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Influence of weaning age, restraint stress, and cortisol addition in vitro on immune function in swine

Marsha Elaine Brown

To the Graduate Council:

I am submitting herewith a thesis written by Marsha Elaine Brown entitled "Influence of weaning age, restraint stress, and cortisol addition in vitro on immune function in swine." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

H.G. Kattesh, Major Professor

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INFLUENCE OF WEANING AGE, RESTRAINT STRESS, AND CORTISOL ADDITION IN VITRO ON IMMUNE FUNCTION IN SWINE

A Thesis

Presented for the

Master of Science

Degree

The Univeristy of Tennessee, Knoxville

Marsha Elaine Brown

August, 1994

DEDICATION

This thesis is dedicated to my beloved husband

John David Crutchfield

and my parents

Mr. and Mrs. Edward B. Brown, Jr.

who have made my life complete and given me the encouragement to accomplish this educational goal.

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ABSTRACT

This research attempted to examine the influence of restraint stress in confinement housing in gilts and age of weaning on cellular immune function in the pig. Peripheral blood mononuclear cell (PBMC) blastogenic response to mitogen, neutrophil elastase degranulation, and neutrophil phagocytic ability were examined as indicators of cell mediated immunity. Traditional measures of stress including total and percent free cortisol, total leukocyte number, and leukocyte differential counts were used as another measure of stress. *In vitro* examinations were made to establish a culture system using cortisol concentrations that were suppressive and those that were not for PBMC blastogenic response to mitogens. Using this system the effects of vitamin C were tested to determine if coincubation of PBMC with cortisol, at suppressive levels, and vitamin C would decrease the suppressive effects seen in blastogenic response to mitogens.

The results indicated that restraint stress causes suppression of PBMC blastogenic response for at least 14 d indicating that the animal is still experiencing stress. The other measures of stress, i. e. total and percent free cortisol only indicated a stress response for 4 d. Weaning before 5 wk of age causes a suppression of PBMC blastogenic response to mitogens. However, weaning age did not appear to affect neutrophil function. Based on these results it would appear that several measures of physiological response to stressors need to be used if the effects of stress are to be reported.

Cortisol addition *in vitro* can cause suppression of PBMC blastogenic response

at various concentrations. Vitamin C did not appear to have any stimulatory effects on PBMC response to mitogens regardless of the presence or concentration of cortisol. Cortisol can be added at various concentrations to PBMC cultures and not suppress blastogenic response to mitogens.

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CHAPTER I

INTRODUCTION

Swine production systems employ management procedures that may be stressful (Kelley, 1988). Stress, as seen in these systems, can be defined by several parameters including physiological conditions, behavior, and animal productivity. An animal's response to physical and behavioral stress is a complex series of events involving an acute or chronic response. The mechanisms of these responses most likely involves a series of cascading events involving the neural, endocrine, and immune systems (Ader, 1990). To examine physiological responses of swine to management stress, more than one physiological parameter must be examined and the acute and chronic effects should be considered.

The objective of these studies was to examine effects of two management stressors, confinement housing and early weaning, on several physiological parameters in swine. The endocrine response to each stressor included measurement of percent free cortisol and total cortisol. Measures of immune system function included peripheral blood mononuclear cell (PBMC) blastogenic response to mitogens, polymorphonuclear cell degranulation of elastase, and polymorphonuclear cell phagocytic ability. Also, behavioral observations were used to determine the animal's perception of and adaptability to it's environment.

Finally, an attempt to alleviate physiological symptoms of stress in an in vitro

study was performed. Research suggests that dietary supplementation of Vitamin C may act in one of two ways to ameliorate of stress related events. Effects of vitamin C on cortisol suppressed PBMC blastogenic response *in vitro* was examined in an attempt to test one of the theories of vitamin C amelioration of immunosuppression.

CHAPTER II

REVIEW OF LITERATURE

Stress

Stress represents the reaction of an animal to some stimuli, physical or psychological, that disrupts homeostasis (Siegel, 1985). One unifying concept of stress physiology is that any stress resulting in a threat to homeostasis stimulates an increase in the secretion of glucocorticoids (Munck et al., 1984). Siegel (1985) classified this generalized reaction as part of the long term adjustment of an animal to environmental change. He further explained that physical and behavioral stressors illicit responses involving integration of neural and endocrine systems (Siegel, 1985).

Two systems are stimulated in response to a stressor, 1) the central nervous system combined with the adrenal medullary tissue, and 2) the hypothalamus-pituitary-adrenal axis (HPA axis) (Siegel, 1985). The first system rapidly responds to stress with a generalized increase in energy reactions through stimulation of cyclic adenosine monophosphate (cyclic AMP) production (Robinson and Sutherland, 1971). The second system concludes with a general increase in adrenal cortical steroid secretion respresenting a more long term adjustment (Siegel, 1985). Control mechanisms and funtion of the HPA have been well defined for both mammals and

birds (Ganog, 1963; Holmes and Phillips, 1976). The function of the HPA has been described as a "cascading" series of events, both nerual and endocrine, which are mediated by anterior pituitary secretion of ACTH which stimulates adrenal cortical steroid production (Siegel, 1985). Siegel (1985) suggested that continued stimulation such as seen during stressful times can lead to chronically high levels of corticosteroids which may be responsible for many of the physiological symptoms of chronic stress including cardivascular and gastrointestinal diseases, metabolic disorders, antibody suppression, and some anti-inflammatory activity.

Glucocorticoids are the family of stress hormones examined most frequently . Cortisol is considered an acceptable measure of stress due to its increased production associated with stress induced increases in corticotropoin-releasing factor (CRF) (Becker et al., 1985).. Many stressful conditions associated with farm animal management such as shipping, handling, restraint, weaning, mixing, and environmental conditions have been associated with increased levels of cortisol in plasma of animals under one or more of these conditions (Becker et al., 1985).

Stress and the Endocrine System

Stress can alter the endocrine profile of an animal with regards to glucocorticoid secretion (Siegel, 1985). Kelley (1988) suggested that when an animal encounters a stressor it attempts to maintain homeostasis by making adjustments, which most likely involve hormone secretions. One such example is the HPA axis response to acute, adverse stimuli by increasing production of CRF which in turn

stimulates pituitary release of ACTH (Kelley, 1988). Pituitary release of ACTH then stimulates the adrenal cortex to increase production of glucocorticoids (Kelley, 1988).

There are many theories which explain the relevance of this glucocorticoid secretion in response to stress. The 1940's saw the influential publication of Selye's Genearl Adaptation Syndrome or G.A.S. (Seyle, 1946). In this 1946 article Selye ascribed the function of glucocorticoids to the resistance to stress. Most current endocrine texts no longer discuss the G.A.S. as a viable theory. However, the 1950's work of Ingle is still mentioned in texts today (Munck et al., 1984). Ingle (1954) suggested that glucocorticoids act in a permissive or normalizing way. Permissive actions probably play an important role in glucocorticoid function at basal levels, however, this theory is hard to define and analyze (Munck et al., 1984). The third theory proposed by Munck et al. (1984) suggests a more suppressive function of glucocorticoids in stress situations. Most glucocorticoid actions have a delay of hours before having an effect and, that this delay allows the bodies defense mechanisms to respond to the stressor, with glucocorticoids then stopping the defense reaction from becoming too aggressive and damaging healthy tissue (Munck et al., 1984). Corticosteroid binding globulin (CBG) is the primary carrier protein for the glucocorticoid hormone cortisol. When cortisol is bound to CBG it is physiologically inactive and therefore unavailable for action (Faltys et al., 1987). Cortisol can also be bound to albumin, which is such a loose bond that the cortisol can be considered biologically active and has been measured in such a way (Kattesh et al., 1990). When examing the effect of stress on increased cortisol levels it is important to

examine the percent of cortisol that is "free" or unbound versus bound. For example, it has been demonstrated that after weaning in pups the concentrations of CBG decrease casuing an increase in free cortisol which may stimulate the return of estrous (Gala and Westphal, 1965). A rise in plasma cortisol may not be attributed to increased production but rather a change in distribution of biologically active and inactive forms. For these reasons, bound and unbound levels of cortisol should be considered when examing the influence of stress on plasma cortisol concentrations.

Stress and The Immune System

The physiologcial response to stress involves a complex interaction of neural, endocrine and immune systems (Ader et al., 1990). These systems might also share common messengers and receptors (Weigent and Blalock, 1987). Corticosteroid hormones, specifically cortisol, are not the only "hormones" altered during stress (Kelley, 1988). There is no generally accepted definition of a hormone, but most agree that is is produced by a specific cell and that it must regulate the biological activity of another cell (Kelley, 1988). Given this definition, neurotransmitters and cytokines could both be classified as having hormonal activity (Kelley, 1988). The fact that the immune response alone is capable of affecting circulating levels of hormones and neurotransmitters supports the theory that this complex reaction to stress includes many feedback and feedforward mechanisms all of which are not fully understood (Ader et al., 1990). Thus the study of physiological effects of stress should acknowledge the complex interaction between several systems, and not just a

direct cause and effect relationship. Each physiological parameter examined is most likely the result of combined efforts of neural, endocrine, and immune factors working to restore homeostasis.

Effects of corticosteroids on the immune system are related directly to decreased protection from invasion of pathogenic organisms, coupled with general suppression of several immune protective functions (Siegal, 1985). Stress related increases in cortisol secretion are related directly to a reduction in T cell proliferation in swine, cattle, and chickens (Westly and Kelley, 1984; Blecha and Baker, 1986; and Franklin et al, 1987), depression in the number of circulating lymphocytes (Siegal and Latimer, 1970); and an increase in neutrophil granulocytes (Siegal and Latimer, 1970; and Garren and Shaffner, 1954). Reduction of lymphocyte proliferation can be explained partially by interleukin-1 (IL-1) inhibition caused by increased levels of cortisol (Ader et al., 1990). High levels of corticosteroids also appear to reduce macrophage function by inhibition of enzymes necessary for phagocytosis and cell to cell interaction (Siegal, 1985). Rey et al. (1987) suggested that there was a feedback circuit involving components of immune cells (i.e. IL-1) and the HPA axis and speculated that this glucocorticoid-associated immune regualtion system may serve as continual surveillance of immunological cells and their activities.

There is a physiological relationship between the immune response and glucocorticoids as demonstrated by Rey et al. (1987) who showed that animals undergoing immunological reactions to various antigens had increased blood glucocorticoid levels in proportion to the magnitude of the immune response. The *in*

vitro addition of various levels of cortisol to porcine peripheral blood mononuclear cells resulted in the suppression of mitogen stimulated blastogenic response (Westly and Kelley, 1984). Elevated cortisol values in mice have also been shown to suppress cellular immune function (Blecha et al., 1983). Munck et al. (1984) suggested that this glucocorticoid-associated regulatory mechanism is a form of surveillance of immunological cells and their activity.

Polymorphonuclear neutrophilic leukocytes are the primary phagocytic cell and serve as the first line of cell-mediated defense against infection (Ainsworth, 1991). Neutrophils may also serve an important role in defense of immunodeficient early weaned pigs (McCauley and Hartmann, 1984). McCauley and Hartmann (1984) showed that weaning at 3 weeks of age caused a transient rise in cortisol which was associated with the beginning of a rise in neutrophil numbers that steadily reached adult levels 21 days post-weaning. Once neutrophils are activated in response to a foreign organism several major events occur including: chemotaxis, phagocytosis, secretion of enzymes (degranulation), and an oxidative burst of metabolism (Spitznagel, 1983). Research has shown that neutrophils may be present in the blood in sufficient numbers, but unable to adequately perform one or more of these funtions involved with host defense (Klebanoff and Clark, 1978; and Brown, 1991). Many factors can be attributed to this compromised function including stress (Kelley, 1980), corticosteroids (Cooper et al, 1972; Heine et al, 1983), and viruses (Abramson and Mills, 1988).

Phagocytosis refers to the act of engulfment of foreign particles by a cell, such

as neutrophils (Ainsworth et al., 1991). The first interaction of host cells with a foreign organism initiates the influx of phagocytic cells into the offending area (Ainsworth et al., 1991). Once phagocytic cells are in the area an attack is mounted that consists of chemotaxis, adherence, engulfment, and destruction (Ainsworth et al., 1991). Research has shown a direct link between stress increased cortisol levels stimulating changes in the phagocytic ability as well as other immunological alterations (Ellsaesser and Clem, 1986; Bly et al, 1990; and Ainsworth et al., 1991). Impaired *in vitro* neutrophil function was correlated with clinical syndromes in humans (Klebanoff and Clark, 1978). The fact that an increase in the neutrophil/lymphocyte ratio has been documented in several studies in association with stress may lend further support to the importance of neutrophils in the physiological response to stress (Moss and McMurray, 1979; Nienaber and Hahn, 1989). McCauley and Hartmann (1984) recognized the potential importance of the increase in neutrophils associated with stress of early weaning in pigs and proposed that by late lactation the piglet has adapted to its environment and the immune system is under minimal stress. Futhermore, the multitude of stressors associated with weaning, coupled with the loss of maternal immunoglobulin protection may increase the antigenic load on pigs immune system which responds with an increase in neutrophils acting as the first line of defense against this new immune challenge (McCauley and Hartmann, 1984).

Degranulation or release of destructive enzymes, is another defense mechanism used by neutrophils. The primary serine protease released during degranulation is

elastase. Neutrophil elastase can degrade the extracellular matrix, denature proteins including immunoglobulins, complement, and clotting factors, and destroy intact cells (Ras et al., 1992). Elastase is downregulated by alpha 1-proteinase inhibitor (A1-PI) which acts by cleaving and binding to elastase preventing further binding with substrates, and the complex is then cleared from the metabolism (Janoff, 1985). Elastase may play a role in recycling of connective tissue macromoloecules, however, it's primary role most likely reflects a pathological process (Janoff, 1985; and Janoff, 1983). PMN elastase is also involved with hydrolysis of plasma proteins, lymphocyte activation, and platelet aggregation (Janoff, 1985). It would appear that the primary functions of elastase are harmful unless contained in the phagocytic vacuole where research has demonstrated its effectiveness in bacterial-digesting functions (Janof, 1985; Rest et al, 1982; and Blondin et al., 1978). The ability of neutrophils to produce and release elastase ccan be compromised by certain disease states and stressful conditions (Savi et al., 1981).

Another interesting aspect of elastase is its interaction with CBG and cortisol. Research demonstrating that CBG and A1-PI are structurally related and that CBG is cleaved by PMN elastase indicates that they might have a common role in determining the amount of physiologically free cortisol (Hammond et al., 1990). Hammond et al. (1990) further stated that it is unlikely that this cleavage occurs in general circulation due to plentiful amounts of A1-PI, but likely occur at sites of inflammation where A1-PI is deactivated. The importance of this relationship is in the destruction of plasma CBG binding capacity due to cleavage by elastase and the possible subsequent rise in

plasma free cortisol (Hammond et al., 1990). This action may represent a mechanism of cortisol delivery to sites of inflammation. Along with the phagocytic action of neutrophils, consequences of production and release of elastase may all be interelated to cellular immune defense in stressed animals.

