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# Plasma and Uterine Insulin-Like Growth Factor-I (IGF-I) and IGF-Binding Proteins in Lactating Dairy Cows Treated with Bovine Somatotropin

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**Abstract:** Lactating Holstein cows (80 day in milk) were utilized in a completely randomized design to examine the effect of bovine somatotropin (bST) on plasma and uterine contents of IGF-I and IGF-binding proteins (IGFBP). Cows were managed using the Ovsynch/TAI program. On the day of expected ovulation, cows were assigned randomly to receive bST (n = 9) or to serve as untreated controls (n = 8). Within each treatment group, cows were sacrificed on day 3 or 7 postovulation. Blood was sampled daily for plasma bST, progesterone (P4) and IGF-I RIAs. At slaughter, the oviducts and uterine horns ipsilateral to the corpus luteum (CL) were collected, trimmed free of the broad ligament, and flushed with PBS. Plasma P4 profiles and the weights of CL at day 7 postovulation were similar for control and bST-treated cows. Short-term bST treatment had no detectable effects on IGF-I concentration in plasma. Total IGF-I contents in oviductal flushings (OLF) were low and did not differ between stages of the estrous cycle or treatments. In contrast, uterine IGF-I luminal contents increased ($P < 0.01$) between days 3 and 7 postovulation, and tended to be higher ($P < 0.08$) in the bST-treated group. All major IGFBPs were present in day-3 ULF, but could not be detected in day-7 ULF. Results indicate that bST effects on reproductive responses in dairy cows may involve peripheral as well as local uterine regulation of IGFs and their binding proteins.

**Key words:** BST, IGF, plasma, uterus, cow

## Introduction

The insulin-like growth factor (IGF) system, which consists of IGFs, IGF receptors, and IGF-binding proteins (IGFBPs), has been characterized in uterine and conceptus tissues of several mammalian species including the cow (Geisert *et al.*, 1991; Kirby *et al.*, 1996; Watson *et al.*, 1992), sheep (Reynolds *et al.*, 1997; Watson *et al.*, 1994), pig (Letcher *et al.*, 1989; Song *et al.*, 1996), human (Nonashita *et al.*, 1994;Zhou *et al.*, 1994), and rodents (Bondy *et al.*, 1990; Croze *et al.*, 1990). In the uterus and placenta of the pig and rat, IGF-I mRNA predominates in early pregnancy, whereas IGF-II mRNA accumulation occurs primarily after implantation (Pescovitz *et al.*, 1991; Simmen *et al.*, 1992), suggesting that these mitogens may have distinct actions at the embryo-maternal and feto-maternal interphases, respectively. The higher periimplantation expression of IGF-I gene in the pig uterus coincides temporally with elevated uterine luminal fluid (ULF) IGF contents (Simmen *et al.*, 1989), elongation of spherical blastocysts to filamentous morphology (Geisert *et al.*, 1992), and onset of conceptus secretion of estrogen (Green *et al.*, 1995), which is a paracrine regulator of endometrial function.

The actions of IGF-I and IGF-II on the reproductive tract are modulated by a family of at least six structurally-related IGFBPs (Wathes *et al.*, 1998). These proteins bind IGF-I and IGF-II with high affinity, and often are associated with cell membranes and extracellular matrix where they influence IGF-IGF receptor interactions and possibly induce responses that are independent of IGFs (Jones and Clemmons, 1995; Rechler, 1993). Although uterine expression of IGFBPs has been characterized in several mammalian species, the basic information regarding the role of these binding proteins in the control of uterine differentiation and conceptus development in cattle is lacking.

The recent observation that bST administration at a timed insemination increased pregnancy rates in lactating dairy cows (Moreira *et al.*, 2000; Moreira *et al.*, 2001) suggested that exogenous bST may play an important role in the control of oviductal and uterine IGF availability at the time of blastocyst formation and subsequent pregnancy establishment in cattle. The objective of this study was to examine the effect of exogenous bST on plasma and uterine IGF-I and IGFBPs in lactating Holstein cows, when administered at a synchronized ovulation.

