Effect of increasing graded doses of formaldehyde on human embryonic kidney cells

Banerjee A.[A-F], Dholey M.[E], Mukherjee S.[A,C,D,E], Maji B.K.*[A,C,D,E,F]

Department of Physiology (UG & PG), Serampore College, Serampore, West Bengal, India

---

A-Conception and study design; **B-**Collection of data; **C**-Data analysis; **D-**Writing the paper; **E**-Review article; **F** –Approval of the final version of the article

---

ABSTRACT

Purpose: In the present study, we aimed to identify the effect of three increasing doses of most widely preservative, formaldehyde (FA) on the urinary system using human embryonic kidney cells (HEK-293) *in vitro*.

Materials and methods: The HEK-293 cells were grown in Dulbecco's Modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% of fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml of streptomycin (Gibco, USA) using 25cm^2 flasks (Nunc, Denmark), in a CO_2 incubator (Heal Force) at 37°C are treated with 19 % formaldehyde in DMEM supplemented with 10% of fetal bovine serum to yield final concentrations of 0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/mlof 19% FA.

Results: The result showed that statistically significant dose dependent decrease in cell viability of HEK-293 cells with exposure to increasing concentration of FA (0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/ml) on MTT assay. Extremely huge dose dependent DNA damage with a dose dependent up regulation of mRNA expression of pro-apoptotic iNOS and TNF-α gene was observed when HEK-293 cells are treated with the increasing concentration of FA. In addition, the protein expression levels of Bcl-2 and Bax revealed that increasing concentration of FA on HEK-293 cells down regulated the anti-apoptotic Bcl-2 and up regulated pro-apoptotic Bax gene.

Conclusions: Our result indicates that, high dose of FA is more dangerous than the low dose on HEK-293 cells with cellular damage.

Keywords: Formaldehyde, human embryonic kidney cells, high and low dose

---

DOI: 10.5604/01.3001.0012.1126

***Corresponding author:**
Dr Bithin Kumar Maji, Associate Professor
Department of Physiology (UG & PG), Serampore College, 9 William Carey Road,
Serampore, Hooghly-712201 West Bengal, India
e-mail id: bm_scp@yahoo.in; Tel. +91- 9433509890

Received: 17.04.2018
Accepted: 13.05.2018
Progress in Health Sciences
Vol. 8(1) 2018 pp 36-45

INTRODUCTION

Most widely used water soluble, simplest naturally occurring organic compound formaldehyde (FA) is an indoor air waste matter aldehyde and a potent carcinogen. Usually FA is widely used in the production of business resins in constructive insulations, dyes and plastics, textiles and the wood and plywood industries, and it is present in concrete and plaster, glass frames, fire extinguishers, platinum electrodes, cables, rubber, furniture, carpet and wallboards in industries, laboratories for medical purpose and in some routinely used materials, e.g., liquid-based home cleaning agents, deodorants, toothpaste and cosmetic products, steam disinfectants and other disinfectants, ink, photographs, cartoons, paper and adhesives. It has been reported that cigarette smoke also contains FA; thus, both active and passive smokers are affected by FA via inhalation [1-2].

FA may cause in the urinary tract infection when it is employed in dental coating materials and as a protecting agent in some medicine. Haemodialysis solutions contain FA. It also causes air pollution as a result of it exists within the exhaust of diesel vehicles, in heating oil, in gas and in fossil fuel, and it's discharged once the burning of trees and firewood. It has been reported that cigarette smoke conjointly contains FA; so, each active and passive smokers square measure plagued by FA via inhalation [3-5].

Formaldehyde, when taken into organisms, is metabolized into formic acid in the liver and erythrocytes and is then excreted, either with the urine and faces or via the respiratory system. Industrial to medical fields, employees in these fields are frequently exposed to it. Students working in medical laboratory are affected frequently by formaldehyde gas during dissection lessons. Because full protection from formaldehyde is impossible for employees in industrial plants using this chemical and for workers in laboratory conditions, several measures can be implemented to prevent and/or reduce the toxic effects of formaldehyde. The genitourinary system includes a major role in termination of noxious materials in most routine protocols. It is a significant role in filtration, metabolism and deification of xenobiotic or their metabolic product. Chemical materials or their active metabolic varieties will transfer from plasma to urinary organ tubules and compared to alternative tissues will have a manifold concentration. Kidneys ought to receive twenty fifth of heart output so as to possess an honest distribution of chemical materials. Some people expertise no discomfort from FA, not withstanding exposed to moderate levels, whereas terribly low levels of FA will have an effect on others. However, there is a detailed relationship between doses of FA with affected organ damage. Low doses of FA exposure will have an effect on the eyes, nervous system and upper respiratory tract, whereas higher doses of FA exposure may end up in lower tract damage. In cases of terribly high doses of FA exposure, death will occur. FA, that causes a rise in cytotoxic effects by compromising the intracellular balance, includes a sturdy tendency to mix with proteins, nucleic acids (DNA and RNA) and unsaturated fatty acids by non-enzymatic processes. These combos lead to inflammatory reactions, allergic reactions, toxicity in cells, necrosis and mutagenic and malignant effects by developing denaturation in proteins. Moreover, free oxygen radicals are elevated within the tissues plagued by FA, and this increase accelerates the processes cause cell death [6].

