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Published in:

BJOG: An International Journal of Obstetrics & Gynaecology

DOI:

[10.1111/j.1471-0528.2004.00049.x](https://doi.org/10.1111/j.1471-0528.2004.00049.x)

2004

[Link to publication](#)

Citation for published version (APA):

Steinwall, M., Hansson, S., Bossmar, T., Larsson, I., Pilka, R., & Åkerlund, M. (2004). Oxytocin mRNA content in the endometrium of non-pregnant women. *BJOG: An International Journal of Obstetrics & Gynaecology*, 111(3), 266-270. <https://doi.org/10.1111/j.1471-0528.2004.00049.x>

Total number of authors:

6

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Oxytocin mRNA content in the endometrium of non-pregnant women

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Objective To study oxytocin mRNA in the human endometrium at different phases of the menstrual cycle.

Design An exploratory study in non-pregnant women.

Setting The Department of Obstetrics and Gynecology, Lund University Hospital, Sweden.

Participants Thirty-three women of fertile age undergoing hysterectomy or endometrial curettage on routine benign gynaecologic indications.

Methods Endometrial tissue was obtained throughout the menstrual cycle. The presence of oxytocin mRNA was investigated by *in situ* hybridisation and by real time PCR.

Main outcome measures Oxytocin mRNA signalling intensity found by *in situ* hybridisation of tissue obtained at different times of the menstrual cycle. Relative amounts of oxytocin mRNA measured by real time PCR.

Results The signal for oxytocin mRNA obtained by *in situ* hybridisation was more pronounced in glandular epithelial cells than in stromal cells. Furthermore, it was most marked around mid-cycle. The expression of oxytocin mRNA was confirmed by real time PCR.

Conclusions The results indicate that oxytocin may be synthesised in the endometrium of non-pregnant women, particularly in the glandular epithelial cells. Hormone released from these sources may have a paracrine action on the uterus. Oxytocin mRNA expression seems to be ovarian hormone dependent with the highest concentration around mid-cycle.

INTRODUCTION

In the pregnant human uterus, oxytocin mRNA has been demonstrated in amnion, chorion and decidua.¹ In non-pregnant conditions, immunoreaction to oxytocin has previously been shown, particularly in the isthmus part of the cervix.² This peptide has also been detected in human follicular fluid.³ These findings may indicate a uterine origin of oxytocin. The possible synthesis of oxytocin in the non-pregnant endometrium has, however, not to our knowledge been studied previously. In a pilot study, we demonstrated oxytocin mRNA in the endometrium of non-pregnant women (unpublished). Oxytocin of endometrial origin may stimulate myometrial activity and, thereby, be involved in sperm and egg transport, implantation and menstruation. We investigated the content of endometrial oxytocin mRNA by *in situ* hybridisation and real time PCR in samples obtained throughout the menstrual cycle.

METHODS

Endometrial tissue was obtained from a total of 33 regularly menstruating, parous women, with a median age of 44 years (range 34–50 years). Sampling was performed at diagnostic curettage or hysterectomy. The diagnoses were leiomyoma in 13 patients, menorrhagia in 10, ovarian cyst in 4, adenomyosis in 3, cervical dysplasia in 2 and uterine prolapse in 1 patient. Only women without hormonal treatment or intrauterine device were included in the study. All subjects were well informed about the purpose and procedure of the investigation and gave their written consent to the sampling. The study was approved by the Ethics Committee at Lund University.

Tissue aliquots measuring approximately 3 × 3 × 3 mm were snap frozen on dry ice and stored at –80°C. The samples were also examined by a histopathologist for exclusion of endometrial pathology and for identification of the menstrual phase.^{4,5} Samples were classified as belonging to early, mid and late proliferative phases, and early, mid and late secretory phases of menstruation.

Tissue sections measuring 12 µm were cut on a cryostat, thaw-mounted on to silanised slides, and stored at –80°C prior to hybridisation. Fresh frozen tissue, rather than fixative-treated material, was used to maximise sensitivity for mRNA detection. Thawing of tissue did not occur prior to sectioning to ensure the best possible tissue integrity.