## Materials and Methods

**Animal Management and Treatment:** Seventeen lactating Holstein cows were managed using the

Ovsynch/TAI program as described previously (Moreira *et al.*, 2000). At approximately 44 days postpartum, all experimental animals were presynchronized with an injection of GnRH (Cystorelin®, Merial Ltd., Iselin, NJ; 100 µg, i.m.) followed 7 day later with PGF2 (Lutalyse®, Pharmacia Animal Health, Kalamazoo, MI; 25 mg, i.m.; Badinga *et al.*, 1994). Twelve days after PGF2 injection, the Ovsynch program was initiated with an injection of GnRH followed 7 days later with PGF2 (Moreira, *et al.*, 2001). At 48 h after PGF2, cows received a second injection of GnRH to induce ovulation. At 16 h after the second GnRH administration, cows were assigned randomly to receive bST (Posilac®, Monsanto Co., St Louis, MO; 500 mg, i.m.; n = 9) or serve as untreated controls (n = 8). Within each treatment group, cows were sacrificed on day 3 or 7 following initiation of bST treatment. Ovulation was verified by ultrasonography within 48 h of the second GnRH injection and later confirmed at slaughter. Blood samples were collected daily for bST, IGF-I and P4 radioimmunoassays (RIA). At slaughter, the oviducts and uterine horns ipsilateral to the corpus luteum (CL) were collected, trimmed free of the broad ligament, and flushed with 5 and 20 ml of phosphate-buffered saline (PBS, pH 7.4), respectively. Oviductal (OLF) and uterine (ULF) flushings were stored at -20oC until analyzed for protein, IGF-I and IGFBP contents.

**Quantification of Hormones in Plasma and Flushings:** Concentrations of bST (Badinga *et al.*, 1991), IGF-I (Lee *et al.*, 1991) and P4 (Knickerbocker *et al.*, 1986) in plasma were measured by RIA. Assay sensitivities were 0.1 ng, 2.5 pg, and 0.03 ng for bST, IGF-I and P4 RIAs, respectively. Corresponding intra- and interassay coefficients of variation (CV) were 7.2 and 11.5%, 5.1 and 7.6%, and 6.9 and 9.5%. Insulin-like growth factor-I contents in OLF and ULF were determined by a double antibody RIA (Simmen *et al.*, 1989). Samples were extracted with acid-ethanol and neutralized with Tris-HCl prior to RIA to remove IGFBPs. Human recombinant IGF-I (rhIGF-I; Upstate Biotechnology, Lake Placid, NY) was used as the standard and monoiodinated tracer. The minimum detectable concentration for IGF-I assay was 2.5 pg/ml. Intra- and interassay CV were 5.1 and 7.6%, respectively. Values for immunoreactive IGF-I were expressed as total ng IGF-I in OLF and ULF. Protein concentrations in flushings were determined using the Bradford method (Bradford, 1976).

**Analysis of Plasma and Uterine IGFBPs:** The relative abundance of IGFBPs in plasma and ULF was examined by ligand blot analysis (Hossenlopp *et al.*, 1986). Samples of flushings were centrifuged briefly to remove insoluble material and then concentrated using Centriprep Centrifugal Filter Devices (Millipore, Bedford, MA). One hundred micrograms of plasma or ULF proteins were subjected to a 12.5% SDS-PAGE under non-reducing conditions. Proteins then were transferred to a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech, Piscataway, NJ) by electrotransfer. The filters were blocked for 1 h with Tris-buffered saline (TBS, pH 7.4) which contained 1% Carnation non-fat dry milk. The membranes were incubated in 30 ml of TBS containing 1 x 106 cpm/ml of [125I]-labeled rhIGF-II for 24 h at 4oC. The filters were washed with four to five changes of TBS, blotted dry and exposed to X-ray film (X-OMAT, Eastman Kodak Co., Rochester, NY) for 24 to 48 h. Signals for IGFBPs were quantified by densitometric analysis.