Experimental studies have incontestable the toxic effects of FA on the urinary system. It absolutely was found that FA administered orally to rats caused membrane ulcers, necrosis, hemorrhage and perforation within the gastrointestinal system and afterward, the event of acidosis, cardiovascular disease, hematuria, anuria and renal papillary necrosis [7]. Previous studies showed that exposure of FA on renal cell cultures from monkeys resulted in depression of RNA synthesis and failure of DNA transcription-termination [8]. FA is a potential bio-marker of bladder and prostate cancer and may also leads to bladder cancers [9]. According to the National Toxicology Program, it was reported that higher levels of formaldehyde exposure caused kidney cancers. DNA-protein cross links (DPCs) are produced endogenously as intermediates during normal DNA metabolism and as by products of abortive base excision repair [10]. DPCs are also produced upon exposure to exogenous DNA-damaging agents such as ionizing radiation, metal compounds, x-rays, oxygen radicals, and reactive aldehydes [11-16]. Major form of DNA damage occurs by the generation of DPCs from FA [17]. The genotoxicity of the formaldehyde is incontrovertible in that it generates DPCs and induces varied chromosomal alterations as antecedent delineate. FA was conjointly found to cause various histopathological changes like loss of cytoplasm and hyperchromatic nuclei in livers [18]. Expression of genes related to macromolecule metabolism, apoptosis, and metabolism, regulation was conjointly altered aldehyde exposure [19-20]. It has been reportable that FA has harmful effects on the skin, respiratory system, nervous system, digestive and urinary apparatus, also as allergic effects. In the present study, we aimed to identify the toxic effect of

FA on the urinary system using human embryonic kidney cells (HEK-293) *in vitro*.

MATERIALS AND METHODS

Cell cultures

The HEK-293 cells were grown in Dulbecco's Modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% of fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml of streptomycin (Gibco, USA) using 25cm^2 flasks (Nunc, Denmark), in a CO_2 incubator (Heal Force) at 37°C.

Formaldehyde solution preparation

19 % formaldehyde was prepared in distilled water from analytical reagent grade stock solutions. After preliminary trials to identify appropriate concentration ranges, serial dilutions of formaldehyde was made in DMEM supplemented with 10% of fetal bovine serum to yield final concentrations of 0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/mlof 19% FA [21].

MTT assay

Trypan blue was utilized to determine the viability of cells. Exponentially growing cells were harvested, counted by hemocytometer and diluted with medium, yielding a concentration of 1×10^6 cells/ml. From this cell suspension, 100 µl was pipetted into 96-well microtiter plates (Nunc, Denmark) and incubated for 24 hours in a 5% CO_2 incubator (Heal Force) at 37°C. Cells were then treated with 0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/mlof FA solution. After adding the samples (FA), new medium was added to make the final volume of 200 µl per well. The plate was then incubated in the 5% CO_2 incubator at 37°C for 24 hours. At the end of treatment time, the medium was replaced by 100 µl MTT (Sigma, St Louis, MO) per well and incubated for another 4 hours at 37°C. The reaction was stopped by adding 100 µl DMSO, AR grade (Sigma) to each well to dissolve the purple-blue MTT formazan precipitate. Finally, the absorbance was read with the ELISA reader (LX-800) and calculates the percentage of viable and non-viable cells [22].

Comet assay

Trypsin (0.025%) was added to the cells after removing media. At that time point, the cells were kept at 37°C for 5 minutes to detach cells. Clear window frosted microscopic slide was coated with 1% low melting agarose. The well was punched at one side of the slide the aliquot of 10 µl cell suspensions (~10,000 cells in 10 µl or less volume) was added and to this 20 µl of lysis solution was added followed by another coat of 0.5% of low melting agarose at 37°C. The slide was placed in moisture chamber for overnight. After lysis, the slide was immersed in the freshly prepared electrophoresis buffer (10 mM Tris, 0.08 mM Boric acid, 0.5 M EDTA, pH 8.2) and electrophoresed for 1 h at 50 V after electrophoresis slide was dehydrated by immersing in absolute alcohol for 3-5 times. Then slide was stained with ethidium bromide followed by observation under trans-illuminator for comet formation and image was photographed and analyzed [22].

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted using TriZol reagent (Invitrogen, Carlsbad, CA). Oligo (dT)-primed RNA (1µg) was reverse transcribed using the Super Script II transcriptase kit (RR047A, Takara, Shiga, Japan) according to the manufacturer's instructions. cDNA obtained was amplified by PCR with Taq DNA polymerase (Fermentas, Burlington, Canada). The presence of possible target genes iNOS and TNF-α were determined using the obtained cDNA and β-actin as the internal control (Table 1). The PCR products were then resolved on 1% agarose gel [22]. The bands were identified based on the product size using DNA ladder. Gel images were analyzed by scanning densitometry (Image J, NIH) and values were normalized to quantity of β-actin and presented as percentage mRNA relative to control.

