

The expression of the IGF system in the bovine uterus throughout the oestrous cycle and early pregnancy

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Abstract

The IGF system is expressed in the uterus during the oestrous cycle and early pregnancy and is likely to play an important role in regulating the development of the embryo and uterus. The IGF peptides (IGF-I and -II) mediate their effects through the type 1 IGF receptor (IGF-1R), while the IGF-binding proteins (IGFBP-1 to -6) modulate their interaction with the receptor. In this study, the expression of the IGF system in the bovine uterus was determined throughout the oestrous cycle and on day 16 of pregnancy.

Endometrial biopsy samples were collected from four cows over three cycles such that there were samples for every 2 days from day 0 (oestrus) to day 14 and then every day until day 21. To assess the effect of pregnancy, uterine horn cross-sections were collected on day 16 from 15 pregnant (PREG), five inseminated non-pregnant (INP) and nine unseminated cyclic controls (CONT). The expression of mRNA for the IGFs, IGF-1R and IGFBP-1 to -5 was determined by *in situ* hybridisation and the results were quantified by measuring the optical density units from autoradiographs. The main region of IGF-I mRNA expression was the sub-epithelial stroma underlying the luminal epithelium. The expression of IGF-I mRNA was highest at oestrus and lowest during the early and late luteal phases. On day 16, IGF-I mRNA levels were low in all groups, with pregnancy having no effect on

the IGF-I mRNA concentrations. The strongest expression of IGF-II mRNA was in the caruncular stroma, with pregnancy having no significant effect in this region. IGF-1R mRNA was also present in the caruncles and was strongly expressed in all epithelial cells both throughout the oestrous cycle and during early pregnancy. The expression of IGFBP-1 mRNA was confined to the luminal epithelium, with the strongest expression seen on day 14 of the cycle. On day 16 the expression of IGFBP-1 mRNA was higher in the PREG group compared with the CONT group. The expression of IGFBP-2 mRNA was localised to the sub-epithelial stroma with more INP than PREG cows showing detectable levels of IGFBP-2. The strongest expression of IGFBP-3 mRNA was in the caruncular stroma; expression in the endometrial stroma was similarly decreased during early pregnancy. IGFBP-5 mRNA was mainly expressed in the inner ring of myometrium and was not affected by pregnancy on day 16.

In conclusion, these results show that many components of the uterine IGF system are differentially regulated during the oestrous cycle and early pregnancy and suggest that modulation of the IGF system may influence uterine activity during this period.

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Introduction

Insulin-like growth factors (IGF)-I and -II are single chain polypeptides which are structural analogues of proinsulin. IGF-I and -II promote DNA synthesis, cell proliferation and differentiation as well as having acute anabolic effects on protein and carbohydrate metabolism (Jones & Clemmons 1995, Hossner *et al.* 1997). IGF-I and -II are expressed in the bovine uterus (Geisert *et al.* 1991, Kirby *et al.* 1996) and conceptus (Watson *et al.* 1992) and are also present in ovine uterine luminal fluid (Ko *et al.* 1991)

during the pre-implantation period. During this time the ruminant embryo is free living *in utero* and is dependent on the maternal glandular secretions for all metabolic requirements. The embryo undergoes elongation beginning on day 12. This is associated with the production of the anti-luteolytic hormone interferon-tau (IFN- τ) (Farin *et al.* 1990). IGF-I and -II have been shown to stimulate embryonic production of IFN- τ *in vitro* (Ko *et al.* 1991) and it is likely that IGF-I and -II play an important role in development of both the embryo and uterus during early pregnancy (Wathes *et al.* 1998). The actions of IGF-I and

-II are mediated through the IGF type 1 receptor (IGF-1R). IGF-1R has been localised to the pre-implantation embryo (Watson *et al.* 1992) and to the glandular epithelium of the ovine uterus (Stevenson *et al.* 1994, Reynolds *et al.* 1997a) supporting a role for IGFs in embryonic and uterine development.

The actions of IGFs are modulated by the IGF-binding proteins (IGFBPs) of which there are at least six different proteins (reviews: Jones & Clemmons 1995, Hossner *et al.* 1997). In the circulation, IGFs are predominately carried by IGFBP-3, which establishes and maintains a large circulatory pool of IGF. The IGFBPs are able to target the IGFs to a particular tissue. In the target tissue the IGFBPs generally attenuate IGF activity by preventing IGF interacting with its surface receptors, although stimulatory effects for IGFBP-1, -2, -3 and -5 have been reported. IGFBPs are able to associate with cells, IGFBP-1 and -2 to the $\alpha_5\beta_1$ integrin receptor (Jones *et al.* 1993a), IGFBP-3 with glucosaminoglycans and IGFBP-5 to collagen, laminins and fibronectin components of the extracellular matrix (Jones *et al.* 1993b). The cell-associated IGFBP has reduced affinity for the IGF, thereby enhancing IGF delivery to IGF-1R. Target cells can also increase the IGF bioavailability by either dephosphorylation of IGFBP-1 or proteolytic cleavage of the IGFBPs.

The current calving rate per insemination for dairy cows is less than 55% with a substantial loss of embryos occurring between days 1 and 42 (Peters 1996). In cattle, poor fertility may in part be due to the cow at service being in negative energy balance as the energy requirement for lactation at this stage is higher than can be supported by feed intake, particularly in high-yielding cows (Kruip *et al.* 1998). Many components of the IGF system are modulated by nutrition; for example serum IGF-I is decreased during dietary energy restrictions (Thissen *et al.* 1994). It is possible that the poor fertility seen during negative energy balance may in part be due to an inadequate IGF system within the uterine environment.

Previous studies have shown that around maternal recognition of pregnancy in cows the expression of both IGF-I and -II mRNA was increased, while IGFBP-3 mRNA was decreased in pregnant (PREG) endometrium (Geisert *et al.* 1991, Kirby *et al.* 1996, Keller *et al.* 1998). In the present study we have extended these findings on the expression of the IGF system in the bovine uterus by examining the localisation of most of the components throughout the oestrous cycle and during early pregnancy. This should aid our understanding of the regulation of the uterine IGFs and the role that the IGF system may play in the establishment of pregnancy in cows.

Materials and Methods

Animals

Experiment 1 Endometrial biopsy samples were collected over three oestrous cycles from four Friesian/

Holstein non-lactating cows as described by Mann & Lamming (1994). The biopsy samples were collected on days 14, 15, 16, 17 and 18 over the first cycle; days 6, 8, 10 and 12 over the second cycle and days 0 (oestrus), 2, 4, 19, 20 and 21 over the third cycle. The samples were frozen immediately in isopentane in liquid nitrogen and stored at -80°C for subsequent analysis. Blood samples were collected daily from the jugular vein for subsequent oestradiol and progesterone analysis.

Experiment 2 Twenty-nine barren Friesian/Holstein cows at the end of lactation were synchronised with two injections of estrumate (prostaglandin $F_{2\alpha}$ analogue; Coopers Animal Health, Crewe, Cheshire, UK) 12 days apart. The cows were then doubly inseminated 72 and 96 h after the second prostaglandin injection or left as controls. The cows were slaughtered on day 16 and the uterus flushed for embryos. Uterine cross-sections, approximately 5 cm in length were taken and frozen in isopentane in liquid nitrogen and stored at -80°C for subsequent analysis. In PREG animals the cross-sections were taken from the horn with the embryo present.