Stress and Decreased Disease Resistance

Effects of stress induced increases in cortisol on components of the immune system can be directly linked to disease resistance. Siegal (1985) suggested that the sum effect of the influence of stress on the immune defense mechanism results in increased resistance to bacterial infection and a decreased resistance to viral infections. The apparant increase in resistance to diseases of bacterial origin may be related to decreases in the number of circulating lymphocytes and increases in polymorphonuclear white blood cells in animals subjected to various stressors (Siegal and Gross, 1965; Gross and Siegal, 1983; and Siegal, 1985). Both acute and chronic stressors decreased the number of circulating lymphocytes as a result of increased cortisol concentrations (Hardie et al., 1991). These times of decreased circulating lymphocytes and increased cortisol are associated directly with increased disease susceptibility (Hardie et al., 1991). Recently, Hardie et al. (1991) demonstrated that stressing Atlantic salmon caused plasma cortisol to increase and that chronically high levels of cortisol increased disease susceptiblity. Blecha et al. (1983) speculated that cortisol was linked to immunosuppression they found in early weaned pigs.

Stress can cause diminished immune function for extended periods of time and

if the right sequence of conditions is present stress can play a role in decreased disease resistance. In humans, it was found that the loss of a spouse, separation from a spouse, and examination periods for medical students suppressed mitogen response of PBMC for the duration of the stressful period (Ader et al., 1990). Increased reports of illness were also noted in medical students with suppressed mitogen response (Glaser et al., 1987). In a detailed examinatoin of literature relating to behavioral stress in adult animals, the increase of disease susceptibility depended greatly on many factors including the quality and quantity of stress, age, sex, and the immune factors measured (Glaser et al., 1987). Stress acting through its potent neural, neurochemical, and hormonal effects can alter susceptibility of animals to many types and kinds of diseases.

Dietary Supplementation of Vitamin C as a Means of Alleviating Cortisol Induced Immunosuppression

Beneficial effects of dietary supplementation of vitamin C in alleviating some of the immunosuppressive effects of stressed induced increases in cortisol are well documented. Thaxton and Pardue (1984) found amelioration of immunosuppression, as measured by antibody response to sheep erythrocytes in chickens supplemented with vitamin C . These beneficial effects were seen under conditions of environmental stress as well as exogenous cortisol injections (Thaxton and Pardue, 1984). Hardie, et al (1991) showed that animals maintained on diets supplemented with vitamin C had fewer immunosuppressive effects related to stress and decreased

disease susceptibility as compared to animals under the same conditions with no supplemental vitamin C.

Bains (1988) has suggested two theories or mechanisms by which vitamin C ameloirates stress/cortisol induced immunosuppression. The first mechanism is a reduction in the synthesis of adrenal glucocorticoids (specifically cortisol) with vitamin C acting as a brake on steriodogenesis (Bains, 1988; Dvorak, 1984). The second mechanism is that of vitamin C acting in some protective capacity on lymphoid tissue (Bains, 1988). The exact mechanism by which dietary supplementation of vitamin C produces beneficial effects during times of stress remains uncertain. However, evidence shows that dietary supplementation of vitamin C reduces immunosuppressive effects of stress in several species, including swine (Hardie et al., 1991; Dvorak, 1984; Pardue and Thaxton, 1984).

STRESS AND SWINE MANAGEMENT PROCEDURES

The Stress of Early Weaning

Early weaning has been herold as an economically sound improvement for modern swine production systems. However, as far back as 1979, Lecce et al. described the diarrhea, deaths, low feed intake, and depressed weight gain associated with early weaning as bearing "grim testimony to the 'state of the commercial art' of early weaning." These neonatal pigs have been described as being born immunodeficient in that they lack a sufficient level of active immunity to act as their

defense mechanism (McCauley and Hartmann, 1981). This fact coupled with the loss of lactational protection associated with colostrum and milk leaves the early weaned piglet in a precarious situtation (McCauley and Hartmann, 1984). Poor postweaning performance has been attributed to low disease resistance or depression of the immune system (Inoue et al., 1978) and other factors such as environmental stress (Kelley, 1980). Weaning has been described as stressful and immunosuppressive at whatever age it occurs (Kelley, 1988; and Blecha et al., 1983). The early weaning, before 5 wk of age, of a piglet imposes multiple stressors that the animal must adapt to if it is to have a chance of survival.

Early weaning has a direct effect on the immune system that has been described as leaving the piglet "immunologically vulnerable" (Blecha et al., 1983). Blecha et al. (1983) described this vulnerablity as a decrease in physiological maturity and its effect on immunological parameters which in turn may account for decreased survival of early weaned pigs. Many factors contribute to this vulnerability beginning with the loss of lactational protection consisting of maternal immunoglobulins (McCauley et al., 1984). Researchers have also shown a decrease in antibody synthesis as a result of the decreased physiological maturity of the early weaned pig (Haye and Kornegay, 1979; Blecha and Kelley, 1981). Research in compromised antibody synthesis associated with early weaning has revealed indirect evidence to support suppressed cellular immune function as well (Blehca and Kelley, 1981). Blecha et al. (1983) found a decrease in PBMC blastogenic response to a mitogen 24 hours after early weaning at younger than 5 weeks of age. McCauley and

Hartmann (1984) also found that the change of environment associated with weaning, along with loss of "protection of the gastrointestinal tract by specific and non-specific antimicrobial factors in milk" may increase the strain on a piglets immune system as marked by dramatic changes in leukocyte population distribution.

Weaning has also been shown to cause transient rises in plasma cortisol levels. Worsaae and Schmidt (1980) found that weaning animals at an older age increased cortisol levels immediately following weaning. McCauley and Hartmann (1984) also noted a "slight" but not significant rise in cortisol attributed to the stress of weaning younger animals (3 wk). Bonnette et al. (1990) reported large increases in cortisol and attributed them to the stress of a combination of factors including weaning, moving, bleeding, injection, and mixing with non-littermates.

The Stress of Confinement Housing

Confinement or restraint immobilization can be considered stressful and used as a means to study the reactions of the HPA axis to such stressors (Becker et al., 1989). Specifically, plasma cortisol can be measured as a determination of HPA activation and the animal's perception of its environment as stressful (Becker et al., 1989). Transient rises in plasma cortisol and disruption of the circadian rhythm of cortisol can be used as indicators of stress in swine (Rampacek et al., 1984).

In many modern swine facilities, the means of confinement results in a restriction of the animal's ability to turn around or move anywhere while allowing enough space to stand, sit, lie down, drink, eat, and eliminate waste (Becker et al.,

1989). Restrictive penning (i.e. tether stalls) caused changes in plasma cortisol and disrupted the circadian rhythm (Becker et al., 1984). Other researchers have also shown that restrictive penning caused a stress response but clarified this to be an acute response that diminished within a few days as the transient rises in plasma cortisol diminish (Barnett et al., 1984; Friend et al., 1984). Restraint stress has also been associated with a depression in cell-mediated immunity in mice (Sheridan et al., 1991). A positive correlation between high levels of plasma corticosteroid and lymphopenia, altered lymphocyte circulation, and decreased adhesion molecules could explain decreased cellular immunity (Sheridan et al., 1991).

McFarlane et al. (1988) suggested that depriving swine of the ability to move freely might affect their health, performance and overall well-being. Behavioral data are often used to indicate the animal's perception of its environment and give some indicatione as to the animal's perceived well-being. In the case of restrictive penning Becker et al. (1984) observed that aggravated behavior (i.e. biting of bars) abated within a few days. Becker et al. (1984) further showed that as aggravated behavior decreased the transient rises in plasma cortisol observed previously also abated suggesting the animal had adjusted to its envrionment.

To date, research indicates that stress associated with management procedures of weaning and confinement housing are acute or temporary stressors that abate within a few days. These researchers suggested that animals adjust to their environment and this adjustment accounts for the diminished physiological indicators of stress, such as cortisol levels.

Research is needed to examine the duration of stress induced suppression of PBMC blastogenic response to mitogens and other physiological indicators of suppressed cellular immune function. Means of alleviating the existing stress of management procedures should be examined and determine lab techniques that might be helpful in assessing an animals response to certain environmental stressors. For these reasons the short and long term effects of restraint stress on PBMC blastogenic response to mitogens, total and free plasma cortisol, total leukocyte numbers, and differential leukocyte numbers were examined. Also, the effects the another management stressor, weaning, were examined on PBMC blastogenic response to mitogens 10 to 12 d following treatment, and nuetrophil elastase degranulatin and phagocytosis. In conclusion an attempt was made to determine suppressive and nonsuppressive concentrations of cortisol on PBMC blastogenic response to mitogens and the effects of vitamin C on cortisol induced suppression.

CHAPTER III

EFFECT OF DEGREE OF RESTRICTED MOVEMENT ON PLASMA TOTAL AND FREE CORTISOL CONCENTRATIONS AND PERIPHERAL BLOOD MONONUCLEAR CELL BLASTOGENIC RESPONSE IN GILTS

Introduction

Gestation stalls of various dimensions and style are used widely in the swine industry and have made many management procedures more efficient (McFarlane et al., 1988). Housing gilts/sows in stalls which restrict movement has generated a great deal of public concern that such practices may affect the well-being of the animal. It has also been suggested that depriving pigs of the opportunity to move freely might adversely affect health and performance (Becker et al., 1984). To date there is a lack of consensus as to what effect social restriction has on selected biological responses in gestating females.

The physiological response to a stressor begins with stimulation of the hypothalamic-pituitary (HPA) axis which results in increased adrenal corticosteroid secretion (Siegel, 1985). Increased secretion of cortisol and disruption of the circadian rhythm of cortisol has been associated with different types of stressors in different species including restrictive penning in swine (Becker et al., 1984; Siegel,

1985). Further evidence indicates that cortisol is capable of suppressing several aspects of immune function including peripheral blood mononuclear cell (PBMC) blastogenic response to mitogens (Belcha et al., 1982; Kelley et al., 1982b; Siegel, 1985). However, the duration of this suppressive response has not been clearly defined.

Objectives of this study were to examine total and free plasma cortisol, behavior, and PBMC blastogenic response to mitogens in gilts subjected to two degrees of restricted movement.

Materials and Methods

Animals. Landrace x Yorkshire F1 gilts (n=28), 8 mo of age, were allotted equally to four pens in a total confinement facility. Each pen was equipped with nine breeding/gestation stalls (Moor Comfort® Gestation System, Moorman Mfg. Co., Quincy, IL) having adjustable side panels that permit an animal to turn around. Animals were fed 1.1 kg twice a day (0800 and 1600 h) of a 18% crude corn-soybean protein meal ration. Animals recieved water ad libitum via nipple waterers located on one end of the stall near the feeder.

Twelve gilts (3/pen) were cannulated surgically in the jugular vein to facilitate frequent collection of blood with minimal stress to the animals (Knight et al., 1982). Initially, all animals were kept in stalls where animals could turn around (movable sided). On day one of the trial, animals in two of the four pens had their stalls adjusted so that they could not turn around (fixed sided). Animals were kept in stalls

for 14 d, at which time the stalls were adjusted to the alternate style. The experiment was conducted as a switchback trial with 3 repetitions.

Sample Collection. Blood samples was collected via the jugular catheter and placed in tubes containing 1.5% EDTA beginning immediately prior to stall adjustment and at 2 h intervals for 8 h. Subsequent blood samples were taken twice daily (0800 and 1600 h) over the following three days. Plasma was isolated by centrifugation at 2200 rpm for 20 min at 4 °C. Blood samples (25 mL) were also collected via jugular puncture on twelve non-canulated gilts (6 animals/stall style) immediately prior to adjusting stalls for each trial. Whole blood was collected into 30 mL syringes with 1.5% EDTA and returned immediately to the laboratory for isolation of PBMC.

Cell Isolation. Peripheral blood mononuclear cells were isolated using the method described by Hoskinson et al. (1990) and outlined in Appendix A. Briefly, whole blood (10ml) diluted with 30 mL of phosphate buffered saline (PBS) was layered over 10 ml of Ficoll/sodium diatrizoate gradient with a density of 1.077 (Histopaque 1077, Sigma Chemical Company St Louis, MO). Tubes were centrifuged at 400 x *g* for 50 min at 18 °C. PBMC were collected from the plasma/Histopaque interface and washed with lysing solution to destroy any contaminating erythrocytes. Cells were then pelleted by centrifugation at 300 x *g*, for 15 min at 4 °C. PBMC concentration and viability were determined by Trypan blue exclusion test with resulting viability > 95%. PBMC were adjusted to a concentration of 5 x 10^6 cells/mL by diluting with RPMI 1640 culture media (Grand

Island Biological Co. [GIBCO], Grand Island, NY) supplemented with 25 mM Hepes buffer, 2mM l-glutamine, 50,000 U penicillin and 50 mg streptomycin in an antibiotic solution, 0.05 mM 2-mercaptoethanol, and 10% fetal bovine serum (FBS; Sigma Chemical Co., St Louis, MO).

PBMC Blastogenic Response to Mitogens. Peripheral blood mononuclear cell proliferation in response to three different mitogens; concanavalin A (Con A; a T cell mitogen), pokeweed (PWM; a B cell mitogen), or phytohemagglutin (PHA; a T or B cell mitogen)(Grand Island Biological Co., Grand Island, NY) was evaluated as one measure of cellular immune function. The assay was performed as described by Hoskinson et al. (1990) and outlined in Appendix A. Briefly, 100 μl of PBMC suspension at a concentration of 5 x 10^6 cells/mL were plated in triplicate onto 96 well flat bottom well culture plates. A volume of 100 μl of either, Con A, PWM, PHA, or RPMI media (control) was added to sample wells. A final concentration of 25 μg/mL for Con A and PHA, and 5 μg/mL for PWM was determined following initial dose response studies testing 6 different levels of each mitogen. Plates were incubated at 39 °C in a humidified, 5% CO_2 incubator for 48 h. A 50 μl aliquot of [^{3}H]-thymidine (1 μCi; Dupont, New England Nuclear Research Prod., Boston, MA) was added to each well and the plates incubated for an additional 24 h. Cells were then harvested onto glass fiber filters and [^{3}H]-thymidine incorporation determined as mean counts per minute (cpm) $\pm$ SEM for the triplicate values using a scintillation counter. The coefficient of variation for triplicate Con A, PHA, and PWM cultures for all determinations were 6.6, 5.7, and 6.5%, respectively.

Total Leukocyte Number. Leukocytes were isolated using a Unopette® microcollection system (Becton-Dickinson, Rutherford, NJ) and total number determined by hemacytometer. Ten percent of the total number of cells counted was added to adjust for the depth of the chamber. This figure was then multiplied by 100 to obtain total leukocyte numbers. Duplicates of each sample were read and the average of the two taken as the actual count.

Leukocyte Differentials. Blood smears were prepared in duplicate for each animal. Differential counts were obtained by counting the number of each cell type observed under microscopic power up until 100 cells had been counted. The percentages for each cell type for duplicate samples were averaged and the mean used as the reported results.

Total Cortisol. Total cortisol concentration was determined using a commercial radioimmunoassay kit (Coat-A-Count, [^{125}I]) purchased from Diagnostic Products Corporation, Los Angeles, CA). Samples were analyzed in duplicate and counted for 1 min on a gamma counter. Cortisol concentration was expressed as ng/mL of plasma. Intraassay and interassay coefficient of variation was 6.3% and 9.8%, respectively.

Percent Free Cortisol. The percentage of unbound or free cortisol (UBC) was determined as described by Hammond et al. (1980) and adjusted for use in swine by Kattesh et al. (1990) as outlined in Appendix B.

