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Investigation of the Neurotoxic Effects of Dimethyl Phthalate and Diisobutyl Phthalate on Sh-Sy5y Neuroblastoma Cells

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ABSTRACT

Objective: Endocrine disruptors, particularly phthalates like Dimethyl phthalate and Diisobutyl phthalate, are prevalent environmental contaminants posing significant health risks.
Methods: This study investigates the combined neurotoxic effects of DMP and DiBP on SH-SY5Y neuroblastoma cells by analyzing cytotoxicity, oxidative stress, and apoptosis. Using MTT and Neutral Red Uptake assays, we determined the IC50 values for DMP and DiBP as 11.35 mM and 1.307 mM, respectively. Flow cytometry revealed increased Reactive Oxygen Species levels, indicating oxidative stress, while apoptosis assays showed enhanced cell death with combined phthalate exposure.
Results: The results demonstrate a synergistic effect, exacerbating cytotoxic and oxidative damage beyond individual exposures.
Conclusion: This study highlights the compounded risk of phthalate mixtures, urging comprehensive risk assessments and regulatory policies to mitigate human health risks from combined chemical exposures.

Keywords: Apoptosis, Endocrine Disruptors, Neurotoxicity, Oxidative Stress, Phthalates

Introduction

Endocrine disruptors are chemical substances that can interfere with the body's normal hormonal balance by mimicking or blocking hormones, and they are mostly man-made. Among these chemicals, phthalates are used as plasticizers to increase the flexibility and softness of plastics (Y. Wang & Qian, 2021). Belonging to the phthalic acid esters group, these substances can cause serious harm to human health with prolonged exposure. Phthalates, with their wide range of applications, pose a significant threat to both human and environmental health. Their presence in various everyday products makes them a major risk factor. Research has shown that exposure to phthalates can lead to numerous health issues such as endocrine system disorders, changes in systolic blood pressure, neuronal degeneration, growth and development disorders, and premature births. Additionally, they have been reported to cause significant changes in parameters related to neurological development in children (Hlisníková et al., 2021; Meeker, 2012).

Dimethyl phthalate (DMP) is the simplest and lowest molecular weight member of the phthalic acid esters group and is frequently detected in various environmental samples. DMP and its metabolites exert toxic effects by disrupting endogenous hormones and their receptors (Cong et al., 2020). High doses of DMP have been reported to have carcinogenic, teratogenic, and mutagenic effects. Diisobutyl phthalate (DiBP) is another commonly detected phthalate in the environment, known for its severe toxic effects, particularly on the male reproductive system. Both types of phthalates can induce cellular stress mechanisms and lead to cell death(G. Wang et al., 2024a).

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The neurotoxic effects and mechanisms of phthalates remain unclear. In conducted with Zebrafish embryos study, they were exposed to six phthalates [dimethyl phthalate (DMP), diethyl phthalate (DEP), butyl benzyl phthalate (BBzP), di(2-ethylhexyl) phthalate (DEHP), di-n-octyl phthalate (DnOP), and diisononyl phthalate (DiNP)] and their locomotor activities were examined. Exposure to BBzP, DEHP, and DiNP affected larval behaviors and some gene expressions, while DMP, DEP, and DnOP did not cause any changes. These findings suggest that phthalates can disrupt neurological development in zebrafish embryos, but the mechanisms vary depending on the type of phthalate(Chen et al., 2014; Tran et al., 2021).

While the toxic effects and mechanisms of DMP and DiBP on various organs and systems are documented in the literature, data on their combined neurotoxic potential is limited (Nahla et al., 2024). No studies have investigated cancer in animals exposed to DMP and DiBP. The few mutagenicity tests found in the literature generally yielded negative results. However, genotoxicity tests on primary human mucosal cells treated with DIBP showed DNA damage (N. Kleinsasser et al., t.y.; N. H. Kleinsasser et al., 2000, 2001). This data is insufficient to evaluate the carcinogenic potential of DIBP, thus the evidence regarding cancer risk remains inconclusive (Yost et al., 2019).

In our study, we have evaluated the cytotoxicity, combined exposure cytotoxicity, oxidative stress and apoptosis parameters to understand the effects of Dimethyl phthalate (DMP) and Diisobutyl phthalate (DiBP) on SH-SY5Y neuroblastoma cells. By analyzing these parameters, our study aims to provide a comprehensive understanding of the toxicological effects of DMP and DiBP on neuronal cells. The insights gained from this research could contribute to the broader knowledge of how phthalates impact human health, particularly in relation to their neurotoxic potential.

