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Effects of 3-monochloropropane-1,2-diol (3-MCPD) and its metabolites on DNA damage and repair under in vitro conditions

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ABSTRACT

3-monochloropropane-1,2-diol (3-MCPD) is a food contaminant that occurs during industrial production processes and can be found mainly in fat and salt containing products. 3-MCPD has exhibited mutagenic activity in vitro but not in vivo, however, a genotoxic mechanism for the occurrence of kidney tumors has not so far been excluded. The main pathway of mammalian 3-MCPD metabolism is via the formation of β − chlorolactatic acid and formation of glycidol has been demonstrated in bacterial metabolism. The aim of this study was to investigate genotoxic and oxidative DNA damaging effects of 3-MCPD and its metabolites, and to provide a better understanding of their roles in DNA repair processes. DNA damage was assessed by alkaline comet assay in target rat kidney epithelial cell lines (NRK-52E) and human embryonic kidney cells (HEK-293). Purine and pyrimidine base damage, H_2O_2 sensitivity and DNA repair capacity were assessed via modified comet assay. The results revealed in vitro evidence for increased genotoxicity and H_2O_2 sensitivity. No association was found between oxidative DNA damage and DNA repair capacity with the exception of glycidol treatment at 20 μg/mL. These findings provide further insights into the mechanisms underlying the in vitro genotoxic potential of 3-MCPD and metabolites.

1. Introduction

As a consequence of food production processes, cooking and storage conditions, numerous chemicals can end up as contaminants in the final food products. Unlike food additives, contaminants are found in food unintentionally and may pose risks for human and animal health due to their toxic properties (Raiola et al., 2015; Peshin et al., 2002).

3-Monochloropropane-1,2-diol (3-MCPD) is a common food contaminant, which belongs to the chemical group of chloropropanols. 3-MCPD is formed under high temperature during the production of food products containing fat, salt, sugar, food emulsifiers and can be found in products such as infant formulas, oils and fats, and bread (Breitling-Utzmann et al., 2005; Buhrke et al., 2011). According to current knowledge, 3-MCPD formation can occur via 3 pathways: acid hydrolysis, heat processing, and release of 3-MCPD from its esters (Baer et al., 2010; Hwang et al., 2009).

In male rats 3-MCPD is rapidly absorbed following oral administration and distributed to various tissues. It is primarily metabolized in the liver and excreted from the body via the kidneys (Xiao et al., 2003).

3-MCPD biotransformation leads to β-chlorolactic acid formation which can be further oxidized to oxalic acid, the major urinary

Abbreviations: EndoIII, Endonuclease III; FBS, Fetal Bovine Serum; FPG, Formamido Pyrimidine Glycosylase; IARC, The International Agency for Research on Cancer; LOEL, Lowest Observed Effect Level; 3-MCPD, 3-monochloropropane-1,2-diol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NOEL, No Observed Effect Level; SCF, European Scientific Committee on Food; SD, Standard deviation; TD, DNA in tail.

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metabolite of 3- MCPD in mammalian metabolic pathways. In bacteria, glycidol formation occurs which is metabolized to glycerol and mercapturic acid. Oxidative metabolism of 3-MCPD in mammals inhibits glycidol formation (Habermeyer et al., 2011; Jędrkiewicz et al., 2015).

3-MCPD (CAS 96-24-2) does not have a harmonized classification and labeling under the Regulation 1272/2008/EC (CLP). Nevertheless, manufactures and importers in EU self-classify the substance for various health hazards, such as Carcinogen Category 2, Toxic for Reproduction Category 1B, Mutagen Category 2 etc. In addition, the International Agency for Research on Cancer (IARC) characterizes 3-MCPD as possibly carcinogenic to humans, based on cancer incidents caused by 3-MCPD in laboratory animals (renal tubular tumors, Leydig cell adenomas). 3-MCPD's metabolite glycidol is also characterized by IARC as possibly carcinogenic to humans (IARC Monographs, 2012).

