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Toxicokinetics of di(2-ethylhexyl) phthalate (DEHP) and its effects on luteal function in sheep

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ABSTRACT

The aim of the present study was to determine the toxicokinetics of short-term exposures to di(2-ethylhexyl) phthalate (DEHP) and its effects on ovarian cyclicity and luteal function using a sheep experimental model. For establishing the model, we examined the clearance of DEHP after intravenous (i.v.) and intramuscular (i.m.) administration of a single dose of 25 mg/kg body weight (b.w.) and after i.m. administration of two different doses (25 and 50 mg/kg b.w.; DEHP25 and DEHP50, respectively) three times a week for two months. Results showed a significant, dose-dependent effect of DEHP administration, when compared to the control group (CTL; untreated ewes; $n = 6$), on the duration of the ewes' estrous cycles (17.1 $\pm$ 0.5 days, CTL; 15.1 $\pm$ 0.9 days, DEHP25; 12.0 $\pm$ 0.8 days, DEHP50; $p < 0.05$); 94.9% of the cycles were of regular duration (15–19 days) in CTL, but only 51.1% and 25.4% in DEHP25 and DEHP50, respectively. Corpora lutea (CL) were smaller in DEHP50 than in DEHP25 ($p < 0.05$) and were smaller in both groups than in CTL ($p < 0.005$), but the maximum plasma concentrations of progesterone were greater ($p < 0.05$) in DEHP25 and DEHP50 than in CTL. In conclusion, the exposure of cycling ewes to DEHP causes shortening of the ovulatory cycles due mainly to a reduction in the size and lifespan of CL. However, the exposure to the phthalate is also associated with an increase in circulating concentrations of progesterone, suggesting the influence of DEHP on steroid metabolism.

1. Introduction

Di(2-ethylhexyl) phthalate (DEHP) is widely used as a plastic softener in manufacturing of various products made of PVC (polyvinyl chloride) such as toys, medical devices and food containers. Therefore, it can be found in different PVC-containing items [1–4], including food present in PVC-containers or dust present on the containers [5]. DEHP is loosely chemically bonded with PVC, thus readily leaches into blood [2,3,6–8]. DEHP is considered an endocrine disrupting chemical or contaminant (EDC). EDCs are xenobiotics, a class of substances including pesticides, industrial chemicals and their derivatives, some metals and an array of natural

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compounds, which produce deleterious effects on reproductive processes in animals and humans through an endocrine-like action [9–11].

There is a great deal of controversy regarding the reproductive effects of DEHP but several studies have reported the alterations induced by DEHP in the endocrine function of rodents [12–16] and pigs [10,17]. Various consequences of the exposure to DEHP in animals and humans have been described but the significance of such exposures for reproductive health has not been completely elucidated. Most of these studies have evaluated the effects of long-term exposures in males. Information on the possible effects on female reproductive function are scarce, although the exposure to high levels of phthalates in humans was associated with a decreased pregnancy and higher miscarriage rate [18], and with impeded gestational development [19]. Studies of DEHP reproductive toxicity in females have been performed in rodents [20–24], mainly using long-term exposure periods and/or high doses during fetal, perinatal and prepubertal periods [25–30]. Studies of short-term exposures of adult cycling females to xenobiotics require the integration of large data sets about the internal dose and exposure assessment, toxicokinetics, health outcomes and individual variations, which necessitates the availability of appropriate large animal experimental models [25,31].

The sheep is regarded as a good model for human reproductive processes due to close similarities in many aspects of reproductive physiology including the precise control of the ovulatory cycle [32,33]. Moreover, the monitoring of ovarian function by ultrasonography and measurements of hormones in blood samples are well-established techniques in ewes [34,35]. The body size and temperament of sheep, unlike that of small laboratory rodents, allow for frequent examinations and blood collections. In view of these considerations, the aim of the present study was to determine the toxicokinetics of DEHP during short-term exposures to the xenobiotic as well as to examine its effects on ovarian cyclicity and the morphometric and functional characteristics of the corpora lutea, using the cyclic ewes treated with DEHP as a model for human situation.

