J. Exp. Biomed. Sci. 11 (2005) 441-446

Role of Poly (ADP-ribose) Polymerase Activation in Chemical Hypoxia-Induced Cell Injury in Renal Epithelial Cells

Soon-Hee Jung†

Department of Clinical Pathology, Jinju Health College, Jinju 660-757, Korea

The molecular mechanism of ischemia/reperfusion injury remains unclear. Reactive oxygen species (ROS) are implicated in cell death caused by ischemia/reperfusion in vivo or hypoxia in vitro. Poly (ADP-ribose) polymerase (PARP) activation has been reported to be involved in hydrogen peroxide-induced cell death in renal epithelial cells. This study was therefore undertaken to evaluate the role of PARP activation in chemical hypoxia in opossum kidney (OK) cells. Chemical hypoxia was induced by incubating cells with antimycin A, an inhibitor of mitochondrial electron transport. Exposure of OK cells to chemical hypoxia resulted in a time-dependent cell death. In OK cells subjected to chemical hypoxia, the generation of ROS was increased, and this increase was prevented by the H_2O_2 scavenger catalase. Chemical hypoxia increased PARP activity and chemical hypoxia-induced cell death was prevented by the inhibitor of PARP activation 3-aminobenzamide. Catalase prevented OK cell death induced by chemical hypoxia. H_2O_2 caused PARP activation and H_2O_2-induced cell death was prevented by 3-aminobenzamide. Taken together, these results indicate that chemical hypoxia-induced cell injury is mediated by PARP activation through H_2O_2 generation in renal epithelial cells.

Key Words: Chemical hypoxia-induced cell injury, Poly (ADP-ribose), Renal epithelial cell, Opossum kidney cell

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radical cause DNA damage which can lead to loss of cell viability through several mechanisms. One of these involves activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) by DNA damage. Several studies have shown that an inhibitor of PARP, 3-aminobenzamide, prevents oxidant-induced cell death in renal proximal tubular cells (Chatterjee et al., 1999; Filipovic et al., 1999; Min et al., 2000; Park et al., 2003). However, the protective effect of PARP inhibitors against oxidant-induced cell injury is not universally acknowledged (Yamamoto and Farber, 1992). Yamamoto et al. (Yamamoto et al., 1993) observed that the PARP inhibitors in hepatocytes did not affect the cell death induced by tBHP and H_2O_2. Studies with opossum kidney (OK) and LLC-PK_1 cells have shown that H_2O_2-induced cell death was prevented by 3-aminobenzamide, but that tBHP-induced death was not affected by the inhibitor (Jung et al., 2000; Min et al., 2000; Park et al., 2003). Similar results are reported by other investigators in LLC-PK_1 cells (Filipovic et al., 1999) and rabbit proximal tubular cells (Schnellmann et al., 1993).

Previous studies in renal epithelial cells have shown that the DNA damage is responsible for cell death-induced hypoxia/reperfusion (Ueda et al., 1995) and chemical hypoxia (Hagar et al., 1996). These effects resulted from ROS generation. However, it is unclear whether PARP activation is involved in chemical hypoxia-induced cell death. The present study was carried out to examine the role of PARP activation in chemical hypoxia-induced alterations in OK cells, an established proximal tubular cell line.

*Received: November 28, 2005
Accepted after revision: December 12, 2005
†Corresponding author: Soon-Hee Jung, Department of Clinical Pathology, Jinju Health College, Jinju 660-757, Korea.
Tel: 055-740-1847, Fax: 055-740-1846
e-mail: jungsh740@hanmail.net

MATERIALS AND METHODS

1. Culture of OK cells

OK cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained by serial passa-

ges in 75-cm^2 culture flasks (Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium/ Ham's F12 (DMEM/F12, Sigma Chemical Co.) containing 10% fetal bovine serum (FBS) at 37℃ in 95% air/5% CO_2 incubator. When the cultures reached confluence, a subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on 24-well tissue culture plates in DMEM/F12 medium containing 10% FBS. All experiments started 3~4 days after plating when a confluent monolayer culture was achieved. Cells were treated with antimycin A in a medium without serum.