Based on the results from genotoxicity studies conducted in vitro and in vivo, the European Scientific Committee on Food (SCF) concluded that the 3-MCPD genotoxicity determined under in vitro conditions was not expressed in the in vivo situation (SCF, 2001; Cho et al., 2008). Based on current data 3-MCPD is therefore considered to be a non genotoxic chemical under in vivo conditions.

The World Health Organization (WHO) indicated that renal tubular hyperplasia is the most sensitive endpoint for the setting of the tolerable intake of 3-MCPD. In a long-term toxicity and carcinogenicity study in Fischer 344 rats the LOEL (Lowest Observed Effect Level) value for renal tubule hyperplasia was 1.1 mg/kg body weight/day, which was deemed to be close to the NOEL (No Observed Effect Level) value for renal tumors (WHO, 2001).

However, there is not enough data to unequivocally determine its genotoxic potential. Oxidative stress is a cause or effect of many disorders and plays a distinct role in 3-MCPD toxicity. In kidney and testis, suspected 3-MCPD toxicity mechanisms are found to be related with glycolysis inhibition and oxidative stress (Sawada et al., 2015). The exact role of oxidative DNA damage and repairing capacity after exposure to 3-MCPD, and its metabolites, β-chlorolactic acid and glycidol is still unclear. Oxidative DNA damage can be assessed with the modified comet assay using bacterial endonucleases. Endo III and FPG are lesion-specific endonucleases, which allow the determination of pyrimidine and purine base damage, respectively. DNA repair capacity assessment is measured by inducing DNA damage with a known DNA damaging agent and repairing capacity could be assessed by allowing cells for repair. Also, using Endo III and FPG have applications in calculating specific pyrimidine and purine base damage repair capacity (Collins, 2011; Gunasekarana et al., 2015).

In this project, we aimed to clarify oxidative DNA damaging potentials of 3-MCPD and its metabolites, β-chlorolactic acid and glycidol, as well as their effect on DNA repair processes. Since the main target organ of 3-MCPD tumourigenicity is known to be the kidneys, NRK-52E and HEK-293 cells were selected in order to represent rat and human kidney cells, respectively.

2. Materials and methods

2.1. Chemicals

3-MCPD (CAS number 96-24-2, purity 98%), glycidol (CAS number 556-52-5) and β-chlorolactic acid (CAS number 1713-85-5) were provided by Sigma–Aldrich Co. Other chemicals for cell culture were all provided by Multicell.

2.2. Cell cultures

NRK-52E, a rat kidney proximal tubular epithelial cell line, and HEK-293, an embryonic kidney cell line, were both obtained from the American Type Culture Collection (ATCC, USA). These cell lines were chosen as representative target cells, as the kidneys are one of the main target organs affected from 3-MCPD.

2.2.1. NRK 52E cell culture

Standard conditions were used for growing NRK 52E cells. 5.5 mmol/L D-glucose (319-075-CL, Multicell) containing Dulbecco's modified Eagles medium: Nutrient mixture F12 (DMEM/F12) was supplemented by 10% heat-inactivated Fetal Bovine Serum-FBS (095-110, Multicell) and 1% antibiotic (100 U/mL penicillin and 100 mg/mL streptomycin). Cell cultures were maintained at 37 °C in a humidified 5% CO_2 incubator and sub-cultured after trypsinization with 0.2% trypsin-0.04% EDTA in PBS. Cells were sub-cultured 3 times per week and treatment of logarithmically growing cells was performed 24 h after seeding.

2.2.2. HEK-293 cell culture

HEK-293 cells were maintained at 37 °C, 5% CO_2 and >90% relative humidity in Dulbecco's modified Eagle's medium (319-005-CL, Multicell) supplemented with 10% fetal bovine serum (095-110, Multicell) and 1% antibiotic (100 U/mL penicillin and 100 mg/mL streptomycin). HEK-293 cells were sub-cultured when they reach 70% confluence and chemical treatments performed 24 h after seeding.