Methods

Cell Culture

Cell culture applications were carried out at the Cell Culture Laboratory of the Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Istanbul University. SH-SY5Y (CRL2266) neuroblastoma cells were obtained from the American Type Culture Collection (ATCC) and are available in our laboratory. The cells were cultured at 37°C with 5% CO2 in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). When the cells reached a confluent state, they were passaged every 3-4 days. All analyses in this study were performed in triplicate and on three separate days.

Cytotoxicity Analyses

In this study, cytotoxicity was evaluated using the "MTT assay" and the "Neutral Red Uptake (NRU) assay". Cells were seeded in 96-well microplates at a density of 1x104 cells/well and incubated overnight to allow attachment. Separate 24-hour exposures to DMP and diisobutyl phthalate DiBP were conducted. Changes in absorbance were measured using an Epoch microplate reader spectrophotometer (BioTek, USA). The concentration that inhibited 50% of the cells (IC50) was calculated from the MTT assay results.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Assay (Determination of IC50 Doses)

To determine the IC50 doses for DMP and DiBP in our experimental groups, SH-SY5Y (CRL2266) neuroblastoma cells were seeded into 96-well culture plates using automated multi-pipettes at a density of 3000-5000 cells/well. After approximately 16 hours, serial dilutions were made in the range of 10-1000 µM for DMP and DiBP, and incubated in the plates at nine different concentrations for 24 hours. While analyzing cell viability in the MTT assay, the outer wells of the culture plates were excluded to minimize experimental error. Each agent and vehicle control group was arranged in six wells. After incubation, the MTT assay was applied to analyze the surviving cells. Based on the results of the MTT analysis, the effects of different concentrations of DMP and DiBP on cells in the control and experimental groups were calculated using SPSS 20 statistical software and probit analysis according to the following formula (Sevim et al., 2024).

$$\text{Viable cells}\% = \frac{\text{Optical density of treated samples}}{\text{Optical density of control samples}} \times 100$$

Figure 1. Formula of cell viability rate

Neutral Red Uptake (NRU) assay

The neutral red dye accumulates in the lysosomes of viable cells, and the neutral red uptake (NRU) assay measures the dye retention capacity of these cells. The intensity of the red color can be quantified using a

spectrophotometer (Rodrigues et al., 2023). The NRU assay was conducted following the method described by Mahmoud et al (Mahmoud et al., 2016). The cells were exposed to decreasing concentrations of zoledronic acid, starting from 1 mM.

The half-maximal inhibitory concentrations (IC50), representing the concentration required to inhibit 50% of enzyme activity in the MTT assay and lysosomal capacity in the NRU assay, were determined from concentration-inhibition curves. Additionally, IC20 values were calculated based on the MTT assay, and lower concentrations were selected for subsequent experiments.

Determination of Reactive Oxygen Species by Flow Cytometry (Oxidative Stress Analysis)

After determining the exposure concentrations of phthalates, both individually and in mixtures, on SH-SY5Y cells based on MTT assay results, the total ROS (Reactive Oxygen Species) analysis was measured using flow cytometry to identify the potential for intracellular oxidative stress development. 2',7'-dichlorofluorescein diacetate (H2DCF-DA) was used to evaluate ROS formation. The measurement results were calculated as %MFI (mean fluorescence intensity) (Kara et al., 2022).

Apoptosis Assay (Annexin V-FITC/PI)

An Annexin V Apoptosis Detection Kit with Propidium Iodide was utilized to assess the pattern of apoptosis and necrosis in cells using flow cytometry. Annexin V was used to detect phosphatidylserine translocation to the cell surface, a hallmark of early apoptosis, while Propidium Iodide (PI) staining was employed to identify necrotic cells. This dual staining approach allowed the differentiation of four cell populations: viable cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), late apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V-/PI+). For the experiment, cells were plated in 6-well plates at a density of 5×10^5 cells per well in 2 mL of medium and incubated overnight for attachment. The cells were exposed to benomyl at non-cytotoxic concentrations (1, 2, 4, and 6 µM) for 24 hours, with 1% DMSO serving as a negative control. After treatment, cells were harvested using trypsin-EDTA, washed twice with staining buffer, and resuspended in binding buffer at a concentration of 3×10^5 cells per 100 µL. Subsequently, 5 µL of Annexin V-FITC and 5 µL of PI were added to the suspension. The cells were incubated in the dark at room temperature for 15 minutes. Fluorescence intensities were analyzed using an ACEA NovoCyte flow cytometer (San Diego, CA, USA), with data acquired from 10,000 events. Results were expressed as percentages of the total cell population (Kara et al., 2020).

