ABSTRACT

POOLPERM, PARIWAT. Factors Influencing Semen Quality and Fertility in Boars. (Under the direction of Drs. Glen W. Almond and William L. Flowers)

The purpose of this research was to evaluate factors that influence semen quality and fertility in boars. The main objectives were to 1) determine the influence of antibiotics on semen quality, 2) determine the association among IGF-I in seminal plasma, semen quality, and fertility, and 3) retrospectively study the association among semen characteristics and sow fertility on commercial pig farms. In the first study, semen was diluted in BTS extender to 3x10^9 spermatozoa/80 ml. The effects of gentamicin (GM), amikacin (AM), neomycin sulfate (NM), and penicillin-streptomycin (PS) on the percentages of motile (MOT), morphologically normal sperm (MOR), and sperm with normal acrosome (NAR) were examined on day 0, 1, 2, 3, 5, and 7 of storage. An in vitro penetration assay was conducted using the diluted semen with the four types of antibiotics on day 0 and day 5 of storage. MOT differed (p<0.05) among treatments, independent of boars, but MOR and NAR were similar. GM and NM groups showed higher (p<0.05) MOT compared to other groups and control (CT). All treatments showed the same trend of declining MOT from day 1 to day 5. However, after day 5 of storage, MOT in PS and CT declined at a faster rate. The concentrations of gentamicin and amikacin sulfate did not change during 120 hours of storage. No differences in in vitro penetration rate were found among treatments. However, the penetration rate decreased (p<0.05) on day 5 of storage.
In the second study, an aliquot (5 ml) of seminal plasma was collected from each ejaculate after centrifugation. The rest of the ejaculate was extended in a commercial extender (Vital™) to 2.5-3.5x10^9/dose, and stored at 17°C. Gilts (n=113) and sows (n=375) in six nucleus herd farms were inseminated with the extended semen in homogenetic-homospermic regimens. MOT, MOR and NAR were determined for each ejaculate. Insulin-like growth factor-I (IGF-I) was determined in the seminal plasma by RIA. Concentration of IGF-I from a total of 204 ejaculates was 95.38 ± 3.56 ng/ml (mean ± SE) and total amount of IGF-I per ejaculate was 23.50 ± 1.20 µg. IGF-I concentration and total amount of IGF-I per ejaculate differed (p<0.05) among genetic lines. IGF-I levels in boar seminal plasma had no effect (p>0.05) on MOT, MOR and NAR. However, IGF-I was associated (p<0.05) with semen volume, sperm concentration and total number of sperm/ejaculate. No association between IGF-I levels in seminal plasma and fertility indices was found. IGF-I levels were associated with sperm production parameters. However, no associations with sperm characteristics and fertility parameters were evident.

The third study retrospectively studied the associations among insemination parameters with subsequent fertility of boar semen. Farrowing rate (FR), total pigs born (TB) and born alive (TBA) were recorded for 388 inseminations from 98 ejaculates. Only MOR was associated (p<0.05) with fertility parameters. Insemination with semen, stored for 0-2 days (SAGE), had higher (p<0.05) TB and TBA than semen stored ≥ 3 days. Furthermore, insemination with ≥ 3x10^9 sperm/dose (AIDOSE) had higher (p<0.05) TB and
TBA than $< 3 \times 10^9$ sperm/dose. With stepwise regression analysis, it was evident that FR was associated with semen volume, MOR and SAGE. Meanwhile, TB and TBA were associated with SAGE, AIDOSE and total number of spermatozoa per ejaculate. In conclusion, the assessment of semen characteristics may not necessarily delineate fertility between boars.
FACTORS INFLUENCING SEMEN QUALITY AND FERTILITY IN BOARS

by

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GENERAL INTRODUCTION

To achieve fertilization, the male and female gamete need to be fertile and exposed to each other in the optimal environment. Production of the male gametes, the spermatozoa, takes place in a small-convoluted tubule, namely the seminiferous tubule. Spermatogenesis is the process of producing spermatozoa from stem cell or spermatogonia, which undergoes meiosis to produce spermatocytes and ultimately spermatozoa. Spermatogenesis is regulated by endocrine and paracrine systems. Gonadotropins (luteinizing hormone and follicle-stimulating hormone) are the most important endocrine hormones that initiate and maintain spermatogenesis. Androgens, produced by Leydig cells, regulate spermatogenesis through Sertoli cells. To be fertile, spermatozoa need to go through maturation processes, which consist of epididymal maturation, sperm capacitation and acrosome reaction. Acrosome reacted spermatozoa are ready to fertilize a matured oocyte.

The fertility of spermatozoa is difficult to estimate or predict. Researchers have tried to find the best indicator or predictor for sperm fertility. Many sperm quality and function tests were evaluated to find correlations of those tests and fertility. Semen analysis, including percentage of motile spermatozoa (MOT), percentage of morphologically normal spermatozoa (MOR) and percentage of spermatozoa with normal acrosome (NAR) are used worldwide to estimate quality of semen. Recently, other assays, such as in vitro penetration and in vitro fertilization (IVF), were applied to predict fertility of spermatozoa. Association
between semen quality parameters and IVF results was reported for humans. IVF is used as a fertility predictor of human spermatozoa; however, there is insufficient evidence to suggest that IVF can be used as a fertility predictor for pigs.

Many factors can interfere with the fertility of matured spermatozoa. Bacterial contamination of semen intended for artificial insemination was reported to have a detrimental effect on sperm quality, longevity and ultimately fertility. Inclusion of antibiotics in boar semen extender is to reduce those detrimental effects. Many types of antibiotics were studied on semen quality in bacterial-contaminated conditions. However, in minimal-contaminated conditions, there is no report of the impact of antibiotic on sperm cells and concentration of antibiotic in extended semen after prolonged storage. In artificial insemination, semen handling and management also can counteract semen fertility. Reducing number of spermatozoa per insemination dose or prolonged storage time of diluted semen has the potential to improve seminal efficiency. Unfortunately, these methods could have deleterious effects to fertility outcome.

Insulin-like growth factors (IGFs) consist of IGF-I and IGF-II. IGFs are mediator of growth hormone and can be found in several cell types, especially in high prolific cells. IGF-I was found in seminal plasma in men, bulls and boars, and was believed to be of testicular origin. Association of IGF-I in seminal plasma with semen quality was reported in men and in bulls. IGF-I was found in Leydig cells and Sertoli cells in prepubertal pigs and believed to play some roles.
in spermatogenesis. IGF-I in boar seminal plasma was not intensively studied for its association with semen quality and fertility.

The objectives of the studies reported in this dissertation were to 1) determine the influence of antibiotics on semen quality, 2) determine the association among IGF-I in seminal plasma, semen quality and fertility, and 3) retrospectively study the association among insemination quality parameters and fertility in commercial pig farms.
LITERATURE REVIEW

I. Spermatogenesis:

Mammalian testis:

The gonads in vertebrates develop along the ventral cranial part of the mesonephros, a fetal kidney-like organ (Balinsky, 1970). In early embryo development, the gonad is initially formed by the thickening of mesodermal cells lining the body cavity laterally to the dorsal mesentery, the coelomic epithelium. This thickening is called the germinal ridge (Balinsky, 1970; Setchell, 1978). In pig embryos, genital ridges can be seen at 26 days (Guraya, 1998). The primordial germ cells, which are alkaline phosphatase positive cells (Buehr, 1997), develop in the yolk sac (extraembryonic endoderm) and migrate into the genital ridges (Kierszenbaum, 1994). The migration of primordial germ cells toward the gonadal ridges depends on two factors: 1) the transient interaction between fibronectin molecules and corresponding primordial germ cell receptors, presumably integrins, and 2) the release of chemo-attractant factors by gonadal ridges (reviewed by Kierszenbaum, 1994). The gonadal ridges are transformed into undifferentiated gonads for both sexes. The number of primordial germ cells originally start at less than a hundred cells in the mouse embryo, however, during migration, the cells undergo repeated mitosis to increase in number. Eventually 5,000 cells or more are present in the germinal ridges (Balinsky, 1970). The primordial germ cells then migrate into the primitive sex cords of the medulla of the gonad, which is formed from germinal ridges. The primitive sex cords are converted into seminiferous tubules. The primordial germ cells give rise to the
spermatogonia and subsequently spermatozoa (Balinsky, 1970). The testis is comprised of two main parts, the seminiferous tubules and the intertubular cells. Spermatogenesis takes places in the seminiferous tubule. The intertubular cells consist of interstitial cells, fibroblast cells and Leydig cells, which produce steroid hormones used in spermatogenesis.

The seminiferous tubules can be divided into three compartments; basal, adluminal and luminal compartment. The basal compartment consists of basal cell membrane, spermatogonia and Sertoli cell. The adluminal compartment, in which differentiation of spermatocyte occurs, consists of primary and secondary spermatocytes, round spermatids and elongate spermatids. The luminal compartment consists of spermatozoa that were released from the Sertoli cells. The basal and adluminal compartment are separated by intercellular structures between adjacent Sertoli cells, which forms a continuous lock, so called the “blood-testis barrier” (Ritzen, 1983).

The spermatogonia lie at the base of the seminiferous epithelium. The spermatogonia act as stem cells, the type A spermatogonia are proliferative and transform into differentiating spermatogonia and simultaneously renew their population by mitotic replication (de Kretser et al., 1998). The replication of the spermatogonia are dependent on the expression of c-kit protein synthesized by Sertoli cells (de Kretser et al., 1998). The type A spermatogonia can be classified as A1, A2, A3, A4 and In (Intermediate), according to the differentiation process (Fig. 1). Type B spermatogonia are the final differentiated stage before the meiotic phase occurs in the spermatocytogenesis process (Russell et al.,
The differences among these types of spermatogonia are based on structure and morphology, using either light or electron microscopy.

**The Sertoli cell**, a fibroblast-like cell, provides mechanical support for the germ cells during spermatogenesis. Sertoli cells act as functional and structural bridges linking the blood-lymphatic intertubular space to protect seminiferous luminal compartment where the spermatozoa are transported to the epididymis (Kierszenbaum, 1994). There are several chemical messengers between Sertoli cells and germ cells (Ritzen, 1983). Some properties of Sertoli cells that affect germ cells include: the tight junction between the Sertoli cells, which prevents all macromolecules and some electrolytes from circulation to germ cells, and all hormones exert their effects on germ cells through Sertoli cells. Sertoli cells secrete large quantities of lactate, which is the substrate for glycolysis used by germ cells for energy metabolism. Sertoli cell activity is regulated by testosterone from Leydig cells and follicle-stimulating hormone (FSH) secreted from the anterior pituitary gland. Also, the Sertoli cell produces androgen-binding protein (ABP), plasminogen, transferrin, insulin-like growth factor I (IGF-I) and II (IGF-II) (Dym, 1994).

**The Leydig cells**, located in the inter-tubular space, produce and secrete androgens, mainly testosterone, in response to stimulation by luteinizing hormone (LH), released from the anterior pituitary gland (Saez, 1994; Sharpe, 1994). The main substrate for the synthesis of steroid hormones in Leydig cells is cholesterol (Saez, 1994). There are three enzymes, which are cytochrome P-450scc (cholesterol side chain cleavage), adrenodoxin, and adrenodoxin
reductase, involved in the conversion of cholesterol to pregnenolone. Then the biosynthetic pathway of androgen formation from pregnenolone is regulated by enzymes $3\beta$-hydroxysteroid dehydrogenase ($3\beta$-HSD), $17\alpha$-hydroxylase ($P450\alpha$), and $17\beta$-hydroxysteroid dehydrogenase (see Saez, 1994, for detail). Testosterone regulates spermatogenesis in the seminiferous tubules through the Sertoli cells. LH is absolutely required for maintenance of Leydig cell-specific functions, and this hormone is the main factor controlling testosterone secretion from Leydig cell under physiological condition (Saez, 1994; Sharpe, 1994). LH receptors, a G-protein-coupled receptor, were reported on the Leydig cells (Saez, 1994). The activation of steroid biosynthesis in Leydig cells by LH is regulated through cAMP as a second messenger (Saez, 1994). Exposure of Leydig cells to LH results in a sharp increase of cAMP and steroid production (Saez, 1994). Testosterone production by Leydig cells was dependent on LH stimulation from the anterior pituitary gland, and FSH stimulation through the Sertoli cell secretion, which suggested endocrine/paracrine regulation (Saez, 1994; Sharpe, 1994).

**Spermatogenesis** is the sequence of cellular divisions and developmental changes that occur within the seminiferous tubules of the testes (de Kretser and Kerr, 1988; Eddy and O'Brien, 1994). The spermatogenetic process is comprised of two major processes: 1) *spermatocytogenesis* which contains two processes; mitotic process of stem cells (spermatogonia) to form spermatocytes and meiosis to reduce the number of chromosomes to form spermatids, and 2) *spermiogenesis*, which is the transformation of spermatids in regards to metamorphic changes. Spermatocytogenesis and spermiogenesis are
closely associated with Sertoli cells, the nurse cells for spermatozoon inside the seminiferous tubules. The Sertoli cells contribute to the blood-testis barrier and supply the nutrients needed for the spermatogenic process. The spermatogonia or stem cells comprise A0, A1, A2, A3 and A4 type of spermatogonia. The division of type A4 forms either A1 spermatogonia or intermediate spermatogonia (In) and another division to form type B spermatogonia. The type B then divide mitotically to form pre-leptotene spermatocytes. When the spermatocytes enter meiotic division, the stage is then referred to the leptotene stage. At the end of the leptotene, the cells are called primary spermatocytes, which have duplicate number of chromosomes (4N). The primary spermatocyte undergoes progressive nuclear changes of meiotic prophase, to form secondary spermatocyte (2N). Then, the secondary spermatocyte undergoes meiosis II to reduce the chromosomal number to haploid (1N). The completion of meiosis required the presence of heat-shock protein 70 (HSP-70) (de Kretser et al., 1998). Genetically modified animals with HSP-70 gene deletion failed to complete meiosis and increased apoptotic rate of spermatocytes. At the end of the meiotic process, spermatocytes form round spermatids, which will undergo a progressive and complex series of structural and developmental changes to form spermatozoa (de Kretser and Kerr, 1988; de Kretser et al., 1998). Spermiogenesis is a series of progressive changes in structure and morphology of spermatids. The changes include 1) formation of spermatozoa flagella and development of a core of microtubules, 2) nuclear chromatin condensation and movement of the nucleus to the periphery of the cell, 3) formation of the
acrosomal cap to form the acrosome, and 4) formation of residual body by shedding a large part of cytoplasm, which is phagocytosed by the Sertoli cell (de Kretser and Kerr, 1988; de Kretser et al., 1998). Spermatozoon, a mature spermatozoa, is released to luminal compartment of the seminiferous tubule, called spermiation (Eddy and O'Brien, 1994).

**Maturation process of spermatozoa:**

Mature spermatozoa are released from the Sertoli cell into the luminal compartment in the seminiferous tubules. The spermatozoa are transported to epididymal duct through the vasa efferentia by the flow of testicular fluid secreted from Sertoli cells and Rete epithelium and intrinsic smooth muscle contractions of the tubules and testicular capsule (Moore, 1995). The epididymis is a single highly convoluted duct, which can be divided into three segments, gross anatomically, of head (caput), body (corpus), and tail (cauda). A number of functions of the epididymis have been reported, which include absorption of large volumes of fluid secreted into the rete testis by the seminiferous tubules, secretion of a variety of ions, small organic molecules, and glycoproteins into its lumen, biosynthesis and metabolism of steroids, and spermiophagy, and the phagocytosis of degenerate or dead spermatozoa (Robaire and Hermo, 1988). The epididymis also forms tight junctions throughout the duct, which is believed to be “blood-epididymis barrier” (Robaire and Hermo, 1988).

The main function of the epididymis is the maturation of spermatozoon released from seminiferous tubule. The process of epididymal maturation involves changes in several morpho-functional aspects of the spermatozoon (Briz
et al., 1995). The epididymal maturation is a gradual process, not an abrupt event (Holtz and Smidt, 1976). The basic maturation process involves the following: 1) progressive loss of water by absorption of large volumes of fluid secreted into the rete testis by the seminiferous tubules, 2) changes in the metabolic patterns and the structural condition of the tail, 3) modifications in patterns of movement, 4) detachment of the cytoplasmic droplet, 5) modifications of the plasma membrane, and 6) changes in the nuclear chromatin and modification of the acrosome (Briz et al., 1995). A number of spermatozoa structures become stabilized by disulfide bond formation, including flagellar structures that contributed to gain motility during epididymal transit (Katz, 1983). The capacity to fertilize is increased as the spermatozoa move down the epididymal duct. Incubation of caput spermatozoa with either cauda fluid or seminal plasma did not increase fertilizing capacity of spermatozoa (Holtz and Smidt, 1976). The spermatozoa require transit through the entire epididymal duct to achieve fertilizing capacity.

**Post ejaculatory maturation:**

The epididymal spermatozoon acquired the progressive motility and fertilization competence during epididymal maturation (Visconti et al., 1998). However, to gain complete fertilization capacity, the spermatozoon needs to undergo maturation process after ejaculation. These maturation processes include sperm capacitation and acrosome reaction, which involve a series of physiological and functional changes of the spermatozoa plasma membrane (Flesch et al., 2000; Visconti et al., 1998). Both maturation processes are critical
to the spermatozoa in fertilization of oocytes (Chang, 1984; Flesch et al., 2000). The maturation of the spermatozoa starts after it is released from the seminiferous tubules and undergoes physiological modification of the cell surface (Flesch et al., 2000). Capacitation, first described in 1952 by Austin, is necessary for acrosome reaction, which occurs at the site of fertilization. The capacitation involved increasing fluidity of plasma membrane by cholesterol efflux through albumin (Flesch et al., 2000; Visconti et al., 1998). The increase of intracellular Ca\(^{2+}\) (Carrell, 2000; Okamura et al., 1992; Visconti et al., 1998) and efflux of hydrogen ions (Carrell, 2000) also were reported in some species. The increases of protein kinase A (PKA) activities and cAMP concentrations as well as an elevation in bicarbonate were implicated in the regulation of adenylyl cyclase activity (Okamura et al., 1992; Visconti et al., 1998). In in vitro capacitation, the media required serum albumin, Ca\(^{2+}\) and bicarbonate to support the phosphorylation process during capacitation. Capacitation is believed to be the preparation step for hyperactivation and acrosome reaction, which are required for fertilization (Carrell, 2000; Chang, 1984).

The acrosome reaction is a morphological change of spermatozoa during transportation in the female reproductive tract (Chang, 1984). The acrosome reaction mostly occurs close to the site of fertilization, namely, the oviductal-ampulla. The acrosome develops during spermiogenesis in the seminiferous tubules, and contains hydrolytic enzymes, such as acrosin, hyaluronidase, proteases (Eddy and O’Brien, 1994). Acrosin is located between the inner and outer acrosomal membranes that cover the anterior portion of the spermatozoa.
(Eddy and O'Brien, 1994; Meizel, 1985). The inner membrane is adjacent to the nuclear membrane and the outer membrane underlies the sperm plasma membrane.