2.3. Cell viability assessment

NRK-52E and HEK-293 cells were seeded at a density of 1×10^4 cells per well of 96 well plates and cultured for 24 h, respectively. Test compounds (3-MCPD, glycidol and β-chlorolactic acid) were added to 96 well plates in various concentrations in the absence of FBS. All test compounds were dissolved in serum free medium.

Cell viability determination was performed by using MTT assay as previously described (Alley et al., 1988) with slight modifications. Cells were exposed to test compounds for 24 h at 37 °C in a humidified 5% CO_2 incubator. Purple colored formazan formation from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was measured spectrophotometrically in order to assess cell viability. Yellow colored MTT is reduced to purple colored formazan derivatives by mitochondrial succinate dehydrogenase and this reduction occurs only in viable cells. Cell viability was calculated as the percentage of the viable cells compared to the untreated control cells. Three independent experiments were performed in quadruplicate.

The trypan blue exclusion assay is also used for determining cell viability prior to performing the alkaline comet assay. In order to perform trypan blue exclusion assay; equal aliquates of cell suspensions and trypan blue (10 μl:10 μl) were mixed and assessed in a Neubauer' haemocytometer under a microscope at 40 × magnification. Percentage of cell viability was recorded for each 24 h compound exposed cell lines.

2.4. Treatment of cultured cells

Cultured NRK-52E and HEK-293 cells were washed with PBS and trypsinization performed with 0.2% trypsin-0.04% EDTA in PBS in order to dissociate adherent cells. Trypsin was immediately removed in order to avoid causing extra damage and the cells were washed with medium and PBS. In pilot pre-studies, several doses of 3-MCPD, glycidol and β-chlorolactic acid were tested for determining appropriate concentrations for treatment. Solubility of the compounds and viability were the major parameters for selecting appropriate doses. Consequently, cells were treated with the

following selected concentrations of test compounds (1.25–2.5–5–10 mg/mL for 3-MCPD, 5–10–20–40 μg/mL for glycidol and 0.25–0.5–1–2 mg/mL for β-chlorolactic acid) for 24 h. Medium pH was checked for large variations in order to avoid non-specific genotoxicity. Viability check was performed by trypan blue exclusion test again for each sample before alkaline and modified comet assays. Cells were adjusted to the density in order to contain approximately 2×10^4 cells per gel.

2.5. Alkaline comet assay

A conventional alkaline comet assay protocol, as described by Tice et al. (2000), was followed with minor modifications. All experiments were done in triplicate and slide reading was performed blindly by one slide reader. The DNA damage assessment was performed with percentage of DNA in tail (TD %) in 50 cells per slide by using Comet Assay IV image analysis system (Perceptive Instruments).

2.6. Modified comet assay

By using the modified comet assay, base oxidization was detected with bacterial repair endonucleases as previously described (Collins et al., 1993). 3-MCPD, glycidol and β-chlorolactic acid induced oxidative DNA damage were assessed by modified comet assay on HEK-293 cells. NRK-52E and HEK-293 cells show similar trends in terms of viability and DNA damage. HEK-293 cells have been found in our lab to be more sensitive in representing DNA damage and are therefore selected for determining this type of adverse effect. The modified alkaline comet assay was followed for selected concentrations of each compound. Dose selection was performed according to the alkaline comet assay results and lowest significantly DNA damage induced concentrations were selected for each compound. HEK-293 cells were treated with 2 mg/mL 3-MCPD, 20 μg/mL glycidol and 0.8 mg/mL β-chlorolactic acid for 24 h and modified comet assay was applied accordingly.