2. Induction of chemical hypoxia

Chemical hypoxia in cultured cells was induced by incubating cells with antimycin A, the inhibitor of mitochondrial electron transport, in a glucose-free medium as previously described (Hagar et al., 1996). The composition of the incubation medium was 115 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 2 mM NaH_2PO_4, 1 mM $MgSO_4$, and 1 mM $CaCl_2$ (pH 7.4). Following the incubation, cell viability and lipid peroxidation were measured as described below. Unless otherwise stated, cells were treated with 20 μM antimycin A for 120 min at 37℃.

3. Measurement of cell viability

The viability in cultured cells was determined by trypan blue exclusion assay. Cells were harvested using 0.025% trypsin, incubated with 4% trypan blue solution, and counted using a hemocytometer under light microscope. Cells failing to exclude the dye were considered nonviable, and the number of nonviable cells was expressed as a percentage of the total cells.

4. Measurement of ROS production

The intracellular generation of ROS in OK cells was measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Bass et al., 1983; Lebel et al., 1992; Rosenkranz et al., 1992). The nonfluorescent ester penetrates into the cells and is hydrolyzed to DCFH by the cellular esterases. The probe (DCFH) is rapidly oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF) in the presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Cells were cultured in 35-mm tissue culture petri dishes. The culture medium was removed, and the cells were collected from flasks using trypsin-EDTA solution. The cells were washed twice with DMEM/F12, and were suspended in glucose-free HBSS for fluorescence analysis. The reaction was carried out in a fluorescent cuvette. The cells were preincubated for 10 min at 37℃ in a fluorescent cuvette containing 3 ml of glucose-free HBSS with 20 μM DCFH-DA (from a stock solution of 20 mM DCFH-DA in ethanol). After the preincubation, the cells were treated with antimycin A and incubated for up to 120 min during which the fluorescent intensity was monitored on a spectrofluorometer (SPEX1681, SPEX Co., USA) with excitation wave length 485 nm and emission wave length 530 nm. The net increase in DCF fluorescence (arbitrary units) was calculated by taking the difference between the values before and after antimycin A addition.

5. Chemicals

Antimycin A, hydrogen peroxide (H_2O_2), and 3-aminobenzamide were purchased from Sigma Chemical (St. Louis, MO). DCFH-DA was obtained from Molecular Probes (Eugene, OR, USA). All other chemicals were of the highest commercial grade available.

6. Statistical analysis

Data are expressed as mean ± SEM. Comparison between two groups was made using the unpaired t test. Multiple group comparison was done using one-way analysis of variance followed by the Dunnett's test. $P<0.05$ was considered statistically significant.

RESULTS

1. Time course of cell death in OK cells subjected to chemical hypoxia

In order to determine the time course of antimycin A-induced cell injury, OK cells were exposed to 20 μM antimycin A, and cell viability was determined at various time points (0~120 min). Antimycin A increased the loss of cell viability in a time-dependent manner. The loss of cell viability had reached significant level at 30 min after exposure of cells to antimycin A, with cell death increasing up to 120 min (Fig. 1).

2. ROS generation in cells exposed to chemical hypoxia

In the next series of experiments, the production of ROS

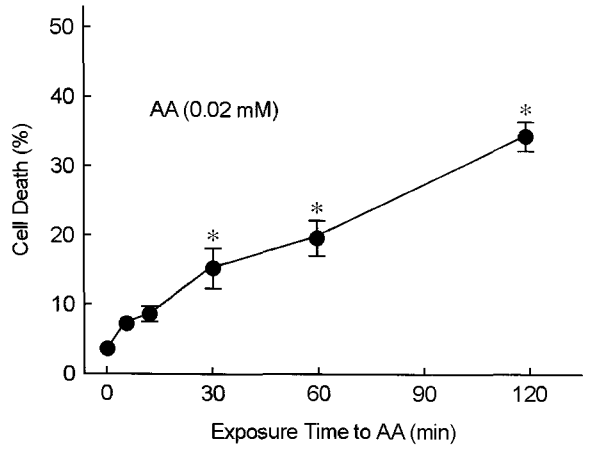

Fig. 1. Time courses of cell death in OK cells subjected to chemical hypoxia. Cells were exposed to 20 μM antimycin A (AA) for 0~120 min before measurement of cell death. Data are mean ± SE of four experiments. *P<0.05 compared with zero (0) time.

in antimycin A-treated OK cells using DCFH-DA was examined. Exposure of cells to antimycin A induced an increase in ROS generation in a time-dependent manner (Fig. 2A). The effect was significantly reduced by the addition of catalase, a scavenger of hydrogen peroxide (Fig. 2B). This may support the notion that hydrogen peroxide is the principle ROS responsible for the oxidation of DCFH to DCF (Bass et al., 1983; Frenkel and Gleichauf, 1991). Catalase did not affect DCF fluorescence in control cells (data not shown).

