MIDDLE POMERANIAN SCIENTIFIC SOCIETY OF THE ENVIRONMENT PROTECTION

Rocznik Ochrona Środowiska

Volume 21. Year 2019 ISSN 1506-218X 1217-1226

Cytotoxic Effects of Two Parabens Determined in Surface Waters and Sewage Sludge on Normal (Senescent) Human Dermal Fibroblasts

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1. Introduction

Parabens, i.e. esters of p-hydroxybenzoic acid (also referred to as nipagins or aseptins), are commonly known preservatives frequently used in cosmetics and medicines due to their broad spectrum of antimicrobial activity (Andersen 2008; Cashman & Warshaw 2005; Dębowska 2016; Muszyński & Ratajczak 2009; Soni et al. 2005). Another advantage of nipagins is their low toxicity and activity over a wide pH range (4-8). As parabens do not demonstrate drug incompatibilities, they can be added to medicines.

Parabens are used to preserve such cosmetic products as creams, soaps, perfumes and deodorants, hair care products, shaving and depilation products, makeup products, nail care products, and agents with UV filters. Almost all leave-on cosmetic products (intended to stay in prolonged contact with skin) are preserved with parabens. They are also frequently found in rinse-off cosmetic products (Bojarowicz et al. 2012).

Mixtures of parabens (in particular methyl paraben and propyl paraben due to the synergism of their actions) are most frequently used.

A concentration of a single paraben in cosmetic products in EC must not exceed 0.4%, while the maximum permitted content of a paraben mixture is 0.8%, according to the Annex V of EC Regulation on cosmetics (Regulation (EC) No 1223/2009 of the European Parliament and of the Council). The most commonly used preservative mixture is a combination of 0.2% methyl paraben with 0.1% propyl paraben.

The use of esters of p-hydroxybenzoic acid in cosmetics may be also due to their properties other than an antimicrobial action; e.g. in soaps they also serve as anti-perspirants, and in shampoos they are used as anti-dandruff agents as well.

Methyl and propyl esters of p-hydroxybenzoic acid have also been used in ophthalmic medicines at concentrations of 0.065-0.15%, and in ointments and oral medicines at a concentration of 0.5%.

Widespread use of parabens is bothering because concerns include endocrine disruption, carcinogenicity (specifically breast cancer), neonatal and perinatal exposure risks, fertility, spermatogenesis disturbance, emotional disorders, and environmental impact (Fransway et al. 2019).

Parabens are present in the natural environment as its significant contaminant, although data on their dissemination in the environment and the secondary sources of exposure to both humans and animals are scarce.

Kijeńska et al. (2016) report that the total content of (methyl, ethyl, propyl and benzyl) parabens amounted to 10.43 ng/g dry matter in sewage sludge collected from five Polish municipal sewage treatment plants. The highest content in all analysed sewage sludge samples was noted for methyl paraben (2.31-2.83 ng/g dry matter).

In surface waters, paraben concentrations ranging from 15 to 400 ng/l were observed (Brausch & Rand 2011), and it should be noted that parabens contaminating surface waters may pose a hazard to aquatic organisms since even their low levels may exhibit estrogenic effects (Dobbins et al. 2009). In the muscle tissue of marine fish inhabiting the Manila Bay waters, a concentration of methyl paraben ranging from 605 to 3,450 ng/g was determined, while that of propyl paraben ranged from 46 to 1,140 ng/g (Haman et al. 2015).

Methyl paraben and propyl paraben were also detected in human urine at concentrations of 43.9 and 9.05 ng/ml, respectively (Ye et al. 2006).

For this reason, studies into toxic effects of parabens on human cells may also be of significance in terms of the environmental exposure of humans to these 4-hydroxybenzoic acid derivatives.

This article presents the results of the study into the cytotoxic effects of (methyl and propyl) parabens of the normal fibroblasts isolated from human skin.

2. Material and methods

2.1. The analysed parabens, cell line, reagents and culture medium

A study was carried out on two esters of p-hydroxybenzoic acid, namely methyl paraben (MePB) (CAS 99-76-3) and propyl paraben (PrPB) (CAS 94-13-3).

The control group included cells incubated in a xenobiotic-free culture medium (untreated cells = 100% viability).

The cytotoxicity study was carried out on an ageing cellular line of diploid human dermal fibroblasts CCD-1136Sk (ATCC® CRL-2697™) purchased through LGC Standards, the exclusive European distributor of the American Type Culture Collection (ATCC) products. The study into the effects of preservatives on the cells were carried out on passages numbered from 8 to 20.

