Toxicology Research

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/toxicology

Oxidatively DNA damaged and formalin-fixation procedures

Marco E. M. Peluso [1,*], Armelle Munnia [1], Mirko Tarocchi [2], Roger W. Giese [3], Laura Annaratone [4], Gianni Bussolati [4], and Roberto Bono [5]

[1] Cancer Risk Factor Branch, Cancer Prevention and Research Institute, Florence, Italy

[2] Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy

[3] Department of Pharmaceutical Sciences in the Bouve College of Health Sciences, Barnett Institute, Northeastern University, Boston, Massachusetts

[4] Department of Medical Sciences, University of Turin, Turin, Italy

[5] Department of Public Health and Pediatry, University of Turin, Turin, Italy

* Correspondence: Dr. M.E.M. Peluso, M.Sc. PhD. Cancer Risk Factor Branch, Cancer Prevention and Research Institute, Via Cosimo il Vecchio, 2, 50139, Florence, Italy. Tel: 39 (0)55 32697867; Fax: + 39 055 326 97 879. Email: m.peluso@ispo.toscana.it

Table of contents entry

An experimental study of how formaldehyde-fixation is capable to induce excess of oxidative DNA damage in formalin-fixed paraffin-embedded tissues.

Abstract

Formaldehyde is the most commonly used fixative for the preparation of formalin-fixed paraffin-embedded tissues (FFPETs) in the "reduction rooms" of pathology wards. Therefore, we analysed for the generation of 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-α]purin-10(3H)-one adducts (M_1dG), an exocyclic DNA adduct considered to be a biomarker of oxidative stress and lipid peroxidation, in DNA extracted from the FFPETs of six liver and lung of C57BL/6 control mice and from the FFPETs of four cancer patients in respect to paired flash-frozen tissues using ^{32}P-postlabeling. When the experimental animals were examined, the percentage of M_1dG adducts was about 4-6 fold greater with the FFPET mouse samples as compared to flash-frozen mouse samples. Specifically, 4.75 M_1dG ± 0.21 (SE) per 10^6 normal nucleotides (nn) were detected in the FFPET liver samples, and 1.07 M_1dG ± 0.08 (SE) per 10^6 nn in the flash-frozen liver(*p=0.02*). Then, 3.80 M_1dG ± 0.73 (SE) per 10^6 nn were measured in the FFPET lung samples, and 1.02 M_1dG ± 0.07 (SE) per 10^6 nn in the flash-frozen lung (*p=0.02*). Also, significantly increased levels of oxidatively damaged DNA were detected in the human colon DNA from the FFPETs in respect to the flash-frozen tissues. There were 30.2 M_1dG ± 7.7 (SE) per 10^8 nn in the colon mucosa DNA from the FFPETs and 4.4 M_1dG ± 0.7 (SE) per 10^8 nn in the corresponding flash-frozen human tissues (*p=0.016*). Formalin penetration through cell membrane components induces excess oxidative stress, causing both direct oxidation in DNA and increased lipid peroxidation, which in turn produces M_1dG adducts, a kind of DNA damage that can partially block DNA synthesis and induce error prone translesion synthesis. Excess of exocyclic DNA adducts in formalin-fixed specimens can stall DNA polymerases and contribute to the induction of artefactual sequence alterations during PRC amplification.

7

Introduction

Formaldehyde is the most commonly used fixative in the "reduction rooms" of pathology wards (1). On the one hand, the chemical reactivity of this aldehyde fixative has been world-wide exploited to "fix" human and animal cells and tissues to arrest biological degradation by forming chemical "cross-links" between and within proteins (2). Furthermore, the use of archives of formalin-fixed paraffin-embedded tissues (FFPETs) stored around the world represents an extensive source of material in DNA analysis (3). On the other hand, formaldehyde has genotoxic and carcinogenic activity (4-6), consistent with the increased incidence of nasopharyngeal cancer in industrial workers, embalmers and pathologists (7, 8). Indeed, this aldehyde has been classified from the International Agency for Research on Cancer as human carcinogen in 2006 (9). An association with lymphohematopoietic malignancies has been also suggested (10).

Oxidative damage and genotoxic responses to formaldehyde have been shown in *in vitro* experiments (5, 6, 11, 12), but conflicting results have been reported in related human studies (13-15). Lu et al. have recently analyzed the *in vitro* reactions between formaldehyde and all of the deoxynucleosides and their oligomers (16). This study has shown that formaldehyde reacted predominantly with deoxyguanosine, and readily forms cross-links between lysine or cysteine with deoxyguanosine (16). Formaldehyde has been additionally reported to induce the generation of N^2-hydroxy-methyl-dG adducts (17). Nevertheless, the genotoxic responses to this chemical can also be indirect and derive from oxidative stress and peroxidation of lipids (LPO). Indeed, formaldehyde induces the generation of reactive oxygen species (ROS) (12, 18, 19), highly reactive compounds that interact with DNA and lipids of inner cell membrane, leading to oxidative damage and LPO (20). LPO produces malondialdehyde (MDA) or its tautomer, β-hydroxyacrolein (20), a highly reactive aldehyde capable to interact with DNA(20). MDA is not only a product of physiological metabolism, but arises in the form of a base propenal when DNA is oxidized (21). Consequently, elevated amounts of 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-α]purin-10(3H)-one, known as M_1dG, are produced. Exocyclic DNA adducts, such as M_1dG, tend to induce base pair and frameshift mutations in repeated sequences (22). M_1dG is considered a biomarker of corresponding environmental exposures (23, 24), dietary habits (25-28), specific methylation aberrations (29, 30), and predictive of cancer risk and tumor

progression (27, 31-34). A relationship between inflammation, M_1dG and myeloperoxidase catalysed production of hypochlorous acid has been shown in lung of rodents (35, 36).

