Chinese Journal of Physiology 58(1): 1-8, 2015
DOI: 10.4077/CJP.2015.BAD276

Effect of Methoxychlor on Ca^{2+} Homeostasis and Apoptosis in HA59T Human Hepatoma Cells

Chi-Ting Horng[1, #], Chiang-Ting Chou[2, 3, #], Hui-Wen Tseng[4], Jin-Shiung Cheng[5], Hong-Tai Chang[6], Po-Min Chang[6], I-Li Chen[7], Ming-Chi Hung[7], Yi-Jen Tsai[7], Peng-Chih Tsai[7], Wei-Zhe Liang[8], Chun-Chi Kuo[9], Daih-Huang Kuo[7], Chin-Man Ho[8], Jia-Rong Lin[8], Pochuen Shieh[7], and Chung-Ren Jan[8]

[1]*Department of Ophthalmology, Kaohsiung Armed Force General Hospital, Kaohsiung 80284*
[2]*Department of Nursing, Division of Basic Medical Sciences, Chang Gung University of Science and Technology, Chia-Yi 61363*
[3]*Chronic Diseases and Health Promotion Research Center, Chang Gung University of Science and Technology, Chia-Yi 61363*
[4]*Department of Dermatology, Kaohsiung Veterans General Hospital, Kaohsiung 81362*
[5]*Department of Medicine, Kaohsiung Veterans General Hospital, Kaohsiung 81362*
[6]*Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung 81362*
[7]*Department of Pharmacy, Tajen University, Pingtung 90741*
[8]*Department of Medical Education and Research, Kaohsiung Veterans General Hospital Kaohsiung 81362*
and
[9]*Department of Nursing, Tzu Hui Institute of Technology, Pingtung 92641*
Taiwan, Republic of China

Abstract

Methoxychlor, an organochlorine pesticide, is thought to be an endocrine disrupter that affects Ca^{2+} homeostasis and cell viability in different cell models. This study explored the action of methoxychlor on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and apoptosis in HA59T human hepatoma cells. Fura-2, a Ca^{2+}-sensitive fluorescent dye, was applied to measure $[Ca^{2+}]_i$. Methoxychlor at concentrations of 0.1-1 μM caused a $[Ca^{2+}]_i$ rise in a concentration-dependent manner. Removal of external Ca^{2+} abolished methoxychlor's effect. Methoxychlor-induced Ca^{2+} influx was confirmed by Mn^{2+}-induced quench of fura-2 fluorescence. Methoxychlor-induced Ca^{2+} entry was inhibited by nifedipine, econazole, SK&F96365, and protein kinase C modulators. Methoxychlor killed cells at concentrations of 10-130 μM in a concentration-dependent fashion. Chelating of cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid/AM (BAPTA/AM) did not prevent methoxychlor's cytotoxicity. Methoxychlor (10 and 50 μM) induced apoptosis concentration-dependently as determined by using Annexin V/propidium iodide staining. Together, in HA59T cells, methoxychlor induced a $[Ca^{2+}]_i$ rise by inducing Ca^{2+} entry *via* protein kinase C-sensitive Ca^{2+}-permeable channels, without causing Ca^{2+} release from stores. Methoxychlor also induced apoptosis that was independent of $[Ca^{2+}]_i$ rises.

Key Words: apoptosis, Ca^{2+}, hepatoma, methoxychlor

Corresponding authors: [1] Dr. Pochuen Shieh, Department of Pharmacy, Tajen University, Pingtung 90741, Taiwan; and [2] Dr. Chung-Ren Jan, Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan R.O.C. Tel: +886-7-3422121 ext. 1509, Fax: +886-7-3468056, E-mail: crjan@isca.vghks.gov.tw.
[#]Contributed equally to this work.
Received: February 14, 2014; Revised (Final Version): May 8, 2014; Accepted: May 9, 2014.

Introduction

Methoxychlor is an organochlorine pesticide used worldwide and is thought to be an endocrine disrupter that affects many systems (11, 16, 17). Copious evidence shows that methoxychlor exerts various actions on many cell types, mainly related to the reproduction system. Methoxychlor was shown to induce death in mouse ovarian antral follicles (1, 29) that might involve inhibition of growth by altering cell cycle regulators and causing mitochondrial dysfunction and oxidative damage through Bcl-2- and Bax-mediated pathways (26). *In vivo* data suggest that methoxychlor causes immunotoxicity in female ICR, BALB/c, and C3H/He mice (12, 19).

Methoxychlor was shown to induce apoptosis in different cells such as mouse thymocytes (13), rat testis cells (37), immunocytes (14), human oral cancer cells (35) and canine renal tubular cells (5). However, methoxychlor was shown to enhance growth of human breast cancer cells and alteration of the expression of cell cycle-related genes, cyclin D1 and p21, *via* an estrogen receptor-dependent signaling pathway (21). In a previous study, methoxychlor interfered with hormone signaling in ovary *via* two mechanisms: altering the availability of ovarian hormones, and altering binding and activity of the hormone at the receptor level (8). In addition, methoxychlor interacted with membrane receptors that modulate ovarian steroidogenesis (8). In sum, it appears that methoxychlor has different effects on various cells depending on the experimental conditions.

Methoxychlor is metabolized in the liver (33). *In vivo* studies show that methoxychlor is hepatotoxic (27). *In vitro* evidence shows that methoxychlor causes death or alters growth in several cell lines including murine Hepa-1c1c7 cells (18), human HepG2 cells (9), frog hepatocytes (32), fish hepatocytes (30), and chicken embryo hepatocytes (24). Although methoxychlor has been shown to induce intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) rises in human granulosa-lutein cells (38) or human umbilical vein endothelial cells (40), the effect of methoxychlor on Ca^{2+} signaling is unclear in hepatocytes from humans or animals. Thus the aim of this study was to explore the effect of methoxychlor on Ca^{2+} movement and viability in human HA59T hepatoma cells.