Cultured cells were treated with enzyme buffer to examine the basal DNA damage and with enzymes Endo III or FPG in order to determine pyrimidine or purine base damage, respectively. Pyrimidine or purine base damage was assessed according to Collins et al. (1993). Damage evaluation was performed as previously described for the alkaline comet assay. In order to assess DNA repair capacity, 50 mM H_2O_2 induction was performed on ice for 5 min, then cells were either lysed immediately or incubated at 37 °C for 30 min prior to lysis. This incubation period allows cells to repair damaged DNA. 50 mM H_2O_2 induction without repair process gives information about H_2O_2 sensitivity. FPG or Endo III sites represented damage was calculated as described by Collins et al. (1993). In order to assess Endo III and FPG site repair, 1 h incubation was performed after H_2O_2 induction and then cells were treated with Endo III or FPG. The DNA repair capacity percentages were calculated for all groups according to Kadioglu et al. (2010).

$$\text{DNA repair capacity \%} = \frac{\text{TD\%}(H_2O_2 \text{ induced}) - \text{TD\%}(H_2O_2 \text{ induced and repaired})}{\text{TD\%}(H_2O_2 \text{ induced})} \times 100$$

2.7. Statistics

Results were summarized as mean (standard deviation) or as percentage depend on variable measurement level. Each treatment group was compared to control by using Mann Whitney U test. Comparison of each treatment groups with each other was out of the study scope, as the potency of each of the tested compounds was not investigated Correlation between parameters was evaluated Spearman linear correlation test. Type 1 error was set as 5% for each comparison. IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp. Released 2012, Armonk, NY: IBM Corp) was used for the analyses.

3. Results

3.1. Cell viability assessment

The cell viability assessment was performed with MTT and trypan blue exclusion assays for each compound in order to determine appropriate doses to assess genotoxicity by alkaline comet assay. Appropriate treatment concentrations are the concentrations which render measured cell viability higher than 70% after treatment. Possible concentrations determined in the MTT test were also tested with the trypan blue exclusion assay. Fig. 1 represents reduced cell viability percentages of 3-MCPD, glycidol and β-chlorolactic acid on NRK 52E and HEK-293 cells following 24 h treatment assessed by MTT assay. As shown in Fig. 1, relatively similar cytotoxicity results were obtained for both NRK-52E and HEK-293 cells exposed to various concentrations of test compounds.

3.2. DNA damage assessment by alkaline comet assay

According to the MTT and trypan blue exclusion assay results, the concentrations of 4–2–1–0.5 mg/mL for 3-MCPD, 40–20–10–5 μg/mL for glycidol and 0.8–0.4–0.2–0.1 mg/mL for β-chlorolactic acid were determined as the appropriate working doses for alkaline comet assay in order to assess DNA damage. For each concentration, following 24 h treatment of NRK-52E and HEK-293 cells, cell viability was checked and found to be higher than 80%.

Alkaline comet assay results are summarized in Fig. 2. The extent of the DNA damage was statistically increased ($p < 0.05$) at 2 mg/mL 3-MCPD for both NRK-52E and HEK-293 cells compared to control groups. 4 mg/mL 3-MCPD also caused statistically increased DNA damage in NRK-52E and HEK-293 cells.

In both cell lines, 20 and 40 μg/mL glycidol treatment caused statistically significant elevation ($p < 0.01$) in DNA damage compared to control groups. β-Chlorolactic acid treatment did not increase DNA damage significantly in NRK-52E cells at any tested concentrations, while treatment of HEK-293 cells with 0.8 mg/mL β-chlorolactic acid caused a significant increase ($p < 0.05$) in DNA damage compared to control group.

According to the alkaline comet assay results, HEK-293 cells are more sensitive to tested compounds in terms of DNA damage. Therefore HEK-293 cells were selected for conducting modified comet assay.

