Pregnancy and Bovine Somatotropin in Nonlactating Dairy Cows: I. Ovarian, Conceptus, and Insulin-Like Growth Factor System Responses

T. R. Bilby,¹ A. Guzeloglu,¹ S. Kamimura,² S. M. Pancarci,¹
F. Michel,¹ H. H. Head,¹ and W. W. Thatcher¹

¹Department of Animal Sciences,
University of Florida, Gainesville 32611

²Department of Veterinary Medicine,
Faculty of Agriculture, Kagoshima University,
Kagoshima, Japan

ABSTRACT

Nonlactating dairy cows were used to examine effects of bovine somatotropin (bST) on components of the insulin-like growth factor (IGF) system. Estrus was synchronized in cows with a Presynch + Ovsynch protocol and timed AI (TAI; $n = 55$) or not TAI (cycling, C; $n = 23$) on d 0 (time of synchronized ovulation). On d 0 and 11, cows received bST (500 mg) or no bST, and were sacrificed on d 17. Pregnancy rates were less in bST cows (27.2%, 9 of 33) than in controls (63.6%; 14 of 22). In contrast, conceptuses were larger in bST-treated cows (39.2 ± 4.8 cm) than in controls (20 ± 4.3 cm). Total interferon- τ in uterine luminal flushings (ULF) was greater in bST-treated cows ($7.15 > 2.36$ μ g). Number of class 2 follicles (6 to 9 mm) was less in bST-C cows on d 7 and 16. On d 17, corpus luteum (CL) weight tended to be greater in bST-treated cows. Concentrations of progesterone were greater after d 10 in C than in pregnant (P) cows. In the ULF, IGF-binding protein-3 was greater in bST-P cows than in pregnant cows. A tendency for an increase in IGF-I hormone concentrations in the ULF was detected on d 17 in bST-treated and cyclic cows. Endometrial mRNA for IGF-I, IGF-II, IGFBP-2, and IGFBP-3 increased in bST-C, but not in bST-P cows. Treatment with bST increased plasma concentrations of insulin, IGF-I, and growth hormone (GH). In conclusion, bST may have hyperstimulated plasma IGF-I and insulin to cause asynchrony between conceptus and uterus that was detrimental to pregnancy.

(Key words: pregnancy, bovine somatotropin, insulin-like growth factor system)

Abbreviation key: C = cyclic, CL = corpus luteum, GH = growth hormone, GHR = growth hormone receptor, IGFBP = insulin-like growth factor binding protein, P = pregnant, TAI = timed AI, ULF = uterine luminal flushings.

INTRODUCTION

Since approval of bST for use in dairy cows to increase milk production, the effects of bST on reproductive function have garnered considerable interest. An increase in pregnancy rate was detected when bST was administered in conjunction with a timed AI (TAI) program. When lactating dairy cows were treated with bST at the initiation of the Ovsynch protocol, pregnancy rates were higher than in controls at d 27 and 45 after insemination (Moreira et al., 2000). In addition, when bST was injected at the initiation of the Ovsynch program or at the time of insemination, lactating dairy cows had higher pregnancy rates than controls (53.2, 44.9, and 38.8%, respectively; Moreira et al., 2001). Additional studies documented the beneficial effect of bST on pregnancy rates when given during the period approaching insemination and to cows considered subfertile (Morales-Roura et al., 2001; Santos et al., 2004).

In an in vitro study (Moreira et al., 2002b), growth hormone (GH) added to the maturation media increased cleavage rates of fertilized ova, but had no significant effect on blastocyst development. Culturing bovine embryos in the presence of GH or IGF-I, however, accelerated embryo development by d 8 postfertilization and increased the number of cells per embryo. Moreira et al. (2002a) reported that bST treatment of superovulated donor cows reduced the number of unfertilized oocytes, increased the number of embryos that developed to the blastocyst stage, and increased the number of transferable embryos. Although several studies examined bST effects on pregnancy rates and early embryonic development, little is known regarding the

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Corresponding author: W. W. Thatcher; e-mail: thatcher@animal.ufl.edu.

physiological mechanisms altered by bST that may increase embryo development up to d 7. Recently, Pershing et al. (2002) examined the effects of bST, given at the time of insemination of the Ovsynch protocol, on expression of oviductal and uterine genes encoding components of the IGF system. Lactating dairy cows were slaughtered at d 3 or 7 following a synchronized estrus (d 0), and oviductal and uterine tissues were analyzed. Steady-state concentrations of IGF-II mRNA were greater in oviducts collected from bST-treated cows than from control cows. Uterine insulin-like growth factor binding protein (IGFBP)-3 mRNA concentrations were greater in bST-treated cows than controls, on d 3 and 7 of the estrous cycle. The mRNA for growth hormone receptor (GHR) was decreased in bST-treated cows by d 7. This study revealed the bST regulatory complexity in tissue-specific gene expression during early pregnancy in lactating dairy cows.

These findings, as well as others, give conclusive evidence that bST has direct effects on the oviduct, uterus, and early embryo development (Lucy et al., 1995; Spicer et al., 1995; Kirby et al., 1996; Izadyar et al., 1996, 1997). However, little is known of bST effects after d 7 and before d 32 postinsemination, which appears to be a critical period for bST to exert a direct embryonic effect, or an indirect effect via the maternal unit (uterus) and/or peripheral responses (Moreira et al., 2001). Another important event within this critical window, on d 16 to 17 after estrus, is maintenance of the corpus luteum (CL). This process is established by the ability of the conceptus to secrete IFN- τ , which regulates secretion of PGF $_{2\alpha}$ in the uterine endometrium (Thatcher et al., 2001). At least 40% of total embryonic losses have been estimated to occur between d 8 and 17 of pregnancy (Thatcher et al., 1994). This high proportion of embryonic losses seems to occur around the same time as the inhibition of PGF $_{2\alpha}$ secretion by the conceptus.