Ca^{2+} is a highly versatile intracellular signal, controlling a wild range of cellular events, such as proliferation, development, division, migration, contraction, fertilization, gene expression, secretion and death (2). The HA59T cell is a useful model for hepatocyte research. In this cell, it has been shown that several ligands can cause a $[Ca^{2+}]_i$ rise, such as calmidazolium (23) and carvedilol (6), *via* causing Ca^{2+} entry and Ca^{2+} release.

In this study, fura-2 was used as a Ca^{2+}-sensitive dye to measure $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ rises were characterized, the concentration-response plot was established, and the pathway underlying methoxychlor-evoked Ca^{2+} entry was explored. The cytotoxic effect of methoxychlor and the role of apoptosis were assessed.

Materials and Methods

Chemicals

The reagents for cell culture were purchased from Gibco® (Gaithersburg, MD, USA). Fura-2/AM and BAPTA/AM were purchased from Molecular Probes® (Eugene, OR, USA). Methoxychlor and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

HA59T human hepatoma cells obtained from Bioresource Collection and Research Center (Taiwan) were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solutions Used in $[Ca^{2+}]_i$ Measurements

Ca^{2+}-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, and 5 mM glucose. Ca^{2+}-free medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 0.3 mM EGTA, 10 mM HEPES, and 5 mM glucose. Methoxychlor was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability or basal $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ Measurements

Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^6 cells/ml. Cell viability was determined by trypan blue exclusion. The viability was greater than 95% after the treatment. Cells were subsequently loaded with 2 μM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed with Ca^{2+}-containing medium twice and was made into a suspension in Ca^{2+}-containing medium at a density of 10^7 cells/ml. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 1 million cells. Fluorescence was

monitored with a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 ml cell suspension was added to 0.9 ml Ca^{2+}-containing or Ca^{2+}-free medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. During the recording, reagents were added to the cuvette by pausing the recording for 2 sec to open and close the cuvette-containing chamber. For calibration of $[Ca^{2+}]_i$, after completion of the experiments, the detergent Triton X-100 (0.1%) and $CaCl_2$ (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 mM) was added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette had a viability of 95% after 20 min of fluorescence measurements. $[Ca^{2+}]_i$ was calculated as previously described (3, 4, 6, 15).

Mn^{2+} quenching of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$. $MnCl_2$ was added to cell suspension in the cuvette 30 sec before the fluorescence recoding was started. Data were recorded at excitation signal at 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as described previously (25).

Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increases in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a density of 10,000 cells/well in culture medium for 24 h in the presence of methoxychlor. The cell viability detecting tetrazolium reagent 4-[3-[4-lodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 μl pure solution) was added to samples after methoxychlor treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, cells were treated with 5 μM BAPTA/AM for 1 h prior to incubation with methoxychlor. The cells were washed once with Ca^{2+}-containing medium and incubated with/without methoxychlor for 24 h. The absorbance of samples (A_{450}) was determined using an enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value.

Alexa ®Fluor 488 Annexin V/PI Staining for Detection of Apoptosis

Annexin V/PI staining assay was employed to further detect cells in early apoptosis stage. Cells were exposed to methoxychlor at concentrations of 0, 10 and 50 μM for 24 h. Cells were harvested after incubation and washed in cold phosphate buffered saline (PBS). Cells were resuspended in 400 μl reaction solution with 10 mM of HEPES, 140 mM of NaC1, 2.5 mM of $CaC1_2$ (pH 7.4). Alexa ®Fluor 488 annexin V/PI staining solution (Probes Invitrogen, Eugene, OR, USA) was added in the dark. After incubation for 15 min, the cells were collected and analyzed in a FACScan flow cytometry analyzer. Excitation wave was at 488 nm and the emitted green fluorescence of Annexin V (FL1) and red fluorescence of PI (FL2) were collected using 530 nm and 575 nm band pass filters, respectively. A total of at least 20,000 cells were analyzed per sample. Light scatter was measured on a linear scale of 1024 channels and fluorescence intensity was on a logarithmic scale. The amount of apoptosis was determined as the percentage of Annexin V^+/PI^- cells.

Statistics

Data are reported as mean ± SEM of three separate experiments. Data were analyzed by one-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significantly difference) procedure. A *P*-value less than 0.05 were considered significant.

Results

Concentration-Dependent Effect of Methoxychlor on $[Ca^{2+}]_i$

Fig. 1A shows that the basal $[Ca^{2+}]_i$ was 51 ± 4 nM (n = 3). At concentrations between 0.1 and 1 μM, methoxychlor induced a $[Ca^{2+}]_i$ rise in a concentration-dependent manner in Ca^{2+}-containing medium. The Ca^{2+} response saturated at 1 μM methoxychlor because at a concentration of 2.5 μM, methoxychlor evoked a similar response as that induced by 1 μM. At 1 μM, methoxychlor induced a $[Ca^{2+}]_i$ rise that attained to a net maximal increase of 95 ± 2 nM (n = 3). Fig. 1B shows that in Ca^{2+}-free medium, 1 μM methoxychlor failed to induce a $[Ca^{2+}]_i$ rise. Fig. 1C shows the concentration-response plot of methoxychlor-induced responses in Ca^{2+}-containing medium.

