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Impact of Nanotechnology-Based Semen Purification on Reproduction of Gilts and Developmental Performance of Offspring

Casey Lynn Durfey

Impact of nanotechnology-based semen purification on reproduction of gilts and developmental performance of offspring

By

Casey Lynn Rockwell Durfey

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Casey Lynn Rockwell Durfey

Approved:

_____
Jean M.N Nguekam Feugang
(Major Professor)

_____
Shengfa Liao
(Minor Professor)

_____
Mark Crenshaw
(Committee Member)

_____
Robert W. Wills
(Committee Member)

_____
Scott T. Willard
(Graduate Coordinator)

_____
John Blanton Jr.
Department Head

_____
George M. Hopper
Dean
College of Agriculture and Life Sciences

Name: Casey Lynn Rockwell Durfey

Date of Degree: August 11, 2017

Institution: Mississippi State University

Major Field: Agricultural and Life Sciences

Major Professor: Jean M.N. Feugang

Title of Study: Impact of nanotechnology-based semen purification on reproduction of gilts and developmental performance of offspring

Pages in Study 127

Candidate for Degree of Master of Science

Semen contain a heterogeneous population of viable and non-viable (damaged) spermatozoa. Proportions of non-viable spermatozoa interfere with male fertility, with available techniques unable to selectively remove prior to breeding. Nanobiotechnology may allow removal, enriching semen with high quality spermatozoa for improved productivity. Here, we applied double nanopurification with boar semen using functionalized magnetic nanoparticles.

Non-viable and viable spermatozoa were magnetically separated and verified through various microscopy imaging. Viable (nanopurified) spermatozoa showed no additional damages. Nanopurification did not interfere with sperm motility and viability, with beneficial effects on motion parameters. Nanopurified spermatozoa maintained fertility following insemination, with resulting offspring indicating no impaired growth or health performance. Pork quality was unaffected showing comparable characteristics to the control.

In summary, the use of magnetic nanopurification in boar spermatozoa showed sperm viability and fertility improvements with successful offspring performance. This

study shows promise for large-scale commercial applications to enhance male fertility and offspring performance.

DEDICATION

This thesis is dedicated to my family, for without them I would not have had the strength to undertake this project. I would like to thank my parents, sister, uncles, and Aunt C for all of their encouragement and motivation. You are my rock and the strength behind all of my accomplishments, I am eternally grateful for all of your love and support.

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CHAPTER I

LITERATURE REVIEW

1.1 Spermatogenesis and semen production

In sexually reproducing species, the comprehension of biological processes leading to sperm production is critical when trying to understand the proper physiological functions of gametes that are produced through gametogenesis. This process comprises oogenesis, for the production of oocytes in females and spermatogenesis, for the production of spermatozoa in males. The understanding of these biological processes is key to forming a solid comprehension for how the male and female reproductive systems function together for successful copulation and conception.

1.1.1 Testicles and accessary sex glands

Spermatogenesis is housed in the testis (or testicle), the primary site of androgen hormone production. The testis can be divided into four subsections, testicular capsule, parenchyma, mediastinum, and efferent ducts (Figure 1.1).

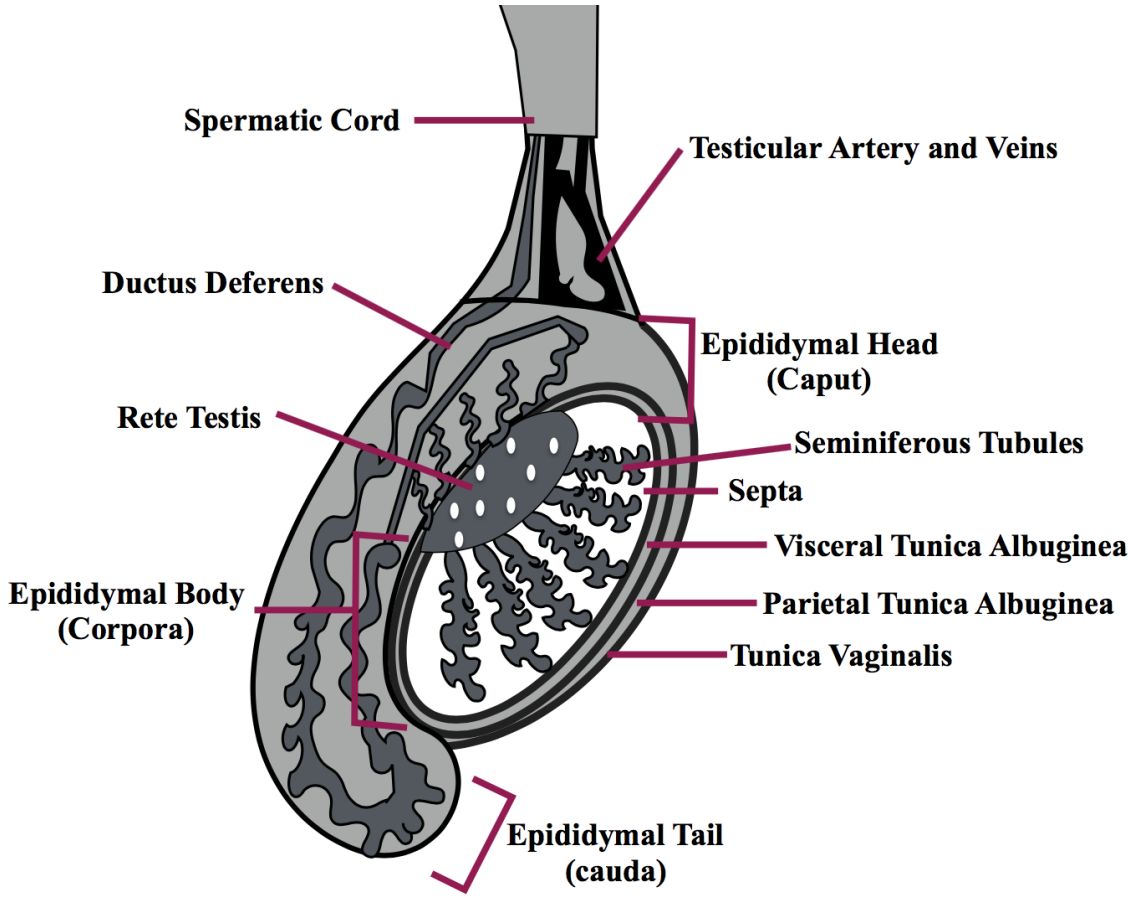

Figure 1.1 Schematic representation of the basic internal structure of the male testis (Recreated from: University of Virginia, 2013)

The testicular capsule is comprised of two layers of connective tissue (visceral vaginal tunic and tunica albuginea) to provide structure and protection to the testicle. The parenchyma consists of glandular tissue supported by the tunica albuginea. This structure is comprised of a tubular compartment housing seminiferous tubules and leydig cells. The mediastinum is the central core of the testicle and is home to ductus tissue of the rete tubules, providing transport channels for developing spermatozoa. Additionally, the mediastinum houses developing germ cells, peritubular cells, and sertoli cells for the

support and development of spermatogenesis. Finally, the efferent ducts provide an important link from the rete testis to the epididymis for fluid resorption and enzymatic activities (Senger 2012). Due to the external anatomical location of the testis, an important vascular arrangement is required for thermoregulation. This arrangement, known as the pampiniform plexus, describes the functional arrangement of arteries (testicular artery) interweaving with the testicular veins, forming a cone-like shape structure to enable cooling of arterial blood entering the testis from the exiting venous blood (Ashdown 1987). This temperature decline of arterial blood ranges between 2-4 ºC in mammals and is essential for thermoregulation of sperm. Additionally, two groups of muscles play important roles in the retraction (dartos muscle) and relaxation (cremaster muscle) of the testis to pull them closer or further from the body during periods of hot or cold weathered months.

Male accessory sex glands consist of the seminal vesicles, prostate, and bulbourethral (cowpers) gland, and work together with the epididymis to provide nutrients and transport medium for mature spermatozoa in the form of seminal plasma. The lobular shaped seminal vesicles in the boar provide essential salts, sugars, and protein to the seminal plasma for sperm nutrient and energy maintenance. Seminal vesicles also work to add volume for additional fluidity space for ease of sperm transport. The prostate gland provides the largest amount of fluid during ejaculation, with alkaline components to neutralize the acidic vaginal secretions (Setchell et al. 1993). Similarly, the bulbourethral gland provides the gel fraction component to the seminal plasma. It is important to note, that although the functions of the accessory sex gland aid in

spermatozoa function, they do not house spermatozoa, but instead function as a reservoir for seminal plasma fluid.

1.1.2 Regulation of the testicular function

The leydig and sertoli cells found within the testis initiating and assisting in spermatogenesis, are regulated by the hypothalamic pituitary testicular axis (HPT). This network of paracrine stimulations is projected from the brain hypothalamic region through the stimulation of gonadotropin releasing hormone (GnRH), controlling the release and production of luteinizing (LH) and follicle-stimulating (FSH) hormones from the anterior pituitary (Hafez and Hafez 2000). These two hormones are responsible for testicular regulation and function through endocrine and paracrine mechanisms.

The release of LH from the pituitary gland stimulates the production of androgens from the leydig cells within the testes, primarily stimulating testosterone production that acts in a variety of functions to aid spermatogenesis and sexual behavior in males. Similarly, FSH stimulates the sertoli cells within the testicular mediastinum to produce specific androgen-binding proteins that move the testosterone trapped within the seminiferous tubules for their conversion into dihydrotestosterone and allow spermatogenesis (Bartke, et al. 1978). The release of FSH activates the production of androgen-binding proteins and inhibin (sertoli cells), with the release of LH activating testosterone (leydig cells) to combine with androgen binding proteins initiating a negative feedback mechanism back to the anterior pituitary to regulate FSH and LH secretion.

1.1.3 Spermatogenesis and sperm maturation

Spermatogenesis occurs entirely within the seminiferous tubules of the testis. Starting with diploid spermatogonia cells, spermatogenesis describes the process of all cellular divisions and morphological changes which occur to developing germ cells, and can be divided into three phases, proliferation, meiotic, and differentiation (Senger 2012).

The *proliferation phase* describes all diploid spermatogonia mitotic divisions cascading through a series of generations of A-type spermatogonia to create a large number of B-spermatogonia generational cells. A key element during this phase is the ability for a portion of cells to undergo loss of intercellular bridges and revert back to stem cells for continuous rejuvenation.

The *meiotic phase* describes the two meiotic divisions spermatocytes undergo following the proliferation phase, creating genetic diversity from DNA replication and chromosomal crossing over for haploid spermatid formation. This process lead to the formation of haploid cells containing only half of the total chromosomes (18 somatic + 1 sex chromosome) in swine.

The *differential phase* of spermatogenesis describes the processes of transformation an undifferentiated spermatid undergoes to create a differentiated highly specialized haploid spermatozoon.

Sperm maturation occurs within the epididymis, surrounding the testicle. Upon transport through the epididymis sperm maturation occurs, causing progressive structural and biochemical modifications. During this process changes to the acrosome and plasma membrane alterations occur to allow compatibility and fertilization with the female gonad, the oocyte. As spermatozoa enter the epididymis, their concentration is low due

their dilution in the rete fluid. The epididymis works to concentrate spermatozoa through absorption of this rete fluid as it migrates through the system. In addition, a large variety of proteins are also changed along the spermatozoal head and body with the increased degree of disulfide crosslinking as spermatozoa migrate towards the epididymis tail. More often, spermatozoa enter the head of the epididymis with a cytoplasmic droplet near their head. Aiding in their ability for motility, as sperm travel through the body of the epididymis these cytoplasmic droplets migrates towards the tail. Spermatozoa can be stored within the body of the epididymis (corpora), but once their progression reaches the tail (cauda), they are released through the vas deferens during ejaculation or urination.

1.1.4 Semen Production

Semen ejaculates comprise both a liquid (or seminal plasma) and solid (cells) phases.