Results

MTT Cytotoxicity Assay

The findings from the MTT cytotoxicity assay revealed the cytotoxic potential of dimethyl phthalate and diisobutyl phthalate on SH-SY5Y cells. The IC50 value for dimethyl phthalate was determined to be 11.35 mM, while diisobutyl phthalate exhibited a significantly lower IC50 value of 1.307 mM, indicating its higher cytotoxic potency. These results provide a quantitative measure of the concentration-dependent toxicity of the two compounds, with diisobutyl phthalate being more toxic at lower concentrations.

NRU Cytotoxicity Assay

The NRU cytotoxicity values, expressed as percentages of cell viability, are provided in the figures below. The Figure 2 illustrates the effect of varying concentrations of dimethyl phthalate on the viability of SH-SY5Y cells, as determined by the NRU assay. For dimethyl phthalate, concentrations ranging from 0.3125 mM to 10 mM were tested. The results demonstrated minimal toxicity at concentrations up to 1.25 mM, where cell viability remained above 90%. However, as the concentration exceeded 2.5 mM, a marked decrease in viability was observed, with approximately 50% cell viability recorded at the highest concentration of 10 mM.

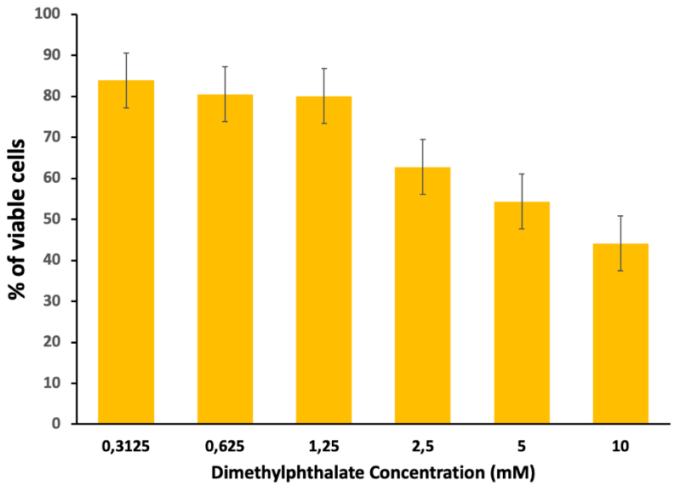

Figure 2. Cytotoxic Effect of Dimethyl Phthalate on SH-SY5Y Cells

The Figure 3 illustrates the effect of varying concentrations of diisobutyl phthalate on the viability of SH-SY5Y cells and diisobutyl phthalate exhibited minimal toxicity at its lowest tested concentration of 0.3125 mM,

maintaining cell viability above 95%. As the concentration increased, cell viability progressively declined, reaching about 50% at 10 mM. These findings highlight the dose-dependent cytotoxicity of both compounds, with diisobutyl phthalate showing a slightly decline in viability compared to dimethyl phthalate.

