

# Comparison of Methods Used for Evaluation of Mutagenicity/Genotoxicity of Model Chemicals - Parabens

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## Summary

Growing worldwide efforts to replace (reduce) animal testing and to improve alternative *in vitro* tests which may be more efficient in terms of both time, cost and scientific validity include also genotoxicity/mutagenicity endpoints. The aim of the review article was to summarize currently available *in vitro* testing approaches in this field, their regulatory acceptance and recommended combinations for classification of chemicals. A study using the combination of Comet Assay performed on two cell lines and the Chromosomal Aberration test on human peripheral lymphocytes was performed with the aim to predict the genotoxic potential of selected paraben esters, serving as a model chemical group. Parabens are widely used in consumer products as preservatives and have been reported to exhibit inconclusive results in numerous genotoxicity studies. The Comet Assay identified Ethylparaben and Benzylparaben as potentially genotoxic. The Chromosomal Aberration test revealed weak genotoxic potential in case of Ethylparaben and positive genotoxicity in case of Butylparaben, Propylparaben and Isopropylparaben. The main reasons for variability seem to be limited water solubility of parabens, determining their bioavailability at the cellular level, and absence of metabolic activation in the Comet Assay. The results confirmed that the Comet Assay should serve as a screening test and should not be used as a stand-alone method for classification of genotoxicity. The weight of evidence approach in risk assessment should be supported with data generated with the use of human relevant *in vitro* methods based on cells / tissues of human origin.

## Key words

Genotoxicity • Mutagenicity • Alternative Toxicological Testing • *in vitro* Methods • Test Batteries • Parabens

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## Introduction

Genotoxicity is one of the most critical toxicological endpoints, highly relevant for public health and environmental protection, including the safety of consumers. The principles of the 3Rs, defined as Replacement, Reduction and Refinement of animal testing by Russell and Burch (1959), represent the ethical basis for the use of laboratory animals in research projects. The Council Directive 2010/63/EU on the protection of animals strongly supports efforts to find alternative methods to animal testing (Beken *et al.* 2016, Directive 2010/63). Numerous *in vitro* testing approaches have been developed with the use of preferentially human relevant biological systems, which often consist of human derived tissue cultures or cell lines (OECD 2017). Unfortunately, the acceptance of *in vitro* toxicological methods alternative to animal testing for genotoxicity endpoints lags behind the progress in technological

development and animal tests remain to be required (Kerecman *et al.* 2017, Coleman *et al.* 2012). Despite of numerous guidelines and publications, considering the benefits of the 3Rs for both animals and science, and substantial advances in the development, validation, and employment of alternative non-animal methods, scientists traditionally tend to prefer the use of animal-based approaches (Combes and Balls 2014). Difficulties in evaluation of *in vitro* genotoxicity endpoints, particularly the physiological background of induction, manifestation and persistence of the genotoxic effects, and also difficulties in transposing and linking the *in vitro* results to *in vivo* conditions may be suggested as the reasons of low acceptance of *in vitro* data. However, a few *in vitro* tests have been already accepted for regulatory purposes, i.e., *in vitro* mammalian chromosomal aberration test (OECD TG 473), *In vitro* mammalian cell micronucleus test (TG 487), and Bacterial reverse mutation test in *Salmonella typhimurium* and *Escherichia coli* (Ames test) (TG 471). Furthermore, *in vitro* systems have been included into OECD TGs designed originally for *in vivo* conditions, e.g. the *in vivo* Mammalian Alkaline Comet Assay considers *in vitro* systems to be useful for further investigation of genotoxicity (OECD 2016d). In order to classify a substance as non-genotoxic, a combination of appropriate *in vitro* technologies should be employed (Kerecman *et al.* 2017). Individual *in vitro* methods may exhibit specific advantages and limitations, while their combination may increase sensitivity of safety evaluation (Bhagat 2018). Nevertheless, so far no single *in vitro* test allows detection of the wide range of specific changes in the DNA structure manifested as adverse effects associated either with genotoxicity or mutagenicity (Nesslany 2017). Therefore, combinations of 2 or 3 validated *in vitro* tests of sufficient sensitivity and specificity are currently recommended to be used. Chemical substances may be evaluated as showing no genotoxic potential if all *in vitro* endpoints used are clearly negative while at least two *in vitro* endpoints showing positive results may predict genotoxic potential (EFSA 2011). International working groups and experts from OECD, EURL ECVAM, EPAA, ICCVAM, etc., have been involved in promotion and validation of alternative toxicological methods for regulatory acceptance to replace, reduce or refine (3Rs) *in vivo* testing. Additional data are needed, particularly related to toxicological mechanisms and absorption, distribution, metabolism and excretion (ADME) properties. The development of new approach methodologies (e.g. the

development of the OECD QSAR Toolbox, supported by the European Chemical Agency ECHA) should bring high throughput assessment methods, supporting current alternative approaches with human relevant information. Scientific recommendations of testing approaches, and combinations of *in vitro* and *in vivo* methods, have resulted in binding legislation or non-binding recommendations, e.g.: the constantly updated Council regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 (REACH) (EC 2008), OECD Overview on Genetic Toxicology TGs (OECD 2017), ECHA Report on Non-animal Approaches - Current Status of Regulatory Applicability under the REACH, CLP and Biocidal Products Regulations (ECHA 2017), EFSA Statement on Genotoxicity Assessment of Chemical Mixtures (EFSA 2018) and EFSA Scientific Opinion on Genotoxicity Testing Strategies Applicable to Food and Feed Safety Assessment (EFSA 2012).

#### *In vitro* approaches available for genotoxicity testing

*In vitro* endpoints which may be manifested as adverse effects *in vivo* include changes in DNA structure, i.e. numerical chromosomal aberrations (aneuploidy), structural changes (clastogenicity), (double) strand breaks, translocations, deletions, point mutations, gaps, etc. (Turkez *et al.* 2017). A test battery designed for initial screening of genotoxic potential should allow detection of three important genotoxic endpoints, i.e., gene mutations, structural chromosomal aberrations i.e., clastogenicity, and numerical chromosomal aberrations (aneuploidy), in order to understand the genotoxic mode of action (genotoxic endpoint) of the tested substance (EFSA 2011). *in vitro* cell systems mostly included in the OECD TGs, such as mouse lymphoma cells L5178Y, CHO-AS52 and V79 cell lines have been derived from laboratory animals. However, human relevant cell lines derived from malignant cells, such as the e.g. TK6 human lymphoblastoid cells, have been recommended to be used for *in vitro* testing OECD TG 473, 476, 490 (EFSA 2011, OECD 2016b, Ranganatha *et al.* 2016, OECD 2015).

