

Studies on formation and repair of formaldehyde-damaged DNA by detection of DNA-protein crosslinks and DNA breaks

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1. ABSTRACT

Formaldehyde (FA) is a genotoxic and mutagenic substance. In 2004, IARC (International Agency for Research on Cancer) concluded that FA is carcinogenic in humans after reevaluating the available evidence on the carcinogenicity of FA. Although many studies have shown that FA had extensive genotoxicity including DNA-protein crosslinks (DPC) and DNA single strand breaks (DSSB), most of these studies only discussed the effects of FA at high levels. In this study, KCl-SDS assay and single cell gel electrophoresis (SCGE) were used to investigate the formation and repair process of FA-induced DPC and DSSB in human peripheral blood lymphocytes and HeLa cell lines. KCl-SDS assay was applied to detect DPC induced by liquid FA in human peripheral blood lymphocytes *in vitro*. The results showed that FA could induce DPC at high levels (≥ 50 micro M). By combining the results of KCl-SDS assay and SCGE, it could be determined that FA would induce DNA-DNA crosslinks (DDC) when FA concentration was more than 25 microM. The repair process of FA-induced DPC was studied with KCl-SDS assay in HeLa cell lines and the results demonstrated that FA-induced DPC could be significantly repaired after 18 hours. The SCGE was also used to determine FA-induced DSSB and its repair process in HeLa cell lines. The results demonstrated that DNA breakages, which is capable of being induced by FA at a low level (< 30 microM), enabled to be repaired completely in 90 minutes.

2. INTRODUCTION

FA is a flammable, colorless, and readily polymerized gas at ambient temperature. It is present in the environment as a result of natural processes and from man-made sources such as motor vehicle exhaust, wood burning, and cigarette smoke. FA is also an endogenous biological compound, derived from the metabolism of serine, glycine, sarcosine, choline, and methionine (1). As a highly reactive chemical, FA was widely used in the manufacture of construction materials, resins, textile, leather goods, paper and pharmaceutical product (2). Due to its wide existence, it is important to study FA toxic effect and mechanism. Studies have shown that FA could induce the formation of DPC. As a key event of tumors induced by FA, DPC is the primary and directly genotoxic effect of FA (1-7). In normal cells there is a basic level of DPC, which is related with DNA replication and transcription, and is necessary for the cell growth (8). However, excessive DPC produced by environmental pollutants and carcinogens is a high risky toxic factor. Unlike DNA strands breaks, DPC is more difficult to be repaired in the cells. Since the poor repair ability, DNA-protein complexes may be presented during DNA replication, resulting in a loss and inactivation of the important genes such as tumor suppressor genes (9). DNA breaks, including DNA single strand breaks and DNA double strands breaks, are usually regarded as biomarkers of the phenomenon that cell life-force is threatened and the integrality of gene is destroyed (8). In the early studies, some researchers demonstrated that FA could induce DSSB

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(10-14). In 2000, Frenzilli *et al* found out that FA could mainly induce DNA breaks at a low level (≤ 50 microM), but DNA crosslinks at a high level (≥ 400 microM) (15). Merk and his associates have extensively studied the FA genotoxicity; conversely, they did not discover the DNA breakages caused by FA (2, 16, 17). In China, researchers have investigated the FA genotoxicity. Tang and Xi's studies showed that FA cause DNA strands breaks at 5 microM for HL60 cell lines and at 10 microM for hepatic cells of rats (18-20).

Although some studies have demonstrated that FA could induce DPC and DSSB, few studies focused on the repair process of them. Moreover, there is also controversial on the persistence of DPC in cells. In this study, KCl-SDS assay and SCGE were used to quantitatively analyze DPC and DSSB, and further to investigate the repair ability of DPC and DSSB in HeLa cells. In addition, despite many studies demonstrated that FA could induce DPC *in vitro* or *in vivo*; most of them only investigated the FA-induced DPC over high concentration range (≥ 100 microM) of FA. In order to deeply assess FA genotoxicity, KCl-SDS assay and SCGE were applied to explore the FA-induced DPC at a relatively low concentration range in the present study.