1.1.4.1 Seminal Plasma

Seminal plasma is produced through glandular secretions from the accessory sex glands and fluids from the cauda epididymis. Seminal plasma provides a protective media for sperm nutrients and transport. During ejaculation seminal plasma is released from the accessory sex glands in a sequential matter before deposition into the female. The first released fraction or pre-ejaculate is rich in bulbourethral gland secretions, followed by the sperm-rich fraction containing spermatozoa (stored within the cauda or corpora epididymis) and finally the last fraction, composed of prostate and seminal vesical secretions (Rodríguez-Martínez, et al. 2011). Seminal plasma is critical for the transport of spermatozoa, but in the boar also works to provide a cervical plug within the female

tract to minimize the loss of spermatozoa following copulation and ejaculation. (Senger 2012).

1.1.4.2 Spermatozoa

The solid phase of the ejaculate is composed of various somatic and sperm cells. The final formation and structure of a differentiated spermatozoon is comprised of a head, mid-piece, principal piece, and end-piece (Figure 1.2).

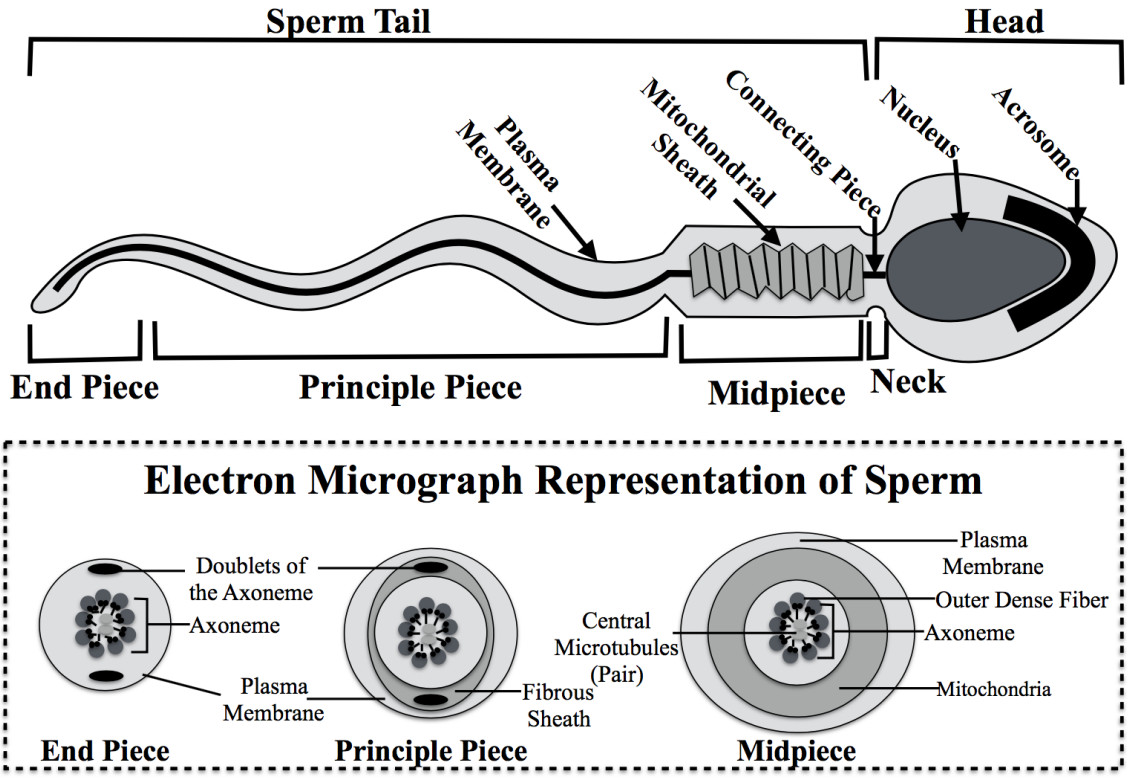

Figure 1.2 Schematic diagram and ultrastructure of spermatozoa (Recreated from: Borg, et al. 2009)

The *head* is the site of nuclear content, acrosome, and the post-nuclear cap. The acrosomal structure is a critical site for receptors and enzymes essential for compatible

interactions with the female oocyte. Specific to the boar, the nuclear shape of the head is species-specific dependent upon genotype, and comprises the majority of the head. The acrosomal region accompanies the nucleus in a sac-like structure capping the spermatozoal head. Acrosome is a membrane-bound organelle comprised of two layers essential for accurate binding to the female oocyte. The inner acrosomal layer is positioned over the nuclear envelope on top of the perinuclear space whereas the outer layer sits just beneath the plasma membrane structure surrounding the entire head. Between these two layers (outer acrosomal layer and plasma membrane) sits a homogeneous mixture of hydrolytic enzymes essential for compatibility binding to the oocyte (Briz and Faberga 2013).

The *connecting piece* on the boar spermatozoa is the region connecting the base of the nucleus (head) to the first mitochondrion of the tail. This region primarily functions to keep the tail region attached to the head.

The *tail* is the final characterized formation of the boar spermatozoa, comprising the mid-piece (site of mitochondrial synthesis), principal piece, and terminal or end-piece. Various primer proteins within the *mid-piece* regulate mitochondrial synthesis allowing for versatility in metabolism regulation for energy production dependent upon substrates available (Piomboni et al., 2012). Glycolysis and oxidative phosphorylation specifically have widely been considered energy regulating pathways within the mitochondrial spermatozoa, with glycolysis functioning as the primary metabolic pathway for maintaining sperm survival (Storey, 2004). The mid-piece itself is categorized into the axoneme, mitochondrial sheath, coarse fibers, and peripheral granules. The axoneme is positioned in the center of the mid-piece consisting of axial

filaments arranged in a classic 9+2 arrangement, with “spokes” and “arms” and extends down the entire length of the tail becoming increasingly disorganized as it reaches the more distal portions of the tail (Figure 1.2). Additionally, the axoneme is surrounded by the mitochondrial sheath where several mitochondria are stationed end-to-end along the length of the mid-piece (Briz and Faberga 2013). The *principal piece* is the longest region of the tail, comprised of a variety of fibrous axes that aid in spermatozoal movement. The *terminal or end piece* is the last and shortest region of the sperm tail, having no accessory cytoskeletal structures like the principle piece, but is instead comprised of a disorganized axonome and a plasma membrane. This region is thought to work in conjunction with the principal piece to provide efficient flagellar movements for movement and propulsion through the female tract (Omoto and Brokaw 1982).

1.2 Fertilization and embryo development

1.2.1 Sperm migration

Spermatozoa stored in the male reproductive tract are unable to move by themselves due to the acidity of the environment. Their transportation within the epididymis is facilitated by the cilia movements of the epididymis epithelial cells. Once they approach release by the ejaculation process, they progressively gain of motility by the presence of oxygen and increased pH by the sex gland secretions at the moment of ejaculation. Following semen deposition during copulation, spermatozoa are subjected to an entirely new environment (i.e., chemical composition, female immune system), with their transport being divided into two phases: sustained and rapid. This migration process is explained in further detail in Figure 1.3.

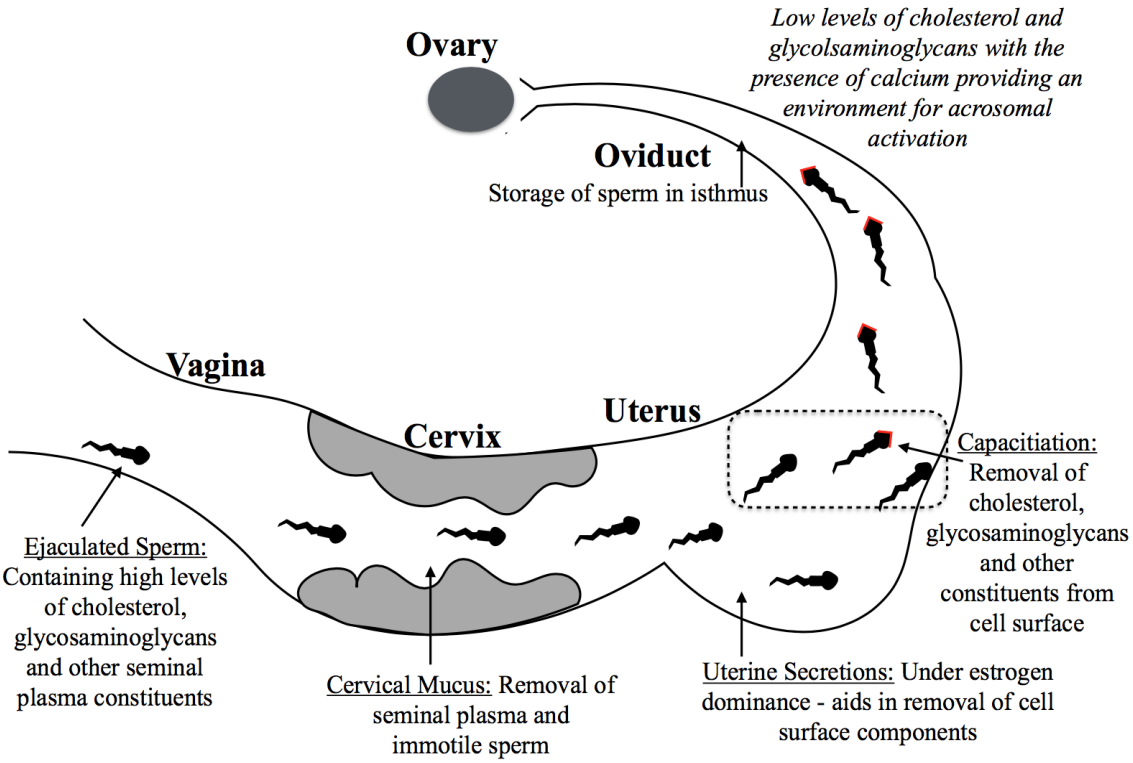

Figure 1.3 Schematic representation of sperm migration and capacitation during transport through the female genital tract (Recreated from: Geisert 1987)

During the *sustained transport phase*, spermatozoa move through the cervix, where the majority of seminal plasma and immotile sperm are lost, to the uterine body and horns with the help of uterine secretions and muscular contractions acting as a selective barrier against non-viable sperm (Suarez 2016). Upon transport to the oviduct, spermatozoa undergo a capacitation reaction (Figure 1.3). The secretions in the female genital tract contribute to destabilize the phospholipid bilayer surrounding the spermatozoal head surface and permitting fusion of the plasma membrane with the outer acrosomal membrane. This fusion allows the release of acrosomal hydrolytic enzymes necessary for acrosome activation when sperm come in contact with the oocyte (Figure

1.4). Various studies have shown this capacitation process can be reversed through the presence of seminal plasma (decapitation) (Figure 1.3). During capacitation, spermatozoa progress within the oviductal lumen to bind with epithelial cells in the lower isthmus region (sperm reservoir). Here, spermatozoa are able to gain further motility (hyper-activation) through interactions with specific molecules in this reservoir.

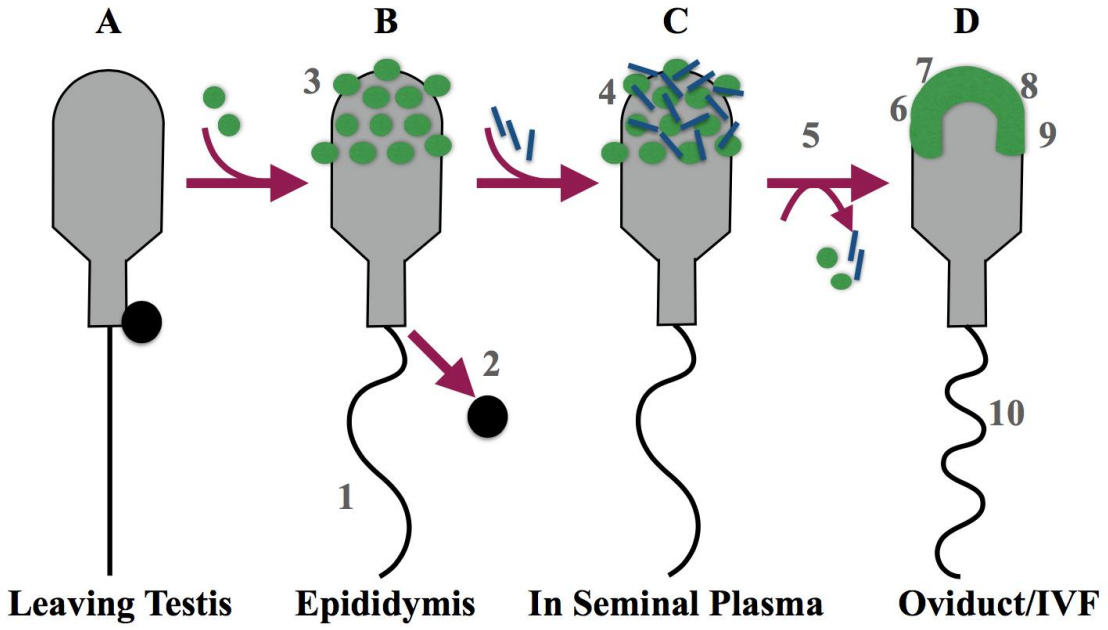

Figure 1.4 Sperm Capacitation

Sperm capacitation. (A) Upon leaving the testis, spermatozoa are morphologically complete but infertile due to the presence of a cytoplasmic droplet. (B) In the epididymis, spermatozoa (1) acquire motility characteristics through tail modification, (2) lose the cytoplasmic droplet and (3) undergo enzymatic protein surface alterations (green) essential for sperm–zona binding, and surface stabilizing factors. (C) During ejaculation, spermatozoa are mixed with seminal plasma and glycoproteins (blue) adhering to sperm surface (4). (D) When spermatozoa reach the site of fertilization, they are triggered to capacitate through surface changes: (6, 7 & 8) epididymal, lipid, and semi-lipid proteins (green) move to the tip of the sperm head. At the cytosolic site, the sperm head is now efficiently and stably docked to the outer acrosomal membrane (9). Additionally, the sperm tail, generates hyperactivated motility (10). (Recreated from: Leahy and Gadella, 2011).

