Diverse cellular actions of *tert*-butylhydroquinone, a food additive, on rat thymocytes

Norio Kamemura[1], Keisuke Oyama[2,3], Kaori Kanemaru[1], Kumio Yokoigawa[1], Yasuo Oyama[1,*]

[1] Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima 770-8513, Japan

[2] Tokushima Prefectural Central Hospital, Tokushima 770-8539, Japan

[3] Present address: Osaka University Hospital, Suita 565-0871, Japan

[*]Corresponding author: Yasuo Oyama, Ph.D.

E-mail: oyamay@tokushima-u.ac.jp

Tel: 81-88-656-7256

Highlights

• *tert*-Butylhydroquinone (TBHQ), a food additive, is assumed to be bioactive.

• Cellular actions of TBHQ at sublethal levels were examined in rat thymocytes.

• TBHQ prompted apoptosis process and elevated intracellular Zn^{2+} levels.

• Inhibitory and facilitatory actions of TBHQ on H_2O_2 cytotoxicity were observed.

• TBHQ at sublethal levels may cause cytoprotective and adverse actions.

Abstract

Tertiary butylhydroquinone (TBHQ) is a food additive that possesses antioxidant activity. Its alternative applications have been explored in recent studies. However, there is controversy regarding safety. In this study using rat thymocytes, the cellular actions of TBHQ at sublethal concentrations were examined. TBHQ at concentrations of 3 μM or more elevated intracellular Zn^{2+} concentration ($[Zn^{2+}]_i$) in a dose-dependent manner, by increasing membrane Zn^{2+} permeability and releasing Zn^{2+} from cellular stores. TBHQ at 30 μM significantly increased side scatter (cell density) and the exposure of phosphatidylserine (PS) on cell membrane surfaces. It also decreased cellular glutathione (GSH) content without affecting cell lethality. Forward scatter was attenuated by 100 μM TBHQ. Thus, it is considered that TBHQ at sublethal concentrations (30 μM or less) exerts some adverse actions on cells. TBHQ at 10–30 μM attenuated the increase in cell lethality induced by hydrogen peroxide (H_2O_2), while potentiation of H_2O_2 cytotoxicity by 100 μM TBHQ was observed. The range of concentrations of TBHQ from benefit to toxicity under in vitro conditions may be 10–30 μM. Although TBHQ exhibits antioxidative actions at concentrations that are lower than those which elicit adverse cellular effects, sublethal levels of TBHQ cause some adverse actions that may be clinically concerned.

Keywords: tert-butylhydroquinone; food additive; cytotoxicity; lymphocytes; zinc

1. Introduction

Tertiary butylhydroquinone (TBHQ, Chemical Abstracts 1948-33-0) is a food additive that possesses antioxidant activity.[1] The European Food Safety Authority has evaluated the toxicity of TBHQ and determined safe levels of TBHQ as a food additive.[2] At concentrations less than 0.02%, TBHQ extends the shelf life of edible fats and oils.[3] In addition, beneficial actions of TBHQ as an antioxidant have been reported as follows. Under in vitro conditions, TBHQ exhibited protective actions on neuroblastoma SH-SY5Y cells against 6-hydroxydopamine-induced oxidative stress, on rat striatal slices damaged by quinolinic acid, on PC12 cells suffering oxidative stress caused by NaF, and on human keratinocytes under arsenic-induced stress.[4–7] TBHQ under in vivo conditions ameliorated testicular toxicity of dibromoacetonitrile, inflammation following brain injury in mice, brain injuries after both subarachnoid and cerebral hemorrhage in mice, and doxorubicin-induced cardiotoxicity.[8–12] Thus, it is expected that TBHQ will have clinical applications. However, there is controversy regarding the safety of TBHQ because cytotoxic and/or DNA-damaging effects of TBHQ have also been documented.[13–16] Therefore, it is important to accumulate information on TBHQ-induced adverse actions under in vitro and in vivo conditions in order to ensure its safe use. Recently, it was reported that TBHQ elevated $[Zn^{2+}]_i$ in 16HBE cells, stabilizing nuclear factor erythroid 2related factor 2 as a result by inhibiting phosphatase activity.[17] Our previous studies have shown that Zn^{2+} is a determinant of cytotoxicity induced by clotrimazole, H_2O_2, polysorbate 80, and zinc pyrithione.[18–22] For example, $ZnCl_2$ significantly potentiated the cytotoxicity of H_2O_2 while a chelator of intracellular Zn^{2+} almost completely abolished it.[20] Thus, the various cellular actions of TBHQ should be explored. In this study, we used flow-cytometric techniques with fluorescent dyes to examine the cytotoxic actions of TBHQ in rat thymocytes. Second, we examined the cellular actions of TBHQ that were presumably related to the mechanism of cytotoxicity. Finally, the range of concentrations of TBHQ transitioning from

benefit to toxicity was explored.

2. Materials and methods

2.1. Chemicals

TBHQ was purchased from Tokyo Chemical Ind. (Tokyo, Japan). The purity was 99.1%. Annexin V-FITC, 5-chloromethylfluorescein diacetate (5-CMF), FluoZin-3-AM, and propidium iodide (PI) were supplied by Molecular Probes, Invitrogen (Eugene, OR, USA). Chelators for extracellular and intracellular Zn^{2+} were, respectively, diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid and N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (DTPA and TPEN; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Unless mentioned, all other reagents were products of Wako Pure Chemicals (Osaka, Japan).

2.2. Cell preparation

Experiments were performed under the approval (No. 05279) of the Tokushima University Committee for Animal Experiments. The thymus glands were obtained from male Wistar rats (8–12 weeks old, Charles River Laboratories, Yokohama, Japan) that were anesthetized with ether. The number of scarified rats was 14. To dissociate individual cells, the glands were triturated in chilled Tyrode's solution (2–4°C) consisting of 150 mM $NaCl_2$, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose with 5 mM HEPES and appropriate amount of NaOH to adjust pH at 7.3–7.4. The Tyrode's solution containing dissociated cells (cell suspension) was incubated at 36–37°C for 1 h before experiments. The cell suspension contained trace amounts (216.9 ± 14.4 nM; mean ± standard deviation of six samples) of zinc from cell preparation.[23] The zinc concentration in distilled water was 3.6 ± 5.0 nM while it was 32.4 ± 4.0 nM in the case of Tyrode's solution. A flame atomic absorption spectrometer equipped with a polarized Zeeman's-effect background corrector (Hitachi Model Z-5300,

Hitachi High-Technologies Co., Tokyo, Japan) was used for determination of zinc. A hollow-cathode lamp of zinc (Hitachi part No. 208-2034) was used as a radiation source.

TBHQ (1–100 mM in 2 μL dimethyl sulfoxide) were added to plastic test tubes (Watson Bio Lab, Tokyo, Japan; 1.998 mL of cell suspension) for achieving final concentrations of 1–100 μM by a vortical mixing and then the cells were treated with TBHQ for 1–4 h, depending on experimental purpose. Thereafter, a sample from each cell suspension (100 μL) was cytometrically analyzed. The starting, sample, and sheath flow rates were manually adjusted to set the measurement of 195–205 cells/s and the interval of about 180 μs between measurements of forward and side scatters. The measurement started after achieving constant flow of cells. Data acquisition from 2500 cells took approximately 15 s. Cell lethality estimated from 2500 cells was quite similar to that estimated from 10000 cells. Therefore, 2500 cells were enough to examine cellular actions of TBHQ.

Thymocytes spontaneously undergo apoptosis during the prolonged incubation.[24,25] Experiments, including cell preparation, were performed within 8 h after the dissection of thymus from experimental animals to avoid possible contribution of spontaneous apoptosis. Therefore, the treatment time of TBHQ should be within 5 h.

