Int. J. Cancer: **81,** 820–828 (1999)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

SODIUM SELENITE-INDUCED OXIDATIVE STRESS AND APOPTOSIS IN HUMAN HEPATOMA $HepG_2$ CELLS

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The mechanisms involved in the anti-carcinogenic activity of selenium remain to be elucidated. In the present study, we examined sodium selenite-induced oxidative stress and apoptosis in a human hepatoma cell line ($HepG_2$). Sodium selenite (10 μM) exerted clear cytotoxic effect, as shown by the significant increase of lactate dehydrogenase leakage. Selenite-induced DNA alterations in apoptosis were studied by: 1. comet assay; 2. TdT-mediated dUTP nick end-labeling assay. In addition, characteristic apoptotic morphological alterations were also observed in selenite-treated cells. Our results clearly show that Se-induced cell death occurs predominantly in the form of apoptosis. Selenite-induced oxidative stress was evaluated by the measurement of reactive oxygen species production using lucigenin-dependent chemiluminescence. The involvement of glutathione in selenite-induced oxidative stress was further demonstrated by the concurrent decline of intracellular reduced glutathione and increase of oxidized glutathione contents in Se-treated cells. Moreover, the finding that selenite-induced oxidative stress and apoptosis was significantly attenuated by superoxide dismutase, catalase and deferoxamine provides additional evidence to suggest that Se-induced oxidative stress mediates the induction of apoptosis, a mechanism related to the anti-carcinogenic and chemopreventive effect of Se. *Int. J. Cancer* 81:820–828, 1999.
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Selenium (Se) is an essential trace element in mammals. Numerous epidemiological studies have implicated a chemopreventive role of Se in many types of human cancers (Combs and Gray, 1998). Experimental studies on animal models also demonstrate the anti-carcinogenic effects of Se (El-Bayoumy, 1991). However, the nature of the anti-carcinogenic activity remains unclear.

One of the important mechanisms related to the anti-carcinogenic effects of Se is believed to be its potent cytotoxicity and inhibitory effects on cell proliferation observed in many types of malignant cells. For instance, Wilson *et al.* (1992) have found that sodium selenite as well as methylated forms of Se reduced the cell viability and cell proliferation of a murine leukemia cell line (L1210). Similar results were obtained in many other cancer cells treated with Se *in vitro* (Thompson *et al*., 1994; Wu *et al*., 1995).

At present, there is some preliminary evidence suggesting that oxidative stress is involved in Se cytotoxicity and cell growth inhibition on cancer cells. Seko *et al.* (1989) have reported that the superoxide anion ($O_2^{\cdot-}$) was generated by the reaction of sodium selenite with reduced glutathione (GSH) in a cell-free system. Subsequent studies found similar results by the determination of reactive oxygen species (ROS), mainly $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2), produced in cells with the addition of exogenous GSH (Yan and Spallholz, 1993; Spallholz, 1994). So far, there is no study on ROS production or oxidative stress in selenite-treated cells, in which selenite reacts only with intracellular GSH, without the addition of exogenous GSH.

On the other hand, Se (organic and inorganic) is able to induce apoptosis in various cancer cells such as mouse leukaemia cells, mouse mammary epithelial cancer cells, human lung cancer cells, and human colonic cancer cells (Thompson *et al*., 1994; Wu *et al*., 1995; Stewart *et al*., 1997). Nevertheless, no systematic studies are available in linking Se-induced oxidative stress with apoptosis. In order to obtain a better understanding of the mechanisms related to the anti-carcinogenic effect of Se, it would be of interest to further explore Se-induced oxidative stress and apoptosis in the same cell system.

MATERIAL AND METHODS

Chemicals

Sodium selenite, sodium dodecyl sulfate (SDS), reduced glutathione (GSH) and oxidized glutathione (GSSG), RNase A, superoxide dismutase (SOD), catalase, deferroxamine (DFO), penicillin and streptomycin, paraformaldehyde, and o-phthalaldehyde (OPT) were all from Sigma (St. Louis, MO). Wright-Giemsa stain was from Fisher Scientific (Pittsburgh, PA). Minimum essential medium (MEM) and foetal bovine serum (FBS) were from GIBCO BRL (Gaithersburg, MD). Lucigenin and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR). TdT-mediated dUTP nick end labeling (TUNEL) assay kit was from Boehringer Mannheim (Germany).

Cell culture

The human hepatoma cell line ($HepG_2$) was from the ATCC (Rockville, MA) and cultured in MEM supplemented with FBS (10%), penicillin (100 units/mL) and streptomycin (100 μg/mL) in culture flasks. Cells normally reached about 80% confluence at the time of various designated treatment. Morphological alterations of $HepG_2$ cells were evaluated under light microscope after cells were cultured in chamber slides and stained with Wright-Giemsa.

Determination of LDH leakage

Activity of lactate dehydrogenase (LDH) was measured using an Abbott VP Biochemical Analyzer with the test kit (Chicago, IL), as described earlier (Shen *et al*., 1995), with % LDH leakage = (LDH activity in medium/total LDH activity) × 100.

Comet assay

Comet assay or single cell gel electrophoresis was carried out as described by Zhuang *et al*. (1996), mainly for the measurement of DNA strand breaks. In each cell the percentage of DNA existing in the comet tail according was estimated and graded according to the following criteria: Grade 0: <5%, grade I: 5–20%, grade II: 20–40%, grade III: 40–90% and grade IV: >90%.

TUNEL assay

DNA fragmentation in apoptosis was determined by the TUNEL assay. The experiment was conducted according to instructions of the manufacturer. Briefly, after being incubated in culture flasks for

Grant sponsor: The National Medical Research Council of Singapore (NMRC); Grant number: RP3970301N; Grant sponsor: Centre for Environmental and Occupational Health.

