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■ Original Research Article

The Effects of Di-2-Ethylhexyl Phthalate (DEHP) on Sex Hormones in Female Wistar Rats: A Randomized Single-blinded Trial

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Abstract

Background: Di-2-ethylhexyl phthalate (DEHP) is a ubiquitous environmental contaminant. Most rodent studies showed sex hormones abnormalities following exposure to high levels above the estimated daily human exposure. The effects of exposure on female rat sex hormones at levels similar to daily human exposure is unknown. **Aim:** To determine the effects of DEHP at environmentally relevant level on the sex hormones of female Wistar rats. **Methodology:** Forty adult female Wistar rats were randomized into two groups, an experimental group A and control group B. Rats in the experimental group were administered DEHP in oil daily for 12-15 days, those in the control group were administered equal amounts of oil daily for 12-15 days. All rats were housed under similar environmental conditions and sacrificed during oestrus. Serum levels of sex hormones were determined and compared. **Results:** The mean serum FSH levels were 39.266 ±14.469 IU/ml and 47.243 ± 3.310 IU/ml in the study and control groups respectively (p = 0.029). The mean serum LH values were 22.994 ± 11.336 IU/ml and 20.529 ± 8.878 IU/ml in the study and control groups respectively (p = 0.477). The median serum oestradiol levels were 240.000pg/ml and 306.700pg/ml (p = 0.632) in the study and control groups respectively. The median serum progesterone levels were 52.860ng/ml and 55.940ng/ml (p = 0.448) in the study and control groups respectively. **Conclusion:** Even at low level of exposure, DEHP is associated with significant reduction in FSH, with a non-significant increase in LH, and non-significant reduction in oestradiol and progesterone.

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INTRODUCTION

Phthalates are diesters of 1,2-benzenedicarboxylic acid also called phthalic acid.[1] They are a group of man-made chemicals with a wide spectrum of applications. Some of the commonly available phthalates include di-2-ethylhexyl phthalate (DEHP), di-isononyl phthalate (DiNP), di-n-octyl phthalate (DnOP) Diethyl phthalate (DEP), and dibutyl phthalate. They are generally used to make plastics more flexible and harder to break. Phthalates have been called 'the everywhere chemicals.[2] Diethyl-hexyl phthalate is an organic compound and is the most common phthalate available with about 3-4 million tons of the chemical produced annually.[3] It has been shown to alter the function of the endocrine systems and cause adverse effects in organisms.[4] It has high levels of human exposure. It has been detected in a wide range of consumer products, including personal care products and cosmetics,[5] infant toys,[6] food wraps, medical consumables, nutritional supplements, cleaning materials, lubricants, insecticides, solvents, adhesives and paints.[7]

The most common exposure to DEHP comes through food.[8] It can also leach into a liquid that comes

in contact with the plastic; it extracts faster into nonpolar solvents (e.g., oils and fats in foods packed in polyvinyl chloride packages). Fatty foods that are packaged in plastics that contain DEHP are more likely to have higher concentrations such as milk products, fish or seafood, and oils.[9]

Human exposure can also occur through the use of medical devices and medical procedures. DEHP is the most common phthalate that has been used as a plasticizer in medical devices such as intravenous tubing and bags, IV catheters, nasogastric tubes, dialysis bags and tubing, blood bags and transfusion tubing, and air tubes. For this reason, concern has been expressed about leachates of DEHP transported into the patient, especially for those requiring extensive infusions or those who are at the highest risk of developmental abnormalities, such as newborns in intensive care nursery settings, haemophiliacs, kidney dialysis patients, neonates, premature babies, lactating, and pregnant women.[10] Exposure can also occur through dermal contact and by inhalation.[11] It is also detected in indoor air,[12,13] indoor dust,[14,15] and air inside vehicles.[16]

Women are exposed to chemicals daily using personal care products such as cosmetics, deodorants and shampoo. Multiple phthalate metabolites have been found in the urine of women,[5] and the levels correlate positively with increasing use of these personal care products.[17] As a result of this, there are concerns about the potential health hazards of the chemical exposure in women, especially to DEHP women are exposed to daily.