Because bST increases the rate of embryo development to the blastocyst stage and increases cell number *in vitro*, bST may subsequently enhance conceptus development, allowing for a greater secretion of IFN- τ at d 17 of pregnancy. This increase of IFN- τ may contribute to an increase in the number of animals establishing pregnancy and decrease the percentage of early embryonic loss.

The objective of this study was to characterize the effects of exogenous bST on ovarian function, conceptus development, and regulation of the IGF system in the uterus on d 17 of the estrous cycle in nonlactating Holstein dairy cows as an experimental model.

MATERIALS AND METHODS

Materials

Gonadotropin-releasing hormone (Fertagyl; Intervet Inc., Millsboro, DE), PGF $_{2\alpha}$ (Lutalyse; Pfizer Animal

Health, Kalamazoo, MI), and bST (Posilac; Monsanto Co., St. Louis, MO) were used for synchronization of ovulation and experimental treatment. Recombinant bovine IFN- τ (1.08×10^7 units of antiviral activity per milligram used as a standard) for the antiviral assay was a generous gift from Michael Roberts (University of Missouri, Columbia, MO). The cDNA of GHR-1A, IGF-I, IGF-II, IGFBP-2, and IGFBP-3 were a generous gift from Mathew Lucy (University of Missouri, Columbia, MO). All other materials were purchased as follows: Trizol, Random Primers DNA Labeling System (Invitrogen Corporation, Carlsbad, CA); Taq polymerase (cat # M166A; Promega Corp., Madison, WI); ultrasensitive hybridization buffer (ULTRAhyb, cat # 8670; Ambion Inc., Austin, TX); dCTP α - 32 P (cat # 33004 $\times$ 01), Biotrans Nylon membrane (ICN, Irvine, CA); Centriprep Centrifugal Filter Devices (Millipore, Bedford, MA); nitrocellulose membranes (Hybond, Amersham Biosciences Corp., Piscataway, NJ); recombinant human IGF-I and IGF-II (Upstate Biotechnology, Lake Placid, NY); and Modified Eagles medium, Vesicular Stomatitis virus, and immortalized bovine kidney cells (MDBK) (American Type Culture Collection, Manassas, VA). All other general materials used were from Fisher Scientific (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO).

Animals and Experimental Design

The experiment was conducted at the University of Florida Dairy Research Unit (Hague, FL) from October 2001 through February 2002. Nonlactating Holstein dairy cows in good body condition (≥ 3.0) were housed together in a free-stall facility with grooved concrete floors, and were fed a TMR twice daily throughout the experiment. The barn was equipped with fans and sprinklers that were operated when the temperature exceeded 25°C. Estrus was presynchronized (Presynch) in 78 cows starting on d -27 (d 0 = TAI) with a GnRH (2 mL, 86 μ g; i.m.) injection and on d -20 with an injection of PGF $_{2\alpha}$ (5 mL, 25 mg; i.m.) (DeJarnette and Marshall, 2003; Figure 1). Estrus was detected between d -20 and -10 using the Heatwatch electronic estrus-detection system (DDx Inc., Denver, CO; Rorie et al., 2002). The Ovsynch protocol (Pursley et al., 1997) was administered beginning on d -10 with an injection of GnRH (2 mL, 86 μ g, i.m.) followed 7 d later (d -3) by an injection of PGF $_{2\alpha}$. At 48 h after injection of PGF $_{2\alpha}$, GnRH (d -1) was administered, and 55 cows were inseminated 16 h later. All inseminations were administered by the same technician with semen from one Holstein bull of known fertility (Select Sires; 7H05379). The cycling group (C; n = 23) was not inseminated. Cows received a recommended commercial dose of bST

Figure 1. Experimental protocol illustrating the sequence of injections, collection of samples, and day of ultrasonography. Day 0 represents the time of ovulation from an induced LH surge. PG = PGF_{2α}, TAI = timed AI.

(500 mg) or no bST on d 0 (when cows were either inseminated or not) and again on d 11. The bST injections were given 11 d apart, instead of 14 d, to allow sustained continual exposure to GH until d 17. The bST injections were given subcutaneously in the space between the ischium and tail head. Ovaries were evaluated by real-time ultrasonography (Aloka SSD-500, Aloka Co. Ltd., Tokyo, Japan) with a 7.5-MHz linear-array transrectal transducer on d 0, 7, and 16. Follicular responses examined were: number of class 2 (6 to 9 mm) follicles, class 3 (>10 mm) follicles, and corpora lutea (CL), diameter of the largest follicle (mm), and CL tissue volume (mm³). The tissue volume (V) was calculated using the length (L) and width (W) of the CL to calculate the average diameter and volume (V), with the formula $V = 4/3 \times \pi \times R^3$ using a radius (R) calculated by the formula $R = (L/2 + W/2)/2$. For CL with a fluid-filled cavity, the volume of the cavity was calculated and subtracted from the total volume of the CL. Blood samples were collected daily from d 0 to 17 to be analyzed for various hormone concentrations. A follicular cyst was detected on d 7 in 5 cows and CL regression before d 16 was observed in 2 cows. These 7 cows were excluded and not slaughtered. Cows (n = 71) were slaughtered on d 17 after TAI to collect tissue samples and verify presence of a conceptus. Pregnancy rates were defined as number of cows classified pregnant based upon visualization of a conceptus in the flushing at slaughter divided by number of cows inseminated.