2.3. Fluorescence measurement

Fluorescence of membrane and cellular constituents was measured with a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan) by using fluorescent dyes, and analyzed using the JASCO software package.[20,26] Chemical reagents, except for fluorescent dyes, exhibited no fluorescence under the experimental conditions described. To assess cell lethality, 5 μM PI was added to the cell suspension. Because PI stains the DNA of dead cells, the fluorescence detected provides information on cell lethality. Phosphatidylserine (PS) exposure on outer membrane surfaces, a phenomenon occurring during the early stages of apoptosis, was examined via annexin V-binding.[27] The cells were treated with FITC-labeled annexin V (10 μl/mL) for 30

min before the assessment. FluoZin-3-AM was applied to the cell suspension to monitor the change in $[Zn^{2+}]_i$.[28] Cells were incubated with 500 nM FluoZin-3-AM for 50–60 min before any fluorescence measurement. FluoZin-3 fluorescence, without PI fluorescence, of the cells (living cells with intact membranes) was measured. 5-CMF-DA was used to estimate changes in cellular GSH content.[26] The cells were treated with 1 μM 5-CMF-DA 30 min before fluorescence measurement. 5-CMF fluorescence reflects cellular GSH content with a correlation coefficient of 0.965. Excitation wavelength for fluorescent probes was 488 nm, and emissions were detected at 530 ± 20 nm for FITC, FluoZin-3, and 5-CMF fluorescence, and at 600 ± 20 nm for PI fluorescence.

2.4. Statistical analysis and presentation

Statistical analysis was carried out through Tukey's multivariate analysis. P-values of < 0.05 indicate statistical significance. Values are expressed as mean and standard deviation of 4–12 samples. Each experimental series was conducted twice or three times unless stated otherwise.

3. Results

3.1. Effect of TBHQ on cell lethality

The cells were treated with various concentrations of TBHQ (1–100 μM) for 3 h. TBHQ at 100 μM increased the population of cells with PI fluorescence (Fig. 1A and 1B). TBHQ at 30 μM or less did not increase the population (Fig. 1C). Thus, a small but significant increase in cell lethality was observed in the case of 100 μM TBHQ while the treatment with 1–30 μM TBHQ for 3–4 h did not significantly increase the cell lethality.

(Figure 1 near here)

3.2. Effect of TBHQ on forward and side scatters

Effects of TBHQ on forward and side scatter were examined since the cytotoxic actions of some chemicals that induce apoptosis are associated with changes in these indicators (cell size and density, respectively) as assessed *via* flow cytometry. Treatment of cells with 100 μM TBHQ for 3 h slightly decreased the intensity of forward scatter and profoundly raised the intensity of side scatter (Fig. 2A). TBHQ at 30 μM or less for 3 h did not affect the intensity of forward scatter, although the agent at 100μM significantly attenuated forward scatter (Fig. 2B), indicating a decrease in cell size. As to the effect on side scatter, TBHQ at 30–100 μM significantly raised the intensity of side scatter while it was not the case for 10 μM TBHQ (Fig. 2C).

(Figure 2 near here)

3.3. TBHQ-induced increase in living cells with exposed PS

The attenuation of forward scatter in the presence of 100 μM TBHQ suggested cell shrinkage (Fig. 2B). Cell shrinkage is one of the initial events in apoptosis.[29] PS exposure on outer membrane surfaces, one of events during early stage of apoptosis, was detected using annexin V-FITC.[27] Treatment with 100 μM TBHQ for 3 h profoundly increased the number of living cells with FITC fluorescence (Fig. 1A). FITC fluorescence from the cells indicated annexin V-binding to PS exposed on cell membrane surfaces. Thus, treatment with 100 μM TBHQ may induce apoptosis in rat thymocytes. The concentration–response relationship for TBHQ-induced cellular changes, as determined by PI and annexin V-FITC, is shown in Fig. 3B. Threshold TBHQ concentration to increase the number of viable PS-exposing cells was 30 μM when cells were treated with TBHQ for 3 h.

3.4. TBHQ-induced change in $[Zn^{2+}]_i$

Changes in some cellular parameters precede cell death. The $[Zn^{2+}]_i$ is one of them. [30–33]

Changes in intracellular levels of Zn^{2+} caused by TBHQ were examined using Zn^{2+}-sensitive FluoZin-3 fluorescence. Treatment with 30 μM and 100 μM TBHQ for 1 h profoundly shifted the fluorescence histogram to the direction of higher intensity (Fig. 3A). As shown in Fig. 3B, there was a dose-dependent augmentation of FluoZin-3 fluorescence by 10–100 μM TBHQ. The results suggest that the treatment of rat thymocytes with 10–100 μM TBHQ significantly elevated $[Zn^{2+}]_i$ in a dose-dependent manner. The threshold TBHQ concentration to increase FluoZin-3 fluorescence ($[Zn^{2+}]_i$) was determined to be 3 μM. However, the increase at this concentration was very small.

(Figure 3 near here)

3.5. Effects of $ZnCl_2$ and Zn^{2+} chelators on TBHQ-elicited elevation in $[Zn^{2+}]_i$

The cell suspension contained a small amount (216.9 ± 14.4 nM) of zinc from the cell preparation process.[23] To identify the source of Zn^{2+} responsible for the increase in intracellular Zn^{2+} levels by TBHQ, the effects of 3 μM $ZnCl_2$ and Zn^{2+} chelators (10 μM DTPA and 10 μM TPEN) on TBHQ-induced changes were examined (Fig. 3C). $ZnCl_2$ and Zn^{2+} chelators were added to the cell suspension 1 min before the application of TBHQ. The addition of 3 μM $ZnCl_2$ raised the control level of FluoZin-3 fluorescence, and 100 μM TBHQ further augmented FluoZin-3 fluorescence. The augmentation by TBHQ with $ZnCl_2$ was greater than that without $ZnCl_2$. Although the removal of external Zn^{2+} by DTPA slightly decreased the control level of FluoZin-3 fluorescence, TBHQ still augmented the FluoZin-3 fluorescence. The augmentation by TBHQ after removal of extracellular Zn^{2+} was less than that under control conditions. These results suggest that TBHQ releases intracellular Zn^{2+} and increases membrane Zn^{2+} permeability (Zn^{2+} influx), resulting in elevation of $[Zn^{2+}]_i$. Removal of intracellular Zn^{2+} by TPEN completely abolished the augmentation of FluoZin-3 fluorescence by TBHQ.

3.6. TBHQ-induced change in cellular GSH content

Intracellular release of Zn^{2+} is associated with oxidative stress that converts thiols to disulfide.[34,35] Therefore, the changes in cellular thiol content induced by TBHQ were examined using 5-CMF fluorescence, an indicator of GSH content. Treatment with TBHQ at 30–100 μM for 3 h profoundly shifted the histogram of 5-CMF fluorescence to the direction of lower intensity (Fig. 4A). Concentration-related change in the intensity of 5-CMF fluorescence is shown in Fig. 4B. Treatment with 10 μM TBHQ for 3 h slightly augmented 5-CMF fluorescence. Further increases in the concentration of TBHQ (30–100 μM) produced dose-dependent attenuation of 5-CMF fluorescence. The attenuation by TBHQ was statistically significant. Results suggest that TBHQ at 30–100 μM, but not at 10 μM, significantly decreased the cellular content of GSH.