The acrosome reaction is composed of five morphological processes; 1) fusion of the plasma membrane and the underlying outer acrosomal membrane; 2) the fused membrane breaking; 3) the release of the acrosomal enzymes; 4) fusion of the outer membrane with the plasma membrane along the equatorial segment; and 5) the loss of reacted membrane (Meizel, 1985). In mammals, the AR is required for penetration through the zona pellucida layer of the oocyte. The regulation and site of acrosome reaction are still debatable. AR could occur in cumulus oophorus cells or on the zona pellucida (Meizel, 1985). In in vitro, the AR could be stimulated by serum albumin, hydrolytic enzymes and glycosaminoglycans (GAGs) (Meizel, 1985). Acrosome-stabilizing factor (ASF) was found in cells of the corpus epididymis and believed to inhibit acrosome reaction stimulation by follicular fluid (Oliphant et al., 1985).

Semen is deposited into the female reproductive tract near the os cervix in cattle and sheep, into the uterus in pigs, and partially into the uterus in horses (Hafez and Hafez, 2000). The movement of flagella or tail of spermatozoa expresses the vitality of a sperm cell, and is important for its reproductive function (Katz et al., 1989). To reach the site of fertilization, namely, the oviductal-ampulla, spermatozoa need to migrate through many barriers in the uterus, and oviduct. In many species, including humans, cervical mucus is the first barrier for spermatozoa transportation. The cervical mucus is a fluid with
complex physical properties, which contributes to important functions of sperm cells (Hunter, 1981; Hunter, 1999; Katz et al., 1989). The penetration of spermatozoa through the mucus might be dependent upon three factors; the motility and number of spermatozoa, hydrolytic enzymes in seminal plasma, and contractility of the female reproductive tract. Hunter (1981) found that spermatozoa could reach the oviduct within 30 minutes after insemination and the study suggested the presence of the spermatozoa reservoir in the female reproductive tract, particularly at the uterine-tubal junction or UTJ. The UTJ environment is composed of glycoprotein secretion that accumulates as mucus-like material in the caudal part of isthmus (Hunter, 1999). The function of the mucus-like material include: 1) deprivation of seminal macromolecules on spermatozoa surface; 2) diminishing the motility of spermatozoa; 3) preventing uterine leukocytes entry; and 4) reducing metabolism rate of spermatozoa by protecting the exposure of ampullary fluid (Hunter, 1999). During ovulation, local influence of pre-ovulatory progesterone was believed to stimulate the releasing of spermatozoa from the UTJ into the oviduct (Hunter, 1999). More spermatozoa were found at the fertilization site and more fertilized eggs were recovered when insemination occurred during or after ovulation (Hunter, 1981).

**Endocrine/paracrine control mechanisms:**

Testicular function, which includes spermatozoa production and the production of androgens, is regulated by the endocrine system. The endocrine input is provided by the hypothalamic-pituitary axis. Interactions within the hypothalamic-pituitary-gonadal axis involve a complex array of endocrine,
paracrine and autocrine mechanisms. The anterior pituitary gland secretes gonadotrophins, namely luteinizing hormone (lutropin; LH) and follicle-stimulating hormone (follitropin; FSH) (Halvorson and Chin, 1999). Gonadotropins are released following stimulation of the pituitary by pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus. The GnRH is a short half-life (less than 10 min) decapeptide produced from neurons in the hypothalamus, and acts through interaction with a specific receptor. A group of neuroendocrine cells in the preoptic hypothalamus secrete pulses of GnRH into the portal blood system. The GnRH is transported by the portal system to the anterior pituitary gland, binds to specific membrane receptors in the gonadotrophs. The GnRH enhances the pituitary gland to release tonic concentrations and pulses of LH, and a relatively continuous stream of FSH to the peripheral blood (Blache et al., 2000; Halvorson and Chin, 1999).

The pituitary gonadotropins, LH and FSH, are critical for the regulation of gonadal function and reproduction in mammalian species. Both FSH and LH are important in the regulation of the production of spermatozoa and hormones in the testis (Blache et al., 2000; Halvorson and Chin, 1999; Leung and Steele, 1992; Weinbauer et al., 2000). LH and FSH are glycoprotein hormones and share similar chemical and structural features. They are composed of similar α-subunits, which are identical polypeptide backbone and apoprotein. The difference between these two hormones is the β-subunit, which has unique protein sequences. The β-subunit confers specific activity on the αβ-heterodimer of each hormone (Halvorson and Chin, 1999).
The LH is released in a pulsatile fashion from the pituitary gland to the blood circulation. The pulses of LH bind to specific receptors in Leydig cells and stimulate production of testosterone and other androgens. The FSH associates with seminiferous tubular epithelium and Sertoli cell receptors to produce androgen-binding protein (ABP) and to stimulate spermatogenesis. In Sertoli cells, testosterone, produced in the testes, is converted to dihydrotestosterone (DHT) and estrogen for spermatid maturation. The role of FSH in spermatogenesis has not been fully described. Boars with low plasma FSH concentrations had smaller testicle size, but greater length and greater volume of seminiferous tubules (Zanella et al., 1999). A reduction in spermatozoa daily output and a decrease in spermatozoa concentration were found in boars and men, respectively, with high serum FSH concentrations (Novero et al., 1997; Zanella et al., 1999).

The biosynthesis and secretion of gonadotropins are regulated by several factors (Fig. 2). Gonadotropin expression is modulated by hypothalamic factors, gonadal feedback and intrapituitary factors. The GnRH is the primary hypothalamic factor controlling gonadotropin expression. The GnRH was first identified as LH-releasing hormone (LHRH); however, it is now known that stimulates release of LH and FSH. GnRH is released as a pulsatile signal, which is correlated to the release of LH. Two gonadal feedback systems; namely, gonadal steroid and activin-inhibin-follistatin systems, modulate gonadotropin biosynthesis. The gonadal steroids include the estrogens, progesterones, and androgens. These steroids show their effects at both the hypothalamus and the
anterior pituitary gland. Estrogens have both positive and negative effect on gonadotropin secretion. The inhibitory effects of estrogen likely are mediated through the pituitary gland by decreasing GnRH-mediated LH secretion. The positive feedback effect of estrogen on gonadotropin secretion is shown as the mid-cycle surge of LH in female (Halvorson and Chin, 1999).

Androgens are likely to show impacts at both hypothalamus and pituitary. Testosterone treatment of orchiectomized animals decreases $\alpha$-subunit and LH-$\beta$-subunit but not FSH $\beta$-subunit mRNA levels. However, within the pituitary, androgens stimulate FSH $\beta$-subunit mRNA levels and FSH secretion with no effects, if any, on $\alpha$-subunit or LH $\beta$ gene expression. Activin, inhibin and follistatin are three polypeptides secreted from the gonad. Inhibin is currently believed to be the most important peptide for the feedback regulation of gonadotropin gene expression. Inhibin and follistatin significantly decreased FSH $\beta$-subunit mRNA levels and FSH secretion (Halvorson and Chin, 1999).

Androgens are important hormone in male. They play important roles during sexual differentiation, during development and maintenance of secondary sex organs, and during the initiation and maintenance of spermatogenesis. The two most important androgens are testosterone and 5$\alpha$-dihydrotestosterone (DHT) (Brinkmann, 2001). The Leydig cell is the steroidogenic cell in the testis. The Leydig cell synthesizes and secretes testosterone and a small amount of DHT locally (Saez, 1994). DHT is an active metabolite form of androgen. Testosterone is transformed into DHT or estradiol by enzymes 5$\alpha$-reductase and
cytochrome P450 aromatase, respectively. Androgens also play an important role in male brain during sexual differentiation (Pouliot et al., 2001). Androgen is reported to involve in male reproductive behaviors and non-reproductive behaviors, such as aggressive, cognitive function and mood (O'Connor et al., 2001; Pouliot et al., 2001). In testis, the Sertoli cells produce androgen-binding protein (ABP), which binds to androgen. The androgen is then metabolized to DHT in the Sertoli cell used in spermatogenesis. The production of ABP by Sertoli cell is regulated by hormones, including FSH and androgens (Joseph et al., 1990). DHT bound ABP was reported to be a major source of testosterone and DHT in epididymal function (Joseph et al., 1990).

O'Donnell et al (2001) reviewed the impact of estrogen hormone on spermatogenesis. The review showed that estrogen receptor (ER) has found in many cell types of testis during development and adult period. Both ER and aromatase were found in Sertoli cell and Leydig cell as well as spermatocytes and spermatids. A very high level of estrogen and ER also found in efferent ductules and epididymis, which could point to a role of estrogen in spermatozoa maturation process (O'Donnell et al., 2001).

II. Assessing Fertility of Semen:

Fertility is described as the capacity or ability to conceive or induce conception and thus generate or produce offspring (The Online Medical Dictionary). Fertility in males requires “production of a sufficient number of mature and motile spermatozoa that can undergo capacitation and acrosome reaction to bind and penetrate the zona pellucida of the oocyte, which
subsequently divides and forms a viable fetus” (Naz and Minhas, 1995).
Defective sperm functions can lead to infertility in males. Sperm functions can be
determined from assessment of semen parameters, which include concentration,
maturation, motility, capacitation, acrosome reaction, binding and penetration of
zona pellucida (Naz and Minhas, 1995). Two parameters, namely, spermatozoa
production and maturation contribute to male fertility. To achieve fertilization,
spermatozoa need to complete the maturation process, which include epididymal
maturation, capacitation in the female reproductive tract, and acrosome reaction
at the fertilization site. Defects in any process can lead to spermatozoa infertility
or subfertility.

**Semen analysis** is a standard starting point in assessment of semen
quality. In general, the analysis composed of semen volume, concentration,
density, and spermatozoa motility, morphology and acrosome evaluations.
These relatively low cost of semen analysis, with the sufficiency of practical
information gained, makes the assay very cost effective (Carrell, 2000).
However, many steps of fertilization were not evaluated by the semen analysis,
such as the ability of spermatozoa to penetrate the oocyte plasma membranes,
also the analysis did not evaluate the capability of spermatozoa to undergo
In general, the quality assessments in semen have been: the percentage of
motile spermatozoa, percentage of morphologically normal spermatozoa and
percentage of spermatozoa with normal acrosome (Colenbrander and Kemp,
1990; Flowers, 1997; Martin Rillo *et al.*, 1996; Tardif *et al.*, 1999; Woelders,
1991), DNA and lipid content of spermatozoa, ability to pass through a sephadex-glass wool filter (Saacke, 1982), and seminal fluid characteristics (Carrell, 2000). The most correlated parameter with fertilizing ability is the percentage of morphologically normal spermatozoa (Kruger et al., 1986; Rogers et al., 1983). However, the semen analysis is not a complete or accurate predictor of fertilization ability (Carrell, 2000; Clark et al., 1989; Flowers, 1997; Kruger et al., 1986; Popwell and Flowers, 1998; Rogers et al., 1983). Semen analysis has some limitations as reviewed by Carrell (2000). Semen quality is substantially variable and unique in each ejaculation. Spermatozoa with normal semen analysis results might be defect in the functional ability, for example, capacitation or acrosome reaction defects, which may contribute to subfertility or infertility in male. Actual event involved in normal fertilization process was not directly measured by semen analysis (Carrell, 2000). Therefore, a major focus of recent research of semen and spermatozoa has been the development of assays that help in assessing the spermatozoa functions (Carrell, 2000).

In human, over thirty percent of couples with infertility were associated with male related problems (Minhas and Ripps, 1996). Subfertility in boars has received limited attention (Clark et al., 1989). Subfertility boars may be detrimental to farm breeding performance. Breeding performance in farms is not dependent only on semen fertility, but also female fertility is an important factor. However, in this review, the main focus will be on semen fertility. In semen, fertility assessment can be done with several methods. The tests of sperm function include the evaluation of sperm viability, motility profiles, ability of
acrosome reaction and oocyte penetration (Tardif et al., 1999). The in vitro fertilization (IVF) technique has been used as an estimation of the fertilizing ability of the spermatozoa (Tardif et al., 1999). In boars, fertility assessment is debatable. In general, semen characteristic parameters were used as a semen quality predictor, not fertility. Percentage of motile spermatozoa (MOT), percentage of morphologically normal spermatozoa (MOR) and percentage of normal acrosomal ridge (NAR) were used as the first criteria to assess semen quality (Flowers, 1997). Mostly the utilization of these parameters was used as a screening assessment for semen quality. It was established that semen with acceptable quality should contain MOT of not less than 60%, MOR of not less than 80% and NAR of not less than 90% (Johnson et al., 2000). However, for semen with acceptable levels of these parameters, the distinguishability of fertility among the semen could not be achieved. To achieve that, more sophisticated methods have been introduced.

**Sperm function assays** include the sperm penetration assay, the hemizona assay, and various forms of acrosomal reaction assays (Carrell, 2000). These assays are more advanced in the validation and standardization process than semen analysis. However, the usefulness of these assays is still debatable. Sperm function assays can be divided into three categories; biochemical or ultrastructure analyses, zona binding and oocyte penetration, and postpenetration function of spermatozoa (Carrell, 2000). The most common assay used extensively to determine spermatozoa function is the zona binding and oocyte penetration category, such as in vitro fertilization (IVF), spermatozoa
penetration, capacitation, and acrosome function tests. However, these assays are laborious and needed more evidence to prove their relationships to fertilizing ability of the spermatozoa.

*In vitro* fertilization (IVF) has been using in human as a predictor for sperm fertility. In pig, there is insufficient evidence to suggest that IVF can be used as sperm fertility estimator (Popwell, 2000; Popwell and Flowers, 1998; Xu et al., 1998). Xu *et al* (1998) reported that IVF results could be used to predict the onset of subfertility in boars.

Seminal plasma is the acellular fraction of semen and composed of the mixture of secretions from accessory male reproductive glands, including prostate, bulbourethral glands, and seminal vesicle. Role of seminal plasma in reproduction is unclear (Carballada and Esponda, 1997). However, seminal plasma maintained decapacitated stage of spermatozoa. The uterine contractions were stimulated by estrogens and prostaglandins in seminal plasma, which promoted a passive transport of the spermatozoa (Claus, 1990; Clavert *et al*., 1990). In rat, the seminal plasma proteins were found mostly in the uterus within few minutes and were eliminated within 12 hours after insemination (Carballada and Esponda, 1997). However, the seminal plasma proteins did not pass through the oviducts in detectable amount (Carballada and Esponda, 1997). Carballada and Esponda (1997) suggested that the seminal plasma components seemed to play mainly roles in the lower regions of the female reproductive tract. The total protein concentration of seminal fluid is approximately half that in serum (Montagnon *et al*., 1990). About 70% of proteins in seminal plasma are secreted
by seminal vesicle, such as lactoferrin, basic proteins, and albumin (Montagnon et al., 1990). Protein components in seminal plasma have been studied to find whether or not it shows relationship to sperm fertility. Flowers (1997) reported mixing seminal plasma from a “non-dominant” boar with spermatozoa from a “dominant” boar decreased the dominant effect of the spermatozoa when heterogeneous inseminations were studied. Some proteins in seminal plasma might play roles in fertility of sperm cell. Seminal plasma was also reported promoting spermatozoa transportation through the uterine horn into the oviducts of pigs. The numbers of spermatozoa found in oviduct were higher when inseminated with seminal plasma compares to inseminate spermatozoa with buffer (Viring and Einarsson, 1980).

Follicular fluid enhances sperm motility, capacitation, acrosome reaction, and fertilization rate (Minhas and Ripps, 1996). Spermatozoa treated with follicular fluid showed increasing hyperactivation when compared to control. Follicular fluid was reported to contain glycosaminoglycans, platelet activating factor (PAF), serum albumin, and xanthine derivatives, which act as phosphodiesterase inhibitors to elevate intracellular cAMP (Minhas and Ripps, 1996). Follicular fluid may be playing an important role in vivo in modulating sperm function as the concentration of follicular fluid increase in proximity of the oocyte. Also, during transportation of spermatozoa to fertilization site, exposure to progesterone in the female reproductive tract can be stimulated to synthesize and secrete PAF. PAF stimulates sperm function in an autocrine fashion through
a cascade of secondary signalling pathways by phosphatidylinositol 4,5-bisphosphate breakdown and Ca$^{2+}$ mobilization (Minhas and Ripps, 1996)

Cytokines are polypeptide hormones produced mainly from immunogenic cells. The cytokines respond to various stimulations including foreign antigens (Naz and Minhas, 1995). The cytokines, including transforming growth factor (TGF)-β, interleukin (IL)-1, IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), and B-cell growth factor (BCGF) affected spermatozoa without adverse effects on sperm functions and subsequent fertilization (Naz and Minhas, 1995). In contrast, high concentrations of interferon-alpha and gamma (IFN) and tumor necrosis factor-alpha (TNF-α) showed deleterious effects on sperm motility and penetration rate (Minhas and Ripps, 1996). However, the addition of cytokines to improve sperm functions was done only in vitro studies, and requires further study.

Maturation after ejaculation plays an important role in fertility of spermatozoa. Seminal plasma was reported to have a decapacitation effect on spermatozoa (Flesch et al., 2000; Meizel, 1985; Way et al., 2000) and provide spermatozoa with the ability to transport through the female reproductive tract and ultimately fertilization. Female reproductive fluid plays an important role on sperm transportation and fertilization.

III. Insulin-like Growth Factor-I in male reproduction:

The discovery of insulin-like growth factors (IGFs) was first reported as somatomedin C by Salmon and Daughaday (1957). The somatomedins then were investigated and found to have similarities with insulin, so they were called
“insulin-like”. The insulin-like growth factors (IGFs) are single-chain polypeptides of about 7.5 kDa. The IGFs consist of peptide domains, B, C, A and D, in which domains A and B are structural homologues of the insulin A and B chains. Domain C is analogous to the connecting peptide in proinsulin, and a unique D domain, which is not found in insulin (Baxter et al., 1982; Gnessi et al., 1997; Humbel, 1990; Van Wyk et al., 1980). The two major forms of the IGFs are IGF-I and IGF-II, which share about 62% homologous sequence (Gnessi et al., 1997). The primary structure of IGF-I in animals consists of 70 amino acid residues, meanwhile IGF-II consists of 67 amino acid residues (Humbel, 1990). The original somatomedin hypothesis stated that growth hormone acts on peripheral tissues not directly but by the intermediary of a plasma growth factor, namely somatomedin, which is produced under the influence of GH (see Humbel, 1990). However, this hypothesis has been challenged by recent discoveries (Humbel, 1990; Le Roith et al., 2001). Administration of GH locally into epiphyseal plates led to significant bone growth without IGF-I involved (see Humbel, 1990). Also IGF-I was found to be locally produced in many tissues, which might be regulated by GH or by other hormones (Le Roith et al., 2001). These studies provided additional evidence to challenge the original hypothesis. A new concept concerning the roles of GH and IGF-I in growth and differentiation was proposed by Green et al (1985), so called the “dual effector hypothesis” (Green et al., 1985). The dual effector hypothesis involved direct effects by GH on peripheral tissues, not mediated by IGF-I, and GH-stimulated local IGF-I production for autocrine/paracrine action of IGF-I.
Growth hormone was reported physiological roles in the control of male sexual maturation and adult reproductive functions (Bartke, 2000). GH deficiency did not affect fertility of males, however, various reproductive defects were reported, including delayed puberty, subnormal development of the male reproductive system, and reduced sexual behavior. Meanwhile, GH overexpressed mice showed abnormalities in gonadotropin release, including LH suppression, and reduced FSH levels (reviewed by Bartke, 2000). GH treatment was found to advance the onset of spermatogenesis (Fanua et al., 2001), and stimulate testicular growth and functions (Bartke, 2000). The role of IGF-I in mediating the effect of GH on Leydig cells and on the testis is shown in figure 3. Since Leydig cells express both IGF-I and IGF-I receptor. Thus, the Leydig cells may be responding not only to GH-induced elevation of both systemic and local IGF-I production, but also to gonadotropins (Bartke, 2000). Some studies proposed the relationship between GH and hypothalamus-pituitary-gonadal axis (see Humble, 1990; Shoham et al., 1994; Bartke, 2000). GH treatment in GH deficiency restored normal pubertal development and Leydig cell functions. In GH-deficient boys, treatment with somatotropin increased the production of testosterone and dihydrotestosterone, response to human chorionic gonadotropin (hCG), from Leydig cell (Kulin et al., 1981). Bartlett et al (1990) also found that GH was important in prepubertal testicular development in GH-deficient rat. The mechanism of GH on testicular function was believed to mediate locally through gonadal IGF-I, which is likely to be GH dependent, rather than gonadotropin dependent (Bartlett et al., 1990; Shoham et al., 1994).
The biological actions of the IGFs are modulated by a family of at least six IGF-binding proteins (IGFBPs) and proteases (Gnessi et al., 1997; Humbel, 1990; Hwa et al., 1999; Jones and Clemmons, 1995; Lamson et al., 1991; Whitley et al., 1998). The IGFBPs, a class of proteins, which have high affinity and specificity for the IGFs, can enhance or inhibit effects and may also have a ligand-dependent effect (Lamson et al., 1991). The functions of IGFBPs modulates IGF-I actions include: 1) directly bind to IGFs and transport into the circulation, 2) regulate IGFs efflux from the vascular, 3) extend half-life and metabolic clearance rates, 4) prevent hypoglycemia induced by IGFs, 5) locally modulate interactions of IGFs with IGF receptors interactions within target tissues, and 6) modulate cellular functions, independent of their ability to bind IGFs (Poretsky et al., 1999). The IGF-I binding proteins (IGFBPs) were reported in semen (Hoeflich et al., 1999), however, no significant roles were revealed. The IGFBPs might or might not modulate the mechanism of IGF-I in seminal plasma. IGFBPs are present in the uterus throughout the estrous cycle (Henemyre and Markoff, 1999) and are modulated follicular function (Whitley et al., 1998). Meanwhile, IGFs are expressed in the mouse uterus during preimplantation period. However, IGF-I receptor transcriptions were found to be at low level (Henemyre and Markoff, 1999). These findings might suggest that regulation of IGF system can occur at the level of the ligand, binding protein, and/or receptor, in the female reproductive tract (Henemyre and Markoff, 1999).

