Assessment of genotoxicity of four volatile pollutants from cigarette smoke based on the in vitro γH2AX assay using high content screening

Article · July 2017
DOI: 10.1016/j.etap.2017.07.005

CITATIONS
0

READS
14

6 authors, including:

Sen Zhang
Hefei Institute of Physical Sciences, Chinese Academy of Sciences
9 PUBLICATIONS **34** CITATIONS
SEE PROFILE

An Wang
27 PUBLICATIONS **53** CITATIONS
SEE PROFILE

Yong Liu
Chinese Academy of Sciences
37 PUBLICATIONS **85** CITATIONS
SEE PROFILE

Environmental Toxicology and Pharmacology 55 (2017) 30–36

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Assessment of genotoxicity of four volatile pollutants from cigarette smoke based on the *in vitro* γH2AX assay using high content screening

Sen Zhang[a,b,c], Huan Chen[c], An Wang[a], Yong Liu[a], Hongwei Hou[c,*], Qingyuan Hu[c,*]

[a] Institute of Applied Technology, Hefei Institutes of physical Science, Chinese Academy of Sciences, Hefei 230088, PR China
[b] University of Science and Technology of China, Hefei 230026, PR China
[c] China National Tobacco Quality Supervision & Test Center, Zhengzhou 450001, PR China

ARTICLE INFO

Keywords:
γH2AX
High content screening
Aldehyde
Benzene
Dose/time-response

ABSTRACT

To evaluate the genotoxic effects of formaldehyde, acetaldehyde, acrolein and benzene on A549 cells, the *in vitro* γH2AX assay was used in combination with high content screening (HCS) technology. All aldehydes showed a significant genotoxicity in a dose/time-dependent effect on the induction of γH2AX. Benzene failed to show a significant genotoxicity based on the γH2AX assay. However, hydroquinone (one of metabolites of benzene) showed a significant genotoxicity in vitro. Based on the dose-response of γH2AX and Hill model, the ability to induce DNA double-strand break can be evaluated as acrolein > formaldehyde > acetaldehyde > benzene. The slow DNA damage/repair mechanism may be more important than the fast one for aldehydes based on time-course of γH2AX and two-component model. Overall, all toxicants were genotoxic in a dose- or time-dependent manner based on the *in vitro* γH2AX HCS assay, and acrolein had a strong potential to induce DNA damage followed by formaldehyde, acetaldehyde and benzene in sequence.

1. Introduction

Double strands breaks (DSBs) are highly deleterious lesions in genomic DNA (Smart et al., 2008). When DSBs occur, DNA damage responses (DDRs) will be triggered, for example, histones which surround the break site are massively phosphorylated (phosphorylated H2AX is called γH2AX) by ATM and form the nuclear foci (Podhoreck et al., 2010). Therefore, γH2AX was widely used as a biomarker of DSBs in the field of toxicology and clinical medicine. To date, various techniques and methods have been developed to detect γH2AX, such as gel electrophoresis, western blot, flow assay, enzyme linked immunosorbent assay, fluorescence microscopy, high content screening (HCS) and liquid chromatography-triple quadrupole tandem mass spectrometry (Zhang et al., 2016). Among these technologies, HCS has been thought as one of most promising technologies for the *in vitro* γH2AX assay, because of a number of competitive advantages, such as performance of high-throughput, small requirement of sample, and high sensitivity and accuracy (Garcia-Canton et al., 2013a; Zhang et al., 2016). At present, the HCS-based *in vitro* γH2AX assay has successfully used to assess the genotoxicity of individual toxicants in cigarette smoke and aerosol of cigarette smoke. Furthermore, it has been highlighted that the *in vitro* γH2AX HCS assay has a promising potential as a complement to current regulatory genotoxicity battery of the *in vivo* assays (Garcia-Canton et al., 2013a).

Formaldehyde (FA), acetaldehyde (AA), acrolein (ACR) and benzene are common volatile organic pollutants in the environment. These pollutants mainly come from the incomplete combustion of organic compounds, such as industrial production, automobile exhaust, trees burning, cooking fume and cigarette smoke. Apart from occupational factors, the most common sources of these toxicants are automotive exhaust and cigarette smoking. In particular, tobacco smoke has substantial concentrations of these substances (Cosma and Marchok, 1988). For example, FA has been found in concentrations ranges between 20 and 100 μg per cigarette; AA in 400–1400 μg per cigarette; ACR in 60–240 μg per cigarette and benzene ranges between 6 and 70 μg per cigarette (Davis and Nielsen, 1999). They all have been classified as carcinogen by the International Agency for Cancer Research (IARC), and have been recommended to compulsorily reduce the content in cigarette smoke by the World Health Organization Framework Convention on Tobacco product regulation (WHO FCTC) in 2008 (WHO, 2012). Genotoxicities of these toxicants have been evaluated a lot in vivo and in vitro based on various genotoxic endpoints using different detection methods, but it is rarely reported for comparing the genotoxicity using an identical genotoxic endpoint and detection method in the same cell line. However, this is important to contribute the prioritization of toxicant reduction controlling research in tobacco products.

* Corresponding authors.
E-mail addresses: qsfctc@163.com (H. Hou), huqy1965@163.com (Q. Hu).

http://dx.doi.org/10.1016/j.etap.2017.07.005
Received 11 February 2017; Received in revised form 13 July 2017; Accepted 14 July 2017
Available online 18 July 2017
1382-6689/

Quantitative risk assessment is essential and it can suggest the existence of some unexpected profiles and their possible explanations (Murado et al., 2002). The nature of the dose response can be obtained by mathematically modelling of observed effects and subsequent interpretation of the model parameters (Walker and Yang, 2005). Although there have been a number of descriptive models applied to model the toxicity of chemicals, the four-parameter sigmoidal Hill model is one of the models most frequently used to model dose-response and to compare the toxicity of toxicants. For example, it has been employed to model dose-response of γH2AX for PHAs (Polycyclic aromatic hydrocarbons) and genotoxicity of PAHs was evaluated by Genotoxic Equivalent Factor (GEF) based on Hill model (Audebert et al., 2012). In addition, the kinetics of formation and loss of γH2AX foci may reflect the rate of DSBs formation/repair at the cellular level. Thus, γH2AX is often used to study DNA damage/repair of charged particles and radiation through two-component model. Therefore, it is very helpful and meaningful for understanding toxicokinetics and the DNA damage/repair mechanism through the kinetics of γH2AX induced by aldehydes and benzene.

