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Original Article

Effects of acrylonitrile on apoptosis of rat cerebral nerve cells

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Abstract: The brain, especially the hippocampus, is sensitive to damage caused by anoxic chemicals. In this study, we established a rat model of acrylonitrile poisoning with administration by gavage, aiming to determine the influence of acrylonitrile on rat cerebral nerve cells. Transmission electron microscopy observation and TdT-mediated dUTP nick-end labelling (TUNEL) staining were used to explore preliminarily the apoptotic changes of cerebral nerve cells. The pathogenesis revealed by transmission electron microscopy indicated that apoptosis in the control group was more serious than that of the exposure groups. The results of TUNEL staining showed the apoptotic rate was significantly higher in the control group than that of other exposure groups. All the results indicated that acrylonitrile can inhibit the apoptosis of rat cerebral nerve cells, which is closely related to its animal carcinogenicity.

Keywords: Acrylonitrile, nerve cells, apoptosis

Introduction

Acrylonitrile is a colourless, almond-flavoured, highly toxic chemical. It is an important organic substance in the petrochemical industry and can be used to produce plastic, synthetic fibre, rubber, resin and other materials, such as acrylonitrile-butadiene-styrene (ABS) plastic, and acrylic. Due to the development of the automotive and household electrical appliances industries, ABS plastic has exceeded acrylic as the most common downstream polymer of acrylonitrile, and it is expected that demand will continue to rise steadily in the next few years, which means that the output and consumption of acrylonitrile will continue to maintain the current level and grow year by year [1]. During the process of production, transportation and usage, acrylonitrile can cause occupational exposure, resulting in a large number of acrylonitrile occupational exposures to the population in China.

Chronic toxicity by acrylonitrile mainly affects the central nervous system, liver, and reproductive system. Studies have shown that acrylonitrile can enter the body through the respiratory tract, skin, and gastrointestinal tract. Chronic acrylonitrile poisoning is the most common consequence of occupational exposure. Since studies on acrylonitrile have not been able to obtain valid evidence for a causal relationship between acrylonitrile and human malignant tumours due to design, sample size, and confounding factors [2-4], in 1999, the International Agency for Research on Cancer (IARC) reclassified acrylonitrile as a 2B substance, which is an animal carcinogen and human suspected carcinogen [2-4]. Our study established a subchronic animal model, using Sprague Dawley rats, to explore the mechanism of chronic acrylonitrile poisoning effects on rat cerebral nerve cells. According to correlational animal studies, acrylonitrile can cause pre-gastric and brain tumours in rats through drinking and gavage administration [6]. This study focused on the effects of acrylonitrile on nerve cells, aiming to determine its influence on nerve cell apoptosis.

Materials and methods

Materials

Acrylonitrile (Identification by gas chromatography, purity > 99.9%, density 0.8000~0.8070)

was purchased from Shanghai Chemical Reagent Co., Ltd. We used freshly prepared acrylonitrile aqueous solution. We also used the TUNEL apoptosis detection kit (KeyGEN BioTECH, KGA7073), Hoechst33258 (Beyotime Biotechnology, C1011), and the laboratory instruments included a fluorescence microscope and a microplate reader.

Rats

A total of 220 clean grade SD rats (half male and half female, 6-8 weeks old) were purchased from Shanghai Jiesijie Experimental Animals Co., Ltd. The male rats weighed 200-220 g, and females weighed 160-180 g. All animals were adaptively fed for one week, and 208 SD rats were finally included for the experiment. We used a random number table, dividing 208 rats into a 0 mg/kg group, a 5 mg/kg group, a 10 mg/kg group, and a 20 mg/kg group.

Poisoning model and sampling

The rats were administered poison by gavage daily from Monday to Friday, taking rests at weekends. The process lasted for 13 weeks, for a total of 91 days. According to the mentioned frequency, a subchronic exposure model was established. We also adjusted the dose according to the change in body weight every week. We ensured that the daily exposure time was fixed. During the gavage administration, behavioural changes were observed and recorded. We female rats of 20 mg/kg died during gavage administration. After 13 weeks of exposure, 206 SD rats were executed by chloral hydrate. Six SD rats were randomly selected from each group, half male and half female, for a total of 24 rats. We carried out perfusion fixation on these 24 rats. One side of the semi-brain fixed sample was used to make paraffin-embedded sections and TUNEL staining, and the other side of the hippocampal fixed sample was used to make transmission electron microscopy sections. All fixed samples were stored in glutaraldehyde.