**Statistical Analysis:** Plasma hormone responses to bST were evaluated using the MIXED procedure of SAS (SAS, 1996). Fixed effects included treatment, day of the estrous cycle, and treatment x day interaction. The variance for cow, nested within treatment, was used as random error term to test the main effect of treatment. Total protein and IGF-I contents in flushings were analyzed using the GLM procedure of SAS (SAS, 1996). The mathematical model included effects of stage of the estrous cycle, treatment, tissue and all 2- and 3-way interactions. The variance for cow, nested within stage x treatment interaction, was used as random error term to test effects of stage, treatment and stage x treatment interaction.

## Results

A treatment x day interaction was detected ($P < 0.007$) for plasma somatotropin (ST) concentration (Fig. 1A). Peripheral ST concentrations were similar for control and bST-treated cows between days 1 and 4 postovulation, and then increased between days 5 and 7 in the bST-treated group. Plasma ST concentrations did not change across days in control cows (Fig. 1A). Due to considerable variations among cows, bST treatment had no detectable effects on plasma IGF-I concentrations (Fig. 1B). Average concentrations of IGF-I in plasma were 126.6 ± 11.3 ng/ml and 137.0 ± 10.8 ng/ml in control and bST-treated

cows, respectively.

Concentration of P4 in plasma increased ($P < 0.0001$) from $1.3 \pm 0.6$ ng/ml at d 1 to $8.1 \pm 0.8$ ng/ml at day 7 postovulation (Fig. 2). Exogenous bST had no detectable effects on plasma P4

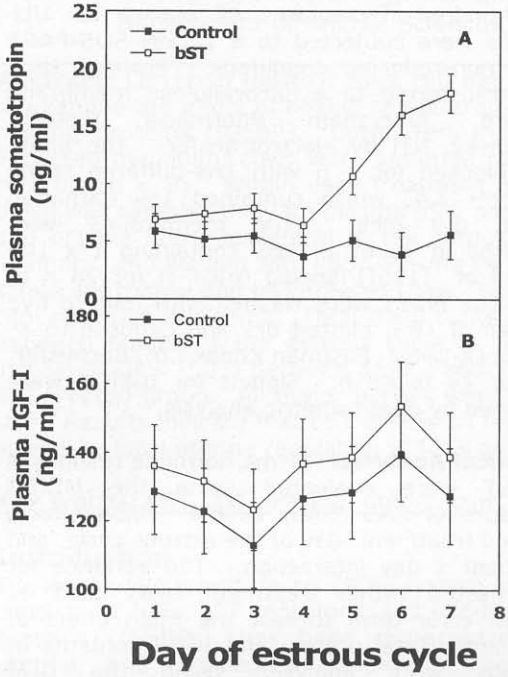


Fig. 1: Plasma somatotropin (A) and IGF-I (B) concentrations in control (■) and bST-treated (□) cows (treatment x d, $P<0.007$).

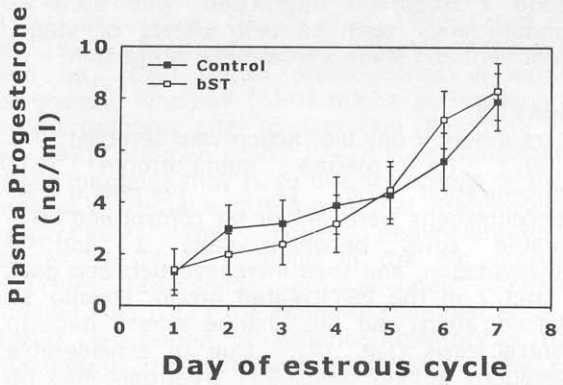


Fig. 2: Plasma P4 concentrations in control (■) and bST-treated (□) cows.

