THE POTENTIAL OF DIETHYLHEXYL-PHTHALATE AND GENISTEIN TO INDUCE TESTICULAR CHANGES IN RAT'S OFFSPRING

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Abstract

Objective. To study the effect of diethylhexyl phthalate (DEHP) alone or in combination with genistein (GEN) on the reproductive system of offspring rats, focus on the induction of reproductive outcomes.

Method. 180 Wistar rats were divided in 6 groups (30 animals per group): DEHP 250 mg/kg/day group, DEHP 1000 mg/kg/day group, DEHP 2500 mg/kg/day group treated with DEHP 2500 mg/kg/day, DEHP (2500 mg/kg) + GEN (50 mg/kg) group, DEHP (2500 mg/kg) + GEN (500 mg/kg) group and control group treated with the same quantity of corn oil. The differences in sperm quality and reproductive organs were observed.

Results. After DEHP administration we observed an increase in rat's abestrus, metaestrus and all estrus cycle ($P < 0.05$), a decrease in rat testicle's organ coefficient and relative energy of testis Sertoli cells and an increase in the early, late and total apoptotic rate of testicular Sertoli cells in a dose dependent manner ($P < 0.05$). When combine DEHP with GEN the sperm density, sperm quality, the cell activity rate and testis tissue's changes will decrease compared with the group that receive only DEHP in a dose dependent manner.

Conclusion. DEHP exposure induces cryptorchidism in offspring rats and this is aggravated by adding GEN.

Key words: DEHP, DEHP+GEN, endocrine disruptors, testicular changes, rat.

INTRODUCTION

Cryptorchidism, a common birth defect in males is characterized by the absence of one or both testicles in the scrotum (1). Together with hypospadias, the abnormal positioning of the opening of the urethra, are considered as risk factors for several reproductive adult-onset disorders and even testicular cancer. There is an upward trend in the incidence of cryptorchidism in developed countries lately, leading to an increasing interest of researchers to elucidate the factors and the

mechanism which are involved in the occurrence of this disease. Many studies show that the occurrence and development of cryptorchidism and hypospadias in males are closely related to the exposure to different xenobiotics which act as endocrine disruptors (2-4). The environmental endocrine disruptors are synthetic chemical pollutants, pesticides, additives that affect the endocrine system, leading to a large number of health issues and diseases, especially on the reproductive system (5, 6). In some cases, the effects could be also dictated by genetic polymorphisms in genes encoding enzymes of cytochrome P450 that affect the susceptibility and the response to endocrine disruptors (7, 8). Among them, diethylhexyl phthalate (DEHP) (9) is an organic compound used in plastic products, such as food packaging materials, medical equipment, dolls, toys, shoes and as fragrance additive in a wide range of cosmetic products. It is a well-known air and food pollutant with endocrine disruptor properties especially on male reproductive system, acting as an androgen antagonist.

Humans can be exposed to DEHP through diet, air, drinking water and invasive methods such as intravenous procedures. DEHP toxicokinetics is complex. Once DEHP enters the body, a part of it is absorbed while the rest is metabolized mainly to mono-2-ethylhexyl phthalate (MEHP) and phthalate salts, by pancreatic and hepatic enzymes (10). This metabolic pathway is blocked in various liver conditions such as nonalcoholic fatty liver or hepatitis C virus infection (11, 12). DEHP produces a lot of dose-dependent toxic effects, mostly related to MEHP metabolite. Exposure to DEHP led mice, rats, and common poultry to death, and this was attributed mainly to MEHP (13).

Previous studies have shown the detrimental effect of DEHP on the male reproductive system since it acts as an androgen antagonist and stimulates the secretion of estradiol (14, 15). Moreover, others

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report that estradiol promotes Insulin-like peptide 3 (InsI3) mRNA expression (16), leading to damages to the sperm function. Leucine-rich repeat containing G protein-coupled receptor 8 (Lgr8) (17) is the only receptor of InsI3 that mainly affects testicles and gubernaculum testis. Lgr8 receptor and InsI3 play an important role in testicular descent. Changes to Lgr8 receptor and its agonist InsI3 could lead to increased incidence of cryptorchidism.

