

Estradiol-17 β Alters Trophectoderm Proliferation in Pig Embryos^a

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Abstract: Around day 12 of gestation, the porcine embryo undergoes a dramatic morphological change in which the trophectoderm elongates and begins producing and secreting estradiol-17 β . Placental size in late gestation is related to the size of the embryo at the time of elongation. Embryos of the Chinese Meishan secrete approximately one-half the amount of estradiol-17 β produced by embryos of domestic large white breeds. Larger litters with smaller, more efficient placentae are associated with the Chinese Meishan. Meishans treated with estradiol-17 β at the time of elongation exhibited an increased placental size. The aim of this study was to determine the effects of treatment with exogenous estradiol-17 β at the time of elongation on the proliferation of the trophectoderm of the embryo, as well as the changes in uterine luminal growth factors, hormones and ions. Pregnant gilts (N = 12) were randomly selected to receive either estradiol-17 β or vehicle every 6 h for 48 h, beginning on day 12 of gestation. On day 14, embryos and uterine luminal contents were surgically collected. The proliferation index of the trophectoderm and the concentrations of growth factors, hormones and ions were determined. Estradiol-17 β treatment resulted in a doubling of the proliferation index. There was an increase in PGE₂ and PGF_{2 α} in the uterine flushings of treated gilts. There were no changes in the concentrations of estradiol-17 β , IGF-1 or calcium. In conclusion, treating gilts with estradiol-17 β resulted in a doubling of the proliferation rate in the trophectoderm and likely an increased length of the embryo at elongation.

Key words: Elongation, estradiol-17 β , swine, trophectoderm proliferation, growth factors, placental size

INTRODUCTION

Establishment of pregnancy in the pig is dependent upon the production and secretion of estradiol-17 β from the elongating blastocyst and the secretion of histotroph from the uterine glandular epithelium. Around day 12 of gestation, the embryo undergoes a dramatic morphological change in which it is transformed from a sphere of about one cm in diameter to a filamentous strand of up to one m in length (Geisert *et al.*, 1982a). At this time, approximately day 11.5, the embryo will begin to produce and secrete estradiol-17 β into the uterine environment (Geisert *et al.*, 1982a). The secretion of estradiol-17 β causes a series of responses in the uterine environment, including increases in protein, calcium, Prostaglandin F (PGF) and Prostaglandin E (PGE) (Geisert *et al.*, 1982b) as well as acting as the signal for maternal recognition of pregnancy.

Placentation in the pig is not complete until day 18 of pregnancy (Steven, 1975), therefore the blastocysts rely on histotrophic nutrition. The development of secretory vesicles and the release of their contents are coincident with blastocyst elongation and the initiation of estradiol-17 β by the blastocyst (Geisert *et al.*, 1982b). The action of estrogen on the release of vesicular secretory products is perhaps mediated by calcium. Calcium may also be activating phospholipase A₂ and therefore increasing arachidonic acid and the production of eicosanoids, such as PGE and PGF (Geisert *et al.*, 1982b). Prostaglandin E₂ and PGF_{2 α} have antileutolytic and luteotrophic effects on the corpora lutea, impact the growth and differentiation of endometrial cells, embryo spacing within the uterus, blood flow to the uterus, vascular permeability and implantation (Stefańczyk-Krzymowska *et al.*, 2005). Insulin like Growth Factor-1 (IGF-1) is an important regulator for both fetal and postnatal growth (Geelhoed *et al.*, 2008).

Increasing litter size of domestic breeds would have a positive impact on the pork industry by increasing production without increasing the size of the sow herd. Average litter size for commercial swine breeds in North America is approximately 9.5 pigs L⁻¹. The Chinese Meishan has larger litters than North American breeds of approximately 13 pigs L⁻¹ (Wilson and Ford, 1997). When Meishan and Yorkshire gilts are compared, they have similar ovulation and fertility rates and similar uterine size but exhibit marked differences in placental size and efficiency (as described by the fetal weight-to-placental weight ratio (Wilson *et al.*, 1999). This smaller, more efficient placenta allows the Meishan to have a larger litter, as compared to the larger, less efficient placenta of the Yorkshire. The smaller placenta occupies less space in the uterus, allowing more space for conceptus development.

