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Roles of Necrosis, Apoptosis, and Mitochondrial Dysfunction in *S*-(1,2-Dichlorovinyl)-L-Cysteine Sulfoxide-Induced Cytotoxicity in Primary Cultures of Human Renal Proximal Tubular Cells

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Running Title:

S-(1,2-Dichlorovinyl)-L-Cysteine Sulfoxide in Human Kidney

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Abbreviations: β-lyase, cysteine conjugate β-lyase; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DCVCS, DCVC sulfoxide; DMEM:F12, Dulbecco's Modified Eagle's Medium : Ham's F12; FACS, flow activated cell sorter; FITC, fluorescein isothiocyanate; FMO, flavin-containing monooxygenase; GSH, reduced glutathione; HPLC, high-pressure liquid chromatography; hPT, human proximal tubular; IARC, International Agency for Research on Cancer; LDH, lactate dehydrogenase; NTP, National Toxicology Program; PBS, phosphate-buffered saline; rPT, rat proximal tubular; TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine; TRI, trichloroethylene.

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ABSTRACT

S-(1,2-Dichlorovinyl)-L-cysteine (DCVC) is the penultimate nephrotoxic metabolite of the environmental contaminant trichloroethylene. Although metabolism of DCVC by the cysteine conjugate β-lyase is the most studied bioactivation pathway, DCVC may also be metabolized by the flavin-containing monooxygenase (FMO) to yield DCVC sulfoxide (DCVCS). Renal cellular injury induced by DCVCS was investigated in primary cultures of human proximal tubular (hPT) cells by assessment of time- and concentration-dependent effects on cellular morphology, acute cellular necrosis, apoptosis, mitochondrial function, and cellular GSH status. Confluent hPT cells incubated with as little as 10 μM DCVCS for 24 hr exhibited morphological changes, although at least 100 μM DCVCS was required to produce marked changes. Acute cellular necrosis did not occur until 48 hr with at least 200 μM DCVCS, indicating that this is a high-dose, late response. The extent of necrosis was similar to that with DCVC. In contrast, apoptosis occurred as early as 1 hr with as little as 10 μM DCVCS and the extent of apoptosis was much less than that with DCVC. Mitochondrial function was maintained with DCVCS concentrations up to 100 μM, consistent with hPT cells only being competent to undergo apoptosis at early time points and relatively low concentrations. Marked depletion (> 50%) of cellular GSH content was only observed with 500 μM DCVCS. These results, combined with earlier studies showing protection from DCVC-induced necrosis and apoptosis by the FMO inhibitor methimazole, suggest that formation of DCVCS plays a significant role in trichloroethylene-induced renal cellular injury in hPT cells.

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Trichloroethylene (TRI; also known as trichloroethene) is a major environmental contaminant that is of concern for both workers who use it, primarily in metal degreasing, and for the general population, because of widespread contamination of soil and surface and ground water. TRI produces acute toxicity or tumors in several tissues, with the target organ specificity and sensitivity exhibiting species-, strain-, and sex-dependent differences. Based on the overall weight of evidence, the International Agency for Research on Cancer (IARC, 1995) designated TRI as a "probable human carcinogen" and the National Toxicology Program (NTP, 2001) listed TRI in its Ninth Report on Carcinogens as "reasonably anticipated to be a human carcinogen."

Most TRI toxicity is associated with its metabolism, which occurs by either cytochrome P450-dependent oxidation or glutathione (GSH) conjugation (Lash et al., 2000a). One of the established target organs for TRI is the kidneys and renal toxicity is associated with metabolism by the GSH conjugation pathway (Lash et al., 2000b). *S*-(1,2-Dichlorovinyl)-L-cysteine (DCVC), the cysteine conjugate of TRI, was initially considered to be the primary, if not sole, penultimate nephrotoxic metabolite that produces renal cellular injury after bioactivation by the cysteine conjugate β-lyase (β-lyase). However, DCVC can also be metabolized by the flavin-containing monooxygenase (FMO) to produce DCVC sulfoxide (DCVCS) (Krause et al. 2003; Ripp et al., 1997; Sausen and Elfarra, 1990), which is highly reactive and is a potent nephrotoxicant in rats and is cytotoxic in isolated rat proximal tubular (rPT) cells (Lash et al., 1994).

Isolated rPT cells are susceptible to DCVC-induced necrosis at relatively high doses (i.e., > 0.2 mM) (Cummings et al., 2000a, 2000b; Lash and Anders, 1986; Lash et al., 1995a, 2001a, 2001b). Primary cultures of rPT cells (van de Water et al., 1996, 1999) and LLC-PK_1 cells (Chen et al., 2001) also undergo apoptosis when exposed to DCVC. The initial step in the GSH conjugation pathway for TRI occurs at comparable rates in both rodent (Lash et al., 1995a, 1998) and human (Lash et al., 1999) kidney and liver. β-Lyase activity, however, is much higher in rat kidney than in human kidney (Lash et al., 1990). While this might be interpreted as meaning that bioactivation of DCVC is much greater in rat kidneys than in human kidneys and that rats are

much more susceptible to DCVC-induced renal injury than humans, the role of FMO-dependent bioactivation and toxicity in human kidney has not been previously considered.

Freshly isolated human proximal tubular (hPT) cells undergo necrosis when incubated with relatively high concentrations (> 200 μM) of DCVC (Cummings and Lash, 2000) but primary cultures of hPT cells can also undergo apoptosis at relatively low concentrations of DCVC (i.e., • 100 μM) and at relatively early incubation times (i.e., • 8 hr). Comparison of the potency of DCVC and DCVCS in rats shows that the sulfoxide is the more potent nephrotoxicant both *in vivo* and *in vitro*. The potency of DCVCS raises the question of the role of this reactive species in the nephrotoxicity of TRI and DCVC. Although studies with selective inhibitors of both the β-lyase and FMO suggested that the β-lyase plays a more prominent role in DCVC-induced cytotoxicity in rPT cells (Lash et al., 1994), FMO appears to play the more prominent role in bioactivation in hPT cells (Cummings and Lash, 2000; Lash et al., 2001a). This apparent discrepancy highlights the difficulty of extrapolating data from rodents to humans, and is a major issue in human health risk assessment for chemicals such as TRI (Maull and Lash, 1998). Furthermore, this suggests that FMO-dependent bioactivation may account for some of the species-dependent differences in the nephrotoxicity of TRI and DCVC. We recently showed that human kidney expresses multiple isoforms of FMO (Krause et al., 2003), supporting the potential for this pathway to function in hPT cells.

In the present study, the importance of the FMO-dependent bioactivation pathway in human kidney was investigated further by studying mechanisms of renal cellular injury in primary cultures of hPT cells induced by DCVCS. The results show that DCVCS is a potent cytotoxicant in hPT cells, inducing acute cellular necrosis and apoptosis. Although the pattern of cellular injury in hPT cells induced by DCVCS and DCVC differs somewhat, the results support the conclusion that FMO-dependent bioactivation of DCVC, along with the β-lyase, plays an important role in TRI- and DCVC-induced renal cellular injury in the human kidney.

Methods

Chemicals. TRI (reported to be 98.6% pure, as judged by electron impact ionization mass spectrometry), collagenase type I (EC 3.4.24.3), ascorbate, TMPD, and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). Annexin V-fluorescein isothiocyanate (FITC) was purchased from BD PharMingen (San Diego, CA). DCVC was first synthesized as described previously (Elfarra et al., 1986) by reacting TRI with L-cysteine in sodium and liquid ammonia. DCVCS was then made by reacting DCVC with H_2O_2, as described previously (Ripp et al., 1997). Purity (> 95%) was determined by high-pressure liquid chromatography (HPLC) analysis, and identity was confirmed by proton nuclear magnetic resonance spectroscopy and mass spectrometry. Impurities were likely degradation products of TRI and cysteine and are unlikely to contribute significantly to the observed cytotoxic responses. As it is very laborious and impractical to try and improve purity of the DCVCS, no additional purification was attempted.

Isolation of hPT cells from human kidneys. hPT cells were derived from human kidney cortical slices obtained from the Human Tissue Resources Core of Wayne State University (Department of Pathology, Harper Hospital, Detroit, MI) or from whole human kidneys procured by International Bioresearch Solutions (Pasadena, CA). Kidneys or kidney slices were obtained from a total of 17 donors, with the following characteristics: 7 female (mean age ± SE, 56.9 ± 3.5; age range: 40-68; 6 Caucasian, 1 Hispanic); 10 male (mean age ± SE, 54.9 ± 6.6; age range: 16–75; 6 Caucasian, 2 African American, 1 Hispanic, 1 Pacific Islander). All tissue was scored by a pathologist as normal (i.e., derived from non-cancerous, non-diseased tissue). Cell isolation procedures are based on those originally described by Todd et al. (1995) and modified (Cummings and Lash, 2000; Cummings et al., 2000c). Whole kidneys were perfused with Wisconsin medium and were kept on wet ice until they arrived at the laboratory, which was usually within 24 hr of removal from the donor. All buffers were continuously bubbled with 95% O_2/5% CO_2 and were maintained at 37°C. Tissue slices were washed with sterile phosphate-buffered saline (PBS), minced, and the pieces were placed in a trypsinization flask filled with 40

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ml or 300 ml (for slices from the Tissue Resources Core or slices from the whole kidneys, respectively) of sterile, filtered Hanks' buffer, containing 25 mM $NaHCO_3$, 25 mM HEPES, pH 7.4, 0.5 mM EGTA, 0.2% (w/v) bovine serum albumin, 50 μg/ml gentamicin, 1.3 mg/ml collagenase, and 0.59 mg/ml $CaCl_2$, which was filtered prior to use. Minced cortical pieces from either slices or whole kidneys were subjected to collagenase digestion for 60 min, after which the supernatant was filtered through a 105-μm mesh filter to remove tissue fragments, centrifuged at 150 x *g* for 7 min, and the pellet resuspended in Dulbecco's Modified Eagle's Medium : Ham's F12 Medium (DMEM:F12; 1:1). Approximately 5 to 7 x 10^6 cells were obtained per 1 g of human kidney cortical tissue.

Culturing of hPT cells. Isolation of hPT cells was achieved as explained above with use of sterile conditions (i.e., all instruments and glassware were autoclaved and all buffers were filtered through a 0.2-μm pore-size filter). After isolation, cells were resuspended in 2 ml of DMEM:F12 and diluted to 500 ml with cell culture media. Basal medium was a 1:1 mixture of DMEM:F12. Standard supplementation included 15 mM HEPES, pH 7.4, 20 mM $NaHCO_3$, antibiotics for day 0 through day 3 only (192 IU penicillin G/ml + 200 μg streptomycin sulfate/ml or 50 μg gentamicin/ml) to inhibit bacterial growth, 2.5 μg amphotericin B/ml to inhibit fungal growth, 5 μg bovine insulin/ml (= 0.87 μM), 5 μg human transferrin/m1 (= 66 nM), 30 nM sodium selenite, 100 ng hydrocortisone/ml (= 0.28 μM), 100 ng epidermal growth factor/ml (= 17 nM), and 7.5 pg 3,3',5-triiodo-DL-thyronine/ml (= 111 nM) (Lash et al., 1995b). Cells were seeded at densities of 50–100 μg protein per cm^2 (0.5–1.0 x 10^6 cells/ml) on either collagen-coated polystyrene culture dishes or collagen-coated polystyrene tissue culture flasks (T-25, T-75, or T-175). Cultures were grown at 37°C in a humidified incubator under an atmosphere of 95% air/5% CO_2 at pH 7.4. Cultures were grown to confluence (generally 5 to 7 days) prior to treatment with any agent. Cells were harvested from the dishes by either scraping the plates with a Teflon scraper or by brief incubation with Cellstripper (Cellgro, Herndon, VA) (in Ca^{2+}- and Mg^{2+}-free Hanks' buffer).

