Connective Tissue Reaction to White and Gray MTA Mixed With Distilled Water or Chlorhexidine in Rats

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INTRODUCTION: The purpose of this study was to compare the histocompatibility of white (WMTA) and gray (GMTA) mineral trioxide aggregate mixed with 0.12% chlorhexidine (CHX) and distilled water (DW) in subcutaneous connective tissues of rats.

MATERIALS AND METHODS: The freshly mixed WMTA and GMTA with CHX or DW were inserted in polyethylene tubes and implanted into dorsal subcutaneous connective tissue of 50 Wistar Albino rats; tissue biopsies were collected and were then examined histologically 7, 15, 30, 60 and 90 days after the implantation procedure. The histology results were scored from 1-4; score 4 was considered as the worst finding. Data were analyzed using one-way ANOVA tests.

RESULTS: All experimented materials were tolerated well by the connective tissues after 90-day evaluation, except for the WMTA/CHX group that had significantly more mean inflammatory scores (P<0.001). There was a statistically significant difference in the mean inflammation grades between experimental groups in each interval (P<0.001). After 90 days, GMTA/CHX group had the lowest inflammatory score.

CONCLUSION: Although adding CHX to WMTA produces significantly higher inflammatory response, it seems a suitable substitute for DW in combination with GMTA. Further research is necessary to recommend this mixture for clinical use.

Keywords: Biocompatibility; Chlorhexidine Gluconate; Mineral Trioxide Aggregate

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found similar results with those reported for the GMTA formulation (13).

Chlorhexidine (CHX) is an antimicrobial agent belonging to a group of N5 derivatives of 1,6 bis- biguanidohexane (16). It has been widely used as an antiseptic and is active against gram-positive and gram-negative bacteria, facultative anaerobes and aerobes, moulds, yeasts and viruses (17). It acts by adhering to cell wall of the microorganisms and causing leakage of intracellular components and eventually leading to cell death (16,17).

In endodontics, CHX has been found to be an effective antimicrobial agent when used as a root canal irrigant (17). Stowe et al. have shown that substitution of 0.12% CHX gluconate with sterile water in tooth colored Pro-Root MTA enhanced the antimicrobial effect of this material in vitro (11). However, Hernandez et al. showed that this mixture induced apoptosis of macrophages and fibroblasts in vitro (18); however there are as yet no in vivo studies to evaluate its cytotoxicity.

The aim of this in vivo study was to compare the histocompatibility of WMTA and GMTA when mixed with CHX 0.12% or distilled water (DW), in subcutaneous connective tissue of rats.

MATERIALS AND METHODS

Fifty male, 5-6 months old Wistar Albino rats weighting 270±20 g were used in this study. The criteria of the Helsinki Declaration regarding laboratory animals were considered in all steps of the project (19). The study has been approved by Ethics Committee of Tabriz University of Medical Science.

The following materials were investigated:

Group 1: WMTA (Angelus, Londrina, Brazil) mixed with DW
Group 2: GMTA (Angelus, Londrina, Brazil) mixed with DW
Group 3: WMTA Angelus/CHX 0.12%
Group 4: GMTA Angelus/CHX 0.12%
Group 5: Control group (empty tubes)

Freshly mixed test materials were placed in clean, sterile, polyethylene tubes (Eastern Medikit LTD, Gurgaon, India) with 1.1 mm inner diameter and 0.8 mm length. MTA was mixed with distilled water according to manufacturer’s recommendation and was applied with a plastic carrier. For groups 3 and 4 CHX 0.12% was substituted with DW. Fifty empty polyethylene tubes served as controls.

The dorsal skins of animals were shaved under general anesthesia using diethyl ether (Parchemie, Tehran, Iran) and anesthetic chamber technique and disinfected with 5% iodine solution. Five incisions were made on the backs of the albino rats; incisions were made over a length of 1 cm using no.15 blade in a head-to-tail alignment. The skin was reflected, and the implantation materials were inserted into spaces created by blunt dissection. To prevent interactions of materials, the tubes were placed at least 2 cm far from each other (2 tubes in one side of the rats back and 3 in the other). The evaluations were made 7, 15, 30, 60 and 90 days after surgical implantation (20,21).

