Single and combined cytotoxicity research of propiconazole and nano-zinc oxide on the NIH/3T3 cell

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Abstract

This study chose the propiconazole and nano-zinc oxide of different particle size as the study object. The single and combined toxicity on the mouse embryonic fibroblast cell (NIH/3T3) was researched. The results showed that the cell growth was inhibited by propiconazole and nano zinc oxide of different concentrations, and it presents a dose-response relationship. When the two substances are combined, the combined of nanoscale particles produces the antagonism effect, and the combined of micron scale particles could generate synergy effect.

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Keywords: Propiconazole; Nano-zinc oxide; Combined cytotoxicity; Single cytotoxicity; NIH/3T3 cell line

1. Introduction

Propiconazole (1-(2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl)-1H-1,2,4-triazole, PPZ), as antiseptic sprayed on the foliar appearance, is widely used on the grasses, fruits, grains and seeds in the agriculture. And it has been listed by the European Union as a compound of persistent and potential toxicity \cite{1}, but it could be possibly the carcinogen to the human, which has been confirmed by the Environmental Protection Agency in the USA. Stephen \cite{2} has found that propiconazole could induce the mouse to produce the hepatoma and the hepatocellular adenoma, which promotes the liver tumor in rats. Propiconazole is a mouse liver hepatotoxicant and a hepatocarcinogen that has adverse reproductive and developmental toxicities in experimental animals \cite{3-4}.

Nano materials have their unique properties such as volume effect, quantum size effect, surface effect and so on, which makes nano-ZnO widely used in industrial production, rubber manufacture, cosmetics, food additives, environmental protection and medicine. It is security, stable and has a good biological

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activity, so it is considered as the most ideal source zinc because of the high dissolution rate of $\text{Zn}^{2+}$. More research has shown that nano-ZnO could result in the cytotoxicity of many types of cells, such as human myeloblastic leukemia cells[5], human bronchial epithelial cells (BEAS-2B)[6], human lung epithelial cells (A549)[7], human keratinocyte cells HaCaT[8], primary mouse embryo fibroblasts (PMEF)[9] and so on. Propiconazole is widely existed in the soil and vegetables, and nano-ZnO has also been considered to be environmental pollutants. There are rare researches about the combined cytotoxicity of propiconazole and nano-ZnO which can occur unexpectedly in the use of these compounds. So propiconazole and ZnO nanoparticles were chosen to be the model, single and combined cytotoxicity to the NIH/3T3 cells have been studied. This paper would provide a theoretical foundation for the further research of the cytotoxicity effect and the cytotoxicity mechanism of propiconazole and nano-ZnO.

2. Materials and methods

2.1. Chemicals and reagents

Propiconazole, 95% purity and oil was purchased from Jiangsu Limin pesticide factory. Dimethyl sulfoxide (DMSO) was purchased from Amresco, US.

Stock solutions of propiconazole, below the solubility, were prepared in DMSO because this carrier has low toxicity to cells, and all of them were dispersed by ultrasonic. The medicine were prepared fresh, they were diluted to the desired concentrations with the culture medium. The final DMSO concentration in the medium was lower than 1%.

Nano ZnO power was prepared with complete cell medium, and was dispersed for 20 minutes by the supersonic wave. Before each experiment, the medicine was prepared fresh, and they are diluted to the desired concentrations with the culture medium. Visualize particles size and shape of ZnO particles was measured by transmission electron microscopy (TEM, H7650).

2.2. Cell culture

NIH/3T3 cell was purchased from China Center for Type Culture Collection (CCTCC). The cells were cultured in the complete growth medium (DMEM) with 10% fetal bovine serum (FBS), antibiotics (100 U/ml penicillin, 100μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

2.3. Cytotoxicity assay

2.3.1. Single cytotoxicity assay of propiconazole and nano-ZnO

Cellular viability was determined using the CCK-8 assay (Beyotime). 100μl of the cell culture medium were seeded in each well of 96-well plates (10⁴ cells per well), and incubated for 24 hours at 37°C. Then cells were treated with different concentrations of propiconazole and nano-ZnO for 24 hours at 37°C. In addition, the blank and the DMSO comparison experiments were set in each experiment. At the same time, another 96-well plate with different concentrations of propiconazole and nano-ZnO but without the cell was also used to eliminate the effect of the test solutions. At the end of the treatment, 10μl CCK-8 was added to each well and incubated for another 2 hours at 37°C. The solution in each well of plates was quantitatively transferred to another plate. Subsequently, the absorbance were measured by dual wavelength spectrophotometry (DNM-9602G, Prolong, China) at 450nm and 630nm using a microplate reader. Each experiment should be repeated five times.
A preliminary experiment is conducted to obtain the appropriate range when the inhibition rate of the propiconazole on the cell growth is from 10% to 100%. Based on the preliminary results, seven propiconazole concentrations (50, 60, 70, 80, 90, 100, 110 μg/ml) and five nano-ZnO concentrations (2.5, 5, 10, 15, 20 μg/ml) are chosen as the experiment concentration range.