3.3. Oxidative DNA damage and repair assessment by modified comet assay

Basal DNA damage, Endo III sensitive sites (pyrimidine base

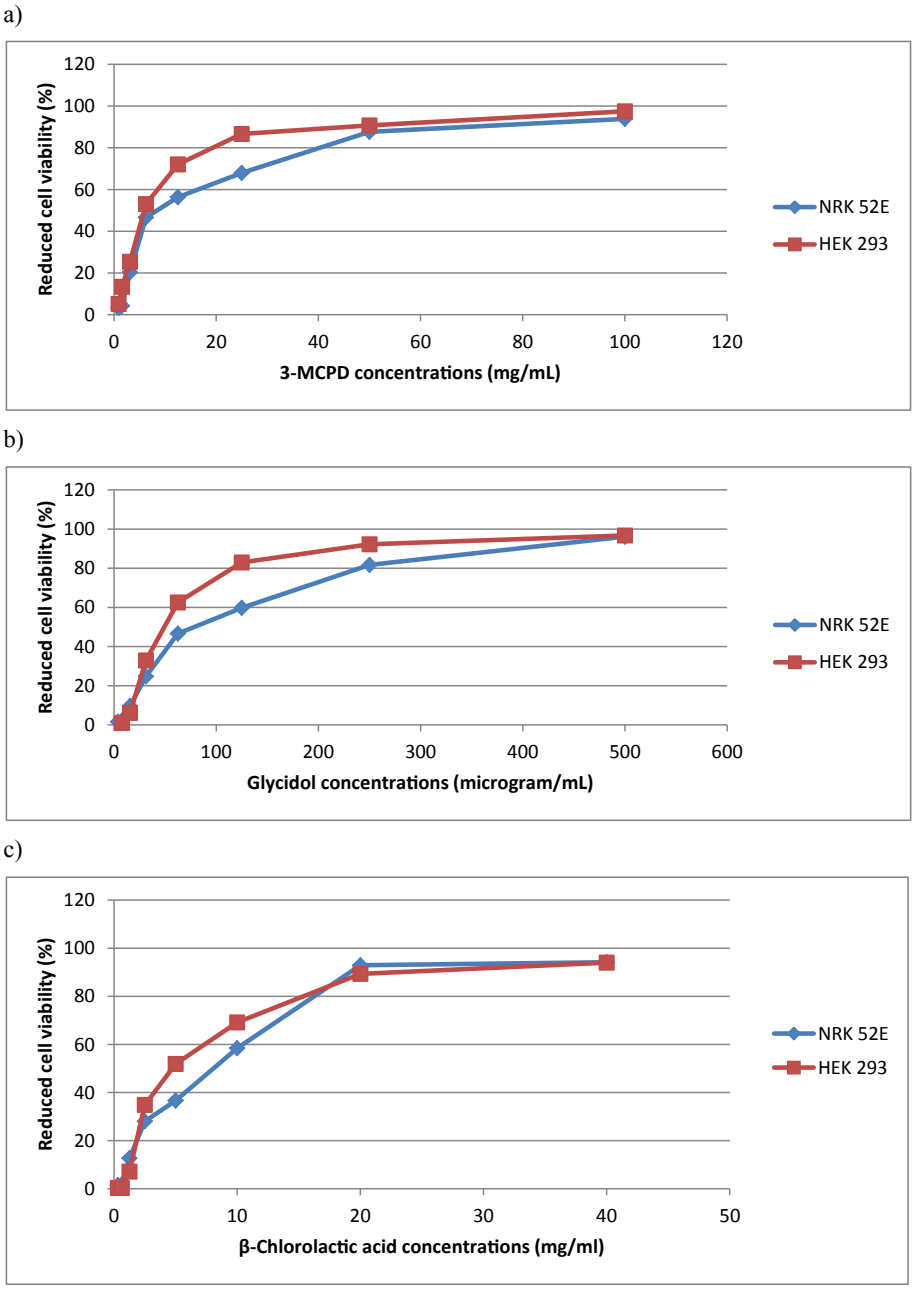

Fig. 1. Reduced cell viability percentages of 3-MCPD (a), glycidol (b) and β-chlorolactic acid (c) on NRK 52E and HEK-293 cells following 24 h treatment assessed by MTT assay.

damage), FPG sensitive sites (purine base damage), H_2O_2 sensitivity, DNA repair capacity (%), pyrimidine base damage repair (%) and purine base damage repair (%) were all assessed by using the modified comet assay. The results are summarized in Table 1.

