Trance Nutrients Researth23:35–41
(2006)

Relation of Excessive Accumulation of Calcium and Endonuclease Activation in the Organotin-Exposed Olfactory System

Ken-ichi TOMIYAMA[1)], Takao KURIYAMA[1)], Akiko YAMAGUCHI[1)], Moritoshi NIHIRA[1)], Yurie SAYAMA[1)], Takayuki TAKEUCHI[2)], Yukihiro NAKANO[2)], Harunobu NAKASHIMA[3)], Yoshikazu MATSUDA[4)] and Yasuaki ARAKAWA[1)]

[1)] *Department of Hygiene & Preventive Medicine, Faculty of Health Sciences, The University of Shizuoka**

[2)] *Research Reactor Institute, Kyoto University***

[3)] *Osaka Prefectural Institute of Public Health****

[4)] *Central Research Institute, Japan Clinic Co., Ltd*****

Summary

Organotin exposure induced severe olfactory lesions. In a series of kinetic analysis of the mechanism in the induction of the lesions, we found remarkable results that organotin exposure induced extensive destruction and cell loss in the areas of the olfactory tubercle (olfactory cortex) and pyriform cortex which normally contain many small granule neurons, and further induced excessive accumulation of calcium and DNA fragmentation in the olfactory system such as olfactory epithelium and olfactory bulb. Therefore, it was investigated whether or not the excessive accumulation of calcium would play a significant role in cell death and might contribute to toxic cell death (necrosis or apoptosis) associated with endonuclease activation, by determining Ca^{2+}-requred DNase I. The result was that the endonuclease activation was parallel with excessive accumulation of calcium in the olfactory system, and that the both were related closely each other.

Organotin exposure induced severe olfactory lesions. Recently, we found that, in addition to trimethyltin, other trialkyltins, particularly tributyltin, impaired the sensory organs when the compound was given intraperitoneally[1-14)]. In particular, tributyltin produced damage and neuronal destruction in the pyriform and olfactory cortices after intraperitoneal exposure. Extensive destruction and cell loss were observed in the areas of the olfactory tubercle (olfactory cortex) and pyriform cortex which normally contain many small granule neurons[1-14)]. Further, in a series of this study, we found that the tributyltin induced an excessive accumulation of calcium in the olfactory system such as olfactory epithelium and olfactory bulb. These results suggest that the abnormal calcium accumulation induces severe destruction of the cortical neurons of the olfactory system[1-14)].

Therefore, in this study, it was investigated whether or not the excessive accumulation of calcium would play a significant role in cell death and might contribute to toxic cell death (necrosis or apoptosis) associated with endonuclease activation, by determining Ca^{2+}-requred DNase I[15)].

*Address：52-1, Yada, Suruga-ku, Shizuoka 422-8526, Japan

**Address：Kumatori-cho, Sennan-gun, Osaka 590-0494, Japan

***Address：1-3-69, Nakamichi, Higashinari-ku, Osaka 537-0025, Japan

****Address：10-1, Kainichi-cho, Uzumasa, Ukyo-ku, Kyoto 616-8555, Japan

Materials and Methods

Animals: A randomized group of five male rats (Wistar-derived, 8 weeks ages, weighing about 200 g, obtained from Japan SLC) was used.

Intraperitoneal treatment: For a single or repeated intraperitoneal administration to rats, tributyltin chloride was dissolved in corn oil. The dose level used once a day was 2.0 mg (3.07 mM) tributyltin chloride per kilogram of body weight. The rats were killed 1, 2, 3 and 4 day after treatment. Olfactory bulb was prepared for primary culture and various assays.