Tissue Sample Collection

All cows were sacrificed in the abattoir of the Meats Laboratory at the University of Florida. Reproductive tracts were collected within 10 min of slaughter, placed on ice and taken to the laboratory. Conceptuses and uterine secretions were recovered as described by Lucy

et al. (1995). Briefly, 40 mL of PBS was injected into the uterine horn at the uterotubal junction contralateral to the CL and massaged gently through the uterine horns, exiting through an incision in the horn ipsilateral to the CL. Uterine luminal flushing (ULF) media and conceptus were recovered into 250-mL beakers. The CL were removed from the ovaries, weighed, and their diameter (mm) was measured to calculate total tissue volume as described above. The uterine horn ipsilateral to the CL was cut along the mesometrial border, and the endometrium was dissected from the myometrium. Endometrial tissue from the antimesometrial border of the ipsilateral horn was cut (1 × 1 cm) and frozen in liquid nitrogen for Northern blot analyses. Endometrial tissues were collected from 14 cycling (7 C and 7 bST-C) and 16 pregnant (P) cows (7 P and 9 bST-P). Uterine luminal flushing media was recovered from 19 cycling (12 C and 7 bST-C) and 18 pregnant (9 P and 9 bST-P) cows.

IFN- τ Antiviral Assay

Activity is expressed in antiviral units per milliliter assessed in a standard cytopathic effect assay (Familletti et al., 1981). Three-fold dilutions of ULF from pregnant cows were incubated with Madin-Darby bovine kidney (MDBK) cells in 96-well plates for 24 h at 37°C. Following incubation, inhibition of viral replication was determined in a cytopathic effect assay using vesicular stomatitis virus as challenge. Antiviral units per milliliter (defined as the dilution causing a 50% reduction in destruction of the monolayer) were converted to micrograms per milliliter of IFN- τ using a standard curve with known amounts of rbIFN- τ . Total amount of IFN- τ ($\mu\text{g}/\text{total volume}$) in the ULF was calculated by multiplying the IFN- τ concentration by total amount of flushing fluid recovered for each pregnant cow.

RNA Isolation and Northern Blotting

Total RNA was isolated from endometrial tissues (300 mg; $n = 30$) with Trizol according to the manufacturer's specifications. Total cellular RNA (30 μ g) was loaded onto a 1.0% agarose-formaldehyde gel and blotted to a nylon membrane. Following blotting, RNA was crosslinked by UV irradiation and baked at 80°C for 1 h. The blots were prehybridized with ULTRAhyb buffer for 1 h at 42°C. Filters were then hybridized with random primer [P^{32}]-labeled bovine specific cDNA (GHR-1A, IGF-I, IGF-II, IGFBP-2, IGFBP-3, and glyceraldehyde-3-phosphate dehydrogenase; Feinberg and Vogelstein, 1983) overnight at 42°C. The next day, the blots were washed once in 2 $\times$ saline sodium citrate/0.1% SDS for 20 min and twice in 0.1 $\times$ saline sodium citrate/0.1% SDS for 20 min each at 42°C. The blots were gently patted dry with tissues and exposed to x-ray film at -80°C. The autoradiographs were quantified using densitometric analysis (AlphaImager, Alpha Innotech Corp., CA). Once all blots had been labeled with their respective probes, blots were stripped, probed for glyceraldehyde-3-phosphate dehydrogenase, and quantified.

Analysis of Hormones in Plasma and Uterine Luminal Flushings

Blood samples (7 mL) were collected daily from TAI (d 0) until slaughter (d 17) using a 20-gauge Vacutainer blood collection needle (Benton Dickinson, Franklin Lakes, NJ) from the coccygeal vein in 3 locations, which were rotated at each bleeding to minimize irritation. Samples were collected in evacuated heparinized tubes (Vacutainer; Becton Dickinson). Immediately following sample collection, blood was stored on ice until it was returned to the laboratory for centrifugation (3000 $\times g$ for 20 min at 4°C) for collection of plasma within 6 h. Plasma was stored at -20°C until assayed for GH, IGF-I, insulin, and progesterone. Concentrations of progesterone were analyzed using a solid phase radioimmunoassay kit (Coat-a-count, DPC, Diagnostic Products Co., Los Angeles, CA). Plasma samples were analyzed for GH (Badinga et al., 1991), insulin (Malven et al., 1987; Badinga et al., 1991), and IGF-I (Badinga et al., 1991) by specific radioimmunoassay. The extraction procedure used for the IGF-I assay (Badinga et al., 1991) was modified slightly using a 6:3:1 ratio of ethanol:acetone:acetic acid. Uterine luminal flushings were concentrated with a Centriprep Centrifugal Filter Device fitted with a 3000-MW filter (Millipore, Bedford, MA) and then analyzed for IGF-I and GH using the same radioimmunoassay procedures. Values for immunoreactive IGF-I and GH were expressed as total nanograms in ULF. Protein concentrations in ULF were

determined using the Bradford method (Bradford, 1976). The minimum detectable concentrations for GH, IGF-I, insulin, and progesterone were 0.1, 10, 0.02, and 0.1 ng/mL, respectively. The intra- and interassay coefficients of variation were 9.7 and 5.4%, 5.3 and 1.9%, 1.7 and 3.5% for plasma GH, IGF-I, and insulin, respectively. Plasma concentrations of progesterone were completed in one assay with intraassay coefficients of variation calculated from duplicate samples in 3 ranges of low (0.5 to 1 ng/mL; 12.0%), medium (1 to 3 ng/mL; 8.24%) and high (>3 ng/mL; 7.27%) progesterone concentrations. The intraassay coefficient of variation was 9.7% for the luteal phase plasma reference sample (5.8 ng/mL). A reference pool for ULF resulted in intraassay coefficients of variation of 15.8 and 10.8% for GH and IGF-I, respectively. The intraassay coefficients of variation for duplicate samples within the complete assay for the ULF GH and IGF-I were 10.0 and 11.5%, respectively.