Oligonucleotide probes

All probes used were single-stranded oligonucleotides 45 bases in length (Brabraham Institute, Cambridge, UK). Sense probes were always included as negative controls and any signal from these was regarded as non-specific (Table 1).

Localisation of mRNA by in situ hybridisation

The method was based upon that previously described by Stevenson *et al.* (1994). Sections (10 μm thick) were cut and thaw mounted onto 1 mg/ml poly-L-lysine ($M_r > 300\,000$) coated glass slides. The sections were fixed in 4% (w/v) paraformaldehyde in 0.01 M PBS, pH 7.0 for 5 min at room temperature and then washed three times with 0.01 M PBS. The slides were sequentially dehydrated in ethanol and stored at 4°C in 95% ethanol. The oligonucleotide (5 ng) was end-labelled with [^{35}S]dATP (Amersham International, Aylesbury, Bucks, UK) using deoxynucleotidyl transferase (Pharmacia Biotech, St Albans, Herts, UK) at 34°C for 1 h. The labelled probe was diluted to a final concentration of 1 100 000 c.p.m./ml in hybridisation buffer (50% (v/v) deionised formamide, $4 \times \text{SSC}$, 25 mM sodium phosphate, 1 mM sodium pyrophosphate, $5 \times \text{Denhardt's}$ solution, 0.2 mg/ml denatured salmon sperm DNA, 120 $\mu\text{g}/\text{ml}$ sodium heparin, 100 $\mu\text{g}/\text{ml}$ polyadenylic acid and 100 mg/ml dextran sulphate) and 100 μl added to each section. The sections were incubated in a humidified box at 42.5°C overnight. The slides were washed in $1 \times \text{SSC}$, 2 g/l sodium thiosulphate at room temperature for 30 min, then in fresh $1 \times \text{SSC}$, 0.2 g/l sodium

Table 1 The sequence of the sense probes and exposure time for *in situ* hybridisation X-ray films and emulsions

Probe	Sense sequence	Exposure time (days)		Homology to bovine gene (%)
		X-ray film	Emulsions	
IGF-I	82–126 of ovine IGF-I gene (Wong <i>et al.</i> 1989) 5' GTC ACA TCC TCC TCG CAT CAC TTC TAT CTG GCC CTG TGC TTG CTC 3'	18	24	98
IGF-II	352–396 of ovine IGF-II gene (O'Mahoney & Adams 1989) 5' CCA GCG AGA CTC TGT GCG GCG GGG AGC TGG TGA CAC CCT CCA GTT 3'	18	24	100
IGF-1R	338–382 of human IGF-1R gene (Ullrich <i>et al.</i> 1986) 5' CTC ACG GTC ATC CGC GGC TGG AAA CTC TTC TAC AAC TAC GCC CTG 3'	18	24	ND*
IGFBP-1	779–823 of bovine IGFBP-1 gene (Sneyers <i>et al.</i> 1991) 5' GGA GAG CCT GGG CTC TGT TGG TGT GTC TAC CCT TGG AGT GGG AAG 3'	24	36	100
IGFBP-2	487–531 of bovine IGFBP-2 gene (Bourner <i>et al.</i> 1992) 5' GAG GTG CTG TTC CGC TGC CCG CCC TGC ACG CCC GAG AGC CTG GCC 5'	30	42	100
IGFBP-3	534–578 of bovine IGFBP-3 gene (Spratt <i>et al.</i> 1991) 5' GAG AGC GAG GAG GAC CAC AGC ATG GGG AGC ACA GAG AAC CAG GCC 3'	24	36	100
IGFBP-4	118–162 of ovine IGFBP-4 gene (Shimasaki & Ling 1991) 5' CTG GGC AAG GGA ATG CGC TGT GGG GTG TAC ACC CCC GAC TGC GGC 3'	30	42	91
IGFBP-5	291–335 of bovine IGFBP-5 gene (Moser <i>et al.</i> 1992) 5' CTA CTC GCC CAA GAT CTT CCG GCC CAA GCA CAC CCG CAT CTC CGA 3'	14	21	100

*ND: This region of the bovine gene has not been determined yet, but the region selected is highly homologous between other species.

thiosulphate at 57.5 °C for 60 min. The sections were washed for 1 min in 1 × SSC, 0.1 × SSC, 75% ethanol and 95% ethanol and air-dried. The slides were exposed to β-max hyperfilm (Amersham International) for the time indicated in Table 1.

Photographic emulsions

Slides previously exposed to X-ray film were coated with photographic emulsion LM1 (Amersham International) according to the manufacturer's instructions and left at 4 °C for the time indicated in Table 1. The slides were developed in 20% phenisol, fixed in 1.9 M sodium thiosulphate and counterstained with haematoxylin and eosin to confirm cellular localisation of the radioactive signal.

Optical density (OD) measurements

OD measurements were made using a Seescan image analysis system (Seescan plc, Cambridge, UK) as described by Stevenson *et al.* (1994). In brief, autoradiographs were projected onto a computer screen, the region of interest was circled and the OD reading was measured. The background OD, from a blank autoradiograph, was first automatically subtracted. The OD value for the sense section was subtracted from the corresponding antisense section. Readings were obtained from two sections per cow and a mean value of specific hybridisation for each animal was then calculated. The limit of detection was taken as an OD value of 0.01. Coefficients of variation between two separate slides for duplicate absorbance measurements were as follows: IGF-I, 12.9%; IGF-II, 17.5%; IGF-1R, 9.2%; IGFBP-1, 13.6%; IGFBP-2,

18.9%; IGFBP-3, 12.7%; and IGFBP-5, 13.5%. In Experiment 1, all sections from the same cow treated with a particular probe were analysed in the same batch. In Experiment 2, sections from all animals treated with a particular probe were analysed together.

Statistical analysis

The data were subjected to ANOVA using the general linear model procedures of SPSS version 7.0. The data were tested for heterogeneity of variance by Levene's test and log transformed if appropriate. In Experiment 1, the data were analysed by randomised block ANOVA with day as the fixed factor and cow as the random factor. The error degrees of freedom were adjusted for the repeated measures design using the Greenhouse–Geisser epsilon value. The concentrations of the same probe in different regions were compared using a paired *t*-test. In Experiment 2, the data were analysed by one-way ANOVA followed by Fisher's least significant difference multiple comparison to elucidate where differences lay. A non-parametric Kruskal–Wallis ANOVA was performed on data for IGF-II mRNA in the caruncular stroma as the data did not show a normal distribution. The numbers of animals which were expressing detectable levels of IGF-I and IGFBP-2 mRNA between PREG and inseminated non-pregnant (INP) groups were compared using Fisher's exact test. All data are presented as OD units ± s.e.m.

Results

The bovine uterine endometrium consists of stromal tissue lined by a luminal epithelium and penetrated by glands.

