

Chlorhexidine possesses unique cytotoxic actions in rat thymic lymphocytes: Its relation with electrochemical property of membranes

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## ABSTRACT

Chlorhexidine (CHX) is an antibacterial agent used in various types of pharmaceutical products. Therefore, CHX is easily found around us. Owing to its positive charge, the electrochemical property of cell membranes was assumed to be a key point of cytotoxic action of CHX. Depolarization of membranes attenuated the cytotoxic action of CHX in rat thymic lymphocytes. CHX interfered with annexin V binding to membranes. Manipulations to induce exposure of phosphatidylserine on the outer membrane surface augmented the cytotoxic action of CHX, indicating that changes in the electrochemical property of membranes affected the cytotoxic action of CHX. Hence, CHX might kill cells physiologically undergoing apoptosis, resulting instead in necrotic cell death. However, the threshold CHX concentration in this in vitro study was slightly higher than blood CHX concentrations observed clinically. Therefore, these results may support the safety of CHX use although CHX possesses unique cytotoxic actions described in this study.

Keywords:

Chlorhexidine

Hydrogen peroxide

Cytotoxicity

Lymphocytes

Phosphatidylserine

## 1. Introduction

Chlorhexidine (CHX) is a widely used antibacterial agent commonly found in disinfectants, cosmetics, and pharmaceutical products (Lowbury and Lilly, 1973; Fardai and Turnbull, 1986; Opstrup et al., 2015). Therefore, CHX is easily found in our environment. Mouthwash typically contains 0.02–0.2% CHX digluconate as an antimicrobial agent (for a review, Jones, 1997), while vaginal wash during labor is performed usually with 0.2% CHX digluconate (Burman et al., 1992). In gel formulation for single topical application, the concentration of CHX ranges from 1–2% (Jones, 1997). CHX digluconate or CHX diacetate of 2–4% is used for full body bath in hospitalized patients (Cowen et al., 1979; Climo et al., 2013) and medical device sterilization (Chapman et al., 2013). No significant adverse events induced by CHX have been reported except for contact dermatitis, and there are no clinical data to discourage the use of CHX. Therefore, the effect of CHX on intact cells may be ignored. The number of cells in multicellular organisms is regulated by controlling the rates of cell division and cell death. Such cell death that is programmed is called as apoptosis. Apoptosis occurs in developing and adult tissues (Elmore, 2007). Therefore, the chemical compounds affecting the process of apoptosis may disturb normal development.

At physiological pH range, CHX is positively charged and binds non-specifically to negatively charged membrane phospholipids (Hjeljord et al., 1973; Jones, 1997). Distribution of phospholipids in the membrane lipid bilayer of eukaryotic living cells is asymmetric under normal physiological conditions, where phosphatidylcholine and phosphatidylserine are found in the outer and inner monolayers, respectively. However, phosphatidylserine is exposed on the outer monolayer in cells at the early stage of apoptosis. Apoptosis occurs normally during development and aging, and is a homeostatic mechanism to maintain cell populations in tissues (Elmore, 2007; Tiwari et al., 2015). Phosphatidylcholine is zwitterionic while phosphatidylserine is anionic. Therefore, electrochemical property of membranes is changed in cells undergoing apoptosis. If positively charged CHX binds to anionic phosphatidylserine in high preference to zwitterionic phosphatidylcholine, CHX would induce a more profound effect on apoptotic living cells, leading to necrosis. Necrosis is an uncontrolled and passive process that usually affects large fields of cells, whereas apoptosis is controlled, energy-dependent, and can affect individual or clusters of cells (Kono and Rock, 2008). Cells undergoing necrosis lose membrane integrity and leak their intracellular components, some of which serve as danger signals that stimulate inflammation (Kono and Rock, 2008; Moriwaki and Chan, 2013). To test the hypothesis, we examined the effects of CHX on rat thymocytes simultaneously incubated

with agents that induce exposure of phosphatidylserine on the outer surface of membranes. This study may provide new insights into the toxicological profile of CHX for its safe use.

