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# Cell death process induced by hydrogen peroxide is accelerated by clioquinol in rat thymocytes

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Abstract

We examined the effect of clioquinol on the process of cell death induced by hydrogen peroxide ($H_2O_2$) using a flow cytometric technique with propidium iodide and annexin V-FITC in order to see if clioquinol augments the toxicity caused by oxidative stress. Clioquinol (100 nM) alone did not change the process of spontaneous cell death. However, the agent accelerated the process of cell death induced by 300 μM $H_2O_2$. Result indicates that clioquinol augments the cytotoxicity induced by $H_2O_2$. Therefore, the use of clioquinol may be inadequate for the treatment of some diseases related to oxidative stress.

Keywords: clioquinol; hydrogen peroxide; cytotoxicity; lymphocyte

In our previous studies (Oyama et al., 2012, 2014), clioquinol (10–300 nM) increased intracellular $Zn^{2+}$ levels. However, the effect induced by 1 μM clioquinol was less than that by 300 nM clioquinol. Removal of extracellular $Zn^{2+}$ by $Zn^{2+}$ chelators abolished the clioquinol-induced increase in intracellular $Zn^{2+}$ levels. The increase in intracellular $Zn^{2+}$ levels augmented the cytotoxicity of hydrogen peroxide ($H_2O_2$) (Matsui et al., 2010). We observed a bell-shaped relationship between the clioquinol concentration and changes in $H_2O_2$ cytotoxicity in the presence of clioquinol; $H_2O_2$-induced cytotoxicity was the highest when clioquinol concentration was 100 nM (Oyama et al., 2014). In this study, we examined the effect of clioquinol on the process of cell death induced by $H_2O_2$ in order to see if clioquinol augments the toxicity elicited by oxidative stress.

This study was approved by Tokushima University (No. 05279). Methods employed in this study were described in our previous papers (Chikahisa and Oyama, 1992; Oyama et al., 1999; Matsui et al., 2008). In brief, thymus glands dissected from ether-anesthetized Wistar rats were sliced with a blade under ice-cold conditions. The slices were triturated in chilled Tyrode's solution to dissociate the thymocytes. Thereafter, the beaker containing the cell suspension was incubated in a water bath at 36–37°C for 1 h before the experiment.

Propidium iodide was used to stain dead cells or the cells with compromised membranes (Yeh et al., 1981). The exposure of phosphatidylserine on the outer surface of cell membranes, which is a phenomenon that occurs during the early stages of apoptosis, was detected with annexin V-FITC (Koopman et al., 1994). The cells were incubated with annexin V-FITC (10 μl/mL) and propidium iodide for 30 min before the measurement (Oyama et al., 1999). Statistical analysis was performed with Tukey's multivariate analysis. A $P$ value of $<0.05$ was considered significant. Values are expressed as the mean and standard deviation, respectively.

Rat thymocytes were incubated with 100 nM clioquinol, 300 μM $H_2O_2$, and their combination for 1.5 h, and then annexin V-FITC and propidium iodide were applied to respective cell suspension. Therefore, the measurement of FITC and propidium fluorescence was made at 2 h after respective drug

application.

As shown in Fig. 1, clioquinol did not change the population of cells classified by propidium and FITC fluorescence. Thus, 100 nM clioquinol did not exert the cytotoxic action on rat thymocytes. $H_2O_2$ at 300 μM greatly increased cell population of area A (annexin V-positive living cells) and those of areas AP and P (dead cells). The combination of clioquinol and $H_2O_2$ further decreased the cell population of area N (intact living cells) and increased the population of annexin V-positive living cells and dead cells.