The mechanism of formalin-fixation has extensively been investigated (37), nevertheless the effects of this process on DNA are unclear. To better understand the consequences on DNA caused from formalin-fixation, we analysed the levels of oxidatively damaged DNA in DNA from the FFPETs and the corresponding flash-frozen tissues in both experimental animal and human tissues. Our interest in this area was a result of two cross-sectional studies that analyzed the genotoxic effects due to formaldehyde exposures in industrial and hospital settings by measuring the levels of two biomarkers of oxidative stress and LPO (1, 38). In those studies, high levels of urinary 15-F_{2t} isoprostane and leukocyte M_1dG adducts were observed in formaldehyde exposed workers. In particular, the pathologists working in the "reduction rooms", where the preparations of FFPETs are commonly performed, were at higher risk of oxidatively damaged DNA (1).

In details, we measured the amounts of M_1dG adducts, a specific type of exocyclic DNA adducts, in DNA extracted from the FFPETs and the flash-frozen paired samples of the liver and the lung of six C57BL/6 control mice. The production of oxidatively damaged DNA was also analyzed in paraffin-embedded tissues as additional" real" control. Subsequently, the levels of M_1dG adducts were determined in DNA extracted from the FFPETs and the flash-frozen paired tissues of the colon mucosa of four patients undergoing surgical resection of colon cancer. This was done using the ^{32}P-postlabeling assay (23, 25), a highly sensitive technique widely employed for the analysis of DNA damage caused from carcinogen exposures (23, 39, 40), including occupational exposures to formaldehyde (1).

Results

Reference standard by ^{32}P-postlabeling and mass-spectrometry

Our results show that the levels of DNA damage, expressed such as RAL, were 5.0 M_1dG adducts ± 0.6 per 10^6 nn in MDA-treated CT-DNA based on ^{32}P-postlabeling. The presence of the M_1dG adduct in the MDA treated CT-DNA sample was confirmed by mass-spectrometry as already reported (41, 42), and we are using the nomenclature reported by Goda and Marnett for this adduct (43). Subsequently, a calibration curve was set up by diluting the reference standard with control DNA and measuring the decreasing levels of M_1dG by using the ^{32}P-postlabeling assay, r-squared = 0.99.

M_1dG adducts in animal and human tissues

To look for potential oxidatively DNA damage caused by the formalin-fixation process, we analyzed the levels of M_1dG adducts, a specific type of exocyclic DNA adducts, in the DNA from the FFPET samples as compared to the flash-frozen tissues using the ^{32}P-postlabeling assay (23, 39). A typical pattern of M_1dG adduct spot was detected in the chromatograms of both experimental mice and humans. As expected, the intensity of M_1dG adduct spots was stronger in the chromatograms of the FFPETs, as compared to the flash-frozen procedure, indicating that the DNA extracted from the FFPET samples contained higher levels of oxidatively damaged DNA in respect to those isolated from the flash-frozen specimens (Figure 1). Therefore, differences in the intensity pattern of M_1dG adduct spots between DNA extracted from the FFPETs and the flash-frozen tissues should reflect different sampling preparation because the same tissue was analysed. Next, we confirmed by co-chromatography the presence of M_1dG adducts in the DNA of experimental animals and human samples.

When we analysed the levels of DNA damage in the liver and the lung specimens of C57BL/6 mice according to the FFPET and the flash-frozen procedures, the results showed that the percentage of M_1dG adducts was about 4-5 fold greater with the FFPET specimens as compared to flash-frozen tissues (Table 1). Specifically, we found that 4.75 M_1dG ± 0.21 (SE) per 10^6 nn were detectable in the FFPET liver samples, whereas 1.07 M_1dG ± 0.08 (SE) per 10^6 nn in the untreated liver DNA ($p < 0.02$). Then, significant increased amount of DNA damage was also observed in the FFPET samples of lung tissues ($p < 0.02$). In detail, 3.80

$M_1dG \pm 0.73$ (SE) per 10^6 nn were measured in the FFPET lung samples, conversely 1.02 $M_1dG \pm 0.07$ (SE) per 10^6 nn in the flash-frozen mouse lung DNA.

In the case of the human colon mucosa, the findings of the ^{32}P-postlabeling analysis revealed that the adduct frequencies were approximately 7 fold greater with the FFPETs in respect to the flash-frozen tissues (Table 1). In particular, the RAL levels of human colon FFPETs were 30.2 $M_1dG \pm 7.7$ (SE) per 10^8 nn, whereas the RAL of flash-frozen human colon DNA used as control were 4.4 $M_1dG \pm 0.7$ (SE) per 10^8 nn ($p < 0.016$).

Since the observed adduct, the M_1dG, is promoted by a secondary reaction and can be induced by any species capable of promote ROS and LPO, the adduct formation in tissue fixed in formaldehyde and embedded in paraffin was compared with tissue embedded in paraffin-only such as additional "real" control. When we looked at the levels of DNA damage caused by the paraffin embedded procedure alone, any increased M_1dG adduct production was not observed in the DNA extracted from the paraffin embedded samples in respect to the fresh-frozen tissues (data not shown), suggesting that the increase formation of DNA damage was associated to formaldehyde fixation in the FFPET samples.