3. MATERIAL AND METHODS

3.1. Reagents and apparatus

10% formalin, calf thymus DNA and fluorescence dye Hoechst 33258 were purchased from Sigma. Reagents for separating human peripheral blood lymphocytes were purchased from the Institute of biomedical engineering of Chinese Academy of Science. RPMI 1640 and fetal bovine sera (FBS) were received from Gibco. SDS and proteinase K were purchased from Merck. Acridine Orange, Triton X-100 and N-lauroylsarcosine were purchased from Amresco. Normal melting point agarose and low melting point agarose were purchased from Promega. The Cell counting kit used in the experiments was purchased from Dojindo.

CO₂ incubator (HH. CP-T80L, Yiheng Scientific Limited Company, China), low temperature centrifuge (Eppendorf-5415R), Nikon fluorescence microscope (E600), Enzyme linked immunoassay detector (DG5031, Hua Dong Vacuum Tube Factory, China), and Fluorescence spectrophotometer (RF-5301PC, Shimadzu) were used in the experiments.

3.2. Cell separation and culture

The blood samples were taken from a male healthy adult. The separation of peripheral blood lymphocytes was conducted according to the procedure described by the supplier's manual. The HeLa cell lines were purchased from the Center for Cell Culture Collection at Wuhan, China. The HeLa cell lines were cultivated in RPMI 1640 supplemented with 10% FBS in a humidified atmosphere of 95% air, 5% CO₂ at 37C. The medium was changed every other day.

3.3. Cell exposure to FA

The separated or cultured cells were compressed gently to suspend cells with RPMI1640 to give a

concentration of about 1×10^6 cells/ml. The resultant suspended cells were distributed into the Eppendorf tubes with 0.5 ml per tube. FA was subsequently added into each tube to make the final concentration consistent with the design in advance. After that, the tubes with cells were incubated for 1 h at 37C.

3.4. KCl-SDS assay

The KCl-SDS assay was initially developed by Liu *et al* and modified by Zhitkovich and Costa in 1992 for detecting DPC in whole cells. The method uses harsh treatments to dissociate the non-covalent DNA-protein binding (2% SDS, at 65C) and selectively precipitates stable DNA-protein complexes by adding KCl (3). In this study the KCl-SDS assay was based on Zhitkovich and Chakrabarti methods (3, 21) with some modification to detect FA-induced DPC. In our experiments, the incubated cells were harvested by centrifugation at 6000 rpm for 3min. The cells were resuspended in a 0.5 ml of PBS, pH 7.5, followed by lysis with a 0.5 ml of 2% SDS solution with gentle vortexing. The lysate solution was heated at 65°C for 10 min and then 0.1 ml of pH 7.4 10 mM Tris-HCl containing 2.5 M KCl was added, followed by passing the resultant mixture six times through a 1-ml polypropylene pipette tip to favor shearing of DNA for a uniform length. Since SDS binds tightly to protein but not to DNA, the free protein and protein-DNA complexes are precipitated with added SDS while free DNA is remained in the supernatant. The SDS-KCl precipitate (containing the protein and DNA-protein crosslinks complexes) was formed by placing the samples in ice for 5 min and was then collected by centrifugation at 10000 rpm for 5 min in an Eppendorf microfuge. The supernatants containing the unbound fraction of DNA were collected in different labeled tubes. The pellets (containing DPC) were washed three times by resuspending in 1 ml washing buffer (0.1 M KCl, 0.1mM EDTA, and 20 mM Tris-HCl, pH 7.4) followed by heating at 65°C for 10 min, chilling in ice for 5 min, and centrifugating as that described above. The latter supernatants from each wash were added into the previous one with unbound fractions of DNA. The final pellet was resuspended in 1 ml proteinase K solution (0.2mg/ml soluble in a wash buffer) and digested for 3 h at 50°C. The resultant mixture was centrifuged at 12000 rpm for 10 min and the supernatant was collected (the supernatant contained the DNA previously involved in DNA-protein crosslinks). 1 ml of either the supernatant containing the unbound fraction of DNA or the supernatant containing the DNA previously involved in DNA-protein crosslinks was then mixed with 1 ml freshly prepared fluorescent dye Hoechst 33258 (400 ng/ml soluble in a 20 mM Tris-HCl), and then the tubes were allowed to stand for 30 min in the dark. The sample fluorescence was measured using a RF-5301PC fluorescence spectrofluorimeter with excitation wavelength at 350 nm and emission wavelength at 450 nm. The DNA contents of the samples were determined quantitatively through a corresponding DNA standard curve generated from a set of calf thymus DNA. The DPC coefficient was measured as a ratio of the percentage of the DNA involved in DPC over the percentage of the DNA involved in DPC plus unbound fraction of DNA.