For cell culturing, the following were used: culture medium Iscove's Modified Dulbecco's Medium (ATCC® 30-2005™) with 10% of Foetal Bovine Serum (catalogue No. 10084-150) manufactured by Gibco BRL (Life Technologies Ltd. Paisley, Scotland), and a trypsin solution (0.25%) and EDTA (catalogue No T-4049) manufactured by Sigma (Sigma Chemical Company, St. Louis, Mo USA).

Cells were maintained in monolayer cultures, at 37°C in a humidified atmosphere with 95% air and 5% CO_2.

2.2. Cytotoxicity assessment methods

The cytotoxicity assessment was carried out in accordance with INVITTOX protocols No 17 and 64 for the MTT and NRU test, respectively (INVITTOX 1990 and 1992). The MTT test involves the assessment of the metabolic activity of cells, expressed by the capability of absorbing a dye, namely the yellow tetrazolium salt (MTT), and reducing it, mainly in the mitochondria with the participation of succinate dehydrogenase, to a formazan compound with a purple-navy blue colour.

The principle of the NRU test which assesses the cell membrane integrity is based on the viable, undamaged cells' capability of absorbing a dye, namely neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) which accumulates in lysosomes.

Based on the MTT and NRU tests, the analysed substances' concentrations inhibiting the viability of cells by 50% (IC_{50}) were determined. IC_{50} values (median inhibitory concentration - concentration required for 50% inhibition of cells viability compared to the negative control, which was accepted as 100%: % cell viability = {(Absorbance value of treated cells - Absorbance value of blank)/(Absorbance value of untreated cells - Absorbance value of blank)} x100). The IC_{50} values for each compound were calculated with a computer program using curve interpolation (four-parameter logistics), Gen5™ Data Analysis, manufactured by BIO-TEK INSTRUMENTS, INC.

2.3. Methodology for statistical analyses of results

For testing of general hypotheses in the analysis of variance, F-Snedecor test was applied, while in multiple comparisons for simple effects, the Bonferroni test was applied.

3. Results and discussion

The obtained IC_{50} values for the analysed parabens on subsequent passage human dermal fibroblasts are presented in Fig. 1.

Having compared the cytotoxicity of both analysed parabens on CCD-1136Sk cells (ATCC® CRL-2697™) based on the IC_{50} values determined in the MTT and NRU tests, it was found that propyl paraben (PrPB) was more toxic; however, none of the tests found a consistent trend for a change in the cells' sensitivity to the analysed parabens as they passaging, that would be reflected in the determined IC_{50} values (Fig. 1).

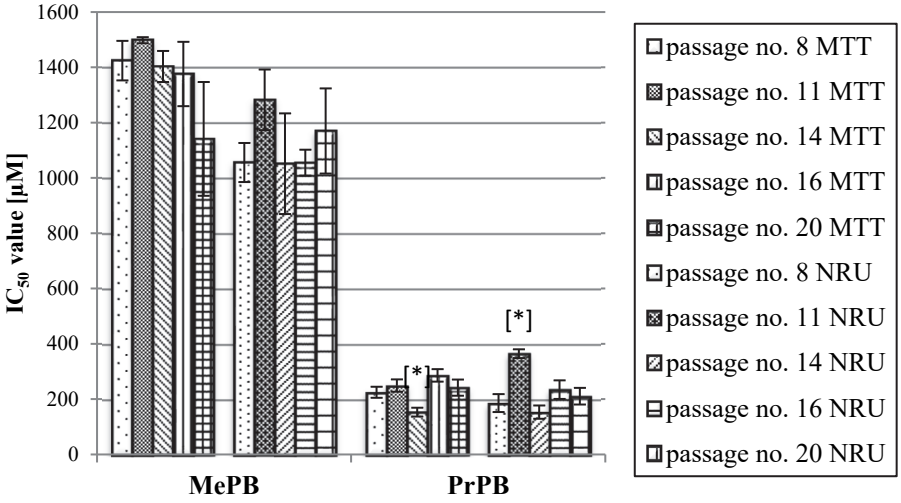

Fig. 1. The comparison of cytotoxicity of the analysed parabens based on the IC50 values determined on subsequent passages of CCD-1136Sk cells (ATCC® CRL-2697™) in the MTT and NRU tests. Each bar represents an average and a standard deviation from 9 measurements in 3 independent experiments. Statistically significant averages ($p < 0.05$) are marked

On the other hand, having analysed the course of the curves of relationships between the cell viability and the preservative concentration, it was found that the exposure of normal dermal fibroblasts to methyl paraben assessed using the NRU test (Fig. 2) indicates a stronger effect of the preservative at a concentration of 50 and 200 µg/ml on the earliest cells passage (no. 8) compared to the subsequent passages of these cells. At the same time, the compound with a concentration of 50 and 800 µg/ml was the least toxic to the latest passage cells (no. 20). No such changes were noted in the MTT test (Fig. 3) except for a weaker

effect of the compound at a concentration of 800 µg/ml on the older passage cells (no. 20) compared to the earlier cell passages.