IGF-I was first discovered in media conditioned with rat seminiferous tubule cultures. During puberty, there is a rapid increase in the number of
spermatogenic cells showing IGF-I immunoreactivity, which is localized in the cytoplasm of spermatocytes (Gnessi *et al.*, 1997). The expression of the mRNA for IGF-I was reported in immature rat testis, and Leydig cells were shown to express this gene. The expression was enhanced by growth hormone treatment (Gnessi *et al.*, 1997). In the human testis, the immunohistochemical staining of IGF-I is localized primarily in Sertoli cells. In prepubertal pig, IGF-I was found in Leydig cells and gonocytes in earlier of age (30-90 days of age) compared to IGF-I in Sertoli cells which was found at a later age (100 days of age) (Fanua *et al.*, 2001). IGF-I and IGF-II stimulate the proliferation of Sertoli cells in the rat and pig; however, IGF-I had little effect on immature Leydig cells. Both IGFs stimulate spermatogonial DNA synthesis in rats under *in vitro* conditions (Gnessi *et al.*, 1997). IGF-I also was found to stimulate the differentiation of type A spermatogonia in mice and the production of testosterone by cultured Leydig cell in rats and pigs.

IGF-I is well documented to be a mediator for most actions of growth hormone (GH). GH signaling is not required for male fertility but it plays a physiological role in the control of male sexual maturation and reproductive functions. Leydig cells express both GH and IGF-I receptors as well as IGF-I. GH can stimulate IGF-I expression on Leydig cells but GH did not affect testosterone levels in normal men (Bartke, 2000). In addition, Sertoli cells express both GH and IGF-I receptors and produce IGF-I. The IGF-I secreted by Sertoli cells can act on Leydig cells, in a paracrine fashion, synergistically with LH to regulate steroidogenesis (Shoham *et al.*, 1994). The Leydig cell-secreted
IGF-I can act as an autocrine factor for the cell. The IGF-I in testis was believed to be locally produced rather than hepatic IGF-I production.

In vasectomized men, IGF-I level in seminal plasma was reduced by 33% compared with pre-vasectomy levels without any changes in serum IGF-I (Ovesen et al., 1995). IGF-I in seminal plasma is most likely originated from Sertoli cells (Baxter et al., 1984; Glander et al., 1996; Ovesen et al., 1995). IGF-I was found in human seminal plasma, however, the association of the IGF-I and semen quality or fertility has not been well documented. In humans, Glander et al. (1996) found that IGF-I in seminal plasma had correlation to semen concentration and percentage of morphologically normal spermatozoa (r=0.301 and 0.748, respectively, p<0.02). In bull semen, addition of IGF-I increased percentage of motile spermatozoa and straight-line velocity. In the same study also showed that concentration of IGF-I in seminal plasma was dependent on animal genetic line (Hendricks et al., 1998; Kouba, 1995). IGF-I receptor was found on bovine spermatozoa (Kouba, 1995). However, the mechanism of the receptor was not revealed. In boar, the IGF-I in seminal plasma was first reported in 2001, and no correlation of IGF-I level and semen parameters was found (Hirai et al., 2001).

In female reproductive system, IGF-I was shown to be beneficial to porcine oocyte maturation when presented in the maturation medium (Xia et al., 1993) but not in the presence of follicular fluid (Illera et al., 1998). IGFs were found in oviductal fluid during estrous cycle (Wiseman et al., 1992). The IGFs in the oviductal fluid did not originate from blood circulation but likely to be secreted
by the oviductal cells and ovarian cells (Wiseman et al., 1992). The mechanism of IGFs in the oviduct is not clear. These findings suggested that IGF system in the reproductive system were locally secreted and acted in paracrine/autocrine fashions.

There are two known IGFs receptors, namely, type 1 IGF receptor and type 2 IGF receptor. The type 1 IGF receptor (also known as the IGF-I receptor) has a high degree of homology with the insulin receptor. The type 2 IGF receptor is identical to the mannose 6-phosphate receptor (see Jones and Clemmons, 1995, for review). The IGF-I receptor is the only receptor to have IGF-mediated signaling functions, both IGF-I and IGF-II (Jones and Clemmons, 1995; Le Roith et al., 2001). The IGF-I receptor and insulin receptor are very similar in structure, especially in the tyrosine kinase domain, which shows about 85% homology. Both receptors are consisted of α- and β-subunits with α-subunit localized extracellularly and the β-subunit localized primarily intracellularly (reviewed by Le Roith et al., 2001). The binding of IGF-I to the extracellular domain of the receptor causes autophosphorylation on both tyrosine and serine residues. The autophosphorylation of the IGF-I receptor recruits endogenous substrates forming docking sites. Insulin receptor substrate 1 (IRS-1) is believed to be a docking protein that forms a large protein complex activating multiple cascades (see Jones and Clemons, 1995; Le Roith et al., 2001, for review). The affinity of the IGF-I receptor for IGF-I is higher than for IGF-II and for insulin. Most of the actions of both IGF-I and IGF-II are mediated by the type 1 IGF receptor. IGF-I receptors are present in a wide variety of cell types. Fanua et al (2001) found the
expression of IGF-I in Sertoli cells, Leydig cells, and gonocytes. IGF-I and IGF-II stimulated DNA synthesis in rat spermatogonia in serum-free culture of seminiferous tubules (Soder et al., 1992). Hormone secretion from many cell types is regulated by the IGFs, such as ovarian granulosa cells, theca cells, Leydig cells and thyroid follicular cells.

**IV. Characteristics of Insemination Doses and Fertility:**

Bacterial contamination in semen occurs accidentally during semen processing for AI, which showed detrimental effects on sperm quality, longevity and fertility (Althouse et al., 2000; Paulson and Polakoski, 1977; Sone, 1990; Sone et al., 1992; Sone et al., 1989; Sone et al., 1982; Teague et al., 1971). More than 13 genera of bacteria was found contaminated in boar semen (Althouse et al., 2000; Sone et al., 1982). Semen could be contaminated from animal source, such as feces, preputial diverticulum, skin, or from non-animal source, such as the floor, bench area in the laboratory (Althouse et al., 2000; Thacker et al., 1984). The detrimental effect from bacterial contaminated might be from endotoxin produced by bacteria (Sone et al., 1992; Teague et al., 1971), or from direct attachment of bacteria to spermatozoa (Paulson and Polakoski, 1977).

Antibiotic has been used in boar semen extender for over 40 years. The most common used antibiotic in the past was a combination of streptomycin and penicillin (Paredis, 1962; Sone et al., 1982), however, the increase of resistant bacteria in boar semen to these drugs was reported (Waltz et al., 1968). Aminoglycosides, such as gentamicin sulfate, amikacin sulfate, are the most
The most common antibiotic used in boar semen extender (Althouse et al., 2000; Sone, 1990; Sone et al., 1992; Sone et al., 1982). The aminoglycosides are broad-spectrum antibiotics, which contain an aminocyclitol moiety (Hooper, 1982). The aminoglycosides bind the bacterial cell wall by ionic interaction and transport into the cytoplasm of the bacteria by an active transport, which is linked to terminal electron transport (Dworzack, 1984). Once inside, the aminoglycosides bind and interfered protein synthesis of bacterial ribosome (Dworzack, 1984). The rate of membrane transportation of the antibiotics is dependent upon the concentration of antibiotic outside the cell. A low concentration of divalent cations, such as Ca\(^{2+}\) and Mg\(^{2+}\), were capable of antagonizing the aminoglycosides transport (Dworzack, 1984). The aminoglycoside antibiotics, such as gentamicin, amikacin sulfate and polymyxin B were reported to be the most effective antibiotics in E.coli-contaminated diluted semen (Sone et al., 1992; Sone et al., 1982).

Antibiotics are added to semen extenders at different concentrations depending on the type that is used. The most common antibiotic used in commercial semen extender is gentamicin sulfate. The concentration of gentamicin of 200 µg/ml was found to be effective against E.coli contamination (Sone et al., 1992). However, there is no report on the efficacy and stability of the antibiotics in the extended semen. The aminoglycosides were significantly affected by the pH of the solution (Hare and Miller, 1984). They found that gentamicin was more effective at pH 8.0 than at pH 7.0 or 5.0. The affinity of antibiotic was affected by proteins in the solution. For aminoglycosides, up to 30% of drug was bound to serum and plasma albumins (Hare and Miller, 1984).
In extended semen, antibiotics might be able to bind to the sperm plasma membrane by ionic interaction, which might reduce the efficiency of antibiotics. Concentration of divalent cations, Ca2+ and Mg2+, also affected the efficiency of the aminoglycosides by increasing ionic strength of the medium, which affected the uptake of aminoglycosides (Hare and Miller, 1984).

In artificial insemination (AI), semen is extended in semen extender to provide the number of insemination per ejaculate. To store the extended semen, it needs to store at lower temperature, 15-20°C. As temperature declines, proportion of the spermatozoa is not able to maintain its normal membrane integrity, biochemical and ultrastructure components (Johnson et al., 2000). The same physiological changes of spermatozoa during cryopreservation and capacitation has been reported (Bailey et al., 2000; Watson and Green, 2000), which could reduce longevity and fertility of spermatozoa.

Semen characteristics, such as percentage of motile spermatozoa (MOT), percentage of morphologically normal spermatozoa (MOR), percentage of spermatozoa with normal acrosome (NAR), total number of spermatozoa/ejaculate, semen concentration and semen volume, provide qualitative assessment of semen. MOT was reported to affect breeding performance when insemination with semen of less than 60% motility (Flowers, 1997). However, the in vivo fertility were not different when MOT was greater than 60% (Flowers, 1997; Popwell, 2000). MOR is considerably used as the best indicator for the fertility in humans (Barratt et al., 1995; Barratt and St John, 1998; Coetzee et al., 1998). Popwell (2000) also found that MOR was the only
parameter differed among boars that differed in \textit{in vivo} fertility. However, this parameter is not seriously considered as an important parameter in animals.

In AI, semen is diluted in semen extender and stored at $17^\circ\text{C}$ until insemination. In general, semen extenders contain a source of energy, such as glucose; pH buffers, such as sodium bicarbonate, HEPES; and chelating agents, such as EDTA. Recently, the semen extenders were commercialized and improved longevity of storage time. In the past, the recommendation was established for AI to use the diluted semen within 1-2 days. With long-term extender, the diluted semen can be stored for up to 5 days (Althouse, 1997; Kuster and Althouse, 1999; Sone \textit{et al.}, 1992; Waberski \textit{et al.}, 1994; Weitze, 1990). However, most of the studies were carried on in vitro study or in university facilities. However, in practical, about 85\% of all inseminations made on farm were conducted within one day after collection (Johnson, 1998), regardless of types of extender.

It was suggested that the concentration of spermatozoa should be greater than $10 \times 10^6$ /ml to achieve optimal fertility in AI (Baker and Polge, 1976; Polge, 1978). However, in general, the number of spermatozoa per insemination dose was recommended at least $3 \times 10^9$ /dose (Johnson, 1998; Paredis, 1962). Attempt to lower the number of spermatozoa/dose was studied, to improve the efficiency of seminal usage per ejaculate, but the results are debatable and many factors confound the results (Park \textit{et al.}, 2000; Rath \textit{et al.}, 2000). In fact, only few spermatozoa are able to reach the fertilization site. Polge (1978) showed that at least $10^7$ spermatozoa needed to reach at the tip of the uterine horn to
achieve maximum fertilization. Meanwhile, Rath et al (2000) reported that $5 \times 10^6$ spermatozoa inseminated at the tip of the uterine horn were sufficient for normal fertilization. Park et al (2000) found that inseminated sows with different number of spermatozoa per dose ($1.5, 2, 2.5$ and $3 \times 10^9$ /dose) had no effect on farrowing rate and litter size. However, lower the number of spermatozoa per dose is not widely recommended.
LITERATURE REVIEW SUMMARY

The literature review illustrates that many factors influence semen quality and fertility in boar semen. Many researchers attempted to determine differences among males in terms of fertility by using several methods and criteria. Determination of semen fertility after collection would enhance the efficiency of seminal usage and reduce using, so called, sub-fertile animals.

Not only spermatogenesis, but also sperm maturation, significantly contribute to semen quality and fertility. Hormonal regulation, namely, the hypothalamus-pituitary-gonadal axis, initiates and regulates spermatogenesis in the seminiferous tubules by endocrine, paracrine and autocrine mechanisms. IGF-I, produced by Leydig cells under stimulation of LH, acts in an autocrine and paracrine fashion to regulate Sertoli cell function in spermatogenesis. The IGF-I level in seminal plasma may reflect the efficiency of spermatogenesis and quality of semen.

Semen characteristic parameters are used to assess ejaculate quality. There is some evidence that showed the relationship among those parameters and fertility. Furthermore, in AI, factors associated with semen handling and management play a role in breeding performance as well. These factors might contribute to the prediction of sperm fertility prior to fertilization, which would be beneficial to swine producers.

A small negligence in semen handling and processing in AI could have a big impact on breeding performance of farms. For example, bacterial
contamination in semen could cause a tremendous reduction in fertility and breeding performance in several farms. Thus, utilization of antibiotic in semen extender is recommended. However, the effect of antibiotics on sperm cells under a minimal-contaminated condition is unknown.

The following research was conducted to attempt to elucidate the factors that influence the semen quality and fertility in boars.
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ANTIBIOTICS IN BOAR SEMEN EXTENDER: INFLUENCE ON SPERM QUALITY PARAMETERS AND IN VITRO PENETRATION RATE

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Abstract:

The objectives of this study were to evaluate the effects of antibiotics on the quality of stored semen and the ability of its spermatozoa to penetrate oocytes. Semen ejaculates, from three mature boars, were collected and extended in BTS extender to \(3 \times 10^9\) sperm in 80 ml. Each of the following antibiotics was added to the extended semen: gentamicin (GM, 200 \(\mu\)g/ml); amikacin (AM, 200 \(\mu\)g/ml); neomycin sulfate (NM, 1 mg/ml); and penicillin (1000 IU/ml)-streptomycin (PS, 1 mg/ml). The control treatment (CT) received no antibiotics. Each sample was examined for the percentages of motile (MOT) and morphologically normal (MOR) sperm, and the proportion of spermatozoa with normal acrosomes (NAR) on day 0, 1, 2, 3, 5, and 7 of storage. For GM and AM, a fluorescence polarization immunoassay was used to determine antibiotic concentrations over 120 hours of storage. On day 0 and 5 of storage, an in vitro penetration assay was conducted using semen from two of the boars. The results showed that MOT differed (p<0.05) among treatment, regardless of boars. However, MOR and NAR were similar. GM and NM treatments had higher (p<0.05) MOT compared to other treatment groups. Across treatments, a progressive decrease (p<0.05) in MOT was observed from day 0 to day 7. The concentrations of GM and AM in extended semen did not change during 120
hours of storage. No differences were found among treatments in in vitro penetration rate. However, the penetration rate was less (p<0.05) on day 5 than on day 0 of storage. Based on routine methods to assess boar semen and the experimental conditions, few differences in semen quality were evident when various antibiotics were added to semen extender. Because the experimental conditions may differ from on-farm semen processing, these results must be viewed with cautious.

Introduction:

Semen is a mixture of spermatozoa, fluids derived from the epididymis, and secretions from the seminal vesicles, prostate and bulbourethral glands. These secondary sex organs typically are sterile, and thus, semen is initially free of bacteria. In humans, bacterial contamination of semen can be caused by prostate gland infection (Gopalkrishnan et al., 1994) or urogenital tract infections (Kohn et al., 1996). Bacterial contamination in semen intended for artificial insemination (AI), occurs accidentally during collection and extension. Sources of bacterial contamination in semen from animal sources, such as feces, preputial sac, skin, or non-animal origins, such as the floor, laboratory area, and extension equipment (Althouse et al., 2000). The bacterial contaminants rarely cause illness in male animals, but transmission of bacteria to the female at breeding may result in subfertility and infectious endometritis (Varner et al., 1998).
Sone et al (1982) reported 13 genera of bacteria in boar semen. The study showed 80.4 % of semen sample were contaminated with *Pseudomonas* spp, 63 % with *Micrococcus* spp, and greater than 50 % with *Staphylococcus* and *Klebsiella* spp. *Escherichia coli* was found in 41.3% of the samples. Althouse et al (2000) also reported 15 species of bacteria in extended boar semen in the U.S. Makler et al (1981) isolated 5 genera and 8 species of bacteria in human semen. Bacteria in extended semen has direct effects on spermatozoa, including reduced motility (Teague et al., 1971), fertility and viability, and severely damaged acrosomal membranes (Sone et al., 1992). The mechanisms by which bacteria affect spermatozoa are debatable. Bacteria might affect spermatozoa by attachment to spermatozoa directly (Teague et al., 1971; Sone et al., 1992) or by endotoxin production (Paulson and Polakoski, 1977). Antibiotics have been used to protect or minimize detrimental effects of bacteria on spermatozoa. However, the exact impact of antibiotics on spermatozoa has not been well documented. Some studies reported positive effects of antibiotics on porcine sperm survival and motility (Sone et al., 1982; Sone et al., 1992). In contrast, Makler et al (1981) reported antibiotics had no effect on sperm viability and motility in contaminated human semen.