(Figure 4 near here)

3.7. Effect of TBHQ on cells under oxidative stress induced by H_2O_2

Cellular thiols are cytoprotective against oxidative stress. Therefore, the decrease in cellular GSH content increases cell vulnerability to oxidative stress. Although TBHQ possesses antioxidant activity,[36] treatment with 100 μM TBHQ increased cell lethality (Fig. 1). This finding suggested the possibility that TBHQ might affect the cytotoxicity of H_2O_2. To test this hypothesis, we examined the effect of TBHQ (1–100 μM) on cells suffering from oxidative stress induced by 100 μM H_2O_2. The cells were simultaneously treated with H_2O_2 and TBHQ for 3 h. As shown in Fig. 5, the concentration–response of TBHQ-induced effects resembles a reversed bell shape. TBHQ at 10–30 μM attenuated the H_2O_2-induced increase in cell lethality by while it was significantly augmented by a dose of 100 μM.

(Figure 5 near here)

4. Discussion

4.1. Characteristics of cell preparation used in this study

Thymocytes were used for this model study of chemical cytotoxicity for the following reasons. Thymus is most active during neonatal and pre-adolescent periods and this organ begins to atrophy by early teens. It is also the case for rat.[37] Since many people are concerned about the adverse effects of compounds on the health of their children, the results obtained from thymocytes scientifically draw interest of people.

4.2. Cellular adverse actions of sublethal levels of TBHQ

Treatment with 30 μM TBHQ significantly augmented the intensity of side scatter of rat thymic lymphocytes (Fig. 2). It was shown that the staining with PI correlated with changes in forward and side scatter characteristics (decreased and increased intensity, respectively). These changes occurred because the dead cells stained with PI became smaller and more granular.[38] However, 30 μM TBHQ significantly increased side scatter intensity without affecting forward scatter in cells that were not stained with PI. It is not surprising that cytotoxic chemicals affect some cellular parameters in viable cells before or during cell death. In the presence of 30–100 μM TBHQ, exposure of PS on outer membrane surfaces of living cells was detected as augmented side scatter intensity (Fig. 2). Thus, TBHQ at higher concentrations may induce apoptosis in rat thymocytes.

TBHQ elevated $[Zn^{2+}]_i$ (Fig. 4). The threshold concentration of TBHQ to increase $[Zn^{2+}]_i$ (Fig. 4) was lower than those to augment the intensity of side scatter (Fig. 2) and to increase the number of cells expressing PS (Fig. 3). In the presence of $ZnCl_2$, TBHQ further elevated $[Zn^{2+}]_i$ (Fig. 4). Excessive increase in $[Zn^{2+}]_i$ has been reported to induce oxidative stress.[22,39] Since TBHQ greatly decreased the cellular content of GSH (Fig. 5), the ability to buffer intracellular Zn^{2+} seems to be lost. Therefore, the influx of Zn^{2+} in the presence of TBHQ may result in the elevation of $[Zn^{2+}]_i$, presumably leading to Zn^{2+}-dependent cytotoxicity.

The European Food Safety Authority estimated the exposure of TBHQ.[40] According to

the statement (regulatory maximum level exposure assessment scenario), the 95th percentile exposure of TBHQ as a food additive is 0.009–0.555 mg/kg body weight/day in infants (4–11 months), 0.084–0.798 mg/kg body weight/day in toddlers (12–35 months), 0.116–0.929 mg/kg body weight/day in children (3–9 years), and 0.040–0.420 mg/kg body weight/day in adolescents (10–17 years). Although it may be unrealistic to predict blood concentration of TBHQ from the assessment scenario, it is unlikely that TBHQ as a food additive affects thymocytes of infants–children since the minimum *in vitro* concentration of TBHQ to exhibit cellular actions was 3–10 μM (0.49–1.66 mg/L).

4.2. Contrasting actions of TBHQ

TBHQ is used as an antioxidant food additive. TBHQ exerts beneficial actions for medical purposes because reactive oxygen species are involved in many diseases.[41,42] As described in the Introduction, many experiments have been performed to prove the beneficial actions of TBHQ. However, in this study using rat thymocytes, TBHQ at 10–30 μM seems to significantly affect some cellular parameters (Figs. 2–5) related to cytotoxicity. TBHQ use resulted in a reversed bell shaped response in cells suffering from oxidative stress induced by 100 μM H_2O_2 (Fig. 6). TBHQ at 10–30 μM significantly reduced H_2O_2-induced cell lethality in a dose-dependent manner. This was not the case at a concentration of 100 μM TBHQ. Thus, a concentration of 10 μM TBHQ is appropriate to exert an antioxidant effect without affecting cell lethality. However, there is another explanation for the protective action of 30 μM TBHQ. The treatment with 100 μM H_2O_2 significantly increases the population of annexin V-positive living cells. TBHQ at 30 μM increased the population of annexin V-positive living cells. Therefore, the protective action of 30 μM TBHQ may be pseudo. TBHQ is supposed to delay the transition from annexin V-positive living cells (apoptotic living cells) and propidium-stained cells (dead cells).

4.3. Clinical consideration

From *in vitro* and *in vivo* studies ,[4–6,9,11] it is proposed that TBHQ is used as a therapeutic agent for brain disorders induced by oxidative stress and hemorrhage-associated brain injuries. In this *in vitro* study, the threshold concentration of TBHQ to induce possible adverse effects was 30 μM. Since the range of concentrations of TBHQ from benefit to toxicity under in vitro conditions may be 10–30 μM, it may be desirable that blood concentration of TBHQ does not exceed 1.66 mg/L.

There are experimental limitations on the interpretation on our results. Experiments were carried out within 5 h (short period) because of rat thymocytes that are spontaneously undergo apoptosis during prolong incubation.[24,25] Therefore, the primary use of present results would be in understanding cytotoxicity in primary thymocytes under these specific applications although the results may reveal new aspects of adverse TBHQ action.

Conflict of interest

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

Acknowledgements

This study was supported by Grant-in-Aids for Scientific Research (C26340039) from the Japan Society for the Promotion of Science.

References

1 World Health Organization, Evaluation of national assessments of intake of tert-butylhydroquinone, The International Programme on Chemical Safety, 1999, http://www.inchem.org/documents/jecfa/jecmono/v042je26.htm

2 European Food Safety Authority, Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commission related to tertiary-butylhydroquinone (TBHQ), *EFSA J.*, 2004, **84**, 1–50.

3 S. Kashanian and J.E.N. Dolatabadi, DNA binding studies of 2-tert-butylhydroquinone (TBHQ) food additive, *Food Chem.*, 2009, **116**, 743–747.

4 H. Hara, M. Ohta, K. Ohta, S. Kuno and T. Adachi, Increase of antioxidative potential by tert-butylhydroquinone protects against cell death associated with 6-hydroxydopamine-induced oxidative stress in neuroblastoma SH-SY5Y cells, *Molec. Brain Res.*, 2003, **119**, 125–131.

5 I. Tasset, D. Elinos-Calderón, P. Carrillo-Mora, I. González-Herrera, A. Luna-López, M. Konigsberg, J. Pedraza-Chaverrí, P.D. Maldonado, S.F. Ali, I. Túnez and A. Santamaría, Protective effect of tert-butylhydroquinone on the quinolinic-acid-induced toxicity in rat striatal slices: role of the Nrf2-antioxidant response element pathway, *Neurosignals*, 2009, **18**, 24–31.

6 J. Wu, M. Cheng, Q. Liu, J. Yang, S. Wu, X. Lu, C. Jin, H. Ma, and Y. Cai, Protective role of tert-butylhydroquinone against sodium fluoride-induced oxidative stress and apoptosis in PC12 cells, *Cell. Molec. Neurobiol.*, 2015, **35**, 1017–1025.

7 X. Duan, J. Li, W. Li, X. Xing, Y. Zhang, L. Zhao, S. Sun, X. Gao, and B. Li, Antioxidant tert-butylhydroquinone ameliorates arsenic-induced intracellular damages and apoptosis through induction of Nrf2-dependent antioxidant responses as well as stabilization of anti-apoptotic factor Bcl-2 in human keratinocytes, Free Radical Biol. Med., 2016, **94**, 74–87.