Discussion

In the present study, we examined the relationship between the FFPET procedures and the generation of M_1dG adducts, a specific type of exocyclic DNA adducts (20), in both experimental animal and human tissues. We find a striking association between formalin-fixation process and a significant increased DNA damage in different specimens, including lung tissues, which are those of concern for occupational exposures. Indeed, the levels of M_1dG adducts were significantly increased in DNA extracted from the mouse liver and the lung FFPETs as well as from the human colon FFPETs as compared to the flash frozen tissues and to the paraffin-embedded tissues. According to Fox and colleagues (2), there is a paradox in the working mechanism of formalin because it penetrates tissues rapidly, but it fixes them very slowly. During this lapse of time, oxidative stress and ROS formation can occur, causing both direct oxidation in DNA and increased LPO, which in turn can produce exocyclic DNA adducts, including M_1dG adducts. Therefore, our results indicated that, in performing its protective role against biological degradation, this aldehyde fixative alters the structure of DNA by inducing oxidative DNA damage.

The reaction of formaldehyde with DNA has been studied for many years (17). A number of reaction products were reported but the main adduct observed was the addition of a hydroxymethyl-substituent to primary and secondary amine groups of the respective base. DNA–protein crosslinks have been found in primary and immortalized formaldehyde exposed cells (6, 11), and in the nasal epithelium of treated Fischer 344 rats (44, 45). The formaldehyde treatment has been associated with free radical production and increased LPO in red blood cells, hepatocytes, testes and cerebellum specimens of experimental animals (18, 46-48). The formaldehyde toxicity is thought to be partially based on the activation of ROS producing enzymes, and the inhibition of scavenging systems, thereby enhancing the cellular levels of oxidative stress (49). In particular, formaldehyde is a substrate for cytochrome P-450 monooxygenase system II E1 isozyme, and it may be oxidized by peroxidase, aldehyde oxidase and xanthine oxidase with subsequent ROS generation (18). Recently, we conducted a cross-sectional study of a group of pathologists working in three pathology wards in Italy (1). In that study, pathologists working in the “reduction rooms” were significantly exposed to air-formaldehyde relative to the controls. A significant difference for the levels of M_1dG adducts between exposed pathologists and controls was also observed. Furthermore, the association of formaldehyde with

oxidatively damaged DNA becomes stronger when the evaluation of external exposure to air-formaldehyde was based on the use of personal dosimeters (1).

Considering that formaldehyde is a fixative commonly used in the “reduction rooms”, our study emphasizes the necessity to avoid improper work behavior in pathology wards. Although this result can intensify concern about the genotoxic risk for pathology ward workers, the dose received by the latter is lower compared to that obtained by dipping a tissue in a 4-10% formalin solution for 24 hrs. Nevertheless, in our previous work (1), the analysis of the dose-response relationship showed an increased M_1dG formation only in pathology-ward workers exposed to external air-formaldehyde amonut higher than 66 $\mu g/m^3$, but not in those exposed to lower air-formaldehyde values. Thus, there can be a threshold in effects in vivo for this type of oxidative damage. Furthermore, the effect of air-formaldehyde exposure was more evident in the group of non smokers, suggesting that the role of formaldehyde exposures in the M_1dG adducts can be confounded by cigarette smoking habits (1). Tobacco smoke has a confounding role in the expression of other biomarkers (30, 32, 50, 51) as well as in the framework of environmental exposures (29, 52, 53). For instance, passive tobacco smoke exposure of children has been associated with physiologic evidence of systemic biochemical alterations (54). In particular, apart the demonstrated relationship between passive tobacco smoke and generation of oxidative stress (55), passive tobacco smoke exposure has been reported to represent a confounding factor with respect to many altered health conditions (56).

Since its introduction as a histological fixative back in the 19th century, the formaldehyde solution in water has been adopted as the fixative of choice for biological specimens. The FFPETs are commonly prepared by "dipping" the tissues in a formalin solution for up to 24 hrs, and, then, embedded in paraffin for long term storage, as stated in widely accepted guidelines (57). The archival FFPETs are a precious source of genetic data, because the detection of mutational biomarkers can predict clinical response in patients with colon (58) and breast cancer (59). Their utilization in biomedical research has been challenging due to the propensity of the FFPET DNA to block of polymerase extension and induced non-reproducible sequence artefacts when it is used as template for PCR amplification (60). For an example, a meta-analysis of somatic EGFR mutations in the *epidermal growth factor receptor* (*EGFR*) in about 12,000 non-small cell lung cancer

patients reported that most of the *EGFR* mutations were detected in a single case alone (61), suggesting that many of these measurements can be artefactual. In our investigation, the levels of M_1dG adducts in the FFPETs were comparable to that of most abundant form of base oxidation, 7,8-dihydro-8-oxo-2'-deoxyguanosine (20), therefore, it is conceivable that the generation of high levels of M_1dG adducts, a kind of DNA damage capable to block DNA synthesis and induce error prone translesion synthesis (62), can contribute to stall DNA polymerase elongation and to be subjected to error prone translesional interpretation/misinterpretation by DNA polymerase during PCR.

The presence of M_1dG adducts have been detected in several tissues as liver, breast, colon, bronchi, gastric mucosa and peripheral leucocytes from healthy human beings at levels ranging from undetectable to 160 per 10^8 nn (1, 23, 27, 33, 34, 63, 64). Herein, the levels of colon mucosa M_1dG adducts were in the range of those found in the colon mucosa of 162 volunteers participating in both the United Kingdom Flexiscope Sigmoidoscopy Screening Trial and the EPIC study, with adduct levels ranging from undetectable to 122.3 M_1dG per 10^8 nn (27).

A strength of the present study is that the levels of M_1dG adducts were analyzed by the ^{32}P-postlabeling, a technique known to be sensitive, to 1 adduct in about10^9-10^{10} nn, for the detection of a wide range of carcinogens (39, 65, 66). A high repeatability of the measurements of aromatic DNA adducts has been also reported for this assay (67). Nevertheless, ^{32}P-postlabeling is a technique that is unable to determine the structure of the adducts under study; higher specificity may be obtained if the assay is combined with the use of appropriate internal standards (68, 69), or coupled with MALDI-TOF mass spectrometry, such as in the case of the M_1dG adducts (1, 24).