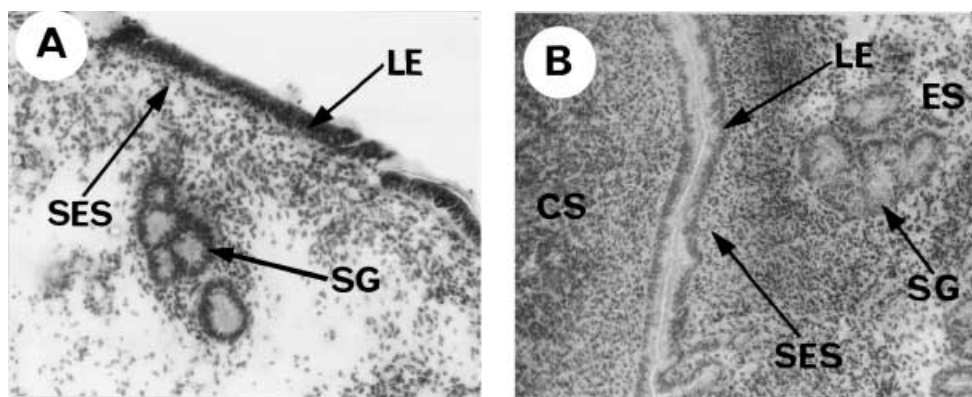


Figure 1 (A) Cryostat section of a bovine endometrial biopsy sample obtained on day 0 (oestrus) of the oestrous cycle stained with haematoxylin and eosin to show the morphology of the different regions examined. LE, luminal epithelium; SG, superficial glands; SES, sub-epithelial stroma underlying the luminal epithelium. (B) Cryostat section of bovine uterine horn cross-section obtained on day 16 of pregnancy stained with haematoxylin and eosin to show the morphology of the different regions examined. CS, caruncular stroma; ES, endometrial stroma. The sections also contained deep glands and myometrium (not shown). Magnification A \times 300, B \times 180.

Three regions of the stroma can be distinguished: the sub-epithelial stroma, a band of dense connective tissue underlying the luminal epithelium; the caruncular stroma, dense connective tissue forming the caruncles (preferred areas of placental attachment) and the endometrial stroma, the loose connective tissue between the luminal epithelium and myometrium. The glands nearest to the lumen are termed superficial glands, whilst those adjacent to the myometrium are the deep glands. The endometrial biopsy samples collected in Experiment 1 routinely only included luminal epithelium, sub-epithelial stroma and superficial glands (Fig. 1A). They also occasionally contained caruncular stroma and myometrium. The complete uterine horn cross-sections available in Experiment 2 contained the following regions: luminal epithelium, sub-epithelial stroma, superficial glands, deep glands, endometrial stroma and myometrium. Caruncular stroma was also present in some cross-sections (Fig. 1B).

Expression of IGF-I mRNA

IGF-I mRNA was localised to the sub-epithelial stroma underlying the luminal epithelium throughout the oestrous cycle and during early pregnancy (Figs 2A and 3A). IGF-I mRNA was expressed in a cyclical manner ($P=0.05$) with the highest expression observed during oestrus. Immediately after oestrus, IGF-I mRNA levels fell to a nadir on day 2, gradually rose to a lower peak around day 10, then declined again by day 16 (Fig. 4). On day 16 of pregnancy, low levels of IGF-I mRNA in the sub-epithelial stroma were detectable (i.e. OD >0.01) in 9 out of 15 PREG (OD 0.04 ± 0.01) compared with only 1 out of 5 INP animals ($P=0.14$) and in 3 out of 9 cyclic control (CONT) cows. In all other animals expression was below

the detection limit of 0.01. IGF-I mRNA was also expressed in the stroma and myometrium in all animals but at very low levels (Fig. 3A).

Expression of IGF-II mRNA

On day 16 of pregnancy, the strongest expression of IGF-II mRNA was observed in the caruncular stroma (Figs 2B and 3B). Caruncles were generally not present in the endometrial biopsy samples and so IGF-II mRNA expression through the cycle was not investigated. IGF-II mRNA was also expressed in the endometrial stroma, myometrium and in the glands, although at much lower levels (Fig. 2B). PREG animals showed lower IGF-II mRNA expression in the endometrial stroma and myometrium compared with the CONT day 16 animals ($P<0.05$) but were not different from the INP group (Fig. 5).

Expression of IGF-1R mRNA

IGF-1R mRNA was expressed at moderate levels in the luminal epithelium and at higher levels in the superficial glands ($P<0.001$) (Figs 2C and 3C). Concentrations of IGF-1R in these regions were relatively constant throughout the oestrous cycle ($P>0.05$), although the highest expression was seen on day 8 in the luminal epithelium and on day 10 in the superficial glands (Fig. 6A). On day 16 of pregnancy, IGF-1R mRNA was expressed in all epithelial cells with the strongest expression observed in the deep glands next to the myometrium ($P<0.001$ versus the luminal epithelium) (Fig. 2C). There were no significant differences between the PREG, CONT and INP

