# Effect of stress due to ultrassonography on plasma cortisol and progesterone (P4) concentrations during oestrous cycle in ewes

ARTÍCULO DE INVESTIGACIÓN

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**ABSTRACT:** The primary purpose of this study was to observe the effects of repeated transrectal ultrassonography on plasmatic concentrations of cortisol and progesterone ($P_4$) in ewes during estrous cycle. Seven Bergamasca ewes (2-5 years of age), received two luteolytic doses of cloprostenol at 7 day intervals during the breeding season. Estrous was detected using a vasectomized ram. Ovaries were examined daily using transrectal ultrassonography. The ewes were studied starting one day before the second $PGF_{2\alpha}$ injection until 10 days after ovulation (day 0 = ovulation). Jugular vein blood samples were collected daily and after ultrassonography for plasma cortisol and $P_4$ measurement. There was no effect of the repeated transrectal ultrassonography on plasma concentrations of cortisol. However, analysis of variance indicated that cortisol concentrations varied over time. Higher plasma concentrations were detected after ultrassonography on day-1 (1.74±0.76 µg/dL) and day-0 (2.9±0.76 µg/dL). Rise in plasma cortisol during the first days of stress reflected the emotional reaction to the stressor. However, mean $P_4$ concentrations were unaffected by transrectal ultrassonography on day-1 (1.28±0.32 ng/mL) and day-2 (1.53±0.32 ng/mL) when compared to those before ultrassonography on the same days (0.95±0.32 and 1.10±0.31 ng/mL, respectively). In summary, endocrine responses of cortisol and $P_4$ plasma concentration to repeated transrectal ultrassonography were unaffected during follicular and early luteal phase in ewes.

**Key words:** ACTH, corticotropin, estrous, ovulation, reproduction

# Efecto del estrés provocado por la ecografía sobre las concentraciones plasmáticas de cortisol y progesterona (P4) durante el ciclo estral en ovejas

**RESUMEN:** El objetivo de este estudio fue observar los efectos de la ecografía transrectal en las concentraciones plasmáticas de cortisol y progesterona ($P_4$) durante el ciclo estral en ovejas. Siete ovejas Bergamasca (2-5 años de edad) recibieron dos dosis luteoliticas de cloprostenol con intervalo de siete días durante la estación reproductiva. El estro se detectó usando un carnero vasectomizado. Los ovarios fueron examinados diariamente por medio de ecografía transrectal. Las ovejas fueron estudiadas desde un día antes de la segunda inyección de $PGF_{2\alpha}$ hasta el día 10 después de la ovulación (día 0 = ovulación). Las muestras de sangre de la vena yugular se tomaron diariamente después de la ecografía para la determinación de cortisol y $P_4$. No hubo efecto de la ecografía transrectal repetida sobre las concentraciones plasmáticas de cortisol. Sin embargo, el análisis de varianza indicó que el cortisol varió en el tiempo. Altas concentraciones plasmáticas se detectaron después de la ecografía en el día 1 (1,74±0,76 µg/dL) y en el día 0 (2,9±0,76 µg/dL). Aumentos en las concentraciones plasmáticas de cortisol durante los primeros días del estrés reflejaron la reacción emocional ante el factor estresante. Sin embargo, las concentraciones medias de $P_4$ no fueron afectadas por la ecografía transrectal en el día 1 (1,28±0,32 ng/mL) y en el día -2 (1,53±0,32 ng/mL) cuando comparado con aquellas antes de la ecografía en los mismos días (0,95±0,32 and 1,10±0,31 ng/mL, respectivamente). En conclusión, las respuestas endocrinas del cortisol y $P_4$ plasmáticas a la ecografía transrectal repetida no fueron afectadas durante la fase folicular e inicio de la fase luteal en ovejas.

**Palabras clave:** ACTH, corticotropina, estro, ovulación, reproducción

## Introduction

Stress can be defined as the inability of an animal to cope with its environment. ‘‘Stress’’ is responsible for many things, including subfertility. Many agricultural advisers and veterinarians are very familiar with those intangible factors that reduce fertility on farms but often they are unable to pinpoint precise contributory causes and hence blame ‘‘stress’’. This, in itself, provides a definition of ‘‘stress’’, that is, the inability of an animal to cope with its environment, a phenomenon that is revealed by a failure to achieve genetic potential, e.g. for growth rate, milk yield, disease resistance, or fertility (Dobson & Smith, 2000).

