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Complete List of Authors:	Nunes, Helder; Universidade dos Acores Departamento de Ciencias Agrarias, ; Faheem, Marwa; University of Azores, Department of Animal Production Dinis, Marleen; University of Azores, Department of Animal Production Borba, Alfredo; University of Azores, Department of Animal Production Moreira da Silva, F.; University of Azores, Department of Animal Production
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**Effect of feed with *Pittosporum undulatum* in vivo on bovine progesterone levels and
embryos produced in vitro**

H.P. Nunes^{1,*}, M. Faheem^{1,2}, M. Dinis¹, A. Borba¹ and F. Moreira da Silva¹

¹ University of the Azores, Faculty of Agrarian Sciences, Department of Animal Reproduction, CITA-A, Angra do Heroísmo, Portugal

² Animal Production Department, Faculty of Agriculture, Cairo University, Giza, Egypt

*Corresponding author: helder.pb.nunes@uac.pt

Abstract

Feeding ruminants with *Pittosporum undulatum* can adversely affect the immune, endocrine and reproductive systems. The aim of the present study was to evaluate how feeding with *P. undulatum* alters progesterone levels during the oestrus cycle, and fertilization and development of oocytes after in vitro fertilization (IVF). Heifers were fed a diet containing (n=4) or not containing (n=4) this plant. Peripheral blood samples were collected every three days and progesterone levels were analysed by ELISA. The heifers' ovaries were recovered after slaughter and the oocytes were removed, matured, in vitro fertilized and cultured for seven days, during which time their development was evaluated every two days. Results indicated that plasma concentrations of progesterone during the oestrus cycle were significantly higher ($p < 0.01$) in the luteal and follicular phases of the animals fed without *Pittosporum* when compared with those fed with *Pittosporum*. Statistical differences ($p < 0.05$) in maturation, cleavage rate and embryonic development were observed between oocytes belonging to the experimental and control group. Rates of maturation, fertilisation and embryo development in the experimental group were, respectively, 78.3 ± 5.8 , 29.92 ± 4.31 and 7.30 ± 3.1 as compared to 90.5 ± 3.0 , 41.86 ± 5.8 and 21.88 ± 6.85 in the control group. These differences can be explained as compounds from this plant can directly or indirectly inactivate cyclooxygenase 2 (COX-2), which may diminish follicular development through the inhibition of prostaglandins synthesis, oocyte maturation and, consequently, reduce the ability of oocytes to be fertilized and develop.

Keywords: *Pittosporum undulatum*; reproduction; heifers; progesterone; embryo (in vitro)

Introduction

In the Azores there are two periods during the year where animal production is limited due to low grass production, therefore, *P. undulatum* Vent. 1800, one of the most successful invader plants of the Azores archipelago, is used mainly as an alternative animal feed (Lourenço et al., 2011). This species derives from the *Pittosporaceae* family, and is characterized by their highly competitive ability. Biochemically, plants of the genus *Pittosporum* contains a large group of secondary compounds such as terpenes, phenols, and alkaloids (Medeiros et al., 2003, Feng et al., 2010, Zhao et al., 2012, Nunes et al., 2014) that possess anti-inflammatory, antimicrobial and antithrombin properties; however, these compounds also show toxic effects on the immune, endocrine and reproductive systems (Mendes et al., 2011, Medeiros et al., 2003). As far as reproductive performance is concerned, some breeders have the perception that animals fed with this species show a decline in their reproductive performance due to the increase of early embryonic mortality or even abortion, although there remains no direct evidence for this. Progesterone is important for the establishment and maintenance of pregnancy, thus ensuring normal embryo development (Spencer et al., 2013). Suboptimal production of progesterone during the luteal phase is likely to adversely affect the viability of the embryo by increasing embryonic loss in the early days of gestation (McNeill et al., 2006, Lüttgenau et al., 2011). Therefore, the present study was designed to evaluate how a diet of *P. undulatum* can influence plasma progesterone levels, ovarian follicle quality, and oocyte competence in heifers.

Materials and Methods

Chemicals

All the chemicals and reagents used in this study were obtained from Sigma-Aldrich (St. Louis, Mo, USA) unless stated otherwise.

Animals

Eight Holstein pubescent heifers, 17 ± 3 months old, with active ovaries confirmed by palpation were divided into two groups: experimental (n=4) and control (n=4). Animals belonging to the experimental group were subject to a specific feed: 5kg grass silage, 3kg of commercial

concentrate and *P. undulatum ad libitum* during a period of 5 weeks. The animals in the control group were fed with the same feed as the other animals with the exception of *P. undulatum* which was replaced with grass *ad libitum*.

Experiment 1: effect on progesterone concentrations

Blood collection

Blood samples were collected every three days from the coccygeal blood vessel into evacuated tubes containing Heparin (S-Monovette®; Sarstedt, Nümbrecht, Germany). Tubes were immediately placed on ice and plasma was separated by centrifugation ($3000 \times g$ for 15 min), and frozen at -20°C until progesterone was determined.