Protein determination was done using the bicinchoninic acid (BCA) protein determination kit from Sigma, using bovine serum albumin as a standard.

Confocal microscopy. hPT cells were grown on collagen-coated, 35-mm culture dishes and were viewed with a Zeiss Triple-Laser Scanning Confocal Microscope (LSM 310) with integrated workstation at the Confocal Imaging Core Facility in the School of Medicine at Wayne State University (Detroit, MI). This is a core facility of the NIEHS Center for Molecular Toxicology with Human Application at Wayne State. Initial magnification was 196X.

Measurement of necrosis by LDH release. hPT cells were incubated with either medium or medium containing the indicated concentrations of DCVCS for up to 48 hr. Cell viability at the conclusion of these incubations was estimated by determining the release of LDH from cells after various incubations and at various times (Lash et al., 1995b). LDH release from cells was measured by determining lactate dehydrogenase (LDH) activity (measured spectrophotometrically as NADH oxidation at 340 nm) in media and, after removal of media, washing cells with phosphate-buffered saline (PBS), and solubilization of cells with 0.1% (v/v) Triton X-100, in total cells. The fraction of LDH release was an index of irreversible injury or necrosis:

$$\%\text{LDH release} = \text{LDH activity in media}/(\text{LDH activity in media} + \text{LDH activity in total cells}) \times 100\%.$$

Flow cytometry analysis of cell cycle. Cell cultures were washed twice with sample buffer (PBS plus glucose (1 g/l) filtered through a 0.22-μm filter), dislodged by trypsin/EDTA (0.1% w/v) incubation, centrifuged at 400 x g for 10 min, and resuspended in sample buffer. Cell concentrations were adjusted to 1 to 3 x 10^6 cells/ml with sample buffer and 1 ml of the cell suspension was centrifuged at 400 x g for 10 min. All of the supernatant except 0.1 ml/10^6 cells was removed and the remaining cells were mixed on a vortex mixer in the remaining fluid for 10 sec. Next, 1 ml of ice-cold ethanol (70%, v/v) was added to the sample drop by drop, with samples being mixed for 10 sec between drops. The tubes were capped and fixed in ethanol at

4°C. After fixing, the cells were stained in propidium iodide (50 µg/ml) containing RNase A (100 U/ml). Samples were then mixed, centrifuged at 1,000 x g for 5 min and all the ethanol except 0.1 ml was removed. Cells were mixed in the residual ethanol and 1 ml of the propidium iodide staining solution was added to each tube. After mixing again, cells were incubated at room temperature for at least 30 min. Samples were analyzed within 24 hr by flow cytometry using a Becton Dickinson FACS*Calibur* Flow Cytometer, which is a core facility of the NIEHS Center for Molecular Toxicology with Human Application at Wayne State. Analysis was performed with 20,000 events per sample using the ModFit LT v. 2 for Macintosh data acquisition software package (Verity Software House, Inc., Topsham, ME; distributed by Becton Dickinson Immunocytometry System BDIS, San Jose, CA). Propidium iodide was detected by the FL-2 photomultiplier tube. Fractions of apoptotic cells were quantified by analysis of the sub-G_1 (sub-diploid) peak with ModFit cell cycle analysis. The percent distribution of cells in the various stages of the cell cycle (G_o/G_1, S, G_2/M) were also calculated. Cell aggregates were discarded in the flow cytometry analysis by post-fixation aggregate discrimination.

Annexin V-FITC binding assay. Induction of apoptosis was also analyzed by annexin V-FITC binding and flow cytometry. hPT cells grown in T-25 flasks were washed with 3 ml of ice-cold PBS. Ice-cold binding buffer (1.5 ml; 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) was added to each flask, to which was then added 5 µl of annexin V-FITC. After a 15-min incubation with shaking in the dark at room temperature, the annexin was removed, 1 ml of ice-cold binding buffer was added. Cells were gently scraped from the flasks, 1 ml of cells was placed in the flow cytometry tubes, 18 µl of a 20X stock solution of propidium iodide (50 µg/ml in water, final concentration) was added, and tubes were placed on ice in the dark for at most 1 hr until analysis on a Becton Dickinson FACSC*alibur* Flow Cytometer. Control samples included unstained cells, cells stained with annexin V-FITC but no propidium iodide, and cells stained with propidium iodide but no annexin V-FITC.

Measurement of mitochondrial membrane potential (•ψ) with JC-1. Confluent, primary cultures of hPT cells were grown on collagen-coated, glass cover slips in 35-mm tissue

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culture dishes and were incubated for 2 hr or 4 hr with 0 (= Control), 10, or 50 μM DCVCS. JC-1 fluorescence was measured by confocal microscopy assessing the emission shift from green (~530 nm) to red (~590 nm) in polarized mitochondria using 488-nm excitation. Polarized mitochondria are indicated by yellow-red punctate staining.

Measurement of cellular respiration. Confluent, primary cultures of hPT cells were grown on collagen-coated T-25 flasks. Cells were incubated for various times up to 48 hr with either PBS or the indicated concentration of DCVCS. At the end of the incubation period, cells were released from the culture surface by brief trypsinization and resuspended in PBS at a concentration of 2 x 10^6 cells/ml. Succinate-, (glutamate + malate)-, or (ascorbate + TMPD)-stimulated O_2 consumption was measured in a Gilson 5/6H Oxygraph as described previously (Lash and Anders, 1986; Lash and Tokarz, 1989).

Measurement of cellular ATP concentrations. Intracellular contents of adenine nucleotides were measured in neutralized perchloric acid extracts by the HPLC method of Jones (1981), as described previously (Lash and Tokarz, 1989). Separation of ATP, ADP, and AMP was achieved by reversed-phase HPLC with a Waters μBondapak C_{18} cartridge (8 mm X 10 cm) (Waters Associates, Milford, MA) and detection was by absorbance at 260 nm.

Measurement of cellular GSH concentrations. Cellular content of GSH was determined by ion-exchange HPLC on a Waters μBondapak amine cartridge (8 mm X 10 cm) (Waters Associates, Milford, MA) after derivatization of thiols with iodoacetate and amine groups with 1-fluoro-2,4-dinitrobenzene (Fariss and Reed, 1987; Visarius et al., 1996). Derivatives were detected by absorbance at 365 nm and were compared to authentic standards (limit of detection = 50 pmol).

Data analysis. Measurements of apoptosis, necrosis, and cell proliferation were performed on at least 3 separate cell cultures, except where indicated. Results are expressed as means ± SE, except where inclusion of error bars made the figures unclear. In those cases, the degree of variation is indicated in the legends. Significant differences for means were first assessed by a one-way or two-way analysis of variance. When significant *F* values were obtained

with the analysis of variance, the Fisher's protected least-significance *t* test was performed to determine which means were significantly different from one another, with two-tail probabilities < 0.05 considered significant.

Results

Cellular morphology. Incubation of confluent primary cultures of hPT cells for 24 hr with a range of concentrations of DCVCS produced obvious cell injury **(Fig. 1)**. Significant changes in cell shape (i.e., elongation, irregularity) and an increase in intracellular vesicularization were observed at a concentration of DCVCS as low as 10 μM. These changes progressed in severity with increasing concentrations of DCVCS, such that at a concentration of 500 μM, little recognizable cellular structure was evident.

Time and concentration dependence of LDH release from hPT cells. Confluent primary cultures of hPT cells were incubated with a wide range of concentrations of DCVCS, ranging from 10 μM to 500 μM, and release of LDH was determined at various times as a measurement of acute cellular necrosis **(Fig. 2)**. Little effect of DCVCS on LDH release was observed at concentrations of 100 μM or less throughout the 48-hr incubation. The lowest concentration of DCVCS and time point that showed a significant increase in LDH release was 200 μM at 48 hr (approximately 35% LDH release as compared to 10% for control cells) whereas the earliest significant increase in LDH release occurred after a 2-hr incubation with 500 μM DCVCS (approximately 19% LDH release as compared to 12% for control cells). A DCVCS concentration of at least 200 μM was thus required to elicit any significant increase in LDH release.

Time and concentration dependence of apoptosis in hPT cells. In contrast to cell swelling and the collapse of membrane permeability that is characteristic of acute cellular necrosis, cells may also undergo apoptosis. The process of apoptosis differs from that of cellular necrosis in several ways, such as a requirement for metabolic energy (i.e., ATP), formation of apoptotic bodies, nuclear condensation, and regulation by expression of specific proteins and/or signaling molecules. Confluent hPT cells were stained with propidium iodide and subjected to FACS analysis and flow cytometry to quantitate the proportion of cells that are sub-diploid. DCVCS was shown to cause time- and concentration-dependent increases in the proportion of

hPT cells undergoing apoptosis **(Fig. 3)**. The general pattern was that the proportion of cells undergoing apoptosis increased up to a DCVCS concentration of 50 μM to 100 μM and increased with time up to 4 hr to 8 hr; at higher concentrations and later incubation times, the proportion of cells undergoing apoptosis decreased. A concentration of DCVCS of as low as 10 μM induced a significant increase in the proportion of apoptotic cells as early as 1 hr. Maximal levels of apoptotic cells occurred in the range of 4 hr to 8 hr of incubation, with approximately 4% to 10% apoptotic cells as compared to untreated hPT cells, which exhibited less than 1% apoptotic cells. The proportion of apoptotic cells tended to decrease after 24 hr or 48 hr of incubation with concentrations of DCVCS of • 100 μM. This response is likely due to cells undergoing necrosis rather than apoptosis when exposed to higher concentrations of toxicant and/or for longer incubation times, when cells are likely to be incompetent to undergo apoptosis.

From the plot in Fig. 3, the 4-hr incubation time appears to elicit the optimal response for cells to undergo apoptosis. An example of the DNA histograms for hPT cells incubated with various concentrations of DCVCS for 4 hr is shown in **Fig. 4**. Note that G_0/G_1 peaks comprise the majority of cells in most incubations and are somewhat broad because the cells are confluent. A progressive increase in the proportion of subdiploid cells up to 100 μM DCVCS was observed. This was followed by a modest decline in the proportion of subdiploid or apoptotic cells. The only significant changes in the proportion of hPT cells in the other phases of the cell cycle were observed in cells incubated with 500 μM DCVCS, which exhibited a nearly doubling in the proportion of cells in the G_2/M phase and a decrease by > 50% in the proportion of cells in the S phase. Also note that the total number of viable cells, as indicated by the scale for the Y-axes in each panel of Fig. 4, was significantly decreased with DCVCS concentrations • 100 μM. This is consistent with cell loss due to necrosis or aggregation at higher DCVCS concentrations.

