

## Effects of stabilized chlorine dioxide and chlorhexidene mouthrinses in vitro on cells involved in periodontal healing

Clinicians may elect to prescribe the use of an antimicrobial mouthrinse as an adjunct to manual oral hygiene procedures and after oral surgical procedures when physical oral hygiene may be limited by surgical dressings, sutures, and tender gingival tissues. Medicated mouthrinses might improve taste, odor, and comfort for patients during the postsurgical period. The problem for the clinician is balancing the need for plaque suppression and a possible detrimental effect on healing tissues, especially for regenerative procedures. Sluss<sup>1</sup> stated the problem, "Antisepsis, whatever the agent employed, must always be regulated by two cardinal principles: the maximum germicidal efficiency; the minimum injury to the tissue cells." His answer to the problem was Dakin's solution. Prepared from sodium carbonate, chlorinate lime, and boric acid, Dakin's was a mild 0.5% neutral sodium hypochlorite. The Carrel-Dakin treatment used Dakin's solution extensively in World War I and achieved a remarkable improvement in saving life and limb.<sup>2</sup>

Chlorine dioxide (ClO<sub>2</sub>) is a molecular free radical found in chlorite solutions.<sup>3</sup> Its antimicrobial activity may be by inhibiting protein synthesis.<sup>4</sup> It oxidizes methionine, removing it from use in the lead triplet of bacterial messenger RNA.<sup>5</sup> Ridenour et al. reported its effectiveness against polio virus, *E. coli* and other water pathogens, and spores.<sup>6-8</sup> Ratcliff and Bolin reported its effectiveness on suspensions of periodontal pathogens.<sup>9</sup> As an active ion, ClO<sub>2</sub> offers little chance that any microorganism could develop resistance.

ClO<sub>2</sub> has been used in community water systems to maintain low counts of bacteria and to remove, by oxidation, the bad tastes, colors, and odors found in some lake and river sources.<sup>10</sup> In water treatments, it has an advantage over chlorine in that it generates no trihalomethanes or haloacetic acids that might be carcinogenic.<sup>11</sup> ClO<sub>2</sub> has been found useful in control of biofilms in food preparation,<sup>12</sup> paper processing,<sup>13</sup> and irrigation water systems.<sup>14</sup> ClO<sub>2</sub> has benefit in control of waterline contamination in dental units<sup>15,16</sup> and hospital water supplies.<sup>17</sup>

ClO<sub>2</sub> and chlorite ingestion has been studied in humans. In prospective clinical trials, Lubbers et al. found no physiological problems in 10 men who drank chlorine dioxide solutions for 12 weeks. They had no relevant alterations of vital signs, serum clinical chemistry, thyroxine levels, or hematological parameters.<sup>18-20</sup> In a companion study, three men with glucose-6-phosphate deficiency had no alterations after an additional 8 weeks.<sup>21</sup> In a community-based prospective study, Michael<sup>22</sup> found no hematological or serum chemistry exposure-related effects in 198 persons, compared with 118 non-exposed controls. Tuthill et al. reviewed infant health and found no effect on fetal, neonatal, or infant mortality, nor on proportion of premature infants.<sup>23</sup> Kanitz<sup>24</sup> followed births for 2 years and found no statistically significant difference from controls for low birth weight or preterm delivery. A review of toxicity found no known risk in using ClO<sub>2</sub>-treated water in dialysis units.<sup>25</sup>

There are a few reports on use of ClO<sub>2</sub> for plaque control or gingivitis therapy. A freshly mixed form of ClO<sub>2</sub> had some beneficial effects, but its pH was too low for comfort or continued use.<sup>26</sup> A phosphate buffer-stabilized ClO<sub>2</sub> in open-label use had the benefits of reduced bleeding and probing depths.<sup>27,28</sup> There is no information on use in postsurgical situations and very little information on stabilized ClO<sub>2</sub>

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The authors have no financial relationship to the manufacturers of any products used or mentioned in this article.

and its effect on cells. Tests on equine dermal fibroblasts compared a freshly mixed chlorous acid-chlorine dioxide solution with chlorhexidine (CHX).<sup>29</sup> All concentrations of CHX were toxic to the fibroblasts, and concentrations of 0.05% and greater were lethal, as determined by Trypan blue exclusion. The chlorous acid product diluted 10:1 was not toxic, but when used full strength or diluted 1:1, was lethal. Companion tests against *Staphylococcus aureus* found that only undiluted chlorous acid-chlorine dioxide and CHX at 0.5% and greater strengths were effective.<sup>29</sup> That chlorous acid-chlorine dioxide solution had a low pH of about 3-4; there are no reports concerning cell toxicity by a neutral, buffer-stabilized ClO<sub>2</sub>.

