Milk and blood progesterone concentration in ewes (Ovis aries) under different physiological states and progestogen treatment

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ABSTRACT
The aim of this study was to quantify the concentration of milk and blood progesterone (P4) from lactating ewes under different physiological states and the possible harmlessness of their milk in human consumption. Progesterone concentration was determined in ovariectomized and intact ewes during the oestrous cycle, while implanted with a controlled internal drug release (CIDR) containing 0.3 g of natural P4 (OxCIDR; ICIDR) or not (Ox; I), and during pregnancy (G). Mean P4 concentration was found to be significantly greater in blood ($P < 0.0001$) than in milk sources. Concentrations also varied according to treatments ($P < 0.0001$). As expected, P4 concentration from Ox ewes was the lowest compared to the rest of the treatments ($0.31 \pm 0.22$ ng ml$^{-1}$; $P < 0.0001$), Ewes with CIDR showed greater P4 concentration than the respective group without implant (OxCIDR vs. Ox; $P < 0.0001$, and ICIDR vs. I; $P < 0.01$). In addition, G ewes showed the highest P4 concentration when compared with the rest of the treatments ($P < 0.03$). There was an interaction between P4 source and treatment ($P < 0.0001$). It was concluded that as milk of cycling and G animals is considered safe for human consumption, then the milk of CIDR-treated ewes should also be considered safe, based on P4 concentration.

1. Introduction
Maruyama et al. (2010) found that humans absorb progesterone (P4) by the ordinary intake of cows’ milk. The high consumption of this hormone by humans may result in suppression of gonadotropin and testosterone secretion, increasing the risk of hormone-sensitive cancers in adults (Farlow et al. 2009; Malekinejad and Rezabakhsh 2015), and jeopardizing sexual maturation of pre-pubertal children (Maruyama et al. 2010). People absorb significant amounts of P4 particularly from dairy products because P4 concentrates within the fat fraction of milk. According to Hoffman et al. (1975), the measurable level of P4 can range from 1.4 to 300 ng ml$^{-1}$ in skim milk and butter, respectively, with similar values between unprocessed and pasteurized/homogenized milk and dairy products (Baumrucker and Magliaro-Macrina 2011).

The physiological status of the animal affects the amount of P4 that can be found in the milk (Goodson 2008). During oestrus, cows and does have a half concentration of P4 in milk than at their mid-cycle and pregnancy (Rioux and Rajotte 2004; Reyes et al. 2012).

The United States Food and Drug Administration (FDA 2010) determined that human food safety for P4 residues in milk can be demonstrated by showing that concentrations of P4 residues in milk as the result of the concurrent treatment with the CIDR do not exceed the concentrations in the untreated pregnant cows. However, no conclusions have been made about other milk sources, like sheep.

In several regions of the world, sheep’s milk is very important for manufacturing some dairy products (Pollott and Wilson 2009), while in others it is also consumed by people as their main source of animal protein (Haenlein 2001). Milk from ewes has more fat than cows’ milk (Gunzler et al. 1975), which might affect the concentration of P4. In addition, ewes’ reproductive seasonality has led to the use of hormonal treatments based on progestogens to uniform milk production during the year. These treatments release progesterone at a controlled rate into the bloodstream after insertion, which might also affect its concentration in milk. However, the concentration of P4 in the ewe’s blood and milk and how it is affected by the physiological status of the animal and hormonal treatments have not been quantified. Thus, the purpose of the present study was to quantify the concentration of milk and blood P4 from lactating ewes under different physiological states and the possible harmlessness of their milk in human consumption. In addition, a P4 implant device was used to simulate a traditional hormonal reproduction programme commonly used in this species (Todaro et al. 2015).

2. Materials and methods
All procedures were approved by the Ethical Committee for Animal Experimentation of the University of the State of Morelos, Cuernavaca, Mexico. All animals were handled according to the principles stated in the EC Directive 86/609/EEC is
The study was performed in the State of Morelos, Mexico, 18°58′53″ N, 99°13′58″ W, located at 1804 m asl. Average annual rainfall is 977.5 mm, and average annual maximum and minimum temperatures are 39 and 22°C, respectively (García 2004). The experiment was conducted during September, which corresponds to the beginning of the sheep breeding season in the northern hemisphere (Keefe and Wichtel 2000).