In order to investigate genotoxicity characteristics of these common volatile organic pollutants in air, a novel and high throughput technology based on γH2AX HCS assay was used here to detect DNA damage induced by FA, AA, ACR and benzene. In addition, Hill model and two-component model is further used to analyze the DNA damage based on dose/time-response in this study.

2. Material and methods

2.1. Chemicals

Formaldehyde (purity: 36.5%–38%, solubility: 0.60 g/mL H_2O), benzene (purity: 99.9%, solubility: 1.88 mg/mL H_2O) and etoposide (purity: ≥98%, solubility: 0.08 mg/mL H_2O) were all purchased from Sigma-Aldrich (St. Louis, USA). Acetaldehyde (purity: 99%, solubility: > 0.50 g/mL H_2O) and hydroquinone (purity: ≥99%, solubility: 50 mg/mL H_2O) were purchased from Aladdin (Shanghai, China). Acrolein (purity: 98%, solubility: 212.5 mg/mL H_2O) was obtained from Puyang Shengdehua Chemical industry Co., Ltd. (Puyang, China). All toxicants were dissolved in water except that benzene and etoposide were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 1% (v/v) in cell culture medium. The maximum concentration of these tested compounds is less than either 1 mmol/L or 500 μg/mL, in accordance with the concentration currently used in the guidance on in vitro genotoxicity tests and data interpretation for pharmaceuticals intended for human use (ICH, 2011). The minimum concentration must be sure that it is sufficiently low to cover the no significant effective concentration.

2.2. Cell culture and treatment

Human pulmonary adenocarcinoma A549 cells were purchased from Cell resource center of Shanghai Institute of life sciences, Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 culture media (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA). Cells were seeded into 25-cm^2 culture flask and maintained at 37 °C and 5% CO_2 in a CO_2 incubator. The day prior to treatment, cells were seeded into 96-well culture plates with 1×10^4 cells per well in 100 μL culture media. Next, the cells were treated with the tested chemicals. For benzene, rat hepatic S9 (Moltox, Boone, USA) was added into the culture media to a final concentration 1% (v/v).

2.3. Cell survival

The CCK8 assay (Dojindo, Shanghai, China) was conducted following manufactures instruction to assess the relative cell counts (RCC). Following exposure to toxicants, 10 μL CCK8 solution was added to each well of 96-well plates and further incubated for 2 h at 37 °C. Then absorbance was measured at a wavelength of 450 nm with microplate reader (Molecular Devices, Santa Clara, USA).

2.4. Immunofluorescence staining of γH2AX

The media was aspirated and each well was washed twice with phosphate buffered saline (PBS). The cells were fixed with 4% paraformaldehyde (Solarbio, Beijing, China) for 15 min. After plates were washed twice with PBS, the cells were permeabilized for 15 min with 0.5% TritonX-100 (Amresco, Houston, USA). The cells were washed twice with PBS, and were blocked with 3% fetal bovine serum for 1 h at 37 °C. After the blocking solution was aspirated, the cells were incubated with 50 μL 0.5% (V/V) phospho-specific (Ser-139) histone H2AX mouse monoclonal antibody (BioLegend, San Diego, USA) in 1% bovine serum albumin (BSA) overnight at 4 °C or for 2 h at 37 °C. After the cells were washed three times with PBS, they were incubated with 50 μL 0.5% (V/V) Alexa Fluor 488 conjugated goat anti-mouse IgG (Wuhan Jiayuan, Wuhan, China) in PBS for 2 h at 37 °C in the dark. 50 μL 1 μg/mL DAPI (Biosharp, Hefei, China) were added into each well for 10 min. After the wells were washed three times with PBS, the wells were filled with 100 μL of PBS again and stored at 4 °C until use.

2.5. Imaging with HCS and data analysis

γH2AX and nuclei was imaged with a HCS platform (Thermo Scientific, Waltham, USA). Image analysis of each well at 20× magnification was performed using HCS Studio software. At least 400 cells were counted per field and nine fields in each well were analyzed. Channel 2 measured the whole nuclei fluorescence intensity of the secondary antibody in the valid nuclei identified by channel 1. “Mean average intensity in channel 2” was measured in intensity units and was reported as γH2AX frequency (intensity units). Genotoxicity evaluation criteria for the in vitro γH2AX assay were based on the 1.5-fold criteria described by Smart et al. (Smart et al., 2011). ANOVA and Turkey's Post Hoc Test were used for further statistical analysis using SPSS 20.0. Z'-factor was tested for evaluating the stability of γH2AX HCS assay and the formula is as follows (Iversen et al., 2006):

$$\mathrm{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \quad (1)$$

where σ_p and σ_n are the standard deviations of γH2AX frequency induced by positive and negative control, respectively. μ_p and μ_n are the average values of γH2AX frequency induced by positive and negative control, respectively.

2.6. Genotoxicity modelling

The four-parameter sigmoidal Hill model was used to model dose-response of γH2AX and its general form is as follows (Audebert et al., 2012):

$$E = 1 + (E_{max} - 1)\ \frac{C^n}{C^n + C_{50}^n} \quad (2)$$

$$NC_{50} = C_{50}/E_{max} \quad (3)$$

where the baseline activity when the dose is equal to 0 equals 1, E_{max} is the maximum genotoxicity level, C_{50} is the dose resulting in 50% of the maximum genotoxicity, n is the Hill coefficient defining the shape of the dose-response curve, NC_{50} is the normalized C_{50} and c is the concentration of toxicant.