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Received 23 October 1998; Revised 29 December 1998

the designated period of time, cells were collected, washed and resuspended in PBS with 1% BSA before being fixed with 4% paraformaldehyde for 30 min. The fixed cells were incubated with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 3 min on ice. The TUNEL reaction took place with the addition of reaction mixture (containing nucleotides and TdT enzyme), which was incubated for 60 min at 37°C in the dark. After wash with PBS, cells were finally resuspended in PBS for flow cytometry analysis (Coulter Epics Elite ESP, Miami, FL). The conditions for the analysis were as follows: air-cooled argon 488 mm laser with 15 mW power, a 525 nm band pass filter and a 550 nm dichroic mirror as detectors. The data rate was 150 to 200 cells per sec, with at least 10,000 cells in each group. A negative control, without the addition of TdT enzymes, was always included in each experiment. The data obtained from flow cytometry were analyzed using WinMDI 2.3 software for subtracting the histogram and calculating the percentage of apoptotic cells in each group.

Lucigenin-dependent chemiluminescence test

Lucigenin-dependent chemiluminescence (CL) in $HepG_2$ cells was measured by a modified method of Peters *et al.* (1990), which was believed to be specific for $O_2^{\cdot-}$. The stock solution of lucigenin (10 mM) was prepared in PBS and stored at −20°C in the dark. The reaction mixture (total volume 1 mL in PBS) contained 1×10^6 cells, 100 μM lucigenin, with or without the presence of selenite. The reaction was initiated by the addition of lucigenin and the CL level was monitored as relative light units in a luminometer (Trans Orchid, Tampa, FL) for total period of 10 min (30 sec intervals).

Determination of intracellular GSH and GSSG content

The determination of intracellular GSH and GSSG content was conducted according to the method of Hissin and Hilf (1976). Briefly, after various designated treatments, cells were collected using cell scrapers and washed with PBS twice before being resuspended in 0.1 M sodium phosphate, 5 mM EDTA (pH 8.0). After ultra-sonication, 0.75 mL of cell homogenate was mixed with 0.2 mL 25% meta-phosphoric acid to precipitate proteins. After centrifugation, the supernatant was then diluted with 0.1 M sodium phosphate, 5 mM EDTA (pH 8.0) or 0.1 N NaCl, for the determination of GSH and GSSG, respectively. The fluorescent intensity of OPT was monitored at 420 nm (em) with excitation wavelength set at 350 nm. The concentration of GSH and GSSG was expressed as nmol/10^6 cells.

Statistical analysis

All data were presented as means ± SD from at least 3 sets of independent experiments. The differences among different groups were examined using one-way ANOVA with Scheffe's test. A p value below 0.05 was considered statistically significant.

RESULTS

Selenite-induced LDH leakage

Selenite-induced cytotoxicity, measured as the percentage of LDH leakage, is shown in Figure 1. The increase of LDH leakage was both selenite-concentration and culture-time dependent. A significant increase of LDH leakage was observed when cells were treated with 25 μM selenite for 12 hr. When cells were treated with selenite for more than 24 hr, a significant increase of LDH leakage was observed in all doses. The LDH leakage reached about 55% when cells were treated with 25 μM selenite for 48 hr.

Selenite-induced apoptosis in $HepG_2$ cells

Figure 2 shows the morphological changes of $HepG_2$ cells when treated with sodium selenite (10 μM × 24 hr). It was observed that selenite treatment caused evident chromatin condensation, cell membrane blebbing and formation of apoptotic body, all being characteristic morphological alterations of apoptosis.

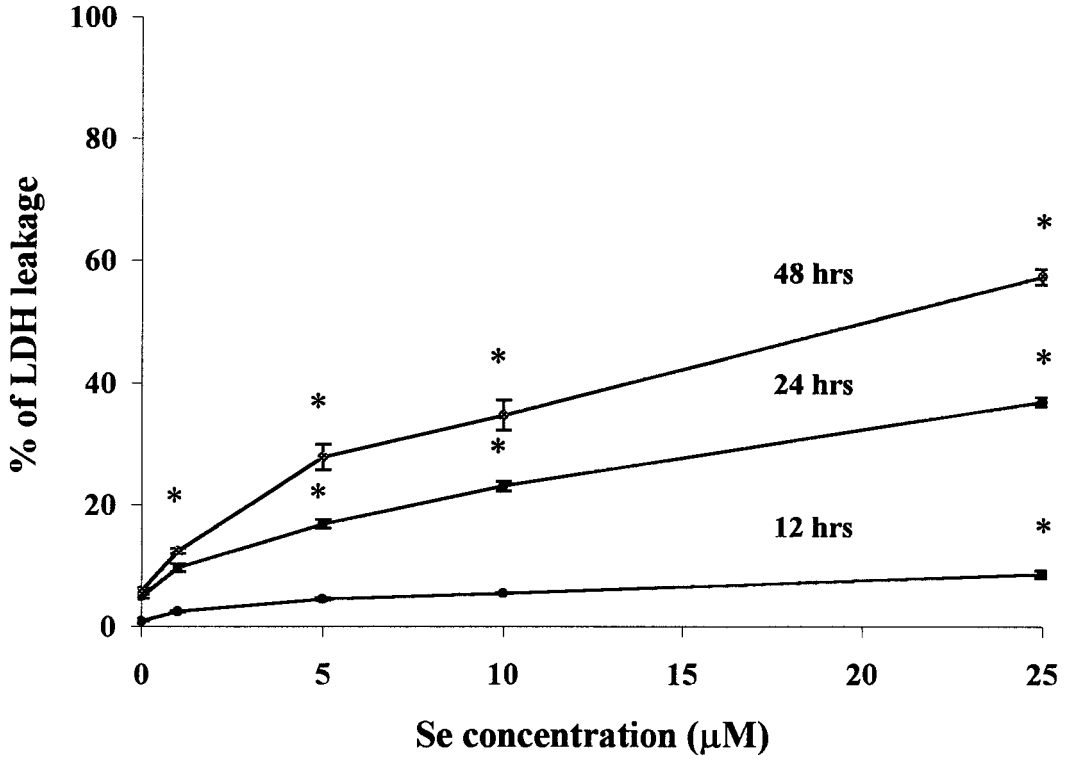

FIGURE 1 – Sodium selenite-induced LDH leakage in $HepG_2$ cells. Data are means ± SD (n=6). * indicates significant difference ($p<0.05$) compared with respective controls for each time group (one-way ANOVA with Scheffe's test).