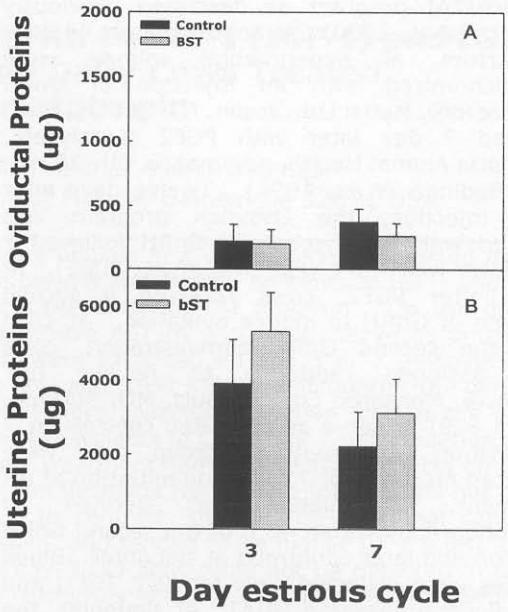


Fig. 3: Protein contents in oviductal (OLF, A) and uterine (ULF, B) lluminal flushings from control (■) and bST-treated (□) cows.

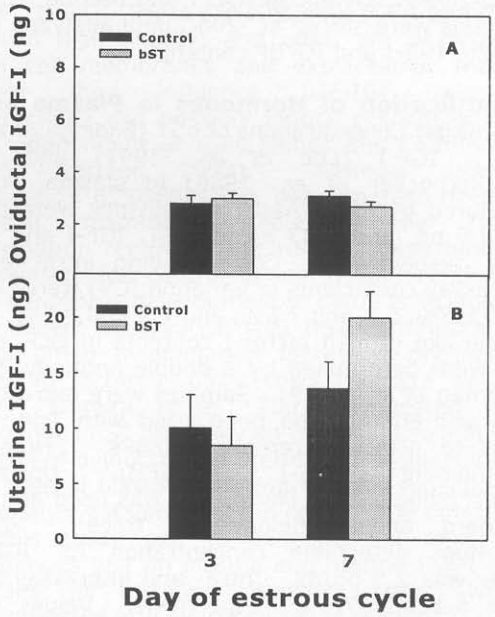


Fig. 4: Oviductal (A) and uterine (B) luminal IGF-I centents in control (■) and bST-treated (□) cows (tissue x d, $P<0.01$).

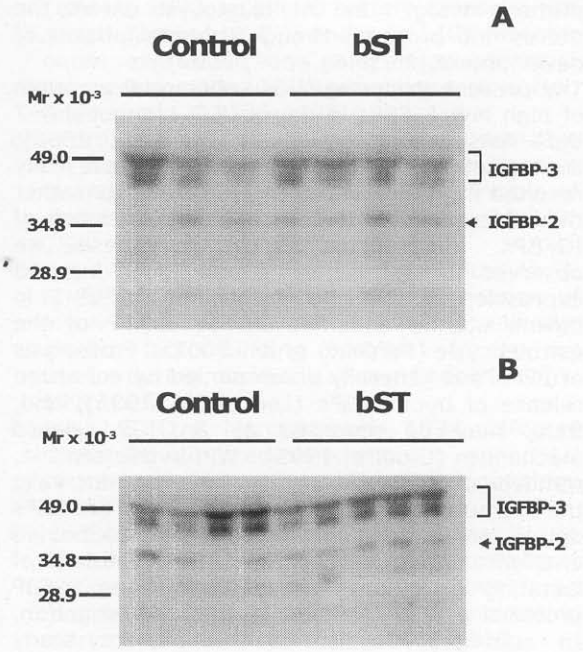


Fig. 5: Plasma IGFBPs at d 3 (A) and 7 (B) postovulation in control and bST-treated cows.

