Measurement of oxytocin concentrations in plasma and ovarian extracts during the oestrous cycle of mares

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Summary. Jugular venous blood samples were collected throughout a complete oestrous cycle from 9 mares for measurement of progesterone and oxytocin by radioimmunoassay. Mean oxytocin concentrations remained at ~1 pg/ml throughout, with no evidence of cyclic variation in the release pattern. Extracts of corpus luteum and follicles obtained from a further 33 mares at different stages of the cycle all contained oxytocin concentrations of <10 pg/g wet weight of tissue. We conclude that the ovaries are not a source of circulating oxytocin during the oestrous cycle in this species. The plasma oxytocin concentrations reported here are substantially lower than those found by other groups.

Keywords: oxytocin; mare; oestrous cycle

Introduction

Previous publications on the plasma concentration of oxytocin in mares have produced contradictory results. Burns *et al.* (1981) reported that concentrations were higher at oestrus and early dioestrus than in the mid and late luteal phases, whereas Tetzke *et al.* (1987) found greatest concentrations in the late luteal phase. A possible role for oxytocin in the control of luteolysis in this species was suggested by Goff *et al.* (1987), who found that the release of prostaglandin F-2 α (PGF-2 α) in response to an oxytocin challenge varied during the cycle, the greatest response being achieved during luteolysis. However, oxytocin injections in either the early or midluteal phases did not affect the time of return to oestrus (Neely *et al.*, 1979; Goff *et al.*, 1987). The present study determined plasma oxytocin concentrations in cyclic mares using a well-defined radioimmunoassay (RIA), and investigated whether the equine ovary is a source of oxytocin as it is in domestic ruminants.

Materials and Methods

Sample collection

Jugular venous blood samples were collected daily from 9 Thoroughbred mares into 10-ml heparinized vacutainers from 19 June to 17 July 1990, inclusive. The blood was kept on ice and centrifuged at 1000 g within 15–30 min of collection. The plasma was divided into two aliquants, 1 ml for progesterone RIA and the remainder (~4 ml) for oxytocin measurement. The plasma samples were frozen immediately on solid CO₂. Progesterone samples were stored at –20°C and assayed weekly. Oxytocin samples were stored at –80°C and extracted within 1 week of collection.

Radioimmunoassays

Progesterone. Measurements were performed on 0.1-ml aliquants of plasma using petroleum ether extraction as described previously (Wathes *et al.*, 1986). The detection limit was 22 pg/tube, the extraction efficiency was 75 ± 7.5%, and the inter- and intra-assay coefficients of variation were 14.1 and 6.4%, respectively.

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Oxytocin. Oxytocin was measured after extraction of 4-ml plasma samples on C18 cartridges (Jones Chromatography Ltd, Hengoed, Mid Glamorgan, UK) with minor modifications to a method described by Wathes *et al.* (1986). Columns were pretreated with 3 ml 80% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) followed by 5 ml H₂O. Plasma samples were prepared by dilution in an equal volume of phosphate buffer (1.4 g Na₂HPO₄·2 H₂O, 0.26 g KH₂PO₄, 8.65 g NaCl/l) and centrifugation for 10 min at 4°C and 1500 *g*. They were then applied slowly to the columns and washed through with 20 ml 10% ACN in 0.1% TFA. Oxytocin was eluted with 3 ml 80% ACN in 0.1% TFA. The ACN was removed by centrifugation in a rotary film evaporation centrifuge set at 35°C (Univap Uniscience, Cambridge, UK) and the remaining extract was freeze dried. The extract was redissolved in 0.5 ml phosphate buffer containing 1 mg/ml bovine serum albumin (Sigma Chemical Co., Poole, Dorset, RIA grade). Aliquants of 50 µl of this solution were measured in an RIA for oxytocin using a well-characterized antiserum 85/2 (Gilbert *et al.*, 1991). Separation was achieved using 0.15 ml anti-rabbit precipitating antibody (UCB BioProducts, Belgium). The sensitivity of the assay was 0.25 pg/tube and the inter- and intra-assay coefficients of variation were 10.8 and 8.5%, respectively.

Validation of this assay for use in horses was achieved using addition of oxytocin to the plasma both *in vitro* and *in vivo*. For the *in-vitro* method, oxytocin standard was added to a pool of either assay buffer or mare plasma at low and high concentrations. Buffer samples were measured without extraction and buffer and plasma samples were also extracted and assayed in triplicate. Results (all in pg/ml with recovery percentages in parentheses) were as follows: low buffer (unextracted) 15.8, low buffer (extracted) 15.9 (100%), high buffer (unextracted) 41.6, high buffer (extracted) 39.1 (94%), plasma (no additions) 1.1, low plasma 16.1 (95%), high plasma 39.9 (93%).

For the *in-vivo* experiment, 2 mares were fitted with jugular vein cannulae under local anaesthesia and injected with 1 unit synthetic oxytocin (Syntocinon: Sandoz Ltd, Basle, Switzerland) directly into the jugular vein. The cannulae were filled with heparinized saline. Blood samples (10 ml) were collected through the cannulae at -10, -5, 0, 1, 2, 5, 10, 20 and 30 min relative to the time of the injection. This procedure was performed twice on each horse at an interval of 2 h. Samples were treated as described above.