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For the human oxytocin mRNA, a probe was used corresponding to 194 NT (3-125), Genbank accession No. M25650.⁶ DNA template was generated by PCR amplification, using bipartite primers consisting of either a T7 RNA promoter and a downstream gene-specific sequence or a T3 RNA promoter and upstream, gene-specific sequence. PCR reactions using 1 ng cDNA, 0.5 μ M primers, 200 μ M dNTPs, 3 mM $MgCl_2$, 10 mM Tris, pH 8.3, 50 mM KCl, and 5 units Taq polymerase (Roche, Basel) were amplified at 95°C for 1 min, 62°C for 1 min and 72°C for 1 min for 30 cycles with a final extension at 72°C for 10 min. DNA templates were purified from agarose gels using GeneClean (Bio101) and thereafter sequenced using a cycle sequencing reaction kit (ABI PRISM, Big dye). cRNA probes were transcribed from 40 ng of gel-purified DNA template using 800 Ci/mmol of ³⁵S-UTP (Dupont NEN, Paris) and either T3 or T7 RNA polymerase according to manufacturer's

instructions (Ambion MAXIscrip, Ambion Europe, Cambridge, UK) to generate sense and antisense probes.

Tissue sections were fixed, dehydrated and delipidated as previously described.⁷ Sections were hybridised for 20–24 hours at 55°C with 2×10^6 cpm of denatured ³⁵S-cRNA probe per 50 μ L hybridisation buffer consisting of 20 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 300 mM NaCl, 50% formamide, 10% dextran sulphate, 1 \times Denhardt's, 250 μ g/mL yeast tRNA, 100 μ g/mL salmon sperm DNA, 250 μ g/mL yeast total RNA (fraction XI, Sigma-Aldrich, St Louis, Missouri), 150 mM dithiothreitol (DTT), 0.15% sodium thiosulphate (NTS), and 0.15% sodium dodecyl sulphate (SDS).

Following washing to remove excess probe, slides were opposed to Kodak Hyperfilm Biomax MR for three days and then coated with nuclear track emulsion (NTB-3, Kodak, New York). After four weeks of exposure at 4°C,

