

FINAL REPORT FOR NPPC

Project Title Hormonal Profiles in sows infected with Atypical Porcine Reproductive and Respiratory Syndrome Virus (PRRSV); a Mechanism of Virus -Induced Abortion. **NPPC project #98-020**

Principal Investigator Dr. Tom Molitor/ Tracey Papenfuss
Dr. Han Soo Joo

Institution University of Minnesota

1. ABSTRACT

Porcine reproductive and respiratory syndrome (PRRSV) is a devastating disease affecting the pig industry. Reproductive failure, which includes abortions, stillbirths, weak-born piglets and delayed return to estrus, cause significant economic loss to the swine industry. The hallmark signs of a typical PRRSV outbreak has been primarily abortions, stillbirths and mummified fetuses in late gestation (80 days gestation). However, a new clinical picture emerged in a variety of farms in the Midwestern region. The abortions that are occurring involved early gestational losses (less than 50 days) with the most significant loss occurring nearly 30 days of gestation. In an attempt to more thoroughly understand the basis for the change in clinical picture and attempt to delineate the pathogenic mechanisms of reproductive failure an the proposed work was undertaken. The hypothesis was that atypical PRRSV induces alterations in reproductive hormones (progesterone and $\text{PGF2-}\alpha$) which may adversely affect the fetus. Progesterone and $\text{PGF2-}\alpha$ (along with estrone sulfate levels) could serve as a diagnostic indicator of impending reproductive failure seen in atypical PRRSV infections. Two objectives were proposed to test the hypothesis. The experimental findings revealed that an atypical PRRSV strain could cross the placenta and infect early gestational fetuses but the abortion outcome was not

reproduced. Whereas hormonal measures change during pregnancy levels of estrone sulfate and progesterone were not significantly different from uninfected sows.

2. INTRODUCTION:

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically devastating disease affecting the swine industry, with significant income loss due to reproductive failure. Many infectious agents are known to cause reproductive pathology (e.g. PRRSV, porcine parvovirus, Leptospirosis, etc.). Currently, there is very little information concerning how many of these infectious agents induce abortion, particularly in the area of hormonal-immune alterations induced by these infections. There is much evidence to indicate that hormones are influenced by infectious agents and vice versa. Specific and directed immune alterations by microorganisms occur on many levels (Kotwal GJ 1997). It has already been shown that hormonal levels estrone sulfate, prostaglandin and PFG2a are altered during porcine parvovirus infection (also a reproductive pathogen) Meyers PJ et al. 1987. HIV-1 infection of human placental cultures resulted in a significant decrease (90% and 70%, respectively) of hCG & progesterone production (Bourinbaiar AS. et al 1992) - Prostanoids are also altered during acute sarcocystiosis infection in growing pigs (Dauguschies A, et al 1989). Newcastle Disease virus activates HPA axis (via IL-1?) during experimental infection (Besdovsky OH and del Rey 1989)

Evidence also indicates that hormones themselves can have effects (i.e. Immunomodulatory) on the course of infectious disease. This is most likely due to the effects of hormones on the immune system. Glucocorticoids (eg. the "stress" hormone cortisol) can suppress the immune system which explains why stressed individuals (eg. shipping, crowding, etc.) are more susceptible to disease. For example, glucocorticoids alter the ability of certain immune cells to migrate to the areas of infection during influenza infection (Herman G 1995) Sex steroid hormones such as progesterone and testosterone are well documented to be immunomodulatory, primarily serving to suppress the immune system. Progesterone and estrogen inhibit HIV-1 release at physiological concentrations (Bourinbaiar AS. et al 1992) and hCG (human Choriogonadotrophin) can actually inhibit HIV infection at physiological concentrations but may increase virus production at higher levels (Bourinbaiar

AS et al 1995). As evidence, there is a definitive interaction between infectious agents and hormone levels and this interaction is complex. Therefore, alterations in hormone levels may serve as an indicator, not only of an infected individual, but indicate individuals that may be more susceptible to disease.

For these reasons, that there is likely a definitive interrelation between immunological defenses against infectious agents such as the reproductive pathogen PRRSV and the fetal-maternal hormonal system.

The hallmark signs of a typical PRRSV outbreak have been primarily abortions, stillbirths and mummified fetuses in late gestation (80 days gestation). However, a new clinical picture is emerging in a variety of farms in the midwestern region. The abortions that are occurring involve early gestational losses (less than 50 days) with the most significant loss occurring nearly 30 days of gestation. The aborted fetuses seem to suffer from no evident pathological lesions, simply that they were expelled prematurely.