Progesterone assay

Progesterone concentrations in plasma were measured in triplicate using a commercial ELISA kit (Abnova KAO299, Heidelberg, Germany). For plasma samples with progesterone levels between 0.15 and 10ng/ml, the intra-assay and inter-assay coefficients of variation were 4.9% and 8.3%, respectively, and the sensitivity was 0.08ng/ml.

Statistical Analysis

Differences in plasma progesterone concentration were determined with a Mann-Whitney non-parametric test using the IBM SPSS Statistics 20 program (SPSS Inc., Chicago, Illinois, USA). Values expressed as mean $\pm$ standard error of the mean (SEM) and comparisons were considered statistically significant if the *p* value was less than 0.05.

Experiment 2: in vitro embryo production assessment

Ovaries

After the feeding period, all animals were slaughtered and the ovaries were collected, trimmed of adhering tissue and transported within 2 hours post slaughter to the laboratory in Phosphate Buffered Saline (PBS) at temperatures ranging from 36 to 38°C.

Tissue collection

Ovaries were rinsed in 70% alcohol and washed with fresh PBS in a sterile laminar airflow. Each ovary was placed in a sterile tissue culture dish and the outer 1 mm cortex was removed

with a scalpel blade as previously described by Faheem et al. (2011). Then the slices were cut into squares approximately 1 cm × 1 cm and washed twice in isolation medium consisting of PBS supplemented with 10% fetal bovine serum (FBS), 50 µg/ml gentamycin and 20 µg/ml nystatin. For follicular puncture, ovarian slices were stabilized in a Petri dish containing 2 ml of warm isolation medium (38°C) by a scalpel blade. Under a stereomicroscope, follicles visible on the surface ranging from 2.0 to 6.0 mm in diameter were punctured using an 18 gauge needle. The whole procedure was completed within 30 minutes.

In vitro embryo production

Collected cumulus-oocyte complexes (COCs) from each group were separately counted and only those of good quality, based on their morphological appearance (Santos et al., 2008) were used. COCs were then washed twice in TCM 199 medium supplemented with 2% FBS, 0.3 mg/ml glutamine, 50 µg/ml gentamycin, 20 µg/ml nystatin, and matured in TCM 199 supplemented with 10% FBS, 5 µg/ml FSH-LH (Stimufol, Belgium), 1 µg/ml estradiol-17β, 0.15 mg/ml glutamine, 22 µg/ml Na-pyruvate, 50 µg/ml gentamycin and 20 µg/ml nystatin. After 24 h of maturation under 5% CO₂ in a humidified atmosphere at 38.5°C, oocytes were placed in fertilization TALP medium for insemination (Faheem et al., 2011). Concisely, thawed semen was washed three times by centrifugation, twice in sperm-TALP medium (4 ml each time) and finally washed in IVF-TALP medium supplemented with 10 µg/ml heparin, 6 mg/ml BSA (essentially fatty acid free), 22 µg/ml Na-pyruvate, 50 µg/ml gentamycin and 20 µg/ml of nystatin. After removing the supernatant, the sperm pellet was resuspended in 0.25-0.5 ml of remaining IVF-TALP medium to the desired sperm concentration of 1x10⁶sperm/ml. Oocytes and sperm were co-cultured in 50 µl of fertilization medium (10-15 oocytes/ droplet) for 22-24h at 38.5°C in 5% CO₂. Presumptive zygotes were denuded by vortexing, then washed and cultured in TCM-199 with Hepes supplemented containing 3 mg/ml BSA (Fr. V), 22 µg/ml Na-pyruvate, 10 µl/ml NEAA (MEM, non-essential amino acids), 20 µl/ml EAA (BME, essential amino acids), 50 µg/ml gentamycin and 20 µg/ml nystatin in an incubator at 38.5°C in 5% CO₂ in air. The cleavage rate was determined after 3 days of fertilization (day 0) and the embryonic development was evaluated 7 days after IVF.

Statistical Analysis

Data was analysed by *Student's* t-test and expressed as mean $\pm$ standard error of the mean (SEM) calculated from the collected oocytes for each group. Comparisons were considered significantly different if P value of less than 0.05. All analyses were performed using the IBM SPSS Statistics 20 program (SPSS Inc., Chicago, Illinois, USA).

Results

Feeding heifers with *P. undulatum* significantly decreased ($p < 0.01$) plasma progesterone concentrations during the luteal phase of the cycle compared with the animals in the control group (Fig 1). The minimum values for progesterone levels were 0.77 ± 0.23 and 0.30 ± 0.04 ng/ml, on the day of estrus for experimental and control groups, respectively, while the maximum values were 1.85 ± 0.48 and 5.34 ± 0.23 ng/ml, respectively. When heifers were slaughtered, we observed that in the experimental group 3 of the 4 animals had corpora lutea while in the control group 2 of 4 heifers had corpora lutea ; the average progesterone levels throughout the study period were 1.18 ± 0.37 and 2.83 ± 0.63 ng/ml for experimental and control groups, respectively.