Primary culture of olfactory bulb neurons: Primary culture of olfactory bulb cells was performed as follows; 25 mm dishes were coated with poly-L-lysine solution (Sigma). Olfactory tissue homogenates and primary neurons were collected by trypsinization and lysed with lysis buffer (50 mM Tris-HCl (pH 7.8), 10 mM EDTA·4Na, 0.5 % (w/v) sodium-N-lauroylsarcosinate) containing 0.01 % DNase I for 2 h at 37℃. Cells were dissociated and triturated with a Pasteur pipette. Cells were plated at a density of 1.0～1.5 x 10^6cells/dish, respectively. Cells were cultured with MEM solution supplemented with 10 % (v/v) fetal calf serum (Gibco) at 37℃ in 5 % CO_2. After 3 days, the medium was replaced with culture medium[16].

Determination of cytotoxicity: Tributyltin-induced cytotoxicity in the rat olfactory bulb was evaluated by trypan blue (TB) exclusion assay. Cells were collected by trypsinization and satained with 0.4 % TB solution before counting. Cell samples were counted in duplicate under a light microscope at 100 x[16].

Preparation of tissue homogenates, cell nuclei, and extracts: DNA extraction from tissues, homogenates, or nuclei was performed by using the method described in literature (18).

Detection of Ca^{2+}-requred DNase: DNase was detected by DNA substrate-SDS-PAGE using 10 % SDS - ployacrylamide gels containing 200 μg/ml of native calf thymus DNA (Sigma), as decribed previously[17, 18]. After electrophoresis, The gels was washed with 10 mM Tris-HCl (pH 7.8) and 5 mM 2-mercapoethanol at 50℃ for 1 h to remove SDS, and then with 10 mM Tris-HCl (pH 7.8) at 4 ℃ overnight. The gel was then incubated in 10 mM Tris-HCl (pH 7.8) containing 1 mM 2-mercapoethanol and 3 mM $CaCl_2$, 3 mM $MgCl_2$ and/or 3 mM $ZnCl_2$ at 37℃ for 6 h. After staining with 0.5 μg/ml ethidium bromide, DNase was identified as a black band on a bright red background of undigested DNA[18].

Determination of endonuclease activity: Endonuclease activity was measured by an ELISA kit (ORGENTEC) for determining the activity of Ca^{2+}-requred DNase I[18]. The DNase activity ELISA from ORGENTEC GmbH is a novel and absolutely innovative assay for the determination of DNase I activity. The principle of the procedure is as follows; 1) the specific DNase substrate is coated in microplates, 2) any present DNase activity reacts with the specific immobilized DNase substrate coated onto the wells, 3) a DNase substrate horseradish peroxidase conjugate solution is pipetted into the wells of the microplate to recognize the remaining DNase substrate immobilized on the microplate, 4) a chromogenic substrate solution is dispensed into the wells, after incubation, colour development is stopped by adding a stop solution, 5) the amount of colour is inversely proportional to the DNase activity.

Isolation of DNA and detection of DNA fragmentation: Olfactory tissue homogenates and primary cortical neurons were collected by trypsinization and lysed with lysis buffer (50 mM Tris-HCl (pH 7.8), 10 mM EDTA·4Na, 0.5 % (w/v) sodium-N-lauroylsarcosinate) for 1 h at 37℃. RNase (10 mg/ml) was added to the samples, followed by incubation for 30 min at 37℃. Proteinase K was added to the samples, followed by incubation for 60 min at 37℃. Lysed cell samples were centrifuged for 15 min at 13000 x g. The supernatants were extracted with equal volumes of phenol/chloroform (1: 1). The DNA samples were loaded onto 2.0 % agrose gels and electrophoresis was performed at 100 mV. Gels were visualized by UV tranceilluminater after staining with ethidium bromide.

Results

Extensive destruction and cell loss of olfactory neurons by organotin exposure

Organotin induced extensive destruction and cell loss in the areas of the olfactory tubercle (olfactory cortex) and pyriform cortex which normally contain many small granule neurons after intraperitoneal exposure (Fig. 1). Figure 2 shows cell viability of tributyltin-exposed olfactory neurons. The cell viability decreased with exposure time and reached 53.5 % after 2 days, and 17.0 % after 3 days. Figure 3 shows the reproducibility of olfactory neurons having a past record of tributyltin-induced damage. The reproducibility decreased with exposure time and still survived after 2 days, and did not subsist after 3 days.

