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Cartap-induced cytotoxicity in mouse C₂C₁₂ myoblast cell line and the roles of calcium ion and oxidative stress on the toxic effects

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Abstract

Our previous study has demonstrated that instead of neuromuscular blockage cartap, an organonitrogen insecticide, could cause a marked irreversible Ca²⁺-dependent contracture in both isolated mouse and rabbit phrenic nerve-diaphragms. We further examined the potential of direct myocytotoxicity of cartap and the possible roles of calcium ion and oxidative stress on cartap-induced muscle cell injury using the mouse myoblast cell line, C₂C₁₂. Cartap exerted a dose- and time-dependent cytotoxic effect in C₂C₁₂ cells measured by MTT colorimetric assay and trypan blue dye exclusion. The extracellular activities of both creatine kinase (CK) and lactate dehydrogenase (LDH) were elevated in the cartap-treated groups at or greater than 100 μM. The isoenzymatic profiles showed that the elevations were mainly due to CK-3, LDH-3, and LDH-4. Following the addition of 0.5–2.5 mM EGTA, a Ca²⁺ chelator, or 30–100 μM verapamil, an L-type Ca²⁺ channel blocker, the cartap-induced reduction in MTT metabolic rate of C₂C₁₂ cells was significantly restored in a dose-dependent manner in both EGTA and verapamil-treated cells. Furthermore, EGTA could significantly reduce the cartap-induced elevation in the levels of total extracellular CK and LDH activities. Additionally, cartap significantly increased the level of endogenous reactive oxygen species (ROS) in C₂C₁₂ cells in a dose- and time-dependent manner. The cartap-induced ROS generation could be significantly inhibited by antioxidants, including Vitamins C and E, catalase, and superoxide dismutase, with catalase the most effective. EGTA could significantly inhibit cartap-induced ROS generation in a dose-dependent manner. The results suggested that cartap could induce ROS generation in C₂C₁₂ cells via a Ca²⁺-dependent mechanism resulting in subsequent cytotoxicity, at least partially, to C₂C₁₂ cells. It is speculated that both Ca²⁺ and Ca²⁺-induced ROS may also play the central role on the myogenic contracture and myofiber injury of the diaphragm leading to respiratory failure and subsequent death in rabbits exposed ocularly to cartap.

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1. Introduction

Cartap, an organonitrogen insecticide, has long been recognized as an analogue of nereistoxin and categorized as a safe compound since it was first synthesized and used in Japan in 1964 (Chiba et al., 1967; Ray, 1991; Tomlin, 2000). However, our previous study has shown that cartap could induce acute intoxication with subsequent death in rabbits and quails via ocular exposure (Liao et al., 1998). Among the toxic signs, severe muscular fasciculation, tremor, and convulsion were the most consistent findings. By using isolated mouse (Liao et al., 2000) or rabbit (Liao et al., 2003) phrenic nerve-diaphragms, we have demonstrated that cartap could cause marked contracture in either stimulated or quiescent diaphragms; the contracture was not nerve-evoked but myogenic and was possibly related to the alterations in Ca^{2+} flow.

Muscle necrosis, such as diaphragm and intercostal muscle, has been reported in insecticide intoxications such as organophosphate (Karanth et al., 2004; Santos et al., 2002) and carbamate (Milatovic et al., 2005). Reactive oxygen species (ROS)-induced cellular injury has been implicated in the development of many forms of muscle dysfunction (Supinski et al., 1997, 1999). It has been further demonstrated that the propensity of a muscle to generate ROS is Ca^{2+} -dependent (Supinski et al., 1999). Our previous study has shown that cartap could induce the influx of extracellular Ca^{2+} and release of internal Ca^{2+} from the sarcoplasmic reticulum with subsequent induction of muscular contracture in the isolated mouse phrenic nerve-diaphragm (Liao et al., 2000). To our knowledge, there has been no report indicating the involvement of ROS generation in organonitrogen insecticide-treated animals, tissues, or cells. The C_2C_{12} myoblast cell line has been used widely to study chemical and drug-induced skeletal muscle toxicities and the associated mechanisms (Matsuki et al., 1999; Wakayama and Sobue, 1977). The objective of the present study was to use mouse myoblast C_2C_{12} cells as a model to evaluate the myocytotoxicity of cartap and the possible roles of Ca^{2+} and ROS on the toxic effects.

2. Materials and methods

2.1. Chemicals

Cartap, *S,S'*-(2-dimethylaminotrimethylene)-bis (thiocarbamate) ($\text{C}_7\text{H}_{15}\text{N}_3\text{O}_2\text{S}_2$, MW = 237, CAS 15263-53-3), and its hydrochloride salt ($\text{C}_7\text{H}_{16}\text{ClN}_3\text{O}_2\text{S}_2$, MW = 273.8, CAS 22042-59-7) are normally used in formulation (Tomlin, 2000). Technical grade of cartap hydrochloride (98% purity in powder) was kindly provided by Lihnung Agricultural Chemical

Industrial Ltd. in Taiwan (Yunlin, Taiwan, ROC). Its purity was double-checked as described previously (Liao et al., 2000). The reagents and materials used for the measurement of the activities of creatine kinase (CK), lactate dehydrogenase (LDH), and their isoenzymes were obtained from Chiron Diagnostics Corp. (Oberlin, OH, USA) and REP Corp. (Helena Lab., TX, USA), respectively. The 2',7'-dichlorofluorescein diacetate (DCFH.DA) was purchased from Fluka Chemie (AG, Buchs, Switzerland). Other chemicals used, including 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyl-tetrazolium bromide (MTT), Vitamin (Vit.) C and E, 1,3-dimethyl-2-thiourea (DMTU), catalase, superoxide dismutase (SOD), ethylene glycol-bis (β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), verapamil, FeSO_4 , and NaH_2PO_4 , were all analytical grade and purchased from Sigma Chemicals (St. Louis, MO, USA).