Electron microscopy section preparation and observation

The hippocampal tissue block was cut into a shape of 1×1×3 mm^3 and placed in 0.25% glutaraldehyde. After fixing the tissue at 4°C for 2 h, we replaced the glutaraldehyde fixative with 0.1 mol/L phosphate buffer (PBS) at pH 7.2. Then, we fixed the tissue with 1% citric acid for 2 h and washed the tissue with PBS. After the process of dehydration, resin impregnation and embedding, we dyed the tissue with 3% uranyl acetate and lead citrate and took photographs using an FEI-T12 transmission electron microscope (FEI Company, USA).

TUNEL staining

Paraffin-embedded hemi-brain sections were dewaxed with 0.1% Triton X-100 at 37°C for 20 min, rinsed and dried the peripheral tissue. Then, TUNEL reaction solution was added at 37°C for 60 min, rinsed and dried for Hoechst staining. Finally, we took fluorescence photographs, in which green fluorescence indicated apoptotic cells.

Data analysis

We use the software SPSS22.0 to input and analyse the data. If the data were subject to normal distribution, the data were analysed by one-way ANOVA. If the data did not conform to the normal distribution, the data were analysed by a nonparametric method. The results are expressed in the form of $\bar{x} \pm sd$, and the significance level is 0.05.

Results

Basic outcomes

During the gavage administration period, we observed the condition of SD rats. From the middle stages of the poisoning, the rats from the 10 mg/kg and 20 mg/kg groups were resistant to effects of acrylonitrile by gavage administration, and the 20 mg/kg groups showed irritability and irascibility. Compared with the 0 mg/kg groups, there was a higher level and frequency of biting and attacking each other in this group.

The average weight of females was 231.64 grams and 269.95 grams for males during the first week. The average weight was 317.20 grams for females and 520.24 grams for males in thirteenth week. We calculated the rate of weight growth for males and females based on data collected every Monday. The formula used is the final weight minus initial weight divided by the initial weight. We analysed the initial and final weights, weight increases, and increase

Table 1. Weight changes and rates of the exposed and control groups

Sex	Group	Amount	Initial Weight ($\bar{x}$ ± s)	Final Weight (x ± s)	Increase Rate (x ± s)
Female	0 mg/kg	26	233.96±10.56	312.15±35.41	0.37±0.11
	5 mg/kg	26	230.62±8.21	319.85±21.26	0.39±0.10
	10 mg/kg	26	229.46±10.27	320.16±33.45	0.39±0.13
	20 mg/kg	24	232.50±9.65	304.24±26.26	0.33±0.10
Male	0 mg/kg	26	270.73±10.29	536.54±60.30	0.98±0.17
	5 mg/kg	26	269.00±8.85	509.12±39.65	0.89±0.13
	10 mg/kg	26	271.39±9.58	517.69±66.70	0.91±0.22
	20 mg/kg	26	268.69±10.99	517.62±44.57	0.93±0.17

rates of weight, at the *p*-value of 0.05. According to the statistical analysis, there were no differences on initial and final weights among the four groups (P > 0.05). After 13 weeks of poisoning by gavage administration, the rat weights increased to different extents (P<0.05), and the increase rates had no significant differences (P > 0.05). The results on weight changes were consistent with the results of Ya et al. [7]. The key data of weight change are listed in **Table 1**.

Hippocampus electron microscopy observation

Electron microscopy sections (**Figure 1**) were observed under a microscope with a magnification of 13,000. It was found that the 0 mg/kg, 5 mg/kg, and 10 mg/kg groups had apoptotic bodies, which decreased with increasing dose. The apoptosis of the 0 mg/kg group was the most affected, and this group shows a series of typical apoptotic morphological changes, such as nuclear chromatin edge accumulation, vacuolation, nucleolus disappearance, and cell member dissolution. The 5 mg/kg group and 10 mg/kg group had a small number of apoptotic bodies and some baseline apoptotic changes, chromatin condensation and nucleolus disappearance. The 20 mg/kg group had the fewest apoptotic bodies among all groups. We found that the 20 mg/kg group had a homogeneous distribution of chromatin, clear nucleolus, and obvious double-layer karyolemma, with intact margins.