Viability of spermatozoa in extended semen depends on several factors, such as type of extender (Sone et al., 1992; Waberski et al., 1994), type of antibiotic and degree of contamination in semen (Sone et al., 1992). The half-life of antibiotics has been established for serum. However, there are no reports on concentrations of antibiotics in diluted semen during storage or on the influence
of the antibiotics on spermatozoa in a non-contaminated situation. Therefore, the objectives of this study were to determine 1) the influence of antibiotics on semen quality, 2) the interaction between type of antibiotic and boar on semen quality, 3) the concentration of selected antibiotics in diluted semen during storage, and 4) whether antibiotics used in extended semen affect spermatozoa by using in vitro penetration assay as a sperm fertility test.

**Material and methods:**

**Animals:**

Each boar used in this study was housed in an individual crate, given unlimited access to water, and fed a corn-soybean meal ration (13% crude protein) supplemented with vitamins and minerals according to NRC guidelines (NRC, 1998). A mature boar (Newsham terminal line; 2 years of age, 400 lbs), used routinely at the swine educational unit, NCSU, was collected seven times as preliminary investigation during July-September 1997. Two crossbred (Duroc x Hampshire; PIC Blackline) boars, 2-3 years of age and approximately 300-500 lbs., were added to the study after the preliminary results. Seven more ejaculates were collected from each of the 3 boars during October-December 1997. Two boars from the first study were selected for the collection of sperm-rich fraction semen, during February-April 1998 for the in vitro penetration study.

**Semen collection and processing:**

Semen was collected by the hand gloved technique into a plastic semen collection bag (Minitube of America, Verona, WI), which was inside a thermos.
The collection equipment was kept at $37^\circ$C prior to use. The mouth of the thermos was covered with 3 layers of clean cotton gauze. Semen was evaluated for volume, concentration, and progressive motility. Then, semen was transferred to the laboratory for evaluation. Volume and concentration were used to calculate the dilution rate. The final spermatozoa concentration of diluted semen was $37.5\times10^6/ml$ or $3\times10^9$ per insemination dose (80 ml) in Beltsville Thawing Solution (BTS; 37.0 g/l glucose, 6 g/l sodium citrate, 1.25 g/l sodium bicarbonate, 1.25 g/l EDTA.2Na, 0.75 g/l potassium chloride), which was prepared from laboratory grade ingredients one day before use. The extender was maintained in a waterbath at $37^\circ$C. The temperature of extender was adjusted, as necessary, to the same temperature as the semen at the time of dilution. The diluted semen was left at room temperature at least 15 min before the addition of antibiotic solutions.

**Antibiotic solutions:**

The following antibiotic solutions (ICN Biomedical, Costa Mesa, CA) were prepared as stock solutions in BTS one day before semen collection:

1. Penicillin G (1,000 IU/ml) and streptomycin sulfate (700 IU/ml)
2. amikacin sulfate (200 µg/ml)
3. neomycin sulfate (1 mg/ml)
4. gentamicin sulfate (200 µg/ml)

The concentration of each antibiotic was based on the results of previous studies (Sone, 1990; Sone et al., 1992). For each semen collection, the diluted semen was divided into 10 aliquots and dispensed into commercial AI bottles.
(80ml). Each antibiotic solution was added to two aliquots (duplicate samples). Antibiotic solutions were not added to two aliquots, which served as controls. The antibiotics were added to each aliquot of diluted semen in a blind-study fashion, briefly, the investigator was unaware of treatments during semen characteristic evaluations. The aliquots were then incubated at 17°C in a semen storage unit (Minitube of America, Verona, WI).

**Semen quality evaluation:**

**Percentage of motile spermatozoa:**

Twenty to twenty five microliters of well-mixed semen were dropped on a caffeine-coated slide on a slide warmer. A warmed glass coverslip (25x25 mm) was gently placed over the semen sample. The slide was evaluated immediately under a phase-contrast microscope (Zeiss, West Germany), equipped with a warming stage. At least 5 fields under 400x magnification were evaluated for spermatozoa that moved forward and had a strong beat of tail movement. Percentages were calculated by division of the number of motile spermatozoa by the total number of sperm counted.

**Percentage of morphologically normal spermatozoa:**

Two hundred microliters of well-mixed semen were placed into an Eppendorf tube (2 ml) containing 1 ml of 10% formalin. From this mixture, 20-25 µl were placed on a cleaned microscope glass slide (Fisherbrand, Fisher) and covered with a plastic coverslip. The slide was evaluated with phase-contrast microscopy. At least 200 sperm cells were evaluated from at least 5 different fields with 400X magnification. Spermatozoa with normal heads and tails were
recorded as normal. Spermatozoa with abnormal head shape, tail or midpiece, and spermatozoa with proximal or distal cytoplasmic droplets, were recorded as abnormal (Briz et al., 1995). The percentage of morphologically normal spermatozoa was calculated from number of normal spermatozoa divided by the number of total sperm counted.

**Percentage of spermatozoa with normal acrosomes:**

Two hundred microliters of well-mixed semen were placed into an Eppendorf 2 ml tube containing 1 ml of 10% formalin. To proceed with the acrosome evaluation, 20-25 µl of well-mixed semen were dropped on a cleaned microscope glass slide covered with a plastic coverslip. The slide was evaluated under a phase-contrast microscope. At least 200 spermatozoa were counted from at least 5 different fields with 400x magnification. Spermatozoa with normal shape and smooth surface of acrosomal ridges were recorded as normal. Spermatozoa with a broken acrosome or loose acrosome were recorded as abnormal (Briz et al., 1995; Pursel et al., 1972). The percentage of spermatozoa with normal acrosomes was calculated by division of the number of normal spermatozoa by the number of total sperm counted.

**Concentration of antibiotic in semen extender:**

Concentrations of selected antibiotics, gentamicin sulfate and amikacin sulfate, were determined by fluorescence polarization immunoassay (FPIA; Beck et al., 1995) at the Clinical Pharmacology Laboratory, College of Veterinary Medicine, NCSU. Four milliliters of diluted semen sample were centrifuged at high speed and the supernatants were collected in 2 ml Nalgene™ cryotubes
and frozen at –20°C before being transferred to a –70°C freezer. The samples were maintained at –70°C until assayed.

**Sample Collection Schedule:**

Samples were evaluated for percentages of motile spermatozoa (MOT), morphologically normal spermatozoa (MOR) and spermatozoa with normal acrosomes (NAR) on day 0, 1, 2, 3, 5 and 7 of storage. Samples were collected for the antibiotic concentration assay at 0, 6, 12, 24, 36, 48, 72, and 120 hours of storage. The *in vitro* penetration assay tested extended semen on day 0 and day 5 of storage.

**In vitro Culture Media:**

Chemicals and media were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. Two media were used, recovery media (M 199 Sigma 2520) and maturation media (M 199 Sigma 3769). The recovery media was used for recovery and washing of oocytes prior to maturation and used for washing and capacitation of spermatozoa prior to fertilization. The maturation media was modified with supplements and used as fertilization media. Both recovery and maturation media were supplemented with 26.18 mM sodium bicarbonate, 8.25 mM, calcium lactate, 0.90 mM pyruvate, 3.05 mM glucose, 0.04% BSA, and 20mg/L gentamicin sulfate. The maturation medium was also supplemented with 0.68 mM glutamine. The final maturation medium consisted of maturation medium supplemented with 2000 IU LH, 1 mg FSH, 100 µl (insulin 5 µg/ml, transferrin 5 µg/ml, sodium selenite 5 ng/ml), 1 µg estradiol-17 β, and 1 ml porcine follicular fluid (v/v) (Yoshida, 1987). All media were prepared fresh.
and sterilized using 0.22 µm cellulose acetate filter system (Bottle top filter, Corning, Inc., Acton, MA). All media were preincubated at 37°C in 5 % CO₂ in humidified air.

**In vitro Collection and Maturation of Oocytes:**

Prepubertal gilt ovaries were collected from a local slaughterhouse with minimal contamination. The ovaries were removed from the reproductive tract, washed twice in D-PBS (Dulbecco’s phosphate buffer saline) containing 1% gentamicin sulfate solution and maintained at a temperature between 30-35°C. The ovaries were transported to the laboratory in a warmed container. Upon arrival, the ovaries were washed 2-3 additional times with D-PBS with gentamicin sulfate. A jar containing the ovaries was then placed in a 35°C waterbath. Oocyte aspiration was done by using a modified-air suction system. All ovarian follicles 3-7 mm in diameter were aspirated with a 18 gauge needle with air suction into sterile 12x100 glass test tubes in a 37°C water bath. Oocytes were placed in a 37°C incubator for 10 min to let oocytes settle to the bottom of the tube. Sediments were collected using a sterile transfer pipette and transferred to a 100x15 polystyrene petri-dish (Fisherbrand, Fisher Scientific, Pittsburgh, PA) containing 5 ml of preincubated recovery medium. Oocytes were retrieved using a dissecting microscope (Olympus SZ40, Olympus, USA) and placed in 5 ml culture dishes (Falcon 60x15 mm; Becton, Dickson; Franklin Lakes, NJ) containing warmed recovery medium. After the recovery procedure, oocytes were washed 5 times in recovery medium. Oocytes were then placed in 3 ml culture dishes (Falcon 35x10 mm; Becton, Dickson; Franklin Lakes, NJ) and
washed 10 times in base maturation medium before transfer into final maturation medium. The final maturation medium was prepared fresh prior to the washing of the oocytes. Oocytes were transferred to the final maturation medium in 3 ml culture dishes (50 oocytes per dish). The dishes were preincubated at least 30 min at 39°C in 5% CO₂ in humidified air. All maturation dishes were incubated at 39°C in 5% CO₂ in humidified air for 22 hours. After 22 hours of maturation, all oocytes were removed and placed in the base maturation medium without hormones and incubated an additional 18-20 hours at 39°C in 5% CO₂ in humidified air (maximum total incubation time 42 hours). Matured oocytes, with expanded cumulus, were recovered from the maturation dishes using a small amount of hyaluronidase enzyme and mouth pipettes. Recovered oocytes were placed in base maturation medium and mature oocytes were transferred to new dishes for fertilization. The fertilization medium contained base maturation medium plus 0.2% caffeine (Wang et al., 1992).

*In vitro Fertilization*

Two batches of semen were used for *in vitro* fertilization. The first batch was the diluted semen collected and processed with antibiotic and stored at 17°C for 5 days. The second batch considered as day 0 spermatozoa, was fresh semen collected from the same boar on the day of fertilization.

Semen samples were centrifuged to remove seminal fluid or semen extender and washed twice in BTS and twice in capacitation medium (recovery medium). Pellets were resuspended in 9 ml (ratio 1:9, pellet:medium) of the capacitation media. All samples were placed in a 37°C incubator with 5% CO₂ in
humidified air and allowed to capacitate for 3 hours. The sperm suspension then was diluted to $1 \times 10^5$ cells/ml. Fifty selected oocytes from maturation dishes were transferred into 60x15 mm organ tissue culture dishes with center wells (Falcon 3037, Becton, Dickson; Franklin Lakes, NJ), containing 2 ml of fertilization medium, which was preincubated at 39°C in 5% CO₂ in humidified air for at least 30 min. Ten microliters of sperm suspension was added into each fertilization dish. The final sperm/oocyte ratio was 20. All fertilization dishes were incubated at 39°C in 5% CO₂ in humidified air for at least 4 hours (4-5 hours of fertilization time). After the fertilization period, all oocytes/embryos were transferred into 3 ml culture dishes containing 2 ml of base maturation medium and incubated at 39°C in 5% CO₂ in humidified air overnight.

**Assessment of Oocyte Penetration**

After fertilization, oocytes/embryos were washed twice in D-PBS and placed on glass microscope slides, covered with a cover slip; and fixed in acetic acid/alcohol (1:3 v/v) for 24 hours. The slides were stained with 1% aceto-orcein and examined under a microscope (Bausch & Lomb Scientific Optical Production Devision, Rochester, NY.) at 20x and 40x magnification (Yoshida, 1987). All oocytes were evaluated for maturational status and the presence of spermatozoa within the cytoplasm of oocytes. Oocytes in metaphase I and metaphase II were scored as matured; those with an intact germinal vesicle (GV) or in the initial stages of germinal vesicle breakdown (GVBD) were considered immature. Oocytes were designated as penetrated when at least one sperm head or male pronucleus was present in the oocyte. Oocytes with more than one sperm
nucleus or male pronucleus were classified as having polyspermic penetration. Degenerated, underdeveloped and immature oocytes were excluded from the calculation.

**Statistical Analysis:**

Semen quality data were normalized with an arc sine transformation prior to analysis. Analysis of variance procedures for repeated measures procedures (SAS, 1988) were used to determine effects of time, treatment and individual boar on semen quality parameters and antibiotic concentrations. Duncan’s multiple range test was used to determine differences among independent variables when significance was observed. The statistical models included treatment, boar, run, and day of storage as independent variables, and percentage of motile, percentage of morphologically normal sperm and percentage of sperm with normal acrosomes as dependent variables with appropriate interactions.

Percentage of penetration was calculated from the number of oocytes presenting either one or more spermatozoa and/or second pronuclear along with polar body, divided by total number of matured oocytes. Germinal vesicle stage and underdeveloped oocytes were excluded from the calculation. The percentage of penetrated oocytes was normalized with an arcsine transformation prior to analysis. Differences among boars and treatments for oocyte penetration were analyzed using the analysis of variance procedures with general linear model estimations of SAS (SAS, 1988). The statistical model consisted of
treatment and day of storage as independent variables and penetration rate as a dependent variable. Boar was treated as a block. Duncan’s multiple range test was used to determine difference between day of storage when significance was observed.

Results:

The MOT, MOR, and NAR were used as spermatozoa characteristic parameters in this experiment. Fourteen ejaculates were used from the first boar, and seven ejaculates collected from the other two boars. The results showed that all parameters differed among boars across treatments and storage time (Table 1). The MOT showed differences among types of antibiotics (p<0.05). However, the MOR and NAR were not influenced by antibiotic treatments. The MOT in gentamicin sulfate (GM) and neomycin sulfate (NM) groups were higher (p<0.05) than MOT in amikacin sulfate (AM), penicillin-streptomycin (PS) and control (CT) groups (Table 2). The differences in MOT among treatments were consistent regardless of boars. The MOT and NAR were reduced (p<0.05) from day 0 to day 7 of storage (Table 3). The MOR differed among times of storage but the differences were not consecutive over storage time.

All treatments showed the same trend in declining MOT from day 1 to day 3 of storage (Figure 1). After day 3, the MOT in CT and PS groups tended to be less compared to the other groups. On day 7, the GM treatment showed MOT around 60%; however, the MOT did not differ among the GM, NM and AM groups.
Table 1. Descriptive statistics values (mean ± SEM) of the percentages of motile spermatozoa, morphologically normal spermatozoa and spermatozoa with normal acrosomes among boars (n=3) across treatments (n=5) and storage time (0, 1, 2, 3, 5 and 7 days).

<table>
<thead>
<tr>
<th>Boar</th>
<th>Motile spermatozoa (%)</th>
<th>Morphologically normal (%)</th>
<th>With normal acrosomes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>77.8 ± 0.6 a</td>
<td>82.9 ± 0.5 a</td>
<td>98.6 ± 0.1 a</td>
</tr>
<tr>
<td></td>
<td>(n=512)*</td>
<td>(n=404)</td>
<td>(n=404)</td>
</tr>
<tr>
<td>2</td>
<td>74.6 ± 1.2 b</td>
<td>80.4 ± 1.0 b</td>
<td>99.2 ± 0.1 b</td>
</tr>
<tr>
<td></td>
<td>(n=162)</td>
<td>(n=45)</td>
<td>(n=45)</td>
</tr>
<tr>
<td>3</td>
<td>64.6 ± 1.5 c</td>
<td>75.7 ± 1.2 c</td>
<td>99.1 ± 0.1 b</td>
</tr>
<tr>
<td></td>
<td>(n=187)</td>
<td>(n=70)</td>
<td>(n=70)</td>
</tr>
</tbody>
</table>

Values (within column) with different superscripts differ at p<0.05

* Values in parentheses represent the number of observations. Fourteen ejaculates were used from boar 1, and seven ejaculates evaluated for boars 2 and 3. All samples were evaluated in duplicate. Due to time constraints, some spermatozoa parameters were not determined for each ejaculate at all storage times.
Table 2. Effect of treatments (antibiotic) (mean ± SEM) on the percentages of motile spermatozoa, morphologically normal spermatozoa and spermatozoa with normal acrosomes across boar (n=3) and storage time (0, 1, 2, 3, 5 and 7 days).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motile spermatozoa (%)</th>
<th>Morphologically normal (%)</th>
<th>With normal acrosomes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>78.0 ± 0.9 a (n=178)*</td>
<td>81.8 ± 0.9 (n=105)</td>
<td>98.8 ± 0.1 (n=105)</td>
</tr>
<tr>
<td>Neomycin</td>
<td>77.4 ± 1.0 a (n=178)</td>
<td>81.8 ± 0.9 (n=105)</td>
<td>99.0 ± 0.1 (n=105)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>74.0 ± 1.2 b (n=164)</td>
<td>82.1 ± 0.9 (n=103)</td>
<td>98.7 ± 0.1 (n=103)</td>
</tr>
<tr>
<td>Pen-strep</td>
<td>71.8 ± 1.4 c (n=180)</td>
<td>81.2 ± 0.9 (n=105)</td>
<td>98.5 ± 0.1 (n=105)</td>
</tr>
<tr>
<td>Control</td>
<td>69.9 ± 1.6 c (n=161)</td>
<td>81.8 ± 0.9 (n=101)</td>
<td>98.6 ± 0.1 (n=101)</td>
</tr>
</tbody>
</table>

Values (within column) with different superscripts differ at p<0.05

* Values in parentheses represent the number of observations. Due to time constraints, some spermatozoa parameters were not determined for each ejaculate at all storage times.
Table 3. Effect of storage time (days) (mean ± SEM) on the percentage of motile spermatozoa, morphologically normal spermatozoa and spermatozoa with normal acrosomes across boars (n=3) and antibiotic treatments (n=5).