Phytoestrogens (PEs) are compounds that can be found in plant foods (18). Once digested, they are converted to compounds with a similar structure to human estrogens (19) with anti-androgenic effect. PEs include flavonoids (genistein and daidzein) and wood esters (20). Exposure to PEs is mainly through diet. Exposure to PEs has a dual effect, both estrogenic and anti-estrogenic. They can be beneficial in some hormone-related conditions (21-23) but also detrimental to some reproductive system diseases, especially in males with sperm quality problems (24).

Currently, the cumulative exposure to these substances, and other xenobiotics, especially endocrine disruptors, which may have additive effects on our health, have become a matter of growing concern (25-27). Therefore, a new methodology for risk assessment of chemical mixtures at recommended doses has been proposed, in order to elucidate the dangers to which consumers are exposed to (28, 29).

Considering current advances in the field, the aim of this study was to evaluate the effect of DEHP, and the combination of DEHP and GEN, on the occurrence of cryptorchidism in rats.

MATERIALS AND METHODS

General materials

All the experiments were performed according to the protocols approved by the Ethical Committee of Medicine College of Hebei University of Engineering, Handan, Hebei, China and in accordance with the practices outlined in the American Veterinary Medical Association Panel. One hundred eighty male Wistar rats offspring (from Medicine College of Hebei University of Engineering, Handan, Hebei, China experiment animal center) from 3rd day in gestation (GD3) to 42 day during lactation (PND42) were divided into 6 groups (30 animals per group): DEHP 250 mg/kg/day group was treated by gavage with DEHP 250 mg/kg/day in corn oil [(1/120) × (median lethal dose)], DEHP 1000 mg/kg/day group was treated by gavage with DEHP 1000 mg/kg/day in corn oil [(1/20) × (median lethal dose)],

DEHP 2500 mg/kg/day group was treated by gavage with DEHP 2500 mg/kg/day in corn oil [(1/8) × (median lethal dose)], DEHP (2500 mg/kg) + GEN (50 mg/kg) group was treated by gavage with DEHP (2500 mg/kg/day) + GEN (50 mg/kg/day) in corn oil, DEHP (2500 mg/kg) + GEN (500 mg/kg) group was treated by gavage with DEHP (2500 mg/kg/day) + GEN (500 mg/kg/day) in corn oil, and control group treated with the same quantity of corn oil by gavage. There was a post-treatment period of two weeks, prior to sacrifice. The animals were kept at a standard temperature of 20±2°C, relative humidity of 40-60%, free access to drinking water. The food used was soy and alfalfa-free (SAFD). DEHP (Tianjin Kermel Chemical Reagent Company Limited, China); optical microscope (Olympus Company, Japan); fetal bovine serum (Gibco Company, U.S); pregnant blood serum (Ningbo Second Hormone Factory, China); 24-hole culture plate and 96-hole culture plate (was Costar Company, U.S); RNA extraction reagent Trizo and DNA maker (Dalian TaKaRa Biological Company Limited); Annexin V-FITC/PI (Shanghai Biyuntian Company); microplate reader (Thermo Company, U.S); FC500 flow cytometry (Beckman Coulter Company, U.S); Lgr8 primers (Shanghai Sangon Biotech Company Limited, China); GEN (Shaanxi Huike Botanical Development Company Limited, China); Mettler AG285 electronic balance (Mettler-Toledo Company, Switzerland).

Experimental Methods

We designed two experiments:

Experiment 1 was to study whether DEHP has any dose-dependent effect on the male reproductive functions of estrus cycle, sperm density, sperm quality and sperm cell activity rate.