In each examination period, 10 animals were euthanized by administering high doses of anesthetics. The dorsal skin was shaved, and the tubes were excised together with the surrounding connective tissues. The specimens were kept in a 10% formalin solution for two weeks (Merk, Darmstadt, Germany) until histological processing. Sections with 5µm thickness were taken from specimens and placed in paraffin blocks and stained with hematoxylin and eosin. Evaluations were made in a light microscope (Leitz, Oberkochen, Germany) at ×400 and ×800 magnifications. Quantitative evaluations of inflammatory cells (lymphocytes, plasmocytes, polymorpho- nuclear leukocytes [PMN], macrophages, and giant cells) were made in ten separate areas of sections at ×400 magnifications. An average value for each material was obtained from the sum of cells counted in ten separate areas (22,23).

Reactions were scored and evaluated as:
Score 1: few inflammatory cells without edema
Score 2: <25 inflammatory cells, wavy collagen fibrils deposition and fibrosis
Score 3: 25-125 inflammatory cells, edema and vascular congestion
Score 4: >125 inflammatory cells, edema and vascular congestion and fibrin deposition (20,21).

Statistical analysis was carried out using one-way ANOVA. To determine differences between groups, LSD test was performed. Statistical significance was defined as P<0.05.
RESULTS

Histologic findings are presented in Table 1. There was a statistically significant difference in the mean inflammation scores among groups in each interval (P<0.001) (Figure 1), (Table 2). In 7-day and 15-day specimens there was no statistically significant difference between the mean inflammation scores of all groups. 30-day specimens (Figure 2) showed statistically significant difference between the mean inflammation scores of all groups (P<0.001). In 60-day specimens WMTA/CHX showed the highest mean inflammation score, however, there was no statistically significant difference between the mean inflammation scores of the others (P<0.05). In 90-day specimens WMTA/CHX showed the highest mean inflammation score and there was no statistically significant difference between the mean inflammation scores of the other groups (P<0.05).

DISCUSSION

Although test materials were directly applied subcutaneously in some studies (24), the implantation of the materials in tubes is advocated in others (4,22,23,25-27); in these cases silicon (22,23), polyethylene (25,28), teflon (29), or dentin tubes (4) have been utilized. Applying the test materials in tubes simulates the clinical conditions (28). When compared with the direct application of the material, this method helps to provide stabilization of the material placement and to achieve the standardization of the material-tissue interfaces (28). In this study, polyethylene tubes with 1.1 mm inner diameter were used. The reactions to empty tubes in this study were similar to others (25,28,30), who found polyethylene tubes caused few or no reactions in subcutaneous connective tissues. Researchers reported that there were some inflammation around the tubes until the end of second week, and this inflammatory infiltration subsided after the third week (25,30). This reaction was the result of the trauma produced during the placement of tubes (30). In the present study MTA-Angelus manufactured in Brazil was chosen because it presents a similar composition to ProRoot MTA according to the manufacturer. Duarte et al.