The *rapid transportation phase* of spermatozoa starts with the accelerated beats of their flagellum within the reservoir, which frees them from epithelium attachments. These hyper-activated spermatozoa move very fast in a disorganized manner through the oviduct to meet the oocyte.

1.2.2 Fertilization

Hyper-activated and capacitated spermatozoa must penetrate through the cells (cumulus cells and radiata corona) surrounding the zona pellucida before direct interaction with the oocyte plasma membrane. First, spermatozoa must bind to the zona pellucida through specific interaction with the zona pellucida protein 3 or ZP3 (receptor on sperm exposed from capacitation), leading to acrosomal reaction and fusion of the zona pellucida and the outer acrosomal membranes (formation of vesicles) and liberation of acrosomal enzymes (i.e., acrosin) allowing the sperm to pass through the zona pellucida and reach the perivitelline space. Following, the oocyte plasma membrane fuses with fusion proteins on the sperm surface to digest the post nuclear cap and enable sperm membrane to fuse with the oocyte membrane. Thereafter, the cortical granules beneath the zona pellucida initiate a “zona block’ disenabling the penetration of further spermatozoa within the oocyte and preventing polyspermy.

At ovulation or prior to fertilization, the oocyte is resting in the metaphase of the second meiotic division, with chromosomes aligned along the meiotic spindle of the equator. During the time of sperm penetration, the oocyte resumes meiosis (anaphase, telophase) ending with expulsion of the secondary polar body and formation of the haploid female pronuclear (Hunter, 2000). As the sperm nucleus enters the cytoplasm of the egg, the nucleus decondenses to form the male pronucleus that fuses with its female

counterpart (syngamy) and allowing both maternal (oocyte) and paternal (sperm) chromosomes to pair up and form a new diploid cell with two sets of chromosomes (2N chromosome or 38 in pigs) called zygote.

1.2.3 Embryo development and preimplantation

The formed diploid zygote (1-cell stage embryo) undergoes successive mitotic divisions to multiple individual and smaller cells (blastomers) within the zona pellucida, in a process called *cleavage*. Around the third or fourth cycle of divisions, cells will begin to form a solid ball of compact cells called *morula*, that creates two cell-lineages (inner cell mass or ICM and trophectoderm or TE) delimiting a central fluid-filled cavity, called blastocoel. This fluid is primarily secreted by the tightly connected TE blastomers that are ranged around the outer morula, beneath the zona pellucida, providing a seal to the blastocoel and protection to the ICM. This developmental stage is referred to as a *blastocyst*, with ICM giving rise to the fetus and TE that will form the future extra-fetal portion of the placenta (chorion). As the blastocyst pursues its growth through mitosis and fluid fills the blastocoel (*expanded blastocyst*), pressure continues to rise and weakening the zona pellucida, causing its rupture and allowing escape of the blastocyst to free-float (*hatched blastocysts*) within the lumen of the uterus for implantation. A chart of pig oocyte maturation and preimplantation embryo development is shown in Figure 1.5.

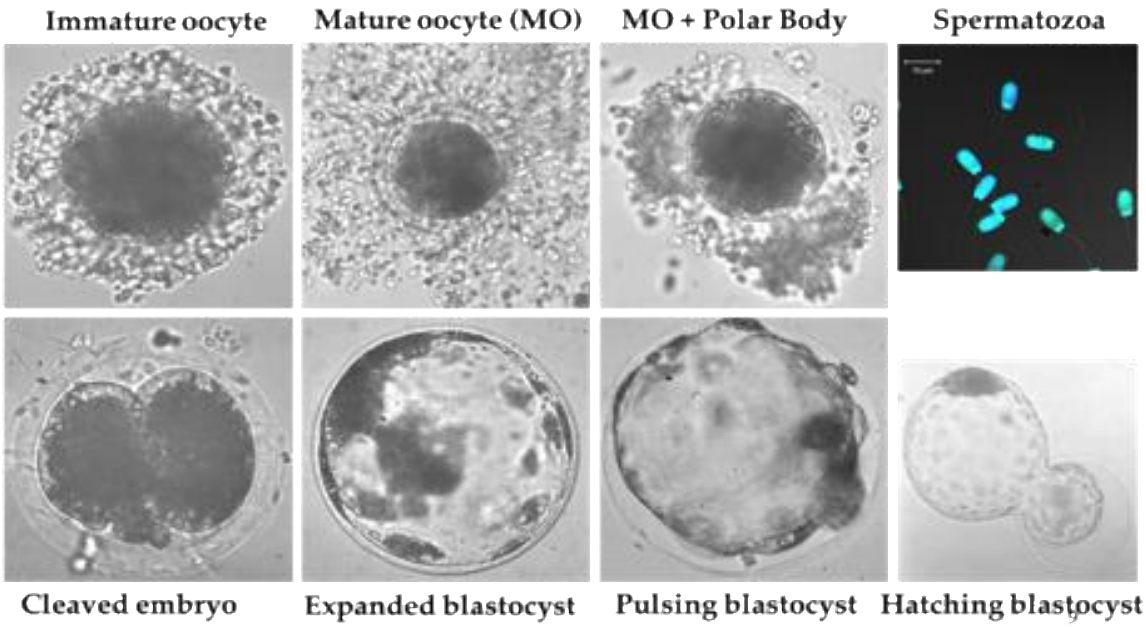

Figure 1.5 Oocyte maturation and preimplantation embryo development

Image curtesy of Jean Feugang (Unpublished data).

In swine, maternal recognition of pregnancy is established through endocrine secretions of estradiol causing prostaglandin PGF2α to be secreted towards the uterine lumen to prevent luteolysis. Other secretions such as prostaglandin, relaxin, and estrogens stimulate contractions in the myometrium to distribute multiple conceptuses along the uterine horn, properly spaced apart for implantation. Upon implantation in the sow, formation of a diffuse epitheliochorial placenta develops. This placenta type consists of many chorionic villi distributed over the entire surface of the chorion, penetrating into the endometrium to form a fetal-maternal interface for nutrient transfer throughout gestation for approximately 114 days.

Spermatozoa's influence in sexual reproductive animals is tremendously consequential on the successful fertilization, normal embryo development and beyond.

Number of studies have evidenced these impacts with various semen/sperm quality and paternal origins (Borini et al., 2006 and Sakkas et al., 1998).

1.3 Application of artificial insemination in swine

1.3.1 Demand for efficient productivity

Increasing efficiency in today's meat and production industries is essential to comply with the overwhelming consumer demand and lack of farmland. Worldwide overpopulation is becoming an unfortunate reality, with an expected world population of over 9 billion people by 2050 (FAO, 2017). With the majority of human populations growing currently occurring in developing countries, the need for efficiency in meat and production industries is not only becoming a necessity but a requirement (Thornton 2010). The ability to provide a functional non-invasive way to increase the amount of quality offspring in production animals is the key to help keep up with increasing consumer demand.

1.3.2 Advantages and limits of artificial insemination

1.3.2.1 Advantages

There are many advantages for the use of artificial insemination (AI) as a means of an assisted reproductive technique (ART) in livestock. Within the swine industry, the high output of semen produced from boars would allow multiple AI doses from a single ejaculate for a higher commercial index boar. One of the largest advantages for the use of artificial insemination is the dramatic improvements on genetic quality and diversity, using fewer sires (Knox 2016).

Previous to artificial insemination, genetic advancements were limited to locally disposable high quality sires within the surrounding area. With the use of AI, semen of highly selected males can be collected from high quality sires and (cryo) preserved for prolonged periods for world-wide transportation to desired females. Moreover, AI allows for more breeding flexibility for pre-timed breeding. Therefore, farmers can concentrate solely on the period of time when the sow will come into heat and not when the boar will be available. Another advantage of AI is the lack of contact between boars and sows, preventing biosecurity concerns and possible exposure to diseases (Singleton 2001).

1.3.2.2 Limits

Although artificial insemination offers great advantages, it can also introduce a variety of limitations.

1) The need for skilled personnel is essential to provide the most effective insemination technique, as the success of artificial insemination is dependent upon the quality of semen used and the insemination protocol (i.e., proper supervision of the sow for accurate heat detection for more effective AI since a boar is not required).
2) The upfront costs associated with semen purchased for AI, skilled personnel, labor, and needed supplies.
3) Higher risk of sexually transmitted diseases associated with accidental use of infected semen (PPRS, leptospirosis, etc.) that could cause serious harm to numerous inseminated females, resulting in reproductive failures or illnesses (Maes, et al. 2008) and serious economic loss for the producer.
4) Better management practices that must be suitable for AI incorporation into a breeding program. Unfortunately, successful cryopreservation of boar semen is still

faced with many limitations due to various factors related to individual boars, ejaculates and environment (Maes, et al. 2011).

1.3.3 Sperm characteristics

Sperm characteristics are dependent on a variety of intrinsic and extrinsic factors. Various genetic factors associated with specific genes and hereditability have shown to effect sperm viability characteristics. Semen with poor genetic value often possess higher concentrations of abnormal spermatozoa with possibly damaged DNA fragments, making them unsuitable for AI (Lopes 1998). Similarly, extrinsic factors such as environmental and management conditions can have a significant impact on sperm quality. Stress (noise, space, etc.), temperature, nutrition, and social interactions can all have an influence on the fertilization potential of boars, through inhibition or interruption of spermatogenesis (Kekalainen, et al. 2015; Wettemann, et al. 1976).

As sperm quality is altered, it creates a heterogeneous population of viable and non-viable (damaged) sperm within the ejaculate dose. In various species, studies have demonstrated that poor semen contain higher amounts of spermatozoa with sub-lethal molecular damages, that may fault the prognosis of semen fertility following the use of routine laboratory techniques (Sakkas, et al. 1998; Seli, et al. 2004). Differences within spermatozoal performance in a single ejaculate dose can be attributed to morphological differences affecting motility, velocity, and directionality; and examination before AI can help prognostic whether semen samples are likely to lead to successful fertilization. As the population of non-viable spermatozoa within an ejaculate dose is concentrated, it can create clumps of damaged cells, blocking a straightforward path for viable sperm to successfully fertilize.

1.3.3.1 Morphological and molecular defects

Sperm abnormalities can arise during spermatogenesis and maturation and can be seen in the form of bent or coiled tails, detached tails, and/or cytoplasmic droplets. Additional forms of semen abnormalities may be established from low sperm count (oligozoospermia), low sperm motility (asthenozoospermia), or the absence of spermatozoa all together (azoospermia). A high proportion of abnormal (teratozoospermia) or immobile/dead (necrozoospermia) may arise when extreme interruption or inhibition to spermatogenesis is present due to intrinsic and extrinsic factors. These morphological characteristics can be indicative of molecular damages as well, due to various genetic abnormalities providing a clear evaluation of the fertility potential of the boar. Indeed, numerous molecular defects of spermatozoa can negatively affect male fertility.