Time-course modelling was analyzed using two-component model and its formula is as follows (Bucciantini et al., 2011; Niu et al., 2014):

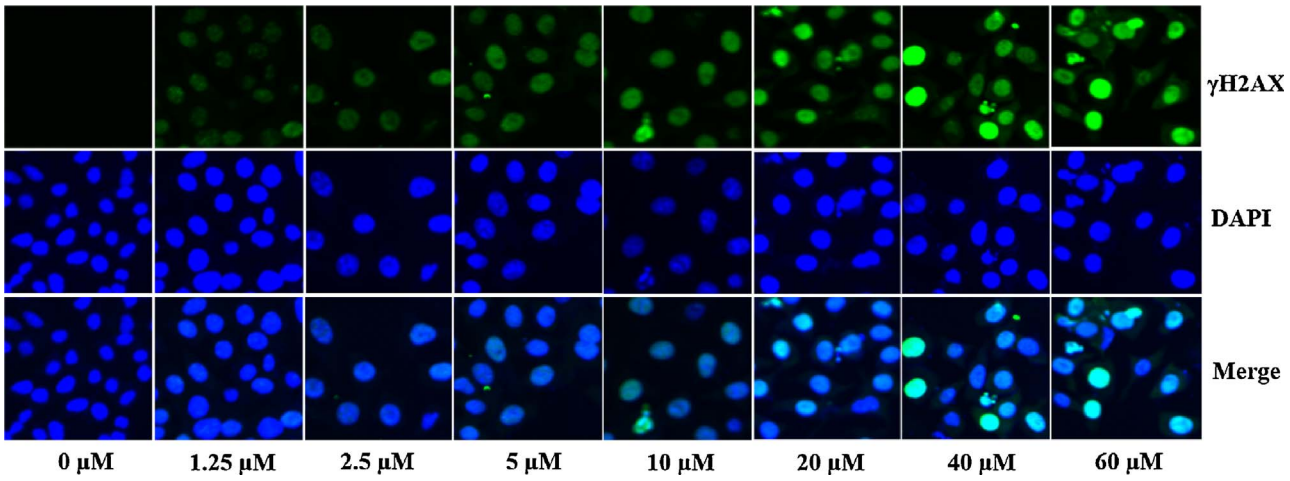

Fig. 1. Image (20 ×) of γH2AX in A549 cells after 24 h treatment with etoposide and vehicle control using HCS. The cell nuclei was stained with DAPI (blue fluorescence) and γH2AX with immunofluorescence staining (green fluorescence). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$$E = a\,e^{-\lambda_f t} + b\,e^{-\lambda_s t} \quad (4)$$

where the baseline activity when the dose is equal to 0 equals 1, E is the genotoxicity level. λ_f and λ_s are the rate constants for simple (fast) and complex (slow) components, respectively. a and b are the normalized coefficients, t is the time.

3. Results

3.1. Stability and sensitivity of the in vitro γH2AX HCS assay

Although HCS has successfully been applied to the *in vitro* γH2AX assay for genotoxicity evaluation and has shown a high accuracy and specificity (Garcia-Canton et al., 2013a), it was necessary to confirm the sensitivity of the instrument due to the differences of cell lines, such as cell size, cell shape and cell segmentation. Etoposide is one of most frequently used positive substances to induce DSBs, so it is used here as a positive inducer of DSBs for instrument sensitivity and calibration. As shown in Fig. 1, fluorescence intensity of γH2AX significantly increased with the increasing doses of etoposide after 24 h treatment. Furthermore, a significant dose-dependent manner was observed based on quantitative analysis of fluorescence intensity (Fig. 2). In addition, Z-factor values were all beyond 0.6 (0.74, 0.78, 0.75, 0.73, 0.82, 0.74 and 0.65 at 1.25, 2.5, 5, 10, 20, 40 and 60 μmoL/L of etoposide, respectively). Thus, the *in vitro* γH2AX HCS assay had a good stability. Therefore, the same image analysis and immunostaining method could be used further for the analysis of aldehydes and benzene.

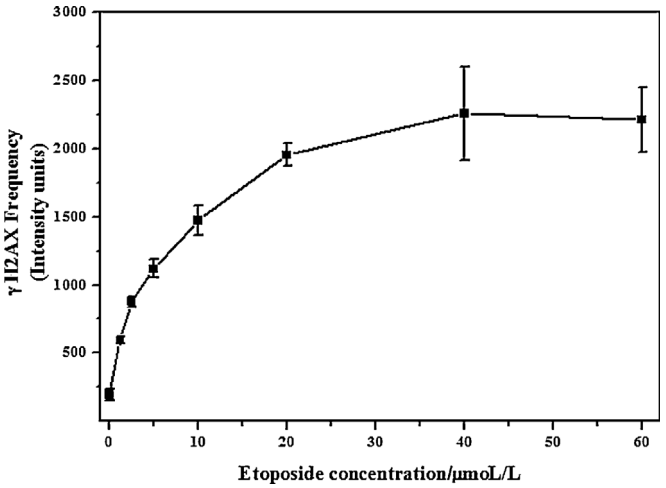

Fig. 2. γH2AX frequency in A549 cells after 24 h treatment with etoposide.

3.2. Effects of formaldehyde, acetaldehyde and acrolein on the induction of γH2AX

There were statistically significant effects ($p < 0.001$) on the induction of γH2AX for FA, AA and ACR. As shown in Fig. 3A, γH2AX frequency induced by FA monotonously increased at 0–250 μmoL/L and stabilized above 250 μmoL/L. Meanwhile, RCC of FA decreased monotonously with the increasing concentrations. γH2AX frequency at 250 μmoL/L of FA was 1.5 times higher than that of the control group (RCC > 60%), and the lowest effective concentration (LEC) was 125 μmoL/L (3.75 mg/L) obtained by statistical analysis. Meanwhile, there was a significant time-dependent increase in the induction of γH2AX at 500 μmoL/L of FA. However, there was a first increase and then decrease in the induction of γH2AX at 125 and 250 μmoL/L of FA. Moreover, the peak γH2AX levels arrived at 4 h and 12 h for 250 μmoL/L and 125 μmoL/L of FA (Fig. 3B), respectively.

There was a monotonic increase for the induction of γH2AX and a monotonic decrease for RCC, when the cells were treated with AA (Fig. 3C). Furthermore, a monotonic time-dependency was also observed for all concentrations (Fig. 3D). 1 mmoL/L (44.05 mg/L) AA induced 1.5-fold higher level of γH2AX than control group with more than 80% of cell viability, and this concentration was also just the LEC.