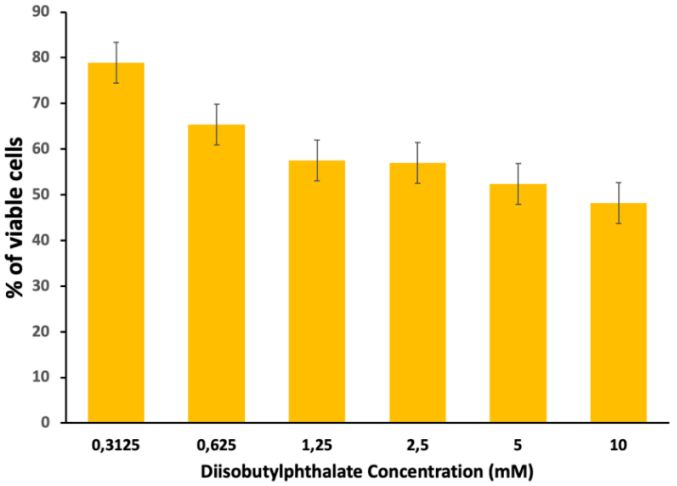

Figure 3. Cytotoxicity of Diisobutyl Phthalate on SH-SY5Y Cells

The cytotoxicity data obtained from the MTT assay were analyzed using CompuSyn software, a widely used tool for quantifying drug interactions. CompuSyn computes the Combination Index (CI) for various combinations of compounds. A CI value less than 1 indicates a synergistic interaction, meaning the combined effect is greater than the sum of the individual effects. A CI value equal to 1 reflects an additive effect, while a CI value greater than 1 suggests antagonism, where the combined effect is weaker than expected.The analysis demonstrated a synergistic effect on cytotoxicity when SH-SY5Y cells were exposed to dimethyl phthalate and diisobutyl phthalate together, particularly at concentrations below their respective IC50 values. The IC50 for dimethyl phthalate was determined to be 11.35 mM, and for diisobutyl phthalate, it was 1.307 mM. At these sub-IC50 concentrations, the combined exposure resulted in a significantly greater reduction in cell viability than what would be predicted by simply adding the effects of the two compounds when administered individually. For instance, combinations where dimethyl phthalate was present at 5 mM and diisobutyl phthalate at 0.625 mM resulted in nearly a 60% reduction in cell viability, far exceeding the expected additive effect. Such results highlight the potential for these compounds to interact in ways that amplify their toxic impact. This synergistic interaction suggests a possible underlying mechanism where the two compounds either enhance each other's ability to disrupt cellular processes or affect overlapping pathways that amplify cytotoxicity. For example, one compound might increase the permeability of the cell membrane, facilitating greater uptake of the other compound, or they might jointly contribute to oxidative stress and mitochondrial dysfunction, both of which are hallmarks of cytotoxicity. (Table 1).

Table 1. Cytotoxicity of Combined Exposure to Dimethyl Phthalate and Diisobutyl Phthalate on SH-SY5Y Cells

Dimethyl phthalate Concentration (mM)	Dimethyl phthalate Concentration (mM)	% of Viable Cells
25.0	1.56	92.93
12.5	0.78	82.67
6.25	0.39	55.47
3.125	0.195	49.33
1.5625	0.0975	41.28
0.78125	0.04875	39.4

Flow Cytometry Analysis of Reactive Oxygen Species (Oxidative Stress Analysis)

Exposure to dimethyl phthalate and diisobutyl phthalate at concentrations determined from the IC50 values resulted in a significant increase in reactive oxygen species (ROS) levels in SH-SY5Y cells. ROS measurements were conducted using the fluorescent probe 2',7'-dichlorofluorescein diacetate (H2DCF-DA), which fluoresces upon oxidation, allowing for quantification of ROS levels via flow cytometry. When SH-SY5Y cells were exposed to dimethyl phthalate alone, a significant elevation in ROS levels was observed compared to the control group ($p<0.05$). Similarly, diisobutyl phthalate exposure also led to a statistically significant increase in ROS levels ($p<0.05$). These results indicate that both compounds individually induce oxidative stress in SH-SY5Y cells.

Combined exposure to dimethyl phthalate and diisobutyl phthalate resulted in a more pronounced increase in ROS levels compared to individual exposures. This synergistic effect suggests that the two compounds interact to exacerbate oxidative stress beyond the effects observed for each compound alone. ROS levels under combined exposure were significantly higher than the sum of their individual effects, indicating a potential interaction that enhances oxidative stress. These findings provide quantitative evidence of increased ROS levels under combined exposure, as measured by the fluorescence intensity of the H2DCF-DA probe.

Table 2. Mean Fluorescence Intensity (MFI) of Reactive Oxygen Species (ROS) in SH-SY5Y Cells Following Exposure to Phthalates

Phthalate Concentration (mM)	Mean Fluorescence Intensity (%MFI) ± SD
Control	720.44 **± 4.07**
Dimethyl phthalate 1.5625 mM	935.38 **± 3.44**
Dimethyl phthalate 0.39 mM	830.36 **± 3.07**
Dimethyl phthalate 1.5625 mM + Diisobutyl phthalate 0.39 mM	776.40 **± 0.76**
Dimethyl phthalate 0.78125 mM + Diisobutyl phthalate 0.195 mM	807.48 **± 4.80**

Table 3. Increase in Apoptosis Percentage in SH-SY5Y Cells Following Exposure to Phthalates