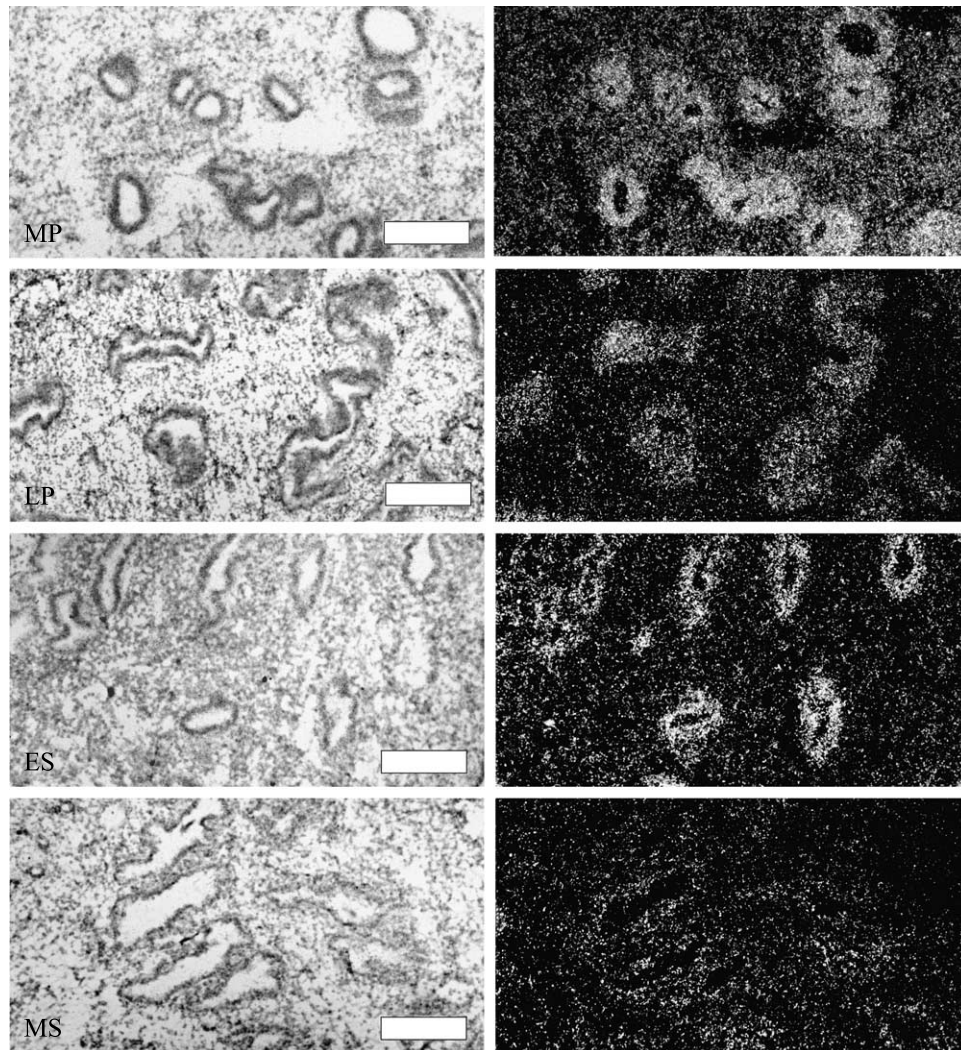


Fig. 1. Brightfield (left column) and darkfield (right column) microscopy showing expression of oxytocin mRNA in endometrial samples obtained from women in different parts of the menstrual cycle. Sections are from mid proliferative (MP), late proliferative (LP), early secretory (ES) and mid secretory (MS) phases of the menstrual cycle. The glandular expression is mainly seen from MP to ES, after which a weak signal remains (MS). Scale bars: 220 μ m.

Table 1. Oxytocin mRNA content in endometrial samples obtained from women in different phases of the menstrual cycle. The oxytocin mRNA signalling intensity in average for the respective menstrual phase was graded from negative (–), equal to background (+), distinguishable from background (++), less than half of maximum (+++) and maximal signalling (++++).

Type of tissue	Early proliferative (n = 7)	Mid proliferative (n = 5)	Late proliferative (n = 4)	Early secretory (n = 3)	Mid secretory (n = 3)	Late secretory (n = 6)	Menstruation (n = 3)
Glandular cells	–	++++	++++	+++	++	+	–
Stromal cells	–	+++	++	++	+	+	–

slides were developed in Dektol (Kodak), fixed and counterstained with a Giemsa stain. All slides were examined by two independent investigators blinded for the experimental conditions (SH and MS) and the signal intensity was graded in five steps from negative to maximal intensity.

Microphotographs were prepared using an Axiophot microscope (Olympus, Tokyo, Japan) equipped for darkfield and brightfield microscopy and a digital camera (Olympus OP50-CU). Captured images were assembled electronically using Adobe Photoshop 5.0. Figures were printed on matte-finished paper by a Fujix Pictography 3000 (Fuji Photo Film, New York) printer at 400 dpi resolution.

Total RNA was extracted using TRIzol reagent (Invitrogen, Invitrogen AB, Lidingö, Sweden) according to manufacturer's instructions. RNA integrity was confirmed on a denaturing formaldehyde gel. RNA was reversely transcribed according to protocols from Applera (Stockholm, Sweden) in a 50 µL reaction containing: 0.5 µg total RNA, and a final concentrations of 1× TaqMan RT buffer, 5.5 mM MgCl₂, 500 µM dNTPs, 2.5 µM random hexamers, 0.4 U/µL RNase inhibitor, and 1.25 U/µL MultiScribe Reverse Transcriptase. The samples were incubated at 25°C for 10 min, at 48°C for 30 min and then 5 min of inactivation at 95°C. They were then stored at –20°C until further used.

Gene transcripts were quantified using real time PCR on ABI PRISM 7000 sequence detection system (Applera). Primers and Fam-labeled probes were obtained from Assays on-Design (Applera).

PCR reactions were carried out in a 25 µL final volume containing final concentrations: 1× Universal PCR Master Mix (Applera), 1× Assaymix (Applera), 0.25 µM probe, 0.9 µM of forward and reverse primers, respectively, and 1 µL of 10 ng/µL of a DNA aliquot. The thermal cycling conditions were initiated by UNG activation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 seconds, annealing at 60°C for 1 min. Two negative controls without template were included in every amplification. RNA samples were tested for genomic DNA contamination prior to further investigation. For each reaction, triplicate or duplicate assay was carried out. Transcript of β-actin as a housekeeping gene, with Gene bank accession number NM_001101, was quantified as endogenous RNA of reference to normalise each sample. Quantification was achieved through a calibration curve obtained by serial fourfold dilutions of the template DNA (0.08–80 ng). Results are expressed as relative values.