Analysis of Uterine Luminal IGFBP

Ligand blot analysis (De la Sota, 1996) determined the relative abundances of IGFBP in the ULF. One hundred micrograms of concentrated ULF protein was subjected to a 12.5% SDS-PAGE under nonreducing conditions. Proteins were then transferred to a nitrocellulose membrane by electrotransfer. The filters were blocked for 1 h with Tris-buffered saline (pH 7.4) which contained 1% NDM. The membranes were washed and then incubated in 30 mL of Tris-buffered saline containing 1×10^6 cpm/mL of [125 I]-labeled rhIGF-II for 24 h at 4°C. Filters were washed 5 times, for 10 min each in Tris-buffered saline, blotted dry, and exposed to x-ray film for 48 to 72 h. Signals for IGFBP were quantified by densitometric analysis and the total content of IGFBP in ULF was calculated. The IGFBP (arbitrary units/100 μ g) were calculated back to the total amount of protein in the ULF recovered, and units expressed as arbitrary units of IGFBP/total ULF.

Statistical Analyses

Pregnancy rates were analyzed using the χ^2 and Logistic Regression procedure examining the main effect of bST. The main effect of bST was also tested for conceptus size (cm) and IFN- τ content of ULF using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The ovarian responses on d 17 at slaughter were also analyzed using the GLM procedure of SAS testing the main effect of bST, pregnancy status, and the interaction of bST and pregnancy status. Number of CL was used as a covariate for analysis of CL volume on d 17.

Numbers of class 2 (6 to 9 mm) and class 3 (>10 mm) follicles, and CL, as well as largest follicle size, and CL

tissue volume were analyzed using the Mixed Model procedure of SAS (Littell et al., 1996). Cow within bST and pregnancy status was a random effect in the model. The model included effects of bST, pregnancy status, day, and the higher order interactions. The CL tissue volume was adjusted for CL number as a covariate.

Plasma hormone concentrations were analyzed using the homogeneity of regression procedure and the repeated measures analysis in the Mixed Model of SAS. This procedure applies methods based on the mixed model with special parametric structure on the covariance matrices. The dataset was tested to determine the covariance structure that provided the best fit for the data. Covariance structures tested included compound symmetry, autoregressive order 1, and unstructured. The covariance structure used was autoregressive order 1. The model included effects of bST, pregnancy status, and day with the higher order interactions using a statement specifying cow within bST and pregnancy status as being random. Day 0 plasma hormone concentrations were used as a covariate for all respective plasma hormone concentrations. The PDIF statement was used for bST $\times$ pregnancy status $\times$ day to obtain the probability values for differences between treatments on a particular day.

Abundances of IGF-I, IGF-II, IGFBP-2, and IGFBP-3 mRNA in Northern blots were analyzed using the GLM procedure of SAS. The main effects of treatment (C, P, bST-C, and bST-P), gel, and the interaction of treatment $\times$ gel were examined with the abundance values for glyceraldehyde-3-phosphate dehydrogenase mRNA used as a covariate to adjust for loading gel differences. Predesigned orthogonal contrasts were used to compare treatment means (bST, pregnancy status, and bST $\times$ pregnancy status).

Total contents of GH, IGF-I, IGFBP-3, IGFBP-4, and IGFBP-5 in ULF were analyzed using the GLM procedure of SAS. The mathematical model included the main effects of treatment (C, P, bST-C, and bST-P), and orthogonal contrasts were used to compare treatment means (pregnancy status, bST, and bST $\times$ pregnancy status interaction).

RESULTS

Pregnancy Rates, Conceptus Sizes, and Total Amount of IFN- τ in ULF

Pregnancy rate at d 17 of nonlactating dairy cows was decreased ($P < 0.01$) by bST (27.2%; 9 of 33) compared with nontreated (63.6%; 14 of 22) cows. Although pregnancy rates were decreased in bST-treated cows, the conceptuses that survived to d 17 in the bST-P cows had a greater ($P < 0.01$) average conceptus length ($n = 8$; 39.2 ± 4.8 cm) than P cows ($n = 10$; 20.0 ± 4.3 cm).

Furthermore, the amount of IFN- τ in the ULF from bST-P ($n = 8$) cows was almost 3 times greater ($P < 0.05$) at $7.15 \mu\text{g}/\text{total ULF}$ compared with $2.36 \mu\text{g}/\text{total ULF}$ in P ($n = 10$) cows (Table 1). However, differences in IFN- τ were not significant when adjusted for conceptus length as a covariate.

Ovarian Responses on D 7, 16, and 17

Ovarian responses were measured by ultrasonography on d 7 and 16 in 7 C, 6 P, 7 bST-C, and 4 bST-P cows. No significant differences among treatments were detected for the number of class 3 follicles (1.7 ± 0.2), size of the largest follicle (18.0 ± 1.1 mm), number of CL (1.2 ± 0.2), and CL tissue volume ($8667 \pm 2069 \text{ mm}^3$). An interaction ($P \leq 0.05$), however, was detected between bST and pregnancy status for number of class 2 follicles, which decreased in bST-C cows compared with C, P, and bST-P cows on d 7 and 16 ($2.4 < 4.9$, 4.2 , and 5.5 , respectively).