Behavioral Observations. Animal behavior was recorded by video cameras mounted from the ceiling in an unobtrusive manner. Representative recordings were taken over a 5 min period at 0800 and 1600 h, resulting in a total of 10 min of

observation. These times reflected optimum intervals for peak swine activity. Certain behaviors were selected for examination based on those reported earlier by Taylor and Friend (1987) and McFarlane et al. (1988). These consisted of aggravated butting of sides and top of crate, attempting to turn around, chain chewing, drinking, standing, and lying down.

Statistical Analysis. Statistical analysis was performed using General Linear Mixed Model analysis procedure (Blouin and Saxton, 1990). Least square means and standard errors were calculated for all variables. Treatment effects were tested with surgical effects being adjusted for. Also, day and sample time effects were examined. Generalized least square means and standard errors are reported for all variables except behavior. Behavior results are the mean number of times the observation occurred in the 10 min observation time.

Results

PBMC Blastogenic Response to Mitogens. Peripheral blood mononuclear cells isolated from gilts who began the trial maintained in fixed sided stalls demonstrated a reduced ($P<.01$) ability to proliferate in response to all three mitogens as compared to their PBMC response 14 d following stall adjustment to movable sides (Table 1). Gilts who began the trial in movable sided stalls had an increased ($P<.05$) blastogenic response to all mitogens after 14 d of treatment as compared to blastogenic response 14 d following crate adjustment to fixed sided. The switchback experiment demonstrated a pattern for increased ($P<.05$) PBMC response to Con

Table 1. Porcine PBMC blastogenic response to mitogens 14 d after beginning the trial in fixed stalls and 14 d after stalls were adjusted to movable[a].

MITOGEN	FIXED [b]	MOVABLE [c]
ConA	167.32 ± 21.31	255.00 [d] ± 21.31
PHA	158.28 ± 21.96	235.23 [d] ± 21.96
PWM	095.53 ± 12.99	139.58 [d] ± 12.98

[a] Data are represented as mean ± SEM of net cpm x 10^3 of ^{3}H-thymidine incorporation = mitogen stimulated cpm - background cpm.

[b] Fixed refers to crate style which did not permit the animal (n=11) to turn around.

[c] Movable refers to crate style which did permit the animal (n=12) to turn around.

[d] Significant ($P<.01$) treatment affect.

A, PHA, and PWM from gilts in movable sided stalls across the three repetitions and this is represented by Figures 1, 2, and 3 respectively.

Total Leukocyte Number. Total leukocyte numbers for animals in fixed versus movable sided stalls are shown in Table 2. There was no difference ($P > .10$) in total leukocyte numbers among gilts due to treatment.

Leukocyte Differentials. There was no difference ($P > .10$) in differential leukocyte counts between animals in fixed versus movable sided stalls (results not shown).

Total and Percent Free Cortisol. Surgical catheters could only be maintained for the first 4 d following placement, therefore results are only presented for the first replicate. Gilts in fixed sided stalls had higher ($P < .05$) total plasma cortisol levels over the eight hours following stall adjustment (Figure 4). Although not significant ($P > .10$), the 1600 h measure of total cortisol for animals maintained in fixed sided stalls was higher relative to the 0800 h sample over the first 2 d of treatment. This is in contrast to the consistently lower afternoon levels observed for gilts confined to movable sided stalls. Total cortisol was not different ($P > .10$) between animals in different stall styles after 14 d of treatment (results not shown).

Percent free cortisol for the 0800 and 1600 h samples taken on d 1-4 is shown in Table 3. The percent free cortisol for gilts in fixed sided stalls was likewise elevated ($P < .05$) over the first 3 d of treatment for both the 0800 and 1600 h samples.

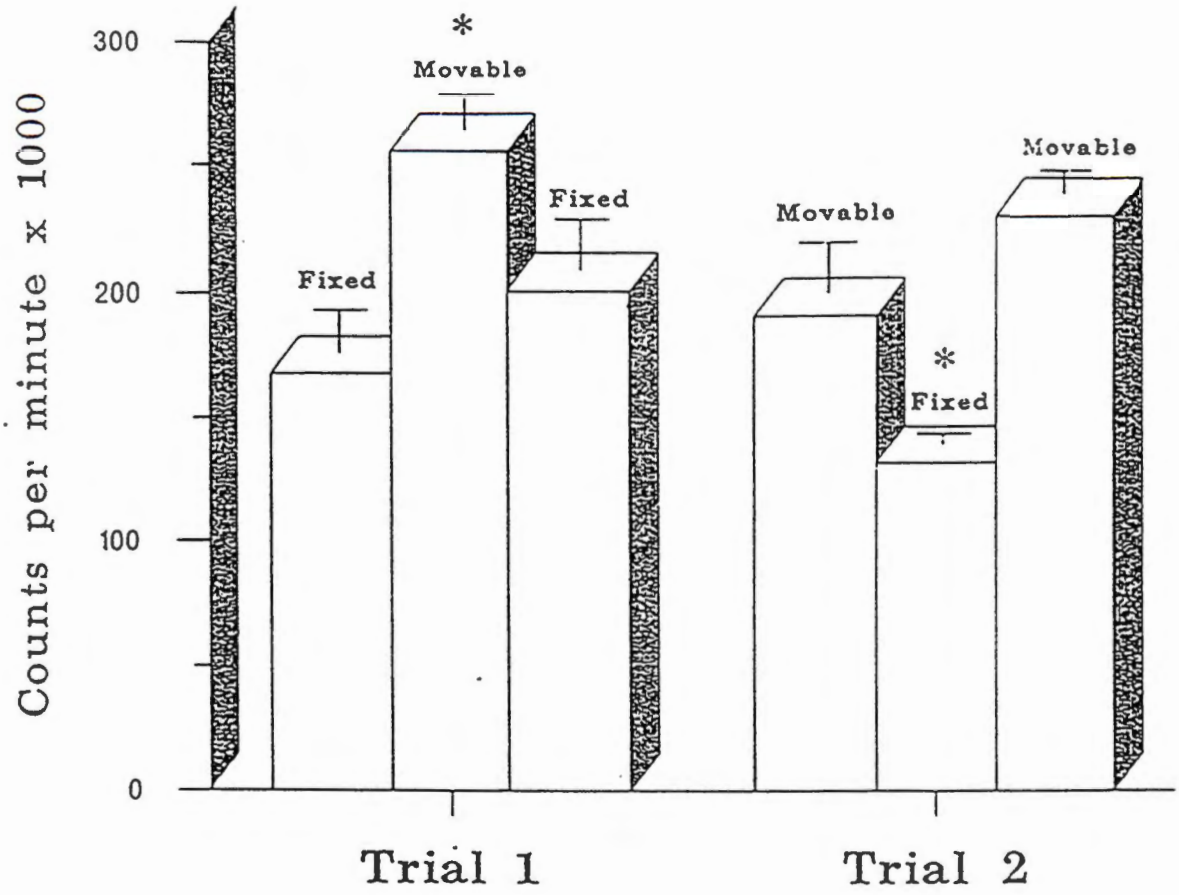

Figure 1. Porcine PBMC blastogenic response to Con A for animals beginning treatment in a fixed stall (Trial 1) and animals beginning treatment in a movable stall (Trial 2). Each bar represents the mean ± SE (n=6). "*" = $P<.05$.

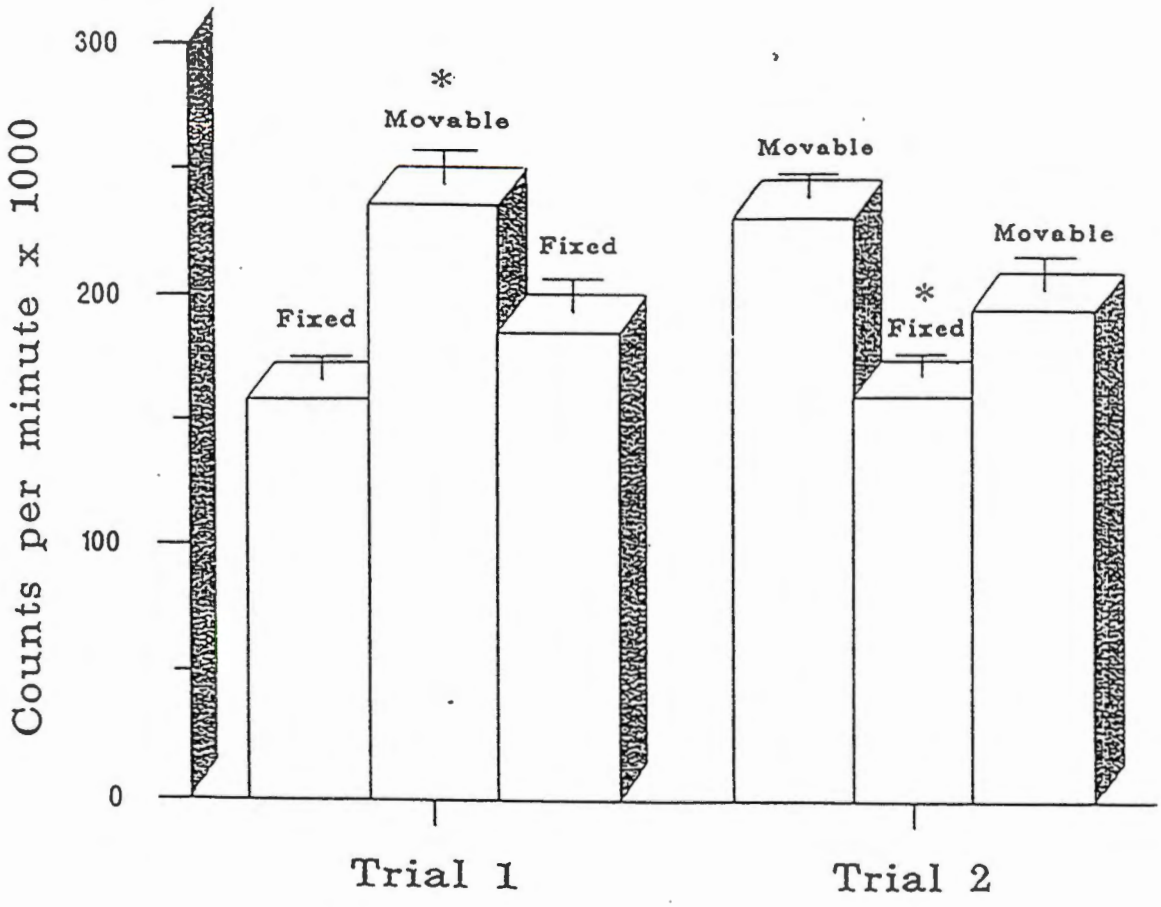

Figure 2. Porcine PBMC blastogenic response to PHA for animals beginning treatment in a fixed stall (Trial 1) and animals beginning treatment in a movable stall (Trial 2). Each bar represents the mean ± SE (n=6). "*" = $P<.05$.

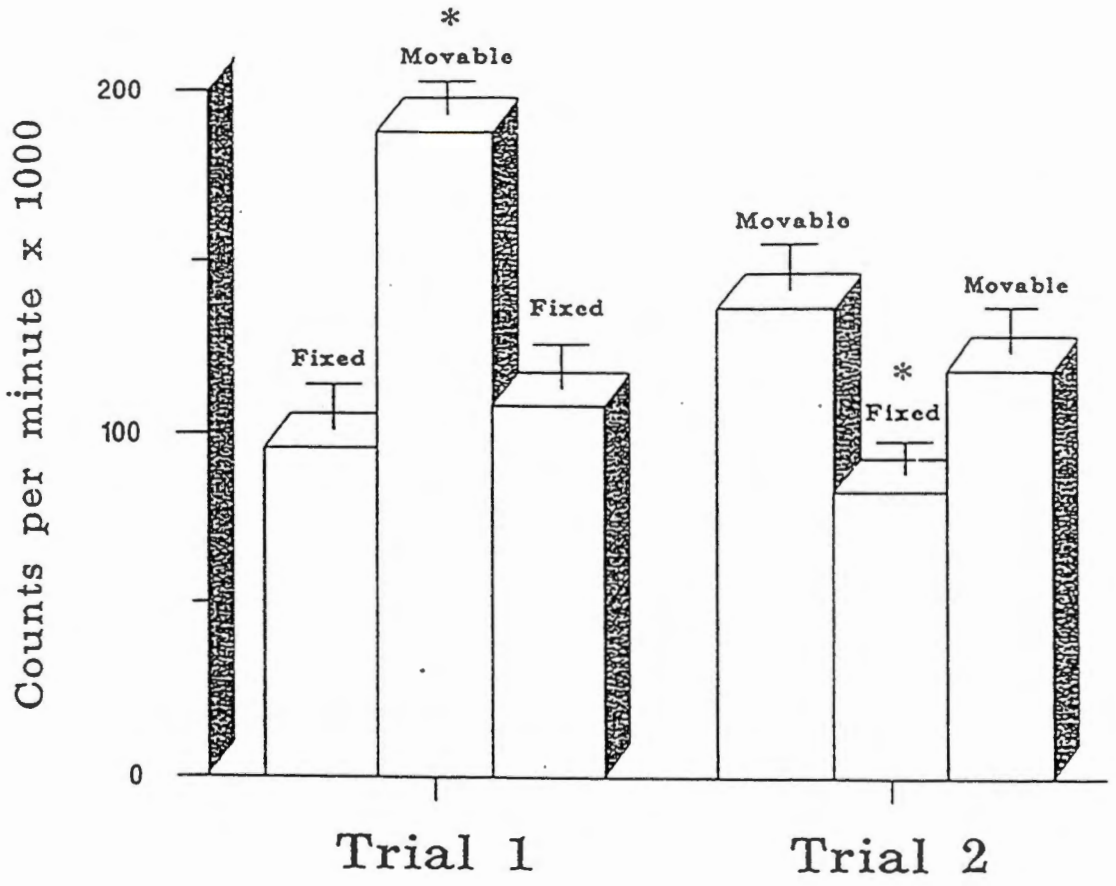

Figure 3. Porcine PBMC blastogenic response to PWM for animals beginning treatment in a fixed stall (Trial 1) and animals beginning treatment in a movable stall (Trial 2). Each bar represents the mean $\pm$ SE (n=6). "*" = $P<.05$.

Table 2. Total leukocyte numbers for gilts (n=28) maintained in fixed vs movable sided stalls as measured on days 1 and 14 of treatment[a].

	FIXED [b]		MOVABLE [c]	
	Day 1	Day 14	Day 1	Day 14
TOTAL LEUKOCYTE NUMBER cubic/mm	14,203	13,560	15,690	18,846

[a] Means were not different (P>.10) between treatment or day of sampling.
[b] Fixed refers to crate style which did not permit the animal (n=11) to turn around.
[c] Movable refers to crate style which did permit the animal (n=12) to turn around.

