

Letter to the Editor

Effects of Low Concentrations of Di-(2-ethylhexyl) and Mono-(2-ethylhexyl) Phthalate on Steroidogenesis Pathways and Apoptosis in the Murine Leydig Tumor Cell Line MLTC-1*

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The aim of this study was to evaluate the effects of low concentrations of DEHP and MEHP on steroidogenesis in a murine Leydig tumor cell line (MLTC-1) *in vitro*. The result of flow cytometry analysis revealed that the proportion of apoptotic cells was significantly increased after the exposure to DEHP. All three genes (*P450scc*, *P450c17*, and *3βHSD*) under study showed an increased expression following exposure to DEHP or MEHP, although some insignificant inhibitory effects appeared in the 10 μmol/L treatment group as compared with the controls. It was also found that DEHP or MEHP stimulated *INSL3* mRNA and protein especially in the 0.001 μmol/L treatment group. Testosterone secretions were stimulated after the exposure to DEHP or MEHP. Alterations of steroidogenic enzymes and *INSL3* in MLTC-1 cells might be involved in the biphasic effects of DEHP/MEHP on androgen production.

Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizer which is commonly used as polyvinyl chloride (PVC) and flexible plastics in food-packaging materials, plastic flooring, carpet material, indoor decorations, cable packaging materials and children's toys. DEHP is rapidly metabolized to 2-ethylhexanol and mono-(2-ethylhexyl) phthalate (MEHP) in the intestines, and it has been suggested that MEHP is the main active anti-androgenic metabolite responsible for the testicular toxicity of DEHP^[1]. Given the testicular toxicity of phthalate plasticizers, they are able to cause a variety of male reproductive disorders, such as decreased sperm counts, hypospadias, cryptorchidism, reproductive tract abnormalities. High concentrations of DEHP metabolites have been thought to be associated with low levels of human sex steroid hormones. However, phthalates do not directly bind the androgen receptor; their effects on testosterone biosynthesis in the rat are hypothesized as resulting

from the inhibition of enzymes expression involved in cholesterol uptake/transport and steroidogenesis. However, few documents have been published about the effects of low level of DEHP or MEHP. Indeed, much attention should be paid to the effects of low-dose endocrine disrupting chemicals (EDCs), since it is a common phenomenon for humans to be exposed to low-doses of phthalates^[2].

Phthalates may exert antiandrogenic effects by impairing testosterone synthesis in the testis, probably through diminishing expression of several genes in the testosterone biosynthesis pathways. When cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane, a six-carbon moiety is cleaved from cholesterol by the side-chain cleavage enzyme (*P450scc*), subsequently progesterone and testosterone are generated gradually, which is regulated by 3β-hydroxy-steroid dehydrogenase (3βHSD) and cytochrome *p450c17* (*CYP17*), respectively^[3-4]. In the male reproductive system, androgenic hormone biosynthesis mainly occurs in the Leydig cells, which are the main sources of testosterone and insulin-like hormone 3 (*INSL3*)^[5]. *INSL3* is responsible for the first phase of testicular descent during entire fetal development and is believed to be involved in germ cell development and bone metabolism as well as cryptorchidism.

The aim of this study was to determine the effects of relatively low concentrations of DEHP and MEHP on steroidogenesis in mouse Leydig tumor cells (MLTC-1), focusing on the dose-response relationship between phthalates and the expression levels of key enzymes and *INSL3* secretions in testosterone biosynthesis pathways. In addition, the effects of DEHP and MEHP on testosterone production and apoptosis in MLTC-1 were further evaluated.

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MLTC-1 cells were incubated with various concentrations (10, 0.1, 0.001, and 0 $\mu\text{mol/L}$ in DMSO) of DEHP and MEHP respectively for 24 h. Testosterone concentrations were measured by direct chemiluminescence assay. Apoptotic frequency of MLTC-1 cells induced by phthalates was detected by flow cytometry. The expression of mRNA and protein of side-chain cleavage enzyme (P450scc), cytochrome p450c17 (P450c17), 3 β -hydroxy-steroid dehydrogenase (3 β HSD) and insulin-like hormone 3 (INSL3) in MLTC-1 were also investigated.

DEHP and MEHP were purchased from AccuStandard, Inc. (New Haven, CT, USA) and dissolved in dimethylsulfoxide (DMSO). DMSO was purchased from Sigma-Aldrich (St. Louis, MO USA). Human chorionic gonadotrophin (hCG) was obtained from PROSPECT (Ness-Ziona, Israel). The DMSO concentration in the culture medium is less than 0.1%.