8 M.H. Abdel-Wahab, Testicular toxicity of dibromoacetonitrile and possible protection by

tertiary butylhydroquinone, *Pharmacol. Res.*, 2003, **47**, 509–515.

9 W. Jin, J. Kong, H. Wang, J. Wu, T. Lu, J. Jiang, H. Ni and W. Liang, Protective effect of tert-butylhydroquinone on cerebral inflammatory response following traumatic brain injury in mice, *Injury*, 2011, **42**, 714–718.

10 T. Li, K.J. Sun, H.D. Wang, M.L. Zhou, K. Ding, X.Y. Lu, W.T. We, C.X. Wang and X.M. Zhou, Tert-butylhydroquinone ameliorates early brain injury after experimental subarachnoid hemorrhage in mice by enhancing Nrf2-independent autophagy, *Neurochem. Res.*, 2015, **40**, 1829–1838.

11 S. Sukumari-Ramesh and C.H. Alleyne Jr, Post-injury administration of tert-butylhydroquinone attenuates acute neurological injury after intracerebral hemorrhage in mice. *J. Molec. Neurosci.*, 2016, **58**, 525–531.

12 L.F. Wang, S,W, Su, L. Wang, G.Q. Zhang, R. Zhang, Y.J. Niu, Y.S. Guo, C.Y. Li, Y. Jiang, and H.C. Guo, Tert-butylhydroquinone ameliorates doxorubicin-induced cardiotoxicity by activating Nrf2 and inducing the expression of its target genes, *Amer. J. Trans. Res.*, 2015, **7**, 1724–1735.

13 N. Gharavi, S. Haggarty and A.O. El-Kadi, Chemoprotective and carcinogenic effects of tert-butylhydroquinone and its metabolites. *Curr. Drug Metab.*, 2007, **8**, 1–7.

14 A. Braeuning, S. Vetter, S. Orsetti and M. Schwarz, Paradoxical cytotoxicity of tert-butylhydroquinone in vitro: What kills the untreated cells? *Arch. Toxicol.*, 2012, **86**, 1481–1487.

15 M. Eskandani, H. Hamishehkar and J. Ezzati Nazhad Dolatabadi, Cytotoxicity and DNA damage properties of tert-butylhydroquinone (TBHQ) food additive, *Food Chem.*, 2014, **153**, 315–320.

16 W. Barfield and B. Burlinson, *p*-Chloroaniline, *t*-butylhydroquinone, and methyl carbamate: Rat in vivo comet test, JaCVAM trial phase 4.2., *Mutat. Res. – Gene. Toxicol. Environ. Mutag.*, 2015, **786–788**, 98–103.

17 Y. Chen, S. Wang, X. Fu, W. Zhou, W. Hong, D. Zou, X. Li, J. Liu, P. Ran and B. Li, tert-Butylhydroquinone mobilizes intracellular-bound zinc to stabilize Nrf2 through inhibiting phosphatase activity, *Amer. J. Physiol. - Cell Physiol.*, 2015, **309**, C148–C158.

18 Y. Oyama, H. Matsui, M. Morimoto, Y. Sakanashi, Y. Nishimura, S. Ishida and Y. Okano, Synergic cytotoxic action induced by simultaneous application of zinc and clotrimazole in rat thymocytes, *Toxicol. Lett.*, 2007, **171**, 138–145.

19 M. Mittal, M.R. Siddiqui, K. Tran, S.P. Reddy and A.B. Malik, Reactive oxygen species in inflammation and tissue injury, *Antioxid. Redox Signaling*, 2014, **20**, 1126–1167.

20 H. Matsui, T.M. Oyama, Y. Okano, E. Hashimoto, T. Kawanai and Y. Oyama, Low micromolar zinc exerts cytotoxic action under H_2O_2-induced oxidative stress: excessive increase in intracellular Zn^{2+} concentration, *Toxicol.*, 2010, **276**, 27–32.

20 H. Matsui, Y. Sakanashi, Y. Nishimura, T. Kawanai, Y. Oyama, S. Ishida and Y. Okano, Effects of Zn^{2+} chelators, DTPA and TPEN, and $ZnCl_2$ on the cells treated with hydrogen peroxide: a flow-cytometric study using rat thymocytes. *Nat. Sci. Res. Tokushima Univ.*, 2009, **23**, 12–18.

21 T.M. Oyama, K. Oyama, T.B. Oyama, S. Ishida, Y. Okano and Y. Oyama, Zinc at clinically-relevant concentrations potentiates the cytotoxicity of polysorbate 80, a non-ionic surfactant, *Toxicol. In Vitro*, 2010, **24**, 737–744.

22 T.M. Oyama, M. Saito, T. Yonezawa, Y. Okano and Y. Oyama, Nanomolar concentrations of zinc pyrithione increase cell susceptibility to oxidative stress induced by hydrogen peroxide in rat thymocytes, *Chemosphere*, 2012, **87**, 1316–1322.

23 Y. Sakanashi, T.M. Oyama, Y. Matsuo, T.B. Oyama, Y. Nishimura, S. Ishida, S. Imai, Y. Okano and Y. Oyama, Y. (2009). Zn^{2+}, derived from cell preparation, partly attenuates Ca^{2+}-dependent cell death induced by A23187, calcium ionophore, in rat thymocytes, *Toxicol. In Vitro*, 2009, **23**, 338–345.

24 I.K. Poon, C.D. Lucas, A.G. Rossi and K.S. Ravichandran, Apoptotic cell clearance: basic

biology and therapeutic potential, *Nat. Rev. Immunol.*, 2014, 14, 166–180.

25 I. Rinner, P. Felsner, D. Hofer, A. Globerson and K. Schauenstein, Characterization of the spontaneous apoptosis of rat thymocytes in vitro. *Intl. Arch. Allergy Immunol.*, 1996, **111**, 230–237.

26 L. Chikahisa, Y. Oyama, E. Okazaki and K. Noda, Fluorescent estimation of H_2O_2-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. *Jpn. J. Pharmacol.*, 1996, **71**, 299–305.

27 G. Koopman, C.P. Reutelingsperger, G.A. Kuijten, R.M. Keehnen, S.T. Pals and M.H. Van Oers, Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis, *Blood*, 1994, **84**, 1415–1420.

28 K.R. Gee, Z.L. Zhou, W.J. Qian and R. Kennedy, Detection and imaging of zinc secretion from pancreatic beta-cells using a new fluorescent zinc indicator, *J. Amer. Chem. Soc.*, 2002, **124**, 776–778.

29 C.D. Bortner and J.A. Cidlowski, A necessary role for cell shrinkage in apoptosis. *Biochem. Pharmacol.*, 1998, **56**, 1549–1559.

30 J.Y. Koh, S.W. Suh, B.J. Gwag and Y.Y. He, The role of zinc in selective neuronal death after transient global cerebral ischemia, *Science*, 1996, **272**, 1013–1016.

31 A.Q. Truong-Tran, L.H. Ho, F. Chai and P.D. Zalewski, Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death, *J. Nutri.*, 2000, **130**, 1459S-1466S.

32 A. Kumari, K.P. Singh, A. Mandal, R.K. Paswan, P. Sinha, P. Das, V. Ali, S. Bimal and C.S. Lal, Intracellular zinc flux causes reactive oxygen species mediated mitochondrial dysfunction leading to cell death in Leishmania donovani, PLoS One, 2017, **12**, e0178800.

33 D. Guo, Y. Du, Q, Wu, W. Jiang and H. Bi, Disrupted calcium homeostasis is involved in elevated zinc ion-induced photoreceptor cell death, *Arch. Biochem. Biophys.*, 2014, **560**, 44–51.

34 W. Maret, Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange,

Proc. Natl. Acad. Sci. USA., 1994, **91**, 237–241.

