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Effect of Phthalates residues in plastic bottled waters on reproductive toxicity and oxidative stress in male rabbits

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Abstract.

Phthalates are a genealogy of man-made chemical compounds improved in the last hundred to be employment in the devise of plastics, solvents, and personal care products. They are colorless, odorless, oily liquids that do not evaporate easily and do not. Phthalate esters are widely used as plasticizer. They can migrate from plastic materials to the environment. Exposure to some of these compounds may increase the risk of getting cancer. The experiment was designed to study toxic effects of Di-(2-ethylhexyl) phthalate (DEHP) on semen characteristics, testosterone levels, testicular lipid peroxidation and testicular antioxidants in male New-Zealand white rabbits for 12 weeks. Results obtained showed that DEHP significantly ($P<0.05$) decreased libido (by increasing the reaction time), ejaculate volume, sperm concentration, total sperm output, sperm motility (%), total motile sperm per ejaculate (TMS), packed sperm volume (PSV), total functional sperm fraction (TFSF), normal and live sperm and semen initial fructose. Also, testosterone levels, body weight (BW), relative weights of the testes (RWT) and epididymis (RWE) were decreased. Thiobarbituric acid-reactive substances and lactate dehydrogenase were increased, while glutathione S-transferase, transaminases and phosphatases were decreased in seminal plasma of rabbits treated with DEHP compared to control. Chemically bind to the material they are added to quality assurance scheme for residue monitoring in water is quite important. Although, one cannot avoid phthalate contamination in bottled waters due to the manufacturing process, but at least special care should be taken regarding their storage conditions.

Keywords: *Phthalates (DEHP), New-Zealand white rabbits, male and testosterone.*

الملخص

الفثالات هي مجموعة من المركبات الكيميائية التي صنعها الإنسان والتي تم تحسينها في العقود الأخيرة حيث تم تواجدها في المواد البلاستيكية والمذيبات ومنتجات العناية الشخصية. فهي عديمة اللون والرائحة و هي من السوائل الزيتية التي لا تتبخر بسهولة. استرات الفثالات تستخدم على نطاق واسع مثل اللدائن يمكن لهذه المواد الانتقال من المواد البلاستيكية إلى البيئة. قد يزيد التعرض لبعض هذه المركبات من خطر الإصابة بالسرطان. صُممت هذه التجربة لدراسة التأثيرات السمية لفثالات دي-(2-إيثيل هكسيل) (DEHP) على خصائص السائل المنوي ومستويات التستوستيرون ودهون بروتينية في الخصية ومضادات الأكسدة الخصوية في الأرانب البيضاء النيوزيلندية الذكور لمدة 12 أسبوعًا. أظهرت النتائج أن DEHP قد انخفض بشكل معنوي ($P <0.05$) من الرغبة الجنسية (بزيادة وقت التفاعل) ، حجم السائل المنوي ، تركيز الحيوانات المنوية ، إجمالي إنتاج الحيوانات المنوية ، حركة الحيوانات المنوية (٪) ، الحيوانات المنوية الكلية لكل قذف (TMS) ، حجم الحيوانات المنوية المعبأة (PSV) ، إجمالي جزء الحيوانات المنوية الوظيفية (TFSF) ، والحيوانات المنوية العادية والحيوية و الفركتوز الأولي للسائل المنوي أيضا ، انخفضت مستويات هرمون التستوستيرون ، ووزن الجسم (BW) ، والأوزان النسبية من الخصيتين (RWT) والبربخ (RWE). تم زيادة المواد المتفاعلة مع حمض الثيوباربيتوريك ونزع الهيدروجين اللاكتات ، في حين انخفضت الجلوتاثيون S-ترانسفيراز ، الترانسامينيسات والفوسفاتازات في البلازما المنوية للأرانب المعالجة بـ DEHP مقارنة بالسيطرة. عليه فان اضافة المواد التي ترتبط به ارتباطا كيميائيا تعتبرمن خطط ضمان الجودة لرصد بقاياه في الماء. وعلى الرغم من ذلك ، لا يمكن تجنب تلوث الفثالات في المياه المعبأة في زجاجات بسبب عملية التصنيع ، ولكن يجب على الأقل توخي الحذر بشأن ظروف التخزين الخاصةبهم.