Experiment 2 examined whether the combination of GEN and DEHP could have an additive effect on the weight of testis and epididymis and sperm quantity and functionality. The effect of DEHP on the estrous cycle phases was assessed by observing morphological changes on the penis. Estrous cycle can be divided into four stages: proestrus, estrus, metaestrus, anestrus, which share distinct morphological features. Urine with sperm was collected from rats, twice a day (5 a.m in the morning and 5 p.m in the afternoon) one week before sacrifice, in order to observe changes in sperm cell morphology and identify the estrus phase of rats (EC) by cell smearing test. After 6 weeks of exposure, the rats were sacrificed by decapitation and the testis and epididymis were collected. After the

surrounding fat and connective tissue were removed, a part of separated testis and epididymis tissues were put in a Petri dish and washed dry with a filtered paper, and weighed. A part of testis and epididymis tissues was washed with phosphate buffer and put in a Petri dish with 1mL of phosphate buffer saline (PBS), pricked with a 25-gauge needle for releasing the granular cells. The granular cells were further isolated by 0.1% trypsin digestion. The supernatant was collected after centrifugation (2500 r/min, 5 min) and washed with PBS. 200mL of diluted suspension solution was added to the supernatant.

For DEHP 250 mg/kg/day group, DEHP 1000 mg/kg/day group, DEHP 2500 mg/kg/day group and control group apoptosis rate in granular cells was determined by Annexin V-FITC/PI and cell vitality by MTT assay. Briefly the cell apoptosis rate was determined in accordance with the Annexin V-FITC/PI apoptosis kit instructions. 100 ml of cell suspension solution used were incubated with 6 μ L of Annexin V and 12 μ L propidium iodide (PI) in the dark for 15 min. 400 mL PBS was added and apoptosis was detected by flow cytometry.

For methyl thiazolyl tetrazolium (MTT) assay we collected cell suspensions from cultures incubated with different concentrations of DEHP for 24 h, 48 h, and 72 h. 20 mL MTT solution was added and plates were placed in CO₂ incubator for 4 hours at 37°C. After incubation, the supernatant was removed, 180 mL of dimethyl sulfoxide (DMSO) solution was added in each well and put in the oscillator for 10 min, leading the violet crystals of cells completely dissolved. Colorimetric measurement was performed at 500 nm wavelength to detect absorbance (OD). Ratio of ODsample/ODcontrol was used to estimate cell viability.

Experimental data were expressed as means $\pm$ standard deviation and statistical analysis of variance of one way ANOVA test was performed using SPSS18.0 statistical package. For data following normal distribution, Student - Newman - Keuls was used, otherwise, we used the Games-Howell test. $P < 0.05$ denoted a statistically significant difference between the groups.

RESULTS

Effects of DEHP exposure on estrous cycle

The duration of offspring rats' anestrus cycle in DEHP 250 mg/kg/day group, DEHP 1000 mg/kg/day group and DEHP 2500 mg/kg/day group were significantly prolonged ($p < 0.05$) compared to the control group, while the duration of anestrus cycle in the 1000 mg/kg/day and 2500 mg/kg/day groups was obviously higher than that of 250 mg/kg/day group. Moreover, the anestrus cycle was longer in the 2500 mg/kg/day group than in the 1000 mg/kg/day group ($P < 0.05$). Differences in metaestrus phase duration were also statistically significant ($P < 0.05$) among the groups. In 1000 mg/kg/day and 2500 mg/kg/day groups metaestrus stage was significantly longer than in the 250mg/kg/day group ($P < 0.05$), and compared to the control all groups showed significantly increased metaestrus stage duration ($P < 0.05$) (Table 1).

