

2-Monokloropropandiol (2-MCPD)'nin Sitotoksik Etkilerinin Fare TM3 Leydig ve TM4 Sertoli Hücreleri Üzerinde *in vitro* Değerlendirilmesi

Evaluation of Cytotoxic Effects of 2-Monochloropropanediol (2-MCPD) on Mouse TM3 Leydig and TM4 Sertoli Cells in vitro

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ÖZET

Amaç: 2-monokloropropandiol (2-MCPD), 3-monokloropropandiol (3-MCPD) ve esterleri ile glisidil yağ asidi esteri (GE), gıda veya gıda işlem kaynaklı kontaminantlar olarak adlandırılmaktadır. Bu gıda kontaminantlarının başta palmye yağı olmak üzere bitkisel yağlar, margarinler, pasta ve kek gibi işlem görmüş gıdalarda ve bebek mamalarında tespit edilmesi, güvenlikleri konusundaki ilgiyi arttırmıştır. Avrupa Gıda Güvenliği Otoritesi'nin (European Food Safety Authority, EFSA) Mayıs 2016 yılında yayınlamış olduğu bilimsel görüşe göre bitkisel yağ ve işlenmiş gıdalarda bulunan GE, 3-MCPD, 2-MCPD ve bunların yağ asidi esterlerinin insan sağlığı için zararlı olduğu raporlanmıştır. Ancak 2-MCPD toksisitesi hakkında yeterli veri olmadığı için tam bir değerlendirme yapılamamıştır. Bu amaçla 2-MCPD'nin, TM3 Leydig ve TM4 Sertoli hücre hatları üzerinde *in vitro* sitotoksik, apoptotik etkileri ve deoksiribonükleik asit (DNA) hasarı yapma potansiyeli değerlendirilmiştir.

Gereç ve Yöntem: Hücre canlılığı, 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromid (MTT) ve nötral kırmızısı alım (NRU) yöntemleri ile değerlendirilmiştir. Akış sitometrisi ile hücre ölüm tipinin belirlenmesinde aneksin V-FITC/PI ikili boyama kiti kullanılmıştır. Genotoksisite değerlendirmesinde komet yöntemi uygulanmıştır.

Bulgular: Hücre canlılık yöntemleri sonucu 2-MCPD'nin test edilen konsantrasyonlarda 1000 µM'a kadar sitotoksik olmadığı tespit edilmiştir. Aynı zamanda 2-MCPD'nin, sub-toksik konsantrasyonlarda (1-1000 µM) her iki hücre hattında da (TM3 Leydig ve TM4 Sertoli hücreleri) DNA hasarını indüklediği bulunmuştur.

Sonuç: 2-MCPD, her ne kadar test edilen konsantrasyonlarda (1-1000 µM) sitotoksik ve apoptotik etki göstermese de daha yüksek konsantrasyonlarda hücre ölümüne ve DNA hasarına neden olabilir. Ancak bu yüksek konsantrasyonlar günlük maruziyet dozlarından çok uzaktır.

Anahtar kelimeler: 2-MCPD, TM3 Leydig hücre hattı, TM4 Sertoli hücre hattı, apoptozis

ABSTRACT

Aim: 2-Monochloropropanediol (2-MCPD), 3-monochloropropanediol (3-MCPD), their esters and glycidyl fatty acid esters (GE) are important food contaminants originating mainly during food processing. Recently, the detection of these contaminants in different foods and vegetable oils, especially in palm oil, margarines, and processed foods such as pastries and cakes and in baby foods raises concerns about their safety. According to the European Food Safety Authority (EFSA) scientific report

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published in May 2016, foods and vegetable oil contaminated with 3-MCPD, 2-MCPD and their fatty acid esters and GE were reported as harmful to human health. However, limited data on 2-MCPD toxicity preclude a complete assessment of its toxicity. For this reason, the cytotoxic, apoptotic effects and deoxyribonucleic acid (DNA) damage potential of 2-MCPD were evaluated on TM3 Leydig and TM4 Sertoli cells.

Material and method: The cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) assays. Annexin V-FITC/PI double staining kit was used to indicate the type of cell death by flow cytometry. The alkaline comet assay was used to estimate the genotoxic effect.

