Effects of γ-hexachlorocyclohexane on apoptosis induced by serum deprivation in PC12 cells

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ABSTRACT

The effects of γ-hexachlorocyclohexane, one of the endocrine disrupters, widely used for agricultural and medicinal purpose, on apoptosis in PC12 cells were investigated using western blotting analysis and RT-PCR method. Apoptosis is a fundamental process necessary for development of individuals and organs. Although γ-HCH at high concentration did not effect on cell viability and apoptosis, DNA fragmentation was slightly enhanced, and apoptotic factors; Bax, Bad, cytochrome c and caspase-3 showed tendency of increase by addition of low γ-HCH to the cell medium. However these changes were not statistically significant. It was concluded that γ-HCH did not affect on apoptosis in the PC12 cell line system, although γ-HCH has been reported to induce apoptosis

in other cell lines.

Keywords: apoptosis, γ-HCH, PC12 cell, caspase, cytochrome C, Bcl-2 family

INTRODUCTION

1,2,3,4,5,6-hexachlorocyclohexane (HCH) is widely used for agricultural and medicinal purpose. Mixtures of HCH isomers have been used extensively since the 1940s to control malaria mosquitoes, and an isomer of HCH is still used in the USA as a component of pediculicide shampoos for head lice and scabies, [1] but in many countries it is banned for public use. Technical graded HCH is eight stereoisomers (α, β, γ, σ, ε, ι, ο, η). Lindane, the γ-isomer possesses the most significant insecticidal activity. [2] γ-HCH is the most persistent and, therefore, accumulative isomer whilst the α-and γ-isomer are mostly converted into the β-isomer in biological systems. [3] However, usage of the α-and β-isomers has fallen more rapidly than that of lindane itself. [4]

The primary route of HCH exposure in the general population is through dietary intake [5] particularly via meat and dairy products. [6] HCH has been reported to induce reproductive toxicities in rat including decrease in sexual receptivity, [7] and induction of degenerative changes in seminiferous tubules. In such animal models lindane-induced damage may result from the generation of superoxide anion radicals, and/or DNA single-strand breaks and epigenetic mechanisms. [8]

On the other hand, it has been reported that some of endocrine disrupters act as chemical substances caused apoptosis in cells. [9-13] In addition, γ-HCH induced apoptosis in HL-60 cells through intracellular Ca2+ release pathway. [14] Apoptosis is a kind of physiological cell death that plays a key role in the maintenance of homeostasis of various animal species. [15] Apoptotic cell death is characterized by chromatin condensation, DNA fragmentation, and cellular shrinkage resulted in the formation of apoptotic bodies. [15]

Apoptosis is mediated by member of caspase family of proteases

and eventually causes the degradation of chromosomal DNA. [16] The effector caspase represented by caspase-3 is frequently activated in response to various apoptosis inducers and essential for the execution and completion of apoptosis in many, but not all, cell types or death stimuli. Pathways leading to activation of the effector caspase have been identified to be either dependent or independent on the release of cytochrome c from mitochondria into the cytosol. [17,18] In addition, activation of the effector caspase appears to be tightly regulated by a signaling mechanism including the members of Bcl-2 family, [18] which include pro-apoptotic proteins such as Bax and Bad is induced the mitochondrial cytochrome c release. [19]

PC12 is a rat pheochromocytoma clonal cell line that responds to nerve growth factor by extending neurites, thus acquiring the appearance of neurons. [20] Over the past 30 years, PC12 cells have become a very suitable model to study neuronal function and differentiation. [21] In this study, to understand the neuronal damage of endocrine disrupters such as γ-HCH, effects of γ-HCH on apoptosis induced by serum deprivation in PC12 cells were

investigated. Experiments were conducted at low-dose and high-dose concentrations of γ-HCH to study the effect of the chemical substance.