A stressful stimulus triggers a complex response in the body. The pattern of responses depends on the nature of the stress, its intensity and duration, as well as the nature and current state of health of the individual (Buckingham et al., 1997). Perception of a stressful stimulus causes an acute response by the immediate release of noradrenaline and adrenaline (catecholamins) from sympathetic nerve endings and the adrenal medulla. Repeated exposure to a stressor often results in adaptation or habituation. The time for this to occur depends on the type of stress, as well as on severity and duration (Vellucci, 1997). Via a complex array of nervous pathways, the perception of a stressful stimulus also triggers the release of CRH (corticotropin-releasing hormone) from the hypothalamus. CRH acts on the anterior pituitary gland to release ACTH (adrenocorticotropin hormone). ACTH return causes the adrenal cortex to produce and release glucocorticoids (such as cortisol) (Buckingham et al., 1997). ACTH also causes the release of other hormones from the adrenal glands, *e.g.* progesterone (Bolaños et al., 1997).

Studying the effects of stress on reproduction is beset with difficulties. The complex nature of some stressors in the modern farm environment simultaneously exposes animals to several different stimuli. Furthermore, there is considerable variability between individuals in response to a given stimulus. Added to this, is the overriding importance of the reproductive system to pass genes on to the next generation. This last issue means that animals have developed several strategies to cope with environmental problems including alternative responses to compensate for failure of any part of the protection mechanism. The sheep is often used as a model for mammalian reproduction and this species provides a more manageable, less expensive, experimental animal than the cow. In addition, mechanisms controlling normal reproductive endocrinology of the sheep are well characterized and are similar to those in the cow (Dobson & Smith, 2000).

A connection between stress and reproductive function has been known for many decades. Stress can impair various aspects of reproduction, including gonadotropin secretion, estrus, and ovulation. In females, stress has been shown to disrupt the natural follicular phase of the ovarian cycle in sheep (Battagglia et al., 1999). Female reproductive cycles depend upon a series of synchronized endocrine events that allow cyclic generation of ovulation and estrus, and this finely tuned sequence of events is clearly susceptible to perturbation by stress (Morberg, 1987). In some species stress may suppress gonadotropin secretion and this may involve increased activity in the pituitary-adrenal axis (Moberg, 1984; Uribe-Velásquez et al., 1998; Oba et al., 2000). This could have practical implications in terms of interference with estrus and ovulation (Doney et al., 1976). Stress disrupts the preovulatory luteinizing hormone (LH) surge in females, but the mechanisms are unknown. Cortisol reduced the incidence of LH surges irrespective of season (Pierce et al., 2009). Progesterone in the ewes is mainly produced in the ovaries by the corpora lutea. Progesterone is formed from all steroidogenic tissues as an intermediate in the production of other steroid hormones (Burris, 1998). Therefore progesterone is also released from the adrenal glands after ACTH stimulation (Tsuma et al., 1998). Progesterone also has an inhibitory effect on LH pulse frequency in cyclic ewes (Uribe-Velásquez et al., 2008b).

In farm animals, plasma cortisol has become a widely used parameter for measuring stress responses. However, only few studies have dealt with basal levels of concentration of cortisol in sheeps during reproductive ultrassonography. The primary purpose of this study was to look at the effects of repeated transrectal ultrassonography commonly used in reproductive endocrine studies, on plasma concentrations of cortisol and progesterone ($P_4$) in ewes during follicular and early luteal phases.

## Materials and methods

The study was performed at the Laboratory of Animal Reproduction and Veterinary Endocrinology – UNESP (Universidade Estadual Paulista, Botucatu, São Paulo, Brazil).

Seven multiparous Bergamasca ewes (2-5 years of age) were used with a body condition score (BCS) of 4.0±0.3 (scale 0-5) and a body weight of 60.4±8.2 kg. All ewes received two luteolytic doses of cloprostenol (125 µg intramusculary; Mallinckrodt, São Paulo, Brazil) at 7 day intervals during the breeding season. All females were housed outdoors in a sheltered pen (30 m x 30 m) and submitted to this feeding routine 2 months before the onset of the trial. The ewes had free access to water and were fed a maintenance diet. Furthermore, all ewes in this study were confirmed cycling by ultrasonography. Ewes were monitored daily for estrus with vasectomized ram every 8 h. Estrus was defined as that time when a ewe stood to be mounted by a vasectomized ram.