35 W. Maret, Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals, Biometals, 2009, **22**, 149–157.

36 C. André, I. Castanheira, J.M. Cruz, P. Paseiro and A. Sanches-Silva, Analytical strategies to evaluate antioxidants in food: a review, *Trend. Food Sci. Technol.*, 2010, **21**, 229–246.

37 C.F. Kuper, R.B. Beems and V.M.H. Hollanders, Development and Aging, Thymus, Rat. In: T.C. Jones, J.M. Ward, U. Mohr and R.D. Hunt. (Eds) Hemopoietic System. Monographs on Pathology of Laboratory Animals. Springer, Berlin, Heidelberg, pp. 257–263, 1990.

38 N. Pick, S. Cameron, D. Arad and Y. Av-Gay, Screening of compounds toxicity against human monocytic cell line-THP-1 by flow cytometry. *Biol. Proced. Online*, 2004, **6**, 220–225.

39 B.K. Singh, A. Kumar, I. Ahmad, V. Kumar, D.K. Patel, S.K. Jain and C. Singh, Oxidative stress in zinc-induced dopaminergic neurodegeneration: implications of superoxide dismutase and heme oxygenase-1, *Free Radic. Res.*, 2011, **45**, 1207–1222.

40 European Food Safety Authority Panel on Food Additives and Nutrient Sources added to Food, Statement on the refined exposure assessment of tertiary-butyl hydroquinone (E 319), *EFSA J.*, 2016, **14**, 4363, 26 pp.

41 K. Sugamur and J.F. Keaney, Reactive oxygen species in cardiovascular disease. *Free Radic. Biol. Med.*, 2011, **51**, 978–992.

42 D.P. Rosanna and C. Salvatore, Reactive oxygen species, inflammation, and lung diseases, *Curr. Pharmaceut. Design*, 2012, **18**, 3889–3900.

Figure legends

Fig. 1 Changes in cell lethality and cell population caused by TBHQ. (A) TBHQ-induced change in proportion of cells classified with annexin V-FITC and PI. Effect of 100 μM TBHQ on fluorescent cytogram (PI fluorescence versus FITC fluorescence). N: Intact living cells, A: Annexin V-positive living cells, P: Annexin V-negative dead cells, and AP: Annexin V-positive dead cells. The cytogram was constructed with 2500 cells. (B) TBHQ-induced increase in the number of cells exhibiting PI fluorescence. Histogram was constructed with 2000 cells. Dotted line under the cytogram indicates cells stained with PI. (C) Change in cell proportions due to TBHQ. Column and bar indicate mean (%) and standard deviation of population of four samples. The experiment was repeated twice. Asterisks (**) indicate significant difference ($P < 0.01$) between control group (CONTROL) and groups of cells treated with TBHQ.

Fig. 2 Change in cytogram (forward scatter versus side scatter) due to TBHQ. (A) Cytograms in absence (left panel) and presence (right panel) of 100 μM TBHQ. The cytogram was constructed with 2500 cells. (B and C) Dose-dependent changes in forward and side scatter intensity due to TBHQ. Column and bar indicate mean and standard deviation of intensity of four samples. The experiment was repeated three times. Asterisks (**) indicate significant differences ($P < 0.01$) between control group (CONTROL) and groups of cells treated with TBHQ.

Fig. 3 TBHQ-induced change in FluoZin-3 fluorescence. (A) TBHQ-induced change in histogram of FluoZin-3 fluorescence. The histogram was constructed with 2500 cells. (B) TBHQ dose-dependent change in mean intensity of FluoZin-3 fluorescence. Column and bar indicate mean and standard deviation of intensity of four samples. The experiment was repeated twice. Asterisks (**) indicate significant difference ($P < 0.01$) between control group

(CONTROL) and groups of cells treated with TBHQ. (C) Effects of $ZnCl_2$, DTPA, and TPEN on the TBHQ-induced augmentation of FluoZin-3 fluorescence. Column and bar indicate mean and standard deviation of intensity of four samples. The experiment was repeated twice. Asterisks (**) indicate significant difference ($P < 0.01$) between control group and groups of cells treated with TBHQ in absence and presence of $ZnCl_2$, DTPA, or TPEN.

Fig. 4 TBHQ-induced change in 5-CMF fluorescence. (A) TBHQ-induced change in histogram of 5-CMF fluorescence. The histogram was constructed with 2500 cells. (B) TBHQ dose-dependent change in mean 5-CMF fluorescence intensity. Column and bar indicate mean and standard deviation of intensity of four samples. The experiment was repeated twice. Asterisks (**) indicate significant difference ($P < 0.01$) between control group (CONTROL) and the groups of cells treated with TBHQ.

Fig. 5 Change in lethality of cells treated with H_2O_2 and TBHQ. Column and bar indicate mean and standard deviation (%) of lethality of four samples. The experiment was repeated three times. Asterisks (**) indicate significant difference ($P < 0.01$) between control group and the groups of cells treated with TBHQ in the absence and presence of H_2O_2. Symbol (##) shows significant differences between the groups of cells treated with respective concentrations of TBHQ in the absence and presence of H_2O_2.

FIGURE 1

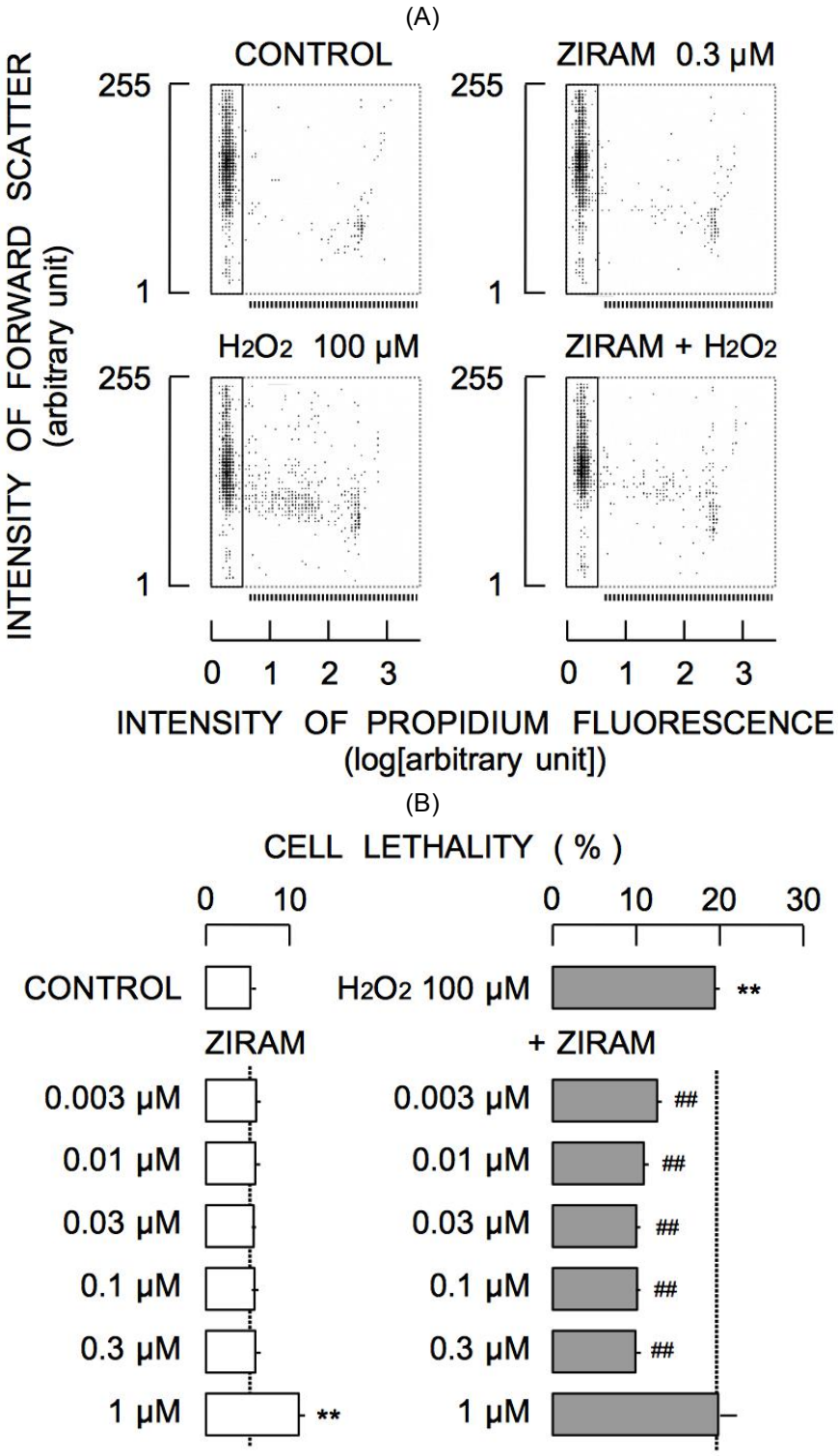
(A)
CONTROL
ZIRAM 0.3 μM
H2O2 100 μM
ZIRAM + H2O2
255
1
INTENSITY OF FORWARD SCATTER
(arbitrary unit)
0 1 2 3
INTENSITY OF PROPIDIUM FLUORESCENCE
(log[arbitrary unit])
(B)
CELL LETHALITY (%)
0 10
0 10 20 30
CONTROL
H2O2 100 μM
**
ZIRAM
+ ZIRAM
0.003 μM
0.01 μM
0.03 μM
0.1 μM
0.3 μM
1 μM
##