As an additional determination of apoptosis, we assessed staining of hPT cells with annexin V-FITC **(Fig. 5)**. Significant increases in the proportion of annexin V-positive/propidium iodide-negative cells was observed at all concentrations of DCVCS (i.e., 10

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μM to 500 μM) after both 2- and 4-hr incubations, providing additional evidence of the induction of apoptosis.

Alterations in mitochondrial function of hPT cells induced by DCVCS. Early stages of induction of apoptosis involve, in many cases, alterations in mitochondrial function. Some of the effects serve as signaling processes in the induction of apoptosis. For cells to be competent to undergo apoptosis, adequate supplies of ATP and the ability to generate a mitochondrial membrane potential (•ψ) must exist. Hence, delineation of the time and concentration dependence at which changes in mitochondrial function occur can provide information about competence of cells to undergo apoptosis.

Mitochondrial •ψ in confluent hPT cell cultures was qualitatively assessed with the fluorescent dye JC-1 and confocal microscopy **(Fig. 6)**. JC-1 accumulates in mitochondria of cells and undergoes a fluorescence emission shift from green (~ 530 nm) to red (~ 590 nm) in polarized mitochondria. Accordingly, a cell population with polarized and functional mitochondria will exhibit red punctate staining whereas mitochondria that are depolarized exhibit yellow-green staining. Photomicrographs from an exemplary set of JC-1 fluorescence measurements of hPT cells treated for 2 hr with various concentrations of DCVCS indicate little effect at DCVCS concentrations of • 100 μM and maintenance of some •ψ even at 200 μM DCVCS **(Fig. 6A)**. In contrast, a marked decrease in the appearance of red staining in cells incubated for 4 hr with • 50 μM DCVCS was observed and at 200 μM DCVCS, no yellow or red staining was observed. Hence, this shows that hPT cells treated with up to 200 μM DCVCS for 2 hr or up to 50 μM DCVCS for 4 hr retain the ability to generate a mitochondrial •ψ.

Mitochondrial integrity of hPT cells treated with DCVCS was also assessed by measurement of cellular respiration **(Fig. 7)**. hPT cells incubated for up to 48 hr with 10 μM to 500 μM DCVCS exhibited time- and concentration-dependent decreases in the rate of succinate-stimulated oxygen consumption. Maximal decreases in respiration rate of approximately 30% were observed after up to 2 hr of incubation with as much as 500 μM DCVCS. This modest extent of functional decrement suggests that the mitochondria and the cells are still largely

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functional. Incubations with DCVCS concentrations of • 100 μM for • 4 hr produced > 50% decreases in respiration rates, suggesting that mitochondrial and cellular function are compromised. These time and dose dependence patterns are consistent with the diminished ability of hPT cells treated with • 100 μM DCVCS at later incubation times to undergo apoptosis (cf. Fig. 3).

Although succinate-stimulated respiration was clearly altered by DCVCS in a time- and concentration-dependent manner and respiration coupled to site II (i.e., succinate:ubiquinone oxidoreductase) substrates such as succinate were previously shown to be the primary form inhibited by DCVC in rat kidney cells (Lash and Anders, 1986), we also examined respiration coupled to site I (i.e., NADH dehydrogenase) and site III (i.e., ubiquinol:cytochrome c oxidoreductase) substrates **(Fig. 8)**. hPT cells were incubated for up to 4 hr with either PBS or 50 μM DCVCS. At the indicated times, effects on respiration coupled to either glutamate + malate (site I substrates), succinate (site II substrate), or ascorbate + TMPD (site III substrates) was determined. As previously shown with DCVC, succinate-stimulated respiration was the earliest and most potently inhibited form. While respiration coupled to glutamate + malate was also inhibited by DCVCS, the extent of inhibition was less and appeared to occur on a later time scale that that with succinate as the respiratory substrate. Respiration coupled to ascorbate + TMPD was not inhibited by 50 μM DCVCS at any incubation time.

Finally, mitochondrial function was assessed by measurement of cellular concentrations of ATP **(Fig. 9)**. Incubation of hPT cells for 0.5 hr with 10 μM to 200 μM DCVCS exhibited relatively modest decreases in ATP concentration of • 50%. In contrast, incubation of hPT cells with as low a concentration of DCVCS as 10 μM for 1 hr caused approximately 60% ATP depletion whereas incubation with 10 μM or higher concentrations of DCVCS for 2 hr or longer caused > 70% ATP depletion. As with mitochondrial respiration, the large extent of ATP depletion induced by DCVCS concentrations > 100 μM at early time points and • 10 μM at later time points is consistent with a diminished competence of the cells to undergo apoptosis under these conditions.

Effects of DCVCS on GSH concentrations of hPT cells. Although DCVC does not markedly deplete cellular GSH concentrations in rPT cells (Lash and Anders, 1986), DCVCS is more chemically reactive than its precursor and reacts directly with GSH (Sausen and Elfarra, 1991). Incubation of hPT cells with up to 200 μM DCVCS produced relatively modest decreases in cellular GSH content **(Fig. 10)**. In each case, the modest depletion of GSH, which was maximal after 2 hr or 4 hr of incubation, was followed by a rebound by 8 hr to near initial levels. Only in cells incubated with 500 μM DCVCS was more than 50% depletion of GSH observed. Additionally, cells incubated with 500 μM DCVCS did not exhibit a rebound in cellular GSH content by the 8-hr incubation time.

Discussion

We show here that DCVCS is a potent cytotoxicant in primary cultures of hPT cells, as judged by effects on several parameters of cell function and toxicity, including morphology, LDH release, apoptosis, mitochondrial function, and GSH status. These findings agree with previous studies in rats and in isolated rPT cells (Lash et al., 1994). Three critical issues on which the present studies shed some light are the (1) temporal relationship between the various effects of DCVCS, (2) comparative toxicity of DCVCS and DCVC, and (3) role of DCVCS in TRI- and DCVC-induced nephrotoxicity in human kidney.

DCVCS produced both necrosis and apoptosis. Similar to the case with DCVC (Lash et al., 2001a), apoptosis induced by DCVCS was primarily a low-dose, early-incubation time response whereas necrosis, as indicated by LDH release, was primarily a high-dose, late-incubation time response. Maintenance of mitochondrial function, as assessed by •ψ, respiration, and ATP concentrations, was also associated with the capability to undergo apoptosis, although the relationship was not always obvious.

With respect to membrane potential, hPT cells incubated for 2 hr with up to 200 μM DCVCS still exhibited red-yellow JC-1 fluorescence, indicating maintenance of some degree of mitochondrial integrity. In contrast, cells incubated for 4 hr with • 50 μM DCVCS exhibited little to no red-yellow JC-1 fluorescence, indicating an inability to generate a membrane potential. Thus, while a statistically significant increase in the induction of apoptosis by 10 μM DCVCS occurred as early as 1 hr, maximal induction of apoptosis did not occur until 4–8 hr. While the JC-1 data suggested little change in mitochondrial membrane potential by 10 μM DCVCS at 4 hr, this is a relatively qualitative assay. It is conceivable that enough of a decrease in membrane potential occurred in the overall cell population or that membrane potential decreased in enough individual cells to cause the observed small, but statistically significant increase in apoptosis.

Regarding mitochondrial respiration, hPT cells needed to be incubated with at least 100 μM DCVCS for at least 4 hr to elicit > 30% inhibition of cellular oxygen consumption with succinate as respiratory substrate, again indicating maintenance of mitochondrial function at lower toxicant concentrations and at early incubation times. While significant inhibition of site I (i.e., NADH dehydrogenase)-coupled respiration was also observed, site II (i.e., succinate:ubiquinone oxidoreductase)-coupled respiration was clearly the most sensitive and site III (i.e., ubiquinol:cytochrome c oxidoreductase) respiration was insensitive to inhibition by DCVCS.

Although ATP depletion was a highly sensitive indicator of mitochondrial dysfunction, hPT cells required at least a 2-hr incubation with • 50 μM DCVCS to elicit > 70% ATP depletion. However, the observations that cellular ATP concentrations were < 2 nmol/mg protein with DCVCS concentrations • 50 μM after 2 or 4 hr and apoptosis was not maximal until 4–8 hr could suggest a temporal discrepancy between apoptosis and ATP supply. While a minimum level of intracellular ATP is needed for cells to remain competent to undergo apoptosis (Kroemer et al., 1998), it is unclear what precisely this value is, although an ADP/ATP ratio of 0.2 has been suggested as the critical level (Richter et al., 1996). It would appear that cellular ATP concentrations of 1-2 nmol/mg protein are still sufficient to allow apoptosis to occur in hPT cells.

Cellular responses to DCVCS differed from those to DCVC in several important ways. Experiments with DCVCS reported in this study were performed on different cell cultures from different kidney donors than those with DCVC that were previously published (Lash et al., 2001a). This is potentially important because of possible interindividual differences in bioactivation and susceptibility, although there have been no documented interindividual differences in rates of DCVC metabolism. We previously noted that hPT cells from males were more sensitive to DCVC than were hPT cells from females but that there were no observable gender-dependent differences in apoptosis (Lash et al., 2001a). In terms of acute cellular necrosis, hPT cells from male donors appeared to be slightly less sensitive to DCVCS than to DCVC, particularly at concentrations < 100 μM. Specifically, the time course for cell death was

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slower in hPT cells incubated with DCVCS. hPT cells also exhibited significantly less apoptosis when incubated with DCVCS than with DCVC. Thus, whereas the maximal extent of apoptosis, as indicated by FACS analysis, was only about 12% in hPT cells incubated with DCVCS, the maximal extent of apoptosis due to DCVC was approximately 30%. Similarly, whereas the maximal amount of apoptosis as indicated by annexin V-FITC staining was about 12% above control cells for DCVCS, it was about 30% above control cells for DCVC. While effects of DCVCS on mitochondrial •ψ and respiration rates were generally comparable to those of DCVC, DCVCS caused a much greater extent of ATP depletion than DCVC (> 90% for DCVCS and only about 60% for DCVC; Lash, L.H., Putt, D.A., and Hueni, S.E., unpublished observations). The more rapid and extensive ATP depletion caused by DCVCS is consistent with its diminished ability relative to DCVC to cause hPT cells to undergo apoptosis.

Finally, although DCVCS only produced marked GSH depletion at the highest concentration tested (i.e., 500 μM), the amounts of GSH depletion observed with DCVCS were still markedly higher than those produced by DCVC (Lash, L.H., Putt, D.A., and Hueni, S.E., unpublished observations). This is likely due to the chemical reactivity of DCVCS with soft nucleophiles such as GSH (Sausen and Elfarra, 1991). DCVC selectively depletes mitochondrial GSH in rat kidney by oxidation and does not have a measurable impact on total renal cellular GSH content (Lash and Anders, 1986, 1987).

