six experiments). Control values have been subtracted from experimental values. * = significantly greater than ZOE ($P < 0.01$).

Both Kri 1 paste and ZOE were highly cytotoxic on contact of the fresh medicaments with L929 mouse fibroblast cells (Fig 3) and significantly different from the control ($P < 0.01$). There was no significant difference between ZOE and control in the 1-, 7-, 14-, and 28-day experiment groups. The cytotoxicity of Kri 1 paste was statistically higher than ZOE and the control in the 1- and 7-day experimental groups ($P < 0.01$). Between 7 and 14 days, the cytotoxicity of Kri 1 paste dropped to a level that was not significantly different from either ZOE or the control and remained at this level to termination of the experiment at 28 days.

Discussion

The finding that ZOE produced better antimicrobial activity than Kri 1 paste contrasts with several other studies showing Kri 1 paste to be the superior antimicrobial.[14, 15] The increased antimicrobial action of ZOE in this study, however, may reflect the increased amount of eugenol incorporated into the zinc oxide to approximate the consistency of Kri 1 paste. The limitations of the method of antimicrobial testing employed in this study and in the studies of Orstavik[14] and Seow[15] must be recognized. The results may represent a variation in the diffusion rate of the medicaments through the agar or the effectiveness of a particular medicament in a gaseous phase.[1] A better assessment of antimicrobial action such as the methods of Spångberg, et al.[19] or Hill, et al.,[20] in which antimicrobial action is determined by the action of serial dilutions of an antibacterial solution on a test organism, was not possible in this study since neither Kri 1 paste or ZOE formed a homogenous solution in an appropriate solvent.

S. faecalis was used in this experiment because it has been shown to occur in pulpal infections in permanent teeth[21, 22] and, as a group, the betahemolytic streptococci have been shown to infect the primary root canal.[23–25] In addition, the organism is easy to grow and it has been used extensively in experimental antiseptic susceptibility studies in endodontics. Most importantly, *S. faecalis* efficiently and rapidly colonizes dentinal tubules compared with other species[26] and therefore is particularly difficult to eradicate. It represents a standard against which the antimicrobial action of a medicament should be tested.

The ^{51}Cr release method of cytotoxicity testing is a standard biocompatibility test, accurate and sensitive in assessing cell injury.[27] L929 mouse fibroblast cells are commonly used in biocompatibility tests and were used in this study for comparison of cytotoxicity results from other studies.

The 7-day set used in this study is an acceptable maximum in the direct exposure experiments because one week is considered sufficient for any material to reach optimal setting.[27] These time points were chosen to investigate the initial cytotoxicity of the medicaments and the time period over which they might lose

their cytotoxic effect. The cells were exposed to the ZOE and Kri 1 paste for 4 and 24 hr as prescribed in the ANSI/ADA document No. 41.[28] Four hours has been shown to be sufficient time for the cells to attach (under normal conditions) and to react to a material.[29] The 24-hr contact time was chosen to detect the effects of prolonged exposure to the material. The ^{51}Cr release value recorded for the L929 mouse fibroblast cells after a 4-hr contact with fresh medicaments in the direct contact experiments was expectedly high for both Kri 1 paste and ZOE. The eugenol in the ZOE and the iodoform, camphor, p-chlorophenol, and menthol in Kri 1 paste, are free to interact and exert their cytotoxic effect upon the mouse fibroblast cells. The significant decrease in ZOE cytotoxicity after 1 and 7 days, over which time the toxicity of Kri 1 paste remained high, may be explained by the fact that ZOE sets while Kri 1 paste does not. The eugenol in set ZOE is bound and mostly unavailable to exert its cytotoxic effect.

Although there were significant differences between Kri 1 paste and ZOE toxicities in the 24-hr direct contact experiments, the high levels of ^{51}Cr release for both Kri 1 paste and ZOE indicate the medicaments are equally cytotoxic when fresh, and after 1 and 7 days. These results differ from the 4-hr direct contact experiments and indicate no difference in cytotoxicity of the two medicaments if they are allowed to contact the target cells for a long enough period of time. The difference between the cytotoxicity of Kri 1 paste and ZOE — expressed as a higher release of ^{51}Cr caused by Kri 1 paste — may be due to the greater toxicity of the individual constituents of Kri 1 paste.[30]

The indirect method of cytotoxicity testing provides an additional assessment of material cytotoxicity and loss of cytotoxic effect with time. The decrease in ZOE cytotoxicity to control levels at 1-day set and beyond in the indirect contact experiments may be explained by the setting of this material in the same manner as explained above for the direct contact experiments. The dramatic decrease in the cytotoxicity of ZOE in this study, however, implies that over the first few days, direct contact is a more important aspect of ZOE cytotoxicity than is the set of the material. As expected, the cytotoxicity of Kri 1 paste remains high for a long period of time. The decline to control levels by 14 days despite the lack of a set state, may occur due to leaching of toxic constituents of the medicament into the PBS.

Browne and Friend's implant studies in rabbits[12, 13] suggest the in vivo effects of Kri 1 paste and ZOE are represented better by the indirect experiment rather than the direct experiments. Kri 1 paste reportedly caused considerable tissue necrosis as an immediate response compared with a small zone of tissue necrosis localized to the material surface caused by freshly mixed ZOE, with the amount of ZOE-induced necrosis dependent on the set of the ZOE.[12, 13] In a clinical situation, it might be expected that periodontal ligament (PDL) cells in direct contact with either Kri 1 paste or ZOE may be killed, while PDL cells not directly in contact may be affected to a greater degree by Kri 1 paste than ZOE.

Conclusion

This study indicates Kri 1 paste is less effective than ZOE in its antibacterial activity. Kri 1 paste is at least as cytotoxic as ZOE on cells in direct contact with the medicament and is more cytotoxic than ZOE on cells not in direct contact.

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