Results: The cell viability assays showed that 2-MCPD was not cytotoxic within the tested concentrations up to 1000 μM on both cell lines. These sub-toxic concentrations of 2-MCPD (1-1000 μM) also did not induce DNA damage both on TM3 Leydig and TM4 Sertoli cells.

Conclusion: Although results demonstrate that tested concentrations of 2-MCPD (1-1000 μM) were neither cytotoxic nor apoptotic but may cause cell death and DNA damage at higher concentrations. On the other hand, those high concentrations are far from the daily exposure doses.

Keywords: 2-MCPD, TM3 Leydig cell line, TM4 Sertoli cell line, apoptosis

INTRODUCTION

2-monochloropropanediol (2-MCPD), 3-monochloropropanediol (3-MCPD), and their fatty acid esters and glycidyl fatty acid esters (GE) are contaminants found in processed foods and vegetable oils. These substances mainly occur during the refining process where the raw oils were boiled at high temperatures, above 200 °C, to remove the natural aromas (1). In 2016, these three contaminants were assessed by European Food Safety Authority (EFSA), in terms of their impact on public health. Glycidol, the main compound of GE, was reported to be genotoxic and carcinogenic (2,3), while 3-MCPD, another contaminant, was documented to damage kidneys and male reproductive organs (4-6). 2-MCPD which is an isomer of 3-MCPD occurs in lower levels than GE and 3-MCPD in refined vegetable oils (7). Thus, toxicologists have focused on 3-MCPD for numerous years and 2-MCPD has gained scientific attention much more lately.

Data on the effects of 2-MCPD on target organs are limited. Studies on the repeated-dose oral toxicity studies have reported that striated muscle and myocardium were the main organs/tissues affected by 2-MCPD, followed by kidneys. Although the reproductive toxicity of 3-MCPD has been summarized by the EFSA, there are no studies evaluating the effects of 2-MCPD on the reproductive system (2). Therefore, in the present study, we evaluated the *in vitro* toxic effect of 2-MCPD on mouse TM3 Leydig and TM4 Sertoli cells which represent the main male reproductive system cells. First, the cells were treated with a wide concentration range of 2-MCPD (1-1000 μM) to determine possible cytotoxic effect by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) assays. Later, cell viability results and the cell death pathway were confirmed by flow cytometry assay using Annexin V-FITC/PI dyes. Finally, non-cytotoxic concentrations of 2-MCPD (1, 10, 100 and 1000 μM) were chosen for testing its genotoxic potential.

MATERIALS AND METHODS

Cell Culture

The mouse TM3 Leydig and TM4 Sertoli were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were grown in DMEM/Ham's F12 medium (ATCC, Manassas, VA, USA) supplemented with 5% horse serum (ATCC, Manassas, USA), 2.5% fetal bovine serum (ATCC, Manassas, VA, USA), 1% penicillin (100 U/mL) and streptomycin (100 µg/mL) (Wisent, St. Bruno, QC, Canada). Both cells were cultured at 37 °C with 5% CO₂ in an incubator. At 90% confluence cells were treated by Tyripsin-EDTA (Wisent, St. Bruno, QC, Canada) for passage processes or sub-cultured into 25 cm² flasks, 6 or 96 well plates according to the assay. The passage number of both TM3 and TM4 cells were 2 to 10.

Cytotoxicity Assay

TM3 Leydig and TM4 Sertoli cells were incubated in 96-well plates in quantities of 5x10³/well for 24 h. 2-MCPD (Toronto Research Chemicals, North York, ON, Canada) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). At the end of incubation, the culture medium was replaced with exposure medium including different concentrations of 2-MCPD (1, 10, 100, and 1000 µM), and incubated for another 24 h. After the exposure period, the medium was removed for MTT assay and 100 µL fresh serum-free medium containing 0.5 mg/mL MTT (Serva, Germany) was added to each well (8). For NRU assay, fresh serum-free medium containing 50 µg/mL NR (Sigma-Aldrich, St. Louis, MO, USA) was added to each well (9). Cells were incubated at 37 °C for 3 h more. MTT and NR dye-containing medium was gently removed. In MTT plates, the formazan crystals were dissolved in 150 µL DMSO, and the plates were shaken for 10 min at room temperature. In NRU plates, wells were washed twice with phosphate buffered saline (PBS) and 150 µL glacial acetic acid/water/ethanol (1:49:50, v/v/v) were added to each well. The absorbances were

measured at 490 nm and 540 nm by using a microplate reader (Thermoscientific, Waltham, MA, USA) for MTT and NRU, respectively.