*In vitro* mammalian cell gene mutation test using the thymidine kinase gene has been recently included into the OECD TG 490 database (OECD 2016e) to identify substances potentially causing gene mutations at the thymidine kinase (*tk*) reporter locus (OECD 2015). The use of *in vitro* systems has indeed been seriously considered useful and included in updated OECD Test Guidelines, such as the OECD TG 489 (*In vivo* Mammalian Alkaline Comet Assay).

The most commonly used methods to assess the genotoxic potential of substances *in vivo*, with possible inclusion of *in vitro* test systems are listed below, on the basis of their principal endpoint of genotoxicity:

*Gene mutations:*

- Transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488).

*Chromosome damage:*

- Mammalian erythrocyte micronucleus test (OECD TG 474),
- Mammalian bone marrow chromosome aberration test (OECD TG 475).

*Primary DNA damage:*

- *in vivo* mammalian alkaline Comet assay (OECD TG 489)

Toxicological test methods designed exclusively as *in vitro* approaches have been developed to be used for detection of the relevant genotoxic endpoints:

*Mutations:*

- Bacterial reverse mutation test in *Salmonella typhimurium* and *Escherichia coli* (Ames test) (OECD TG 471),
- *in vitro* mammalian cell gene mutation test (OECD TG 476),
- *in vitro* gene mutation assays using the *tk* locus (OECD TG 490).

*Chromosome aberrations:*

- *in vitro* mammalian chromosomal aberration test (OECD TG 473),
- *in vitro* mammalian cell micronucleus test (OECD TG 487) (*clastogenicity*),
- *in vitro* mammalian alkaline Comet assay (performed with the use of *in vitro* test system, in compliance with OECD TG 489) (*strand breaks*).

The most widely used tests include Bacterial reverse mutation test (OECD TG 471), *in vitro* mammalian cell micronucleus test (OECD TG 487) and *in vitro* mammalian chromosome aberration test (OECD 473).

The Bacterial reverse mutation test (Ames test) has been designed to evaluate the mutagenic potential of test chemicals or mixtures. Ames test is generally used as

the first screening method to assess genotoxicity and detection of gene mutations (Bhagat 2018). The assay is based on specific strains *Salmonella typhimurium* and *Escherichia coli* containing identified mutations in amino acid biosynthesis genes at the reporter locus (i.e., histidine [His] or tryptophan [Tryp], respectively) (OECD 1997, OECD 2015). The test detects mutations which revert the identified mutations in the test strains. The revertants are identified by their restored functional capability to synthesize a specific amino acid. The limitation of this method is the use of prokaryotic cells which differ from mammalian cells in factors such as uptake, metabolism, chromosome structure and DNA repair processes. There have been developments to use it in high throughput screening (Flückiger-Isler *et al.* 2004) but the methods have not been developed to a point where they can be routinely used. Substances not directly interacting with DNA may not be detected as mutagenic, which may be relevant especially for substances that have been shown to decrease genomic stability by indirect mechanisms, such as DNA repair systems, cell cycle control and apoptosis (EFSA 2011).

The *in vitro* mammalian cell micronucleus test (MN) has been used for evaluation of cytogenetic damage for decades (Morita *et al.* 1997) in numerous types of test systems, including rodent bone marrow or peripheral blood lymphocytes (Benigni *et al.* 2012, Morita *et al.* 2016, Soeteman-Hernández *et al.* 2016) to identify chemical substances that cause primarily structural chromosomal damage (Corvi *et al.* 2008). The assay detects micronuclei in the cytoplasm of interphase cells and typically employs human or rodent cells lines or primary cell cultures (OECD 2016c). The MN test is almost as sensitive as the chromosomal aberration test (CA) for the detection of clastogens and has the additional advantage of detecting aneugenic substances. The *in vitro* MN test detects the fragments of chromosomes formed into micronuclei, whereas the chromosomal aberration test records visible numerical changes (aneuploidy), translocations and other complex chromosomal rearrangements, that may provide additional mechanistic information (Corvi *et al.* 2008, Kimura *et al.* 2013). Automated systems used for measuring the frequency of micronucleated cells include approaches of flow cytometry (De Boeck *et al.* 2005, Dertinger *et al.* 2011), fluorescent microscopy and image analysis (Parton *et al.* 1996), or laser scanning cytometry (Styles *et al.* 2001). The *in vitro* mammalian cell micronucleus test (OECD TG 487) can be conducted in

the presence or in the absence of cytochalasin B (cytoB), which is used to block cell division and generate binucleated cells and may be combined e.g. with kinetochore staining or fluorescence in situ hybridisation (FISH) (EFSA 2011).

The *in vitro* mammalian chromosome aberration test, based on a proliferating cell population of established cell lines or primary cell cultures in metaphase (Bhagat 2018, OECD 2015), has been used for decades as a biomarker of the early effects of genotoxic carcinogens (Norppa *et al.* 2006, Güzel Bayülken *et al.* 2019). The assay primarily allows the detection of structural aberrations of chromosomes, resulting in specific numerical chromosome aberrations (aneuploidy) in cultured mammalian cells. Additional mechanistic information can be provided using FISH or advanced chromosome staining techniques.

Increasing number of scientific studies have criticized the human relevance of using laboratory animals (e.g. rodents, as the preferred animal model) for assessing hazard and risk of chemicals in humans, as it has become scientifically debatable after consideration of data generated with the use of molecular biology approaches (Phalen *et al.* 2008, Creton *et al.* 2010, Chamanza and Wright 2015, Mowat *et al.* 2017).

