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LETTER TO EDITOR

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THE BEST SAMPLING TIME IN BUCCAL MICRONUCLEUS CYTOME ASSAY

RE: Cassini C, Calloni C, Bortolini G, Garcia SC, Dornelles MA, Pêgas Henriques JA, et al. Occupational risk assessment of oxidative stress and genotoxicity in workers exposed to paints during a working week. Int J Occup Med Environ Health 2011;24(3):308–19

Dear Sir,

I have read the paper of Cassini et al. "Occupational risk assessment of oxidative stress and genotoxicity in workers exposed to paints during a working week" [1] with interest, particularly because the validation and standardization of the micronucleus (MN) test in exfoliated buccal mucosal cells is in progress at present time. Comments and suggestions in this regard are certainly desirable for designing proper methods of the application of this assay in human biomonitoring studies.

The authors describe the results of biochemical and genotoxicological investigations in workers exposed to paints containing toluene and lead during five full working days (Monday-Friday). They applied 3 genotoxicological assays, namely the comet assay, the MN tests in lymphocytes and in exfoliated buccal mucosa cells (BMC). Based on the philosophy of the two first mentioned tests, the investigators can obtain changes (if any) in DNA-damage (migration of DNA) and MNi frequencies (and also other nuclear anomalies) in lymphocytes, because genetic damage (DNA and chromosomal aberrations in the two abovementioned tests, respectively) may be found within a short period of time after exposure, i.e. in some hours in case of the comet assay and after one cell cycle in the MN test in lymphocytes (ca. 24 hours).

As for the MN test in BMC, the time of exposure (five days) is possibly not enough for the formation of MNi (and possibly other nuclear anomalies) and, therefore, it is too short to find any changes in this type of cells. The right time for sampling BMC after exposure is not known yet. Recently, this problem has been briefly discussed [2]. It is assumed that MNi appear in basal cells, and then the cells from the basal layer migrate through the prickle cell and the intermediate layer to the surface layer where they can be obtained. The reported turnover times for BMC are not consistent and vary from 5 to 25 days (cited by Paetau et al. [3]). But this group reported that the turnover of BMC is > 14 days in their human intervention trial with tomato juice or lycopene supplements [3]. According to Squier and Kremer [4], the median turnover time of BMC is 14 days. This data makes one hesitate as to whether the time of sampling is chosen by Cassini et al. [1] correctly.

However, certainly more important are the results obtained in the experiments which studied the formation of MNi as a function of time after exposure. Sarto et al. [5] studied the MNi formation in two cancer patients under radiotherapy with squamous cell carcinomas of the mouth. They presented the results of only one patient with a linear increase of the levels of MNi after receiving 400 rads of gamma-rays (3rd day). Nevertheless, the significance of the difference between the background level and the one after exposure was not presented. Arutyunyan et al. [6] studied MNi levels in 14 patients with oral cavity cancer under radiotherapy every 6 days (after 5 fractions of radiation 2 Gy each). Although the numbers of MNi increased on 13th day, only

on 20th day the frequencies of this endpoint were statistically significant compared with the background levels. The maximum one was registered on 27th day. This data was confirmed by Cao et al. [7] who studied some genotoxicological endpoints after radiotherapy in nasopharengeal cancer patients. They found a significantly increased number of MNi and chromosomal aberrations (CAs) in lymphocytes on 5th and 15th day, respectively, after the first application of radiotherapy. However, a significant elevation of the frequencies of MNi in BMC was registered only on 19th day. In another study, Ramirez et al. [8] suggested analyzing the MNi levels between 3rd and 4th week after exposure based on their study devoted to the treatment of cancer patients with radioactive iodine.

The MN-test in BMC was also applied in patients with various diseases treated with highly mutagenic cytostatic drugs. Sarto et al. [9] studied the MNi level in 7 cancer patients under chemotherapy. Both CAs in lymphocytes and MNi in BMC were found in 5 patients. The remaining two got only interferon which is not genotoxic, and therefore they had no cytogenetic alterations. In one patient, during polychemotherapy of lung cancer, the frequencies of MNi increased on 8th day after three days of consecutive injections of cytostatics and the level remained the same until 14th day. On 21st day the level decreased significantly and reached the value close to the background level. After the second round of injections (28th, 29th and 30th day), the number of MNi increased from 38th until 46th day, and then decreased significantly. Twelve days after the third round of therapy the number of cells with MNi in BMC increased significantly. Hence, in this case, the formation of MNi was found after 8–12 days following the exposure. In other patients, a statistically increased number of MNi in BMC was found on 8th, 10th, 11th and 21st day after injections. Aceves-Avila et al. [10] studied MNi in BMC of patients with systemic lupus erythematosus (not cured before) treated with cyclophosphamide bolus. They found a significantly increased number of cells with MN on 14th day (the only time point studied) after the application of the drug.