On d 17, at the time the reproductive tract was recovered, CL (15 C, 10 P, 5 bST-C, and 9 bST-P) were counted, measured, and weighed. No significant differences were detected between treatment groups for the number of CL and the volume of the CL. However, CL tended ($P \leq 0.10$) to be heavier in bST-treated animals than in nontreated animals (Table 1).

Plasma and ULF Hormone Concentrations

Daily blood samples were collected from d 0 (day of synchronized estrus) to 17 after estrus from 22 cows (5 C, 6 P, 7 bST-C, and 4 bST-P). No main effect of bST on progesterone concentrations in plasma was detected, however, there was a tendency ($P < 0.10$) for a treatment $\times$ day interaction, with C cows having greater progesterone on d 12, 14, and 16 ($P < 0.05$) and a tendency ($P < 0.10$) to have greater progesterone on d 11 and 15 (Figure 2) compared with P cows. The bST injections increased ($P < 0.01$) plasma GH concentrations in bST-C and bST-P cows compared with the nontreated C and P cows (8.5 ± 0.6 and $7.9 \pm 0.8 \text{ ng/mL}$ vs. 3.1 ± 0.7 and $2.9 \pm 0.8 \text{ ng/mL}$, respectively; Figure 3). Associated with an increase in plasma GH was an increase ($P < 0.01$) in IGF-I in bST-C and bST-P groups in contrast with C and P groups (601 ± 42 and $635 \pm 55 \text{ ng/mL}$ vs. 413 ± 50 and $345 \pm 45 \text{ ng/mL}$ respectively; Figure 4). Concentrations of plasma insulin increased ($P < 0.01$) in bST-C and bST-P animals compared with C and P animals (3.68 ± 0.3 and 3.76 ± 0.4 vs. 1.85 ± 0.4 and 1.97 ± 0.3 respectively; Figure 5) with a tendency for a bST $\times$ status $\times$ day interaction ($P \leq 0.10$; Figure 5) with the quadratic curves being different ($P \leq 0.05$) for bST-C and bST-P cows. Inspection of the curves indicated

Table 1. Least square means and pooled SE for conceptus length, INF- τ (μ g/total uterine luminal flushing), number of corpora lutea (CL), CL tissue volume (mm^3), and CL weight (g) on d 17 after a synchronized estrus (d 0) in nonlactating cyclic (C) and pregnant (P) cows injected with bST (+/-) on d 0 and 11.

Response	Treatments ¹				SE	Contrasts		
	C	P	bST-C	bST-P		Pregnancy status (P)	bST	bST $\times$ P
Pregnancy rate	...	64%(14/22)	...	27%(9/33)	...	...	**	...
Conceptus size, ² cm	...	20.0	...	39.2	4.6	...	**	...
INF- τ , ² μ g/total ULF	...	2.36	...	7.15	1.7	...	*	...
Number of CL ³	1.1	1.1	1.2	1.0	0.1	NS	NS	NS
CL volume, ³ mm^3	6555	7474	7669	7773	761.5	NS	NS	NS
CL weight, ³ g	5.7	5.5	7.2	5.9	0.5	NS	†	NS

† $P = 0.10$.

* $P = 0.05$.

** $P = 0.01$.

¹bST-C = bST-cyclic, bST-P = bST-pregnant.

²n = 18.

³n = 39.

that insulin concentrations of bST-C cows were greater before d 9 compared with bST-P cows, and insulin concentrations were greater after d 9 in bST-P animals, compared with bST-C animals.

Uterine luminal flushings were collected at the time of slaughter on d 17 from 37 cows (12 C, 9 P, 7 bST-C, and 9 bST-P) for analysis of GH and IGF-I concentrations. Volumes and protein content of ULF did not differ due to pregnancy status or bST treatment. Total amount of GH in the ULF did not differ. A tendency ($P = 0.07$) for an increase in ULF content of IGF-I in bST-C and bST-P cows was detected compared with C and P cows (202 ± 32 and 161 ± 28 vs. 157 ± 25 and 101 ± 28 ng, respectively; Table 2). There was also a tendency ($P < 0.10$) for an increase in ULF content of IGF-I in C cows compared with P cows (Table 2).

Figure 2. Profiles of plasma progesterone concentrations of cyclic (C) cows (Δ) and pregnant (P) cows ($\square$) from d 0 to 17 of a synchronized estrous cycle (* $P < 0.05$; $^aP < 0.10$).

Endometrial mRNA Expression of the GH/IGF-I System

Northern blot analysis was used to probe the endometrial tissue from d 17 cows for GHR-1A, IGF-I, IGF-II, IGFBP-2, and IGFBP-3. Growth hormone receptor 1A was undetectable in the endometrium of all animals. The mRNA transcript sizes (kb) for IGF-I, IGF-II, IGFBP-2, and IGFBP-3 were 7.5, 4.6, 1.7, and 2.8 kb, respectively. There were significant and consistent interactions ($P < 0.01$) between status (P vs. C) and bST (+/-) for IGF-I, IGF-II, and IGFBP-3 mRNA and a comparable ($P < 0.10$) trend for IGFBP-2 (Table 2). In general, a slight increase in expression was detected for P cows in the absence of bST. In contrast, bST treatment stimulated the respective responses of C cows. How-

Figure 3. Profiles of plasma growth hormone (GH) concentrations of C (Δ), P ($\square$), bST-C (Δ), and bST-P ($\blacksquare$) cows from d 0 to 17 of a synchronized estrous cycle. Both bST-C and bST-P cows had greater GH concentrations when injected with bST on d 0 and 11 than C and P cows ($P < 0.01$). C = cyclic (no bST); P = pregnant (no bST); bST-C = cyclic with bST injection; bST-P = pregnant with bST injection.