Based on the present work and previous studies, we conclude that sulfoxidation of DCVC does indeed play a significant role in the mechanism of toxicity in human kidney. Previous demonstrations in both freshly isolated and primary cultures of hPT cells (Cummings and Lash, 2000; Lash et al., 2001a) showing marked protection from DCVC-induced necrosis or apoptosis by preincubation of cells with the FMO substrate methimazole directly implicate DCVCS formation in the mechanism of toxicity. Moreover, the relatively low amount of total β-lyase activity in the human kidney (Lash et al., 1990) and the lack of or marginal ability of aminooxyacetic acid to protect hPT cells from DCVC-induced necrosis or apoptosis, suggest further that FMO-dependent bioactivation is important. We also recently demonstrated

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expression of FMO isozymes in human kidney (Krause et al., 2003). These studies, although derived from a limited number of samples, also provided data suggesting that humans exhibit a wide range of levels of expression of FMOs in the kidneys. This may indicate a correspondingly wide range of susceptibility in the general population to nephrotoxicity from chemicals that undergo FMO-dependent bioactivation. Additional studies with higher sample numbers and specific genotyping of individuals are needed to confirm this finding.

In comparing DCVCS and DCVC, it is critical to note that effects due to reactive species generated from both DCVC and DCVCS contribute to the overall effects of incubations with DCVC. In contrast, one only observes effects due to reactive species generated from DCVCS when cells are incubated with DCVCS. Furthermore, the molecular targets for reactive species generated by β-lyase and FMO may differ. These considerations make a direct comparison of DCVC and DCVCS and an assessment of the role of DCVCS formation in the cytotoxicity induced by DCVC difficult. The markedly higher amount of apoptosis induced by DCVC as compared to that induced by DCVCS and the consistent findings that apoptosis is a relatively low-dose, early response, suggest that for DCVC bioactivation and cytotoxicity, β-lyase-dependent processes may be more important at lower doses and during the early stages of exposure whereas a role for FMO may become more prominent at higher doses and at later exposure times. This conclusion is also supported by the apparent kinetics of DCVC metabolism by the two enzymes: Whereas the β-lyase from human kidney cytosol exhibits a K_m of approximately 1.5–2 mM for DCVC (Lash et al., 1990), the reported K_m value for DCVCS formation from DCVC by cDNA-expressed rabbit FMO3 is > 50 mM (Ripp et al., 1997). While this kinetic difference suggests that FMO may be only important at very high concentrations, this will be modified by the apparent, higher reactivity of reactive species derived from DCVCS than those derived from β-lyase-dependent metabolism of DCVC.

In conclusion, we have shown that DCVCS is a potent cytotoxicant in primary cultures of hPT cells that elicits both apoptosis and necrosis, according to a specific temporal and dose-dependent pattern. This pattern appears to correlate with the ability of DCVCS-treated cells to

maintain mitochondrial integrity, such that the ability to generate a membrane potential and to maintain some minimal level of intracellular ATP are required for the cells to be capable of undergoing apoptosis. Although a correlation between cellular GSH status and these responses was not specifically addressed, lower concentrations of DCVCS induce a modest, but reversible depletion of GSH, suggesting a transient oxidative stress. At high DCVCS concentrations, however, the oxidative stress is irreversible and cells undergo necrosis.

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Footnotes

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Figure Legends

Fig. 1. Morphology of primary cultures of hPT cells incubated with DCVCS.

Confluent, primary cultures of hPT cells were incubated for 24 hr with either media (= Control) or the indicated concentrations of DCVCS. Photomicrographs were obtained on a Zeiss Triple Laser Scanning confocal microscope at an initial magnification of 196X. Bar = 5 μm.

Fig. 2. DCVCS-induced LDH release in primary cultures of hPT cells.

Cellular necrosis was measured as LDH release after incubation of confluent, primary cultures of hPT cells with 10 to 500 μM DCVCS for various times up to 48 hr. Results are means of measurements from 3 cell cultures. SEM values, which were omitted for clarity, were generally 5% to at most 20% of means. Significantly different ($P < 0.05$) from corresponding control: 1-hr: none; 2-hr: 500 μM; 4-, 8-, 24-, and 48-hr: 200 and 500 μM.

Fig. 3. DCVCS-induced apoptosis in primary cultures of hPT cells as indicated by FACS analysis and flow cytometry.

Confluent hPT cells were grown on 35-mm polystyrene culture dishes and treated for the indicated times with PBS (= Control) or DCVCS. Cells were then processed as described in Methods and analyzed within 24 hr by flow cytometry with a Becton Dickinson FACS*Calibur* flow cytometer. Results are means ± SEM of measurements from 4 separate cell cultures. The percentages of subdiploid (i.e., apoptotic) cells in samples incubated with DCVCS (10 μM to 500 μM) were significantly greater ($P < 0.05$) than the control value at each incubation time. Percentage of cells in subdiploid, G_0/G_1, G_2/M, and S phases: Control– 1.39, 71.08, 12.29, 16.63; 10 μM– 7.98, 77.10, 12.64, 10.26; 50 μM– 12.17, 75.37, 12.37, 12.26; 100 μM– 12.41, 74.97, 11.24, 13.79; 200 μM– 7.22, 75.50, 11.95, 12.54; 500 μM– 6.09, 72.31, 21.58, 6.10.

Fig. 4. Cell cycle analysis of hPT cells incubated for 4 hr with DCVCS.

Cells were incubated with PBS (= Control) or the indicated concentration of DCVCS for 4 hr and were processed for analysis of DNA content as described in Methods and the legend to Fig. 3. Peaks from left to right represent apoptotic cells, cells in G_0/G_1, cells in S phase, and cells in G_2/M. Results are from a single preparation and are typical of those from 4 separate experiments. The coefficient of variation (CV) for these peaks ranged between 3% and 14%, with approximately 80% of the peaks in samples exhibiting CV values of 5% to 8%. *Insets:* Distribution of cells according to fluorescence intensity. Cells outside the box are those that were excluded from the analysis due to aggregation. Values above the arrows indicate the percentage of cells that are subdiploid.

Fig. 5. DCVCS-induced apoptosis in primary cultures of hPT cells as indicated by annexin V-FITC staining and flow cytometry.

Confluent hPT cells were grown in T-25 flasks and were treated for 2 hr or 4 hr with either PBS (= Control) or the indicated concentrations of DCVCS. Samples were processed for analysis of annexin V-FITC staining as described in Methods and were analyzed by flow cytometry. Values shown are the percentage of cells that are apoptotic, and represent cells in the lower right quadrant that are annexin V-positive and propidium iodide-negative. Results are the means ± SEM of measurements from 3 separate cell cultures. All values for cells incubated with 10 μM to 500 μM DCVCS for either 2 hr or 4 hr were significantly greater ($P < 0.05$) than those for the corresponding control.

Fig. 6. Effect of DCVCS on mitochondrial membrane potential in primary cultures of hPT cells.

Confluent, primary cultures of hPT cells were grown on collagen-coated, glass cover slips in 35-mm tissue culture dishes and were incubated for 2 hr or 4 hr with 0 (= Control), 10, 50, 100, or 200 μM DCVCS. JC-1 fluorescence was measured by confocal microscopy assessing the emission shift from green (~530 nm) to red (~590 nm) in polarized mitochondria using 488-nm excitation. Composite red and green fluorescence is shown. Polarized mitochondria are indicated by yellow-red punctate staining. White bars = 5 μm. Results are from one cell culture and is representative of those from 3 separate cultures.

Fig. 7. Inhibition of cellular respiration in hPT cells by DCVCS.

Confluent, primary cultures of hPT cells were incubated with the indicated concentrations of DCVCS for up to 48 hr. At specified times, succinate-stimulated O_2 consumption was measured in a Gilson 5/6H Oxygraph. Results are averages of measurements from 2 separate cell cultures.

Fig. 8. Respiration coupling site specificity of DCVCS in hPT cells.

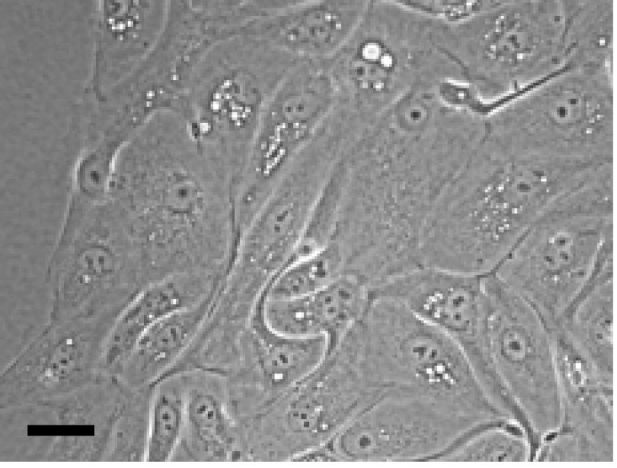
Confluent, primary cultures of hPT cells were incubated with 50 μM DCVCS for up to 4 hr. At specified times, O_2 consumption stimulated by either 1 mM L-glutamate + 1 mM malate, 3 mM succinate, or 1 mM ascorbate + 0.2 mM TMPD was measured in a Gilson 5/6H Oxygraph. Results are means ± SEM of measurements from 3 separate cell cultures. *Significantly different ($P < 0.05$) from corresponding control sample.

Fig. 9. ATP depletion in hPT cells incubated with DCVCS.

Confluent hPT cells grown on collagen-coated, polystyrene T-25 flasks were incubated for up to 4 hr with either medium (= Control) or the indicated concentration of DCVCS. Cellular contents of ATP were measured in perchloric acid extracts of cells by reversed-phase HPLC. Results are means ± SEM of measurements from 4 separate cell cultures. Significantly different ($P < 0.05$) from corresponding control: 0.5-hr: 50 μM and higher; 1-, 2-, and 4-hr: 10 μM and higher.

Fig. 10. GSH depletion in hPT cells incubated with DCVCS.

Confluent hPT cells grown on collagen-coated, polystyrene T-25 flasks were incubated for up to 8 hr with either medium (= Control) or the indicated concentration of DCVCS. Cellular contents of GSH were measured in perchloric acid extracts of cells after derivatization with iodoacetate and 1-fluoro-2,4-dinitrobenzene by reversed-phase HPLC. Results are means ± SEM of measurements form 4 separate cell cultures. Significantly different ($P < 0.05$) from control: 1-hr: 50, 200, 500 μM; 2-hr: 100 μM and higher; 4-hr: 100 μM and higher; 8-hr: 500 μM.