## 2. Materials and Methods

### 2.1. Animal and cell preparation

This study was approved by the Committee for Animal Experiments of Tokushima University, Tokushima, Japan (No. 14124). Experimental methods were similar to those described in previous papers (Chikahisa et al., 1996; Oyama et al., 1999; Matsui et al., 2010).

Male rats (Wistar strain, Charles River Laboratories, Yokohama, Japan) were provided with water automatically and a commercial diet (MF, Oriental Yeast, Tokyo, Japan) ad libitum. The animal room was maintained at a temperature of  $23 \pm 2$  °C and a relative humidity of  $55 \pm 5\%$ , and it was artificially illuminated with fluorescent light on a 12-h light/dark cycle (08:00–20:00 h). The total number of 8–12 week old rats sacrificed under ether inhalation anesthesia was 27.

Cell suspension was prepared as previously reported (Chikahisa et al., 1996). In brief, thymus glands dissected from ether-anesthetized rats were sliced under cold conditions. The slices were triturated in Tyrode's solution to dissociate the thymocytes. The cell suspension was incubated at  $36\text{--}37$  °C for 1 h before the experiment. The cell suspension (60–80 mL) was prepared from one experimental animal.

We used ether inhalation anesthesia for sacrifice. One may argue the possibility that the ether anesthesia change the effect of drug on rat thymocytes under the in vitro condition. We previously confirmed no difference between the results obtained from the cells isolated from rats sacrificed with decapitation and ether-anesthetized ones (Matsui et al., 2008).

### 2.2. Chemicals

CHX digluconate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Annexin V-FITC, propidium iodide, and 5-chloromethylfluorescein diacetate (5CMF-DA) were obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless mentioned.

### 2.3. Fluorescence measurements of cellular parameters

The methods for measurements of cellular and membrane parameters using a flow cytometer equipped with an argon laser (CytoACE-150, JASCO, Tokyo, Japan) and fluorescent probes were similar to those previously described (Chikahisa and Oyama, 1992; Chikahisa et al.,

1996). The fluorescence was analyzed by JASCO software (JASCO). As to chemicals used in this study, there was no fluorescence detected under our experimental condition.

To assess cell lethality (population of dead cells) using propidium iodide, the dye was added to the cell suspension at a final concentration of 5  $\mu\text{M}$  at 5 min before the measurement. Exposure of phosphatidylserine on the outer surface of cell membranes, a marker of early stage apoptosis, was detected using 10  $\mu\text{L/mL}$  annexin V-FITC (Koopman et al., 1994). AnnexinV-FITC was added to the cell suspension at 30 min before the measurement. Excitation wavelength for these fluorescent probes was 488 nm elicited by argon laser. Fluorescence of FITC and propidium was detected at  $530 \pm 20$  nm and  $600 \pm 20$  nm, respectively.

#### 2.4. Experimental protocols

The agents were added to the cell suspension (2 ml cell suspension in each 10ml test tube). The cells were incubated with the agent(s) at 36°C for 1–3 h under room air condition. The incubation time was dependent on each experimental purpose. The data acquisition of fluorescence from 2500 cells by a flow cytometer required 30 sec at maximum.

#### 2.4. Statistical analysis

Statistical analyses were performed by ANOVA with post-hoc Tukey's multivariate analysis. P-values of less than 0.05 were considered significant. Values (including columns and bars in figures) were expressed as mean and standard deviation of 4–8 samples.

### 3. Results

#### 3.1. CHX-induced change in cell lethality of rat thymocytes

The incubation of rat thymocytes with 30  $\mu\text{M}$  CHX for 1 h increased the population of cells exhibiting propidium fluorescence (Fig. 1), indicating CHX-induced increase in cell lethality. The dose-response relationship of CHX-induced increase in cell lethality shown in Tab. 1. revealed statistically significant increase in cell lethality when the cells were incubated with CHX concentrations of 10  $\mu\text{M}$  and higher.