Material and Methods

Experimental animals and histopatology

The normal liver and the lung specimens were collected from six C57BL/6 mice (Harlan Laboratories S.r.l. Udine, Italy), which were kept on a regular dark/light cycle, and received regular chow, 17.0% kcal from fat (crude oil) (TD.2018, Harlan Laboratories S.r.l. Udine, Italy) and water ad libitum. After eight weeks of standard chow, mice were weighed and the liver and the lung tissues were harvested, weighed, and apportioned for DNA extraction as flash-frozen tissue or preserved in 10% buffered formalin. Experimental animal procedures were performed in accordance with the guidelines of the General Hospital Institutional Committee that reviewed and approved the protocol. Formalin-preserved liver and lung tissue samples were first embedded in paraffin, then, the mouse FFPETs were deparaffinized, hydrated and used for DNA extraction applying standardized protocols of the Department of Experimental and Clinical Biomedical Sciences of the University of Florence (70, 71). Both flash-frozen and paraffin-embedded tissues were used such as controls.

Human specimens and histopatology

The colon mucosa specimens were obtained from four cancer patients undergoing colon cancer surgery after informed consent. The study was approved by the relevant ethical committee (37). Normal colon mucosa specimens were obtained near the noninvolved surgical margin of the colon of the patients. Biological samples were immediately transported on ice to the pathology laboratory. From each specimen, two samples were taken and processed as follows: a) the FFPET procedure, where the sample (4 mm thick) was fixed for 24 hrs in 4% at room temperature, routinely processed to paraffin embedding with an automatic processor, and embedded in paraffin wax; b) the freezing procedure, where the sample (4 mm thick) was embedded in Tissue-Tek® OCT™ compound, flash-frozen in isopentane immediately after dissection and stored at -80°C. Before DNA extraction and purification, the FFPET samples were deparaffinized and rehydrated using standardized protocols (37).

Preparation of reference adduct standard

A reference adduct standard was prepared as follow: calf-thymus (CT)-DNA was treated with 10 mM MDA (ICN Biomedicals, Irvine, CA, USA), as previously reported (1). Then, MDA treated DNA was diluted with untreated CT-DNA to obtain decreasing levels of the reference adduct standard to generate a calibration curve.

DNA extraction and purification

Extraction columns were treated according to the manufacturer's protocol for the QIAamp DNA minikit (37, 71). The concentrations of DNA extracted from FFPETs and flash-frozen samples were measured spectrophotometrically and DNA was stored at -80°C until laboratory analysis (50, 68).

Mass-spectrometry

The presence of DNA adducts in MDA treated CT-DNA sample was analyzed by mass-spectrometry (Voyager DE STR from Applied Biosystems, Framingham, MA), as reported elsewhere (41, 42), through the following sequence of steps: (1) reaction of DNA with $NaBH_4$ followed by precipitation with isopropanol (43); (2) digestion with snake venom phosphodiesterase and nuclease P1; (3) extraction of DNA adducts that are less polar than normal nucleotides on an OASIS cartridge (Waters Corp.); (4) tagging with an isotopologue pair of benzoylhistamines (d_o and d_4) in a phosphate-specific labeling reaction in the presence of carbodiimide (41, 42); (5) removal of residual reagents by ion exchange solid-phase extraction; (6) resolution of tagged adducts by capillary reversed-phase HPLC with a collection of drops onto a MALDI plate; (7) addition of matrix (α-cyano-4-hydroxycinnamic acid); and (8) analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

^{32}P-DNA postlabeling

The generation of M_1dG adducts in DNA from the FFPETs and the fresh-frozen tissues were measured by the ^{32}P-DNA postlabeling technique (23), a highly sensitive method used for the analysis of DNA adducts induced from a wide variety of carcinogens (39, 72, 73), including exocyclic DNA adducts (74). In brief, DNA (2 μg) was hydrolyzed by incubation with micrococcal nuclease (21.45 mU/μl) and spleen phosphodiesterase (6.0 mU/μl) at 37°C for 4.5 h (72). Hydrolyzed DNA was treated with nuclease P1

(0.1 U/µl) at 37°C for 30 min. The samples were incubated with 25 µCi of carrier-free [γ-^{32}P]ATP (3000 Ci/mM) and polynucleotide kinase T4 (0.75 U/µl) to generate ^{32}P-labeled adducts at 37°C for 30 min (72), followed by spotting onto polyethyleneimine cellulose thin layer chromatography plates (Macherey-Nagel, Germany). ^{32}P-labeled adducts were applied on polyethyleneimine (PEI) cellulose thin-layer chromatography plates (Macherey-Nagel, Germany) and processed as previously described (25). This chromatographic modification of the ^{32}P-postlabeling method has been developed from our laboratory for the specific detection of this specific kind of exocyclic DNA adducts (25) by using a low-urea solvent system known to be effective for the detection of low molecular weight and highly polar DNA adducts. In brief, ^{32}P-labeled products were applied to the origin of chromatograms and developed with 0.35 $MgCl_2$ up to 2.0 cm filter paper wick. Plates were developed in the opposite direction with 2.1 M lithium formate, 3.75 M urea, pH 3.75, and then run at the right angle to the previous development with 0.24 M sodium phosphate, 2.4 M urea, pH 6.4. Detection and quantification of M_1dG adducts and normal nucleotides (nn) were performed by storage phosphor imaging with intensifying screens from Molecular Dynamics (Sunnyvale, CA, USA). The intensifying screens were scanned using a Typhoon 9210 (Amersham). Software used to process the data was ImageQuant (version 5.0) from Molecular Dynamics. After background subtraction, the levels of M_1dG adducts were expressed such as relative adduct labeling (RAL): pixels for adducted nucleotides / pixels for normal nucleotides (nn). The RAL levels of M_1dG adducts were corrected across experiments based on the recovery of the reference standard, the MDA treated CT-DNA sample.