2.2. C_2C_{12} cell preparation

The C_2C_{12} cells (ATCC #CRL1722) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemicals) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 2 mM L-glutamine, 100 U/ml of penicillin, and 100 $\mu\text{g}/\text{ml}$ of streptomycin (all from Sigma Chemicals) (DMEM-C) and incubated at 37 °C in 5% CO_2 . The C_2C_{12} cells usually formed monolayers after 3–5 days of culture in 75-T flasks (Corning-Costar, Corning, NY, USA). Before each experiment, the monolayers were digested with 0.25% trypsin for 3–5 min followed by DMEM wash twice. The cell viability was determined by trypan blue dye exclusion and adjusted to 5×10^4 or 5×10^5 cells/ml in DMEM-C as needed. The overall cell viability was always greater than 98%. The cell suspension at 5×10^4 cells/ml was then seeded onto 24 or 96-well culture plates (Corning-Costar) at 0.5 ml or 100 $\mu\text{l}/\text{well}$, respectively; or 12-well culture plates containing sterile glass coverslips (0.5 cm in diameter) (Assistant, Deckglaser, Germany) at 1 ml/well. Following incubation at 37 °C in 5% CO_2 for 3–4 days to form monolayers, the non-adherent cells were removed by gentle wash with cold DMEM-C. The plates or plates containing coverslips covered with monolayers were then used for various assays.

2.3. Cell morphological examination

Cartap at 10–1000 μM or an equal volume of DMEM-C was placed into 12-well culture plates containing monolayer-covered coverslips and incubated at 37 °C in 5% CO_2 for 24 h. Morphological alterations of C_2C_{12} cells were then evaluated by scanning electron microscopy. Briefly, cartap-treated or medium control coverslip preparations were rinsed with PBS, fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Washington, PA, USA) for 30 min, post-fixed in 2% osmium tetroxide (Electron Microscopy Sciences, Washington, PA, USA), dehydrated in graded ethanol solutions (35, 50, 70, 85, 90, 95, and 100%), and immersed in isoamyl acetate for 30 min. The coverslips were then affixed to aluminum stubs with silver conducting paint. After dried in a critical point

drying apparatus (Tousimis Research, Rockville, MD, USA), the cells were coated with gold–palladium in a sputter coater (SPI Supplies, West Chester, PA, USA) and examined with a JEOL® JSM-5410 electron microscope (Jeol, Tokyo, Japan) at 5–20 kV.

2.4. Trypan blue dye exclusion assay

To evaluate the direct cytotoxic effect of cartap on C_2C_{12} cells, cartap at the final concentration of 0, 10, 30, 100, 300, and 1000 μM or an equal volume of DMEM-C was placed into 12-well culture plates containing monolayer-covered coverslips and incubated at 37 °C in 5% CO_2 for 24 h. Following the removal of the culture supernatant and staining with 0.2% trypan blue dye in phosphate-buffered saline, the coverslips were then mounted on slides the rates of positivity were calculated in 200 cells out of 10 randomly selected fields at 400 \times by light microscopy.

2.5. MTT assay

To measure the direct cytotoxic effect of cartap, C_2C_{12} monolayers prepared on 96-well culture plates were incubated with cartap at the final concentration of 0, 10, 30, 100, 300, and 1000 μM or an equal volume of DMEM-C at 37 °C in 5% CO_2 for 24 h. The cell activity was then analyzed by the MTT colorimetric assay (Mosmann, 1983). Briefly, an MTT solution at 2 mg/ml in PBS was added to the 96-well culture plates at 50 μl /well on each time point. Following another 3 h of incubation, 100 μl of DMSO (Merck, Darmstadt, Germany) were added in each well and mixed thoroughly to dissolve the dark blue formazan crystals. Thereafter, the plates were read on an ELISA reader (MRX, Dynatech Medical Products, Guernsey, Channel Island, Great Britain) at the wavelength of 540 nm using absorbance (A) at 630 nm as a reference. Data were expressed as a percentage of control (ΔA) = [(A value of cartap-treated group)/(A value of medium control)] \times 100. The median lethal concentration (LC50) was further calculated as described by Mosmann (1983).

2.6. Activity measurement for extracellular creatine kinase (CK) and lactate dehydrogenase (LDH) and their isoenzymes

The activities of total extracellular CK and LDH and their isoenzymes were measured according to the methods reported by Gupta et al. (1994), and Harauchi and Hirata (1993). Monolayers of C_2C_{12} cells prepared on 24-well culture plates were incubated with cartap at the final concentration of 0, 10, 30, 100, 300, and 1000 μM or an equal volume of DMEM-C at 37 °C in 5% CO_2 for 24 h. At the end of incubation, the culture supernatants were collected for extracellular enzyme activity measurement. The total extracellular activities of CK and LDH were determined according to the spectrophotometry of Chiron Diagnostics Corporation (Oberlin, OH, USA) using the Express Plus Automatic Clinical Chemistry Ana-

lyzer. The isoenzymes of CK, including CK-1 (CK-BB), CK-2 (CK-MB), and CK-3 (CK-MM) and the isoenzymes of LDH, including LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5, were separated by electrophoresis, and the activity of each isoenzyme was quantified according to the manufacturer's instructions (REP, Helena Lab, Beaumont, TX, USA) using the Rapid Electrophoresis Analyzer and densitometer. The activities of total extracellular CK and LDH, and their isoenzymes (A_{CK} and A_{LDH}) were expressed as IU/l and a percentage of total activity (ΔA_{CK} or ΔA_{LDH}) for each isoenzyme was further calculated, where ΔA_{CK} or ΔA_{LDH} = [(A_{CK} or A_{LDH} value of a particular group)/(total A_{CK} or A_{LDH} value of the corresponding group)] \times 100.

2.7. Effect of cartap on intracellular ROS generation in C_2C_{12} cells

To measure the effect of cartap on the generation of ROS in C_2C_{12} cells, a DCFH.DA oxidation-based fluorescence test was used (Lebel et al., 1992). DCFH.DA is a stable molecule that is readily to cross cell membrane and oxidized to highly fluorescent DCF in the presence of intracellular ROS. A 50 μM working solution of DCFH.DA was freshly prepared by mixing 250 μl of 1 mM DCFH.DA in methanol with 1 ml of 0.01 N NaOH at room temperature for 30 min followed by neutralization with the addition of 25 mM NaH_2PO_4 at pH 7.4 to a final volume of 5 ml. This DCFH.DA working solution was then kept on ice in the dark until use. For ROS measurement, the reaction was performed with monolayers of C_2C_{12} cells prepared on 24-well culture plates in 0.1 mM Tris–buffer at pH 7.4 in a total volume of 1 ml, either containing cartap at various concentrations, 2.5 μM DCFH.DA, and 0.5 mM FeSO_4 , at 37 °C for 20 min on a shaker at 200 rpm, or containing 1000 μM cartap, 2.5 μM DCFH.DA, and 0.5 mM FeSO_4 , at 37 °C for 0–120 min on a shaker at 200 rpm. After placing in –20 °C freezer for 20 min to terminate the reaction, the reaction mixture was then transferred to a cuvette and the fluorescence intensity (FI) of DCF was determined by a HITACHI F-4010 Fluorescence Spectrophotometer (HITACHI, Tokyo, Japan) at the excitation wavelength of 488 nm (bandwidth 3 nm) and emission wavelength of 525 nm (bandwidth 20 nm). Data were expressed as the differences in the fluorescence intensity (ΔFI) between the cartap-treated or medium control group and the corresponding cartap-treated or medium control group without the presence of C_2C_{12} cells, where ΔFI = (FI value of cartap-treated or medium control group) – (FI value of each corresponding group without the presence of C_2C_{12} cells). A percentage of control was further calculated as [(ΔFI value of a particular cartap-treated group)/(ΔFI value of the medium control group)] \times 100.

