Gaseous Formaldehyde-induced DNA-protein Crosslinks in Liver, Kidney and Testicle of Kunming Mice

Guangyin Peng, Xu Yang, Wei Zhao, Junjun Sun, Yi Cao, Qian Xu, Junlin Yuan, Shumao Ding

College of Life Science, Central China Normal University, Wuhan 430079, China

Abstract: To explore the effect of distant-site toxicity, this study detected the amount of DNA-protein crosslinks (DPC) with KCl-SDS assay in liver, testicle and kidney of the purebred Kunming mice treated with gaseous formaldehyde (FA). The results showed that gaseous FA couldn't cause DPC or could cause few DPC at the lower concentration (0.5 mg/m^3), while could cause significant DPC at higher concentrations (1.0 mg/m^3 and 3.0 mg/m^3) ($P<0.01$). The results suggested that FA could induce DPC in the distant organs (liver, testicle and kidney) of mice at relatively high concentrations, which indicated that FA might induce distant-site toxicity. [Life Science Journal. 2006;3(3):82-87] (ISSN: 1097-8135).

Keywords: formaldehyde; distant-site toxicity; DNA-protein crosslinks; KCl-SDS assay

Abbreviations: DPC: DNA-protein crosslinks; FA: formaldehyde; DSB: DNA strand breaks; IARC: International Agency for Research; NO: nitric oxide; SDS: sodium dodecyl sulfate

1 Introduction

Formaldehyde (FA) is a colorless, highly flammable gas at ambient temperature, which is present in the environment from both natural processes and manmade sources. As a major industrial chemical, it can be found in construction materials, resins, textiles, leather goods, paper, and consumer products. At the same time, as a naturally occurring biological compound, it is present in tissues, cells, and body fluids. In addition, some reviews have reported that FA is a genotoxic and mutagenic compound and has been classified as a human carcinogen (class AI) by IARC (International Agency for Research) recently[1]. Due to its extensive sources, high level, long-term and high toxicity, it is important to study FA toxic effect and mechanism.

Studies have shown that FA has extensive genotoxicity, including DNA-protein crosslinks (DPC) and DNA strand breaks (DSB)[2,3,4]. DPC is the primary genotoxic effect of FA, which is formed through covalent bond where deoxyribonucleic acid is linked to an endogenous protein. Regions of DNA that are covalently linked with protein are typically considered to be non-functional and can block normal functions of the nuclear matrix, such as replication and transcription, and can form the foci for double strand breaks, which can lead to chromosomal aberrations and sister-chromatid exchanges. Furthermore high amounts of DPC can cause the expression of critical regulatory genes change. For the significance of DPC, the content of it may be of value in the assessment of FA-induced genotoxicity. Casanova *et al* have proved that acute FA inhalation could induce DPC in nasal mucosa of rats and monkeys by experiments *in vivo*[5,6,7]. Kuykendall exposed rat nasal epithelial cells to FA and found DPC content increased remarkably above the concentration of 100 μmol/L[4]. In addition, in our laboratory, Liu *et al* exposed human peripheral blood lymphocytes to FA and found that there was no significant difference in the DPC coefficient between the groups treated with 5 μM, 25 μM FA and the control group, while there was a significant difference between the groups treated with 125 μM, 625 μM FA and the control group ($P<0.01$). The results showed that FA could not induce DPC at low concentrations but could induce DPC significantly at high concentrations[8].

These studies above either chose organs located at exposure site, or were conducted *in vitro* to estimate FA toxicity. Few of them proved whether FA could induce distant-site toxicity. At present, there are still controversies on whether inhaled FA can induce distant-site toxicity, mainly because of the rapid metabolism and removal of FA *in vivo*[9,10]. Recently, Franks developed a mathematical model for estimating the absorption and metabolism of FA by humans. This model was used to analyze the increase of FA concentration in the blood after exposure to FA, and results showed that the increase was insignificant, indicating FA

could be removed rapidly in the blood[11]. This is consistent with previous reports declaring that inhaled FA could be removed rapidly[9,10]. However, some work also support FA has the distant-site toxicity. For example, Shaham *et al* examined DPC in peripheral blood lymphocytes of workers exposed to FA, and they found a significantly positive correlation between FA and DPC concentrations. In their early work, they also found a linear relationship between years of FA exposure and the amount of DPC[12,13]. Epidemiological studies on workers also suggested that the possible relations between FA exposures and leukemia[14,15]. These authors proposed that although inhaled FA was metabolized rapidly at contact sites, FA might be transported by unknown mechanism and cause cancers like leukemia subsequently.