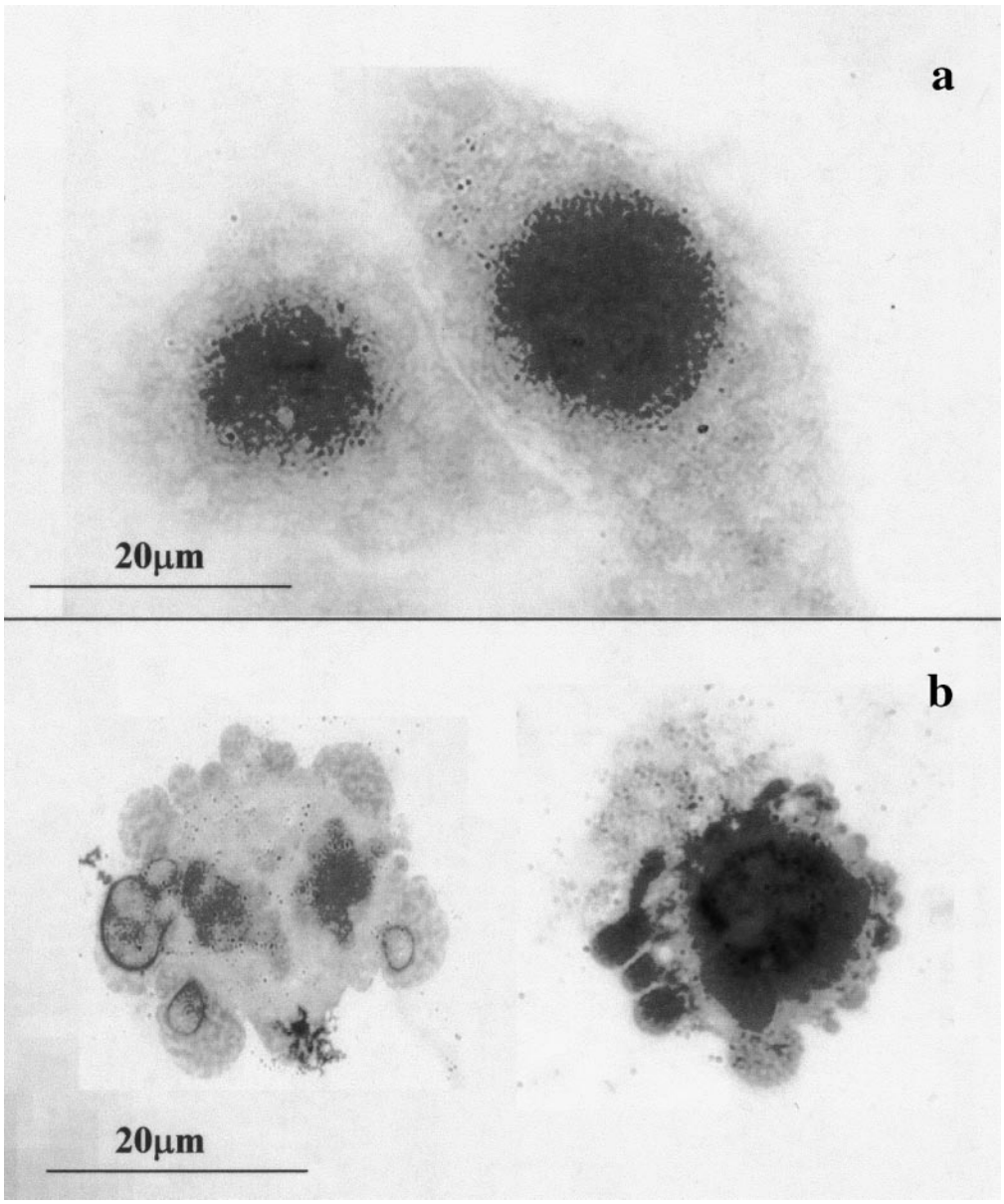

FIGURE 2 – Morphological changes in $HepG_2$ cells treated with sodium selenite. *(a)* Control cells, and *(b)* cells treated with selenite (10 μM × 24 hr). Cells were stained with Wright-Giemsa and examined under light microscope. Clear chromatin condensation, cell membrane blebbing and formation of apoptotic bodies were observed in Se-treated cells.

Se-induced DNA strand breaks were investigated using comet assay and the results are presented in Figure 3. Significant increase of grade III and IV cells was observed when treated with 5 μM selenite for 24 hr. For cells treated with 10 or 25 μM selenite, almost all cells were in the status of total DNA damage (grade IV).

DNA fragmentation occurred during apoptosis was further studied using the TUNEL assay. The percentage of apoptotic cells was calculated by the positive staining of nuclei with fluorescein and the results are summarized in Figure 4. It was found that selenite-induced apoptosis was both time and dose dependent. No obvious changes were observed when cells were treated with 1 μM Se for 12 or 24 hr. Substantial increase of apoptotic cells was observed from 5 μM onwards. The highest percentage was noted with 25 μM selenite for 12 hr (about 73%), even slightly higher than the value at 24 hr (about 70%).