Detection of endonuclease activation in the organotin-exposed olfactory system

DNase activity could be detected in total extracts of tributyltin-exposed olfactory and nuclear extracts of both control and tributyltin-exposed olfactory, whereas it could not be detected in total extracts of control olfactory (Fig. 4-ABCD). Especially, the DNase activity was strong in total extracts of tributyltin-exposed olfactory. DNA substrate gel analysis revealed the presence of a DNase with an apparent molecular mass of 25 to 30 kD. Although DNase in nuclear extracts of tributyltin-exposed olfactory was inactive at the presence of known inhibitors of Ca^{2+}-requred endonuclease, i.e. zinc[17, 18], DNase in total extracts of tributyltin-exposed olfactory was active at the presence of zinc (Fig. 4-EF).

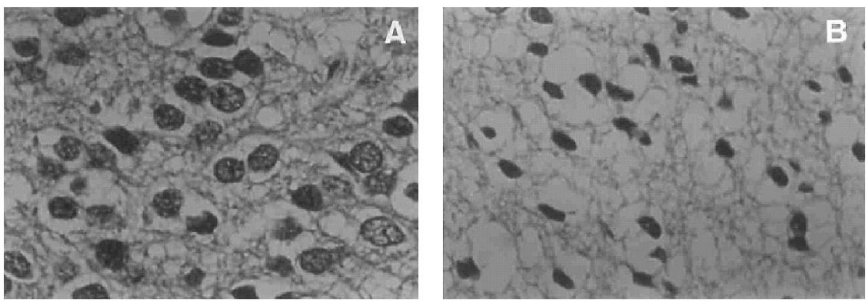

Fig. 1 Part of the olfactory bulb cortex from a control rat (A) and a rat (B) of 3 days after the exposure with a single intraperitoneal injection of tributyltin chloride (3.07 mM/kg body weight).

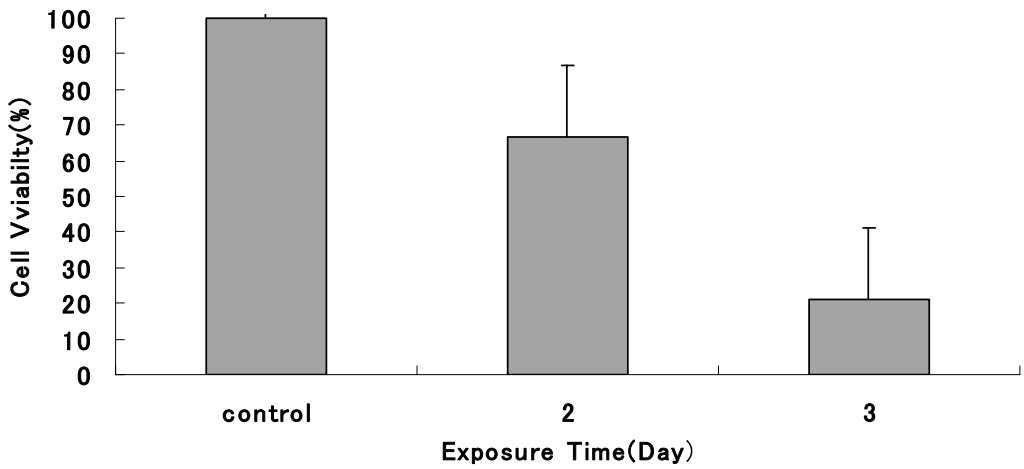

Fig. 2 Changes with the passage of exposure time in the cell viability of cortical neurons in the olfactory bulb of rats given a single intraperitoneal injection of tributyltin chloride (2.0 mg/kg body weight).

