Van Sag Bil Derg
2022;15(1):51-59

Orjinal Araştırma Makalesi/ Original Paper

TheEffect of Di (2-Ethylhexyl) Phthalate on Lipid Peroxidation and Antioxidant Levels in Rats

Di (2-Etilhekzil) Fitalatın Ratlarda Lipid Peroksidasyonu ve Antioksidan Düzeylerine Etkisi

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Cited: Eskici Kaymak M, Altın E, Ertekin A. The effect of di (2-Ethylhexyl) phthalate on lipid peroxidation and antioxidant levels in rats. *Van Sag Bil Derg* 2028, *15,*(1) 51-59. https://doi.org/10.52976/vansaglik.1008005.

Arrived date: 11/10/2021
Accepted date: 16/03/2022
Published date: 30/04/2022

ÖZET

Amaç: Kağıt, karton, kozmetik, gıda ambalajı, çocuk oyun malzemeleri, deterjan, şampuan, sabun ve boya üretimi gibi çok çeşitli sektörlerde hammadde veya yardımcı kimyasal olarak sıklıkla kullanılan fitalatlar kanserojen ve endokrin bozucu kimyasallar arasında yer almaktadır. Bu çalışmada Di (2-Etilheksil) Fitalat (DEHP) maruziyetinin, ratlardan alınan kan örneklerinde lipid peroksidasyonu ve antioksidan seviyeleri üzerine etkileri incelendi.

Materyal ve Metot:Çalışma için 250-300 gr ağırlığında 40 adet dişi Wistar-Albino rat kullanıldı. Ratlar 5 gruba ayrıldı; grup 1: kontrol grubu, grup 2: pozitif kontrol (mısır yağı bazlı diyet), grup 3: 20 mg DEHP/kg canlı ağırlık (c.a.) (test edilen en düşük doz), grup 4: 100 mg DEHP kg/c.a. (orta doz), grup 5: 500 mg DEHP kg/c.a. (test edilen en yüksek doz). Çalışma süresi 14 gün olarak belirlendi. Fitalatratlara mısır yağı içerisinde gastrik gavaj yöntemiyle verildi. Çalışma sonunda alınan kan örneklerinde malondialdehit (MDA), glutatyon (GSH), seruloplazmin, C vitamini ve toplam protein düzeyleri ölçüldü**.**

Bulgular: DEHP verilen tüm gruplarda GSH seviyeleri kontrol grubuna göre azaldı ($p < 0.05$). En düşük GSH düzeyi 100 mg DEHP grubunda gözlendi. MDA, seruloplazmin, C vitamini ve total proteinde gözlenen değişiklikler istatistiksel olarak anlamlı değildi.

Sonuç: Fitalat uygulanan gruplarda GSH, MDA, seruloplazmin, C vitamini ve total protein seviyelerinde gözlenen değişiklikler, hücrelerde oksidatif stresle ilgili hasarın oluşmuş olabileceğini göstermektedir.

Anahtar Kelimeler: Antioksidan maddeler, C vitamini, Glutatyon, Malondialdehit, Seruloplazmin, Total protein.

ABSTRACT

Objective: Phthalates, which are frequently used as raw materials or auxiliary chemicals in a widevariety of industries such as paper, cardboard, cosmetics, food packaging, children's play materials, detergents, shampoo, soap and paint production, are among the carcinogenic and endocrine disrupting chemicals. This study was conducted to investigate the effects of Di(2-ethylhexyl) phthalate on lipid peroxidation and antioxidant levels in rats.

Material and Method: Forty female Wistar-Albino rats weighing 250-300 g were used for the study. The rats were divided into 5 groups; group 1: control group, group 2: positive control (cornoil-based diet), group 3: 20 mg DEHP per kg body weight (bw) (lowest dose tested), group 4: 100 mg DEHP kg/bw (mediumdosetested), group 5: 500 mg DEHP kg/bw (highest dose tested). The working period was determined as 14 days. Phthalate was given to rats by gastric gavage method in cornoil. Malondialdehyde (MDA), glutathione (GSH), ceruloplasmin, vitamin C and total protein levels were measured in blood samples taken at the end of thes tudy.