Phthalate Concentration (mM)	Apoptosis Increase (%)
Control	2.54
Dimethyl phthalate 1.5625 mM	4.6
Dimethyl phthalate 0.39 mM	4.34
Dimethyl phthalate 1.5625 mM + Diisobutyl phthalate 0.39 mM	5.53
Dimethyl phthalate 0.78125 mM + Diisobutyl phthalate 0.195 mM	4.46

Apoptosis Assay (Annexin V-FITC/PI) Analyses

The data indicate an increase in cellular apoptosis in SH-SY5Y cells following exposure to dimethyl phthalate and diisobutyl phthalate, as shown in Table 3. Apoptotic cells were quantified using flow cytometry with Annexin V-FITC and PI staining, which differentiates between apoptotic and necrotic cell populations. Exposure to dimethyl phthalate alone resulted in a significant elevation in the percentage of apoptotic cells compared to the control group ($p<0.05$). Similarly, diisobutyl phthalate exposure also significantly increased apoptosis levels relative to the control ($p<0.05$), indicating that each compound independently induces apoptosis in SH-SY5Y cells.

When SH-SY5Y cells were exposed to a combination of dimethyl phthalate and diisobutyl phthalate, a more pronounced increase in apoptosis was observed compared to individual exposures. This combined exposure led to a significantly higher percentage of apoptotic cells, suggesting a synergistic effect between the two compounds. These results demonstrate that the interaction of dimethyl phthalate and diisobutyl phthalate enhances apoptotic responses, as quantified through flow cytometry analysis.

Discussion

This study aimed to elucidate the neurotoxic effects of DMP and DiBP on SH-SY5Y neuroblastoma cells by evaluating cytotoxicity, oxidative stress, and apoptosis. Our findings reveal significant insights into the potential health risks associated with these common environmental contaminants. The MTT and NRU assays were employed to assess the cytotoxicity of DMP and DiBP. The IC50 values for DMP (11.35 mM) and DiBP (1.307 mM) indicate that DiBP is considerably more toxic to SH-SY5Y cells at lower concentrations compared to DMP. This higher cytotoxicity of DiBP is consistent with previous reports highlighting its potent toxic effects on various cell types, particularly neuronal cells. The combined exposure to DMP and DiBP showed a synergistic effect, resulting in greater cytotoxicity than expected from the sum of their individual effects. This finding underscores the importance of evaluating the combined effects of multiple phthalates, as their interactions can exacerbate toxicity(Sellinger et al., t.y.).

The flow cytometry analysis using the H2DCF-DA probe demonstrated a significant increase in ROS levels in cells exposed to DMP and DiBP, both individually and in combination. The observed rise in ROS levels indicates that these phthalates induce oxidative stress, which can lead to cellular damage. Notably, the combined exposure resulted in a more pronounced increase in ROS compared to individual exposures, suggesting a synergistic interaction that enhances oxidative stress. This exacerbation of oxidative stress by combined phthalate exposure highlights the potential for increased cellular damage and underscores the need for further investigation into the mechanisms underlying this interaction (Chi et al., 2022; G. Wang et al., 2024b; Zhang et al., 2022a).

The apoptosis assay results showed a significant increase in the percentage of apoptotic cells following exposure to DMP and DiBP, with the combined exposure

leading to an even higher rate of apoptosis. This synergistic effect on apoptosis suggests that the interaction between DMP and DiBP enhances their ability to trigger cell death pathways. The increased apoptosis observed in combined exposures aligns with the elevated oxidative stress levels, indicating that oxidative stress may play a critical role in mediating the apoptotic response to phthalate exposure (Zhang et al., 2022b).

The findings of this study provide critical insights into the neurotoxic potential of dimethyl phthalate (DMP) and diisobutyl phthalate (DiBP), two widely used phthalates with significant environmental and consumer product prevalence. The results reveal the alarming health implications of exposure to multiple phthalates, particularly through the observed synergistic effects on cytotoxicity, oxidative stress, and apoptosis. This interaction suggests that combined phthalate exposures may pose more severe health risks compared to exposures to individual compounds, calling attention to the potential underestimation of health hazards in current safety evaluations.

These results underscore the urgency for regulatory frameworks that incorporate the cumulative effects of phthalate exposure. Policies should aim to mitigate human health risks by addressing not only the individual toxicities of these chemicals but also their interactive and amplified effects when present in mixtures.