Methoxychlor-Induced Mn^{2+} Influx

Experiments were performed to confirm that

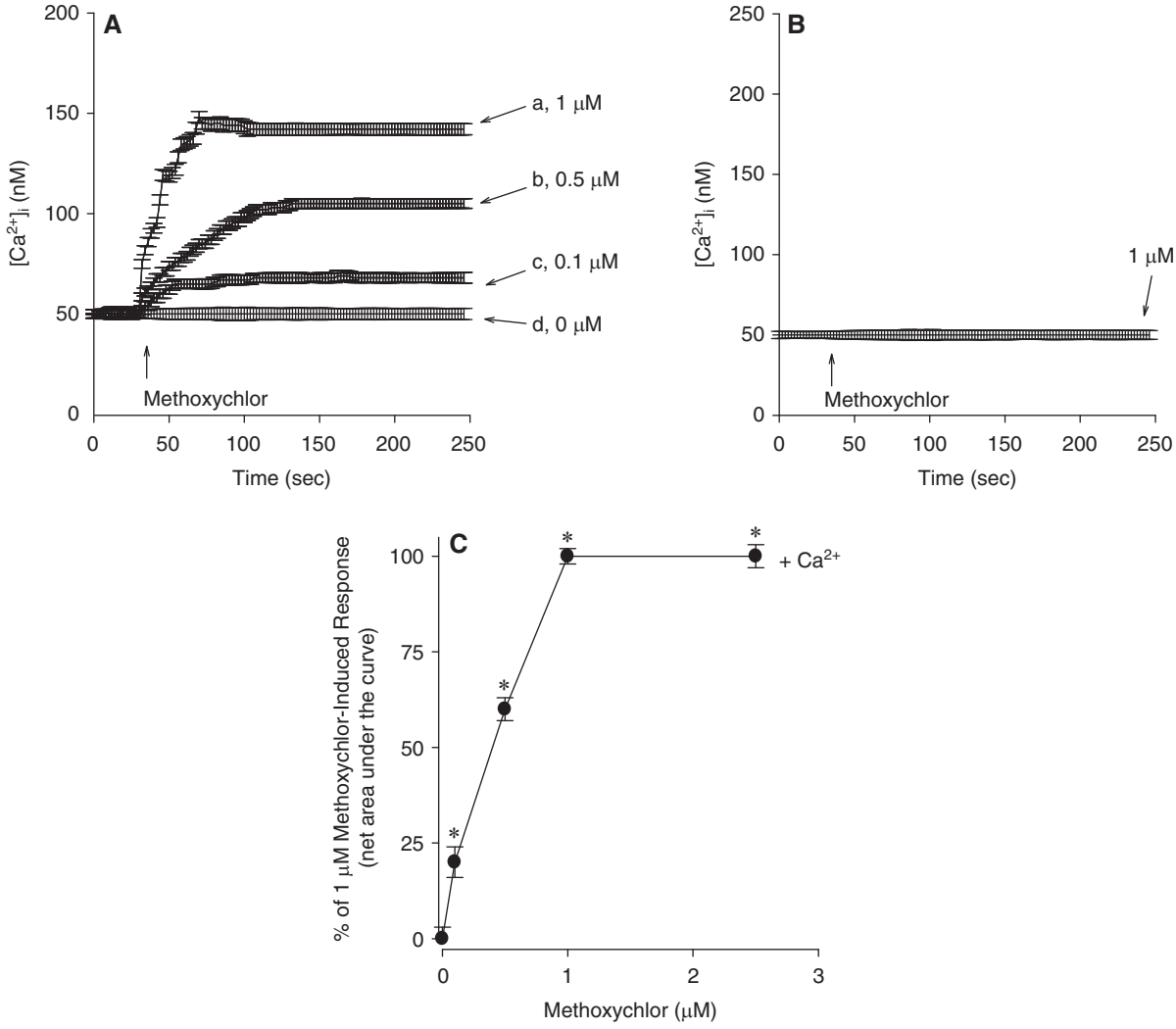

Fig. 1. Effect of methoxychlor on $[Ca^{2+}]_i$ in fura-2-loaded cells. (A) Methoxychlor was added at 25 sec. The concentration of methoxychlor was indicated. The experiments were performed in Ca^{2+}-containing medium. (B) Effect of methoxtchlor on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Methoxychlor was added at 25 sec in Ca^{2+}-free medium. (C) A concentration-response plot of methoxychlor-induced $[Ca^{2+}]_i$ rises. Y axis is the percentage of the net (baseline subtracted) area under the curve (25-250 sec) of the $[Ca^{2+}]_i$ rise induced by 1 μM methoxychlor. Data are mean ± SEM of three separate experiments. $*P < 0.05$ compared to control (no methoxychlor added).

methoxychlor-evoked $[Ca^{2+}]_i$ rise involved Ca^{2+} influx. Mn^{2+} enters cells through similar mechanisms as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths. Therefore, quenching of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} implicates Ca^{2+} influx. Fig. 2 shows that 1 μM methoxychlor evoked an instant decrease in the 360 nm excitation signal that reached a maximum value of 89 ± 2 arbitrary units at 200 sec. This suggests that Ca^{2+} influx participates in methoxychlor-evoked $[Ca^{2+}]_i$ rise.

Modulations of Methoxychlor-Induced $[Ca^{2+}]_i$ Rise

Fig. 1 shows that methoxychlor-induced Ca^{2+} response saturated at 1 μM; thus in the following experiments the response induced by 1 μM methoxychlor was used as control. Nifedipine (1 μM), econazole (0.5 μM) and SK&F96365 (5 μM); phorbol 12-myristate 13 acetate (PMA; 1 nM; a protein kinase C activator); and GF109203X (2 μM; a protein kinase C inhibitor) were applied 1 min before 1 μM methoxychlor in Ca^{2+}-containing medium. The concentrations of these modulators have been used to potently inhibit Ca^{2+} entry through different Ca^{2+} channels in other cell types (3, 4, 7, 23). Furthermore, the concentrations of these modulators did not affect the basal $[Ca^{2+}]_i$ level before addition of methoxychlor (data not shown). These agents all significantly inhibited methoxychlor-induced $[Ca^{2+}]_i$ rise to different degrees (Fig. 3A).