Ovarian follicular dynamics were monitored by transrectal ultrasonography using Aloka 500 with a 7.5-MHz linear array transducer designed for human prostate (Aloka Co., Ltd, Tokyo, Japan). This technique has been validated for monitoring ovarian follicular dynamics in sheep (Schrick et al., 1993; Ravindra et al., 1994; Uribe-Velásquez et al., 2008a; Letelier et al., 2009). Ovarian ultrassonography was performed from 1d before second $PGF_{2\alpha}$ injection until 10d after ovulation (day 0 = ovulation). The procedure to locate the ovaries was similar to that described by Ginther and Kot (1994). A follicular wave was identified using the same criteria as described by Menchaca and Rubianes (2004). The day of emergence of the follicular wave was determined retrospectively as the day on which the largest follicle of the wave reached a diameter of 3 mm. Ovulation was detected with ultrassonography by the sudden disappearance of a large follicle (≥ 5 mm in diameter) that had been identified for several days. Follicular data were combined for both ovaries of each ewe (Barrett et al., 2004).

Blood samples for plasma progesterone and cortisol determinations were taken by jugular venipuncture into Vacutainer tubes containing heparin daily from Day -1 to Day 10 (Day 0 = Ovulation). Blood was allowed to clot at 4° C for 24 h and centrifuged at 1500 x g for 15 min at room temperature. Plasma was removed and frozen at -20° C until assay for progesterone and cortisol.

Plasma progesterone concentrations were determined using a commercial Kit by direct solid-phase Radioimmunoassay $Iodo^{125}$ (Coat-a-Count, DPC, Los Angeles, CA, USA). The intra-assay CV was 8%. The analytical sensitivity limit of the assay was 0.01 ng/ml. Plasma cortisol concentrations were determined using Radioimmunoassay (Coat-ACountR Cortisol-Medilab). The intra-assay CV was 9%.

Analysis of variance was conducted using GLM procedures of SAS (1996). All data were summarized as means and standard errors of the means. Mean plasma concentrations of progesterone and cortisol are presented for each sampling period. Plasma progesterone and cortisol concentrations were compared by ANOVA for repeated measures. The differences were considered to be significant when $P < 0.05$.

## Results and Discussion

The administration of prostaglandin ($PGF_{2\alpha}$) to cyclic ewes in which 4-14 days old luteum corpus

CL are present results in regression of the CL and a decline in serum progesterone concentrations (Chamley et al., 1972; Sheldrick & Flint, 1985). A decline in plasma progesterone concentrations leads to a preovulatory surge in LH and the cascade of events that result in ovulation of a follicle (Niswender et al., 1986; Alila & Dowd, 1991). The timing of estrus and ovulation in PG-treated cyclic ewes in this study was similar to that in others studies in small ruminants (Uribe-Velásquez et al., 2008a, 2010).

The use of $PGF_{2\alpha}$ or its analogues in cycling ewes, caused rapid luteolysis and achieved well-synchronized behavioral estrus (López-Sebastian et al., 2007). So, all the ewes in this study were detected in oestrous at 48 h after cessation of treatment.

In a previous study in normally cyclic Western White Face ewes, all ewes ovulated follicles from the final follicular wave of the cycle and only 10% ovulated follicles from the penultimate wave (Bartlewski et al., 1999). In the present study, all ewes ovulated follicles from final wave.

There was no effect due to repeated transrectal ultrassonography on plasma concentrations of cortisol. However, analysis of variance indicated that cortisol concentrations varied over time (Figure 1). Higher plasma concentrations were detected after ultrassonography on day-1 (1.74±0.76 µg/dl) and day-0 (2.9±0.76 µg/dl). Rise in plasma cortisol during the first days of stress reflects the emotional reaction to the stressor (Uribe-Velásquez et al., 1998). The reasons for the dominant cyclic pattern in plasma cortisol is unclear, suggesting a cycle of the pituitary adrenal axis synchronized to the estrous cycle (Rawlings & Cook, 1991).

All stressors result in increases in corticoids and catecholamines from the adrenal gland medulla but it is unlikely that these have a major influence on LH secretion because stress effects on GnRH have been observed in adrenalectomized animals (Rivier & Rivest, 1991). An increase in hypothalamic-pituitary-adrenocortical activity indicates a physiological response to different stressors, and measurement of plasma corticosteroids is frequently used to study stress response (Sapolsky et al., 2000; Pacak & Palkovits, 2001). The secretion of stress hormones (glucagon, catecholamins, cortisol and growth hormone - GH) and especially, cortisol increases during the acute stress and emotional stimuli (Barglow et al., 1985; Goldstein et al., 1994). Imposition of an experimental stressor (shearing, transport, isolation from other sheep, injection of endotoxin or insulin or cortisol infusion) suppresses GnRH/LH pulse frequency and amplitude in the presence of lesser or greater oestradiol concentrations. Part of the effects on amplitude are mediated via Type II glucocorticoid receptors at the pituitary that, in the short-term, interrupt GnRH stimulation of LH release, as well as more long-term, by suppressing GnRH and GnRH receptor synthesis (Phogat et al., 1999; Breen & Karsch, 2006).