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Motile spermatozoa (%)</th>
<th>Morphologically normal (%)</th>
<th>With normal acrosomes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>0</td>
<td>85.7 ± 0.4&lt;sup&gt;a&lt;/sup&gt; (n=172)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>81.3 ± 0.9&lt;sup&gt;c&lt;/sup&gt; (n=109)</td>
<td>99.8 ± 0.0&lt;sup&gt;a&lt;/sup&gt; (n=109)</td>
</tr>
<tr>
<td>1</td>
<td>80.3 ± 0.6&lt;sup&gt;b&lt;/sup&gt; (n=151)</td>
<td>82.5 ± 1.1&lt;sup&gt;b&lt;/sup&gt; (n=93)</td>
<td>98.9 ± 0.1&lt;sup&gt;b&lt;/sup&gt; (n=93)</td>
</tr>
<tr>
<td>2</td>
<td>77.3 ± 0.8&lt;sup&gt;c&lt;/sup&gt; (n=145)</td>
<td>80.0 ± 1.1&lt;sup&gt;d&lt;/sup&gt; (n=84)</td>
<td>98.6 ± 0.1&lt;sup&gt;c&lt;/sup&gt; (n=84)</td>
</tr>
<tr>
<td>3</td>
<td>77.4 ± 0.9&lt;sup&gt;c&lt;/sup&gt; (n=124)</td>
<td>83.7 ± 0.9&lt;sup&gt;a&lt;/sup&gt; (n=84)</td>
<td>98.4 ± 0.1&lt;sup&gt;c,d&lt;/sup&gt; (n=84)</td>
</tr>
<tr>
<td>5</td>
<td>68.3 ± 1.1&lt;sup&gt;d&lt;/sup&gt; (n=175)</td>
<td>82.5 ± 0.8&lt;sup&gt;b&lt;/sup&gt; (n=109)</td>
<td>98.0 ± 0.1&lt;sup&gt;e&lt;/sup&gt; (n=109)</td>
</tr>
<tr>
<td>7</td>
<td>46.3 ± 2.5&lt;sup&gt;e&lt;/sup&gt; (n=94)</td>
<td>78.7 ± 1.3&lt;sup&gt;e&lt;/sup&gt; (n=40)</td>
<td>98.2 ± 0.2&lt;sup&gt;d,e&lt;/sup&gt; (n=40)</td>
</tr>
</tbody>
</table>

Values (within column) with different superscripts differ at p<0.05

* Values in parentheses represent the number of observations. Due to time constraints, some spermatozoa parameters were not determined for each ejaculate at all storage times.
Figure 1. The percentage of motile spermatozoa among different antibiotic treatments at different storage time across boars. The 60% line represented the number of MOT that should be used to inseminate sows (Flowers, 1997).
The concentrations of gentamicin sulfate and amikacin sulfate determined by FPIA did not change during storage from 0 hour to 120 hours (Table 4).

No differences were found in the in vitro sperm penetration of boar semen among treatments (Figure 2). The penetration rate was less (p<0.05) on day 5 (11.4%) than on day 0 (24.2%) of storage. However, each boar showed a different penetration rate on day 5 (p<0.05) but not on day 0 (p=0.28, Figure 2). The interaction between boar and day was found as shown in Figure 2. Boar 2 showed higher (p<0.05) penetration rate than boar 1 on day 5 of storage but not on day 0.
Table 4. Antibiotic concentration (µg/ml; mean ± SEM) in diluted semen at different times of storage.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Amikacin sulfate (n=9)</th>
<th>Gentamicin sulfate (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>116.17 ± 10.80</td>
<td>392.80 ± 58.46</td>
</tr>
<tr>
<td>6</td>
<td>141.17 ± 10.74</td>
<td>383.33 ± 65.81</td>
</tr>
<tr>
<td>12</td>
<td>127.67 ± 16.15</td>
<td>380.67 ± 68.65</td>
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<tr>
<td>24</td>
<td>125.67 ± 24.40</td>
<td>364.83 ± 62.06</td>
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<tr>
<td>36</td>
<td>142.67 ± 22.33</td>
<td>364.00 ± 64.39</td>
</tr>
<tr>
<td>48</td>
<td>116.25 ± 26.20</td>
<td>471.25 ± 36.33</td>
</tr>
<tr>
<td>72</td>
<td>140.50 ± 18.01</td>
<td>375.67 ± 62.90</td>
</tr>
<tr>
<td>120</td>
<td>95.17 ± 24.71</td>
<td>355.17 ± 56.33</td>
</tr>
</tbody>
</table>
Figure 2. The penetration rate of diluted semen from boars 1 and 2 on day 0 and day 5 of storage. a,b statistically differ within the same boar (p<0.05). x,y statistically differ within day of storage (p<0.05).
Discussion:

Antibiotics are added to semen extender to control bacterial growth and to protect spermatozoa from detrimental effects from bacteria (Teague et al., 1971) and their toxins (Paulson and Polakoski, 1977). The present study examined the effect of different types of antibiotics on sperm cells in an environment with minimal contamination.

Semen characteristics were used to assess semen quality in this study. The results showed that percentage of motile sperm was affected by boars, antibiotics, and storage time. The MOT is used to indicate quality of semen before extension for artificial insemination. Flowers (1997) reported that insemination of semen with MOT less than 60% could reduce reproductive performance (farrowing rate and litter size). Thus, 60% could be used as the critical point for spermatozoa motility in AI programs. The results of the present study showed that treatment with antibiotics affected the MOT of spermatozoa when stored for seven 7 days. Regardless of treatment (antibiotic), MOT decreased for the first three days of storage. However, after day three of storage, GM, NM and AM groups showed a difference in the trend of declining MOT compared to PS and CT. Therefore, the inclusion of GM, NM or AM in semen extender may extend the shelf-life of extended semen.

In this experiment, considerable efforts were used to prevent contamination. However, in CT there were microorganisms and agglutinated sperm cells in the diluted semen after day 3 of storage. These were taken as indirect evidence of contamination. The PS and CT differed in MOT on day 3
compared to the others. Even in the minimal contamination environment, the combination of penicillin and streptomycin could not preserve diluted semen in the same level of the more recently developed antibiotics. Sone et al (1992) reported the same result that PS was not effective in extending sperm life. Under more severe contamination, GM and AM as well as polymyxin B and dibekacin were reported to prolong shelf-life and be effective against bacterial contamination (Sone et al., 1992). PS was recommended to use in semen extenders in the past. Many reports found the resistance of bacteria to PS was increased (Sone et al., 1992; Sone, 1990; Althouse et al., 2000). The new generation of antibiotics, such as gentamicin, neomycin, amikacin and polymyxin B, has been used in semen extender (Sone et al., 1992; Althouse et al., 2000).

Storage time had an effect on all semen characteristic parameters. MOT and NAR both decreased from day 1 through 7 of storage. However, MOR was not consistent in decreasing trend. Day 3 semen had the highest MOR compared to day 0 to 2 of storage. However, day 7 semen had the lowest MOR. This might be due to the random error in evaluating sperm cells. The results suggested that the diluted semen with BTS could be stored up to five days with acceptable level of semen characteristic parameters in an environment with minimal contamination.

There has not been a published study on changes in concentrations of antibiotic in diluted semen during storage. The GM and AM concentrations were stable in diluted semen for five days. The minimally contaminated environment may, at least in part, contribute to this stability. The efficiency of antibiotics,
especially aminoglycosides, was affected by pH, concentration of albumins and
divalent cations, Ca^{2+} and Mg^{2+}, in the solution (Hare and Miller, 1984). In
general, the pH of semen extenders was 6.8-7.2 (Waberski et al., 1994; Weitze,
1990). The pH of diluted semen decreased over storage time (personal
observation), which could reduce the efficiency of aminoglycosides (Hare and
Miller, 1984). However, semen extender does not contain calcium and
magnesium. Furthermore, EDTA was used in most extenders to chelate metallic
ions. It was anticipated that the antibiotic stability would be compromised by the
proteins in seminal plasma and pH changing over storage time. However, the
results in this study showed that the concentrations of gentamicin and amikacin
sulfate were not affected by the storage time.

It should be noted that the concentrations of gentamicin and amikacin
sulfate measured in diluted semen were different from the initial concentrations
added to the extender. In this study, the concentration of antibiotics used was
calculated from the activity of antibiotic labeled in the manufacturer's leaflet. The
FPIA technique is considered a precise and reliable procedure (Barnes, 1984). It
is possible that either one or more components of the extender affect the
outcome of the FPIA or a dilution error occurred during preparation for the FPIA.
Since the upper detection limit of the FPIA is 10 µg/ml, the extender required a
10 fold dilution. However, the main purpose of the FPIA was to determine if the
antibiotic concentrations changed during 120 hours of storage at 17^\circ C. The
results showed that there was no change in the concentrations of both antibiotics
in the diluted semen stored for 5 days. However, the test did not quantify the
potency of the antibiotics. From the concentration standpoint, it could suggest that antibiotics added in semen extenders, particularly gentamicin and amikacin sulfate, were maintained in stored semen over time. These results might not be applicable if the semen or extender was contaminated with bacteria.

Two boars used in *in vitro* penetration study were selected based on the results of MOT at day 5 of storage (72% and 57%, respectively). Antibiotics in semen extender had no influence on sperm penetration rate. The present study showed that treatment of diluted semen with antibiotics did not result in differences among antibiotics and control semen. The results of the first phase of this study indicated that, in minimal-contaminated environment, antibiotics in semen extender prolong motility of spermatozoa. In pig reproduction laboratories, utilization of a homologous penetration assay is used to estimate the fertility of semen. The *in vitro* penetration assay in swine is unable to predict the fertility of semen (Popwell, 2000; Xu *et al.*, 1998). However, to test the fertilization capacity of spermatozoa, the penetration rate is appropriate to test spermatozoa efficiency.

The penetration rate of the low MOT semen was lower (p=0.002) than the other boar on day 5 of storage. Boar semen that trended to show lower characteristic quality also showed lower penetration rate when stored for 5 days. In conclusion, treatment of diluted semen with antibiotics did not influence *in vitro* sperm penetration rate. However, storage of semen in BTS extender for five days had reduced *in vitro* sperm penetration rate. This conclusion must be
viewed with caution, as the *in vitro* sperm penetration rate does not necessarily predict subsequent fertility of the semen.

In conclusion, addition of particular antibiotics in semen extenders could prolong the motility of spermatozoa when stored up to 5 days. In this study, BTS, a 3-day extender, was used. The BTS extender has been used in swine AI program worldwide, and it was recommended for storage up to 3 days. Sperm quality characteristics in this study could be prolonged over 3 days with particular antibiotics, namely, gentamicin, neomycin and amikacin sulfate, but not with penicillin-streptomycin combination. In the control group, the MOT decreased rapidly and an increase in microorganisms and agglutination was observed after day 3 of storage.

Contamination with live bacteria or endotoxin reduces sperm motility, with agglutination or clumping of spermatozoa (Paulson and Polakoski, 1977; Teague *et al*., 1971). The present study showed that there were differences among types of antibiotics in terms of their effect on semen quality (*p*<0.05). Gentamicin, neomycin and amikacin apparently prolong motility of spermatozoa for a period of up to 7 days. Currently, swine practitioners and producers store diluted semen up to 5 days, so the aminoglycosides would be the antibiotics most recommended for the practitioners. It could be hypothesized that different effects of the antibiotics would be observed in semen quality for each boar; however, in the present study the changes in semen quality were similar for the three boars, despite differences in semen quality at the onset of the experiment.
References:


INSULIN-LIKE GROWTH FACTOR I (IGF-I) IN BOAR SEMINAL PLASMA AND ITS ASSOCIATION WITH SEMEN CHARACTERISTICS AND SUBSEQUENT FERTILITY

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Abstract:

The objectives of the study were to characterize insulin-like growth factor I (IGF-I) in boar semen and evaluate its association with semen characteristics and subsequent fertility. Semen from 105 boars (204 ejaculates) in a commercial boar stud was collected and processed for artificial insemination on six nucleus sow farms using a homogenetic-homospermic regimen. IGF-I concentrations in aliquoted seminal plasma were determined by RIA. IGF-I in seminal plasma was 95.38 ± 3.56 ng/ml (mean ± SEM). The total amount of IGF-I per ejaculate was 23.05 ± 1.20 µg. There were no associations among IGF-I level and percentages of motile spermatozoa, morphologically normal spermatozoa, spermatozoa with normal acrosomes, and subsequent farrowing rate and litter size. IGF-I levels were associated (p<0.05) with semen volume, sperm concentration and total number of spermatozoa per ejaculate. The results suggested that IGF-I in seminal plasma had no association with fertility. The association of IGF-I and sperm concentration as well as total number of spermatozoa might be used as a predictor for sperm production in the pig.
Introduction:

Insulin-like growth factor I (IGF-I) is a single-chain polypeptide and produced by many cell types. Salmon and Daughaday (1957) reported that growth hormone (GH) regulates growth of cells through a factor, called somatomedin, in serum. The biological actions and structures of the somatomedin were similar to insulin, and thus, it was renamed insulin-like growth factor I (Jones and Clemmons, 1995). Insulin-like growth factor I is a potent mitogenic protein (Hendricks et al., 1998) and can be found in many cells, especially highly proliferative cells. Sertoli and Leydig cells express IGF-I locally in testes and receptors for IGFs also were found in Leydig cells, Sertoli cells, spermatogonia, spermatocytes, and spermatids (Hendricks et al., 1998). The expression of IGFs receptors showed an association of IGFs and testicular function (Hendricks et al., 1998). The IGF-I was reported in seminal plasma in humans (Glander et al., 1996), bulls (Hendricks et al., 1998; Kouba, 1995) and boars (Hirai et al., 2001). Its concentration in seminal plasma was associated with germ cell development in humans (Glander et al., 1996) and motility of bull spermatozoa (Hendricks et al., 1998; Hoeflich et al., 1999).

Boar fertility is one of the most important components of reproductive performance in swine farms. To determine fertility of pig spermatozoa, the percentage of motile spermatozoa showed a correlation to in vivo fertility, but not to the results of the chlortetracycline fluorescence assay, internal ATP content and sperm viability test (Tardif et al., 1999). The percentage of motile spermatozoa distinguished fertility among boars when motility was less than 60%
(Flowers, 1997). Currently, there is no test to distinguish boars with different, yet acceptable levels of motility. Thus, IGF-I concentrations in the boar seminal plasma may be of possible clinical value as indices of seminiferous tubule function. The objectives of this experiment were to characterize IGF-I concentrations in boar seminal plasma and examine the relationship of IGF-I concentration with semen quality characteristics and ultimately, fertility.

**Materials and methods:**

**Animals:**

Semen from 105 boars in a commercial boar stud was collected on Mondays, Tuesdays, and Fridays from December 13 through 31, 1999. All boars were housed individually in pens in a temperature-controlled building. Boars were fed a corn-soybean meal base ration (12.5% crude protein) with free access to water. Twenty-six Hamline boars (a hybrid sire line boar), 54 Yorkshire boars and 25 Landrace boars (9-30 months of age) were included in this study. A total of 204 ejaculates were collected, including 48 ejaculates from the Hamline, 103 ejaculates from Yorkshire and 53 ejaculates from Landrace boars. Semen was processed for artificial insemination using a commercial extender (Vital™, IMV International Corp., Minneapolis, MN) at a dilution rate of 2.5-3.5 x 10⁹ spermatozoa per dose. Gilts (n=113) and sows (n=275), parity 1-6, were inseminated in homogenetic-homospermic regimens. Briefly, gilts and sows were inseminated with the extended semen from the same genetic line and all services performed in one estrous cycle used the extended semen from the
same boar and same collection. The inseminations were performed with more than 98% multiple inseminations, including 20 Ham line gilts and 55 sows, 77 Yorkshire gilts and 190 sows and 16 Landrace gilts and 30 sows.

**Semen and seminal plasma collection:**

Semen was collected by the hand-gloved technique into a plastic semen collection bag (Minitube of America, Verona, WI), which was inside a thermos preincubated at 37°C. The mouth of the thermos was covered with 3 layers of clean cotton gauze. Semen was evaluated for volume, concentration using a calibrated spectrophotometer, and progressive motility. A commercial extender (Vital™, IMV International Corp., Minneapolis, MN) was prepared fresh. The extender was warmed to 37°C. The temperature of the extender was adjusted, as necessary, to the same temperature as that of semen. The final number of spermatozoa in diluted semen was 2.5-3.5x10^9 per dose. The extended semen was placed in an 82 ml cochette with an automatic filling system (Automatic cochette filling system, IMV International Corp., Minneapolis, MN) and stored at 17°C until transported to sow farms.

**Semen quality evaluation:**

**Percentage of motile sperm cell:**

Twenty to twenty five microliters of a well-mixed semen were dropped on a caffeine-coated slide on a slide warmer stage. A warmed glass coverslip (25x25 mm) was gently placed over the semen sample. The slide was evaluated immediately under a phase-contrast microscope (Zeiss, West Germany), equipped with a warming stage. At least 5 fields under 400x magnification were
evaluated for spermatozoa that moved forward and had a strong beat of tail movement. Percentages were calculated from the number of motile spermatozoa divided by total number of spermatozoa counted.

**Percentage of morphologically normal spermatozoa:**

Two hundred microliters of well-mixed semen were placed into an Eppendorf tube (2 ml) containing 1 ml of 10% formalin. From this mixture, 20-25 µl of the semen sample was placed on a cleaned microscope glass slide (Fisherbrand, Fisher) and covered with a plastic coverslip. The slide was evaluated with phase-contrast microscopy. At least 200 sperm cells were evaluated from at least 5 different fields with 400X magnification. Also, spermatozoa with head and tail were recorded as normal spermatozoa. Other morphological appearance included abnormal in head shape, tail and midpiece, proximal and distal cytoplasmic droplets, were recorded as abnormal spermatozoa (Briz et al., 1995). The percentage of morphologically normal spermatozoa was calculated from number of normal spermatozoa divided by number of total spermatozoa counted.

**Percentage of spermatozoa with normal acrosome:**

Two hundred microliters of well-mixed semen were placed into an Eppendorf 2 ml tube containing 1 ml of 10% formalin. To proceed with the acrosome test, 20-25 µl of well-mixed semen was dropped on a cleaned microscope glass slide covered with a plastic coverslip. The slide was evaluated under a phase-contrast microscope. At least 200 sperm cells were counted from at least 5 different fields with 400x magnification. Normal shape and smooth
surface of acrosomal ridge were recorded as normal acrosomal ridge spermatozoa. Spermatozoa with broken acroome or loose acroome was recorded as abnormal acrosomal ridge spermatozoa (Briz et al., 1995; Pursel et al., 1972). The percentage of normal acrosomal ridge spermatozoa was calculated from number of normal acrosomal ridge spermatozoa divided by number of total spermatozoa counted.

**Artificial Insemination:**

Cochettes of extended semen were stored in 17°C room at the laboratory until transport to 6 nucleus sow farms (total of 6,000 sows), on a same-day transportation basis. Gilts and sows were assessed for estrus once daily in the morning using nose-to-nose boar exposure and a back-pressure test. The insemination was performed by a trained technician in a pen with the presence of boars in an adjacent pen. Sows were inseminated beginning the morning of detected estrus. The second insemination was performed on the next morning and the third insemination was performed on the afternoon of the second day of insemination. The third service was performed when sows were still in standing heat. Gilts were inseminated twice on the day (in the morning and afternoon), that they were found in standing heat. The third insemination, if necessary, was performed the next morning. 93.17% of the inseminations were triple matings and 5.46% were double matings. Age of semen at the first insemination was ranged from day 0 (day of collection) to day 5. Semen cochettes were discarded if they were not used on the day of removal from the storage unit.
Sows and gilts were immediately moved to gestation crates after breeding and fed 4.5 lbs/head/day. Commencing at 35 days and throughout gestation, they were fed to maintain a body condition score of 3 in a 5-score system. The feed ration was a typical corn based gestation diet of 12.5% protein. Three weeks prior to farrowing, sows were given an additional one pound until they were moved to the farrowing house.

Radioimmunoassay for IGF-I

Sample extraction:

Seminal plasma samples were extracted using an acid degeneration method (Houseknecht et al., 1988) modified to extract IGF-I from its binding proteins. The extraction was conducted in polystyrene or polypropylene tubes (Sarstedt Inc., Newton, NC). Briefly, 20 µl of sample was added into 380 µl of 1M Glycine solution (pH 3.2), which was prepared fresh before use. Then, 400 ml of RIA buffer (0.03M NaH2PO4, 0.02% Protamine sulfate, 0.01 M EDTA.2H2O, and 0.05% Tween 20, pH 7.5) was added, and the tubes were vortexed, capped and incubated at 37°C for 72 hours. After the incubation, 100 µl of 0.05M NaOH was added to neutralize the extraction reaction. The extracted samples then were vortexed and kept at 4°C until assay.