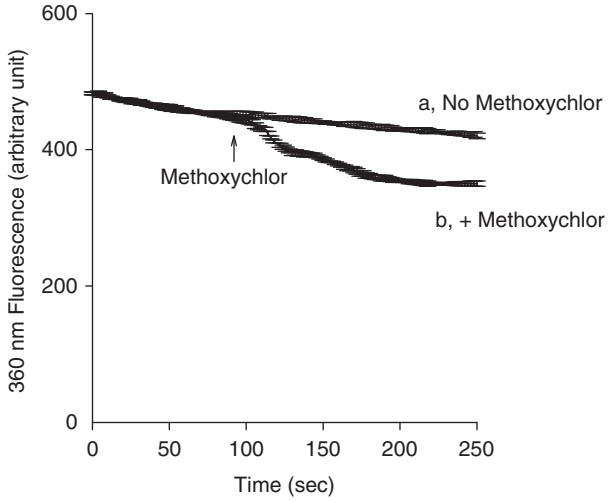

Fig. 2. Effect of methoxychlor on Ca^{2+} influx by measuring Mn^{2+} quenching of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells 1 min before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Trace a: control, without methoxychlor. Trace b: methoxychlor (1 μM) was added as indicated. Data are mean ± SEM of three separate experiments.

Fig. 3B shows the original tracings of GF109203X and PMA-induced inhibition of methoxychlor-induced $[Ca^{2+}]_i$ rise.

Effect of Methoxychlor on Cell Viability

Because acute incubation with methoxychlor (0.1-1 μM) induced a substantial $[Ca^{2+}]_i$ rise, and that unregulated $[Ca^{2+}]_i$ rise may change cell viability (2), experiments were performed to examine the effect of methoxychlor on viability of cells. Cells were treated with 0-130 μM methoxychlor for 24 h, and the tetrazolium assay was performed. Methoxychlor at 1 μM did not alter cell viability. In the presence of 10-130 μM methoxychlor, cell viability decreased in a concentration-dependent manner (Fig. 4).

Lack of a Relationship between Methoxychlor-Induced $[Ca^{2+}]_i$ Rise and Cell Death

An important question was whether the methoxychlor-induced cytotoxicity was caused by a preceding $[Ca^{2+}]_i$ rise. The intracellular Ca^{2+} chelator BAPTA/AM (36) was used to prevent a $[Ca^{2+}]_i$ rise during methoxychlor treatment. Fig. 4 shows that 5 μM BAPTA/AM loading did not change the control value of cell viability. BAPTA/AM at 5 μM effectively prevented 1 μM methoxychlor-induced $[Ca^{2+}]_i$ rise in HA59T cells (data not shown). This suggests that

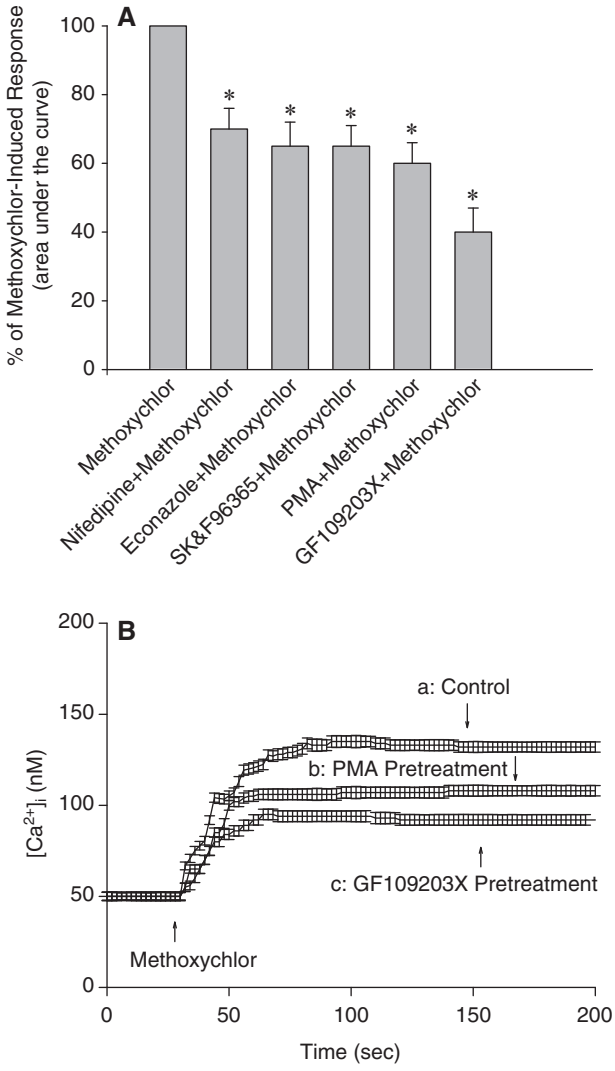

Fig. 3. Effect of Ca^{2+} channel modulators on methoxychlor-induced $[Ca^{2+}]_i$ rise. (A) The experiments were performed in Ca^{2+}-containing medium. In modulator-treated groups, the modulator was added 1 min before methoxychlor (1 μM). The concentration was 10 nM for phorbol 12-myristate 13-acetate (PMA), 2 μM for GF109203X, 1 μM for nifedipine, 0.5 μM for econazole, 5 μM for SK&F96365. Data are expressed as the percentage of control (1st column) that is the area under the curve (25-250 sec) of 1 μM methoxychlor-induced $[Ca^{2+}]_i$ rise, and are mean ± SEM of three separate experiments. $*P < 0.05$ compared to the 1st column. (B) Trace a: control (1 μM methoxychlor). Trace b: The PMA was added 1 min before methoxychlor. Trace c: The GF109203X was added 1 min before methoxychlor. Data are mean ± SEM of three separate experiments.