Results: GSH levels decreased in all groups given DEHP compared to the control group ($p < 0.05$). The lowest level of GSH was observed in the 100 mg DEHP group. The changes observed in MDA, ceruloplasmin, vitamin C and total protein were not statistically significant.

Conclusion: The changes observed in GSH, MDA, ceruloplasmin, vitamin C and total protein levels in thephthalate-administeredgroupsindicatethatoxidativestress-relateddamagemayhaveoccurred in the cells.

Keywords: Antioxidantsubstances, ceruloplasmin, glutathione, malondialdehyde, total protein, vitamin C.

INTRODUCTION

Di(2-ethylhexyl) phthalate is the most widely used phthalate ester as a plasticizer in flexible vinyl products. Plastics can contain up to 40% of their weight in DEHP (Durusoy Onmuş and Karababa, 2011). Phthalates are preferably used in food pac-kaging, children's play materials, cosmetics, medi-cines, solvents, adhesives, pipe materials, dyes, pesticides, detergents and other fields (Schet-

tler, 2006). Due to its widespread use in today's industry, regulations have been enacted to control the nega-tive effects on nature and human health (İmren, 2011). Depending on the dose, phthalates have short-term and long-term adverse effects on the liver, kidneys, thyroid, and testes. The acute toxicity dose is reported as 1-30 g / kg body weight (LD50) (Heudorf et al., 2007). It has been reported to cause liver damage, liver failure, peroxisome proliferation, fetal death, malformations, anti-androgenic hor-mone effects, testicular damage, poor sperm quality, and reproductive toxicity in laboratory animals (Foresta et al., 2010). Since DEHP is not covalently bound to PVC, it is released from plastics, causing chronic exposure in humans (Koch et al., 2006). Exposure can occur by oral route, inhalation and skin contact (Johns et al., 2015). Once taken, DEHP is metabolized into different compounds; one of its main metabolites is mono (2-ethylhexyl) phthalate (MEHP) (Kessler et al., 2012). In a study, DEHP was shown to cause liver cancer in male and female mice and rats (David et al., 2000). In another study with rats, the incidence of DEHP-induced Leydig cell tumors was increased and this increase was asso-ciated with early onset doses (Voss et al., 2005). There is an increasing concern about the impact of DEHP on human health due to its negative effects on the reproductive system. DEHP has been re-ported in a study to cause a decrease in pregnancy, an increase in spontaneous abortions and congeni-tal malformations (Lovekamp-Swan and Davis, 2003).

Free radicals are formed within the body as a result of natural metabolic processes involving intermo-lecular electron exchange (Öğüt and Atay, 2012). Herbicide and pesticide residues, solvents, and petrochemicals used in various fields can increase the formation of free radicals, leading to diseases such as rapid aging, heart disease, and cancer (Temur, 2006). Free radicals formed due to oxidative stress are considered to be a factor in almost all di-seases, can lead to protein modification, lipid pe-roxidation, DNA fragmentation, irreversible da-mage and cell death (Cakatay and Kayalı, 2014). Accumulated oxidizing agents can oxidize DNA, protein and lipids, thereby changing their structure, activity and physical properties. Such widespread oxidative damage can disrupt the body's redox ba-lance, severely affecting biological homeostasis and causing various diseases (Ricciotti and FitzGerald, 2011; Turini and DuBois, 2002). MDA, the final product of lipid peroxidation, is used to measure the rate of chain reactions in tissues and to deter-mine the level of ROS. The level of MDA in plasma is also an indicator of non-enzymatic oxidative degradation of lipid peroxides (Yarıktaş et al., 2003). MDA, which is involved in the pathogenesis of many diseases, is considered to be mutagenic, car-cinogenic and cytotoxic (Mendes et al., 2009; Yonny et al., 2016).