3. Role of PARP activation in chemical hypoxia-induced cell death

To determine the role of PARP activation in chemical hypoxia-induced cell death, the effect of 3-aminobenzamide, an inhibitor of PARP activation was examined. The addition of 3-aminobenzamide prevented antimycin A-induced cell death dose-dependently (Fig. 3). Indeed, antimycin A induced PARP activation and the effect was prevented by 3-aminobenzamide (Fig. 4). These results indicate that PARP activation is responsible for the antimycin A-induced cell death.

4. Role of ROS in chemical hypoxia-induced cell death

Although antimycin A induced ROS generation, it is unclear if the ROS generation is involved in antimycin A-induced cell death. To test the possibility, the effects of scavengers of hydrogen peroxide were examined in cells exposed to antimycin A. As shown in Fig. 5, two scaven-

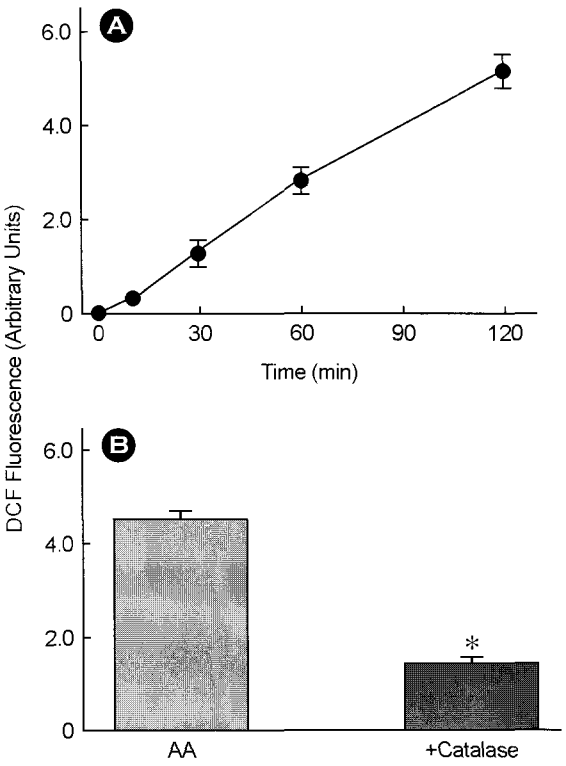

Fig. 2. A, Time course of the generation of reactive oxygen species in OK cells during exposure to 20 μM antimycin A (AA). The net increase in DCF fluorescence (arbitrary units) shown was calculated by subtracting the values for the control cells from the corresponding values for AA-treated cells. Data are mean ± SE of five experiments. **B,** Effect of catalase on chemical hypoxia-induced increase in DCF fluorescence in OK cells. Cells were exposed to 20 μM AA for 120 min in the presence and absence of 800 units/ml catalase. Data are mean ± SE of four experiments. *P<0.05 compared with AA alone.

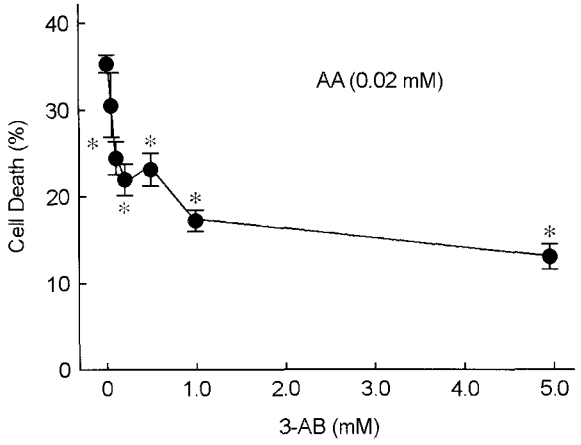

Fig. 3. Effect of an inhibitor of PARP activation superoxide dismutase (SOD), catalase (Cat), and dimethylthiourea (DMTU) on chemical hypoxia-induced cell death in OK cells. Cells were exposed to 20 μM antimycin A (AA) for 120 min in the presence and absence of 5 mM 3-aminobenzamide (3-AB). Data are mean ± SE of five experiments. *P<0.05 compared with control (absence of 3-AB).