Basal DNA damage was assessed by using enzyme buffer. Basal DNA damage values were significantly higher ($p < 0.05$) in 2 mg/mL 3-MCPD, 20 μg/mL glycidol and 0.8 mg/mL β-chlorolactic acid treated groups compared to control. These results are in line with alkaline comet assay results. Both pyrimidine base damages (Endo III sensitive sites) and purine base damages (FPG sensitive sites) were not determined to be induced with 3-MCPD, and β-chlorolactic acid ($p > 0.05$) treatments. 20 μg/mL glycidol treatment statistically induced ($p < 0.05$) purine base damages compared to control group, while no induction was observed for pyrimidine base damage.

H_2O_2 sensitivity gives insight for chemical sensitivity in the exposed cells. H_2O_2 sensitivity was determined to be higher compared to control group in all treated groups (2 mg/mL 3-MCPD, 20 μg/mL glycidol and 0.8 mg/mL β-chlorolactic acid) with statistical significance ($p < 0.05$). Pyrimidine, purine base damage repair and calculated DNA repair capacity were decreased in all treated groups, however differences were not statistically significant ($p > 0.05$) compared to control group. Negative weak linear correlation was observed between DNA repair capacity and H_2O_2 sensitivity (correlation coefficient: −0.203, $P = 0.527$).

4. Discussion

There are different carcinogenicity prescreening approaches for identifying genotoxic/epigenetic carcinogenic potential of a