Around day 12 of gestation when the embryo elongates, it will also begin producing estradiol-17 β . The amount of estradiol-17 β secreted by Meishan embryos is approximately 1/5 that of Yorkshire embryos and approximately 1/2 that found in uterine flushings of Yorkshire embryos (Anderson *et al.*, 1993). In addition, Meishan embryos are smaller before and shorter after elongation than Yorkshire embryos (Anderson *et al.*, 1993). When Meishan sows were treated with exogenous estradiol-17 β at the time of elongation, the result was a 40% increase in placental size, a decreased placental efficiency and a decreased litter size (Wilson and Ford, 2000).

Therefore, the objective of the current experiment is to determine how administration of exogenous estradiol-17 β at the time of elongation might alter trophoblast proliferation and the uterine environment.

MATERIALS AND METHODS

Animal breeding: Twelve Smithfield Hamline gilts were checked twice daily for estrous behavior with a boar and were artificially inseminated 12 and 24 h following detection of estrus (day 0). Gilts were bred no earlier than their third estrus. Twelve days following insemination (day 12) gilts were randomly assigned to receive either one mg of estradiol-17 β or vehicle every 6 h for 48 h as previously described by Wilson and Ford (2000) to increase placental size approximately 40%. All procedures were approved by the West Virginia University Animal Care and Use Committee (ACUC # 03-0603).

Surgical collection of the embryos: On day 14, embryos and uterine flushings were surgically collected from the gilts. Embryos and flushings were collected through a

borosilicate glass canula (10 mm O.D. and 10 cm long), which was inserted into the tip of the uterine horn. Using a flushing syringe, which was inserted into the base of the same uterine horn, each uterine horn was flushed with 10 mL of phosphate buffered saline (pH 7.4). The flushings were massaged down the uterine horn and through the canula into a sterile petri dish. This was performed separately on each uterine horn.

Immunohistochemistry: Embryos were separated from uterine flushings by centrifugation and fixed in formalin. Embryos were first dehydrated in a series of alcohols and xylenes and then embedded in paraffin. Embryos were sectioned at 5 μ m and fixed to slides for staining. The embryos were immunohistochemically stained for proliferating cell nuclear antigen and counterstained with hematoxylin according to procedures previously determined by Wilson and Ford (1997) (Fig. 1). One slide was stained for each gilt and two microscopic fields were counted for each slide. The proliferation index was then calculated by dividing the number of PCNA positive cells by the total number of cells counted.

Uterine flushings: After removal of the embryos, the uterine flushings were clarified by centrifugation and the supernatant harvested. The flushings were frozen for later determination of growth factors, hormones and ions utilizing commercially available assay kits. Uterine flushings were analyzed for PGF α and PGE $_2$ (Cayman Chemical, Ann Arbor, MI), estradiol-17 β and IGF-1 (Diagnostic Systems Laboratory, Webster, TX) and calcium (Sigma Aldrich, St. Louis, MO).

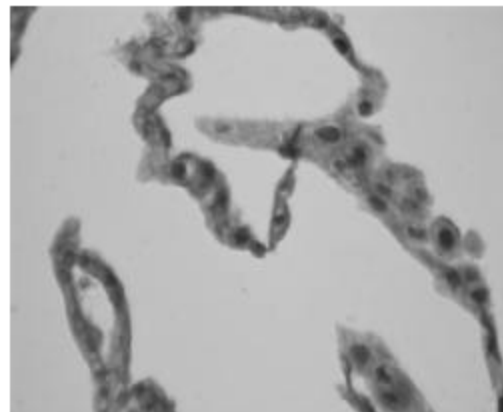


Fig. 1: Single field image of d 14 pig embryos that have been immunostained for PCNA. Black arrows indicate PCNA positive cells and the arrow heads indicate PCNA negative cells

Statistics: The effects of treatment on proliferation of the trophoctoderm and composition of uterine luminal fluid were determined by analysis of variance, using the GLM procedures of SAS.