3.5. Single cell gel electrophoresis (Comet assay)

SCGE is also called Comet assay. It was initially developed by Ostling and Johanson in 1984, and modified

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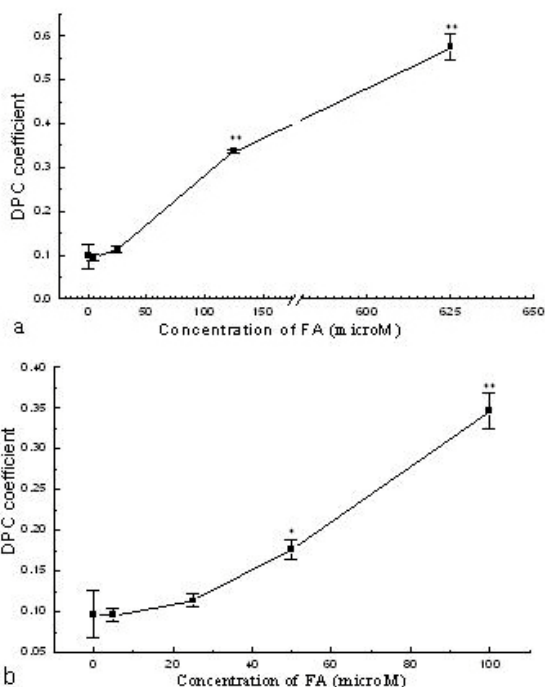


Figure 1. Relationship between DPC coefficient and FA concentrations in human peripheral blood lymphocytes (*: $P < 0.05$; **: $P < 0.01$, FA treated groups compared with control group)

by Singh *et al* to improve the detection sensitivity for DNA damages in 1988. In our experiments, it was performed to detect FA-induced DNA damages and repair process according to Singh's report (22). The protocol is described as below in brief: 180 micro liter of 1% normal melting point (NMP) agarose was formed on fully frosted slides (75mm \times 25mm) and 100 micro liter low melting point (LMP) agarose containing cells (the ratio of solution over suspended cells was 5:3) was formed on the top of NMP agarose layer. After formation of the cell-containing layer, additional LMP agarose was added onto it to fill in any residual holes and to form additional layer for increasing the distance between the cells and the gel surface. After the agarose gel was solidified, the slides were placed in a lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 1% N-lauroylsarcosine, 1% Triton X-100, 10% DMSO) for 2 hrs. After the lysis, the slides were incubated in an alkaline (pH>13) electrophoresis buffer (1 mM Na₂EDTA and 300 mM NaOH) for 20 min to produce single strand DNA, followed by performing electrophoresis with the condition of 20V, 220mA for 20min. After the electrophoresis, the alkali in the gels was neutralized and the slides were rinsed with a suitable buffer (0.4M Tris at pH7.5) for 30min. At last, the Acridine orange (fluorescent dye) was used to stain the resulting DNA, followed by comet visualization under fluorescence microscope, and the CCD was used to image the comets for analyzing DNA damage with assistance of the software of CASP.

3.6. Statistical analysis

Experimental results were analyzed by software Origin 6.0. Student's-test was applied to evaluate the

significance of the differences in the results between treated and control groups. A level of $P < 0.05$ was considered to be statistically significant.

