Cytotoxicity of Direct Current with Antibacterial Agents against Host Cells In Vitro

Yuko Nakamura, DDS, Keiso Takahashi, DDS, PhD, Akiko Shimetani, DDS, Hiroshi Sakagami, PhD,†† and Hirofumi Nishikawa, DDS, PhD

Abstract

The purpose of this study was to investigate the cytotoxicity of iontophoresis treatment using direct current (DC) with or without antibacterial agents. The following antibacterial agents were used: diamine silver fluoride (AgF); sodium fluoride (NaF); and iodine zinc iodide (JJZ). The cytotoxic activity of DC with or without antibacterial agents against human polymorphonuclear cells (PMNs) was evaluated by the 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. It was noted that DC (2 mA) killed PMNs in a time-dependent manner and the cytotoxicity was enhanced when DC was combined with antibacterial agents. The toxic effect of antibacterial agents was in the order: AgF > JJZ > NaF. The death of PMNs by DC was evaluated by flow cytometry using annexin V-FITC/propidium iodide staining. DC appeared to induce necrosis rather than apoptosis of PMNs. These results suggest that iontophoresis treatment using DC and antibacterial agents may induce necrotic cytotoxicity in host cells around periapical lesions.

Key Words

Iontophoresis, direct current, antibacterial agents, cytotoxicity, necrosis

From the *Department of Endodontics and ††Department of Dental Pharmacology, Meikai University School of Dentistry, Saitama, Japan.

Address requests for reprints to Dr. Keiso Takahashi, Department of Endodontics, Meikai University School of Dentistry, Keyaki-dai 1-1, Sakado 350-0283, Japan. E-mail address: okeiso@dent.meikai.ac.jp.

The mechanical debridement and instrumentation of root canal system are essential for the reduction of intracanal infection. In addition, intracanal medicaments are still used in some cases because of the difficulties eradicating microorganisms from dentinal tubes by instrumentation alone (1).

For similar reasons, iontophoresis with antibacterial agents has been used as an adjunct tool for endodontic therapy (2) and as a desensitizing procedure for hypersensitivity (3). In general, iontophoresis is a drug delivery method that uses electric current to drive water-soluble ionized drugs and this method can deliver drugs locally at concentrations sufficiently enough to provide therapeutic effects (4). For example, iontophoresis of dexamethasone is routinely used for the treatment and rehabilitation of patients with various inflammatory conditions (5, 6).

Antibacterial agents, when applied to infected root canals by iontophoresis, pass through the main and lateral canals, and finally reach the dentinal tubes and the surface of tooth root. The effectiveness of iodide-potassium iodide and diamine silver fluoride has been reported (7, 8) and they appear to have improved therapeutic management of apical periodontitis. In addition, antibacterial effects of several agents, such as iodide-potassium iodide, iodine-iodate and sodium fluoride, have been investigated (9–12). The clinical effectiveness of these combinations is still controversial because of the difficulty in determining the combinatorial antibacterial and cytotoxic effects. In fact, many patients experience postoperative pain and prolonged inflammation following this treatment.

It is feasible that direct current (DC) and antibacterial agents have adverse effects on periapical lesions. Neutrophils play a crucial role in pulpal and periapical inflammation (13, 14). PMNs contain a lot of granules which can damage the inflammation-inducing agents such as bacteria, but are potentially cytotoxic and may amplify the inflammatory response by enzymatic cleavage of matrix proteins (15). Degradation of PMNs in inflamed sites and the release of superoxide and proteases to the extracellular environment also induces tissue damage. To assess these biological effects, we determined the cytotoxic activity of DC and antibacterial agents used for iontophoresis singly and in combination, against host cells in vitro, using human peripheral blood polymorphonuclear cells (PMNs) as a model system.