2.1. Animals

A total of 30 multiparous Saint Croix cycling ewes were injected with two doses of 7.5 mg of PGF2α im (5 mg of dinoprost tromethamine, Lutalyse®, Pfizer, Mexico) with a 9-day interval. Oestrous detection was performed every 6 h for 72 h after the second injection. All ewes came into oestrus, were sired by the same ram and became pregnant. Pregnancy was confirmed by ultrasound revision 60 days after mating.

At four months of gestation, 12 ewes were ovarioectomized (Ox), remaining pregnant until birth. During ovarioectomy procedure, ewes were subjected in the supine position to midventral laparotomy under general anaesthesia induced with 0.15 ml/kg xylazine hydrochloride 2% im (Rompum, Bayer, Mexico) and 10 min later, 4 mg/kg ketamine chloride iv (Anesket Vet, Pisa, Mexico). Bilateral ovarioectomy was performed, with care being taken to ligate doubly the ovarian ligament, artery and vein. Postoperative pain medication was given applying 2 ml every 24 h for 3 days of flunixin meglumine im (Flucavet, Bayer, Mexico), according to a modification of the technique proposed and validated in sheep (Sigrist et al. 2007).

At day 30 postpartum, the Ox and 12 intact (I) ewes were randomly assigned to two of four groups. Two groups that received no treatment (n = 6; Ox and n = 6; I, respectively) and two groups where a controlled internal drug release containing 0.3 g of natural P4 (Eazy-breed CIDR®, Pfizer, New Zealand) were inserted into the vagina of each ewe for a 20-day period (n = 6; OxCIDR and ICIDR, respectively). The rest of the ewes were induced again into oestrus at day 30 postpartum by insertion of a CIDR implant that remained in situ for 9 days. On the day of withdrawal, 500 IU of PMSG was applied im, and oestrous detection took place every 6 h for 72 h after implant removal. Ewes were mated and become pregnant while still lactating. This fifth group of pregnant ewes (n = 6; G) was used as a second control to compare P4 values under different physiological conditions.

2.2. Progesterone determination

Blood and milk samples were collected from all ewes in all groups. Samples from groups: Ox, OxCIDR, I and ICIDR were obtained starting at day 30 postpartum and finishing at day 50, while ewes in the G group were sampled starting at 60 days postpartum when they were 20-day pregnant. Pregnancy in all ewes from the G group was confirmed a posteriori by ultrasound evaluation 80 days postpartum, corroborating the findings evidenced by the high levels of P4 found in all animals during the sampling period.

Blood samples were taken by jugular venipuncture in vacutainer tubes every third day for 17 days between 7:30 and 8:00 h, immediately after milking each individual. All samples were centrifuged at 3000 rpm during 15 min at room temperature within the first hour after collection (Pulido et al. 1991), and the serum remained frozen at –20°C until analysis of P4 concentration. Serum P4 concentrations were determined by Radioimmuno-analysis (Coat-a-count, DPC, Diagnostic Products Co., Los Angeles, CA, USA) at the end of the experiment. Results of P4 concentration are expressed as ng ml−1, obtained in tests performed in the laboratory of Animal Reproduction, National Autonomous University of Mexico. The sensibility of the assay was 1.1 ng ml−1, with intra- and inter-assay coefficients of variation of 3.9% and 4.4%, respectively.

Milk samples were taken at the beginning, middle and end of the milking from both treatments. The milk was mixed and shaken, and a subsample of 5 ml was obtained, refrigerated and centrifuged at 3000 rpm during 15 min to separate and remove fat, and then immediately frozen at –20°C until analysed. Serum P4 concentrations were determined by Radioimmuno-analysis (Coat-a-count, DPC, Diagnostic Products Co., Los Angeles, CA, USA) at the end of the experiment. The inter-assay coefficient of variation was 8% and intra-assay CV was 12%, from results obtained in tests performed in the laboratory of Animal Reproduction, National Autonomous University of Mexico.

2.3. Statistical analysis

Data were analysed using the mixed model of SAS, including as main factors the sources (blood vs. milk) and the treatment (Ox, I, OxCIDR, ICIDR and G), as well as their interaction as fixed effects; the day as a repeated measure, and the animal as a random effect. Data are presented as least squares means.