BAPTA loading for 25 h still effectively chelated cytosolic Ca^{2+}. In the presence of 10-130 μM methoxychlor, BAPTA/AM loading did not reverse methoxychlor-induced cell death ($P > 0.05$).

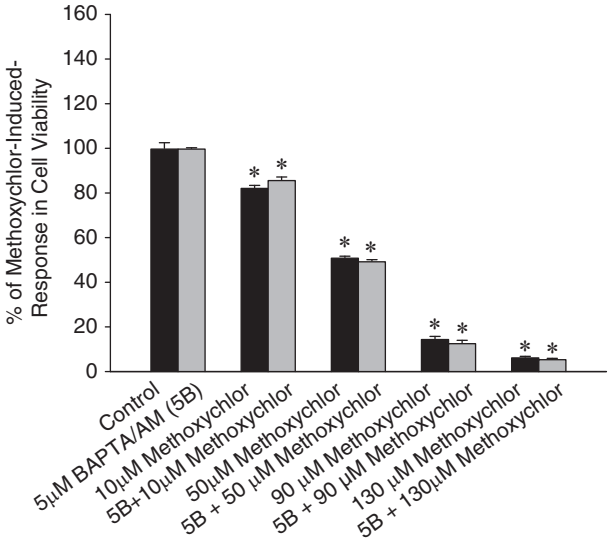

Fig. 4. Effect of methoxychlor on viability of cells. Cells were treated with 0-130 μM methoxychlor for 24 h, and the cell viability assay was performed. Data are mean ± SEM of three separate experiments. Each treatment had six replicates (wells). Data are expressed as percentage of control that is the increase in cell numbers in methoxychlor-free groups. Control had 10,125 ± 712 cells/well before experiments, and had 13,968 ± 702 cells/well after incubation for 24 h. $*P < 0.05$ compared to control. In each group, the Ca^{2+} chelator BAPTA/AM (5 μM) was added to cells followed by treatment with methoxychlor in Ca^{2+}-containing medium. Cell viability assay was subsequently performed.

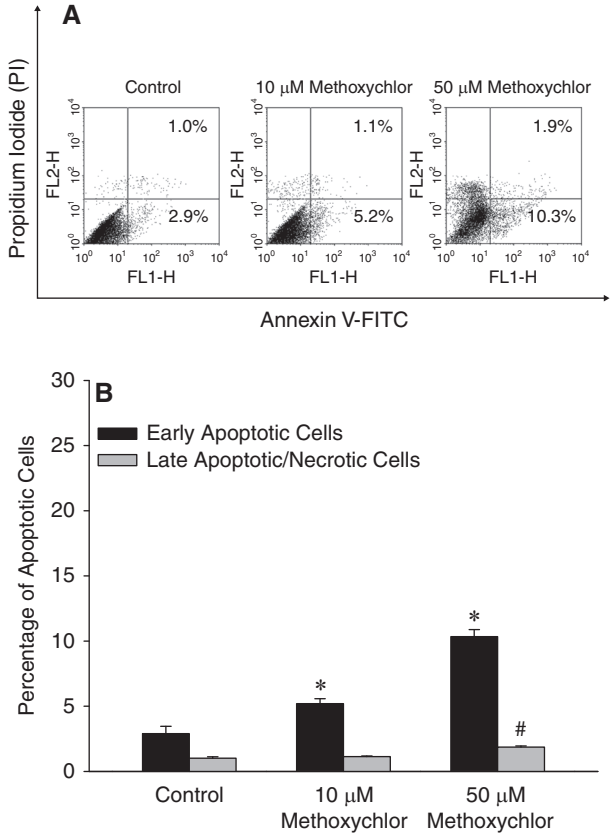

Fig. 5. Apoptosis induced by methoxychlor as measured by Annexin V/PI staining. (A) Cells were treated with 0, 10 μM, 50 μM methoxychlor, respectively, for 24 h. Cells were then processed for Annexin V/PI staining and analyzed by flow cytometry. (B) The percentage of apoptotic cells. #, $*P < 0.05$ compared with control. Data are mean ± SEM of three separate experiments.

A Possible Involvement of Apoptosis in Methoxychlor-Induced Cell Death

Because the cytotoxic response was most significant between 10 μM and 50 μM methoxychlor, these concentrations were chosen for apoptotic experiments. Annexin V/PI staining was applied to detect apoptotic cells after methoxychlor treatment. Figs. 5A and B show that treatment with 10 μM or 50 μM methoxychlor significantly induced apoptosis in a concentration-dependent manner in HA59T cells.

Discussion

The results show that methoxychlor induced $[Ca^{2+}]_i$ rises and cell death in human hepatoma cells. The data are notable because methoxychlor is metabolized in the liver. Previous studies showed that methoxychlor induced $[Ca^{2+}]_i$ rises from extracellular space in human granulosa-lutein cells (38). In our study, methoxychlor appeared to increase $[Ca^{2+}]_i$ solely by inducing Ca^{2+} entry from extracellular medium without involvement of Ca^{2+} release from stores. Previous evidence showed that several stimulants induced $[Ca^{2+}]_i$ rises in HA59T cells by causing Ca^{2+} influx and also Ca^{2+} release (from the endoplasmic reticulum) (6, 8, 23). Furthermore, methoxychlor (10-20 μM) was shown to increase $[Ca^{2+}]_i$ in other cell types such as human oral cancer cells (35) and renal tubular cells (5) by inducing both Ca^{2+} influx and Ca^{2+} release. However, in our study, methoxychlor (0.1-1 μM) evoked a $[Ca^{2+}]_i$ rise by inducing Ca^{2+} entry in HA59T cells. Thus it appears that the pathways of the effect of methoxychlor on Ca^{2+} movement were characteristic of hepatoma cells and vary among different cell types.