2.3.2. Combined cytotoxicity assay of propiconazole and nano-ZnO to the NIH/3T3 cell

Four propiconazole concentrations (50, 70, 90, 110 μg/ml) and two nano-ZnO concentrations (5, 10 μg/ml) are chosen for the combined concentration of the two nano material. Single and combined poisoned concentrations of propiconazole and nano-ZnO are shown in table 1.

Table 1. Single and combined poisoned concentrations of propiconazole and nano-ZnO.

| Propiconazole Concentration (μg/ml) | Nano-ZnO Concentration (μg/ml) | Combined Effect |
|------------------------------------|-------------------------------|----------------|
| 50                                 | 5                             | Combine        |
| 70                                 | 10                            | Combine        |
| 90                                 |                               | Combine        |
| 110                                |                               | Combine        |

2.3.3. Statistical analysis

For statistical analysis, the experimental values are compared to their corresponding control values. A statistical analysis is done by SPSS 16.0 (SPSS Inc., Chicago, USA). The significant difference is judged at \( p < 0.05 \).

3. Results

3.1. Single toxicity result of propiconazole

As shown in Fig.1. (b), cytotoxicity effect of propiconazole on NIH/3T3 cells was characterized by the dose-dependent pattern. The cytotoxicity of propiconazole would be higher with the increase of concentrations. The median of the propiconazole inhibition concentrations (IC50) to the cells at 24 hours are determined, it was 73.36 μg/ml (71.874 μg/ml, 74.293 μg/ml).

3.2. Cytotoxicity result of nano-ZnO on the NIH/3T3 cell

As shown in Fig.1. (a), cytotoxicity effect of propiconazole on NIH/3T3 cells was characterized by dose-dependent pattern. The cytotoxicity of nano-ZnO would be significantly higher with the increase of concentrations (\( p < 0.05 \)). The IC50 of 10-30nm ZnO, 30nm ZnO, 100nm ZnO and fine-ZnO are listed in Table 2. T-test was used to test the differences of the measure data. The different letters (A, B and C) represent significant difference among the IC50 values of ZnO (\( p < 0.05 \)).

It was found that there was significant difference between different sizes of ZnO particles. 10–30nm ZnO particles showed the highest toxicity while fine-ZnO particles have the lowest toxicity at the same concentration. The fine ZnO would promote growth when the concentration was lower than 2.5 μg/ml. The inhibition rate of 10-30nm ZnO was changed quickly in 2.5-10 μg/ml, while the rate of 100nm and fine ZnO in 10-20μg/ml were increased promptly.

Combined cytotoxicity of propiconazole and nano-ZnO were shown in Fig. 1. (b) and Fig. 1. (c) At 5μg/ml, all size of nano-ZnO appeared significant antagonism with propiconazole, while this effect was decreased at 10μg/ml. But fine-ZnO was different with nano-ZnO, it has additive effect with
propiconazole. When the concentration of Fine-ZnO was increased to 10μg/ml, it has seriously caused the cell death.

Table 2. IC50 (24h) of NIH/3T3 cells exposed to various ZnO.

| Size  | IC50 (μg/ml) | 95% confidence (μg/ml) |
|-------|-------------|------------------------|
| 10-30nm | 6.814 A     | 5.261 - 10.639         |
| 30nm   | 13.232 B    | 11.813 - 14.558        |
| 100nm  | 15.101 C    | 14.583 -15.644         |
| fine   | 15.220 C    | 14.231 - 15.434        |

Fig. 1. (a) Growth inhibition ratio of NIH/3T3 cell exposed to different sized ZnO particles; (b) Growth inhibition ratio of NIH/3T3 cell exposed to propiconazole single and compound with 5μg/ml different sized ZnO particles; (c) Growth inhibition ratio of NIH/3T3 cell exposed to propiconazole single and compound with 10μg/ml different sized ZnO particles.

4. Discussion

Propiconazole has been listed by the European Union as a persistent and potential toxic compound. As we all know that propiconazole is toxic substance of mouse liver cell. It is reported that it could induce reactive oxygen species (ROS) in the liver cell of immortalized mouse AML12 [10]. And it could also increase the glutathione-S-transferase (GSTα) protein levels and the levels of thiobarbituric acid reactive substances (TBARS) in AML12 cells. What’s more, propiconazole can damage cell membranes [11]. In this paper, the toxic effect of propiconazole rise with the increase of concentrations. It may be caused by the high liposolubility, which caused the cell membrane composed by the phospholipid bilayer to influence the structure and function of the inner organelles. Mitochondria played an important role in the
cell function. When it was damaged, the cell respiratory chain would be disrupted. And then active oxygen radical was produced, which had damaged the cell composition, such as the protein, the lipids, and the nucleotides [12]. This can lead to the lost of the cell structure and function, and then the apoptosis and necrosis of the cells occurs.

When the particle size of Nano-ZnO is below 100nm, the radio of the surface atom and the total atom would increase promptly due to the lack of the neighboring atoms, and there are many dangling bonds of unsaturated nature which is likely to form a stable structure in combination with other atoms, so it has large chemical activity. Some researches have shown that nano-ZnO results in many types of cell toxicity, and it has steep dose-cytotoxicity relationship and does not depend on the cell sort. It may disrupt cell membrane, causing oxidative stress and DNA damage [13]. Lin has found that nano-ZnO may reduce the vitality of human lung adenocarcinoma cell (A549), and particles were observed in the cells through the scanning electron microscopy (SEM). In this research, the cytotoxicity of 10-30nm ZnO was the biggest, and the fine ZnO is the smallest. It was maybe cause of size effect. Nanoscale ZnO of small size could travel into the cells easily, affected the organelle and lead to the cell damage. Meanwhile, many other researches suggested that the toxic mechanism of nano-ZnO was related to the dissolved zinc ions. Our previous study has found that the toxicity of ZnO nanoparticles was mainly due to the dissolved zinc ions [14]. The concentration of zinc ions was decreased with the increase of the particle size of ZnO, which was consistent with the result of this article. So it can be inferred that the law could be applied to most of cells.

Combined cytotoxicity of propiconazole and nano-ZnO has few researches, but the research suggests that dissolved organic substance is able to adsorb on the surface of the nano oxide particles, which might affect the toxicity of both the nano oxide particles and propiconazole[15]. For example, a previous research has demonstrated that propiconazole could absorb on the PtO surface[16]. In this research, antagonism appears when propiconazole is combined with nano-ZnO. It may be caused by the change of physicochemical properties. Nano-ZnO could easily combine with other atoms to form a stable structure because of the large surface area. It could infer that nano-ZnO could absorb some propiconazole which would make the propiconazole concentration decrease. At the same time, if the diameter of nano material combined with propiconazole is larger, the toxicity on the cell would become smaller. When propiconazole covers on the surface of nano-ZnO, the contact area of nano material with the culture medium will become smaller, which must influence the dissolve of zinc ions, so their combined cytotoxicity would be decreased. With the increase of the nano-ZnO size, the specific surface area would decrease, which make the absorption of propiconazole become weak. The adsorption could be ignored in large-size ZnO, so it has additive effect with propiconazole(Fig.1.(c)).

5. Conclusions

The single and combined cytotoxicities of propiconazole and ZnO nanoparticles to the NIH/3T3 cells have been determined in this paper. The results showed that the single toxicity of propiconazole and nano-ZnO was characterized by a steep response pattern. But the combined cytotoxicity depends on the ZnO size, and antagonism appears between Nano ZnO and propiconazole while micron ZnO could cause additive reaction. The possible toxicity mechanism of propiconazole is that it can pass through cell membrane and affect the organelles. Thus, the dissolved Zn$^{2+}$ and particle size plays the main role in the toxic effect of ZnO particles. When the two compounds are existed together, nano-ZnO could adsorb part of propiconazole, and the concentrations of free propiconazole and dissolved zinc would be declined, so the toxicity to NIH/3T3 cell would be lower. Whereas, ZnO of micron scale could adsorb little propiconazole because of the small surface area, so the additive effect appeared when combined with
propiconazole. This study would provide information about the safety use of propiconazole and nano-ZnO.

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