### Table 1. Distribution of inflammation grades (IG) percentage in groups in five intervals

| Groups         | IG | Intervals (Day) | 7  | 15  | 30  | 60  | 90  |
|----------------|----|----------------|----|-----|-----|-----|-----|
| WMTA/DW        | I  | 0              | 0  | 0   | 42  | 93  |
|                | II | 3              | 0  | 57  | 55  | 7   |
|                | III| 30             | 3  | 51  | 43  | 3   | 0   |
|                | IV | 67             | 49 | 0   | 0   | 0   |
| GMTA/DW        | I  | 0              | 0  | 39  | 72  | 97  |
|                | II | 0              | 0  | 61  | 28  | 3   |
|                | III| 48             | 54 | 0   | 0   | 0   |
|                | IV | 52             | 46 | 0   | 0   | 0   |
| WMTA/CHX       | I  | 0              | 0  | 0   | 16  | 54  |
|                | II | 0              | 5  | 84  | 36  |
|                | III| 38             | 76 | 100 | 0   | 0   |
|                | IV | 62             | 19 | 0   | 0   | 0   |
| GMTA/CHX       | I  | 0              | 0  | 0   | 51  | 99  |
|                | II | 0              | 0  | 78  | 49  | 1   |
|                | III| 46             | 94 | 22  | 0   | 0   |
|                | IV | 54             | 6  | 0   | 0   | 0   |
| Control        | I  | 0              | 0  | 63  | 82  | 100 |
|                | II | 8              | 36 | 18  | 0   |
|                | III| 92             | 91 | 1   | 0   | 0   |
|                | IV | 0              | 0  | 0   | 0   | 0   |

have demonstrated that both materials released calcium and provide alkaline environment. Moreover, when used in direct pulp capping or pulpotomy, both materials were biocompatible and effective to produce complete pulp healing (31). Menezes et al. also showed that the tissue reactions were identical for ProRoot MTA and MTA-Angelus (30). Xavier et al. showed that MTA-Angelus presented the best marginal adaptation in comparison with super EBA and vitremer (32). The toxic effects of white or GMTA in mixture with CHX 0.12% or DW were examined at 7, 15, 30, 60 and 90 days (20,21). When assessing the biocompatibility of a material, the delayed detrimental effects were considered to be more important that its initial effects (28). Seven-day results of both materials showed that moderate inflammatory response developed in subcutaneous connective tissues of rats, but these reactions subsided by the 60th day and were further reduced on the 90th day. There are further studies that support these findings demonstrating fibrous connective tissue formation around MTA and amalgam (28); which indicates that these materials are well tolerated by tissues. Interestingly we showed that MTA (white or gray) in combination with
Table 2. Result of statistically analysis between groups in 5 intervals. (mean inflammation grades± standard deviation)

| Interval | Groups    | 7-day     | 15-day     | 30-day     | 60-day     | 90-day     | P value |
|----------|-----------|-----------|------------|------------|------------|------------|---------|
|          | WMTA/DW   | 3.54±0.50 | 3.06±0.23  | 2.22±0.41  | 1.49±0.50  | 1.01±0.10  | <0.001  |
|          | GMTA/DW   | 3.48±0.50 | 3.06±0.67  | 1.61±0.49  | 1.29±0.47  | 1.03±0.17  | <0.001  |
|          | WMTA/CHX  | 3.62±0.48 | 3.14±0.47  | 3±0.00     | 1.84±0.36  | 1.56±0.67  | <0.001  |
|          | WMTA/CHX  | 3.64±0.54 | 3.24±0.43  | 2.43±0.49  | 1.61±0.54  | 1.07±0.25  | <0.001  |
|          | Control   | 2.92±0.28 | 2.91±0.28  | 1.18±0.38  | 1.38±0.38  | 1±0.00     | <0.001  |

Figure 1. Mean inflammation in experimental and control groups in different intervals.

CHX or DW, could decrease the mean inflammatory response significantly from 7 to 15 days, while the empty tubes (control group) could not. This adds further weight to the argument that MTA may be used as a biocompatible material with CHX and DW to reduce inflammatory response particularly in early tissue contact.

Moretten et al. examined the biocompatibility of MTA by subcutaneous and intra-osseous implantation (33). MTA initially elicited severe reactions with coagulation necrosis and dystrophic calcification; the reactions however subsided gradually to a moderate level. The subcutaneous implantation results of our study concur with Moretten et al. (33).