To investigate FA-induced genotoxicity in the distant organs such as liver, kidney and testicle of mice, in this study, the content of FA-induced DPC in the organs of mice was detected by KCl-SDS assay. At the same time, the results will give more information to understand whether FA has distant-site toxicity. Additionally, this study may be helpful to understand the genotoxicity and the carcinogenicity of FA comprehensively and systematically, providing a scientific basis for constituting secure professional concentration standard of FA.

2 Materials and Methods

2.1 Reagents and apparatus

10% formalin, calf thymus DNA and fluorescence dye Hoechst 33258 were purchased from Sigma(USA). Sodium dodccyl sulfate (SDS) and proteinase K were purchased from Merck(Germany). PBS (without Ca^{2+} and Mg^{2+}), trypan blue solution of 0.4% and other chemicals were of analytical grades.

A WH-2 type environmental chamber (WH-2, Yu-Xin Inc, China) as FA generator, a 4160 type digital electrochemical FA analyzer (Interscan Inc, USA), glass low inhalation chamber, temperature centrifuge (Eppendof-5415R), fluorescence spectrophotometer (RF-4500, HITACHI, Japan) were used in the experiments.

2.2 Animal

24 male Kunming mice were supplied by the Experimental Animal Center of Hubei Province, China. Animals' weight were 19 ± 1 g.

2.3 Mice exposure to gaseous FA

24 male Kunming mice were divided randomly into 4 testing groups ($n = 6$ each) and were exposed to different concentrations of FA: 0, 0.5, 1.0 and 3.0 mg/m^3. The inhaled groups were exposed to gaseous FA for 72 hours continuously. During the exposure the mice were allowed to drink and eat twice at fixed time each day. FA inhaled groups were placed into glass inhalation chambers. The chamber temperature was 23 ℃ ± 0.5 ℃, and the humidity was 45% ± 0.5%, and the gas flux was 1 ± 0.01 L/min. A 4160 type digital electrochemical analyzer was used to measure the concentrations of gaseous FA.

2.4 Preparation of cells

Mice were executed immediately after exposure and livers, kidneys and testicle were obtained. The tissues were minced with scissors and homogenized with seven to eight strokes in PBS (pH 7.5). The homogenate was filtered through four layers of cheesecloth and then the cells were collected by centrifugation at 1500 rpm for 5 min. After re-suspending, the cell density was regulated with PBS and cell viability was analyzed with the method of trypan blue exclusion. Cell viability was above 95% and density was $10^5 - 10^6$ cells per ml.

2.5 KCl-SDS assay

In this study the KCl-SDS assay was based on Zhitkovich and Chakrabarti methods with some modification to detect FA-induced DPC[16,17]. Cells were harvested by centrifugation at 1500 rpm for 5 min. The cells were resuspended in 0.5 ml of PBS, pH 7.5, followed by lysis with 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 and 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 10,000 rpm for 5 min. 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, 0.1 mM 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 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 (0.2 mg/ml soluble in a wash buffer) and digested for 3 h at

50 °C. The resultant mixture was centrifuged at 12,000 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 20 mM Tris-HCl), and then the tubes were allowed to stand for 30 min in the dark[18]. The sample fluorescence was measured using a RF-4500 fluorescence spectrofluorimeter with excitation wavelength 350 nm and emission wavelength 450 nm. The DNA contents of the samples were determined quantitatively through a corresponding DNA standard curve (as Figure 1 shows and regression equation is $y = 2.4423 + 0.0028x$, $r^2 = 0.9974$) 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.

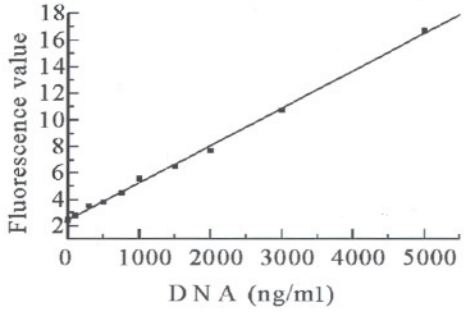

Figure 1. The standard curve of DNA concentration

2.6 Statistical analysis

Results were analyzed by software Origin 6.0. Student's t-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 defined to be statistically significant.

3 Results

3.1 Effect of FA-induced DPC in the livers of mice

Figure 2 showed the effect of gaseous FA exposure on DPC levels in livers of mice. There was no significant difference in DPC coefficient between 0.5 mg/m^3 FA inhaled group and 0 mg/m^3 control group. However, the DPC levels at 1.0 mg/m^3 and 3.0 mg/m^3 groups were significantly ($P<0.01$) higher than that in the control group, demonstrating that as the inhaled gaseous FA concentrations increased, the DPC levels ascended.