MATERIALS AND METHODS

Materials

PC12 cells, a cell line of rat pheochromocytoma cells, were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM) and *o*-phenylendiamine dihydrochloride (OPD) were obtained from Sigma (St. Louis, MO USA). Fetal bovine serum (FBS) was bought from HyClone (Rockville, MD USA). Caspase assay kit and SV total RNA isolation system were from Promega (Madison, WI USA). Trypan blue stain solution (0.5%) and 1,2,3,4,5,6-Hexachlorocyclohexane (γ-HCH) were from Nacalai Tesque (Kyoto, Japan). Biotin-16-2'-deoxy-uridine-5-triphosphate, proteinase K and the blocking reagent were from Roche Diagnostics (Mannheim, Germany). Terminal deoxynucleotidyl

transferase (TdT) was from Toyobo (Osaka, Japan). Biotinylated anti-mouse immunoglobulin was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Antibody of cytocrome c was purchased from Promega (Madison, WI USA). Other chemicals were of analytical regent grade.

Cell Culture

PC12 cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator at 37°C and 5% CO_2. The cells were preincubated in 25 cm^2 flasks for over night, and then the medium was replaced with serum/serum-free DMEM with or without γ-HCH. When the medium was changed to serum deprivated medium, cells in the flask were washed twice with serum-free DMEM.

Cell Viability Measured by Trypan Blue Exclusion Assay

PC12 cells were incubated in the serum/serum-free medium with 0 to 5,000 ng/mL γ-HCH for 72 hr. After the incubation, cells were stained in 0.25% trypan blue solution in phosphate-buffered saline.

Total cells and trypan blue staining cells were counted by a hemocytometer. Cell viabilities were expressed as percentage against the total cell number in each experiment.

Quantification of DNA Fragmentation in PC12 Cells by the TdT-Mediated dUTP-biotin Nick End Labeling (TUNEL) Method

After that the PC12 cells were cultured in the serum/serum-free medium with 0 to 5,000 ng/mL γ-HCH for 6 to 72 hr, genomic DNA was isolated by the method of Yamanoshita et al. [11] The obtained DNA was resuspended in 1X TBE buffer and equal amount of DNA was put into 96-well plate wells. Quantification of DNA fragmentation in PC12 cells was measured by the method of Kurasaki et al. [22] In briefly, the plate was incubated at 4°C for over night. After the incubation, the plate was washed with the 40 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl. Nonspecific binding sites were blocked with 2% blocking reagent in the same buffer for 30 min. Then the plate was washed twice with same buffer. TdT reaction was performed in a reaction mixture consisting of 30 mM Tris-HCl buffer, pH 7.4, containing 140 mM

sodium cacodylate, 1 mM cobalt chloride, 0.1 units TdT/μL and 2 nM biotinylated-dUTP at 37°C for 1.5 hr. After the reaction, the plate was washed. The plate was incubated with streptavidin-conjugated peroxidase for 1 hr. After washing, 0.1% OPD in 50 mM phosphate-citrate buffer, pH 5.0, containing 0.03 % sodium perborate was added to each well and incubated for 0.5 hr at 37 °C. Then, HCl was added to stop the enzyme reaction. Absorbance at 495 nm was measured with a Microplate Reader model 450 (BIO-RAD).

Gene Expression Determined by Reverse Transcriptase Chain Reaction (RT-PCR)

The PC12 cells were incubated with 0 to 100 ng/mL γ-HCH for 6 hr. The total RNA of these cells was extracted using the SV total RNA isolation kit according to the instruction manual. The obtained total RNA was verified using a RNA 6000 nano assay with the Agilent 2100 bio analyzer according to the instruction manual. Expression of Bad, bax and bcl-2 mRNA was detected using the access RT-PCR kit and access RT-PCR introductory kit according

to the instruction manual. Total RNA of PC12 cells cultured in the serum/serum-free medium with 0 to 100 ng/mL γ-HCH for 6 hr was prepared by using of SVTotal RNA Isolation kit. The PCR primers of Bad, Bax, Bcl-2 and β-actin were synthesized according to the their DNA sequences. PCR condition was as follows; 48°C for 45 min, 94°C for 2 min. The cycles were 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min. Final extension was for 7 min at 72°C. The cycles performed were 30 or 40. An annealing temperature of 60°C was used for β-actin and Bax, 55°C for Bad, and 47°C for Bcl-2. PCR of β-actin that was chosen as an internal control. PCR products were verified using a DNA 7500 assay with an Agilent 2100 Bio Analyzer according to the instruction manual.

Western Blot Analysis Using Antibodies Against Cytochrome c.