الكلمات المفتاحية: الفثالات (DEHP) ، ذكورالأرانب البيضاء النيوزيلندية ، التستوستيرون

1. INTRODUCTION

The use of bottled water is really widespread. For instance, more than 53 000 m^3were drunk in Europe in 2004, which was the biggest annual consumption, thus far "(Gleick et al., 2006)". Polyethylene terephthalate is a semi-crystalline polymer belonging to the family of polyesters. It is the most widespread polymer used for the manufacture of food contact packaging and moving pictures, especially for beverages and drinking water "(ILSI, 2000)". Numerous studies have investigated the interaction of phthalates bottles in contact with drinking water. These studies focused on the release of phthalates initial reactants, reaction by products and plastic additives into bottled water. The monitoring of many substances of migration controlled processes that simulate actual storage conditions with respect to time, temperature, and sunlight exposure has also been widely covered "(Franz et al., 2004; Feigenbaum et al., 2005; Vitrac et al., 2007; Welle and Franz, 2008; Franz and Welle, 2009)". Phthalates can be absorbed through food or inhaled through contaminated air. Dermal contact with care products that contain phthalates and medical devices contaminated with phthalates are another possible source of exposure "(Hernandez-Diaz *et al.,* 2009)".Endocrine disruptors are compounds that mimic or antagonize the actions of natural estrogens, and are the most common form of endocrine disruptor activity "(NRC, 1999; ICCVAM, 2003, 2006)". These compounds alter the hormone system involved in many biological metabolisms and can produce many health-related problems, such as early puberty in females, reduced sperm counts, altered functions of reproductive organs, obesity, altered gender-specific behaviors, and increased rates of some breast, ovarian, testicular, and prostate cancers "(Kabuto *et al*., 2004; Newbold *et al*., 2004; Della Seta *et al*., 2006;Patisaul *et al*., 2009)".Phthalates are suspected to interfere with the thyroid hormone system "(Ghisari and Bonefeld-Jorgensen, 2009)", a system vital to normal brain development in the fetus and infant "(Berbel *et al.,* 2010)".Contamination of bottled water by endocrine disruptors could happen at the different steps of the bottling process, namely: untreated groundwater from a spring, supply pipes or the filling and cleaning of containers in the bottling process "(Montuori et al., 2008; Wagner and Oehlmann, 2009; Sax, 2010)". Furthermore, for some authors plastic bottle stress (UV radiation and heat) could also be a source of endocrine disruptors "(Yang *et al.,* 2011)".Di-2ethylhexyl phthalate (DEHP) is by far the most commonly used plasticizer, annual production being 1-4 million tons. From the late 1960s, leaching of DEHP from PVC formulations, human exposure and, more recently, tissue deposition have been documented "(Latini, 2000 and Tickner*et al.,* 2001)". In particular, DEHP induces a wide range of developmental and reproductive toxicities in mammals"(Parmar*et al.,* 1986)", while its ability to produce toxicity to humans remains uncertain. Limited studies in human populations suggest an association between phthalate exposure and adverse reproductive health outcomes in both genders. In fact, chronic occupational exposure to high levels of phthalates is associated with decreased rates of pregnancy and higher rates of miscarriage and an ovulation in female factory workers"(Aldyreva *et al.,* 1999; Hoyer, 1999)". DEHP is a well-characterized reproductive system toxicant; it is a member of the phthalate chemical family, plasticizers that have potential endocrine-disrupting effects. DEHP and its metabolites alter proper testicular development in fetal rat models "(Chauvigne, 2009)".It also is capable of disturbing the reproductive process by mimicking antagonizing steroid hormone action "(Sharpe, 2001)", and its effects on testosterone, luteinizing hormone or estrogen-like activity have been reported "(Akingbemi *et al.,* 2004; Latini *et al.,* 2004)". After entering the body, phthalates undergo rapid metabolism to monoesters and can also be oxidized further to oxidative metabolites "(Engel *et al.*, 2010)". Phthalate esters can migrate from plastic materials to the environment, and often found in water, soil, air, food products and the human body "(Castillo *et al.*, 1998)". The fact that some phthalates including DEHP has been found in environmental samples, shows it can be released during use and migrate from the packaging to contaminate the contents such as foods and beverages "(Guart, *et al.*, 2011)".This work was therefore carried out in view of the action of DEHP residues in plastic bottled waters on reproductive toxicity and oxidative stress in male rabbits.