The following laboratory animals have been conventionally recommended to be used for genotoxicity testing or as a primary source of *in vitro* cell systems:

- Chinese hamster (*Mesocricetus auratus*) in the Syrian Hamster Embryo (SHE) cell transformation test, Chinese hamster ovary (CHO) and Chinese hamster lung (CHL) cells
- Deer mouse (*Peromyscus leucopus*, *Peromyscus maniculatus*) (He and Toth 2017)
- Laboratory mouse (*Mus musculus* var. *alba*)
- Rat (*Rattus rattus*), (*Rattus norvegicus* var. *alba*)

Rodent cell lines currently used in genotoxicity tests may exhibit specific deficiencies. CHO cells have been reported to carry a mutant p53 sequence and lack of G1 checkpoint; CHL cells have been reported to exhibit altered regulation of p53 expression; V79 and L5178Y mouse lymphoma cells were found to synthesize a dysfunctional p53 protein (Chaung *et al.* 1997, Storer *et al.* 1997). These issues may increase the test sensitivity, but, in return, induce false positive results (Kirkland *et al.* 2011). The specific cell type used in the bioassay may strongly affect the cellular response and *in vitro* predictivity (Fowler *et al.* 2012). The deficiencies and

limits of rodent cell lines have led to efforts to develop and prefer *in vitro* systems based on cells of human origin, which are efficient for DNA repair and for p53 (Kirkland *et al.* 2005, Kirkland *et al.* 2007, Nesslany 2017). An increasing number of studies based on human relevant *in vitro* systems, have demonstrated promising results, indicating significant sensitivity and specificity of the novel approaches. Computational modeling approaches have been suggested for predicting the genotoxic potential and carcinogenicity of chemicals, and have been included in testing strategies in pharmacology (Wu and Wang 2018). Human derived reconstructed 3D epithelium tissue models (RhE) consisting of reconstructed epidermis and functional stratum corneum have been used to establish novel *in vitro* human reconstructed skin micronucleus (MN) and Comet (CMT) assays designed to measure genotoxicity endpoints induced in dividing human keratinocytes after dermal exposure (Kirsch-Volders *et al.* 2011, Reisinger *et al.* 2018, Roy *et al.* 2016, Pfuhrer *et al.* 2020a, Pfuhrer *et al.* 2020b). Examples of three-dimensional organ models, multicellular spheroids of liver cells (Xu *et al.* 2003) and bone marrow cell cultures (Dal Negro *et al.* 2006) as well as novel technologies using human organ models on-a-chip have been recently introduced (Andersen *et al.* 2014, Alépée *et al.* 2014, Marx *et al.* 2016) with the aim to maximally mimick physiological conditions (Rossini and Hartung 2012). Experimental studies have used e.g. exfoliated oral epithelium cells from buccal smears of healthy donors in the micronucleus test. Buccal smears have been reported as a valuable source of primary cells, however, of limited standardization (Bortoluzzi *et al.* 2014, Jyoti *et al.* 2012, Jyoti *et al.* 2015). For routine *in vitro* toxicological testing, standardized (non-malignant) cell lines may provide more relevant response in standardized conditions. Human bronchial epithelial cell line BEAS-2B was used for detection of genotoxicity of engine emissions in *in vitro* MN assay (Cervena *et al.* 2017). Human derived cell lines that have been used in MN assay include primary human lymphocytes, lymphoblastoid cell line TK6, HepaRG cell line, HT29 (Human colon cancer cell line), Caco-2 cell line (Human heterogenous epithelial colorectal adenocarcinoma), HepG2 (hepatocellular carcinoma) (Allemang *et al.* 2018, Wiczerzak *et al.* 2019, Puerto *et al.* 2018, McCarrick *et al.* 2019). Primary human peripheral lymphocytes have been found to potentially generate fewer false positive results than e.g. V79, L5178Y and TK6 cells (Kirkland *et al.* 2007, OECD 2015). However, not all the systems

have been extensively validated so far, and their use is recommended only if their performance meets the acceptance criteria. Conflicting data have been often obtained in genotoxicity studies, depending on the substances analyzed and methods used, indicating low analytical sensitivity to fully cover the complex causation of genotoxicity, mutagenicity and carcinogenicity. Comparison of the results may be compromised by variability in practical procedure steps or test systems used. Therefore, combinations of toxicological methods (*in vitro*, *in vivo*) have been proposed in order to increase the sensitivity of genotoxicity testing.

#### *Combinations of methods recommended for genotoxicity/mutagenicity/carcinogenicity testing*

Combinations of tests for the screening of carcinogenicity were proposed already in 1974 (Stoltz *et al.* 1974). Most recently, the combination of Ames test, *in vivo* MN, *in vivo* CA and CMT assay has been reported to increase the sensitivity of a testing battery proposed for detection of carcinogens (Kang *et al.* 2013a, Kim and Margolin 1999, Morita *et al.* 2016), alternatively the combination of Ames test and *in vivo* MN has been also reported to show promising sensitivity (Shelby and Zeiger 1990) or combination of MN and CMT assays (Recio *et al.* 2010, Vasquez 2010, Kang *et al.* 2013a). Significant sensitivity was suggested for combination of *in vivo* CMT (90 %) and *in vitro* CMT (86.9 %), *in vivo* MN + *in vivo* CA (88.6 %), *in vivo* MN + *in vivo* CMT (92.5 %), *in vitro* CMT + *in vivo* MN (95.6 %) (Bhagat 2018, Kimura *et al.* 2013). The highest sensitivity has been observed in combination of *in vivo* CMT + *in vivo* CA (96.7 %) (Bhagat 2018, Madrigal-Bujaidar *et al.* 2008, Pfuhrer *et al.* 2009, Bowen *et al.* 2011). However, the general development of toxicological testing, performed according to the 3R principles, allows the inclusion of exclusively *in vitro* endpoints in suitable combinations into testing batteries without the use of animals. Assuming that a stand-alone single *in vitro* test cannot be expected to predict all key events potentially resulting in persistent genotoxicity and/or mutagenicity, the combination of *in vitro* assays should include tests with sufficient sensitivity (in order to avoid false negative results) and specificity (in order to avoid false positive results). A standard battery of *in vitro* genotoxicity tests, recommended to be suitable for regulatory purposes, should include 2 or 3 validated tests with at least one test on bacteria and one test on cell cultures (Nesslany 2017). The complex aspects of the induction and causation of the

adverse effects, representing potential connections between genotoxicity, mutagenicity and carcinogenicity have to be deeply considered. Genotoxicity has been referred to as an induced damage to DNA, including alterations of the DNA structure, information content and segregation, without direct connection to mutagenicity. Therefore, *in vitro* genotoxicity testing was recommended to include tests detecting DNA strand breaks, DNA adduct formation or mitotic recombination, in addition to specific tests for mutagenicity (Dearfield *et al.* 2011, EFSA 2011, UK Committee on Mutagenicity COM 2011).