The PC12 cells were cultured in the serum/serum-free medium with 0 to 100 ng/mL γ-HCH for 6 hr. The same protein amounts of the obtained lysate were separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes with a semidry blotting system type-AE6678 (ATTO, Japan). After

transfer, the membranes were incubated for 2 hr at room temperature with against cytochrome c antibodies in 40 mM Tris-HCl buffer, pH 7.4, containing 150mM NaCl and 1% blocking reagent (1% blocking buffer). The membranes were washed three times for each 3 min in TBTS consisting of 40 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 0.3% Tween 20, and then incubated at room temperature for 1 hr with anti-mouse IgG biotinylated secondary antibody (dilution; 1:1000) in the 1% blocking buffer. The membranes were washed three times in TBTS and were washed twice for each 3 min in Tris-HCl buffer of 40 mM Tris-HCl, pH 7.4, 150mM NaCl. Finally, streptavidin-conjugated peroxidase (1:400 diluted with the same buffer) was added and incubated for 1 hr. Protein bands responded to antibodies were detected with an enhanced chemiluminescence system.

The Activity of Caspase-3-like Protease

The activity of caspase-3-like protease was detected using the caspase assay system kit according to the instruction manual. This kit was using DEVD-pNA as substrate and this chemical was

cleaved by DEVDase such as caspase-3 protease. The activity of caspase-3 like protease in the PC12 cells was indirectly measured as absorbance at 405 nm deputing on released pNA from the cleaved substrate. The caspase-3 like activity was expressed as relative contents against that in the cells incubated in the medium containing serum without γ-HCH.

Statistical Analysis

Each value is expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by the Fisher's multi comparison test.

RESULTS AND DISCUSSION

To confirm whether γ-HCH affects on cell viability, the viability was measured by trypan blue staining after PC12 cells exposed to 0 to 5,000 ng/mL γ-HCH for 72 hr. No significant difference of viabilities among the cells exposed to 0 to 5,000 ng/mL γ-HCH was observed (data not shown). These results indicated that γ-HCH did

not show cytotoxicity against the PC12 cells.

With TUNEL method, DNA ends generated by endonucleases activity are specifically labeled. [23] Quantification of DNA ladder was carried out to evaluate the degree of apoptosis in the cells treated with 0 to 5,000 ng/mL γ-HCH. As shown in Fig. 1a, TUNEL signals were not significantly different between PC12 cells with and without γ-HCH (100 to 5,000 ng/mL). On the other hand, as shown in Fig. 1b, there is a tendency of increase ($p<0.1$) in the DNA fragmentation in PC12 cells exposed with 10 ng/mL γ-HCH as compared with that in control cells. From these results, there is a possibility of enhancement of the apoptosis in PC12 cells by addition of γ-HCH at low concentration. To examine weather γ-HCH enhances apoptosis induced by serum deprivation in PC12 cells, apoptotic factors such as Bcl-2 family, cytochrome c and caspase-3 were measured.

As shown in Fig. 2, contents of Bax and Bad mRNAs in the cells exposed with γ-HCH did not increase significantly as compared with those in the control PC12 cells (Fig. 2). In addition, from the

results of western blotting analyses using the cell lysis from PC12 cells treated with γ-HCH was performed using antibodies against cytochrome c, the contents of cytochrome c was slightly increased in comparison with that in cells treated without γ-HCH (Fig. 3). Although Liu et al. [24] reported that apoptosis is induced by releasing cytochrome c from mitochondria to cytosol, the amounts of cytochrome c in our experiment was not significantly increased by addition of γ-HCH as expected ($p>0.1$, $n=3$).

Moreover, by addition of γ-HCH, the caspase-3 like activity in the cells incubated in the serum/serum-free medium did not also increase (Fig. 4).

In this study, the DNA fragmentation under the apoptotic condition was slightly enhanced by the treatment with γ-HCH at low concentration (Fig. 1), although significant difference was not observed in cell viability and DNA fragmentation at high concentration (100-5,000 ng/mL γ-HCH) in the PC12 cells.