Epidemiological investigation showed that the average daily dose of DEHP an adult from the general population is exposed to is 2.1mg/kg.[18] One study showed a positive correlation between DEHP levels and negative endocrine effects in females.[19]

The exact effect of the chemical in humans is not known, and most of the observations of its toxicity are obtained from animal studies.[18] Exposure to DEHP represents a public health concern, as it has been identified as one of the top contaminants present in human tissues. DEHP and its metabolites are present in over 95% of human blood samples and nearly 100% of human urine samples tested in different studies.[20,21] Also, DEHP and its metabolites have been found in human ovarian follicular fluid, indicating its ability to reach the ovary, even though they were not found to accumulate in the fluid in that study.[22]

Animal studies have shown decreased levels of serum oestradiol, FSH and LH in rats exposed to oral DEHP. However, in these studies the rats were exposed to DEHP doses of 300 mg, 1000mg and 3000mg daily and 1400mg twice weekly.[23,24] For the rats, these doses are well above the estimated levels of human exposure at about 2.1mg/kg/day.[18] The animal equivalent dose (AED) to this level of exposure for rats would be determined by multiplying the human exposure value by 6.2.[25] This would give an AED of 13.1mg/kg. With increasing knowledge about the endocrine disrupting effects of the chemical, there is increasing anxiety associated with the use of products containing DEHP especially in developed countries.

The choice of animal for this study is the female Wistar rat. Both rats and humans are mammals. The reproductive system of the female rat is under hormonal control and undergoes cyclical changes during the oestrus cycle based on the serum levels of these hormones, similar to what is found in humans.

An advantage of rats over mice is that rats are generally bigger and larger amounts of blood can be obtained from them for analysis. An advantage of rats over bigger animals like monkeys is that even though the overall physiology of primates is closer to that of humans, bigger animals are generally more difficult to obtain, handle and house. Also, it is easier to study a larger number of rats due to their smaller size. It is for these reasons that rats are suitable animal models for toxicological studies in reproductive physiology.

Despite the known effects of DEHP in animal studies, its use continues till today. This study was done with the aim of determining the effects of DEHP on the sex hormones of adult female Wistar rats with similar levels of the daily human exposure to the chemical, to determine the effects of such level of exposure. The objectives were to determine the serum sex hormone levels in DEHP exposed female Wistar rats, to determine the serum levels of sex hormones in female Wistar rats unexposed to DEHP, and to compare the serum sex hormone levels between the female Wistar rats exposed to oral DEHP and the female Wistar rats not exposed to DEHP.

It was hoped that this would give an insight into the possible effect of daily exposure to the chemical in humans, so as to help make recommendations regarding the safety of the chemical or allay some fears by showing its safety.

MATERIALS AND METHODS

Animals

Adult female Wistar rats, 2 months-6 months old each weighing 200 ± 20 g were purchased from the Human Physiology Department of Bayero University Kano. The rats of appropriate age were weighed and sequentially recruited if their weight was within 200 ± 20 g till the desired number of rats was obtained. The rats were housed in cages (5 rats per cage) with clean bedding made of saw dust and maintained under the same environmental temperature, humidity and light-dark cycle in the same laboratory.

Chemical

The DEHP was obtained from Sigma-Aldrich Corporation®, United States of America. It was 99.7% pure, at a concentration of 1g/ml. It was mixed with corn oil 10ml/kg before administration by oral gavage

daily at a dose of 13.1mg/kg. This dose was obtained by multiplying the average daily adult human exposure of 2.1mg/kg by the constant 6.2 to determine the AED.[25] Since the weight of the rats were about 200g, this gave a dose of 2.6mg per day. This was then rounded up and a dose of 3mg was administered daily. The chemical was administered daily in the morning between 9-10am.