1) *Pre-mature acrosome reaction* (hyperactivation) occurs during early capacitation, resulting in the inability to be compatible with the female oocyte for successful fertilization, often leading to cellular apoptosis.
2) *Apoptosis incidence* in spermatozoa can be caused by DNA-damages, morphological impairments, or a release of toxic amounts of reactive oxygen species (ROS). The occurrence of ROS removes cholesterol from the sperm plasmalemma and increases its permeability (mitochondrial and plasma membrane), inducing a cascade of reactions leading to apoptosis (Aitken and Baker 2013).
3) *Decreased motility* can result from the formation of lipid adducts on spermatozoal head proteins and impairments in mitochondrial energy regulation, causing limited movement potentials and induced apoptosis.

1.3.3.2 Current status of semen evaluation manipulation

Semen evaluation are conducted using a variety of subjective and objective methods. Now processing semen subjectively involves routine laboratory techniques which vary according to technicians. In contrast, objective semen evaluation are conducted with high quality and often costly equipment to obtain non-bias data. For example, the use of a Computer-Assisted-Sperm-Analyzer (CASA) has allowed for objective evaluation of sperm motility, velocity, DNA-fragmentation, morphology, etc. In addition, CASA analysis in other species provides further insight in the directionality and efficiency of sperm movements (Figure 1.5) (Amann and Waberski 2014). Sperm movements are important to estimate the amount of sperm that would efficiently travel through the female tract and provide a better chance for fertilization. Numerous viability tests are available and have been nicely summarized in numerous reviews (Niżański, et al. 2015; Sutovsky 2015). Commonly used staining tests permit the evaluation of sperm membrane integrity (i.e., propidium iodide, eosin), acrosome reaction (i.e., Peanut sativum/peas agglutinin or PSA/PNA), DNA fragmentation or apoptosis (i.e., Annexin-V), ROS accumulation (i.e., DCHF-DA), and mitochondrial potential (i.e., JC-1, mito traker).

The aforementioned techniques are mainly applied in laboratory settings, while many farmers choose to conduct a breeding soundness exam, determining the male fertility potential through parameters such as evaluation of external scrotal circumference. Semen color, sperm characteristics, and seminal plasma.

The combination of these evaluation techniques (objective, subjective, and BSE) are excellent predictors of semen quality, their routine applications in large commercial

studs are still limited. The current progress in understanding the sperm biology allows for the development of molecular-based technical approaches to improve semen manipulation for accurate recognition of non-viable spermatozoa within the semen ejaculate. The combination of these techniques for sperm evaluation in large commercial farms are still limited, and the fertility predictors capability remain.

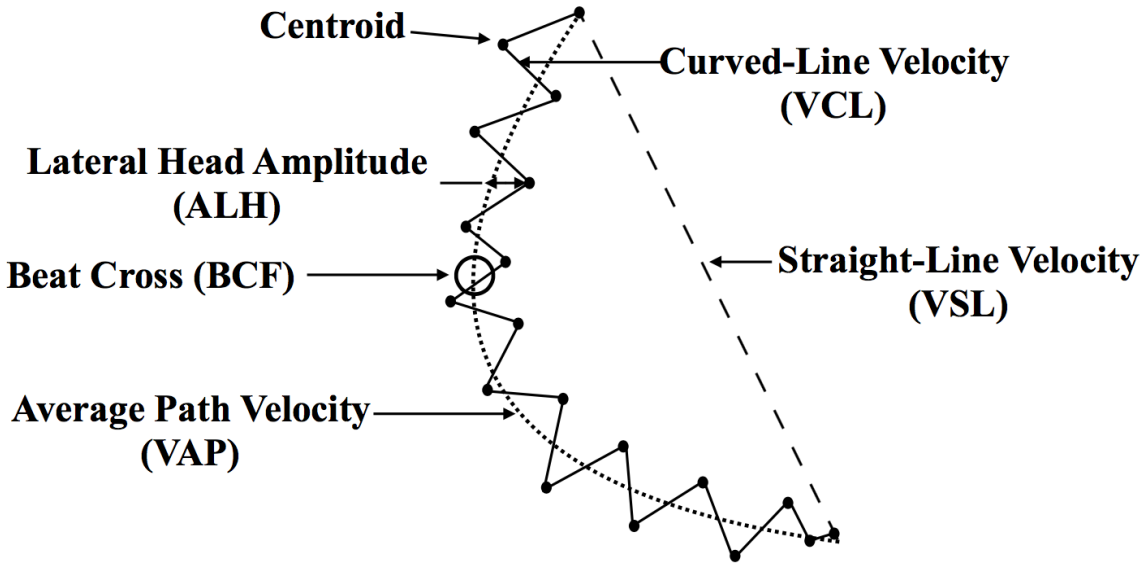

Figure 1.6 CASA analysis of sperm.

The centroids represent the actual path sperm would take. Curved-line velocity (VCL) represents the total point-to-point distance traveled over a period of time. Average path velocity (VAP) represents the average velocity traveled over time through averaging VCL measurements. Straight-line velocity (VSL) represents the straight-line distance traveled. Beat-cross frequency (BCF) is the frequency that the sperm tail moves (back and forth). Additionally, the lateral head amplitude (ALH) describes the deviation of the sperm head to each side (Recreated from: Amann and Waberski, 2014)

1.4 Nanobiotechnology

1.4.1 Nanoparticle: definition, types, and characteristics for biomedical applications

Nanobiotechnology is a novel area of science allowing nano-scale particles and structures of 1 to 100 nanometers to be created for remarkable biomedical applications (Sun, et al. 2014). Nanoparticles generally consist of a core and an outer polymer layer (i.e., polyethylene glycol or PEG, PLGA, chitosan, dextran) tailored for customized cell targeting and biosensing (Koo, et al. 2005).

Nanoparticles can be individually developed or combined as a group (composites) into a variety of shapes including spheres, cubes, rods, tubes and stars (Sun, et al. 2014), whereas nanoparticle structures can be carbon-based arranged in a variety of shapes to create a hollow vesicle (dendrites or liposomes) or may be comprised of a single core made from a variety of metals (silver, gold, metal oxides, titanium oxides, etc.) (Farokhzad and Langer 2009). A metal-based core can possess supplementary characteristics to enhance the ability for use in particular applications. For example, a nanoparticle with a cadmium selenium (CdSe) core possesses a broad spectrum of fluorescence properties, ideal for imaging techniques (Sounderya and Zhang 2008), while an iron oxide core-based nanoparticle will possess magnetic properties expanding the versatility of applications.

As indicated in Figure 1.6, synthesized nanoparticles for biological applications generally consist of a core or structure coated with a biodegradable element such as lipids, polymers, and PEG that render the structure biocompatible (hydrophilic). This external (preferentially) negatively charged structure can further be functionalized with biomolecules (i.e., targeting ligands, nucleic acids, fluorescent dyes, tunable surface

charges) for various bio-applications such as targeting, imaging, and therapy (Whitesides 2003).

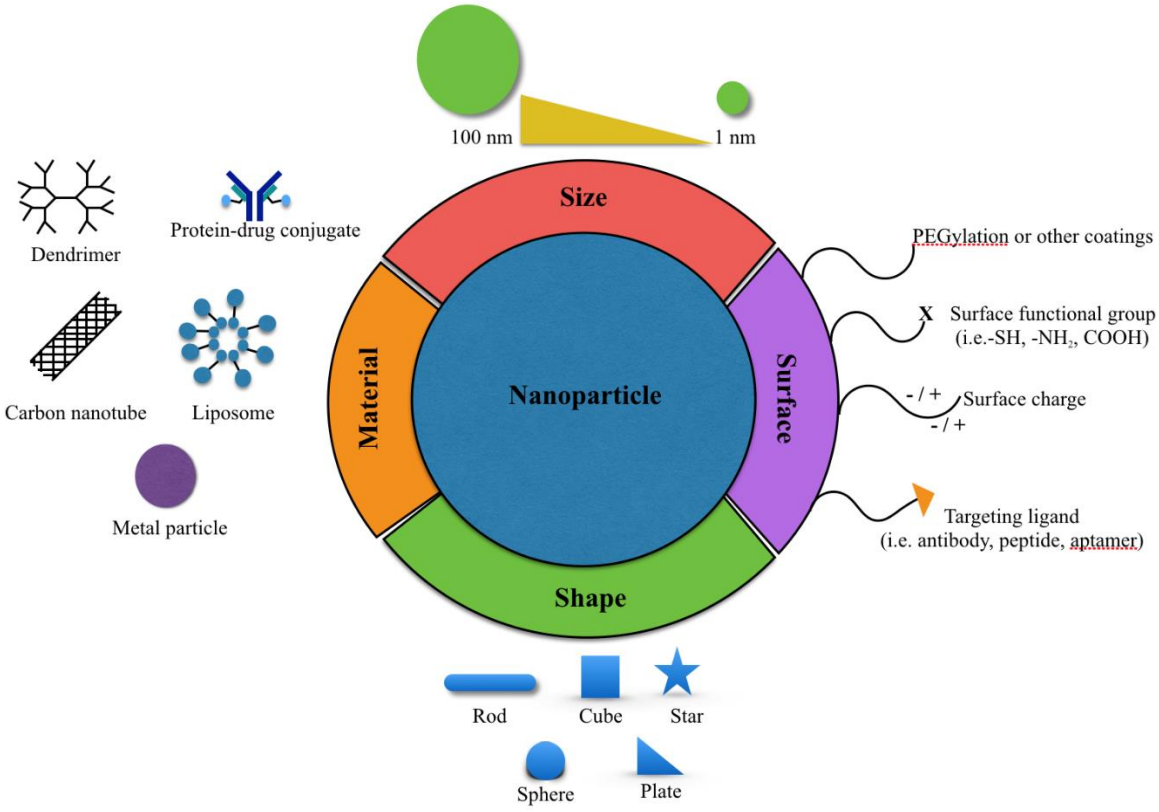

Figure 1.7 General schematic representation of a nanoparticle structure.

A summary of nanoparticles used for biomedical techniques, with illustrations of bio physicochemical properties. (Recreated from: Sun et al., 2014)

1.4.2 Applications in medicine

The small size and large surface-to-volume ratio of functionalized nanoparticles make them excellent tools for various applications in biomedicine, due to their greater payload allowing them to target more surface area (i.e., receptors) in even the most discrete locations of the tissue (Gupta and Wells 2004). The biomedical aspects of

nanotechnology have shown a dramatic increase due to the diverse array of applications in disease research and biomedicine such as:

1) *Drug delivery for disease therapies.* Numerous strategies have been developed using liposomes and carbon-based nanotubes for therapeutic drug delivery for various brain diseases such as Alzheimer's and Parkinson's diseases (Re, et al. 2012; Sercombe, et al. 2015; Taylor, et al. 2011; Trapani, et al. 2011; Ulbrich, et al. 2009).
2) *Gene delivery.* DNA encapsulated nanomaterials (i.e., carbon nanotubes) are able to invade the cell and target the nucleus through endocytosis, creating a non-viral delivery vesicle potentially less harmful than a traditional viral source (Dizaj, et al. 2014). Delivered DNA are functional and capable of gene expression (Bianco, et al. 2005).
3) *Diagnosis and imaging*. Functionalized fluorescent or magnetic nanoparticles such as gold, quantum dots, and iron oxides allow accurate identification or localization of target tissues (i.e., tumor) for adequate treatment (i.e., potential drug or gene therapy therapies) (Reddy, et al. 2006).

1.4.3 Applications in Reproductive Biology

Nanotechnology was introduced in the field of reproductive biology less than two decades ago, primarily for toxicology studies (Lafuente, et al. 2016; Pankhurst, et al. 2003; Wiwanitkit, et al. 2009; Zhang, et al. 2014). Since, the current developments in nanotechnology are allowing novel applications for gamete targeting and imaging, which are believed to ultimately benefit livestock productivity through the improvement of our understanding of the reproductive processes and fertility of animals. Below are few aspects of current progress utilizing nanoparticles for gamete investigations.

