Original Paper

ORL

ORL 2001;63:141–147

Received: May 15, 2000
Accepted after revision: November 15, 2000

Genotoxicity of Nitroso Compounds and Sodium Dichromate in a Model Combining Organ Cultures of Human Nasal Epithelia and the Comet Assay

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Key Words
Genotoxicity · Mini organ cultures · Comet assay · Chromates · Nitroso compounds

Abstract
Genotoxic effects of xenobiotics are a possible step in tumor initiation in the mucosa of the upper aerodigestive tract. Using the comet assay, detecting genotoxicity in human tissue has been restricted to single incubations in vitro, but in vivo most xenobiotics harm their target in a repetitive or chronic manner. Therefore, we propose a model, which provides repetitive incubations in human upper aerodigestive tract mucosa cultures. Samples of human inferior nasal turbinate mucosa (n = 25) were cultured according to a modified version of a technique originally described by Steinsvåg. On day 1 fresh samples and on days 7, 9 and 11 organ cultures were incubated with N-nitrosodiethylamine (NDEA), sodium dichromate ($Na_2Cr_2O_7$) and N′-methyl-N-nitro-N-nitrosoguanidine (MNNG). Mucosa samples and organ cultures, respectively, underwent a modified comet assay on days 1, 7 and 11. Genotoxicity could be shown for NDEA, $Na_2Cr_2O_7$ and MNNG on days 1, 7 and 11. Duration of tissue culture and repetitive incubations did not significantly influence the results for NDEA. Nevertheless, $Na_2Cr_2O_7$ and MNNG caused higher genotoxic effects on cultures subjected to the comet assay on day 11. This model may help to assess genotoxic hazards posed by environmental pollutants that have a cumulative character in repetitive or chronic exposure in vivo.

Introduction

Environmental xenobiotics may strike their human targets either in a repetitive or chronic manner. In estimating the in vivo potential of possible genotoxic agents, single incubations with the agent on separated mucosa cells or lymphocytes are used in a variety of in vitro models. Nevertheless, separated cells have a restricted metabolism [1, 2], with possibly altered genotoxic sensitivity and DNA repair mechanisms compared to cells in a tissue

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0301–1569/01/0633–0141$17.50/0

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formation. Moreover, lymphocytes may not be the cell type of first contact with the xenobiotic; thus, they may be exposed to metabolites but not to the original agent. Consequently, the amount of DNA damage measured after a single incubation of separated mucosa cells or lymphocytes and after no time interval of DNA repair does not necessarily reflect the complex in vivo situation of a possible imbalance in DNA damage and repair.

An in vitro model for human mucosal cells should allow repetitive incubations with possible genotoxic agents on cells in their epithelial formation combined with a reliable method of detecting genotoxicity. Therefore, mini organ cultures of human nasal mucosa were established by a modified method based on one originally described by Steinsvåg for investigating the influence of nasal sprays on adenoid tissue [3, 4]. Structurally intact, fully coated cultures with partly ciliated pseudostratified epithelium were incubated with known genotoxic agents for one versus three times. The genotoxic impact was investigated applying a modified comet assay. It was the aim of the present study to prove whether it is possible to use the comet assay on cells harvested from mini organ cultures of human upper aerodigestive tract epithelial cells. Furthermore, possible differences in the amount of DNA damage after one versus three incubation periods with xenobiotics were investigated. In addition, results should be compared with those of noncultured, isolated cells derived from fresh specimens. N-nitrosodiethylamine and sodium dichromate were chosen as xenobiotics in this test series because they are well-known habitual and occupational pollutants with genotoxic and carcinogenic effects.

Materials and Methods

Donors

Specimens of approximately 25 mm by 5 mm by 1 mm of human mucosa from the lower ridge of the inferior nasal turbinates (n = 25, 9 female, 16 male patients, average age 38.8 years, 12 smokers, 13 nonsmokers, all donors had an anamnesis of no or of up to 50 mg of alcohol per day) were harvested during surgery on the nasal air passage. The patients were otherwise healthy, and only as much mucosa was resected as was necessary for the benefit of the patient as regards ease of nasal breathing to prevent any additional risk to the patient. The patients had signed an informed consent statement. The study was approved by the Ethic Commission of the Medical School, Ludwig-Maximilian University, Munich.

Culture

Specimens were dissected into cubes of 1 mm^3 using only mucosa excluding any deeper layers and bony structures. Specimens were washed in bronchial epithelial cell growth medium (BEGM; Promocell, Heidelberg, Germany) three times and placed in 24-well plates, one fragment in each well, that were coated with 0.75% Agar Noble dissolved in Dulbecco's modified Eagle medium (Gibco, Eggenstein, Germany), 10% fetal calf serum (FCS) and nonessential amino acids, streptomycin und amphotericin B. Mini organ cultures floated in their medium and did not adhere to the plates.

250 µl BEGM served as medium per mucosa fragment. Cultures were subjected to a temperature of 37°C, in an atmosphere of 5% CO_2 with 100% relative humidity. BEGM was renewed every other day, and the multiwell plates were replaced after incubations on days 7 and 9 since the supplements of the Agar Noble are used up with time and the plates are contaminated by the xenobiotics. After 5 days, the initial mucosa fragments appeared completely coated with partly ciliated epithelium [3, 5–7] (fig. 1).

Incubation

A portion of the fresh samples of each donor was incubated for 60 min on day 1 before transferring the remaining portion to cultures of mini organs. Subsequently the comet assay was applied on these first portions of mucosal cells according to the standard protocol [8, 9].

Mini organs were incubated for 60-min periods on days 7, 9 and 11. Earlier incubations did not appear to be appropriate since it takes approximately 5 days until the mini organs are fully coated by superficial epithelial cells. 50 µmol/ml N-nitrosodiethylamine (NDEA; Sigma, Deisenhofen, Germany), 1 µmol/ml sodium dichromate ($Na_2Cr_2O_7 \times 2H_2O$, Sigma) and 0.07 µmol/ml of N′-methyl-N-nitro-N-nitrosoguanidine (MNNG; Fluka, Buchs, Switzerland) were tested as xenobiotics. After 60-min incubation periods with the xenobiotics, cultures were washed in BEGM three times and placed onto new plates to exclude further genotoxic reactions. Dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) was used as solvent for MNNG and NDEA. Sodium dichromate was solved in in aqua bidest. DMSO being the more aggressive chemical was used as the negative control (166 µmol/ml). Concentrations of the substances were chosen according to pilot tests on mini organs, dose-response relations and prior studies. Viability tests during culture were performed recording the cilia function by inverse microscopy.

Comet Assay

Aliquots of fresh samples (approx. one third of each individual sample) were applied to the comet assay on day 1 after cell isolation and incubation with the xenobiotics. The alkaline comet assay was performed on days 7 and 11 immediately after incubation on the remaining aliquots of approximately half of the mini organs on each day. 5×10^4 cells could be harvested per mini organ. The alkaline version of the microgel electrophoresis technique (comet assay) was applied to primarily detect DNA strand breaks in single cells. This technique was originally described by Oestling and Johanson [10] in a neutral version, where it is supposed to detect double-strand breaks. The samples underwent enzymatic digestion with collagenase P (1 mg/ml), hyaluronidase from bovine testes (1 mg/ml; Boehringer, Mannheim, Germany) and pronase E type XIV from *Streptomyces griseus* (5 mg/ml; Sigma) for 45 min in a 37°C shaking water bath. Enzymes were dissolved in BEGM and digestion was neutralized by FCS before cells were washed in phosphate-buffered saline twice. Cell isolation from human epithelia for the use in the comet assay was first described by Kuchenmeister [11] and Pool-Zobel et al. [12].

Slides with a frosting of 5 mm along the long edges were developed with the Langenbrinck company (76 mm × 26 mm; Langenbrinck, Emmendingen, Germany) [13]. They were prepared with 85 µl of 0.5% normal melting agarose (Biozym, Hameln, Germany). The viability of the cells was examined using trypan blue staining, obtaining between 80 and 100% viable cells. The remaining aliquots were embedded in 75 µl of 0.7% low melting agarose (Biozym) and applied to the prepared slides. Alkaline lysis (10 ml DMSO, 1 ml Triton-X, 89 ml alkaline lysis buffer: 2.5 *M* NaCl, 10 m*M* Tris buffer, 100 m*M* EDTA-Na_2; pH 10) followed for 1 h. Slides were placed into a horizontal electrophoresis chamber (Renner, Dannstadt, Germany), positioned close to the anode and covered with alkaline buffer solution containing NaOH (10 m*M*) and Na_2-EDTA (200 m*M*) with pH 13.2. After a 20-min DNA 'unwinding' period, electrophoresis was started with 25 V and 300 mA for 20 min. Following neutralization (Trizma base, pH 7.5, Merck), the DNA was stained with ethidium bromide (Sigma).

Analysis

The slides were examined using a DMLB fluorescence microscope (Leica, Heerbrugg, Switzerland) with an adapted CCD camera (Cohu Inc., San Diego, Calif., USA).

Depending on the degree of strand break induction, the DNA fragments demonstrate different types of migration within the electric field, creating a comet with tail configuration when fragmentation, incomplete excision repair or alkali-labile sites occur (fig. 2, 3). The head of the comet contains the portion of DNA with no or minor fragmentations and shorter running distance in electrophoresis, whereas its tail represents the fragmented DNA after induced strand breaks. The comets were measured using an image analysis system (Komet 3.1, Kinetic Imaging, Liverpool, UK). To quantify the induced DNA damage we used the Olive tail moment (OTM), which is described as the amount of DNA in the tail relative to the amount of DNA in the head of the comet multiplied by the median of migration distance [14].

Statistics

The DNA migration of 80 cells per substance and donor were examined. For statistical analysis, the Wilcoxon test of the SPSS™ 8.0 program was applied since the tested specimens of the different days were achieved from the same donors (matched pairs). Genotoxic impacts on day 1 on single cells of fresh samples according to

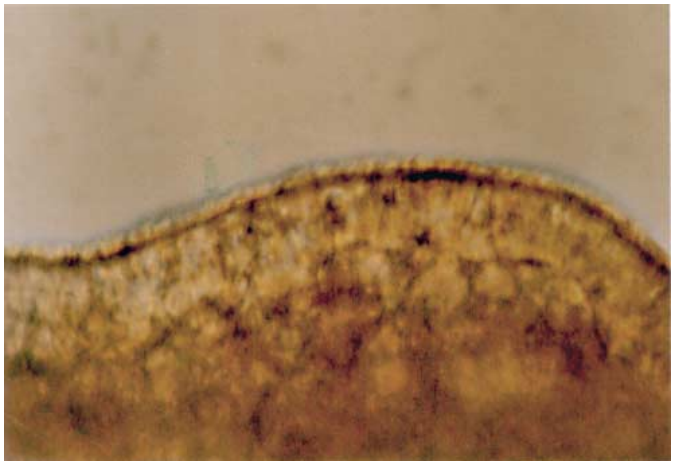

Fig. 1. Mini organ culture of human nasal epithelium on day 7 of culture. Note the ciliated pseudostratified epithelium covering the mini organ. Since this photomicrograph does not represent a slice through but a microscopic view onto the mini organ in its culture medium, there are many layers superposed in that photograph. However, the photograph demonstrates viable cells of the mini organs in their epithelial formation. ×400 magnification at inverse microscopy.

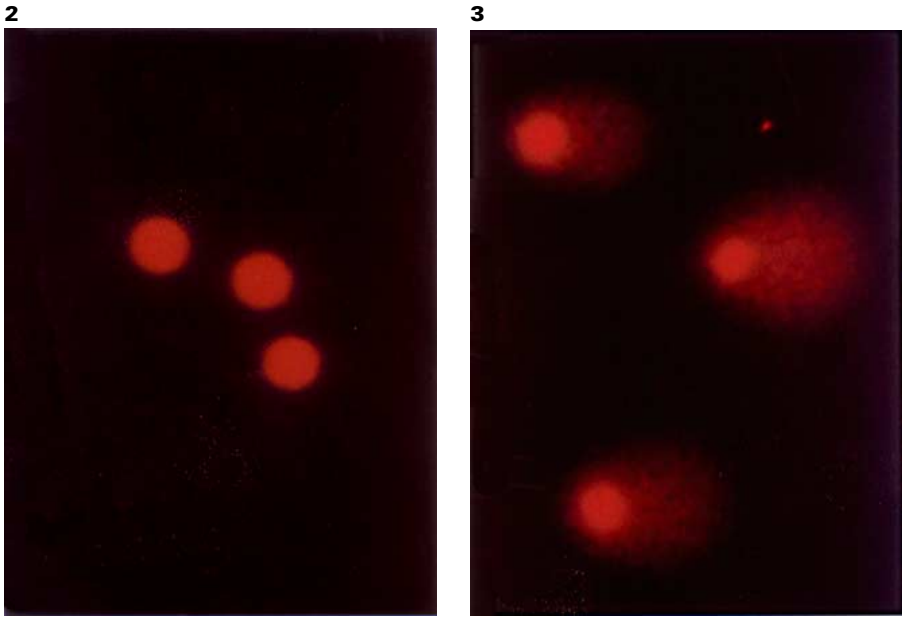

Fig. 2. DNA of epithelial cells after incubation with DMSO as negative control, electrophoresis and ethidium bromide staining. Note the round structure and the clear borders of the unfragmented DNA in the nuclei. Fluorescence microscopy, ×400 magnification.

Fig. 3. DNA of epithelial cells after incubation with sodium dichromate, electrophoresis and ethidium bromide staining. Note the DNA fragments that are pulled out of the DNA conglomerate by electrophoresis after fragmentation. Fluorescence microscopy, ×400 magnification.

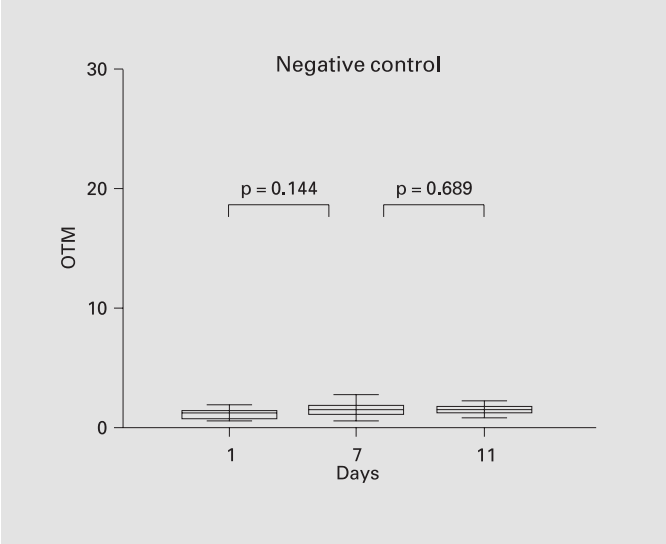

Fig. 4. Box plots showing the lowest and highest values of OTMs as well as the 1st and 3rd quartiles and the median of the negative control on days 1, 7 and 11. There were no significant differences between the tests on fresh specimens on day 1 and the tests on day 7 on mini organ cultures nor between day 7 and day 11. p values of the Wilcoxon test were 0.144 and 0.689, respectively. OTM values below 2 represent undamaged DNA.