To prepare the DEHP-corn oil mixture, dose calculation was done as follows:

- Concentration of DEHP was 1g/ml = 1000mg in 1ml
- Daily dose administered per rat was 3mg = 0.003ml
- Total dose of DEHP for 20 rats over 15 days = 0.003x20x15=0.9ml
- Daily dose of corn oil per rat was 10ml/kg (approximate weight was 200mg) = 2ml
- Total dose of corn oil for 20 rats over 15 days (approximate) = 2x20x15=600mls
- Therefore, 0.9mls of DEHP was added to 599.1mls of oil and mixed thoroughly, making a concentration of 3mg of DEHP in 2mls of the mixture. This was placed in a clean jar, covered and labelled as A. In a similar jar, 600mls of corn oil without the chemical was poured and labelled as B.

Study Design

It was a randomized controlled study.

Sample Size Determination

Since the endpoint was quantitative, the following formula was used to calculate the sample size for comparison between the two groups in animal studies:[26]

$$n = \frac{2SD^2(Z_\alpha + Z_\beta)^2}{d^2}$$

Where:

- n was the minimum sample size
- SD was the standard deviation. The standard deviation of oestradiol obtained from a previous study was 9pg/ml.[21]
- Zα was determined from a statistical table based on the value of the α-level of significance. For this study, it was set at 0.05. Therefore, Zα = 1.96.
- Zβ was determined from a statistical table based on the acceptable power of comparison between 2 groups. For this study, power of 95% (0.95) was used. Therefore Zβ = 1.64
- d was the difference between the mean values that was considered significant. For this study a change in serum oestradiol level by 10pg/ml or more was considered significant.

Substituting the values:

$$n = \frac{2\ x\ 9^2(1.96 + 1.64)^2}{10^2}$$

$$n = 17.96$$

To this value, 10% was added as attrition to account for any animal that might die during the experiment or that might be later excluded, giving a sample size of 19.75. This was rounded up and 20 rats were assigned to each group.

Randomization and Blinding

The 40 rats were housed in groups of 5. There were 8 cages numbered 1-8. The individual rats were numbered 1-1 to 8-5 indicating the cage number and the animals' identification number. The animals were marked using a marker. The rats were then grouped into 2, with 4 cages (20 rats) in each group. The cages were numbered serially from 1 to 8, and each cage was assigned a group by balloting by a research assistant. The same research assistant prepared 8 opaque envelopes that contained pieces of paper marked as A or B, and had no further role in the study. The research assistant responsible for administration of the chemical drew an envelope for each of the cages. Rats in the cages belonging to group A comprised the study (experimental) group while those in group B were the control group. The cages were then labelled as A or B. Rats in group A received the chemical-oil mixture daily by oral gavage while rats in group B only receive the corn oil daily at the same dose of 2ml. A different instrument was used to administer the oil in the two groups to avoid contamination.

The research assistants administering the chemical was aware of the group allocation of the rats and this was recorded in a book which was kept at the laboratory. Also, there was daily recording of the chemical administered to each animal. At the end of the experiment period, the research assistant who administered the chemical and oil euthanized the animals by decapitation and the trunk blood was collected for the serum hormones in labelled plain bottles, then transported to the laboratory immediately. The researcher and research assistants testing for the serum hormones were blinded to the rats' group. This was only known by the researcher after completing the experiment (form the record book at the laboratory) before data analysis.

Data Collection Techniques

All rats were allowed free access to food and water from the same source and of the same composition. A study protocol was discussed with the research assistants and a copy of the study protocol was given to them. Daily administration of the chemical-oil mixture to rats in group A and oil to rats in group B

was done for 12-15 days, and ended on oestrus day of the oestrus of the cycle. This was confirmed before euthanasia by noting the presence of lordosis and vulva swelling. Examination for features of oestrus was commenced on 12th day with daily euthanasia of those found to be in oestrus till the 15th day. A picture guide was placed in the laboratory to guide identification of vulva swelling and to ensure accurate timing of oestrus. Twelve to fifteen days was used since the duration of the oestrus cycle is 4-5 days. The chemical was administered for 3 cycles. Rats found to be in oestrus by the 12th day were euthanized, those not in oestrus were administered the chemical for 2 more days and euthanized once in oestrus. Those who failed to achieve oestrus after 15 days were to be excluded from the study, though all had reached oestrus by the 15th day. A fixed timing (oestrus) was chosen because hormone levels vary at different times during the oestrus cycle.