Removal of extracellular Ca^{2+} abolished the methoxychlor-induced $[Ca^{2+}]_i$ rise throughout the measurement interval of 250 sec, implying that Ca^{2+} entry happened during the whole stimulation period. Three Ca^{2+} entry blockers (nifedipine, econazole and SK&F96365) were applied to explore the methoxychlor-induced Ca^{2+} influx pathways. Regarding plasmalemmal Ca^{2+} channels in HA59T hepatoma cells, only store-operated Ca^{2+} channels were reported (6,

8, 23); the existence of other types of Ca^{2+} channel is unclear. However, in our study, because methoxychlor did not induce Ca^{2+} release, it is unlikely that the methoxychlor-induced Ca^{2+} influx was *via* store-operated Ca^{2+} channels. In other hepatoma cell lines, Ca^{2+}-permeable channels such as receptor-operated channels, Ca^{2+}-activated Cl^- channels, and transient potential channels (TRP) (20) have been studied in hepatocytes *in situ*, isolated hepatocytes and hepatoma cell lines (22).

The spatial and temporal parameters of the cytoplasmic Ca^{2+} signals and the entry of Ca^{2+} through plasmalemmal Ca^{2+}-permeable channels are critical to the regulation of hepatocyte function by Ca^{2+}. Accumulated evidence shows that nifedipine (39) or econazole (28) inhibits Ca^{2+}-permeable channels in several cell types. The effect of SKF96365 on Ca^{2+}-permeable channels is unclear. Our results show that nifedipine, econazole or SK&F96365 inhibited methoxychlor-induced $[Ca^{2+}]_i$ rise. Therefore, methoychlor might induce Ca^{2+} influx *via* Ca^{2+}-permeable channels in our study. Furthermore, our data show that both activation and inhibition of protein kinase C suppressed methoxychlor-evoked $[Ca^{2+}]_i$ rise. This may be because normal protein kinase C activity is required for methoxychlor-induced $[Ca^{2+}]_i$ rise, and enhancement or decrease of this activity both decreased the $[Ca^{2+}]_i$ rise. Regulation of protein kinase C activity has been shown to modulate Ca^{2+}-permeable Ca^{2+} entry in different cells such as cultured hippocampal neurons (34) and smooth muscle cells (31).

Our study shows that methoxychlor was cytotoxic to HA59T cells in a concentration-dependent manner between 10 and 130 μM. The concentration range of methoxychlor used in $[Ca^{2+}]_i$ measurements (0.1-1 μM) and cytotoxicity assays were not comparable. This was because in $[Ca^{2+}]_i$ measurements cells were exposed to methoxychlor for only a few min; whereas in cytotoxicity assays cells were exposed to methoxychlor overnight. Ca^{2+} overloading is known to initiate processes leading to alteration in cell viability. Because methoxychlor induced both $[Ca^{2+}]_i$ rises and cell death in HA59T cells, the relationship between death and a preceding rise in $[Ca^{2+}]_i$ was explored. Our data show that methoxychlor-induced cell death was not altered when cytosolic Ca^{2+} was chelated by BAPTA/AM. This implies that methoxychlor-induced cell death was not triggered by a $[Ca^{2+}]_i$ rise. Furthermore, apoptosis appeared to be involved in methoxychlor-induced cell death based on Annexin V/PI staining assays. Although 50 μM methoxychlor caused cell death by 50% in viability experiments, the same concentration of methoxychlor only induced apoptosis in 13% of cells. Thus it is possible that the significant loss of cell viability is through other pathways such as necrosis or autophagy.

Previous studies explored the plasma concentration of methoxychlor after oral ingestion. Bio-Response methoxychlor (BR-methoxychlor)-related adverse effects were reported at doses up to 1 mg. A single 1 mg dose of BR-methoxychlor resulted in a mean C_{max} of ~10 μM after 24 h (10). In contrast, our data show that methoxychlor at concentrations between 0.1-1 μM evoked $[Ca^{2+}]_i$ rises without altering cell viability in HA59T cells. In addition, methoxychlor was metabolized in the liver. den Tonkelaar and van Esch (10) also show that in elderly or liver impaired patients, the plasma concentration of methoxychlor after oral administration might be 3-fold higher than in healthy adults. The local concentrations in the liver may be even much higher than in the plasma. Thus, our study may have clinical relevance.

Together, the data show that methoxychlor induced $[Ca^{2+}]_i$ rises in HA59T human hepatoma cells solely by causing Ca^{2+} entry without inducing Ca^{2+} release. Methoxychlor evoked Ca^{2+}-independent cell death that might involve apoptosis. The $[Ca^{2+}]_i$-elevating and apoptotic effects of methoxychlor should be considered in other hepatocyte research.

Acknowledgments

This work was supported by Kaohsiung Veterans General Hospital (VGHKS101-019) to CR Jan. The authors report no declarations of interest.