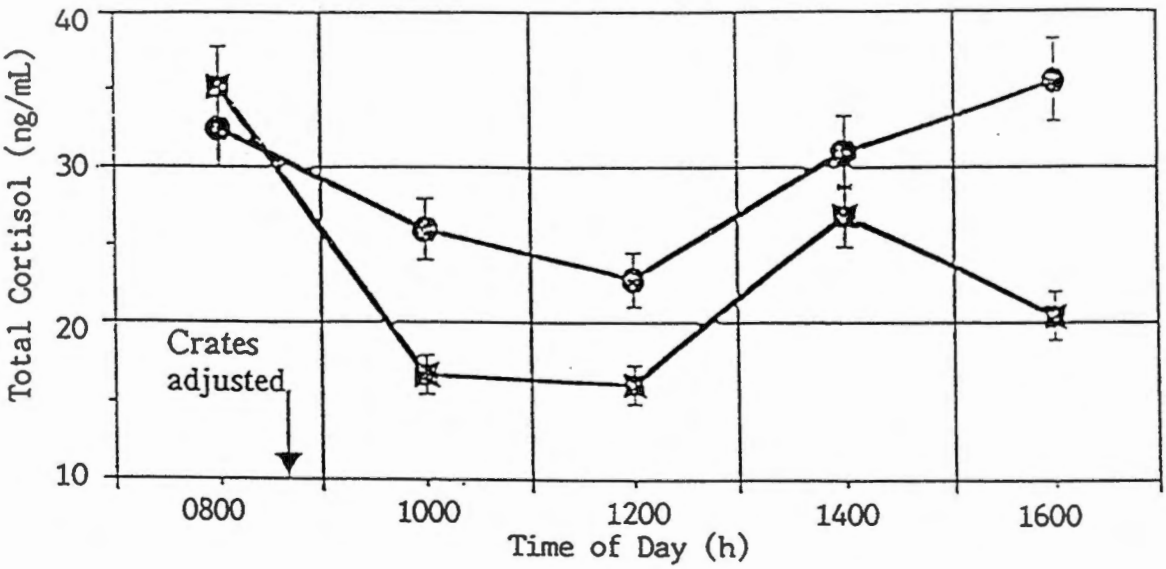

Figure 4. Total plasma cortisol in gilts over 8 h following crate adjustment. Gilts in movable sided crates are represented by square markers and for gilts in fixed sided crates are represented by circular markers. Gilts in fixed sided crates had lower ($P<.05$) plasma total cortisol over the 8 hour time period following crate adjustment.

Table 3. Influence of degree of restricted movement on plasma free cortisol levels in gilts over the first four days of treatment[a].

Day	Time (h)	Free Cortisol (%) Fixed [b]	Movable [c]	P
1	0800	13.2 ± 0.5	8.8 ± 1.8	<.05
	1600	10.3 ± 0.1	9.1 ± 0.6	<.05
2	0800	13.7 ± 0.5	10.0 ± 0.3	<.05
	1600	11.2 ± 0.4	9.9 ± 0.7	<.05
3	0800	16.2 ± 0.2	9.0 ± 1.5	<.05
	1600	11.2 ± 0.3	8.5 ± 1.7	<.05
4	0800	8.0 ± 2.1	8.6 ± 0.5	>.10
	1600	6.8 ± 1.0	10.2 ± 0.2	>.10

[a] Data are represented as mean ± SEM

[b] Fixed refers to crate style which did not permit the animal (n=11) to turn around.

[c] Movable refers to crate style which did permit the animal (n=12) to turn around.

Behavior. Animals placed in fixed sided stalls expressed aggravated behavior immediately following stall adjustment and continued to do so for 48 h (Table 4). Aggravated behaviors were those described as "butting the crate" and attempting to turn around (i.e. fixed sided stalls).

Discussion

Confinement or restraint/immobilization of swine can be employed as a type of stressor in an attempt to assess the role of HPA axis in stress and in evaluating an animal's perception of its environment (Becker et al., 1989). Elevated plasma cortisol is one measure of HPA axis stimulation. Results of the present study suggest that restricting a gilt's movement so that it is unable to turn around, after having previously been permitted to do so, elicits an acute stress response as measured by the transient elevation of total and percent free cortisol, and disruption of normal circadian rhythm of cortisol. This pattern of higher afternoon cortisol concentrations for animals in fixed sided stalls is in contrast to the normal lower afternoon level of cortisol secretion in the pig (Whipp et al., 1970). Rampacek et al. (1984) also found that transient rises in plasma cortisol and disruption of circadian rhythm are indicators of an acute stress in swine in response to confinement housing. They further stated that results did not indicate chronic stress response based on data related to different hormone levels. Gilts placed in fixed-sided stalls returned to a normal circadian rhythm of serum cortisol within 72 h of the imposed restriction, which is consistant with the results of Becker et al. (1985) who found that 96 h were

Table 4. Observed behavior in gilts immediately following initiation of treatment and two days later (n=16)[a].

Behavior	FOLLOWING CRATE ADJUSTMENT		2 DAYS LATER	
	Fixed [b]	Movable [c]	Fixed	Movable
	%	%	%	%
Aggravated butting of crate not associated with turning around	86.2	13.8	0	- [d]
Turning around/or attempting to turn around	61.0	39.0	-	63.1
Drinking	51.2	48.8	49.1	50.9
Chain chewing[e]	-	80.0	-	86.0
Contact with neighbor	27.8	72.2	36.2	53.8
Facing front of crate[f]	100.0	1.1	100.0	2.4

[a] Gilt behavior recorded by video camera and examined for 5 min period beginning at 0800 and 1600h.

[b] Fixed refers to crate style which did not permit the animal (n=11) to turn around.

[c] Movable refers to crate style which did not permit the animal (n=12) to turn around.

[d] A "-" means that variable was not measured because it did not apply to that treatment.

[e] Chains are located at rear of crate and only accessible when crate is adjusted to movable position.

[f] Animals in fixed sided crates can only face forward.

required for a normal hormonal pattern to return after restrictive penning. Barnett et al. (1984) and Friend et al. (1984) showed that long-term restrictive penning did not elicit a chronic stress response in swine. They did find that luteinizing hormone secretion decreased which is consistent with that expected under conditions of stress. Results from the present study further support the contention that swine adjust to restrictive conditions within 4 d and apparently experience only an acute stress response.

Depriving swine of the abilty to move freely might affect their health, performance and overall well-being (McFarlane et al., 1988; Becker et al., 1984). McFarlane et al. (1988) found that when given the opportunity gilts turned around in movable sided stalls an average of 13 times/d, and that this did not seem to be motivated by need or desire to face the feeder or waterer since they were located at both ends of the stall. They speculated that gilts turned around simply to face the other direction. Behavioral research suggests that when behaviors occur apparently unrelated to some environmental stimulus, it is probably driven by internal motivations, often defined as a needed behavior (Becker, 1984). McFarlane et al. (1988) reasoned that while turning around might be a behavioral need of a gilt, it could not clearly be defined as such and most likely represented the gilt's desire to stimulate herself in one of the few ways open to her. Results shown in Table 2 clearly agree with findings of McFarlane et al. (1988) in that the ability to turn around did not influence the drinking of water, and probably was not driven by such a need. Present results show that aggravated behavior stops within 48 h of stall adjustment which parallels the decrease in plasma total cortisol and plasma free

cortisol within 72 h of stall adjustment and the normal circadian rhythm of plasma cortisol secretion within 4 d of stall adjustment. Aggravated behavior seen after adjusting stalls to fixed sides was similar to the short term bar-biting in gilts placed in tether stalls (Becker et al., 1984). Restrictive penning (tether stalls) disrupted the cortisol circadian rhythm for 4 d following penning, with high transient increases in cortisol secretion immediately following penning (Becker et al., 1984).

Gilts, in the present study, spent a great deal of time "chain chewing" on the loose chains located at the rear of the crate. This activity, like that of turning around, is probably an effort on the part of the gilt to stimulate herself in a barren environment and is equivalent to playing with a toy and can lead to reduced cortisol levels (Dantzer and Mormede, 1981).

Results of the present study suggest that restricting an animals freedom of movement (i.e. fixed sided stalls) decreases PBMC blastogenic response to mitogens for up to 14 d. The ability of PBMC to proliferate in response to mitogens is considered to be a viable measure of cellular immunity (Blecha et al., 1983). Results of studies using mice indicate that stress-induced increases in glucocorticoids are involved in suppression of cellular immunity (Blecha et al., 1982). Furthermore, *in vitro* studies suggest that physiological concentrations of cortisol decreased response to mitogen stimulated blastogenesis (Kelley et al., 1982b).

Restraint stress lasting 16 h has been associated with a depression in cell-mediated immunity in mice (Sheridan et al., 1991). Plasma corticosterone increased to approximately 179 ng/mL within 30 min after the onset of a stressful restraint

cycle. At the end of the 16 h cycle, corticosterone was at baseline levels. The positive correlation between high levels of corticosterone and lymphopenia, altered lymphocyte circulation, and decreased adhesion molecules could explain decreased cellular immunity (Sheridan et al., 1991). The transient rise in plasma corticosteroid level observed in the present study agree with Sheridan et al. (1991), with baseline levels returning within 2 wk. Likewise, a depression in cellular immunity was found in both studies. While Sheridan et al. (1991) measured viral infection rates instead of PBMC blastogenic response, they are both considered reliable measures of cellular immunity. Research in humans has found that stressful events like loss of a spouse, separation from a spouse, and course examinations for medical students resulted in suppressed mitogen response of PBMC for the duration of the stressful period (Ader et al., 1990). It would appear that suppression of the cellular immune response continues for the duration of the stress.

In this study, gilts maintained in fixed sided stalls experienced stress for the duration of confinement based on suppression of cellular immune response as measured by PBMC blastogenic response to mitogens. Absence of elevated plasma cortisol and aggravated behavioral characteristics by day 4 of treatment suggests that animals to their environment. Recent research has found a link between transient increases in plasma cortisol , suppression of cellular immune function, and an increase in disease susceptibility (Hardie et al., 1991; Sheridan et al., 1991). Effects of corticosteroids on immunological defense mechanisms and its relationship to disease resistance is extremely important (Siegal, 1985). Before any final conclusions

can be drawn about effects of increased restraint/confinement on gilts more research is needed to examine the potential long-term effects on the immune system and the relationship to disease resistance.

Implications

Swine perceive restriction of movement as an acute stress based on measured changes in plasma cortisol and behavior. Animals appear to adjust to their new environment within 48 h based on the nonsignificance of cortisol values between treatments after 3 d and the abating of "aggravated" behavior in gilts confined to fixed sided stalls. However, cell mediated immunity as measured by PBMC blastogenic response was suppressed for as long as 14 d in animals subjected to greater restriction of movement. Confinement of gilts in fixed sided versus movable sided stalls apparently causes a chronic suppression of PBMC blastogenic response. One component of the cellular immune defense system is compromised and may indicate that animals are not as well adapted to their environment as we would like to think, but are resigned to exist in it under stressful conditions.

CHAPTER IV

INFLUENCE OF AGE OF WEANING ON CELLULAR IMMUNE FUNCTION IN THE PIG

Introduction

Early weaning, usually before 5 wk, has been herald as an economically sound improvement for modern swine production systems due to an increase in the number of pigs born/sow/year and efficient use of facilities and equipment. However, Lecce et al. (1979) reported the increased diarrhea and deaths, lowered feed intake, and depressed weight gain associated with early weaning as bearing "grim testimony to the 'state of the commercial art' of early weaning." Poor postweaning performance can be related to low disease resistance or depressed immune response (Inoue et al., 1978) and other factors like environmental stress (Kelley, 1980).

Early weaning has a direct effect on the immune system leaving the piglet "immunological vulnerable" (Blecha et al., 1983). Research has shown that weaning is directly responsible for decreased antibody-mediated cellular immunity (Haye and Kornegay, 1979; Blecha and Kelley, 1981). Blecha et al. (1983) also found that weaning before 5 wk of age suppressed cell-mediated immunity, as measured by PBMC blastogenic response to mitogens, 24 h following weaning. However, Nienaber and Hahn (1989) determined that weaning at 2 and 3 wk of age did not appear to affect the ratio of neutrophils to lymphocytes (N:L) when measured 16 d

following weaning. The function of neutrophils may still be affected even though the circulating numbers do not appear to decrease. The loss of lactational protection associated with maternal antibodies from colostrum and milk and indirect evidence of suppressed cell mediated immunity suggests early weaned pigs are in a precarious situation (McCauley and Hartmann, 1984).

Pigs first line of defense during this time are polymorphonuclear cells (PMN), primarily neutrophils, which protect against bacterial and fungal invasion (McCauley and Hartmann, 1984). The ability of neutrophils to protect against invading organisms involves several functions, two of which are phagocytosis which involves ingestion of invading organisms and elastase degranulation which inclues chemotactic destruction of invading organisms (Thomsen et al., 1991). McCauley and Hartmann (1984) reported that the number of neutrophils increased around 3 wk, the time of weaning, and that this trend continued for 21 d. Research indicates that neutrophils are in plentiful numbers in the neonatal pig and that for this reason they may serve as the primary line of defense for the immunodeficient pig. While neutrophil numbers may be plentiful their defensive capacity could be compromised.

Objectives of this study were to examine effects of age of weaning on cell mediated immune response in the piglet as measured by PBMC blastogenic response to mitogens, neutrophil elastase degranulation, and neutrophil phagocytic ability 10 to 12 d following weaning. The study will indicate if weaning before a certain age can alter cellular immune defense mechanisms and if more than one component, or cell type, of cellular immunity it affected.

Materials and Methods

Animals. Duroc x (Yorkshire x Landrace) piglets (n=32) were allotted to one of four groups consisting of weaning at 3, 4, 5, or 6 wk of age. An additional 32 pigs of similar breeding were used to evaluate effects of weaning age on polymorphonuclear cell function. Pigs remained with the sow in a conventional farrowing system and , upon weaning, kept together in nursury pens according to weaning age. All pigs were provided 22% crude protein pelleted creep feed beginning a 14 d of age. All pigs were given iron dextran (2mL i.m.) on d 7, and all males were castrated on d 14.

Blood samples (5-10 mL) were collected via jugular puncture into syringes containing 1 ml of 1.5% EDTA in PBS. Animals were bled immediately prior to weaning and 10 to 12 d after weaning for pigs weaned at 3, 4, and 5 wk of age. In addition, all animals were bled at 3, 18, and 24 wk of age. Animals of the same age and litter that had not been weaned were bled as controls.

PBMC Blastogenic Response. PBMC were isolated and their response to con A and PWM was determined as described in the previous study and outlined in Appendix A. Intrassay coefficient of variation for sample triplicates was < 6% for both mitogens.

Neutrophil Isolation, elastase activity, and phagocytosis. Neutrophils were isolated from whole blood as described by Roth and Kaeberle (1981) and outlined in Appendix C. Neutrophil elastase degranulation was measured as outlined in Appendix D. Briefly, neutrophils were stimulated with cytochalasin B and the cell

free supernatant was analyzed for elastase release using a spectrophotometric procedure. Total cellular content was determined by disrupting cells by freeze/thawing at least 3 times and then measuring cell free supernatant also by a spectophotometric procedure. Neutrophil phagocytic ability was analyzed as described by Roth and Kaeberle (1981) and outlined in Appendix E. The procedure was modified for use with [^{125}I]-K88^{+} *E. coli*, a common bacteria that causes problems for pigs.

Statistical Analysis. Data for lymphocyte blastogenic response to Con A and PWM, neutrophil elastase degranulation and phagocytosi were analyzed using General Linear Mixed Models procedure (Blouin and Saxton, 1990). Generalized least square means with standard errors are reported. Contrast statements were used for treatment comparisons. General Linear Models (GLM) were used for comparison of age related effects among least square means.