Table 1. The sequences of primers used for amplification

Primer	Forward primer	Reverse primer
β-actin	5′-CATGTACGTTGCTATCCAGGC-3′	5′-CTCCTTAATGTCACGCACGAT-3′
iNOS	5′-TCTTGGTCAAAGCTGTGCTC-3′	5′-CATTGCCAAACCTACTGGTC-3′
TNF-α	5′-CTTCTC CTT CCT GAT CGT GG-3′	5′-GCT GGT TAT CTC TCA GCT CCA-3′

Western blotting

Protein was extracted by Minute TM Total Protein Extraction Kit (For Animal Cultured Cells and Tissues). To detect changes in apoptosis-related

proteins (Bcl-2, Bax), HEK-293 cells (1 $\times 10^7$ cells) seeded on a 10 cm^2 dish were treated with formaldehyde at different concentrations. The cells were harvested and lysed using a lysis buffer, and the protein concentrations of the cell lysates were quantified with a protein assay kit. The equivalent of 20-50 µg of total proteins were separated on 10% SDS-PAGE, then electro blotted on PVDF membrane; the membranes were blocked with 10% non-fat dry milk in TBS-T overnight at 4°C, then incubated with primary antibodies against with Bcl-2 and Bax, GAPDH (1:1000) for 1.5 h at room temperature. After a wash with TBS-T, membranes were incubated with HRP-conjugated secondary antibodies (dilution 1:1,000) for 1 h at room temperature. Finally, the protein-antibody complexes were visualized by chemiluminescence (ECL system, Pierce). Protein expression was normalized to that of GAPDH [22].

Statistical analysis

Data were expressed as Mean ± SE. Kruskal-Wallis non parametric one-way analysis of variance (ANOVA) test was performed to find whether or not scores of different groups differ significantly and to test intergroup significant difference, Mann-Whitney U multiple comparison tests was performed by using Stat Direct Software (UK). Differences were considered significant if $P<0.05$.

RESULTS AND DISCUSSION

The footmarks of FA on the viability of HEK-293 cells were tested by the MTT assay. The result showed that different concentrations of FA could potently suppress the cell growth. MTT assay exhibited a significant dose dependent decrease in cell viability of HEK-293 cell line. Cell viability reduced from 100% to 72.6%, 55.3%, 40.3% and 10.3% as the dose of FA was increased from 0µl/ml to 0.05µl/ml ($P<0.05$), 0.1 µl/ml ($P<0.01$) to 0.5 µl/ml ($P<0.01$) and1.0 µl/ml ($P<0.001$), respectively pointing out to the fact that the cell morphology was diminished with the increasing concentration of the FA (Figure 1). Contrasted with the control group, the number of surviving cells decreased over incubation time and with increasing FA concentrations. This recommends presentation to low concentrations for longer exposure could result in cytotoxicity comparable to presentation to higher concentrations for shorter exposure. In this manner, this outcome unmistakably shows that the inhibitory effect of FA on HEK-293 cell growth was exerted in a time and concentration dependent manner.

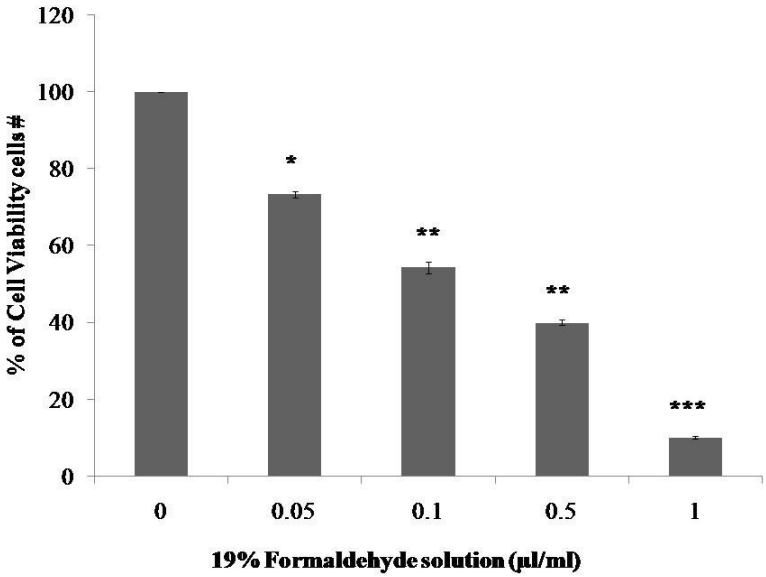

Figure 1: Effect of FA (0µl/ml, 0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/ml) on HEK-293 cell line on cell viability assay (MTT assay). #Significance based on Kruskal-Wallis test ($P<0.05$).*Significance based on Mann-Whitney U multiple comparison test: Control vs 0.05 µl/ml FA ($P<0.05$), Control vs 0.1 µl/ml FA ($P<0.01$), Control vs 0.5 µl/ml FA($P<0.01$), Control vs 1.0 µl/ml FA ($P<0.001$).

Usually utilized system for the estimation of apoptosis is comet test which is a quick and touchy strategy for the recognition of DNA damage in individual cells, instigated by various genotoxic

specialists. In the present study, FA prompted DNA damage of HEK-293 cells with expanding length of comet tail were likewise inspected and analyzed by software (Figures 2 and 3).