Apoptosis Assay

For the quantitative evaluation of apoptosis, the cells were seeded on 6 well plates in quantities of 2x10⁵/well for 24 h. The cells were then treated by 10 and 1000 µM 2-MCPD for 24 h. After that, TM3 cells were trypsinized and washed with cold PBS. The apoptosis was detected using an Annexin V-FITC/PI kit (cat. no. 556570; BD Pharmingen, San Jose, CA, USA) according to the manufacturers' protocol. Briefly, cells were suspended in 500 µL binding buffer and 5 µL Annexin V-FITC, and 5 µL PI were added to this suspension. Then, cells were incubated in dark at room temperature for 15 min. From each sample, 1.5x10⁴ cells were analysed within 1 h, using a flow cytometry (Accuri C6, BD Biosciences, San Jose, CA, USA).

Genotoxicity Assay

The alkaline comet assay was based on the standard method with minor modifications (10). TM3 Leydig and TM4 Sertoli cells were seeded in 6 well plates in quantities of 2x10⁵/well for 24 h. Cells were then treated by 10 and 1000 µM 2-MCPD for 24 h. After treatment, TM3 cells were detached with Trypsin-EDTA, and were collected in separate 15 mL centrifuge tubes. The positive control group cells were treated by H₂O₂ (Tekkim Kimya San., Turkey) at 100 µM for 1 h at 37 °C. The unexposed cells were accepted as negative control group. For all groups, the cells suspended in PBS were expected to be 5x10⁴ cell/100 µL. The cells were gently mixed with 100 µL of low melting agar (LMA) (0.5%) (HiMedia, Marc, Mumbai, India) and immediately pipetted onto the NMA (1%) (HiMedia, Marc, Mumbai, India) pre-coated slides. Thereafter, the slides were covered by coverslip and put on an ice-pack for a couple of times for solidification. After

removal of the coverslip, the slides were placed on a cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, and 1% sodium sarcosinate at pH=10) containing freshly added 1% Triton-X 100 (Sigma-Aldrich, St. Louis, MO, USA), and 10% DMSO for at least 1 h at 4 °C. The lysis solution was removed and the slides were washed with the electrophoresis buffer. Then, the slides were placed on a horizontal electrophoresis tank filled with freshly prepared electrophoresis solution (1 mM Na₂EDTA and 300 mM NaOH, pH=13). The slides were proceeded to alkaline denaturation and electrophoresis (20 V, 350 mA) during 20 min. Afterwards, the slides were washed with PBS and neutralized with Tris buffer (0.4 M Tris, pH=7.5) for 15 min at room temperature. The slides were fixed with 50%, 75% and 90% ethanol, respectively, before stained with ethidium bromide (EtBr) (Sigma-Aldrich, St. Louis, MO, USA). On the day of analysis, 100 µL of EtBr (2 µg/mL in water) was added on each slide and covered with a coverslip. The slides were examined on a fluorescence microscope (Eclipse E800, Nikon, Japan) and images were analysed with a software (TriTek Corp, Sumerduck, VA, USA). Hundred cells on each slide randomly selected and analysed. DNA tail intensity (%) was used for expressing the DNA damage.

Statistical Analysis

All assays were done in triplicates and repeated in different three days (total repetition 3x3=9). Data was expressed as mean ± standard deviation (SD). The analysis of variance (ANOVA) and post-hoc Tukey tests were performed using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). A p-value less than 0.05 ($p < 0.05$) was considered to represent statistically significant results.