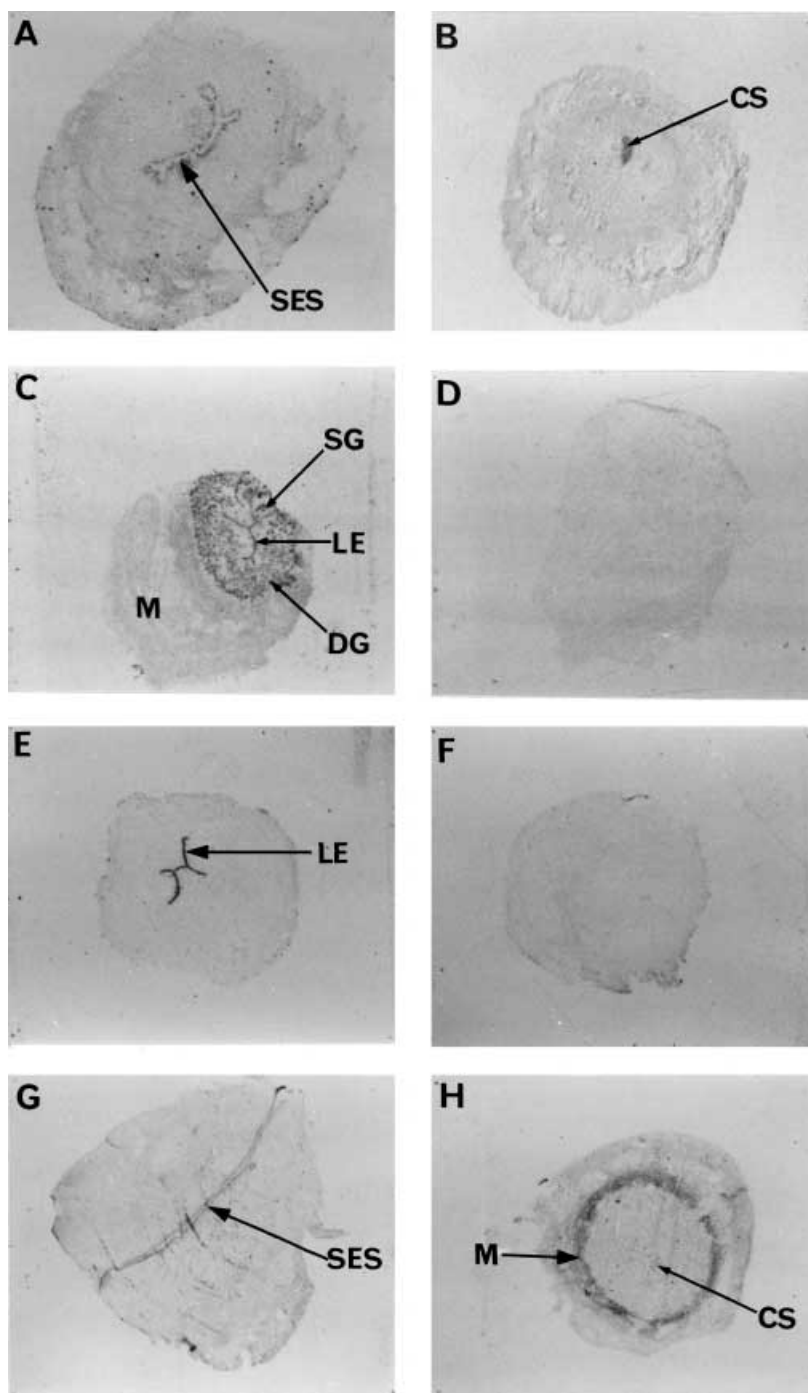


Figure 2 The localisation of mRNA encoding components of the IGF system in the day 16 PREG bovine uterus. The photographs are complete uterine horn cross-sections from autoradiographs showing the distribution of (A) IGF-I; (B) IGF-II; (C) IGF-1R; (E) IGFBP-1; (G) IGFBP-2 and (H) IGFBP-5 mRNA. (D and F) Control sections probed with sense oligonucleotides to IGF-1R and IGFBP-1 mRNA respectively. The sense controls for IGF-I, IGF-II, IGFBP-2 and IGFBP-5 were similarly blank (not shown). There was moderate expression of IGF-1 mRNA in the sub-epithelial stroma (SES) underlying the luminal epithelium (LE). IGF-II mRNA was strongly expressed in the caruncular stroma (CS). IGF-1R mRNA was present in luminal epithelium, superficial glands (SG), deep glands (DG) and myometrium (M). IGFBP-1 mRNA was strongly expressed in the luminal epithelium, while IGFBP-2 mRNA was localised to the sub-epithelial stroma region underlying the luminal epithelium in some PREG cows. The strongest expression of IGFBP-5 mRNA was observed in the inner ring of myometrium (M), but it was also expressed in the caruncular stroma, although at much lower concentrations. Magnification $\times 2$.

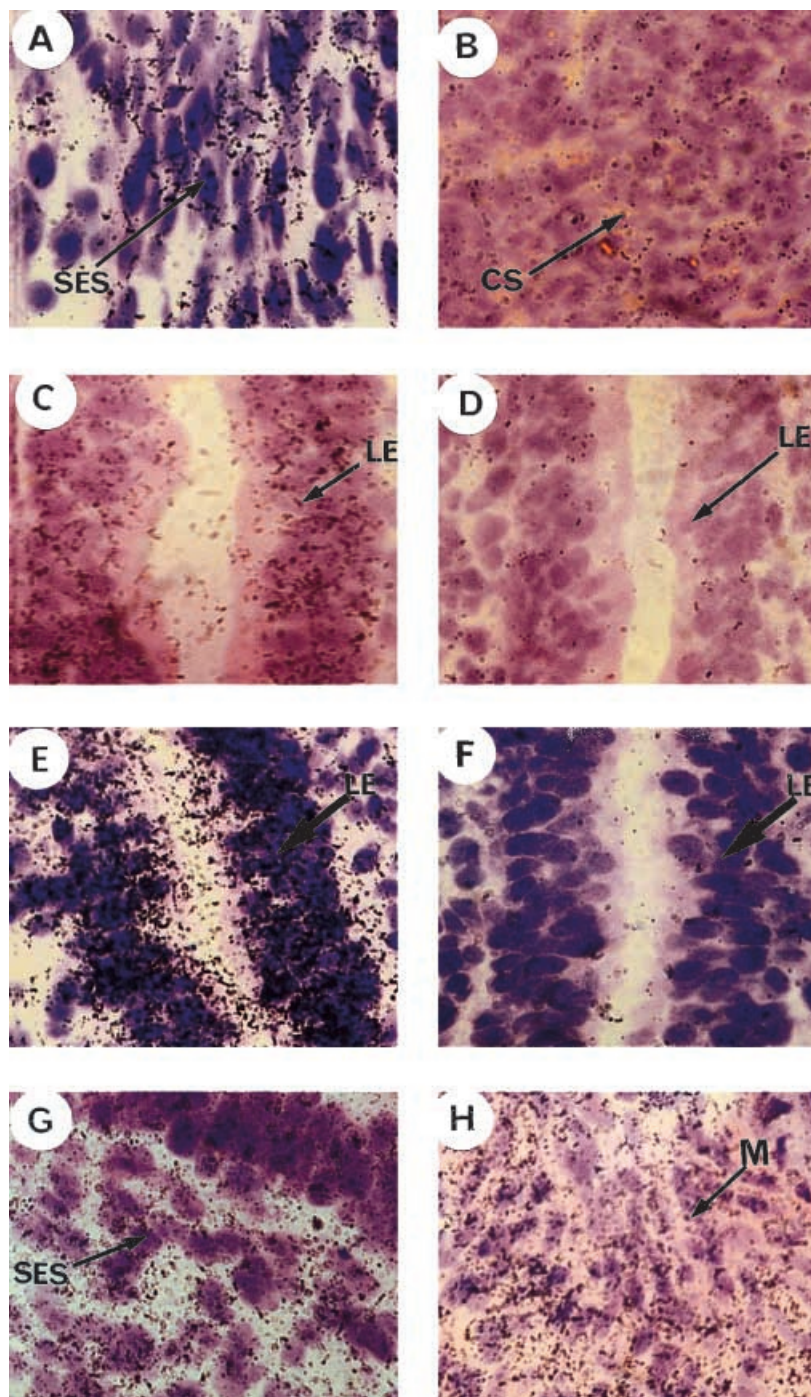


Figure 3 The distribution of the IGF system in the bovine uterus on day 16 of pregnancy. The photographs show sections of uterine horn coated with photographic emulsion and counterstained with haematoxylin and eosin. The cellular localisation of mRNA encoding for (A) IGF-I; (B) IGF-II; (C) IGF-1R; (E) IGFBP-1; (G) IGFBP-2 and (H) IGFBP-5 are shown. The sections were probed with antisense oligonucleotides. (D and F) Show control sections probed with sense oligonucleotides to IGF-1R and IGFBP-1 mRNA respectively. The sense controls for IGF-I, IGF-II, IGFBP-2 and IGFBP-5 were similarly blank (not shown). The silver grains confirmed the localisation for IGF-I mRNA in the sub-epithelial stroma (SES) underlying the luminal epithelium (LE), IGF-II mRNA in the caruncular stroma (CS) and IGF-1R mRNA in luminal epithelium. IGFBP-1 mRNA was strongly expressed in the luminal epithelium, whereas IGFBP-2 mRNA was localised to the sub-epithelial stroma region. The strongest expression of IGFBP-5 mRNA was observed in the inner ring of the myometrium (M). Magnification $\times 1300$.