This study aimed to examine the effects of di (2-Ethylhexyl) phthalate on lipid peroxidation and antioxidant levels in rats.

MATERIAL and METHOD

This study was carried out at the Laboratory Animal Research Center of the Ondokuz Mayis University (OMU-DEHAM) and was approved by the Local Ethics Committee for Animal Experiments of the Ondokuz Mayis University, No. 68489742-604-E.2002, dated January 14, 2019.

Experimental Animals: Forty female Wistar-Albino rats, 3 to 4 months old, weighing 250 to 300 grams, were used in the study. Rats were provided and maintained by OMU DEHAM. Divided into 5 groups with 8 animals in each group. The animals were kept at 21±3°C ambient temperature, 50±5% relative humidity, 12-hour day and 12-hour night cycles. During the study, rats were given ad libitum standard rat food and water. The content of corn oil used to dissolve phthalates is as follows: energy 900 kcal/100g, fat 100g/100g, carbohydrate 0, protein 0 (refined corn oil CIGDEM, Kucukbay Oil and Detergent Industry Inc., Izmir, Turkey).

Experimental Design: The duration of the experiment was determined to be 14 days. Phthalate

(Sigma-Aldrich, Darmstadt, Germany) was dissolved in 2.5 µl/g/body weight (body weight) corn oil. Dissolved phthalate was administered by gastric tube at doses of 20 mg/kg bw (lowest), 100 mg/kg bw/day (moderate), and 500 mg/kg bw/day (highest), respectively (Göktekin, 2016).

Grouping and intervention:

Group 1 (Control group): No treatment was applied to the animals.

Group 2 (Positive control-Corn oil-based group): Corn oil (2.5 µl / g / bw) was administered to the rats by gastric gavage.

Group 3 (20 mg DEHP group): 20 mg/kg bw/day DEHP dissolved in corn oil (2.5 µl / g / bw) administered to the rats by gastric gavage.

Group 4 (100 mg phthalate group): 100 mg/kg bw /day DEHP dissolved in corn oil (2.5 µl / g / bw) administered to the group by the gastric gavage.

Group 5 (500 mg phthalate group): 500 mg/kg bw/day DEHP dissolved in corn oil (2.5 µl / g / bw) administered to the group by gastric gavage.

Collection and preparation of blood samples for analysis: The rats were anesthetized with i.p. (intraperitoneal) anesthetics [2% basilazine (Bavet, Istanbul, Turkey) (2-5 mg/kg/bw) and 10% ketasol (Richter Pharma-Interhas, Ankara, Turkey) (0.8-1.3 ml/kg/bw) at the end of the 14th day. Blood samples were taken from the hearts of the rats. The plasma was obtained by centrifuging the collected blood samples in EDTA tubes at 3000 rpm for 15 minutes at 40C (Nüve NF 800R Centrifuge, Ankara, Turkey). All analyses were carried out without freezing the plasma once it was collected.

Methods: MDA was measured using the method of (Jain et al., 1989). GSH measurement was carried out according to method (Beutler et al., 1963). The vitamin C analysis was performed according to the method of (Omaye et al., 1979). The modified Ravin method (Ravin, 1956) was used to determine ceruloplasmin, and the Biuret method (Tiftik, 1996) were performed for the determination of total protein.

Statistical analysis: The mean values of MDA, GSH, ceruloplasmin, vitamin C, and total protein in the experimental groups were analyzed using one-way analysis of variance, and Duncan multiple comparison tests were used to analyze differences between groups (John, 1971). The SPSS 21.00 software package program for Windows (IBM, Armonk, NY, USA) was used to perform statistical analyzes. Results are expressed as means and standard deviations (means ± SD).

RESULTS

The plasma concentrations of MDA, GSH, ceruloplasmin, vitamin C, and total protein in each groups are shown in Figure 1.