It has been reported that serum deprivation induced apoptosis

accompanied by rapid down-regulation of Bcl-2, and up-regulation of Bax and Bad. [24] However, as shown in Fig. 2, the Bax and Bad mRNA contents were not changed in PC12 cells treated with γ-HCH. Cytochrome c released into the cytosol activates the caspase cascade, and was generated during apoptosis from procaspase-3. [25] In this study, although content of cytochrome c was slightly increased in the cells cultured in the serum-free medium contains low concentration of γ-HCH, the caspase-3 like activity did not increase under the same condition (Figs. 3 and 4). As results, it was concluded that γ-HCH affected on apoptosis induced by serum deprivation in the PC12 cells.

Kang et al. [14] reported that γ-HCH at high concentration induced apoptosis in HL-60 cells through intracellular Ca ion release pathway. In addition γ-HCH was shown as an inhibiter of gap junction formation through apoptotic pathway. [26] These reports dose not agree with this study. The reason of the discrepancy is still unknown. However these phenomena depend on the characteristics of the cell type. PC12 cells were used to study neuronal function and differentiation. It was suggested that a

neuronal cell had a resistance against the toxic substances such as pesticide.

On the other hand, γ-HCH is thought as one of the endocrine disrupters. We had reported the relationship between the endocrine disrupters and apoptosis. Tributyltin and 2,4,5 Trichlorophenoxyacetic acid inhibited completely apoptosis induced by serum deprivation. [11,12] Aoki et al. [13] reported that nonylphenol enhanced apoptosis induced by serum deprivation.

Apoptosis is a fundamental process necessary for development of individuals and organs, and differentiation of the nervous system. [27] Since apoptosis is essential for cells in development and in elimination of harmful cells, the adverse effects of endocrine disrupting chemicals on apoptosis would cause serious damages to organisms. [28] According to this study and our previous reports, it was suggested that the effect of endocrine disrupters on apoptosis was independent from the reaction of estradiol receptor.

Therefore, the methods presented in this study, are expected to be a

useful tool for evaluating of endocrine disrupting chemicals which dose not only relate to disrupt endocrine function by the binding to the estrogen receptor, but also relate to affect on the other functions such as apoptosis.

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FIGURE LEGENDS

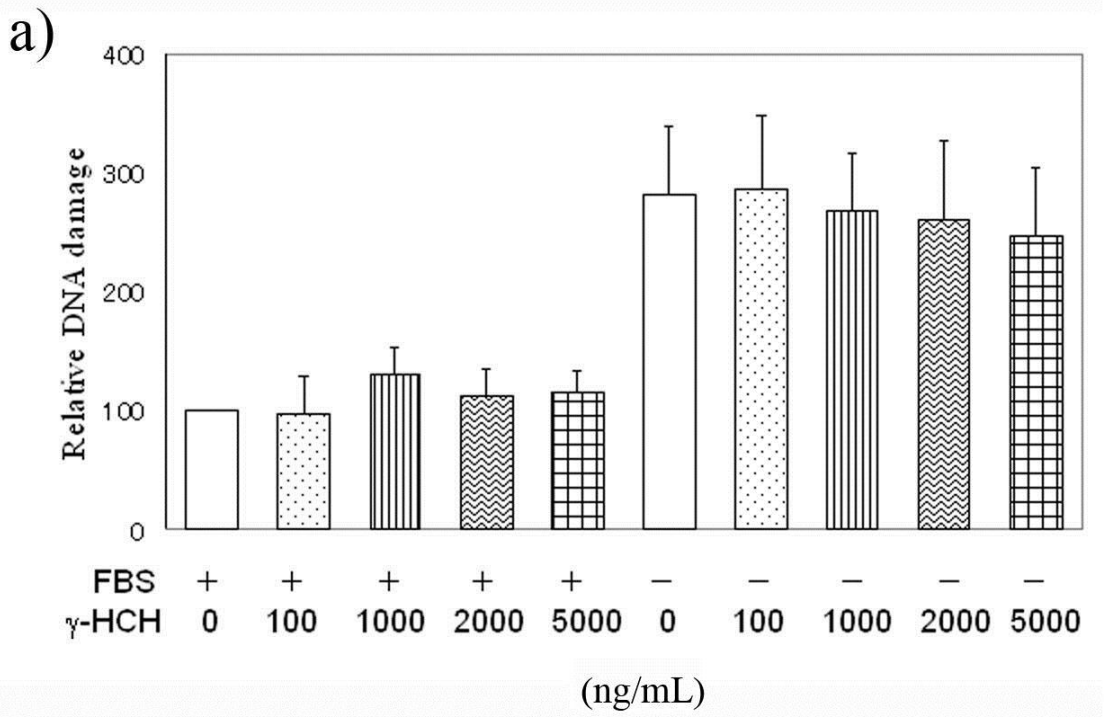
Fig. 1 Relative contents of DNA fragmentation in PC 12 cells treated with γ-HCH : 0 to 5000 ng/mL (a), 0 to 100 ng/mL (b) in serum-containing medium or serum-free medium, for 72 hr. Error bars indicate SEM (a:n=6, b:n=3).