Effects of DEHP exposure on body weights of offspring rats

Table 2 demonstrates the increase in the body weight of rats. Body weight was significantly decreased in rats exposed to DEHP in a dose and time-dependent manner. In the first week there was little difference between the groups ($P > 0.05$), but 2, 3 and 4 weeks

Table 1. Effects of DEHP exposure on estrous cycle on offspring rats

Group	Number of animals	Proestrus	Estrus	Metaestrus	Anestrus
250mg/kg/day DHEP	30	(16.57 $\pm$ 4.37)d	(20.88 $\pm$ 3.21)d	(24.73 $\pm$ 7.67)d ^{&}	(31.45 $\pm$ 9.70)d ^{&}
1000 mg/kg/day DHEP	30	(18.57 $\pm$ 4.77)d	(19.72 $\pm$ 3.76)d	(37.50 $\pm$ 9.62)d ^{*&}	(42.33 $\pm$ 6.95)d ^{*&}
2500 mg/kg/day DHEP	30	(20.16 $\pm$ 3.61)d	(19.84 $\pm$ 3.44)d	(43.21 $\pm$ 7.23)d ^{*#&}	(65.42 $\pm$ 7.12)d ^{*#&}
Control	30	(14.36 $\pm$ 3.72)d	(19.34 $\pm$ 2.92)d	(19.85 $\pm$ 7.23)d	(27.28 $\pm$ 9.25)d

Compared with group of 250 mg/kg DEHP, * $P < 0.05$; Compared with group of 1000 mg/kg DEHP, # $P < 0.05$; Compared with control group, & $P < 0.05$.

Table 2. Effect of DEHP on body weight of offspring rats

Groups	Exposure Time (weeks)			
	1	2	3	4
250mg/kg/day DEHP	(203.82 $\pm$ 2.84)g	(215.62 $\pm$ 2.24)g ^{&}	(222.37 $\pm$ 2.21)g ^{&}	(224.46 $\pm$ 1.20)g [#]
1000mg/kg/day DEHP	(202.37 $\pm$ 2.45)g	(206.13 $\pm$ 4.65)g ^{*&}	(217.67 $\pm$ 4.21)g ^{*&}	(220.27 $\pm$ 1.58)g [#]
2500mg/kg/day DEHP	(200.52 $\pm$ 2.76)g	(196.75 $\pm$ 1.96)g ^{*&}	(208.25 $\pm$ 3.76)g ^{*&}	(215.72 $\pm$ 2.11)g ^{&}
Control	(201.55 $\pm$ 2.24)g	(220.47 $\pm$ 2.37)g	(227.66 $\pm$ 2.43)g	(243.53 $\pm$ 1.78)g

Compared with group of 250 mg/kg DEHP, * $P < 0.05$; Compared with group of 2500 mg/kg DEHP, # $P < 0.05$; Compared with control group, & $P < 0.05$.

after the exposure the increase of body weight of rats in 250 mg/kg/day, 1000 mg/kg/day and 2500 mg/kg/day groups was obviously slower than in the control group ($p<0.05$). Body weight growth of 1000 mg/kg/day group and 2500 mg/kg/day group was slower than that of 250 mg/kg/day group ($p<0.05$).

Effects of DEHP exposure on organ coefficient in offspring rats

Organ coefficient is significantly decreased in the exposure groups compared to the control ($P<0.05$) as depicted in Table 3. The weight of offspring rats' testis and epididymis in the three groups were reduced according to DEHP doses ($p<0.05$), and organ coefficient of 1000 mg/kg/day and 2500 mg/kg/day groups was significantly decreased compared to 250 mg/kg/day group ($P<0.05$).

Effects of DEHP exposure on sperm cell viability

As shown in Figure 1, the difference of early, late and total apoptotic rate of testicular sperm cells in DEHP exposed groups was statistically significant ($P<0.05$) compared to the control group. The diagram is divided into four squares, increase of the two square

Figure 1. Sperm cell death after DEHP exposure.

