Theriogenology 80 (2013) 684–692

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Coumestrol and its metabolite in mares' plasma after ingestion of phytoestrogen-rich plants: Potent endocrine disruptors inducing infertility

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ARTICLE INFO

Article history:
Received 19 September 2012
Received in revised form 3 June 2013
Accepted 4 June 2013

Keywords:
Phytoestrogens
Coumestrol
Clover-mixed pastures
Alfalfa
Mare
Hyperestrogenicity

ABSTRACT

Phytoestrogens exist in plants that are present in forages fed to horses. They may compete with 17-β estradiol and influence the estrous cycle. Therefore, the objective was to determine whether coumestrol from clover-mixed pastures is present in mare's plasma after their ingestion (experiment I), and when this phytoestrogen was present in mare's plasma after ingestion (experiment II). The effect of a long-term ingestion of phytoestrogens on estrous cycle disruption was assessed (experiment III; clinical case). Experiment I was carried out in nonpregnant anestrous and cyclic Lusitano mares ($n = 14$) kept on clover and grass-mixed pastures, and supplemented with concentrate and hay or cereal straw. Blood and feedstuff were obtained from November to March. In experiment II, stabled cyclic Lusitano mares ($n = 6$) were fed for 14 days with increasing amounts of alfalfa pellets (250 g to 1 kg/day). Sequential blood samples were obtained for 8 hours after feed intake on Day 0 (control) and on Days 13 and 14 (1 kg/day alfalfa pellets). Experiment III mares were fed with a mixture of alfalfa and clover haylage for 5 months (group 1; $n = 4$) or for 9 months (group 2; $n = 12$). Estrous cycle was determined on the basis of plasma estradiol (E_2), progesterone (P_4), and ultrasound (experiment III). Concentrations of phytoestrogen coumestrol and its metabolite methoxycoumestrol were determined by high-performance liquid chromatography coupled with mass spectrometry. Phytoestrogens decreased in pasture from November until March ($P < 0.01$) (experiment I), but were always detected in mares' plasma. In experiment II, plasma-conjugated forms of coumestrol and methoxycoumestrol were higher on Days 13 and 14 than in control ($P < 0.05$). The highest concentrations of conjugated form of coumestrol were at 1.5 and 4 hours ($P < 0.001$), whereas its free forms peaked at 1 and at 3.5 hours after ingestion ($P < 0.05$). Methoxycoumestrol-conjugated form concentration was the highest at 1.5 and 5 hours ($P < 0.001$), whereas its free form peaked at 1 hour ($P < 0.05$) and at 1.5 hours ($P < 0.001$). Long-term intake of coumestrol caused lack of ovulation, uterine edema, and uterine fluid accumulation (experiment III). Coumestrol and methoxycoumestrol in both forms were higher in group 2 (while still ingesting haylage) than in group 1, after haylage withdrawal ($P < 0.001$). These data show that in the mare, coumestrol and its metabolite increase in

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0093-691X/$ – see front matter
http://dx.doi.org/10.1016/j.theriogenology.2013.06.002

blood after ingestion of estrogenic plants and can influence reproduction in mares as potent endocrine disruptors.

1. Introduction

Some plants present in pasture may contain "estrogenic compounds" known as phytoestrogens. Phytoestrogens similarity with 17β-estradiol (E_2), due to the presence of phenolic rings in their structure, enables their binding to estrogen receptors with the strongest affinity to estrogen receptor β (over 80%) and the weakest to estrogen receptor α [1]. Because phytoestrogens may compete with the endogenous estrogens, they may act as endocrine disrupters by influencing several endocrine mechanisms during the estrous cycle [2]. As reviewed by Burton and Wells [3], a number of studies on both animal and humans have reported the effects of the ubiquitous phytoestrogens on the development of abnormalities of male and female reproductive tracts and infertility. Numerous reproductive pathologies, such as cervical and uterine lesions and paraovarian cysts, have been referred in lambs and ewes fed a diet primarily consisting of alfalfa, a legume containing high levels of coumestrol [4]. In addition, ovariectomized sows supplemented with the phytoestrogen ginestein showed uterus hypertrophy and proliferation of endometrium epithelial cells [5].