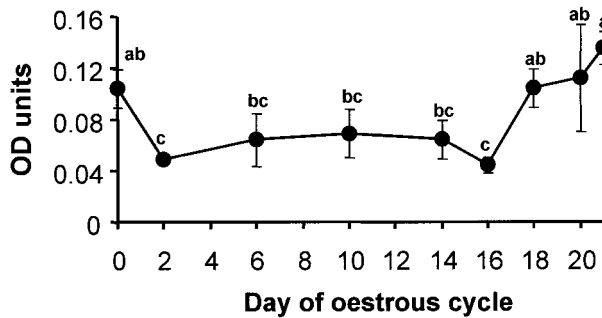


Figure 4 The expression of IGF-I mRNA in the sub-epithelial stroma of endometrial biopsy samples throughout the oestrous cycle. Values are mean OD units \pm S.E.M. ($n=3-4$ per time point). There was a significant effect of day of cycle (ANOVA). All significant differences are indicated with different letters ($a>b>c$) ($P<0.05$).

groups in any region (Fig. 6B). IGF-1R mRNA was also expressed in the caruncular stroma at similar levels in all animals.

Expression of IGFBP-1 mRNA

IGFBP-1 mRNA was localised solely to the luminal epithelium in all samples tested (Figs 2E and 3E). IGFBP-1 mRNA was expressed in a cyclical manner throughout the oestrous cycle ($P=0.04$). IGFBP-1 mRNA expression was low but detectable at oestrus in three out of the four cows, but was not detected during the early luteal phase in all cows. IGFBP-1 mRNA concentrations increased between days 8 and 12, peaked on day 14 and then decreased to become not detected again by day 20 (Fig. 7A). On day 16, the expression of IGFBP-1 mRNA was significantly higher in PREG than in CONT cows ($P=0.02$) with intermediate values in the INP group ($P>0.05$, Fig. 7B).

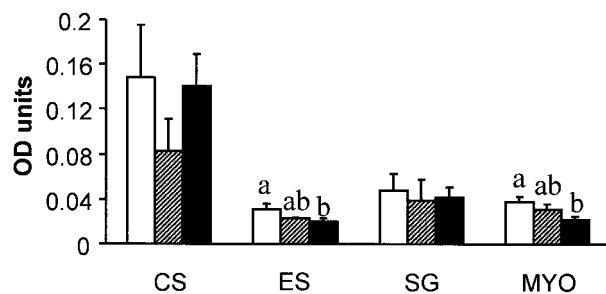


Figure 5 The expression of IGF-II mRNA in the bovine uterus on day 16 measured as OD units. Values are mean OD \pm S.E.M. of CONT (open bars; $n=9$), INP (hatched bars; $n=5$) and PREG (solid bars; $n=15$) cows. The regions shown are caruncular stroma (CS), endometrial stroma (ES), superficial glands (SG) and myometrium (MYO). ($a>b$, $P<0.05$).

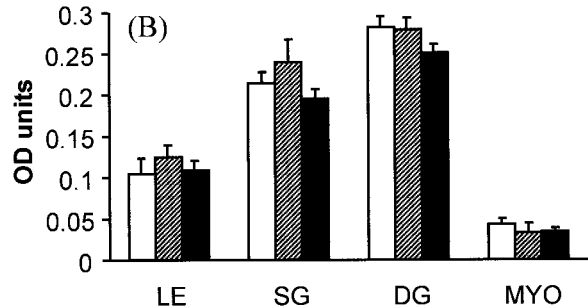
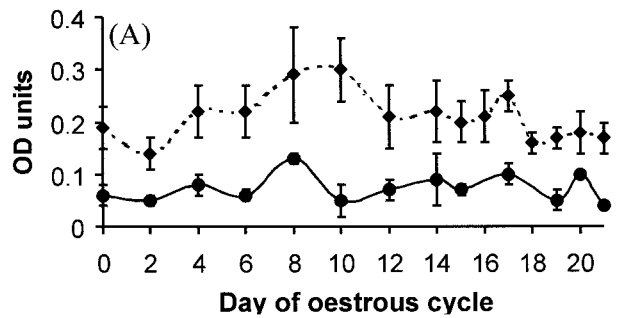


Figure 6 The concentration of IGF-1R mRNA in the bovine endometrium: (A) throughout the oestrous cycle and (B) on day 16 of pregnancy. Values are mean OD units \pm S.E.M. (A) Concentrations of IGF-1R mRNA in the luminal epithelium (continuous line, ●) and in the superficial glands (dotted line, ◆) of bovine endometrial biopsy samples throughout the oestrous cycle ($n=3-4$ per time point). There were no significant differences between oestrus (day 0). (B) Expression of IGF-1R mRNA in the bovine uterus from CONT (open bars; $n=9$), INP (hatched bars; $n=5$) and PREG (solid bars; $n=15$) cows in the luminal epithelium (LE), superficial glands (SG), deep glands (DG) and myometrium (MYO). There were no significant differences between the PREG, CONT and INP groups.

Expression of IGFBP-2 and -4 mRNA in the bovine uterus during early pregnancy

The expression of IGFBP-2 mRNA was the last to be analysed, and since there was not enough tissue left in the biopsy sample we were unable to analyse its expression during the cycle. In early pregnancy, low concentrations of IGFBP-2 mRNA were observed in the sub-epithelial stroma underlying the luminal epithelium with expression confined to this region on day 16 (Figs 2G and 3G). Concentrations were detectable in four out of five INP cows compared with only 3 out of 15 PREG animals ($P=0.03$). The CONT group with three out of seven animals expressing detectable levels was not different from the INP or PREG groups. Preliminary attempts to localise IGFBP-4 mRNA using ovine probes showed no hybridisation in the PREG or cyclic bovine uterus on day 16. Further investigation is required to determine if there was no IGFBP-4 mRNA on day 16 in the bovine uterus or if the ovine probe does not hybridise to the bovine IGFBP-4 mRNA. The homology of the probe to the equivalent region of the bovine gene was 91%.

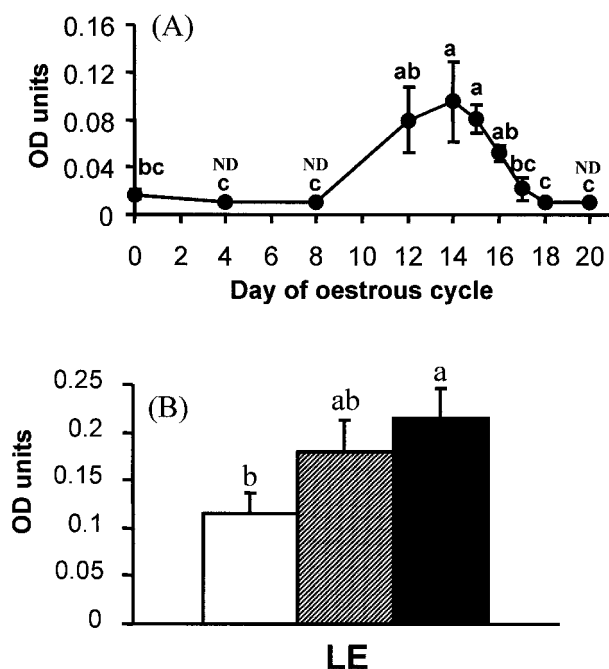


Figure 7 The expression of IGFBP-1 mRNA: (A) throughout the oestrous cycle and (B) on day 16 of pregnancy. Values are mean OD units \pm S.E.M. (A) Concentrations of IGFBP-1 mRNA in the luminal epithelium of bovine endometrial biopsy samples throughout the oestrous cycle ($n=3-4$ per time point). There was low expression of IGFBP-1 mRNA at oestrus (day 0) with non-detected levels (ND) on day 4, 8 and 20. All significant differences are indicated with different letters ($a>b>c$) ($P<0.05$). (B) Expression of IGFBP-1 mRNA in the bovine uterus from CONT (open bars; $n=9$), INP (hatched bars; $n=5$) and PREG (solid bars; $n=15$) cows. The expression of IGFBP-1 mRNA was confined to the luminal epithelium (LE) and was significantly higher ($a>b$; $P<0.05$) in the PREG than in the CONT group.

Expression of IGFBP-3 mRNA

In Experiment 1, IGFBP-3 mRNA was expressed in the luminal epithelium, sub-epithelial stroma and superficial glands (Fig. 8A and B). There was no significant variation in IGFBP-3 mRNA expression throughout the oestrous cycle in any region ($P>0.1$) (Fig. 9A). This may in part be due to the large variation in endometrial IGFBP-3 mRNA expression between cows. In Experiment 2, IGFBP-3 mRNA was absent from the luminal epithelium (except for one INP cow), sub-epithelial stroma and glandular epithelium, in contrast to the results from Experiment 1 (Fig. 8C). IGFBP-3 mRNA was, however, expressed in the endometrial and caruncular stroma as well as in the myometrium (Fig. 8C and D). IGFBP-3 mRNA expression in the endometrial stroma and myometrium was significantly lower in PREG and INP cows than in the CONT cows ($P<0.001$) (Fig. 9B). IGFBP-3 mRNA was also expressed in the blood vessel walls but this was not quantified.

Expression of IGFBP-5 mRNA

The photographic emulsions suggested that there was very low expression of IGFBP-5 mRNA in the glandular epithelium, which was below the detection limit of the Seescan instrument. There was strong IGFBP-5 mRNA expression in the caruncular stroma and myometrium, although these regions were not present in the majority of the biopsy samples and so we were unable to study the expression of IGFBP-5 mRNA throughout the oestrous cycle. On day 16 of pregnancy, the expression of IGFBP-5 mRNA was higher in the inner ring of myometrium compared with the outer ring ($P<0.001$) (Figs 2H and 3H). IGFBP-5 mRNA was also expressed in the caruncular stroma and sub-epithelial stroma although at low concentrations. On day 16, there was no difference in the expression of the IGFBP-5 mRNA between the PREG, CONT and INP groups (for example in the inner ring of myometrium CONT 0.06 ± 0.01 vs INP 0.07 ± 0.01 vs PREG 0.08 ± 0.01 ; $P=0.24$).

Discussion

In this study, we have determined the expression patterns of the various members of the IGF system in the bovine uterus throughout the oestrous cycle and early pregnancy. The different members of the IGF system were localised to different cell types in the endometrium and expression was also influenced both by the stage of the cycle and by the presence of an embryo.

In the bovine endometrium, the strongest expression of IGF-I mRNA was observed in the sub-epithelial stroma underlying the luminal epithelium, which is in agreement with IGF-I mRNA localisation in the sheep (Stevenson *et al.* 1994, Reynolds *et al.* 1997a) and human (Zhou *et al.* 1994) uterus. The expression of IGF-I mRNA in this region would enable IGF-I to stimulate the proliferation/differentiation of the luminal epithelium and/or embryo. A number of studies have demonstrated that uterine IGF-I mRNA concentrations can be markedly increased by the administration of oestradiol to either ovariectomised rats (Murphy *et al.* 1988) or pigs (Simmen *et al.* 1990). Furthermore, Stevenson *et al.* (1994) showed that in sheep IGF-I mRNA expression was highest at oestrus and strongly correlated with oestrogen receptor protein levels. In the present study, IGF-I mRNA expression in the sub-epithelial stroma was highest around oestrus and lowest during the early and late luteal phases with a minor peak on day 10. Ohtani *et al.* (1996) previously reported a similar pattern of IGF-I protein expression in the bovine uterine stroma as determined by immunocytochemistry. Collectively, these results suggested that uterine IGF-I mRNA expression in the cow may be up-regulated by oestradiol. However, Geisert *et al.* (1991) observed no changes in endometrial IGF-I mRNA expression throughout the oestrous cycle as determined by Northern