(Figure 1 and Table 1 near here)

#### 3.2. Effects of $\text{Na}^+$ , $\text{K}^+$ , and $\text{Ca}^{2+}$ on CHX-induced increase in cell lethality

The cytotoxicity of positively charged CHX may be influenced by the changes in membrane potentials or negative surface charge of membranes. We examined the effects of KCl, NaCl, and  $\text{CaCl}_2$  on the cytotoxicity of CHX. First, to assess the influence of membrane

potential, the effect of CHX on the cells was examined upon the addition of 50 mM KCl and 50 mM NaCl. Since the concentration of KCl in cell suspension was 11 times higher than the control (5 mM KCl), the Nernst equation predicts large depolarization. 50 mM NaCl was used to consider the change in osmolarity. The presence of the monovalent cations, Na<sup>+</sup> and K<sup>+</sup>, significantly reduced the cell lethality of CHX (Fig. 2A). However, CHX cytotoxicity was significantly lower under high K<sup>+</sup> condition compared to high Na<sup>+</sup> condition. Secondly, to assess the screening effect of Ca<sup>2+</sup> on the negative membrane charge, the effect of CHX on the thymocytes was also studied after the addition of 25 mM CaCl<sub>2</sub>. Although a slight reduction in CHX-induced cell lethality was observed in the presence of 25 mM Ca<sup>2+</sup>, it was not statistically significant (Fig. 2B).

(Figure 2 near here)

### 3.3. Effect of CHX on cells treated simultaneously with H<sub>2</sub>O<sub>2</sub>

As shown in Fig. 3A, the incubation of thymocytes with 0.3 and 1 μM CHX for 3 h did not increase the population of dead cells exhibiting propidium fluorescence. In contrast, incubation with 3 μM CHX for 3 h slightly, but significantly, increased the population of dead cells (Fig. 3A). Thus, the presence of CHX at 3 μM seems to induce cytotoxic action on rat thymocytes. Similarly, the incubation with 100 μM H<sub>2</sub>O<sub>2</sub> for 3 h also slightly increased the population of dead cells and was statistically significant (Fig. 3A). The simultaneous treatment of 100 μM H<sub>2</sub>O<sub>2</sub> with 1 and 3 μM CHX for 3 h induced further significant increase in the population of dead cells. However, this was not the case with 0.3 μM CHX.

Fig. 3B shows the effect of 30–300 μM H<sub>2</sub>O<sub>2</sub> treatment on cells for 3 h in the absence and presence of 3 μM CHX. The treatment of cells with H<sub>2</sub>O<sub>2</sub> significantly increased the population of living thymocytes with phosphatidylserine exposed on outer membrane surface (Oyama et al., 1999). Since phosphatidylserine is anionic, the treatment with H<sub>2</sub>O<sub>2</sub> was expected to increase anionic membrane property. While incubation with 30 μM H<sub>2</sub>O<sub>2</sub> did not significantly change the population of dead cells, significant increase in cells lethality was observed with 100 and 300 μM H<sub>2</sub>O<sub>2</sub>. Furthermore, the increase in dead cell population upon simultaneous treatment of 3 μM CHX with 30–300 μM H<sub>2</sub>O<sub>2</sub> was also statistically significant in comparison with treatment in the absence of 3 μM CHX (Fig. 3B).

(Figure 3 near here)

### 3.4. CHX-induced increase in population of living cells positive to annexin V

In the presence of 3  $\mu\text{M}$  CHX, propidium fluorescence was not affected while FITC fluorescence produced by annexin V-FITC bound to cell membranes was greatly reduced (Fig. 4). Thus, CHX attenuated the binding of annexin V to phosphatidylserine exposed on the outer surface of membranes.

Fig. 5 shows cytograms of cells treated with 3  $\mu\text{M}$  CHX, 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , and their combination for 2.5 h prior to the application of annexin V-FITC. The cells were incubated with Annexin V-FITC for 0.5 h after the removal of CHX from cell suspension. The combination greatly decreased the population of living cells with exposed phosphatidylserine (area A of Fig. 5) and increased the population of dead cells (area AP of Fig. 5). The changes in cell population induced by 3  $\mu\text{M}$  CHX, 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , and their combination are summarized in Fig. 6. Thus, it is hypothesized that the living cells with phosphatidylserine exposure are vulnerable to CHX.