Ovarian extraction

Ovarian tissue samples were collected from 33 mares at the abattoir within 10 min of slaughter, snap frozen in liquid nitrogen and stored at -80°C. They were classified according to appearance at the time of collection into corpora haemorrhagica (*n* = 5), corpora lutea (*n* = 13), corpora albicantia (*n* = 5), follicular wall (*n* = 5) and follicular fluid (*n* = 10). Samples were partially thawed, weighed (0.7–1.3 g) and chopped into 5 ml ice-cold extraction medium (15% v/v TFA, 5% v/v formic acid, 1% w/v NaCl in 1 mol HCl/l). The tissue was homogenized and centrifuged at 20 000 *g* for 30 min at 4°C and the resulting supernatant was applied to C18 columns as described above. Freeze-dried extracts were reconstituted in 0.5 ml assay buffer and the oxytocin concentration was estimated at 2 dilutions (neat and 1:10) as described.

Results

All 9 mares were exhibiting regular oestrous cycles as judged by their plasma progesterone profiles (Fig. 1). However, the oxytocin concentrations measured were consistently low (<0.6–4.0 pg/ml), the majority (68%) being at or below the detection limit of the assay (Fig. 1). All ovarian extracts contained negligible amounts of oxytocin. Of the 38 samples measured, 18 were at or below the detection limit of the assay (2 pg/g wet weight) and the remainder were all <10 pg/g. In contrast, there was a clear variation in the ovarian progesterone concentration measured at different stages of the cycle. Values (in µg/g) were 0.3 in follicle walls (*n* = 2), 12.6 ± 1.2 in corpora haemorrhagica (*n* = 4), 74.7 ± 14.1 in corpora lutea (*n* = 11) and 0.6 in a corpus albicans (*n* = 1). There was no apparent correlation of either blood or ovarian oxytocin concentrations with the stage of the cycle.

As these results differed from previous reports, 2 further experiments were undertaken to validate the assay and extraction procedure. Recovery of exogenous oxytocin added to mare plasma *in vitro* was ~95% (see Materials and Methods). Injection of 1 unit of oxytocin to mares *in vivo* resulted in the expected increase in circulating concentrations (Fig. 2). Values peaked at 19.7 ± 2.34 pg/ml at 1 min after injection and returned to basal values of 0.8 pg/ml within 30 min. This maximum value is close to the estimated concentration of 48 pg/ml based on an even distribution of 1 unit of oxytocin (1.68 µg) within a blood volume of ~35 l.

Discussion

These results indicate that the ovary of the mare is not a source of oxytocin, and that there is no major change in the circulating concentration of oxytocin during the oestrous cycle. In this respect,

Fig. 1. Patterns of (a) progesterone (■) and (b) oxytocin (□) secretion measured in daily jugular venous samples collected from 9 mares. Values are centred around the day on which the plasma progesterone first dropped to <2.5 ng/ml during luteolysis. The number of estimates is shown above each point. Concentrations are means $\pm$ s.e.m.

horses clearly differ from domestic ruminants, in which there is substantial evidence that the corpus luteum synthesizes and secretes oxytocin during the cycle (Wathes & Swann, 1982; Ivell & Richter, 1984; Swann *et al.*, 1984) and that this peptide is involved in the regulation of luteolysis (Flint & Sheldrick, 1983).

Our results also differ from previous reports of oxytocin concentrations in mares' plasma (Burns *et al.*, 1981; Tetzke *et al.*, 1987). According to Burns *et al.* (1981), concentrations peaked at ~ 80 pg/ml on Day 2 of oestrus and declined to ~ 5 pg/ml on Day 10. In direct contrast, Tetzke *et al.* (1987) found that concentrations were highest on Day 15 of the cycle with a mean of 170 pg/ml and lowest at oestrus (83 pg/ml). In both these reports concentrations were therefore at least one order of magnitude higher than those reported here. The most obvious difference in methodology relates to the assay procedure, as Burns *et al.* used unextracted plasma, whereas Tetzke *et al.* (1987) employed an acetone-HCl extraction procedure with Fuller's earth. It is possible that these procedures produced high levels of nonspecific binding; or the differences could relate to the different antisera used. The present paper also employed a lower sampling frequency than Tetzke *et al.*

Fig. 2. Concentrations of oxytocin measured in jugular venous blood following the injection of mares with 1 unit of Syntocinon i.v. at time zero. Concentrations are means $\pm$ s.e.m. of 4 samples at each point.

(1987). Whilst daily samples are clearly inadequate to characterize a pulsatile release pattern, previous work in ewes has demonstrated that this is sufficient to pick up cyclic variations of oxytocin release from the corpus luteum (Wathes *et al.*, 1991).

Although the present results indicate that ovarian oxytocin is not involved in regulating reproductive tract activity in horses, this does not rule out the possibility that oxytocin of pituitary origin could play a role during the oestrous cycle. Goff *et al.* (1987) found that injections of large doses of oxytocin (10 iu/kg body weight) on various days of the cycle caused an increase in plasma 15-keto-13,14-dihydro PGF-2 α , with a maximum response on Day 16 of the cycle and a reduced response in pregnant vs. nonpregnant mares. As oxytocin release during luteolysis in ewes is pulsatile (Flint & Sheldrick, 1983; Hooper *et al.*, 1986) a similar pattern could have been missed by the once a day sampling protocol employed here. We have also reported a possible role for very short (1–4 min) oxytocin pulses in regulating motility of the ovine reproductive tract at oestrus (Gilbert *et al.*, 1991). Evidence for a similar function for oxytocin in mares could best be provided by measurements of oxytocin receptors in the reproductive tract during the cycle.

We thank Professor A. E. Goodship and the Janssen Research Foundation for allowing use of their mares, J. Allen for excellent care of the animals, M. L. Wild for skilled technical assistance, the Journals of Reproduction and Fertility Ltd for provision of a vacation scholarship for K. R. Stevenson and the Wellcome Trust for financial support.

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Received 9 November 1990