Elevated level of ROS formation in selenite-treated cells, lucigenin-dependent CL test

The temporal changes of CL level in $HepG_2$ cells was monitored up to 10 min and the results are presented in Figure 5*a*. A relatively low level of CL was observed in control cells without the presence of selenite. The addition of selenite enhanced the CL level markedly. Moreover, the increase of CL level in $HepG_2$ cells appeared to be selenite-dose dependent (Fig. 5*b*). In cells treated with the highest concentration of selenite (25 μM), the integral value of relative light units increased by nearly 50%, compared with control cells.

Changes of intracellular GSH and GSSG content

Figure 6*a* shows that sodium selenite was able to deplete the intracellular GSH concentration even at the concentration of 1 μM.

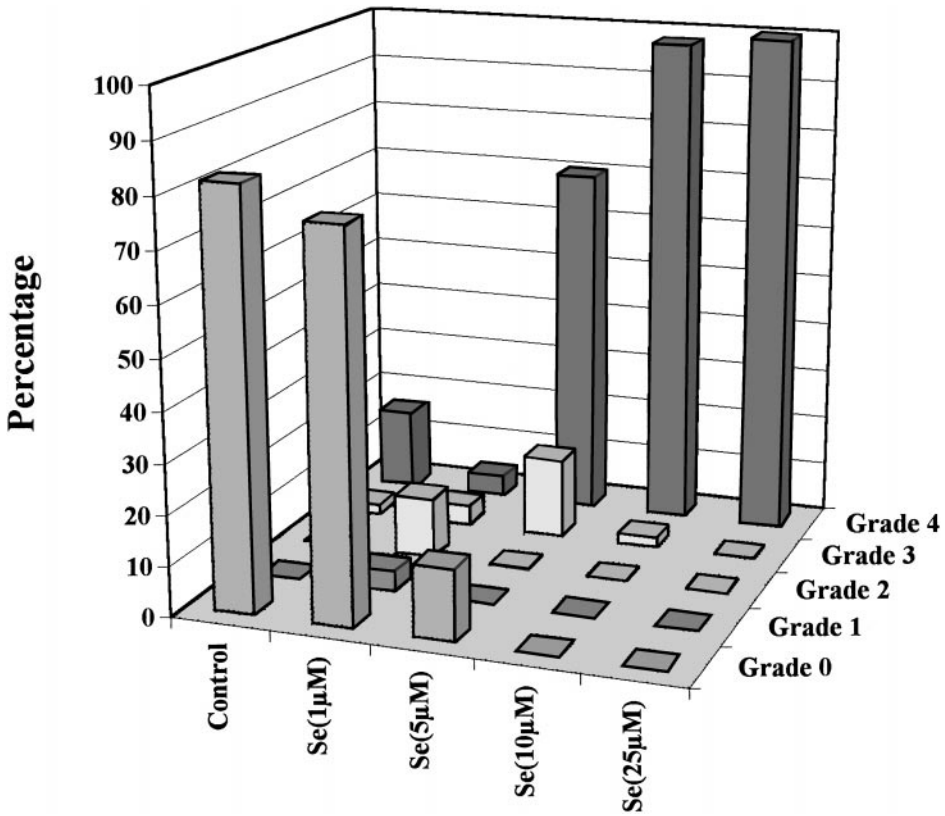

FIGURE 3 – Selenite-induced DNA damage examined by comet assay. Cells were treated with selenite for 24 hr before being harvested for analysis. The percentage of cells in each grade was calculated based on 50 cells counted on each slide.

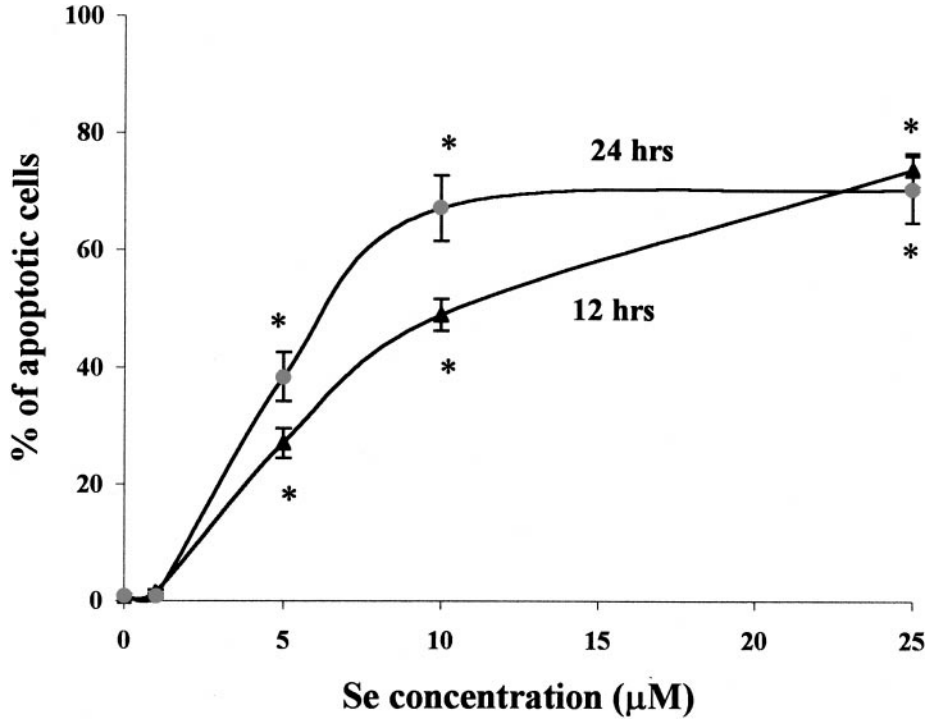

FIGURE 4 – Increase of the percentage of apoptotic cells induced by selenite treatment in $HepG_2$ cells. The data were obtained from a TUNEL assay using flow cytometry based on at least 10,000 events for each group and are presented as means ± SD (n=3). * indicates significant difference ($p<0.05$) compared with a control group (one-way ANOVA with Scheffe's test).