Radiolabeled IGF-I hormone:

Two micrograms of rhIGF-I (R&D Systems Inc., Minneapolis, MN) was iodinated with 0.25 mCi Na-I\textsuperscript{125} (Amersham Pharmacia Biotech, Piscataway, NJ) using the chloramine-T method (Blum and Breier, 1994; Houseknecht et al., 1988). Briefly, Na-I\textsuperscript{125} (2.5 µl) was added directly to the hormone solution in a 0.2
ml Eppendorf tube, and gently mixed. Ten microliters of 0.05M chloramine-T was added to the reaction for 45 seconds, followed by 200 µl of RIA buffer in order to stop the oxidization reaction. The mixture was then purified by using a column of sephadex G-50 beads (Houseknecht et al., 1988). Each sample was collected as a 1 ml fraction into a polystyrene tube (5 ml) containing 500 µl of RIA buffer. The radioactivity of each fraction was counted using a gamma counting system (Packard Multi-prias 4, United Technologies Packard, Downers grove, IL) to determine the peak with the highest radioactivity count. These fractions with the highest radioactivity were tested for purification by using the talc-resin-trichloroacetic acid test (Tower et al., 1980). All tubes were stored for less than a month at 4°C until assayed.

Insulin-like growth factor I assay:

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. The RIA buffer contained 0.03M NaH$_2$PO$_4$, 0.01M EDTA, 0.02% protamine sulfate and 0.05% Tween 20 in distilled water adjusted pH to 7.5. Polyethylene glycol (MW=8,000) was prepared at 12.5%, pH 8.6. The anti-hIGF-I, used as primary antibody (Lot # AFP 4892898), was obtained through the National Hormone and Peptide Program, NIDDK (Torrance, CA). The final dilution of the primary antibody was 1:150,000 in the final volume of the reaction. The goat anti rabbit IgG, used as secondary antibody (Lot# 5060-20), was purchased from Linco Research Inc. (St. Charles, MO). The IGF-I standards were prepared from IGF-I stock at a concentration of 200 µg/ml. Twenty microliters of working stock was aliquoted and frozen at −20°C. The standard
curve was prepared in 1:2 serial dilution in RIA buffer from 0.031 to 1.00 ng/tube (50 µl). Twenty microliters of standards or extracted samples were assayed (see appendices). Radioactivity of the pellets in each tube was counted for 1 min in a gamma counting system (Packard Multi-priias 4, United Technologies Packard, Downers Grove, IL). All data were calculated using RIA calculating software (Reese, 1987). The software calculated data in LOGIT/LOG fashion.

Validation of the assay:

The parallelism test was determined by comparing different volumes (20, 40, 60, 80 and 100 µl) of sample or control sample. Recovery of added IGF-I was determined by adding rhIGF-I (0, 100, 200 and 500 ng/ml) to RIA buffer and seminal plasma. Recovery of added rhIGF-I was 105%. The interassay and intraassay covariances were less than 15%.

Definitions:

Semen characteristic parameters included semen volume, sperm concentration, total number of spermatozoa/ejaculate, percentage of motile spermatozoa, percentage of morphologically normal spermatozoa and percentage of spermatozoa with normal acrosomes. AI semen management parameters included number of spermatozoa/insemination dose and age of semen at the first insemination. Sperm fertility parameters included percentage of sow farrowing after insemination, total number piglets born and total number of piglets born alive. Concentration of IGF-I in seminal plasma is reported in nanogram/ml (ng/ml) and total amount of IGF-I /ejaculate is reported in microgram/ml (µg/ml).
**Statistical analysis:**

Data from all ejaculates collected were used to analyze association among IGF-I levels and semen characteristic parameters. Only data of ejaculates that were utilized for insemination of more than one gilt or sow were used to analyze association among IGF-I levels and semen fertility parameters.

Total amount of IGF-I per ejaculate and amount of IGF-I per insemination dose were calculated to determine the effect of IGF-I on spermatozoa. Total amount of IGF-I/ejaculate represented indirect effect of IGF-I on spermatozoa in whole ejaculate. The amount of IGF-I/dose represented the direct effect of IGF-I on spermatozoa that used in insemination.

Descriptive statistical values included all semen characteristics, as well as IGF-I concentration, and total amount of IGF-I /ejaculate. The associations among level of seminal IGF-I, semen characteristic parameters, and fertility parameters were analyzed.

The associations were determined between IGF-I concentration and semen characteristics and fertility parameters; and between total amount of IGF-I per ejaculate and semen characteristics and fertility parameters. Data were categorized into groups of low, medium and high by using mean ± 0.5 (SD) as a medium group (see Figure 1). One way analysis of variance in general linear model procedures (SAS, 1988) were used to analyze the association among groups and semen characteristic and fertility parameters. The farrowing rate was analyzed using appropriated contingency tables Chi-square (SAS, 1988).
Since the insemination regimen was homogenetic and homospermic insemination, all data were analyzed based on breed of animal. Therefore, the genetic line was treated in the analyses as a block.

**Figure 1.** Data were categorized into 3 groups; low, medium and high, using mean ± 0.5 SD as a medium group.
Results:

IGF-I in seminal plasma and semen characteristic parameters:

The total number of boar semen collection was 204 ejaculates from 105 boars (Table 1). The overall concentration of IGF-I in boar seminal plasma was $95.38 \pm 3.56$ ng/ml (mean ± SEM) and total amount of IGF-I was $23.50 \pm 1.20$ µg/ejaculate (Table 2). Data were then categorized into three groups, low, medium and high, based on IGF-I concentration or total amount of IGF-I/ejaculate by using mean ± 0.5 SD as a medium group (Figure 1).

IGF-I concentration (IGF) showed differences among groups in total amount of IGF-I per ejaculate (TOTAL), semen volume (VOL), sperm concentration in semen (CONC) and total spermatozoa per ejaculate (TSP) (Table 3). The medium and high groups showed higher (p<0.05) VOL, and TSP compared to the low group.

TOTAL showed differences among groups (Table 4) in IGF, VOL, CONC and TSP as well. For each variables, except CONC, the high group had greater (p<0.05) values than the other groups. CONC was less (p<0.05) in the high category than the low category.

The descriptive statistical values and association among breeds are shown in Table 5. Landrace showed the highest (p<0.05) concentration of IGF-I in semen and total amount of IGF-I per ejaculate compared to that of Hamline and Yorkshire. No effect of breed was present (p>0.05) for percentage of motile spermatozoa and percentage of morphologically normal spermatozoa.
IGF-I in seminal plasma and subsequent sperm fertility:

The total of 98 ejaculates from 60 boars were used to inseminate gilts/sows (Table 1). Semen used to inseminate less than two sows were excluded from the analysis. Four hundred and forty seven sows originally were in this study but only 388 sows were included in the analysis. The subsequent fertility was assessed by farrowing rate (FR) and litter size (LS), including total number of pigs born (TB) and number of pigs born alive (TBA). The FR was 82%, TB was $11.7 \pm 3.5$ (mean ± SD) and TBA was $10.7 \pm 3.4$ (Table 6). The average of age of diluted semen at the first insemination (SAGE) was $1.7 \pm 1.1$ days and average number of spermatozoa per insemination dose was $2.85 \pm 0.35 \times 10^9$. Semen that was processed and used to inseminate gilts/sows had MOT of $78\% \pm 7$, and MOR of $85\% \pm 13$. Sow parity was $2.9 \pm 1.7$ and number of services was $2.9 \pm 0.3$ times per estrus.

IGF showed differences among groups (Table 7) in total amount of IGF-I per insemination dose (AIIGF) only. There were no other differences found. The “high” group had greater ($p<0.05$) AIIGF compared to the low and medium groups.

TOTAL showed differences among groups (Table 8) in number of sperm/dose (AICONC), AIIGF and SAGE. AIIGF showed statistical differences among groups (Table 9) in AICONC, AIIGF and SAGE. The high group showed the higher value in each variable. Among breeds, there were significance differences in AIIGF, AICONC, TB and TBA (Table 10). Ham-line had the highest FR but it was not significantly different when compared with that of the
other breeds (91 VS. 81, 78%; $\chi^2=4.678$ p=0.096). Meanwhile, Ham-line showed the lowest in LS, both TB and TBA, compared to Yorkshire and Landrace.
Table 1. Described number of boars and ejaculates used in the study

<table>
<thead>
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<th>Breed</th>
<th>Number of boars (head)</th>
<th>Number of ejaculates</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Total collected</td>
<td>Total used for insemination</td>
</tr>
<tr>
<td>Ham-Line</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>Landrace</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>Yorkshire</td>
<td>54</td>
<td>23</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>60</td>
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Table 2. Descriptive values for pooled semen characteristic parameters and IGF-I data

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Mean ± SEM</th>
</tr>
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<tbody>
<tr>
<td>IGF-I Concentration (ng/ml)</td>
<td>204</td>
<td>95.38 ± 3.56</td>
</tr>
<tr>
<td>Total amount of IGF-I/ejaculate (µg)</td>
<td>204</td>
<td>23.05 ± 1.20</td>
</tr>
<tr>
<td>Semen concentration (x10⁶/ml)</td>
<td>200</td>
<td>410.78 ± 10.85</td>
</tr>
<tr>
<td>Semen volume (ml)</td>
<td>204</td>
<td>229.78 ± 6.34</td>
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<tr>
<td>Total number of sperm/ejaculate (x10⁹)</td>
<td>200</td>
<td>87.89 ± 2.43</td>
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<tr>
<td>Percentage of motile spermatozoa (%)</td>
<td>199</td>
<td>78 ± 1</td>
</tr>
<tr>
<td>Percentage of morphologically normal spermatozoa (%)</td>
<td>199</td>
<td>85 ± 1</td>
</tr>
<tr>
<td>Percentage of spermatozoa with normal acrosomes (%)</td>
<td>199</td>
<td>99.9 ± 1</td>
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</table>
Table 3. Association of seminal IGF-I concentration and semen characteristics (mean ± SEM)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=71</td>
<td>n=74</td>
<td>n=59</td>
</tr>
<tr>
<td>IGF-I concentration (ng/ml)</td>
<td>44.83 ± 2.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.35 ± 1.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>160.02 ± 4.18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total amount of IGF-I (µg)</td>
<td>9.67 ± 0.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.04 ± 0.97&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.68 ± 2.33&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>207.21 ±10.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>227.42 ± 9.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>259.92 ± 12.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Semen concentration (x10&lt;sup&gt;6&lt;/sup&gt;/ml)</td>
<td>395.08 ± 17.33&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>443.92 ± 19.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>387.76 ± 18.95&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>% of motile spermatozoa</td>
<td>77 ± 1</td>
<td>77 ± 1</td>
<td>79 ± 1</td>
</tr>
<tr>
<td>% of morphologically normal spermatozoa</td>
<td>83 ± 2</td>
<td>86 ± 1</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>% of spermatozoa with normal acrosomes</td>
<td>99.9 ± 0</td>
<td>99.8 ± 0</td>
<td>99.9 ± 0</td>
</tr>
<tr>
<td>Total sperm/ejaculate (x10&lt;sup&gt;9&lt;/sup&gt;)</td>
<td>74.45 ± 3.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94.35 ± 4.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.74 ± 4.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>boar age (month)</td>
<td>16.6 ± 0.7</td>
<td>17.0 ± 0.7</td>
<td>16.0 ± 0.7</td>
</tr>
</tbody>
</table>

Values (within row) with different superscripts differ at p<0.05
Table 4. Association among total amount of IGF-I and semen characteristics (mean ± SEM)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=69</td>
<td>n=86</td>
<td>n=49</td>
<td></td>
</tr>
<tr>
<td>IGF-I concentration (ng/ml)</td>
<td>49.20 ± 2.91</td>
<td>96.14 ± 3.06</td>
<td>159.10 ± 5.43</td>
</tr>
<tr>
<td>Total amount of IGF-I (µg)</td>
<td>7.76 ± 0.48</td>
<td>21.52 ± 0.56</td>
<td>47.29 ± 2.21</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>165.78 ± 7.81</td>
<td>238.31 ± 7.84</td>
<td>304.94 ± 12.42</td>
</tr>
<tr>
<td>Semen concentration (x10^6/ml)</td>
<td>456.10 ± 20.33</td>
<td>404.68 ± 16.50</td>
<td>359.40 ± 17.21</td>
</tr>
<tr>
<td>% of motile spermatozoa</td>
<td>77 ± 1</td>
<td>78 ± 1</td>
<td>79 ± 1</td>
</tr>
<tr>
<td>% morphologically normal spermatozoa</td>
<td>85 ± 2</td>
<td>86 ± 1</td>
<td>84 ± 1</td>
</tr>
<tr>
<td>% of spermatozoa with normal acrosomes</td>
<td>99.9 ± 0</td>
<td>99.9 ± 0</td>
<td>99.8 ± 0</td>
</tr>
<tr>
<td>Total sperm/ejaculate (x10^9)</td>
<td>68.93 ± 2.78</td>
<td>92.17 ± 3.60</td>
<td>106.27 ± 5.41</td>
</tr>
<tr>
<td>boar age (month)</td>
<td>15.8 ± 0.7</td>
<td>17.5 ± 0.6</td>
<td>15.9 ± 0.7</td>
</tr>
</tbody>
</table>

Values (within row) with different superscripts differ at p<0.05
Table 5. Association among boar breeds and semen characteristics as well as IGF-I (mean ± SEM)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hamline (n=48)</th>
<th>Yorkshire (n=103)</th>
<th>Landrace (n=53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I concentration (ng/ml)</td>
<td>86.56 ± 7.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.33 ± 3.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>120.98 ± 8.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total amount of IGF-I (µg)</td>
<td>16.96 ± 2.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.51 ± 1.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.59 ± 3.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>177.36 ± 9.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>244.30 ± 8.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>249.04 ± 13.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Semen concentration (x10&lt;sup&gt;6&lt;/sup&gt;/ml)</td>
<td>415.58 ± 20.38</td>
<td>415.41 ± 16.55</td>
<td>397.45 ± 19.53</td>
</tr>
<tr>
<td>% of motile spermatozoa</td>
<td>77 ± 1</td>
<td>78 ± 1</td>
<td>79 ± 1</td>
</tr>
<tr>
<td>% of morphologically normal spermatozoa</td>
<td>86 ± 2</td>
<td>84 ± 1</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>% of spermatozoa with normal acrosomes</td>
<td>99.9 ± 0</td>
<td>99.8 ± 0</td>
<td>99.9 ± 0</td>
</tr>
<tr>
<td>Total sperm/ejaculate (x10&lt;sup&gt;9&lt;/sup&gt;)</td>
<td>70.20 ± 4.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.46 ± 3.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>93.05 ± 4.73&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>boar age (month)</td>
<td>18.0 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.8 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.8 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values (within row) with different superscripts differ at p<0.05
Table 6. Descriptive statistic values for pooled semen data used for insemination

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Mean ± SEM (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I Concentration (ng/ml)</td>
<td>98</td>
<td>94.97 ± 4.87 (0.00-259.68)</td>
</tr>
<tr>
<td>Total amount of IGF-I/ejaculate (µg)</td>
<td>98</td>
<td>22.35 ± 1.57 (0.00-79.17)</td>
</tr>
<tr>
<td>Total IGF-I in insemination dose (µg)</td>
<td>97</td>
<td>0.79 ± 0.06 (0.08-2.75)</td>
</tr>
<tr>
<td>Semen volume (ml)</td>
<td>98</td>
<td>225.83 ± 8.86 (59.00-476.00)</td>
</tr>
<tr>
<td>Semen concentration (x10⁶/ml)</td>
<td>98</td>
<td>425.61 ± 16.22 (136.24-823.96)</td>
</tr>
<tr>
<td>Total number of sperm/ejaculate (x10⁹)</td>
<td>98</td>
<td>89.00 ± 3.37 (31.10-165.17)</td>
</tr>
<tr>
<td>Percentage motile spermatozoa (%)</td>
<td>97</td>
<td>78 ± 1 (58-92)</td>
</tr>
<tr>
<td>Percentage of morphologically normal spermatozoa (%)</td>
<td>96</td>
<td>85 ± 1 (24-98)</td>
</tr>
<tr>
<td>Percentage of spermatozoa with normal acrosomes (%)</td>
<td>96</td>
<td>99.9 ± 0 (93-100)</td>
</tr>
<tr>
<td>Boar age (month)</td>
<td>98</td>
<td>16.8 ± 1.2 (9.00-29.00)</td>
</tr>
<tr>
<td>Concentration of AI dose(x10⁹)</td>
<td>97</td>
<td>2.85 ± 0.04 (2.50-4.00)</td>
</tr>
<tr>
<td>Semen age (day)</td>
<td>370</td>
<td>1.7 ± 0.6 (0.00-5.00)</td>
</tr>
<tr>
<td>Farrowing rate (%)</td>
<td>388</td>
<td>82</td>
</tr>
<tr>
<td>Total pigs born</td>
<td>318</td>
<td>11.7 ± 0.2 (2.00-23.00)</td>
</tr>
<tr>
<td>Total pigs born alive</td>
<td>318</td>
<td>10.7 ± 0.2 (0.00-19.00)</td>
</tr>
<tr>
<td>Sow parity</td>
<td>388</td>
<td>2.9 ± 0.1 (1.00-7.00)</td>
</tr>
<tr>
<td>Number of service/estrus</td>
<td>366</td>
<td>2.9 ± 0.0 (1.00-3.00)</td>
</tr>
</tbody>
</table>
Table 7. Association of seminal IGF-I concentration and semen fertility (mean ± SEM)

<table>
<thead>
<tr>
<th>variables</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=129</td>
<td>n=166</td>
<td>n=93</td>
</tr>
<tr>
<td>Semen conc/dose (x10^9)</td>
<td>2.8 ± 0.0</td>
<td>2.9 ± 0.0</td>
<td>2.9 ± 0.0</td>
</tr>
<tr>
<td>IGF-I/dose (µg)</td>
<td>0.48 ± 0.0³</td>
<td>0.67 ± 0.0³</td>
<td>1.37 ± 0.07³</td>
</tr>
<tr>
<td>Semen age (day)</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Farrowing rate (%)</td>
<td>79</td>
<td>84</td>
<td>83</td>
</tr>
<tr>
<td>Total pigs born</td>
<td>11.4 ± 0.3</td>
<td>11.5 ± 0.3</td>
<td>12.3 ± 0.3</td>
</tr>
<tr>
<td>Total pigs born alive</td>
<td>10.3 ± 0.4</td>
<td>10.5 ± 0.3</td>
<td>11.4 ± 0.3</td>
</tr>
</tbody>
</table>

Values (within row) with different superscripts differ at p<0.05
### Table 8. Association of total amount of seminal IGF-I/ejaculate and semen fertility (mean ± SEM)