TUNEL staining, observation and cell counting

TUNEL fluorescence staining was used to observe the apoptosis of nerve cells. The results were similar to those observed by electron microscopy. The 0 mg/kg group had the most apoptotic cells. We also found the same trend as that with the dose increase, i.e., the quantity of apoptotic cells gradually decreased, and the 20 mg/kg group only had very few apoptotic cells. **Figure 2**, representing the 0 mg/kg group to the 20 mg/kg group, is the results of TUNEL staining, consisting of three pictures. Picture A includes images from picture B and C. Picture B is Hoechst nuclear staining to count the total number of cells, and picture C is TUNEL staining, in which green fluorescence represents apoptotic cells.

To accurately ensure whether there is an inter-group difference in apoptosis, we did cell counting for each sample, and each sample was repeated three times (**Table 2**). The apoptotic rate of the 0 mg/kg group was 0.27, 0.24 for the 5 mg/kg group, 0.17 for the 10 mg/kg group, and 0.07 for the 20 mg/kg group. Statistical analysis showed that there was no difference on the total number of cells between the groups (P > 0.05). There was no significant difference on inter-group apoptosis rates (P > 0.05). The apoptotic cells and apoptotic rate between groups were significantly different; the rates for the 10 mg/kg and 20 mg/kg groups were lower than those of the others (P<0.05). It was not confirmed whether the 5 mg/kg is different from the 0 mg/kg group. We transformed the data into abar graph (**Bar 1**) in order to illustrate the change in trend. **Bar 1** shows that the 20 mg/kg group had the lowest apoptotic rate.

Discussion

The central nervous system is one of the important targets of acrylonitrile. The electron microscopic observations and TUNEL staining results of this study indicate that acrylonitrile can cause anti-apoptosis in nerve cells, and there is a dose-response relationship. The apoptotic rate of the 10 mg/kg group and the 20 mg/kg group were significantly different from that of the 0 mg/kg group. There was no sufficient evidence to judge whether the apoptotic rate of the 5 mg/kg group was decreased. Apoptosis is a normal physiological process of the body that