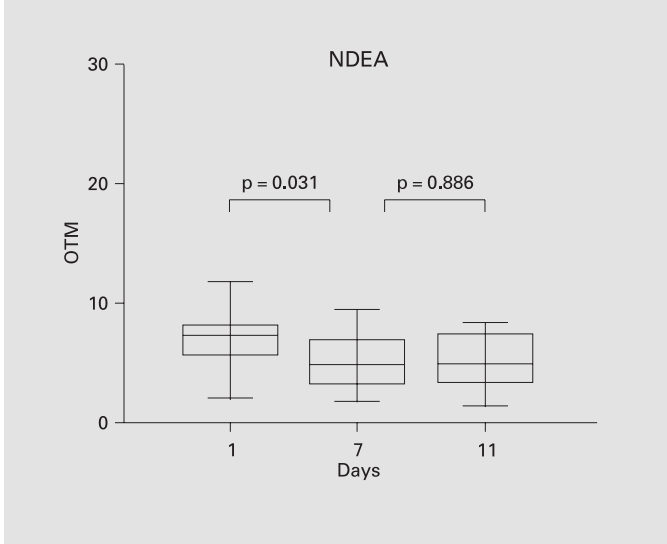

Fig. 5. OTMs obtained after the incubation with NDEA on fresh specimens (day 1) and mini organ cultures (days 7 and 11). Results for day 7 were significantly lower than on day 1 (p = 0.031). No differences could be detected between day 7 and day 11 (p = 0.886). For the definition of the box plots, see figure 4.

standard protocols and on day 7 on mini organs with single incubations were compared. Furthermore, DNA strand breaks on day 7 with single incubations versus day 11 with threefold incubations in mini organ cultures were analyzed. The Bonferroni-Holm method was performed to correct for multiple testing. The overall level of statistical significance was 0.05.

Results

For the first time, the comet assay was applied on cells harvested from mini organ cultures of human nasal epithelia. Results of the genotoxicity tests varied depending on the substances tested. Nevertheless, cell viability and cilia function remained uncompromised for all substances. The following differences could be shown after incubation with xenobiotics.

Control

Concerning the negative control incubated with DMSO, no significant differences between tests on neither fresh samples (day 1) versus mini organ cultures (day 7) nor between tests on mini organ cultures on day 7 and 11 day, respectively, were found (fig. 4).

N-Nitrosodiethylamine

In testing the genotoxicity of NDEA, results from fresh samples (day 1) were higher than the results from organ cultures on day 7. No differences could be detected between the results on day 7 (single incubation with the xenobiotic) and day 11 (three 60-min incubations of the mini organ cultures; fig. 5).

Sodium Dichromate

Assessing genotoxicity for this chromate, there were no differences found between day 1 and day 7. Nevertheless, the genotoxic impact of 3 incubation periods on mini organs harvested on day 11 proved to be significantly higher than that of a single incubation on the mini organs harvested on day 7 (fig. 6).

N′-Methyl-N-Nitro-N-Nitrosoguanidine

For the directly alkylating MNNG, genotoxicity levels presented as equal on days 1 and 7, whereas day 11 had significantly higher genotoxic impacts after 3 incubations with this agent compared to day 7 with only a single incubation (fig. 7).

