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Mechanism of Flame Retardants Causing Damage to Chinese Hamster Ovary Cells

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Abstract: *Phosphorus flame retardants are harmful to human body, but the research on them is not enough. This study focuses on the damage mechanism of phosphorus flame retardant to human body by selecting Chinese hamster ovary cells which similarity of DNA sequences of human is ninety-five percent, mainly from the aspects of calcium ion and caspase. The possible results of the experiment are listed in this research.*

Keywords: *phosphorus flame retardant, caspase, calcium*

1. Introduction

The use of flame retardants can effectively delay the combustion propagation speed of polymer materials and inhibit the probability of being ignited by heat, which is of great significance for the study of the combustion characteristics of polymer composites and fire prevention technology. Because brominated flame retardants tend to accumulate in the human body and affect the endocrine, immune, and nervous systems, the EU and other countries have gradually replaced brominated flame retardants with phosphorus-based flame retardants (PFRs), which have less threats. Previous studies have shown that the exposure to nonhalogen PFRs has potential to cause neurological effects, endocrine disruption and oxidative stress in different organisms.[1][2] PFRs are becoming more and more widely used today, but we still lack solid evidences to prove its safety to humans. A recent study has revealed that PFRs have the potential to induce oxidative stress, DNA damage, neurotoxicity, and cardiotoxicity. [3][4] A total of four flame retardants were studied in this experiment by testing the degree of damage to Chinese hamster ovary (CHO-k1) cells. It is found that they could cause different degrees of damage to mitochondria and show different toxicity mechanisms to cells. In this work, the mechanism of cell damage induced by phosphorous flame retardants was studied. It is conducive to people's understanding of the disease and research on treatment methods.

2. Materials and methods

2.1 Chemicals

Trihexyl phosphate (THP), tri-p-cresyl phosphate (TPCP), PBS (Phosphate Buffered Saline), phosphorus insecticides, DMSO (dimethyl sulfoxide) are prepared.

2.2 Cell culture and exposure

The cells are maintained at 37 ℃ in a 5% CO_2 atmosphere, and the medium was refreshed every three or four days. During all experiments, a control culture was treated with an equivalent amount of DMSO. CHO-k1 cells were seeded at a density of 3000 cells/well in 96-well microtiter plates. After 24 h of pre-culture, cells were exposed to 5 concentrations (50, 100, 200, 300, 500μM) of 4 kinds of flame retardants (TNBP, THP, TPCP, TPHP). The cells treated with PBS were used as negative control, and the cells treated with phosphorus insecticides which were known to be toxic to cells were used as positive control. Three parallels were set for each concentration and t-test was used to conduct data analysis.

2.3 Western blot analysis

Caspase is closely related to eukaryotic cell apoptosis, and is involved in cell growth, differentiation

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and apoptosis regulation. Changes in protein in caspase 3,8,9 can be detected using western blot. The tissue is cut into pieces, reagents are added in order and centrifuged to extract the protein. After preparing a standard curve, the protein content is measured according to the curve.

2.4 Cell viability assay

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay measures the real-time exposure of phosphatidylserine (PS) on the outer leaflet of cell membranes during the apoptotic process. Annexin V luciferase fusion proteins supplied in the assay reagent bind to PS during early apoptosis and are detected with a simple luminescence signal. The assay reagent also includes a DNA-binding dye, which enters the cell and generates a fluorescent signal upon loss of membrane integrity. Then, flow cytometry was used to analyze each cell to determine the amount of cell necrosis and apoptosis.

2.5 Measurement of Ca2+

Overload of Ca^{2+} in the cell will cause a series of metabolic disorders until the cell is necrotic or apoptosis. After Fluo-3 / AM enters the cell, the ester group is hydrolyzed. Fluo-3 binds to free Ca^{2+} in the cell, so its fluorescence intensity can reflect the free Ca^{2+} concentration in the cytoplasm. Fluo-8 is used as a fluorescent probe for calcium, followed by a fluorescence microplate reader to detect changes in mitochondrion calcium levels.