The ^{32}P-postlabeling technique is an assay that is unable to determine the structure of the DNA adducts under exams, but higher specificity may be achieved when the assay is coupled with the use of appropriate reference standards. (68, 69, 75).Thus, co-chromatography on PEI-cellulose thin layer chromatography plates was used to confirm the identity of adduct spots observed in the FFPET tissues. Briefly, the FFPET and the reference standard ^{32}P-labeled samples were spotted onto the same PEI-cellulose thin layer chromatograms and analyzed using the following chromatographic solvent system: 2.1 M lithium formate, 3.75 M urea, pH 3.75 (first direction) and 0.24 M sodium phosphate, 2.4 M urea, pH 6.4 (second direction) or 0.24 M sodium phosphate, 2.7 M urea, pH 6.4 (second direction). After storage phosphor

imaging with intensifying screens, the chromatographic mobility of the FFPET adduct spots was compared with that of the reference standard onto PEI-cellulose plates.

Statistical analysis

The levels of M_1dG adducts were expressed as adducted nucleotides per 10^6 nn (experimental animal tissues) or per 10^8 nn (human tissues). Statistical analyses were performed using Mann-Whitney U test to examine the differences between FFPET and control unprocessed samples. All statistical tests were two-sided and p less than 0.05 was considered to be statistically significant. The data were analyzed using SPSS 13.0 (IBM SPSS Statistics, New York, NY).

Conclusion

In the present study, we showed a significant association between formalin-fixation procedures and the generation of oxidatively damaged DNA. We provided data that formalin-fixed DNA contains increased levels of M_1dG adducts, a kind of DNA adducts that can also partially block DNA polymerase extension and induce error prone translesion synthesis (62). Thus, it is possible that high levels of M_1dG adducts in FFPET tissues can partially block DNA synthesis and to be subjected to error prone translesional interpretation/misinterpretation by DNA polymerase during PCR. Our results can have important implications in biomedical research, where the FFPETs are widely used such as source of DNA for mutational studies. The extrapolation of the genotoxic risk is certainly difficult from the drastic conditions commonly used for the formalin-fixation, but one could argue that if the continuous exposures to formaldehyde persist in reduction rooms, the risk of DNA damage increases. Therefore, we should consider the present genotoxic finding as an important knowledge for the future preventive actions aimed to reduce formaldehyde exposures and to avoid improper work behaviour in the pathology-wards.

Acknowledgments

The study was partially supported from the Tuscany Region, Tumor Institute of Tuscany, and INAIL Piemonte.

References

1. Bono R, Romanazzi V, Munnia A, Piro S, Allione A, Ricceri F, et al. Malondialdehyde-deoxyguanosine adduct formation in workers of pathology wards: the role of air formaldehyde exposure. Chem Res Toxicol. 2010;23(8):1342-8.
2. Fox CH, Johnson FB, Whiting J, Roller PP. Formaldehyde fixation. J Histochem Cytochem. 1985;33(8):845-53.
3. Frankel A. Formalin fixation in the '-omics' era: a primer for the surgeon-scientist. ANZ J Surg. 2012;82(6):395-402.
4. Hansen J, Olsen JH. [Occupational exposure to formaldehyde and risk of cancer]. Ugeskr Laeger. 1996;158(29):4191-4.
5. Speit G, Schutz P, Hogel J, Schmid O. Characterization of the genotoxic potential of formaldehyde in V79 cells. Mutagenesis. 2007;22(6):387-94.
6. Speit G, Schmid O, Frohler-Keller M, Lang I, Triebig G. Assessment of local genotoxic effects of formaldehyde in humans measured by the micronucleus test with exfoliated buccal mucosa cells. Mutat Res. 2007;627(2):129-35.
7. Hauptmann M, Lubin JH, Stewart PA, Hayes RB, Blair A. Mortality from solid cancers among workers in formaldehyde industries. Am J Epidemiol. 2004;159(12):1117-30.
8. Duhayon S, Hoet P, Van Maele-Fabry G, Lison D. Carcinogenic potential of formaldehyde in occupational settings: a critical assessment and possible impact on occupational exposure levels. Int Arch Occup Environ Health. 2008;81(6):695-710.
9. IARC. Formaldehyde, 2-butoxyethanol and 1-tert-butoxypropan-2-ol. IARC Monogr Eval Carcinog Risks Hum. 2006;88:1-478.
10. Beane Freeman LE, Blair A, Lubin JH, Stewart PA, Hayes RB, Hoover RN, et al. Mortality from lymphohematopoietic malignancies among workers in formaldehyde industries: the National Cancer Institute Cohort. J Natl Cancer Inst. 2009;101(10):751-61.
11. Speit G, Schmid O, Neuss S, Schutz P. Genotoxic effects of formaldehyde in the human lung cell line A549 and in primary human nasal epithelial cells. Environ Mol Mutagen. 2008;49(4):300-7.
12. Wang F, Li C, Liu W, Jin Y. Oxidative damage and genotoxic effect in mice caused by sub-chronic exposure to low-dose volatile organic compounds. Inhal Toxicol. 2013;25(5):235-42.
13. Viegas S, Ladeira C, Nunes C, Malta-Vacas J, Gomes M, Brito M, et al. Genotoxic effects in occupational exposure to formaldehyde: A study in anatomy and pathology laboratories and formaldehyde-resins production. J Occup Med Toxicol. 2010;5(1):25.
14. Jiang S, Yu L, Cheng J, Leng S, Dai Y, Zhang Y, et al. Genomic damages in peripheral blood lymphocytes and association with polymorphisms of three glutathione S-transferases in workers exposed to formaldehyde. Mutat Res. 2010;695(1-2):9-15.
15. Zeller J, Neuss S, Mueller JU, Kuhner S, Holzmann K, Hogel J, et al. Assessment of genotoxic effects and changes in gene expression in humans exposed to formaldehyde by inhalation under controlled conditions. Mutagenesis. 2011;26(4):555-61.
16. Lu K, Ye W, Zhou L, Collins LB, Chen X, Gold A, et al. Structural characterization of formaldehyde-induced cross-links between amino acids and deoxynucleosides and their oligomers. Journal of the American Chemical Society. 2010;132(10):3388-99.
17. Swenberg JA, Moeller BC, Lu K, Rager JE, Fry RC, Starr TB. Formaldehyde carcinogenicity research: 30 years and counting for mode of action, epidemiology, and cancer risk assessment. Toxicologic pathology. 2013;41(2):181-9.
18. Saito Y, Nishio K, Yoshida Y, Niki E. Cytotoxic effect of formaldehyde with free radicals via increment of cellular reactive oxygen species. Toxicology. 2005;210(2-3):235-45.
19. Yan Y, Yang JY, Mou YH, Wang LH, Zhang H, Wu CF. Possible metabolic pathways of ethanol responsible for oxidative DNA damage in human peripheral lymphocytes. Alcohol Clin Exp Res. 2011;35(1):1-9.
20. Marnett LJ. Oxy radicals, lipid peroxidation and DNA damage. Toxicology. 2002;181-182:219-22.