References

1. Basavarajappa, M.S., Karman, B.N., Wang, W., Gupta, R.K. and Flaws, J.A. Methoxychlor induces atresia by altering *Bcl2* factors and inducing caspase activity in mouse ovarian antral follicles *in vitro*. *Reprod. Toxicol.* 34: 545-551, 2012.
2. Bootman, M.D., Berridge, M.J. and Roderick, H.L. Calcium signalling: more messengers, more channels, more complexity. *Curr. Biol.* 12: R563-R565, 2002.
3. Chang, K.H., Tan, H.P., Kuo, C.C., Kuo, D.H., Shieh, P., Chen, F.A. and Jan, C.R. Effect of nortriptyline on Ca^{2+} handling in SIRC rabbit corneal epithelial cells. *Chinese J. Physiol.* 53: 178-184, 2010.
4. Chen, W.C., Chou, C.T., Liou, W.C., Liu, S.I., Lin, K.L., Lu, T., Lu, Y.C., Hsu, S.S., Tsai, J.Y., Liao, W.C., Liang, W.Z. and Jan, C.R. Rise of $[Ca^{2+}]_i$ and apoptosis induced by *M*-3M3FBS in SCM1 human gastric cancer cells. *Chinese J. Physiol.* 57: 31-40, 2014.
5. Cheng, H.H., Lu, Y.C., Lu, T., Cheng, J.S., Mar, G.Y., Fang, Y.C., Chai, K.L. and Jan, C.R. Effect of methoxychlor on Ca^{2+} movement and viability in MDCK renal tubular cells. *Basic Clin. Pharmacol. Toxicol.* 11: 224-231, 2012.
6. Cheng, J.S., Huang, C.C., Chou, C.T. and Jan, C.R. Mechanisms of carvedilol-induced $[Ca^{2+}]_i$ rises and death in human hepatoma cells. *Naunyn-Schmiedebergs Arch. Pharmacol.* 376: 185-194, 2007.
7. Cheng, J.S., Lo, Y.K., Yeh, J.H., Cheng, H.H., Liu, C.P., Chen, W.C. and Jan, C.R. Effect of gossypol on intracellular Ca^{2+} regulation in human hepatoma cells. *Chinese J. Physiol.* 46: 117-122, 2003.
8. Craig, Z.R., Wang, W. and Flaws, J.A. Endocrine-disrupting chemicals in ovarian function: effects on steroidogenesis, metabolism and nuclear receptor signaling. *Reproduction* 142: 633-646, 2011.
9. Dehn, P.F., Allen-Mocherie, S., Karek, J. and Thenappan, A. Organochlorine insecticides: impacts on human HepG2 cytochrome

P4501A, 2B activities and glutathione levels. *Toxicol. In Vitro* 19: 261-273, 2005.

