Toxicological Characterisation of Dermal Exposure to DBPs by an In Vitro Skin Model

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Abstract
The possible relationship between disinfection by-products (DBPs) of swimming pool water chlorination and bladder cancer is the background of our work. The applied methodology is a combination of the human reconstructed skin micronucleus (RSMN) assay and of the analysis of the micronuclei frequency with a complex flow cytometry staining and analysis protocol. First experiments with the positive control vinblastine sulphate (VB) show a dose dependent increase of micronucleus frequency and IL1-alpha release of the cells of the skin model. The results with mitomycin C (MMC) were not clear. In the next experiments it will be analysed, if the solvent acetone is responsible for this. The exposure of the skin model to 2,5-Dichloro-1,4-benzoquinone and 2,6-Dichloro-1,4-benzoquinone, a new identified toxic DBP, showed an increase in micronucleus and hypodiploid DNA formation.

Keywords: skin model, dermal exposure, disinfection by-products, micronucleus, flow cytometry

Introduction

The central issue regarding the quality of drinking water and swimming pool water has been and still is to keep it free of pathogens. The main approach is to add chemical substances with a long term effect for disinfection. In recent years the focus has also moved to possible adverse effects of that water treatment. In many cases the consequence of water chlorination and brominating is the formation of a vast number of disinfection by-products (DBPs). Many of them are already known to be genotoxic (Richardson et al. 2007, Kogevinas et al. 2010). A possible relationship between exposure to DBPs and bladder cancer has been discussed by Villanueva et al. 2004 and 2007.
A further step to explore this issue is to receive evidence whether DBPs have the ability to migrate through the upper skin layers and produce certain negative effects on the lower layers regarding genotoxicity and pro-inflammatory stimulations or not. A possible approach has been found by applying a skin model with a flow cytometry based analysis. The methods used for the in vitro skin model for testing the effect of DBPs in water samples and first attempts of its validation are the contents of this communication.

Work Description

The test protocol has been set up based on the OECD guideline No. 487 (2010) for the in vitro mammalian cell micronucleus test and the work of Curren et al. 2006, Mun et al. 2009, Hu et al. 2009 and Bryce et al. 2008 and 2010. More detailed the EST1000 Epidermal Skin Test (CellSystems®, Troisdorf, Germany) is applied to simulate the absorption of DBPs through the upper skin layers and its effects on the lower ones. A 48 h repeated dosing regimen is used. The first treatment with the test substances is applied topically about 3 h after the skin model tissue has arrived in the laboratory. The second treatment is performed 24 h later. Depending on the experiment the skin model is trypsinized directly after the incubation time of the second treatment or after further 24 h incubation for recovery. The protocol to gain a single cell suspension of the layers below the stratum corneum has been described by Curren et al. 2006 and Dahl et al. 2011. In the next steps the In Vitro MicroFlow™ Kit (Litron Laboratories, Rochester, New York, USA) is used to measure micronucleus frequencies caused by the test substances via flow cytometry. The protocol of the kit enables the user to distinguish between viable and already apoptotic or necrotic cells by the stepwise staining and lysing of the cells and its DNA. By adding a defined number of beads to every sample preparation, the cells per beads ratio can be calculated as a measure for cell viability.
Samples of cell culture medium are taken after every treatment and stored at -20 °C. IL1-alpha concentration in the samples is determined with the Quantikine® Elisa Kit (R&D Systems GmbH, Wiesbaden, Germany) according to the relating protocol.

For test validation MMC (clastogen activity) and VB (aneugen activity) dissolved in acetone have been chosen as positive controls (Aardema et al. 2010, Hu et al. 2009). The newly in drinking water identified toxic 2,6-Dichloro-1,4-benzoquinone (2,6DCBQ) (Qin et al. 2010) and its derivate 2,5-Dichloro-1,4-benzoquinone (2,5DCBQ) have been selected to be tested with this method.