(Figures 4, 5, and 6 near here)

### 3.5. Effect of CHX on lethality of cells treated simultaneously with A23187

A23187, a calcium ionophore, at 100 nM greatly increases the population of living cells with phosphatidylserine exposed on the outer surface of membranes (Yamaguchi et al., 2005; Sakanashi et al., 2008). To test the hypothesis described above, the effect of CHX on the cells treated with A23187 was examined. As shown in Fig. 7, the combination of 3  $\mu\text{M}$  CHX and 100 nM A23187 significantly increased the population of dead cells, in comparison to treatment with the individual agents.

(Figure 7 near here)

## 4. Discussion

At physiological pH, CHX is a large dicationic molecule with its positive charge distributed over nitrogen atoms on either side of the hexamethylene bridge. Thus, CHX has the ability to adsorb onto negatively charged surfaces such as cell membranes and bacterial cell walls. Positive charged compounds are absorbed into membranes as membranes are hyperpolarized (Waggoner, 1979). Membrane depolarization releases these dyes from membranes. This seems to apply to CHX because membrane depolarization by the addition of KCl reduced the cytotoxicity of CHX (Fig. 2A). Respective addition of NaCl and KCl significantly reduced the lethal action of CHX (Fig. 2A), whereby the potency of KCl was greater than that of NaCl. While the application of KCl causes membrane depolarization, it is

not the case for NaCl. The difference in potency between NaCl and KCl is due to the difference between their actions on membrane potential. The positively charged CHX is considered to become less absorbable to the membranes when the membranes are depolarized. The negative surface charge of membranes is also critical factor affecting the actions of positive charged compounds.  $\text{Ca}^{2+}$  has a screening effect on the membrane's negative surface charge (McLaughlin et al., 1971). Thus, reducing its negativity by adding  $\text{CaCl}_2$  slightly attenuated the cytotoxicity of CHX (Fig. 2B). Since the effect of  $\text{CaCl}_2$  on CHX-induced cell lethality was not statistically significant (Fig. 2B), modification of negative membrane surface charges does not appear to be involved in the attenuation of lethal action of CHX by NaCl and KCl. One may argue that the electrical property of membranes under *in vitro* conditions differs from that under *in vivo* conditions. Membrane surface charge is also modified by pH (Gilbert and Ehrenstein, 1984) and proteins containing polycationic domains in their amino acid sequence (Goldenberg and Steinberg, 2010). Therefore, our results have informational limitation.

In addition, CHX appeared to interfere with the binding of annexin V to the negatively charged membrane because FITC fluorescence emitted by annexin V-FITC bound to cell membranes was greatly attenuated in the presence of CHX (Fig. 4). Thus, CHX appeared to interfere with the binding of annexin V to the negatively charged membrane. Membrane phosphatidylserine is reported to regulate surface charge in the inner surface under normal conditions (Yeung et al., 2008). However, it is exposed on the outer surface of membranes during the early stage of apoptosis (Fadok et al., 1992). Since annexin V demonstrates several physiological roles (Gerke and Moss, 2002), CHX may directly or indirectly inhibit the physiological functions of annexin V. During the early stage of apoptosis, the membrane may become a more attractive binding target for positively charged molecules. CHX increased the population of dead cells when the cells were simultaneously treated with  $\text{H}_2\text{O}_2$  and A23187 (Figs. 3 and 7), which increased the population of living rat thymocytes with phosphatidylserine exposed on the outer surface of membranes (Oyama et al., 1999; Yamaguchi et al., 2005; Sakanashi et al., 2008). It has been suggested that CHX kills apoptotic living cells, resulting instead in necrotic cell death. Apoptosis is an important event in various processes such as tissue development and maintenance (Elmore, 2007), whereas necrosis induces release of endogenous adjuvants from dying cells (Kono and Rock, 2008). Therefore, the lethal action of CHX on living cells with exposed phosphatidylserine may induce inflammation.