Effects on GnRH/LH pulse frequency are mediated at hypothalamus, as well as alterations in the onset of the GnRH/LH surge. If stressors are imposed during the follicular phase in intact ewes, there is a decrease in oestradiol concentrations, as well as a delay/abolition of the preovulatory GnRH/LH surge (Dobson & Smith, 1998; Battaglia et al., 2000; Saifullizam et al, 2010). Results of other studies conducted in the follicular phase of intact ewes using insulin or small dose endotoxin treatments could be interpreted as interfering with the transmission or surge release phases (Smith & Dobson, 2002; Smith et al., 2003).

However, means $P_4$ concentrations were unaffected by transrectal ultrassonography on day-1 (1.28±0.32 ng/ml) and day-2 (1.53±0.32 ng/ml) compared to those before ultrassonography on the same days (0.95±0.32 and 1.10±0.31 ng/mL, respectively). The changes in $P_4$ plasma concentrations among follicular and early luteal stages in the present study were expected.

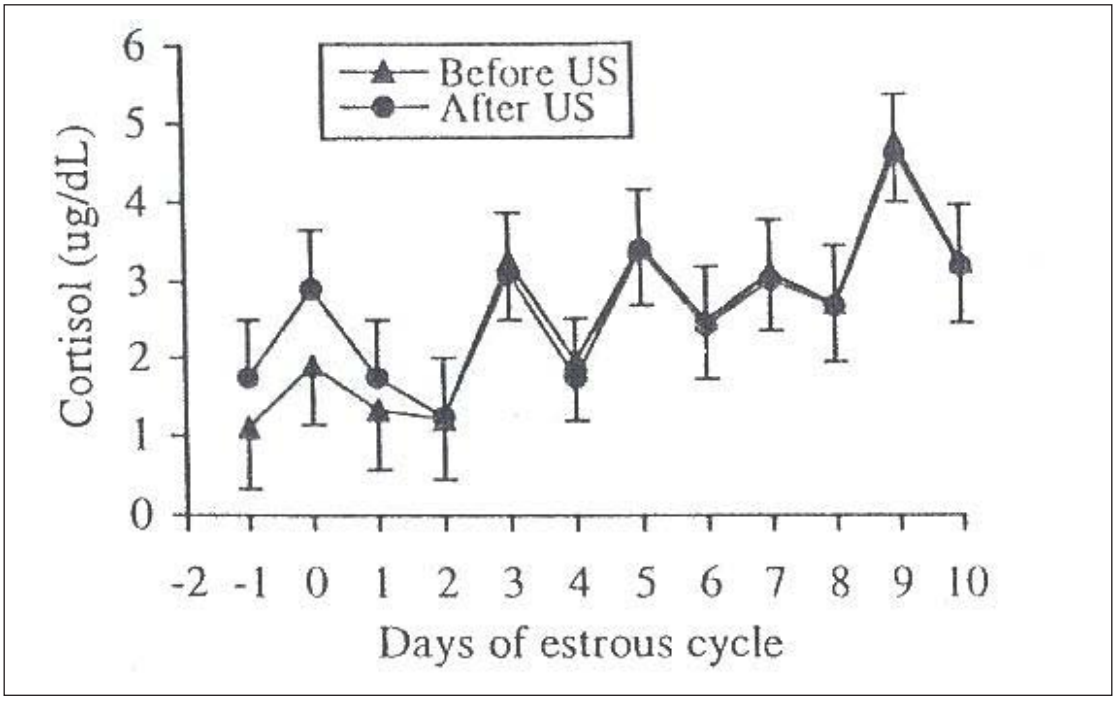


**Figure 1.** Cortisol plasma concentrations before and after ultrassonography during follicular and early luteal phases.

Stress-induced changes in reproductive hormone profiles are the result of altered neurotransmitter activities mediated by different neurons in several locations. In the early follicular phase of intact ewes, there is a gradual decline in progesterone after the onset of luteolysis simultaneous with a gradual increase in oestradiol stimulated from follicles by increasing LH pulse frequency. During this phase, progesterone acts via GABA neurones to hold GnRH pulse frequency in check in the face of increasing oestradiol stimulatory influence (Robinson et al., 1991). Plasma hormone patterns change with the stage of the estrous cycle, therefore stress effects may vary too.

## Conclusion

In summary, endocrine responses of cortisol and $P_4$ plasma concentration to repeated transrectal ultrassonography were unaffected during follicular and early luteal phase in ewes.

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