2.8. Effect of antioxidant on cartap-induced changes in MTT metabolic rate and ROS generation in C_2C_{12} cells

To evaluate the effect of various antioxidants on cartap-induced changes in MTT metabolic rate and ROS generation,

monolayers of C_2C_{12} cells prepared on 24-well culture plates were treated with 50 μM Vit. C or Vit. E (Barrett et al., 1999; Chow et al., 1999); 300 U/ml catalase (a H_2O_2 scavenger) (Hsieh et al., 1998); 0.1 mM DMTU (a OH^\bullet scavenger) (Hsieh et al., 1998); or 36.85 U/ml superoxide dismutase (SOD, a O_2^- scavenger) (Lebel et al., 1992) for 30 min. Following the addition of 1000 μM cartap for another 24 h, the MTT metabolic rate was then measured as described above. For ROS generation, 1000 μM cartap, 2.5 μM DCFH-DA, and 0.5 mM FeSO_4 were then added and the reactions were performed in 0.1 mM Tris–buffer at pH 7.4 in a total volume of 1 ml at 37 °C for 20 min on a shaker at 200 rpm. Changes in the fluorescence intensity of DCF were then determined as described above.

2.9. Effects of Ca^{2+} antagonist on cartap-induced ROS production in C_2C_{12} cells

To determine the effect of Ca^{2+} on cartap-induced ROS production, monolayers of C_2C_{12} cells prepared on 24-well culture plates were incubated with 0–2.5 mM EGTA for 30 min prior to the addition of 1000 μM cartap, 2.5 μM DCFH-DA, and 0.5 mM FeSO_4 . All of the reactions were performed in 0.1 mM Tris–buffer at pH 7.4 in a total volume of 1 ml at 37 °C for 20 min on a shaker at 200 rpm. Changes in the fluorescence intensity of DCF were then determined as described above.

2.10. Effect of Ca^{2+} antagonist on cartap-induced cytotoxicity and enzymatic leakage in C_2C_{12} cells

The role of Ca^{2+} on cartap-induced cytotoxicity was evaluated by the incubation of C_2C_{12} monolayers prepared on 96-well culture plates with 0.5–2.5 mM EGTA, a Ca^{2+} chelator (Fleckenstein, 1983), or 0.01–0.1 μM verapamil, an L-type Ca^{2+} channel blocker (Fleckenstein, 1983), for 30 min prior to the addition of various concentrations of cartap followed by incubation for another 24 h. Changes in the MTT metabolic rate were then analyzed as described above. To measure the effects of Ca^{2+} on cartap-induced CK and LDH leakage, C_2C_{12} monolayers prepared on 24-well culture plates were incubated with various concentrations of cartap in the presence or absence of 2.5 mM EGTA for 24 h. Changes in extracellular activities of CK and LDH were then analyzed as described above.

2.11. Statistical analysis

Values, expressed as mean \pm S.D., presented in figures and tables and used in statistical analyzes represent at least three independent trials with 4–8 replicates each per main experiment run concurrently. The data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test. Statistical analyzes were carried out by Statistical Analysis System procedures (Statistical Analysis System; SAS for windows 6.12; SAS Institute Inc., Cary, NC, USA). The difference was considered statistically significant when $p < 0.05$.

3. Results

3.1. Effects of cartap on C_2C_{12} cell morphology, MTT metabolic rate, and viability

Following 24 h of incubation, the C_2C_{12} cells of the 10 μM cartap-treated group remained their normal morphology as that of the medium control group, characterized by ruffled membrane surface with many long, radiating, finger-like pseudopodia. However, when the cartap concentration reached 30 μM , some of the cells were lysed and many of the remaining cells became swollen and/or rounded with formation of surface blebs and cytoplasmic vacuoles. The number of cells undergoing lysis increased as the concentration of cartap increased. When measured by the MTT colorimetric assay, an apparent dose-dependent reduction in the metabolic rate was seen in the cartap-treated group (Fig. 1). When compared with the medium control, the average MTT metabolic rate of C_2C_{12} cells in the cartap-treated group following 24 h of incubation reduced from $89.6 \pm 4.1\%$ of control at 10 μM to $63.9 \pm 5.6\%$, $38.7 \pm 6.4\%$, $28.2 \pm 3.0\%$, and $8.0 \pm 0.4\%$ at 30, 100, 300, and 1000 μM , respectively. Statistically significant difference appeared in cartap-treated group at 30 μM or higher. The corresponding dose of cartap to induce 50% inhibition in MTT metabolic rate was 52.8 μM . When stained with trypan blue, there were only scattered positive cells in the medium control and 10 μM cartap-treated groups; a 20–25% positive rate occurred in 30 μM cartap-treated group; however, nearly 60–70% positive rate was seen in the cartap-treated group at 100 μM or a higher concentration (data not shown).

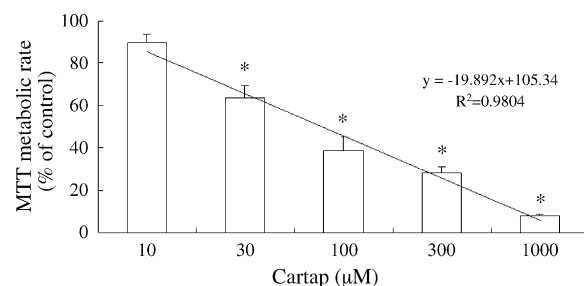


Fig. 1. Effect of cartap on the MTT metabolic rate of C_2C_{12} cells. C_2C_{12} cells were incubated with cartap at various concentrations for 24 h. The MTT metabolic rate of each group was determined according to the procedure outlined in Section 2. The data were expressed as percentage of control and are presented as the mean \pm S.D. of three different experiments with eight replicates each. Asterisks indicate significant differences ($p < 0.05$) between cartap-treated and medium control groups. The linear correlation between cartap concentration and MTT metabolic rate of C_2C_{12} cells was calculated and shown at the up right hand corner.