Exclusive *in vitro* approaches are strongly recommended in case of dermal exposure and are the only available testing approaches in case of cosmetics and cosmetic ingredients. In case of cosmetic ingredients, the evaluation of the potential for mutagenicity to be annexed in the Regulation (EC) No 1223/2009 should include tests to provide information on three genotoxic endpoints, i.e.: mutagenicity at the gene level, chromosome breakage and/or rearrangements (clastogenicity) and numerical chromosome aberrations (aneuploidy).

The European Scientific Committee on Consumer Safety recommended two tests for the base level testing of cosmetic ingredients, represented by the following test systems:

- Bacterial Reverse Mutation Test (OECD 471) as a test covering gene mutations,
- *In vitro* Micronucleus Test (OECD 487) as a test for both structural (clastogenicity) and numerical (aneugenicity) chromosome aberrations.

Confirmative or supportive evidence may be obtained with the use of tests detecting primary DNA damage (Kirkland *et al.* 2011).

### **Metabolic activation**

Certain chemicals may exhibit genotoxicity only after metabolic activation (indirect genotoxins), therefore it is recommended to include metabolic activation system (MAS) to mimic the situation *in vivo* (Tice *et al.* 2000, Nesslany 2017). Cultivation *in vitro* without MAS may lead to downregulation of enzyme genes and low function of detoxification systems involved in reduction of reactive oxygen species (Oesch *et al.* 2019). The most common system used for metabolic activation is the S9 fraction, prepared traditionally from the liver of rodents (usually rats), but nowadays also human S9 fractions are

commercially available (EFSA 2011, Hong *et al.* 2018). S9 fraction has, however, been reported to compromise the *in vitro* results, as the efficacy of metabolic activation may be specific (depending on e.g. age or sex) and cytotoxicity or variable mutagenic response may be observed (Nishimuta *et al.* 2013, Cox *et al.* 2016, Tweats *et al.* 2007a). Moreover, S9 fractions lack cellular compartmentalization and transporter functions. Human HepaRG cell line, instead, exhibit major CYP activities and other liver specific functions comparable to primary hepatocytes, and has unique potential for competent metabolic activation and may be used as the source of MAS (Josse *et al.* 2012).

### Aim of the study

More data from the use of *in vitro* bioassays and test systems are necessary to be generated in order to evaluate the predictivity, sensitivity and specificity of alternative methods suitable for genotoxicity assessment, as still a limited amount of data has been available. Therefore, we carried out a study using the combination of CMT (Comet) assay performed using two non-malignant keratinocyte cell lines HaCaT and SVK14, and the CA (Chromosomal aberration) test using human peripheral lymphocytes, with the aim to predict the genotoxic potential of selected paraben esters, used as

a model chemical group. Parabens are widely used in consumer products as preservatives and have been reported to exhibit inconclusive results in numerous genotoxicity studies. We describe the advantages and limits of the combined *in vitro* approaches and also introduce practical issues and details which may help to develop a standardized approach.

### Materials and Methods

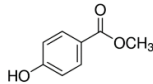
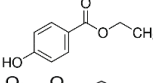
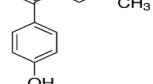
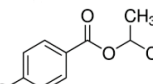
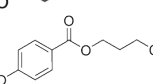
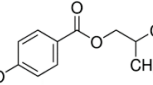
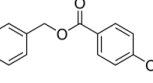
#### Tested chemicals

Paraben esters (methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, isobutylparaben, benzylparaben) were supplied from (TCI) TOKYO Chemical industry CO., LTD and used as test substances (Table 1).

#### Cell lines and culture conditions

Commercially available non-tumor cell lines derived from human keratinocytes, i.e. HaCaT cell line and SVK14 cell line (both from ATCC, USA) were used in the *in vitro* CMT assay. For the CA assay, human peripheral blood was obtained by venepuncture from a healthy female human volunteer (non-smoker, non-drinker who was not exposed to radiation, infections or medication for less than one month before the date of collection). The study, performed in compliance

**Table 1.** Tested chemicals and their formulas.

Substance	IUPAC Name	CAS	Formula	Structural Formula	Molecular weight
<i>Methylparaben</i>	methyl 4-hydroxybenzoate	99-76-3	C <sub>8</sub> H <sub>8</sub> O <sub>3</sub>		152.15
<i>Ethylparaben</i>	ethyl 4-hydroxybenzoate	120-47-8	C <sub>9</sub> H <sub>10</sub> O <sub>3</sub>		166.17
<i>Propylparaben</i>	propyl 4-hydroxybenzoate	94-13-3	C <sub>10</sub> H <sub>12</sub> O <sub>3</sub>		180.21
<i>Isopropylparaben</i>	propan-2-yl 4-hydroxybenzoate	4191-73-5	C <sub>10</sub> H <sub>12</sub> O <sub>3</sub>		180.20
<i>Butylparaben</i>	butyl 4-hydroxybenzoate	94-26-8	C <sub>11</sub> H <sub>14</sub> O <sub>3</sub>		194.23
<i>Isobutylparaben</i>	2-methylpropyl 4-hydroxybenzoate	4247-02-3	C <sub>11</sub> H <sub>14</sub> O <sub>3</sub>		194.23
<i>Benzylparaben</i>	benzyl 4-hydroxybenzoate	94-18-8	C <sub>14</sub> H <sub>12</sub> O <sub>3</sub>		228.25

with the WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects (1964, amended 2013) and the International Ethical Guidelines for Health-related Research Involving Humans (CIOMS 2016), was approved by the Ethical Review Committee of the National Institute of Public Health.