Materials and Methods

Reagents

The following three iontophoresis agents were purchased from the indicated companies: diamine silver fluoride solution (AgF) (Saforaide Toyo Pharmaceutical Ind. Co., Osaka, Japan): sodium fluoride (NaF) (Sigma Chemical Co., NY): iodine zinc iodide solution (JJZ) (Showa Pharmaceutical Ind. Co., Tokyo, Japan). Various concentrations of fluoride were added to the cultures as an aqueous NaF solution. Phosphate-buffered saline (PBS) was added to the control cultures. The three electrodes, stainless steel (Dentsply-Sankin, Tokyo, Japan), Zn, and silver (Showa Pharmaceutical Ind. Co.) were used during electrophoresis for NaF, AgF, and JJZ, respectively.

Preparation of peripheral polymorphonuclear leukocytes (PMNs)

Peripheral blood PMNs were isolated by Mono-Poly density gradient centrifugation of heparinized venous blood, as previously described (16). The red cells in the PMNs preparation were lysed by hypotonic shock for 30 s (17). This procedure yielded a PMN population of >99% viability and >98% purity, as judged by trypan blue dye

exclusion and cytospined staining with Diff-Qick (Sysmex, Kobe, Japan), respectively. The cells were resuspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS, GIBCO, Grand Island, NY) and subjected to electric stimulus and/or antibacterial agents for different times at 25°C.

Treatment of DC

The PMNs were suspended at 2×10^6/ml in RPMI 1640 medium supplemented with 10% heat inactivated FBS in the 24-well culture plate (Falcon 3047, Becton Dickinson, Franklin Lakes, NJ), and subjected to direct current (2 mA) for the indicated periods with occasional agitation (Fig. 1*A*). Two hundred μl of the cell suspensions were transferred to the 96-microwell culture plate (Falcon 3072, Becton Dickinson) for assessing the cytotoxic activity of the DC and antibacterial agents by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay described below. The three antibacterial agents and three electrodes (Zn, silver, stainless) were used in the following combinations: Zn + JJZ; silver + AgF; and stainless + NaF.

Assessment of Cytotoxicity by MTT Assay

Cytotoxic activity of DC was evaluated by MTT assay using cell counting kit as described previously (18). In brief, DC-treated PMNs were seeded at a concentration of 4×10^5 cells/200 ml/well in the 96-microwell culture plate (Falcon, Becton Dickinson) and treated with antibacterial agents. After 1 h culture, MTT was added and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 3 h. Viable PMNs generate insoluble crystal, the absorbance of which was measured at 450 nm by using microplate reader (Dainippon pharmaceutical Co., Laboratory Products, Osaka, Japan).

Flow Cytometric Analysis for Cell Death

Cell death of DC-treated PMNs was also evaluated by flow cytometry. The PMNs were stained with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit (Immunotech, Beckman Coulter, Marseille, France). Briefly, PMNs were treated by DC for indicated periods (1 or 5 min) as described in Fig. 1*A* and then stained with 2 μl/sample annexin V-FITC and PI for measuring apoptosis and necrosis of the cells. After staining, cells were analyzed with FACS flow cytometer (EPICS System II; Coulter) and EXPO 32 soft ware, according to the manufacturer's instructions. For each sample, 20,000 cells in the PMN region were analyzed and gated onto a fluorescence dot plot, where the fraction of total annexin V and/or PI-positive cells was determined. Cell death was expressed as a percentage of dead cells in relation to the total number of counted cells. The same conditions for forward/side scatter, fluorescence gains, color compensation and fluorescence threshold were adopted throughout the study. These conditions were determined by assaying unstained and single-stained cells (with annexin V or PI alone) in our preliminary study.

Statistical Analysis

The 50% concentration (C_{50} value) was calculated for each drug two sequential concentrations: one that caused >50%, and the other that caused <50% of the control. In the MTT assay, the result of each experiment was expressed as mean ± SD from three independent wells. Most of data are the representative of three individual experiments with similar results. The absorbance value at each time compared with that of control was calculated. Results were analyzed by ANOVA with the value of statistical significance being set at $p < 0.05$.