4. RESULTS AND DISCUSSION

4.1. FA-induced DPC

FA-induced DPC in human peripheral blood lymphocytes and Hela cell lines was investigated. Figure 1-a and 1-b show the results obtained from human peripheral blood lymphocytes. It can be seen from the figure 1-a, there is no significant difference in the DPC coefficient between the groups treated with 5 microM, 25 microM of FA and the control group. However, significant difference between the groups treated with 125, 625 microM of FA and the control group ($P < 0.01$) is demonstrated in the figure. Figure 1-b also shows very similar results to that demonstrated in figure 1-a. There is no significant difference between the groups treated with 5, 25 microM FA and the control in DPC coefficient; but the DPC coefficient of groups treated with 50 and 100 microM FA is significantly higher than that of the control group (50 microM FA-treated group compared with the control, $P < 0.05$; 100 microM FA-treated group compared with the control, $P < 0.01$). Furthermore, it also can be determined from the figures that the inflexion of FA-induced DPC is about 50 microM in human peripheral blood lymphocytes.

The induced DPC in Hela cell lines after exposures to different concentrations of FA is illustrated in Figure 2, demonstrating that there is no significant difference between the group treated with 25 microM FA and the control group in DPC coefficient; but the DPC coefficient of 50 μ M FA-treated group is significantly higher than that of control group ($P < 0.05$), and there are significant difference in DPC coefficient between 100 microM, 200 microM FA-treated groups and the control group ($P < 0.01$). It indicated clearly a dose-dependent relationship between the DPC coefficient and the concentration of FA.

Both results showed that FA could not induce DPC in two cell lines at low concentration range, but at a higher concentration range (≥ 50 microM) it could. Although many researchers have disclosed that FA could induce DPC, they only investigated the effect of FA on FA-induced DPC at high concentration range. In this study, we experimentally confirmed that FA could induce DPC at a relatively low concentration, and discovered that the inflexion of FA-induced DPC in human peripheral blood lymphocytes and Hela cells occurred at 50 microM FA. By combining the results obtained from KCI-SDS and SCGE as shown in figure 1 and 5, we could determine that a concentration of about 25 microM FA enabled to induce DNA-DNA crosslinks. Therefore, FA could induce DNA damages at a relatively low concentration. This discovery provides important information for deeply understanding the FA genotoxicity.

4.2. The repair of DPC in Hela cells

In order to explore the repair of FA-induced DPC in Hela cells, it is necessary to select an appropriate FA

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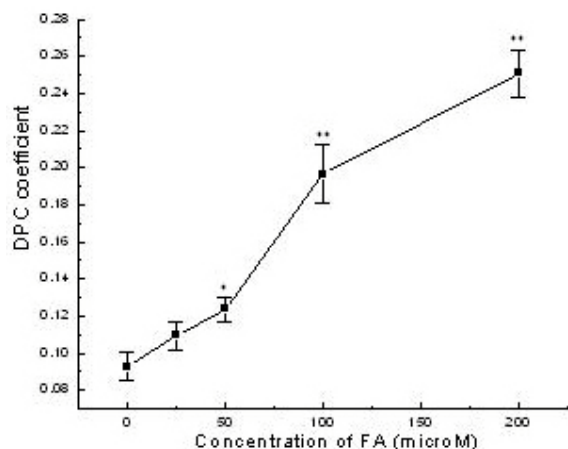


Figure 2. Relationship between DPC coefficient and concentration of FA in Hela cell lines. (*: $P < 0.05$; **: $P < 0.01$, FA treated groups compared with control group).