The MLTC-1 cell line was obtained from the Cell Institute of Shanghai (Shanghai, China) and maintained in Roswell Park Institute-1640 medium (RPMI-1640; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (pH 7.2), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin.

Data were presented as mean \pm SD. For group comparisons, statistical analysis was performed using one-way ANOVA or the Wilcoxon Mann-Whitney test (when parameters were not met), followed by the Student-Newman-Keuls posttest or the Dunnett multiple comparison test (when parameters were not met). A two-sided test was conducted, and the statistical significance was set at $P < 0.05$. All statistical analyses were performed using the SAS version 9.1 software.

Compared with the control group (MLTC-1 cells were incubated with 0 $\mu\text{mol/L}$ of DMSO), the proliferation of MLTC-1 cells was not altered when cultured with different concentrations of DEHP or MEHP. When the dose of DEHP or MEHP was increased to 100 $\mu\text{mol/L}$, the growth of MLTC cells was significantly inhibited. In order to verify whether DEHP or MEHP could cause mLTC-1 apoptosis, the PI and Annexin V-labeled FACS assays were performed. The result of flow cytometry analysis revealed that the proportion of apoptotic mLTC-1 cells was significantly increased after exposure to different concentrations of DEHP. Although the proportion of apoptotic mLTC-1 cells in MEHP incubated groups had a trend to increase, there was no statistical

difference except for the dose of 0.1 $\mu\text{mol/L}$. It has been shown that Leydig cells are capable of producing testosterone. Indeed, exposure to many types of chemical agents is known to induce Leydig cell apoptosis with no change in testosterone concentration^[6].

Testosterone secretions of MLTC-1 cells were stimulated after exposure to DEHP or MEHP. However, with the increase of treatment doses, testosterone was inhibited and reached the lowest at the highest treatment concentration (10 $\mu\text{mol/L}$). In contrast to the anti-androgenic effect induced by high dose of DEHP [over 500 mg/(kg-d)], Akingbemi et al.^[7] reported that prepubertal exposure to DEHP at a dose of 10 mg/(kg-d) enhanced androgen biosynthesis in Leydig cells and increased testosterone levels in rats. Another study provided evidence that pubertal exposures to low and high levels of DEHP had different effects on T production in Leydig cells^[8]. Many environmental endocrine disruptor chemicals can induce U or invert U dose-response, resulting in a low-dose stimulation response^[9].

As shown in Figures 1A-C, all three genes investigated demonstrated an increased expression following exposure to DEHP or MEHP, although non-significant inhibition appeared in the 10 $\mu\text{mol/L}$ treatment group compared with the control group. For P450scc, the mRNA expression was stimulated in the 0.001 $\mu\text{mol/L}$ DEHP treatment group however, it was decreased in the 10 $\mu\text{mol/L}$ DEHP treatment group. No significant changes of mRNA expression were observed after exposure to MEHP. For 3 β HSD, stimulated effects of DEHP or MEHP were witnessed in the three treatment groups. In addition, the 3 β HSD mRNA increased significantly in the DEHP treatment groups at doses of 0.001 $\mu\text{mol/L}$ and 0.1 $\mu\text{mol/L}$ compared with the control group. For P450c17, mRNA expression was increased in the DEHP treatment group by raising the lower concentration; however, the MEHP treatment group had no such significant stimulating effect on mRNA expression, instead it decreased mRNA expression more significantly at the dose of 10 $\mu\text{mol/L}$ than at the dose of 0.001 $\mu\text{mol/L}$.

INSL3 mRNA and protein levels in MLTC-1 after exposure to DEHP and MEHP are shown in Figure 1D and Figure 2. The same trend between mRNA and protein expression after the exposure to DEHP or MEHP was observed, which stimulated INSL3 mRNA and protein especially at the dose of 0.001 $\mu\text{mol/L}$.