RESULTS AND DISCUSSION

No difference ($p > 0.15$) was detected in the uterine flushings concentration of estradiol-17 β between the control and treated group (84.4 ± 28.0 versus 145.3 ± 27.1 pg mL $^{-1}$, respectively). Treatment of pregnant gilts with exogenous estradiol-17 β at the time of elongation resulted in a doubling ($p < 0.04$) of the proliferation index in the trophoctoderm (Fig. 2). Coincident with the increase in trophoctoderm proliferation in embryos from treated gilts, there was a near doubling ($p = 0.06$) of uterine flushings concentration of PGE $_2$ (Fig. 3a) and a trend ($p = 0.08$) for an increase in the uterine flushings concentration of PGF $_2\alpha$ (Fig. 3b). Treatment of pregnant gilts with exogenous estradiol-17 β did not affect ($p = 0.89$) the uterine flushings concentration of IGF-1 (29.5 ± 6.3 ng mL $^{-1}$) or calcium (0.9 ± 0.2 μ g mL $^{-1}$, $p = 0.29$). Overall, there was a strong positive correlation observed between PGF $_2\alpha$ and PGE $_2$ ($r = 0.94$, $p < 0.05$). Furthermore, estradiol-17 β and IGF-1 concentrations in uterine flushings exhibited a positive correlation ($r = 0.52$, $p = 0.08$).

The Chinese Meishan was imported into the United States to investigate their large litter size compared to domestic control breeds. Though this breed has a uterine size similar to that of domestic breeds, the Meishan is more prolific, having three to four more pigs per litter. Biensen *et al.* (1999) compared Meishan and Yorkshire gilts and demonstrated that the two breeds had dramatic differences in placental size, though uterine size was the same. The Meishan has a smaller, more vascularized placenta than the larger, less vascularized placenta of domestic breeds. These researchers proposed that the larger placenta is what leads to the restriction of litter size in domestic commercial breeds. Wilson and Ford (1997) showed that the concentration of estradiol-17 β was greater in the Yorkshire compared to the Meishan uterine environment. Meishan embryos were found to be smaller than Yorkshire embryos at the same physiologic state and the trophoctoderm of Meishan embryos elongated to a much shorter length as a result of a reduced mitotic rate. Wilson and Ford (2000) found that treatment of Meishan gilts with estradiol-17 β at the time of elongation resulted in an increase in the placental size, including weight, surface area and length. This led the researchers to suggest that estradiol-17 β was responsible for the variation in the

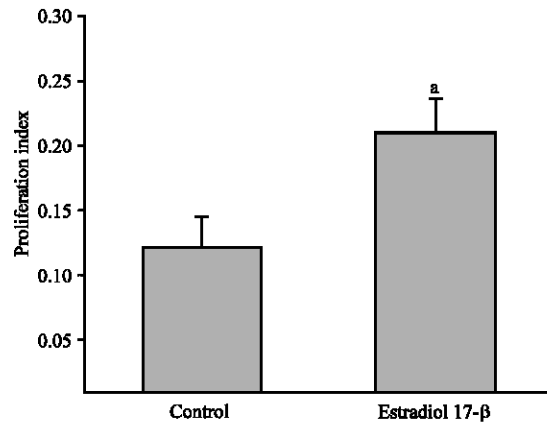


Fig. 2: Proliferation index (as measured by proportion of PCNA positive nuclei), for control and estradiol-17 β treated groups. *Mean \pm SEM between treatments, differ ($p < 0.05$)