#### *MTT assay*

The *in vitro* cytotoxic effect of the parabens used on the two cell lines SVK14 and HaCaT (both human keratinocytes; ATCC, USA) was measured by MTT assay (Mosmann, 1983). Each paraben (diluted in DMEM) was applied to cells at selected concentrations and incubated for 24 hours. After incubation, DMEM (Sigma Aldrich, USA) was replaced by a solution containing 50  $\mu\text{l}$  of 0.5  $\text{mg ml}^{-1}$  MTT (Sigma Aldrich, USA) dissolved in 1x PBS. Afterwards, the cell lines were incubated with MTT for 4 hours at 5 %  $\text{CO}_2$  and 37 °C. The MTT solution was then replaced with 100  $\mu\text{l}$  of DMSO to ensure the formazan crystals to be properly dissolved. The absorbance level was measured by a Tecan Infinite Pro200 reader (Tecan, Switzerland).

#### *In vitro Comet assay (CMT)*

The method was used based on OECD TG 489 with modifications, i.e. using human relevant non-tumor cell lines derived from human keratinocytes (Jiravova *et al.* 2016). Test substances at pre-tested selected concentrations in DMEM were applied to the cell culture for 24 hours. As a positive control, 1 %  $\text{H}_2\text{O}_2$  in PBS was applied to the cell culture for 15 minutes at 4 °C. Culture medium (DMEM) served as negative control. After incubation, cells were harvested using TrypLE (Gibco, 12604-021) for +/- 10 min. DMEM with fetal bovine serum was applied to stop the trypsinization. Harvested cell suspension was centrifuged and the cell pellet was collected. A 1 % low melting point (LMP) agarose solution, tempered at 37 °C, was mixed with the cell pellet. The resulting dense agarose suspension was pipetted onto pre-coated microscopic slides and quickly covered with coverslips. The agarose was solidified at 4 °C in the refrigerator and then the coverslips were carefully removed, preventing the damage of the sample. Slides were immersed in lysis buffer with 1 % Triton X-100 and incubated in glass Coplin Jars for 60 minutes at 4 °C in order to ensure sufficient cell lysis. In the next step, slides were placed in an electrophoretic tank (Sub-Cell Model 192 Cell, 2,000 ml) and embedded in

sufficient volume (slightly covering the slides) of chilled (4 °C) alkaline electrophoretic solution for 40 minutes. Electrophoresis was performed under the following conditions: 350 mA, 0.8  $\text{V cm}^{-1}$ , for 20 minutes. After electrophoresis, slides were carefully transferred and rinsed twice for 10 minutes with neutralization buffer (pH 7.5, 4 °C). The slides were stained with SYBR Green I and evaluated microscopically using a fluorescence microscope with a CCD camera (Olympus). The experiment was repeated in three runs in triplicates. From each sample, 100 cells were selected for evaluation using the CometScore 1.5 software. The median values from each measurement were used for mathematical and statistical analysis, and mean values of the amount of DNA in the head of the comet (directly proportional to the intact, non-fragmented DNA), the Olive momentum and DNA in the tail were evaluated. The values of the DNA in the head of the comet were used for interpretation of results. Analysis of variance (*ANOVA*) and Dunnett *t*-tests were used for statistical analysis. A *p* value < 0.05 was used as the level of statistical significance. All statistical analyzes were performed using IBM SPSS Statistics for Windows, version 23.0. Armonk, NY: IBM Corp.

#### *In vitro mammalian chromosome aberration test (CA)*

The test was performed in accordance with OECD TG 473 (OECD 2016a). Peripheral blood lymphocytes were collected from a healthy donor and cultured in RPMI-1640 medium with L-glutamine and  $\text{NaHCO}_3$  (SIGMA) supplemented with 10 % heat-inactivated bovine serum (Bioveta, Czech Republic). Exogenous source of metabolic activation system (MAS), i.e. a cofactor-supplemented post-mitochondrial fraction prepared from rodent livers (Wistar rats, males, 7-8 weeks old) treated with an enzyme-inducing agent (polychlorinated biphenyl Delor), was used to model mammalian metabolic activation. Cells were exposed to the test substances with or without MAS. 48 hours after the start of the culture, the cells were treated for 4 hours with the test substance and positive control chemicals (Thio-TEPA  $10^{-6}\text{M}$  without MAS and cyclophosphamide  $10^{-4}\text{M}$  with MAS) and cultured for 24 hours. Culture medium was used as negative control. Then, the cell cultures were exposed for 2 hours to a metaphase arresting agent (colchicine), harvested and subjected to hypotonic cell treatment, fixation, and staining (5 % Giemsa). Metaphase cells (at least 200 well-spaced metaphases with  $46 \pm 2$  centromeres) were analyzed

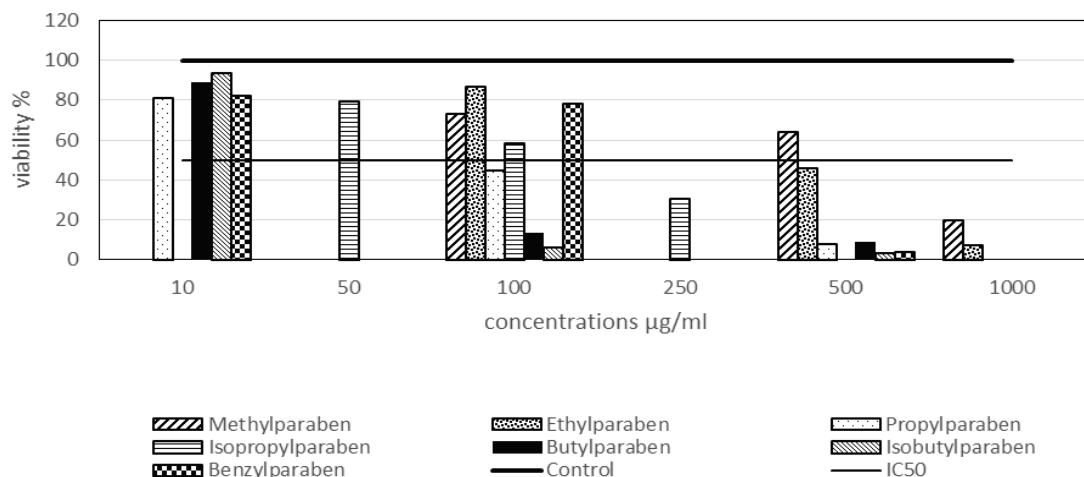
microscopically for the presence of chromosomal aberrations. Gaps were recorded separately and were not included in the total frequency of aberrations. The percentage of cells with structural chromosomal aberrations was evaluated. At least a 2-fold increase in aberration cells compared to the negative control was a criterion for determining a positive result including a concentration-related increase in the number of cells with chromosomal aberrations.