It is well known that many pharmacological agents can be used to induce parturition. Similarly, parturition can also be prevented, even parturition induced by infectious disease (e.g. preventing LPS induced abortion in early pregnant gilts by flunixin meglumine by Cort N et al 1990). Parturition itself is characterized by an increase in an increase in estrogen, rapid fall in progesterone, elevations of prostaglandins and oxytocin and elevations in both maternal and fetal cortisol levels. By manipulating the levels of some of these hormones, it may be feasible to prevent abortions for a reproductive pathogen such as PRRSV.

The involvement of estrogens is extremely complex, due to their mechanisms of action as well as the multitude of potential players. Estadiol is luteotropic in the pig and cannot be used as an abortifacient (Clark et al 1986). The majority of estrogens (estradiol 17b, etc) become significantly elevated only several days prior to parturition. However, estrone sulfate levels manifest with a biphasic peak with the initial peak in the first trimester (30+ days) and the second peak occurring during the third trimester (80+ days). The initial estrone sulfate during the first trimester originates from a viable fetus and its membranes (Wise TH et al 1992). This fact has been used to determine fetal viability in horses and could potentially be used as a diagnostic indicator of fetal demise in pigs (Kassman LH et al 1988).

Prostaglandins serve to augment the drop in progesterone and increase oxytocin that occurs near term and the trophoblast serves to prevent a drop in progesterone throughout gestation (Meyer HH 1994, Sander-Richter H et al 1988). Prostaglandin analogs (cloprostenol and/or dinoprost tromethamine) given during midgestation induce abortion (Meyers PJ et al 1987). If PGF2 α is blocked by administration of a β -blocker (carazolol), abortion is prevented (Holtz W, 1990). Prostaglandins given after day 12 of gestation can induce abortion (Clark LK et al 1986).

Decreased progesterone levels are known to be associated with the onset of parturition. False elevation of progesterone levels with medroxyprogesterone acetate has been used to slightly delay parturition (Whitely JL et al 1990) while Epostane, a 3-beta-hydroxysteroid dehydrogenase inhibitor which decreases progesterone levels, is used clinically to induce parturition (Silver M et al 1988).

3. OBJECTIVES

The hypothesis is that atypical PRRSV induces alterations in reproductive hormones (progesterone and PGF2- α) which may adversely affect the fetus. Progesterone and PGF2- α (along with estrone sulfate levels) could serve as a diagnostic indicator of impending reproductive failure seen in atypical PRRSV infections. Two objectives were proposed to test the hypothesis; (1) to determine the hormonal pattern during early gestation in normal sows and in sows infected with atypical PRRSV under field conditions of abortion; (2) to determine the hormonal pattern in sows experimentally infected with an atypical strain of PRRSV.

4. PROCEDURES

Animals

Pregnant sows of objective 2 studies were obtained from a commercial farm. They were free of PRRSV, PRV and porcine parvovirus. The sows were housed in College of Veterinary medicine animal isolation facilities and cared for according to the guideline of the University of Minnesota animal care committee.

Experimental design-Objective 2

Due to the evidence that "atypical" PRRSV infection result in the most significant fetal loss in early gestation (the peak occurring at 30 days of gestation) sows at 30 days of gestation were experimentally infected. For this reason, four (2 control and 4 infected) artificially inseminated PRRSV-negative sows will be placed into the isolation facilities at the University of Minnesota campus on day 23 of gestation. Pregnancy was verified at 30 days of gestation using ultrasound. Jugular catheters were placed and animals will be allowed to adjust to housing conditions (socialization/ handling/ blood collection procedures) for one week. Baseline (pre-infection) blood samples were obtained every 12 hours and stored at -20°C until analysis.

Following environmental acclimatization and verification of pregnancy, experimental treatments were performed. Intranasal inoculation was performed by administration of 0.5 ml of virus into each nostril of virus (1×10^{-5} TCID₅₀). "Atypical" PRRSV was obtained from submitted samples (challenge virus RFLP 1-4-2 pattern from Joo, 1997) and will be used to infect the 4 sows of the 'infected group'. Blood was drawn every 12 hours for 2 weeks following infection and hormone levels determined.

Fetal tissue was obtained from infected and uninfected sows through sacrifice and necropsy 14 days post-infection (day 44 of gestation). Fetal tissues was collected (lung, liver, spleen, kidney, heart, mesenteric lymph nodes, brain, tonsil) in order to assess histopathological lesions. A pooled tissue homogenate of fetal tissues was prepared for virus isolation and PCR (polymerase chain reaction)-based detection for the presence of PRRSV (i.e. Taqman™). The PRRSV-status of the sow was determined with standard virus isolation and PCR-based detection.