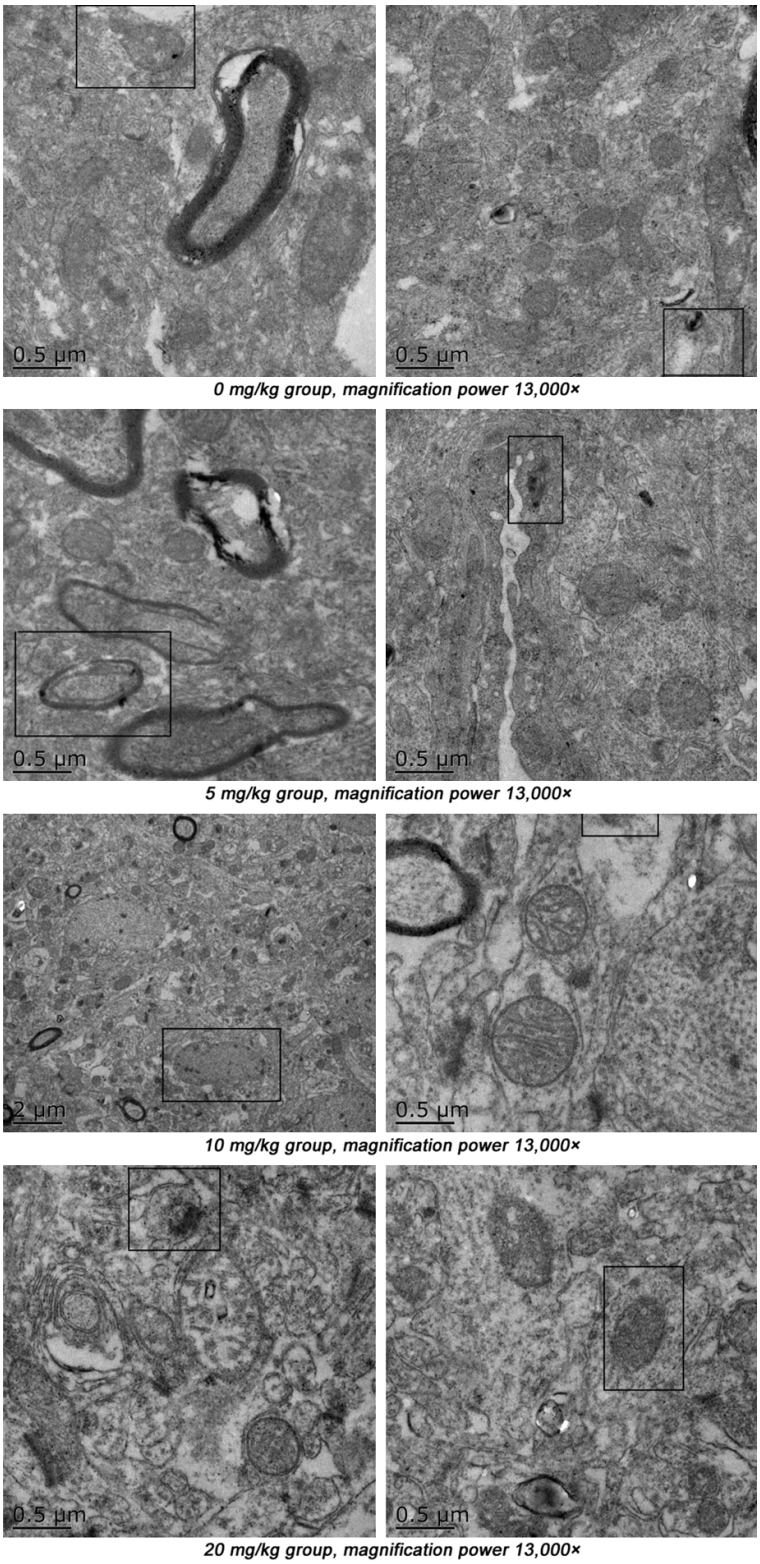


Figure 1. 0 mg/kg group of electron microscopy showed that there were many mitochondria and clear structures. We could also find phagocytic vacuoles, presynaptic membranes and synaptic vesicles. Electron microscopic observation also showed that vacuoles appeared, the nucleolus disappeared, the apoptotic bodies formed, and the cell membrane disappeared.

The apoptotic bodies could be observed by electron microscopy in the 5 mg/kg group. Electron microscopy revealed that the chromatin of the nerve cells was condensed and the nucleolus disappeared. In the 10 mg/kg group, we found that the synaptic structure is abnormal, and the nucleolus and the edge structure of the nuclear membrane is damaged. Electron microscopy of this group showed that the chromatin of the nerve cells was condensed, the nucleus was broken, and apoptotic bodies were formed. The cell membrane dissolved and disappeared. The mitochondria were significantly damaged, and the rough endoplasmic reticulum were abnormal in 20 mg/kg group. The electron microscopy of this group showed that the nucleolus was clear, the double-layer nuclear membrane was obvious, the margin was intact, and the number of apoptotic bodies was significantly less than in the other three groups.

can eliminate ageing and damage cells, thereby achieving homeostasis of viable cells. Anti-apoptosis is an abnormal state of apoptosis that is closely related to the formation, development, and metastasis of tumours. The effect of acrylonitrile, inhibiting apoptosis, shows new evidence demonstrating the accuracy of animal carcinogenicity.

After entering the human body, acrylonitrile is oxidized to 2-cyanooxirane (CEO) by cytochrome P450, especially isozyme CYP2E1, and a small amount of acrylonitrile is excreted as cyanate [7]. Both 2-cyanooxirane and acrylonitrile can bind to glutathione (GSH), resulting in a decrease in the body's antioxidant capacity [9]. In addition, due to the leakage enzyme characteristic of CYP2E1, more active oxygen will be generated in the process of metabolizing acryloni-