Extremely huge dose dependent DNA damage was observed after treatment of HEK-293 human embryonic kidney cell lines with FA. Aftereffects of this study uncovered that HEK-293 cells demonstrated most astounding comet tail length when these cells were treated with 1.0 ul/ml ($P<0.001$) FA. To decide the molecular mechanisms of FA induced apoptosis in HEK-293 cells, the expression levels of several apoptosis-related genes and proteins were inspected. Results revealed that there was a dose dependent up regulation of mRNA expression of pro-apoptotic iNOS and TNF-α gene (Figure 4, 5) was observed when HEK-293 cells are treated with the increasing concentration of FA (0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/ml). The endogenous control β-actin showed strong positive bands.

Further, the protein expression levels of Bcl-2 and Bax were verified by western blotting. Results revealed that increasing concentration of FA down regulated the anti-apoptotic Bcl-2 gene as evidenced by dose dependent decreased protein expression (Figure 6). Whereas, dose dependent up regulation of protein expression of pro-apoptotic Bax gene (Figure 7) was observed when HEK-293 cells are treated with the increasing concentration of FA(0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/ml). The endogenous control GAPDH showed strong positive bands.

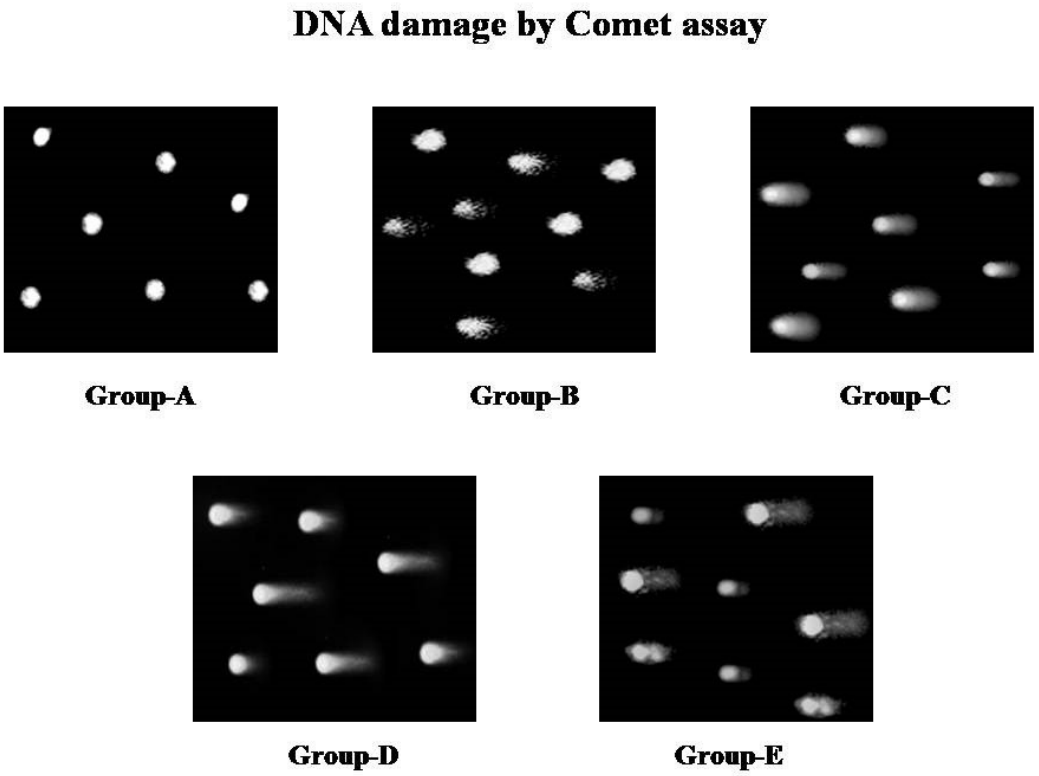

Figure 2. Effect of FA on HEK-293 cell line onDNA damage by Comet assay at the doses of 0µl/ml (A), 0.05 µl/ml (B), 0.1 µl/ml (C), 0.5 µl/ml (D) and 1.0 µl/ml (E)

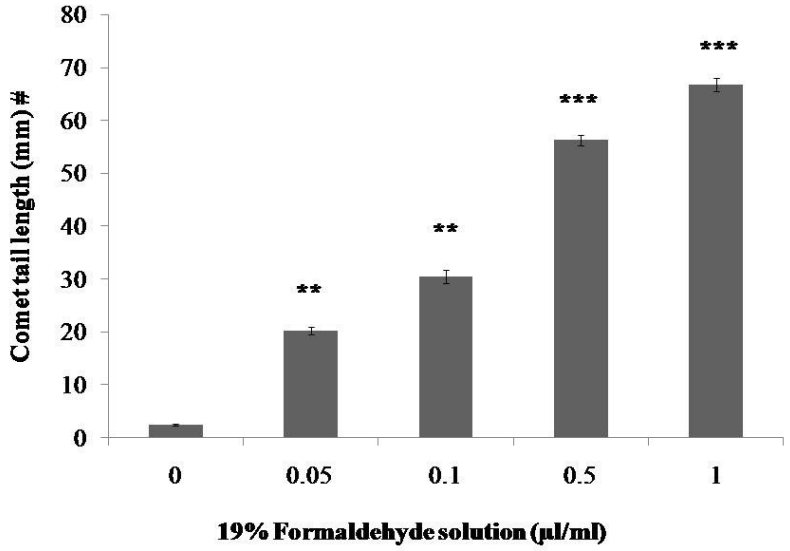

Figure 3. Effect of FA (0µl/ml, 0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/ml) on HEK-293 cell line on DNA damage by Comet assay with respective DNA tail length. #Significance based on Kruskal-Wallis test (P<0.05).*Significance based on Mann-Whitney U multiple comparison test: Control vs 0.05 µl/ml FA (P<0.05), Control vs 0.1 µl/ml FA (P<0.01), Control vs 0.5 µl/ml FA(P<0.001), Control vs 1.0 µl/ml FA (P<0.001)