Table 1

Changes in the levels of total extracellular activities of creatine kinase (CK) and its isoenzymes in C₂C₁₂ cells following incubation with various concentrations of cartap for 24 h

Cartap (μ M)	CK (IU/l)	Isoenzymes (IU/l)		
		CK-1	CK-2	CK-3
0	2.0 \pm 0.2	–	–	–
10	1.8 \pm 0.5	–	–	–
30	2.0 \pm 0.5	–	–	–
100	3.5 \pm 0.7*	–	0.2 \pm 0.1 ^{a,*} (6.2 \pm 0.1) ^b	3.0 \pm 0.7* (93.8 \pm 0.1)
300	3.5 \pm 0.3*	–	0.1 \pm 0.0* (1.4 \pm 0.6)	3.5 \pm 0.3* (98.6 \pm 0.6)
1000	4.0 \pm 0.7*	–	0.1 \pm 0.0* (2.5 \pm 0.2)	3.9 \pm 0.7* (97.5 \pm 0.2)

–: Not detectable.

^a Data are expressed as mean \pm S.D. of three independent experiments and each experiment was done in quadruplicates.

^b Data in the parentheses represent the percent activity of CK of each isoenzyme in a particular group.

* Significantly different from the control group at $p < 0.05$.

3.2. Effects of cartap on the activities of total extracellular CK and LDH and their isoenzymatic profiles in C₂C₁₂ cells

In the medium control, the level of total extracellular activity of CK was significantly lower than that of LDH. The average total extracellular CK activity of the medium control was 2.0 \pm 0.2 IU/l and there was no detectable activity for all CK isoenzymes based on the method used in the present study (Tables 1 and 2). As for the cartap-treated group, the levels of the total extracellular CK activity at 10 and 30 μ M cartap were about the same as those of the medium control group, but it increased significantly as the dose of cartap reaching 100 μ M or higher, ranging from 3.5 \pm 0.3 to 4.0 \pm 0.7 IU/l (Table 1). Similar to the medium control group, there was no detectable activity of CK isoenzymes in the cartap-treated group at lower doses. The elevated total extracellular CK activity in the cartap-treated group at 100 μ M or higher was contributed to the increase in CK-2 and CK-3 activities with CK-3 predominant (Table 1).

The average total extracellular LDH activity in the medium control was 50.8 \pm 0.3 IU/l; the percentage activity in each isoenzyme was LDH-1 35.4%, LDH-2 21.6%, LDH-3 16.4%, LDH-4 18.9%, and LDH-5 7.7%. In cartap-treated group, the levels of total extracellular activities of LDH and its five isoenzymes at 10 and 30 μ M cartap were all about the same as those of the medium control group. When the cartap concentration reached 100 μ M or higher, there was a significant dose-dependent increase in the level of total extracellular LDH activity, ranging from 78.3 \pm 8.3 to 157.8 \pm 5.7 IU/l (Table 2). The elevation was mainly resulted from the increase in the levels of LDH-3 and LDH-4 activities, although there was a concomitant slight increase in the

level of LDH-5 activity and decrease in the levels of LDH-1 and LDH-2 activities (Table 2).

3.3. Effects of antioxidant and Ca²⁺ antagonist on cartap-induced cytotoxicity in C₂C₁₂ cells

When incubated with cartap alone for 24 h, the MTT metabolic rate of C₂C₁₂ cells dropped markedly in a dose-dependent manner from 90% to 8% of the medium control group as the dose of cartap increased from 10 to 1000 μ M, respectively. The cartap-induced cytotoxicity to C₂C₁₂ cells could be effectively protected by the pretreatment of the cells with various antioxidants. Interestingly, antioxidant-pretreatment in 10–30 μ M cartap-treated groups could promote the MTT metabolic rate significantly from 64–90% up to 102–126% of the medium control group; however, following similar antioxidant-pretreatment the MTT metabolic rate remained around 100–102% in the medium control group. Among the five tested antioxidants, it is shown that C₂C₁₂ cells pretreated with Vit. E, catalase, and SOD at the selected doses could constantly maintain a significantly higher MTT metabolic rate than the cartap-alone group at the doses of 10–1000 μ M (Fig. 2A); in contrast to the rather low MTT metabolic rates, 28% and 8% of the medium control group, in cartap-alone groups at the higher doses of 300 and 1000 μ M, respectively, the MTT metabolic rates of the two groups receiving the two higher doses of cartap following the pre-treatment with these three antioxidants raised to 59–62% and 40–50% of the medium control group, respectively. As for the less effective Vit. C- and DMTU-treated cartap groups, instead of continuous decline as seen in cartap-alone group the MTT metabolic rate retained 26–31% and 34–42%, respectively, when the doses of cartap reaching 100–1000 μ M; significant difference between

Table 2
Changes in the levels of total extracellular activities of lactate dehydrogenase (LDH) and its isoenzymes in C₂C₁₂ cells following incubation with various concentrations of cartap for 24 h

Cartap (μ M)	Isoenzymes (IU/l)					
	LDH (IU/l)	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
0	50.8 \pm 0.3 ^a	17.9 \pm 0.5 (35.4 \pm 0.9) ^b	11.0 \pm 0.6 (21.6 \pm 1.3)	8.4 \pm 0.3 (16.4 \pm 0.4)	9.6 \pm 0.3 (18.9 \pm 0.5)	3.9 \pm 0.7 (7.7 \pm 1.4)
10	50.8 \pm 0.5	15.7 \pm 1.1 (30.6 \pm 3.2)	10.2 \pm 0.4 (20.1 \pm 1.0)	8.7 \pm 0.3 (17.1 \pm 0.6)	11.4 \pm 1.3 (22.4 \pm 2.5)	4.7 \pm 0.5 (9.3 \pm 0.9)
30	61.0 \pm 8.0	18.0 \pm 0.7 (31.1 \pm 2.3)	9.9 \pm 0.5 (17.2 \pm 2.4)	12.9 \pm 2.1* (20.9 \pm 0.7)	16.1 \pm 6.7 (23.9 \pm 6.3)	4.1 \pm 0.7 (7.3 \pm 1.6)
100	78.3 \pm 8.3*	15.0 \pm 0.5* (19.9 \pm 2.3)	10.9 \pm 0.3 (14.5 \pm 2.0)	17.9 \pm 2.1* (22.8 \pm 0.7)	28.7 \pm 6.4* (34.9 \pm 5.4)	5.8 \pm 0.3* (7.9 \pm 1.4)
300	81.8 \pm 1.9*	16.8 \pm 0.3 (20.6 \pm 0.2)	10.9 \pm 0.3 (13.3 \pm 0.6)	22.7 \pm 1.1* (27.9 \pm 2.0)	26.0 \pm 3.3* (31.6 \pm 3.5)	5.4 \pm 0.4 (6.6 \pm 0.8)
1000	157.8 \pm 5.7*	16.6 \pm 0.5 (10.6 \pm 0.2)	7.8 \pm 0.6* (4.9 \pm 0.3)	87.4 \pm 2.6* (55.4 \pm 0.6)	46.0 \pm 2.3* (29.1 \pm 0.5)	–

–: Not detectable.