The molecular weight of CHX digluconate is 897.76. In our *in vitro* study, 3  $\mu\text{M}$  CHX was used to characterize its cytotoxic action, which was equivalent to 0.000269%. This

concentration of CHX was much lower than those (0.2-4%) used in gel formulation for topical application (Jones, 1997), full body bath in hospitalized patients (Cowen et al., 1979; Climo et al., 2013) and medical device sterilization (Chapman et al., 2013). Detected concentrations of CHX in human blood after its application were reported to range from 1 ng/mL (about 2 nM) to 460 ng/mL (about 900 nM) (Cowen et al., 1979; Wilson et al., 2004; Chapman et al., 2013). Since previously reported concentrations of CHX in the blood are lower than 3  $\mu$ M, this study may support the safety of CHX use.

#### Conflict of Interests

All authors affirm that there are no conflicts of interest to declare.

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## Figure Legends

### Figure 1

CHX-induced increase in population of cells exhibiting propidium fluorescence (dead cells). The dotted bar under each cytogram indicates the population of cells stained with propidium iodide.

### Figure 2

Effects of NaCl and KCl (A), and CaCl<sub>2</sub> (B) on CHX-induced cell lethality. Asterisks (\*\*) indicate significant difference ( $P < 0.01$ ) between the control group and the groups of cells treated with CHX. Symbols (##) indicate significant difference ( $P < 0.01$ ) between the arrowed groups.

### Figure 3

Concentration-dependent changes in cell lethality induced by CHX, H<sub>2</sub>O<sub>2</sub>, and their combinations. (A) Changes in CHX-induced cell lethality in the absence and presence of H<sub>2</sub>O<sub>2</sub>. (B) Changes in H<sub>2</sub>O<sub>2</sub>-induced cell lethality in the absence and presence of CHX. Asterisks (\*, \*\*) indicate significant difference ( $P < 0.05$ ,  $P < 0.01$ ) between the control group and the groups of cells treated with CHX, H<sub>2</sub>O<sub>2</sub>, and their combinations. Symbols (##) indicate significant difference between the groups of cells treated with either H<sub>2</sub>O<sub>2</sub> or CHX alone, and those treated with their combinations.

### Figure 4

Cytograms of cells stained with propidium iodide and annexin V-FITC in the absence and presence of CHX. Area N: intact living cells exhibiting neither propidium fluorescence nor FITC fluorescence, area A: living cells with exposed phosphatidylserine exhibiting FITC fluorescence but not propidium fluorescence, areas P and AP: dead cells having propidium fluorescence without and with FITC fluorescence, respectively.

### Figure 5

Changes in the cytogram (propidium fluorescence versus FITC fluorescence) of cells upon treatment with CHX, H<sub>2</sub>O<sub>2</sub>, and their combination. Classification (N, A, P, and AP) was the same as described in Fig. 4.

Figure 6

Changes by CHX, H<sub>2</sub>O<sub>2</sub>, and their combination in cell population classified with propidium iodide and annexin V-FITC. Asterisks (\*\*) indicate significant difference ( $P < 0.01$ ) between the control group and the groups of cells treated with respective agents.

Figure 7

Changes by CHX, A23187, and their combination in cell lethality. Asterisks (\*\*) indicate significant difference ( $P < 0.01$ ) between the control group and the groups of cells treated with respective agents.