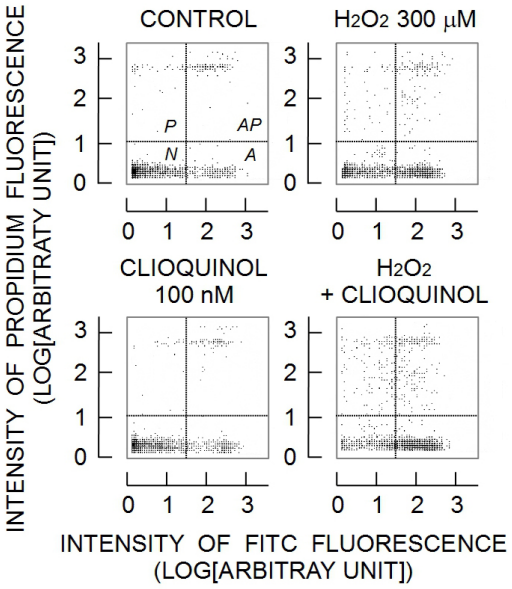


**Figure 1**. Changes induced by clioquinol, $H_2O_2$, and their combination in cells that were labeled by annexin V- FITC and propidium iodide. Changes in the fluorescence cytogram (propidium fluorescence versus FITC fluorescence) by 300 μM $H_2O_2$, 100 nM clioquinol (CLIOQUINOL), and their combination ($H_2O_2$ + CLIOQUINOL). The effects were examined 2h after their application. Each cytogram consisted of 2,000 cells. The N, A, P, and AP areas show the intact living cells, annexin V-positive living cells, dead cells, and annexin V-positive dead cells, respectively

The results of Fig. 1 are summarized in Fig. 2. The result of Fig.2 suggests that simultaneous application of clioquinol and $H_2O_2$ increased the number of living cells with phosphatidylserine exposed on their outer membrane surfaces (area A of Fig. 1), which is a marker for the early stages of apoptosis (Koopman et al., 1994). Thus, it is likely that clioquinol accelerate the process of apoptosis.

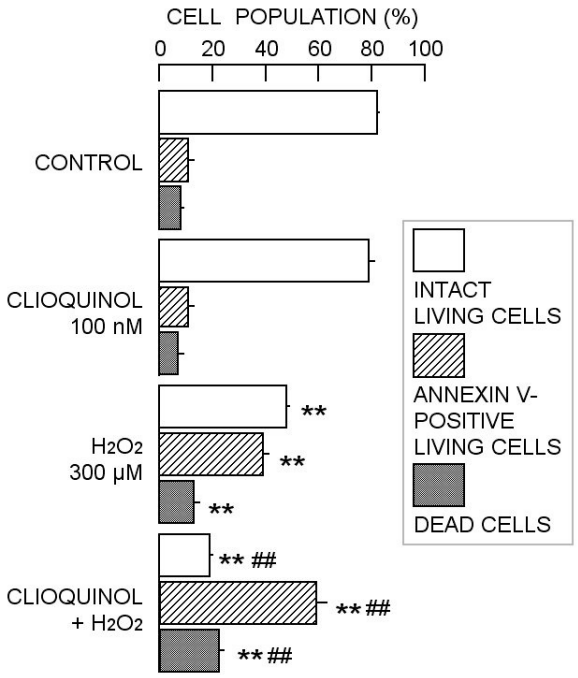


**Figure 2**. Changes in the percentage population after treatment with clioquinol (CLIOQUINOL), $H_2O_2$, and their combination. The dead cells consisted of the P and AP areas as shown in Fig. 1. The asterisk (**) indicates a significant difference ($P < 0.01$) between the control group (CONTROL) and the test groups. The symbol (##) also shows a significant difference ($P < 0.01$) between the group of cells treated with $H_2O_2$ and that with the combination (CLIOQUINOL + $H_2O_2$).

In our previous studies (Oyama et al., 2012, 2014), clioquinol increased intracellular $Zn^{2+}$ levels, depending on the presence of extracellular $Zn^{2+}$. The result indicates that clioquinol accelerated the process of cell death induced by $H_2O_2$. Therefore, it is suggested that the agents modifying intracellular $Zn^{2+}$ homeostasis may affect the toxicity caused by oxidative stress. The use of clioquinol may be inadequate for the treatment of some diseases related to oxidative stress.

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