RESULTS

Cytotoxicity Assays

2-MCPD did not exhibit cytotoxic effect in TM3 Leydig and TM4 Sertoli cells up to 1000 µM. The viability of cells was more than 95% in all tested concentration groups both MTT and NRU assays (Figure 1).

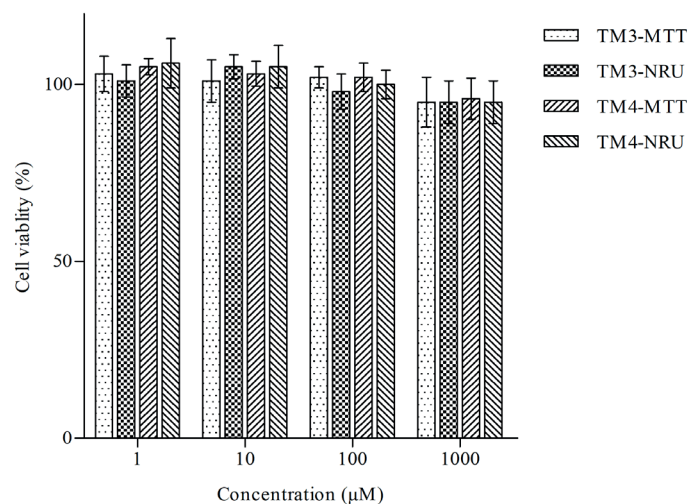


Figure 1. Results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) assays showed the cytotoxic effect of 2-MCPD in TM3 Leydig cells and TM4 Sertoli cells ($N = 3$). The cells were treated with different concentrations (1-1000 µM) of 2-MCPD for 24 h. Results are given as median of cell viability (%). The error bar represents ± standard error.

Apoptosis Assay

The percentage of TM3 Leydig and TM4 Sertoli cells undergoing early apoptosis was not significantly changed by 10 and 1000 µM of 2-MCPD exposure (Figure 2).