2. Materials and methods

2.1. Animals and experimental procedures

This study consisted of two consecutive experiments that employed a total of 30 adult (4–7 years old) ewes. Sheep were in good body condition, and were kept as a part of the larger herd outdoor with free access to indoor facilities, on the experimental farm of the INIA (Madrid, Spain; latitude 40°N). The farm meets the requirements of the European Union for Scientific Procedure Establishments and the experiment was performed under the Project Licence from the INIA Scientific Ethics Committee.

In the first experiment aimed to determine the kinetics of DEHP after administration of a single dose, a two-phase cross-over study was performed, with an interval of three months to avoid any carry-over effects. Sixteen animals were randomly allocated to two equal experimental groups ($n = 8$) and received DEHP (25 mg/kg b.w., active ingredient; Sigma–Aldrich, St. Louis, MO, USA) either by an i.v. (jugular vein) or i.m. (semitendinosus muscle) injection. All animals were examined for the occurrence of inflammation or tissue damage at the site of DEHP injection by visual inspection, palpation and temperature measurements. No pathological changes were observed that could alter the absorption of the drug.

In the second experiment designed to determine the kinetics of DEHP after administration of multiple i.m. doses and the effects of DEHP on ovarian cyclicity, ewes were treated with 25 mg/kg b.w. (DEHP25, $n = 8$) or 50 mg/kg b.w. (DEHP50, $n = 8$), whilst saline was administered to the control animals (CTL, $n = 6$). All solutions were injected deeply in the semitendinosus muscle, three times a week for eight weeks. The ensuing effects of DEHP on ovarian cyclicity were assessed by serial morphometric (ovarian ultrasonography) and functional (circulating progesterone concentrations) assessments of spontaneous (non-synchronized) ovulations and resultant corpora lutea, for the period of two months (*i.e.*, 3–4 cycles, since the mean duration of the estrous cycle in sheep is ~17 days).

2.2. Blood sampling

In the first experiment, serial blood samples were obtained at 0, 5, 10, 15, 20, 30, 45 and 60 min, 2, 4, 6, 8, 10, 12 and 24 h, and 2, 3, 4, 6, 8, 15, 22 and 29 days after DEHP administration to determine plasma DEHP concentrations. Afterwards, a control sample was collected at day 45 post-DEHP injection. In the second experiment, blood sampling was carried out three times a week for two months, just before each injection of DEHP, for determining plasma DEHP and progesterone concentrations.

In all groups, jugular blood samples of 5 ml each (in the first experiment drawn from the jugular vein contralateral to the vein into which DEHP was injected) were collected into lithium heparinized vacutainers (Vacutainer™ Systems Europe, Becton Dickinson, Meylan Cedex, France). Blood samples were immediately centrifuged at 2000 × *g* for 15 min and the plasma was stored at −20 °C until assayed.

2.3. DEHP analysis

Plasma samples (1 ml each) were placed in glass test tubes and mixed with 1 ml of NaOH (1 M) solution, 500 μl of acetonitrile and 2 ml of *n*-hexane. The mixture was vigorously vortexed for 2 min and centrifuged at 2500 × *g* for 5 min. The upper phase was transferred to another glass test tube and the lower portion was extracted again with 2 ml of *n*-hexane. The upper (organic) phase from the second extraction was added to the first one and the mixed samples were dried under nitrogen atmosphere at 40 °C. The dried extracts were reconstituted in 250 μl of acetonitrile, vortexed, mixed for 20 s, and then transferred to autosampler vials for HPLC analysis.

Measurements of DEHP concentrations was performed by a reversed-phase HPLC-UV method modified from the technique previously described [36]. The HPLC system consisted of Spectra-physic Series (Thermo Scientific, Essex, UK) components including a pump (P100), an autosampler (AS1000), and a