Embryo Survival on Day 25 of Generation in the Gilt is Not Affected by Exogenous Progesterone but is Correlated with Levels of Insulin-Like Growth Factor-I (IGF-I) mRNA in the Uterus

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ABSTRACT : The present study was undertaken to determine the effect of administration of exogenous progesterone early in gestation on uterine levels of IGF-I mRNA and on embryo survival at day 25 of gestation in the pig. Forty-one prepubertal gilts were induced into oestrus with PG600 and artificially inseminated at their subsequent naturally occurring oestrus. Gilts were then randomly assigned to one of three groups. Gilts in the two treatment groups were injected intramuscularly with 50 mg of progesterone either from day 2 to 14 (N=14) or from day 4 to 14 (N=15) after breeding while those in the control group (N=12) were given corn oil (0.5 ml) from day 2 to 14. Between days 25 and 28 of gestation, gilts were slaughtered and reproductive tracts were recovered. Endometrial tissue (1 g) was collected and analysed for IGF-I mRNA levels using a reverse transcription-polymerase chain reaction. Progesterone treatment, starting either on day 2 or 4 after breeding, neither significantly increased embryo survival rate by day 25 of gestation nor altered IGF-I mRNA levels in ulterine tissue. However, across all samples, the IGF-I mRNA level in the uterus was highly correlated with embryo survival rate ($r=0.8193$, $p<0.01$), supporting the involvement of IGF-I in the regulation of porcine embryo development. (*Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 6 : 862-867*)

Key Words : Progesterone, Gilt, Embryo, Survival, IGF-I

INTRODUCTION

Ovarian proesterone is believed to be essential for maintaining a successful pregnancy in the pig. It prepares a receptive endometrium to which the conceptus can attach and also maintains an appropriate uterine environment in which the conceptus develops to term. Supplementation of exogenous progesterone, early in gestation, has been reported to increase the number of viable embryos, by day 30 of gestation, in gilts exhibiting an increased ovulation rate due to flush-feeding prior to mating (Ashworth, 1991). However, the cellular and molecular mechanisms by which progesterone regulated uterine function, and thus influences embryo development, are not fully understood. An emerging concept is that the actions of ovarian steroid hormones both progesterone and oestradiol, are partially mediated by various peptide and polypeptide growth factors, including IGF-I, in paracrine/autocrine manners.

In swine, IGF-I is a single-chain polypeptide of 70 amino acid residues (Tavakkol et al., 1988). Structurally, IGF-I is related to insulin. Functionally, IGF-I is a potent mitogen for a variety of cell types *in vitro* and may promote cell proliferation, cell differentiation and cellular remodelling *in vitro* (Zapf and Froesch, 1986). Although the liver has been recognized as the major source of circulating IGF-I, the IGF-I gene is expressed in many other tissues including the female reproductive tract. Murphy et al. (1987) reported that IGF-I mRNA levels in the uterus of the rat were very high, second only to the liver among tissues tested. Expression of the IGF-I gene has also been demonstrated in the pig uterus, where the endometrial levels of IGF-I mRNA vary during pregnancy, with the highest levels being recorded on day 12 of gestation (Tavakkol et al., 1988; Letcher et al., 1989; Simmen et al., 1990).

IGF-I, synthesized by the endometrium is released into the uterine lumen where it presumably come into contact with the conceptus and thereby affects its development. Parallel to these changes in IGF-I mRNA levels in the endometrial tissues, the concentration of IGF-I polypeptide in the uterine luminal fluid changes during early pregnancy, with maximal concentrations detected between days 10 and 12 of gestation (Simmen et al., 1989). In contrast, mRNA levels for IGF-I receptors within the pig conceptus are constitutive across days 1-15 of pregnancy and are similar among conceptuses of various sizes (Green et al., 1995). Taken together, it appears that changes in IGF-I mRNA levels and IGF-I receptors, limit the effects of IGF-I on embryo development in the pig. In agreement with this, higher IGF-I mRNA levels in the endometrium (Simmen et al., 1992) and higher IGF-I polypeptide contents in the uterine luminal fluids (Simmen et al., 1989) have been observed in highly prolific Chinese Meishan gilts compared with Large White gilts. These results suggest the possibility of a distinctive relationship between uterine expression of the IGF-I gene and prolificacy in the pig. However,

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no information is available on the differential expression of the IGF-I gene in the pig endometrium in relation to embryo survival between individuals within the same breed. Therefore, the present study was conducted to evaluate the effect of exogenous progesterone supplementation early in pregnancy in embryo survival and on the endometrial levels of IGF-I mRNA and to compare endometrial IGF-I mRNA levels in relation to embryo survival.