Hormone assays

Progesterone, PGF2- α and estrone sulfate levels were determined and quantified by the endocrinology lab at the veterinary teaching hospital. The endocrinology laboratory uses the radioimmunoassay, a standard technique for measuring hormone levels that is significantly more sensitive than ELISA detection of hormone levels. The radioimmunoassay is a superior test to ELISA in this

experiment due to our desire to quantify potentially small changes in hormone levels. Validation of hormone detection in swine has been established by the University of Minnesota's endocrinology lab (progesterone) and other investigators (estrone sulfate and PGF2- α).

Virus Isolation

Virus isolation was attempted using the CL2621 cell line according to methods previously reported (Bautista et al 1993). Briefly sera were inoculated directly or diluted 1:5 with minimal essential medium into 4-6 day old CL2621 cell monolayers prepared in 48 well plates. After 1.5 h absorption, cells were washed with Hanks, and cultured with fresh MEM at 37 C. Cells were observed for characteristic cytopathic effects for 7 days and presence of virus was confirmed by IFA with monoclonal antisera to PRRSV (SDOW) Two additional blind passages were performed before designating a sample as negative.

Taqman RT-PCR

Primers and probes corresponding to PRRSV ORF6 were synthesized by Perkin Elmer Applied Biosystems (Foster City, CA 94404, U.S.A.). Primer mix was added to samples, melted at 70°C for 5 min and ramped slowly to room temperature (25°C). Samples were then placed on ice and 35 μ l of PCR reaction mix was added to each sample. The samples were then placed into a PE-9700 thermocycler with the following cycle conditions: 50°C for 30 min, 95°C for 10 min, 60 cycles of: 94°C (30 sec), 54°C (1 min), 72°C (1 min), followed by a 72°C hold for 7 min and a hold at 25°C. Samples were then removed and a post-PCR read was performed. The TaqmanTM reader used an excitation wavelength of 488 nm, and intensity of the reporter and dye was measured at 518 nm. FAM fluorescent fluctuations were normalized by dividing the emission intensity of the reporter dye by the emission intensity of a passive reference measured at 602 nm and determining the change in this normalized reporter value (ΔR_n) following PCR amplification with and without target. The TaqmanTM 7200 reader displayed this ΔR_n value and a standard deviation from the samples run in duplicate.

Cutoffs values for the ΔRn values were determined by using the formula: $Cutoff = x + (t \text{ value}) 2 \text{ S.D.}$ where x and $S.D.$ were obtained from the Taqman™ reader values.

5. RESULTS.

Objective 1: Hormonal and virus in naturally infected sows.

A herd was identified that was experiencing the characteristic signs of atypical PRRSV: abortions occurring early in gestation (less than 50 days, the majority near 30 days). The aborted fetuses seem to suffer from no evident pathological lesions (routine diagnostic analysis) and seem to be simply expelled prematurely. In addition, clinical signs (fever, anorexia, sudden death, etc.) were seen in sows. Thirty sows on this farm were identified and blood sampled over a week period of time. Ten of these sows aborted. Thus, we were successful in collecting blood samples for hormonal profiling before and after abortion. Sera samples were subjected for hormonal profiles for progesterone and estrone sulfate. Tissues were subjected to PRRSV identification by virus isolation and PCR.

Virus was identified in the sera of selective sows experiencing abortion. Not all of 10 sows were virus positive. Hormone assays performed on the sera from these sows need not reveal any differences in values of either progesterone or estrone sulfate from the aborting sows compared to the nonaborting sows.

Objective 2: Hormonal profiles in experimentally infected sows.

We first identified a source of PRRSV negative pregnant sows for experimental infection.

Following environmental acclimatization and verification of pregnancy, experimental treatments were performed. Intranasal inoculation was performed by administration of 0.5 ml of virus into each nostril of PRRSV virus (1×10^{-5} TCID₅₀). "Atypical" PRRSV was obtained from submitted samples (challenge virus RFLP 1-4-2 pattern from Jo, 1997) and was used to infect the 4 sows of the 'infected group'. Blood was drawn every 12 hours until for 2 weeks following infection or abortion occurs.

Four sows at 30 days of gestation were infected with an atypical strain of PRRSV. Two sows remained uninfected. The period of observation following infection was extended to 21 days. Following the 21 period of observation sows were euthanized and the fetuses collected and examined for evidence of fetal infection with PRRSV and any fetal abnormalities and the presence of virus. Sows were catheterized to allow for repeated samples. Throughout the period of observation no abortions occurred either in the infected or noninfected sows. In all four of the inoculated sows virus was detected in the sera ranging from 2 to 10 days following infection. Virus was not detected in noninfected sows. Collections of fetuses from the sows at 14 and 21 days following infections revealed no gross abnormalities. Taqman PCR revealed virus was detected in tissues from fetuses of 2 of the infected sows. Thus virus did cross the placenta in two of the 4 sows. Hormone, progesterone and estrone sulfate measures on the sera from the sows demonstrated changes in the hormone levels over the 3-week gestation period for all sows. However there were no differences in either progesterone or estrone sulfate levels from infected sows compared to uninfected sows.