^a Data are expressed as mean \pm S.D. of three independent experiments and each experiment was done in quadruplicates.

^b Data in the parentheses represent the percent activity of LDH of each isoenzyme in a particular group.

* Significantly different from the control group at $p < 0.05$.

antioxidant-treated and cartap-alone groups was also seen at 1000 μ M cartap.

As for the two Ca²⁺ chelators, EGTA and verapamil, they both also possessed statistically significant protective effect on cartap-induced cytotoxicity to C₂C₁₂ cells (Fig. 2B and C). Similar to antioxidants, EGTA at 0.5–2.5 mM and verapamil at 0.03–0.1 mM could promote the cartap-reduced MTT metabolic rate from 64–90% up to 106–125% of the medium control group in a dose-dependent manner. Statistically significant elevation in MTT metabolic rate was constantly seen in all 10–1000 and 10–300 μ M cartap-treated groups pre-incubated with 1.25 or 2.5 mM EGTA and 0.5 mM EGTA, respectively; and also in 10–300 and 10–100 μ M cartap-treated groups pre-incubated with 0.1 and 0.03 mM verapamil, respectively.

3.4. Effect of cartap on intracellular ROS generation in C₂C₁₂ cells

The level of intracellular ROS generation in the medium control group, as expressed by the fluorescence intensity (FI) of DCF, was 440.9 \pm 23.4. A dose-dependent elevation in DCF formation was seen in the cartap-treated group, ranging from 536.1 \pm 50.1 at 10 μ M to 735.1 \pm 14.8 at 1000 μ M; when the values were expressed as percentage of control, it was 122.0 \pm 13.9% at 10 μ M and reached 167.0 \pm 6.0% at 1000 μ M (Fig. 3A). The level of cartap-induced intracellular ROS generation was also time-dependent. When C₂C₁₂ cells were incubated with 1000 μ M cartap for 0–120 min, the FI value was 418.8 \pm 28.8 at 0 min and it gradually increased from 623.3 \pm 37.3 at 10 min to 1768.4 \pm 212.3 by 120 min; when the values were expressed as percentage of 0 min, it was 149.1 \pm 8.1% at 10 min and reached 424.0 \pm 56.3% at 120 min (Fig. 3B). The differences either between the cartap-treated group at all tested doses and medium control group or between all tested incubation periods and 0 min at 1000 μ M cartap were all statistically significant.

3.5. Effects of antioxidant on cartap-induced intracellular ROS generation in C₂C₁₂ cells

To further evaluate the role of ROS on cartap-induced cell injury, various ROS scavengers, including Vit. C, Vit. E, DMTO, catalase, and SOD, at each selected dosage were added in C₂C₁₂ cells prior to the addition of 1000 μ M of cartap. The value of FI was then monitored after the reaction mixture was incubated for 20 min. The results of various treatments are shown in Fig. 3C. Catalase at 300 U/ml was the most effective in the inhibition

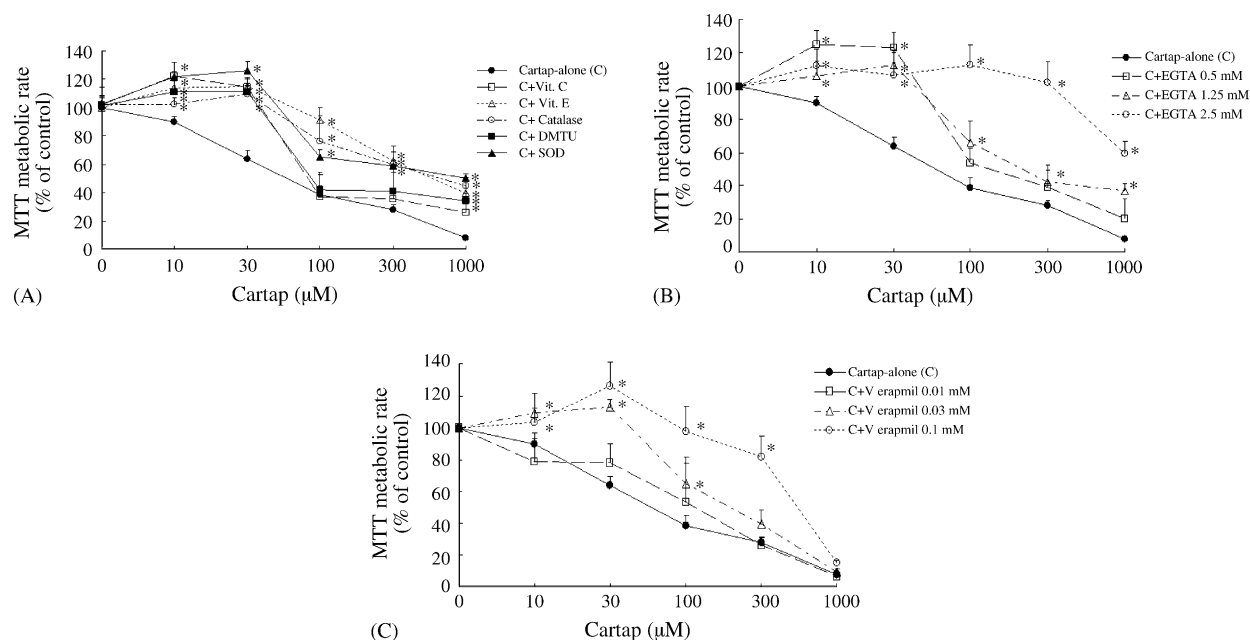


Fig. 2. Effects of antioxidant and Ca^{2+} antagonist on cartap-induced cytotoxicity measured by MTT metabolic rate in C_2C_{12} cells. Under each tested condition, the MTT metabolic rate of each group was determined according to the procedure outlined in Section 2. Changes in MTT metabolic rate in C_2C_{12} cells by incubation with (A) various antioxidants, including Vit. C (50 μM), Vit. E (50 μM), catalase (300 U/ml), 1,3-dimethyl-2-thiourea (DMTU) (100 μM), and superoxide dismutase (SOD) (36.85 U/ml); (B) various concentrations of ethylene glycol-bis (β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) (a Ca^{2+} chelator); or (C) various concentrations of verapamil (an L-type Ca^{2+} channel blocker) for 30 min prior to the addition of various concentrations of cartap for another 24 h. The data were expressed as percentage of control and are presented as the mean \pm S.D. of three different experiments with eight replicates each. Asterisks indicate significant differences ($p < 0.05$) between cartap-alone and antioxidant- or Ca^{2+} antagonist-treated groups.