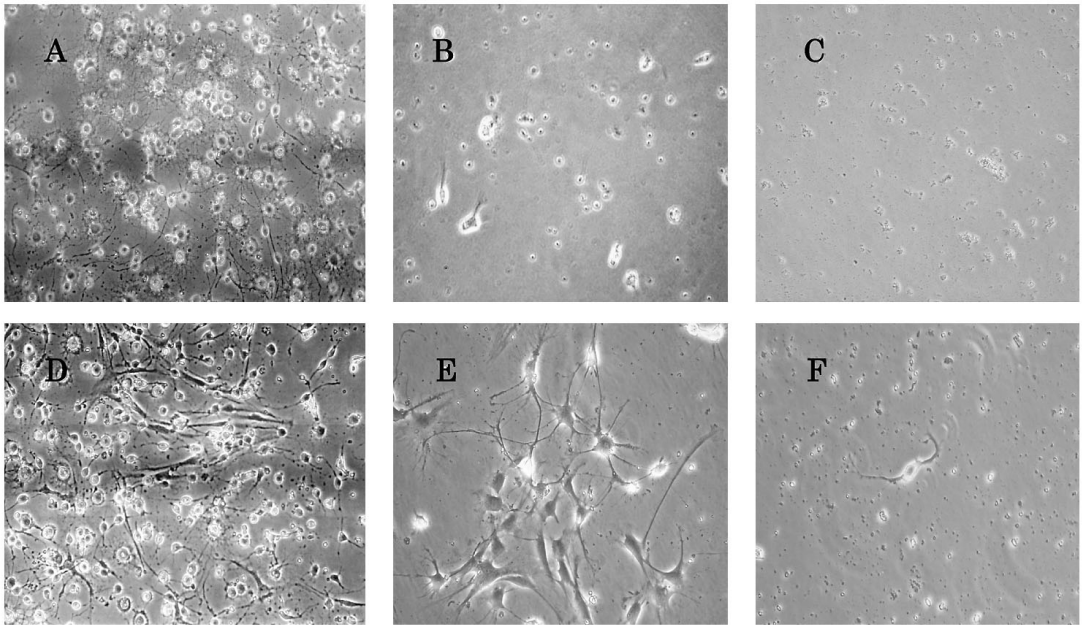

Fig. 3 The reproducibility of olfactory neurons having a past record of tributyltin-induced damage. Primary culture of control neurons (A), neurons exposed for 2 days (B) or for 3 days (C) by tributyltin were conducted for 2 days to examine their reproducibility. Similarly, the primary culture of control neurons (D), tributyltin-exposed neurons for 2 days (E) or for 3 days (F) were conducted for 12 days.

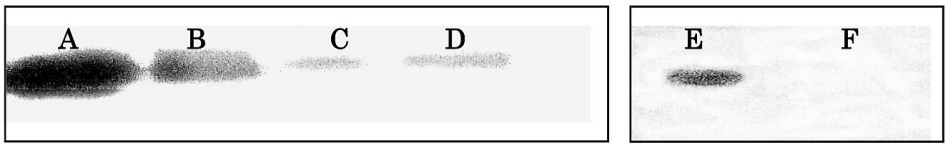

Fig. 4 Analysis of rat olfactory bulb DNase by substrate gel electrophoresis. Lane A: DNase in the total protein extract (10 μg protein/well) from tributyltin-exposed olfactory bulb, Lane B: DNase in the nuclear extract (10 μg protein/well) from tributyltin-exposed olfactory bulb, Lane C: DNase in the total protein extract (10 μg protein/well) from control olfactory bulb, Lane D: DNase in the nuclear extract (10 μg protein/well) from control olfactory bulb, Lane E: DNase in the total protein extract (5 μg protein/well) from tributyltin-exposed olfactory bulb under the presence of zinc, Lane F: DNase in the nuclear extract (5 μg protein/well) from tributyltin-exposed olfactory bulb under the presence of zinc.

These results suggested that DNase detected in total extracts of tributyltin-exposed olfactory would be a cytoplasmic protein such as DNase I which was Ca^{2+}-requred endonuclease and did not require zinc as the inhibitor.