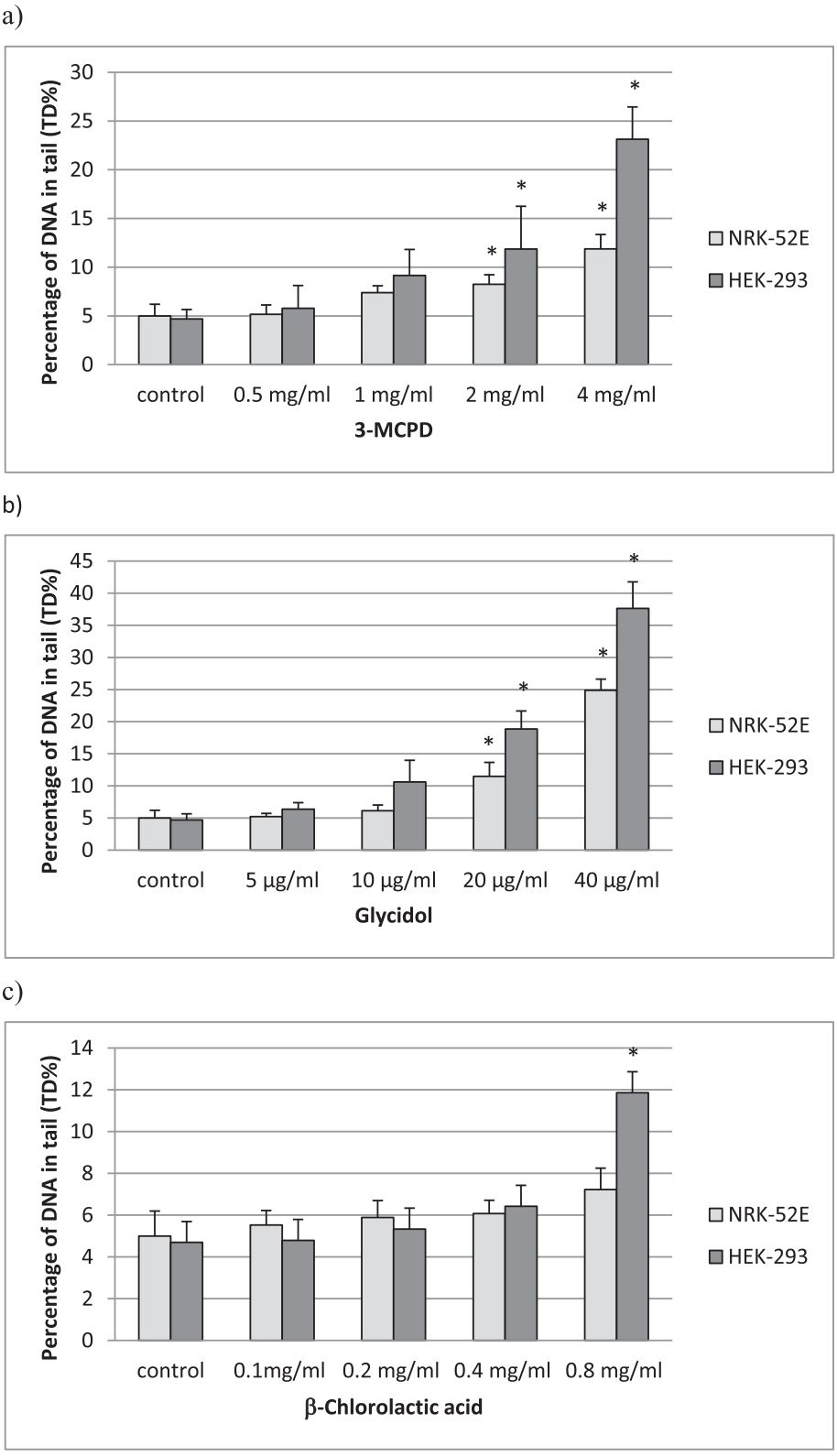

Fig. 2. Alkaline comet assay results of a) 3-MCPD, b) glycidol and c) β-chlorolactic acid induced NRK-52E and HEK-293 cells (*$p < 0.05$, compared to control).

compound (Benigni, 2014). In vitro and in vivo genotoxicity tests are widely used for carcinogenicity prediction. According to the CLP Regulation, in vivo mutagenicity data could lead to classification of a substance as Carcinogen 2, while at least one positive in vivo test with supporting in vitro data on mutagenic effects could be regarded sufficient to classify a substance as Mutagen category 2. Typically, in vitro tests are performed with cultured bacterial cells, human or other mammalian cells and the comet assay can provide supporting evidence for the purposes of classification according to expert judgement. In vivo test results for mutagenicity should be

Table 1
Parameters of oxidative DNA damage (TD%) and repair % in HEK-293 cells for selected concentrations of 3-MCPD, glycidol and β-chlorolactic acid.

Mean (standard deviation)	Control	3-MCPD (2 mg/mL)	Glycidol (20 μg/mL)	β-Chlorolactic acid (0.8 mg/mL)
Basal DNA Damage	5.99 (0.33)	**11.83 (0.83)***	**19.47 (0.61)***	**12.67 (1.00)***
Endo III sensitive sites (Pyrimidine base damage)	2.48 (0.41)	2.82 (1.85)	2.98 (1.22)	2.03 (0.92)
FPG sensitive sites (Purine base damage)	2.37 (0.65)	2.86 (0.14)	**5.26 (0.92)***	2.23 (0.95)
H_2O_2 Sensitivity	21.09 (1.84)	**28.46 (3.91)***	**36.67 (2.84)***	**35.19 (1.62)***
DNA repair capacity (%)	33.75 (9.66)	32.45 (6.87)	28.72 (1.88)	30.79 (5.74)
Pyrimidine base damage repair (%)	34.42 (8.83)	29.76 (8.59)	30.25 (10.14)	33.18 (10.25)
Purine base damage repair (%)	29.52 (2.43)	27.56 (6.57)	28.44 (1.82)	29.00 (3.69)

*Mean (standard deviation) values that are statistically significant different from control are indicated bold in table. ($p < 0.05$ compared to control).

evaluated with regards to the route of administration. In case of negative in vivo results by other than intraperitoneal application, it could be argued that mutagenicity/genotoxicity can only be shown at internal body substance concentrations, which cannot be achieved using application routes like oral, dermal or inhalative substance application. It also has to be taken into account that there is generally no threshold for mutagenicity, unless there is specific proof for the existence of such a threshold, as may be the case for aneugens.