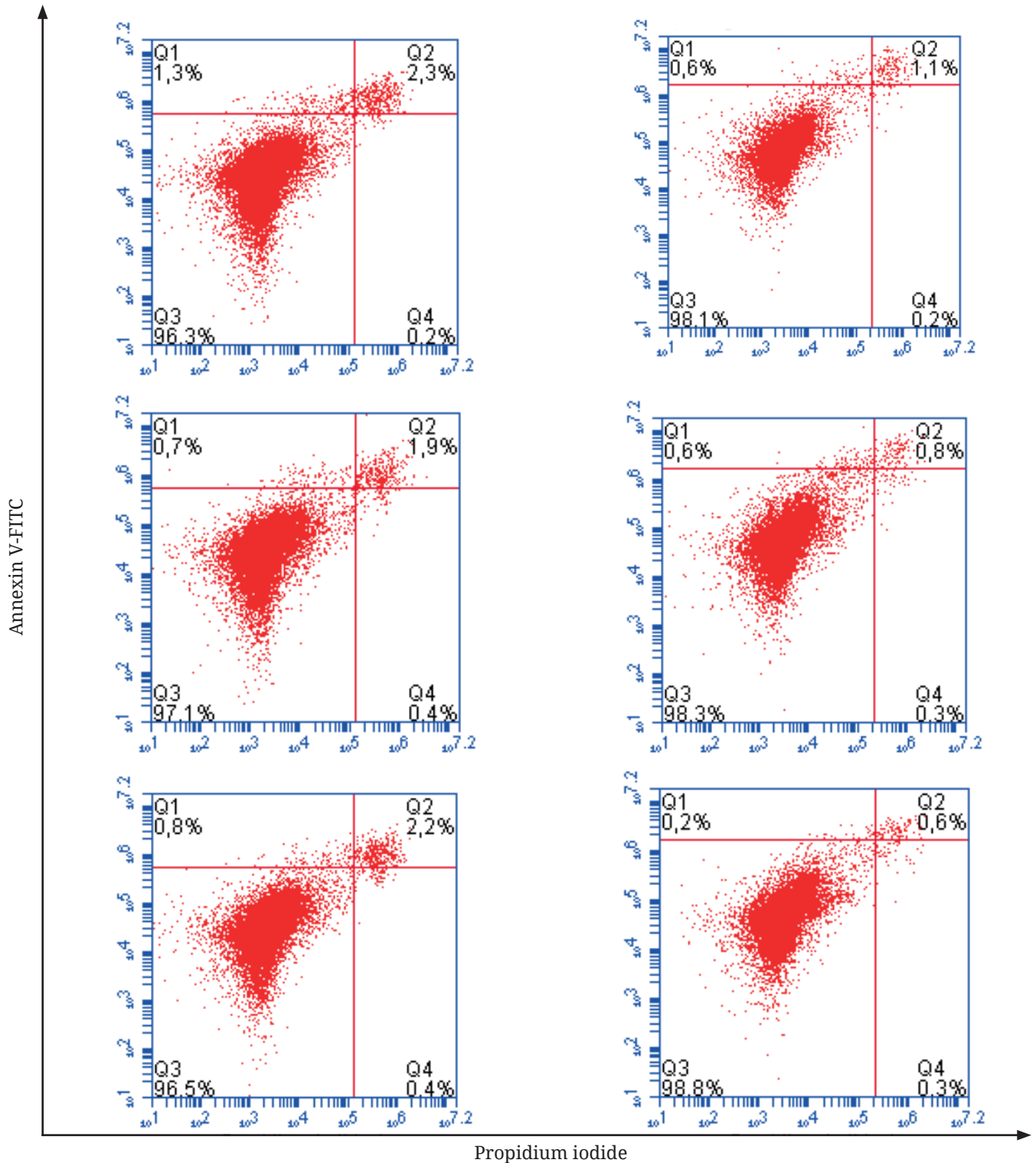


Figure 2. Flow cytometry analysis of apoptotic/necrotic TM3 Leydig and TM4 Sertoli cells (N = 3). (a) Negative control group of TM3 cells. (b) TM3 cells treated with 10 μM and (c) 1000 μM of 2-MCPD. (d) Negative control group of TM4 cells. (e) TM4 cells treated with 10 μM and (f) 1000 μM of 2-MCPD.

Genotoxicity Assay

The results of the alkaline comet assay indicated that 2-MCPD did not induce DNA damage in both TM3 Leydig and TM4 Sertoli cells at tested concentrations. In the positive control (PC) cells, 100 μM H_2O_2 treatment for 1 h significantly increased the DNA tail intensity (%) in both TM3 and TM4 cells (Figure 3).

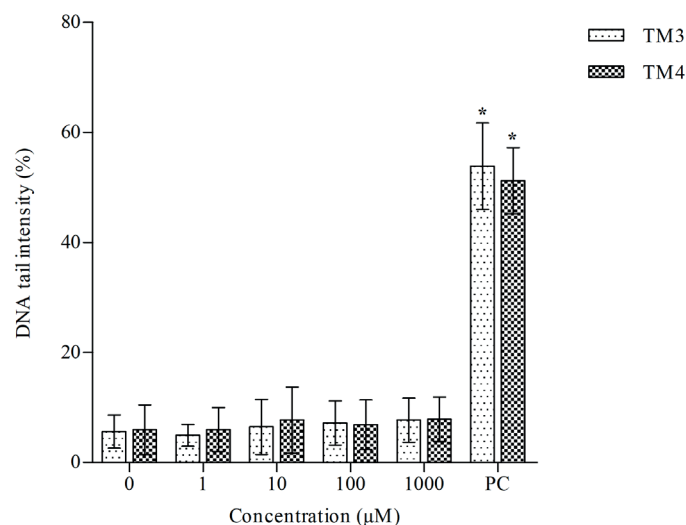


Figure 3. The level of the genotoxicity in TM3 Leydig and TM4 Sertoli cells treated by 2-MCPD (1-1000 μM) and 100 μM H_2O_2 for positive control (PC) cells. “DNA tail intensity (%) \pm SD” was used to evaluate the genotoxicity. *Statistically significant ($p < 0.05$).