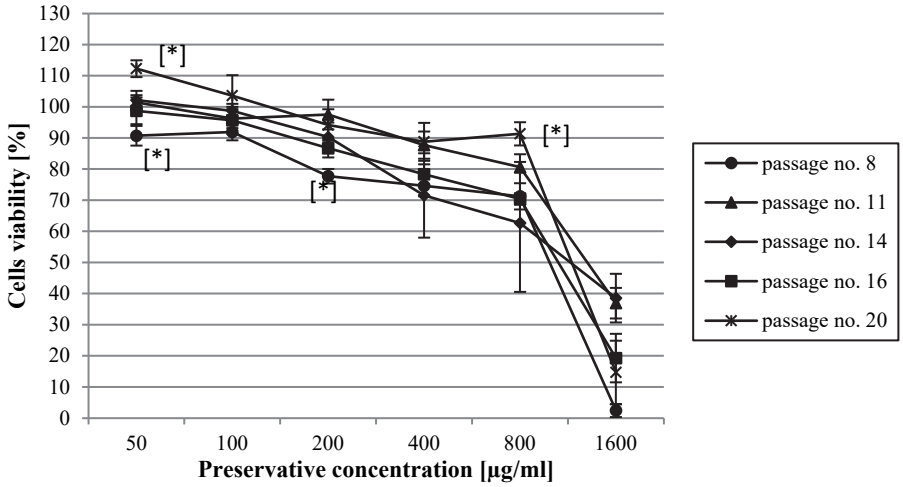

Fig. 2. The effect of methyl paraben on the viability of CCD-1136Sk cells (ATCC® CRL-2697™) determined using the NRU test. Each point represents an average and a standard deviation from 9 measurements in 3 independent experiments. Statistically significant averages ($p < 0.05$) are marked

No different sensitivity of consecutive passages of cells was noted at the exposure of CCD-1136Sk fibroblasts to propyl paraben in any of the two tests.

The obtained results enable the conclusion that propyl paraben exhibits a stronger cytotoxic effect on normal (senescent) diploid human fibroblasts CCD-1136Sk (ATCC® CRL-2697™) than methyl paraben.

These results are consistent with those obtained by Carvalho et al. (2012), who found that propyl paraben exhibited a stronger cytotoxic effect on human dermal fibroblasts (HDF) than methyl paraben, as the IC_{50} value for propyl paraben in the NRU test was determined at a level of 0.25%, while for methyl paraben this value amounted to 2.35%.

The results of the comparison of toxicity of MePB and PrPB, carried out in this study, are also consistent with the Cosmetic Ingredient Review (2008) according to which propyl paraben exhibits a stronger cytotoxic effect than methyl paraben; a study carried out on the cells of a specified cell line originating from the cervical cancer cells (HeLa) determined the IC_{50} values for methyl paraben and propyl paraben at a level of 1.3 mM and 0.22 mM, respectively. The cited IC_{50} value for propyl paraben falls within the range of the values determined in this project on dermal fibroblasts.