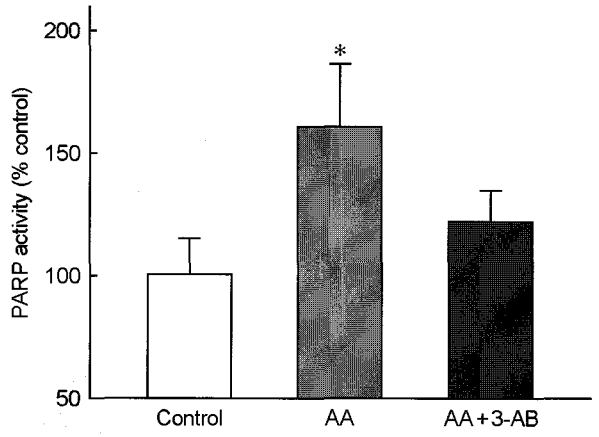

Fig. 4. Effect of chemical hypoxia on PARP activation in OK cells. Cells were exposed to 20 μM antimycin A (AA) for 120 min in the presence and absence of 5 mM 3-aminobenzamide (3-AB). Data are mean ± SE of four experiments. *P<0.05 compared with control.

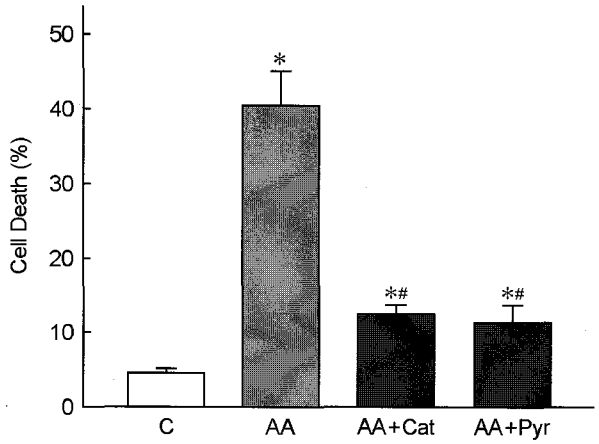

Fig. 5. Effects of the hydrogen peroxide scavengers on chemical hypoxia-induced cell death in OK cells. Cells were exposed to 20 μM antimycin A (AA) for 120 min in the presence and absence of 800 Units/ml catalase (Cat) and 10 mM pyruvate (Pyr). Data are mean ± SE of four experiments. *P<0.05 compared with control (C); #P<0.05 compared with AA alone.

gers of hydrogen peroxide, catalase and pyruvate, significantly prevented the antimycin A-induced cell death.

The last series of experiments investigated whether H_2O_2 induces directly PARP activation and whether H_2O_2-induced cell death results from the PARP activation. As expected, H_2O_2 caused PARP activation and H_2O_2-induced cell death was prevented by 3-aminobenzamide (Fig. 6).