Results

PBMC Blastogenic Response. Lymphocyte blastogenic responses of pigs weaned at 3, 4, and 5 wk are presented in Table 5. Data are presented as counts per minute (cpm), and represent ^{3}H-thymidine incorporation during the last 24 h of incubation as a measure of lymphocyte proliferation. PBMC response to con A and PWM was lower ($P<.001$) for pigs 10 to 12 d following weaning at 3 wk of age compared to nonweaned controls. The PBMC response to Con A for pigs weaned at 4 wk was less ($P<.01$) than nonweaned counterparts.

The PBMC blastogenic response to mitogens at 18 and 24 wk of age was not

Table 5. Peripheral blood mononuclear cell blastogenic response for pigs weaned at 3 (n=7), 4 (n=8), and 5 (n=8) wk of age measured 10 to 12 d after weaning[a].

Age of weeking		Mitogen	
wk	Treatment	Concanavalin A	Pokeweed mitogen
3	Weaned	96 ± 20	64 ± 15
	Nonweaned	315 ± 26	147 ± 15
	P<[b]	.001	.001
4	Weaned	156 ± 21	143 ± 14
	Nonweaned	242 ± 26	144 ± 13
	P<	.01	.99
5	Weaned	215 ± 14	117 ± 10
	Nonweaned	236 ± 21	103 ± 16
	P<	.41	.44

[a] Data are generalized least-square means ± SEM of net cpm x 10^3 = mitogen stimulated-cpm background cpm.

[b] Probability of observing a greater F-value between treatments.

different ($P > .10$) between pigs regardless of age of weaning (Table 6). There was no age effect ($P > .05$) for mitogen stimulation by Con A between the three bleeding ages. An age effect ($P < .05$) for mitogen stimulation by PWM occurred with increasing responses as age increased.

Elastase Degranulation. Elastase degranulation results are expressed as percentages of total cellular elastase content. Results for elastase degranulation are shown in Table 7. There was no difference ($P > .10$) between weaned and nonweaned animals for each age group. There was also no age effects ($P > .05$) between the three weaning ages.

Phagocytosis. The phagocytic ability of porcine polymorphonuclear cells, primarily neutrophils, to ingest labeled *E. coli* was not different ($P > .10$) between the weaned and nonweaned controls for each of the three weaning ages (Table 8). There was an age effect ($P < .05$) with pigs weaned at 5 wk of age having greater phagocytic ability (results not shown).

Discussion

Blecha et al. (1983) found that PBMC response to PWM decreased ($P < .01$) in pigs 24 h after weaning at 2 wk of age but not in pigs weaned at 3 or 5 wk. However, Blecha et al. (1983) reported a decrease in PBMC response to phytohemagglutin, another T cell mitogen, in 2 ($P < .001$) and 3 ($P < .01$) wk old but not 5 wk old weaned pigs. These researchers also found that PBMC blastogenic response increased with age which is supported by the results of the present study. The present findings show a suppression in cellular immune function in pigs weaned

Table 6. Peripheral blood mononuclear cell blastogenic response for gilts bled at 18 and 24 wk of age[a].

Age (wk)	Weaning Age (wk)	Mitogen[b] Concanavalin A	Pokeweed mitogen
18	3	251 ± 37	145 ± 27
	4	250 ± 14	143 ± 10
	5	249 ± 19	139 ± 14
24	3	255 ± 26	149 ± 18
	4	243 ± 14	146 ± 10
	5	247 ± 19	148 ± 14

[a] The blastogenic response to each mitogen was not different ($P < .10$) regardless of age of weaining.

[b] Data are generalized lease-square means ± SEM of net cpm x 10^3 = mitogen stimulated cmp-background cpm.

Table 7. Porcine polymorphonuclear cell elastase degranulation measured 10 to 12 d following weaning at 3, 4 and 5 wk of age [a].

Age at Weaning (wk)	Treatment	Elastase Degranulation (%)[b]
3	Weaned	6.3 ± .04
	Nonweaned	6.8 ± .08
4	Weaned	4.4 ± .03
	Nonweaned	4.3 ± .08
5	Weaned	7.3 ± .07
	Nonweaned	8.4 ± .07

[a] There was no difference ($P > .10$) between treatments at any age of weaning.
[b] Data are generalized least-square means ± SEM of % elastase degranualtion = total cellular elastase content - elastase degranulated from intact cells.

Table 8. Porcine polymorphonuclear cell % phagocytosis of [^{125}I]-K88^{+} *E. coli* measured 10-12 d following weaning at 3, 4, and 5 wk of age [a].

Age at Weaning (wk)	Treatment	Phagocytosis (%) [b]
3	Weaned	32.2 ± 4.8
	Nonweaned	42.3 ± 6.8
4	Weaned	55.2 ± 4.8
	Nonweaned	53.6 ± 5.5
5	Weaned	65.9 ± 4.8
	Nonweaned	73.4 ± 6.9

[a] There was no difference ($P>.10$) in % phagocytosis regardless of age of weaning.
[b] Data are generalized least-square means ± SEM of % phagocytosis labeled *E. coli* = (cpm reaction tube - cpm background) / (cpm standard - cpm background) x 100.

before 5 wk of age, consistant with data reported by Blecha et al. (1983), and also demonstrate that suppression is not a short term (24 h) response but rather remains for at least 10 d.

Research has shown a relationship between stressful psychological factors and decreased PBMC response to mitogens, a measure of cellular immunity. Research in humans has found that stressful events like loss of a spouse, separation from a spouse, and course examinations for medical students result in suppressed mitogen response of PBMC for the duration of the stressful period (Ader et al., 1990). Increased reports of illness have been noted in medical students with suppressed PBMC blastogenic responses (Ader et al., 1990). An experiment examining effects of separation of mice from dams showed that immunological responsiveness was lower in maternally deprived mice and was not related to other factors like weight differences (Michaut and Dechambre, 1981). A similar study in primates showed decreased lymphocyte mitogenic response due to separation of infant monkeys from their mothers (Laundenslager et al., 1982). Thus stress in different forms and among different species, regardless of age, can cause suppression of cellular immune response.

Blecha et al. (1986) found a lower ($P<.05$) PBMC response to mitogens in artificially reared pigs compared to sow raised pigs. Suppression of PBMC blastogenic response was independent of weight differences between artificially reared pigs and the sow reared pigs (Blecha et al., 1986). These researchers speculated that the increase in morbidity and mortality of early weaned or artificially reared pigs associated with impaired immunological function may be related to the

psychological stress of maternal separation. The findings reported in the present study further indicate that impaired immunological function may persist for up to 10 d following weaning before 5 wk of age.

Disease susceptibility has been shown to be correlated positively with stress-induced increases in plasma cortisol (Hardie et al., 1991). Demonstration of a link between stress, cortisol secretion, and disease resistance with a reduction of many cellular immune functions in cattle, swine, chickens, and fish has been reported (Westly and Kelley, 1984; Blecha and Baker, 1986; Franklin et al., 1987; Hardie et al., 1991). Increased cortisol secretion has also been linked to decreased disease resistance in Atlantic salmon (Hardie et al., 1991). Results of other researchers and of the previous study indicate that stress (i.e. restricted movement) causes transient rises in plasma cortisol which may inturn suppress cellular immune function (i.e. decreased PBMC response to mitogens). At least one aspect of cellular immunity is suppressed for up to 10 d that could indicate that the animal has impaired defense mechanisms and may prove to be more susceptible to disease.

Neutrophils, another component of cell-mediated immunity, serve as the first line of defense against bacterial and fungal infection (Thomsen et al., 1991). Research indicates that the neutrophil population is susceptible to change under different condition of stress. Clemens et al. (1986) found that the N:L increased in response to stress (22 - 24 h of feed and water deprivation) in swine. They also found that cortisol increased following stress and suggested that this might play a role in the N:L increase. However, by 14 d following stress, significant differences in cortisol or

N:L among stressed animals were no longer detected (Clemens et al., 1986). McCauley and Hartmann (1984) indicated stress of early weaning at 3 wk of age caused a transient increase in plasma cortisol (24 h following weaning) and increased the number of neutrophils. Increased cortisol concentration was no longer evident 12 d following weaning, but the rise in neutrophil numbers persisted for 21 d following weaning, reaching adult levels. Nienaber and Hahn (1989) reported that weaning at 2 and 3 wk of age did not appear to affect N:L when measured 16 d following weaning.

Phagocytosis by PMNs in the baby pig may serve as the primary defense mechanism against invading organisms. However, the results of the present study showed that phagocytic ability of porcine neutrophils to ingest labeled *E. coli* was not different ($P > .10$) regardless of age at weaning. Results from the present study did indicate an age related effect ($P < .05$) with pigs weaned at 5 wk of age showing a greater phagocytic ability. Hoskinson et al. (1990) reported a decrease ($P < .001$) in phagocytic ability from 1 to 3 wk of age in neonatal pigs. From 3 to 6 wk of age phagocytic ability of neutrophils increased to values observed in the newborn. It was also reported that while phagocytic ability decreased around 3 wk of age, intracellular killing ability was not altered (Hoskinson et al., 1990). Studies in horses have reported no difference between the phagocytic ability or intracellulalr killing ability of neutrophils from mares and foals (Morris et al., 1987; Martens et al., 1988). However, Hauser et al. (1986) found that calves from 4 to 5 wk of age had only about half the neutrophil iodination activity, another measure of neutrophil cell mediated cytotoxicity, of cows 12 to 14 mo of age. Mills et al. (1979) demonstrated

that bactericidal ability of human neonatal neutrophils varied in response to the bacteria:neutrophil ratio. At low ratios (i.e. 1:1) there were no age related differences in neutrophil function. However, at larger ratios such as 100:1, neutrophils from younger individuals performed less than those of older individuals. Effects of age on PMN function in neonatal pigs are not clear at this time. While neutrophil numbers may be changing it also appears that each function of the neutrophil is responding differently in relation to the age of the animal. Neutrophil function in the young pig is undergoing developmental changes dependent on age and other related factors such as stress.

Neutrophil function is related in part to their ability to degranulate, or release the serine protease elastase which causes destruction of invading organisms. Elastase production is an important measure of neutrophil activity in the young pig, especially considering that neutrophils may serve as the primary defense mechanism for the young immunodeficient pig (McCauley and Hartmann, 1984). In the present study, early weaning had no effect ($P > .10$) on neutrophil elastase degranulation. No significant ($P > .10$) age effect was detected for degranulation, however there is an apparant trend that may have biological implications. Elastase degranulation may not be the best measure of neutrophil activity in relation to the stress of weaning.

The stress of weaning before 5 wk of age along with the many physiological changes that occur in the pig during this time could seriously compromise health of the baby pig. The present study has shown that weaning before 5 wk of age resulted in extended suppression of PBMC blastogenic response to mitogens for up to 10 d,

and that PMN function in animals not weaned until 5 wk of age was better in regards to phagocytic ability. Lymphocyte blastogenic response, PMN elastase degranulation, and PMN phagocytosis are all measures of cell-mediated immunity, probably the primary defense mechanisms in the young pig. Extensive research has already shown that weaning decreases antibody-mediated immunity (Haye and Kornegay, 1979; Gwazdauskas et al., 1980; Blecha and Kelley, 1981; Blecha et al., 1983). In addition, decreased physiological maturity associated with the stress of weaning at 3 wk lowered antibody production (Haye and Kornegay, 1979). Early weaning (i.e. at least before 5 wk) impairs antibody-mediated immunity and indirect evidence indicates that cellular immunity is also impaired (Blecha et al., 1983). The present research reports that weaning before 5 wk of age suppresses PBMC blastogenic response to mitogens, and that age effects are present in neutrophil phagocytic response. Results may indicate altered or immature immune function in pigs weaned before 5 wk.

The physiological mechanism responsible for effects of early weaning on cellular immune response are still unclear. Blecha et al. (1983) speculated that elevated plasma corticosteroid may be responsible, based on research in mice which indicated that corticosteroids are involved in stress related immunosuppression (Blecha et al., 1982). Also, in vitro results indicated that physiological concentrations of cortisol added to porcine PBMC caused suppression of blastogenic response to mitogens (Kelley, 1980). Elevated plasma cortisol in early weaned pigs, in comparison to their non-weaned counterparts (Worsaae and Schmidt, 1980), indicates

corticosteroid involvement in reduced cell-mediated immunity. Resposne to adrenal corticosteroids is usually associated with a time span of 24 to 48 h (Ader, 1990) and transient rises in cortisol, often diminishing within 48 h, cannot explain the extended cellular immune suppression (i.e. 14 d) as seen in this study. In channel catfish, the stress of handling and transportation can substantially increase cortisol concentrations to a level capable of causing suppression of PMN phagocytosis and intracellular killing 16 - 18 h following the stress (Ainsworth et al., 1991). Ainsworth et al. (1991) reported that degree of stress and duration of neutrophil exposure to increased cortisol determined if a suppression in neutrophil defense funciton occurred. Cortisol addition *in vitro* to neutrophils did not cause significant differences in phagocytic index, bacteria killing ability, and phagocytosis, whereas cortisol concentration *in vivo* above the normal threshold did cause suppression of neutrophil function (Ainsworth et al., 1991). These results suggest that cortisol does not function alone in stimulating the suppression of neutrophil function observed *in vivo* under conditions of stress increased cortisol concentrations. While there appears to be some link between cortisol and immunosuppression there are other factors that are also involved and are not fully understood at this time.

Implications

Antibody-mediated immune system is compromised in the pig weaned before 5 wk of age, and it also appears that the cell-mediated immune system is not fully functional at this time. Cellular immune function as measured by PBMC blastogenic

response to mitogens remained suppressed for up to 10 d following weaning before 5 wk of age. However, it appears that PBMC blastogenic response to mitogens recovers in swine by market weight (18-24 wk of age) with no differences detectable between different weaning ages. It also appears that the PMN function (i.e. elastase degranulation and phagocytosis) of young pigs undergoes developmental changes that could be especially susceptible to stress of weaning at early ages.

CHAPTER V

IN VITRO EFFECTS OF CORTISOL AND VITAMIN C ON PORCINE PBMC BLASTOGENIC RESPONSE TO CONCANAVALIN A

Introduction

Cell-mediated immunity is seriously compromised by weaning earlier than 5 wk of age as demonstrated in Chapter 4. Dietary supplementation of Vitamin C has been considered as a possible ameliorative measure for cell-mediated suppression associated with stress of early weaning. Stress increased cortisol secretion has been reported to down regulate the immune system, i.e. PBMC proliferation and neutrophil function (Siegel, 1985; Ainsworth, 1991). Stress also depletes vitamin C stores and increases the demand for exogenous supplementation since the body cannot produce enough to meet the demand. Animals maintained on diets supplemented with vitamin C experienced a decrease in the immunosuppressive effects related to stress (Hardie et al., 1991).

Objectives of this study were 1) to examine effects of physiological concentrations of cortisol in vitro on PBMC and 2) to determine if addition of vitamin C to PBMC in vitro could decrease the in vitro suppressive effects of cortisol.

Materials and Methods

Culture Conditions. Peripheral blood mononuclear cells were isolated from whole blood samples (25 mL) collected into syringes containing 3 mL of 1.5% EDTA. PBMC were isolated as described in Appendix A. Lymphocytes were adjusted to a concentration of 4 x 10^6 cells/mL. Fifty microliters each of cells and concanavalin A (con A; 50 μg/mL) were plated onto 96 well flat bottom plates. Serial dilutions of cortisol were performed and resulted in 50 μl of cortisol at concentrations of 0, .0125, .025, .05, .1, .2, .4, .8, or 1.6 x 10^{-6} M/well being plated in triplicate with PBMC and Con A in each well. Plates were incubated, pulsed with [3H] thymidine, harvested, and counted as described for Chapter 4.