1) *Sperm imaging*. Few studies have used boar spermatozoa for targeting and non-targeting imaging through conjugated mesoporous silica (Barkalina, et al. 2014a) and fluorescent quantum dots (Feugang, et al. 2012) nanoparticles. In both studies, spermatozoa were imaged under microscope, and the later use of quantum dots-labeled spermatozoa placed within the female reproductive tract allowed for ex situ imaging (Feugang, et al. 2015). This study was an explorative investigation of the possibility of non-invasive and non-destructive tracking of spermatozoa within their natural physiological environment following artificial insemination (Figure 1.7).
2) *Gene delivery and sex sorting*. Mesoporous silica nanoparticles have been examined as potential carriers for gene delivery in spermatozoa (Barkalina, et al. 2014b). Meanwhile, nucleic acid-conjugated gold nanoparticles are currently tested for bull sperm sex sorting (Rath, et al. 2015).
3) *Sperm targeting*. Among the numerous synthesized nanomaterials, magnetic nanoparticles have been determined as the candidate of choice for sperm separation due to their specific property. Their specific functionalization permit interactions with spermatozoa for isolation under electromagnetic fields, through following Coulomb's law (Pankhurst 2003). These nanoparticles have been successfully applied boar and bull semen to remove acrosome reacted spermatozoa exhibiting carbohydrate receptors that are targeted by the lectin coated-nanoparticles. Interestingly, the authors found no detrimental effects on the function of residual, acrosome intact spermatozoa that were solely exposed to nanoparticles (Feugang 2015; Odhiambo 2014).

In conclusion, nanobiotechnology is a novel platform to improving knowledge in animal sciences. The safe use of nanomaterials provides non-invasive applications in animal reproduction for basic and applied research to enhance livestock productivity.

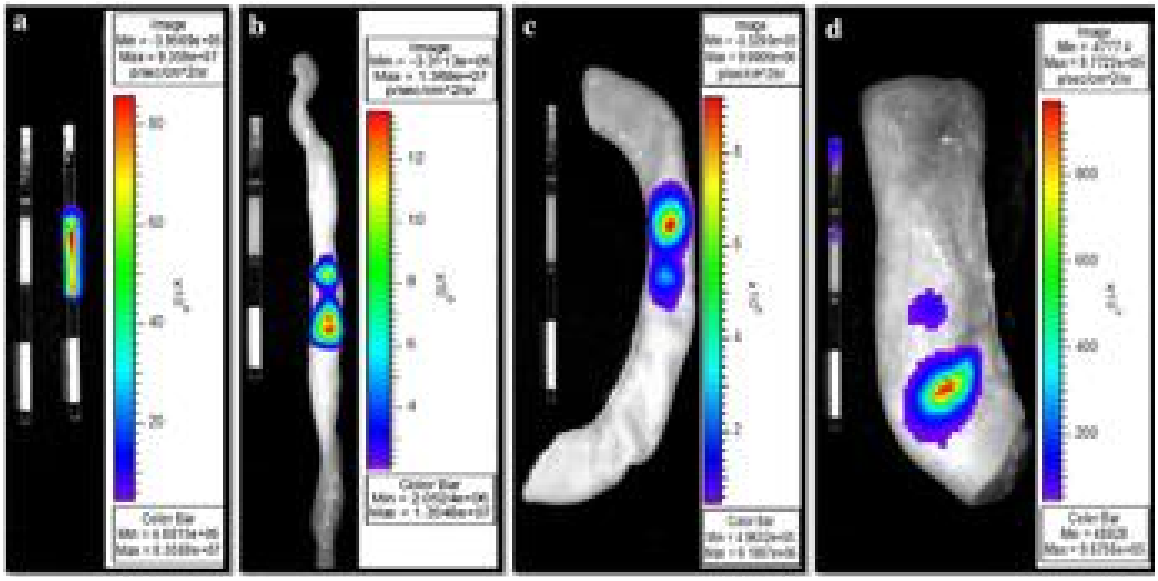

Figure 1.8 Bioluminescence imaging of quantum dot labeled sperm.

Intra-uterine bioluminescence imaging of labeled spermatozoa with quantum dot (QD-1) nanoparticles. a) Boar spermatozoa labeled with QD-1loaded into plastic straws, b) QD-1 loaded spermatozoa injected into the female oviduct, c) uterine horn, d) and uterine body (Feugang, et al. 2015).

1.5 Problem and objectives of the study

1.5.1 Problem

Semen ejaculates routinely contain a balanced proportion of viable and non-viable spermatozoa that is influenced by both intrinsic and extrinsic factors associated with that animal. However, a serious change of factors such as sickness and seasonal variations can create an imbalance in favor of non-viable spermatozoa, jeopardizing semen fertility. For instance, seasonal variations associated with heat stress is still a great concern in boar

studs, where numerous semen ejaculates are generally discarded during hot seasons due for poor sperm quality. This rejection constitutes a great loss for swine producers and there is a crucial need to develop new innovative approach to mitigate the negative effects of seasonal variation in swine.

1.5.2 Objectives

Numerous molecular defects characterize non-viable spermatozoa. Damages induced by boar exposure to heat stress, correspond to higher proportions of spermatozoa exhibiting early acrosome reaction and apoptotic signs. Specific molecules are already available for the detection of acrosome reacted and apoptotic spermatozoa, but the current techniques of evaluation are only informative, and useless as semen that do not pass the tests are still entirely discarded. Here we propose to develop an explorative molecular-based approach to specifically target and remove damaged cells. The proposed approach resides on the use of functionalized magnetic nanoparticles that enable targeted-sperm removal through a process termed "nanopurification", with the expectation that the remaining viable spermatozoa have maintained their function intact. This Master thesis constitutes the first ground work toward the aforementioned overall objective. Therefore, the objectives of the current study are as followed:

1. ***Sperm nanopurification and evaluation of sperm performance***
    a. Designed magnetic nanoparticles used to target apoptotic and acrosome reacted spermatozoa, confirmed through various microscopy imaging.
    b. Examination of residual spermatozoa are examined through various motility and viability tests

c. Finally, *in vivo* fertility is verified through insemination of gilts and evaluation of neonatal pigs born alive.

2. ***Sperm nanopurification and evaluation of post-natal offspring developmental performance***

   a. Selected neonatal pigs representing standard and nanopurified semen litters are grown until market size.

   b. Animals are monitored for growth and numerous developmental and health parameters are collected for comparative analyses.

3. ***Sperm nanopurification and evaluation of carcass and meat quality at market***

   a. Carcass characteristics and meat quality are evaluated

   b. Chemical and Molecular parameters are evaluated.

CHAPTER II

SUMMARY OF METHODOLOGIES

2.1 Sperm Nanopurification and Analyses

2.1.1 Nanoparticle synthesis

Iron oxide (Fe_3O_4) magnetic nanoparticles (MNP) were synthesized and coated with lectins (PNA/PSA) or annexin V (Sigma Aldrich, St Louis, MO, USA) by Clemente Associates (Madison, CT, USA) following an undisclosed proprietary protocol. Stock solutions contained sodium aside and were stored at 4°C until use.

2.1.2 Sperm labeling and purification with nanoparticles

Freshly harvested and extended semen doses (n=18; 3 X 10^9 spermatozoa/dose) were purchased from a local boar stud (Prestage Farms, West Point, MS). A total of 0.3 mg of each annexin V- and lectin-conjugated MNP was successively mixed with each semen dose to target 0.6 X 10^9 moribund or non-viable spermatozoa (equivalent of 2 X 10^9 sperm/mg). Following each mixture, co-incubation was taken at 37°C with a gentle rotation for 30 min to allow sperm-MNP interaction. Thereafter, semen mixtures were placed against a powerful magnet for 10 min incubation at room temperature (4°C) for entrapment of sperm-MNP complexes. Free, unbound, or intact spermatozoa were subsequently eluted into new identified tubes. This separation process was repeated twice for each MNP conjugate, for a total of three purification steps (= semen nanopurification). Nanopurification started with the targeting of apoptotic (annexin V)

followed by acrosome damaged (lectin) spermatozoa, although there was no specification for this order. Single or pooled (of at least 3) boar semen doses were used in at least four independent replicates according to experiments

2.1.3 Characterization of nanoparticles and interactions with spermatozoa

Synthesized nanoparticles were prepared for evaluation through (1) Transmission Electron Microscopy (TEM imaging: TEM-JEOL 2100 Lab6 200kV TEM), (2) Atomic Force and Magnetic microscopy (AFM/MFM; Dimension Icon® atomic force microscope with a Bruker® MESP tip, under magnetic force microscopy mode), and (3) Dynamic Light Scattering (DLS: ZetaPALS instrument @ 659 nm) to measure the size and distribution of MNP using previously reported protocols (Feugang, Youngblood et al. 2012, Feugang, Youngblood et al. 2015, Vasquez, Feugang et al. 2016).

Evaluation of nanoparticle and sperm interactions were performed using standard protocols with slight modifications for TEM (Feugang, Youngblood et al. 2012), AFM/MFM (Vasquez, Monroe et al. 2015), and DLS (Feugang, Youngblood et al. 2015) analyses. Imaging and quantification were performed at the Institute for Imaging and Analytical technology (I2AT, Mississippi State University) and (Dave C. Swalm School of Chemical Engineering, Dr. Santanu Kundu, Mississippi State University).

(Results generated from these analyses are presented in Chapter III)

2.1.4 Analysis of sperm motility and viability

Analyses was performed using a Computer-Assisted Sperm Analyzer (CASA: HTM-IVOS; Hamilton-Thorne Biosciences; Beverly, MA, USA). Sperm viability analysis was performed on nanopurified (n=4) semen using a single staining adapted

from Martinez-Alborcia and colleagues (Martinez-Alborcia, Valverde et al. 2012). Samples were subjected to various staining for viability assessment: 2 µl propidium iodide (PI, 1 mg/ml in PBS) for plasma membrane integrity, 5 µl PNA-FITC (100 mg/ml in PBS) for acrosome reaction, 2 µl JC-1 (500mg/ml; Cayman Chemical Co., Ann Arbor, MI, USA) for mitochondrial integrity, and 2.5 µl H2DCFDA (1 mM in DMSO) for reactive oxygen species (ROS) accumulation within the cells. All samples were analyzed with a flow cytometer (Becton–Dickinson FACS Diva version 6.1.3; Basic Sciences, College of Veterinary School, Mississippi State University) set for 10,000 total events per analysis. For imaging, sample aliquots were mounted onto microscope slides and visualized under an epifluorescence microscope (EVOS FL-Auto, Thermo Fisher Scientific, Hampton, NH, USA) to validate proper staining.

(Results generated from these analyses are presented in Chapter III)

2.2 Estrus detection, synchronization, and insemination (refer to chapter 3.1)

A total of 14 sexually mature gilts used in 2 trials (n=6, n=8) were purchased from Prestage Farms (West Point, MS) and maintained at the Animal Physiology Unit, H.H. Leveck Animal Research Center, Mississippi Agricultural and Forestry Experiment Station, Mississippi State University. All gilts were dewormed and ear tagged upon arrival, and weights were collected. They were individually fed approximately 2-2.5 kg of a commercial swine feed (16% Crude Protein) per day, with ad libitum access to water.

In the first trial, within three days of arriving, gilts were given MATRIX (altrenogest) 0.22 % solution to allow synchronization of estrus. Each gilt was individually fed 6.8 ml of MATRIX once a day soaked in a cookie in the middle of their

feed to insure complete ingestion. After 14 days, gilts were assessed for estrus through the appearance of redness-swollen vulva and responsiveness to the lordosis test or with the help of a teaser boar (trial 2).

Upon confirmation of estrus, all gilts were artificially inseminated two to three times with control standard (non-purified) or magnetic nanopurified semen within 24 hours of estrus detection.

2.3 Pregnancy check, farrowing, and processing of neonate pigs

Inseminated gilts were regularly monitored and were maintained with approximately 2-2.5 kg of feed per day and ad libitum access to water. Pregnancies were confirmed approximately 30 days post-insemination using an ultrasonic detector and approximately 109 days post-insemination, pregnant gilts were transferred to farrowing stalls for delivery. Sows had ad libitum access to water and fed 2 kg per gilt per day a commercial sow diet (Power Sow TC; ADM Alliance Nutrition, Quincy, IL).