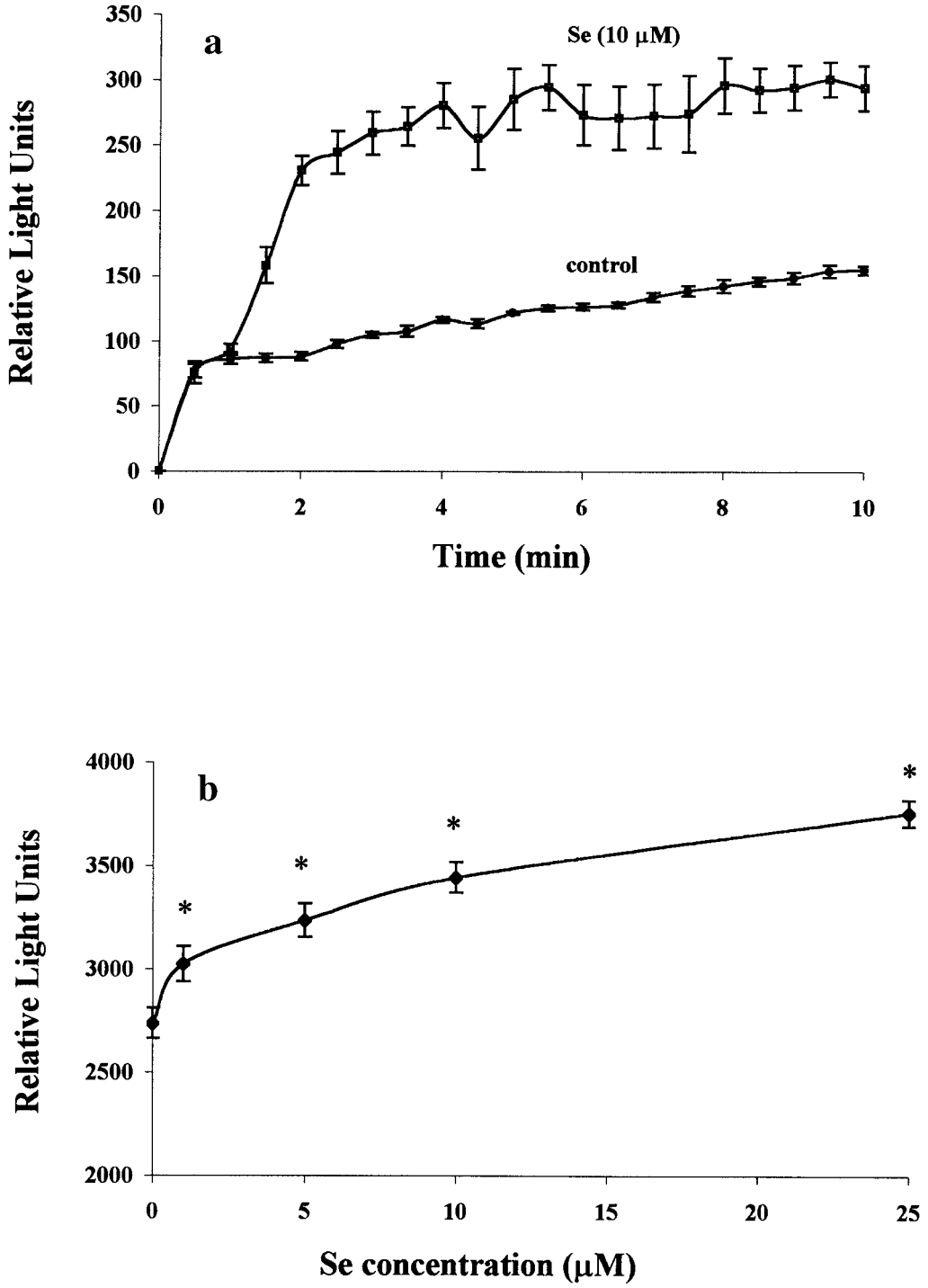

FIGURE 5 – Selenite-induced elevated level of ROS formation in $HepG_2$ cells detected by lucigenin-dependent CL test. The basic reaction mixture contained 1×10^6 cells and 100 μM lucigenin in 1 mL PBS. *(a)* Temporal changes of CL in both control and selenite-treated cells for a total of 10 min and in which the CL level was monitored at the intervals of 30 sec; *(b)* dose-dependent increase of CL represented by the integral value of 10 min. Data are presented as means ± SD (n=4). *$p<0.05$ compared with the control group (one-way ANOVA with Scheffe's test).

Such effect was clearly time and dose dependent. When cells were treated with 25 μM selenite for 48 hr, the GSH concentration was only 0.8 nmol/10^6 cells, which was about 13-fold lower than the control value (10.5 nmol/10^6 cells). The GSSG level in the control cells was only about one third of that of GSH (3.4 vs. 10.5 nmol/10^6 cells, Fig. 6*b*). Exposing cells to Se for 24 or 48 hr caused the decrease of GSSG level. In contrast, the GSSG level tended to increase when cells were treated with selenite for 12 hr. The GSSG content was 5.5 nmol/10^6 cells in cells treated with 25 μM selenite for 12 hr, significantly higher than the control value.