The same cell population was also tested for response to cortisol and vitamin C co-cultured with con A. After serial dilutions were tested on cell populations to determine a maximum dose without killing cells, a concentration of 2.4 x 10^{-5} M for vitamin C was used in the experiment. Cells were plated as described before with the addition of 50 μl of vitamin C added to each of the previously listed cortisol concentrations. Again plates were incubated, pulsed, harvested, and counted as described for Chapter I.

Statistical Analysis. Data for lymphocyte blastogenic response to Con A and cortisol were analyzed using General Linear Models (GLM) procedure (SAS, 1985). Least square means were determined with standard errors. After an analysis of variance determined treatment differences ($P < .05$), Duncan's Multiple Range Test was used to determine significant differences in blastogenic response between different

concentrations of cortisol.

Data for lymphocyte blastogenic response to con A, cortisol, and vitamin C were analyzed using GLM (SAS, 1985). Least square means with standard errors were reported. An analysis of variance was used to determine treatment differences among blastogenic responses of cells treated with cortisol versus cells treated with the same concentrations of cortisol plus vitamin C.

Results

Cortisol Addition In Vitro. Effects of various concentrations of cortisol on PBMC proliferation in response to Con A are illustrated in Figure 5. Mitogen stimulated PBMC proliferation in response to cortisol at .0125 and .025 x 10^{-6} M were not different ($P > .05$) from media alone. A suppression ($P < .05$) of PBMC proliferation in response to Con A occurred following the addition of .05 x 10^{-6} M cortisol. The minimum concentration of cortisol resulting in maximum suppression of PBMC proliferation was .2 x 10^{-6} M.

Cortisol and Vitamin C Addition In Vitro. Vitamin C addition to the culture system containing various concentrations of cortisol was tested and results are presented in Figure 6. There were no ($P > .10$) differences in proliferation of PBMC coincubated with vitamin C. Vitamin C alone had no significant effect on PBMC blastogenic response to any mitogen.

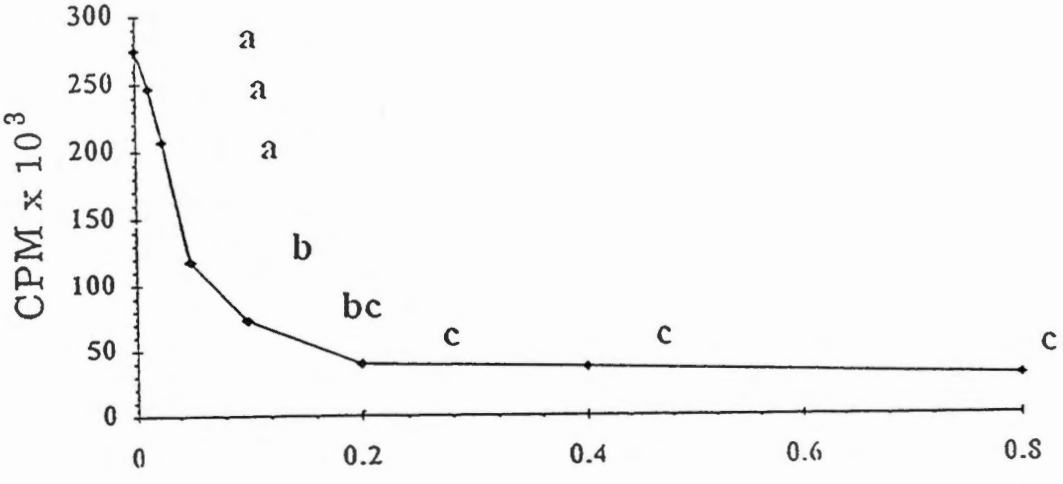

Figure 5. Effect of varying concentrations of cortisol addition to porcine PBMC blastogenic response to Con A. Mean (X± SE) counts per minute with the same letter are not ($P<.05$) different.

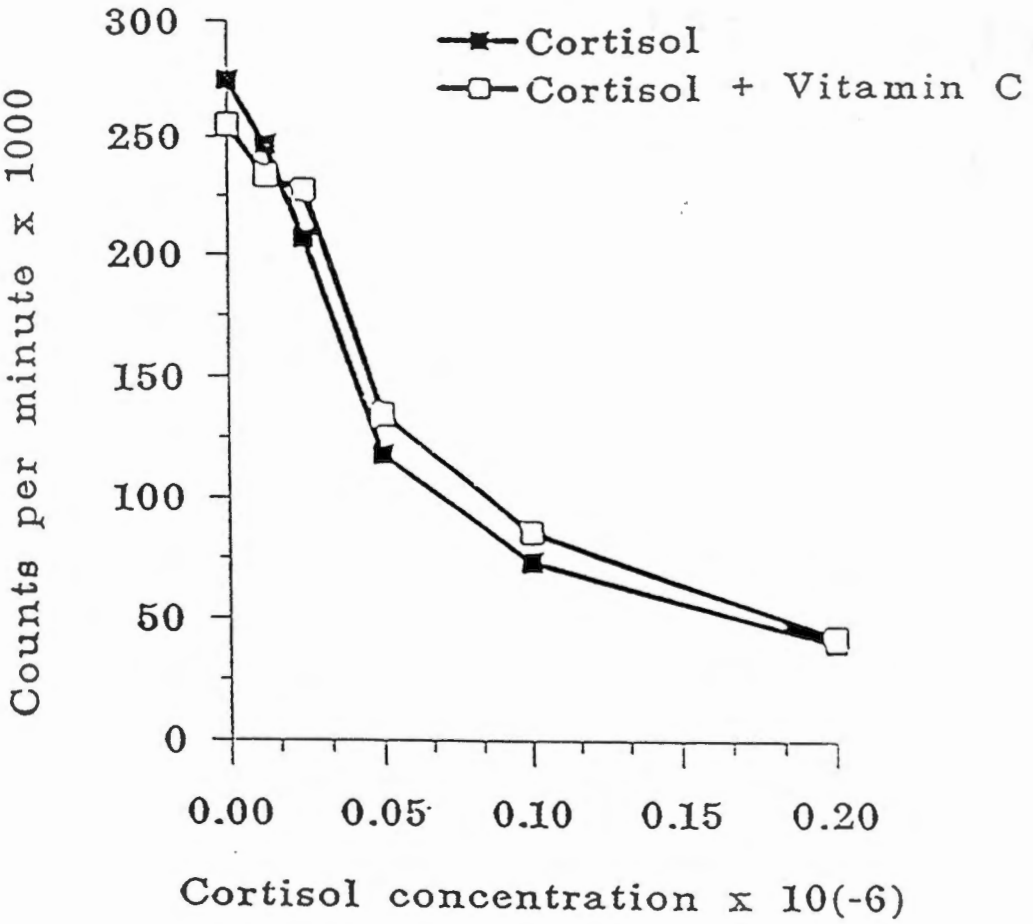

Figure 6. Porcine PBMC blastogenic respone to Con A in the presence of increasing concentrations of cortisol with and without the addition of 2.4 x 10^{-5} M/well of Vitamin C. There was no difference ($P > .10$) between samples with Vitamin C and those without Vitamin C at any concentration of cortisol addition.

Discussion

Animals undergoing immunological response to various antigens had increased blood glucocorticoid levels in proportion to the magnitiude of the immune response (Rey et al., 1987). Immunosuppressive effects of cortisol can be explained partially by inhibition of of interleukin 1 (IL-1) which can result in decreased proliferation of lymphocytes (Rey et al., 1987). Research has shown that production of IL-1 in humans and animals can be inhibited by increased production of glucocorticoids (Rey et al., 1987). Physiologically high levels of cortisol can also decrease interleukin 2 production which is required for clonal T cell expansion (Blecha and Baker, 1986). Rey et al. (1987) suggested that glucocorticoid-associated regulatory mechanisms may act as a surveillance mechanism of immunological cells and their activity.

Overcoming immunosuppressive effects of cortisol would be a very practical and beneficial application of immune modulation (Ainsworth et al., 1991). Previous studies have shown that immunosuppression associated with stress coincide with elevations in cortisol levels (Pardue and Thaxton, 1984; Bains, 1988; Hardie et al., 1991). Animals, including swine, quickly deplete vitamin C stores from adrenal tissue during times of stress, which is most likely associated with increased steroidogenesis (Dvorak, 1984; Hardie et al., 1991). Immunosuppression associated with stress has been reduced with dietary supplementation of vitamin C (Pardue and Thaxton, 1984; Dvorak, 1984).

There are two current theories that explain the mechanism by which vitamin C ameliorates stress related immunosuppression. Reduction of adrenal synthesis of

corticosteroids, and by protection of lymphoid tissue are these theories (Bains, 1988). Based on results presented here it would appear that the beneficial effects of vitamin C on stress related immunosuppression are not detectable or do not exist under *in vitro* conditions of PBMC blastogenic suppression by cortisol. The results presented here support the suggestion by Gower (1984) that the beneficial effects of vitamin C in regards to the immune system may be that vitamin C present in the adrenal gland acts as a brake on steroidogenesis. Dvorak (1984) also noted a depletion of vitamin C in pigs under stress which paralleled decreased stress responses reported in fish (Hardie et al., 1991).

Hardie et al. (1991) found an increase in disease resistance in Atlantic salmon fed diets consisting of normal and high levels of vitamin C. Interestingly, the increase in available vitamin C was not associated with changes in leukocyte numbers or function, or antibody and lymphokine secretion by lymphocytes. These researchers speculated that increased disease resistance may be related to beneficial effects on the complement system. Guinea pigs have also been shown to have increased complement activity as represented by up to a 50% rise in plasma levels of C1q when fed tissue-saturating levels of ascorbate (Johnston et al., 1987). There may be a direct link between supplemental dietary vitamin C, increased resistance to disease, and activation of the complement component of the immune system.

Vitamin C and its effects on increased disease resistance could also be related to the effects of stress. Stress has been shown to evoke increased secretion of glucocorticoids, primarily cortisol (Siegel, 1985; Ainsworth and Bowser, 1985). Such

increases in plasma cortisol have secondary effects such as downregulation of the immune system (Ellsaesser and Clem, 1987). Thaxton and Pardue (1984) recommended that vitamin C be considered as a potential means of decreasing losses due to management stress of poulty based on the ameliorative effects of dietary supplementation with vitamin C they found uder stressful conditions. Results indicate that PBMC blastogenic response to mitogens under suppressed conditions due to cortisol are either not affected by vitamin C addition in vitro or the beneficial effects are not detectable due to other factors involving cell activation and secretion.

Implications

Cortisol can be added to PBMC in culture without causing PBMC blastogenic response to con A to be suppressed at concentrations $< .05 \times 10^{-6}$. Addition of cortisol *in vitro* caused suppression of PBMC blastogenic response to mitogens if added at $.05 \times 10^{-6}$ M or greater. A maximum dose response is reached at $.2 \times 10^{-6}$ M. Vitamin C addition *in vitro* did not alleviate the suppressive effects of cortisol on PBMC proliferative response. It appears that the reported beneficial effects of dietary vitamin C supplementation during times of stress is not detectable in cortisol suppressed PBMC blastogenic response to con A in an *in vitro* culture system.

CHAPTER VI

CONCLUSIONS

Restricting an animal's ability to turn around or move freely causes transient increases in plasma cortisol, a disruption of the circadian rhythm of cortisol, and immunosuppression as demonstrated by a decreased ability of PBMC to respond to mitogens. Other research has linked transient rises in plasma cortisol with immunosuppression and decreased disease resistance. Other studies examined confinement stress and concluded that there was only an acute stress response based on the results of cortisol data and behavioral data. Results from this study agree with the conclusion that animals adjust to their environment based on the decrease in cortisol and aggravated behavior within 4 d following restricted confinement. However, while it may appear that animals have adapted to their environment, immune measures of stress as indicated in this study show that stress related immunosuppression continues for at least 14 days. Since PBMC blastogenic response appears to be suppressed it is possible that the immune system is compromised the animal's disease resistance may also be impaired. Statistical significance was observed between the PBMC blastogenic response of animals under different restraint conditions does not necessarily correlate with a biological significance. Animals in fixed sided stalls maintained a reasonably good PBMC blastogenic response to

mitogens. Further research is needed to examine the effects of confinement stress on several different physiological parameters of stress to determine if the animals are really adapting to their environment, simply resigned to exist in it, and if the immune parameters used are viable indicators of the animal's physiological condition.

Early weaning, before 5 wk of age is another management stress often found in modern swine facilities. Research indicates that weaning before 5 weeks of age causes a temporary (usually 24 hour) suppression in antibody-mediated and cell-mediated immunity. The more long term effects of early weaning in response to cellular immunity have only partially been examined. Based on the findings reported here, cellular immunity appears to be suppressed for at least 10 days following weaning before 5 weeks of age. The young pig is immunologically immature to begin with, and the added stress of early weaning can only contribute to poor postweaning performance. Economic gains of early weaning may not exceed losses in performance and mortality rates by weaning at such early ages. The transient rises in plasma cortisol, continued suppression of PBMC blastogenic response to mitogens, and age related changes in PMN phagocytic and elastase degranulation ability all contribute to impaired immune function in the early weaned pig.

Finally, research has indicated that vitamin C may be a means of ameliorating some of the immunosuppressive effects associated with stress and transient rises in plasma cortisol. Results of this *in vitro* study indicate that the beneficial effect of dietary supplementation of vitamin C are not detectable under conditions of cortisol induced suppression of PBMC blastogenic response to con A. The benefits of dietary

supplementation of vitamin C in relation to immune function during times of stress have been well documented. Results from the current study did not indicate any beneficial effects occurring *in vitro* however, the benefits may be related to other mechanisms, such as decreased steroidogenesis, or they may not be detectable by the assay presented here.

The modern swine industry will continue to use management procedures that are most efficient and economical. However, economic effects of increased disease occurrence, mortality, poor performance, and decreased animal well-being are well recognized. More research is needed to examine the issues and propose possible solutions, such as dietary supplementation of Vitamin C, in order to aid in the production of healthy and economically profitable animals as well as to educate people on the well-being of production animals.

LITERATURE CITED

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Abramson, J. S., and E. L. Mills. 1988. Depression of neutrophil function induced by viruses and its role in secondary mocrobial infections. Rev. Infect. Dis. 10:326-341.

Ader, R., D. Felten, and N. Cohen. 1990. Interactions between the brain and the immune system. Ann. Rev. Pharmacol. Toxicol. 30:561-602.

Ainsworth, A. J., and P. R. Bowser. 1985. Serum cortisol livels in channel catfish from production ponds. Prog. Fish-Cult. 47:176-181.

Ainsworth, A. J., C. Dexiang, and P. R. Waterstrat. 1991. Changes in peripheral blood leukocyte percentages and function of neutrophils in stressed channel catfish. J. Aquatic Ani. Health. 3:41-47.

Bains, B. S. 1988. Role of vitamins in enhancing immune response in chickens. A review. Proc. Poultry Research Foundation Symp. 1988, University of Sydney.

Barnett, J. L., G. M. Cronin, and C. G. Winfield. 1981. the effects of individual and group penning of pigs on total and free plasma corticosteroids and the maximum corticosteroid binding capacity. Gen. Comp. Endoc. 44:219-223.

