Brazilian Journal of Medical and Biological Research (2004) 37: 207-212
ISSN 0100-879X

Cytotoxicity of chlorhexidine digluconate to murine macrophages and its effect on hydrogen peroxide and nitric oxide induction

C. Bonacorsi[1], M.S.G. Raddi[2] and I.Z. Carlos[2]

[1]Instituto de Química de Araraquara, Universidade Estadual Paulista "Júlio de Mesquita Filho", Araraquara, SP, Brasil
[2]Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas de Araraquara, Universidade Estadual Paulista "Júlio de Mesquita Filho", Araraquara, SP, Brasil

Abstract

Chlorhexidine, even at low concentrations, is toxic for a variety of eukaryotic cells; however, its effects on host immune cells are not well known. We evaluated *in vitro* chlorhexidine-induced cytotoxicity and its effects on reactive oxygen/nitrogen intermediate induction by murine peritoneal macrophages. Thioglycollate-induced cells were obtained from Swiss mice by peritoneal lavage with 5 ml of 10 mM phosphate-buffered saline, washed twice and resuspended (10^6 cells/ml) in appropriate medium for each test. Cell preparations contained more than 95% macrophages. The cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay and the presence of hydrogen peroxide (H_2O_2) and nitric oxide (NO) by the horseradish peroxidase-dependent oxidation of phenol red and Griess reaction, respectively. The midpoint cytotoxicity values for 1- and 24-h exposures were 61.12 ± 2.46 and 21.22 ± 2.44 µg/ml, respectively. Chlorhexidine did not induce synthesis or liberation of reactive oxygen/nitrogen intermediates. When macrophages were treated with various sub-toxic doses for 1 h (1, 5, 10, and 20 µg/ml) and 24 h (0.5, 1, and 5 µg/ml) and stimulated with 200 nM phorbol myristate acetate (PMA) solution, the H_2O_2 production was not altered; however, the NO production induced by 10 µg/ml lipopolysaccharide (LPS) solution varied from 14.47 ± 1.46 to 22.35 ± 1.94 µmol/l and 13.50 ± 1.42 to 20.44 ± 1.40 µmol/l (N = 5). The results showed that chlorhexidine has no immunostimulating activity and sub-toxic concentrations did not affect the response of macrophages to the soluble stimulus PMA but can interfere with the receptor-dependent stimulus LPS.

Correspondence
I.Z. Carlos
Departamento de Análises Clínicas
Faculdade de Ciências Farmacêuticas
Rua Expedicionários do Brasil, 1621
14801-902 Araraquara, SP
Brasil
Fax: +55-16-222-0073
E-mail: carlosiz@fcfar.unesp.br

Research supported by FAPESP (No. 00/04305-0) and PADC/FCF (No. 2000/4-I).

Received February 27, 2003
Accepted September 16, 2003

Key words
- Chlorhexidine
- Cytotoxicity
- Macrophages
- Nitric oxide
- Hydrogen peroxide

Introduction

Chlorhexidine is an antibacterial agent widely used for skin disinfection, preoperative whole-body disinfection, and prevention and treatment of oral disease because of its broad-spectrum efficacy, retention by the skin and mucosa, and low risk of allergic complications (1). In recent years, vaginal washing with this compound before or during delivery has been suggested to prevent the development of both maternal and neo-

natal infections (2-4). Studies have shown that small amounts of chlorhexidine are absorbed through the skin and vaginal mucosa (3,5) and that, even at low concentrations, this compound has toxic effects on a variety of eukaryotic cells (6). However, these effects on host immune cells are not well known.

Macrophages are widely distributed in different tissues and play an essential role in the development of the specific and nonspecific immune response. These cells can be activated by a variety of stimuli such as bacterial components, cytokines and chemicals. Once activated, macrophages produce and release numerous secretory products including several cytokines, inorganic reactive radicals, reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), all having biological activity (7). Hydrogen peroxide (H_2O_2) and nitric oxide (NO) are important in cell signaling and are effector molecules for the microbicidal and cytotoxic response of macrophages after stimulation (8). If ROI and RNI may be considered as beneficial intermediates (with respect to their microbicidal and tumoricidal activities), they can also become destructive for the host tissue under certain conditions (9). There is insufficient information about the effect of chlorhexidine on macrophage function.