As for ACR, a sigmoidal dose-response curve was obtained (Fig. 3E). The induction of γH2AX and RCC was almost unchanged between 0 and 100 μmoL/L. Above 100 μmoL/L, a sudden increase and decrease was observed for γH2AX and RCC, respectively. The peak γH2AX level arrived at 160 μmoL/L (RCC > 70%), while γH2AX frequency at 120 μmoL/L (6.73 mg/L) was 1.5 times higher than that of the control group with more than 90% of cell viability and this dose was just the LEC to induce γH2AX. Although time-dependent increase at 160 μmoL/L was obtained (Fig. 3F), the induction of γH2AX firstly increased and then decreased over time and the peak γH2AX levels reached at 2 h both for 80 and 120 μmoL/L group.

3.3. Effects of benzene on the induction of γH2AX

Significant decrease ($p > 0.05$) was observed in the induction of γH2AX for benzene in the absence of S9 (Fig. 4 A) and the LEC was 500 μmoL/L (39.06 mg/L). However, there was no significant effects on the formation of γH2AX in the presence of S9 (Fig. 4C), so the LEC was more than the highest treated concentration (500 mg/L). There were no significant effects ($p > 0.05$) on the induction of γH2AX for exposure time in the absence of S9 (Fig. 4B). Although a time-dependent decrease

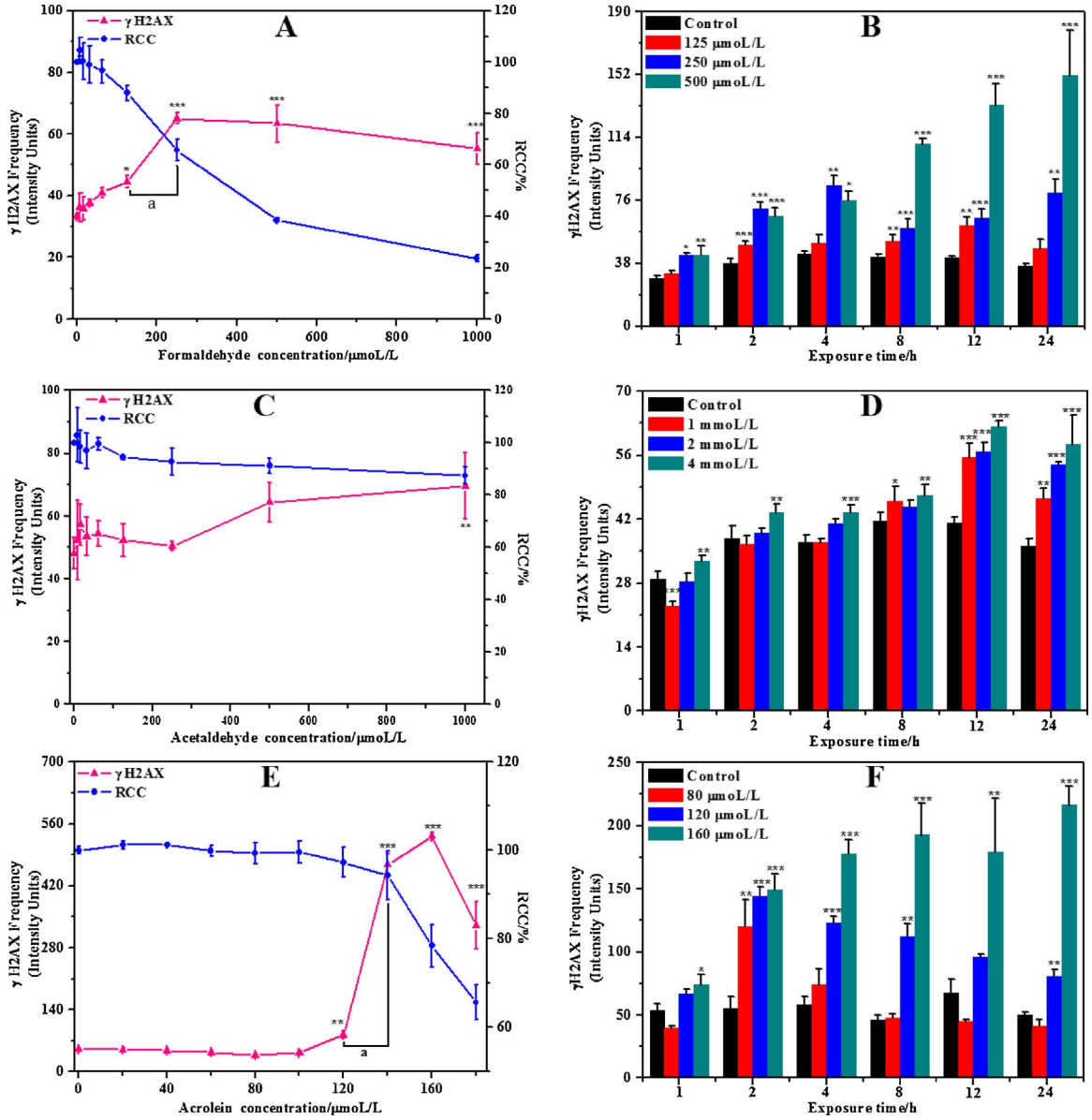

Fig. 3. γH2AX frequency after treatment with formaldehyde, acetaldehyde and acrolein in A549 cells. A, C and E is the dose-response relationship after 24 h treatment with formaldehyde, acetaldehyde and acrolein, respectively. B, D and F is the time-course relationship of γH2AX induced by formaldehyde, acetaldehyde and acrolein, respectively. Significant differences were observed between control and treated groups (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$), as well as between positive groups (a, $p < 0.001$).

was observed in the induction of γH2AX at all concentrations with a similar manner in the presence of S9 (Fig. 4D), benzene failed to show a significant genotoxicity, because there was no significant increase (> 1.5-fold of control group) in the induction of γH2AX. However, benzene is a very clear genotoxic substance in vivo indicated by numbers of animal experiments. In order to avoid the possible defect in the metabolism in vitro, one typical intermediate metabolite of benzene, hydroquinone was used here as a substitute of benzene for further study. A statistically significant effect ($p < 0.001$) was observed on the induction of γH2AX for hydroquinone. As shown in Fig. 4E, significant dose-dependent increase was observed for hydroquinone. When the concentration of hydroquinone was beyond 60 μmoL/L (6.61 mg/L), γH2AX frequencies were 1.5 times higher than that of the control group and this concentration was just the LEC. A non-monotonic time-course relation was observed for hydroquinone (Fig. 4F). The peak γH2AX was induced at 2 h, 2 h and 1 h for 31.25, 62.5 and 120 μmoL/L of hydroquinone, respectively.