of cartap-induced intracellular ROS generation among the antioxidants tested with a reduction rate close to 79% when compared with the group of cartap-alone. A less prominent but statistically significant reduction in cartap-induced ROS generation was also seen in Vit. C-, Vit. E-, and SOD-treated groups at the doses of 50, 50 μM , and 36.85 U/ml, respectively. The efficacy of inhibition in DFC generation was in a gradual reduction fashion as Vit. C > Vit. E > SOD, ranging from 50% to 31%. However, there was only 8% reduction in cartap-induced ROS generation by the OH^\bullet scavenger, DMTU at 0.1 mM, and the reduction was not statistically significant.

3.6. Effects of Ca^{2+} antagonist on cartap-induced changes in extracellular enzymatic profiles and intracellular ROS generation in C_2C_{12} cells

To further explore the role of Ca^{2+} on cartap-induced changes in extracellular enzymatic profiles and intracellular ROS generation, C_2C_{12} cells were incubated with cartap in the presence or absence of 2.5 or 0.5–2.5 mM EGTA, respectively. The addition of 2.5 mM EGTA

could significantly reduce the levels of cartap-induced CK and LDH leakage in C_2C_{12} cells, especially in the groups treated with 100–1000 μM cartap (Fig. 4); when the values were expressed as percentage of medium control group, it reduced from 264–393% to 160–192% for CK and from 169–377% to 99–163% for LDH in cartap-treated groups at 100–1000 μM (Fig. 4). As for the effect of EGTA on the generation of intracellular ROS in C_2C_{12} cells, the result showed that the removal of Ca^{2+} by the addition of EGTA could markedly reduce the level of FI in a dose-dependent manner with 55% reduction at 0.5 mM EGTA, 94% at 1.25 mM EGTA, and up to 99% at 2.5 mM EGTA when compared with cartap-alone group (Fig. 3D).

4. Discussion

Cartap is a nereistoxin analogue pesticide and commonly considered acting like nereistoxin to evoke its acute toxicity by neuromuscular blockage via the inhibition of postsynaptic nicotinic acetylcholine receptor ion channel leading to respiratory failure and subsequent death (Ray, 1991). However, our recent studies

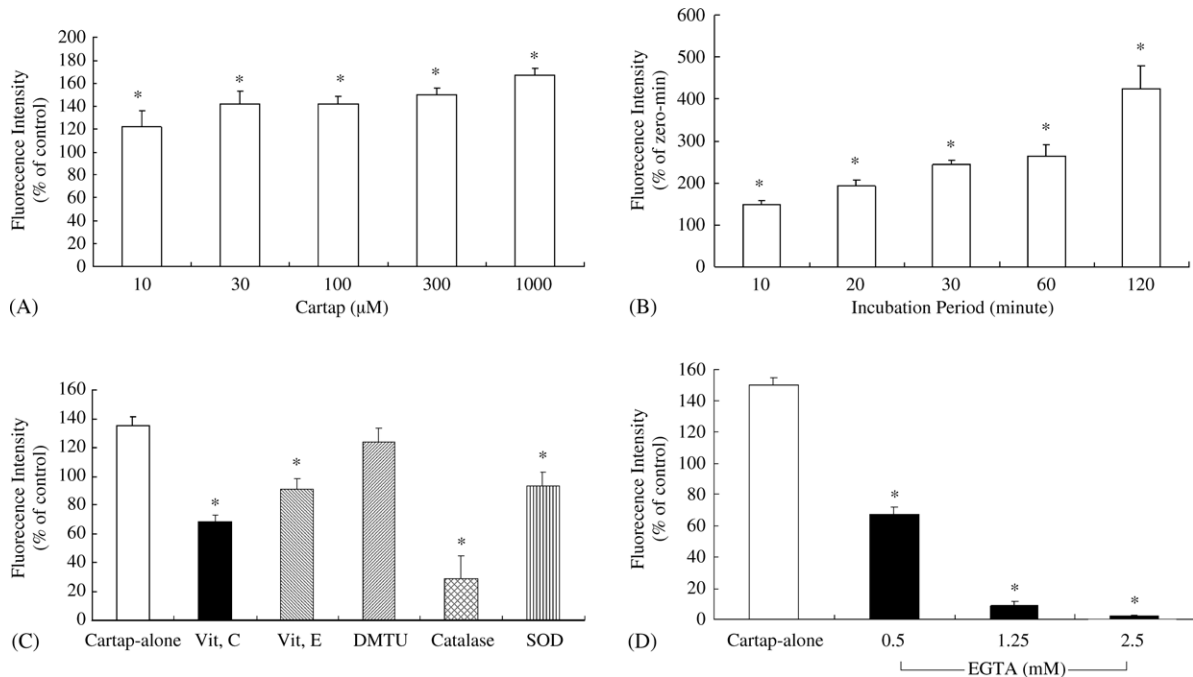


Fig. 3. Effects of antioxidant and Ca²⁺ chelator on cartap-induced reactive oxygen species (ROS) production in C₂C₁₂ cells. Under each tested condition, the total intracellular ROS production was measured by the DCFH₂DA oxidation-based fluorescence test as fluorescence intensity and expressed as percentage of control as outlined in Section 2. (A) Changes in ROS production in C₂C₁₂ cells treated with various concentrations of cartap for 20 min. (B) Changes in ROS production in C₂C₁₂ cells treated with 1000 μM cartap for various time periods. (C) Changes in ROS production in C₂C₁₂ cells by incubation with various antioxidants, including Vit. C (50 μM), Vit. E (50 μM), 1,3-dimethyl-2-thiourea (DMTU) (100 μM), catalase (300 U/ml), and superoxide dismutase (SOD) (36.85 U/ml), for 30 min prior to the addition of 1000 μM cartap for another 20 min. (D) Changes in ROS production in C₂C₁₂ cells by incubation with various concentrations of the Ca²⁺ chelator, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), for 30 min prior to the addition of 1000 μM cartap for another 20 min. Data are presented as mean ± S.D. of three experiments with five replicates each. Asterisks indicate significantly different from the medium control or cartap-alone group at p < 0.05.