Figure 4. Profiles of plasma IGF-I concentrations of C (Δ), P ($\square$), bST-C ($\blacktriangle$), and bST-P ($\blacksquare$) cows from d 0 to 17 of a synchronized estrous cycle. Both bST-C and bST-P cows had greater IGF-I concentrations when injected with bST on d 0 and 11 than C and P cows ($P < 0.01$). C = cyclic (no bST); P = pregnant (no bST); bST-C = cyclic with bST injection; bST-P = pregnant with bST injection.

ever, the bST-induced increases were attenuated in P cows to a level of expression comparable to P cows not injected with bST.

Analysis of ULF for IGFBP

Ligand blot analyses for IGFBP were conducted on d 17 ULF from 37 cows. Total ULF protein did not differ among treatment groups. Five distinct IGFBP bands were detected, from 44 to 24 kDa. There was no significant treatment effect on IGFBP-4 (28 and 24 kDa) and IGFBP-5 (29 kDa). There was an interaction ($P < 0.10$) between status and bST with pregnancy decreasing

IGFBP-3 (44 and 40 kDa), but bST blocked this decrease in bST-P cows (Table 2).

Simple Correlations and Partial Correlations for the GH-IGF System

There was no correlation between IGF-I in ULF and circulating concentrations of IGF-I at d 17 ($r = -0.17$). A series of positive correlations were detected ($P < 0.05$). Plasma concentrations of GH were correlated with IGF-I in plasma ($r = 0.81$), IGF-I in plasma was associated with insulin in plasma ($r = 0.51$), and IGF-I in plasma was associated with GH in ULF (partial correlation adjusted for treatment [$pr = 0.55$]). Concentrations of GH in ULF were associated negatively with IGF-I mRNA (-0.38), and low expression of IGF-I mRNA was related to enhanced expression of IGF-II mRNA ($r = -0.68$; $pr = -0.77$). Growth hormone in the intrauterine environment (i.e., GH in ULF) was correlated with the relative abundance of IGFBP-3 ($r = 0.59$), IGFBP-4 ($r = 0.63$), and IGFBP-5 ($r = 0.63$) in the ULF. Although correlations are not proof of causative effects, significant associations were detected among hormonal components and uterine gene expression of the GH-IGF system at d 17 in cyclic and pregnant animals treated differentially with bST.

DISCUSSION

In the present study, bST significantly decreased pregnancy rates when administered at the time of insemination in nonlactating dairy cows following an Ovsynch protocol. Nonlactating cows were chosen as the model to eliminate the homeorhetic state of lactation in evaluating bST effects and to reduce the expense. Previous studies have shown that pregnancy rates were increased in cyclic, lactating dairy cows when bST was injected at initiation of the Ovsynch protocol or near the TAI (Moreira et al., 2000, 2001; Morales-Roura et al., 2001; Santos et al., 2004). This difference in response may reflect a complex relationship between the physiological and nutritional status (lactating vs. nonlactating) and reproduction in dairy cows. In dairy heifers receiving either a 500-mg dose of bST on d 14 following AI, or on the day of AI and on d 14 after AI, pregnancy rates were reduced compared with controls not receiving bST (66.7, 66.1, and 94.4%, respectively; Rorie et al., 2004). However, in that study, when bST was given only at the time of AI, pregnancy rates did not differ from untreated controls (84.2 vs. 94.4%). This may have been due to timing of bST injections or the amount of IGF-I stimulation after bST injection, or both. Amount of IGF-I stimulation seemed to have detrimental effects when bST was administered immedi-

Figure 5. Profiles of plasma insulin concentrations of C (Δ), P ($\square$), bST-C ($\blacktriangle$), and bST-P ($\blacksquare$) cows from d 0 to 17 of a synchronized estrous cycle. Both bST-C and bST-P cows had greater insulin concentrations when injected with bST on d 0 and 11 than C and P cows ($P < 0.01$). A tendency for bST $\times$ status $\times$ day interaction occurred, with bST-C animals having greater insulin concentrations before d 9 than bST-P animals, and bST-P animals having greater insulin concentrations after d 9 compared with bST-C cows ($P \leq 0.10$). C = cyclic (no bST); P = pregnant (no bST); bST-C = cyclic with bST injection; bST-P = pregnant with bST injection.

Table 2. Least square means and pooled SE for uterine endometrial mRNA, uterine luminal flushings (ULF) protein expression, and hormone concentration at d 17 after a synchronized estrus (d 0) in nonlactating cyclic (C) and pregnant (P) dairy cows injected with bST (+/–) on d 0 and 11. Arbitrary units (AU) were generated by densitometry and mRNA results are adjusted for glyceraldehyde-3-phosphate dehydrogenase as a covariate.