Value of Proposed Research to the Swine Industry:

PRRSV is a disease, which represents significant reproductive loss to the swine industry due to both respiratory disease as well as significant reproductive loss in primarily late gestation. A different picture of PRRSV (atypical PRRSV) of early reproductive loss (i.e. significant fetal loss around 30 days of gestation) is emerging on Midwestern farms. There is little information known concerning the mechanism of PRRSV-induced reproductive failure. Previous investigators have shown some direct effects of cellular infection and destruction, but at present, indirect effects (e.g. hormonal alterations induced by the virus) of PRRSV are currently poorly characterized. The investigation into potential indirect effects (i.e. hormonal alterations) of PRRSV infections may help us better understand the mechanism of reproductive pathogenesis of PRRSV. PRRSV is a disease which represents significant reproductive loss to the swine industry due to both respiratory loss in youngstock as well as significant reproductive loss in primarily late gestation. However, a new clinical picture of PRRSV (atypical

PRRSV) of early reproductive loss (i.e. significant fetal loss around 30 days of gestation is emerging on Midwestern farms. While the mechanism of reproductive pathogenesis of PRRSV includes direct effects of cellular infection and destruction, indirect effects (e.g. hormonal alterations induced by the virus) of PRRSV are currently poorly characterized. The idea that a viral agent can induce reproductive loss in pigs due to hormonal alterations was demonstrated with porcine parvovirus in 1987 by Meters et al. These investigators demonstrated that parvovirus induces hormonal alterations (increased PGF2a, decreased progesterone) which mirrored the hormonal profiles, fetal loss and lack of fetal pathology seen with induced abortion. Atypical PRRSV demonstrates a similar early gestational fetal loss with little apparent fetal pathology. One potential component to Atypical PRRSV is the potential hormone changes induced by the virus. The findings from the work do not support a role for the two selected hormones but other hormones may be involved.

REFERENCES

Besedovsky HO, del Rey A. Mechanism of virus-induced stimulation of the hypothalamus-pituitary-adrenal axis. **Journal of Steroid Biochemistry**. 34(1-6):235-9, 1989.

Bourinbaier AS, Nagorny R, Tan X. Pregnancy hormones, estrogen and progesterone, prevent HIV-1 synthesis in monocytes but not in lymphocytes **FEBS Letters**. 302(3):206-8, 1992 May 18

Bourinbaier AS, Lee-Huang S. Anti-HIV effect of beta subunit of human chorionic gonadotropin (beta hCG) in vitro **Immunology Letters**. 44(1):13-8, 1995 Jan.

Clark LK, S D'allaire, AD Leman. Reproductive System *in* Diseases of Swine 6th ed. (Eds AD Leman, B Straw, RD Glock, WL Mengeling, RHC Penny, E Scholl. Iowa State University Press Ames, Iowa 1986.

Cort N, H Kindahl. Endotoxin-induced abortion in early pregnant gilts and its prevention by flunixin meglumine. **Acta Veterinaria Scandinavica** 31(3): 347-358. 1990

Dauguschies A, M Rommel, H-O Hoppen. 1989. Prostanoids during acute sarcocystiosis in growing pigs. **Parasitology Research** 76 (2): 115-118.

Done SH, DJ Paton, MEC White 1996. Porcine reproductive and respiratory syndrome (PRRS): a review, with emphasis on pathological, virological and diagnostic aspects. *Br. Vet. J.* 152: 153-174.

Hermann G. Beck FM. Sheridan JF. Stress-induced glucocorticoid response modulates mononuclear cell trafficking during an experimental influenza viral infection. ***Journal of Neuroimmunology.* 56(2):179-86, 1995 Feb.**

Kasman LH, JP Hughes, GH Stabenfeldt, MD Starr, BL Lasley. Estrone sulfate concentrations as an indicator of fetal demise in horses. ***Am. J. of Vet Research* 49(2): 184-187, 1988**

Knight JW. 1994. Aspects of placental estrogen synthesis in the pig. *Exper. and Clinical Endocrinology* 102(3) 175-184.

Meyers PJ. Liptrap RM. Miller RB. Thorsen J. Hormonal changes in sows after induced porcine parvovirus infection in early pregnancy. ***Am. J. of Veterinary Research.* 48(4):621-6, 1987**

Wise TH, RK Christenson. Relationship of fetal position within the uterus to fetal weight, placental weight, testosterone, estrogens and thymosin beta 4 concentrations at 70 and 104 days of gestation in swine. ***J. Animal Science* 70(9): 2787-2793. 1992**