After farrowing, lactating sows were individually fed each day a commercial sow diet to the amount they would consume without left over with ad libitum access to water. All pigs born alive were evaluated through weight, and body measurements weekly from birth until weaning (refer to chapter 3). On the first day of life for all offspring, their umbilical cord was tied and cut about one inch from tied knot and sprayed with a 1% iodine solution to prevent infection and blood loss in the piglet. Within 3 days post-natal, all neonate pigs were administrated 200 mg (IM) of iron in their neck to prevent anemia, a common occurrence in neonatal piglets. Additionally, all neonates were processed through tail docking, ear notching, and clipping of the needle teeth. Ear notching was critical to identify each piglet and maintain accurate records, and were performed

according to the Universal Ear notching system (Figure 2.1). All pigs (sow and neonate offspring) were observed twice daily for behavior and body condition.

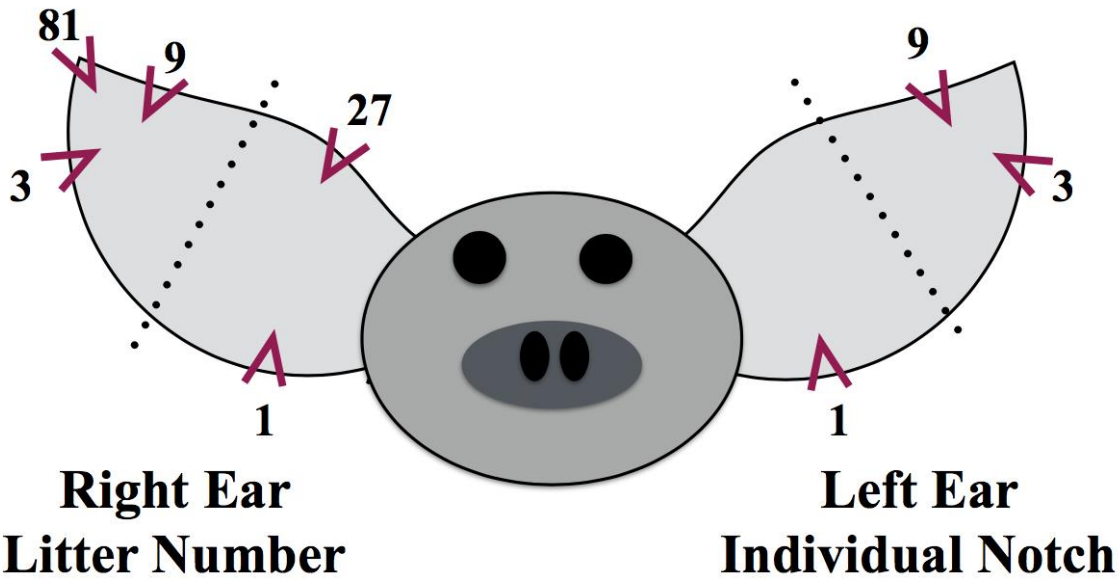

Figure 2.1 General description of the universal ear notching system

2.4 Postnatal growth and health assessments (refer to chapter 3)

Ten offspring pigs of equal genders (5 females and 5 males) born from control and purified (nanopurified or treated) semen from the first trial of gilts, were randomly selected based on weight and grown to market size for growth, development, and health parameter assessments. All offspring were initially dewormed (Ivomec; Merial, Duluth, GA) and vaccinated for Erysipelas (Erysipelothrix Rhnsiopathiae Bacterian; Novartis, Larchwood, IA) at weaning with a booster injection approximately one month after.

2.4.1 Growth and Developmental Parameters

Each pig (n=20) was weighed approximately every 30 days from weaning (day 28) until market (day 164). Upon harvest, reproductive tracts of all females (5 control and 5 treated) were maintained on ice for subsequent morphological analyses. Liver specimens were harvested from all pigs (n=20), immediately snap-frozen in liquid-nitrogen, and stored at -80°C for enzymatic analyses.

(Results generated from these analyses are presented in Chapter IV)

2.4.2 Assessment of health parameters

Blood samples were collected intravenously (jugular vein) from each pig (n=20) approximately every 30 days, from weaning (day 28) until market (day 164). Blood was collected into EDTA-coated tubes and kept cold (on ice or 4°C) for glucose, hematocrit, and white blood cell count analysis. Upon analysis of whole blood, each tube of collected blood was immediately centrifuged (15 rpm for 15 minutes, 4°C), with plasma fractions collected for immunoglobulin G (IgG) and near infrared spectroscopy (NIRS) analysis (refer to chapter 5 for NIR analysis).

(Results generated from these analyses are presented in Chapter IV and V)

2.5 Pork carcass evaluation (refer to chapter 5)

Upon harvest, blood and tissue samples were collected from each 20 pigs (10 females and 10 males). Subcutaneous fat and muscle from the longissimus dorsi were collected post-mortem and immediately frozen in liquid nitrogen and stored at -80°C or fixed in 4% methanol-free paraformaldehyde for protein analyses.

2.5.1 Carcass evaluation

Upon reaching market size at approximately 164 days of age, pigs were harvested at the Mississippi State University Meat Science and Muscle Biology Laboratory to determine carcass characteristics as previously described (Wang et al. 2015). Pigs were stunned by trained staff through electrical shock, and exsanguinated and de-haired. Upon removal of the head and feet, the hot carcass was split longitudinally into two halves and weighed prior to cooling at 4°C. Dressing percentage was calculated by dividing the hot carcass weight by the live weight at slaughter and multiplying by 100.

Approximately 48 hours post-mortem after chilling, the following carcass measurements were taken blindly by the same trained personal each time: chilled carcass weight, carcass length, average back fat thickness (first rib, last rib, and last lumbar vertebrae), ham fat thickness, 10th rib back fat thickness, untrimmed ham weight, trimmed ham weight, loin weight, picnic weight, boson butt weight, belly weight, rib weight, and marbling score. Color and firmness scores were obtained on the ham and longissimus dorsi (loin) muscles subjectively. Objective color and pH measurements of the loin muscle were obtained with a HunterLab MiniScan 4500L, Spectrophotometer (Hunter Associates Laboratory, Inc., Reston, VA) and a Multiparameter pH Meter HI2020 (Hanna Instruments, Woonsocket, RI) with the use of a pH Electrode FC2320 (Hanna Instruments, Woonsocket, RI). The loin eye area (10th rib) was also calculated using a Plastic Grid (Product number AS 0235E, Extension and Outreach, Ames, IA, USA).

(Results generated from these analyses are presented in Chapter V)

2.5.2 Protein analysis

Fatty Acid Synthase (FAS) and myoglobin (MYO) protein levels in muscle and fat tissues were examined using anti-FAS (C-20; sc-1820) and anti-MYO (FL-154; sc-25607) antibodies (Santa Cruz Biotechnology, Inc. Santa Clara, CA). Analyses were performed using standard protocols of western immunoblotting and *in situ* immunofluorescence, as previously described (Feugang et al. 2011, Feugang et al. 2015).

(Results generated from these analyses are presented in Chapter V)

CHAPTER III

OBJECTIVE 1: SYNTHESIZED MAGNETIC NANOPARTICLE CONJUGATES FOR HIGH-THROUGHPUT SEMEN PURIFICATION AND MALE FERTILITY IMPROVEMENT

Durfey, C[1]., Swistek, S[1,2]., Liao, S. F[1]., Crenshaw, M. A[1]., Clemente, H[4].,

Willard, S. T[1,2]., Ryan, P. L[1,3]., Feugang, J.M[1]

1. 1.Departments of Animal and Dairy Sciences, Mississippi State University, Mississippi State, MS
2. 2. Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS
3. 3. Pathobiology and Population Medicine, Mississippi State University, Mississippi State, MS
4. 4. Clemente Associates, Madison, CT

– In Submission (March 2017)

Figure 3.1 Image of a neonatal pig

3.1 Summary

Heterogeneous populations of viable and non-viable spermatozoa within the semen ejaculates can jeopardize male fertility. Yet, the properties of magnetic nanoparticles provide unique features that make them ideal for selective cell targeting and removal under a magnetic field. Here, we have incorporated specifically designed magnetic nanoparticles to target and eliminate damaged (apoptotic and acrosome reacted) spermatozoa from boar semen, and determined subsequent effects on sperm fertility and neonatal offspring performance.

Transmission electron microscopy, atomic force microscopy, and hyperspectral imaging techniques confirmed attachments of specifically designed magnetic nanoparticle conjugates to damaged spermatozoa. The computer assisted-sperm analyzer revealed significant increase of sperm motility characteristics after purification, while flow cytometry analyses indicated comparable viability parameters (reactive oxygen species accumulation and acrosome, plasma, and mitochondrial membrane intactness) between purified and non-purified (control) spermatozoa. Furthermore, sow fertility and performance (growth and health) of baby pigs born from purified spermatozoa were unaffected compared to their non-purified counterparts.

3.2 Introduction

Spermatozoa are haploid cells containing the genetic potential of the male, which makes them essential sources to search for reliable biomarkers of male fertility. Physical and physiological characteristics of spermatozoa can be indicative of semen quality (motility, direction of movement, intact acrosome, etc.); and their examination before

artificial insemination (AI) can help prognostic whether a semen sample is suitable for assisted reproductive techniques (ART), leading to successful fertilization [1].

Male fertility potential varies with genetic, health, nutrition, and environmental conditions (i.e., seasonal variations, stress, and cryopreservation), and such damages can impact the integrity of spermatozoa creating a mixture of viable and non-viable populations within the semen ejaculate [2]. Such heterogeneous populations with large amounts of moribund sperm in ejaculates have shown to negatively affect overall male fertility [3]. Reproductive disorders such as asthenozoospermia (poor motility) and oligospermia (low sperm count) have implied the need for efficient ART applications, requiring delicate in vitro manipulation of gametes or embryos to maximize fertility outcomes of male subjects.

Non-viable or damaged spermatozoa are known to excrete a harmful abundance of reactive oxygen species (ROS), creating aggregates within the semen ejaculate [4]. Such accumulation of moribund spermatozoa can further impede viable and high performance spermatozoa from successful migration through the female reproductive tract, impairing fertilization [5]. Therefore, maximum concentrations of homogenous viable sperm cells within an ejaculate is critical for successful fertilization and optimal male fertility. In humans, infertility is a social and moral devastating issue while in livestock, its occurrence through sub-fertile males has significant economic and genetic impacts, as a single male can sire hundreds to thousands females through AI during its lifespan. Currently available techniques for sperm purification such as swim-up [6], discontinuous percoll [7] and albumin [8] density gradient centrifugations, and microfluidic devices such as magnetic-assisted cell sorting [9,10] yield low numbers of

motile spermatozoa that are only effective in small-scale applications such as in vitro fertilization and intra-cytoplasmic sperm injection. These techniques are time-consuming and lack specificity and sensitivity which make them less suitable for large-scale applications in commercial studs dealing with large volumes of semen. Interestingly, the progress in nanotechnology gives new prospects for developing novel non-destructive techniques to improve post-collection semen handling in livestock.

Nanotechnology is a new field of science dealing with particles synthesized at a nanoscale level, allowing a large surface-to-volume ratio targeting. In addition, nanoparticles can be tailored for specific applications (i.e., non-invasive cell targeting and imaging) in agriculture and biomedicine, with promising applications in reproductive medicine [11-14]. Studies have reported their applications in sperm manipulation and at present, magnetic nanoparticles appear as the most suitable for sperm purification [15]. Traditionally consisting of an iron oxide core (Fe_3O_4) and an outer polymer layer, magnetic nanoparticles can be customized for specific cell targeting and biosensing allowing their use in a variety of bioapplications such as imaging, drug delivery, cell targeting, and cell sorting [16].

Despite reported reproductive toxicity of nanoparticles [17], iron oxide nanoparticles (MNP) have been successfully used for high-throughput and rapid purification of bull and boar semen [18,19]. These pioneering studies used MNP conjugated with lectins to specifically target and remove acrosome reacted spermatozoa from semen doses before AI. The authors termed this purification process as "nanopurification" that has neither affected semen nor dam fertility and resulted in normal live-born offspring (pigs and calves). It is sought that nanopurification with

specific biomarkers would allow maximum achievement in proficient spermatozoa purification leading to enhanced fertility success of sub-fertile males and overall productivity in livestock.