Response ²	Treatments ¹				SE	Contrasts		
	C	P	bST-C	bST-P		Pregnancy status (P)	bST	bST × P
Endometrium (n = 30)								
IGF-I mRNA, AU	58	61	66	58	1.1	*	*	**
IGF-II mRNA, AU	57	61	62	60	1.1	NS	†	**
IGFBP-2 mRNA, AU	57	63	67	65	2.2	NS	**	†
IGFBP-3 mRNA, AU	50	54	60	50	1.1	**	**	**
ULF (n = 37)								
GH, ng/ULF	10	10	11	12	1.3	NS	NS	NS
IGF-I, ng/ULF	157	101	201	161	28	†	†	NS
IGFBP-3, AU	21	14	20	24	2.7	NS	NS	*
IGFBP-4, AU	18	14	20	19	2.3	NS	NS	NS
IGFBP-5, AU	18	14	20	19	2.4	NS	NS	NS

†*P* = 0.10.

**P* = 0.05.

***P* = 0.01.

¹bST-C = bST-cyclic, bST-P = bST-pregnant.

²IGFBP = Insulin-like growth factor binding protein; GH = growth hormone.

ately before the initiation of CL maintenance for establishment of a pregnancy. In other studies using lactating beef cows and heifers (Bilby et al., 1999), which may have more closely mimicked the physiological status of a nonlactating dairy cow, no effect of a low dose (167 mg) of bST (Posilac) on pregnancy rates was detected. In these latter studies, conception rates for bST-treated and control cows were 54.4 and 49.5% (n = 617) for lactating beef cows and 46.0 and 46.3% for heifers (n = 1123), respectively. Dose of bST may determine the ultimate outcome of reproductive responses because administration of a reduced daily dose of bST (5 mg/d) improved first-service conception rates and pregnancy rates in cows (Stanisiewski et al., 1992). In contrast, in that study, cows injected with more bST (14 mg/d) had a significant decrease in conception and pregnancy rates compared with those treated with less bST (5 mg/d). Other dose titration studies reported a reduction in estrus detection rate (Morbeck et al., 1991) and an increase in number of cows not conceiving (Downer et al., 1993) when treated with high doses of rbST.

Dose and timing of bST in which a positive or negative reproductive response occurs may depend upon the stimulatory responses of IGF-I and IGFBP after injection of bST. The primary determinants of plasma IGF-I concentrations are nutrition and body condition (Vicini et al., 1991; McGuire et al., 1992). Plasma concentrations of IGF-I are positively associated with body condition and nutrient intake (Houseknecht et al., 1988; Yelich et al., 1996), and low concentrations of IGF-I are associated with an extended postpartum in-

terval to estrus in beef cows and with delayed puberty (Rutter et al., 1989; Nugent et al., 1993; Roberts et al., 1997). Once plasma IGF-I concentration increases to reach a threshold concentration, follicular sensitivity to LH may increase due to IGF-I induction of LH receptors (Beam and Butler, 1999). With increases in estradiol output by the preovulatory follicle that induces an LH surge, cows resume estrous cycles and can potentially become pregnant. These changes illustrate a positive threshold response of IGF-I on reproduction.

The opposite, however, may be true when IGF-I concentrations are overstimulated. Well-fed cows and heifers have greater blood IGF-I concentrations, whereas undernourished cows have reduced plasma concentrations of IGF-I. The same association exists for nonlactating vs. lactating cows, in which nonlactating cows have greater IGF-I concentrations than lactating cows (De la Sota et al., 1993; Bilby et al., 1999). In the present study, IGF-I concentrations may have been hyperstimulated with a standard dose of bST (Figure 4). It is important to recognize, that in addition to using experimental cows that were nonlactating, cows were injected twice with bST at an 11-d interval. This was done to ensure a high concentration of bST for the entire 17-d period to slaughter. Although IGF-I concentrations were sustained throughout the experimental period, the concentrations in bST-C (601 ng/mL) and bST-P (635 ng/mL) cows were substantially higher than that normally seen in untreated nonlactating cows (214 ng/mL) or bST-treated lactating cows (306 ng/mL; De la Sota, 1993), or growing heifers (117 ng/mL; Lucy et al.,

1994). Collectively, these studies indicate that critical thresholds of GH and IGF-I concentrations may exist that stimulate reproductive performance; exceeding those thresholds may decrease reproductive responses. In the present study, hyperstimulation may have had deleterious effects on embryo development, uterine environment, and gene expression on or before d 17; this is further addressed in the companion paper (Guzeloglu et al., 2004). The reason for the deleterious effects of bST in some inseminated cows and not others, may reflect differences in among-cow sensitivity to GH regarding IGF-I secretion. Another deleterious effect of bST on pregnancy rates in nonlactating dairy cows may be the hyperstimulation of blood insulin secretion. High concentrations of insulin, GH, and IGF-I may be detrimental to early embryo growth. Overstimulation of IGF-I (Armstrong et al., 2001) and probably insulin (Armstrong et al., 2003) is detrimental to follicle and oocyte development. Excess IGF-I in vivo or in vitro had deleterious effects on the preimplantation embryo in rats (Katagiri et al., 1996, 1997). In mice, high concentrations of IGF-I and insulin induced a downregulation of the IGF-I receptor on the blastocyst, with a subsequent decrease in signaling of IGF-I receptor-associated pathways (Chi et al., 2000). This decrease in IGF-I receptor reduced glucose uptake and triggered apoptosis. Women with polycystic ovary syndrome exhibit elevated concentrations of insulin and IGF-I and experience more pregnancy losses (Sagle et al., 1988; Balen et al., 1993; Tulppala et al., 1993). A threshold may exist in which the level of GH, IGF-I, or insulin goes from being beneficial to detrimental on oocyte and embryo development.