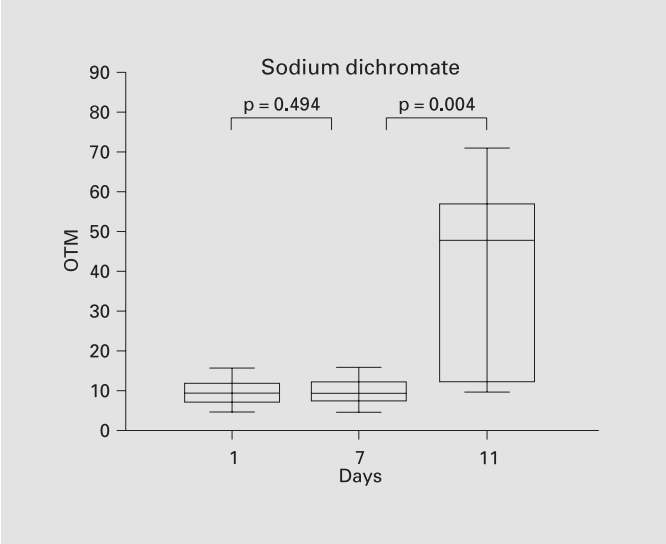

Fig. 6. OTMs obtained after incubation with sodium dichromate ($Na_2Cr_2O_7$) on fresh specimens (day 1) and mini organ cultures (days 7 and 11). Box plots show equal values for tests on day 1 and day 7 (p = 0.494), whereas on day 11 OTMs are significantly higher (p = 0.004), representing additive genotoxic effects of multiple incubations with the xenobiotic.

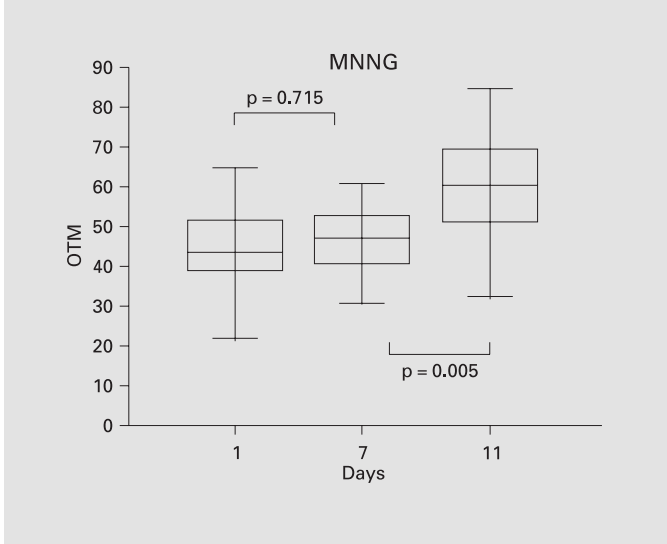

Fig. 7. MNNG showed an additive genotoxic effect after 3 incubations of 60 min each in mini organ cultures (day 11 vs. day 7, p = 0.005), whereas the level of DNA fragmentation on days 7 and 1 was equal (p = 0.715).

Discussion

To what extent in vitro techniques are appropriate to elucidate phenomena in vivo depends on the phenomena to be studied and the conditions under which the investigations are performed. Although observations in vitro should not be taken as hard evidence for the presence or absence of certain properties in vivo, it is generally accepted that tissue cultures may be valuable in the exploration of functional tissue properties [15]. Therefore, tissue cultures have been used widely to investigate, e.g., arteriosclerosis in cultures of the intima of the aorta [16], hormone production in cultures of the thyroid tissue [17, 18], genotoxicity in endometrium cultures [19] and sensitivity against tirapazamine in tumor spheroids [20]. Although the techniques and objectives of these publications are diverse, they share a common aim: ensuring the integrity of cells in their organ-specific structure. Combining mini organ cultures of human nasal mucosa, thus allowing repetitive incubations with xenobiotics, and the comet assay may provide a better surrogate for in vivo conditions than single incubations on separated cells. All the more so as epithelial cells need the epithelial organization for the metabolism of xenobiotics [2].

The equal levels of OTM and persistent good viability of the cells in the negative control incubated with DMSO in the tests on days 1, 7 and 11 proved the ability of the model to provide sufficient vital cells for the comet assay cultured under stable conditions. Additional viability tests were performed by controlling cilia function. There was no evidence of ongoing apoptosis. Nevertheless, incubating with NDEA, the detected damage for day 7 after a single incubation of the mini organ cultures was lower than in the tests with fresh specimens on day 1. This may be possibly due to the fact that in day 7 tests, after incubation of the mini organs, there is a 45-min period for separating the cells, whereas in the fresh samples single cells are incubated. During this interval DNA repair may occur, as shown for lymphocytes or mucosal cells for that time period [21, 22]. Still, there are no data available on DNA repair in conjunction with enzymatic digestion. Further controls will provide additional information on mutagen sensitivities of mini organs and single cells of human epithelia. Even after 3 incubations with NDEA in tests on day 11, no higher OTMs could be detected compared to a single incubation of the mini organs on day 7. Thus, DNA damage caused by the given concentration of this nitrosamine is very likely to be