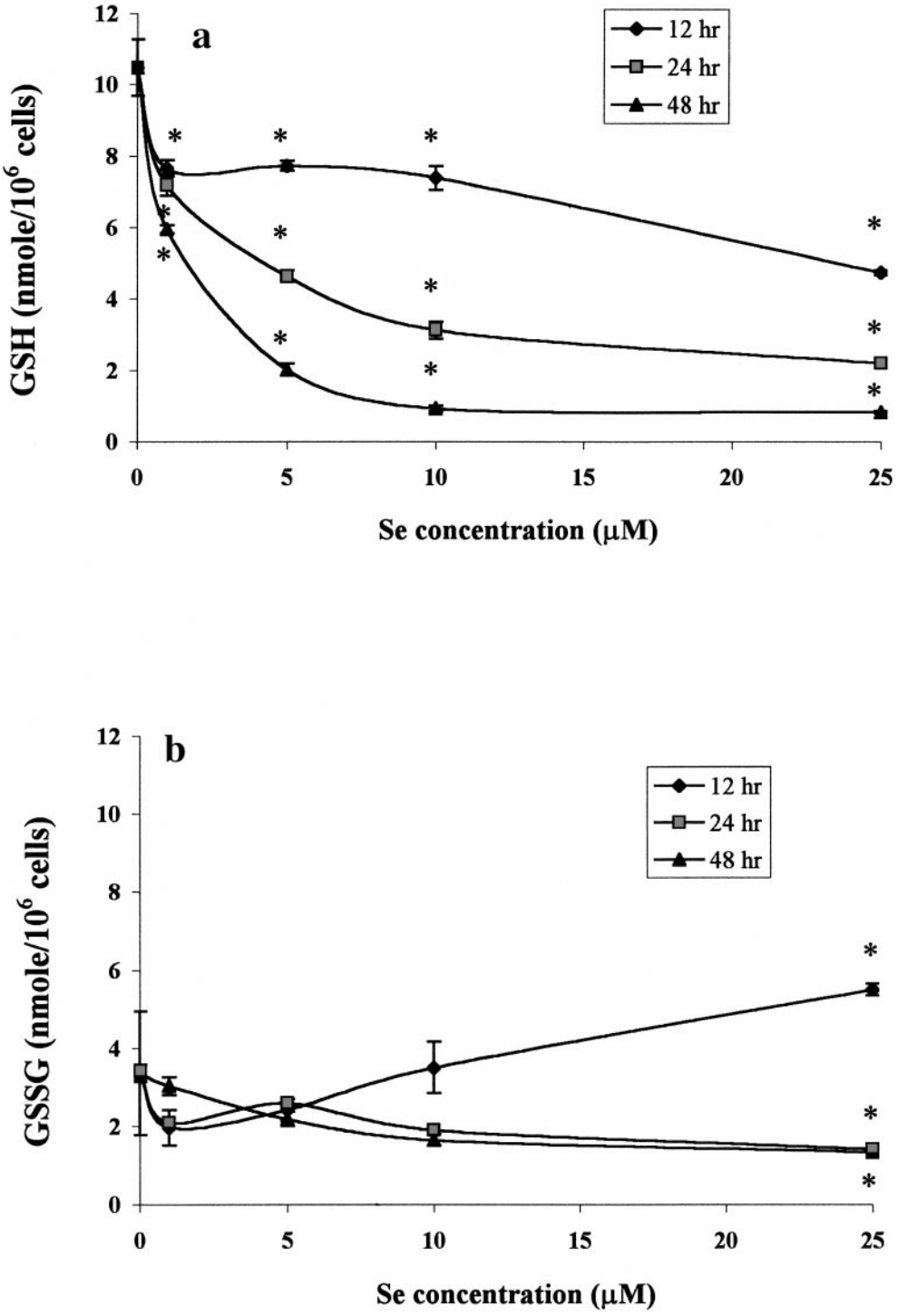

FIGURE 6 – Changes of intracellular glutathione content in selenite-treated $HepG_2$ cells: GSH *(a)* and GSSG *(b)*. Data are presented as means ± SD (n=6). * indicates $p<0.05$ compared with the control group (one-way ANOVA with Scheffe's test).

Inhibitory effects of SOD, catalase and DFO on selenite-induced oxidative stress and apoptosis

In this set of experiments, cells were either pretreated with SOD (250 units/mL), catalase (500 units/mL), or DFO (5 mM) for 6 hr before they were collected, washed and used for lucigenin-dependent CL test, or cells were treated with SOD, catalase or DFO and selenite (10 μM) simultaneously for 24 hr for determining of LDH leakage and apoptosis. Figure 7*a* shows that SOD and DFO significantly reduced the CL level induced by sodium selenite. Lower level of ROS formation was also observed in cells pretreated with catalase although no statistical significant difference was observed. Results in Figure 7*b* clearly demonstrate the inhibitory