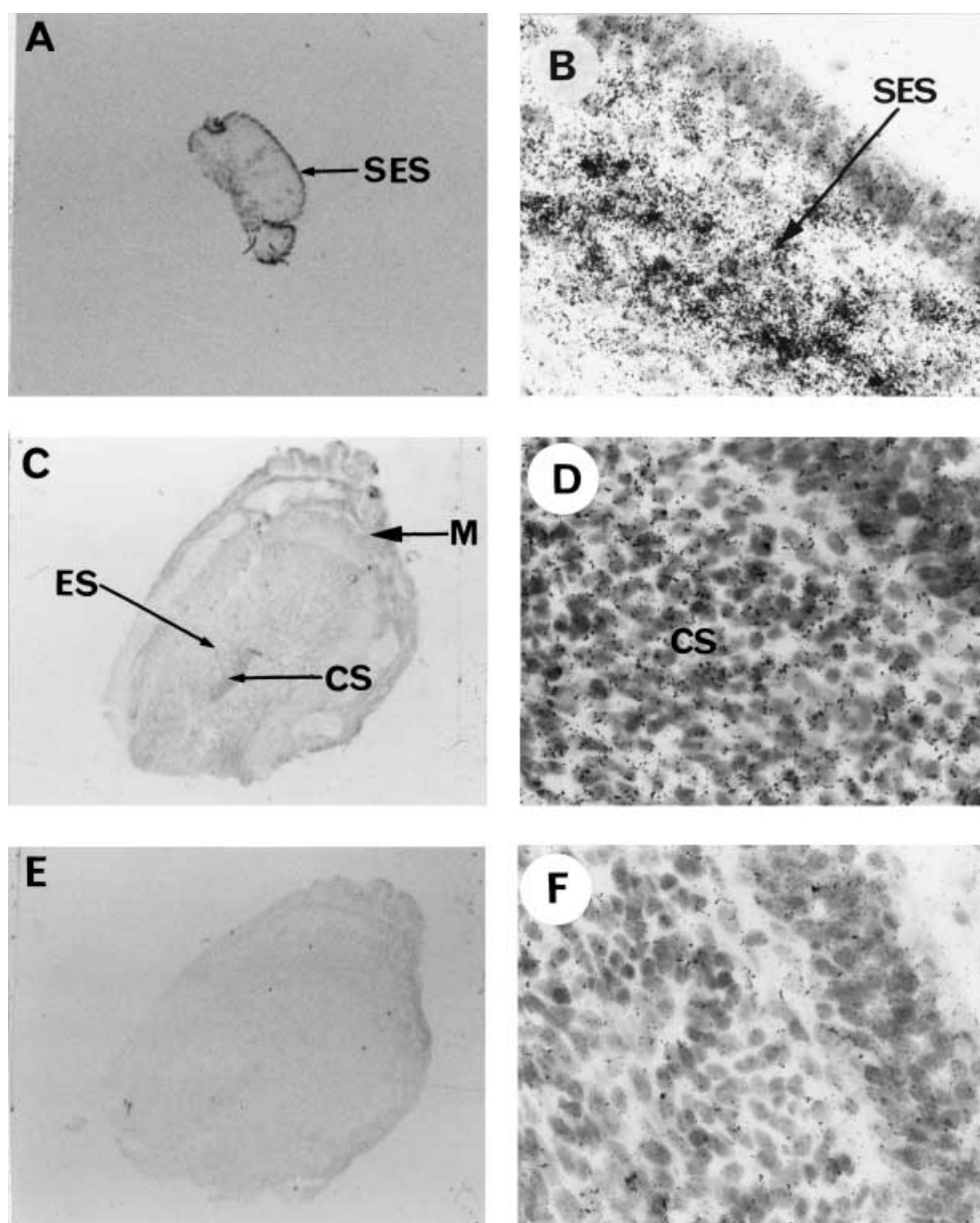


Figure 8 The localisation of IGFBP-3 in the bovine endometrium throughout the oestrous cycle (A and B) and on day 16 of pregnancy (C and D). These sections were probed with antisense oligonucleotides. (E and F) Control sections probed with the corresponding IGFBP-3 sense mRNA. (A, C and E) Photographs of complete uterine cross-sections from autoradiographs; (B, D and F) show sections of uterine horn coated with photographic emulsion and counterstained with haematoxylin and eosin. The strongest expression of IGFBP-3 mRNA in the endometrial biopsies was found in the sub-epithelial stroma (SES) whereas in the day 16 PREG cow (except one non-pregnant cow, not shown) IGFBP-3 mRNA was confined to the endometrial stroma (ES), caruncular stroma (CS) and myometrium (M). Magnification $\times 6$ (A); $\times 2$ (C and E); $\times 1300$ (B, D and F).

blot hybridisation, which would have combined tissue from most endometrial regions. Thus the cyclical regulation of IGF-I mRNA expression may be limited to the sub-epithelial stroma.

The effect of pregnancy on IGF-I mRNA expression in the bovine endometrium is equivocal. Kirby *et al.* (1996) reported higher levels on day 17 of pregnancy, while Geisert *et al.* (1991) showed that pregnancy had no effect

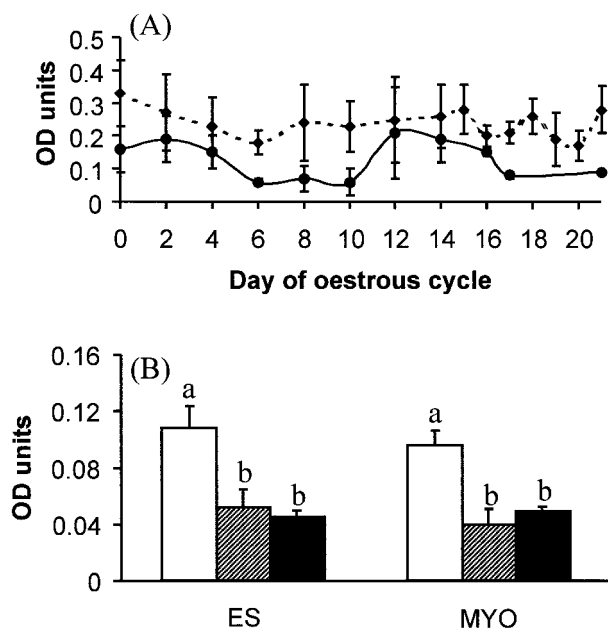


Figure 9 The concentration of IGFBP-3 mRNA in the bovine endometrium: (A) throughout the oestrous cycle and (B) on day 16 of pregnancy. Values are mean OD units \pm S.E.M. (A) Concentrations of IGFBP-3 mRNA in the sub-epithelial stroma (continuous line, ●) and in the superficial glands (dotted line, ◆) of bovine endometrial biopsy samples throughout the oestrous cycle ($n=3-4$ per time point). There were no significant differences with time in any region. (B) Expression of IGFBP-3 mRNA in the bovine uterus from CONT (open bars; $n=9$), INP (hatched bars; $n=5$) and PREG (solid bars; $n=15$) cows. The expression of IGFBP-3 mRNA was significantly ($a>b$; $P<0.01$) lower in the PREG and INP compared with the CONT group. ES, endometrial stroma; MYO, myometrium.

on endometrial IGF-I expression on days 10, 15 and 18. In this study, we observed that 60% of the PREG cows expressed IGF-I mRNA in the sub-epithelial stroma compared with only 20% of the INP group and 33% of the CONT group. These differences were not, however, significant and more studies are required to elucidate the exact regulation of IGF-I mRNA during early pregnancy.

The main site of IGF-II mRNA production in the endometrium was the caruncular stroma, which is the area of embryonic attachment in ruminants. Stevenson *et al.* (1994) similarly reported a strong expression of IGF-II mRNA in the caruncles. Collectively, these results would support a role for IGF-II in the preparation of the caruncle for pregnancy. Geisert *et al.* (1991) reported an increase in IGF-II mRNA expression in the bovine endometrium on days 15 and 18 of pregnancy, but there was no such increase in the uterine luminal fluid and they hypothesised that IGF-II expression was regulated by the conceptus. Because not all sections had a caruncle present, it is difficult to say whether or not the presence of an embryo

had any effect on the caruncular IGF-II mRNA in this study. However, the expression of IGF-II mRNA in the endometrial stroma and myometrium was lower in the PREG cows compared with the CONT.

IGF-1R mRNA was localised to the epithelial cells throughout the oestrous cycle and on day 16 of pregnancy with expression higher in the deep glands than the luminal epithelium. IGF-1R mRNA was also localised to the caruncular stroma and myometrium although at lower levels. A similar distribution of uterine IGF-1R mRNA and IGF-I binding (detected by autoradiography) during early pregnancy has been reported for sheep (Stevenson *et al.* 1994, Reynolds *et al.* 1997a). IGF-1R mRNA was also localised to the glandular epithelium in baboons during the luteal phase and early pregnancy (Hild-Petito *et al.* 1994). This would support a role for either systemic or local IGF-I and -II in the stimulation of glandular secretions, on which the ruminant embryo is dependent during the pre-implantation period. The presence of IGF-1R mRNA in the caruncular stroma would suggest an autocrine action for IGF-II in the proliferation of the caruncle in embryonic attachment. The expression of IGF-1R mRNA was not affected by either hormonal or pregnancy status. Similarly, in sheep and pigs IGF-1R mRNA was expressed throughout the oestrous cycle and early pregnancy (Hofig *et al.* 1991, Stevenson *et al.* 1994). This would suggest that IGF-1R mRNA *in vivo* was constitutively expressed in the bovine uterus.