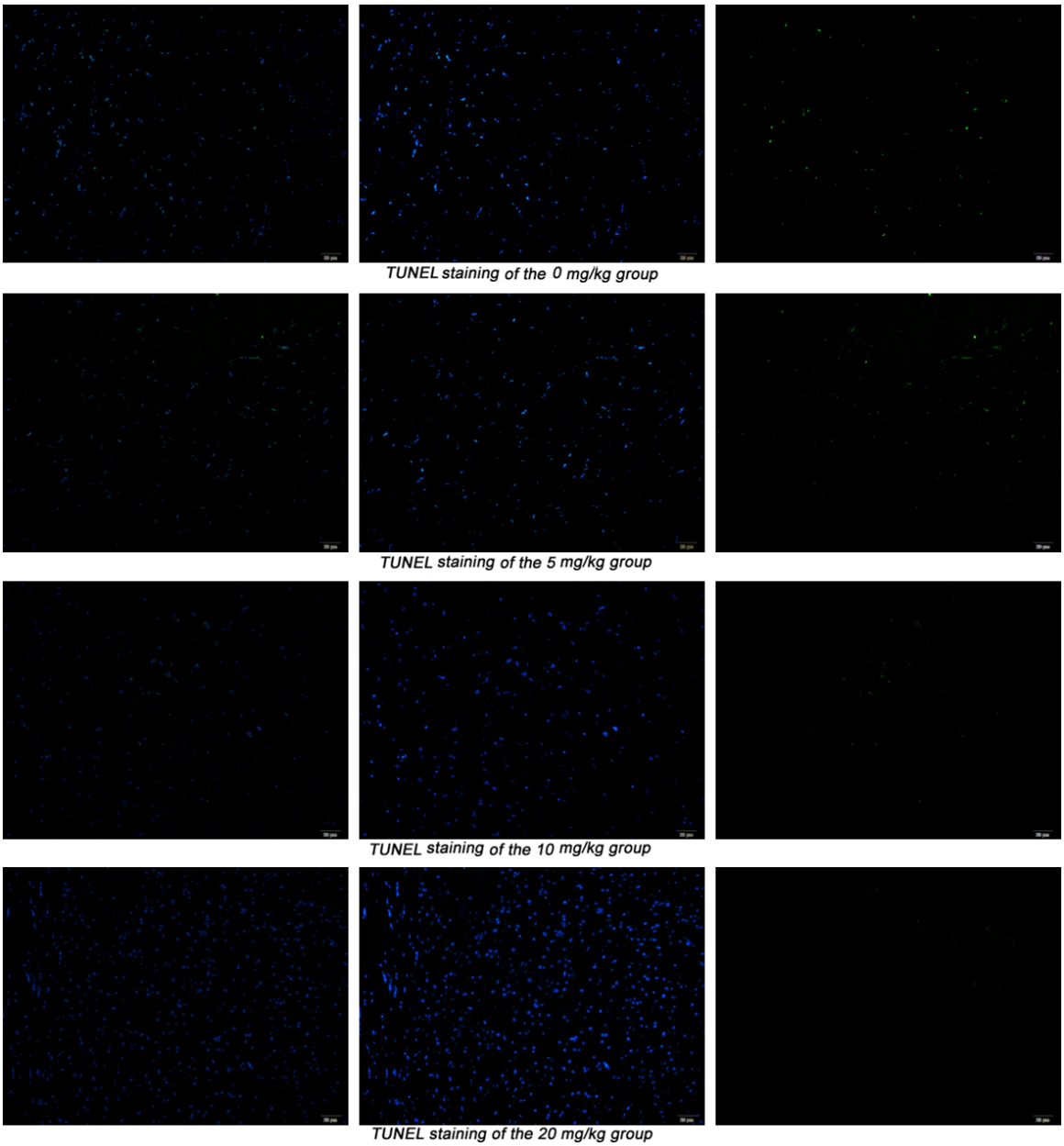


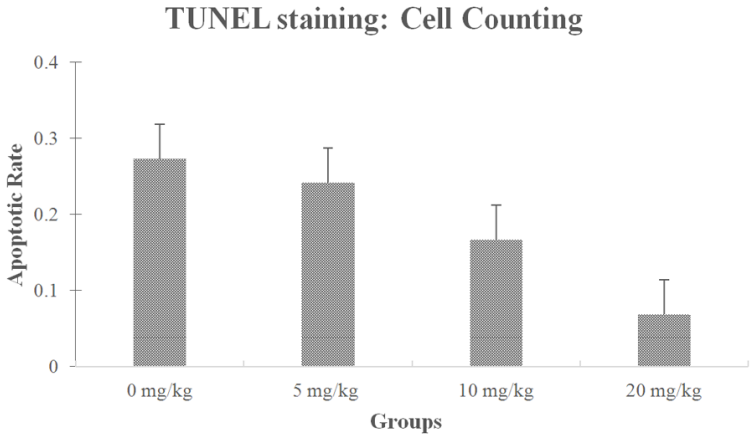
Figure 2. Blue fluorescent dots represent all cells in the field of view, and green fluorescent dots represent apoptotic cells. The TUNEL staining in the 0 mg/kg group indicated that there were many apoptotic cells. TUNEL staining in the 5 mg/kg group found that there was no significant difference between the 0 mg/kg group and 5 mg/kg group on the quantity of apoptotic cells. The TUNEL staining in the 10 mg/kg group showed that apoptotic cells were significantly reduced, compared to the 5 mg/kg group and 0 mg/kg group. We also found that there were almost no apoptotic cells in 20 mg/kg group based on TUNEL staining, which implied apoptosis inhibition in this group.

trile, under the circumstance of the decline in antioxidant capacity, which increases the oxidative stress burden on the body. Currently, researchers generally believe that oxidative stress damage is one of the main toxic effects of acrylonitrile and is the main mechanism of carcinogenicity. Remarkably, other studies show that oxidative stress can promote the expression of proapoptotic genes such as Bax, leading to cell apoptosis [10, 11]. Both PI3k/AKT and MAPK signalling pathways are closely related to cell apoptosis and differentiation [12-14]. Tang and her team found that in an animal oxidative stress model, the signalling pathway PI3k/AKT increased in the early stage of oxidative stress injury, but it decreased rapidly. The expression of downstream target Bcl-2 also gradually decreased and apoptosis increased.

Table 2. Data of cell counting through TUNEL staining

	Amount	Total number of cells (x ± s)	No. of apoptotic cells ($\bar{x}$ ± s)	Apoptotic Rate ($\bar{x}$ ± s)
0 mg/kg	6	318.67±69.91	82.17±21.89	0.27±0.11
5 mg/kg	6	296.67±95.38	73.50±30.20	0.24±0.09
10 mg/kg	6	314.11±35.04	53.78±43.69★	0.17±0.13★
20 mg/kg	6	292.78±46.86	20.00±8.26★,▲	0.07±0.03★,▲