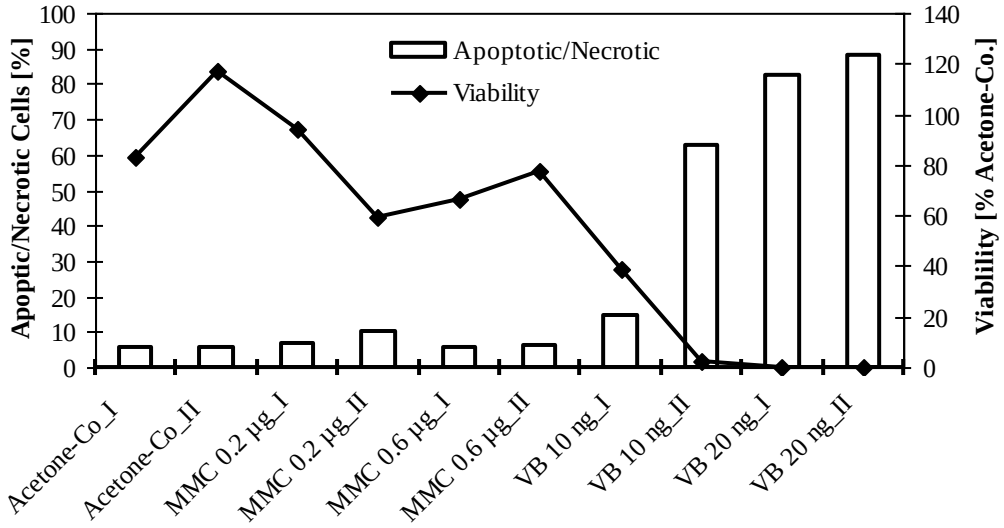

Figure 1 Percent of apoptotic/necrotic cells and viability based on the acetone control according to the given treatment, two exposures with 24 h incubation each and extra 24 h recovery time, results of one experiment.

The test concentrations of MMC (two times 0.1 and 0.3 µg/tissue) caused up to 10 % apoptotic or necrotic cells and 77 to 95 % of the cells were still alive. In contrast VB in concentrations of two times 5 ng and 10 ng per tissue lead to over 99 % death cells and causing 15 to 88 % of the cells to be in an apoptotic or necrotic stage (Figure 1).

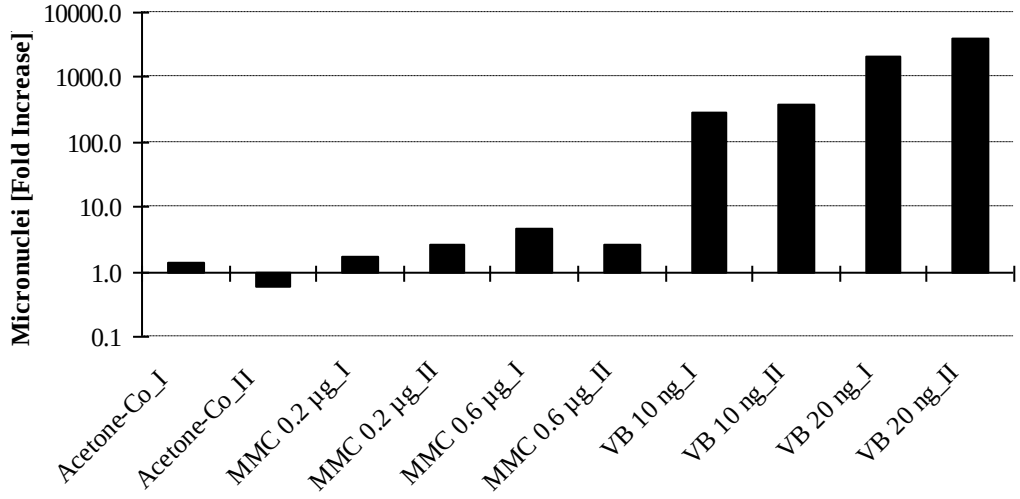

Figure 2 Fold micronuclei increase based on the acetone control according to the given treatment, two exposures with 24 h incubation each and extra 24 h recovery time, results of one experiment.