3.4. Comparison of genetic toxicity based on γH2AX assay and Hill model

All toxicants achieved a goodness value of fit (more than 0.7 in Table 1). The E_{max} values of benzene both in the absence and presence of S9 were less than 1.5, and E_{max} values of the other toxicants were all more than 1.5. These findings were consist with the result described above based on the 1.5-fold criteria. In addition, as the same as IC50 (half-maximal inhibitory concentration), which can be used to compare the potency of cytotoxicity in vitro, NC_{50} was used to compare the potency of genotoxicity in vitro. That is, the lower NC_{50} value, the stronger the genotoxicity. So based on NC_{50} (Table 1), it could be speculated that ACR had the most strong potential to induce DSBs in vitro, which was followed by hydroquinone, FA, AA and benzene in sequence.

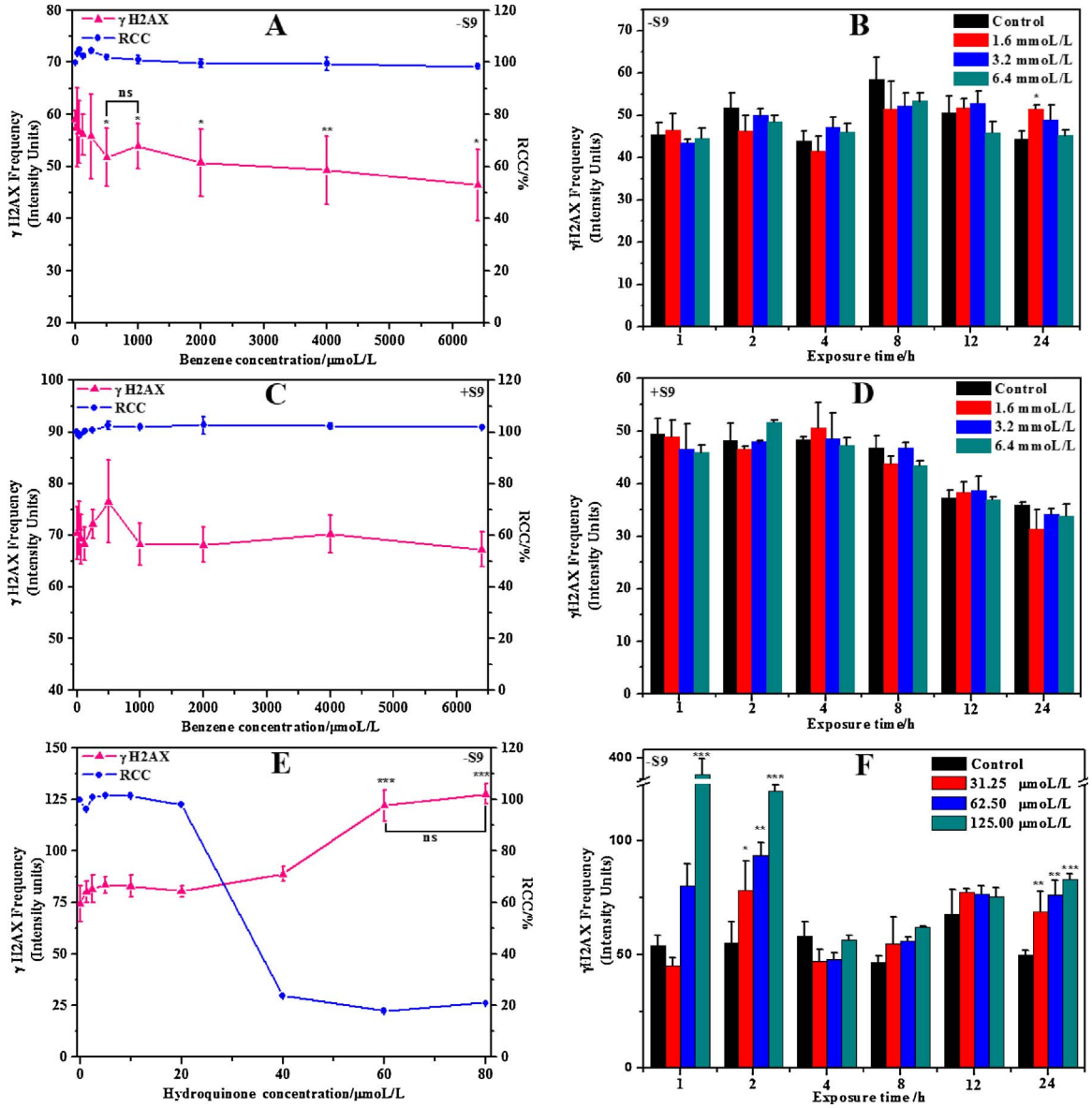

Fig. 4. γH2AX frequency after treatment with benzene and hydroquinone in A549 cells. A, C and E is the dose-response relationship after 24 h treatment with benzene in the absence (A) and presence (C) of S9, and hydroquinone in the absence of S9, respectively. B, D and F is the time-course relationship of γH2AX induced by the absence (B) and presence (D) of S9, and hydroquinone in the absence of S9, respectively. Significant differences were observed between control and treated groups (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$), as well as between positive groups (*ns* not significant).

Table 1
Hill model parameters for the tested toxicants.