The expression of IGFBP-1 mRNA was confined to the luminal epithelium. A similar localisation has been reported by *in situ* hybridisation in cows using a bovine riboprobe (Keller *et al.* 1998) and in sheep by both *in situ* hybridisation (Osgerby *et al.* 1999) and immunocytochemistry (Waites *et al.* 1990). IGFBP-1 mRNA expression was tightly regulated throughout the oestrous cycle, with low expression at oestrus and high expression from days 12 to 16. Similarly in other species higher levels of IGFBP-1 mRNA have been found during the luteal phase of the ovarian cycle compared with the follicular phase (Cann *et al.* 1998 (sheep), Fazleabas *et al.* 1989 (baboon), Ghahary *et al.* 1993 (rats), Zhou *et al.* 1994 (humans)). Since high endometrial IGFBP-1 levels are observed when progesterone levels are high and the human IGFBP-1 gene contains a progesterone response element, it has been suggested that progesterone regulates IGFBP-1 expression in the endometrium (Lee *et al.* 1997, Tseng *et al.* 1997). In the present study, IGFBP-1 mRNA was up-regulated 6 days after plasma progesterone concentrations began to rise. We have previously reported that progesterone receptor mRNA concentrations are high from oestrus to day 8 and then progesterone receptor mRNA levels are up-regulated again on day 16 and continue to rise back to oestrus levels (Robinson *et al.* 1998). This suggested that when progesterone receptor mRNA expression was down-regulated, IGFBP-1 mRNA expression was induced and that as progesterone receptor reappeared

IGFBP-1 mRNA concentrations declined. It is unlikely that progesterone is the sole regulator since IGFBP-1 mRNA expression was also observed at oestrus. The exact role that progesterone plays in the regulation of bovine IGFBP-1 mRNA needs further investigation.

We have reported here that on day 16, the uterine IGFBP-1 mRNA expression was higher in the PREG than in the CONT group. Other studies have shown similar increases in IGFBP-1 in pregnant animals compared with controls during the time of maternal recognition of pregnancy (cow, (Keller *et al.* 1998) and sheep (Waites *et al.* 1990, Cann *et al.* 1998)). In the present study, there was no luteolytic progesterone fall in the non-pregnant cows on day 16 (plasma progesterone levels in PREG 7.4 ± 0.6 ng/ml; CONT 6.7 ± 0.8 ng/ml; INP 5.9 ± 0.7 ng/ml; $P > 0.05$, Mann *et al.* 1996). Osgerby *et al.* (1999) previously reported that in unilaterally pregnant ewes, in which the uterus had been previously transected, IGFBP-1 mRNA levels were higher in the pregnant horn compared with the non-pregnant horn. Collectively, these results suggest that the pregnancy effect on IGFBP-1 mRNA expression was a local embryonic signal rather than a systemic one. Waites *et al.* (1990) hypothesised that since maximal endometrial IGFBP-1 and embryonic IFN- τ production coincided with each other, IFN- τ may stimulate IGFBP-1 production. Further studies need to be carried out to see whether this embryonic signal is IFN- τ .

The localisation of IGFBP-2 mRNA expression to the sub-epithelial stroma underlying the luminal epithelium agreed with previous studies in the cow using a riboprobe generated from a rat cDNA (Keller *et al.* 1998) and sheep (Reynolds *et al.* 1997a). In cows, it has been shown that endometrial IGFBP-2 mRNA expression increased during the late luteal phase in both cyclic and non-pregnant animals, although pregnancy had no effect (Geisert *et al.* 1991, Keller *et al.* 1998). In the present study on day 16, only 20% of PREG animals expressed detectable levels of IGFBP-2 mRNA in the sub-epithelial stroma compared with 80% of the INP group and 43% of the CONT cows. This suggested that the conceptus may suppress IGFBP-2 mRNA expression and thereby increase the bio-availability of the IGFs required for embryonic development.

In the endometrial biopsy samples, IGFBP-3 mRNA was present in the sub-epithelial stroma, luminal and superficial glands, whereas in the cows from Experiment 2, IGFBP-3 mRNA was absent from these regions. In both experiments, IGFBP-3 mRNA was present in the endometrial and caruncular stroma. The difference in the localisation of IGFBP-3 mRNA between the two groups may be explained by the differences in lactational or nutritional status of the animals. IGFBP-3 mRNA has been localised to the stromal cells juxtaposed to the luminal epithelium in rats (Girvigian *et al.* 1994, Cerro &

Pintar 1997) and to the luminal epithelium and caruncular stroma in sheep (Reynolds *et al.* 1997a). There was no significant change in the expression of endometrial IGFBP-3 mRNA throughout the oestrous cycle, which suggested that in cows endometrial IGFBP-3 mRNA was not regulated by ovarian steroid hormones. In rats, the regulation of IGFBP-3 mRNA is unclear with oestradiol reported to decrease uterine IGFBP-3 mRNA in ovariectomised rats (Molnar & Murphy 1994), whereas Girvigian *et al.* (1994) reported the highest IGFBP-3 mRNA expression at oestrus. On day 16 of pregnancy, the expression of IGFBP-3 mRNA was significantly lower in the PREG group compared with the CONT but was not different from INP cows. Keller *et al.* (1998) similarly reported lower levels of IGFBP-3 mRNA in the pregnant endometrium. IGFBP-3 acts primarily as an inhibitor of IGF-induced mitogenesis; thus if the embryo can reduce IGFBP-3 expression, it may increase the mitogenic action of IGF on the endometrial stroma (Cerro & Pintar 1997). IGFBP-3 was moderately expressed in the caruncular stroma on day 16 of pregnancy and is likely to modulate the action of IGF-II on the development of the caruncle. IGFBP-3 mRNA was also strongly expressed in the blood vessel wall and has been similarly localised in both sheep (Reynolds *et al.* 1997a) and cows (Keller *et al.* 1998). Thus IGFBP-3 may either act as a barrier to the infiltration of systemic IGF or aid the transport of systemic IGF to its target site.

The strongest expression of IGFBP-5 mRNA was in the inner ring of the myometrium, with pregnancy having no effect. IGFBP-5 mRNA has also been localised to the myometrium in rats (Girvigian *et al.* 1994, Cerro & Pintar 1997) and sheep (Reynolds *et al.* 1997b). IGFBP-5 mRNA was also expressed in the caruncular stroma, although at low concentrations, with pregnancy having no effect. In sheep, IGFBP-5 mRNA was also expressed in the caruncular stroma, although at much higher concentrations (Reynolds *et al.* 1997b) suggesting that there may be a species difference. Unfortunately, the majority of the endometrial biopsy samples did not contain myometrium or caruncular stroma and thus we were unable to determine IGFBP-5 mRNA regulation throughout the oestrous cycle.

In conclusion, IGF-I mRNA was localised to the sub-epithelial stroma underlying the luminal epithelium and was maximal at oestrus, whereas IGF-II mRNA was located in the caruncular stroma. Locally produced or systemic IGF-I and -II could therefore act on the IGF-1R in the glandular and luminal epithelium and caruncles to influence glandular activity and caruncular development. Their actions would be modulated by the IGFBPs. Expressions of IGFBP-1, -2 and -3 were all influenced by the presence of an embryo but only IGFBP-1 was regulated during the oestrous cycle. Future work is required to determine how this system is influenced by maternal nutrition.

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