Future investigations should prioritize uncovering the precise molecular mechanisms driving the observed synergistic effects. Longitudinal studies are essential to understand the chronic impacts of phthalate mixtures on neuronal function, development, and overall health. Furthermore, research focusing on vulnerable populations, such as children and pregnant women, is vital to develop targeted intervention strategies and inform public health recommendations.

This study highlights the profound cytotoxic, oxidative stress-inducing, and apoptotic effects exerted by DMP and DiBP on SH-SY5Y neuroblastoma cells. The demonstrated synergistic toxicity of combined exposures provides robust evidence of the compounded risk associated with multiple phthalate contaminants. These findings contribute to the broader understanding of phthalate-induced neurotoxicity and emphasize the importance of comprehensive risk assessments and preventative measures to protect public health in the face of widespread phthalate exposure.

Ethics Committee Approval: Since the study is an in vitro cell culture study, Ethics Committee Approval is not required.
Peer-review: Externally peer-reviewed.
Author Contributions: Concept – Kara M; Supervision – Kara M, Sevim Ç; Data Collection and/or Processing – Göker Z, Erdinç A, Gülgen E; Analysis and/or Interpretation – Göker Z, Erdinç A, Gülgen E, Kara M; Literature Review – Sevim Ç; Writing – Sevim Ç; Critical Review – Kara M, Sevim Ç; Statistics – Sevim Ç
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References

Chen, X., Xu, S., Tan, T., Lee, S. T., Cheng, S. H., Lee, F. W. F., Xu, S. J. L., & Ho, K. C. (2014). Toxicity and estrogenic endocrine disrupting activity of phthalates and their mixtures. *International Journal of Environmental Research and Public Health*, 11(3), 3156–3168. https://doi.org/10.3390/IJERPH110303156

Cong, B., Liu, C., Wang, L., & Chai, Y. (2020). The impact on antioxidant enzyme activity and related gene expression following adult zebrafish (Danio rerio) exposure to dimethyl phthalate. *Animals*, 10(4), 717. https://doi.org/10.3390/ANI10040717

Hlisníková, H., Petrovičová, I., Kolena, B., Šidlovská, M., & Sirotkin, A. (2021). Effects and mechanisms of phthalates' action on neurological processes and neural health: A literature review. *Pharmacological Reports*, 73(2), 386–404. https://doi.org/10.1007/S43440-021-00215-5

Kara, M., Boran, T., Öztaş, E., Jannuzzi, A. T., Özden, S., & Özhan, G. (2022). Zoledronic acid-induced oxidative damage and endoplasmic reticulum stress-mediated apoptosis in human embryonic kidney (HEK-293) cells. *Journal of Biochemical and Molecular Toxicology*, 36(8), e23083. https://doi.org/10.1002/JBT.23083

Kleinsasser, N. H., Wallner, B. C., Kastenbauer, E. R., Weissacher, H., & Harréus, U. A. (2001). Genotoxicity of di-butyl-phthalate and di-iso-butyl-phthalate in human lymphocytes and mucosal cells. *Teratogenesis, Carcinogenesis, and Mutagenesis*, 21(3), 189–196. https://doi.org/10.1002/tcm.1007

Kleinsasser, N. H., Weissacher, H., Kastenbauer, E. R., Dirschedl, P., Wallner, B. C., & Harréus, U. A. (2000). Altered genotoxicity in mucosal cells of head and neck cancer patients due to environmental pollutants. *European Archives of Oto-Rhino-Laryngology*, 257(6), 337–342. https://doi.org/10.1007/S004059900220

Mahmoud, A., Ezgi, Ö., Merve, A., & Özhan, G. (2016). In vitro toxicological assessment of magnesium oxide

nanoparticle exposure in several mammalian cell types. *International Journal of Toxicology*, 35(4), 429–437. https://doi.org/10.1177/1091581816648624

Meeker, J. D. (2012). Exposure to environmental endocrine disruptors and child development. *Archives of Pediatrics & Adolescent Medicine*, 166(10), 952–958. https://doi.org/10.1001/ARCHPEDIATRICS.2012.241

Nahla, E., Arya, P., Maneesha, P., & Chitra, K. C. (2024). Exposure to the plasticizer dibutyl phthalate causes oxidative stress and neurotoxicity in brain tissue. *Environmental Science and Pollution Research*, 31(14), 21399–21414. https://doi.org/10.1007/S11356-024-32604-7