Table 1 represents results obtained in experiment 2, where rates of *in vitro* embryonic development of experimental and control groups were evaluated.

On average, for the control group, the number of oocytes produced per ovary was 23.3 ± 3.6 vs 17.0 ± 2.4 for the experimental group. The percentage of matured oocytes attaining the stage of metaphase II was significantly higher when compared to the experimental group (90.5 ± 3.0 vs 78.3 ± 5.8 $p < 0.05$). This tendency was then observed in their ability to produce embryos, as the percentage of cleaved and those progressing to morula/blastocyst were statistically higher in the control than in the experimental group. None of the embryos from the animals fed with *P. undulatum* reached the blastocyst stage, while the control group reached 7.2 % seven days after IVF.

Discussion

The present work was designed as a field study to follow up on blood progesterone concentration levels throughout the oestrous cycle of Azorean heifers after feeding with *P. undulatum*. Moreover, the developmental competence of oocytes from those animals was also examined. To our knowledge, this is the first study demonstrating that ingestion of this plant alters the reproductive performance of heifers. The choice of heifers was primarily due to the fact that in the Azores islands, the farmers use this plant to feed heifers during the months where grass is scarce, particularly during the winter months.

Plasma concentrations of progesterone during the oestrous cycle reflect the development of the corpora luteum. In this study it was found that significant differences occurred ($p < 0.01$) in averages of plasma progesterone concentrations between the control and experimental groups. The control group showed a typical pattern of progesterone concentration, while heifers in the experimental group essentially failed to show a luteal phase increase in progesterone concentrations. These low progesterone values throughout the cycle lead us to infer that, despite the luteal cell formation, there is a metabolite present in *P. undulatum* functioning as antagonist of the production of progesterone. The progesterone values in the experimental group of animals reflects what producers empirically observed, concerning no oestrus external signals and abortions in the early pregnancy when animals are fed with this plant, as this hormone is essential for establishment and maintenance of pregnancy (Sakumoto et al., 2014). The mechanism of action of *P. undulatum* is unknown, although as the enzymes cytochrome P450 (P450_{scc}) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) enzymes are essential for the conversion of cholesterol into progesterone (Diaz et al., 2002, Schams and Berisha, 2004), it is possible that the metabolites produced by this plant may affect the expression and/or activity of these enzymes.

In this study, the number of COCs collected statistically lower in the experimental group when compared to the control group, which suggests that fewer follicles were developing in the experimental group. More interesting, however, is the major inhibition of developmental competence of the oocyte observed after feeding with *P. undulatum*. These effects may be

attributed to the chlorogenic acid compound previously identified in this plant by Nunes et al. (2014), which is known to inhibit the activity of the enzyme COX-2 (Guan et al., 2014). This enzyme is essential for the metabolism of arachidonic acid to prostaglandins, which are important for ovulation and function of the corpus luteum (Tsai and Wiltbank, 1998, Calder et al., 2001, Sales and Jabbour, 2003). This enzyme is also important for development of embryos (Thatcher et al., 1984, Pereira et al., 2006) therefore a suppression of COX2 activity may explain the lower rate of cleavage observed in the experimental group.

In summary, feeding heifers with *P. undulatum* drastically decreased plasma progesterone levels and developmental competence of oocytes. These data indicate that metabolites existing in this plant decrease oocyte development which can lead to reduced embryonic development. Since this is the first study evaluating the effects of *P. undulatum* in reproduction, further research is required to elucidate through which biochemical and physiological mechanisms this plant acts upon fertility.

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Table 1: Rates of development embryonic *in vitro* of experimental and control groups

Treatment	No. of ovaries	No. of collected oocytes	No. of oocytes for maturation	Rate of		
				Maturation	Cleavage	Developed Embryo
Experimental	8	136 ^a	119 ^a	78.3±5.8 ^a	29.92±4.31 ^a	7.30±3.11 ^a
Control	8	186 ^b	148 ^b	90.5±3.0 ^b	41.86±5.58 ^b	21.88±6.85 ^b

Numbers in the same column with different letters (a, b) differ significantly at $P < 0.05$.

Data are expressed in percentage (%) as mean ± SEM, standard error of means.

% of developed embryos classified as morula, early blastocyst and blastocyst stages.

Figure 1-Plasma progesterone concentration in coccygeal vein during the bovine oestrous cycle. The data represents the average the group control (n=4) and experimental group (n=4) during the oestrous cycle. Data obtained were adjusted so that Day 0 represent the value minimum progesterone (oestrus = Day 0). Vertical bars represent the SEM.