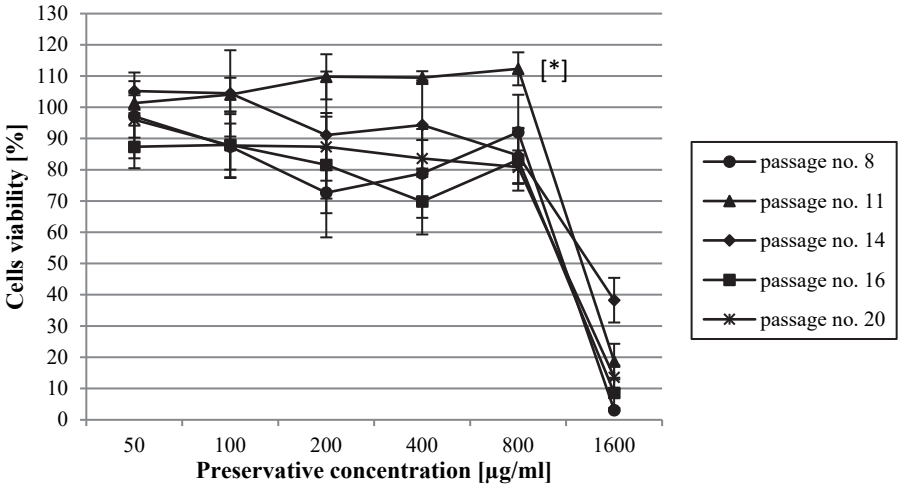

Fig. 3. The effect of methyl paraben on the viability of CCD-1136Sk cells (ATCC® CRL-2697™) determined using the MTT test. Each point represents an average and a standard deviation from 9 measurements in 3 independent experiments. Statistically significant averages ($p < 0.05$) are marked

Taking into account the course of the curves of relationships between the cell viability and the concentrations of the analysed preservatives, it can be observed that these compounds may exert various effects on the cells of the same line which differ in age (the early and late passage).

Having considered the comparison of the sensitivity to cytotoxic effects of parabens between the younger cell passages and the older passages, it was found that the CCD-1136Sk cells of the early passages were characterised by a greater sensitivity to the cytotoxic effects of methyl paraben, which was evident at the exposure to the two lowest of the applied concentrations of the compound. As the cells were passaged, a phenomenon of their increased resistance to the effects of the analysed preservatives occurred; however, it concerned only one of the higher analysed paraben concentrations.

It should be noted that even the cells of immortalised lines that are maintained for a long time may respond differently to the same xenobiotic due to the occurrence of mutations which change the cells' characteristics found in the early passages of a particular line (Wenger et al. 2014). Inter alia, changes (aberrations) in the cell caryotype (a set of cell chromosomes) were found, namely duplications of a chromosomal region and of entire chromosomes. The authors emphasise that the confirmation of the origin of a cell line, and its accurate characterisation (including the passage number) is a prerequisite for obtaining reliable results of experiments which can only be useful for other researchers only on this condition.

The number of passage at which changes occur in the cells is characteristic of a particular cell line. For the Syrian hamster embryo (SHE) cells, a "crisis" was found (Chang-Liu & Woloschak 1997) which occurred at passage 37 and continued to passage 49, and which was characterised by rapid changes in the number of colonies formed from a single cell ("plating efficiency") and by changes in the cell growth parameters. In the testing carried out as part of this study, the phenomenon of the inhibition of the proliferation of CCD-1136Sk cells (ATCC® CRL-2697™) was observed at passage 27 (unpublished data).

The results obtained in this study confirm the findings of many authors about the heterogeneity and variability of the results of studies carried out on the cells of various age or origin. A review of the literature on the effects on the passage number on cell lines indicates that this effect is complex and highly dependent on many factors such as the cell line type, the tissue and species of its origin, the culture conditions (temperature, pH, appropriate mediums and culture additives, or the growth surface) and the uses for which a particular cell line is applied. For example, it was found (unpublished ATCC data) that older passages of Caco-2 cells exhibited the up-regulation of the reporter protein GFP (green fluorescent protein) following the transfection, while older passages of the MCF7 line, compared to the younger ones, exhibit a decrease in the GFP levels (Tech Bulletin of ATCC 2010).

In summary, it can be concluded that the presented results indicate that the analysed parabens used in the cosmetic and pharmaceutical industries may be toxic to skin cells, and their consecutive passaging may be associated with differences in the susceptibility to cytotoxic effects. It therefore appears necessary to take into account the possibility of different later and earlier passages cells' reactivity when interpreting the results of studies into the effects of preservatives on the living body. Furthermore, it should be borne in mind that humans may be exposed to preservatives due to environmental contamination with their residues, although compared to other sources of exposure, such as the direct use of cosmetic products or medicines, neither water bodies nor the biota appear to be the main source of exposure for humans (Ramaswamy et al. 2011).

This paper has been based on the results of a research task I.N.13 carried out within the scope of the fourth stage of the National Programme "Improvement of safety and working conditions" partly supported in 2017-2019 – within the scope of research and development – by the Ministry of Science and Higher Education/National Centre for Research and Development. The Central Institute for Labour Protection – National Research Institute is the Programme's main coordinator.