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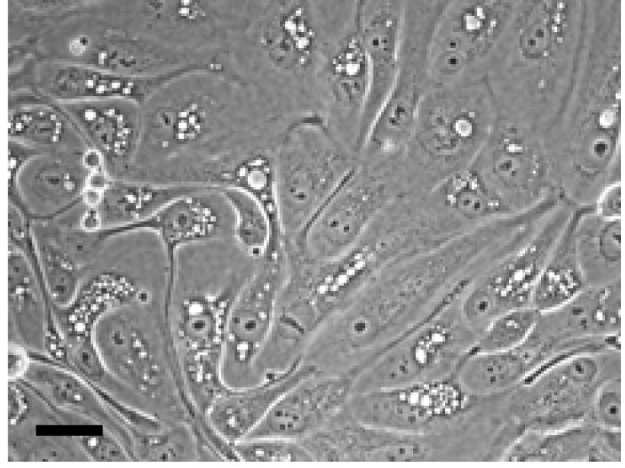
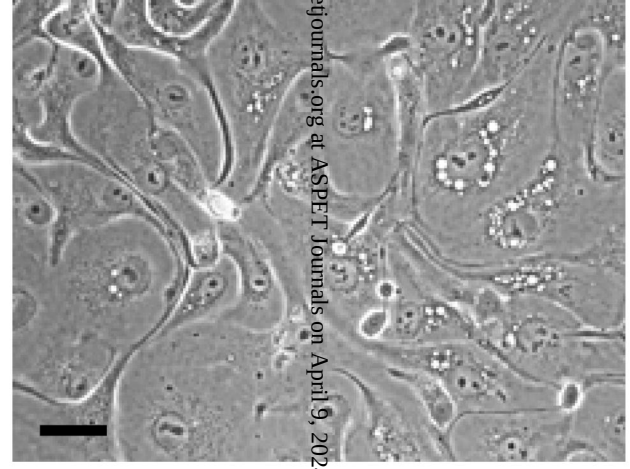


10 μM DCVCS



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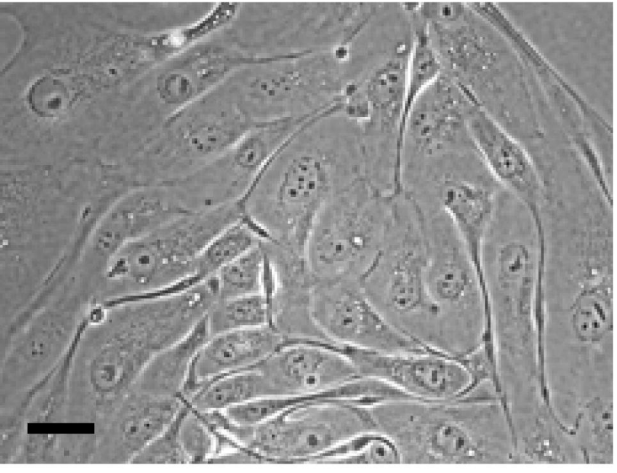

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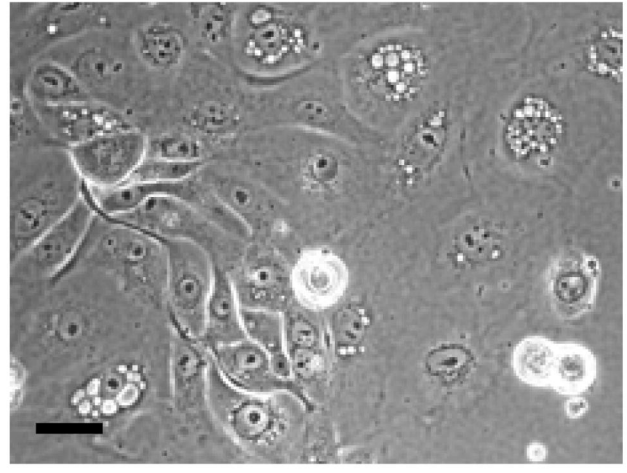
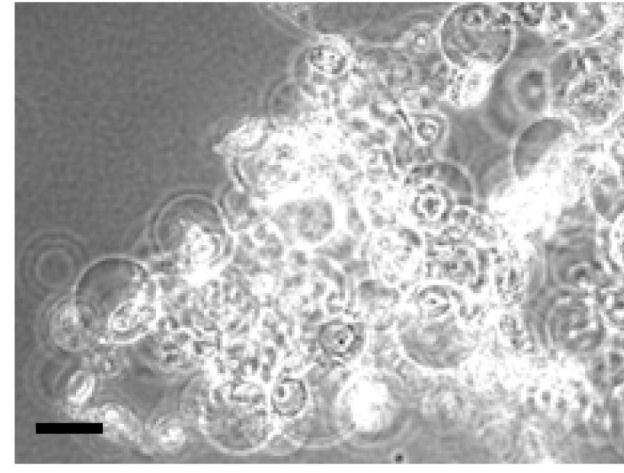
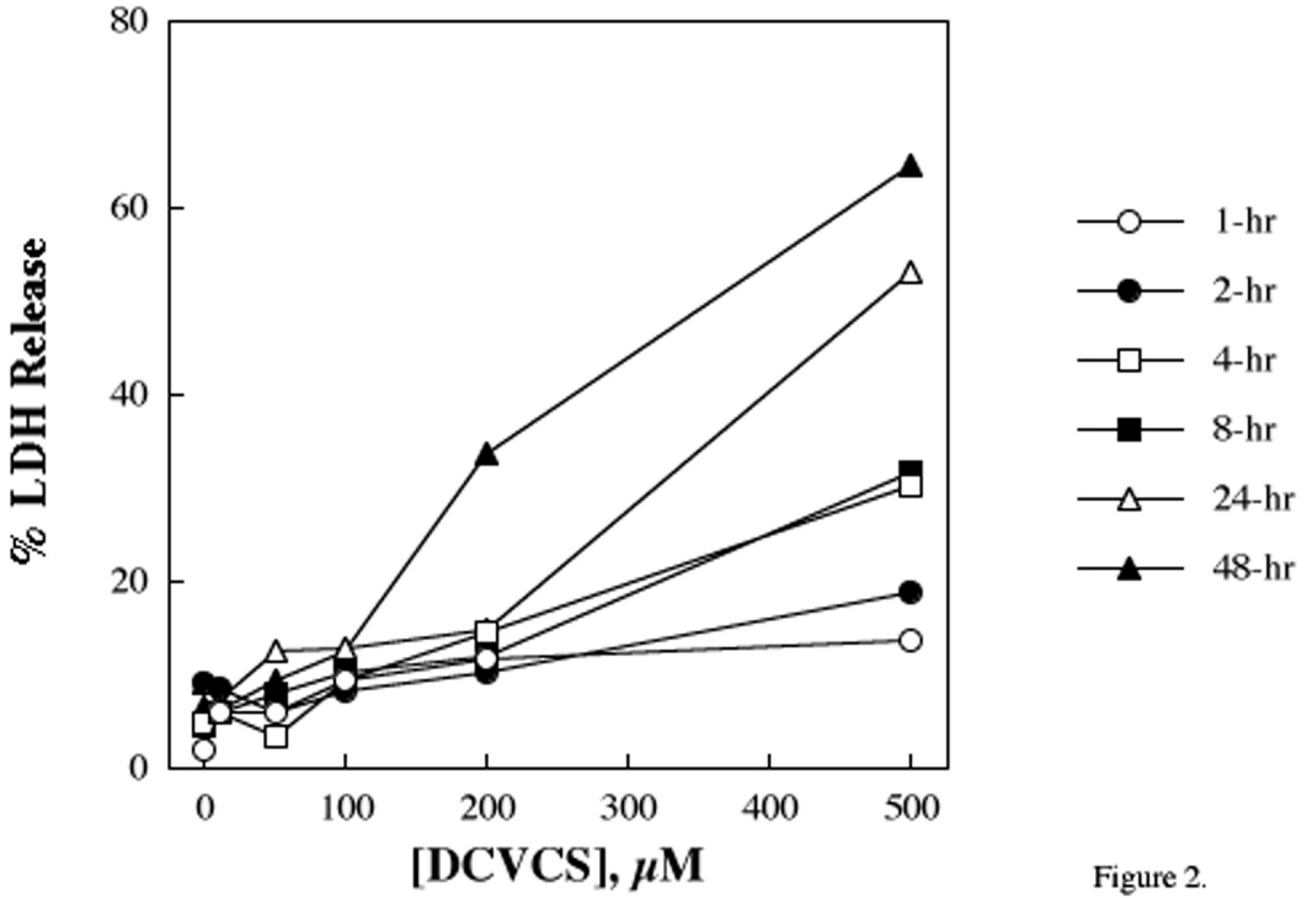


200 μM DCVCS



500 μM DCVCS

Figure 1.


80
60
40
20
0
% LDH Release
0
100
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500
[DCVCS], μM
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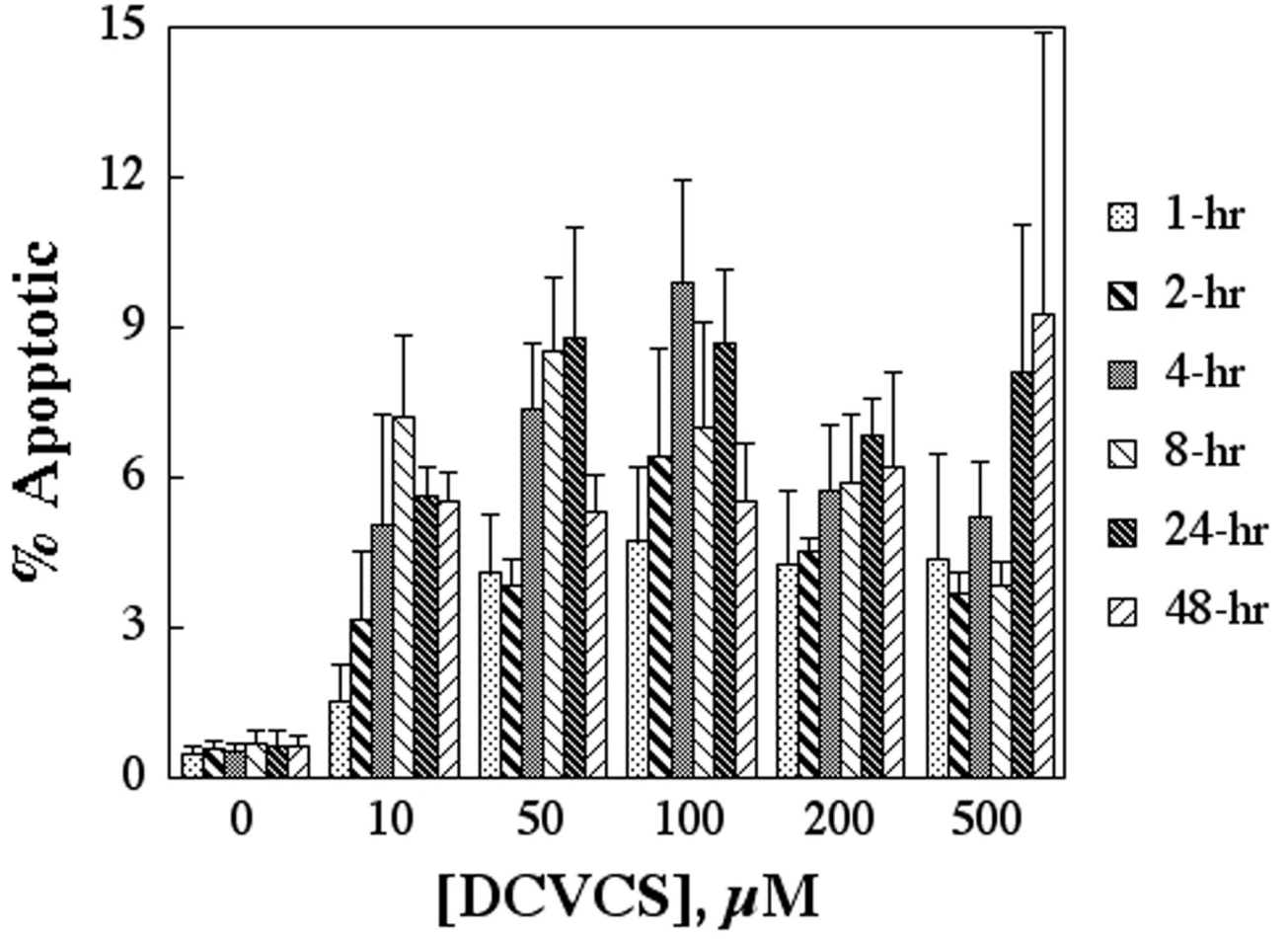
8-hr
24-hr
48-hr

Figure 2.