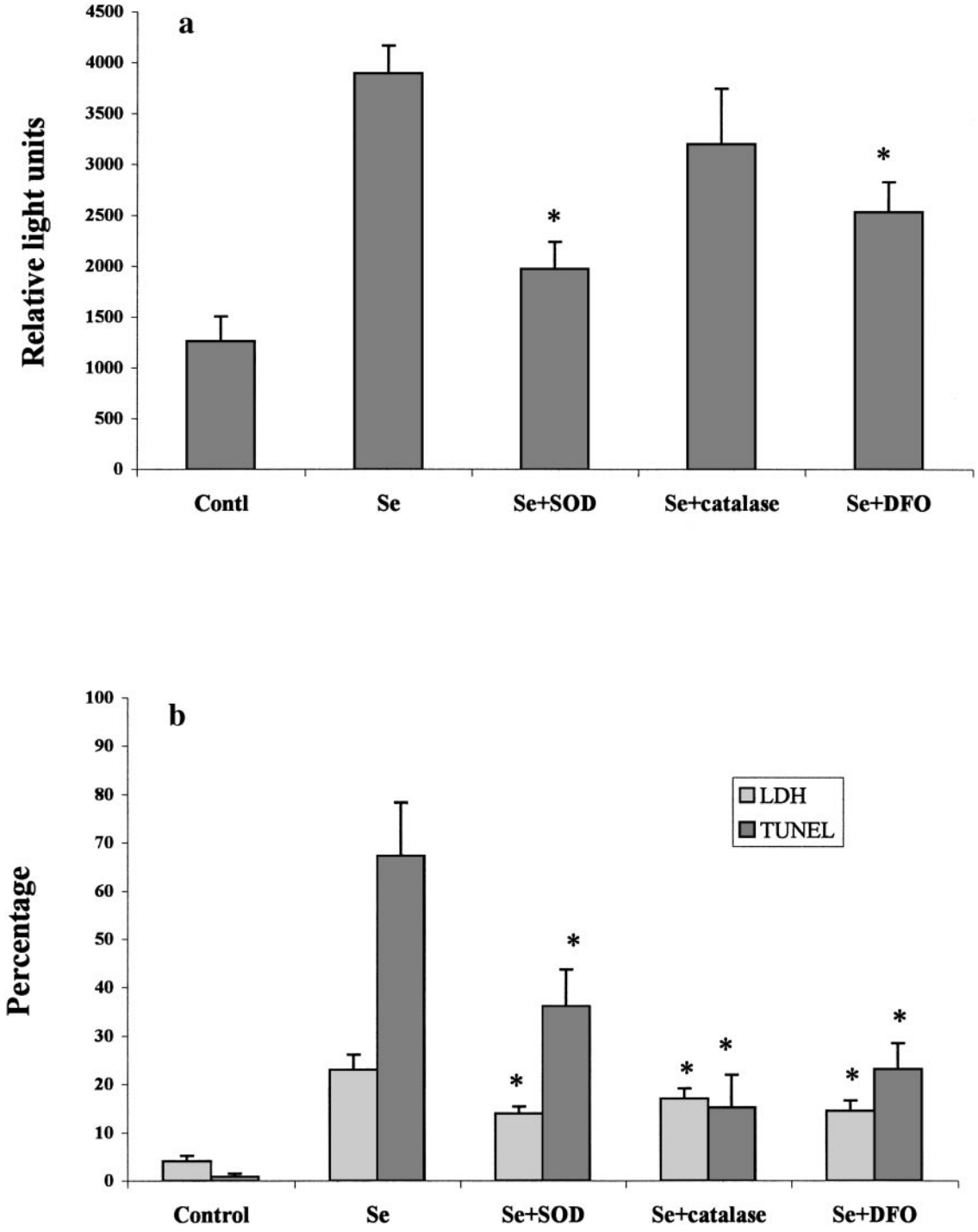

FIGURE 7 – Inhibitory effects of SOD, catalase and DFO on selenite-induced ROS formation *(a)* and LDH leakage and apoptosis *(b)*. Cells were either pretreated with SOD (250 U/mL), catalase (500 U/mL) or DFO (5 mM) for 6 hr before being collected, washed and used for lucigenin-dependent CL test *(b)*, or treated with selenite (10 μM) and SOD, catalase, or DFO simultaneously for 24 hr *(b)*. Data were presented as means ± SD (n=3 to 6). * indicates $p<0.05$ compared with the selenite-only group (one-way ANOVA with Scheffe's test).

effects of SOD, catalase and DFO on selenite-induced LDH leakage and apoptosis. The percentage of apoptotic cells examined by the TUNEL assay in SOD, catalase and DFO treated cells was 36%, 15% and 23%, respectively, which is significantly lower than that in cells treated with selenite only (67%).

DISCUSSION

In this study, we systematically examined selenite-induced oxidative stress and apoptosis in human hepatoma, $HepG_2$ cells. Firstly, the potent cytotoxic effect of selenite was demonstrated by

the significant increase of LDH leakage (Fig. 1). Secondly, selenite-induced apoptosis was evaluated by comet assay and TUNEL assay results and characteristic cell morphological changes. The results demonstrate that apoptosis is the major form of cell death caused by Se exposure (Figs. 2–4). Thirdly, elevated level of ROS in Se-treated cells as measured by lucigenin-dependent CL indicated selenite-induced oxidative stress in $HepG_2$ cells (Fig. 5*a*,*b*). Fourthly, there was a concurrent decrease of intracellular GSH and increase of GSSG content in Se-treated cells (Fig. 6*a*,*b*). And finally, SOD, catalase and DFO were able to protect cells against Se-induced oxidative stress and apoptosis (Figure 7*a*,*b*). As all these observations were obtained from the same cell culture system, we thus believe that they provide more convincing evidence supporting the hypothesis that Se-induced apoptosis, mediated by oxidative stress, constitutes an important mechanism in the anti-carcinogenic activity of Se.

The implication of oxidative stress in Se toxicity has been suspected since early 1980s when cells were found to have increased lipid peroxidation and oxygen consumption with Se treatment (Stacey and Klaassen, 1981, Dougherty and Hoekstra, 1982). Seko *et al.* (1989) and subsequently Yan and Spallholz (1993) and Spallholz (1994) demonstrated the production of ROS ($O_2^{\cdot-}$, H_2O_2) when selenite was reacted with exogenous GSH, using lucigenin or luminol-dependent CL methods. In the present study, we used a similar approach to detect elevated level of ROS production in selenite-treated $HepG_2$ cells. Lucigenin-dependent CL is considered to be highly specific for $O_2^{\cdot-}$ (Peters *et al*., 1990), in spite of recent controversies that lucigenin itself may also act as a source of $O_2^{\cdot-}$ (Fridovich, 1997). However, this may perhaps be negligible (Li *et al*., 1998). Different from the previous studies which either used cell-free system (Seko *et al*., 1989) or added exogenous GSH into cell culture media (Yan and Spallholz, 1993; Spallholz, 1994), our data show significantly increased lucigenin CL in Se-treated cells (Fig. 5*a*,*b*), indicating that selenite actually reacts with intracellular GSH to form ROS.