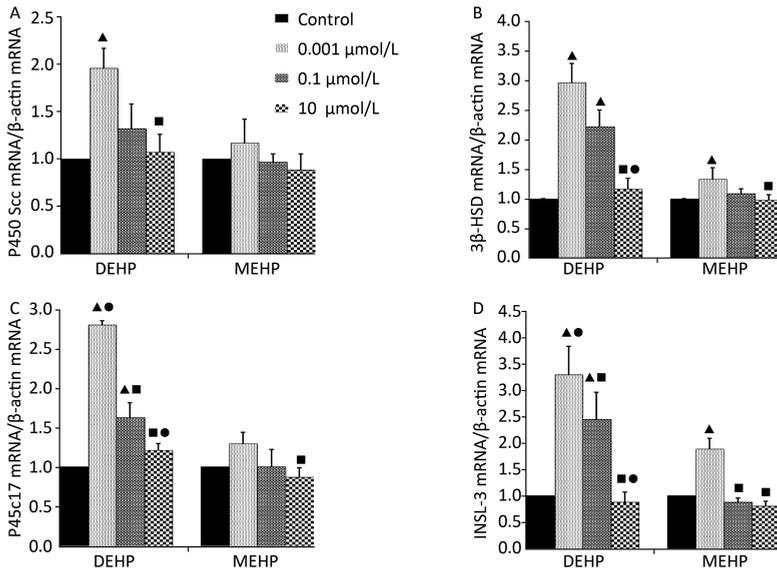


Figure 1. Effects of DEHP or MEHP on mRNA expression of P450scc, 3βHSD, P450c17, and INSL3. MLTC-1 cells were exposed to media containing various concentrations of DEHP or MEHP (10, 0.1, 0.001, and 0 μmol/L in DMSO) for 24 h, and the effects of DEHP and MEHP on mRNA expression of P450scc (A), 3βHSD (B), P450c17 (C), and INSL3 (D) were determined by real-time RT-PCR using β-actin as a housekeeping gene. Data are presented as mean±SD, $n=8$. ▲Significant difference in comparison with the control, $P<0.05$; ■Significant difference in comparison with the 0.001 μmol/L group, $P<0.05$; ●Significant difference in comparison with the 0.1 μmol/L group, $P<0.05$.

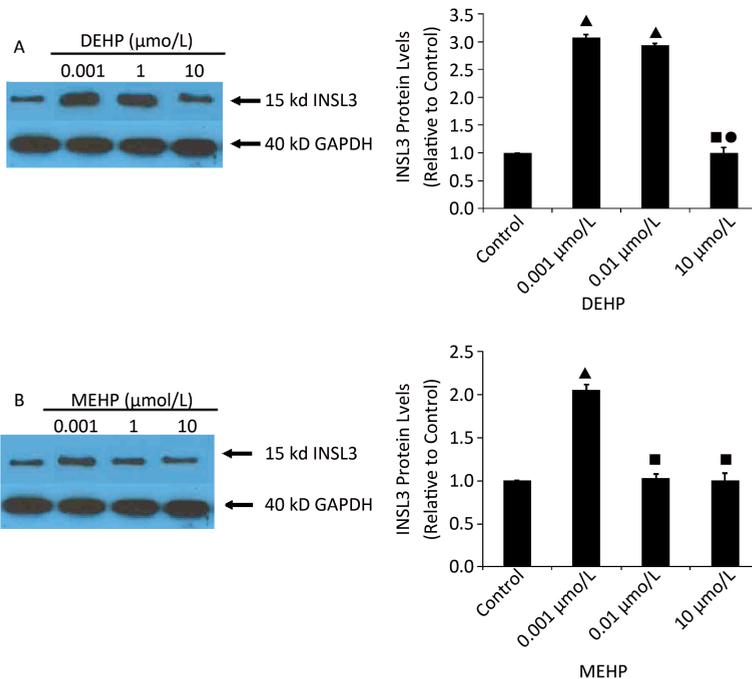


Figure 2. Effects of DEHP (A) or MEHP (B) on INSL3 protein. MLTC-1 cells were treated with DEHP (10, 0.1, 0.001, and 0 μmol/L in DMSO) followed by stimulation with hCG (0.1 U/mL). INSL3 protein was determined by Western blot analyses. Data are presented as mean±SD, $n=8$. ▲Significant difference as compared to the control, $P<0.05$; ■Significant difference as compared to the 0.001 μmol/L group, $P<0.05$; ●Significant difference as compared to the 0.01 μmol/L group, $P<0.05$.

Further studies are needed to clarify the effects of environmental phthalate exposure on canine Leydig cell *in vivo* by comprehensive assessment^[10].

In summary, exposure to relatively low concentrations of DEHP or MEHP appears to increase the expression of genes involved in testosterone biosynthesis and a similar stimulation in INSL3 gene expression, whereas the proportion of apoptotic of MLTC-1 cells is significantly increased by raising the treatment doses.

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