The mean inflammatory score reported in our study differ from those of Yaltirik et al. (28); this may be due to differences in scoring microscopic evaluations, which ranged from 0 to 3 in Yaltirik et al.’s study (28) (1 to 4 in our study). In addition we reported the mean inflammatory scores obtained from the sum of cells which were counted in 10 separate areas. The average value was not rounded and was precisely reported, which is more accurate than the overall mean value. This may be why our results showed significant difference in 15, 30 and 60 days.

Tissue reaction to empty tubes in all intervals was milder than other experimental groups according to our results. On the 60th day no significant difference were present between control and GMTA/DW group; none of the studied groups except for WMTA/CHX showed statistically significant difference in inflammatory response in 90 days. This is consistent with the findings of Yaltirik et al. (28).

Our findings showed that white and gray MTA mixed with DW had significantly different tissue reactions in all intervals except for the 90-day interval. This can be the result of different chemical properties of the two materials. Asgary et al. showed that the major disparity is FeO, compound being omitted from the WMTA formulation (34). It also has more bismuth oxide than GMTA (34). Yamamoto et al. demonstrated that this oxide can cause toxic effects and have negative effects of cell growth.
Perez et al. showed that osteoblasts are more sensitive to WMTA rather than GMTA and cells attached to WMTA were not viable. Furthermore, the various tissue reactions of these two materials may be due to the difference in their surface roughness and topographies. Matt et al. also showed that GMTA had more sealing ability than WMTA to be used as a root end filling material. Furthermore, the various tissue reactions of these two materials may be due to the difference in their surface roughness and topographies. Matt et al. also showed that GMTA had more sealing ability than WMTA to be used as a root end filling material. The mixture of MTA/CHX has been studied in previous in vitro studies. Stowe et al. showed that CHX improved antibacterial activity of MTA. Hernandez et al. showed that WMTA could induce apoptosis of macrophages and fibroblasts; however, in vitro studies are fundamentally different from in vivo ones, as proteins, tissue fluid and other factors can reduce the toxic effects of materials. Sauthard et al. have demonstrated biocompatibility of CHX. Other researchers have reported similar results, but it is not known why this material acts differently when mixed with GMTA and WMTA. In our study CHX showed good biocompatibility with GMTA but not with WMTA; this may be due to the physical and chemical properties of the materials, yet the precise reasons are still obscure.

CONCLUSION

According to results of this in vivo study, CHX can be a good substitute for DW in mixture with GMTA; however, WMTA elicits more inflammatory response in combination with CHX. CHX has no negative effects on MTA-dentin bonding; more studies about physical and chemical properties of MTA/CHX mixture are needed.

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Conflict of Interest: ‘None declared’.

