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Nitric oxide stimulates progesterone and prostaglandin E_2 secretion as well as angiogenic activity in the equine corpus luteum

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Abstract

Cytokines and nitric oxide (NO) are potential mediators of luteal development and maintenance, angiogenesis, and blood flow. The aim of this study was to evaluate (i) the localization and protein expression of endothelial and inducible nitric oxide synthases (eNOS and iNOS) in equine corpora lutea (CL) throughout the luteal phase and (ii) the effect of a nitric oxide donor (spermine NONOate, NONOate) on the production of progesterone (P4) and prostaglandin (PG) E_2 and factor(s) that stimulate endothelial cell proliferation using equine luteal explants. Luteal tissue was classified as corpora hemorrhagica (CH; $n = 5$), midluteal phase CL (mid-CL; $n = 5$) or late luteal phase CL (late CL; $n = 5$). Both eNOS and iNOS were localized in large luteal cells and endothelial cells throughout the luteal phase. The expression of eNOS was the lowest in mid-CL ($P < 0.05$) and the highest in late CL ($P < 0.05$). However, no change was found for iNOS expression. Luteal explants were cultured with no hormone added or with NONOate (10^{-5} M), tumor necrosis factor-α (TNFα; 10 ng/mL; positive control), or equine LH (100 ng/mL; positive control). Conditioned media by luteal tissues were assayed for P4 and PGE_2 and for their ability to stimulate proliferation of bovine aortic endothelial cells (BAEC). All treatments stimulated release of P4 in CH, but not in mid-CL. TNFα and NONOate treatments also increased PGE_2 levels and BAEC proliferation in CH ($P < 0.05$). However, in mid-CL, no changes were observed, regardless of the treatments used. These data suggest that NO and TNFα stimulate equine CH secretory functions and the production of angiogenic factor(s). Furthermore, in mares, NO may play a role in CL growth during early luteal development, when vascular development is more intense.

Keywords: Corpus luteum; Nitric oxide; Progesterone; Prostaglandin E_2; Angiogenesis

1. Introduction

Despite continuous advances in our understanding of luteal function, the complete mechanism has been only partially defined. In mares, soon after ovulation, a complex dynamic process of luteinization leads to the formation of a transient endocrine gland, the corpus

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luteum (CL), which produces progesterone (P4). This essential process of luteal development is characterized by the remodeling of extracellular matrix [1], as well as differentiation and proliferation of cells derived from the postovulatory follicle, such as granulosa, theca, and vascular endothelial cells [2–4]. Although luteinizing hormone (LH) secreted by the anterior pituitary has been considered the main hormone responsible for ovulation and luteal formation for decades [5], some ovarian mediators/factors might also play an auto/paracrine role in CL growth in mares. Because cytokines, like tumor necrosis factor (TNFα), have been shown to exert several physiological roles in the reproductive tract, such as granulosa cell proliferation [6], luteal development and maintenance [7], and angiogenesis [8], they might also be potentially involved in the regulation of equine luteal tissue formation. TNFα is a pleiotropic cytokine whose cellular effects depend on cell type, concentration, and receptor type present [7].

In addition to its vasodilator properties, nitric oxide (NO) is an important signaling molecule that plays various physiological roles in the reproductive system [8–11]. In the genital tract of mares, NO appears to be involved both in follicular growth and ovulation [9] and in endometrial prostaglandin (PG) E_2 and $PGF_{2\alpha}$ secretion [8]. Although in cows NO may serve as a local mediator of luteolytic $PGF_{2\alpha}$ action [10,12], in other species NO prevents luteolysis [13], stimulates PG and P4 production [14–16], and impairs ovarian steroidogenesis [14,15,17]. Nitric oxide synthases (NOS) are the enzymes that convert L-arginine to citruline and NO [18]. In the genitalia of mares, the presence of inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3) forms has been reported in the endometrium under physiologic and pathologic conditions [11,19]. The link between angiogenesis and NO is known. Potent angiogenic growth factors have been able to stimulate endothelial NO production by vascular endothelial cells [20].

Despite some work done in other species, to the best of our knowledge, NOS expression in equine CL and the role of NO and TNFα on equine luteal formation and endocrine function have not yet been studied. In contrast to other farm animals, mares have a prolonged estrus (5–7 days) and a unique pattern of endocrine reproductive events, whereas LH reaches a peak the day after ovulation and then decreases [5,21]. Thus, the hypothesis was that local factors such as NO and TNF might also be involved in CL luteinization and vascular development in mares. Therefore, this study was carried out to evaluate (i) the expression of endothelial and inducible forms of NOS on equine CL throughout the luteal phase and (ii) the effect of a nitric oxide donor (spermine NONOate, NONOate) on the *in vitro* production of P4 and PGE_2, as well as factor(s) that stimulate endothelial cell proliferation by equine luteal explants from early and midluteal stages of the estrous cycle.

2. Materials and methods

2.1. Animals and tissue collection

Ovaries and venous blood were collected post mortem as by-products at an abattoir from randomly designated cyclic mares from the spring equinox until the end of August. The animals were euthanized after being stunned according to Portuguese legislation (DL 98/96, Art. 1) and European Legislation concerning welfare aspects of animal stunning and killing methods (EFSA, AHAW/04-027) approved by the Faculty of Veterinary Medicine Ethics Committee. Reproductive and clinical data of all mares were unknown, but the animals were in good physical condition as assessed by veterinary inspection.

Mares ($n = 15$) were in various stages of the luteal phase based on plasma P4 levels and the presence and size of follicles, as well as on morphological appearance of the CL, as previously described [11]. Luteal tissue was classified as early luteal phase (presence of corpora hemorrhagica; CH; $n = 5$), midluteal phase (CL associated with follicles 15 to 20 mm in diameter and P4 above 6 ng/mL; mid-CL; $n = 5$) and late luteal phase (CL associated with follicles 30 to 35 mm in diameter; P_4 between 1 and 2.5 ng/mL; $n = 5$; late CL). Classification of late luteal phase CL was also based not only on large-diameter follicles, which may also be present in the early luteal phase [22], but also mainly on luteinization of the CL, with a consistent and trabeculated appearance that exists in late CL [5].

2.2. Collection and preparation of luteal tissue

Immediately after exsanguination, ovaries were removed and corpora lutea were collected. Small samples of corpora lutea were cut, rinsed with cold sterile RNAse-free saline solution, and divided into specific solutions depending on further experiments, such as: (i) buffered formaldehyde for immunohistochemistry studies for eNOS and iNOS localization; (ii) RNAlater (AM7020; Ambion, Applied Biosystems, Porto, Portugal) for eNOS and iNOS Western imunoblot analysis,