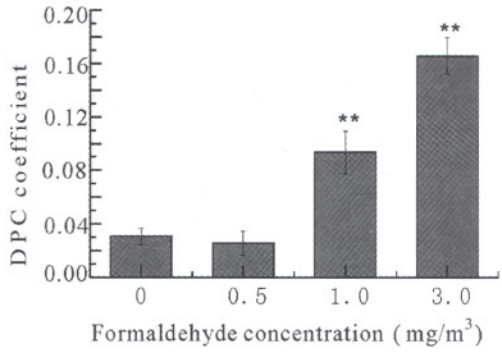

Figure 2. DPC formation in mice liver cells at different FA concentrations

* * : $P<0.01$, compared with control group

3.2 Effect of FA-induced DPC in the kidneys of mice

Figure 3 showed the effect of gaseous FA on DPC levels in kidneys of mice. There was significant difference in the DPC levels between control group and FA inhaled groups($P<0.01$). The results indicated that the DPC levels ascended with the increasing of inhaled gaseous FA concentrations.

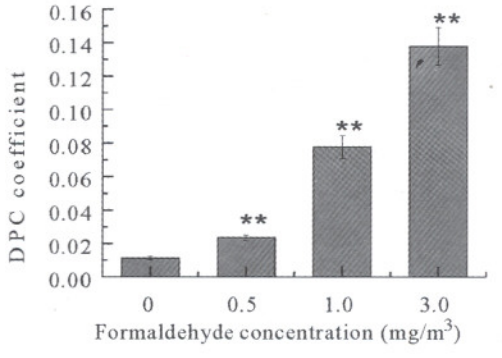

Figure 3. DPC formation in mice kidney cells at different FA concentrations

* * : $P<0.01$, compared with control group

3.3 Effect of FA-induced DPC in the testicles of mice

Figure 4 showed that the DPC coefficient of 0.5 mg/m^3 FA-treated group was significantly higher than that of control group ($P<0.05$), and there were significant difference in DPC coefficient between 1.0 mg/m^3, 3.0 mg/m^3 FA-treated groups and the control group ($P<0.01$). It indicated that there was a clearly dose-dependent rela-

tionship between the DPC coefficient and the concentrations of FA.

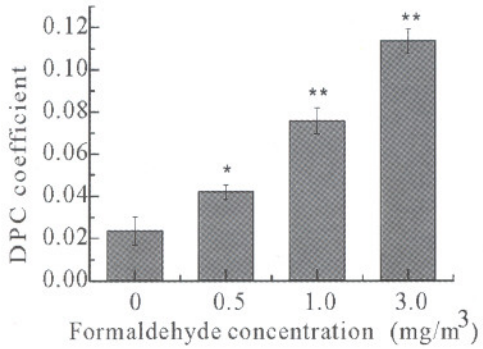

Figure 4. DPC formation in mice testicle cells at different FA concentrations
*: $P<0.05$; **: $P<0.01$, compared with control group

4 Discussion

4.1 Parameters of chamber and the concentrations of gaseous FA

The concentrations of gaseous FA generated by small environmental chamber were measured 3 times per day for 3 days. The measurement results (0.03 ± 0.03 mg/m^3, 0.49 ± 0.03 mg/m^3, 1.03 ± 0.04 mg/m^3 and 3.03 ± 0.08 mg/m^3) were very close to the anticipative concentrations (0 mg/m^3, 0.5 mg/m^3, 1.0 mg/m^3 and 3.0 mg/m^3). It indicated that the gaseous FA from chamber emission was quite stable and reliable.

In the previous studies, gaseous FA was generated from paraformaldehyde. Then high level of FA was diluted with clean, filtered air to achieve the desired gas concentrations[19]. In this way, it was very difficult to control the temperature (T), the humidity (RH) and the gas flux (F) of gaseous FA. It is well-known that the alterations of temperature and humidity could affect the experimental results remarkably. The gas flux of FA is also important to the inhalational quality of mice. In the present study, different concentrations of gaseous FA were generated by small environmental chamber with formalin. In this method, the temperature, the humidity and the gas flux could be set by the chamber before exposure. The parameters of the chamber will be very stable after 3 hours of operation. The results were showed in Figure 5. Thus, this experimental method has great improvement for better reliability and higher reproducibility.