The aim of the present study was to determine the in vitro effects of a buffer-stabilized chlorine dioxide mouthrinse on cultures of cells involved in periodontal wound healing. Assessment was both by quantitative methods of measuring tritiated thymidine DNA labeling as an indirect parameter of cell proliferation and by a colorimetric analysis for quantification of cell death and cell lysis, based on the measurement of lactate dehydrogenase activity released from the cytoplasm of damaged cells into the supernatant.

### Methods and Materials

Human gingival fibroblasts (HGF) were isolated from a specimen taken from the gingiva of a healthy adult patient. Primary cultures of fibroblasts were prepared and grown in Dulbecco's modified Eagle medium (DMEM)+10% fetal calf serum (FCS). The doubling time for these cells was 48 hours. Periodontal ligament cells were isolated from the root surface of a healthy, freshly extracted third molar and were grown in DMEM+10% FCS. These cells grew slower than HGF and had a doubling time of 84 hours. Osteoblasts were obtained from an available mouse cell line (MC3T3-E1), established by sequential collagenase digestion of C57BL/6 embryo/fetal calvaria. They had a doubling time of 48 hours. Osteoblasts were grown in MEM+10% FCS to confluence, trypsinized, and counted in a hemocytometer to adjust cell density for the assays.

Commercially available ClO<sub>2</sub> mouthwash\*, a 0.1% ClO<sub>2</sub>-0.2% phosphate buffer in deionized water, was used full strength and in six 50% serial dilutions combined with the assay medium immediately prior to use to ensure freshness: 0.10, 0.050, 0.025, 0.0125, 0.00625, and 0.00313%. As a control, commercially available CHX mouthwash†, 0.12% chlorhexidine gluconate in 11.6% alcohol, was used full strength and in dilutions (0.12, 0.060, 0.030, 0.015, 0.0075, and 0.00375%). Unchallenged cells were also used.

All cell lines were grown to confluence, released with trypsin, counted, re-suspended in culture media, and divided into 96-well microtiter plates. They were incubated with the test concentrations for 30 minutes. To evaluate the effect of the agents on cell proliferation, following the mouthwash challenge cells were washed with culture media and incubated with media supplemented with [<sup>3</sup>H]-thymidine (50-100,000 CPM per plate). Cells were incubated overnight, washed, and lysed in 0.1% sodium dodecyl sulfate, a detergent that breaks up the cell membrane, and the radioactivity was measured in a scintillation counter. All assays were performed in triplicate.

A lactate dehydrogenase assay‡ was performed using the kit manufacturer's instructions for the measurement of the cytotoxic potential of soluble substances by analysis of cytoplasmic enzyme activity released by damaged cells. Lactate dehydrogenase (LDH) is rapidly released into the cell supernatant upon damage of the plasma membrane. A cell concentration of 10<sup>5</sup> cells/ml in the culture media was used in all assays, which employed the 96-well plate format. LDH activity is determined by a coupled reaction whereby nitroreductase (NBT) is reduced to formazan dye and the resultant color is quantitated by reading absorbance at 500 nm. The sensitivity range of this kit is between 3.91 mU/ml and 62.5 mU/ml. All assays were in triplicate.

A multiple-group analysis of variance was used to detect differences between treatment groups. Significance was established at P<0.05.

\* CloSYS II, Rowpar Pharmaceuticals, Inc., Scottsdale, AZ 85260.

† Periogard, Colgate Oral Pharmaceuticals, Inc., Canton, MA 02021.

‡ Roche Cytotoxicity Detection Kit (LDH), Roche Applied Science, Indianapolis, IN 46250

## Results

In the tritiated thymidine uptake assays, ClO<sub>2</sub> caused significant cell death at 0.1 and 0.05%, but at lesser concentrations, cell death was not significantly increased over that in controls. All CHX concentrations caused significant cell death of all cell types tested ( $P < 0.05$ ). At all CHX concentrations except 0.003%, there was total cell death, and at that concentration, cell death was significantly increased over that in unchallenged controls ( $P < 0.05$ ). These results were consistent for all three cell lines – HGF, periodontal ligament, and osteoblasts.

LDH release from HGF was not different from that in controls at any concentration of ClO<sub>2</sub>. CHX at 0.12 to 0.015% showed significantly more LDH release than ClO<sub>2</sub> or controls ( $P < 0.05$ ). For the periodontal ligament cell line, ClO<sub>2</sub> at all concentrations did not show any significant increase in LDH release over controls, however, CHX at 0.12 to 0.03% had significantly more LDH release than ClO<sub>2</sub> and controls ( $P < 0.05$ ). Osteoblasts did not show any significant increase in LDH after challenge by ClO<sub>2</sub>. CHX at 0.12 to 0.015% showed significantly more LDH release by osteoblasts than ClO<sub>2</sub> and controls ( $P < 0.05$ ).