21. Plastaras JP, Riggins JN, Otteneder M, Marnett LJ. Reactivity and mutagenicity of endogenous DNA oxopropenylating agents: base propenals, malondialdehyde, and N(epsilon)-oxopropenyllysine. Chem Res Toxicol. 2000;13(12):1235-42.
22. VanderVeen LA, Hashim MF, Shyr Y, Marnett LJ. Induction of frameshift and base pair substitution mutations by the major DNA adduct of the endogenous carcinogen malondialdehyde. Proc Natl Acad Sci U S A. 2003;100(24):14247-52.
23. Peluso M, Srivatanakul P, Munnia A, Jedpiyawongse A, Ceppi M, Sangrajrang S, et al. Malondialdehyde-deoxyguanosine adducts among workers of a Thai industrial estate and nearby residents. Environ Health Perspect. 2010;118(1):55-9.
24. Peluso M, Munnia A, Ceppi M, Giese RW, Catelan D, Rusconi F, et al. Malondialdehyde-deoxyguanosine and bulky DNA adducts in schoolchildren resident in the proximity of the Sarroch industrial estate on Sardinia Island, Italy. Mutagenesis. 2013;28(3):315-21.
25. van Helden YG, Keijer J, Heil SG, Pico C, Palou A, Oliver P, et al. Beta-carotene affects oxidative stress-related DNA damage in lung epithelial cells and in ferret lung. Carcinogenesis. 2009;30(12):2070-6.
26. Peluso M, Munnia A, Piro S, Jedpiyawongse A, Sangrajrang S, Giese RW, et al. Fruit and vegetable and fried food consumption and 3-(2-deoxy-beta-D-erythro-pentafuranosyl)pyrimido[1,2-alpha] purin-10(3H)-one deoxyguanosine adduct formation. Free Radic Res. 2012;46(1):85-92.
27. Leuratti C, Watson MA, Deag EJ, Welch A, Singh R, Gottschalg E, et al. Detection of malondialdehyde DNA adducts in human colorectal mucosa: relationship with diet and the presence of adenomas. Cancer Epidemiol Biomarkers Prev. 2002;11(3):267-73.
28. Vanhees K, van Schooten FJ, van Waalwijk van Doorn-Khosrovani SB, van Helden S, Munnia A, Peluso M, et al. Intrauterine exposure to flavonoids modifies antioxidant status at adulthood and decreases oxidative stress-induced DNA damage. Free Radic Biol Med. 2013;57:154-61.
29. Peluso M, Bollati V, Munnia A, Srivatanakul P, Jedpiyawongse A, Sangrajrang S, et al. DNA methylation differences in exposed workers and nearby residents of the Ma Ta Phut industrial estate, Rayong, Thailand. Int J Epidemiol. 2012;41(6):1753-60; author response 61-3.
30. Peluso ME, Munnia A, Bollati V, Srivatanakul P, Jedpiyawongse A, Sangrajrang S, et al. Aberrant methylation of hypermethylated-in-cancer-1 and exocyclic DNA adducts in tobacco smokers. Toxicol Sci. 2013.
31. Wang M, Dhingra K, Hittelman WN, Liehr JG, de Andrade M, Li D. Lipid peroxidation-induced putative malondialdehyde-DNA adducts in human breast tissues. Cancer Epidemiol Biomarkers Prev. 1996;5(9):705-10.
32. Munnia A, Amasio ME, Peluso M. Exocyclic malondialdehyde and aromatic DNA adducts in larynx tissues. Free Radic Biol Med. 2004;37(6):850-8.
33. Munnia A, Bonassi S, Verna A, Quaglia R, Pelucco D, Ceppi M, et al. Bronchial malondialdehyde DNA adducts, tobacco smoking, and lung cancer. Free Radic Biol Med. 2006;41(9):1499-505.
34. Peluso M, Munnia A, Risso GG, Catarzi S, Piro S, Ceppi M, et al. Breast fine-needle aspiration malondialdehyde deoxyguanosine adduct in breast cancer. Free Radic Res. 2011;45(4):477-82.
35. Gungor N, Knaapen AM, Munnia A, Peluso M, Haenen GR, Chiu RK, et al. Genotoxic effects of neutrophils and hypochlorous acid. Mutagenesis. 2010;25(2):149-54.
36. Gungor N, Pennings JL, Knaapen AM, Chiu RK, Peluso M, Godschalk RW, et al. Transcriptional profiling of the acute pulmonary inflammatory response induced by LPS: role of neutrophils. Respir Res. 2010;11:24.
37. Bussolati G, Annaratone L, Medico E, D'Armento G, Sapino A. Formalin fixation at low temperature better preserves nucleic acid integrity. PLoS One. 2011;6(6):e21043.
38. Romanazzi V, Pirro V, Bellisario V, Mengozzi G, Peluso M, Pazzi M, et al. 15-F(2)t isoprostane as biomarker of oxidative stress induced by tobacco smoke and occupational exposure to formaldehyde in workers of plastic laminates. Sci Total Environ. 2013;442:20-5.
39. Phillips DH, Arlt VM. The 32P-postlabeling assay for DNA adducts. Nat Protoc. 2007;2(11):2772-81.
40. Peluso M, Srivatanakul P, Munnia A, Jedpiyawongse A, Meunier A, Sangrajrang S, et al. DNA adduct formation among workers in a Thai industrial estate and nearby residents. Sci Total Environ. 2008;389(2-3):283-8.