Relation of excessive accumulation of calcium and endonuclease activation in the organotin-exposed olfactory system

Figure 5 shows movement of organotin, calcium and DNase I activity in the olfactory bulb of rats given a single intraperitoneal injection of tributyltin chloride. Tributyltin was quickly distributed to the olfactory bulb and the concentration of tributyltin reached a maximum within a day, decreasing rapidly during the subsequent 3 days. Under this tributyltin exposure condition, the concentration of calcium in the olfactory system increased slowly during the first day and then rose steeply to extremely high levels after 2 days, followed by a rapid decrease in a similar pattern. At the same condition, DNase I activity increased slowly during the first day and then rose steeply to extremely high levels after 2 days, followed by a rapid decrease in a similar pattern as well as the movement of calcium.

Both movements were parallel. These results revealed that there was a clear correlation between excessive accumulation of calcium and activation of DNase I.

Similarly, agarose gel electrophoresis revealed that DNA fragmentation, at the same condition, was confirmed most clearly after 2 days (Fig. 6).

Putting all these results together, organotin exposure induced extensive destruction and cell loss in the areas of the olfactory tubercle (olfactory cortex) and pyriform cortex which normally contain many small granule neurons, and further induced excessive accumulation of calcium and DNA fragmentation in the olfactory system such as olfactory epithelium and olfactory bulb.

Further, the endonuclease activation was parallel with excessive accumulation of calcium in the olfactory system, and there was a clear correlation between both.

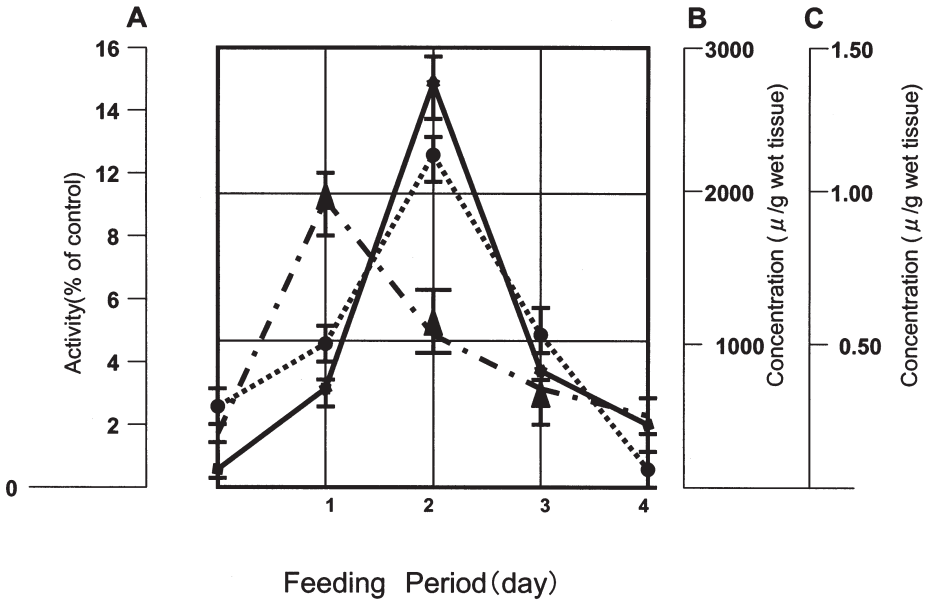

Fig. 5 Changes with the passage of time in the activity of DNase I (●in the A axis), in the concentration of calcium (◆in the B axis) and organotin (▲in the C axis) in the olfactory bulb of rats given a single intraperitoneal injection of tributyltin chloride (2.0 mg/kg body weight). Vertical bars denote the SE of the mean for five determinations.