Compounds	R^2	E_{max}	C_{50} (mg/L)	n	NC_{50} (mg/L)	LEC (mg/L)
Formaldehyde	0.839	1.85	3.40	2.72	1.84	3.75
Acetaldehyde	0.734	1.84	5.06	0.63	2.75	44.05
Acrolein	0.920	8.62	6.88	106.28	0.80	6.73
Benzene	0.880	1.22	9.62	0.82	7.89	39.06
$Benzene_{(+S9)}$	0.737	1.06	19.26	32.26	18.17	> 500
Hydroquinone	0.857	6333.01	11472.84	1.26	1.81	6.61

Note: Subscript "+S9" represents "in the presence of S9"; R^2 is the goodness of fit of Hill model; The formula of Hill model is $E = 1 + (E_{max} - 1)\frac{C^n}{C^n + C_{50}^n}$ and E_{max} is the maximum genotoxicity level in the Hill model; C_{50} is the dose (mg/L) resulting in 50% of the maximum genotoxicity; n is the Hill coefficient defining the shape of the dose-response curve; NC_{50} (mg/L) is the normalized C_{50} and calculated by the formula $NC_{50} = C_{50}/E_{max}$; LEC is the lowest effective concentration (mg/L) observed.

3.5. Expression kinetics of γH2AX induced by formaldehyde, acetaldehyde, acrolein and benzene

Because of bad goodness value of fit (less than 0.5), partial results were not showed in Table 2. For example, the goodness of fit was 0.287 at 80 μmoL/mL of ACR. As showed in Table 2, all λ_f values were higher than λ_s values for the listed toxicants. It suggested that two-component model was suitable to analysis the data of these toxicants. The majority of "b" were more than "a" except for 2000 μmoL/mL of AA and 125 μmoL/mL of hydroquinone, so the slow component dominates in DNA damage/repair for most toxicants.

4. Discussion

Although the *in vitro* γH2AX HCS assay had a high accuracy because of high sensitivity and specificity, the stability has not been descripted before. Z-factor is a common method to evaluate the stability of a technology or an assay (Iversen et al., 2006). When Z-factor is between

Table 2
Two-component model parameters for the tested toxicants.

Dose/μmoL/mL	R^2	a	λ_f	b	λ_s
Formaldehyde					
500	0.739	-4.6667×10^6	1.1257×10^6	1.7258	−0.0382
Acetaldehyde					
1000	0.688	−33.3136	0.0341	34.0456	0.0313
2000	0.712	1.4917×10^5	4.2871×10^4	0.9532	−0.0257
Acrolein					
120	0.876	-5.7872×10^4	10.6624	2.6636	0.0274
160	0.9538	−7.2795	1.3697	3.1944	−0.0092
Benzene $_{(+S9)}$					
1600	0.757	-1.6555×10^4	12.0756	1.0452	0.0205
3200	0.808	−0.2193	0.8150	1.0585	0.0190
6400	0.838	−0.1486	0.6713	1.0727	0.0231
Hydroquinone					
125	0.997	26.4880	1.5433	1.0091	−0.0188

Note: Subscript "+S9" represents "in the presence of S9"; R^2 is the goodness of fit of two components model; the formula of two components model is $E = a\,e^{-\lambda_f t} + b\,e^{-\lambda_s t}$; λ_f and λ_s are the rate constants for simple (fast) and complex (slow) components, respectively; a and b are the normalized coefficients.

0.5 and 1, it is evaluated as an excellent assay. When Z-factor is between 0 and 0.5, it is just a marginal assay. Z-factor equaling to 1 means the assay is ideal, but it can never exceed 1.When Z-factor is less than 0, it indicates that there is too much overlap between the positive and negative controls. Z-factor of the *in vitro* γH2AX HCS assay was observed above 0.5 (0.65–0.82) here, so the *in vitro* γH2AX HCS assay had a good stability and can be evaluated as an excellent assay.

The free aldehyde is highly reactive with amines, thiols, hydroxyls, amides and cysteine to form adducts and crosslinks. DNA strands breaks, chromosome aberration and gene mutation can be induced in cells because of interaction between aldehydes and DNA. FA, AA and ACR were significantly genotoxic at 250, 1000 and 120 μmoL/L, respectively, based on genotoxicity evaluation criteria of the *in vitro* γH2AX assay presented by Smart et al. (Smart et al., 2011). They also can cause cytogenetic toxicity in a significant dose/time-dependent manner in terms of DSBs. However, the dose/time-response relations were not similar for different aldehydes. For example, when concentrations of FA and ACR were below 500 μmoL/L and 160 μmoL/L, respectively, the peak γH2AX levels occurred at an earlier time. However, time-dependent increase was observed for AA at all concentrations.

Although cells have already been treated with a high dose of benzene here, there still have been no significant genotoxicity. Therefore, it meant that benzene might be not a direct clastogen in vitro. Furthermore, low activity and expression of CYP2E1 was found in A549 cells (Garcia-Canton et al., 2013b). So the standardized Aroclor-1254-induced rat liver S9 mix was further added into culture media to activate metabolically benzene. Nevertheless, there were still no significant effects on the induction of γH2AX in the presence of S9. This may be caused by detection method, because significant DNA damage was observed at 200 μmoL/L of benzene using neutral comet assay (Chen et al., 2008). Furthermore, although the *in vitro* γH2AX HCS assay has a high sensitivity (86–92%), it may be not suitable for all genotoxic chemicals. Therefore, the *in vitro* γH2AX HCS assay may be just a good supplemental tool to traditional genetic toxicity tests, and not an alternative tool. In addition, a slightly decreasing in the induction of γH2AX was found with the increasing exposure time in the presence of S9. This might be caused by 400 μmol/L coenzyme II $NADP^+$ in S9 mix. Because it was reported that highly reactive and toxic free radical could be produced and this was one of important way to induce DNA damage, when intermediates of benzene was metabolized. However, $NADP^+$ reductase could be contributed to increase tolerance to reactive oxygen species (ROS) (Giró et al., 2011). So high doses of $NADP^+$ might disrupt the process of the oxidative stress and reduced the DNA damage induced by benzene in the presence of S9. For further investigating the genotoxicity of benzene, hydroquinone was directly used here. Hydroquinone not only could induce significant γH2AX expression, but also had significant dose/time-response relations in the induction of γH2AX, which was similar with the observation of Khoury L. (Khoury et al., 2016). It therefore illustrated that metabolites of benzene were more genotoxic than benzene itself.