FIGURE 2

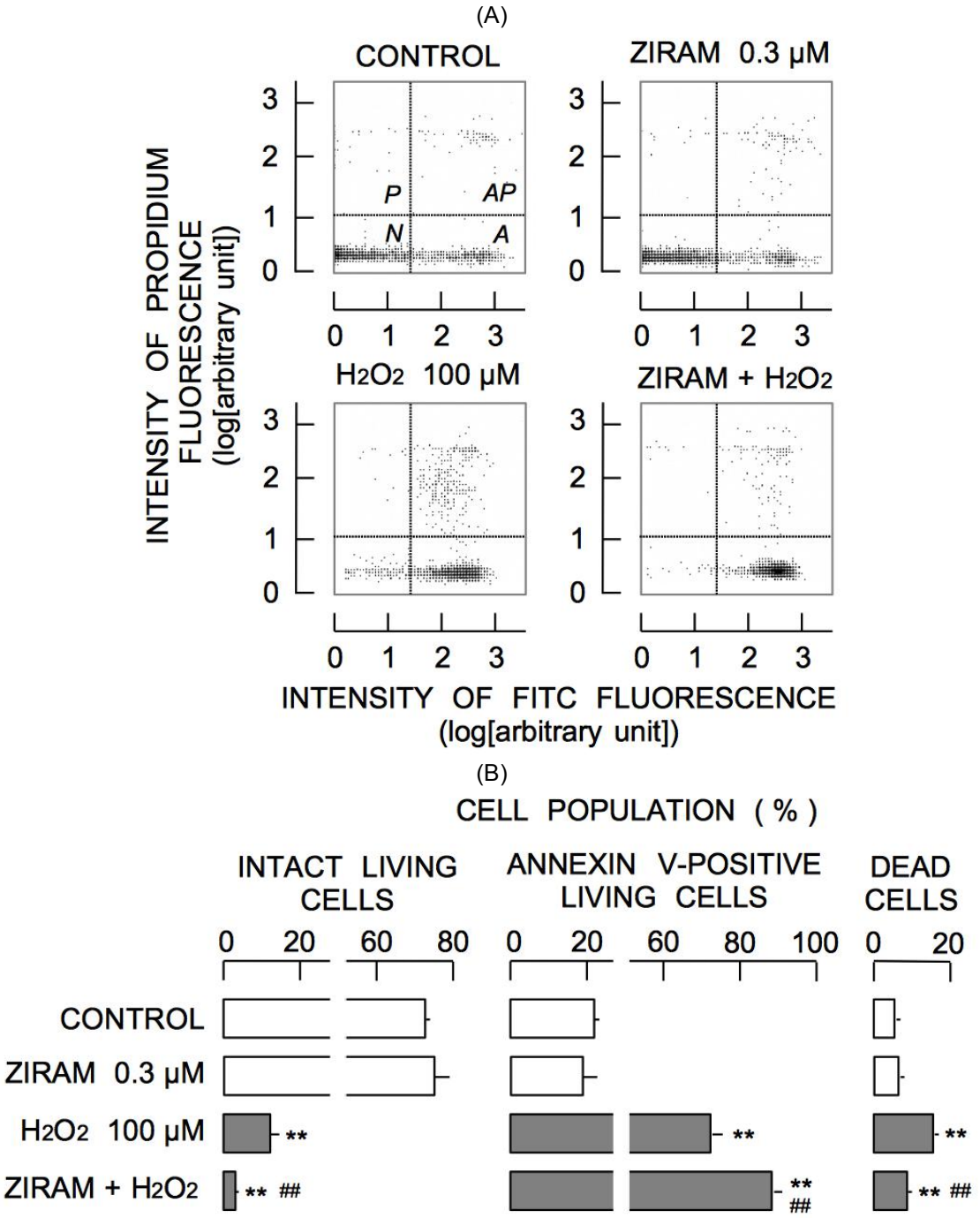
(A)
CONTROL
ZIRAM 0.3 μM
H2O2 100 μM
ZIRAM + H2O2
P
AP
N
A
INTENSITY OF PROPIDIUM FLUORESCENCE (log[arbitrary unit])
INTENSITY OF FITC FLUORESCENCE (log[arbitrary unit])
(B)
CELL POPULATION (%)
INTACT LIVING CELLS
ANNEXIN V-POSITIVE LIVING CELLS
DEAD CELLS
CONTROL
ZIRAM 0.3 μM
H2O2 100 μM
ZIRAM + H2O2
** ##