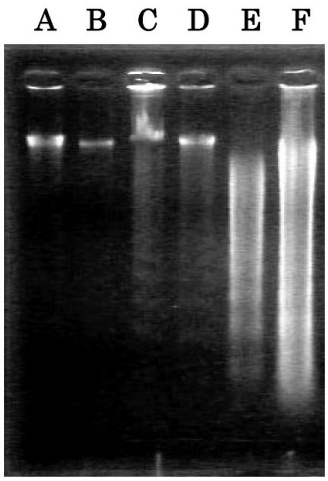

Fig. 6 DNA fragmentation in rat olfactory bulb tissue homogenates. Agarose gel electrophoresis of DNA isolated from the olfactory bulb of control (Lane A and B), of 2 days after (Lane C) or 3 days after (Lane D) the exposure with a single intraperitoneal injection of dibutyltin dichloride (3.07 mM/kg body weight) and of 2 days after (Lane E) or 3 days after (Lane F) the exposure with a single intraperitoneal injection of tributyltin chloride (3.07 mM/kg body weight) were conducted.

Discussion

From our previous results[1-14], a trialkyltin-induced excessive increase of calcium in the olfactory bulb is associated with an increase of olfactory PTH[6-10]. Since the IP_3 level in the olfactory bulb decreases significantly under the tributyltin exposure, which seems to be a consequence of tributyltin-induced inhibition of PI turnover, the excessive increase of olfactory calcium is not due to an influx of Ca^{2+} mediated by a plasma membrane IP_3-gated Ca^{2+} channel and a release of intracellular Ca^{2+} mediated by the IP_3 receptor-channel complex from endoplasmic reticulum (ER)[6-10]. The tributyltin-induced excessive increase of olfactory calcium is perhaps caused by an excessive increase in the formation of cAMP mediated by activation of adenyl cyclase and an excessive influx of extracellular Ca^{2+} mediated by cAMP-activated channels[1-14].

Further, Organotin compounds such as dibutyl-, tributyl- and triphenyltin are selectively transported to and accumulated in the region of Golgi apparatus and endoplasmic reticulum (ER) but not to or in the plasma membrane and the nucleus because of their hydrophobicity[1-4, 14]. Similarly, these hydrophobic organotins selectively destroy the specific stratified structure of the Golgi apparatus and the characteristic reticular structure of the ER. Furthermore, these organotins inhibit such Golgi functions as the lipid metabolism and such ER functions as inositol 1,4,5-triphosphate (IP_3)-induced intracellular Ca^{2+} mobilization by promoting Ca^{2+} release in a similar fashion to IP_3[1-4, 14].

On the other hand, DNase I is localized in the ER, and is released and transported to the nucleus by ER stress which is induced through an impairing of the structure and functions of the ER[20]. Apoptosis induced by ER stress depends on activation of Caspase-3 via Caspase-12[19, 20].

From these results, organotin exposure appears to induce a release of DNase I from the ER and a transport of DNase I to the nucleus by ER stress.

References

1) Arakawa, Y (1998) Chapter 10 Recent studies on the mode of biological action of the di- and tri-alkyltin compounds. In Chemistry of Tin (P. J. Smith ed.). Blackie Academic & Professional, Chapman & Hall, Glasgow, U. K. 388-428.

2) Arakawa, Y (1993) Chapter 4 Biological properties of alkyltin compounds. In Metal Ions in Biological Systems, Vol.29 Biological Properties of Metal Alkyl Derivatives (H. Sigel & A. Sigel ed.). Marcel Dekker Inc., New York, USA. 101-136.

3) Arakawa, Y (1996) Cellular and biochemical aspects of antitumor activity of organotin compounds. In Main Group Elements and Their Compounds (Kumar Das VG ed.). Narosa Publishing House, New Dehli, India. 422-445.

4) Arakawa, Y (1992) Chapter 23 Antitumor activity of organotin compounds and inhibition of membrane signal transduction. In Chemistry and Technology of Silicon and Tin (Kumar Das VG, Gielen M. ed.). Oxford University Press, Oxford. 319-333.