In addition, although FA, AA and hydroquinone have shown positive responses in other cell lines with the *in vitro* γH2AX assay, sensitivity of the different cell lines is obviously different. For example, 1000, 100 and 1000 μmoL/L of FA has induced the significant level of γH2AX in HepG2, LS-174T and ACHN cell lines, respectively. Meanwhile, the LECs were all 10 μmoL/L for hydroquinone to induce γH2AX in these cell lines (Khoury et al., 2016). Such differences in sensitivity may result from the intrinsic nature of cell lines, such as metabolic capabilities and DNA repair capabilities. One hand, recently published recommendations on cell line selection suggest the use of human p-53 (one of most important components in DNA damage and repair pathway) competent cell lines to reduce the incidence of "false positive"(Fowler et al., 2012). For example, 1 moL/L of AA induced 5.1-fold γH2AX expression in lymphoblastic cell lines from XPA patient (abnormal DNA repair exists in the cells of XPA patients) (Marietta et al., 2009). However, AA just induced 1.5-fold γH2AX in A549 cells (which have normal DNA repair function) with the same dose in this study. On the other hand, the deficiencies in the metabolic capabilities of cell lines could lead to inaccurate evaluation of the tested compounds (Kirkland et al., 2007). This is especially important for pro-genotoxicants. For example, benzene is a pro-genotoxicant and is mainly metabolized to di- and tri-hydroxy benzenes by CYP2E1(Gayathri and Kamaraj, 2014). However, activity and expression of CYP2E1 in HepG2 cells is higher than that in A549 cells (Garcia-Canton et al., 2013b). So more sensitive response was observed for hydroquinone (one intermediate of benzene) in HepG2 cells (LEC is 10 μmoL/L in Khoury's study) (Khoury et al., 2016) than that in A549 cells (LEC is 60 μmoL/L in this study).

In this study, Hill model was used to compare the genotoxicity of FA, AA, ACR and benzene. Unlike IC_{50} can be directly used to compare the cytotoxicity of chemicals, C_{50} can not directly be used to compare the genotoxicity. Because maximum survival rates was same (100%) for different chemicals, but maximum genotoxic effect was different for different substances. For that reason, we attempted to calibrate C_{50} with E_{max}, and a novel composite parameter NC_{50} was established and defined as the dose resulting in 50% of the normalized maximum genotoxicity (equal to 1). Based on the NC_{50} , the ability to induce γH2AX was evaluated as ACR > hydroquinone > FA > AA > benzene > benzene (S_9). It is generally considered that one toxicant with lower LEC is more toxic, so the ability to induce γH2AX was also assessed in LEC method, and it could be evaluated as hydroquinone ≈ ACR > FA > AA > benzene (S_9) in terms of LEC value (Table 1). In contrast with other toxicants, benzene has a monotonic decrease response in the absence of S9, so benzene(-S_9) was not evaluated here. This result in terms of LEC value was similar with the result based on NC_{50} value. The only subtle distinction was the order of ACR and hydroquinone between these two evaluation methods. What needs to be pointed out is that "NC_{50} value" is a theoretical calculation value based on dose-response relation and mathematical model. While "LEC value" is an actual measurement value based on dosage settings and statistical calculation. "LEC value" can be easily obtained just by fewer experiments and dosage settings, but "LEC value" may be imprecise because of inappropriate and rough dosage settings. "NC_{50} value" is more accurate than "LEC value",

because more dosages are needed to fit the dose-response curve. However, the accuracy of "NC_{50} value" method seriously depends on the goodness of fit and the selectivity of model. In this study, "NC_{50} value" method may be better, because one previous research suggested that acrolein was more genotoxic than hydroquinone based on Salmonella mutation assays (Claxton et al., 1989). This result was consist with the method based on NC_{50} value. So the ability to induce DNA damage should be evaluated as A-CR > hydroquinone > FA > AA > benzene > benzene (S9). Although little has been reported in previous studies for the comparison of genotoxicity, this is important to contribute the prioritization of toxicant reduction and controlling research in tobacco products.

In general, DNA damage can be classified into two subtypes, complex DNA damage and simple DNA damage. A series of complex DNA damage is closely formed into a cascade or a cluster, and is therefore a slow process that is difficult to be repaired (Hada and Georgakilas, 2008). However, simple DNA damage is distinguishable by the non-continuity of the damage (Niu et al., 2014), and therefore it occurs and can be repaired rapidly. Two-component model was most used to investigate the kinetics of DNA repair for radiations (Niu et al., 2014). Here it was applied to model the kinetics of DNA damage/repair for chemicals based on the mutual biomarker (γH2AX) of DNA damage and DNA repair. Because fitted data of γH2AX were obtained from the continuous exposure to chemicals, the model did not represent DNA repair alone, while it represented the results of the combined effects of DNA damage and DNA repair. Based on the two-component model, DNA damage induced by 500 μmoL/L of FA, 1000 μmoL/L of AA, 120 and 160 μmoL/L of ACR and benzene were complex and a slow DNA damage/repair mechanism was more dominant. While DNA damage induced by 2000 μmoL/L AA and 125 μmoL/L hydroquinone were simple and a fast DNA damage/repair mechanism was more important. Although this cannot clearly reveal their genotoxicity mechanism, it is very helpful for understanding the process of DNA damage/repair induced by these toxicants. Because complex damage and slow mechanisms frequently indicate that a cascade or a cluster signals is involved.

Overall, the present findings demonstrate that the *in vitro* γH2AX HCS assay has an excellent performance and can be applied to pre-screen and assess the genotoxicity of toxicants in terms of DSBs. Benzene failed to show a significant dose/time-response of γH2AX. However, dose/time-dependency in the induction of γH2AX was obtained by aldehydes and metabolite of benzene. Although the time-dependent manners were different for these toxicants, peak γH2AX was observed at an earlier exposure time. Based on the γH2AX assay and Hill model, the ability to induce γH2AX was evaluated as ACR > hydroquinone > FA > AA > benzene > benzene (S_9). Meanwhile, the DNA damages induced by these toxicants were complex and the slow DNA damage-repair mechanism was more important than the fast one for aldehydes based on the analysis of two-component model.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank prof. Yanan Jiang (Zhengzhou University) for using of HCS instruments. The authors acknowledge the financial support from the National Natural Science Foundation of China (No 21347002).