In the 20 mg DEHP, 100 mg DEHP, and 500 mg DEHP groups, changes in MDA, ceruloplasmin, vitamin C, and total protein levels were not statistically significant to the control group. However, GSH levels decreased in all groups to the control group, mainly in the 100 mg DEHP group ($p < 0.05$).

Table 1. Plasma concentrations of MDA, GSH, ceruloplasmin, vitamin C and total protein in the control, positive control (cornoil-treated), 20 mg DEHP, 100 mg DEHP, and 500 mg DEHP groups.

	Groups									
Parameters	**n**	**Control**	**n**	**Positive control (corn oil-treated)**	**n**	**20 mg DEHP**	**n**	**100 mg DEHP**	**n**	**500 mg DEHP**
MDA (nmol/ml)	8	14.594±7.732	8	13.574±3.751	6	44.243±19.261	8	31.936±17.713	7	17.643±5.985
GSH (mg/dl)	8	135.101±19.231^{b}	8	139.393±4.325^{b}	8	121.211±13.437^{ab}	8	92.347±3.777^{a}	7	104.501±11.845^{ab}
Ceruloplasmin (%mg)	8	17.126±4.477	8	21.744±2.361	7	21.074±2.579	8	23.937±5.220	7	21.770±3.198
Vitamin C (mg/dl)	8	27.977±11.939	8	9.730±0.456	6	15.416±2.043	8	10.790±1.449	7	16.112±3.023
Total protein (%g)	8	2.426±0.680	8	1.810±0.059	7	1.921±0.063	8	1.903±0.058	7	1.865±0.084

a,b; The difference between values displayed with different letters for the same property in the sameline is significant (P<0.05)

DISCUSSION

Phthalates are industrial chemicals found primarily in food packaging, children's toys, medical products, cosmetics, gelling agents, dispensers, adhesives, lubricants, emulsifiers, and many consumer products. They are environmental pollutants that humans can be exposed to through ingestion, inhalation, or skin routes (Zarean et al., 2016). Phthalates are endocrine disruptors that interfere with the endocrine system. They are toxic to the reproductive system and have negative effects on reproduction, development, steroidogenesis, neurology, and the immune system (Martinez-Arguelles et al., 2013; Monneret, 2017). A study have shown that some phthalates can impair steroidogenesis (Zoeller et al., 2012). Besides, exposure to DEHP may be associated with precocious puberty, increased body mass and obesity (Kim et al., 2016). In the literature, it has been shown in a group of women that there is an association between observed MEHP concentrations and breast cancer. However, the effects of DEHP and MEHP on mammary gland development and carcinogenicity are not yet fully understood (Parada et al., 2018). Animal studies have shown that phthalate exposure can negatively affect reproductive physiology and the development of estrogen-sensitive target tissues. Phthalates have been reported in the literature toaffect fetal growth and development in pregnant women directly or indirectly by passing through the placental barrier (Kay et al., 2013).

Additionally, some literature studies have shown that exposure to DEHP-containing products may be associated with pancreatic cancer, testicular cancer, respiratory cancer, breast cancer, pediatric hepatoblastoma, and multiple myeloma (López-Carrillo et al., 2010; Reynolds et al., 2004). However, it has been reported that phthalate exposure causes increased respiratory and allergic complaints in children (Buckley et al., 2018), decreased lung function and depression in older ages (Kim et al., 2018) and decreased liver function and cardiovascular metabolism disorders in men (Milošević et al., 2017).

MDA is a reactive aldehyde that covalently binds to biomolecules in the cell, used to measure oxidative stress, which contributes to the pathogenesis of many diseases, is considered mutagenic, carcinogenic, and cytotoxic (Mendes et al., 2009). In our study, MDA levels increased in the phthalate groups. These increases were mainly observed in the 20 mg phthalate group. Increased MDA levels can be due to higher levels of oxidative metabolic activity and concentration of membrane PUFAs subject to oxidation. Biological membranes rich in unsaturated fatty acids are cellular structures that are particularly sensitive to free radical attack. One

study reported that phthalates exposure could induce ROS and oxidative stress biomarkers via the activation of peroxisome proliferator-activated receptors (Zhang et al., 2017). Positive associations between phthalate exposure and increased levels of MDA and 8-OHdG have been reported in some other studies (Ferguson et al., 2011; Høyer et al., 2018).