At the end of experiment, all the rats were weighed and their weights were recorded. The rats were euthanized by decapitation, blood was collected from the trunks into sterile plain well-labelled sample bottles and allowed to clot for about 2 hours at room temperature. At least 5mls of blood was collected from each rat. The clotted blood was then transported to the laboratory where each sample was centrifuged at 1000 revolutions per minute for 10 minutes, and the serum was separated and transferred into sterile plain bottles using a pipette (different pipette for each sample). The serum was then stored in the refrigerator at about -4°C temperature before hormone analysis. The serum levels of FSH, LH, oestradiol and progesterone were determined.

Blood Tests

At the laboratory, serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), progesterone (P4), and oestradiol (E2) were measured by Enzyme-linked Immunosorbent Assay (ELISA) technique. For determination of FSH and LH, rat FSH and ELISA kit and rat LH ELISA kit from Wuhan Fine Biotec Co Ltd, China were used, the manufacturer's instructions were strictly followed. For the determination of oestradiol and progesterone, oestradiol rat/mouse ELISA kit, and progesterone rat/mouse ELISA kit from Demeditec diagnostics, Germany was used strictly adhering to the manufacturer's instructions.

The microplates were read using UNIEQUIP ELISA Reader, United Kingdom.

Calculation of Concentrations

The position of each sample in the wells was recorded. For each test, the standard concentration (obtained from the kit) and absorbance of the standards were put in an Excel spread sheet and a standard curve was plotted with the concentration on the x axis and the absorbance (OD450) on the y axis. This curve was then used to determine the concentration that corresponded to the mean absorbance of each serum sample obtained (computer generated values), giving the serum hormone concentration. The values were then copied onto an SPSS 20.0 spread sheet and analysed.

Statistical Analysis

This was done using Excel and SPSS 20.0 statistical software. The data was presented in tables. Continuous data that was normally distributed was summarized using mean and standard deviation, while Student's T test was used to compare means between the groups for any statistically significant difference. Continuous data that was not normally distributed was summarized as median and inter quartile range (IQR), while Mann-Whitney U test was used to test for any clinically significant difference between the values. The level of significance was set at $p < 0.05$.

Ethical Clearance

The study was conducted at the department of Human Physiology laboratory, Bayero University Kano. Ethical clearance was obtained from the research ethics committee of the College of Health Science, Bayero University Kano (BUK/CHS/REC/01/17). The minimum number of animals needed to obtain a scientifically valid result was used. The rats were allowed free access to food and water and they were not maltreated. Decapitation at the end of the experiment was quick using a very sharp blade to reduce undue suffering in the animals.

Safety while handling the animals and using the chemical was ensured for the researcher and the research assistants. Hand gloves were provided for daily use and disposed daily after handling the animals and chemical, hand sanitizer was also provided. Disposable facemasks were also provided and used when handling the chemical to prevent inhalation.

RESULTS

Five of the rats died by the end of the study, 3 in the study group and 2 in the control group, therefore results were obtained from 17 (85%) rats in the study group and 18 (90%) rats in the control group. Table 1 shows the serum hormone levels of the rats in the two groups. Table 2 show the changes in hormone levels observed in the study group compared to the control group. it shows there was a significant decrease in FSH level, with non-significant increase in LH, decrease in E2 and decrease in P4.

Table 3 shows the mean and median serum levels of the hormones as well as the mean weight of the rats at the end of the experiment, and the p value

after comparing the means/medians. The mean serum FSH level in the study group was 39.266IU/ml while in the control group it was 47.243IU/ml. the value was significantly lower in the study group (p = 0.029). The mean serum LH values were 22.994IU/ml and 20.529IU/ml in the study and control groups respectively, and the difference was not statistically significant (p = 0.477). The median serum oestradiol level was lower in the study group (240.000pg/ml) than in the control group (306.700pg/ml), though the difference was not statistically significant (p = 0.632). The median serum progesterone level was also lower in the study group (52.860ng/ml) than in the control group (55.940ng/ml), again this difference was not statistically significant (p = 0.448). The mean body weight of the rats in the two groups were 198.880g and 204.06g, and the difference was not statistically significant (p = 0.238).