Fig. 2 Relative contents of Bax mRNA (a) and Bad mRNA (b) in PC12 cells treated with γ-HCH (0 to 100 ng/mL) in serum-containing medium or serum-free medium for 6 hr. Error bars indicate SEM (n=3).

Fig.3 Western blot analyses of cytochrome c in PC 12 cells treated with γ-HCH (0 to 100 ng/mL) in serum-containing medium

or serum-free medium for 6 hr.

Fig. 4 Relative contents of Caspase-3 in PC 12 cells treated with γ-HCH (0 to 100 ng/mL) in serum-containing medium or serum-free medium for 6 hr. Error bars indicate SEM (n=3).


a)
Relative DNA damage
400
300
200
100
0
FBS + + + + + − − − − −
γ-HCH 0 100 1000 2000 5000 0 100 1000 2000 5000
(ng/mL)

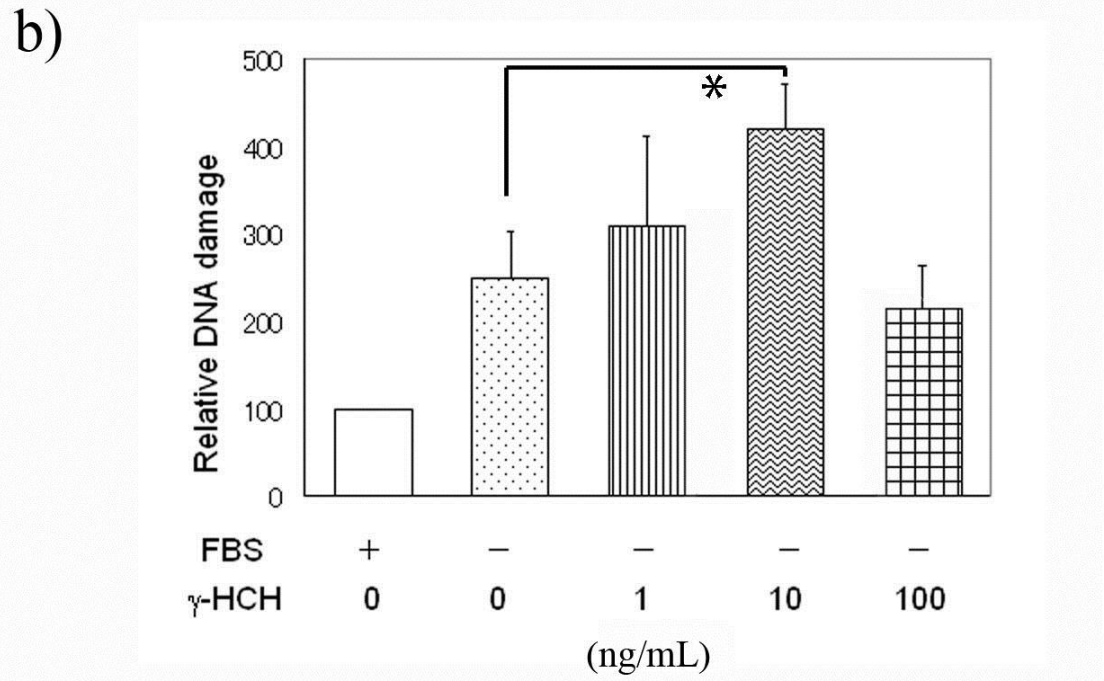
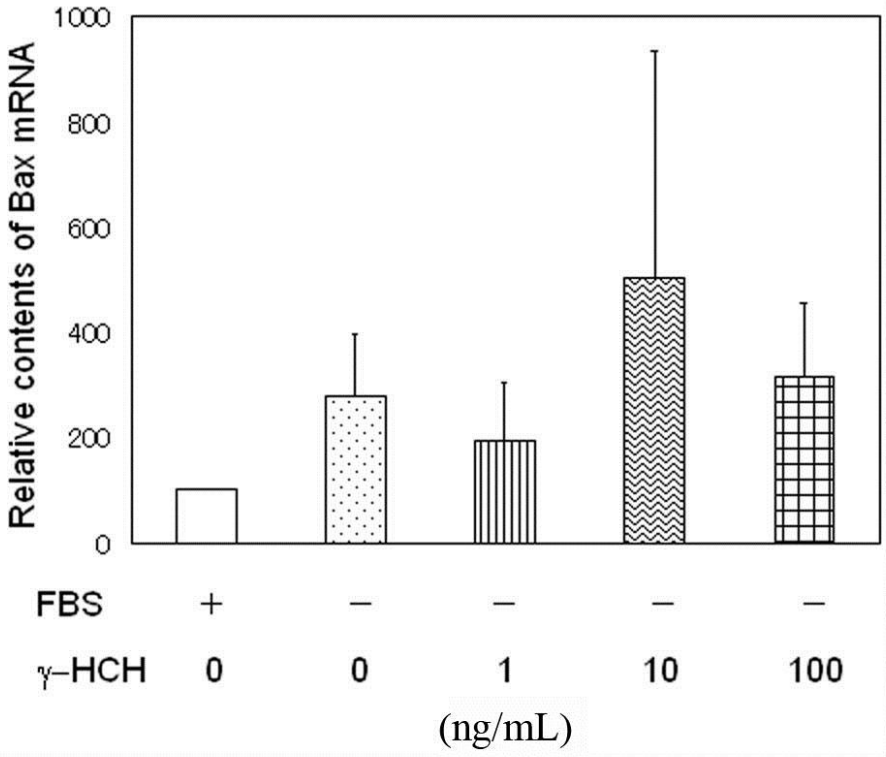