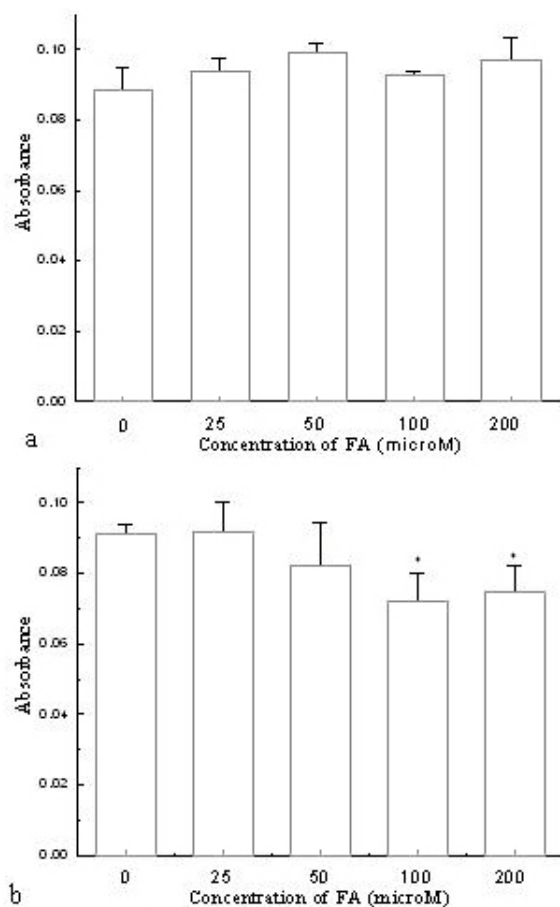


Figure 3. The histograms of cytotoxicity effect of FA on Hela cell lines. Cells suspension was pipetted into the wells of 96-well plate. When cells have expanded on well surface, new media with different concentration of FA were employed in the cells. After 1 or 12 hours the absorbance was measured. (*: $P < 0.05$, treated groups compared with control).

concentration. At this concentration, FA can induce DPC and cannot produce evidently cytotoxicity. Thus, we detected the cytotoxicity of various concentrations of FA to Hela cells after 1 and 12h-treatments, respectively. The results are shown in figure 3, demonstrating that there is no significant difference in cell activities between each FA-treated groups (FA concentration range: 25-200 microM) and the control group treated after 1h. However, after 12h-treatment, the cell activities of the groups treated with 100 and 200 microM FA were apparently decreased in comparison with that of the control group ($P < 0.05$). According to the results of FA-induced DPC in Hela cells, 50 microM is the appropriate concentration for study of the repair of DPC.

The results for repair of DPC after removal of FA in Hela cells is illustrated in Figure 4, indicating that the DPC coefficient had no significant decrease after the groups with FA removal for 6 or 12 hours in comparison to the group without FA removal. However, significant difference of the DPC coefficient between 18, 24 removal hours and 0 removal hour was clear ($P < 0.05$), and the DPC coefficient at 18 and 24 removal hours were close to that of negative control ($P > 0.05$). This shows that FA-induced DPC could be repaired after FA removal for 18 or 24 hrs in Hela cells. The reported research work so far presented conflict conclusions on the persistence of DPC in cells with other reports. Shaham *et al* proposed that FA-induced DPC was irreversible and could persist for a much longer time after removal of the cross linking agents (6, 7). However, Casanova *et al* pointed out that FA-induced DPC could be repaired within 24 hrs after FA removal (23). Furthermore, Speit *et al* studied the repair of DPC in normal and repair-deficient cells and concluded that DPC could be repaired within 24 hrs in both kinds of cells (24). In our study, the Hela cell lines were applied to explore the repair of FA-induced DPC. The results further confirmed that FA-induced DPC could be repaired slowly and could not be accumulated for a long period in cells.

4.3. Formaldehyde-induced DSSB

With SCGE, FA-induced DSSB was detected in human peripheral blood lymphocytes and Hela cells, respectively. The effects of FA on DNA damages are illustrated in figure 5 and 6. Figure 5 showed that FA could induce DSSB significantly after exposure of human peripheral blood lymphocytes to 5 and 25 microM FA (5 microM, $P < 0.01$; 25 microM $P < 0.05$). The induced DSSB decreased rapidly with the increase of FA concentrations (25 microM compared with 5 microM, $P < 0.05$; 125 and 625 microM compared with 5 microM, $P < 0.01$). It could be concluded that FA induced DSSB after exposure to 5 and 25 microM FA, and induced crosslinks after exposure to 125 and 625 microM. Importantly, by combining this result with the result obtained from KCl-SDS assay we can quantitatively determine that FA concentration to induce DNA-DNA crosslinks, which was about 25 microM from our experimental results.