Rodrigues, R. M., Stinckens, M., Ates, G., & Vanhaecke, T. (2023). Neutral red uptake assay to assess cytotoxicity in vitro. *Methods in Molecular Biology*, 2644, 237–245. https://doi.org/10.1007/978-1-0716-3052-5_15

Sellinger, E., Riesgo, V., Brinks, A. S., & Willing, J. (2021). Perinatal phthalate exposure increases developmental apoptosis in the rat medial prefrontal cortex. *Neurotoxicology*, 87, 167–173. https://doi.org/10.1016/j.neuro.2021.09.007

Sevim, C., Taghizadehghalehjoughi, A., Kara, M., Nosyrev, A. E., Nițulescu, G. M., Margină, D., & Tsatsakis, A. (2024). Investigation of the effects of metformin on the miR-21/PTEN/AKT pathway in HT-29 human colorectal adenocarcinoma cell and HUVEC co-culture. *Farmacia*, 72(1). https://doi.org/10.31925/farmacia.2024.1.5

Tran, C. M., Do, T. N., & Kim, K. T. (2021). Comparative analysis of neurotoxicity of six phthalates in zebrafish embryos. *Toxics*, 9(1), 1–11. https://doi.org/10.3390/TOXICS9010005

Wang, G., Shen, J., Lin, Y., Zhai, L., Guan, Q., & Shen, H. (2024). Dimethyl phthalate exposure induces cognitive impairment through COX2-mediated microglial activation. *Research Square*. https://doi.org/10.21203/RS.3.RS-4081530/V1

Wang, Y., & Qian, H. (2021). Phthalates and their impacts on human health. *Healthcare*, 9(5), 603. https://doi.org/10.3390/HEALTHCARE9050603

Yost, E. E., Euling, S. Y., Weaver, J. A., Beverly, B. E. J., Keshava, N., Mudipalli, A., Arzuaga, X., Blessinger, T., Dishaw, L., Hotchkiss, A., & Makris, S. L. (2019). Hazards of diisobutyl phthalate (DIBP) exposure: A systematic review of animal toxicology studies. *Environment International*, 125, 579–594. https://doi.org/10.1016/J.ENVINT.2018.09.038

Chen, X., Xu, S., Tan, T., Lee, S. T., Cheng, S. H., Lee, F. W. F., Xu, S. J. L., & Ho, K. C. (2014). Toxicity and estrogenic endocrine disrupting activity of phthalates and their mixtures. International *Journal of Environmental Research and Public Health*, 11(3), 3156–3168. https://doi.org/10.3390/IJERPH110303156

Chi, Z., Lin, H., Wang, X., Meng, X., Zhou, J., Xiang, L., Cao, G., Wu, P., Cai, Z., & Zhao, X. (2022). Dimethyl phthalate induces blood immunotoxicity through oxidative damage and caspase-dependent apoptosis. *Science of The Total Environment*, 838, 156047. https://doi.org/10.1016/J.SCITOTENV.2022.156047

Cong, B., Liu, C., Wang, L., & Chai, Y. (2020). The impact on antioxidant enzyme activity and related gene expression following adult zebrafish (Danio rerio) exposure to dimethyl phthalate. *Animals*, 10(4), 717. https://doi.org/10.3390/ANI10040717

Hlisníková, H., Petrovičová, I., Kolena, B., Šidlovská, M., & Sirotkin, A. (2021). Effects and mechanisms of phthalates' action on neurological processes and neural health: A literature review. *Pharmacological Reports*, 73(2), 386–404. https://doi.org/10.1007/S43440-021-00215-5

Kara, M., Boran, T., Öztaş, E., Jannuzzi, A. T., Özden, S., & Özhan, G. (2022). Zoledronic acid-induced oxidative damage and endoplasmic reticulum stress-mediated apoptosis in human embryonic kidney (HEK-293) cells. *Journal of Biochemical and Molecular Toxicology*, 36(8), e23083. https://doi.org/10.1002/JBT.23083

Kara, M., Oztas, E., Ramazanoğulları, R., Kouretas, D., Nepka, C., Tsatsakis, A. M., & Veskoukis, A. S. (2020). Benomyl, a benzimidazole fungicide, induces oxidative stress and apoptosis in neural cells. *Toxicology Reports*, 7, 501–509. https://doi.org/10.1016/J.TOXREP.2020.04.001