Results

The cytotoxic effect of the AgF, JJZ, and NaF without DC was investigated. AgF showed the strongest cytotoxic activity, followed by JJZ and NaF (Fig. 1*B*). The AgF, JJZ and NaF killed the half number of PMN, at the dilution of 10^{-6}, 10^{-4}, and 10^{-2}, respectively. DC (2 mA) with three different electrodes showed cytotoxic effect on PMNs, when treatment time with DC exceeded 1 min (Fig. 1*C*) and this effect of DC was further increased for 5 min. The cytotoxicity of 1 min-DC treatment was en-

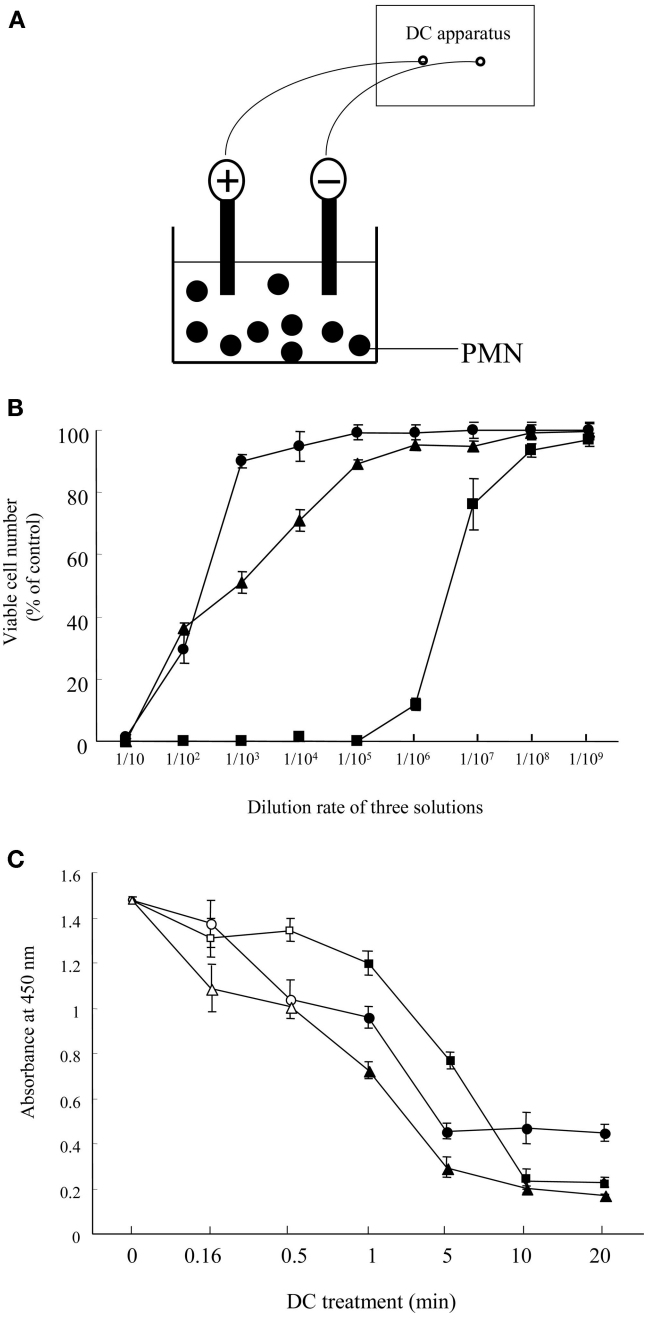

Figure 1. (*A*) Direct current apparatus. PMNs were suspended at 2×10^6/ml in RPMI 1640 medium supplemented with 10% heat inactivated FBS, and treated with DC for the indicated times. (*B*) Concentration-effect curves of AgF, NaF, and JJZ in MTT assay. Three antibacterial agents were serially diluted and their cytotoxicity against PMNs was evaluated by MTT assay and expressed as % of untreated control. ■:AgF, ▲:JJZ, ●:NaF. (*C*) Time course of DC (2 mA)-induced cytotoxicity with three different electrodes. PMNs were treated with DC for the indicated period. *$p < 0.01$, **$p < 0.05$, □ and ■:Ag; △ and ▲:Zn, ○ and ●:St. Filled marks indicate the significant differences from the control (without DC treatment) at $p < 0.05$, while open marks do not.