The purposes of the present study were to determine *in vitro* the cytotoxicity of chlorhexidine digluconate to murine peritoneal macrophages and investigate whether this compound induces the production of ROI and RNI as well as to determine if chlorhexidine treatment affects macrophage functions in the presence of stimulation with phorbol myristate acetate (PMA) and lipopolysaccharide (LPS).

Material and Methods

Chemicals

Chlorhexidine digluconate was obtained from Henri Pharma Produtos Farmacêuticos (São Paulo, SP, Brazil). Dextrose, horseradish peroxidase (HRP), LPS, penicillin, phenol red, PMA, RPMI-1640 and streptomycin were purchased from Sigma (St. Louis, MO, USA). Brewer thioglycollate medium was purchased from Difco (Detroit, MI, USA). Fetal bovine serum (FBS) was obtained from Cultilab (Campinas, SP, Brazil). 2-Mercaptoethanol, sodium hydroxide and sodium chloride were purchased from Merck (Darmstadt, Germany).

Animals

Swiss mice (6-8 weeks old, weighing 18 to 25 g), supplied by the Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara, SP, Brazil, were maintained in a polycarbonate box (at 23 ± 1ºC, 55 ± 5% humidity, 10-18 circulations/h and a 12-h light/dark cycle), with water and food available *ad libitum*.

Peritoneal macrophages

Resident and thioglycollate-elicited peritoneal exudate cells were obtained from mice following intraperitoneal injection of 3 ml thioglycollate medium (3.0 g/100 ml) and lavage of the peritoneal cavity with 5 ml of 10 mM phosphate-buffered saline (PBS), pH 7.2, 3-4 days later. The proportion of macrophages in the peritoneal exudate was determined by cell staining with May-Grünwald-Giemsa. Cell preparations contained more than 95% macrophages. The cells were washed twice with PBS and resuspended in appropriate medium for each test.

Cytotoxicity assay

Macrophages (2 x 10^6 cells/ml) were suspended in RPMI-1640 containing 5% heat-inactivated FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 mM 2-mercaptoethanol. The suspension (100 µl) was added

to each well of a 96-well tissue culture plate and the cells were incubated at 37ºC in a humidified atmosphere containing 5% CO_2. After 1 h, the wells were washed and adhering cells exposed to different concentrations of chlorhexidine for 1 and 24 h. The test was accompanied by a viability positive control (medium + cells) and negative control (medium + chlorhexidine). Finally, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (10) was performed and the absorbance at 540 nm determined with a microplate reader (Spectra III Classic, Tecan Austria Ges.m.b.H, Salzburg, Austria.

Hydrogen peroxide

H_2O_2 was measured by the HRP-dependent oxidation of phenol red (11). Macrophages (2 x 10^6 cells/ml) were suspended in 10 mM potassium phosphate buffer containing 140 mM NaCl, 5.5 mM dextrose, 0.56 mM phenol red and 0.01 mg/ml HRP, pH 7.4. Briefly, 100 µl of this suspension was added to each well of a 96-well tissue culture plate and exposed to different concentrations of chlorhexidine for 1 h (time for the H_2O_2 assay) at 37ºC in a 5% CO_2 atmosphere. The reaction was stopped by the addition of 10 µl of 1 N NaOH and the absorbances were read at 620 nm. The results are reported as nmol/ml calculated from a hydrogen peroxide standard curve. The test was also performed with macrophages previously treated with sub-toxic concentrations of chlorhexidine for 1 and 24 h, washed twice with 10 mM potassium phosphate buffer and stimulated with PMA. The experiment was accompanied by a positive control (medium + cells + 200 nM PMA solution) and negative control (medium + cells).

Nitric oxide

NO synthesis was determined by measuring the accumulation of nitrite (NO_2^-), a stable metabolite of NO, in culture supernatants using the Griess reaction (12). Macrophages at 5 x 10^6 cells/ml in RPMI-1640 containing 5% heat-inactivated FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 mM 2-mercaptoethanol were added to each well of a 96-well cell culture plate and exposed to different concentrations of chlorhexidine for 24 h (time for the NO assay) at 37ºC in a 5% CO_2 atmosphere. After incubation, 50-µl aliquots of culture supernatant were mixed with an equal volume of Griess reagent (1% w/v sulfanilamide, 0.1% w/v naphthylethylenediamine and 3% H_3PO_4), and incubated at room temperature for 10 min. Absorbance at 540 nm was measured using a microplate reader. NO_2^- concentration (µmol/l) was calculated from an $NaNO_2$ standard curve. The test was also performed with macrophages previously treated with sub-toxic concentrations of chlorhexidine for 1 and 24 h, washed twice with RPMI-1640 and stimulated with LPS. Each experiment was accompanied by a positive control (medium + cells + 10 µg/ml LPS solution) and a negative control (medium + cells).