Nevertheless, all the above-mentioned papers concern patients with various diseases under treatment. It is logical to propose that the turnover time (MNi formation time) in subjects with tumors (and maybe with other diseases) should differ from that in healthy subjects.

The only study in which BMC obtained from healthy subjects were investigated after exposure to a genotoxic agent is the study of Kassie et al. [11]. They studied the formation of MNi in BMC of khat (a plant containing genotoxic substances) chewers depending on the time after exposure and found that a significantly increased level of MNi, compared with the background level, was found 28 days after the exposure (beginning of chewing khat). Even 7 days after the exposure no increase in the number of MNi was discovered (to say nothing of 5 days of exposure described by Cassini et al. [1]). Based on this data, it is obvious that the time of exposure (5 days) to paints was too short to result in any cytologic and/or cytogenetic changes in BMC of the workers. The statistically significant increase of the frequencies of cells with MNi and the total number of MNi are obtained only between 14th and 28th day after exposure to genotoxic agent(s).

Thomas et al. [12] proposed a protocol for buccal MN cytome assay. They stated that it is theoretically possible to observe the genotoxic effects of acute exposure approximately 7–21 days after it. “Ideally, repeat sampling, at least once every 7 days after acute exposure, should be performed for 28 d or more so that the kinetics and extent of biomarker induction can be thoroughly investigated”. Multiple samplings and then evaluation of an additional set of buccal cells are quite difficult and time-consuming, therefore it is not possible for many laboratories to conduct them. That is why, if only one time point is chosen, it should be between 14 and 28 days after exposure because the exact time of BMC sampling is not known yet.

Other serious shortcomings of the study are 1) the scoring of only 1000 cells and 2) staining the cells with Giemsa.

In the paper of Thomas et al. [12] it is clearly indicated that to obtain robust results in the MN assay in BMC, 2000 cells should be evaluated. There are keratin bodies in BMC which are stained by Giemsa, but not by DNA-specific stains like Schiff's reagent, acridine orange and DAPI [13]. Taking this into consideration, it can be stated that the significant changes in the numbers of binucleated and kalyolytic cells in the control subjects are due to physiological and/or scoring fluctuations. The scoring especially could play an important role because only 1000 cells were evaluated in this study.

The same concerns the data on MNi in lymphocytes. To obtain robust results in this assay, 2000 cells should be evaluated [14]. As can be seen in Table 5, the numbers of nuclear buds (NB) are significantly higher in the exposed group (Monday sampling). The authors did not indicate that in the control subjects the number of NB in Friday's samples is also significantly higher compared with Monday's samples since it is the same (2.93) as in Monday samples of the workers. It means that changes of NB frequencies are due to subjective reasons, i.e. low number of evaluated cells and possible inter-scorers differences. It is not indicated in the paper how many scorers were involved in the investigation.

The authors found that the level of delta-amonovaleric acid, which is an indicator of exposure to lead, was more than 2-fold increased in the workers (1.63 vs. 0.76). It means that they were exposed to lead during their work. But, at the same time, there were not found changes in the MNi levels in the workers compared with the control subjects. In four studies, namely Diaz et al. [15], Pinto et al. [16], Khan et al. [17], and Celik et al. [18], significantly increased numbers of MNi were discovered in the exfoliated buccal cells of workers exposed to paints containing lead, i.e. 1.9-, 3.7-, 7.0- and 3.7-fold, respectively. It is very important that all these groups of investigators used Schiff's reagent for staining and evaluated 3000 cells from each study participant. In my opinion, the evaluation of 1000 Giemsa stained cells instead of 2000 DNA-specific stained cells (at least) leads to a false-negative result, i.e. no difference in the MNi levels in the exposed and control subjects. Celik et al. [18] also found increased levels of all nuclear anomalies in painters compared with the controls. These examples clearly show that a short-time exposure, low number of evaluated cells and the use of not adequate stain led to false-negative results.

There are also some minor errors. The authors wrote in the abstract that "The exposed group showed higher hippuric acid (HA) and delta-aminolevulinic acid levels (Friday samples)", but there are no signs (*) of significant differences in Table 3. It seems to me that there are also significant differences between the values of HA in the controls and the workers on Monday (0.76 vs. 1.63), Table 3, and in case of the DNA damage between the workers and the controls in Monday samples (61.48 vs. 34.94, Table 4). Binucleation is not an indicator of cytotoxicity, it appears due to cytokinetic defects [12]. Moreover, the authors cited for this statement Collins et al., 2008, but the paper concerns some aspects of the comet assay.

Apart from this, I find strange the data concerning the nuclear division index (control group, Monday sample, Table 5). The index is very low (1.23), and even lower than the indexes in the exposed subjects at two time points (1.42 and 1.41). In healthy persons non-exposed to mutagens it should be not lower than 1.8.

In conclusion, this article is quite interesting and raises indirectly the problem of the proper sampling time of buccal exfoliated cells for human biomonitoring studies. At the same time, it contains some shortcomings which should be clarified.

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