DISCUSSION

Despite numerous experimental and clinical studies on

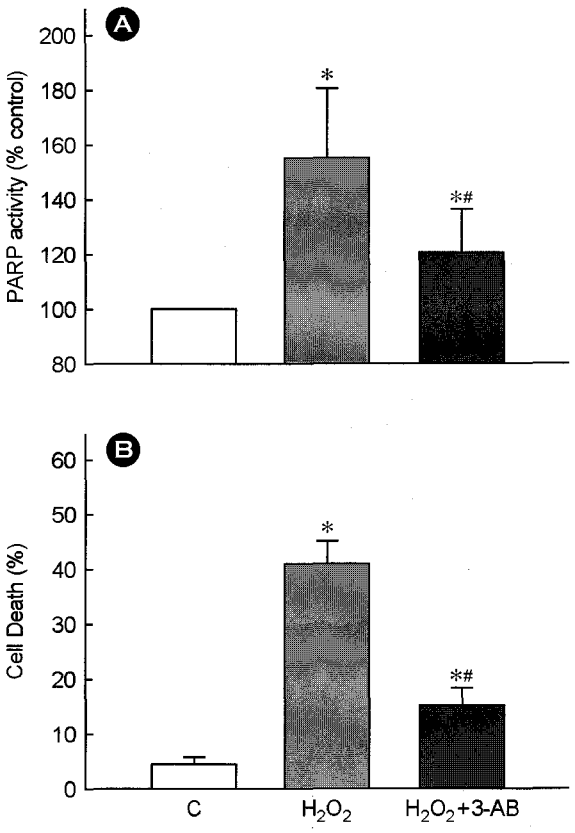

Fig. 6. Effect of H_2O_2 on PARP activation (A) and cell death (B) in OK cells. Cells were exposed to 0.5 mM H_2O_2 for 120 min in the presence or absence of 5 mM 3-aminobenzamide (3-AB). Data are mean ± SE of three experiments. *P<0.05 compared with control (C); #P<0.05 compared with H_2O_2 alone.

renal ischemia, the mechanisms underlying renal dysfunction following ischemia have not been clearly understood. It has been proposed that ROS play a critical role in renal cell injury induced by hypoxia in vitro (Paller and Neumann 1991), by ischemia in vivo (Bratell et al., 1990; Linas et al., 1987; Paller et al., 1984), and by chemical hypoxia (Kim et al., 2002). Although PARP activation has been reported to be involved in H_2O_2-induced cell death to renal epithelial cells (Min et al., 2000; Park et al., 2003), the role of PARP activation in chemical hypoxia-induced cell death remains to be defined. Therefore, the present study was undertaken to investigate the role of PARP activation in chemical hypoxia-induced cell injury in OK cells.

The results of the present study demonstrated that chemical hypoxia induces cell death and generation of ROS in OK cells. Exposure of cells to antimycin A induced a significant increase in DCF fluorescence (Fig. 2A), which was inhibited by the hydrogen peroxide scavenger catalase (Fig

2B). These results are consistent with the hypothesis that hydrogen peroxide is the principle ROS responsible for the oxidation of DCFH to DCF (Carter et al., 1994; Frenkel and Gleichauf, 1991). In addition, these data suggest that ROS production is involved in chemical hypoxia-induced cell death in OK cells.

Chemical hypoxia-induced cell death was prevented by an inhibitor of PARP activation, 3-aminobenzamide (Fig. 3). These results implicate the participation of PARP activation in the cell injury. Indeed, PARP activity was increased in cells exposed to antimycin and its effect was inhibited by 3-aminobenzamide (Fig. 4).

Previous studies in OK cells (Min et al., 2000; Park et al., 2003) have shown that the cell death induced by inorganic hydroperoxide H_2O_2 was not affected by potent antioxidants such as N,N'-diphenyl-p-phenylenediamine (DPPD) and butylated hydroxyanisole, while the cell death induced by the organic hydroperoxide t-butylhydroperoxide (tBHP) was effectively prevented by these antioxidants. The PARP inhibitor 3-aminobenzamide (3-AB) prevented the cell death induced by H_2O_2, but not that by tBHP. The PARP activity was increased in cells exposed to H_2O_2 but not tBHP. These results indicate that the H_2O_2-induced cell death in cultured renal epithelial cells is associated with PARP activation but not lipid peroxidation, whereas the tBHP-induced cell death is mediated by lipid peroxidation. Based on these data, H_2O_2 may induce PARP activation in cells exposed to chemical hypoxia and subsequently lead to cell death. To test this possibility, the effect of the H_2O_2 scavengers on antimycin A-induced cell death was examined. The cell death was prevented by catalase and pyruvate (Fig. 5). H_2O_2 also induced cell death dependent of PARP activation (Fig. 6). These results indicate that H_2O_2 is involved in chemical hypoxia-induced cell death through PARP activation in OK cells.

In summary, chemical hypoxia with antimycin A caused cell death, which was accompanied by an increase in ROS generation and PARP activation. Chemical hypoxia-induced cell death was prevented by 3-aminobenzamide, an inhibitor of the PARP activation, and the two H_2O_2 scavengers, catalase and pyruvate. These results indicate that the PARP activation through H_2O_2 generation is involved in chemical hypoxia-induced cell death in renal epithelial cells.

Acknowledgments

This research was supported by Jinju Health College Grant in 2005.