Poor semen quality causes are multifactorial [20, 21], and current studies have only focused on the lectin binding carbohydrates present on prematurely acrosome reacted sperm as a method of cell targeting [18, 19]. Apoptosis is another defect that characterizes the subset of non-viable sperm within the ejaculate. The proportion of apoptotic sperm cells increases following seasonal variations of temperatures inducing apoptosis or DNA fragmentation through the production ROS concentrations, influencing cellular metabolism [22]. The lack of tools to selectively eliminate damaged spermatozoa from semen ejaculates causes the rejection of entire ejaculates leading to substantial productivity losses in livestock [20, 23]. Here we propose the use of a double or two-step removal (purification) process to respectively target acrosome reacted and apoptotic spermatozoa within semen ejaculates through specifically functionalized MNP. In the present study, MNP were coated with either lectin (PNA/PSA) or annexin V, two common probes used for respective detection of acrosome reaction (lectin) or early apoptosis (annexin V) of spermatozoa [15, 24]. The present study aimed at characterizing the synthesized MNP conjugates (lectin or annexin V), assessing their interactions with boar spermatozoa, and evaluating the nanopurification impacts on sperm motion and viability, field fertility outcomes and offspring performance.

3.3 Methods

Otherwise indicated, all reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA.

3.3.1 Animals and oestrus detection

Sexually mature gilts (n=14) were purchased from a local commercial swine farm (Prestage Farms; West Point, MS) and maintained at Leveck Animal Research Center, Mississippi State University. Gilts were fed and allowed ad libitum access to water. Oestrus was detected through responsiveness to a teaser boar or a lordosis test ("standing reflex"), and positive gilts were artificially inseminated with single sire extended boar semen doses (n=7). Gilts were divided into two trials; trial 1 (n=6) and trial 2 (n=8). Animal care and use were performed according to protocols approved by the Institutional Animal Care and Use Committee of Mississippi State University.

3.3.2 Nanoparticle synthesis and characterization

Iron oxide (Fe_3O_4) magnetic nanoparticles (MNP) were synthesized and coated with lectins (PNA/ PSA) or annexin V (Sigma Aldrich, St Louis, MO, USA) by Clemente Associates (Madison, CT, USA) following an undisclosed proprietary protocol. Lectin and annexin V allowed conjugated nanoparticles to selectively bind to glycan's exposed by damaged acrosome membrane (or premature capacitated) and early apoptotic spermatozoa, respectively. Stock solutions contained sodium azide and were stored at 4^{o}C until use. Synthesized nanoparticles were prepared for (1) transmission electron microscopy (TEM imaging: TEM-JEOL 2100 Lab6 200kV TEM), (2) atomic force and magnetic microscopy (AFM/MFM; Dimension Icon® atomic force microscope with a Bruker® MESP tip, under magnetic force microscopy mode), and (3) dynamic light scattering (DLS: ZetaPALS instrument @ 659 nm) to measure the size and distribution of MNP using previously reported protocols [25-27].

3.3.3 Sperm labelling and purification with nanoparticles

Freshly harvested and extended semen doses (~ 3×10^9 spermatozoa/dose; n=14) were purchased from a local boar stud (Prestage Farms, West Point MS). A total of 0.3 mg of annexin V- and lectin-conjugated MNP was successively mixed with each semen dose to target 0.6 X 10^9 moribund spermatozoa (= 2 X 10^9 sperm/mg). Following each mixture, co-incubation was taken at 37ºC with a gentle rotation for 30 min to allow sperm-MNP interactions. Thereafter, semen mixtures were placed against a powerful magnet for 10 min at room temperature for entrapment of sperm-MNP complexes. Free, unbound, or intact spermatozoa were subsequently eluted into new identified tubes. This separation process was repeated twice for each MNP conjugate, for a total of three purification steps (= semen nanopurification). A simplified illustration of the nanopurification process is shown in Figure. 3.3. Nanopurification started with the targeting of apoptotic (annexin V; single removal) followed by acrosome damaged (lectin; double removal) spermatozoa. Semen samples of four independent replicates using pooled boar semen were kept before and after each nanopurification process for the following experiments.

3.3.4 Experiment 1: Evaluation of sperm-MNP interactions

Sperm aliquots were prepared for Transmission electron microscopy (TEM), atomic force microscopy (AFM/MFM), and Hyperspectral imaging (HI) as previously reported. Briefly, spermatozoa preparations (1) did not include osmium tetroxide fixation and uranyl acetate staining for TEM-JEOL [26], (2) were placed fixed in 4% paraformaldehyde and smeared on histology microscope slides for AFM/MFM [27], and (3) were fixed on histology glass slides and hyperspectral data collected using reference

spectral libraries created by MNP-conjugates (CytoViva® imaging technology; CytoViva Inc., Auburn, AL, USA [25]).

3.3.5 Experiment 2: Analysis of sperm motility characteristics

Analyses were performed using a Computer-Assisted Sperm Analyzer (CASA: HTM-IVOS; Hamilton-Thorne Biosciences; Beverly, MA, USA). Aliquots of nanopurified semen were submitted to analyses using the 20 micron Leja® Count-4-chamber slides (Nieuw Vennep, The Netherlands). Pre-set values of CASA were used (e.g., 60 frames/sec; VAP and STR of progressive cells at 45 μm/sec and 45%, respectively; VAP and VSL cut-offs of slows cells at 20 and 5 μm/sec, respectively; magnification: 1.89X, and 37^{o}C). Each sample aliquot was run in triplicate (3 chambers) with approximately 262 spermatozoa (± 3) analyzed per chamber. The sperm motility (percent of total, progressive, and rapid (≥30 μm/sec) motility and velocity parameters (μm/s; average path or VAP, straight line or VSL, and curvilinear or VCL). Other parameters such as lateral head aptitude (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR; VSL/VAP x 100), linearity (LIN; VSL/VCL x 100), and static spermatozoa (%).

3.3.6 Experiment 3: Analysis of sperm viability

Analyses were performed in single staining adapted from Martinez-Alborcia et al. [28]. Nanopurified spermatozoa were immediately diluted to 30 X 10^6 cells/ml with a pre-warmed phosphate-buffered saline solution (PBS). Aliquots of 0.1 ml of sperm suspensions were allocated to various staining for viability assessment: 2 μl propidium iodide (PI, 1 mg/ml in PBS) for plasma membrane integrity, 5 μl PNA-FITC (100 mg/ml

in PBS) for acrosome reaction, 2 µl JC-1 (500mg/ml; Cayman Chemical Co., Ann Arbor, MI, USA) for mitochondrial integrity, and 2.5 µl H2DCFDA (1 mM in DMSO) for reactive oxygen species (ROS) accumulation within the cells. All samples were incubated at 37^{o}C for 15 min and diluted 6x with pre-warmed PBS before immediate analyses with a flow cytometer (Becton–Dickinson FACSDiva version 6.1.3) set for 10,000 total events per analysis. Sample aliquots were mounted onto microscope slides and visualized under an epifluorescence microscope (EVOS FL-Auto, Thermo Fisher Scientific, Hampton, NH, USA) to validate proper staining.

3.3.7 Field fertility test and offspring performance

Females (gilts) detected in oestrus were artificially inseminated twice within the 24 hours, starting from 6 h post-detection. Inseminations were performed with control (non-purified) and nanopurified semen of single sire doses as described above, with seven females (gilts) per group. Various data (i.e., pregnant and weaning rates, and litter size) were recorded to establish the fertility rate and fecundity of each female (sow).

Additional data on born-piglets (n=8 control; n=8 nanopurified-born) from trial 2 was collected from the moment of birth before nursing. Blood samples were collected directly from each neonatal pig's umbilical cord to measure levels of glucose (Glucose meter, Agamatrix Inc, Salem, NH), pact cell volume or hematocrit (using a standard procedure), and immunoglobulin G (porcine IgG ELISA kit; Bethyl Laboratories, Inc., Montgomery, TX; Cat. No. E101-104).

Weight and growth of all pigs born (n=83) were measured weekly from birth until weaning (approximately 28 days). Additional growth performance was determined by measurements of crown-rump length (CRL, distance from the crown of the head to the

base of the tail), body length (measure from tip of snout to base of tail), head length (measured from tip of snout to base of neck), head circumference, and heart girth.

3.3.8 Statistical analysis

All statistical analysis was performed using the Statistical Analysis Software (SAS) 9.4 (SAS Institute, Inc., Cary, NC). A frequency model was used to determine pregnancy rates, and evaluate fecundity characteristics. A general linear model (Proc GLM) was used to determine differences between control and nanopurified semen (single and double nanopurified). A linear mixed model (Proc MIXED) was used to evaluate offspring growth performance and blood characteristics in trial 2, with treatment, gender, and their two-way interactions as fixed effects. Additionally, a linear mixed model was used to evaluate growth performance from birth until weaning from all neonatal pigs (trial 1 and 2), with trial, treatment, gender, and day as fixed effects. Two-way (treatment*gender, and treatment*day) and three-way (treatment*gender*day) interactions were also included as fixed effects. Repeated measures of growth performance were analyzed using an autoregressive one covariance method. Additionally, litter within trial was considered a random effect when applicable. All data are expressed as mean ± standard error mean (sem), with significant differences set as $p \leq 0.05$.

3.4 Results

3.4.1 Characterization of MNP conjugates.

Results are shown in Figure 3.1. Transmission electron microscopy (TEM) revealed the spherical shape of functionalized magnetic nanoparticles (MNP), with the

iron oxide core diameter of approximately 14 nm from randomly selected MNP (Figure 3.2a). The atomic force microscopy (AFM/MFM) confirmed the MNP structure and indicated the presence of various size aggregate formations, with monomers of approximately 50 to 90 nm in diameters (Figure 3.2b and c). Hydrodynamic diameter measurements by dynamic light scattering (DLS) showed bimodal distribution sizes averaging 183 and 1,463 nm for annexin V- and 146 and 1,226 nm for lectin-MNP conjugates.

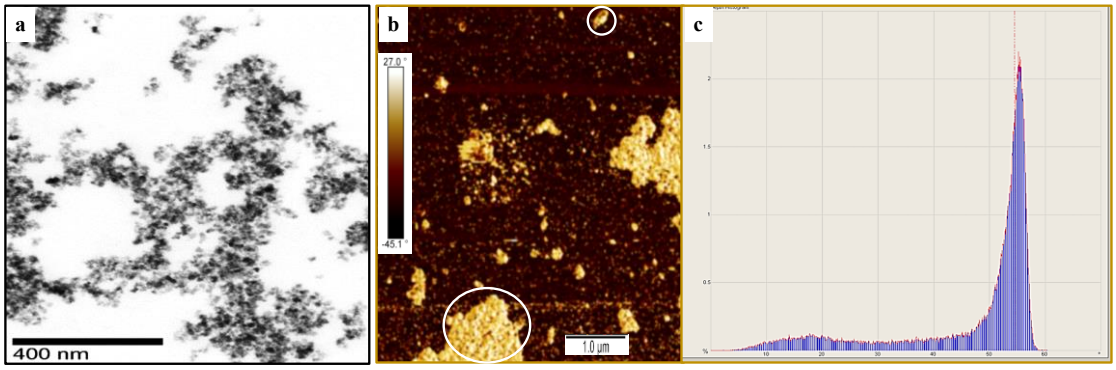

Figure 3.2 Representative micrographs of magnetic nanoparticles.

Representative micrographs of MNP imaging with TEM (a) and AFM/MFM (b and c). Circles indicate few MNP aggregates; AFM/MFM phase image (b) and height size distribution (c) are illustrated.

3.4.2 Sperm nanopurification process.

A simplified purification process of semen is presented in Fig. 3.3. Incubations of sperm-MNP mixture (Figure 3.2a) and subsequent trapping of total MNP (free and sperm-bound) with magnets (Figure 3.2b) led to the elution of unbound spermatozoa, devoid of MNP into new recipients (Figure 3.2c). The MNP trapping indicates the

magnetism of synthesized MNP. The process is called nanopurification and was repeated for each annexin V- and lectin-conjugated MNP.