Table 3. Effects of DEHP exposure on organ coefficient in offspring rats

Groups	Number	Weight of Testicle	Weight of Epididymis	Coefficient
250 mg/kg/day DEHP	30	(2.24±0.35)g ^{&}	(0.55±0.09)g ^{&}	(1.26±0.07)% ^{&}
1000 mg/kg/day	30	(2.02±0.12)g ^{*&}	(0.49±0.03)g ^{*&}	(1.21±0.09)% ^{*&}
2500 mg/kg/day	30	(1.56±0.37)g ^{*#}	(0.42±0.09)g ^{*#}	(1.29±0.19)% ^{*#}
Control	30	(2.24±0.56)g [*]	(0.58±0.13)g [*]	(1.47±0.24)% [*]

Compared with group of 250 mg/kg DEHP, * $P<0.05$; Compared with control group, & $P<0.05$.

number on the right side showed that the number of apoptotic cells is increasing, bottom right of the square denoted apoptosis number of early period, upper right of the square denoted apoptosis number of late period. Along with the continuous increase of DEHP dose, numbers of early and late period's apoptotic sperm cells were increasing and the total number of apoptosis was obviously higher.

Early, late and total apoptotic rates were also increased ($P<0.05$). Apoptotic rate of 250 mg/kg/day group was 5%, 8.5 % was in 1000 mg/kg/day group, and 13% in 2500 mg/kg/day group ($P<0.05$). Early apoptotic rate (5%) of 2500 mg/kg/day group is higher than that of 1000 mg/kg/day group (3%) and 250 mg/kg/day group (1.3%) ($P<0.05$), and late apoptotic rate of 1000 mg/kg/day group (5.5%) was obviously higher than that of 250 mg/kg/day group (3.7%) ($P<0.05$).

Effects of DEHP exposure on testis and epididymis tissue granulosa cells

As shown in Figure 2, different concentrations of DEHP inhibit the activity of testis and epididymis tissue granulosa cells ($P<0.05$) while they did not affect the control group.

The average activity of rats was highly affected by exposure to different doses of DEHP; in the group of 250 mg/kg/day DEHP the average activity was 87.2% and further decreased to 78.7% in the group of 1000 mg/kg/day DEHP and to 76.2% in the group of 2500 mg/kg/day DEHP, compared to the control group (92.4%).

Figure 2. Activity of testis and epididymis tissue granulosa cells after DEHP exposure.

After 24h of exposure, differences in relative energy, among groups of granular cells, were statistically significant, the activity of 250mg/kg/day group was 87.8%, the activity of 1000mg/kg/day group was 80.6%, the activity of 2500mg/kg/day group was 80.3% ($P<0.05$). Cells activity of 2500mg/kg/day group was significantly lower compared to other groups ($P<0.05$). Cell activity gradually decreased with the time of exposure. The average activity after 24h of exposure was 83.4%, the average activity after 48h of exposure was 80.3%, the average activity after 72h of exposure was 75.4% ($P<0.05$). After 48h and 72h of exposure, cells activity of 2500mg/kg/day group was obviously less than cell activity after 24h ($p<0.05$). These findings show that DEHP can inhibit granulosa cell proliferation after long term high dose exposure.

Effects of DEHP +GEN on testis and epididymis weight, organ coefficient, sperm density and sperm activity

As it can be seen in Table 4, exposure at 500 mg/kg/day of GEN decreased the weight of testis and epididymis and organ coefficient compared to the dose of 50 mg/kg/day and to the group exposed to DEHP alone ($P<0.05$). This effect was not statistically significant for the low dose of GEN (50mg/kg/day). 500 mg/kg/day of GEN also a negatively affected sperm counts of testis and epididymis and increased sperm deformity rate compared to the group exposed to 50

mg/kg/day GEN and the group without GEN ($P<0.05$).

Effects of DEHP + GEN on weight percentage of testis and epididymis

As shown in Table 5, there were no significant differences in the weight of offspring rats at 42 days from birth among the groups 2500 mg/kg/day DEHP, 2500 mg/kg/day DEHP + 50 mg/kg/day GEN, 2500 mg/kg/day DEHP + 500 mg/kg/day GEN and control group. Weight of testis and epididymis and organ coefficient in DEHP (2500 mg/kg/day) + GEN (500 mg/kg/day) group was significantly lower than that of DEHP (2500 mg/kg/day) + GEN (50 mg/kg/day) group, DEHP (2500 mg/kg/day) group and control group ($P<0.05$). Prostate and seminal vesicle coefficient in DEHP (2500 mg/kg/day) + GEN (500 mg/kg/day) group was significantly lower than that of DEHP (2500 mg/kg/day) + GEN(50 mg/kg/day) group, DEHP (2500 mg/kg/day) group and control group ($P<0.05$).