Table 1: Serum hormone concentrations of the rats.

Serial number	FSH (IU/ml)	LH (IU/ml)	E2 (pg/ml)	P4 (ng/ml)
Study group A (n = 17)	49.553	31.765	140.000	53.922
	44.158	22.415	156.667	86.546
	48.763	25.865	290.000	2.504
	44.684	22.515	323.333	103.496
	43.105	11.515	173.333	19.950
	51.526	23.715	373.333	103.681
	13.105	45.415	756.667	84.560
	49.158	22.965	123.333	26.687
	7.842	17.015	273.333	45.979
	41.921	11.915	240.000	52.858
	40.210	13.815	123.333	101.227
	50.211	27.165	240.000	47.255
	49.026	23.165	73.333	6.0496
	39.684	2.265	706.667	40.731
	9.0263	18.315	273.333	55.482
	41.526	23.115	156.667	104.277
	44.026	47.965	340.000	42.007
Control group (n = 18)	44.947	22.915	123.333	58.532
	46.526	45.765	356.667	2.504
	51.658	17.965	323.333	40.092
	44.026	22.965	290.000	71.440
	44.421	23.865	73.333	17.326
	38.368	20.365	340.000	15.837
	52.053	28.615	273.333	62.433
	50.079	12.015	390.000	59.099
	47.316	16.865	90.000	78.957
	44.026	22.865	56.667	8.63
	48.632	6.465	406.667	31.865
	49.158	20.765	490.000	62.007
	50.474	22.365	156.667	59.312
	49.158	20.765	490.000	38.887
	48.237	6.465	456.667	103.290
	46.921	10.765	540.000	53.355
	46.263	24.115	40.000	59.312
	48.105	23.615	240.000	1.936

Table 2: Changes in the mean/median hormone levels

Variable	Study group (n = 17)	Control group (n = 18)	Change observed in the study group	P value
Mean FSH (IU/ml)	39.266	47.243	Decrease by 7.977	0.029*
Median E2 (pg/ml)	240.000	306.700	Decrease by 66.700	0.632
Mean LH (IU/ml)	22.994	20.529	Increase by 2.465	0.477
Median P4 (ng/ml)	52.860	55.940	Decrease by 3.080	0.448

* = significant

DISCUSSION

The study showed significantly lower levels of serum FSH following administration of DEHP. Also, serum levels of oestradiol and progesterone were lower following administration of DEHP though the difference was not statistically significant. The level of LH however, was slightly higher in the study group though the difference was also not statistically significant.

Follicle Stimulating Hormone

Few studies of the reproductive effects of DEHP on females have been conducted.[11] However, the lower FSH value found in this study is similar to the findings in other studies.[23,24] One study found a significant reduction in serum FSH levels in rats exposed to high doses of DEHP.[24] The DEHP was found to cause inhibition of proteins necessary for mRNA production in the pituitary gland of the rats, resulting in low FSH levels in the exposed rats.[24] Another study also found reduced FSH levels following prolonged exposure to DEHP in female rats. In that study, DEHP led to increased level of mRNA in the hypothalamus and increased level of gonadotropin releasing hormone (GnRH). This increase in mRNA level was seen in the rats exposed to lower dose of DEHP (300mg/kg) but not in the rats exposed to higher dose (1000mg/kg), the reason for this was unclear.

The authors hypothesized that the increased GnRH could have led to desensitization of the pituitary gland to the effect of GnRH leading to reduction in the level of FSH.[23] FSH is important for growth and maturation of ovarian follicles. Low levels could therefore cause insufficient or delayed oocyte growth and maturation and this could cause anovulation and infertility or subfertility.

Luteinizing Hormone

Serum LH levels in this study were similar in the two groups though slightly higher in the study group. This is different from the findings in earlier studies where levels of LH were found to be lower following DEHP exposure. The reason for this is unclear, perhaps DEHP

acts through different mechanisms to cause endocrine disruption at different doses and different durations of exposure, as evident by increased GnRH levels seen following exposure to lower dose but no such increase was seen following exposure to higher dose in one study.[23] In the previous studies the doses of DEHP used were much higher than the dose used in this study, and the durations of exposure were also much longer. This could be responsible for the observed differences.