Figure 6 exhibits the degrees of FA-induced DSSB in Hela cells, showing that FA significantly induced DSSB after the cells expose to 5, 7.5, 10 and 15 microM of

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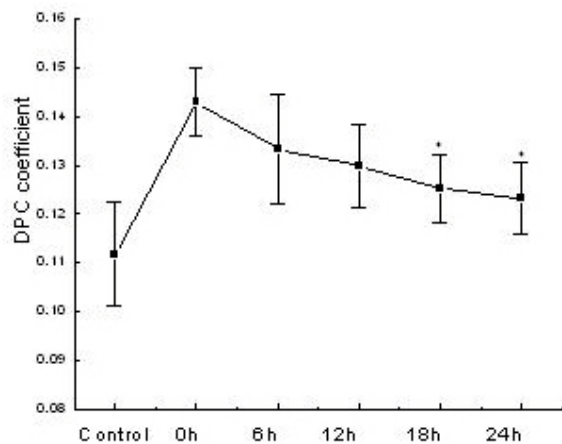


Figure 4. Repair of the FA-induced DPC in Hela cell lines. (*: $P < 0.05$, groups repaired for 18 and 24 hrs compared with group repaired 0 hr, respectively).

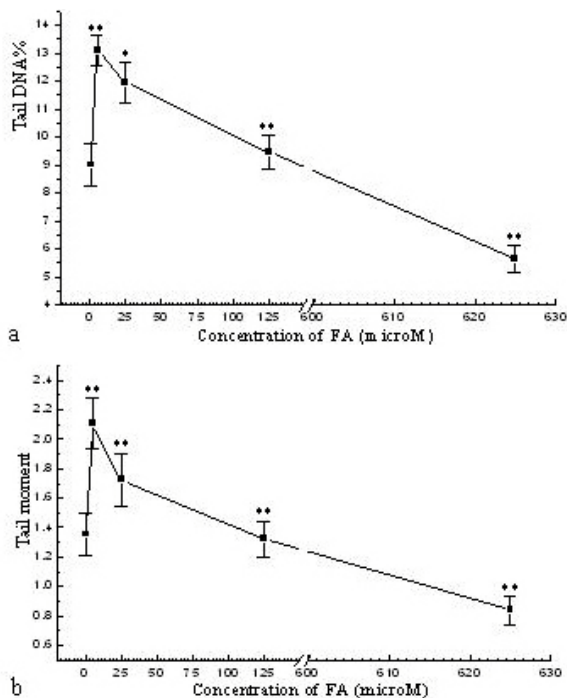


Figure 5. The FA-induced DNA damage in human peripheral blood lymphocytes, 5-a for tail DNA% and 5-b for Tail moment. (**: $P < 0.01$, group treated with 5 microM FA compared with 0 control; Groups treated with 25, 125 and 625 microM FA compared with group treated with 5 microM FA).

FA ($P < 0.01$), and the strongest effect was generated after the exposure to 10 microM FA. Although numerous studies reported that FA could not induce DSSB (2, 16, 17), the results in our studies evidently demonstrated that FA could induce DSSB at a low concentration. In comparison of our study with the other

reports described above, the reason for the conflict conclusions on whether FA could induce DSSB is possibly because of very high concentrations of FA used in the previously reported researches. Interestingly, FA induces crosslinks (DNA-DNA crosslinks or/and DNA-protein crosslinks) but not DSSB when FA at a high level. The biological mechanism needs to be further studied.

4.4. The repair of DSSB in Hela cells

The repair process of FA-induced DSSB in Hela cells was investigated. The results are shown in figure 7, demonstrating that levels of FA-induced DSSB were decreased with the increasing time after FA removal. There were significant differences between FA removal for 30, 60, 90, 120 min groups and 0 min control group in the degree of DSSB ($P < 0.01$). The degree of DSSB had almost no change between 90 and 120 min. Therefore, it could be concluded that FA-induced DSSB could be significantly repaired within 30 min and be almost completely repaired within 90 min after FA removal. In other words, FA-induced DSSB could be easily repaired and cannot be persistent in cells for a long time (less than 90 min). Actually, there was none study on the repair of DSSB from FA-induced damage, and there were only a few reports on the repair of DSSB from non-FA-induced DSSB damages such as the consensus with the reports (25, 26), which studied on the DSSB induced by other DNA damaging agents including H_2O_2 and X-ray.