repaired by epithelial cells to a certain extent; no additional effect of threefold incubation is to be expected. Unfortunately, a protocol with incubations on days 11 vs. 7, 9, 11 and genotoxicity tests on day 11 for both groups was not possible due to the limited number of mini organs per donor not allowing for sufficient amounts of cells for viability tests.

On the other hand, in testing the chromate $Na_2Cr_2O_7$ and the nitrosamide MNNG, OTM results did not differ between tests with the comet assay on fresh mucosa samples and mini organs of mucosa of the same group of donors after a single 60-min incubation. This suggests that DNA damage caused by these two agents will not be sufficiently repaired by the cells during the interval of separating the cells of the mini organs. Although the development of DNA comets in this assay is augmented by fragments of incision repair [23], the significantly higher DNA fragmentation after 3 incubations in tests on day 11 suggests an additive effect of DNA damage caused by the two agents in repetitive incubations.

NDEA and $Na_2Cr_2O_7$ were chosen as xenobiotics because of their known genotoxic effects and their importance as habitual and occupational pollutants [24]. The present data provide additional information on the significance of these effects on human mucosa. Whereas the nitrosamine and the chromate in fresh mucosa samples cause single-strand breaks to an equal degree, as shown in the present investigation, fragmentation due to NDEA was lower in the mini organs of day 1. This may reflect a more pronounced hazard posed by $Na_2Cr_2O_7$ in vivo since this substance proved to significantly damage DNA of integrated epithelial cells. Further investigations on the significance of incubating single cells, as in the fresh samples of day 1, and cells in their epithelial structure, as in the mini organs of days 7 and 11, will provide additional data on this issue.

Obviously, the three tested xenobiotics, NDEA, $Na_2Cr_2O_7$ and MNNG, have different mechanisms for inducing DNA damage. Whereas the nitrosamine NDEA needs to be activated by cytochrome-P450-depending hydroxylation to act genotoxically [25], the nitrosamide MNNG has a direct impact on the DNA by alkylation. Genotoxicity of chromates depends on their bioavailability in the cells, which is closely related to the degree of oxidation, thus a matter of, e.g., the pH of the tissue. The oxidation state 6 is one of the most frequently encountered states in chromates and presumed to be the most reactive state, too, partly due to the ability to readily cross cellular membranes via nonspecific anion carriers [26]. Furthermore, the ability of cells to repair the damage depends not only on the amount of DNA fragmentation, but also on the agent and its specific mechanism of DNA damage.

Conclusion

The test setting of mini organ cultures of human mucosa combined with the comet assay promises to be a beneficial tool in the assessment of genotoxic hazards posed by environmental pollutants in vitro. It allows for detection of DNA damage that may show a cumulative character in repetitive or chronic exposure in vivo. Compared to studies focusing on biomonitoring [27], amounts and concentrations of specific substances, and thereby exposure levels, may be well defined. Nevertheless, refinements of the test setting will provide additional information on the balance or imbalance of DNA damage and DNA repair. Especially tests on incubations of the whole mini organs versus single cells harvested from those organs will be helpful. A higher number of mini organ cultures resulting from larger specimens in selected cases of hyperplasia of the inferior nasal turbinate will also allow additional genotoxicity tests after longer periods of culture with or without further incubation with the xenobiotic. Furthermore, the modality of incubation with the xenobiotic may be elaborated by, e.g., applying defined concentrations of the substance by perfusion of the cultures. However, mini organ cultures and the comet assay have the potential to become a relevant tool in describing genotoxicity of xenobiotics and susceptibilities of individuals. Thereby, they may contribute to preventive measures against upper aerodigestive tract malignancies.

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