MATERIALS AND METHODS

Animal housing and treatment

A total of 41 crossbred (Camborough Line 15, PIC Canada Limited) prepubertal gilts were used in this study. They were housed throughout the entire experiment in groups of four under total confinement conditions at the University of Saskatchewan Research Pig Barn. Procedures for animal breeding and treatment were approved by the University of Saskatchewan Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care.

Gilts weighing 80-90 kg were induced into oestrus by a single intramuscular injection of PG600 (Intervet Canada Ltd., Ontario, Canada). Starting four days after PG600 treatment, gilts were exposed to an intact mature boar on a daily basis to facilitate and detect the onset of oestrus. At their subsequent, naturally occurring oestrus, and again 24 h later, gilts were artificially inseminated with a minimum dose of 3×10^9 sperm cells of mixed origin (Alberta Swine Genetics, Leduc, Alberta, Canada).

After breeding, gilts were randomly assigned to one of three groups. Gilts in the treatment groups were given daily intramuscular injections of 50 mg progesterone (Sigma, St. Louis, MO, USA) dissolved in 0.5 ml of corn oil, from day 2 to 14 (N=14) or from day 4 to 14 (N=15) after breeding while those in the control group (N=12) were injected with corn oil (0.5 ml) alone from day 2 to 14. The choice of 50 mg progesterone as the treatment dose was based on a study by Ashworth (1991).

During the period from PG600 injection to onset of the first oestrus, gilts were fed *ad libitum* a barley-wheat-soybean meal diet formulated to provide 13 MJ DE kg^{-1} and 14% crude protein. From estrus detection, until the completion of the experiment, gilts received 2.0 kg d^{-1} of the same diet.

Between days 25 and 28 of gestation, gilts were slaughtered in a local slaughterhouse. About 20 min after exsanguination, the reproductive tract was removed from each animal and 1 g samples of endometrial tissue were collected from two random sites in each uterine horn. The pooled samples were snap-frozen in liquid nitrogen and stored at -80℃ until use. The number of corpora lutea and number of embryo were recorded for each animal and the embryo survival rate was calculated as the percentage of embryos over corpora lutea.

Total cellular RNA extraction

Total cellular RNA was extracted from the frozen endometrial tissues using the guanidine isothiocyanate-cesium chloride method (Chirgwin et al., 1979). Briefly, the endometrial tissues were homogenised in 4 M guanidine isothiocyanate solution (Gibco, Gaitherburg, MG, USA). Then, the homogenate was overlaid on 5.7 M cesium chloride and ultracentrifuged for 22 h at 20 ℃. The RNA pellet was washed with 70% ethanol, air dried, and then dissolved in diethyl pyro-carbonate-treated water. Further purification of the RNA samples was performed by phenol-chloroform extraction and ethanol precipitation (Chirgwin et al., 1979). The RNA quality was checked by electro-phoresis separation of 30 μg of RNA from each sample on a 1.2% denaturing (formaldehyde) agarose gel followed by transferring the RNA to a nylon membrane which was then stained with methylene blue. RNA was isolated from the livers of two control gilts and used as a positive control for IGF-I mRNA analysis.

Reverse transcription

Two micrograms of total RNA from each sample was reverse transcribed into the first strand complimentary DNA (cDNA) in a total volume of 20 $\mu\ell$ as follows. After ethanol precipitation, the RNA pellet was resuspended in 10.5 $\mu\ell$ of water containing 0.5 $\mu\ell$ of random primer (Promega, Madison, WI, USA), heated at 65℃ for 5 min, and then incubated at room temperature for 2 min before adding 9.5 $\mu\ell$ of a master reverse transcription reaction mixture (4.0 $\mu\ell$ of 5×1st strand buffer, 1.0 mM deoxynucleoside triphosphates, 0.25 mM dithiothreitol, 0.1 mg ml^{-1} bovine serum albumen, 20 U RNase inhibitor [Promega, Madison, WI, USA] and 100 U Moloney Leukaemia Virus reverse transcriptase [Gibco, Gaitherburg, MD, USA]. After incubation at 37℃ for 90 min, the reactions were heated at 95℃ for 5 min, briefly centrifuged and placed on ice.