41. Wang P, Fisher D, Rao A, Giese RW. Nontargeted nucleotide analysis based on benzoylhistamine labeling-MALDI-TOF/TOF-MS: discovery of putative 6-oxo-thymine in DNA. Anal Chem. 2012;84(8):3811-9.
42. Wang P, Gao J, Li G, Shimelis O, Giese RW. Nontargeted analysis of DNA adducts by mass-tag MS: reaction of p-benzoquinone with DNA. Chem Res Toxicol. 2012;25(12):2737-43.
43. Goda Y, Marnett LJ. High-performance liquid chromatography with electrochemical detection for determination of the major malondialdehyde-guanine adduct. Chem Res Toxicol. 1991;4(5):520-4.
44. 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;23(4):525-36.
45. Lu K, Collins LB, Ru H, Bermudez E, Swenberg JA. Distribution of DNA adducts caused by inhaled formaldehyde is consistent with induction of nasal carcinoma but not leukemia. Toxicol Sci. 2010;116(2):441-51.
46. Petushok NE, Petushok BG, El'chaninova MA, Ban'kovskii AA, Trebukhina RV. [Functional activity of blood and liver cells under formaldehyde intoxication via inhalation]. Biomed Khim. 2005;51(1):76-80.
47. Gulec M, Gurel A, Armutcu F. Vitamin E protects against oxidative damage caused by formaldehyde in the liver and plasma of rats. Mol Cell Biochem. 2006;290(1-2):61-7.
48. Zararsiz I, Sarsilmaz M, Tas U, Kus I, Meydan S, Ozan E. Protective effect of melatonin against formaldehyde-induced kidney damage in rats. Toxicol Ind Health. 2007;23(10):573-9.
49. Lino-dos-Santos-Franco A, Correa-Costa M, Durao AC, de Oliveira AP, Breithaupt-Faloppa AC, Bertoni Jde A, et al. Formaldehyde induces lung inflammation by an oxidant and antioxidant enzymes mediated mechanism in the lung tissue. Toxicol Lett. 2011;207(3):278-85.
50. Peluso M, Amasio E, Bonassi S, Munnia A, Altrupa F, Parodi S. Detection of DNA adducts in human nasal mucosa tissue by 32P-postlabeling analysis. Carcinogenesis. 1997;18(2):339-44.
51. Peluso M, Neri M, Margarino G, Mereu C, Munnia A, Ceppi M, et al. Comparison of DNA adduct levels in nasal mucosa, lymphocytes and bronchial mucosa of cigarette smokers and interaction with metabolic gene polymorphisms. Carcinogenesis. 2004;25(12):2459-65.
52. Peluso ME, Munnia A, Srivatanakul P, Jedpiyawongse A, Sangrajrang S, Ceppi M, et al. DNA adducts and combinations of multiple lung cancer at-risk alleles in environmentally exposed and smoking subjects. Environ Mol Mutagen. 2013.
53. Peluso M, Srivatanakul P, Jedpiyawongse A, Sangrajrang S, Munnia A, Piro S, et al. Aromatic DNA adducts and number of lung cancer risk alleles in Map-Ta-Phut Industrial Estate workers and nearby residents. Mutagenesis. 2013;28(1):57-63.
54. Wilson KM, Finkelstein JN, Blumkin AK, Best D, Klein JD. Micronutrient levels in children exposed to secondhand tobacco smoke. Nicotine & tobacco research : official journal of the Society for Research on Nicotine and Tobacco. 2011;13(9):800-8.
55. Bono R, Bellisario V, Romanazzi V, Pirro V, Piccioni P, Pazzi M, et al. Oxidative stress in adolescent passive smokers living in urban and rural environments. International journal of hygiene and environmental health. 2014;217(2-3):287-93.
56. Van Miert E, Sardella A, Bernard A. Biomarkers of early respiratory effects in smoking adolescents. The European respiratory journal. 2011;38(6):1287-93.
57. Goldstein NS, Hewitt SM, Taylor CR, Yaziji H, Hicks DG. Recommendations for improved standardization of immunohistochemistry. Appl Immunohistochem Mol Morphol. 2007;15(2):124-33.
58. Weichert W, Schewe C, Lehmann A, Sers C, Denkert C, Budczies J, et al. KRAS genotyping of paraffin-embedded colorectal cancer tissue in routine diagnostics: comparison of methods and impact of histology. J Mol Diagn. 2010;12(1):35-42.
59. Yost SE, Smith EN, Schwab RB, Bao L, Jung H, Wang X, et al. Identification of high-confidence somatic mutations in whole genome sequence of formalin-fixed breast cancer specimens. Nucleic Acids Res. 2012;40(14):e107.
60. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol. 2002;161(6):1961-71.