FIGURE 3

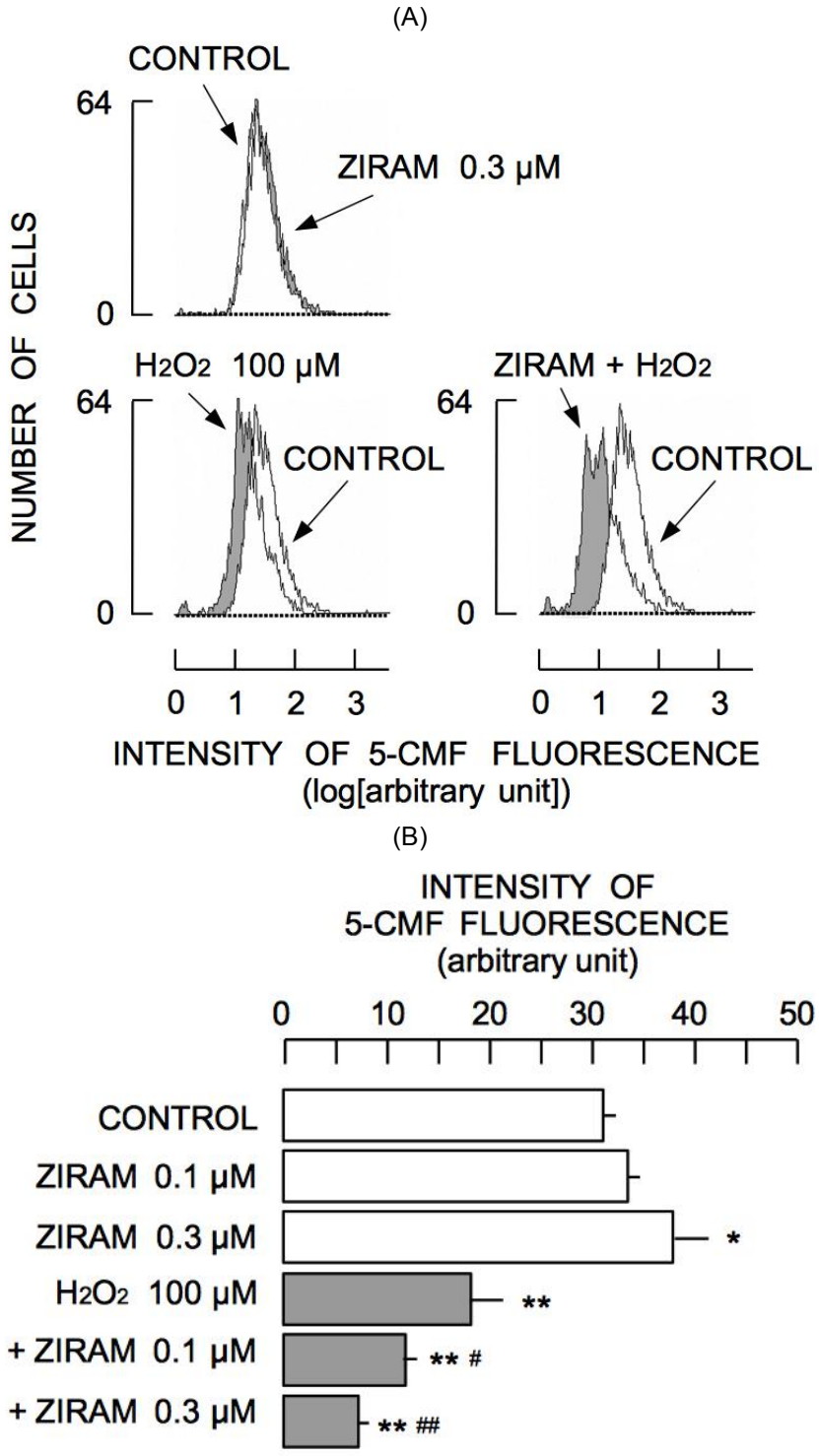
(A)
CONTROL
64
ZIRAM 0.3 μM
0
NUMBER OF CELLS
H2O2 100 μM
64
CONTROL
0
ZIRAM + H2O2
64
CONTROL
0
0 1 2 3
0 1 2 3
INTENSITY OF 5-CMF FLUORESCENCE
(log[arbitrary unit])
(B)
INTENSITY OF
5-CMF FLUORESCENCE
(arbitrary unit)
0 10 20 30 40 50
CONTROL
ZIRAM 0.1 μM
ZIRAM 0.3 μM
*
H2O2 100 μM
**
+ ZIRAM 0.1 μM
** #
+ ZIRAM 0.3 μM
** ##

FIGURE 4

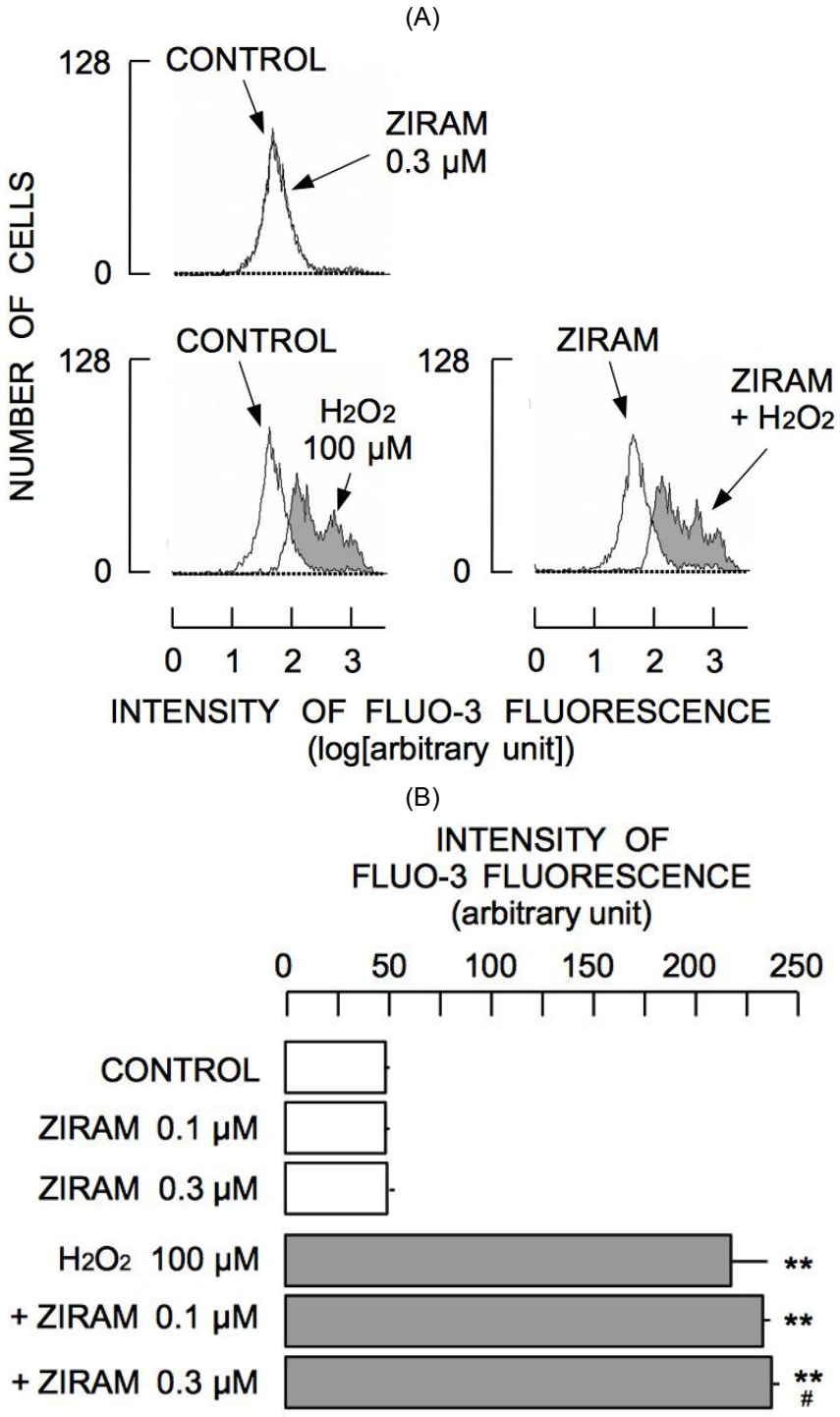
(A)
128
CONTROL
ZIRAM
0.3 μM
0
NUMBER OF CELLS
128
CONTROL
H2O2
100 μM
0
128
ZIRAM
ZIRAM
+ H2O2
0
0 1 2 3
0 1 2 3
INTENSITY OF FLUO-3 FLUORESCENCE
(log[arbitrary unit])
(B)
INTENSITY OF
FLUO-3 FLUORESCENCE
(arbitrary unit)
0 50 100 150 200 250
CONTROL
ZIRAM 0.1 μM
ZIRAM 0.3 μM
H2O2 100 μM
+ ZIRAM 0.1 μM
+ ZIRAM 0.3 μM
**
**
**
#