Statistical analysis

The midpoint cytotoxicity value, the concentration of chemical agent needed to reduce the spectrophotometric absorbance to 50%, was determined by linear regression analysis with 95% confidence limits. H_2O_2 and NO concentrations are reported as means ± SD. Statistical difference between groups was determined by one-way analysis of variance (ANOVA; $P < 0.05$). All experiments were carried out in triplicate and repeated at least three times.

Results

Effect of chlorhexidine on cell viability

Concentration-response cytotoxicity

curves for 1- and 24-h exposures of macrophages to chlorhexidine are shown in Figure 1. Initial toxicity, i.e., a statistically significant reduction in the viability of chlorhexidine-exposed cells relative to unexposed controls, occurred at >20 µg/ml chlorhexidine with 1-h exposure and at >5 µg/ml with 24-h exposure. Concentrations below these were designated as sub-toxic. The midpoint cytotoxicity values were 61.12 ± 2.46 and 21.22 ± 2.44 µg/ml.

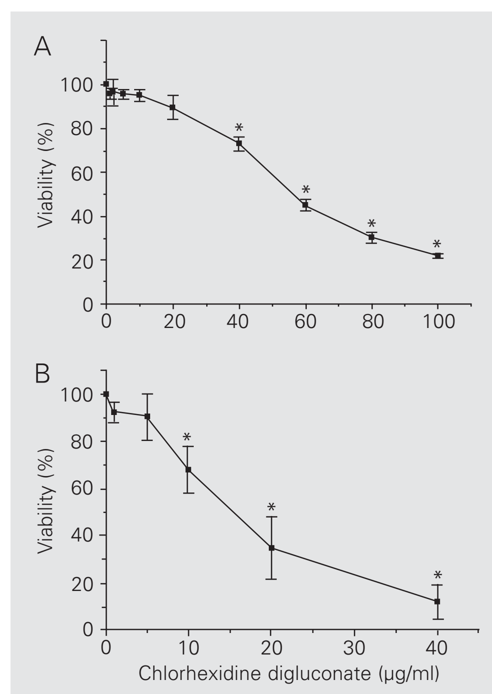

Figure 1. Effect of chlorhexidine on the viability of peritoneal macrophages after 1 h (A) and 24 h (B). Each point and bar represents the mean ± SD for at least three independent experiments carried out in triplicate. *P < 0.05 compared to control (ANOVA).

Effect of chlorhexidine on H_2O_2 and NO induction

To evaluate the ability of chlorhexidine to induce the production and liberation of ROI and RNI, murine macrophages were exposed to various concentrations of the test substance for 1 and 24 h (time for the H_2O_2 and NO assay, respectively). At none of the concentrations tested did chlorhexidine induce the production of H_2O_2 or NO by macrophages (Table 1).

Table 1. H_2O_2 and NO production by peritoneal macrophages stimulated with chlorhexidine.

Chlorhexidine (µg/ml)	After 1 h H_2O_2 (nmol/ml)	After 24 h NO_2^- (µmol/l)
Control	0 ± 0.64	1.21 ± 0.25
1	0.13 ± 0.70	0.61 ± 0.80
10	0.20 ± 0.90	0.57 ± 0.23
60	0.21 ± 0.55	0.43 ± 0.11
100	0.81 ± 0.82	0.52 ± 0.15
PMA	14.40 ± 1.81*	–
LPS	–	37.50 ± 2.85*

Macrophages were exposed to chlorhexidine for 1 and 24 h. Data are reported as the mean ± SD for at least three independent experiments carried out in triplicate. LPS = lipopolysaccharide, PMA = phorbol myristate acetate.
*P < 0.05 compared to control (ANOVA).

Production of H_2O_2 and NO by chlorhexidine-exposed macrophages

To determine the effect of chlorhexidine on H_2O_2 and NO production by macrophages, these cells were treated with sub-toxic concentrations of chlorhexidine for 1 and 24 h and stimulated with both PMA and LPS. Table 2 shows that the H_2O_2 production was not altered in macrophages exposed to chlorhexidine for 1 and 24 h but the NO production induced by LPS was reduced when macrophages were exposed to sub-toxic doses of chlorhexidine (Figure 2).