## Results

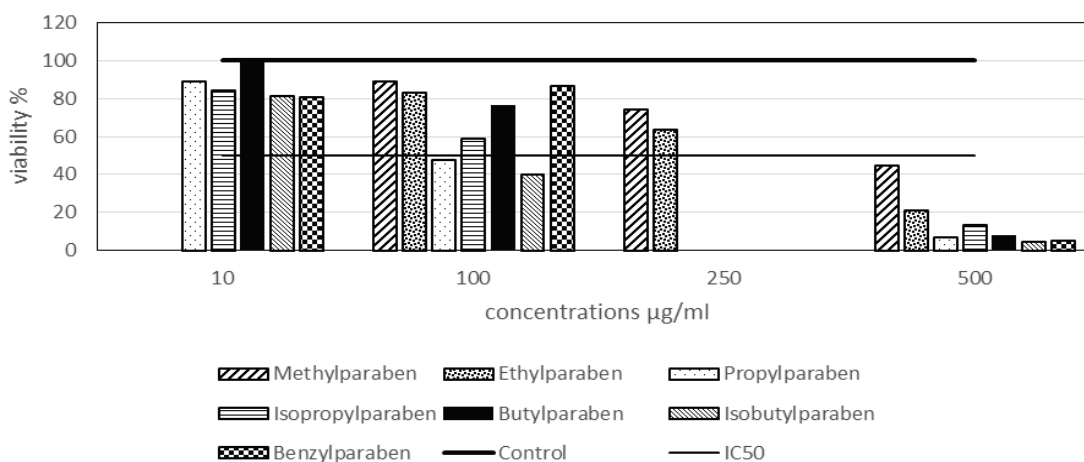
### MTT assay

The results of MTT assay have shown concentration-dependent responses of all samples (Fig. 1, 2). Variability was observed in the used two *in vitro* test systems. Methylparaben was shown to

exhibit the weakest cytotoxicity, as the detected cytotoxic concentration (below IC<sub>50</sub> value) was 750 µg/ml in the HaCaT cell line and 500 µg/ml in the SVK14 cell line. Ethylparaben and Benzylparaben exhibited cytotoxicity at 500 µg/ml in both *in vitro* cell systems. Butylparaben exhibited cytotoxicity at 100 µg/ml in the HaCaT cell line and 500 µg/ml in the SVK14 cell line. Isobutylparaben and Propylparaben have shown similar results, as the detected cytotoxic concentration (below IC<sub>50</sub> value) was 50 µg/ml for both Isobutylparaben and Propylparaben in the HaCaT cell line and 100 µg/ml in the SVK14 cell line. Isopropylparaben exhibited lower cytotoxicity in the SVK14 cell line, as the detected cytotoxic concentration (below IC<sub>50</sub>) was 500 µg/ml, however, in the HaCaT cell line, the first cytotoxic concentration (below IC<sub>50</sub>) was 125 µg/ml (data not shown).



**Fig. 1.** MTT assay, HaCaT cell line. Bold full line represents the value of solvent control (100 %). Full line represents the cut-off value in comparison with solvent control (IC<sub>50</sub>).



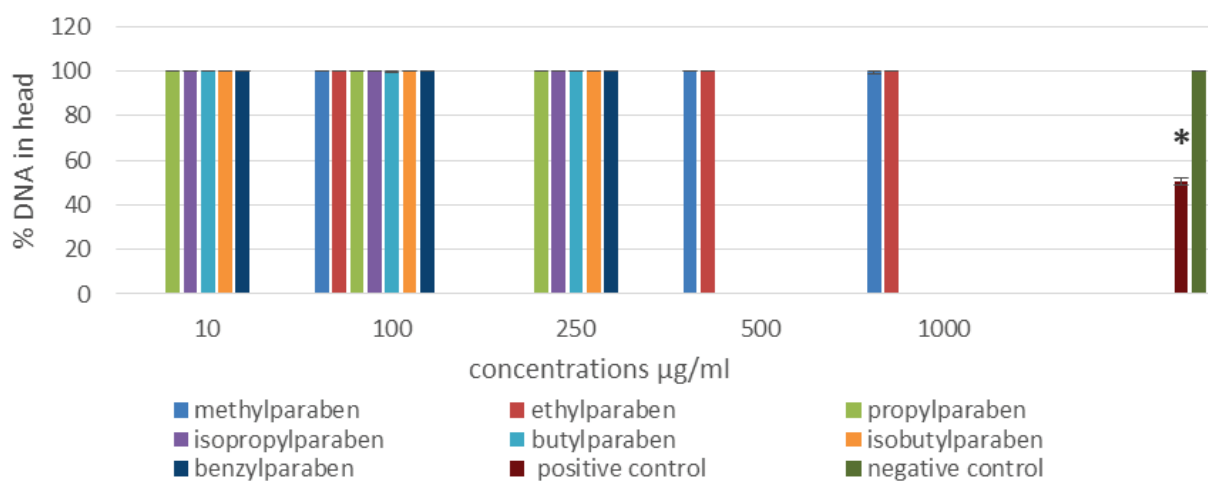
**Fig. 2.** MTT assay, SVK14 cell line. Bold full line represents the value of solvent control (100 %). Full line represents the cut-off value in comparison with solvent control (IC<sub>50</sub>).