4.2 Comparison of the effect of FA-induced DPC in the three different organs of mice

According to the effect of FA-induced DPC in the three different organs of mice, we could conclude that FA could induce DPC with a dose-dependent relationship between the DPC coefficient and the concentrations of FA. We also found that the DPC level of 3.0 mg/m^3 FA inhaled groups of liver was the highest and that of testicle was the lowest, which indicated that gaseous-FA-induced genotoxicity in the liver of mice was the most severe, then was kidney and the least was testicle.

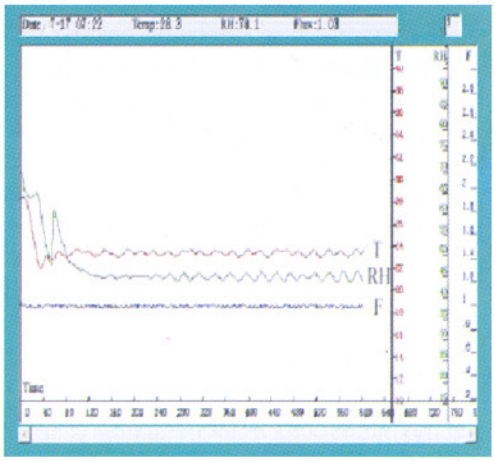

Figure 5. The parameters of the small environmental chamber

4.3 The mechanism of the FA-induced DPC

DPC is relatively permanent in the cells. Due to the poor repair capacity, DNA-protein complexes may be present during DNA replication and possibly cause a loss and inactivity of the important genes such as tumor suppressor genes, and may be responsible for tumor formation. For the electrophilicity of carbonyl and smaller steric hindrance, FA is apt to form DPC. Initially, a hydroxymethyl intermediate is formed by the addition of FA to a primary amine of either DNA or protein. The hydroxymethyl group then condenses with a second primary amine to produce a methylene bridge between DNA and protein. The form of DPC can be expressed as histone-NH-CH_2-NH-DNA. Additionally, as a inhibitor to antioxidases, FA could induce the formation of DPC indirectly, for example, by increasing the content of DPC resulted from the depletion of GSH and the inhibition of glutathioneperoxidase and superoxide dismutase which can clear hydroxy radical and oxygen-derived free radicals[6,13,20,21].

4.4 FA and distant-site toxicity

Whether the FA does have the distant-site toxicity and what is the mechanism still is unknown. Thrasher proposed that rapid removal of FA was insufficient to support that FA could not induce distant-site toxicity, because FA might form adducts with amino acid or other biological molecule in blood, and the adducts could be sent to remote

sites by blood circular system and generate FA again, so these adducts were responsible for tumors caused by FA[22]. In fact, as biochemical active as nitric oxide (NO), it is also able to induce physiological and toxicological effects at distant sites, probably by forming adducts[23,24]. NO is active to sulfhydryl and amino groups, and interestingly, recent work showed that it was also the cause for FA[25]. Therefore, it would be interesting to investigate which kind of adduct formed in blood after exposure to FA, since it had been proposed that the amount of free FA in blood was not altered after exposure.

In the present study, significant DPC could be detected in the three organs of liver, kidney and testicle when the mice were exposed to gaseous FA of higher concentrations, which evidently indicated that FA might induce distant-site toxicity.

5 Conclusions

In this study, KCl-SDS assay was applied to detect the amount of DPC in liver, kidney and testicle of mice. According to the results, we could conclude that FA could induce DPC significantly at the higher concentrations, the results also indicated that FA might induce distant-site toxicity.

Acknowledgments

This work is supported by China National Science Foundation (project coded 30570799).

Correspondence to:
Xu Yang
College of Life Sciences
Huazhong Normal University
No. 152, Luo-Yu Road, Wuhan 430079, China
Email: yangxu@mail.ccnu.edu.cn