Effects of DEHP + GEN on sperm activity level

The comparison of sperm activity between groups has been performed based on four ratings: (a) denoted sperm activity is very good, straight forward movement. (b) denoted sperm activity is ok, straight forward movement. (c) denoted sperm activity level is normal, presented forward curvilinear movement. (d) denoted sperm activity ability is poor, move only in place.

Table 4. Effects of DEHP+ GEN on weight and sperm count of testis and epididymis and malformation sperm rate

Groups	2500 mg/kg/day DEHP	2500 mg/kg/day DEHP + 50 mg/kg/day GEN	2500 mg/kg/day DEHP + 500 mg/kg/day GEN	Control group
Number	30	30	30	30
Weight of Testis	(1.56±0.37)g ^{*#}	(1.53±0.36)g [*]	(0.73±0.22)g	(2.24±0.56)g [*]
Weight of Epididymis	(0.42±0.09)g ^{*#}	(0.41±0.11)g [*]	(0.17±0.03)g	(0.58±0.13)g [*]
Organ Coefficient	(1.29±0.19)% ^{*#}	(1.26±0.15)% [*]	(0.62±0.07)%	(1.47±0.24)% [*]
Sperm count of Testis	1325±152 [*]	1285±220 [*]	670±125	1574±178 [*]
Sperm count of Epididymis	158±38(10 ⁶ /mL) [*]	147±40(10 ⁶ /mL) [*]	70±13(10 ⁶ /mL)	175±42(10 ⁶ /mL) [*]
Deformity rate	(3.0±0.32)% ^{&}	(3.4±0.28)% ^{&}	(7.4±0.65)%	(3.3±0.36)% ^{&}

Compared with group of 2500 mg/kg DEHP+500 mg/kg GEN, ^{*} $P<0.05$; Compared with group of 2500 mg/kg DEHP, [#] $P<0.05$; Compared with group of 2500 mg/kg DEHP+50 mg/kg GEN, group of 2500 mg/kg DEHP, control group, & $P<0.05$.

Table 5. Effects of DEHP + GEN on weight percentage of testis and epididymis

Group	2500 mg/kg/day DEHP	2500 mg/kg/day DEHP + 50 mg/kg/day GEN	2500 mg/kg/day DEHP + 500 mg/kg/day GEN	Control
Number	30	30	30	30
Weight(g)	243.5±28.72 [*]	238.5±16.69 [*]	244.4±15.84	247.2±26.95 [*]
Testis/ weight (%)	0.532±0.111 [*]	0.495±0.050 [*]	0.382±0.04	0.567±0.118 [*]
Epididymis/ weight (%)	0.148±0.015 [*]	0.144±0.007 [*]	0.135±0.005	0.157±0.019 [*]
Prostate/ weight (%)	0.112±0.012 [*]	0.098±0.025 [*]	0.067±0.009	0.118±0.013 [*]
Seminal vesicle/ weight (%)	0.171±0.018 [*]	0.154±0.015 [*]	0.128±0.005	0.178±0.021 [*]

Compared with group of 2500mg/kg DEHP+500mg/kg GEN, ^{*} $P<0.05$.

