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Evaluating the protective effects of melatonin on di(2-ethylhexyl) phthalate-induced testicular injury in adult mice

Nosrat Bahrami[a], Mehdi Goudarzi[b], Azam Hosseinzadeh[c], Susan Sabbagh[d], Russel J. Reiter[e], Saeed Mehrzadi[a,*]

[a] Department of Midwifery, Faculty of Nursing and Midwifery, Dezful University of Medical Sciences, Dezful, Iran
[b] Medicinal Plant Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[c] Razi Drug Research Center, Iran University of Medical Sciences, Tehran, Iran
[d] Department of Anatomy, Faculty of Medicine, Dezful University of Medical Sciences, Dezful, Iran
[e] Department of Cellular and Structural Biology, The University of Texas Health Science Center, San Antonio, TX, USA

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ABSTRACT

Objective: Di (2-ethylhexyl) phthalate (DEHP) is a common phthalate derivative, interfering with normal function of reproductive system. The present study evaluated effects of melatonin on DEHP-induced testicular injury in mice.

Design: Thirty-two adult male mice were randomly divided to four groups; group I received normal saline, group II received DEHP, group III received DEHP and melatonin, and group IV was treated with melatonin alone. Body and testes weights, total antioxidant capacity (TAC), glutathione level and superoxide dismutase, glutathione peroxidase and catalase activities were measured. Serum testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels and interleukin 1 beta (IL-1β) and tumor necrosis factor (TNF-α) concentration were evaluated by ELISA assay. Also, malondialdehyde (MDA) and nitric oxide (NO) levels, sperm characteristics and histological changes of testes were analyzed.

Results: Body and testes weights were decreased in DEHP group. DEHP also reduced the number of spermatogonia, primary spermatocyte and sertoli cells as well as sperm vitality and progressive motility; these toxic effects were associated with alterations in serum hormone levels. Melatonin remarkably inhibited DEHP-induced reduction of body weight and antioxidant capacity. Melatonin reduced DEHP-induced elevation of NO, MDA, IL-1β and TNF-α levels. Melatonin improved DEHP-induced changes in hormonal levels, number of sertoli cells, spermatogonia, and sperm viability and motility.

Conclusion: Melatonin considerably inhibits DEHP-induced gonadotoxicity through reducing oxidative stress and inflammatory responses. These results suggest that melatonin may be considered as a promising agent to reduce toxic effects of endocrine disrupting chemicals such as DEHP on the male reproductive system.

1. Introduction

Endocrine disrupting chemicals are exogenous substances impacting the reproductive system; these compounds can increase the incidence of reproductive anomalies by disturbing the homeostasis of the hormonal balance [1]. The susceptibility of male reproductive system to adverse effects of these toxicants has been shown in various studies. The deleterious effects of endocrine-disrupting compounds on male reproductive functions result from their estrogenic and/or anti-androgenic activities. These toxic agents remain in the environment and exert profound effects on wildlife and human health [2,3]. Phthalates are well known compounds with endocrine disrupting properties; their estrogenic and/or anti-androgenic effects have been reported in *in vivo* studies [4]. There are more than 24 types of phthalates; di(2-ethylhexyl) phthalate (DEHP), di-iso-nonyl phthalate (DiNP), benzyl butylphthalate (BBP), di-iso-hexyl phthalate (DiHP), di-*n*-butyl phthalate (DnBP) and diethyl phthalate (DEP) are the most important phthalates. DEHP has the most reproductive toxicity compared to other phthalates [5–7]. DEHP is the most common phthalate derivative, used as plasticizer in the manufacturing of polyvinyl chloride (PVC) to increase the flexibility of final products. DEHP easily leaches from products into the environment due to the weak non-covalent links with matrix of plastic components [8]. Blood storage bags, intubation tubes and devices used for injection and hemodialysis contain large amounts of DEHP. As a

* Corresponding author at: Razi Drug Research Center, Iran University of Medical Sciences, P.O. Box: 1449614535, Tehran, Iran.
E-mail address: mehrzadi.s@iums.ac.ir (S. Mehrzadi).

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result, DEHP residues are commonly found in the blood and tissues of patients who have had frequent transfusions [9]. Foods are the main sources of human exposure to DEHP, however. The exposure of humans to DEHP is estimated to be 3–30 μg/kg/day, while this increases several-fold in premature infants receiving intensive medical interventions [10]. The carcinogenicity of DEHP has been shown in animal studies but there is no epidemiological evidence indicating the carcinogenic effects of DEHP in humans [11]. In recent years, there is a growing concern about the impact of DEHP on human health due to its adverse effects on reproductive system in animal models. Phthalates, especially DEHP, lead to reduction in pregnancy and increase in spontaneous abortions and congenital malformations [7]. DEHP is metabolized by plasma or tissue esterases to active metabolites including mono-(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2 EH) which have toxic effects on testes [12]. These compounds change the normal function of Sertoli and Leydig cells, disrupt the endocrine system and reduce the production of testosterone, resulting in the infertility. The active metabolite, MEHP, induces oxidative stress in the testis through increasing the generation of ROS and reducing the level of glutathione, free thiol and ascorbic acid. Oxidative stress induced by MEHP contributes to the mitochondrial damage and cytochrome c escape from inner mitochondrial membrane; this results in the induction of spermatocyte apoptosis and testicular atrophy [13]. Recent studies have demonstrated that treatment of animals with antioxidant agents may reduce the toxicity of DEHP on testicular cells [14].