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Abstract

The aim of the study was to compare the cytotoxic effects of (methyl and propyl) parabens likely to be environmental contaminants on early and late passage fibroblasts isolated from human skin. The study was carried out on senescent diploid cell lines, namely normal (senescent) dermal fibroblasts CCD-1136Sk (ATCC®CRL-2697™). In order to assess the cytotoxic effect, the MTT test which determines the cells' metabolic activity and the neutral red uptake assay which assesses the cell membrane integrity (NRU test) were applied. Propyl paraben (PrPB) appeared to be more cytotoxic to the analysed dermal fibroblasts (since it reached lower IC_{50} values); however, none of the tests found a consistent trend for a change in the cells' sensitivity to the analysed parabens as they age, that would be reflected in the determined IC_{50} values. On the other hand, having analysed the course of the curves of relationships between the cell viability and the preservative concentration, it was found that at the exposure of CCD-1136Sk fibroblasts to propyl paraben, none of the two tests observed different sensitivity of late and early cell passages; however, the exposure of dermal fibroblasts to methyl paraben, determined using the NRU test, indicated a stronger effect of the preservative at a concentration of 50 and 200 μg/ml on the earliest cells passage (no. 8), compared to the subsequent passages of these cells. At the same time, the compound with a concentration of 50 and 800 μg/ml was the least toxic to the latest cells passage (no. 20). The presented results indicate that the analysed parabens used in the cosmetic and pharmaceutical industries may be toxic to

skin cells; moreover, it is not excluded that with consecutive passaging, differences in the susceptibility to cytotoxic effects may occur. It therefore appears necessary to take into account the possibility of different later and earlier passages cells' reactivity when interpreting the results of studies into the effects of preservatives on the living body. Furthermore, it should be borne in mind that humans may be exposed to preservatives due to environmental contamination.

Keywords:
preservatives, methylparaben, propylparaben, cytotoxicity, senescent cells, in vitro

Cytotoksyczne działanie dwóch parabenów oznaczanych w wodach powierzchniowych i osadach ściekowych na normalne (starzejące się) ludzkie fibroblasty skórne w badaniach in vitro

Streszczenie

Celem badań było porównanie cytotoksycznego działania parabenów (metylu i propylu), mogących stanowić zanieczyszczenie środowiska naturalnego, na wczesne oraz późne pasaże fibroblastów wyprowadzonych ze skóry człowieka. Badania wykonano na starzejących się diploidalnych liniach komórkowych: fibroblastach skórnych CCD-1136Sk (ATCC® CRL-2697™). Do oceny cytotoksycznego działania zastosowano test MTT, który określa aktywność metaboliczną komórek oraz test pochłaniania czerwieni obojętnej oceniający integralność błon komórkowych (test NRU). Bardziej cytotoksyczny (osiągający niższe wartości IC_{50}) dla badanych fibroblastów skórnych okazał się propyloparaben (PrPB), jednak w żadnym z testów nie stwierdzono spójnej tendencji zmiany wrażliwości komórek na badane parabeny w miarę ich starzenia się, która znalazłaby odzwierciedlenie w wyznaczonych wartościach IC_{50}. Analizując natomiast przebieg krzywych zależności żywotności komórek od stężenia konserwantu stwierdzono, że przy narażeniu fibroblastów CCD-1136Sk na paraben propylowy nie zaobserwowano zróżnicowanej wrażliwości komórek późnych i wczesnych pasaży w żadnym z dwóch testów, natomiast narażenie fibroblastów skórnych na paraben metylowy oceniane testem NRU wskazało na silniejsze działanie konserwantu o stężeniu 50 i 200 µg/ml na komórki najwcześniejszego pasażu (nr 8) w porównaniu z kolejnymi pasażami tych komórek. Jednocześnie, związek o stężeniu 50 i 800 µg/ml był najmniej toksyczny dla komórek najpóźniejszego pasażu (nr 20). Przedstawione wyniki wskazują, iż badane parabeny stosowane w przemyśle kosmetycznym i farmaceutycznym mogą działać toksycznie na komórki skóry, nie wyklucza się także, że wraz z ich starzeniem się mogą być występować różnice w podatności na działanie cytotoksyczne. Konieczne zatem wydaje się uwzględnianie przy interpretacji wyników badań nad odziaływaniem substancji konserwujących na żywy organizm również możliwości odmiennej reaktywności komórek późniejszych i wcześniejszych pasaży. Nie należy ponadto zapominać o możliwości narażenia człowieka na substancje konserwujące wynikające z zanieczyszczenia środowiska naturalnego.

Słowa kluczowe:
konserwanty, paraben metylu, paraben propylu, cytotoksyczność,
starzejące się komórki, in vitro