Becker, B. A., R. K. Christenson, J. J. Ford, J. A. Nienaber, J. A. DeShazer, and G. L. Hahn. 1989. Adrenal and behavioral responses of swine restricted to varying degrees of mobility. Physiol. Behav. 45:1171-1176.

Becker, B. A., J. J. Ford, J. A. DeShazer, and G. L. Hahn. 1984. The effects of tether stalls on the circadian rhythm of cortisol, behavioral activities and estrous response in ovariectomized gilts. Proc. Human-Animal Bonding Conf., 1984, Minneapolis, MN.

Becker, B. A., J. A. Nienaber, R. K. Christenson, R. C. Manak, J. A. DeShazer, and G. L. Hahn. 1985. Peripheral concentrations of cortisol as an indicator of stress in the pig. Am. J. Vet. Res. 46:1034-1037.

Blecha, F. 1988. Immunomodulation: A means of disease prevention in stressed livestock. J. Anim. Sci. 66:2084-2090.

Blecha, F., and P. E. Baker. 1986. Effect of cortisol invitro and in vivo on production of bovine interleukin 2. Am. J. Vet. Res. 47:841-848.

Blecha, F., R. A. Barry, and K. W. Kelley. 1982. Stress-induced alterations in

delayed-type hypersensitivity to SRBC and contact sensitivity to DNFB in mice. Proc. Soc. Exp. Biol. Med. 169:239-246.

Blecha, F., and K. W. Kelley. 1981. Effect of cold and weaning stressors on the antibody-mediated immune response of pigs. J. Ani. Sci. 53:439-441.

Blecha, F., D. S. Pollmann, and E. J. Kluber, III. 1986. Decreased mononuclear cell response to mitogens in artifically reared neonatal pigs. Can. J. Vet. Res. 50:522-525.

Blecha, F., D. S. Pollmann, and D. A. Nichols. 1983. Weaning pigs at an early age decreases cellular immunity. J. of Anim. Sci. 56:396-400.

Blondin, J. A. Janoff, and J. C. Powers. 1978. Digestion of E. coli proteins by human neutrophil elastase and chymotrypsin-like enzyme (cathepsin G): Experiments with a cell-free system and living leukocytes. In: K. Havemann, and A. Janoff (eds) Neutral proteases of human polymorphonuclear leukocytes, Urban & Schwarzenberg, Baltimore, MD.

Blouin, D. C., and A. M. Saxton. 1990. General linear mixed models GLMM (version 1.1). Baton Rouge, LA. Louisiana Agricultural Experiment Station, LSU.

Bly, J. E., N. W. Miller, and L. W. William. 1990. A monoclonal antibody specific for neutrophils in normal and stressed channel catfish. Dev. Comp. Immuno. 14:211-221.

Bonnette, E. D., E. T. Kornegay, M. D. Lindemann, and C. Hammerberg. 1990. Humoral and cell-mediated immune response and performance of weaned pigs feed four supplemental vitamin e levels and housed at two nursery temperatures. J. Anim. Sci. 68:1337-1345.

Bonnette, E. D., E. T. Kornegay, M. D. Lindemann, and D. R. Notter. 1990. Influence of two supplemental vitamin e levels and weaning age on performance, humoral antibody production and serum cortisol levels of pigs. J. Anim. Sci. 68:1346-1353.

Brown, G. B., and J. A. Roth. 1991. Comparison of the response of bovine and human neutrophils to various stimuli. Vet. Immuno. Immunopath. 28:201-218.

Brown, G. B., S. R. Bolin, D. E. Frank, J. A. Roth. 1991. Defective function of leukocytes from cattle persistently infected with bovine viral diarrhea virus, and the influence of recombinant cytokines. Am. J. Vet. Res. 52:381-387.

Canning, P. C., and P. E. Baker. 1990. Selective alteration of bovine neutrophil responses by recombinant bovine interleukin-1. Vet. Immuno. Immunopath. 26:1-12.

Clemens, E. T., B. D. Schultz, M. C. Brumm, G. W. Jesse, and H. F. Mayes. 1986. Influence of market stress and protein level on feeder pig hematologic and blood chemical values. Am. J. Vet. Res. 47:359-362.

Cooper, M. R., L. R. DeChatelet, and C. E. McCall. 1972. The in vitro effect of steroids on polymorphonuclear leukocyte metabolism. Proc. Soc. Exp. Biol. Med. 141:986-990.

Dantzer, R., and P. Mormede. 1981. Can physical criteria be used to assess welfare in pigs? In: The Welfare of Pigs. Martinuss, N. and Nijhoff, A. (Ed.) Netherlands.

Durve, V. S., and R. T. Lovell. 1982. Vitamin C and disease resistance in channel catfish (Ictalurus punctatus). Can. J. Fish. Aquat. Sci. 39:948-951.

Dvorak, M. 1984. Ascorbic acid, stress resistance and reproduction in swine. Proceed. Royal Danish Ag. Soc., Skjoldenaesholm, Sept. 1983.

Ellsaesser, C. F., and L. W. Clem. 1986. Haematological and immunological changes in channel catfish stressed by handling and transport. J. Fish Bio. 28:511-521.

Ellsaesser, C. F., and L. W. Clem. 1987. Cortisol-induced hematological and immunologic changes in channel catfish (Ictalurus punctatus). Comp. Biochem. Physiol. 87A:405-408.

Faltys, G. L., E. M. Convey, R. E. Short, C. A. Keech, and R. L. Fogwell. 1987. Relationship between weaning and secretion of luteinizing hormone, cortisol and transcortin in beef cows. J. Anim. Sci. 64:1498-1505.

Franklin, R. A., D. R. Davila, and K. W. Kelley. 1987. Chicken serum inhibits lectin-induced proliferation of autologous splenic mononuclear cells. Proc. Soc. Exp. Biol. Med. 184:225-232.

Friend, T. H., L. Taylor, D. Knab, and G. R. Dellmeier. 1984. Effect of housing on physiology of gestating gilts. J. Ani. Sci. 59(Suppl. 1):150, Abstract 14.

Gala, R. R., and U. Westphal. 1965. Corticosteroid-binding globulin in the rat: Possible role in the initiation of lactation. Endoc. 76:1079-1083.

Ganong, E. F. 1963. The central nervous system and synthesis and release of adrenocorticotropic hormone. In: A. V. Nalbandov (ed) Advances in neuroendocrinology. University of IL Press.

Garren, J. W., and C. S. Shaffner. 1954. Factors concerned in the response of young New Hampshires to muscular fatigue. Poult. Sci. 33:1095-1099.

Glaser, R., J. Rice, J. Sheridan, R. Fertel, and J. Stout. 1987. Stress-related immune suppression: Health implications. Brain Behav. Immuno. 1:7-20.

Gower, D. B. 1984. Regulation of steroidogenesis. In: H. L. J. Makin (Ed.)Biochemistry of Steroid Hormones, Blackwell, Oxford.

Gross, W. B., and H. S. Siegel. 1983. Evaluation of heterophil, lymphocyte ratio as a measure of stress in checkens. Avian Dis. 27:972-976.

Gwazdauskas, F. C., M. J. Paape, D. A. Peery, and M. L. McGilliard. 1980. Plasma glucocorticoid and circulating blood leukocyte responses in cattle after sequential intramuscular injections of ACTH. Am. J. Vet. Res. 41:1052-1056.

Haye, S. N., and E. T. Kornegay. 1979. Immunogloculin G, A and M and antibody response in sow-reared and artificially-reared pigs. J. Ani. Sci. 48:1116-1121.

Hammond, G. L., J. A. Nisker, L. A. Jones, and P. K. Siiteri. 1980. Estimation of the percentage of gree steroid in undiluted serum by centrifugal ultracentrifugation-dialysis. J. Bio. Chem. 255:5023-5026.

Hammond, G. L., C. L. Smith, N. Am M. Paterson, and W. J. Sibbald. 1990. A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. J. Clin. Endo. Meta. 71:34-39.

Hardie, L. J., T. C. Fletcher, and C. J. Secombes. 1991. The effect of dietary vitamin C on the immune response of the Atlantic salmon (Salmo salar L.). Aqua. 95:201-214.

Hauser, M. A., M. D. Koob, and J. A. Rtoh. 1986. Variation of neutrophil function with age in calves. JAm. J. Vet. Res. 47:152-157.

Heine, K. J., J. C. Shallcross, M. S. Trachtenberg, R. B. Galland, and H. C. Polk. 1983. A reassessment of the influence of steroids on neutrophil phagocytosis. Am. Surg. 49:221-224.

Holmes, W. N., and J. G. Phillips. 1976. The adrenal cortex of birds. In: I. Chester-Jones, and I. W. Henderson (eds) General, comparative and clinical

endocrinology of the adrenal cortex. Vol 1. Academic Press, New York, New York.

Hoskinson, C. D., B. P. Chew, and T. S. Wong. 1990. Age-related changes in mitogen-induced lymphocyte proliferation and polymorphonuclear neutrophil function in the piglet. J. Anim. Sci. 68:2471-2478.

Ingle, D. J. 1954. Permissibility of hormone action. Acta Endocrinol. 8:23-30.

Inoue, T., Y. Yamada, and T. Saito. 1978. Concentrations of immunoglobulins and protein in piglet fecces. Microbiol. Immuno. 22:711-715.

Janoff, A. 1983. Do neutrophils play a major role in elastin turnover of normal tissues? Am. Rev. Respir. Dis. 127:782-783.

Janoff, A. 1985. Elastase in tissue injury. Ann. Rev. Med. 36:207-216.

Johnston, C. S., W. P. Kolb, and B. E. Haskell. 1987. The effect of vitamin C nutrition on complement component C1q concentrations in guinea pig plasma. J. Nutr. 117:764-768.

Kattesh, H. G., S. F. Charles, G. A. Baumbach, and B. E. Gillespie. 1990. Plasma cortisol distribution in the pig from birth to six weeks of age. Bio. Neonate 58:220-226.

Kelley, K. W. 1980. Stress and immune function: A bibliographic review. Ann. Res. Vet. 11:445-451.

Kelley, K. W. 1988. Cytokines and hormones: Views of an immunophysiologists. Animal Science Distinguished Lecture presented at the University of TN, Knoxville, TN.

Kelley, K. W., H. J. Mertsching, B. A. Mathison, and C. R. Kissinger. 1982b Physiologic concentrations of cortisol suppress mitogenesis of porcine lymphoid cells. J. Ani. Sci. 54:191 (Abstract).

Klebanoff, S. J., and R. A. Clark. 1978. The neutrophil: Function and Clinical Disorders. North Holland, Amsterdam.

Knight, J. W., H. G. Kattesh, F. C. Gwazdauskas, H. R. Thomas, and E. T. Kornegay. 1982. Peripheral testosterone in boars after admininstration of hCG, ACTH, and testosterone at three ages. Theriogenology 17:383-392.

Laudenslager, M. L., M. Reite, and R. J. Harbeck. 1982. Suppressed immune

response in infant monkeys associated with maternal separation. Behav. Neural Bio. 36:40-48.

Lecce, J. G., W. D. Armstrong, P. C. Crawford, and C. Ducharme. 1979. Nutrition and management of early weaned piglets. I. Liquid vs dry feeding. J. Ani. Sci. 48:1007-1011.

McCauley, I., and P. E. Hartmann. 1984. Changes in piglet leucocytes, B lymphocytes and plasma cortisol from birth to three weeks after weaning. Res. Vet. Sci. 37:234-241.

McFarlane J. M., G. L. Morris, S. E. Curtis, J. J. McGone, and P. J. Dziuk. 1988. Turning and walking by gilts in modified gestation crates. J. Ani. Sci. 67:244-251.

Martens, J. G., R. J. Martens, and H. W. Renshaw. 1988. Rhodococcus (Corynebacterium) equi: Bactericidal capacity of neutrophils from neonatal and adult horses. Am. J. Vet. Res. 49:295-299.

Michaut, R. J., R. P. Dechambre, S. Doumerc, B. Lesourd, A. Devillechabrolle, and R. Moulias. 1981. Influences of early maternal deprivation on adult humoral immune response in mice. Physiol. Behav. 26:189-91.

Moss, B. W., and C. H. McMurray. 1979. The effects of the duration and type of stress on some serum enzyme levels in pigs. Res. Vet. Sct. 26:1-6.

Morris, D. D., G. Gaulin, P. J. Strzemienski, and P. Spencer. 1987. Assessment of neutrophil migration, phagocytosis and bactericidal capacity in neonatal foals. Vet. Immuno. Immunopath. 16:173-179.

Munck, A., P. M. Guyre, and N. J. Holbrook. 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endoc. Rev. 5:25-44.

Nienaber, J. A., and G. L. Hahn. 1989. Cool nighttime temperature and weaning age effects on 3 to 10 week old pigs. Transactions of the ASAE, March-April 1989, 32:691-694.

Pardue, S. L., and J. P. Thaxton. 1984. Evidence of amelioration of steroid-mediated immunosuppression by asocrbic acid. Poult. Sci. 63:1262-1268.

Rampacek, G. B., R. R. Kraeling, E. S. Fonda, and C. R. Barb. 1984. Comparison of physiological indicators of chronic stress in confined and nonconfined gilts. J. Anim. Sci. 58:401-408.

Ras, G. J., A. J. Theron, R. Anderson, G. W. Taylor, R. Wilson, P. J. Cole, and C. A. Van der Merwe. 1992. Enhanced release of oxidative inactivation of alpha-1-protease inhibitor by stimulated human neutrophils exposed to Pseudomonas Aeruginosa pigment 1-hydroxyphenazien. J. Infect. Dis. 166:568-573.

Rest, R. F., S. H. Fischer, Z. Z. Jones, and J. F. Jones. 1982. Interactions of Neisseria gonorrhoeae with human neutrophils: effects of serum and gonococcal opacity on phagocytic killing and chemiluminescence. Infect. Immuno. 36:737-744.

Rey, Del A., H. Besdovsky, E. Sorkin, and C. A. Dinarello. 1987. Interleukin-1 and glucocorticoid hormones integrate and immunoregulatory feedback circuit, in: B. D. Jankovic, B. M. Markovic, N. H. Spector (eds): Ann. NY Acad. Sci. 496:85-90.

Robinson, G. A., and E. W. Sutherland. 1971. Cyclic AMP and the function of eukaryotic cells: An introduction. Annals N.Y. Acad. Sci. 185:5-7.

Roth, J. A., and M. L. Kaeberle. 1981. Evaluation of bovine polymorphonuclear leukocyte function. Vet. Immuno. Immunopath. 2:157-174.

SAS. 1985. SAS User's Guide: Statistics (Version 5). SAS Inst. Inc., Cary, NC.

Savi, L., H. Zouaghi. A. Carli, and E. A. Nunez. 1981. Serum depletion of corticosteroid binding activities, an early marker of human septic shock. Biochem. Biophys. Res. Commun. 102:411-419.

Schultz, R. D., J. T. Wang, and H. W. Dunne. 1971. Development of the humoral immune response of the pig. Am. J. Vet. Res. 32:1331-1336.

Selye, H. 1946. The general adaptation syndrome and the diseases of adaptation. J. Clin. Endo. Metab. 6:117-123.

Sheridan, J. F., N. Feng, R. H. Bonneau, C. M. Allen, B. S. Huneycutt, and R. Glaser. 1991. Restraint stress differentially affects anti-viral cellular and humoral immune responses in mice. J. Neuroimmuno. 31:245-255.