15
12
9
6
3
0
% Apoptotic
0
10
50
100
200
500
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1-hr
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4-hr
8-hr
24-hr
48-hr

Figure 3.

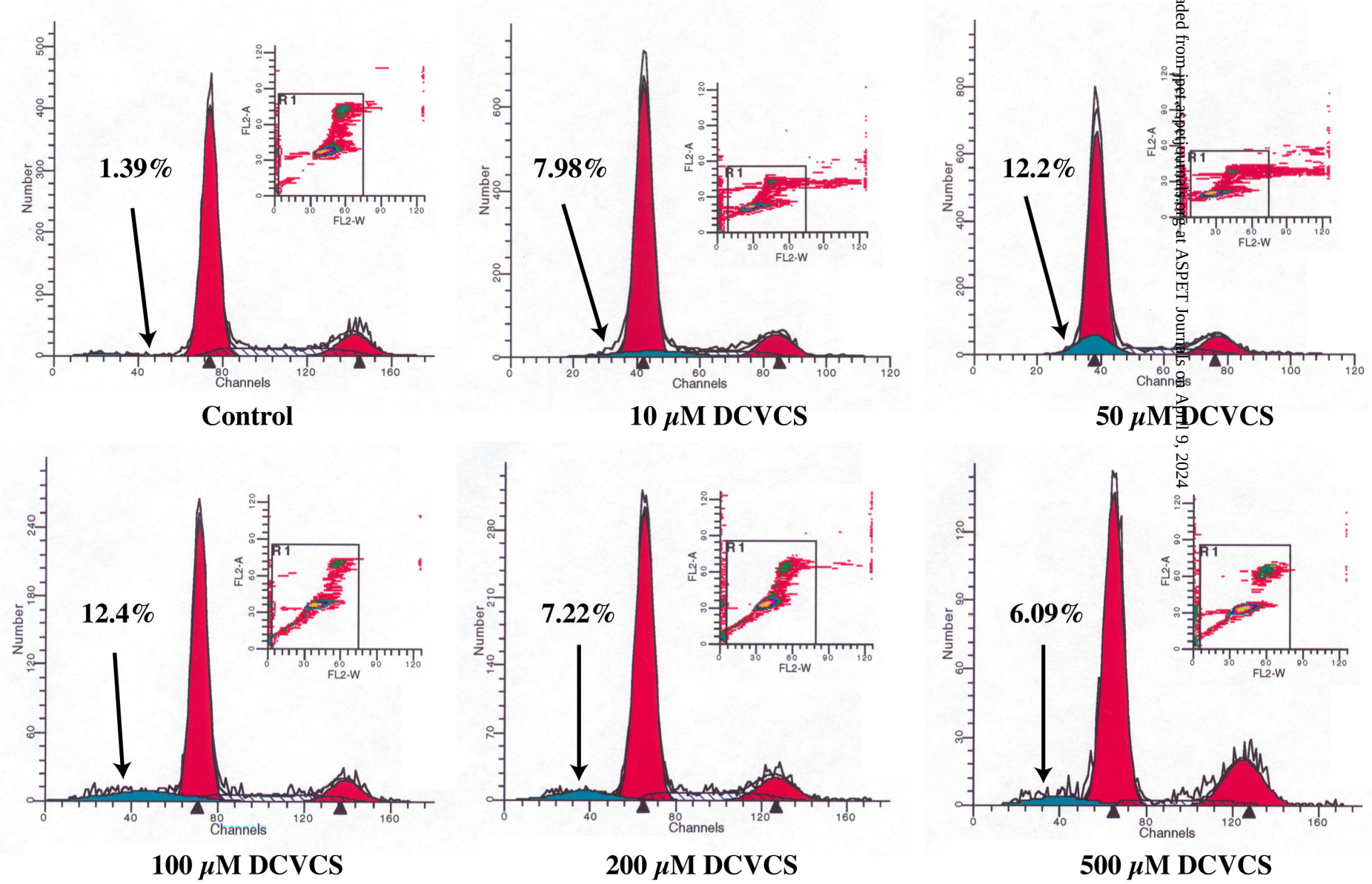


Figure 4.

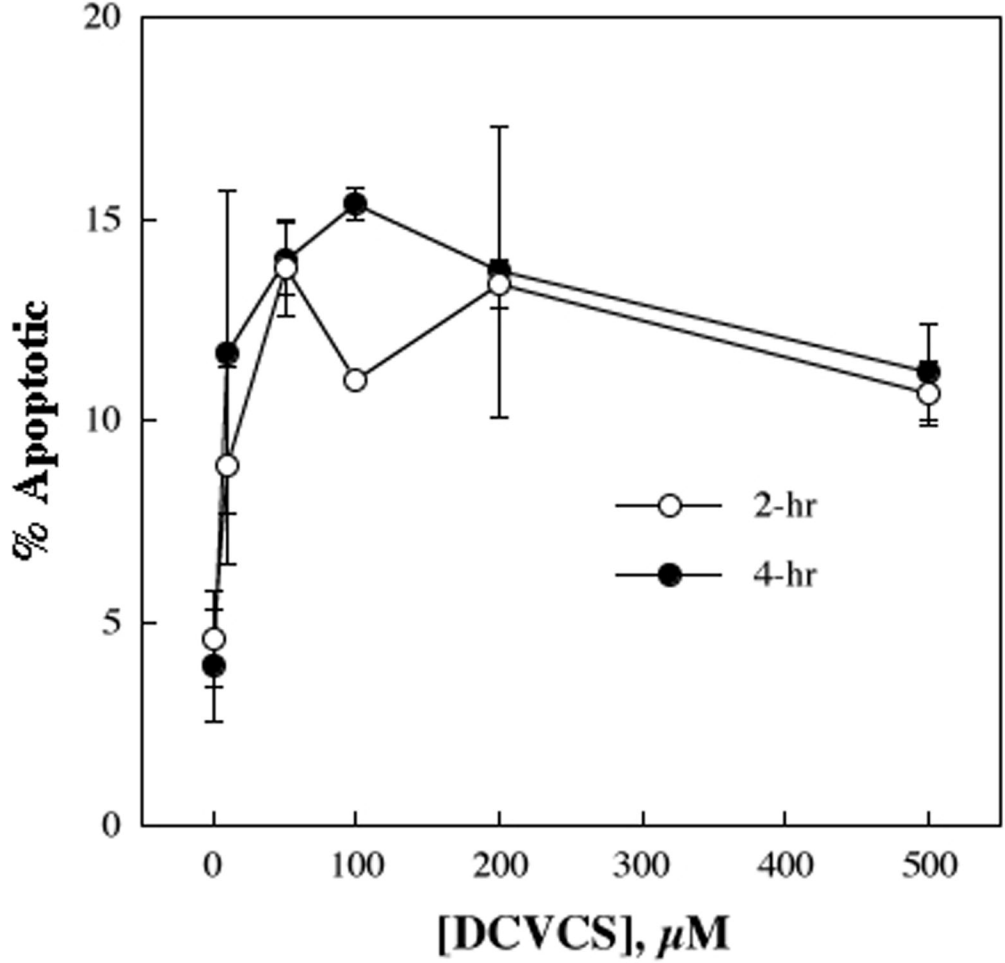
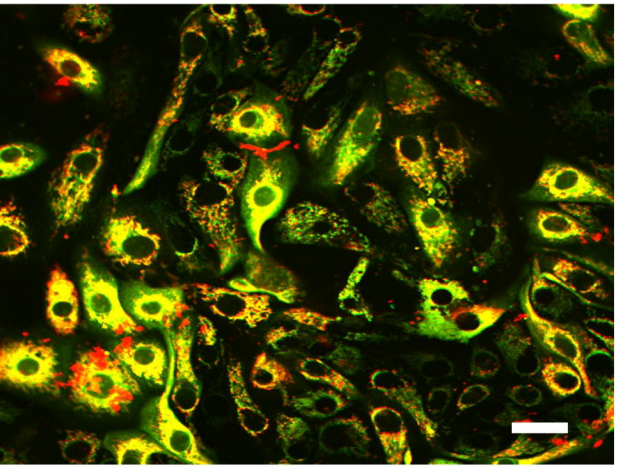
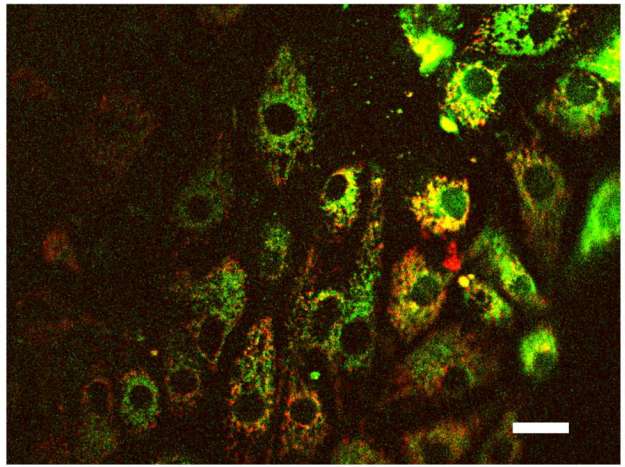


Figure 5.

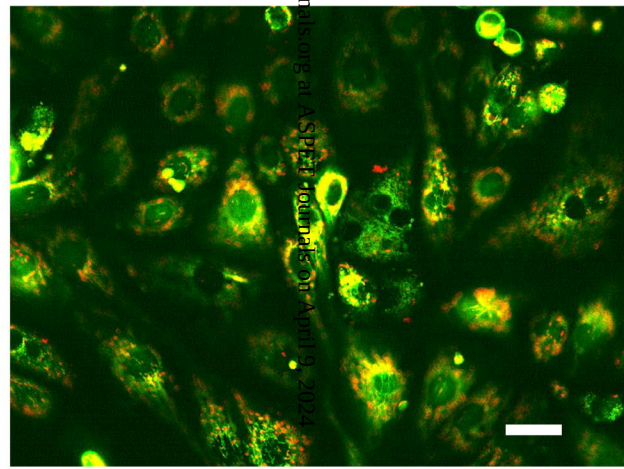
A. 2-hr Exposure.