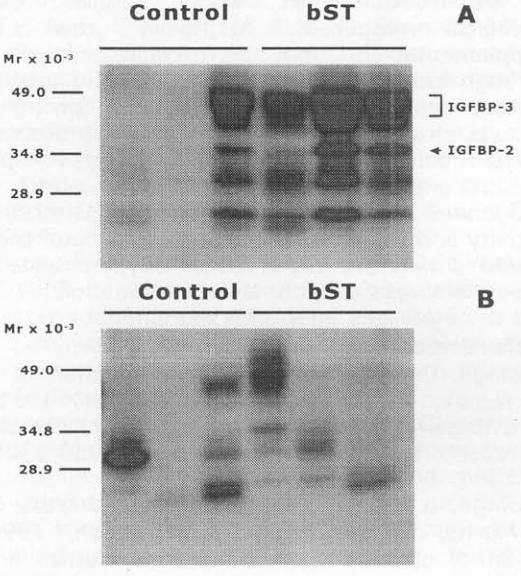


Fig. 6: Uterine luminal IGFBPs at d 3 (A) and 7 (B) in control and bST-treated cows.

concentrations during the seven-day treatment period. Similarly, there was no evidence for bST effect on weight of the CL at day 7 postovulation (Control, 4.7 ± 1.0 g; bST, 4.4 ± 1.0 g).

Total protein contents were higher ($P < 0.0001$) in ULF (3655.7 ± 365.0 µg) than in OLF (271.9 ± 365.0 µg; Fig. 3). In ULF, protein content tended($P < 0.06$) to decrease at day 7 of the estrous cycle, presumably as a result of enhanced intraluminal proteolysis. Short-term bST treatment had no detectable effect on protein contents in OLF or ULF (Fig. 3).

A tissue x stage of the estrous cycle interaction was detected ($P < 0.01$) for luminal IGF-I content (Fig. 4). Within the oviduct, IGF-I content did not change between stages of the estrous cycle (day 3, 2.9 ± 1.4 ng; day 7, 2.9 ± 1.2 ng) or treatments (control, 3.0 ± 1.4 ng; bST, 2.9 ± 1.3 ng). In contrast, uterine IGF-I luminal content increased ($P < 0.01$) between days 3 (9.3 ± 1.4 ng) and 7 (16.9 ± 1.2 ng), and there was a tendency ($P < 0.08$) for higher luminal IGF-I in bST-treated (20.1 ± 1.7 ng) than control (13.7 ± 1.7 ng) cows.

Five distinct IGFBP bands with approximate molecular masses of 44-48, 35, 31, 30, and 28 kDa were detected in plasma (Figure 5). Peripheral IGFBP profiles appeared to be similar for the two stages of estrous cycle and treatments. All major IGFBP bands were present in day-3 ULF but could not be detected in day-7 ULF (Fig. 6).

## Discussion

Bovine somatotropin administration at a timed artificial insemination increases pregnancy rates in lactating dairy cows (Moreira *et al.*, 2000; Moreira *et al.*, 2001), raising the possibility that exogenous bST may modulate the endocrine and paracrine communication beween the conceptus and maternal uterus at the time of blastocyst formation and subsequent pregnancy establishment. In the present study, in spite of small numerical tendencies, concentration of IGF-I in plasma did not differ significantly between bST-treated and control cows. These findings contrasted with earlier observations that plasma IGF-I concentrations were significantly elevated in pigs (Sterle *et al.*, 1995), heifers (Lefebvre and Block, 1992), and cows (Morbeck *et al.*, 1991) treated with ST. The majority of these earlier studies detected significant increases in plasma IGF-I during long-term treatment with bST. Conversely, in the present study, IGF-I response to exogenous bST was examined over a short time span with limited numbers of animals, which may have accounted for lack of bST effect on

plasma IGF-I concentration.