Discussion

Macrophages have been used for immunocytotoxicity testing because they permit the measurement of the cytotoxic response directly in the cell culture and because of their ability to maintain immunological functions in the presence of many different chemical agents (13). The cytotoxicity of chlorhexidine has been assayed using cell lines or primary cultures of mammalian cells (6); however, there is no specific information on the toxicity of this substance to immune cells. In the present study, we demonstrated that macrophages are as sensitive to the toxic

effects of chlorhexidine as other cell types and that the cytotoxic potential of the substance is dependent on the concentration and time of exposure (14). It is important to note that chlorhexidine digluconate is toxic to macrophages *in vitro* at concentrations 100 times below those used in clinical practice, usually 0.2-0.5% (15).

In response to antigens or inflammatory signals generated at sites of tissue injury, macrophages undergo a process of cellular “activation” which is associated with morphological, functional, and biochemical changes in the cells. One prominent characteristic of activated macrophages is their increased capacity to release pro-inflammatory and cytotoxic mediators, which aid in antigen destruction (16).

The present data show that chlorhexidine does not induce the production of ROI/RNI by macrophages. When macrophages were treated with chlorhexidine (sub-toxic concentrations) for 1 and 24 h and stimulated with PMA there was no alteration in H_2O_2 production. However, NO production induced by LPS was decreased in macrophages previously treated with sub-toxic doses of this substance. These observations are probably relevant to the mechanism underlying chlorhexidine-induced cytotoxicity.

Chlorhexidine may adversely affect the eukaryotic plasma membrane by nonspecific electrostatic binding to negative moieties (protein and phospholipid components), causing alteration in the structure of the cell membrane and in the osmotic equilibrium of the cell (6,14). PMA is a soluble stimulus that acts directly on protein kinase C (present in cytoplasm) activating the production of the superoxide anion, which is subsequently converted to H_2O_2 (17). PMA-induced stimulation can be mediated by specific receptors, and also via insertion into the membrane due to its hydrophobic nature (18). Probably, chlorhexidine does not prevent the entry of PMA into the cell, since this substance also acts by a receptor-independent mechanism.

Several types of receptors have been reported to be present on the macrophage surface. The inhibitory effect of several chemicals (ligands) on NO production induced by LPS would appear to be derived from the inhibition of LPS binding to the receptor through ligand-LPS complex formation or ligands may compete for LPS receptor (19). However, in our study, the reduction in the

Table 2. H_2O_2 production by macrophages treated with chlorhexidine for 1 and 24 h and stimulated with PMA.

Chlorhexidine (µg/ml)	1 h (nmol/ml)	24 h (nmol/ml)
Control	7.73 ± 0.84	5.30 ± 0.34
0.5	–	5.07 ± 0.65
1.0	6.75 ± 0.70	5.55 ± 0.57
5	6.90 ± 0.73	5.32 ± 0.37
10	7.91 ± 1.20	–
20	6.96 ± 1.00	–

Data are reported as the mean ± SD for at least three independent experiments carried out in triplicate.

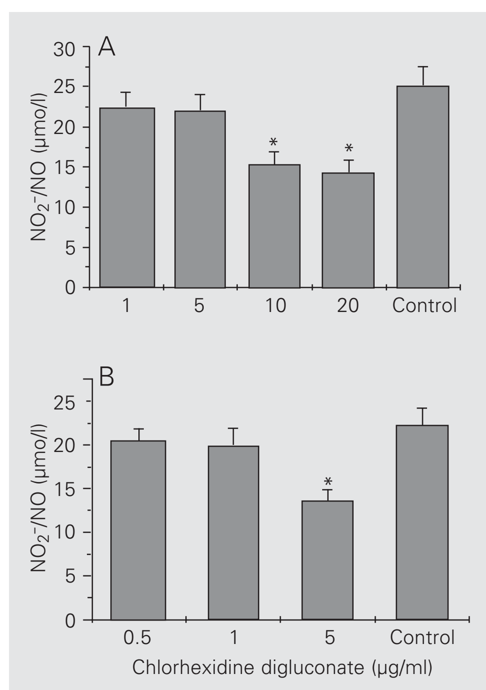

Figure 2. NO production by macrophages treated with chlorhexidine for 1 h (A) and 24 h (B) and stimulated with LPS. Data are reported as the mean ± SD for at least three independent experiments carried out in triplicate. *P < 0.05 compared to control (ANOVA).