REFERENCES

1. Koh ET, McDonald F, Pitt Ford TR, Torabinejad M. Cellular response to Mineral Trioxide Aggregate. J Endod. 1998;24:543-7.
2. Torabinejad M, Hong CU, Lee SJ, Monsef M, Pitt Ford TR. Investigation of mineral trioxide aggregate for root-end filling in dogs. J Endod. 1995;21:603-8.
3. Ford TR, Torabinejad M, McKendry DJ, Hong CU, Kariyawasam SP. Use of mineral trioxide aggregate for repair of furcal perforations. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1995;79:756-63.
4. Holland R, de Souza V, Nery MJ, Otoboni Filho JA, Bernabé PF, Dezan Júni E. Reaction of rat connective tissue to implanted dentin tubes filled with mineral trioxide aggregate or calcium hydroxide. J Endod. 1999;25:161-6.
5. Torabinejad M, Chivian N. Clinical applications of mineral trioxide aggregate. J Endod. 1999;25:197-205.
6. Hayashi M, Shimizu A, Ebisu S. MTA for obturation of mandibular central incisors with open apices: case report. J Endod. 2004;30:120-2.
7. Torabinejad M, Ford TR, Abedi HR, Kariyawasam SP, Tang HM. Tissue reaction to implanted root-end filling materials in the tibia and mandible of guinea pigs. J Endod. 1998;24:468-71.
8. Cohen S, Burns RC. Pathways of the Pulp, 8th Edition. St Louis, Missouri, Inc: Mosby; 2002. pp. 917-26.
9. Torabinejad M, Hong CU, McDonald F, Pitt Ford TR. Physical and chemical properties of a new root-end filling material. J Endod. 1995;21:349-53.
10. Kettering JD, Torabinejad M. Investigation of mutagenicity of mineral trioxide aggregate and other commonly used root-end filling materials. J Endod. 1995;21:537-42.
11. Stowe TJ, Sedgley CM, Stowe B, Fenno JC. The effects of chlorhexidine gluconate (0.12%) on the antimicrobial properties of tooth-colored ProRoot mineral trioxide aggregate. J Endod. 2004;30:429-31.
12. Mah T, Basrani B, Santos JM, Pascon EA, Tjäderhane L, Yared G, Lawrence HP, Friedman S. Periapical inflammation affecting coronally-inoculated dog teeth with root fillings augmented by white MTA orifice plugs. J Endod. 2003;29:442-6.
13. Holland R, Souza V, Nery MJ, Faraco Júnior IM, Bernabé PF, Otoboni Filho JA, Dezan Júni E. Reaction of rat connective tissue to implanted dentin tubes filled with a white mineral trioxide aggregate. Braz Dent J. 2002;13:23-6.
14. Salako N, Joseph B, Ritwik P, Salonen J, John P, Junaid TA. Comparison of bioactive glass, mineral trioxide aggregate, ferric sulfate, and formocresol as pulpotomy agents in rat molar. Dent Traumatol. 2003;19:314-20.
15. Sarkar NK, Caicedo R, Ritwik P, Moiseyeva R, Kawashima I. Physicochemical basis of the biologic properties of mineral trioxide aggregate. J Endod. 2005;31:97-100.
16. Jeffcoat MK, Bray KS, Ciancio SG, Dentino AR, Fine DH, Gordon JM, Gunsolley JC, Killoy WJ, Lowengurt RA, Magnusson NJ, Odenbacher S, Palcanis KG, Proskin HM, Finkelman RD, Flashner M. Adjunctive use of a subgingival controlled-release chlorhexidine chip reduces probing depth and improves attachment level compared with scaling and root planning alone. J Periodontol. 1998;69:989-97.

17. Hauman CH, Love RM. Biocompatibility of dental materials used in contemporary endodontic therapy: a review. Part 1. Intracanal drugs and substances. Int Endod J. 2003;36:75-85.

18. Hernandez EP, Botero TM, Mantellini MG, McDonald NJ, Nör JE. Effect of ProRoot MTA mixed with chlorhexidine on apoptosis and cell cycle of fibroblasts and macrophages in vitro. Int Endod J. 2005;38:137-43.

19. Lloyd M. The ethics of using animals in experiments. In: Wolfen S, Lloyd M, editors. Handbook of laboratory animal Management and Welfare, 3rd Edition. Berlin; Blackwell; 2003. pp. 33–7.

20. N Y: 1979. American National Standards Institute/Revised American National Standards Institute American Dental Association Document No. 41 For recommended standard practices for biological evaluation of dental materials, Am National Standards Institute.

21. Recommended standard practices for biological evaluation of dental materials. Fédération Dentaire International, Commission of Dental Materials, Instruments, Equipment and Therapeutics. Int Dent J. 1980;30:140-88.

22. Zmener O, Guglielmotti MB, Cabrini RL. Biocompatibility of two calcium hydroxide-based endodontic sealers: a quantitative study in the subcutaneous connective tissue of the rat. J Endod. 1988;14:229-35.

23. Zmener O, Guglielmotti MB, Cabrini RL. Tissue response to an experimental calcium hydroxide-based endodontic sealer: a quantitative study in subcutaneous connective tissue of the rat. Endod Dent Traumatol. 1990;6:66-72.