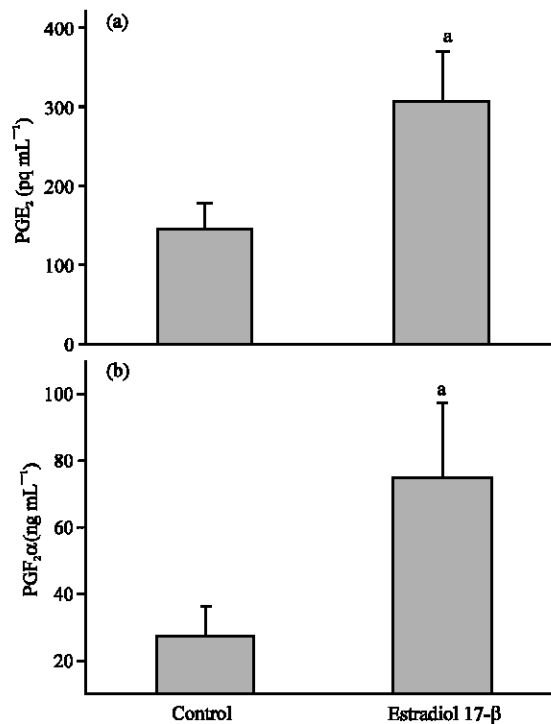


Fig. 3: Representative of uterine flushings concentration of (a) PGE $_2$ and (b) PGF $_2\alpha$ for control and estradiol-17 β treated groups. *Mean \pm SEM between treatments

length of the elongated embryo and that the size of the elongated embryo determined surface area, weight and length of the placenta. The result of the current experiment, which employed a similar treatment to that utilized by Wilson and Ford (2000) that resulted in a 40%

increase in placental size near term, was a doubling of the number of proliferating trophoblast cells in conceptuses of estradiol treated gilts compared to conceptuses of control gilts.

A study by Wilson *et al.* (1998) found that the pattern of placental growth is determined by the conceptus. In this study, Meishan and Yorkshire conceptuses were collected on day 2 of gestation and were reciprocally transferred into the other breed and allowed to gestate. The placenta of the Yorkshire conceptus increased in surface area, weight and length between days 90-110 of gestation, with no change in vascular density, regardless of the uterine environment in which they were gestated. Meishan conceptuses showed no increase in the surface area, size or weight of the placenta but did show an increase in vascular density during the last part of gestation.

The estrogen found in the uterine environment is produced only by the trophoblast of the embryo and serves as the signal for maternal recognition of pregnancy (Geisert *et al.*, 1982b). Exogenous administration of estrogen to cycling gilts causes an increase in endometrial secretory products (Pusateri *et al.*, 1996). The increased concentration of estrogen that is associated with the Yorkshire breed may cause an increase in the growth factors in the uterine environments which would allow for the increased size of the trophoblast of the embryo. Geisert *et al.* (1982a), found that there was a significant increase in PGE₂, PGF₂α, calcium, estrogen and protein concentrations in pregnant versus nonpregnant gilts. Geisert *et al.* (1982b), also found that after exogenous administration of 5 mg of estradiol valerate there was an increase in the PGE₂ and PGF₂α concentrations in the uterine luminal fluid. In this study, we also found that there was an increase in the concentrations of prostaglandins, with a near doubling in the concentration of PGE₂ and a trend for an increase in the concentration of PGF₂α. However, no differences were found in the concentrations of IGF-1 or calcium. No difference was observed in estradiol-17β between control and treated gilts, though treated gilts were receiving exogenous estradiol-17β. This may be explained by the short half life of estradiol-17β and therefore its relatively quick clearance rate. Pusateri *et al.* (1996) treated gilts with differing concentrations of estradiol-17β and found that estradiol-17β concentrations returned to those similar to endogenous production within 18 h.

CONCLUSION

Treatment of gilts with exogenous estradiol-17β resulted in a doubling of the trophoblast at the time of

elongation. This may explain why treatment with exogenous estradiol-17β increased placental size at term. Therefore, the amount of estrogen produced by the embryo appears to regulate the degree of elongation which then regulates placental size.

ACKNOWLEDGEMENT

We would like to thank Smithfield Premium Genetics for donation of the pigs utilized in this study.

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