REFERENCES

Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol. 1983. 130: 1910-1917.

Bratell S, Haraldsson G, Herlitz H, Jonsson O, Pettersson S, Schersten T, Waldenstrom J. Protective effects of pretreatment with superoxide dismutase, catalase and oxypurinol on tubular damage caused by transient ischaemia. Acta Physiol Scand. 1990. 139: 417-425.

Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. J Leukoc Biol. 1994. 55: 253-258.

Chatterjee PP, Cuzzocrea S, Thiemermann C. Inhibitors of poly (ADP-ribose) synthetase protect rat proximal tubular cells against oxidant stress. Kidney Int. 1999. 56: 973-984.

Filipovic DM, Meng X, Reeves WB. Inhibition of PARP prevents oxidant-induced necrosis but not apoptosis in LLC-PK1 cells. Am J Physiol. 1999. 277: F428-F436.

Frenkel K, Gleichauf C. Hydrogen peroxide formation by cells treated with a tumor promoter. Free Radic. Res Commun. 1991. 12-13: 783-794.

Hagar H, Ueda N, Shah SV. Role of reactive oxygen metabolites in DNA damage and cell death in chemical hypoxic injury to LLC-PK1 cells. Am J Physiol. 1996. 271: F209-F215.

Jung JS, Park MY, Lee TH, Jun JS, Kim YK. Protection against hydrogen peroxide-induced injury in renal proximal tubule cell lines by inhibition of poly (ADP-ribose) synthase. Kidney Blood Press Res. 2000. 23: 14-19.

Kim YK, Lee SK, Ha MS, Woo JS, Jung JS. Differential role of reactive oxygen species in chemical hypoxia-induced cell injury in opossum kidney cells and rabbit renal cortical slices. Exp Nephrol. 2002. 10: 275-284.

Lebel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol. 1992. 5: 227-231.

Linas SL, Whittenburg D, Repine JE. O2 metabolites cause reperfusion injury after short but not prolonged renal ischemia. Am J Physiol. 1987. 253: F685-F691.

Min SK, Kim SY, Kim CH, Woo JS, Jung JS, Kim YK. Role of lipid peroxidation and poly (ADP-ribose) polymerase activation in oxidant-induced membrane transport dysfunction in opossum kidney (OK) cells. Toxicol Appl. Pharmacol. 2000. 166: 196-202.

Paller MS, Hoidal JR, Ferris TF. Oxygen free radicals in ischemic acute renal failure in the rat. J Clin Invest. 1984. 74: 1156 -1164.

Paller MS, Neumann TV. Reactive oxygen species and rat renal epithelial cells during hypoxia and reoxygenation. Kidney Int. 1991. 40: 1041-1049.

Park SM, Jung HC, Koak IS, Na HY, Woo JS, Jung JS, Kim YK. Oxidant-induced cell death in renal epithelial cells: Differential effects of inorganic and organic hydroperoxides. Pharmacol Toxicol. 2003. 92: 43-50.

Rosenkranz AR, Schmaldienst S, Stuhlmeier KM, Chen W, Knapp W, Zlabinger GJ. A microplate assay for the detection of oxidative products using 2',7'-dichlorofluorescin-diacetate. J Immunol Methods. 1992. 156: 39-45.

Rush GF, Gorski JR, Ripple MG, Sowinski J, Bugelski P, Hewitt WR. Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. Toxicol Appl Pharmacol. 1985. 78: 473-483.

Schnellmann RG, Swagler AR, Compton MM. Absence of endonuclease activation during acute cell death in renal proximal tubules. Am J Physiol. 1993. 265: C485-C490.

Ueda N, Walker PD, Hsu SM, Shah SV. Activation of a 15-kDa endonuclease in hypoxia/reoxygenation injury without morphologic features of apoptosis. Proc Natl Acad Sci U.S.A. 1995. 92: 7202-7206.

Yamamoto K, Farber JL. Metabolism of pyridine nucleotides in cultured rat hepatocytes intoxicated with tert-butyl hydroperoxide. Biochem Pharmacol. 1992. 43: 1119-1126.

Yamamoto K, Tsukidate K, Farber JL. Differing effects of the inhibition of poly (ADP-ribose) polymerase on the course of oxidative cell injury in hepatocytes and fibroblasts. Biochem Pharmacol. 1993. 46: 483-491.