Melatonin (N-acetyl-5-methoxytryptamine) is a molecule with numerous effects including anti-oxidant, anti-inflammatory and anti-apoptotic activity [15–18]. Melatonin is produced in many organs including the pineal, bone marrow, gut, thymus, retina, placenta, liver, testis and ovary [19–21]. In addition to its hormonal function, melatonin has neurotransmitter-like activity and modulates various physiological processes; melatonin has regulatory effects on the body temperature, sleep and the function of immune and reproductive systems [22]. Due to its lipophilic characteristic, melatonin may easily pass through the cell membrane, where it is distributed in subcellular organelles [20]. There are other routes by which melatonin enters cells [23,24]. Current evidence indicates that melatonin is produced in every animal and plant cells that have mitochondria [25]. The physiological effects of melatonin could be receptor-dependent or receptor-independent; receptor-dependent functions are mediated by its nuclear (RZR) or membrane receptors (MT1 and MT2) and receptor-independent actions are related to its free radical scavenging activity [26]. Following interaction with free radicals, melatonin is converted to its metabolites including N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK); these metabolites also have radical scavenging activity [27]. Moreover, melatonin induces the expression of glutathione as well as the activity and the expression of antioxidant enzymes including superoxide dismutase (SOD), glutathione reductase, glutathione peroxidases (Gpx) and catalase [22,28,29]. Because of its multiple actions, especially at the mitochondrial level, melatonin strongly reduces oxidative stress. The current study was carried out to evaluate the protective effects of melatonin on DEHP-induced testicular injury with an evaluation of the possible mechanisms involved.

2. Materials and methods

2.1. Material

Melatonin, di(2-ethylhexyl) phthalate (DEHP), Bradford reagent, thiobarbituric acid (TBA), 5, 5- dithiobis (2-nitrobenzoic acid) (DTNB) and trichloro acetic acid (TCA) were purchased from Sigma–Aldrich (St Louis, MO, USA). All the other materials used in this experiment were obtained from Merck (Darmstadt, Germany).

2.2. Animals and experimental design

This study was accepted by Institutional Animal Care and Use Committee of the Dezful University of Medical Sciences (Ethic code: IR.DUMS.REC.1397.016). Thirty-two adult male NMRI mice, at the age of 4 weeks, were obtained from animal house, Dezful University of Medical Sciences, Iran. The animals were placed into polycarbonate cages and received standard food and water. The animal room was maintained at 25 ± 2 °C with 12 h light and dark cycle. Animals were randomly divided to four groups; group I received normal saline (5 ml/kg/day) for 14 days, group II received DEHP (2 g/kg/day, dissolved in corn oil, p.o.) for 14 days, group III received DEHP (2 g/ kg/day, dissolved in corn oil, p.o.) and melatonin (10 mg/kg/day, dissolved in ethanol 0.5%, i.p.) for 14 days and group IV was treated with melatonin (10 mg/kg/day, dissolved in ethanol 0.5%, i.p.) for 14 days. Duration of treatment and doses of melatonin and DEHP were according to previous studies [30–32].

2.3. Body weight and testes weights

The body weight (g) and testes weights (mg) of mice were measured before starting the experiment and after sacrifice of animals at the end of experiment.

2.4. Sample collection and preparation

At the end of the treatment, animals were anesthetized with the injection of ketamine and xylazine (60/6 mg/kg; i.p.). The whole blood samples were taken from the heart. Mice were then sacrificed by cervical dislocation. To obtain serum, samples of blood were centrifuged at 2000*g* at 4 °C for 10 min and stored at −20 °C for biochemical analysis. Testes were removed and prepared for experiments. The left testis was used for histological study; tissue was fixed in Bouin's solution and then embedded in paraffin. The right testis was used for biochemical assays; tissue was homogenized (1/10 w/v) in ice-cold Tris − HCl buffer (0.1 M, pH 7.4) and stored at −20 °C. The protein content was determined using the Bradford method [33].

3. Biochemical investigations

3.1. Serum testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels

The levels of testosterone, LH and FSH in the serum of mice were measured using mouse testosterone ELISA kit (MyBioSource Co, cat num: MBS843463), LH ELISA kit (MyBioSource Co, cat num: MBS041300) and FSH ELISA kit (MyBioSource Co, cat num: MBS2507988). Data were expressed as ng of testosterone per ml of serum and milli-international units of FSH and LH per milliliter (mlU/ml) of serum.

3.2. Total antioxidant capacity (TAC)

The TAC of samples was measured using ferric reducing antioxidant power (FRAP) assay as described previously [34]. The fresh FRAP reagent (1.5 ml) containing sodium acetate buffer (0.3 M and pH 3.6), TPTZ (0.01 M, dissolved in 0.04 M HCl) and FeCl3 (0.02 M) was added to supernatant and the absorbance was assessed after 5 min at 593 nm. Ferrous sulphate solution was used to calibrate the instrument response. The results were expressed as μg of TAC per mg of protein.

3.3. The activity of antioxidant enzymes and the content of glutathione (GSH)

Catalase (CAT) activity was determined as described previously [35]. Briefly, tissue supernatant (50 μl) was mixed with phosphate