Results are presented in a box-plot diagram. The relative amounts of oxytocin mRNA obtained by real time PCR at different times of the menstrual cycle were compared using non-parametric Mann–Whitney *U* test.

RESULTS

The sense probe showed no specific hybridisation. Hybridisation was repeated at least twice with consistently reproducible results. The semiquantitative observations of the two examiners were in agreement. Marked signal for oxytocin mRNA was observed in endometrial glands of women, from whom samples were obtained in mid and late proliferative and early secretory phases of the menstrual cycle (Fig. 1, Table 1). Weak signal was seen in mid secretory phase and signals equal to background in late secretory phase, whereas at other parts of the cycle no clear oxytocin mRNA expression was observed (Table 1). In

Oxytocin/β-actin mRNA

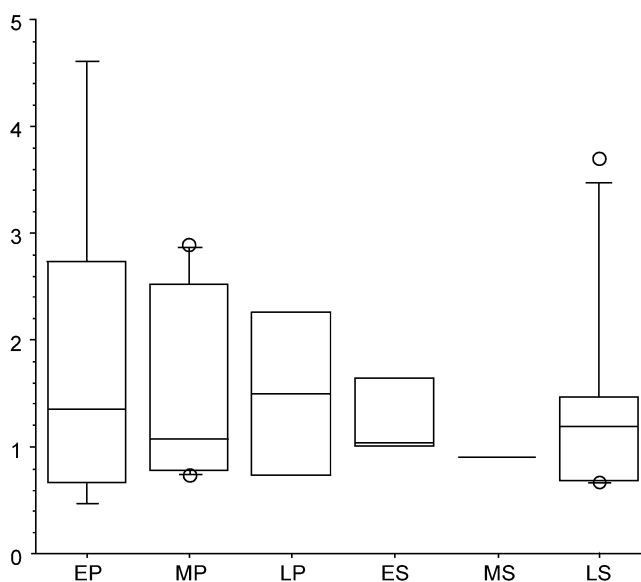


Fig. 2. Real time PCR quantification of oxytocin mRNA. The amount of oxytocin mRNA normalised to the amount of β-actin mRNA. The relative values are presented in a box-plot diagram. No significant changes were obtained between the proliferative and the secretory phases. Early proliferative (EP, number of observations *n* = 5), mid proliferative (MP, *n* = 6), late proliferative (LP, *n* = 4), early secretory (ES, *n* = 4), mid secretory (MS, *n* = 1) and late secretory phases (LS, *n* = 6).

endometrial stromal cells, the signal was weaker, but a cyclical variation in expression of oxytocin mRNA was again observed (Table 1). In the adjacent myometrium, the signal for oxytocin mRNA was absent or scarce.

In samples other than those used for *in situ* hybridisation, but from the same women, the presence of oxytocin mRNA in the endometrium was confirmed (Fig. 2). The expression levels were relatively low, but oxytocin mRNA expression was found in all menstrual phases. No statistically significant difference in expression was found between the different phases (Fig. 2).

DISCUSSION

By *in situ* hybridisation, we demonstrated marked expression of oxytocin mRNA in endometrial samples obtained from non-pregnant women around mid-cycle. The signal was more obvious in glandular epithelial cells than in stromal cells. In endometrium from women at menstruation and in the early proliferative phase, the signal intensity was equal to background. The pattern of oxytocin expression was so unique that an alternative analysis using real time PCR was used for further validation. This method confirmed the presence of oxytocin mRNA, although the expression was comparatively low in all phases. Whether or not the low gene expression level also reflects a low level of functional peptide remains to be determined.

The cyclical variation in oxytocin expression observed by *in situ* hybridisation could not be verified by quantitative PCR. Instead, real time PCR showed expression of low intensity throughout all the examined phases without significant differences. Although this lack of difference may be related to a low number of observations in some groups, a more likely explanation is the difficulty to quantify low transcript gene from heterogenous tissue. Indeed, the appearance of endometrial glands and stroma differs markedly between the proliferative and secretory phases of the menstrual cycle.