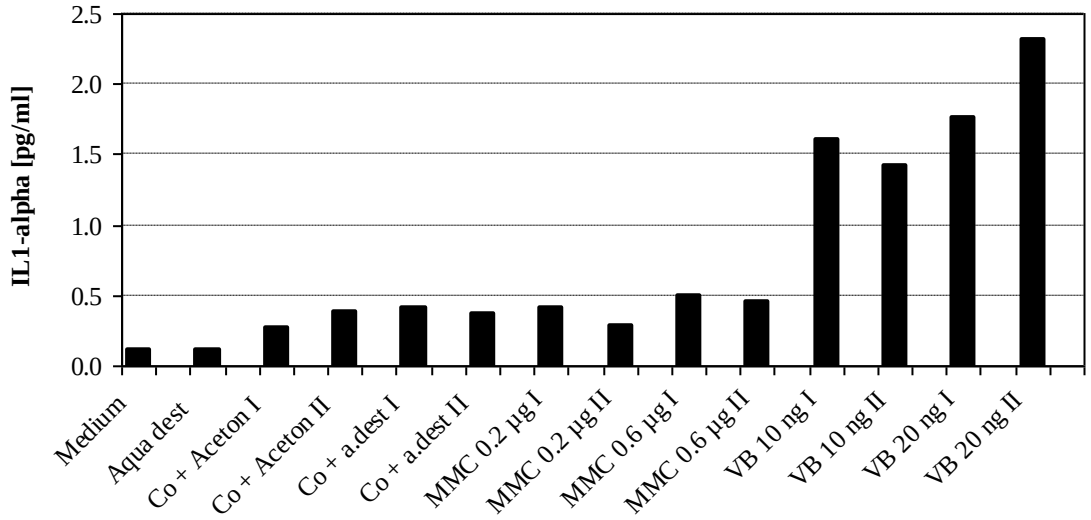

Figure 3 IL1-alpha release from the skin model, measured in the medium below the cells according to the given treatment, samples taken after two exposures with 24 h incubation each and extra 24 h recovery time.

First experiments with the positive control VB show a dose dependent increase of micronucleus frequency and IL-1-alpha release of the cells of the skin model (Figure 2 and 3). The results with MMC were not clear. In the next experiments it will be analysed, if the solvent acetone is responsible for this.

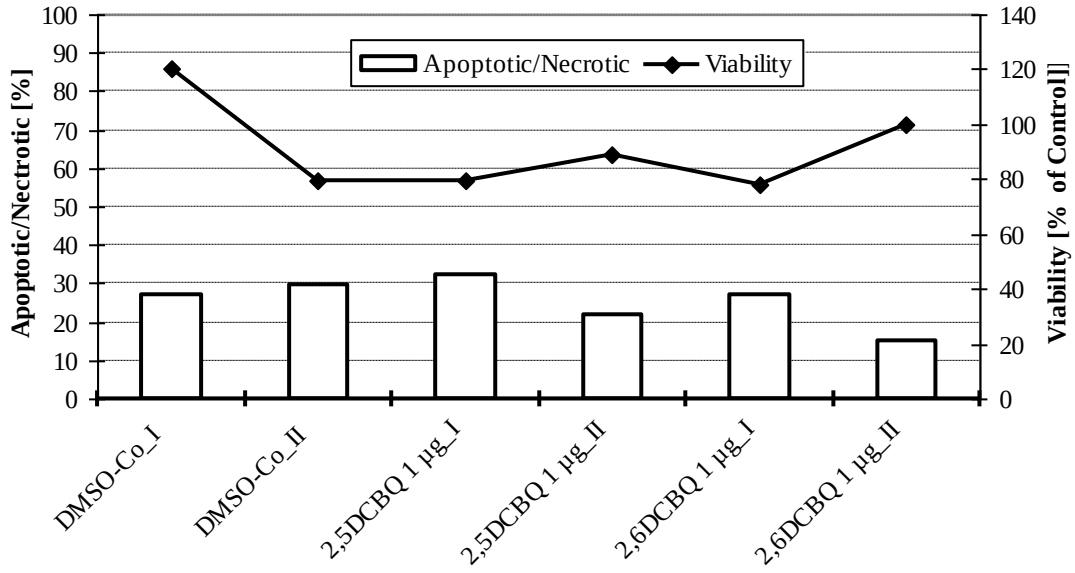

Figure 4 Percent of apoptotic or necrotic cells and cell viability based on the DMSO control after two applications of 10 µl DMSO without or with test substance, 24 h incubation each, results of one experiment.