References

1. International Agency for Research on Cancer. 2004. IARC classifies formaldehyde as carcinogenic to humans. 2004-06-15, http://www.iarc.fr/ENG/Press-Releases/archives/pr153a,html.
2. Bunde RL, Jarvi EJ, Rosentreter JJ. A piezoelectric method for monitoring formaldehyde induced crosslink formation between poly-lysine and poly-deoxyguanosine. Talanta 2000; 51: 159-71.
3. Conaway CC, Whysner J, Verna LK. Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation. Pharmacol Ther 1996; 71: 29-55.
4. Kuykendall JR, Trela BA, Bogdanffy MS. DNA-protein crosslink formation in rat nasal epithelial cells by hexamethylphosphoramide and its correlation with formaldehyde production. Mutat Res 1995; 343: 209-18.
5. Casanova M, Deyo DF, Heck HD. Covalent binding of inhaled formaldehyde to DNA in the nasal mucosa of Fischer 344 rats: analysis of formaldehyde and DNA by high-performance liquid chromatography and provisional pharmacokinetic interpretation. Fundam Appl Toxicol 1989; 12: 397-417.
6. Casanova M, Morgan KT, Gross EA. DNA-protein cross-links and cell replication at specific sites in the nose of F344 rats exposed subchronically to formaldehyde. Fundam Appl Toxicol 1994; 23: 525-36.
7. Casanova M, Morgan KT, Steinhagen WH, *et al*. Covalent binding of inhaled formaldehyde to DNA in the respiratory tract of Rhesus monkeys: pharmacokinetics, rat-to-monkey interspecies scaling, and extrapolation to man. Fundam Appl Toxicol 1991;17: 403-28.
8. Liu YS, Lu ZS, Yang JW, *et al*. Quantification study on the DNA-protein in crosslinks of human blood lymphocytes induced by formaldehyde. Hubei journal of preventive medicine 2004; 15: 4-7.
9. Heck HD, Casanova M. The implausibility of leukemia induction by formaldehyde: a critical review of the biological evidence on distant-site toxicity. Regul Toxicol Pharmacol 2004; 40: 92-106.
10. Collins JJ, Ness R, Tyl RW. A review of adverse pregnancy outcomes and formaldehyde exposure in human and animal studies. Regul Toxicol Pharmacol 2001; 34: 17-34.
11. Franks SJ. A mathematical model for the absorption and metabolism of formaldehyde vapour by humans. Toxicology and Applied Pharmacology 2005;206: 309-20.
12. Shaham J, Bomstein Y, Gurvich R. DNA-protein crosslinks and p53 protein expression in relation to occupational exposure to formaldehyde. Occup Environ Med 2003; 60: 403-9.
13. Shaham J, Bomstein Y, MeltzerA, *et al*. DNA-protein crosslinks, a biomarker of exposure to formaldehyde-*in vitro* and i*n vivo* studies. Carcinogenesis 1996; 17: 121-5.
14. Coggon D, Harris EC, Poole J. Extended follow-up of a cohort of British chemical workers exposed to formaldehyde. J Natl Cancer Inst 2003; 95: 1608-15.
15. Hauptmann M, Lubin JH, Stewart PA. Mortality from lymphohematopoietic malignancies among workers in formaldehyde industries. J Natl Cancer Inst 2003; 95: 1615-23.
16. Zhitkovich A, Costa M. A simple, sensitive assay to detect DNA-protein crosslinks in intact cells and *in vivo*. Carcinogenesis 1992; 13:1485-9.
17. Chakrabarti SK, Bai CJ, Subramanian KS. DNA-protein crosslinks induced by nickel compounds inisolated rat renal cortical cells and its antagonism by specific amino acids and magnesium ion. Toxicol Appl Phamacol 1999; 154: 245-55.
18. Cesarone CF, Bolognesi C, Santi L. Improved microfluorometric DNA determination in biological materical using 33258 Hoechst. Anal Biochem 1979; 100: 188-97.
19. Fujimaki H, Kurokawa Y, Kunugita N, *et al*. Differential immunogenic and neurogenic inflammatory responses in an allergic mouse model exposed to low levels of formaldehyde. Toxicology 2004; 197: 1-13.
20. Speit G, Schutz P, Merk O. Induction and repair of formaldehyde-induced DNA-protein crosslinks in repair-deficient human cell lines. Mutagenesis 2000; 15: 85-

90.
21. Takashima H, Boerkoel CF, John J. Mutation of TDP1, encoding a topoisomerase I-dependent DNA damage repair enzyme, in spinocerebellar ataxia with axonal neuropathy. Nature Genet 2002; 32: 267 – 72.
22. Thrasher JD, Kilburn KH. Embryo toxicity and teratogenicity of formaldehyde. Arch Environ Health 2001; 56: 300 – 11.
23. Hogg N. The biochemistry and physiology of S-nitrosothiols. Annu Rev Pharmacol Toxicol 2002; 42: 585 – 600.
24. Weinberger B, Laskin DL, Heck DE, *et al*. The toxicology of inhaled nitric oxide. Toxicol Sci 2001; 59: 5 – 16.
25. Metz B, Kersten GF, Hoogerhout P, *et al*. Identification of formaldehyde-induced modifications in proteins. Biol Chem 2004; 279: 6235 – 43.

Received June 5, 2006