The mutagenic activity of 3-MCPD and its metabolites has been shown in several in vitro studies (Silhankova et al., 1982; Stolzenberg and Hine, 1979). In order to assess carcinogenic potential of 3-MCPD, long term carcinogenicity studies conducted and in these studies increase in Leydig cell carcinoma, kidney tumors, hyperplasia and adenomas in Fisher 344 and Sprague–Dawley rats were demonstrated (Lynch et al., 1998; Jędrkiewicz et al., 2015; Cho et al., 2008). However, in vivo studies did not indicate genotoxicity in rats (Robjohns et al., 2003; Onami et al., 2014; El Ramy et al., 2007). It is generally believed that 3-MCPD related tumors are occurring via non-genotoxic mechanisms (Jędrkiewicz et al., 2015), while there is not enough data for clarifying its genotoxic/non-genotoxic potential. The aim of our study was to investigate whether oxidative DNA damage and DNA repair mechanisms could be involved in positive in vitro genotoxic results of 3-MCPD.

Our results show statistically significant differences in genotoxic activity as assessed by the alkaline comet assay above the doses of 2 mg/mL for 3-MCPD, 20 μg/mL glycidol and at the dose of 0.8 mg/mL β-chlorolactic acid. In a similar study, increased DNA damage was observed at 2.5 and 5 mg/mL 3-MCPD and at 20 and 40 μg/mL glycidol, while no DNA damaging effect of β-chlorolactic acid was detected up to 1 mg/mL under in vitro conditions (El Ramy et al., 2007). β-Chlorolactic acid has been previously reported as immunotoxic (Lee et al., 2005), however there has not been enough evidence for its genotoxicity up to now.

Glycidol is a known mutagenic and genotoxic compound. Glycidol has a harmonised classification as carcinogen (category 1B) and mutagen (category 2) in CLP Regulation. Induced structural chromosome aberrations with or without metabolic activation has been determined with glycidol (Ikeda et al., 2012). It has been shown that glycidol induces DNA alkylation selectively at the N-7 sites of the guanines (Toshima et al., 2003). Our results revealed an elevated purine base damage in 20 μg/mL glycidol induced HEK-293 cells. FPG protein has been shown to detect N7 guanine DNA alkylation damage (Azqueta and Collins, 2013; Speit et al., 2004). These results may clarify glycidol induced purine base damage detected with FPG enzyme. However, glycidol induction did not affect any of the studied DNA repair parameters. In a recent study, glycidol induced DNA damage was not found to be associated with cytotoxicity in rat urinary bladder (Wada et al., 2014). Thus, the mechanism of glycidol genotoxicity needs further studies in order to clarify the role of purine bases.

H_2O_2 is a well known chemical for the assessment of DNA damage induction and repair under in vitro conditions (Gedik et al., 2005; Barron et al., 2015). In the present study, induction with 3-MPCD and metabolites did not have an effect on DNA repair capacity and base damage repair, while all compounds demonstrate increased chemo-sensitivity with H_2O_2. The absence of DNA repair capacity reduction after treatment with 3-MPCD and its metabolites point to the hypothesis that the in vitro genotoxic potential of these compounds is independent of their effect on DNA repair mechanisms. On the other hand, El Ramy et al. suggested that in vitro DNA damaging effects of 3-MCPD could be related to the production of DNA reactive metabolites (El Ramy et al., 2007). There is a need for further studies clarifying the underlying mechanisms.

In conclusion, our results have provided a new insight regarding in vitro genotoxicity of 3-MCPD and its metabolites, glycidol and β-chlorolactic acid. However, further studies are needed to elucidate the in vitro/in vivo DNA damaging mechanisms of 3-MCPD and whether harmonized classification is warranted for this food process contaminant.

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Transparency document

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