3. Possible results

One result is that both the experimental group and the positive control group have cell death and apoptosis, and cell death in the negative control group. There is no change in the calcium concentration and Caspase-8 protein, the number of Caspase-3 increased with the concentration, and it reached the peak when the concentration was 500 μM. The second result is that there is no change in the calcium concentration and Caspase-3 protein in the experimental group. Besides, the calcium concentration in the experimental group might has no change, but with time passing, Caspase-3 protein had an effect first, and Caspase-8 protein changed again. The final possible result is that the calcium in the negative control group and in the experimental group is higher than that in the cells.

Some studies have shown the apoptotic pathway is triggered by the release of cytochrome c from the mitochondria, leading to sequential activation of Caspase-9 and 3. Caspase-3 will function as the ultimate "executioner" of cell death.[5] Previous studies and this experiment are to study the cause of apoptosis caused by flame retardant. What should be done further is to judge whether the change of calcium ion is directly caused by the flame retardant or indirectly affects other organelles leading to cells death. The increase of intracellular calcium concentration may be due to the increase of membrane permeability, or the outflow of calcium ions in various organelles caused by flame retardants. Some study suggest that mitochondria might be the one of the earliest targets affected by exposure to flame retardants.[6] Some aryl-PFRs, can induce the mitochondria- mediated apoptotic pathway in adult hens.[7] However, Carlson et al. found that 1 mM PFRs could not only activate caspase-3 but also be caused necrotic morphological changes in SH-SY5Y cells.[8] The cell death induced by PFRs seems to depend on an unusual mechanism. A similar conclusion from also indicated that OPs can induce mitochondrial dysfunction and that there is more than one mechanism to explain the toxicity of OPs.[9] PFRs-induced cell death seemed to be highly related to mitochondrial.

If it is the first result, it shows that PFRs can induce apoptosis by activating Caspase-3, which is the main factor of apoptosis. The most important substrate of Caspase-3 is PARP, which is related to DNA repair and gene integrity monitoring. At the initiation of apoptosis, 116kd of PARP was cut into 31kd and 85kd fragments by Caspase-3 between asp216-gly217, which separated the two zinc finger structures combined with DNA from the catalytic region at the carboxyl end of PARP and failed to play its normal function. The activity of Ca2 + / Mg2 + dependent endonuclease, which was negatively regulated by PARP, was increased, and DNA between nucleosomes was cleaved, resulting in apoptosis. In the next experiment, we can add Caspase-3 specific inhibitors to observe whether the cells will apoptosis.

The second result shows that PFRs can induce apoptosis by activating Caspase 8, which can cause downstream caspase "waterfall" to be activated. At the same time, PFRs can transfer apoptosis signal from independent mitochondrial pathway to mitochondrial pathway, link death receptor pathway and mitochondrial pathway, amplify apoptosis signal and cause apoptosis. In the next experiment, we can try to add Caspase-8 specific inhibitors.

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For the third result, we can see that PFRs can induce apoptosis by activating Caspase 8 and 3. The former is the initiator of apoptosis and the latter is the executor of apoptosis. The effect time of PFRs on the former was earlier consider the different time required for PFRs to transcribe from gene level to egg white matter level. Next, RT-PCR can be used to detect the change time of gene level.

PFRs changes the calcium concentration from the last result. Many functions of cells depend on the extremely high concentration difference of calcium ions inside and outside cells. Once the concentration difference is reduced, cell functions are damaged. The next thing to do is to test the concentration of mitochondria and cellular glia, and determine whether only direct mail mitochondria or other organelles are flowing out.

4. Conclusion

In this work, the mechanism of cell damage induced by phosphorous flame retardants was studied. It is conducive to people's understanding of the disease and research on treatment methods. The research on other flame retardants can be further studied.

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