3. Results

Mean P4 concentration was found to be significantly greater in blood than in milk sources (3.77 ng ml−1 vs. 1.73 ng ml−1, pooled SEM = 0.14, respectively; P < 0.0001). Concentrations also varied according to treatments (P < 0.0001). As expected, P4 concentration from Ox ewes was the lowest compared to the rest of the treatments (0.31 ± 0.22 ng ml−1; P < 0.0001). Ewes with CIDR showed greater P4 concentration than the respective group without implant (3.22 ± 0.23 vs. 0.31 ± 0.22 for OxCIDR vs. Ox, respectively; P < 0.0001, and 3.46 ± 0.23 vs. 2.59 ± 0.23 for ICIDR vs. I, respectively; P < 0.01). In addition, G ewes showed the highest P4 concentration when compared with the rest of the treatments (4.17 ± 0.23 ng ml−1; P < 0.03).

There was an interaction between P4 source and treatment (P < 0.0001).

4. Discussion

This study determined P4 concentration in milk and blood of ewes under different physiological states with and without a CIDR. Statistically, P4 concentration was higher in blood than
in milk, perhaps at least among other reasons, due to the slow and inefficient path that leads blood P4 into milk. The kinetics studies of P4 transfer from blood to milk show that the pathways of trans-cellular, simple and facilitated diffusion are involved (Rioux and Rajotte 2004).

The fact that P4 concentration from OX ewes was the lowest compared to the rest of the treatments was expected, since P4 is a naturally produced steroid hormone by the corpus luteum of mammalian ovaries and placenta, and the purpose of the present experiment was to have no P4 from OX ewes to use it as a basal comparison level. On the other hand, P4 concentration from milk or blood in all groups was lower or similar to the level found in the G group. It is necessary to consider that although the sex, number and weight of the foetuses in the G ewes were not determined, this could affect the concentration of P4, particularly in Saint Croix ewes which have high prolificacy. Ewes carrying multiple foetuses have higher P4 concentration than ewes carrying single lambs (Gur et al. 2011). The P4 concentration in ewes carrying male foetuses is higher than in those carrying female foetuses (Ranilla et al. 1994), and ewes bearing foetuses with birth weights of more than 4 kg have greater P4 concentration than ewes with smaller lambs (Bedford et al. 1972).

The CIDR significantly increased P4 compared with untreated OX and I ewes. This is in agreement with previous results from sheep (Hamra et al. 1986), goats (Wheaton et al. 1993) and cows (Macmillan and Peterson 1993), and although the effect is concurrent, according to our results, increases in sheep P4 concentration do not reach P4 levels of pregnant animals. Similarly, other studies have concluded that CIDR device does not significantly increase milk P4 compared with untreated or pregnant goats (Reyes et al. 2012) or cows (Chenault et al. 2003). Contrary, Chenault et al. (2003) found that when CIDR inserts were administered, concentrations of P4 in de-fatted milk from treated cows, over a 10-day interval, consisting of the 7-day CIDR insert administration period and 3 days after insert removal, were similar than from untreated cows. This lack of coincidence with our results may be due to the lower P4 concentration expected on milk than blood sources, as we mentioned above, combined with a reduction in P4 concentration averages due to the fact that in their CIDR groups, average P4 concentration was determined to combine 7 days with the CIDR in place, plus 3 days after implant withdrawal.

It is well known that P4 is metabolized by CYP 450s (CYP3A, CYP2D6) in human. However, the involvement of hydroxy-steroid dehydrogenases, in the biotransformation of the P4 has also been demonstrated. The most important and biologically active metabolites of the P4 are 20α-dihydroprogesterone, 5α-dihydroprogesterone, and 3α-hydroxy-4-pregnen-20-one, which exhibit various effects (Lou et al. 2002; Suzuki et al. 2002). Previous studies demonstrated that following oral administration of P4 in human, circulating concentration of P4 and its active metabolite (20α-dihydroprogesterone) raises. Moreover, it has been also shown that this increase could exert proposed prostaglandin response in target tissues (Padwick et al. 1986). However, other studies indicate that due to rapid absorption following oral administration (equally to consumption via dairy foods) and extensive first-pass effect in the hepatic biotransformation, the oral bioavailability of exogenous P4 is less than 10% (Maxson and Hargrove 1985). Whatever the case, there is lack of knowledge about the kinetics of P4 and its potentially active metabolites in human following consumption of dairy foods in particular fatty foods (butter) which contain high amounts of P4. However, regardless of the pathway that P4 follows, as milk of cycling and G animals is considered safe for human consumption, then it might be concluded that the milk of CIDR-treated ewes, should also be considered safe, based on P4 concentration.

It was concluded that: as milk of cycling and G animals is considered safe for human consumption, then the milk of CIDR-treated ewes should also be considered safe, based on P4 concentration.

**Disclosure Statement**

No potential conflict of interest was reported by the author(s).

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