<table>
<thead>
<tr>
<th>variables</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=139</td>
<td>n=151</td>
<td>n=98</td>
<td></td>
</tr>
<tr>
<td>Semen conc/dose (x10^9)</td>
<td>2.7 ± 0.0 (^{a})</td>
<td>2.9 ± 0.0 (^{b})</td>
<td>3.0 ± 0.0 (^{b})</td>
</tr>
<tr>
<td>IGF-I/dose (µg)</td>
<td>0.40 ± 0.03 (^{a})</td>
<td>0.73 ± 0.03 (^{b})</td>
<td>1.38 ± 0.06 (^{c})</td>
</tr>
<tr>
<td>Semen age (day)</td>
<td>1.5 ± 0.1 (^{a})</td>
<td>2.0 ± 0.1 (^{b})</td>
<td>1.7 ± 0.1 (^{a,b})</td>
</tr>
<tr>
<td>Farrowing rate (%)</td>
<td>81</td>
<td>83</td>
<td>84</td>
</tr>
<tr>
<td>Total pigs born</td>
<td>11.6 ± 0.3</td>
<td>11.5 ± 0.3</td>
<td>11.9 ± 0.3</td>
</tr>
<tr>
<td>Total pigs born alive</td>
<td>10.6 ± 0.3</td>
<td>10.6 ± 0.3</td>
<td>11.0 ± 0.3</td>
</tr>
</tbody>
</table>

Values (within row) with different superscripts differ at p<0.05
Table 9. Association of amount of IGF-I/insemination and semen fertility (mean ± SEM)

<table>
<thead>
<tr>
<th>variables</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=159</td>
<td>n=151</td>
<td>n=78</td>
<td></td>
</tr>
<tr>
<td>Semen conc/dose (x10^9)</td>
<td>2.8 ± 0.0^a</td>
<td>2.9 ± 0.0^b</td>
<td>3.0 ± 0.0^c</td>
</tr>
<tr>
<td>IGF-I/dose (µg)</td>
<td>0.34 ± 0.01^a</td>
<td>0.77 ± 0.01^b</td>
<td>1.67 ± 0.06^c</td>
</tr>
<tr>
<td>Semen age (day)</td>
<td>1.6 ± 0.1^a</td>
<td>1.8 ± 0.1^a</td>
<td>2.1 ± 0.1^b</td>
</tr>
<tr>
<td>Farrowing rate (%)</td>
<td>81</td>
<td>84</td>
<td>81</td>
</tr>
<tr>
<td>Total pigs born</td>
<td>11.8 ± 0.3</td>
<td>11.6 ± 0.3</td>
<td>11.3 ± 0.4</td>
</tr>
<tr>
<td>Total pigs born alive</td>
<td>10.8 ± 0.3</td>
<td>10.7 ± 0.3</td>
<td>10.4 ± 0.4</td>
</tr>
</tbody>
</table>

Values (within row) with different superscripts differ at p<0.05.
Table 10. Association among genetic lines and fertility (mean ± SEM)

<table>
<thead>
<tr>
<th>variables</th>
<th>Ham-line</th>
<th>Yorkshire</th>
<th>Landrace</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=75</td>
<td>n=267</td>
<td>n=46</td>
</tr>
<tr>
<td>Semen conc/dose (x10^9)</td>
<td>2.6 ± 0.0</td>
<td>2.9 ± 0.0</td>
<td>3.1 ± 0.0</td>
</tr>
<tr>
<td>IGF-I/dose</td>
<td>0.63 ± 0.06</td>
<td>0.80 ± 0.02</td>
<td>0.88 ± 0.1</td>
</tr>
<tr>
<td>Semen age (day)</td>
<td>2.1 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Farrowing rate (%)</td>
<td>91</td>
<td>81</td>
<td>78</td>
</tr>
<tr>
<td>Total pigs born</td>
<td>10.2 ± 0.3</td>
<td>12.0 ± 0.3</td>
<td>12.6 ± 0.5</td>
</tr>
<tr>
<td>Total pigs born alive</td>
<td>9.5 ± 0.3</td>
<td>10.8 ± 0.2</td>
<td>12.3 ± 0.5</td>
</tr>
</tbody>
</table>

Values (within row) with different superscripts differ at p<0.05
Discussion:

The objectives of this study were to quantify IGF-I in boar seminal plasma and to determine its relationship to subsequent fertility. The results confirmed the presence of IGF-I in boar semen. The IGF-I concentration in this study was higher (95.38 ± 3.56 ng/ml) than previously reported (14.8 ± 4.1 ng/ml) by Hirai et al (2001). The total amount of IGF-I per ejaculate was 23.05 ± 2.30 µg. The difference in IGF-I concentration between both studies was suspected in sample extraction methods. The sample extractions for the IGF-I assay were reported for several methods (Breier et al., 1991; Lee and Hendricks, 1990). The most common method was acid-ethanol extraction (Baxter et al., 1984; Breier et al., 1991). Recently, utilization of Sep-Pak C18 has been used in several tissue and samples extractions (Breier et al., 1991). In the present study, the acid degeneration method was used since it has been used in the laboratory for pig serum extraction. Hirai et al (2001) referred to the extraction method used in bull semen. The acid-ethanol extraction was not appropriate with pig samples (unpublished observation) since it was 5 times less effective in acid-ethanol compared to acid degeneration with glycine. Therefore, differences in extraction methods may account, at least in part, for differences in IGF-I concentrations (Lee and Hendricks, 1990).

In the present study, data were categorized and analyzed as groups, which can quantify the association among variables more reasonably. Since considerable variation in semen quality among boars and ejaculates is well documented, analyses of data in groups could explain the results with more
confidence and less confounding. The present study investigated the association among IGF-I level in seminal plasma, semen characteristics and subsequent fertility. The results indicated that the IGF-I level in boar seminal plasma had no apparent influence or association on all semen characteristic parameters. However, IGF-I was associated with semen volume, sperm concentration and total spermatozoa per ejaculate. These parameters could determine the capability in sperm production. IGF-I is found in high prolific cells, and could be an indicator for cell growth. In the testicle, spermatogenesis is considered as a prolific process supported by Sertoli cells. IGF-I in semen originates from the testis (Ovesen et al., 1995) and could be used as an indicator for spermatozoa production. Glander (1996) reported the correlation of IGF-I level in seminal plasma and normal spermatozoa morphology and concentration in human. Meanwhile, Kouba et al (1995) and Hendricks et al (1998) reported a correlation between IGF-I level and sperm motility for bovine semen. The present study did not show an association between IGF-I concentration and semen characteristics as well as subsequent fertility. In studies reported in men and bulls, the criteria used in those studies were semen characteristics, which could not determine its association with fertility. In this study, true fertility indices were investigated, including farrowing rate, total number of pigs born and number of pigs born alive. This could suggest that IGF-I level in seminal plasma was not associated with fertility indices in boars.

Historically, Landrace and Yorkshire were recognized as high productivity breeds. Meanwhile, Hamline was developed to serve as lean breed, which
means this breed was intended as a high-lean meat breed and not for productivity purposes. Landrace boars showed the highest concentration of IGF-I and LS compared to the others. It is interesting that the high productivity breed also had the highest level of IGF-I. Meanwhile, the Ham-line had the lowest total amount of IGF-I/ejaculate and LS but numerically had the highest FR. Unfortunately, no significance was detected. These results indicate that IGF-I levels in seminal plasma differed among breeds. Hendricks et al (1998) also reported the difference of IGF-I level in seminal plasma between breeds of bulls (Hendricks et al., 1998). Nevertheless, the results of the present study indicated that there were no differences in MOT, MOR or FR, TB and TBA among the three breeds of boars.

Previous attempts to find a predictive test for semen fertility failed to identify a reliable predictor. Percentage of motile spermatozoa has been used as a predictor for semen subsequent fertility. However, MOT can be used only to discriminate semen fertility at levels lower or higher than 60% (Flowers, 1997). In vitro fertilization (IVF) has been used as a fertility indicator in human and bovine spermatozoa. In pigs, there was no significant correlation between in vitro and in vivo fertility (Popwell, 2000; Popwell and Flowers, 1998; Xu et al., 1998). Farrowing rate and litter size apparently provide the most reasonable prediction of fertility in pigs (Clark et al., 1989); however, it takes more than 113 days to know the result of a particular ejaculate. IGF-I in seminal plasma could be used as predictor for spermatogenesis on spermatozoa production. The correlation between IGF-I level and sperm concentration in another study
(Glander et al., 1996) as well as the results of the present study indicated that IGF-I in seminal plasma might play some role on spermatozoa production. However, IGF-I did not show any association to subsequent fertility on FR and LS. The IGF-I receptor was reported in bovine spermatozoa (Hendricks et al., 1998; Kouba, 1995). Currently, there is a lack of knowledge on mechanisms of how IGF-I acts on spermatozoa.

The IGF-I binding proteins (IGFBPs) were reported for semen (Hoeflich et al., 1999), however, no significant roles were revealed. IGFBPs influences IGF-I activities and could enhance or reduce IGF-I potency (Henemyre and Markoff, 1999). There are at least six IGFBPs, and additional IGFBP related proteins. The IGFBPs might or might not modulate the mechanism of IGF-I in seminal plasma.

Overall, the presence of IGF-I in boar seminal plasma is reported in this study. IGF-I level showed an association to sperm concentration, semen volume and total spermatozoa per ejaculate. The IGF-I concentration might be considered as a sperm production predictor in boar seminal plasma. In contrast, IGF-I level did not show associations with semen characteristic parameters. In addition, boar seminal plasma IGF-I had no association to subsequent fertility, namely, farrowing rate and litter size. Therefore, quantifying seminal IGF-I may have limited practical applications in commercial boar studs.
References:


ASSOCIATIONS AMONG SEMEN CHARACTERISTICS AND SUBSEQUENT FERTILITY IN BOARS

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¹ Department of Farm Animal Health and Resource Management, ² Department of Animal Science, NCSU, Raleigh NC 27695.

Abstract:

The objectives of this study were to study the association among semen characteristics with subsequent fertility of boar semen. Semen from 105 boars, from three genetic lines, was collected from a commercial stud from December 13 to 31, 1999. Each ejaculate was evaluated for volume (VOL), concentration (CONC), percentage of motile spermatozoa (MOT), percentage of morphologically normal spermatozoa (MOR) and percentage of spermatozoa with normal acrosomes (NAR). Then, the semen was extended in a Vital™ semen extender to 2.5-3.0x10⁹ sperm cells per dose, and used for AI in a homogenetic-homospermic regimen at six nucleus sow herds. Age of semen at the insemination (SAGE) was 0-5 days. From a total of 388 inseminations, fertility parameters, including farrowing rate (FR), numbers of pigs born (TB) and pigs born alive (TBA), were recorded. No significant differences were found among groups of MOT and fertility parameters. Insemination with semen 0-2 days of age had higher (p<0.05) TB and TBA compared with that of semen that was syored for 3 or more days. Insemination with the number of spermatozoa per insemination (AIDOSE) of ≥ 3x10⁹ had higher TB and TBA compared to the group with < 3x10⁹ sperm cells per dose. With stepwise regression analysis, it was evident that FR was associated with VOL, MOR and SAGE. Meanwhile, TB
and TBA were associated with SAGE, AIDOSE and total number of spermatozoa per ejaculate. In conclusion, the assessment of semen characteristics may not necessarily delineate fertility between boars.

Introduction:

Several factors are involved or contribute to the fertility of boar semen. In general, the assessment of one or more of these factors does not provide a reliable prediction of the semen fertility. Assessment of semen characteristics, including percentage of motile spermatozoa (MOT), percentage of morphologically normal spermatozoa (MOR) and percentage of spermatozoa with normal acrosomes (NAR) can be used to evaluate semen quality (Colenbrander and Kemp, 1990; Flowers, 1997; Martin Rillo et al., 1996; Tardif et al., 1999; Woelders, 1991) but not semen fertility (Clark et al., 1989; Flowers, 1997; Popwell and Flowers, 1998). To assess boar semen fertility, farrowing rate and litter size were more reliable criteria (Clark et al., 1989); however, there is a significant time lag to assess fertility with these criteria.

Semen quality can be assessed on farms using macroscopic and microscopic evaluations (Martin Rillo et al., 1996). Flowers (1997) reported that inseminating sows with semen with less than 60% motility rate affected the breeding performance of sows. However, the study did not show any differences when semen had more than 60% motility. Sperm function tests, including ability of acrosome reaction, oocyte penetration, and motility assessment by computer-assisted semen analysis (CASA), are not practical, and the results are
controversial (Tardif et al., 1999) and depend on quality control (Barratt and St John, 1998). The in vitro penetration assay was used to estimate subsequent fertility in pigs (Popwell, 2000; Popwell and Flowers, 1998; Xu et al., 1998); however, the studies did not consistently show the correlation of in vivo fertility and the in vitro penetration rate (Popwell, 2000; Popwell and Flowers, 1998; Xu et al., 1998). Most sperm function tests are time consuming, and costly.

In artificial insemination (AI), semen is extended and stored until insemination (Baker and Polge, 1976; Johnson, 1998; Polge, 1978). Number of spermatozoa per insemination dose and age of semen at the insemination play some roles in breeding performance (Park et al., 2000; Waberski et al., 1994). It is recommended to use at least $3 \times 10^9$ sperm/insemination dose (Johnson, 1998). Johnson (1998) reported that about 85% of all inseminations made were conducted within one day and about 90% were used within three days after collection. Each factor described could play a significant role in semen fertility or be used to predict fertility of boar semen. This retrospective study was conducted to determine the associations among semen quality characteristics and AI semen management factors with subsequent fertility.

**Materials and methods:**

**Animals:**

Semen was collected from 105 boars in a commercial boar stud from December 13 through 31, 1999. All boars were housed individually in pens in a temperature-controlled building. Boars were fed a corn-soybean base meal
ration (12.5% crude protein) with free access to water. Twenty Hamline boars (a hybrid sire line boar), 23 Yorkshire boars and 17 Landrace boars (age between 9-30 months) were used in this experiment. A total of 98 ejaculates were used for breeding, including 32 ejaculates from Hamline, 44 ejaculates from Yorkshire and 22 ejaculates from Landrace. Semen was processed for artificial insemination using Vital™ semen extender (IMV International Corp., Minneapolis, MN) at a dilution rate of 2.5-3.5 x 10⁹ spermatozoa per dose. A total of 113 gilts and 275 sows (parity 0-6) were inseminated in homogenetic-homospermic regimens. Briefly, gilts and sows were inseminated with the extended semen from the same genetic line and all services performed in one estrous cycle used the extended semen from the same boar and same collection. The inseminations were performed with more than 98% multiple inseminations, including 20 Ham line gilts and 55 sows, 77 Yorkshire gilts and 190 sows, and 16 Landrace gilts and 30 sows.

**Semen and seminal plasma collection:**

Semen was collected by the hand-gloved technique into a plastic semen collection bag (Minitube of America, Verona, WI), which was inside a thermos preincubated at 37°C. The mouth of the thermos was covered with 3 layers of clean cotton gauze. Semen was evaluated for volume, concentration using a calibrated spectrophotometer, progressive motility, and dilution rate was calculated. The commercial extender was prepared fresh and warmed at 37°C. The temperature of the extender was adjusted, as necessary, to the same temperature as that of semen collected. The final number of spermatozoa in
diluted semen was 2.5-3.5x10^9 per dose. The extended semen was placed in an 82 ml cochette with an automatic filling system (Automatic cochette filling system, IMV International Corp., Minneapolis, MN) and stored at 17°C until transported to sow farms.

**Semen quality evaluation:**

**Percentage of motile sperm cell:**

Twenty to twenty five microliters of well-mixed semen were dropped on a caffeine-coated slide on a slide warmer stage. A warmed glass coverslip (25x25 mm) was gently placed over the semen sample. The slide was evaluated immediately under a phase-contrast microscope (Zeiss, West Germany), equipped with a warming stage. At least 5 fields under 400x magnification were evaluated for spermatozoa that moved forward and had a strong beat of tail movement. Percentages were calculated from the number of motile spermatozoa divided by total number of spermatozoa counted.

**Percentage of morphologically normal spermatozoa:**

Two hundred microliters of well-mixed semen were placed into an Eppendorf tube (2 ml) containing 1 ml of 10% formalin. From this mixture, 20-25 µl were placed on a cleaned microscope glass slide (Fisherbrand, Fisher) and covered with a plastic coverslip. The slide was evaluated with phase-contrast microscopy. At least 200 sperm cells were evaluated from at least 5 different fields with 400X magnification. Also, spermatozoa with normal heads and tails were recorded as normal spermatozoa. Other morphological appearance included abnormal head shape, tail and midpiece, and proximal and distal
cytoplasmic droplets, were recorded as abnormal spermatozoa (Briz et al., 1995). The percentage of morphologically normal spermatozoa was calculated from number of normal spermatozoa divided by number of total spermatozoa counted.

**Percentage of spermatozoa with normal acrosomes:**

Two hundred microliters of well-mixed semen were placed into an Eppendorf 2 ml tube containing 1 ml of 10% formalin. To proceed with the acrosome test, 20-25 µl were dropped on a cleaned microscope glass slide covered with a plastic coverslip. The slide was evaluated under a phase-contrast microscope. At least 200 sperm cells were counted from at least 5 different fields with 400x magnification. Normal shape and smooth surface of acrosomal ridge were recorded as normal acrosomal ridge spermatozoa. Spermatozoa with broken acrosome or loose acrosome was recorded as abnormal acrosomal ridge spermatozoa (Briz et al., 1995; Pursel et al., 1972). The percentage of normal acrosomal ridge spermatozoa was calculated from number of normal acrosomal ridge spermatozoa divided by number of total spermatozoa counted.

**Artificial Insemination:**

Extended semen were stored at 17°C at the laboratory until transportation. The extended semen were transported to 6 nucleus sow farms (total of 6,000 sows), on the same-day transportation basis. Gilts and sows were assessed for estrus once daily in the morning using nose-to-nose boar exposure and a back-pressure test. On each farm, trained technicians performed the insemination in a pen with the presence of boars in an adjacent pen. Sows were
inseminated beginning the morning of detected estrus. The second insemination was performed on the next morning and the third insemination was performed on the afternoon of the second day of insemination. The third service was performed when sows/gilts were still in standing heat. Gilts were inseminated twice on the day (in the morning and afternoon) they were detected in standing heat. The third insemination was performed the next morning, if necessary. With this insemination strategy, 93.17% of the inseminations were triple matings and 5.46% were double matings. Age of semen at the first insemination varied between 0 and 5 days. Insemination doses that were removed from the storage unit were discarded if they were not used.

The sows were immediately moved to gestation crates after breeding and fed 4.5 lbs/head/day. At 35 days, they were fed according to body condition in order to maintain a 3 (in a 5 score system) during gestation. The feed ration was a typical corn based gestation diet with 12.5% protein. Three weeks prior to farrowing, sows were given an additional one pound until they were moved to the farrowing house.

**Definitions:**

Semen characteristic parameters included semen volume, sperm concentration, total number of spermatozoa/ejaculate, percentage of motile spermatozoa, percentage of morphologically normal spermatozoa and percentage of spermatozoa with normal acrosomes. AI semen management parameters included number of spermatozoa/insemination dose and age of semen at the first insemination. Fertility parameters of spermatozoa included
percentage of females farrowing after insemination, total number pigs born, and
total number of pigs born alive.

**Statistical analysis:**

Data were categorized into groups as the following:

A. Percentage of motile spermatozoa (MOT) and percentage of morphologically
   normal spermatozoa (MOR) data were grouped as less than 70%, 70-80%,
   80-90%, and greater than 90%. This scale was chosen to include the range
   of spermatozoa with certain characteristics observed in this study, and so that
   lower end percentages and higher end percentages were grouped together.