Table 6. Effects of DEHP + GEN on sperm activity level

Group	2500 mg/kg/day DEHP	2500 mg/kg/day DEHP + 50 mg/kg/day GEN	2500 mg/kg/day DEHP + 500 mg/kg/day GEN	Control
Number	30	30	30	30
Sperm Density (10 ⁶ /L)	55.75±15.63*	49.56±13.54*	42.75±11.23	58.25±5.23*
Sperm Activity Perc (%)	71.7±12.2*	65.7±11.34*	57.2±15.71	75.34±9.82*
Sperm Perc of (a+b) level (%)	46.7±11.53*	38.7±13.21*	32.7±13.82	43.5±12.32*
Sperm Perc of (c) level (%)	26.6±12.35	22.6±10.13	21.4±7.85	31.54±8.15
Sperm Perc of (d) level (%)	28.7±7.47	33.6±8.27	45.3±13.98 [#]	24.92±9.53

Compared with group of 2500mg/kg DEHP+500mg/kg GEN, *P<0.05; Compared with group of 2500mg/kg DEHP+50mg/kg GEN, group of 2500mg/kg DEHP and control group, [#]P<0.05.

As shown in Table 6, sperm density [(42.75±11.23)×10⁶/L], sperm activity rate [(57.2±15.71)%] in DEHP (2500 mg/kg/day) + GEN (500 mg/kg/day) group was significantly lower than that in DEHP (2500 mg/kg/day) + GEN(50 mg/kg/day) group [(49.56±13.54)×10⁶/L, (65.7±11.34)%] and DEHP (2500 mg/kg/day) group [(55.75±15.63)×10⁶/L, (71.7±12.2)%] and control group [(58.25±5.23)×10⁶/L, (75.34±9.82)%] (P< 0.05), sperm density and sperm activity rate in control group was significantly higher than that in other three groups (P< 0.05). (a + b) level sperm proportion [(32.7±13.82)%] in DEHP (2500 mg/kg/day) + GEN (500 mg/kg/day) group was significantly less than that in other three groups [(46.7±11.53)%, (38.7±13.21)%, (43.5±12.32)%] (P< 0.05), (d) level sperm proportion [(45.3±13.98)%] was significantly higher than that in other two groups [(28.7±7.47)%,(33.6±8.27)%,(24.92±9.53)%] (P< 0.05).

DISCUSSION

Over the years, governments, scientists and physicians are paying more and more attention to the potential link between environmental pollution and chronic diseases and started prevention campaigns in order to prevent the exposure. A big concern for the researchers is related to the exposure to environmental endocrine disruptors. These substances affect the reproductive, endocrine and immune systems and accumulate in the body having detrimental effects on our health, some of which may be inherited to the next generation (30). DEHP, one of the most common plasticizers, found in all types of products, acts as an endocrine disruptor, but the exact mechanism of toxicity remains unclear. There are some studies that correlate the utero and lactational exposure to different kinds of endocrine disruptors with cryptorchidism, hypospadias and decline semen quality in offspring (31-33).

The aim of this study was to evaluate the potential effects of DEHP on the occurrence of cryptorchidism to rats' males offspring and also to test the potential additive toxic effect of GEN on the male reproductive system.

Our findings suggest that exposure to DEHP may affect the estrous cycle and steroidogenesis, decrease sperm counts, sperm production and sperm cells viability in a dose dependent manner, these results being in accordance with the study carried out by Lee JY *et al.* (34).

Genistein (GEN), a weak estrogenic phytoestrogen has a dual impact on the male reproductive system. After low dose exposure, studies have shown that GEN has a protective role in DEHP induced toxicity on male reproductive system as it decreased epididymis and testis weight, decreased organ coefficients and decreased sperm activity (35). Contrary to the low dose effects, GEN in high dose (400 mg/kg.day) when combined with DEHP has additive toxic effects on male reproductive systems, decreasing sperm density, sperm activity, and weight of testis and epididymis (36), findings that are in accordance with our results. Further studies are required in order to investigate the risk of GEN combined with DEHP exposure on the male reproductive system.

This study demonstrates that DEHP exposure leads to the decline of testicular sperm production, affects testicular development, restrains the proliferation of granular cell and increases cell apoptosis. All these effects are aggravated when DEHP is combining with GEN in high doses having an additive effect on the DEHP-induced cryptorchidism in offspring rats.

Conflict of interest

There are no potential conflicts of interest relevant to this article to report.

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