2. MATERIALS AND METHODS

In this study DEHP (purity 99.0%) was purchased from Sigma–Aldrich (USA). All other chemicals used in the experiment were of analytical grade. Mature male New Zealand White rabbits (age of 7 months and initial weight of (2.917 ± 28.9 Kg) were used. Ten mature male rabbits were randomly divided into couple equal groups (each five rabbits): Group I: Rabbits were used as control and received an equivalent volume of the vehicle (corn oil) alone by oral gavage daily for 12 successive weeks. Group II: Rabbits were treated daily with DEHP by gavage at a dose of 500 mg/kg B.W/day (1/50 of DEHP lethal dose "(Dalsenter *et al.*, 2006; Song *et al.*, 2009)" for 12 successive weeks. Semen collection was done weekly and continued throughout the 12-week experimental period. Ejaculates were collected using an artificial vagina and a teaser doe. The volume of each ejaculate (EV) was recorded (using a graduated collection tube) after removal of the gel mass. A weak eosin solution was used for evaluation of sperm concentration (SC) by the improved Neubauer hemocytometer slide (GmbH + Co., Brandstwiete 4, 2000 Hamburg 11, and Germany"(Smith and Mayer 1955)". Total sperm output (TSO) calculated by multiplying semen ejaculate volume and semen concentration. Determination of initial fructose concentration (IF) in seminal plasma was determined immediately after semen collection"(Mann, 1948)". Assessments of dead and normal spermatozoa were performed using an eosin-nigrosine blue staining mixture (Blom, 1950). The percentages of motile sperm (SM) were estimated by visual examination under low-power magnification (10x) using light microscope. Total number of motile sperm (TMS) was calculated by multiplying the percentage of motile sperm and total sperm obtained. Reaction time (RT) was determined as the moment of subjecting a doe to the buck until the completion of erection; it was measured in seconds. Initial hydrogen ion concentration (PH) was determined immediately after collection using PH cooperative paper (Universalindikator PH 0-14 Merck, Merck KgaA, 64271 Darmstadt, Germany). Packed sperm volume (PSV) was recorded. Total functional sperm fraction (TFSF) was calculated as the product of total sperm output, motility (%), and normal morphology (%)"(Correa and Zavos, 1996)".

Seminal plasma thiobarbituric acid-reactive substances (TBARS) were measured in the seminal plasma using the method"(Cho *et al.*, 2002)". Seminal plasma glutathione content (GSH) was determined using commercial glutathione reductase kits according to the method of "(Yang*et al.*, 2011)". Superoxide dismutase (SOD) activity was assayed according to "(Fitzenberger*et al.*, 2014)". Lactate dehydrogenase (LDH) activity was measured. After incubation of the testes or Sertoli cells in the absence or presence of the glyphosate–Roundup at nominal concentrations ranging from 0.72 to 360 ppm for 30 min, the incubation medium was collected for determination of extracellular LDH activity by a spectrophotometric method. The estimation of LDH activity was carried out by measuring the oxidation of NADH and the results were expressed as U/L/mg of protein. Blood samples were spun at 2500 rpm for 10 minutes in a table top centrifuge. The serum samples obtained were analyzed to determine the concentration of testosterone. The analysis was carried via the tube-based enzyme immunoassay (EIA) method. The protocol used for the hormone was according to the method described for the kit (Immunometrics Limited UK) and meet the WHO standards in research programme for human reproduction. Catalase activity was determined using the Luck method involving the decomposition of hydrogen peroxide"(Hwang *et al.*, 2006)".