References

Audebert, M., Zeman, F., Beaudoin, R., Péry, A., Cravedi, J.P., 2012. Comparative potency approach based on H2AX assay for estimating the genotoxicity of polycyclic aromatic hydrocarbons. Toxicol. Appl. Pharm. 260, 58–64.
Bucciantini, M., Nosi, D., Forzan, M., Russo, E., Calamai, M., Pieri, L., Formigli, L., Quercioli, F., Soria, S., Pavone, F., Savistchenko, J., Melki, R., Stefani, M., 2011. Toxic effects of amyloid fibrils on cell membranes: the importance of ganglioside GM1. FASEB J. 26, 818–831.
Chen, C.S., Hseu, Y.C., Liang, S.H., Kuo, J.Y., Chen, S.C., 2008. Assessment of genotoxicity of methyl-tert-butyl ether, benzene, toluene, ethylbenzene, and xylene to human lymphocytes using comet assay. J. Hazard. Mater. 153, 351–356.
Claxton, L.D., Morin, R.S., Hughes, T.J., Lewtas, J., 1989. A genotoxic assessment of environmental tobacco smoke. Mutat. Res./Genet. Toxicol. 222, 81–99.
Cosma, G.N., Marchok, A.C., 1988. Benzo[a]pyrene- and formaldehyde-induced DNA damage and repair in rat tracheal epithelial cells. Toxicology 51, 309–320.
Davis, D., Nielsen, M.T., 1999. Tobacco: Production, Chemistry and Technology. Blackwell Science, Oxford.
Fowler, P., Smith, K., Young, J., Jeffrey, L., Kirkland, D., Pfuhler, S., Carmichael, P., 2012. Reduction of misleading (false) positive results in mammalian cell genotoxicity assays. I. Choice of cell type. Mutat. Res. 742, 11–25.
Garcia-Canton, C., Anadon, A., Meredith, C., 2013a. Assessment of the in vitro γH2AX assay by High Content Screening as a novel genotoxicity test. Mutat. Res. Genet. Toxicol. Environ 757, 158–166.
Garcia-Canton, C., Minet, E., Anadon, A., Meredith, C., 2013b. Metabolic characterization of cell systems used in in vitro toxicology testing: lung cell system BEAS-2B as a working example. Toxicol. In Vitro 27, 1719–1727.
Gayathri, S.B., Kamaraj, P., 2014. Genotoxicity of benzene and soluble benzene substituted organic compounds in mammals—a review. Int. J. Pharm. Sci. Health Care 4, 24–39.
Giró, M., Ceccoli, R.D., Poli, H.O., Carrillo, N., Lodeyro, A.F., 2011. An in vivo system involving co-expression of cyanobacterial flavodoxin and ferredoxin-NADP(+) reductase confers increased tolerance to oxidative stress in plants. FEBS Open Biol. 1, 7–13.
Hada, M., Georgakilas, A.G., 2008. Formation of clustered DNA damage after high-LET irradiation: a review. J. Radiat. Res. 49, 203–210.
ICH, 2011. Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use S2(R1). International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Expert Working Grouppp. 1–25.
Iversen, P.W., Eastwood, B.J., Sittampalam, G.S., Cox, K.L., 2006. A comparison of assay performance measures in screening assays: signal window, Z' factor, and assay variability ratio. J. Biomol. Screen. 11, 247–252.
Khoury, L., Zalko, D., Audebert, M., 2016. Complementarity of phosphorylated histones H2AX and H3 quantification in different cell lines for genotoxicity screening. Arch. Toxicol. 90, 1983–1995.
Kirkland, D., Pfuhler, S., Tweats, D., Aardema, M., Corvi, R., Darroudi, F., Elhajouji, A., Glatt, H., Hastwell, P., Hayashi, M., Kasper, P., Kirchner, S., Lynch, A., Marzin, D., Maurici, D., Meunier, J.R., Muller, L., Nohynek, G., Parry, J., Parry, E., Thybaud, V., Tice, R., van Benthem, J., Vanparys, P., White, P., 2007. How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: report of an ECVAM workshop. Mutat. Res. 628, 31–55.
Marietta, C., Thompson, L.H., Lamerdin, J.E., Brooks, P.J., 2009. Acetaldehyde stimulates FANCD2 monoubiquitination, H2AX phosphorylation, and BRCA1 phosphorylation in human cells in vitro: implications for alcohol-related carcinogenesis. Mutat. Res. 664, 77–83.
Murado, M.A., González, M.P., Vázquez, J.A., 2002. Dose–response relationships-an overview, a generative model and its application to the verification of descriptive models. Enzyme Microb. Technol. 31, 439–455.
Niu, H., Chang, H.C., Cho, I.C., Chen, C.H., Liu, C.S., Chou, W.T., 2014. Spatiotemporal kinetics of γ-H2AX protein on charged particles induced DNA damage. Appl. Surf. Sci. 310, 62–65.
Podhoreck, M., Skladanowski, A., Bozko, Przemyslaw, 2010. H2AX phosphorylation: its role in DNA damage response and cancer therapy. J. Nucleic Acids 2010, 1–9.
Smart, D.J., Halicka, H.D., Schmuck, G., Traganos, F., Darzynkiewicz, Z., Williams, G.M., 2008. Assessment of DNA double-strand breaks and gammaH2AX induced by the topoisomerase II poisons etoposide and mitoxantrone. Mutat. Res.-Fund Mol. Mech. 641, 43–47.
Smart, D.J., Ahmedi, K.P., Harvey, J.S., Lynch, A.M., 2011. Genotoxicity screening via the γH2AX by flow assay. Mutat. Res.-Fund Mol. Mech. 715, 25–31.
WHO, 2012. Agents Classified by the IARC Monographs, vol. 1–105. WHO Grouppp. 81–84.
Walker, N.J., Yang, J.H., 2005. Complexities in understanding the nature of the dose-response for dioxins and related compounds. Dose-Response 3, 267–272.
Zhang, S., Liu, L.J., Chen, H., Hou, H.W., Hu, Q.Y., 2016. Progress on molecular mechanism of phosphorylation/dephosphorylation and detection technology of γH2AX. Chin. J. Anal. Chem. 44, 1291–1299.