10. den Tonkelaar, E.M. and van Esch, G.J. No-effect levels of organochlorine pesticides based on induction of microsomal liver enzymes in short-term toxicity experiments. *Toxicology* 2: 371-380, 1974.
11. Frye, C., Bo, E., Calamandrei, G., Calzà, L., Dessì-Fulgheri, F., Fernández, M., Fusani, L., Kah, O., Kajta, M., Le Page, Y., Patisaul, H.B., Venerosi, A., Wojtowicz, A.K. and Panzica, G.C. Endocrine disrupters: a review of some sources, effects, and mechanisms of actions on behaviour and neuroendocrine systems. *J. Neuroendocrinol.* 24: 144-159, 2012.
12. Fukuyama, T., Kosaka, T., Hayashi, K, Miyashita, L., Tajima, Y., Wada, K., Nishino, R., Ueda, H. and Harada, T. Immunotoxicity in mice induced by short-term exposure to methoxychlor, parathion, or piperonyl butoxide. *J. Immunotoxicol.* 10: 150-159, 2013.
13. Fukuyama, T., Kosaka, T., Tajima, Y., Hayashi, K., Shutoh, Y. and Harada, T. Detection of thymocytes apoptosis in mice induced by organochlorine pesticides methoxychlor. *Immunopharmacol. Immunotoxicol.* 33: 193-200, 2011.
14. Fukuyama, T., Tajima, Y., Ueda, H., Hayashi, K., Shutoh, Y., Harada, T. and Kosaka, T. Apoptosis in immunocytes induced by several types of pesticides. *J. Immunotoxicol.* 7: 39-56, 2010.
15. Grynkiewicz, G., Poenie, M. and Tsien, R.Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440-3450, 1985.
16. Gupta, R.K., Meachum, S., Hernández-Ochoa, I., Peretz, J., Yao, H.H. and Flaws, J.A. Methoxychlor inhibits growth of antral follicles by altering cell cycle regulators. *Toxicol. Appl. Pharmacol.* 240: 1-7, 2009.
17. Gupta, R.K., Schuh, R.A., Fiskum, G. and Flaws, J.A. Methoxychlor causes mitochondrial dysfunction and oxidative damage in the mouse ovary. *Toxicol. Appl. Pharmacol.* 216: 436-445, 2006.
18. Han, E.H., Jeong, T.C. and Jeong, H.G. Methoxychlor suppresses the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible CYP1A1 expression in murine Hepa-1c1c7 cells. *J. Toxicol. Environ. Health A.* 70: 1304-1309, 2007.
19. Hayashi, K., Fukuyama, T., Ohnuma, A., Tajima, Y., Kashimoto, Y., Yoshida, T. and Kosaka, T. Immunotoxicity of the organochlorine pesticide methoxychlor in female ICR, BALB/c, and C3H/He mice. *J. Immunotoxicol.* 10: 119-124, 2013.
20. Kim, J.A., Kang, Y.S., Jung, M.W., Kang, G.H., Lee, S.H. and Lee, Y.S. Ca^{2+} influx mediates apoptosis induced by 4-aminopyridine, a K^+ channel blocker, in HepG2 human hepatoblastoma-cells. *Pharmacology* 60: 74-81, 2000.
21. Lee, H.R., Hwang, K.A., Park, M.A., Yi, B.R., Jeung, E.B. and Choi, K.C. Treatment with bisphenol A and methoxychlor results in the growth of human breast cancer cells and alteration of the expression of cell cycle-related genes, cyclin D1 and p21, *via* an estrogen receptor-dependent signaling pathway. *Int. J. Mol. Med.* 29: 883-890, 2012.
22. Lee, Y.S. Mechanism of apoptosis induced by diazoxide, a K^+ channel opener, in HepG2 human hepatoma cells. *Arch. Pharm. Res.* 27: 305-313, 2004.
23. Liu, S.I., Lin, K.L., Lu, T., Lu, Y.C., Hsu, S.S., Tsai, J.Y., Liao, W.C., Huang, F.D., Chi, C.C., Liang, W.Z., Tseng, L.L., Chiang, A.J. and Jan, C.R. *M*-3M3FBS-induced Ca^{2+} movement and apoptosis in HA59T human hepatoma cells. *Chinese J. Physiol.* 56: 26-35, 2013.
24. Lorenzen, A., Williams, K.L. and Moon, T.W. Determination of the estrogenic and antiestrogenic effects of environmental contaminants in chicken embryo hepatocyte cultures by quantitative-polymerase chain reaction. *Environ. Toxicol. Chem.* 22: 2329-2336, 2003.
25. Merritt, J.E., Jacob, R. and Hallam, T.J. Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J. Biol. Chem.* 264: 1522-1527, 1989.
26. Miller, K.P., Gupta, R.K., Greenfeld, C.R., Babus, J.K. and Flaws, J.A. Methoxychlor directly affects ovarian antral follicle growth and atresia through Bcl-2- and Bax-mediated pathways. *Toxicol. Sci.* 88: 213-221, 2005.
27. Morgan, J.M. and Hickenbottom, J.P. Comparison of selected parameters for monitoring methoxychlor-induced hepatotoxicity. *Bull. Environ. Contam. Toxicol.* 23: 275-280, 1979.
28. Morita, K., Sakakibara, A., Kitayama, S., Kumagai, K., Tanne, K. and Dohi, T. Pituitary adenylate cyclase-activating polypeptide induces a sustained increase in intracellular free Ca^{2+} concentration and catechol amine release by activating Ca^{2+} influx *via* receptor-stimulated Ca^{2+} entry, independent of store-operated Ca^{2+} channels, and voltage-dependent Ca^{2+} channels in bovine adrenal medullary chromaffin cells. *J. Pharmacol. Exp. Ther.* 302: 972-982, 2002.
29. Paulose, T., Tannenbaum, L.V., Borgeest, C. and Flaws, J.A. Methoxychlor-induced ovarian follicle toxicity in mice: dose and exposure duration-dependent effects. *Birth Defects Res. B. Dev. Reprod. Toxicol.* 95: 219-224, 2012.
30. Rankouhi, T.R., Sanderson, J.T., van Holsteijn, I., van Leeuwen, C., Vethaak, A.D. and van den Berg, M. Effects of natural and synthetic estrogens and various environmental contaminants on vitellogenesis in fish primary hepatocytes: comparison of bream (*Abramis brama*) and carp (*Cyprinus carpio*). *Toxicol. Sci.* 81: 90-102, 2004.
31. Robertson, B.E., Schubert, R., Hescheler, J. and Nelson, M.T. cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am. J. Physiol.* 265: C299-C303, 1993.
32. Rouhani Rankouhi, T., Sanderson, J.T., van Holsteijn, I., van Kooten, P., Bosveld, A.T. and van den Berg, M. Effects of environmental and natural estrogens on vitellogenin production in hepatocytes of the brown frog (Rana temporaria). *Aquat. Toxicol.* 71: 97-101, 2005.
33. Stuchal, L.D., Kleinow, K.M., Stegeman, J.J. and James, M.O. Demethylation of the pesticide methoxychlor in liver and intestine from untreated, methoxychlor-treated, and 3-methylcholanthrene-treated channel catfish (Ictalurus punctatus): evidence for roles of CYP1 and CYP3A family isozymes. *Drug Metab. Dispos.* 34: 932-938, 2006.
34. Tan, S.E., Wenthold, R.J. and Soderling, T.R. Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. *J. Neurosci.* 14: 1123-1129, 1994.
35. Tseng, L.L., Shu, S.S., Kuo, C.C., Chou, C.T., Hsieh, Y.D., Chu, S.T., Chi, C.C., Liang, W.Z., Ho, C.M. and Jan, C.R. Effect of methoxychlor on Ca^{2+} handling and viability in OC2 human oral cancer cells. *Basic Clin. Pharmacol. Toxicol.* 108: 341-348, 2011.
36. Tsien, R.Y. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* 19: 2396-2404, 1980.
37. Vaithinathan, S., Saradha, B. and Mathur, P.P. Methoxychlor induces apoptosis *via* mitochondria- and FasL-mediated pathways in adult rat testis. *Chem. Biol. Interact.* 185: 110-118, 2010.
38. Wu, Y., Foster, W.G. and Younglai, E.V. Rapid effects of pesticides on human granulosa-lutein cells. *Reproduction* 131: 299-310, 2006.
39. Young, W., Chen, J., Jung, F. and Gardner, P. Dihydropyridine Bay K 8644 activates T lymphocyte calcium-permeable channels. *Mol. Pharmacol.* 34: 239-244, 1988.
40. Younglai, E.V., Wu, Y. and Foster, W.G. Rapid action of pesticides on cytosolic calcium concentrations in cultures of human umbilical vein endothelial cells. *Reprod. Toxicol*. 21: 271-279, 2006.