The DBPs 2,5DCBQ and 2,6DCBQ (two times 0.5 µg/tissue) as well as the DMSO control induced a low degree of apoptotic or necrotic cells of 15 to 32 %. The viability of the cells treated with 2,5DCBQ and 2,6DCBQ ranged from 79 to 99 % based on the mean of the DMSO controls (Figure 4). The number of micronuclei increased after treatment with 2,5DCBQ by 2.7 to 5.6 fold and with 2,6DCBQ by 6.6 to 11.6 fold. The occurrence of hypodiploid DNA fragments augmented by 2.9 to 17.3 fold (2,5DCBQ) and by 17.7 to 31.6 fold in case of 2,6DCBQ compared to the DMSO-control (Figure 5).

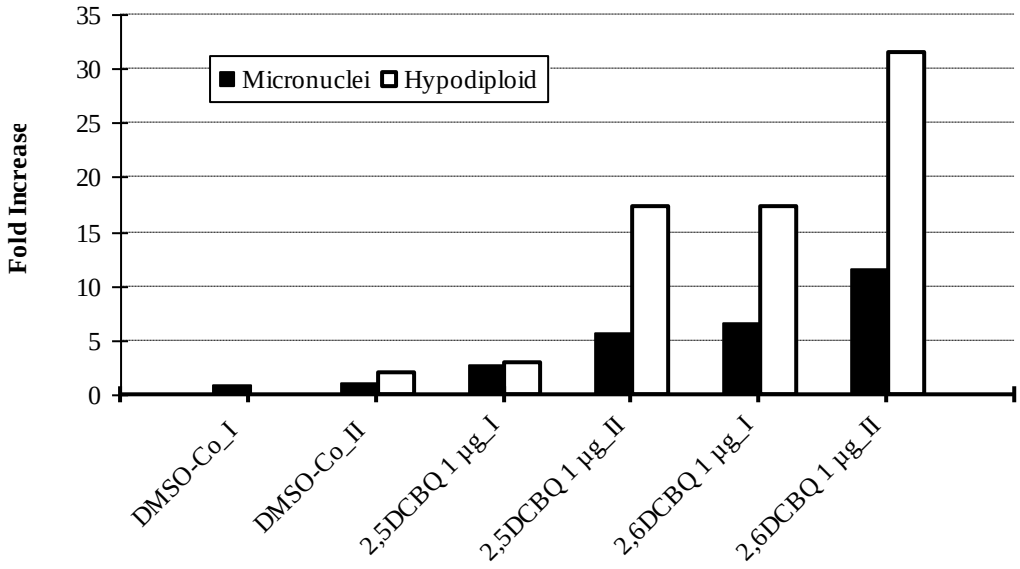

Figure 5 Fold micronuclei and hypodiploid DNA increase based on DMSO control after two applications of 10 µl DMSO without or with test substance, given is quantity per tissue (two times 0.5 µg), 24 h incubation each, results of one experiment.

The results reveal an obvious difference between the percent of apoptotic or necrotic cells and cell viability after the treatment with VB and the other three test substances. In case of VB the very strong increase of micronuclei is connected to a high degree of cytotoxicity in the tested concentrations. 2,6DCBQ, as a member of the DBPs, shows a strong mutagenic potential in combination with a relatively low cytotoxicity. With this characteristic it would also be qualified as a third positive control. Some parallels show, that intra test reliability still has to be improved.

Conclusions

The in vitro skin model is very interesting, as it simulates dermal absorption through the upper skin layer and damage in lower skin layers without animal testing and with human cells. Compared to the microscopic determination of micronucleus numbers, the flow cytometry analysis allows the measurement of a multiple of cells in only a fraction of the time. A first experiment with DBPs in DMSO showed an increase in micronucleus formation. Until it is possible to use the method in the screening of water samples and of single DBPs still some verification experiments are necessary regarding its reproducibility and sensitivity.

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