Polymerase chain reaction (PCR)

Two microliters of reverse transcription products were mixed with 18 $\mu\ell$ of the master polymerase chain reaction mixture containing 2 $\mu\ell$ of 10 × PCR butter, 1.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates, 500 μM of sense and antisense primers, 5 μCi of ^{32}P-dCTP and 2.5 U Tag polymerase (GIBCO, Gaithersburg, MD, USA). After a brief centrifugation, reactions were overlayed with 20 $\mu\ell$ mineral oil, and then amplified by polymerase chain

reaction for 32 cycles under the following conditions: 1) denaturation at 94℃ for 40 sec, 2) primer annealing at 65℃ for 30 sec, and 3) primer extension at 72℃ for 1 min. An initial denaturing cycle of 94℃ for 5 min and a final extension cycle of 72℃ for 5 min were performed, before and after the main cycles. The sense and antisense primers used were 5'-ATGCA CATCACATCCTCTTCG-3' and 5'-CATCTCCAGCCT CCTCAGTC-3' respectively, which were identical to those described previously (Green et al., 1995) and predicated a 252 base pair fragment. Amplification of the 450 base pair actin fragment was used as an internal control to correct for reaction-to-reaction variations in the polymerase chair reaction amplification efficiency by adding a pair of primers (sense: 5'-CTACAATGAGCTGCGTGTGG-3' and antisense: 5'-TAGCTCTTCCCAGGGAGGA-3'), designed by Vaughan et al. (1992) according to the mouse β-action DNA sequence, into the same polymerase chain reaction as for IGF-I at the beginning of the 7th cycle. Polymerase chain reaction products were then separated on a 2% agarose gel containing ethidium bromide and the bands were viewed under ultraviolet light and were cut off the gel. The radioactivity in each collected band was measured on a β-counter and the ratio of counts in the IGF-I band to the action band was then calculated for each sample.

Blood sample collection and progesterone measurement

In order to demonstrate that the dose of progesterone used was effective in elevating serum progesterone concentration, 5 castrate males were injected with a single dose of 50 mg progesterone while an additional 5 castrates served as controls. Blood samples were collected from these animals 1 h, 3 h, 6 h, 9 h and 24 h after treatment and serum progesterone concentration was measured using a progesterone assay kit (Intermedico, Markham, Ontario, Canada). The results from this study were published previously (Yu et al., 1997) and confirmed that the dose of progesterone used was successful in elevating circulating progesterone concentrations for a peiod of at least 24 h.

Statastical analysis

Data on ovulation rate, embryo survival and relative levels of endometrial IGF-I mRNA in different groups were first analysed by ANOVA using General Linear Models procedures (SAS, 1990). Since the average levels of IGF-I mRNA were not different between groups and the value for individual animal showed a tendency to change with embryo survival, data on mRNA levels were then subjected to an analysis of simple linear correlation using Pearson's test (Shott, 1990).

RESULTS

Effect of exogenous progesterone on ovulation rate and embryo survival

The mean values for ovulation rate, number of embryos and embryo survival are shown in table 1. The ovulation rate, as represented by the number of corpora lutea on both ovaries, did not differ between treatment groups ($p>0.05$). Post-breeding supplementation of exogenous progesterone increased the number of embryos from 8.0 in the control group to 8.4 in the group treated with progesterone from days 4 to 14 while the number of embryos increased to 9.1 in the group treated with progesterone from days 2 to 12, but these changes were not significant ($p>0.05$). The average embryo survival rate was 73.9% in gilts supplemented with exogenous progesterone from day 2 to 14 after breeding compared with 71.3% for those treated two days later and 70.7% for gilts without treatment. None of these differences were statistically significant ($p>0.05$).

Endometrial levels of IGF-I mRNA

In order to determine the effect of treatment of pregnant gilts with exogenous progesterone given early in gestation on uterine levels of IGF-I mRNA, total cellular RNA extrated from endometrial tissues was subjected to reverse transcription-polymerase chain reaction analysis (figure 1). RNA isolated from the liver of two control gilts was pooled and used as a positive control. Before the reverse transcription-polymerase chain reaction, the RNA samples were checked for their integrity by methylene blue staining after they were separated and transferred to the membrane. As shown in panel A of figure 1, the 28S and 18S rRNA bands were clearly stained by

Table 1. Influence of exogenous progesterone (50 mg/day) from days 2 or 4 to 14 after breeding on the reproductive performance of gilts

Duration of Treatment	Total Gilts	Number of Corpora Luteum	Number of Embryos	Embryo Survival (%)
Days 2 to 14	14	12.4±2.7	9.1±2.7	73.9±15.6
Days 4 to 14	15	11.7±2.9	8.4±2.7	71.3±14.1
Control	12	11.3±2.8	8.0±1.6	70.7±13.3

methylene blue and no sign of degradation was seen. To adjust for possible differences made in the initial step of pipeting the RNA sample, as well as for variations in polymerase chain reaction amplification efficiency, co-amplification of actin was performed as an internal control. Fragments of the expected sizes were successfully and specifically co-amplified for both IGF-I and actin in samples from all 41 pigs. Panel B of figure 1 shows samples collected from the liver (positive control) and one representative sample from each treatment group.

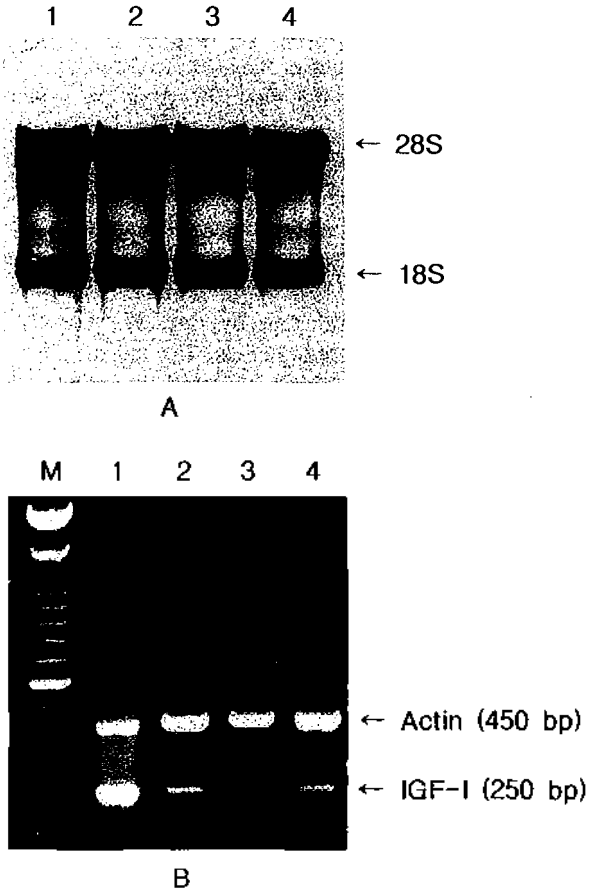

Figure 1. Panel A: Methylene blue staining of RNA after being separated by electrophoresis, transferred and cross-linked to the nylon membrane, showing the integrity of the RNA. Panel B: Reverse transcription-polymerase chain reaction products stained with ethidium bromide on the agarose gel, showing the specific IGF-I and actin bands. Lane M shows 100 base pair DNA maker; lane 1 show a sample isolated from liver; lane 2 shows endometrial tissue of gilt treated with oil (control); lane 3 is endometrial tissue from a gilt treated with 50 mg progesterone daily from 2 to 14 and lane 4 shows sample from a gilt treated from day 4 to 14 after breeding. Only one sample from each group is shown.

Progesterone treatment, starting either on day 2 or 4 after breeding, did not alter IGF-I mRNA abundance in uterine tissues (figure 2). However, across all samples, the IGF-1 mRNA level in the uterus was highly correlated (r=0.8193) with embryo survival rate ($p<0.01$; figure 3).

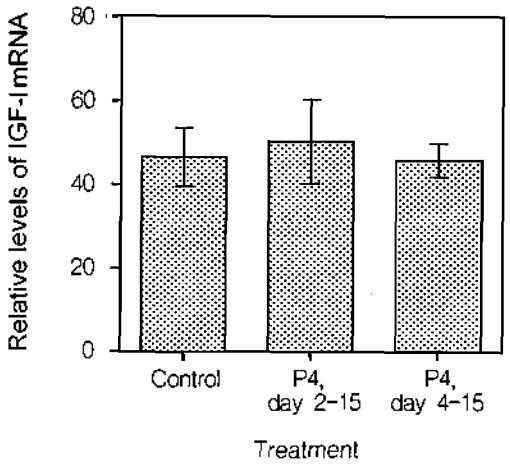

Figure 2. Average levels of endometrial IGF-I mRNA after normalization against the internal control actin in gilts treated with corn oil (control) or progesterone. The lines at the top of the chart represent standard error bars. There were no significant differences between groups ($p>0.05$).