In the present study, the DNA damage in selenite-treated $HepG_2$ cells was evaluated by comet and TUNEL assays, both showing DNA alterations characteristic of apoptosis. Se-induced apoptosis has been studied in a number of cancer cells in *in vitro* conditions and the results generally suggest the involvement of apoptosis in Se-induced cytotoxic and anti-proliferative effects against cancer cells. There, we found that the percentage of apoptotic cells reached more than 70% when treated with 25 µM selenite for 12 or 24 hr, as examined by TUNEL assay (Fig. 4), indicating that apoptosis is the dominant form of cell death caused by selenite. The percentage of apoptotic cells found in TUNEL assay also increased markedly when cells were treated with selenite for 12 hr (Fig. 4), while no significant changes of LDH leakage (except for the group treated with 25 µM selenite) were found (Fig. 1), suggesting that Se-induced DNA damage (strand breaks) precedes the disruption of cell membrane integrity.

The important role of oxidative stress in apoptosis has been well acknowledged, based on the following evidence (Buttke and Sandstrom, 1994): 1. ROS and oxidants induce apoptosis in many cell systems; 2. antioxidants inhibit apoptosis; 3. Bcl-2 and p53, 2 of the most important controllers of apoptosis, act through the antioxidant or pro-oxidant pathways, respectively. Therefore, that Se-induced oxidative stress, demonstrated by the elevated level of ROS, is closely related to the induction of apoptosis in Se-treated cells.

Glutathione is closely involved in Se metabolism, as illustrated in the reactions below (Seko *et al*., 1989; Spallholz, 1994):

$$\underset{}{SeO_3^{2-}} \xrightarrow{4GSH\ GSSG} \underset{}{GSSeSG} \xrightarrow{GSH\ GSSG} \underset{}{GSSeH} \xrightarrow{GSH\ GSSG} \underset{}{H_2Se} \xrightarrow{O_2\ O_2^{\cdot-}} \underset{}{Se^o}$$

However, results from experimental studies on intracellular GSH remain controversial. For instance, studies by Kuchan and Milner (1991, 1992) have shown that selenite treatment was able to increase intracellular GSH level, while other *in vitro* studies demonstrated that selenite treatment lead to decline of intracellular GSH content (Yan *et al*., 1991; Kitahara *et al*., 1993). A similar reduction of intracellular GSH content was found in the present study (Fig. 6). Exposing to 1 µM selenite for 12 hr decreased the GSH concentration significantly, and GSH was almost completely depleted in cells treated with 25 µM selenite for 48 hr. Furthermore, our results show a concomitant increase of GSSG level in selenite-treated cells (25 µM for 12 h), clearly suggesting the conversion of GSH to GSSG caused by the non-enzymatic reaction of GSH with selenite during selenite metabolism. Therefore, changes of GSH and GSSG content as found here, further support the close involvement of glutathione in Se metabolism and toxicity.

The strong inhibitory effects of antioxidants (SOD, catalase and DFO) on Se-induced ROS formation, LDH leakage and apoptosis provides additional evidence indicating a close relationship among oxidative stress and apoptosis in selenite-treated $HepG_2$ cells. According to the Fenton reaction, under the redox cycle of iron radical (Fe^{2+}/Fe^{3+}), both $O_2^{\cdot-}$ and H_2O_2 are the direct predecessors of hydroxyl radical (·OH), the ultimate form of ROS which is highly active and directly reacts with macromolecules to cause oxidative damage. SOD is one of the primary anti-oxidative enzymes which transforms $O_2^{\cdot-}$ to H_2O_2. As $O_2^{\cdot-}$ also acts as a reducing agent for Fe^{3+}, which supplies Fe^{2+} for the Fenton reaction. Therefore, it is believed that SOD and catalase are able to reduce the supply of Fe^{2+} and H_2O_2 and then inhibit the formation of ·OH through the Fenton reaction. On the other hand, DFO, a specific iron chelator, prevents the formation of ·OH by depleting intracellular iron. The above results are generally consistent with the findings by Kitahara *et al.* (1993), in which Se-induced LDH leakage and lipid peroxidation were reduced by DFO and a synthetic SOD mimic. Moreover, the strong protective effects of catalase against Se-induced LDH leakage and apoptosis do not parallel to its inhibitory effect on selenite-induced ROS formation (Fig. 7*a*,*b*). Such discrepancy might be explained by the following: 1. the lucigenin-derived CL test used in the present study is highly specific for $O_2^{\cdot-}$, not H_2O_2; 2. some earlier studies showed the production of H_2O_2 in Se-treated cells using luminol-derived CL (Yan and Spallholz, 1993; Spallholz, 1994), indicating the involvement of H_2O_2 in Se-induced oxidative stress and apoptosis.

In summary, our present work has systematically explored selenite-induced oxidative stress and apoptosis in a human hepatoma cell line. Our results generally support the notion that Se-induced oxidative stress mediates the induction of apoptosis, which may constitutes an important mechanism in the anti-carcinogenic and chemopreventive activity of Se.

ACKNOWLEDGEMENTS

C.-F.Y. is sponsored by a research scholarship by the National University of Singapore. We thank Ms B.L. Ng for her excellent support in flow cytometry analysis. We also thank Mr. H.Y. Ong and Ms L. Jin for their technical support in cell culture.

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