b)
Relative DNA damage
500
400
300
200
100
0
*
FBS + − − − −
γ-HCH 0 0 1 10 100
(ng/mL)

Fig.1

a)


Relative contents of Bax mRNA
1000
800
600
400
200
0
FBS + – – – –
γ–HCH 0 0 1 10 100
(ng/mL)

b)

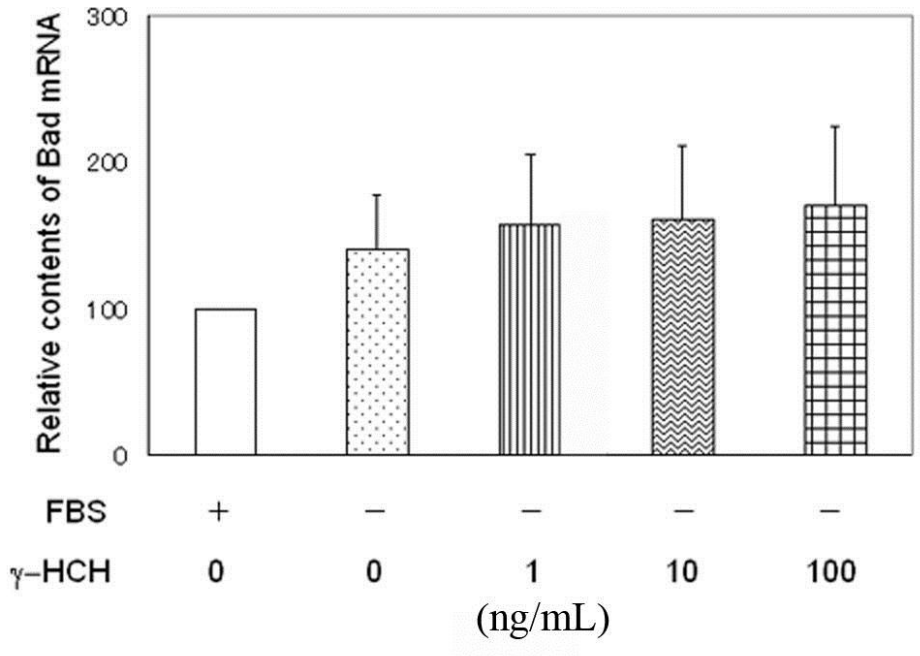

Relative contents of Bad mRNA
300
200
100
0
FBS + – – – –
γ–HCH 0 0 1 10 100
(ng/mL)