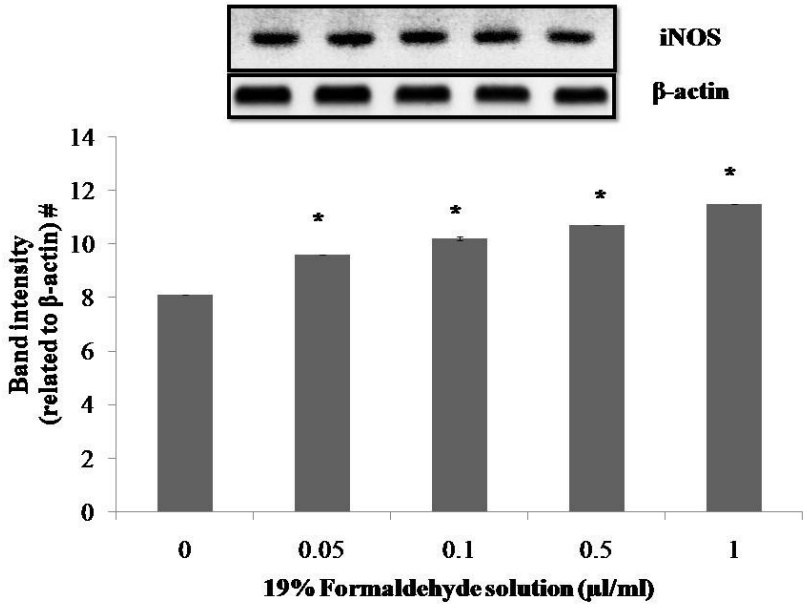

Figure 4. Effect of FA (0µl/ml, 0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/ml) on HEK-293 cell line on mRNA expression level of iNOS. #Significance based on Kruskal-Wallis test (P<0.05).*Significance based on Mann-Whitney U multiple comparison test: Control vs 0.05 µl/ml FA (P<0.05), Control vs 0.1 µl/ml FA (P<0.05), Control vs 0.5 µl/ml FA(P<0.05), Control vs 1.0 µl/ml FA (P<0.05)

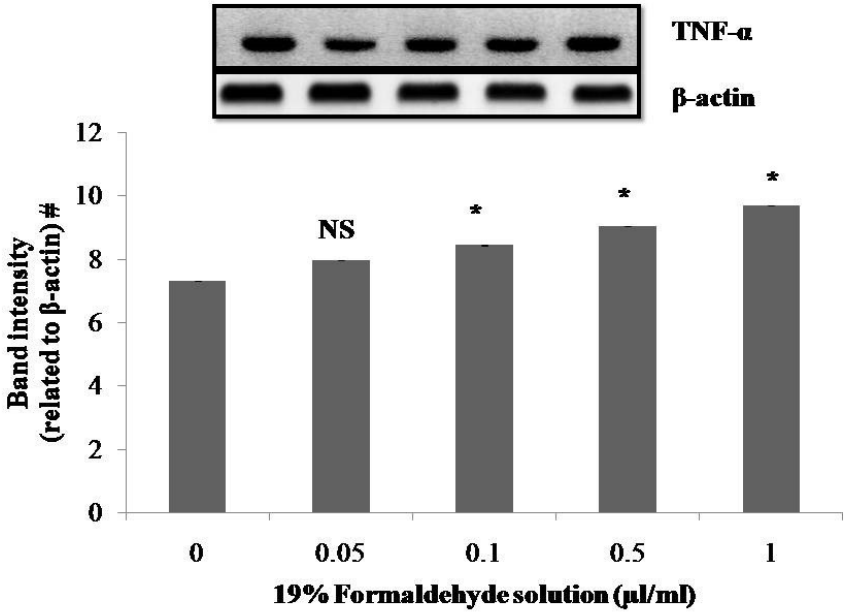

Figure 5. Effect of FA (0µl/ml, 0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/ml) on HEK-293 cell line on mRNA expression level of TNF-α. #Significance based on Kruskal-Wallis test (P<0.05).*Significance based on Mann-Whitney U multiple comparison test: Control vs 0.05 µl/ml FA (NS: not significant), Control vs 0.1 µl/ml FA (P<0.05), Control vs 0.5 µl/ml FA(P<0.05), Control vs 1.0 µl/ml FA (P<0.05)