B. Semen volume (VOL) data were categorized as less than 200 ml, 200-300 ml,
   and greater than 300 ml.

C. Sperm number per insemination dose (AIDOSE) data were grouped as less
   than 3x10^9 and greater than or equal 3x10^9 per dose.

D. Age of semen at the first insemination (SAGE) were grouped as 0-2 days and
   over 3 days.

E. Age of boar (BAGE) data were categorized as less than 12 months, 12-24
   months and over 24 months.

F. Total number of spermatozoa per ejaculate (SPTOTAL) data were grouped
   by using mean ± 0.5 SD as the medium group, plus groups less than and
   greater than the 0.5 SD.

Each parameter was analyzed separately at first by one-way analysis of variance
(ANOVA). The analyses were done to determine associations of these
parameters and subsequent fertility of the semen. The one-way ANOVA does not take into account interactions or confounding. Total number of pigs born (TB), and number of pigs born alive (TBA) were used in a statistical model as dependent variables. One-way analysis of variance (ANOVA) procedure with general linear model (SAS, 1988) was used to analyze association among individual parameter and fertility parameters. Genetic line of boar was treated as block to reduce variation among breeds.

Differences among means were determined by Duncan’s multiple range test when significance was observed. Association between each independent variable and farrowing rate (FR) were determined using the Chi-square test in the frequency table procedure (FREQ) of SAS (SAS, 1988).

The second approach in the statistical analysis was to use a stepwise regression procedure (SAS, 1988) to determine the relative importance of semen characteristics and other variables in the variation observed for fertility. The independent variables examined consisted of MOT, MOR, NAR, VOL, SPTOTAL, CONC, AIDOSE, SAGE and BAGE. The stepwise procedure was selected at a level of entry of p<0.15.

Results:

The results of each parameter analysis are shown in Figures 1-4. Spermatozoa in each MOT category did not show an association to fertility parameters (Figure 1). Spermatozoa with different MOR showed an association to the FR (\(\chi^2 = 8.085, p=0.044\)). The differences in MOR were not consistent
(Figure 2). Age of semen at the first insemination showed differences in TB and TBA. Insemination of semen age 0-2 days showed higher TB and TBA compared to semen of 3 days of age (11.9 and 11.0 vs. 10.6 and 9.6, respectively, p<0.01, Figure 3). Volume of semen did not show any associations to fertility parameters. The number of sperm/insemination dose showed differences in TB and TBA when analyzed regardless of genetic lines (p=0.053 and p=0.021, respectively, Figure 4). However, when genetic lines were included in the model, no significant differences were found. Total sperm/ejaculate did not show association with fertility parameters.

In stepwise regression analyses for FR, only VOL, MOR and SAGE were included in the final model (p≤0.15). For TB and TBA, only SPTOTAL, AIDOSE and SAGE were included in the final model (p≤0.15).
Figure 1. The association among motility and FR. Motility was classified in group.
Figure 2. The associations among MOR, FR, TB and TBA. MOR was classified into groups. MOR had association with FR ($\chi^2 = 8.085$, $p = 0.044$) and TB as well as TBA ($p < 0.05$). a,b,c and x,y bars with different superscripts differ ($p < 0.05$).
Figure 3. The associations between age of semen at the first insemination and TB, TBA as well as FR. Semen older than 2 days produced smaller TB and TBA than semen >2 days (p<0.05) and had a non-significant 6% less FR. Within parameter (TB, TBA), bars with different superscripts differ (p<0.05).
Figure 4. The association between number of spermatozoa per dose and TB and TBA. Sows inseminated with ≥3x10^9 spermatozoa had higher (p<0.05) TBA than females inseminated with the <3x10^9 spermatozoa.
* TB p=0.053.
Discussion:

The objective of this study was to determine the associations among semen characteristics and AI semen management factors with subsequent fertility. The analysis was carried on in two parts; the first was to analyze each semen characteristic and AI semen management factor independently, then a stepwise regression was included to determine the relative importance of semen characteristics and other variables in the variations observed for fertility.

The present results showed that semen in each MOT groups did not show associations with the fertility parameters, FR, TB and TBA. Popwell (2000) found that though boars had differences in in vivo fertility, they had no significant difference in MOT. Motility is used as the first evaluation method on boar semen evaluation protocols. In practice, motility assessment is a simple method to achieve on-farm and is used as the first criteria for acceptance of ejaculates. MOT was reported to be correlated to subsequent fertility of the semen, including FR, penetration rate and TBA (Flowers, 1997). Sows inseminated with semen containing less than 60% of motility had an adverse effect on breeding performance (Flowers, 1997). In this study, MOT did not show significances in FR, TB and TBA. The results of the stepwise regression also indicated that the MOT was not a significant factor in relation to TB and TBA.

In humans, MOR is consistently used as the best indicator of the fertility in males (Coetzee et al., 1998; Kruger et al., 1986; Rogers et al., 1983) but it is not used often in animals. MOR was previously reported in regard to in vivo fertility in boars (Popwell, 2000). In this study, MOR had a statistical association with
farrowing rate and initial ANOVA showed differences in TB and TBA. The differences in TB and TBA are misleading, particularly when the low MOR is similar to >90% MOR. This observation emphasizes the problem with one-way ANOVA. The interactions and confounding factors are removed from the model. In contrast, MOR was dropped from the model using stepwise regression. Therefore, in the present study, MOR presumably exerted minimal influence on TB and TBA.

The number of spermatozoa per insemination of 2.5-3 x 10^9 was used widely in research and in commercial farms (Polge, 1978). Park et al. (2000) reduced the number of spermatozoa per dose to 1.5 x 10^9 and found no difference in FR and TBA. In this study, when data was analyzed on number of spermatozoa per insemination dose, regardless of genetic line and with SAGE as a covariate, the inseminations with spermatozoa number of ≥ 3x10^9 per dose produced higher (p<0.05) numbers pigs born alive compared to insemination with < 3x10^9 per dose. Similarly, stepwise regression indicated that the semen dose contributed to the differences in TB and TBA. However, no association was found on farrowing rate. When genetic line was included in the model, none of the differences were observed. These results are in contrast to those reported by Park et al. (2000), which found no effect of sperm concentration per dose on litter size (1.5, 2, 2.5 and 3 x10^9/dose). However, the same result was shown on farrowing rate. The present results illustrate the effect of genetic line on fertility parameters of boar semen. In addition, the results reinforce the recommendation that was established for AI in pigs, namely, the artificial inseminate should
contain $3 \times 10^9$ spermatozoa per insemination dose, regardless of genetic lines. The attempt to reduce the number of spermatozoa per insemination dose was studied but the results are controversial and many factors confound the results (Park et al., 2000; Rath et al., 2000).

The type of extender has an effect on longevity of spermatozoa after dilution. In commercial farms, at least two types of extender (short-term and long-term) have been sold and used in swine farms. Short-term extenders are used for semen stored for one to two days (Waberski et al., 1994), meanwhile the long-term extenders are used for 3-5 days of semen storage (Kuster and Althouse, 1999; Waberski et al., 1994). In the present study, VitalTM semen extender, a long-term extender, was used. However, the results showed that insemination with semen older than 2 days of age at the first insemination resulted in lower litter size compared to inseminating with semen 0-2 days of age. The farrowing rate of the 0-2 days group tended to be (6%) higher compared to that of more than 2 days group (84% vs. 78%, respectively).

An interesting result was age of boar, which had no association with fertility parameters. The result is in contrasted to Clark et al (1989), who found that younger boars with less than 10 services had lower FR than the others. In the present study, the youngest group’s average age was 10.4 months.

Volume of semen and total number of spermatozoa per ejaculate did not show associations with fertility parameters. In stepwise regression analyses, VOL was associated with FR and SPTOTAL was associated with TB and TBA. However, this result needs to be viewed with caution because the semen was
extended with a semen extender and the volume as well as number of spermatozoa per insemination dose was adjusted to the same level in each insemination as the AI standard protocol. Thus, the associations of VOL and SPTOTAL with fertility indices were not true associations.

The breeding performance and production of swine farms are often measured by farrowing rate (FR) and number of pigs produced per sow per year (PSY). The PSY is reflected by the number of litter size (LS). Therefore, in general, farm breeding performance can be monitored by farrowing rate and litter size (Clark et al., 1989). In this study, none of the semen parameters were associated with TB and TBA only MOR had an association with FR. This result was, at least in part, in contrast with a previous study in which MOR reflected in vivo fertility (Popwell, 2000). AI semen management factors that affected TB and TBA were SAGE and AIDOSE. These results suggested that in AI, age of semen and number of spermatozoa per insemination dose play important roles in numbers of pigs born. However, only an association of SAGE with farrowing rate could be found. To assess breeding performance on farms, not only farrowing rate, but litter size is another important parameter that should be monitored. MOT is the routine assessment methods on farm, and the present results also failed to show its association to fertility.

Other factors that should be considered are SAGE and AIDOSE, which have been debated, in several scientific conferences. A reduction in the number of spermatozoa per insemination dose is one method to increase efficiency of boar usage in boar studs. The general recommendation for the number of
spermatozoa per AI dose is $3 \times 10^9$ (Polge, 1978). Reducing the number of spermatozoa per dose can increase the number of insemination doses per ejaculate. Since LS decreased in sows inseminated with less than $3 \times 10^9$ spermatozoa per dose, the present results did not support the concept of reducing the number of spermatozoa per dose. Extension of semen storage can increase the efficiency of boar usage. Many companies have tried to improve quality of their products to be able to store extended semen for longer periods of time. However, for utilization of the semen extenders for on-farm AI, several factors need to be considered (Kuster and Althouse, 1999). In this study, the Vital™ semen extender was used, and the results showed that the sows inseminated with extended semen age of 0-2 days had higher LS than the semen stored for more than 2 days. Extended semen should be used within 2-3 days regardless of extender types. The number of spermatozoa per AI dose is still as previously recommended, namely $3 \times 10^9$ per dose.

Each parameter in this study could be used to explain its association with subsequent fertility. However, when interactions among parameters were accounted for by using stepwise regression, none of the parameters showed a strong association with fertility parameters. The use of one-way ANOVA does not account for interactions among these parameters and the results, as shown in this study, maybe misleading. Finally, it is possible that the overall “quality” of the semen from boars in this study exceed levels necessary for fertility and thus, few differences were evident.
References:


SUMMARY

The studies illustrated that semen fertility is difficult to predict with semen analysis parameters. Many factors influence quality and fertility of boar semen.

The first study examined the addition of antibiotics in semen extender to affect shelf-life and the ability of spermatozoa to penetrate oocytes. It was shown that type of antibiotics affected semen quality. Aminoglycoside antibiotics maintained the quality of extended semen for 7 days of storage. However, the type of antibiotic had no effect on the ability of spermatozoa to penetrate oocytes. The day of storage had significant effect on penetration rate. The concentrations of gentamicin and amikacin in extended boar semen did not change during storage time. The findings suggested that antibiotics affect semen quality but not penetration rate. Day of storage impacts the penetration rate of spermatozoa.

The second study related the quantity of IGF-I in boar seminal plasma to semen characteristics and fertility. It was shown that levels of IGF-I in boar seminal plasma had no influence on the characteristics of the semen or on subsequent fertility. However, IGF-I levels were associated with semen volume, sperm concentration, and total number of spermatozoa in each ejaculate. The findings show that IGF-I in seminal plasma might be an indicator of the ability of boars to produce spermatozoa.

The third study examined factors affecting semen fertility. Semen characteristics and semen handling factors were associated with subsequent fertility indices. There was an association of the age of semen at the first
insemination with the number of pigs born and the number of pigs born alive. The same association was shown with the quantity of spermatozoa in each insemination dose. Semen age and quantity of sperm/dose play a role in the number of pigs born and the number of pigs born alive. Traditional methods to assess semen quality evidently provide a limited assessment of fertility and cannot delineate differences in fertility of boars.
APPENDICES
Appendix A: Tissue Culture Media:

Recovery Medium***
The recovery medium consisted of M199 Sigma 2520 supplemented with:

- pyruvate (0.90 mM) 0.1 g
- sodium bicarbonate (26.18 mM) 2.2 g
- glucose (3.05 mM) 0.55 g
- calcium lactate (8.25 mM) 0.9 g*
- BSA (0.4%) 4.0 g**
- Gentamicin sulfate solution 2.0 ml

Base Maturation Medium***
The base maturation medium (BM) consisted of M199 Sigma 3769 supplemented with:

- pyruvate (0.90 mM) 0.1 g
- sodium bicarbonate (26.18 mM) 2.2 g
- glucose (3.05 mM) 0.55 g
- glutamine (0.68 mM) 0.1 g
- calcium lactate (8.25 mM) 0.9 g*
- BSA (0.4%) 4.0 g**
- Gentamicin sulfate solution 2.0 ml

Remark:
* dissolve calcium lactate in 100 ml double-distilled water in a beaker, then pour only the top clear solution into the media at the last step.
** add BSA on top of media, let it dissolve by itself.
*** filter all media with 0.22 μm cellulose acetate filter system (Bottle top filter, Corning, Inc., Acton, MA).
Final Maturation Medium:
The final maturation medium consisted of base maturation medium supplemented with:

- LH (2000 IU/100 µl) 100 µl
- FSH (1 mg/100 µl) 100 µl
- ITS 100 µl

(insulin - 5 µg/ml, transferin - 5 µg/ml, sodium selenite - 5 ng/ml)

- estradiol-17β (1 mg/ml) 1 ml
- Porcine follicular fluid (v/v) 1 ml

Fertilization Medium:
The fertilization medium consisted of base maturation medium supplemented with:

- Caffeine (1.02 mM) 10 mg/50 ml
Appendix B: IGF-I Radioimmunoassay:

**Reagents:**

**RIA buffer:**
- Sodium phosphate monobasic: 2.28 g
- EDTA.2H₂O: 7.44 g
- Protamine sulfate: 0.4 g
- Tween 20: 1.0 ml
- Deionized water: 1,800 ml
- Adjust pH to 7.5
- Bring volume up to 2,000 ml with deionized water

**1M Glycine:**
- Glycine: 75.07 g
- Deionized water: 800 ml
- Adjust pH to 3.2 with concentrate HCL
- Bring volume up to 1,000 ml

**12.5% Polyethylene glycol:**
- Polyethylene glycol (MW=8000): 250 g
- Universal PHAB buffer powder*: 1 vial
- Deionized water: 1,800 ml
- Dissolve the powder and adjust pH to 8.6, mix until clear (low heat may be necessary for dissolving)
- Bring volume up to 2,000 ml with deionized water
- Store in refrigerator for up to 3 month

*Remark: Universal PHAB Buffer contains: 16.7 g sodium barbital, 2.5 g barbital, 1.0 g sodium chloride, 0.7 g disodium EDTA, and sucrose octaacetate (cat. no. 470180; Helena Laboratory, Beaumont, TX)
IGF-I Standards:
From a 20 µl IGF-I stock solution (1 µg/ml), a 1:2 serial dilution in RIA buffer was prepared as follow:
Standard G= 1ng/50 µl (stock 20 µl + 980 µl RIA buffer)
Standard F= 0.5ng/50 µl (500 µl of G + 500 µl RIA buffer)
Standard E= 0.25ng/50 µl (500 µl of F + 500 µl RIA buffer)
Standard D= 0.125ng/50 µl (500 µl of E + 500 µl RIA buffer)
Standard C= 0.0625ng/50 µl (500 µl of D + 500 µl RIA buffer)
Standard B= 0.0313ng/50 µl (500 µl of C + 500 µl RIA buffer)
Standard A= 0.015ng/50 µl (500 µl of B + 500 µl RIA buffer)

Radioiodination procedures:
1. To a vial containing 2 µg of IGF-I in 20 µl
2. Add 2.5 µl (0.25 mCi) of Na-I¹²⁵ (Amersham Pharmacia Biotech, Piscataway, NJ)
3. Add 10 µl (2.5 µg) Chloramine T, react for 45 seconds
4. Add 200 µl RIA buffer to stop the reaction
5. Iodinated IGF-I was recovered using a Sephadex G-50 column (0.9x25 cm) equilibrated with RIA buffer
6. Collect 15 fractions of 1 ml each from the column into a polystyrene tube containing 0.5 ml of RIA buffer
7. Monitor radioactivity in each fraction by counting 10 µl for 1 minute in a gamma counter to determine iodination profile
**Talc-resin-trichloroacetic acid stability test:**

**Reagents:**

- Talc powder 100 mg
- Bio-Rad anion exchange resin, AG 1x8, 200-400 mesh, chloride form 150 mg
- 10% Trichloroacetic acid (TCA) 1 ml
- 1% BSA in RIA buffer

**Procedures:**

1. Prepare three 12x75 mm test tubes, each containing 1 ml of IGF-I tracer (18,000 cpm in 1% BSA-RIA buffer)
2. To the first tube, add 100 mg of talc powder
3. To the second tube, add 150 mg of resin
4. To the third tube, add 1 ml of 10% TCA
5. Vortex each tube; walls of the talc and resin tubes may be washed down with an additional 1 ml of 1% BSA-RIA buffer
6. Centrifuge 30 minutes at 3000 rpm
7. Carefully decant supernatants into clean 12x75 mm tubes accordingly
8. Count the supernatant (super.) and precipitate (ppt) for each of the three tests
9. Calculate the percentage of precipitate for each test as
   \[
   \% \text{ ppt} = \frac{\text{precipitate}}{\text{super.} + \text{ppt}} \times 100
   \]

**General considerations:**

Optimal RIA results are obtained from monomeric iodohormones showing:

- greater than 90% absorbed to talc
- less than 25% bound to resin
- greater than 90% precipitate by TCA

With talc and TCA results within 3% agreement
**RIA Procedures:**

1. Using Digiflex-CX automatic pipette (Micromedic systems, Inc., Huntsville, AL), pipette 50 µl of extracted unknown sample or pooled serum or standards plus 250 µl of RIA buffer into polypropylene tubes.

2. Pipet 300 µl of RIA buffer into Bo tubes (total bound)

3. Pipet 400 µl of RIA buffer into NSB tubes (non-specific binding)

4. Add 100 µl of 1:100,000 primary antibody ((Lot # AFP 4892898, NHPP) to all tubes except Tc (total count) and NSB tubes.

5. Add 100 µl of IGF tracer, already diluted to 18,000 cpm/100 µl, into all tubes.

6. Cover tubes, vortex and incubate overnight (at least 20 hours) at 4°C.

7. After the incubation, add 50 µl of 1:100 normal rabbit serum (NRS) to all tubes except Tc tubes.

8. Add 50 µl of 1:80 secondary antibody into all tubes except Tc tubes.

9. Add 250 µl of cold 12.5% polyethylene glycol to all tubes, vortexed and incubated at 4°C for 30 min

10. Add 1 ml of RIA buffer to all tubes except Tc, and centrifuged 3,000 RPM at 4°C for 30 min.

11. Pour off the supernatant in a radiation waste bucket

12. Let tubes upside down for 5 min gently tap tubes with soft paper towel.

13. Count radioactivity in all tubes for 1 min in a gamma counting system (Packard Multi-priors 4, United Technologies Packard, Downers Grove, IL)
Appendix C: Parallelism plot for IGF-I in seminal plasma:

Figure 1. Parallelism plot of analyte concentration found in each dilution and concentration expected. The regression equation was \( Y = 0.98452X + 0.0006 \) with \( R^2 = 0.9765 \). The ideal result should be in a straight line with a slope of one, an intercept of zero, and a correlation coefficient of 1.0 (Perlstein, 1987).

Reference:
