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LETTER TO THE EDITOR

Comments on 'DNA–protein crosslinks, a biomarker of exposure to formaldehyde—*in vitro* and *in vivo* studies' by Shaham *et al.*

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Shaham *et al.* (1) reported that the white blood cells (WBC*) of 12 anatomists and pathologists exposed to formaldehyde (FA) in a laboratory had significantly higher average levels of DNA–protein cross-links (DPC) than those of eight subjects 'who had never been exposed to FA'. Means and coefficients of variation (CV) (%SD) for DPC/total DNA were 0.288 (19%) for the exposed group and 0.227 (30%) for the unexposed group. Ranges of DPC/total DNA were 0.215–0.389 for exposed workers and 0.16–0.329 for the unexposed subjects. The range of exposure concentrations ('personal samples') was 2.8–3.1 p.p.m. There are several reasons to question whether the apparent difference in DPC represents a true difference and is due to FA.

Defects in experimental design and interpretation

(i) The putative cross-linking agent *in vivo* was not shown to be FA.

(ii) Study groups were small and were not matched. The 'unexposed' group was not a proper control group. Numbers of smokers and smoking histories were not reported. Other confounding factors (exposures to other compounds, diet, age and gender) were not considered in the analysis.

(iii) Blood samples were allowed to stand for 3 h prior to isolation of WBC.

(iv) Intrasubject variability over time and analytical variability (within-subject CV) were not determined. The developers of the assay reported a within-subject CV of 33% (2).

(v) Analyses of DPC in exposed and unexposed subjects were not performed in a double blind manner.

(vi) The proposed 'linear' correlation between DPC and years of exposure to FA (1) was not shown to be statistically significant and the interpretation is clouded by the possible effects of aging on DPC (3).

(vii) A lack-of-fit test could not be applied to the dose–response curve for DPC formation *in vitro* to validate the assumed linear model owing to a failure to obtain replicate observations.

Inconsistency with evidence against distant site toxicity

(i) Fifteen years of research, including at least four chronic bioassays, have shown that high concentrations of inhaled FA are cytolethal and carcinogenic in the upper respiratory tract of rats, but no lesions associated with FA exposure have been observed at other sites (4–8). The localization of toxicity to the epithelial lining of the upper respiratory tract is consistent with the high reactivity and rapid metabolism of inhaled FA.

(ii) DPC were not detected in rat bone marrow, even under conditions of glutathione depletion (9,10).

(iii) Exposure of humans (1.9 p.p.m.), monkeys (6 p.p.m.) and rats (14.4 p.p.m.) to FA did not increase the FA concentration of the blood (11,12).

(iv) Although FA can induce sister chromatid exchanges (SCE), chromosomal aberrations (CA) and other clastogenic lesions in human WBC *in vitro* (13), SCE and CA were not detected in rat WBC following an *in vivo* exposure to 15 p.p.m. (6 h/day, 5 days) (14) and CA were not found in WBC of workers exposed to FA (0.45–8.4 p.p.m.) for time periods ranging from 5 to 16 years (15).

(v) Despite the authors' assertion of persistent cross-links from Cr and Ni (1), the available evidence indicates that FA-induced DPC are rapidly repaired in mammalian cells *in vitro*, including human lymphoblasts (16), mouse leukemia L1210 cells (17), human fibroblasts and bronchial epithelial cells (18) and rat tracheal epithelial cells (19). In addition, DPC were rapidly removed from and did not accumulate in the rat nasal mucosa *in vivo* in subchronic (12 weeks) exposures to 6 or 10 p.p.m. FA (20).

Implausibility

An adult man (respiratory minute volume 12.0±3.0 l/min; 21) would absorb 46 μg FA/min if the FA concentration were 3.1 p.p.m. In an 8 h working day, the total amount absorbed would be 22 mg. Pharmacokinetic analyses of DPC formation in the nasal mucosa of rats (22) and monkeys (23) indicate that ~92% of the inhaled FA is eliminated by saturable metabolism in the target tissues of the respiratory tract. The residual 8% is eliminated by non-saturable metabolism, covalently bound to macromolecules in the respiratory tract or distributed to other tissues. If the residual FA were unmetabolized and distributed throughout the total body water (41 l), its concentration after 8 h would be only 0.001 mM, which is well below the concentration of endogenous FA in human blood (0.087±0.005 mM) (11) and lower than the lowest concentration shown on the dose–response curve for DPC formation *in vitro* published by Shaham *et al.* (1). Of course, metabolism in the blood and tissues would reduce the concentration even further. Therefore, FA in blood is highly unlikely to reach the concentrations required to induce an increase in the DPC of WBC.

Taken together, the defects in experimental design, the inconsistency with published evidence on FA toxicity and metabolism and the implausibility of the results call into question the conclusion (1) that inhaled FA forms DPC in peripheral WBC. Shaham *et al.* (1) failed to address the discrepancies between their results and the large body of evidence demonstrating a lack of distant site toxicity, the rapid repair of FA-induced DPC, the absence of genotoxicity in WBC following *in vivo* exposure and the inability of inhaled FA to increase the FA concentration of the blood. Identification of the putative cross-linking agent and supporting data from controlled animal exposures or from a large and carefully conducted human study are required to justify the use of DPC in peripheral WBC as a biomarker of exposure to FA and as an indicator of potential human risk.

*Abbreviations: WBC, white blood cells; FA, formaldehyde; DPC, DNA–protein cross-links; CV, coefficient of variation; SCE, sister chromatid exchange; CA, chromosomal aberrations.

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Response

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The following are our responses which negate the points raised by Casanova *et al.*

Defects in experimental design and interpretation

(i) The putative cross-linking agent *in vivo* was FA only. This was based on the data from the questionnaire that every worker completed. These data included a complete history of exposures and employment, including army service and hobbies. Based on these questionnaires it was established that no one in the exposed group is, or was ever, exposed to a known substance that forms DPC, such as radiation, BCNU, alkylating agents, nickel chromate and *cis-* or *trans*-Pt (II) diamine dichloride. In addition, no one in the control group is, or was ever, exposed to FA or other substances known to form DPC. Based on these findings we can say with certainty that the cross-linking agent was FA.

(ii) The study groups were large enough to show significant differences in the amount of DPC. The group in our article was age matched, as can be seen in Table I (1). The unexposed group was an ideal group for comparison as no one in this group is, or was ever, exposed to FA, as mentioned in (i) above. The number of smokers was also similar in the two study groups. In the exposed group, nine workers were non-smokers and three were smokers and in the control group six workers were non-smokers and two were smokers. This is important, since smoking is the major source of non-occupational exposure to FA. We did not find significant differences in the amount of DPC between men and women. The level of exposure to FA from food (one of the possible confounding factors suggested by Casanova *et al.*) cannot be quantified. According to the questionnaire, none of the study group imbibed excessive amounts of alcohol. As all the results were adjusted for smoking and the group was age matched, it is clear that we took into consideration all the known confounders according to the literature in our statistical evaluation. No other adjustment could be done in the study group, as this would have resulted in very small subgroups.

(iii) Blood samples were examined as soon as they arrived at the laboratory, according to uniform protocols and conditions. According to Cosma *et al.* (2), DPC repair was only evident 4 h after the cessation of FA exposure.

(iv) Every worker included in the study had duplicate or triplicate samples for measuring the amount of DPC. We took the range of the results of everyone and compared the mean of all the ranges obtained with that of each worker. These ranges were tested by *R* chart. The within subject CV was 22%.

(v) The analyses of DPC in exposed and unexposed subjects were performed in a double blind manner, as all the examples were examined for DPC in one session and there was no identification of group members except by ID number.

(vi) The correlation between DPC and years of exposure is linear and is not clouded by ageing as we did not find any correlation between DPC and ageing in our study group. To our knowledge, this correlation was not reported in the literature. The correlation that we found emphasized that DPC can be used as a biomarker for chronic exposure and represents

early changes in the multiple process of carcinogenesis (3) that can continue for 20 years and more (latency period) (4).

(vii) We calculated the dose–response curve for DPC formation *in vitro* by adjusting every result to baseline (before exposure to FA). We subtracted the baseline level from each result so that they were not connected to the first level of DPC.

Based on these facts and the results in our article, the difference in DPC represents a true difference and is due to FA exposure. There are no defects in the experimental design, nor in the interpretation.

Inconsistency with evidence against distant site toxicity

(i) It is not unexpected that studies including inhalation of FA showed evidence of carcinogenicity, usually at higher dose (>6 p.p.m.), since rats are obligatory nose breathers (5,6), and FA is known to be highly reactive. On the other hand, FA-induced lesions in mice were less severe in the nasal cavities than similar lesions in rats from the same exposure group, including a lower incidence of squamous cell carcinoma. These differences in response between the two species is related to differences in their physiological response to FA inhalation. Exposure of mice to 15 p.p.m. FA by inhalation results in a 50% reduction in minute volume, as opposed to a 20% reduction in rats (7). Similar studies in hamsters have shown no evidence of carcinogenicity (8).

Evidence of potentially harmful effects on an organ or tissue which is remote from the site of first contact of inhaled FA, namely the nasal mucosa, came from the data published by Beall (9), who reviewed 84 articles relating to the adverse health effect in animals and humans of subchronic exposure to FA and concluded that animal data reveal a qualitative relationship between FA absorption and hepatotoxicity. Woutersen *et al.* (10) found a significant elevation of aspartate amino transferase, alamine amino transferase and alkaline phosphatase activity in the plasma of male rats exposed to 20 p.p.m. as compared with the controls and concluded that the finding might be indicative of hepatotoxic effect of FA. Murphy *et al.* (11) found increased alkaline phosphatase activity in liver of rats exposed to 35 p.p.m. for 18 h and suggested that FA is hepatotoxic.

Because of its rapid metabolism, by the time FA reaches sites which are distant from the respiratory system, its concentration is much lower and hence it is less toxic in these tissues than in those with which it is in immediate contact.

Migliore *et al.* (12) showed that treatment of rats with *per os* FA causes the induction of micronuclei and molecular anomalies in cells of the stomach, duodenum, ileum and colon. These data suggest that FA not only causes nuclear damage at the site of application (stomach), but is also able to reach other segments of the gastrointestinal tract. Oral administration of FA to rats in drinking water caused a significant increase in leukemia incidence and gastrointestinal tumors compared with the controls (13).

The reason that the main effect of FA is in the nasal cavity in rats after inhalation is a complex of factors: rats are obligatory nose breathers, FA is a highly reactive molecule and the presence of high concentrations of detoxifying enzymes, such as FA dehydrogenase, in the nasal mucosa cause rapid metabolism of inhaled FA.

In humans, where respiration is both oral and nasal, the site and degree of FA toxicity is different from that in rodents, namely increased incidence of cancers in the nasopharynx, nasal cavities and paranasal sinuses associated with occupational exposure and possible brain tumors among embalmers, anatomists and other professionals (14).

(ii) Chang *et al.* (15) and others (16) showed that significant concentrations of ^{14}C are localized in the nasal mucosa and in well-perfused tissue, such as bone marrow, liver and kidney, following inhalation exposure of rats to [^{14}C]formaldehyde. Industrial cohorts (17–19) showed possible toxic effects of FA in these tissues (leukemia and lymphatic/hematopoietic cancer), as did rats after exposure to FA by oral administration (13).

In the bone marrow, the highest concentration of ^{14}C was found in the DNA, indicating that the carbon derived from [^{14}C]CH_2O was mainly utilized for DNA synthesis (20) and was covalently bound to macromolecules. Using this method, Casanova *et al.* failed to detect DPC at concentrations of FA <2 p.p.m. (21), and this may be the reason why they could not detect DPC in the bone marrow in normal as well as glutathione-depleted rats. Later they developed a more sensitive method (22) that was able to detect DPC more accurately, even at lower concentration of exposure (0.3 p.p.m.). It is possible that by using this method they could have detected DPC, even in the bone marrow. Using our method, we were able to detect DPC at even lower concentrations, which is closer to the situation in humans. Another explanation is that the increase in the total quantity of DNA in the bone marrow can cause an apparent decrease in the concentration of DPC and that this amount could not be detected by the methods used by Casanova *et al.*

(iii) We agree that the concentration of FA in blood does not increase after exposure to FA, due mainly to rapid metabolism of FA in the erythrocyte (14), but it must be remembered that FA can escape metabolism and be able to react with macromolecules (14). These facts do not preclude the formation of DPC in peripheral WBC, which are in contact with many tissues, including those exposed directly to FA, which have a very rich blood supply and can provide an integrated measure.