Figure 1

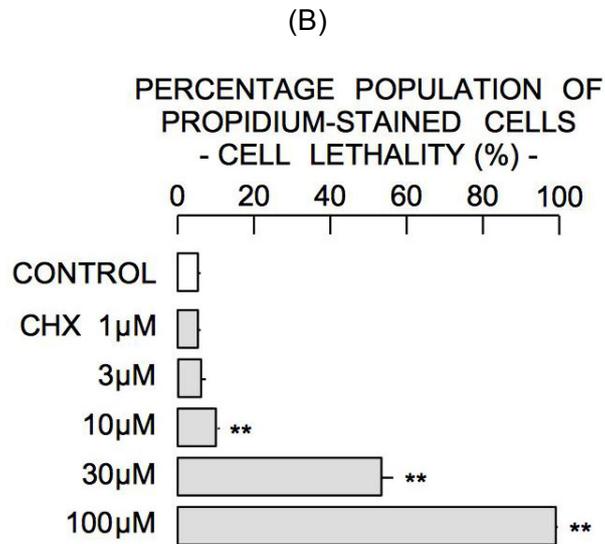
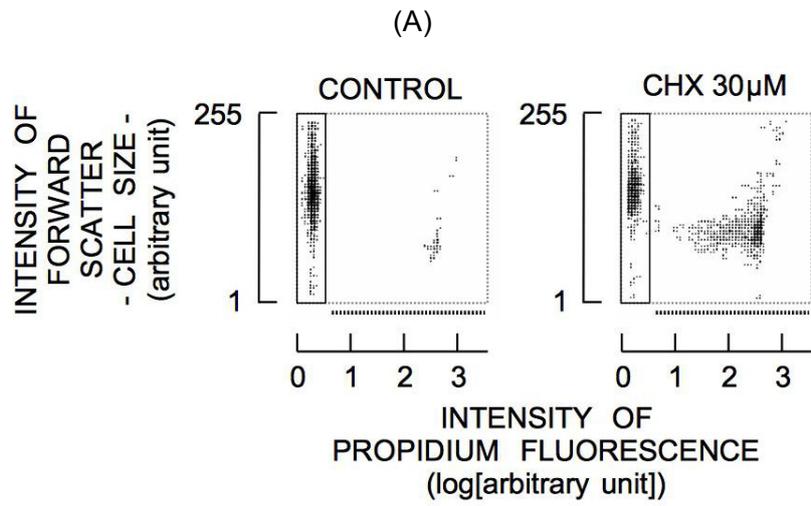


Figure 2

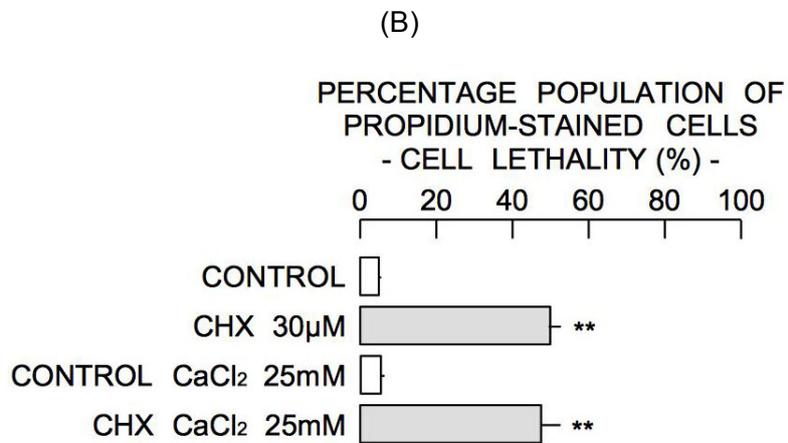
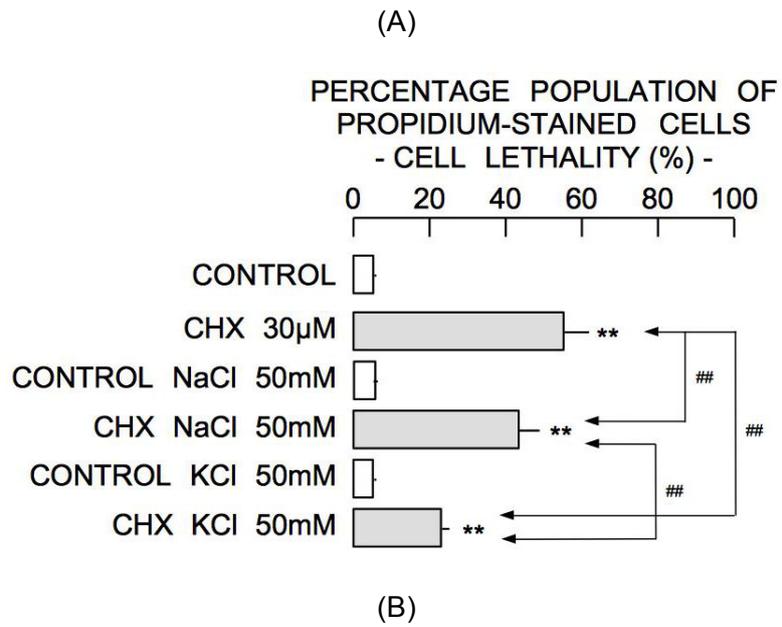