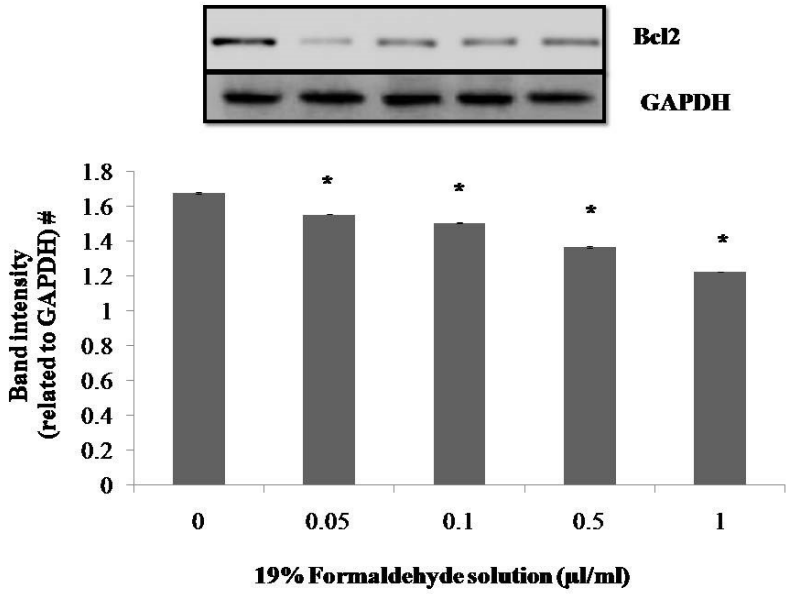

Figure 6. Effect of FA (0µl/ml, 0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/ml) on HEK-293 cell line on Wester blot of Bcl-2. #Significance based on Kruskal-Wallis test (P<0.05).*Significance based on Mann-Whitney U multiple comparison test: Control vs 0.05 µl/ml FA (P<0.05), Control vs 0.1 µl/ml FA (P<0.05), Control vs 0.5 µl/ml FA(P<0.05), Control vs 1.0 µl/ml FA (P<0.05)

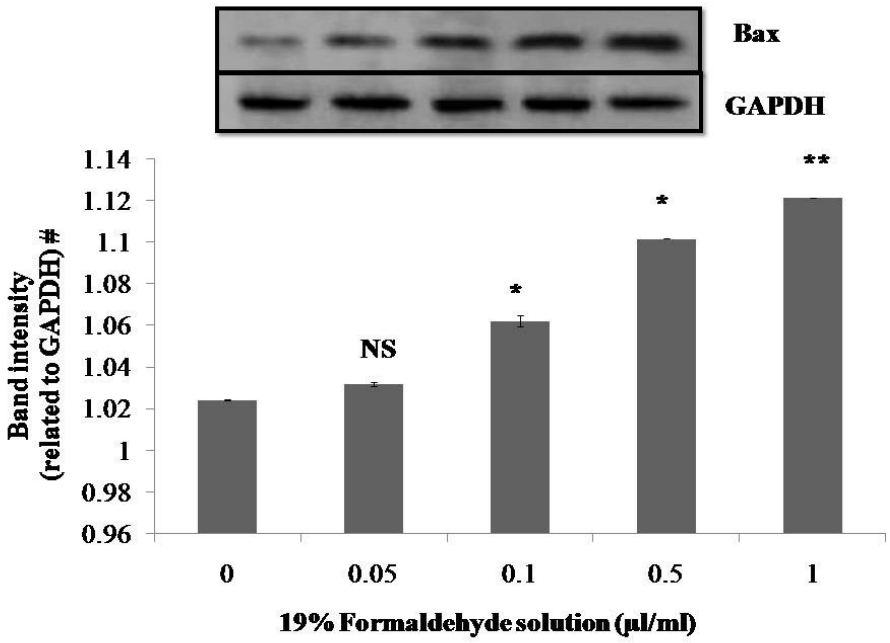

Figure 7. Effect of FA (0µl/ml, 0.05 µl/ml, 0.1 µl/ml, 0.5 µl/ml and 1.0 µl/ml) on HEK-293 cell line on Wester blot of Bax. #Significance based on Kruskal-Wallis test (P<0.05).*Significance based on Mann-Whitney U multiple comparison test: Control vs 0.05 µl/ml FA (NS: not significant), Control vs 0.1 µl/ml FA (P<0.05), Control vs 0.5 µl/ml FA(P<0.05), Control vs 1.0 µl/ml FA (P<0.01)

Enzyme assay provides quantitative approach for evaluating cytotoxicity than more other generalized used techniques such as dye exclusion or uptake and the assay is able to detect any degree of enzyme inhibition. The MTT assay used here is technically simple, especially since the enzyme assay is conducted in the multi well tray without disruption of the cell monolayer [23-27]. Many different cell types have been used for cytotoxicity studies. Ideally, using a human target cell that is affected by the agent in normal clinical use is highly desirable [28]. Apoptosis assumes a vital role in the cancer prevention. A cell fails to undergo apoptosis by means of mutation or biochemical hindrance, can keep dividing and develop to become a tumor [29].