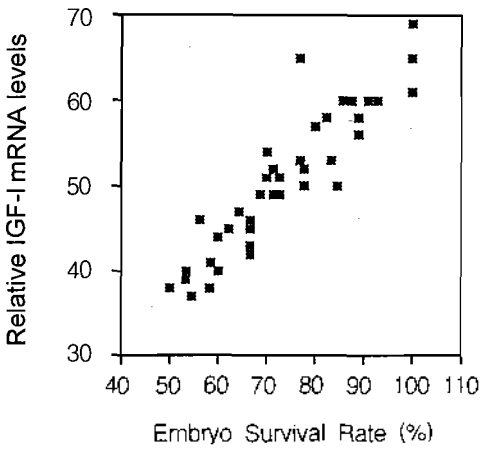

Figure 3. Correlation between embryo survival rate and IGF-I mRNA levels across all 41 samples ($r=0.8193$, $p<0.01$)

DISCUSSION

It has been established that oestrogen and progesterone are the major factors regulating IGF-I gene expression and IGF-I protein production by the endometrium. Oestrogen treatment has been reported to

induce IGF-I mRNA in the uterus of immature and ovariectomised rats (Murphy et al., 1987; Hana and Murphy, 1994). Both oestrogen and progesterone increased IGF-I mRNA levels within the endometrium as well as the IGF-I protein content in the luminal fluid in prepubertal gilts (Simmen et al., 1990). In the present study, we further assessed the possible regulatory effect of exogenous progesterone on the endometrial levels of mRNA encoding IGF-I during early porcine pregnancy by treating pregnant gilts with progesterone early in gestation. When compared with gilts in the control group, daily administration of progesterone between days 2-14 or between days 4-14 did not alter the uterine IGF-I mRNA levels which were determined on day 25-28 of gestation.

Results from other laboratories have demonstrated higher IGF-I mRNA levels in endometrial tissues (Simmen et al., 1992) and higher IGF-I protein contents in the uterine luminal fluids (Simmen et al., 1989) on day 12 of gestation in the highly prolific Chinese Meishan pigs compared with Large White gilts. In the present study, we observed large variations in embryo survival rate between gilts of the same breed and demonstrated that the IGF-I mRNA levels in the endometrium on day 25 were positively correlated with embryo survival. This finding supports the suggestion that IGF-I gene expression and IGF-I protein synthesis/secretion by the endometrium are closely correlated with prolificacy in the pig.

The mechanism by which IGF-I exerts its effects on embryo development and thus influences embryo survival or even litter size has not been well defined. In the pig, material recognition of pregnancy takes place on day 10-12 of gestation during which the conceptus exhibits dramatic morphological changes. Coincident with this rapid morphological transition, the porcine conceptus starts to produce oestrogen. Unlike cattle and sheep, the oestrogen produced by the conceptus is believed to be the primary signal responsible for maternal recognition of pregnancy in the pig (Bazer and Thatcher, 1977). Concomitant with the maximal conceptus oestrogen production, the endometrial expression of IGF-I mRNA and the luminal concentration of IGF-I protein peaks on day 12 of gestation, suggesting that uterine IGF-I may be functioning during early pregnancy in the pig by regulating the conceptus oestrogen production in a paracrine manner. This is supported by recent evidence that *in vitro* additon of IGF-I to day 12 conceptus resulted in increased expression of mRNA encoding the important steriodogenic enzyme, cytochrome P-450 aromatase, in filamentous conceptuses (Green et al., 1995).

In the early work of Ashworth (1991), supplementation of exogenous progesterone between days 4 and 21 after mating increased both the number of viable embryos and the embryo survival rate by day 30 of gestation in gilts exhibiting increased ovulation rate due to flush-feeding prior to mating (Ashworth, 1991). In contrast, in the present study, treatment of pregnant gilts with exogenous progesterone increased neither the number of embryos nor the embryo survival rate on day 25 of gestation. As the duration of treatment in the present experiment was 7 days shorter that the treatment period of Ashworth (1991) it is possible that exogenous progesterone supplementation up to day 14 of gestation might not be long enough to achieve any significant effects on embryo survival. However, our data is in agreement with the study of Webel et al. (1995), which demonstrated a lack of relationship between plasma progesterone concentrations and embryo survival during the first 30 days of gestation in gilts with normal ovulation and supports the conclusion of Flint et al. (1982) that exogenous progesterone is generally ineffective or inconsistent in enhancing preattachment embryo survival in pigs.

In summary, progesterone treatment, starting either on day 2 or 4 after breeding, neither signigicantly increased embryo survival rate by day 25 of gestation nor altered IGF-I mRNA levels in uterine tissues. However, across all samples, the IGF-I mRNA level in the uterus was highly correlated with embryo survival rate ($r=0.8193$, $p<0.01$), supporting the involvement of IGF-I in the regulation of porcine embryo development.

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