Grazing on pastures containing estrogenic plants, such as alfalfa and clover, has been blamed for temporary or permanent infertility in sheep and cattle [6] by decreasing plasma progesterone [7] and estradiol [8]. The different reproductive status of the animal, such as ovarian cycle or pregnancy, can also influence phytoestrogens absorption, metabolism, and biotransformation [9]. Cows fed soy beans during early pregnancy showed higher plasma concentration of isoflavones metabolites [9]. Phytoestrogens appear to impair reproductive efficiency and uterine function due to high levels of prostaglandin F2α production by the endometrium [2]. It appears that not only in domestic animals but also in wild animals the phytoestrogenic compounds in their diet cause infertility. Dietary coumestrol and daidzein, present in plants fed to female southern white rhinos in captivity, activated estradiol receptors in their cervix and ovary, which might explain their infertility [10]. Current information on circulating levels of phytoestrogens in mares is limited. However, other studies have shown that administration of exogenous estradiol may act as an endocrine disruptor in mares, altering hormone profiles and the estrous cycle. Actually, intramuscular injections of exogenous estradiol to ovariectomized mares increased plasma LH and reduced FSH [11–13]. While it increased the duration of estrus and the interovulatory interval, it suppressed follicular development and ovulation [11]. Even though estradiol treatments appear not to affect luteal function [11], diethylstilbestrol when administered at mid-diestrus was able to prolong luteal function in the mare [14]. The longer duration of action of diethylstilbestrol might be responsible for this finding [11]. An incidence of 5% to 20% of mares in a given herd experiencing at least one period of persistence of the corpus luteum during the breeding season has been reported [15], but its relationship to phytoestrogens has not been shown. Therefore, it is very likely that phytoestrogens might affect ovarian and uterine function in the mare.

In Portugal, most Lusitano stud-farms are based on extensive grazing systems. In these systems, mares and foals are bred outdoors throughout the year, pasture being an important part of their diets [16]. The floristic composition of these pastures (natural or sown) includes a mixture of plants, in which several species of grasses, clovers, and other native legumes are commonly found. Thus, our hypothesis is that phytoestrogens in plants fed to mares can also be present in their plasma and act as endocrine disruptors. Therefore, the objective of this study was to determine whether phytoestrogens from clover-mixed pastures were present in mare's plasma after their ingestion (experiment I), and when phytoestrogens were present in mare's plasma after ingestion (experiment II). Moreover, the putative effect of a long-term ingestion of phytoestrogen-rich plants on the disruption of mares' estrous cycle was assessed (experiment III; clinical case).

2. Materials and methods

2.1. Animals and experimental design

This study included two *in vivo* experiments involving 20 nonpregnant Lusitano mares in Portugal and a clinical case of coumestrol-induced infertility in 16 nonpregnant, nonlactating mares of mixed breeds in Poland. Normal husbandry procedures, such as deworming and vaccinations, were routinely performed. The animals were handled with care during all experimental procedures. The protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Technical University of Lisbon, Lisbon, Portugal.

In experiment I, performed from November to March, 14 Lusitano barren mares from two different stud-farms (A and B) were followed. All mares were kept on clover and grass-mixed pastures. They were also supplemented with 2.5 to 3.0 kg of compound feed/mare/day and hay (mixture of grasses and legumes) (stud-farm A) or wheat straw (stud-farm B). Mares had free access to water and to a salt mineral block. Bimonthly, mares blood was collected from the jugular vein (10 mL/mare) into heparinized tubes (monovettes, Sarstedt, Numbrectht, Germany) and plasma was stored at −20 °C until further analysis of coumestrol and hormones. On the same dates, samples of feedstuff (hay, pasture, and compound feed) were randomly collected, lyophilized, and kept at −80 °C.

Experiment II was carried out for 14 days (March) on other Lusitano mares ($n = 6$; stud-farm C) kept on stalls with wood shaving beddings. Each mare was fed 3 kg/day of concentrate (a commercial compound feed) and wheat straw (8 kg). Increasing amounts of alfalfa pellets (from 250 g on Day 4 to 1 kg/day on Days 10–14) were added to