Figure 3

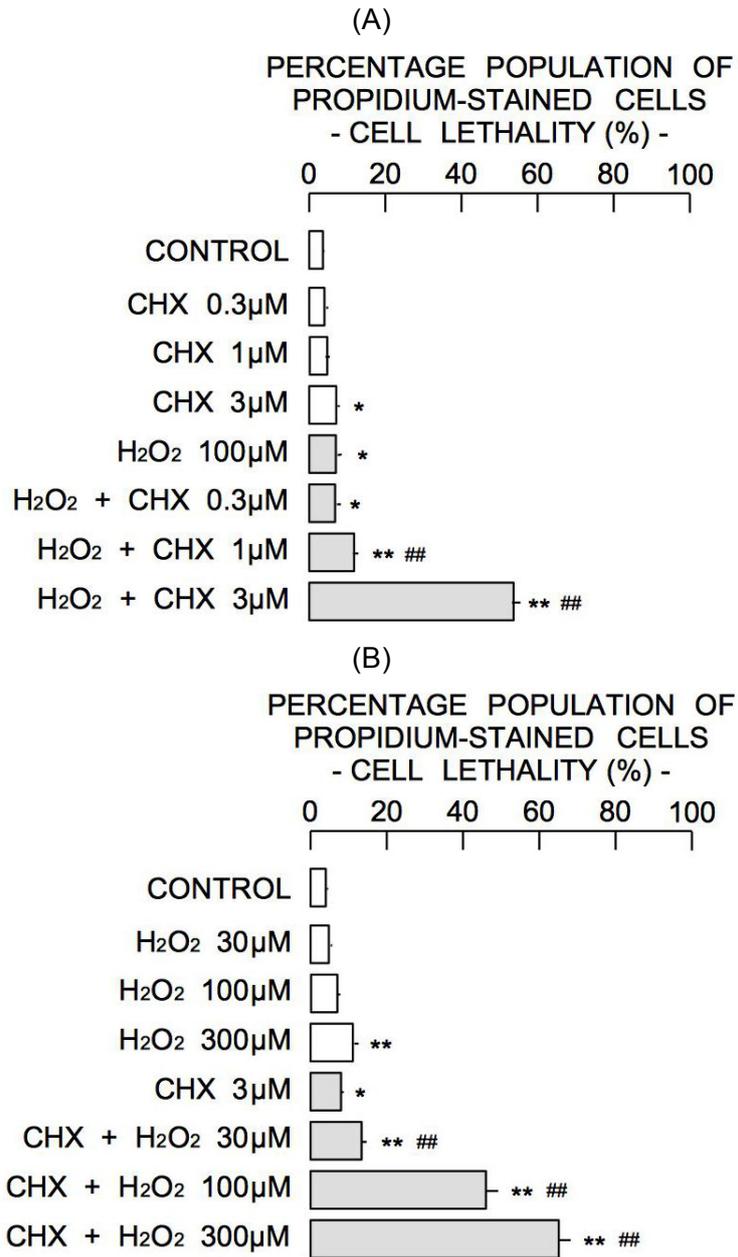


Figure 4

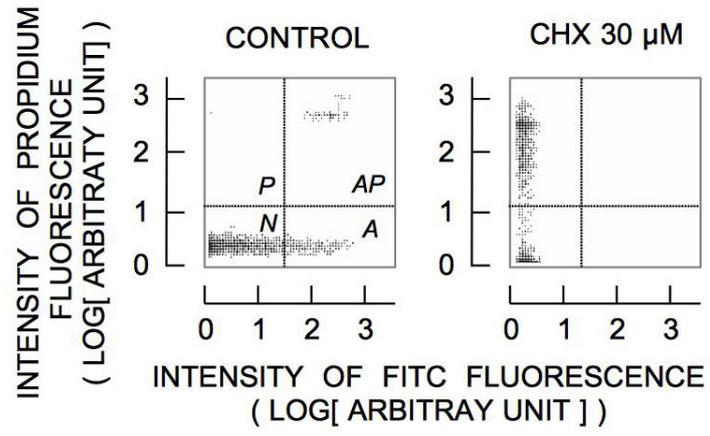