61. Murray S, Dahabreh IJ, Linardou H, Manoloukos M, Bafaloukos D, Kosmidis P. Somatic mutations of the tyrosine kinase domain of epidermal growth factor receptor and tyrosine kinase inhibitor response to TKIs in non-small cell lung cancer: an analytical database. J Thorac Oncol. 2008;3(8):832-9.
62. Stafford JB, Eoff RL, Kozekova A, Rizzo CJ, Guengerich FP, Marnett LJ. Translesion DNA synthesis by human DNA polymerase eta on templates containing a pyrimidopurinone deoxyguanosine adduct, 3-(2'-deoxy-beta-d-erythro-pentofuranosyl)pyrimido-[1,2-a]purin-10(3H)-one. Biochemistry. 2009;48(2):471-80.
63. Chaudhary AK, Nokubo M, Reddy GR, Yeola SN, Morrow JD, Blair IA, et al. Detection of endogenous malondialdehyde-deoxyguanosine adducts in human liver. Science. 1994;265(5178):1580-2.
64. Everett SM, Singh R, Leuratti C, White KL, Neville P, Greenwood D, et al. Levels of malondialdehyde-deoxyguanosine in the gastric mucosa: relationship with lipid peroxidation, ascorbic acid, and Helicobacter pylori. Cancer Epidemiol Biomarkers Prev. 2001;10(4):369-76.
65. Phillips DH, Venitt S. DNA and protein adducts in human tissues resulting from exposure to tobacco smoke. Int J Cancer. 2013;131(12):2733-53.
66. Phillips DH, Castegnaro M. Results of an interlaboratory trial of 32P-postlabelling. IARC Sci Publ. 1993(124):35-49.
67. Peluso M, Hainaut P, Airoldi L, Autrup H, Dunning A, Garte S, et al. Methodology of laboratory measurements in prospective studies on gene-environment interactions: the experience of GenAir. Mutat Res. 2005;574(1-2):92-104.
68. Peluso M, Castegnaro M, Malaveille C, Talaska G, Vineis P, Kadlubar F, et al. 32P-postlabelling analysis of DNA adducted with urinary mutagens from smokers of black tobacco. Carcinogenesis. 1990;11(8):1307-11.
69. Peluso M, Castegnaro M, Malaveille C, Friesen M, Garren L, Hautefeuille A, et al. 32P Postlabelling analysis of urinary mutagens from smokers of black tobacco implicates 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) as a major DNA-damaging agent. Carcinogenesis. 1991;12(4):713-7.
70. Galli A, Crabb DW, Ceni E, Salzano R, Mello T, Svegliati-Baroni G, et al. Antidiabetic thiazolidinediones inhibit collagen synthesis and hepatic stellate cell activation in vivo and in vitro. Gastroenterology. 2002;122(7):1924-40.
71. Tarocchi M, Hannivoort R, Hoshida Y, Lee UE, Vetter D, Narla G, et al. Carcinogen-induced hepatic tumors in KLF6+/- mice recapitulate aggressive human hepatocellular carcinoma associated with p53 pathway deregulation. Hepatology. 2011;54(2):522-31.
72. Munnia A, Saletta F, Allione A, Piro S, Confortini M, Matullo G, et al. 32P-Post-labelling method improvements for aromatic compound-related molecular epidemiology studies. Mutagenesis. 2007;22(6):381-5.
73. Peluso M, Merlo F, Munnia A, Bolognesi C, Puntoni R, Parodi S. (32)P-postlabeling detection of DNA adducts in peripheral white blood cells of greenhouse floriculturists from western Liguria, Italy. Cancer Epidemiol Biomarkers Prev. 1996;5(5):361-9.
74. Phillips DH, Venitt S. DNA and protein adducts in human tissues resulting from exposure to tobacco smoke. Int J Cancer. 2012;131(12):2733-53.
75. Phillips DH, Castegnaro M. Standardization and validation of DNA adduct postlabelling methods: report of interlaboratory trials and production of recommended protocols. Mutagenesis. 1999;14(3):301-15.

Table 1. Mean levels of 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-α]purin-10(3H)-one adducts (M_1dG) ± (SE) in the liver and the lung specimens of mouse and in human colon according to the formalin-fixed paraffin-embedded (FFPTE) procedures and in the respective fresh-frozen tissues, as controls.

Species	**Specimens**	**Buffered formalin**	**M_1dG adducts ± (SE)**		**P-values**
			FFPTEs	**Control tissues**	
Mouse					
	Liver	10%	4.75 ± 0.21 per 10^6 nn	1.07 ± 0.08 per 10^6 nn	0.02
	Lung	10%	3.80 ± 0.73 per 10^6 nn	1.02 ± 0.07 per 10^6 nn	0.02
Human					
	Colon	4%	30.2 adducts ± 7.7 per 10^8 nn	4.4 ± 0.7 per 10^8 nn	0.016

Figure 1. 3-(2-deoxy-β-D-erythro-pentafuranosyl)pyrimido[1,2-α]purin-10(3H)-one adduct-spot in the human colon DNA extracted and purified from the formalin-fixed paraffin-embedded tissues (A) and from the correspondent flash-frozen colon mucosa tissues (B).