5. CONCLUSION

Although many studies suggested that FA could induce different kinds of DNA damages, most of them only discussed the effect of FA at high concentrations (> 100 microM). Moreover, there also has a conflict on whether or not FA-induced DSSB and the DPC persistence in cells. In the present study, KCI-SDS assay and SCGE were employed to detect DPC and other DNA damages from FA. Experimental results showed that FA could induce DPC after exposures to 50 microM or a higher concentration of FA, and could be significantly repaired after removal of FA for 18 hrs. The significance of this investigation demonstrated that FA genotoxicity was produced by exposure to a low concentration of FA and the resulting DPC could be repaired. The results also confirmed that FA could induce DSSB at a low level (< 30 microM), and DSSB could be readily repaired (It is almost completely repaired within 90 min after FA removal). By combining the results obtained from KCI-SDS assay and SCGE, the concentration of FA to induce DNA-DNA crosslinks could be quantitatively determined and it was experimentally found out to be 25 microM FA. It is very important to understand the FA genotoxicity and the repair process of FA-induced DNA damages. This study provides powerful tools and methods to assess the FA genotoxicity and the repair process, allowing to substantially lower FA toxic level and improve the indoor air quality.

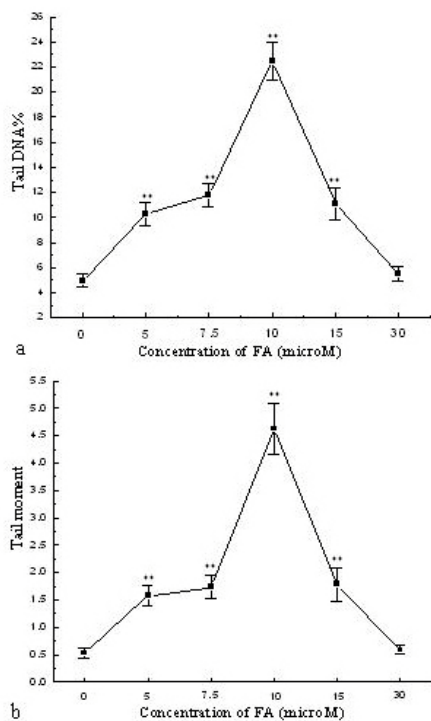


Figure 6. FA-induced DSSB in Hela cells, 6-a for tail DNA% and 6-b for Tail moment. (**P<0.01, Treated group compared with 0 control).

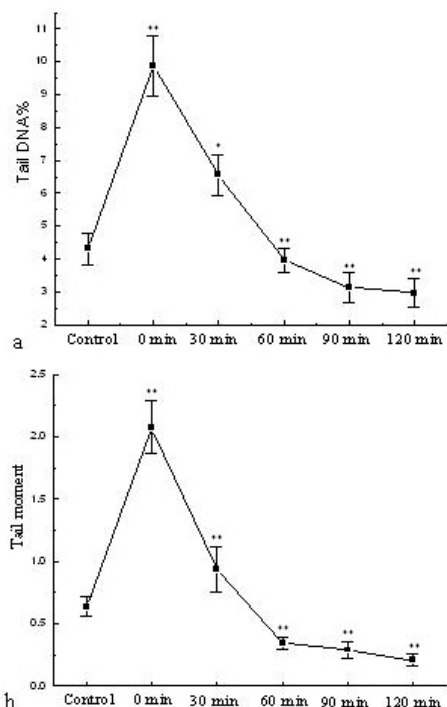


Figure 7. Repair of the FA-induced DSSB in Hela cell lines, 7-a for tail DNA% and 7-b for tail moment. (**: P<0.01, 0 min group compared with control; groups repaired after 30, 60, 90, 120 min removal of FA compared with group repaired after 0 min removal of FA, respectively).

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Abbreviation: FA, formaldehyde; DPC, DNA-protein crosslinks; DDC, DNA-DNA crosslinks; DSSB, DNA single strand breaks; SCGE, single cell gel electrophoresis; FBS, fetal bovine sera; LMP, low melting point; NMP, normal melting point; DMSO, diethyl sulfoxide; SDS, sodium dodecyl sulfate

Key Words: Formaldehyde, Formalin, Genotoxicity, DNA, Protein Crosslink, Single Strand Break, Repair

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