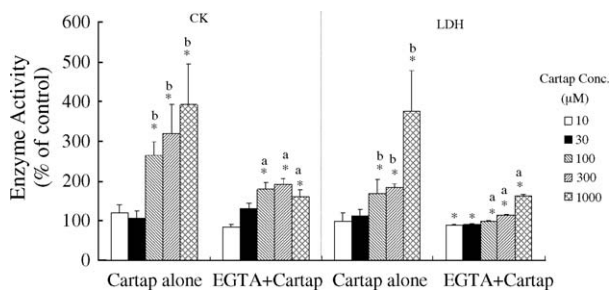


Fig. 4. Effect of calcium chelator on cartap-induced enzyme leakage in C₂C₁₂ cells. C₂C₁₂ cells were incubated with various concentrations of cartap in the presence (EGTA + Cartap) or absence (Cartap alone) of 2.5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) for 24 h. The total extracellular activities of creatine kinase (CK) and lactate dehydrogenase (LDH) in the supernatant were determined according to the procedure outlined in Section 2. Data are presented as the mean ± S.D. of three different experiments with quadruplicate each. Asterisks indicate significantly different from the corresponding medium control within each Cartap alone or EGTA + Cartap group. (a and b) Within the same enzyme group, values with different labels at the same cartap concentration differ significantly (p < 0.05).

have demonstrated that rather than neuromuscular blockage cartap induces a Ca²⁺-dependent severe irreversible contracture in isolated mouse and rabbit phrenic-nerve diaphragms (Liao et al., 2000, 2003); and it is speculated that the cartap-induced contracture is due to the influx of extracellular Ca²⁺ and release of internal Ca²⁺ from the sarcoplasmic reticulum (Liao et al., 2000). Rabbits dying acutely from ocular instillation of cartap displayed prominent hypercontraction bands, fragmentation, and rupture of the myofibers in diaphragm but not in heart and thigh (Liao et al., 2003). In the present study, attempts were made to evaluate the myocytotoxicity of cartap and its related mechanism of action using the mouse skeletal muscle myoblast cell line, C₂C₁₂, to mimic diaphragmatic muscle cells. Reduction of the tetrazolium salt MTT in cells and tissues is regarded as an indicator of “cell redox ability”, attributed to both mitochondria and non-mitochondria (Bernas and Dobrucki, 2002). Because MTT is reduced to the colored formazan only by metabolically active cells, it is generally accepted that the assay detects viable cells exclusively (Kwok et al.,

2004; Satoh et al., 1996). Thus, the data of trypan blue dye exclusion and MTT assay shown in the present study clearly indicate that cartap not only has a cytotoxic effect but also has a direct cytotoxic effect on C₂C₁₂ cells and these effects were dose and time-dependent.

Skeletal muscle is rich in CK and LDH and extracellular release of these enzymes and their isoenzymes is considered to be a sensitive indicator of myocyte injury and frequently used for monitoring damage of muscle cells (Angulo and Lomonate, 2003; Gupta et al., 1994; Gupta and Goad, 2000). The evident extracellular leakage of both CK and LDH further supported the cartap-induced cytotoxic effect on C₂C₁₂ cells. When the concentration of cartap reached 100 μ M or higher, significant changes were consistently present in the MTT metabolic rate, extracellular leakage of CK and LDH, and trypan blue dye exclusion; however, only MTT metabolic rate displayed statistically significant change at 30 μ M cartap. The results suggest that the MTT colorimetric assay is more sensitive than enzyme leakage or dye exclusion to measure cartap-induced cytotoxic effect in C₂C₁₂ cells.

Within cartap-induced enzyme leakage in C₂C₁₂ cells, it appears that the leakage of LDH is slightly more sensitive than CK. Although no significant change was seen in the mean value of the total extracellular CK and LDH activities in the 30 μ M cartap-treated group, significantly increased LDH-3 activity started to appear at this dose. However, no significant change was noted in CK or its isoenzymes at the same dose. The release of CK is generally considered as the single most important indicator of skeletal muscle injury even after minor insults (Katrjji and Al-Jaberi, 2001). Although LDH is present in most cells and its release is non-specific to any organ, its leakage to the culture supernatant is also frequently used in vitro to evaluate the myotoxic activity of various compounds (Angulo and Lomonate, 2003). Similar to the present in vitro study, elevation in the activities of CK, LDH, and their isoenzymes was also observed in the sera of rabbits exposed to a lethal dose of cartap at 12.5 mg/kg by eye instillation (Liao et al., 2003). The increased isoenzymes were mainly CK-2, CK-3, LDH-3, and LDH-4 in C₂C₁₂ cells, and CK-1, CK-2, and CK-3 in rabbits. The cause for the differences in the isoenzyme profiles between cartap-treated C₂C₁₂ cells and rabbits is not known, although the differences due to animal species and/or source of tissue/organ should be considered (Katrjji and Al-Jaberi, 2001).

Microscopically, cartap-induced cell surface blebbing was observed initially at the dose of 30 μ M followed by cell rounding and swelling when the dose was increased. This was in good correlation with the initial significant enzyme leakage of LDH-3 at 30 μ M followed

by subsequent significant release of both CK and LDH as the dose reached 100 μ M or higher. Significant reduction in the ability to exclude trypan blue also became apparent when the dose of cartap reached 100 μ M. The morphological changes, enzyme leakage, and reduced trypan blue dye exclusion are all indicative of membrane injury (Fornelli et al., 2004; Katirji and Al-Jaberi, 2001; Trump and Berezsky, 1995). This suggests that cartap could cause damage to the membrane integrity of C₂C₁₂ cells and lead to cell death.