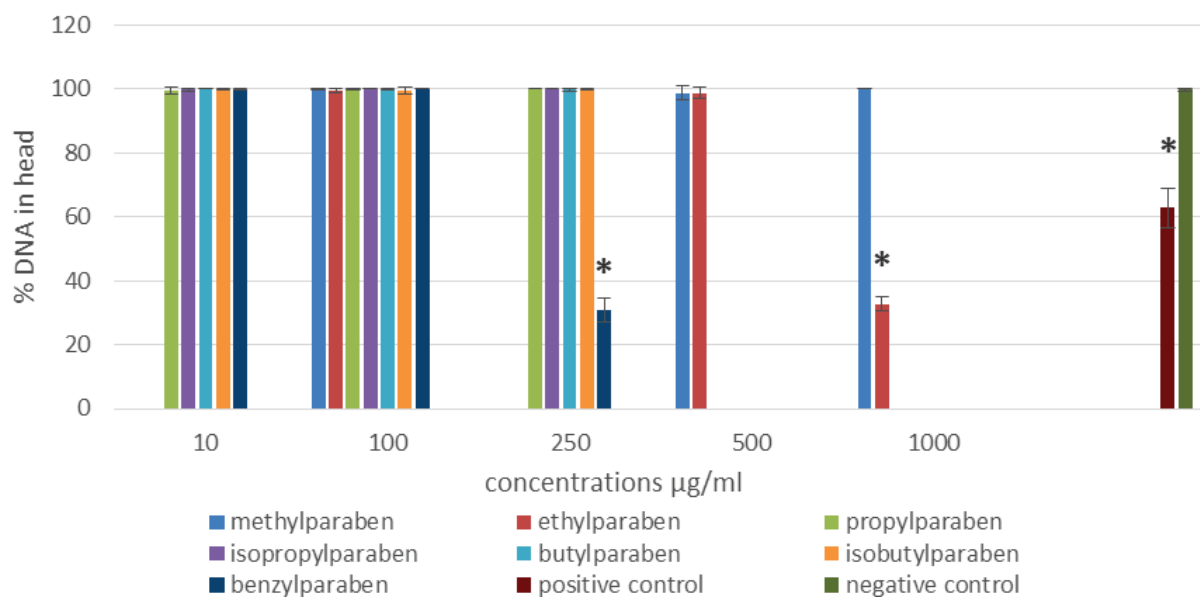
### Comet assay *in vitro* in SVK14 cell line and HaCaT cell line

The observed intensity of DNA damage in the SVK14 cell line exposed to any of the parabens (Methyl-, Ethyl-, Propyl-, Isopropyl-, Butyl-, Isobutyl- or Benzylparaben) did not exceed the values of negative control even at high concentrations which were observed as cytotoxic in the MTT assay (Fig. 3). In the HaCaT cell line, Benzylparaben and Ethylparaben exhibited increased fragmentation of DNA (Benzylparaben, mean value 30.9 % DNA in head at 250  $\mu\text{g}/\text{ml}$ ; Ethylparaben, mean value 32.8 % DNA in head at 1000  $\mu\text{g}/\text{ml}$ ), which

represented values significantly distant from the negative control (99.7 % DNA in head), whereas the fragmentation of DNA exceeded even the values of positive control (0.1 %  $\text{H}_2\text{O}_2$  in 1x PBS, 15 min, 4 °C, mean value 62.9 % DNA in head), as reported in Figure 4. Therefore, Benzylparaben and Ethylparaben were evaluated as suspected genotoxic substances in the *in vitro* Comet assay, as they may potentially exhibit genotoxic effects at higher concentrations. This result was used as an indication for further testing with the aim to confirm the results.



**Fig. 3.** Comet assay *in vitro*, SVK14 cell line. Data (% DNA in head) are presented as median  $\pm$  SD values from three independent measurements. The results were considered statistically significant when  $p < 0.05$  (\*). The DNA in head is proportional to the DNA damage (the higher value, the lower DNA damage).



**Fig. 4.** Comet assay *in vitro*, HaCaT cell line. Data (% DNA in head) are presented as median  $\pm$  SD values from three independent measurements. The results were considered statistically significant when  $p < 0.05$  (\*). The DNA in head is proportional to the DNA damage (the higher value, the lower DNA damage).

**Table 2.** *In vitro* mammalian chromosome aberration test.

Samples / Controls	Concentration µg/ml	% aberrant cells			Evaluation
		4 h with MAS	4 h without MAS	26 h without MAS	
<i>Methylparaben</i>	10	3	4	4.5	negative
	25	4	3	tox	inconclusive result due to cytotoxicity
	50	tox	tox	tox	
	100	tox	tox	tox	
<i>Ethylparaben</i>	10	3	3	3	borderline
	25	5.5	4	tox	inconclusive result due to cytotoxicity
	50	tox	tox	tox	
	100	tox	tox	tox	
<i>Propylparaben</i>	5	4	3.5	2.5	positive
	10	2	3	2	
	25	5	6	9	
	50	tox	tox	tox	
<i>Isopropylparaben</i>	5	3	3	2	positive
	10	2.5	3	3.5	
	25	4	4.5	7.5	
	50	tox	tox	tox	
<i>Butylparaben</i>	5	5	5	3	positive
	10	6	5.5	9	
	25	tox	tox	tox	
	50	tox	tox	tox	
<i>Isobutylparaben</i>	5	3	3	2.5	negative
	10	5	4	4.5	
	25	tox	tox	tox	
	50	tox	tox	tox	
<i>Benzylparaben</i>	1	4	3	2.5	negative
	2.5	5	3	0.5	
	5	2.5	4	1.5	
	10	3.5	3	5	
<i>Negative control (non-treated cells)</i>			3	2	
<i>Positive control (thio-TEPA)</i>			10	15	
<i>MAS Negative control (non-treated cells with MAS)</i>		5.5			
<i>Positive control (cyclophosphamide)</i>		11			

The percentage of aberrant cells is recorded after 4h with/without MAS and 26h without MAS (MAS - metabolic activation system). Due to the toxicity of the test substance at a given concentration, mitoses were not evaluable (designated tox).