The single cell gel electrophoresis (SCGE) or comet assay combines the simplicity of biochemical techniques for detecting DNA single strand breaks, alkali-labile sites and cross linking with the single cell approach typical of cytogenetic assays of DNA damage. In the present study, treatment of HEK-293 cells with FA was resulted in increased comet parameters like tail length, thereby indicating that the level of DNA damage increased in a dose-dependent manner. During the endotoximic episodes, HEK-293 cells are activated to secrete a wide array of inflammatory mediators including cytokins (TNF-α), reactive oxygen, nitrogen intermediates (NO). Among these pro-inflammatory mediators, TNF-α plays a critical role in FA induced kidney injury. NO has recently be recognized as an important molecule in inflammation. In the kidney it is produced by two different isoforms of nitric oxide synthase (NOS), e.g., endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). iNOS has been shown to be implicated in pathogenesis of many kidney injuries such as, renal injury during endotoxemia, ischemia-reperfusion injury, toxin mediated kidney damage. We found an increase in TNF-α due to an increased expression of iNOS, which led to a high production of NO, in the increasing concentration of FA exposure on HEK-293 cells. In our study, increased level of iNOS and TNF-α mRNA expression in the HEK-293 cells were also statistically significant (P<0.05).

Bcl-2 is astounding for imparting its anti-apoptotic role in various cell types. It has been found that Bcl-2 play an important role in resistance of cancer cells to chemotherapy or radiation therapy [30]. Anti-apoptotic Bcl-2 is now a prime focus for

novel anti-cancer therapeutics as a result of its commitment to cancer development and progression by promoting cell survival. On contrary, Bax is pro-apoptotic in nature and is distributed over many tissues of human body. Bax can offer apoptotic signal to a cell by forming homodimer and Bcl-2 forms competing heterodimers with Bax and can inhibit the development of apoptosis [31].

In the present study, the average band intensity of Bcl-2 was lower in HEK-293 cells supplemented with 1.0 ul/ml FA than the cells without supplementation. Conversely, FA dose dependently promotes Bax expression in HEK-293 cells. Lopsidedness of molecular mechanisms of proliferation and apoptosis has been found to be associated with the development of kidney damage, emphasizing the reduction of apoptosis as a major underpinning factor. In the present study, it is evident that FA has promoted the Bax up regulation simultaneously with Bcl-2 down regulation in a dose dependent manner in HEK-293 cells. Therefore, these results are well in line with the earlier report that there are up regulation of pro-apoptotic proteins Bax and Bak and down regulation of anti-apoptotic protein Bcl-2 in cell lines undergoing apoptosis [32]. In this way, we speculate that FA regulate balance between Bcl-2 and Bax expression and induces apoptosis in human embryonic kidney cells in the present study.

Furthermore, our data indicate that the aqueous extract of FA specifically reduce viability of HEK-293 cell lines possibly through G0/G1 or S phase arrest or possibly via induction of sub-G0/G1 DNA fragmentation [22]. Be that as it may, the mechanism of the action is still pending. Along these lines, further investigation of the molecular mechanism involved is needed to fully understand the use of FA as a toxic agent.

CONCLUSIONS

In conclusion, our studies demonstrate that FA has a significant cytotoxic and apoptotic effect on human embryonic kidney cells and this also recommends presentation to low concentrations for longer exposure could result in cytotoxicity comparable to presentation to higher concentrations for shorter exposure. In general, the antagonistic effect of formaldehyde on cell growth and endogenous formaldehyde can be explained by the role of formaldehyde in signaling pathways. These pathways will be studied in detail in future research. Exposure to formaldehyde interferes with the cell's metabolic regulation, changes gene expression and apoptosis.

Conflicts of interest

The authors declared no conflicts of interest.

Acknowledgement

Authors are grateful to Prof. Dhrubajyoti Chattopadhyay, Vice Chancellor of Amity University, Kolkata, for his continuous encouragement and valuable suggestions. We are indebted to Dr. Vansanglura, Principal of Serampore College for his active administrative support and encouragement during the experiment. We are thankful to Mr. Gaurab Banerjee, Mr. Abhishek Kantha, Mr. Aniruddha Sengupta, Ms. Piya Majumdar, Mr. Rajarshi Paul, Mr. Sanjib Misra, Mr. Pradip Kumar Mondal, Ms. Dipali Das, Mr. Prabir Kumar Das, Mr. Gopal Murdinga, Department of Physiology (UG & PG), Serampore College for their technical help during this study.

REFERENCES

1. Smith AE. Formaldehyde. Occup Med (Lond) 1992 May;42(2):83-8.
2. Starr TB, Gibson JE. The mechanistic toxicology of formaldehyde and its implications for quantitative risk estimation. Annu Rev Pharmacol Toxicol 1985 Apr;25:745-67.
3. Bolt HM. Experimental toxicology of formaldehyde. J Cancer Res Clin Oncol 1987 June;113(4):305-9.
4. Upreti RK, Farooqui MY, Ahmed AE, Ansari GA. Toxicokinetics and molecular interaction of [14C]-formaldehyde in rats. Arch Environ Contam Toxicol 1987 May;16(3):263-73.
5. Songur A, Akpolat N, KusI, Ozen OA, ZararsizI, Sarsilmaz M. The effects of the inhaled formaldehyde during the early postnatal period in the hippocampus of rats: a morphological and immune-histochemical study. Neurosci Res Commun 2003 Nov;33(3):168-78.
6. Mehmet I, Ismail Z, Mursel D, Sadik G. Toxic effects of formaldehyde on the urinary system. Turk J Urol 2013 Mar;39(1):48–52.
7. Til HP, Woutersen RA, Feron VJ, Clary JJ. Evaluation of the oral toxicity of acetaldehyde and formaldehyde in a 4-week drinking-water study in rats. Food Chem Toxicol 1988 May; 26(5):447-52.
8. Nocentini S, Moreno G, Coppey J. Survival DNA synthesis and ribosomal RNA transcription in monkey kidney cells treated by formaldehyde. Mutat Res 1980 Apr;70(2):231-4.
9. Yang M. A current global view of environmental and occupational cancers. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2011