Kleinsasser, N. H., Wallner, B. C., Kastenbauer, E. R., Weissacher, H., & Harréus, U. A. (2001). Genotoxicity of di-butyl-phthalate and di-iso-butyl-phthalate in human lymphocytes and mucosal cells. *Teratogenesis, Carcinogenesis, and Mutagenesis*, 21(3), 189–196. https://doi.org/10.1002/tcm.1007

Kleinsasser, N. H., Weissacher, H., Kastenbauer, E. R., Dirschedl, P., Wallner, B. C., & Harréus, U. A. (2000). Altered genotoxicity in mucosal cells of head and neck cancer patients due to environmental pollutants. *European Archives of Oto-Rhino-Laryngology*, 257(6), 337–342. https://doi.org/10.1007/S004059900220

Mahmoud, A., Öztaş, E., Merve, A., & Özhan, G. (2016). In vitro toxicological assessment of magnesium oxide nanoparticle exposure in several mammalian cell types. *International Journal of Toxicology*, 35(4), 429–437. https://doi.org/10.1177/1091581816648624

Meeker, J. D. (2012). Exposure to environmental endocrine disruptors and child development. *Archives of Pediatrics & Adolescent Medicine*, 166(10), 952–958. https://doi.org/10.1001/ARCHPEDIATRICS.2012.241

Nahla, E., Arya, P., Maneesha, P., & Chitra, K. C. (2024). Exposure to the plasticizer dibutyl phthalate causes

oxidative stress and neurotoxicity in brain tissue. *Environmental Science and Pollution Research*, 31(14), 21399–21414. https://doi.org/10.1007/S11356-024-32604-7

Rodrigues, R. M., Stinckens, M., Ates, G., & Vanhaecke, T. (2023). Neutral red uptake assay to assess cytotoxicity in vitro. *Methods in Molecular Biology* (Clifton, N.J.), 2644, 237–245. https://doi.org/10.1007/978-1-0716-3052-5_15

Sellinger, E., Riesgo, V., & Brinks, A. (2021). Perinatal phthalate exposure increases developmental apoptosis in the rat medial prefrontal cortex. *Neurotoxicology*. https://doi.org/10.1016/j.neuro.2021.03.010

Sevim, C., Taghizadehghalehjoughi, A., Kara, M., Nosyrev, A. E., Nițulescu, G. M., Margină, D., & Tsatsakis, A. (2024). Investigation of the effects of metformin on the miR-21/PTEN/Akt pathway in HT-29 human colorectal adenocarcinoma cell line. *Farmacia*, 72(1). https://doi.org/10.31925/farmacia.2024.1.5

Tran, C. M., Do, T. N., & Kim, K. T. (2021). Comparative analysis of neurotoxicity of six phthalates in zebrafish embryos. *Toxics*, 9(1), 1–11. https://doi.org/10.3390/TOXICS9010005

Wang, G., Shen, J., Lin, Y., Zhai, L., Guan, Q., & Shen, H. (2024a). Dimethyl phthalate exposure induces cognitive impairment through COX2-mediated microglial activation. *Research Square*. https://doi.org/10.21203/RS.3.RS-4081530/V1

Wang, G., Shen, J., Lin, Y., Zhai, L., Guan, Q., & Shen, H. (2024b). Dimethyl phthalate exposure induces cognitive impairment through COX2-mediated microglial activation. *Research Square*. https://doi.org/10.21203/RS.3.RS-4081530/V1

Wang, Y., & Qian, H. (2021). Phthalates and their impacts on human health. *Healthcare*, 9(5), 603. https://doi.org/10.3390/HEALTHCARE9050603

Yost, E. E., Euling, S. Y., Weaver, J. A., Beverly, B. E. J., Keshava, N., Mudipalli, A., Arzuaga, X., Blessinger, T., Dishaw, L., Hotchkiss, A., & Makris, S. L. (2019). Hazards of diisobutyl phthalate (DIBP) exposure: A systematic review of animal toxicology studies. *Environment International*, 125, 579–594. https://doi.org/10.1016/J.ENVINT.2018.09.038

Zhang, Y., Lyu, L., Tao, Y., Ju, H., & Chen, J. (2022). Health risks of phthalates: A review of immunotoxicity. *Environmental Pollution*, 313, 120173. https://doi.org/10.1016/J.ENVPOL.2022.120173