Throughout the menstrual cycle there is a proliferation of the endometrial stroma in addition to the morphological changes of the endometrial glands. Therefore, an expression in glandular cells will appear as weaker when measured in the midst of a large amount of negative stromal cells. Both methods used can detect low levels of RNA in tissue, but only *in situ* hybridisation allows identification of low intensity expression in individual cells.

The cyclical variation in endometrial content of oxytocin mRNA could be due to a stimulation by oestradiol in the proliferative phase of oxytocin production, an effect which in the luteal phase is counteracted by progesterone. This concept is in agreement with the finding that oestrogen receptors are well expressed already in the early luteal phase of the menstrual cycle, whereas those for progesterone are developed somewhat later.⁸ The effects of ovarian steroids

on oxytocin mRNA in the endometrium would therefore be similar to those seen in the hypothalamus, regulating the release of oxytocin into the blood. In previous studies in postmenopausal women, we observed a stimulatory influence of oestradiol on oxytocin release, an effect which was counteracted by progesterone.^{9,10} This effect of oestradiol is also in agreement with the finding that the human oxytocin promoter gene has an oestrogen responsive element.¹¹ The endometrial content of vasopressin mRNA was not studied here, but regarding the circulating level of this closely related peptide, a variation with peak plasma concentration at mid-cycle has also been observed by our group.¹² We also observed high vasopressin peptide levels after unopposed oestradiol treatment of postmenopausal women and that addition of progestogen counteracted this effect.^{10,12,13}

Myometrial and endometrial contents oxytocin and vasopressin V_{1a} receptors as well as the *in vivo* sensitivity of the myometrium to these hormones vary in a way, which is opposite to that presently observed for oxytocin mRNA in the endometrium. Thus, maximal density of these receptors and the highest myometrial sensitivity are found at the onset of menstruation.^{14–16} Our results, with high oxytocin mRNA levels in the endometrium at mid-cycle but low oxytocin receptor concentration in the myometrium, could imply different physiological functions of oxytocin in these two tissues. However, regarding receptors it must be kept in mind that individual cells can show a great heterogeneity and rapid changes in their expression of oxytocin receptor.¹⁷

Oxytocin has since long been ascribed a significant role in the start of labour preterm and at term. An important proof of the involvement of oxytocin and vasopressin in mechanisms of preterm labour is the therapeutic effect of atosiban, an oxytocin and vasopressin V_{1a} receptor blocking agent.^{20,21} However, any marked rise in plasma concentration of oxytocin or in uterine receptors at the onset of labour has not been demonstrated.^{18,19} Indeed, data supporting a local synthesis of oxytocin in the pregnant uterus, not reflected in plasma levels, and a paracrine action have accumulated during the latest years.^{1,22,23} The present results suggest a uterine synthesis of oxytocin also in non-pregnant condition. Oxytocin of endometrial origin could possibly induce myometrial contractions indirectly by an effect over endometrial receptors stimulating the synthesis of PGF_{2α}, which would mediate contractions in parallel to the situation in pregnancy.^{24,25} Locally released oxytocin could also directly stimulate contractions of the non-pregnant uterus via oxytocin receptors in adjacent myometrium, as in pregnancy.¹⁴ In fact, the uterine contractility *in vivo* in non-pregnant women in the late follicular phase was shown by ultrasound technique to involve only the subendometrial layer of the myometrium.²⁶ This observation is in agreement with the previous *in vitro* finding of a variation in uterine contractility between different layers of the myometrium in non-pregnant condition.²⁷ In that

study, myometrium closest to the endometrial cavity had the most pronounced activity. It may be that the retrograde transport of sperms towards the fallopian tubes at this time of the menstrual cycle is facilitated by the selective contractility of myometrium close to the endometrium. An involvement of endometrial oxytocin in the uterine hyperactivity of primary dysmenorrhoea is less probable, in view of the lack of observed endometrial oxytocin mRNA around the onset of menstruation.

Acknowledgements

This study was supported by Lund University, the Crafoord Foundation and the Swedish Scientific Council, grant nos. 14358 and 14187.

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Accepted 16 October 2003