Figure 5

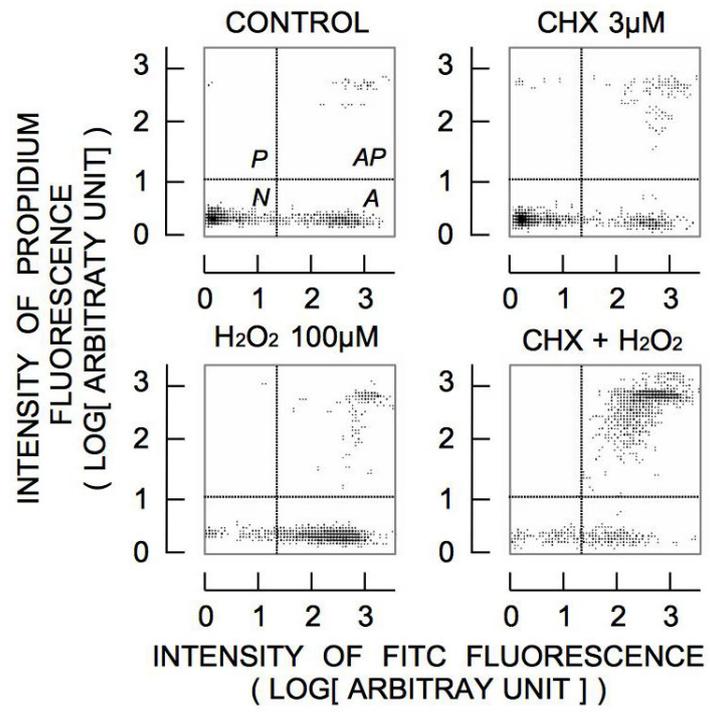


Figure 6

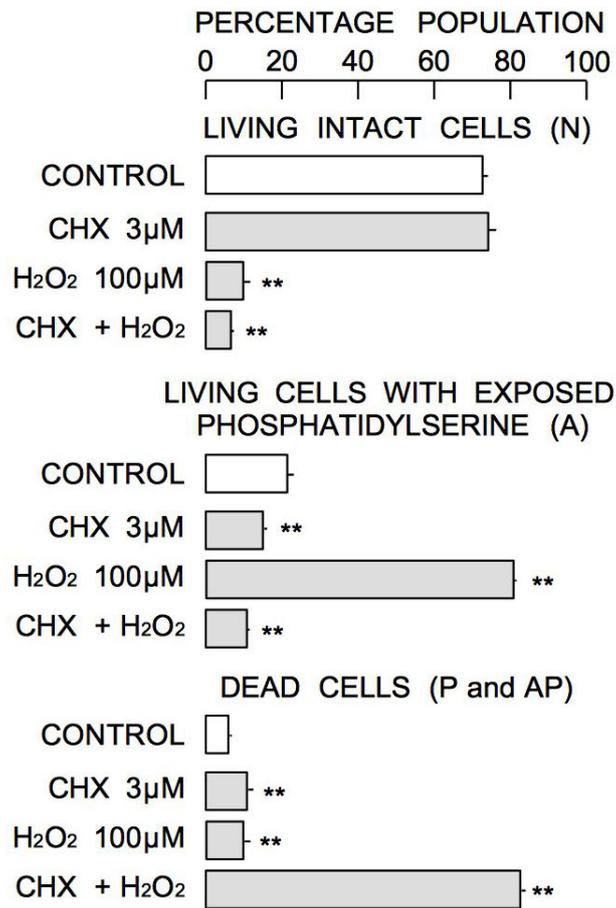


Figure 7