## Discussion

Chlorine dioxide as a buffer-stabilized mouthwash had a minimal effect on HGF, periodontal ligament cells, and osteoblasts. Only at the highest concentrations did ClO<sub>2</sub> have an effect on cell proliferation, and there was no significant effect on LDH release in any cells at any concentrations. The buffer-stabilized ClO<sub>2</sub> mouthwash is active against volatile sulfur compounds, making it a good choice for halitosis<sup>30,31</sup> and possibly in periodontal therapy. While little research has been done with this formulation, its antibacterial activity and low toxicity suggest it would be an effective postsurgical lavage.

Chlorhexidine mouthwashes are currently popular as adjuncts to oral hygiene and plaque control. CHX antimicrobial activity *in vitro* is the disruption of bacterial cell membranes and precipitation of their cytoplasm.<sup>32</sup> Its virucidal effect is through inactivation of DNA polymerase.<sup>33</sup> CHX binds carbonyl, sulfate, and phosphate groups, and so may impede bacterial adsorption.<sup>34</sup> The *in vivo* activity of CHX is seen as clumped bacterial cytoplasm; but what might it do to cells of the host?

CHX has adversely affected different cultures of cells found in periodontal tissues. CHX applied to root surfaces decreased HGF attachment.<sup>35</sup> CHX at 0.001% inhibits [<sup>3</sup>H]-leucine incorporation into protein by HGF<sup>36</sup> and has caused [<sup>51</sup>Cr] release from HGF, foreskin fibroblasts, and HeLa cells.<sup>37</sup> CHX at 2.0% caused hamster cheek pouch epithelial hyperkeratosis, some hyperchromasia, and rete extensions.<sup>38</sup> CHX affected lipid order packing in buccal epithelial cell membranes.<sup>39</sup> CHX at 0.1 mM was lethal to human skin epithelial cells.<sup>40</sup> CHX at 0.02% was toxic to HeLa cells in culture.<sup>41</sup> At 1 mM, CHX lysed human red blood cells.<sup>42</sup> At 0.02%, CHX caused polymorphonuclear leukocyte (PMN) cell damage and inhibited the PMN cell's chemotactic response to fMLP.<sup>43</sup> CHX at 0.2% impeded *in vitro* migration of PMN.<sup>44</sup> Macrophages have shown cytotoxicity by trypan blue uptake at 0.005% CHX.<sup>45</sup>

CHX has affected hamster cheek pouch microcirculation, causing stasis.<sup>46</sup> CHX at 0.02 and 0.12% has reduced the rupture strength of rat skin wounds.<sup>47</sup> CHX used postsurgically on open wounds of the palate inhibited wound closure, resulting in more granulation tissue, a lack of epithelial closure, and a detrimental effect on bone.<sup>48</sup> CHX injected into cellulose sponges implanted subcutaneously in the backs of rats caused an increase in hyaluronic acid, but a delay in collagen formation.<sup>49</sup> When open skin wounds in dogs were lavaged with CHX or lactated Ringers' solution as a control for 24 days, clinically and histologically there were no reported differences.<sup>50</sup> CHX at 0.05% in lactated Ringers' solution was used as a lavage for tarsocrural joints in six horses and caused lameness, effusion, edema, soreness, bloody synovial fluid, synovial ulceration, inflammation, and an increase in macrophage and PMNs that was not resolved in 8 days.<sup>51</sup> Using 0.0005% CHX and EDTA in a Tris buffer as a lavage for tarsocrural joints in horses caused lameness, joint effusion, and bloody synovial fluid that were cleared in 8 days.<sup>52</sup>

Our study confirms the foregoing reports that CHX is toxic to human cells pertinent to periodontal wound healing and includes cultured bone cells not explicitly tested before. There are clinical reports of CHX mouthrinses used postsurgically in humans<sup>53,54</sup> and dogs<sup>55</sup> for control of plaque that describe good results. However, in cases of regenerative procedures where CHX might wick into subgingival areas wherein CHX could destroy the friable new granulations from the periodontal ligament or bone, there could be a risk. One might consider not using CHX until a new epithelial attachment seal has formed. That is usually at 6 to 7 days of healing in a periodontal flap surgery; but after a guided tissue regeneration procedure, there may be no seal until the barrier is removed.

Stabilized chlorine dioxide could be safer on cells during periodontal wound healing. ClO<sub>2</sub> contains no alcohol, as does CHX, which may be why CHX sometimes causes oral mucosal lesions.<sup>56</sup> ClO<sub>2</sub> does not cause staining of teeth, restorations, or soft tissues, as does CHX.<sup>57</sup> ClO<sub>2</sub> does not cause calculus formation as reported for CHX. ClO<sub>2</sub> has not been reported to affect taste or to cause allergic reactions, which have been associated with CHX.<sup>58,59</sup> Unlike with CHX,<sup>60</sup> ClO<sub>2</sub> free radical ions do not allow resistant species to develop. Stabilized chlorine dioxide mouthrinse is less toxic than chlorhexidine to human gingival fibroblasts, periodontal ligament cells, and an osteoblast cell line in vitro. In vitro tests on cell monolayers may not be fully applicable to therapy, so the clinical significance of the findings remains to be explored.

## Conclusions

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