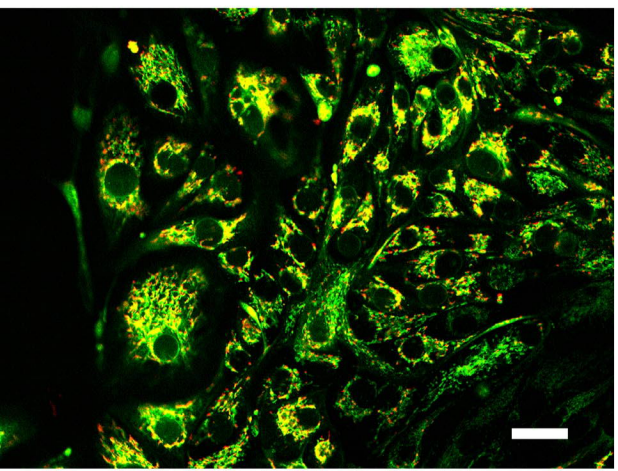
Control



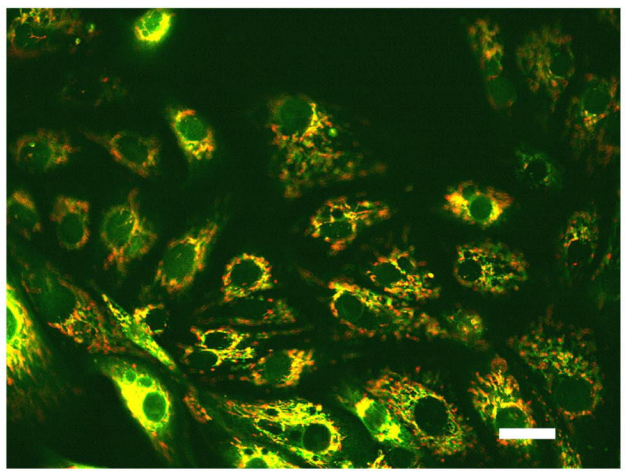
10 μM



50 μM



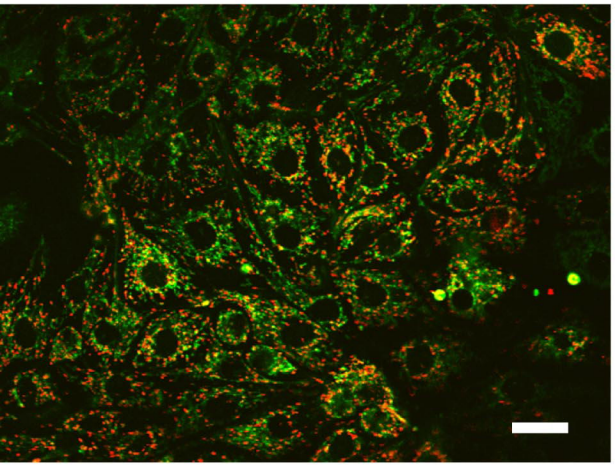
100 μM



200 μM

Figure 6A.

B. 4-hr Exposure.



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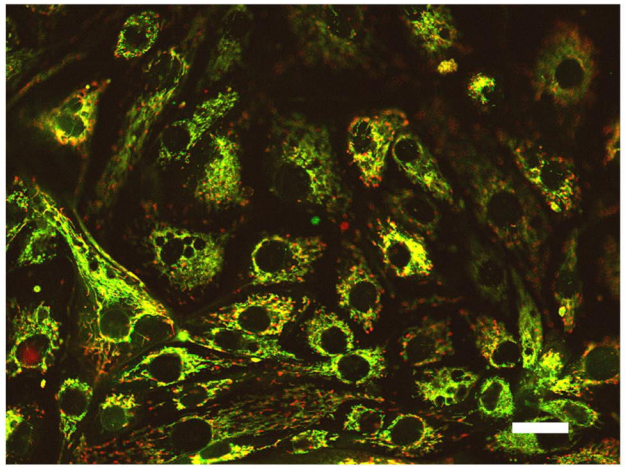
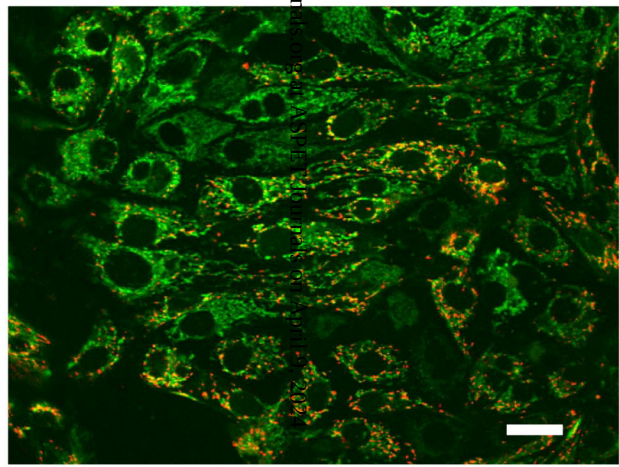
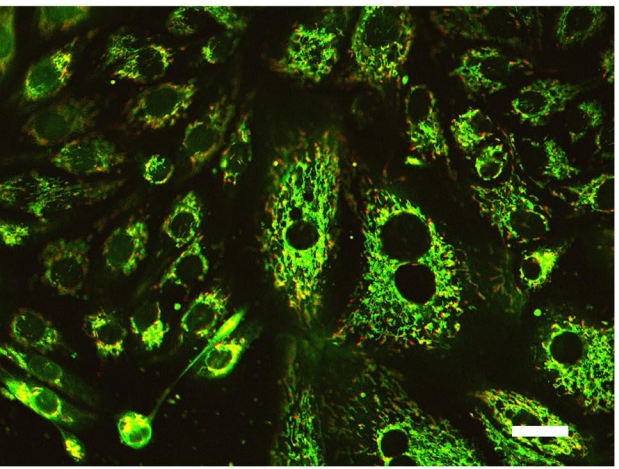


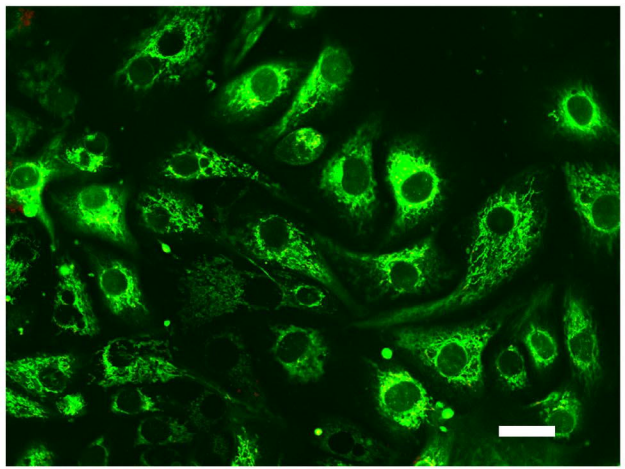
10 μM



50 μM



100 μM



200 μM

Figure 6B.

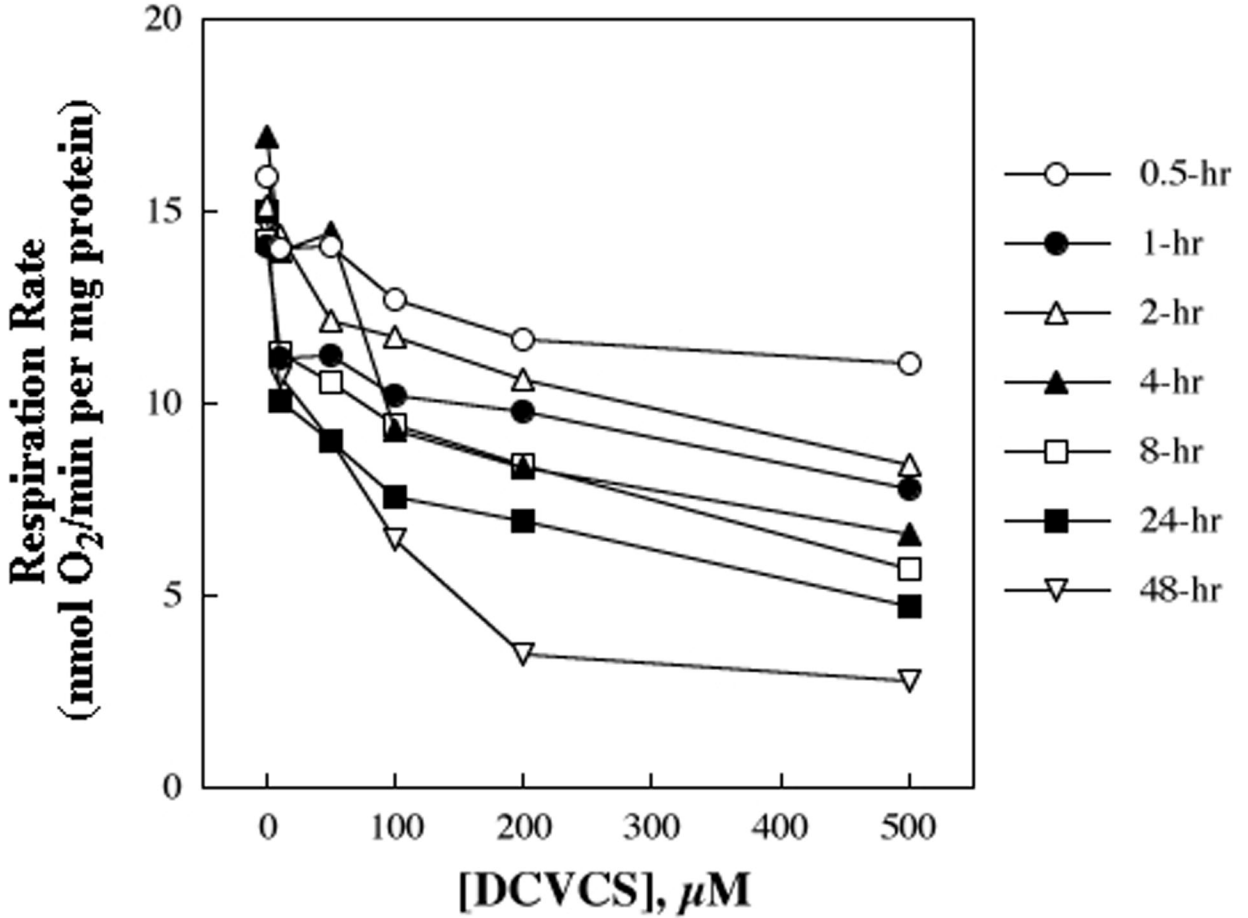

Respiration Rate
(nmol O_2/min per mg protein)
20
15
10
5
0
0
100
200
300
400
500
[DCVCS], μM
0.5-hr
1-hr
2-hr
4-hr
8-hr
24-hr
48-hr

Figure 7.