3. RESULTS

Table 1 was shown the overall means of the data of different parameters. Administration of DEHP caused significantly decreased in most parameters including the body weight, testicular weight, EV, SC, TSO, IFSM, TMS, PH, PSV, TFSF, GSH, SOD, LDH and testosterone compared to control. However, treatment with DEHPcaused significantly increases in the RT and TBARSin the experimental rabbits as compared with the control.

Table 1: The overall means (±SEM) of different parameters in male rabbits which treatment with DEHP compared to control.

Parameters	Groups	
	Control	**(DEHP) (500 mg/kg B.W/day)**
Body weight (gm)	3402 ± 28.2	3019 ± 29.9***↓
Testes weight (g/100 g body weight)	0.207 ± 0.013	0.154 ± 0.007**↓
EV (ml)	0.74 ± 0.017	0.63 ± 0.018**↓
SC	263± 4.5	228 ± 4.6**↓
TSO ($\times 10^6$)	195 ±5.1	147 ± 6.1***↓
IF (mg/dl)	257 ± 3.9	201 ± 5.9***↓
SM (%)	68.2 ± 0.7	61.3 ± 0.9**↓
TMS ($\times 10^6$)	133 ± 4.1	91 ± 4.4***↓
RT (sec.)	4.05 ± 0.099	5.13 ± 0.204*↑
PH	7.83 ± 0.022	8.02 ± 0.031*↓
PSV (%)	15.3 ± 0.16	12.9 ± 0.21***↓
TFSF ($\times 10^6$)	108± 3.4	71 ± 3.7***↓
TBARS (nmol/ml)	1.728 ± 0.025	1.978 ± 0.063*↑

GSH (g/dl)	5.7 ± 0.10	5.2 ± 0.09**↓
SOD (%)	1.154 ± 0.022	1.041 ± 0.029**↓
LDH (U/L)	1268 ± 14.0	1172 ± 16.4***↓
Testosterone (ng/mL)	1.59 ± 0.034	1.05 ± 0.069**↓