FIGURE 5

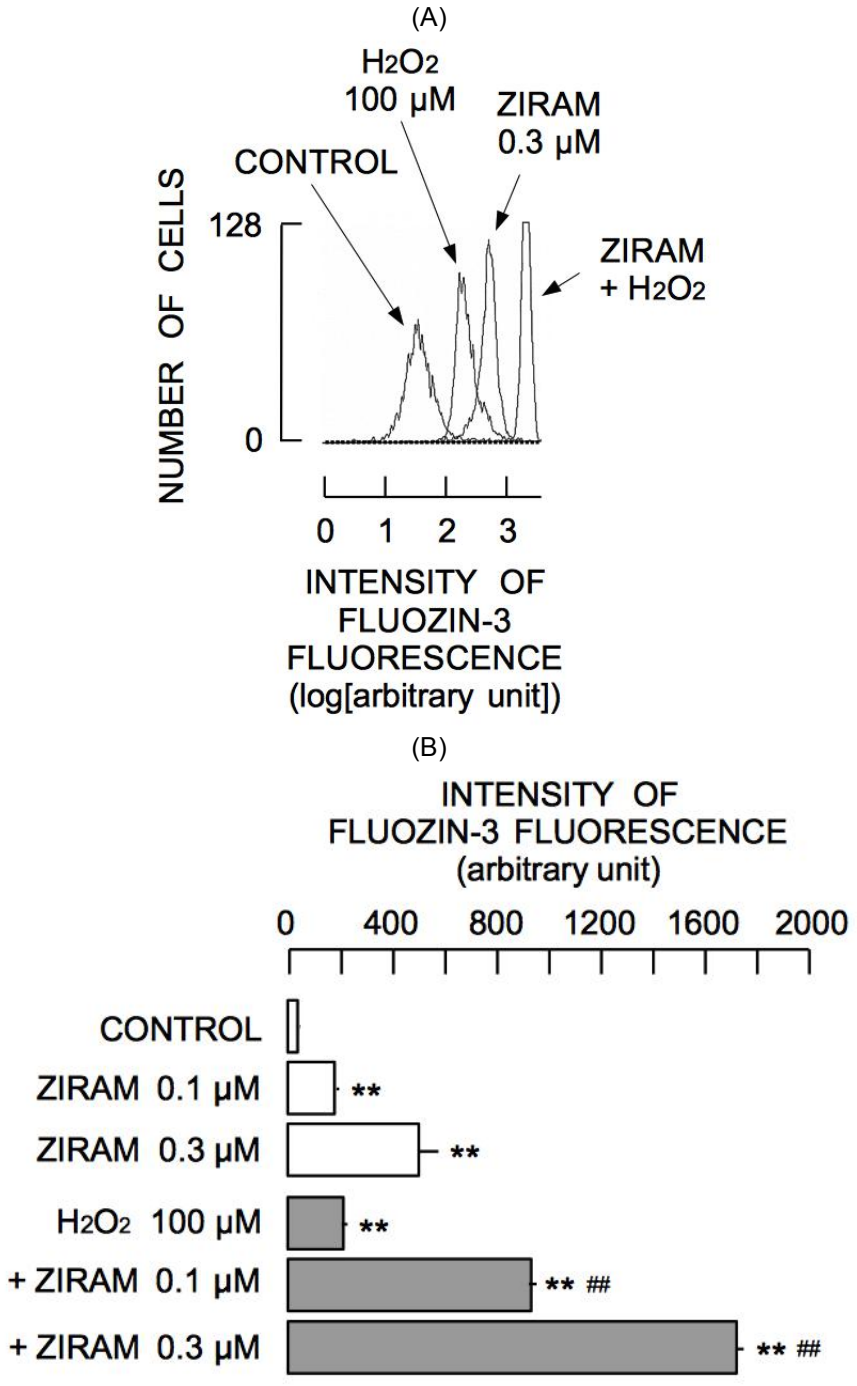
(A)
H2O2
100 μM
ZIRAM
0.3 μM
CONTROL
ZIRAM
+ H2O2
NUMBER OF CELLS
128
0
0 1 2 3
INTENSITY OF
FLUOZIN-3
FLUORESCENCE
(log[arbitrary unit])
(B)
INTENSITY OF
FLUOZIN-3 FLUORESCENCE
(arbitrary unit)
0 400 800 1200 1600 2000
CONTROL
ZIRAM 0.1 μM
ZIRAM 0.3 μM
H2O2 100 μM
+ ZIRAM 0.1 μM
+ ZIRAM 0.3 μM
**
**
**
** ##
** ##

FIGURE 6

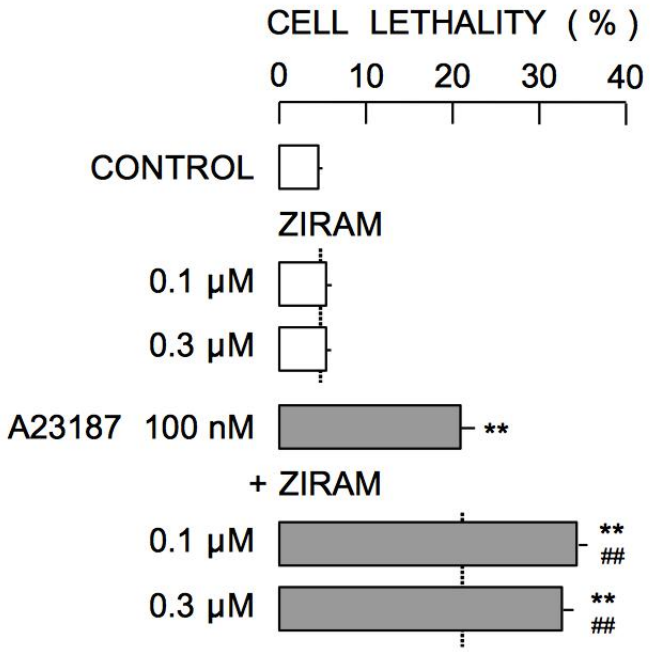

FIGURE 7

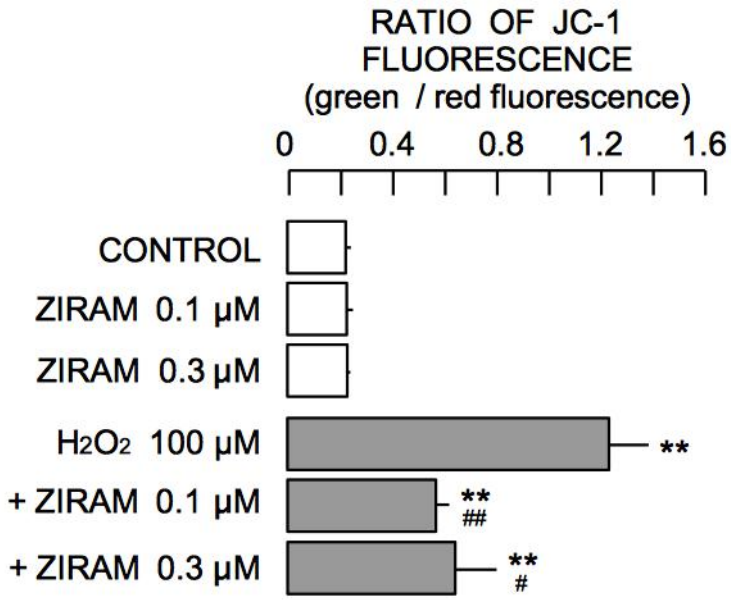

Table 1.

Fluorescent probes used in this study.

Fluorescent probes / Manufacture
Propidium iodide (PI) / Invitrogen (Eugene, OR, USA)
Annexin V-FITC / Invitrogen
FluoZin-3-AM / Invitrogen
Fluo-3-AM / Dojin Chemical Laboratory (Kumamoto, Japan)
5-Chloromethylfluorescein diacetate (5-CMF-DA) / Invitrogen
JC-1 / Invitrogen