Plasma concentrations of progesterone were greater in C compared with P cows after d 11 following synchronized induced ovulation (Figure 2). This is contradictory to earlier reports where noninseminated and inseminated, but not pregnant nonlactating dairy cows, had a slower rise of progesterone compared with pregnant nonlactating dairy cows during the first 16 d following insemination (Mann and Lamming, 2001). In their study, however, cows were administered 2 injections of PGF_{2α}, 11 to 13 d apart, and inseminations occurred 72 or 96 h after the second injection. Their reproductive management system probably did not induce as precise a timing of ovulation as the system used in the present study. A reduced synchrony in CL formation may have contributed to differences in progesterone concentrations between pregnant and nonpregnant cows. Even though progesterone concentrations were less in pregnant cows of the present study, no differences in CL tissue volume were detected on d 7, 16, and 17, or CL weight on d 17. Reduced plasma concentrations of progesterone may reflect a greater clearance rate of proges-

terone by the uterus of pregnant cows. However, a tendency existed for bST-treated cows to have a heavier CL on d 17. Lucy et al. (1995) showed that CL weight was increased when lactating dairy cows were treated with 25 mg/d of bST for 16 d after estrus compared with saline-treated controls. Greater CL weight may be due to increased differentiation of luteal cells by GH or IGF-I (Donaldson et al., 1965), or to increased DNA synthesis of luteal cells, as shown in vitro (Chakravorty et al., 1993).

Although pregnancy rates were decreased in bST-treated cows, the bST-P cows that maintained a conceptus until d 17 had 2-fold longer embryos than nontreated cows. Furthermore, with an increased conceptus length, the amount of IFN- τ was increased 3-fold. Longer conceptuses confirm the observations of Hansen et al. (1988), who stated that elongation of the embryo is associated with increased secretion of IFN- τ , and that most of the increased output of IFN- τ is due to the increased size of the embryo and not increased synthesis per unit weight. In combination with high IFN- τ and IGF-I concentrations in bST-treated cows, profound effects on the luteolytic mechanisms involved with maternal recognition of pregnancy were observed (Guzeloglu et al., 2004).

With the substantial differences in conceptus lengths between bST-treated and nontreated cows, it is possible that bST advanced uterine development such that an asynchronous uterine environment was created (Guzeloglu et al., 2004). Change in intrauterine environment could have occurred by advancing gene expression, or advancing embryonic growth such that the uterine environment was altered. An asynchronous environment is detrimental to embryo survival as shown in an embryo transfer model (Moore and Shelton, 1964). Transfer of embryos to recipients that are not in estrus at the same time as the donors resulted in altered embryo development (Wilmut and Sales, 1981; Lawson et al., 1983). In these studies, embryo development was retarded when embryos were transferred to a less advanced uterus, whereas development was accelerated when embryos were transferred to a more advanced uterus. Perhaps in the present study, bST stimulated a more advanced uterus.

Advancement of the uterus and conceptus may not only be due to the amount of IGF-I in the blood, but may be closely related to the amount of IGF-I in the uterine lumen and its association with regulatory binding proteins. In our study, IGF-I concentrations in the ULF tended to be greater in bST-treated cows, and particularly stimulated in cyclic vs. pregnant cows. However, IGFBP-3 was greater in bST-P vs. nontreated P cows. This increase of IGFBP-3 may be due to maternal mechanisms compensating for high IGF-I concen-

trations and alleviating some of the IGF-I actions on the developing conceptus.

Amounts of IGF-I, IGF-II, IGFBP-2, and IGFBP-3 mRNA in the endometrium had consistent interactions between status and bST such that bST increased expressions in cyclic cows but not in pregnant cows. This appears to be another example in which treatment with bST in pregnant cows caused a hyperstimulation in plasma IGF-I that appeared to induce a local endometrial downregulation in components encoding the IGF system (i.e., IGF-I, IGF-II, and IGFBP-3 mRNA). This may be a coordinated response to maintain homeostasis and a suitable environment for embryo growth. This effect in pregnant cows is likely due to products of the conceptus such as IFN- τ . Bovine IFN- τ regulates gene transcription of endometrial cells via induction of signal transducers and activators of transcription and IFN regulatory factors (i.e., IFN regulatory factor-1; Binelli et al., 2001). Bovine IFN- τ regulates expression of various endometrial proteins, for example, suppression in transcription of oxytocin and estrogen receptors (Spencer et al., 1995), enhanced expression of Mx (Ott et al., 1998), and ubiquitin cross-reactive proteins (Johnson et al., 1999). Spencer et al. (1999) demonstrated that pretreatment of ewes with IFN- τ was necessary to induce endometrial responsiveness to placental lactogen and GH. Although GHR-1A mRNA was not detected in the present experiment, bST stimulated gene expression of the IGF family within the endometrium of cyclic cows. In endometrial tissues of pregnant cows, however, bST stimulation was blocked. Local regulatory effects of the conceptus on endometrial responsiveness to exogenous hormones such as bST warrant further investigation, for example, in lactating dairy cows where bST stimulates pregnancy rates.

CONCLUSIONS

Treatment with bST significantly decreased pregnancy rates in nonlactating dairy cows. This appeared to be due to hyperstimulation of IGF-I in nonlactating dairy cows. As a consequence, the uterus and embryo seem to be advanced in development as reflected by stimulation in conceptus growth. The IGF system within the intrauterine environment (as characterized by gene expression in the uterine endometrium and proteins in the uterine lumen) seems to be quite responsive to treatment with bST. Further investigation is needed to ascertain the mechanisms by which bST increases pregnancy rates in lactating dairy cows during the critical period of CL maintenance necessary to sustain a pregnancy.

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