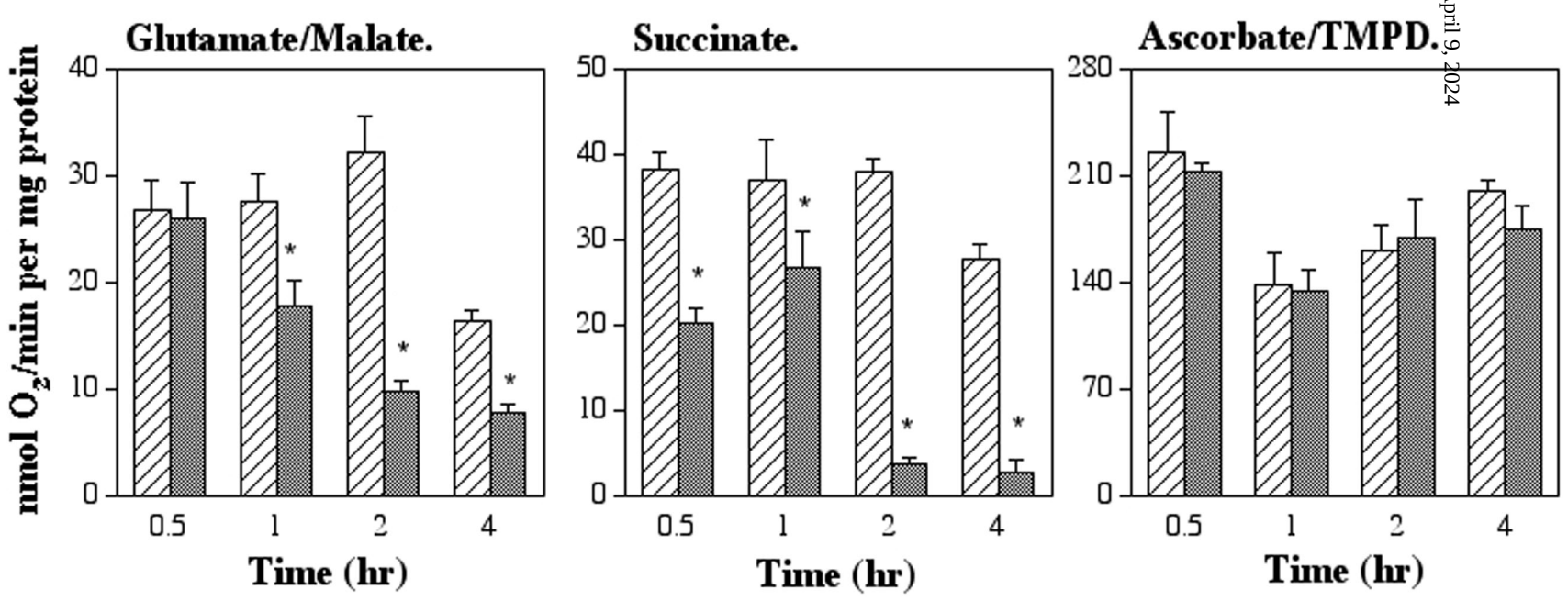


Figure 8.

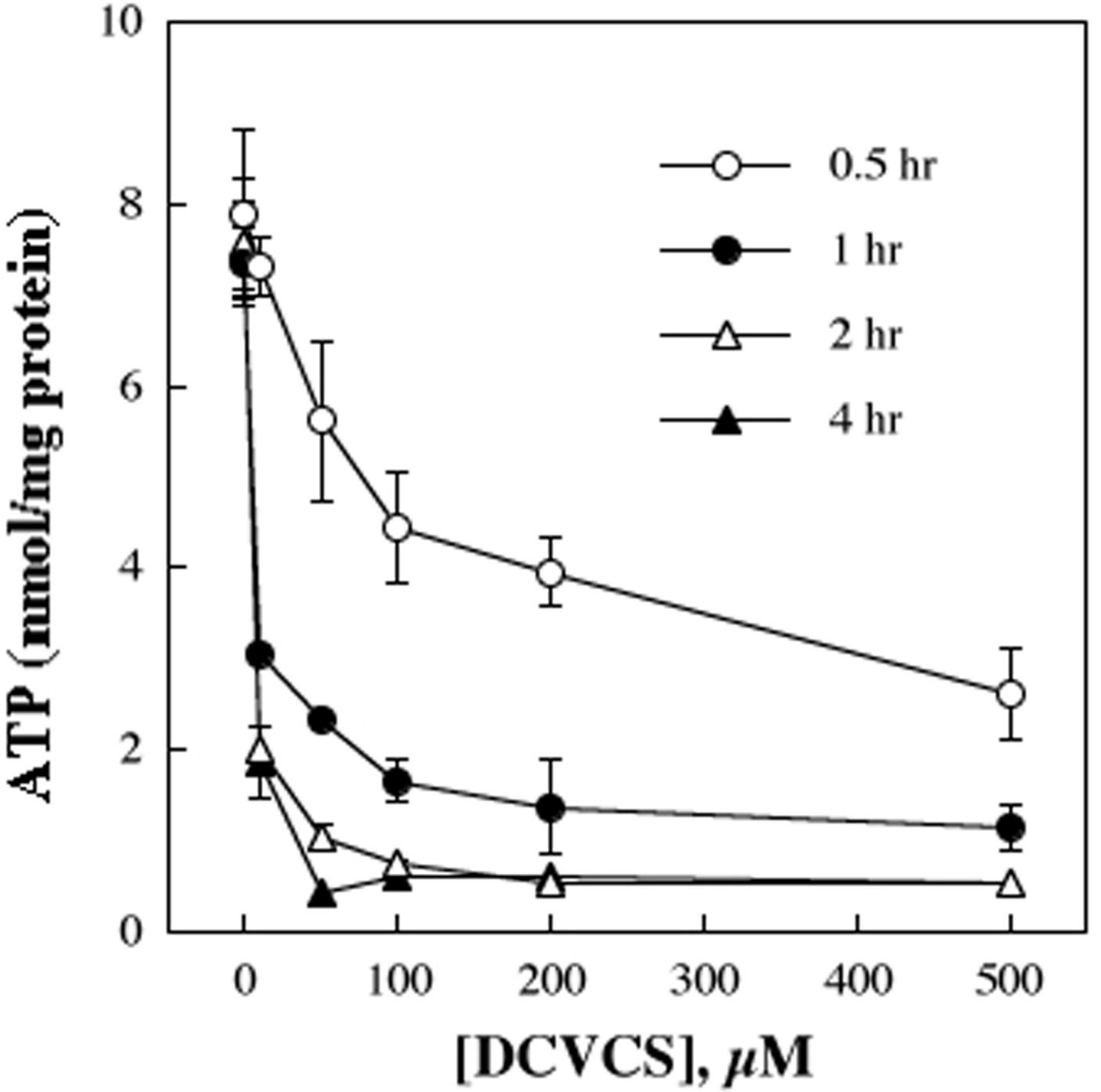

ATP (nmol/mg protein)
10
8
6
4
2
0
0
100
200
300
400
500
[DCVCS], μM
0.5 hr
1 hr
2 hr
4 hr

Figure 9.

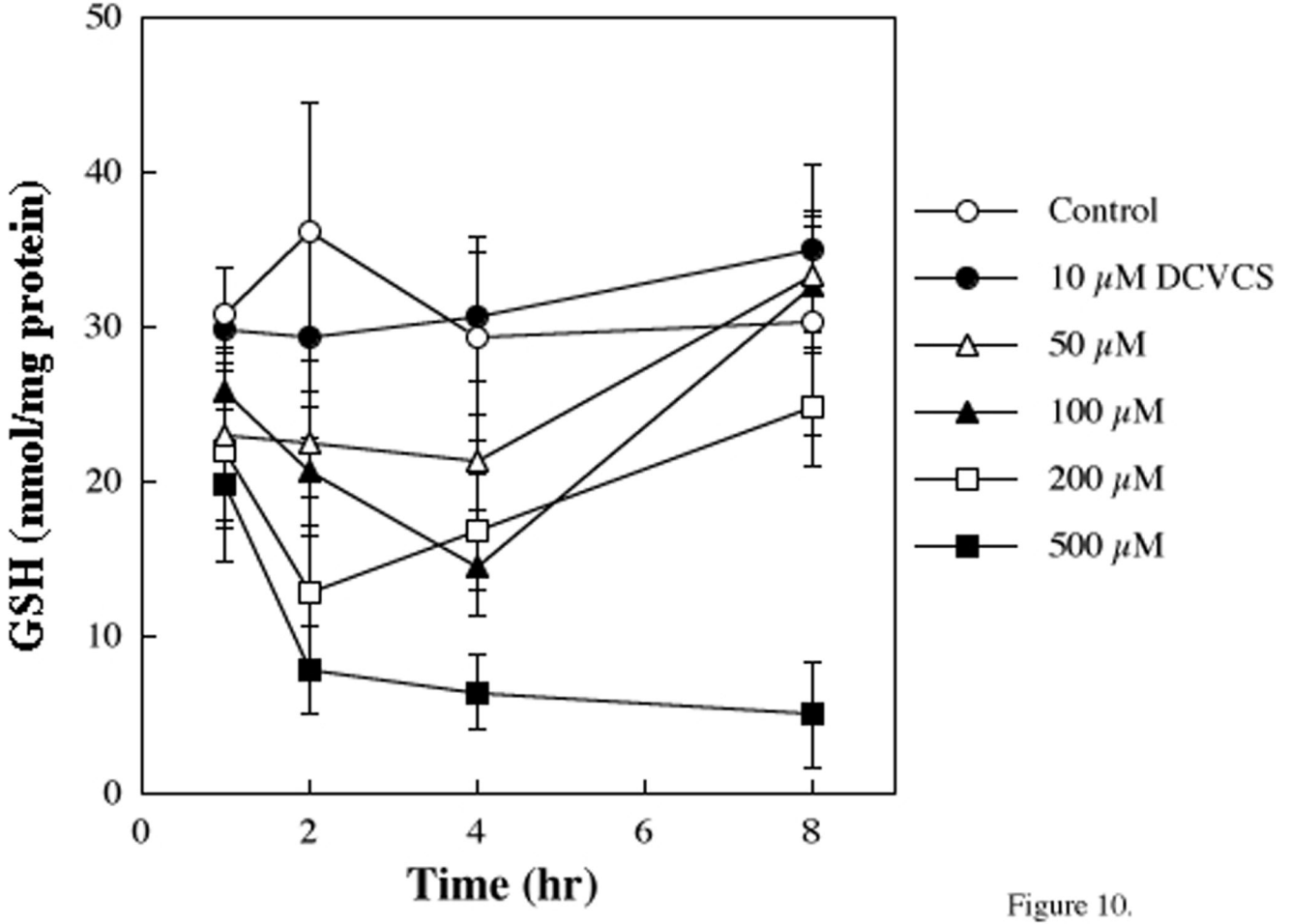

GSH (nmol/mg protein)
50
40
30
20
10
0
0
2
4
6
8
Time (hr)
Control
10 μM DCVCS
50 μM
100 μM
200 μM
500 μM

Figure 10.