DISCUSSION

Food safety has gained importance due to concerns about its effects on public health and economy. Chlorinated propanols such as 2-MCPD and 3-MCPD, and their esters are crucial compounds for the occurrence of foodborne diseases. Prior to entering systemic circulation, esterified forms of 2- and 3-MCPD are cleaved to free 2- and 3-MCPD (11,12). Daily mean dietary exposure in infants, adults and elderly range between 0.2 to 1.2 $\mu\text{g}/\text{kg}$ body weight (bw) for 2-MCPD, and 0.2 to 2.6 $\mu\text{g}/\text{kg}$ bw for 3-MCPD (1). Although 3-MCPD, an isomer of 2-MCPD, has been well studied, limited data are available for the toxicity of 2-MCPD (13).

3-MCPD has been classified under Category 2B (possibly carcinogenic to humans) according to the International Agency of Research for Cancer (IARC) (6,14). Animal studies showed that testicles were the main target organs for 3-MCPD exposure, leading to male infertility and Leydig cell cancer. Other target organs were reported as liver, kidneys and heart (4,15-17). 2-MCPD shows its toxic effects mostly on the striated muscle of the heart, liver, and kidneys (18). In adult male rats, the acute oral lethal dose (LD_{50}) of 2-MCPD and 3-MCPD were estimated as 50-60 mg/kg bw (19) and 150 mg/kg bw, respectively (20), by using the ‘Up and Down’ procedure (OECD 425). The no-observed-adverse-effect level (NOAEL) for 2-MCPD was reported as 2 mg/kg bw per day in male rats (1).

In this study, the toxic effects of 2-MCPD on TM3 Leydig and TM4 Sertoli cells were identified *in vitro*. These mammalian male reproductive system cells are known as the target cells for 3-MCPD exposure. As 3-MCPD is similar to 2-MCPD in terms of molecular structure, we hypothesized that it may also have significant effects on reproductive system. Therefore, TM3 Leydig and TM4 Sertoli cells were exposed to varying concentrations of 2-MCPD (1, 10, 100, and 1000 μM) for 24 h to investigate the effects on cell viability. The cytotoxicity data by MTT and NRU (Figure 1) assays revealed that 2-MCPD was not cytotoxic up to a level of 1000 μM .

Apoptotic/necrotic effect of the highest and lowest sub-toxic two concentrations of 2-MCPD were tested on TM3 Leydig and TM4 Sertoli cells. Data in Figure 2 confirms MTT results, as there was no significant difference between treatment and negative controls of each cell line. 2-MCPD did not induce apoptosis in TM3 Leydig and TM4 Sertoli cells. Similarly, Buhrke et al. (11) found that free forms of 2-MCPD and 3-MCPD did not induce cytotoxicity and caspase 3/7 activity in Caco-2 cells up to 1mM.

Data on the genotoxic effects of 2-MCPD are limited, and most of the available data consist of unpublished study reports. 2-MCPD was found to be mutagenic by Ames test without metabolic activation (unpublished report) (21). In V79 Chinese hamster lung cells, 2-MCPD was not induced at the hprt locus gene mutations up to extremely high concentrations (50 mM) with or without metabolic activation (unpublished report) (2). In *Drosophila melanogaster*, 2-MCPD showed genotoxic effect using the ‘wing spot test’ (22). Furthermore, no studies have been published on *in vitro* genotoxicity of 2-MCPD in mammalian cells. In this study, we demonstrated the genotoxic effects of 2-MCPD on TM3 Leydig and TM4 Sertoli cells *in vitro*. No significant difference was found in the DNA damage of TM3 Leydig and TM4 Sertoli cells treated by 2-MCPD compared to the control group ($p < 0.05$) (Figure 3). However, in the positive control group (100 μ M H₂O₂ treatment for 1 h), DNA tail intensity (%) was approximately ~5-fold of the 1 mM 2-MCPD exposed cells.

The inhibition of glycolysis has an important role in the development of 3-MCPD toxicity but, it is still unknown if 2-MCPD, the isomeric compound of 3-MCPD, has the same toxic mechanism of action (18). If their metabolism occurs through different oxidative steps, the metabolites of 2- and 3-MCPD should also be different which are responsible for the toxicity (23). As a consequence, further studies are needed to explain this difference.

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Conflict of interest: The authors declare that they have no conflict of interest.

KAYNAKLAR

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