#### *In vitro* mammalian chromosome aberration assay

In the validated *in vitro* mammalian chromosome aberration assay, human peripheral blood

lymphocytes did not show an increased rate of chromosomal aberrations compared to the negative control for Methylparaben, Isobutylparaben and

Benzylparaben. Ethylparaben was considered borderline, but due to its high toxicity it was not possible to evaluate the sample in all concentrations. Propylparaben, Isopropylparaben and Butylparaben had a high percentage of aberrations and the concentration dependence at the highest tested concentrations is evident compared to the negative control (more than 2-fold) in the case of the 26-hour incubation without MAS (Table 2).

## Discussion

The aim of our study was to generate data with the use of two *in vitro* toxicological methods in combination, performed in three human relevant non-malignant cell systems. We performed the *in vitro* Comet assay using the HaCaT and SVK14 cell line and the CA assay using human peripheral blood lymphocytes. Our study demonstrated high variability of results between the two *in vitro* methods, and surprisingly also between the two cell lines used in the Comet assay. The observed intensity of DNA damage in the SVK14 cell line did not exceed the values of negative control and all the samples were classified as negative, indicating that the substances neither significantly accumulated in the DNA nor attacked the DNA structure, thus, their cytotoxicity may be attributed to other mechanisms than direct DNA damage. With the use of the HaCaT cell line, most samples were also classified as negative, however, Benzylparaben and Ethylparaben exhibited an increased fragmentation of DNA at one tested concentration. We considered the results from the Comet assay as indicative for further testing and performed a validated CA assay using human peripheral blood lymphocytes. In the CA assay, Methylparaben was classified as negative and Ethylparaben as borderline, however, both exhibited significant cytotoxicity. Propylparaben, Isopropylparaben, Butylparaben were classified as positive, and Isobutylparaben, Benzylparaben as negative. Comet assay has been repeatedly described in other studies to give positive results only at significantly higher concentrations than in the CA or MN test (Giannotti *et al.* 2002, Kimura *et al.* 2013, Kawaguchi *et al.* 2010). As the Comet assay may not give a specific response with satisfactory sensitivity at lower concentrations and lacks metabolic activation, or the CA test may be compromised by higher cytotoxicity, the two tests do not seem to correlate promisingly. Assuming that a negative *in vitro* result should be supported by data

from at least two validated *in vitro* tests, and with regard that only one *In vitro* bioassay has been validated in our laboratory, the result has to be considered with caution. In case of inconclusive results from *in vitro* testing, repeating the test or applying another *in vitro* test is recommended (EFSA 2011). Unfortunately, increasing the number of *In vitro* assays may further reduce specificity and increase false positivity. Inconclusive results may be obtained due to inclusion of metabolic activation system, experimental conditions, specificities of the test system, cell type used, etc. (Nesslany 2017). Various mammalian cell types may exhibit variability in expression of genes involved in DNA repair, cellular defence systems, cell cycle control and apoptosis (e.g. p53), resulting in different response to chemicals. Lymphocytes have been reported to be karyotypically stable, however, when used in the CA test, mitogens have been observed to induce the resting phase. Characterization of *in vitro* test systems should be performed and donor-to-donor variation avoided (Tweats *et al.* 2007b). In order to deeply consider other possible sources of variability, we focused also on water solubility of parabens, determining their bioavailability at the cellular level. We also performed additional cytotoxicity test using 3T3 Balb/c fibroblasts and Neutral Red Uptake assay (3T3 NRU Cytotoxicity Assay), which was performed according to ECVAM DB-ALM Protocol no. 46 (data not published). Interestingly, during microscopic inspection, we observed various paraben-borne formations in the serum-free media, such as clusters or crystals, which were not observed during routine visual inspection. Moreover, the results of 3T3 NRU Cytotoxicity Assay have shown further variability. Therefore, we suggest that solubility in water solutions (i.e., culture media) should be thoroughly inspected in case of parabens, as poor water solubility or crystallization may significantly affect the bioavailability of parabens in both *in vitro* and *in vivo* test systems and compromise the results of genotoxicity testing. We performed a brief data search upon water solubility of parabens and found similar conclusions for individual parabens or their mixtures. Generally, parabens are stable in air and are resistant to hydrolysis in hot or cold water and in acidic solutions. The antibacterial potential of parabens increased with the length of esterified alcohol chain, however, the water solubility decreased (Soni *et al.* 2001). Variable antimicrobial potential and water solubility represents one of the reasons for application of parabens in mixture, to ensure optimal antimicrobial

activities and acceptable solubility (Charnock and Finsrud 2007, Güzel Bayülken and Tüylü 2019, Soni *et al.* 2005, Kapalavavi *et al.* 2014, Giordano *et al.* 1999, Perlovich *et al.* 2005).

The results of our study are in agreement with numerous toxicological studies on parabens mutagenicity, genotoxicity and carcinogenicity, which often present ambiguous or warning conclusions. Potential genotoxicity and carcinogenicity has frequently been attributed to parabens, e.g. paraben esters have been reported to be detected in breast tissue biopsies, and antiperspirants have been suggested to be the source (Darbre *et al.* 2004). However, the risk assessment for humans has become highly challenging, as inconclusive or contradictory results have been demonstrated by numerous studies *in vivo* and *in vitro* or in combined test batteries (e.g., Tayama *et al.* 2008, Roszak *et al.* 2017, Barr *et al.* 2012, Fransway *et al.* 2019, Soni *et al.* 2005, Kang *et al.* 2013b, Darbre and Harvey 2008, Kim *et al.* 2020, Bilal and Iqbal 2019). Official scientific opinions summarize in their studies that the use of parabens is safe for human health as parabens are metabolised to benzoic acid in the human body and excreted in the urine, with

benzoic acid being practically free of toxic effects in numerous tests and unlikely to interfere with chromosomes (SCF 1994, SCCP 2011, SCCP 2013). The results of our study indicate that genotoxicity testing should be strictly interpreted exclusively for the specific *in vitro* test system used in the bioassay, and standard bioavailability of the substance has to be thoroughly ensured. Moreover, as the genotoxicity of parabens is metabolism-dependent, a most relevant MAS should be included. With regard to the above limitations and observations of *in vitro* bioassays, our aim is to implement more *in vitro* methods based on human relevant cells and tissues into our laboratory, to achieve a sufficient battery of non-animal toxicological methods.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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