Gill et al. (2015) reported a negative correlation between oxidative stress and GSH levels. In our study observed that GSH levels were decreased in all groups to the control group, at most in the 100 mg phthalate group. This decrease can be due to either inhibited GSH synthesis or increased the use of GSH for detoxification of toxin-induced free radicals (Singh et al., 2001). Some studies have reported that phthalates exposure decreases antioxidant levels, especially GSH and SOD levels (Sobarzo et al., 2015; Udensi and Tchounwou, 2016). Active phthalate metabolites induce oxidative stress in tissues by increasing the formation of MEHP and ROS and decreasing the levels of ascorbic acid, GSH, and free thiol. Mitochondrial damage occurs as a result of oxidative stress caused by MEHP, and cytochrome c exits through the inner mitochondrial membrane. The mitochondrial damage caused by MEHP causes the efflux of cytochrome c through the inner membrane (Kasahara et al., 2002).

Ceruloplasmin, with its ferroxidase, cuproxidase, and NO-oxidase activities, plays a vital role in preventing the formation and persistence of free radicals (Shiva et al., 2006). Ceruloplasmin exhibits antioxidant activity by removing superoxide and other reactive oxygen species and inhibiting Fenton's reaction (Samokyszyn et al., 1989). Ceruloplasmin, an extracellular antioxidant, neutralizes superoxide radicals, binds free oxygen radicals (Akkuş, 1996), prevents protein and DNA damage, protect against cellular damage and degradation by free radicals (Krsek-Staples and Webster, 1993). In our study, no statistical significance was observed in the changes observed in ceruloplasmin levels. In our literature review, no studies were found to support the ceruplasmin findings on the subject.

Ascorbic acid is known as a good antioxidant, radioprotectant, free radical scavenger, and ascorbic acid increases the antioxidant enzyme activity of cells at the cellular level (Öztürk-Ürek et al., 2001). In clinical studies, vitamin C has been reported to reduce the risk of many chronic diseases such as heart disease, cancer, eye disease, and neurodegenerative disorders (Jacob and Sotoudeh, 2002). In our study, the changes observed in vitamin C amount in all groups were not statistically significant. The observed changes in vitamin C, an antioxidant vitamin, can be due to possible free radical attacks. Changes were also observed in the corn oil group in Vitamin C, these changes may be due to possible stress during the application. No statistical significance was observed in the changes here either.

The liver is the major detoxification organ for DEHP and its metabolites. In a study, DEHP was shown to impair antioxidant balance in rats, increase oxidative stress in the liver, and cause hepatotoxicity (Erkekoglu et al., 2014). Another study showed that exposure to DEHP caused hyperplasia in the liver of rats (Liu et al., 2015). It has also been reported that DEHP can cause hepatic edema by inducing liver enzymes (Ye et al., 2017). In this study, total protein levels decreased. This decrease was mainly observed in the 500 mg phthalate group, and the decrease can be due to possible damage to hepatocytes by phthalate.

In conclusion, the observed changes in MDA, GSH, ceruloplasmin, vitamin C, and total protein levels indicate that damage to cells occurs due to oxidative stress. In order to protect against the harmful effects of many chemicals that we are exposed to in our daily lives, studies on this issue should be brought to the forefront, and the results of epidemiological studies should be investigated in detail.

Acknowledgments

This research was supported by Ondokuz Mayıs University Scientific Research Projects Committee (OMU-BAP), project number PYO.VET.1904.19.014.

*This article is summarized from Master Thesis. Original Article.

Ethical Statement

This study was approved by the Ondokuz Mayis University Animal Experiments Local Ethics Committee (68489742-604-E.2002, January 14, 2019).

Conflict of Interest

The authors declared that there is no conflict of interest.

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