5) Arakawa, Y (1988) Chapter 9 Suppression of ell proliferation by certain organotin compounds. In Tin and Malignant Cell Growth (J. J. Zuckerman ed.) CRC Press, Boca Raton, Florida, USA. 83-106.

6) Arakawa Y, Hirano Y, Murata J, Nakashima H, Takeuchi T, Nakano Y (1995) The relationship between the movement of trace elements and parathyroid hormone (PTH) in the zinc deficiency- and organotin-induced brain lesion. Trace Nutrients Research 12: 107-112. Proceedings of the 12th Symposium on Trace Nutrients Research.

7) Arakawa Y, Igura K, Yoshinari S, Hirano Y, Nakashima H, Ohmori S, Takeuchi T, Nakano Y, Wada O (1996)

An excessive accumulation of olfactory calcium and inhibition of olfactory signal transduction by organotin compounds. Trace Nutrients Research 13: 63－67.

8) Arakawa Y, Igura K, Yoshinari S, Hirano Y, Nakashima H, Ohmori S, Takeuchi T, Nakano Y, Wada O (1996) An excessive accumulation of olfactory calcium and inhibition of olfactory signal transduction by organotin compounds. Biomed Res Trace Elements 7: 121－122.
9) Arakawa Y (1997) Olfactory disorders and calcium localization. Trace Nutrients Research 14: 19－28.
10) Kuriyama T, Tomiyama K, Nihira M, Jyoho T, Takeuchi T, Nakano Y, Nakashima H, Matsuda Y, Arakawa Y (2005) Excessive accumulation of calcium and movement of parathyroid hormone (PTH) in the olfactory system by zinc deficiency. Trace Nutrients Research 22: 19－28.
11) Arakawa Y, Takeuchi T, Nakano Y, Matsuda Y, Yamaguchi M, Takaya H, Shibata Y, Nakasa T, Okinaka Y, Abe K, Miyazawa T (1999) Organotin-induced olfactory lesions and its suppresion. Trace Nutrients Research 16: 41－47.
12) Arakawa Y, Takeuchi T, Nakano Y, Matsuda Y, Yamaguchi M, Takaya H, Shibata Y, Nakasa T, Okinaka Y, Abe K, Miyazawa T (1999) Organotin-induced olfactory lesions and its suppresion by vitamin E. Biomed.Res Trace Elements 10: 217－218.
13) Arakawa, Y (1998) Cellular and biochemical aspects of the activity manifestation of organotin compounds. Proc. of The 2nd Internat. Sympo. on Trace Elements and Food Chain, Wuhan, Hubei, China, November 12－15.
14) Arakawa Y, Iizuka T, Matsumoto C (1991) Intracellular distribution of organotin compounds and its effects on the structure and function of organellae. Biomed Res Trace Elements 2: 321－326.
15) Tanuma S (1999) New molecular medicine of apoptosis. In DNA Endnuclease. 96－109.
16) Choi EJ, Lee BH (2004) Evidence for genistein mediated cytotoxicity and apoptosis in rat brain. Life Siences 75: 499－509.
17) Montague J W, Gaido ML, Frye C, Cidlowski JA (1994) A calcium-dependent nuclease from apoptotic rat thymocytes is homologous with cylophilin. J Biol Chem 269, 18877－18880.
18) Basnakian AG, Ueda N, Kaushal GP, Mikhailova MV, Shah SV (2002) DNase I-like endnuclease in rat kidney cortex that is activated during ischemia/reperfusion injury. J Am Soc Nephrol 13: 1000－1007.
19) Katayama T, Endou M (2001) Endoplasmic reticulum and apoptosis. Experimental Medicine 19 (13): 103－110.
20) Hitomi J, Katayama T, Taniguchi M, Honda A, Imaizumi K, Tohyama M (2003) Apoptosis induced by endoplasmic reticulum stress depends on activation of Caspase-3 via Caspase-12. Neuroscience Letters 357: 127－130.