Siegel, H. S. 1985. Immunological responses as indicators of stress. Worlds. Poult. Sci. J. 41:36-44.

Siegel, H. S., and W. B. Gross. 1965. Social grouping, stress, and resistance to coliform infection in cockerels. Poult. Sci. 44:1530-1536.

Siegel, H. S., and J. W. Latimer. 1970. Bone and blood calcium responses to adrenocorticotropin, cortisol and low environmental temperature in young chickens. Proc. 14th World Poult. Congress, Madrid, 1970.

Spitznagel, J. K. 1983. Microbial interaction with neutrophils. Rev. Infect. Dis. 5(Suppl):S806-S822.

Taylor, L., and T. H. Friend. 1987. Effect of housing on open-field test behavior of gestating gilts. Appl. Ani. Behav. Sci. 17:83-93.

Thomsen, M. K., A. L. Jensen, T. Skak-Nielsen, and J. Kristensen. 1991. Enhanced granulocyte function in a case of chronic granulocytic leukemia in a dog. Vet. Immuno. Immunopath. 28:143-156.

Weicker, H., and E. Werle. 1991. Interaction between hormones and the immune system. Int. J. Sports Med. 12:S30-S37.

Weigent, D. A., and J. E. Blalock. 1987. Interactions between the neuroendocrine and immune systems: Common hormones and receptors. Immuno. Rev. 100:79-88.

Westly, H. J., and K. W. Kelley. 1984. Physiologic concentrations of cortisol suppress cell-mediated immune events in the domestic pig. Proc. Soc. Exp. Bio. Med. 177:156-164.

Whipp, S. C., R. L. Wood, and N. G. Lyon. 1970. Diurnal variation in concentrations of hydrocortisone in plasma of swine. Am. J. Vet. Res. 31:2105-2107.

Worsaae, H., and M. Schmidt. 1980. Plasma cortisol and behavior in early weaned piglets. Acta. Vet. Scand. 21:640-657.

Zarkower, A., M. L. Eskew, W. J. Scheuchenzuber, F. G. Ferguson, and F. Confer. 1982. Antibody-dependent cell-mediated cytotoxicity in pigs. Am. J. Vet. Res. 43:1590-1593.

APPENDICES

APPENDIX A

PROCEDURE USED FOR PBMC BLASTOGENIC ASSAY

I. Reagents

A. Complete RPMI

1. Supplement 477.5 mL of RPMI 1640 (Grand Island Biological Co. [GIBCO],Grand Island, NY) with,
   12.5mL Hepes buffer (1M stock),
   5mL L-glutamine (0.2 M stock),
   5mL antibiotic solution: containing 50,000 U Penicillin and 50 mg streptomycin, and 2 μl 2-mercaptoethanol (30mM stock)
2. Solution good for 2 wk at 4 °C
4. Sterilize

B. Complete RPMI plus Fetal bovine serum (FBS)

1. Add 10% (i.e. 5 mL to FBS to 45 mL complete RPMI) FBS (Sigma Chemical Co., St. Louis, MO) immediately prior to use
2. Solution is good for 2 d at 4 °C
3. Sterilize

C. Phosphate Buffered Saline (PBS)

1. To 100 mL distilled water add:

2.7 g NaCl

.115g Na_2HPO_4

.020g KH_2PO_4

2. Adjust pH to 7.3

3. Sterilize

4. Store at 4 °C

D. Phosphate Buffered H_2O (PB H_2O)

1. To 100mL distilled water add:

.115g Na_2HPO_4

.020g KH_2PO_4

2. Adjust pH to 7.3

3. Sterilize

4. Store at 4 °C

E. 2 x PBS

1. To 100mL distilled water add:

1.6g NaCl

.115g Na_2HPO_4

.020g KH_2PO_4

.040g KCl

2. Adjust pH to 7.3

3. Sterilize

4. Store at 4 °C

II. Isolation of Peripheral Blood Mononuclear Cells (PBMC)

A. Collect 20mL of whole blood in 25cc syringe with 3mL of 1.5% EDTA added to syringe under sterile condition

B. Constantly mix blood well

C. In a sterile hood add 10-11mL of blood into 50mL centrifuge tubes

D. Add PBS to 40mL of whole blood and mix well

III. Ficoll Gradient Centrifugation

A. Mix tubes well immediately before adding Ficoll

B. To 40mL of PBMC and PBS , SLOWLY AND STEADILY layer 10mL of Ficoll (Histopaque 1077) on bottom, using sterile blunt end metal needle tips 8 in in length

C. Centrifuge Ficoll tubes at 400 x *g* at 18 °C for 50 min with NO BRAKE in free swinging buckets designed for the centrifuge

IV. Cell Isolation

A. Aspirate top layer (~ 25mL) and discard

B. With a transfer pipet remove PBMC layer, without disturbing RBC layer in bottom of tube

C. Transfer cells to new 50mL centrifuge tube and wash with PBS

D. Centrifuge at 300 x *g* for 15 min at 4 °C

V. Lysing of any remaining RBCs

A. Remove PBS from previous wash, without disturbing pellet

B. To cell pellet add 0.5mL PB H_2O and vortex lightly

C. After 45 seconds add 0.5mL 2 x PBS and vortex lightly

D. Add PBS up to 50mL and centrifuge at 300g for 15 min at 4 °C

E. Repeat if necessary

F. Resuspend pellet in 3-5mLs complete RPMI + FBS

VI. Cell Counting

A. Dilute cell sample 1:20 (i.e. 50 μl of cell suspension and 950 μl of RPMI)

B. Add 100 μl of cell dilution to 100 μl Trypan Blue dye

C. Let stand at least 5 min and no longer than 15 min

D. Count the 4 corner squares of a hemacytometer

E. Record total cells, viable cells, and non-viable cells

1. Should be greater then 90% viable

F. Calculation:

N/4 x 20 x 2 x 10,000 = # cells/mL

N = number of viable cells

4 = squares counted

20 = cell dilution

2 = dilution factor due to Trypan Blue

10,000 = Hemocytometer dilution factor

G. Resuspension of Cells:

$V_i = C_f \times V_f / C_i$

V_i = initial volume of cells required (unknown)

C_f = final cell concentration needed

V_f = final volume needed

C_i = initial cell concentration (known from above)

VII. Cell Plating

A. For each sample in triplicate add the following to all wells of a 96 well culture plate:

1. 100 μl cells at 2×10^5 cells/well
2. 100 μl mitogen (if more than one mitogen is used each one is plated in triplicate)

B. A triplicate of control wells is plated for each sample that receives no mitogen.

VIII. Pulsing

A. After 48 h of incubation at 39 °C in a humidified, 5% CO_2 air chamber, 50 μl of ^3H thymidine is added to all wells (1 μCi/well)

B. The plate is then incubated for another 24 h

IX. Harvesting and Counting

A. Plates are harvested onto glass fiber filters using an automated cell harvester and added to 6 mL plastic scintillation vials

B. Add 3ml of scintillation fluid (Scintiverse II) to each vial

C. Each vial is counted for 1 min in a beta scintillation counter and readings recorded as counts per minute

APPENDIX B

PROCEDURE FOR ULTRCENTRIFUGATION ASSAY FOR DETERMINATION OF PERCENT DISTRIBUTION OF CORTISOL IN PLASMA

I. Membrane Preparation

A. Boil 1-2ft sections of dialysis tubing (Fisher Scientific; spectra/por, molecular weight 12,000 -14,000 daltons, and a size of 25mm x 15.9mm diameter) for 30 to 45 min in 95% ethanol

B. Rinse entensively with distilled water to remove ethanol

C. Boil membrane twice for 15-20 min in a 2 L solution of 5-10mg of disodium EDTA and 5-10mg of sodium carbonate

D. Rinse with distilled water between boiling and when finished rinse extensively to remove all EDTA (CAUTION - EDTA can interfere with the assay results)

E. Store membrane in a .02% sodium aside solution at 4 °C no longer than 1 mo

II. Capsule Preparation

A. Rinse membrane before use to remove EDTA and sodium azide

B. Cut dialysis membrane into small squares that fit over the fire polished end of a glass capsule and hold it in place with rubber bands made from latex tubing

(Pimeline Industries, Inc., 1/3" x 1/32")

C. Trim any excess membrane from around the rubber band, being careful to check for any tears that might interfere with the assay

D. Store capusules in a 0.02% sodium azide solution for no more than 7 d prior to use

E. Blot tube dry and insert, membrane down, into scintillation vials containing three filter paper discs (Whatman No. 1, 13mm diameter) (Do this step during the 30 min incubation at room temperature in III. A. 5.)

F. Discard capsule in proper place after use

III. Assay Procedure

A. Plasma Preparation

1. In *n* disposable glass test tubes (12 x 74mm) add 5 μl ^{3}H-Cortisol (3 x10^{5} DPM) and evaporate in Vaccum oven

2. Add 5 μl of ^{14}C-glucose (12 x 10^{3} DPM) to each of the *n* tubes

3. Add 500 μl of plasma to each tube and vortex

4. Incubate at 37 °C for 30 min then an

additional 30 min at room temperature

B. Centrifugation of Dialysis Capsules

1. Pipette duplicate alliquots (200 μl) of the plasma onto the dialysis membrane at the bottom of the inner tubes (labling the duplicates as *n*a and *n*b)
2. Centrifuge at 2500 rpm for 1 h at 37 °C
3. After centrifugation carefully remove the inner tubes and pipette 30_l of plasma onto a filter disc at the bottom of another scintillation tube labeled as *n*(a or b)p (p representing plasma; this represents the amount of bound cortisol)
4. If any membranes are torn or any tubes broken make a note of sample number in case the results are affected

C. Counting

1. Add 300 μl of distilled H_2O to all scintillation vials
2. Vortex
3. Add 3mL scintillation cocktail (Scintiverse II),cap, mix, and count on a beta counter with both ^{14}C and ^{3}H channels

APPENDIX C

PROCEDURE FOR ISOLATION OF PORCINE NEUTROPHILS

I. Neutrophil Isolation

A. Place 5-10mL of whole blood with a 1.5% EDTA solution into siliconized 25mL glass centrifugation tubes

B. Centrifuge tubes for 20 min at 2000 rpm in free swinging buckets

C. Remove the plasma, buffy coat and top 2/3 of the RBC layer carefully and discard

D. Lyse RBC's by adding 2 volumes of PB H_2O (solution receipe in Appendix A, I., C.) followed by mixing end to end for 50 sec

E. After 50 sec immediately add 2 volumes of 2 x PBS in Appendix A, I., D.) and mix end to end

F. After lysing, tubes are centrifuged at 1500 rpm for 15 min in free swinging buckets

G. Remove supernatant by aspiration and discard

H. Repeat lysing if necessary

I. Resuspend pellet in 3mL Hanks Balanced Salt Solution (Sigma Chemical Company, St. Louis, MO)

II. Cell Counting

A. Dilute cell sample 1:20

B. Add 100 μl of cell dilution and 100 μl of Trypan Blue

dye (Allow to stand for at least 5 min and no longer than 15 min)

C. Place samples on hemacytometer and count the four corners of the slide

D. Record total cells, viable and non-viable cells

E. Viability should be greater than 85%

F. Calculations:

N/4 x 20 x 2 x 10,000 = # cells/mL

N = number viable cells

4 = squares counted

20 = cell dilution

2 = dilution factor due to Trypan Blue

10,000 = Hemocytometer dilution factor

G. Resuspension of Cells

$V_i = C_f \times V_f / C_i$

V_i = initial volume of cells required (unknown)

C_f = final cell concentration needed

V_f = final volume needed

C_i = initial cell concentration (known from above)

APPENDIX D

PROCEDURE FOR SPECTROPHOTOMETRIC ASSAY OF ELASTASE

I. Elastase Activity of Degranulation

A. Neutrophils are adjusted to a concentration of 1 x 10^7/mL in Hanks Balanced Salt Solution (HBSS) (Sigma Chemical Co., St. Louis, MO)

B. Stimulate neutrophils with 10 μM Cytochalasin B (Sigma Chemical Co.) for 15 min in 37 °C water bath

C. Place tubes on ice to stop reaction

D. Centrifuge for 5 min, 4 °C, at 300 x *g*

E. In a 96 well culture plate, add 125 μl of cell free supernatant and 125 μl substrate (N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide, Sigma Chemical Co.; .7 mM)

F. Run standards of supernatant and substrate

G. All samples are run in triplicate

H. Read on spectrophotometer at 410 nm

I. Readings are adjusted by subtracting the mean value of the standards from the mean value of the samples

II. Total Cellular Elastase

A. Neutrophils are adjusted to a concentration of 1 x 10^7/mL in HBSS and frozen

B. Cell suspensions are freeze-thawed 3 times

C. Cell suspensions are then centrifuged for 15 min, 4 °C,

at 300 x *g*

D. Supernatant is then analyzed as described above in I., E-I.

APPENDIX E

PROCEDURE USED FOR NEUTROPHIL PHAGOCYTOSIS ASSAY

I. Labeling of K88$^+$ *E. coli*

A. *E. coli* K88$^+$ are grown in 500mL of brain heart infusion broth (BHI) containing 250 μCi ^{125}IUdr

B. Incubate for 24 h at 37 °C

C. Aliquot culture into 10 50mL centrifuge tubes and centrifuge at 1000 x *g* for 20 min

D. Remove supernatant by aspiration and discard

E. Add 5 mL of PBS to each vial and heat kill at 56 °C for 1 h

F. Wash vials with PBS and centrifuge at 450 x *g* for 10 min

G. Aliquot culture containing 1.5 x 10^9 colony forming units to vials and store at 4 °C until use

H. Aliquots are thawed and diluted 1:10 for use in phagocytic assay

II. Phagocytic Procedure

A. Adjust neutrophils to a concentration of 5 x 10^7 cells/mL

B. All samples, standard, and backgroud tubes are run in duplicate

C. In 12 x 75 mm plastic tubes add 100 μl of ^{125}I K88$^+$ and 300 μl Earls Balanced Salt Solution (EBSS) (Sigma

Chemical Co., St. Louis, MO) to each tube and incubate for 15 minutes in a shaking water bath at 37 °C

D. Add 50 μl of neutrophils to every tube except the background tubes and incubate exactly 10 min in shaking water bath at 37 °C

E. Add 0.5mL PBS containing 0.5 units of lysostaphin (Sigma Chemical Co.) to every tube except the standard tubes and incubate in shaking water bath at 37 °C for 30 min

F. Add 2 mL of COLD PBS to every tube to stop the reaction

G. Centrifuge at 1250 x *g* for 10 min at 4 °C

H. Aspirate and discard supernatant

I. Wash pellet twice

J. Count vials in a gamma counter and record as counts per minute (cpm)

VITA

Marsha Elaine Brown was born in Memphis, Tennessee on June 7, 1970 to Edward B. Brown, Jr. and Christe Ford Brown. She has one sister, Jenny Diane Brown born on March 11, 1977. In August of 1988 she entered the University of Tennessee, Knoxville in pursuit of a Bachelor of Science degree in Animal Science. In May of 1992 she graduated. By July 1st of 1992 she was enrolled in graduate school at the University of Tennessee, Knoxville in the Department of Animal Science. On December 11, 1993 she was married to John David Crutchfield, another graduate of The University of Tennessee, Knoxville. In August of 1994 she received her Master of Science Degree from The University of Tennessee, Knoxville.