Fig.2

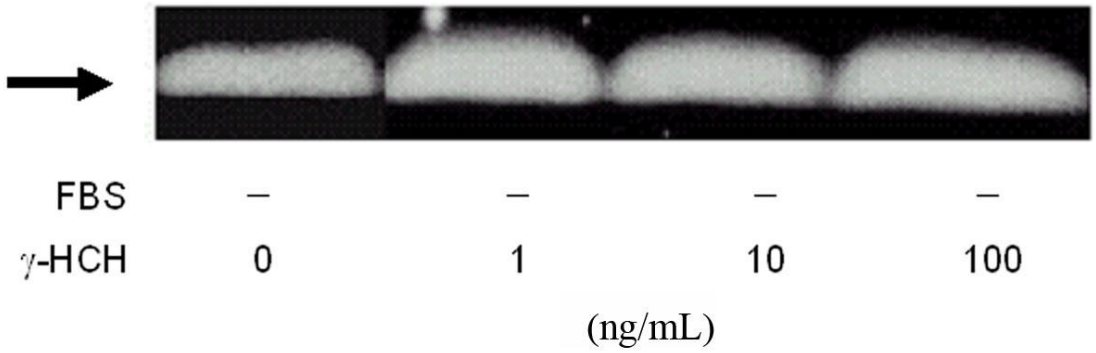


Fig.3

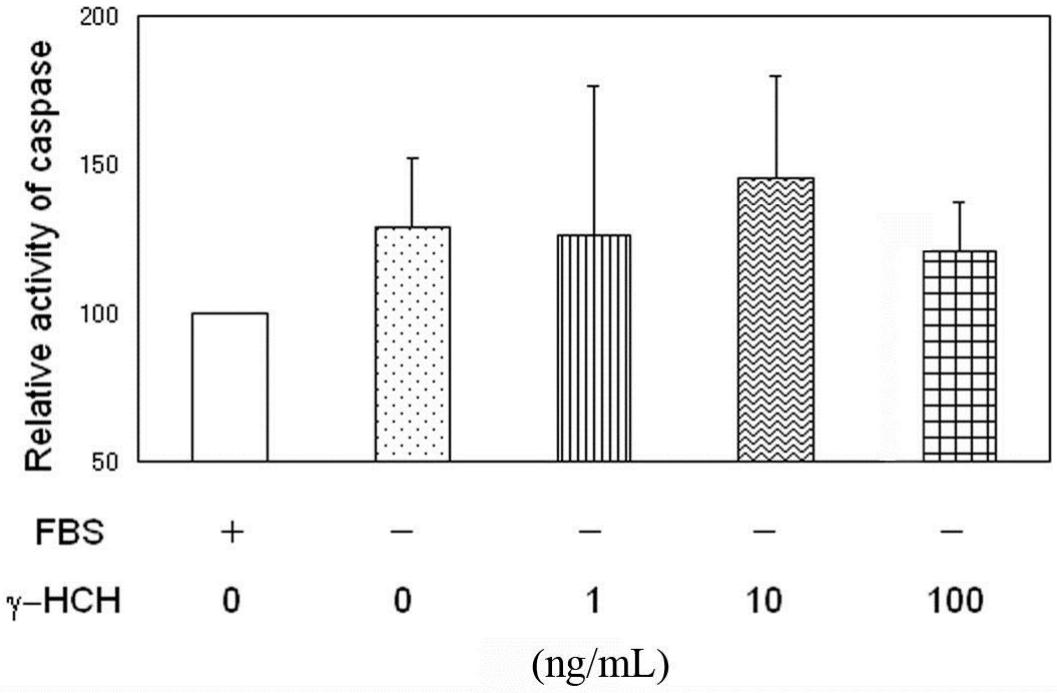

Relative activity of caspase
200
150
100
50
FBS
+
–
–
–
–
γ–HCH
0
0
1
10
100
(ng/mL)

Fig.4

Outline