Jul;29(3):223-49.
10. Reard on JT, Cheng Y, Sancar A. Repair of DNA-protein cross-links in mammalian cells. Cell Cycle 2006 Jul;5(13):1366-70.
11. Fornace AJ, Little JB. DNA cross linking induced by x-rays and chemical agents. Biochim Biophys Acta. 1977 Aug 16;477(4):343-55.
12. Fornace AJ, Seres DS. Repair of trans-Pt(II) diamminedichloride DNA-protein crosslinks in normal and excision-deficient human cells. Mutat Res 1982 Jun;94(2):277-84.
13. Izzotti A, Cartiglia C, Taningher M, De Flora S, Balansky R. Age-related increases of 8-hydroxy-2'-deoxyguanosine and DNA-protein crosslinks in mouse organs. Mutat Res. 1999 Dec; 446(2): 215-23.
14. Kuykendall JR, Bogdanffy MS. Efficiency of DNA-histone crosslinking induced by saturated and unsaturated aldehydes in vitro. Mutat Res 1992 Oct;283(2):131-6.
15. Merk O, Reiser K, Speit G. Analysis of chromate-induced DNA-protein crosslinks with the comet assay. Mutat Res. 2000 Nov; 20;471(1-2):71-80.
16. Olinski R, Nackerdien Z, Dizdaroglu M. DNA-protein cross-linking between thymine and tyrosine in chromatin of gamma-irradiated or H_2O_2-treated cultured human cells. Arch Biochem Biophys. 1992 Aug;297(1):139-43.
17. Casanova M, Morgan KT, Gross EA, Moss OR, Heck HA. DNA-protein cross-links and cell replication at specific sites in the nose of F344 rats exposed subchronically to formaldehyde. Fundam Appl Toxicol 1994 Nov;23(4):525-36.
18. Cikmaz S, Kutoglu T, Kanter M, Mesut R. Effect of formaldehyde inhalation on rat livers: a light and electron microscopic study. Toxicol Ind Health 2010 Mar;26(2):113-9.
19. Li GY, Lee HY, Shin HS, Kim HY, Lim CH, Lee BH. Identification of gene markers for formaldehyde exposure in humans. Environ Health Perspect. 2007 Oct;115(10):1460–6.
20. Neuss S, Holzmann K, Speit G. Gene expression changes in primary human nasal epithelial cells exposed to formaldehyde in vitro. Toxicol Lett 2010 Jul;198(2):289-95.
21. Hsiao WS, Robert JF, Harold HM. Cytotoxicity of glutaraldehyde and formaldehyde in relation to time of exposure and concentration. Pediatr Dent 1990 Sep-Oct;12(5):303-7.
22. Banerjee A, Das D, Maji BK, Mukherjee S. An *in vitro* cytotoxic activity of Bees' honey in Huh-7 cell line. Adv Biotech & Micro 2018 Apr;9(1):555751.
23. Guess WL, Rosenbluth SA, Schmidt B, Autian J. Agar diffusion method for toxicity screening of plastics on cultured cell monolayers. J Pharm Sci 1965 Oct;54(10):1545-7.
24. Tronstad L, Wennberg A, Hasselgren G. Screening tests for dental materials. J Endod 1978 Oct;4(10):304-7.
25. Stanford JW. Recommended standard practices for biological evaluation of dental materials. Int Dent J 1986 Mar;36(1):45-8.
26. Mosman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983 Dec 16;65(1-2):55-63.
27. Edmondson JM, Armstrong LS, Martinez AO. A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. J Tissue Cult Methods. 1988 Mar;11(1):15-17.
28. Rounds DE. General toxicology: target cell approach with differentiated cell systems in Short Term In Vitro Testing for Carcinogenesis. Mutagenesis and Toxicity, Berky J, Serrod PCe ds. Philadelphia: Franklin Institute Press 1978;215.
29. Williams NG, Roberts T.M. Signal transduction pathways involving the Raf proto oncogene. Cancer Metastasis Rev 1994 Mar;13(1):105-16.
30. Terada T, Nakanuma Y. Expression of apoptosis, proliferating cell nuclear antigen, and apoptosis-related antigens (bcl-2, cmyc, Fas, Lewis(y) and p53) in human cholangio-carcinomas and hepatocellular carcinomas. Pathol Int 1996 Oct;46(10):764-70.
31. Naseri MH, Mahdavi M, Davoodi J, Tackallou SH, Goudarzvand M, Neishabouri SH. Up regulation of Bax and down regulation of Bcl2 during 3-NC mediated apoptosis in human cancer cells. Cancer Cell Int 2015 May;15(1):55-63.
32. Hill MM, Adrainm C, Duriez PJ, Creagh EM, Martin SJ. Analysis of thecomposition, assembly kinetics and activity of native Apaf-1 apoptosomes. EMBO J. 2004 May;23(10):2134-45.