NO production by chlorhexidine-treated macrophages is likely the result of the action of this substance on plasma membrane. Chlorhexidine may interact or damage membrane receptors interfering in the binding receptor-LPS.

The products of the oxidative burst are used to kill phagocytic pathogens and also for the extracellular destruction of other cells. Based on the results obtained here we conclude that chlorhexidine does not produce an immunostimulatory effect. However, this substance may have an immunosuppressive effect on exposed macrophages which is related to receptor-dependent particulate stimuli.

References

1. McDonnell G & Russell AD (1999). Antiseptics and disinfectants: activity, action and resistance. *Clinical Microbiology Reviews*, 12: 147-179.
2. Facchinetti F, Piccinini F, Mordini B & Volpe A (2002). Chlorhexidine vaginal flushing versus systemic ampicillin in the prevention of vertical transmission of neonatal group B streptococcus, at term. *Journal of Maternal Fetal Medicine*, 11: 84-88.
3. Nilsson G, Larsson L, Christensen KK, Christensen P & Dykes AK (1989). Chlorhexidine for prevention of neonatal colonization with group B streptococci. V. Chlorhexidine concentrations in blood following vaginal washing during delivery. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 31: 221-226.
4. Stray-Pedersen B, Bergan T, Hafstad A, Normann E, Grogaard J & Vangdal M (1999). Vaginal disinfection with chlorhexidine during childbirth. *International Journal of Antimicrobial Agents*, 12: 245-251.
5. Lafforgue C, Carret L, Falson F, Reverdy ME & Freney J (1997). Percutaneous absorption of a chlorhexidine digluconate solution. *International Journal of Pharmaceutics*, 147: 243-246.
6. Hidalgo E & Dominguez C (2001). Mechanisms underlying chlorhexidine-induced cytotoxicity. *Toxicology in Vitro*, 15: 271-276.
7. Forman HJ & Torres M (2001). Redox signaling in macrophages. *Molecular Aspects of Medicine*, 22: 189-216.
8. Kayser O, Kolodziej H & Kiderlen AF (2000). Immunomodulatory principles of *Pelargonium sidoides*. *Phytotheraphy Research*, 15: 122-126.
9. McBride AG, Borutaité V & Brown GC (1999). Superoxide dismutase and hydrogen peroxide cause rapid nitric oxide breakdown, peroxynitrite production and subsequent cell death. *Biochimica et Biophysica Acta*, 1454: 275-288.
10. Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *Journal of Immunological Methods*, 65: 55-63.
11. Pick E & Mizel D (1981). Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *Journal of Immunological Methods*, 46: 211-226.
12. Green LC, Wagner DA, Glogorwki J, Skipper PL, Wisnok JS & Tannenbowm SR (1982). Analysis of nitrate, nitrite and (^{15}N) nitrate in biological fluids. *Analytical Biochemistry*, 126: 131-138.
13. Barile FA, Dierickx PJ & Kristen U (1994). *In vitro* cytotoxicy testing for prediction of acute human toxicity. *Cell Biology and Toxicology*, 10: 155-162.
14. Babich H, Wurzburger BJ, Rubin YL, Sinensky MC & Blau L (1995). An *in vitro* study on the cytotoxicity of chlorhexidine digluconate to human gingival cells. *Cell Biology and Toxicology*, 11: 79-88.
15. Adriaanse AH (1995). Prevention of neonatal septicemia due to group B streptococci. *Baillieres Clinical Obstetrics and Gynaecology*, 9: 545-552.
16. Laskin DL & Laskin JD (2001). Role of macrophages and inflammatory mediators in chemically induced toxicity. *Toxicology*, 160: 111-118.
17. Komatsu H, Tsukimori K, Hata K, Satoh S & Nakano H (2001). The characterization of superoxide production of human neonatal neutrophil. *Early Human Development*, 65: 11-19.
18. Bender JG, McPhail LC & van Epps DE (1983). Exposure of human neutrophils to chemotactic factors potentiates activation of the respiratory burst enzyme. *Journal of Immunology*, 130: 2316-2323.
19. Matsudo R, Amaraki Y, Arima H & Tsuchiya S (1997). Scavenger receptors may regulate nitric oxide production from macrophages stimulated by LPS. *Biochemical and Biophysical Research Communications*, 237: 601-605.