Table 3: Comparison of mean/median serum hormone concentration and mean weight.

Variable	Study group A (n = 17)	Control group B (n = 18)	Test	P value
Mean FSH ± SD (IU/ml)	39.266 ± 14.469	47.243 ± 3.31	t = -2.279	0.029*
Mean LH ± SD (IU/ml)	22.994 ± 11.336	20.529 ± 8.878	t = 0.719	0.477
Median E_2, IQR (pg/ml)	240.000, 148.300-331.700	306.700, 115.000-419.200	U = -0.086	0.632
Median P_4, IQR (ng/ml)	52.860, 33.710-93.890	55.940, 16.950-62.110	U = 1.102	0.448
Mean weight ± SD (g)	198.880 ± 13.350	204.060 ± 12.129	t = -1.201	0.238

* significant, t = Student's t test, U = Mann-Witney U test

The clinical significance of low FSH in the presence of higher LH levels in unclear. In humans however, high levels of LH with low FSH can be seen in polycystic ovarian syndrome, which is the commonest cause of anovulatory infertility. Perhaps a similar disorder could occur in the rats following exposure to low levels of DEHP as was done in this study. One study showed exposure to DEHP at high levels of 3g/kg/day was associated with anovulation and ovarian morphology of polycystic ovaries, even though those rats exhibited vaginal oestrus.[27]

Oestradiol

The serum levels of oestradiol were lower in the study group than in the control group but the difference was not significantly different. This is in contrast with findings from other studies that noted significant decrease in levels of oestradiol, though much higher doses of DEHP were used for longer durations of time.[23,24] It is possible that prolonging the duration of exposure could cause further decline in the oestradiol levels to significant levels even when the dose of DEHP is low as was done in this study, though further research is needed. The lower levels of oestradiol could be a consequence of the low FSH levels in the study group.

Progesterone

The serum levels of progesterone were statistically similar to the levels in the control group though the levels in the study group were slightly lower. Again this is contrast with the findings from other studies, in which significant lower levels of progesterone were found, suggesting anovulation in the rats.[23,24] The longer periods of exposure and higher doses of the DEHP used in those studies may be responsible for the differences in observation.

The low level of FSH, higher level of LH and lower levels of both oestrogen and progesterone appear to be in keeping with the biochemical findings in the presence of polycystic ovaries and anovulation. It is possible that some of the rats were anovulatory while others were not fully anovulatory due to the shorter period of exposure to the chemical. Perhaps the full biochemical picture would be made clearer by increasing the duration of exposure to DEHP, but further research is needed to confirm this. This would be important as human exposure to the chemical normally occurs over long periods of time. Another possible explanation would be that the increase in LH observed was as a result of a flare effect of the chemical in the early periods of exposure with increase in amplitude of GnRH release in favour of more LH secretion, and the low LH observed in other studies is due to subsequent down regulation of gonadotropin receptors in the pituitary, and this might have occurred if the duration of exposure was increased. Further research is however needed to confirm this.

CONCLUSION

Short term exposure to low dose of DEHP is associated with a significant reduction in the serum level of FSH, with a slight non-significant increase in LH, but no significant reduction in the levels of oestradiol and progesterone. The exact implication of these findings on fertility is not clear, but it may lead to poor follicle growth and maturation, anovulation and polycystic ovaries. Further research is needed to fully evaluate the implication of these findings on fertility in the rats.

Limitations

1. Only the oral route of exposure was tested, even though human exposure to DEHP can occur through other means such as inhalation and intravenous route.

2. The experiment can only determine the short-term effects of exposure to DEHP on the sex hormones, the long effects of exposure were not studied.

Recommendations

1. Awareness should be raised about the possible endocrine toxicity of DEHP even at low levels of daily exposure in humans based on the result of this animal study.
2. Further research can be conducted on the effects of long-term exposure to DEHP at environmentally relevant levels.
3. There is also the need for further research into safer alternative plasticisers that can be used in the plastic industry.

Conflict of Interest

The researchers have no conflict of interest to declare.

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