24. Yesilsoy C, Koren LZ, Morse DR, Kobayashi C. A comparative tissue toxicity evaluation of established and newer root canal sealers. Oral Surg Oral Med Oral Pathol. 1988;65:459-67.

25. Torneck CD. Reaction of rat connective tissue to polyethylene tube implants. I. Oral Surg Oral Med Oral Pathol. 1966;21:379-87.

26. Marcotte LR, Dowson J, Rowe NH. Apical healing with retrofilling materials amalgam and gutta-percha. J Endod. 1975;1:63-5.

27. Maher WP, Johnson RL, Hess J, Steiman HR. Biocompatibility of retrograde filling materials in the ferret canine. Amalgam and IRM. Oral Surg Oral Med Oral Pathol. 1992;73:738-45.

28. Yaltirik M, Ozbas H, Bilgic B, Issever H. Reactions of connective tissue to mineral trioxide aggregate and amalgam. J Endod. 2004;30:95-9.

29. Olsson B, Sliwkowski A, Langeland K. Subcutaneous implantation for the biological evaluation of endodontic materials. J Endod. 1981;7:355-67.

30. Makkes PC, van Velzen SK, Wesselinck PR, de Greeve PC. Polyethylene tubes as a model for the root canal. Oral Surg Oral Med Oral Pathol. 1977;44:293-300.

31. Duarte MA, Demarchi AC, Yamashita JC, Kuga MC, Fraga Sde C. PH and calcium ion release of 2 root-end filling materials. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2003;95:345-7.

32. Xavier CB, Weismann R, de Oliveira MG, Demarco FF, Pozza DH. Root-end filling materials: apical microleakage and marginal adaptation. J Endod. 2005;31:539-42.

33. Moreton TR, Brown CE Jr, Legan JJ, Kafrawy AH. Tissue reactions after subcutaneous and intraosseous implantation of mineral trioxide aggregate and ethoxybenzoic acid cement. J Biomed Mater Res. 2000;52:52-33.

34. Asgary S, Parirokh M, Eghbal MJ, Brink F. Chemical differences between white and gray mineral trioxide aggregate. J Endod. 2005;31:101-3.

35. Yamamoto A, Homma R, Sumita M. Cytotoxicity evaluation of 43 metal salts using murine fibroblasts and osteoblastic cells. J Biomed Mater Res. 1998;39:331-40.

36. Pérez AL, Spears R, Gutmann JL, Opperman LA. Osteoblasts and MG-63 osteosarcoma cells behave differently when in contact with ProRoot MTA and White MTA. Int Endod J. 2003;36:564-70.

37. Matt GD, Thorpe JR, Strother JM, McClanahan SB. Comparative study of white and gray mineral trioxide aggregate (MTA) simulating a one- or two-step apical barrier technique. J Endod. 2004;30:876-9.

38. Southard SR, Drisko CL, Killoy WJ, Cobb CM, Tira DE. The effect of 2% chlorhexidine digluconate irrigation on clinical parameters and the level of Bacteroides gingivalis in periodontal pockets. J Periodontol. 1989;60:302-9.

39. Loe H, Schiott CR. The effect of mouthrinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man. J Periodontal Res. 1970;5:79-83.

40. Brennan SS, Foster ME, Leaper DJ. Antiseptic toxicity in wounds healing by secondary intention. J Hosp Infect. 1986;8:263-7.

41. Sanchez IR, Swaim SF, Nusbaum KE, Hale AS, Henderson RA, McGuire JA. Effects of chlorhexidine diacetate and povidone-iodine on wound healing in dogs. Vet Surg. 1988;17:291-5.

42. Yan P, Peng B, Fan B, Fan M, Bian Z. The effects of sodium hypochlorite (5.25%), Chlorhexidine (2%), and Glyde File Prep on the bond strength of MTA-dentin. J Endod. 2006;32:58-60.