The exposure groups are compared with the control group, and the *p*-values equal 0.05 and 0.001, respectively. Data are considered significant when the *p*-values are less than 0.05. ★equals P<0.05, and ▲equals P<0.001.



Bar 1. Cell counting data through TUNEL staining. The abscissa represents different dose groups, and the ordinate represents apoptotic rate (apoptotic cells/total number of cells).

Other studies also prove that oxidative stress damage leads to genotoxicity and apoptosis [15], suggesting that there may be other mechanisms to promote cell anti-apoptosis and to promote tumour formation. The latest study shows that the PI3k/AKT signal pathway influences oxidative stress, altering the normal levels of apoptosis [16]. According to our results and other research achievements, we speculate that oxidative stress is not the main mechanism of carcinogenicity.

We still cannot confirm whether the related signalling pathways cause cancer; future studies are needed to explore how acrylonitrile affects the apoptotic process, leading to the anti-apoptotic phenomena, and the changes in signal pathways. From this study we conclude that acrylonitrile causes anti-apoptotic effects in cerebral nerve cells.

There are many unregulated factors in population epidemiology studies. Currently, only a few teams are focused on acrylonitrile research for the main reason that epidemiological studies have not made great progress so far [17]. However, many people still suffer occupational exposure. We started to consider whether the toxic effects in animal bodies are different from those in humans, thus causing the different outcomes between animal studies and studies on human populations. To answer the aforementioned question, we should determine the specific mechanisms of causing cancer, which will probably provide new ideas for carcinogenicity studies.

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Disclosure of conflict of interest

None.

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