Calcium has been shown to be an important factor in cell injury resulting from a variety of pathophysiological stresses in various tissues, including skeletal muscle (Jackson et al., 1984; Matucci et al., 1997; Supinski et al., 1999). Our previous studies also showed that Ca²⁺ seems to play an essential role in cartap-induced muscular contracture in isolated mouse and rabbit phrenic nerve-diaphragms, which may be the cause for the acute death of rabbits due to respiratory failure after ocular exposure to cartap (Liao et al., 2000, 2003). The involvement of Ca²⁺ on cartap-induced muscular injury is further proved in the present study at cellular level. Lowering the intracellular level of Ca²⁺ by adding either the Ca²⁺ chelator, EGTA, or the L-type Ca²⁺ channel blocker, verapamil, could effectively, although not completely, restore the cartap-induced decline in the MTT metabolic rate of C₂C₁₂ cells in a dose-dependent manner. Aside from the influx of extracellular Ca²⁺, our previous study has also demonstrated that the release of internal Ca²⁺ from the sarcoplasmic reticulum via a ryanodine receptor channel also plays an important role in the cartap-induced contracture in the isolated mouse phrenic nerve-diaphragm (Liao et al., 2000). Thus, cartap may exert its effect by promotion of the extracellular Ca²⁺ influx and the induction of internal release Ca²⁺ although the underlying mechanism for the changes in Ca²⁺ flow remains unclear.

A sustained increase of cytosolic Ca²⁺ concentration is capable of activating a number of potentially harmful processes in the cell. Some of these involve the activation of hydrolytic enzymes such as phospholipase A₂, phospholipase C, Ca²⁺-activated proteases, and Ca²⁺-activated endonucleases, whereas others include ligand-activated kinases and intracellular messengers, e.g., IP₃, cADPR, and nitric oxide (Supinski et al., 1999; Trump and Berezsky, 1995). The increased cytosolic Ca²⁺ concentration exerts adverse effects on the cytoskeleton, including loss of F actin fibers, loss of the integrity of actin-plasmalemmal attachments, and destabilization of microtubules resulting in the important prelethal reaction to injury, formation of cytoplasmic blebs as seen

in cartap-treated C₂C₁₂ cells (Bennet and Weeds, 1993; Trump and Berezsky, 1995).

Reactive oxygen species (ROS), although are required for certain physiological responses, when produced in excess amounts are known to be important tissue damage causing agents, especially concomitant with elevated cytosolic Ca²⁺ (Gordeeva et al., 2003; Suzuki et al., 2003). A significant dose and time-dependent increase in the amount of total intracellular ROS was seen in cartap-treated C₂C₁₂ cells. Based on the reduction level of fluorescence intensity of DCF in cartap-treated cells by the addition of various antioxidants, it seems that H₂O₂ was produced at the highest level followed by O₂⁻ with OH[•] at the lowest level, although the exact ROS species and their relative ratio generated in cartap-treated C₂C₁₂ cells remain to be clarified. The significance of the increased ROS production on cartap-induced cytotoxicity in C₂C₁₂ cells was further proved by the result of the protective assay of antioxidants. Similar to reducing intracellular level of Ca²⁺ by EGTA or verapamil, the addition of various antioxidants could also effectively restore the cartap-induced decline in the MTT metabolic rate of C₂C₁₂ cells. At the selected doses of all tested antioxidants, it appears that at the lower doses of cartap, 10 and 30 μM, these antioxidants not only completely abolished the cartap-induced adverse effect on the MTT metabolic rate but also further slightly enhanced its metabolic activity in C₂C₁₂ cells. At the higher doses of cartap, it seems that Vit. E, catalase and SOD were more effective than Vit. C and DMU on the protection of C₂C₁₂ cells from cartap-induced decline in MTT metabolic rate. Vitamin E scavenges ROS formed in redox reactions to terminate ROS-generated lipid peroxidation chain reactions, particularly in cellular and subcellular membranes. Catalase and SOD are ROS scavengers selective for H₂O₂ and O₂⁻, respectively. Based on these findings, it is speculated that H₂O₂ may play a relatively more important role than other ROS on cartap-induced cytotoxicity in C₂C₁₂ cells. This speculation requires further elucidation.

The cartap-evoked generation of ROS in C₂C₁₂ cells is believed to be Ca²⁺-dependent. This is because there was a drastic dose-dependent reduction in the level of intracellular ROS and the concomitant restoration of cartap-induced decline in MTT metabolic rate and elevation in extracellular release of CK and LDH in cartap-treated C₂C₁₂ cells by Ca²⁺ removal with EGTA. Accumulated evidence has shown that there is a close relation between stress-induced cytosolic and/or mitochondrial Ca²⁺ influx and ROS production (Goldman et al., 1999; Gordeeva et al., 2003; Supinski et al., 1999; Tan et al., 1998; Zhang et al., 2000). The stimulation

of ROS generation is through the activation of certain Ca²⁺-dependent ROS-generating enzymes such as NADPH-oxidase and phospholipase A₂ (Gordeeva et al., 2003; Supinski et al., 1999). Accumulated evidence has also shown that ROS may increase the intracellular Ca²⁺ via activating various plasmalemmal and/or sarcoplasmic reticular calcium channels (Kourie, 1998; Gordeeva et al., 2003). If the cross-talking phenomenon between Ca²⁺ and ROS in living cells, as suggested by Gordeeva et al. (2003), also occurs in cartap-treated C₂C₁₂ cells, it is reasonable to speculate that this interaction may lead those cartap-treated cells having suffered from initial reversible changes eventually towards the no return death.

Various studies have shown that ROS may play an important, if not central, role in the development of many forms of muscle dysfunction, including that resulting from strenuous muscle contractions (Nethery et al., 1999; Supinski et al., 1997). It has also been demonstrated that ROS generation by the contracting diaphragm is strongly dependent on the level of extracellular Ca²⁺ (Supinski et al., 1999). Our previous studies have demonstrated that cartap induces a Ca²⁺-dependent severe irreversible contracture in isolated mouse and rabbit phrenic-nerve diaphragms, which is possibly due to the influx of extracellular Ca²⁺ and release of internal Ca²⁺ from the sarcoplasmic reticulum (Liao et al., 2000, 2003). Thus, it is reasonable to speculate that there will be a concomitant increase in the generation of ROS not only in the cartap-treated phrenic nerve-diaphragm but also in the diaphragm of a rabbit receiving ocular instillation of cartap due to the increased cytosolic Ca²⁺ with further damage to the diaphragmatic muscle.

In summary, we demonstrated in this study that mouse skeletal muscle myoblast cell line, C₂C₁₂, is a suitable *in vitro* model for studying the myocytotoxicity of cartap. Cartap may exert its cytotoxicity, at least partially, to C₂C₁₂ cells by inducing ROS generation via a Ca²⁺-dependent mechanism. It is speculated that both Ca²⁺ and Ca²⁺-induced ROS may play the central role on the myogenic contracture and myofiber injury of the diaphragm leading to respiratory failure and death in rabbits exposed to cartap ocularly.

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