The observation that bST receptor mRNA transcripts are readily detectable in the bovine CL (Kirby *et al.*, 1996; Lucy *et al.*, 1993) has led to the general speculation that bST may affect pregnancy outcome, in part, through direct stimulation of CL development and function. In the present study, neither plasma P4 profiles nor the weight of CL at day 7 of the estrous cycle differed significantly between bST-treated and control cows. The literature data on bST effects on CL function and development are conflicting, with some studies reporting stimulatory effects (Lucy *et al.*, 1994a; Lucy *et al.*, 1995; Schemm *et al.*, 1990) and others reporting no effects (Dalton and Marcinkoswsky, 1994; De la Sota *et al.*, 1993; Lucy *et al.*, 1994b) of supplemental bST on plasma P4 and CL development. Unlike the present study, which examined P4 and CL responses to bST in lactating dairy cows at early stages of the estrous cycle, the majority of the aforementioned studies evaluated CL response to bST in heifers (Lucy *et al.*, 1994b), dry cows (De la Sota *et al.*, 1993), or lactating cows at later stages of the estrous cycle or pregnancy (Lucy *et al.*, 1995). Consequently, inconsistencies among experiments relative to bST effect on CL development and function may be due, in part, to differences among physiological states of experimental animals utilized in various experiments.

The present study revealed a clear tissue x stage of the estrous cycle interaction for luminal IGF-I content. Within the oviduct, IGF-I content was low and did not differ between stages of the estrous cycle or treatments. In contrast, Uterine IGF-I luminal content increased between days 3 and 7 postovulation, and there was a numerical tendency for higher luminal IGF-I in bST-treated than control cows. The low IGF-I content in OLF is consistent with our recent observation (Pershing *et al.*, 2002) that oviductal IGF-I mRNA expression is low and is not induced by bST at early stages of the estrous cycle. The increased uterine IGF-I luminal content at day 7 of the estrous cycle coincides with high uterine endometrial IGF-I gene expression (Pershing *et al.*, 2002), and points to a role for this mitogen in the control of terminal blastocyst development within the uterus. In further support for this hypothesis, we recently observed that addition of either bST or IGF-I to the maturation medium stimulated development of in vitro-derived pre-implantation bovine embryos (Moreira *et al.*, 2002). Our experimental model would suggest that IGF-II may play an important role in the control of cleavage and blastocyst development within the oviduct, but that IGF-I becomes the primary mitogen as the blastocyst enters the uterus and proceeds though terminal phases of development (Pershing *et al.*, 2002).

The present study revealed substantial amounts of high MW IGFBPs in day-3 ULF, but not day-7 ULF. As suggested previously (Lee *et al.*, 1998), the loss of high MW IGFBPs during diestrus likely resulted from luminal proteolytic cleavage rather than decreased endometrial gene expression of IGFBPs. In support of this hypothesis, we observed constitutive and bST-induced expression of the mRNA encoding IGFBP-3 in bovine uterine endometrium at day 7 of the estrous cycle (Pershing *et al.*, 2002). Proteolysis of IGFBPs is generally accompanied by enhanced release of bound IGFs (Lee *et al.*, 1996), and, thus, may be regarded as an IGF release mechanism (Giudice, 1995). Within this context, removal of IGFBPs via proteolysis may increase the intrauterine availability and action of IGFs during pre-attachment development of bovine embryos. Whether and how bST treatment of lactating dairy cows may alter uterine IGFBP protease activity warrants further investigation.

In summary, results of the present study provided no evidence for bST stimulation of CL function and development during early stages of the estrous cycle in lactating dairy cattle subjected to an Ovsynch program. The tendencies for higher uterine IGF luminal content in bST-treated cows would suggest that biological responses of dairy cows to supplemental bST may be mediated, in part, through local regulation of IGF family of growth factors and possibly their binding proteins. Results also implicate uterine luminal proteases in the regulation of IGF availability and, perhaps, IGF actions within the uterus. The effect of exogenous bST on uterine IGFBP protease activity and the physiological significance of their action during pre-implantation development of bovine embryos await further investigation.

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