(iv) Although SCE or CA were not detected in rats following exposure, these changes were found in WBC of workers exposed to FA. Yager (24) reported increased incidence of SCE among anatomy students after 10 weeks exposure to embalming solution, compared with SCE before exposure. The average short-term breathing zone concentration of FA was 1.2 p.p.m. (24). Goh and Cestero (25) studied chromosomal patterns of direct bone marrow preparations from 40 patients undergoing maintenance haemodialysis. During the period of the study, each patient could have received up to 126±50 mg FA during each dialysis. They found aneuploidies, chromosomal structure abnormalities and chromosomal breaks in metaphase. A study among workers exposed to FA in a paper factory (26) showed a significantly increased frequency of structural chromosomal aberration in lymphocyte of long-term FA exposed paper workers. Suruda *et al.* (27) examined the effect of low level exposure to FA on oral, nasal and lymphocyte biological markers in a group of 29 mortician students. The cumulative FA exposure was 14.8 p.p.m./h, with average air concentration of 1.4 p.p.m. during embalming, and 8 h time-weighted average of 0.33 p.p.m. on days when embalmings were done. Epithelial cells from the buccal area of the mouth showed a 12-fold increase in micronuclei frequency. Nasal epithelial micronuclei increased 22% and the frequency of micronucleated lymphocytes increased 26% during the study period, and a dose–response relationship was observed with cumulative exposure to FA.

We also examined SCE in the same study population in which we examined DPC and found a significant elevation of SCE in the exposed group as compared with the controls (unpublished data).

(v) *In vitro* studies indicate that after 24 h most of the DPC are eliminated (2,28–30). However, in all these studies the assay used was alkaline elution, which lacks sufficient sensitivity at low FA concentrations and cannot discriminate between protein binding to a single DNA strand or protein-mediated DNA interstrand binding. Such qualitatively different forms of DPC may have different consequences with regard to DPC repair and cell survival (30). In contrast to the *in vitro* conditions described (2,28–30), which are different from *in vivo* and mainly concern cell replication and metabolism, DPC from Cr and Ni (31) and FA (1) are persistent *in vivo*. In our study we used WBCs, most of which are in a resting state and differ from the *in vitro* situation with regard to metabolic activation, deactivation and levels of chemical metabolizing enzymes. As the main aim is to assess exposure risk, *in vivo* studies carried out on humans are likely to be more efficacious than *in vitro* studies. Concerning the 'evidence' that DPC are rapidly removed from and do not accumulate in rat nasal mucosa *in vivo* after subchronic exposure to 6 or 10 p.p.m. (32), this is questionable, as the authors themselves said in their article. There are at least three explanations of their findings that do not support the assumption of rapid repair and disaccumulation of DPC *in vivo* (32). There is also a question as to the ability of the assay Casanova *et al.* used in this study to accurately detect the differences in DPC between the study groups at various exposures, as they themselves acknowledge. There is also doubt concerning the results as rats were exposed on single days and, if their theory of removal of the DPC is correct, how much remained on the next day or after the weekend? To prove their theory this study should have been done with continuous subchronic low dose exposure. The theory of Casanova *et al.* of the rapid removal of DPC formed after FA exposure is in contrast to the evidence of the connection between DPC and point mutations in the tumor suppressor gene *p53* in tumors of rats exposed to FA, which may be caused by failure to remove DPC (23).

Implausibility

The concentration of FA in blood after occupational exposure to 3.1 p.p.m. FA estimated by Casanova *et al.* is scientifically questionable, due mainly to the differences in the physiology and anatomy of the respiratory system between rat, monkey and human (see above) (33). Using our assay we can detect DPC at even lower concentrations than 0.001 mM FA, as is shown in our *in vitro* studies (Figure 1). The assay we used is more sensitive and more appropriate to real human life than the method Casanova *et al.* used and it is possible that if they had used our assay, their results would have looked different. As we said above, the formation of DPC in WBC is not influenced by the concentration in the blood. That is why WBCs are widely used in molecular epidemiological studies as surrogate tissues for possible lesions in other tissues, such as the upper respiratory tract, which has presumably received a considerably higher exposure than the peripheral WBC.

The questions Casanova *et al.* posed do not cast any doubt on our results (based on the facts shown). An assessment of human risk should incorporate a collective evaluation of animal toxicity studies and epidemiological studies. At the same time, it must be remembered that not every finding in humans has an explanation in animals, and vice versa.

In order to confirm our results we have recently commenced a larger study.

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