*P<0.05, values expressed as mean ± SEM, n= 5

4. DISCUSSION AND CONCLUSSION

Phthalates have been detected in the atmosphere "(Xie *et al.,* 2006)", in aquatic environments "(Peijnenburg and Struijs, 2006; Oehlmann *et al.*, 2008)". The present results indicated that treatment with DEHP caused significant reductions in body weight (BW) and relative testes weight (RTW) (Table 1). The reduction in BW and RTW of the DEHP treated rabbits is in agreement with those reported in previous studies "(Farombi *et al.*, 2007; Pereira *et al.*, 2008; Zhou *et al.*, 2010)". Also, Api (2001) found that rats treated with DEP at different dose 250, 1250 or 2500 mg/kg bw/d for 2 years caused decrease in body weight gain without without shrinkage in food consumption at 5% throughout the study. Testes weight is largely dependent on the mass of the differentiated spermatogenic cells. The observed reduction in the weight of testes was due to the decreased number of germ cells and elongated spermatids in the testes "(Aly *et al.*, 2009)".The present study showed that DEHP caused deterioration in semen characteristics EV, SC, TSO, IFSM, TMS, PH, PSV, TFSF, GSH, SOD, LDH and testosterone compared to control of male rabbits (Tables 1). This means that DEHP has reproductive toxicity in male rabbits. Previous studies also demonstrated that semen of different animal species was adversely affected by DEHP. The obtained results were in agreement with those reported by "Gray *et al.*, (2000); Parks *et al.*, (2000); Moore *et al.*, (2001)" in rat who reported that DEHP can adversely affect reproduction, including semen quality, Also, "Gray *et al.*, (2000); Akingbemi *et al.*, (2001)" reported that DEHP has anti-androgenic activity and male reproductive toxicity. The present study suggested that the decline in previous parameters can be partly attributed to the DEHP-induced reduction in plasma testosterone level, increased the levels of free radical (TBARS) and decrease in the antioxidant enzymes (Tables 1). "Zhou *et al.*, (2011)" reported that DEHP treatment resulted in increased oxidative stress and TBARS formation in epididymis of adult male rats that resulted in decline in both sperm count and quality. The adverse effects of DEHP on the development of male reproduction might be mediated by the formation of reactive oxygen species (ROS), that have been shown to induce DNA damage and may accelerate the process of germ cell apoptosis, leading to the decline in sperm counts associated with male infertility "(Agarwal *et al.*, 1986)". This work showed increased TBARS and decreased SM, suggesting that a decline could be attributed partly to concomitant reductions in semen fructose and/or testosterone production following DEHP exposure. Fructose formation by the accessory glands is dependent upon testosterone secreted by the testis "(Atterwill and Steele, 1987)". Accordingly, the observed reduction in IF suggests a corresponding decrease in testosterone secretion by DEHP exposure. Motility is critical in enabling the sperm to ascend the female reproductive tract to the site of fertilization and also is necessary to achieve fertilization "(Aitken, 1990)".The present study showed that DEHP caused decreased in the activity and concentration of antioxidant enzyme GSH and SOD in plasma (Tables 1). The decrease in antioxidant enzyme and GSH corroborated the findings of "Acharya *et al*., (2004)" who found a decrease in the activities of testicular antioxidant enzymes in mice. Induced oxidative stress may result in lipid peroxidation and fall in intracellular ATP levels which decrease sperm motility "(Verma and Kanwar, 1999)".The most important biological reaction of spermatozoa is fructolysis (in addition to the motility, concentration, PH, morphology, and live-dead tests) for use as short-time tests in determining the quality of semen "(Herman and Madden, 1950)". Thus, the increase in abnormal sperms in the phthalate-treated rats may be due to both testicular dysfunction and impairment of epididymal function. The fructose formation by the accessory glands is dependent upon the secretion of testosterone by the testes "(Atterwill and Steele, 1987)".

Overall this compound has been shown to effect on reproductive toxicity and oxidative stress in male rabbits. Many studies are directed to determine the correlation between concentration and *in vivo* or *in vitro* estrogenic or anti-estrogenic effects. It is described in the literature that especially phthalate esters may migrate from food contact plastics to the feed or water"(Fasano, *et al*., 2012)".Comparing the results of analyses of bottled water before and after storage, "Casajuana and Lacorte (2003)" concluded that poor storage conditions including 10 weeks outdoors at temperatures of up to 30°C increased the concentrations of DBP, BBP and DEHP in bottled water. After exposure, with mean concentrations of DBP, BBP and DEHP were 0.046 µg/L, 0.010 µg/L and 0.134 µg/L, respectively. It is also difficult to compare the reported results due to the variety of parameters favoring the release of substances (contact time, type of simulant, temperature, sunlight exposure and bottle color) into drink water. Considering all these difficulties and controversies, further investigations are needed to clearly identify the migration products from PEHP and to ensure that the consumption of PEHP bottled water does not involve any health hazards.

Although compounds used for the manufacture of plastic packaging are carefully controlled, the stressing of material during their production can change the chemical structures and generate degradation products, which may have an estrogenic activity "(Yang *et al*., 2011)". Future epidemiological and toxicological studies are needed to determine to what extent human exposure to phthalates impairs reproductive function and hence, human health. It is necessary to combine toxicological data and chemical analysis, especially when the responses are positive, and to determine the possible entry pathways and concentration of compounds to minimize their risk.

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