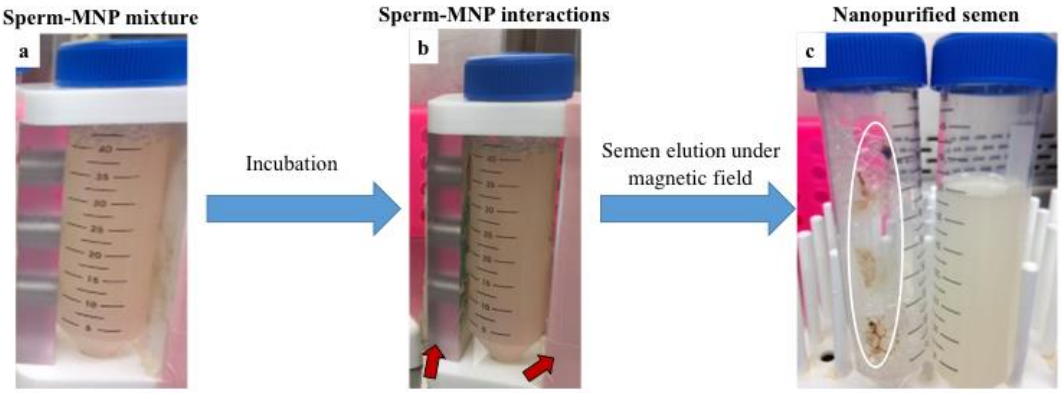

Figure 3.3 Simplified illustration of the semen nanopurification process.

Extended boar semen mixed with magnetic nanoparticle conjugates (MNP) (a) were incubated and placed under a magnet to attract free and bound MNP conjugates (b). Thereafter, unbound or non-damage sperm were eluted into a new recipient while MNP free or bound to spermatozoa remained trapped in the original recipient (c). Eluted semen was reused for a second nanopurification process or are readily available for utilization. Circle shows total trapped MNP. Arrows indicate magnet placed against the sample tube wall.

3.4.3 Evaluation of sperm-MNP interactions.

Subsequently for each experiment (1-3), sperm-MNP interactions were assessed with a routine laboratory microscope (data not shown). Using more advanced microscopy systems, Figure 3.3 shows representative TEM (a), AFM (b), and hyperspectral (c) imaging confirming our prior observations. Successful sperm-MNP interactions were seen as single- (Panel a1) and multi- (Panel a2) point attachments. Sperm-MNP interactions and magnetic properties of MNP conjugates were confirmed by AFM/MFM (B). Sample aliquots exposed to MNP showed positive signals to hyperspectral fluorescence imaging (c3b and c4b). Spermatozoa in the control group and subsets of

spermatozoa following MNP incubation did not show any presence or sign of interactions with MNP.

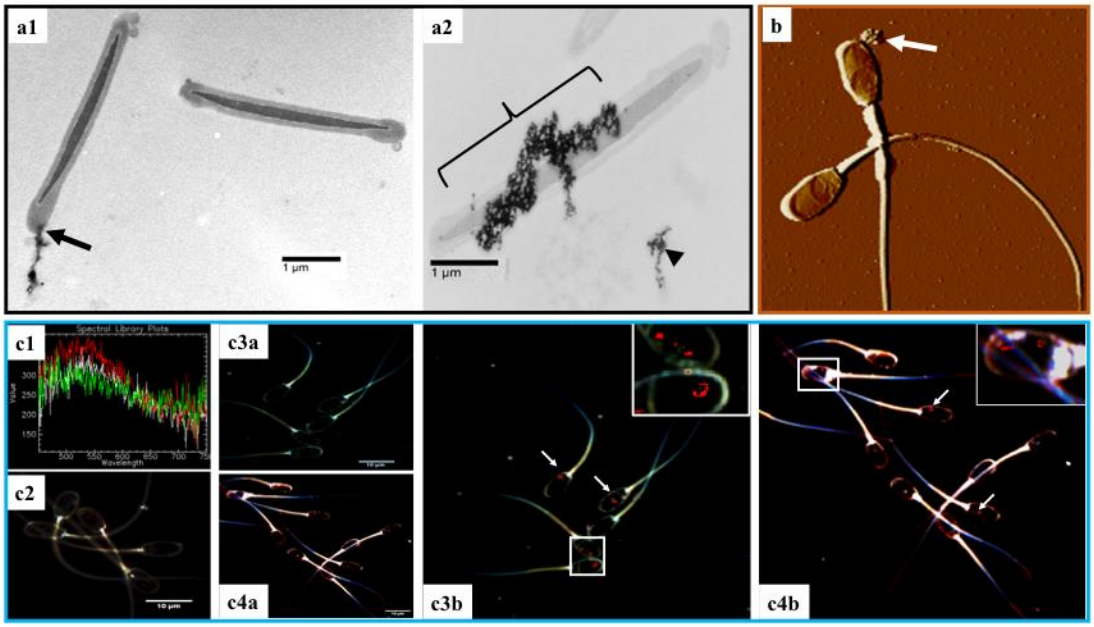

Figure 3.4 Evaluation of sperm-magnetic nanoparticle conjugate (MNP) interactions.

Evaluation of sperm magnetic nanoparticle conjugate (MNP) interactions, using TEM (a), AFM/MFM (b), and Hyperspectral (c) imaging. Single-point (arrow) and multi-point (brace) interactions between MNP and spermatozoa (cross sections of heads) are shown in a1 and a2, respectively. Binding of MNP to the sperm head is shown with AFM/MFM amplitude imaging. Hyperspectral libraries were generated for each MNP to track their presence in sperm samples (c1). Optical images of samples were first taken before screening for the presence of MNP (c3a and c4a, for annexin and lectin, respectively). Fluorescence spectra indicating the presence of MNP (red spots) was not seen in control sperm cells (c2), but in MNP-exposed sperm cells (c3b and c4b for annexin and lectin, respectively). Inserts (boxes) in c3b and c4b highlight the MNP-sperm interactions.

3.4.4 Motility characteristics of nanopurified spermatozoa.

Results are summarized in Figure 3.5 and Table 3.1. In comparison to the control group, nanopurification with apoptotic MNP (single removal) and lectin MNP (double removal) significantly increased spermatozoa proportions of total (Total MOT), forward

(Progressive), and rapid motility, while significantly decreasing proportions of static (non-moving) spermatozoa ($P < 0.05$; Figure 3.5a). Nanopurification globally increased the sperm velocity parameters, compared to the control group (VAP, VSL and VCL, $P < 0.05$; Figure 3.5b). Other motion parameters such as ALH, BCF, straightness, and linearity were variably increased following nanopurification ($P < 0.05$; Table 3.1).

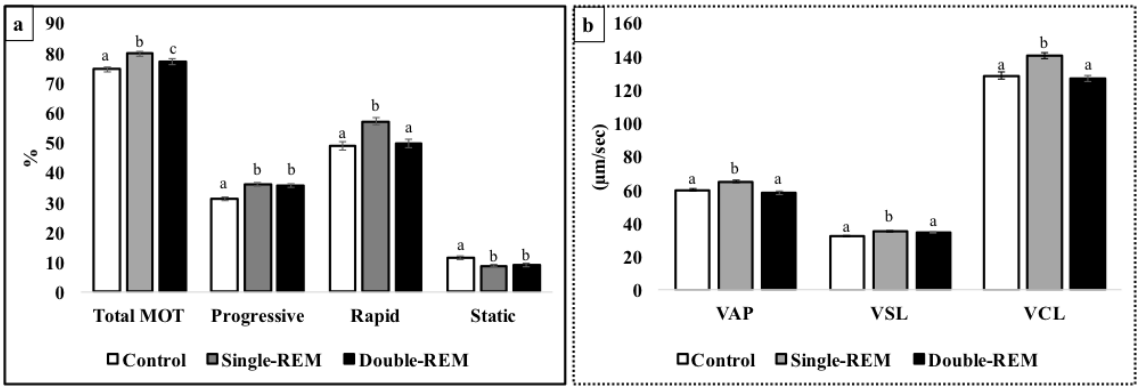

Figure 3.5 Sperm motility characteristics.

Data were obtained after purification with annexin- (Single-REM) and lectin- (Double-REM) magnetic nanoparticle conjugates (MNP). Results characterizing the motility (a) and velocity (b) characteristics of spermatozoa are summarized. Data are represented as mean (± sem) of 4 independent pooled semen dose replicates. Columns that share the same letter are not significantly different ($P > 0.05$).

Table 3.2 Additional sperm motility characteristics

	Lateral Head Amplitude (ALH: µm)	Beat Cross Frequency (BCF; beat/sec or Hz)	Straightness (%)	Linearity (%)
Control (Non-purified)	7.95 ± 0.08[a]	38.14 ± 0.3[ab]	55.7 ± 0.6[a]	27.3 ± 0.4[a]
Single-Removal (Annexin V)	8.06 ± 0.05[a]	37.6 ± 0.3[b]	55.8 ± 0.6[a]	27.3 ± 0.4[a]
Double-Removal (Lectin)	7.5 ± 0.1[b]	38.7 ± 0.3[a]	60.4 ± 0.8[b]	29.8 ± 0.7[b]

Data were obtained after purification with annexin- (Single-Removal) and lectin- (Double-Removal) magnetic nanoparticle conjugates (MNP). Data are means (± sem) of 4 independent replicates using pooled semen doses. Columns that share the same letter are not significantly different (P > 0.05).

3.4.5 Viability assessment of nanopurified spermatozoa.

Results are summarized in Fig. 3.6. All groups (control, single- and double-removal) had comparable proportions of spermatozoa having high mitochondrial membrane potential, low ROS levels, and intact acrosome and plasma membranes (P > 0.05). However, mean relative fluorescence intensities (RFI) associated with ROS production (H2DCFDA; 1,069 ± 200 vs. 864 ± 200) and damaged plasma membrane (PI; 12 ± 26 vs. 9 ± 26) were numerically decreased after nanopurification (single or double removal). Meanwhile, RFI indicating high potential mitochondrial membrane were increased after nanopurification (JC-1; 898 ± 224 vs. 426 ± 200). The successful staining with aforementioned dyes were confirmed with fluorescence microscope imaging (results not shown).

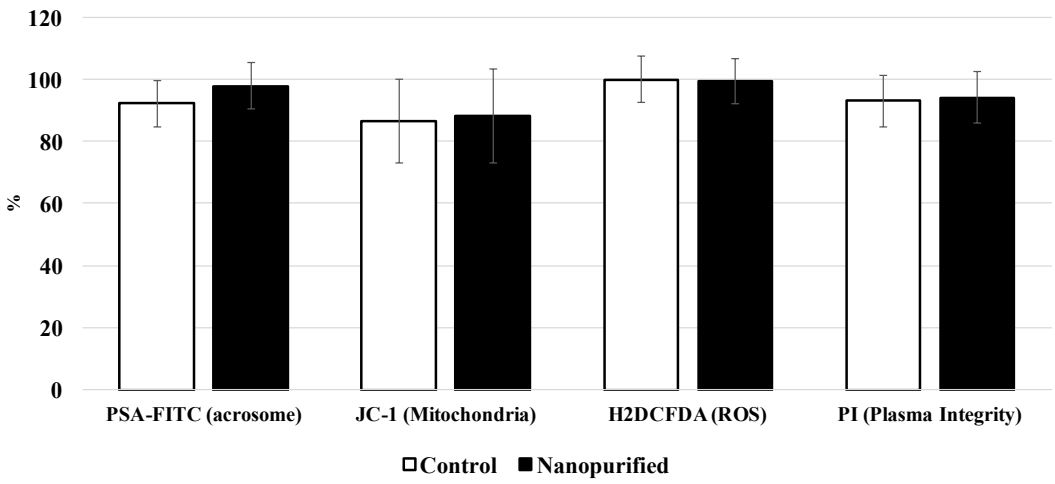

Figure 3.6 Sperm viability assessment.

Spermatozoa were stained with specific dyes to detect the intactness of acrosome (PSA-FITC), mitochondria (JC-1) and plasma (PI) membranes, as well as the metabolism status of cells generating ROS (H2DCFDA). Data are means (± sem) of 4 independent replicates using pooled semen doses. No significant differences were found between groups (control and nanopurified - single removal and double removal; $P > 0.60$).

3.4.6 Reproductive outcomes of nanopurified spermatozoa.

Nanopurified semen (double removal) induced pregnancy (43% vs. 57%) and farrowing (100% vs. 100%) at comparable rates to their control counterparts. Sow fecundity were not affected and comparable litter sizes and weights between control (11.3 ± 5.6 and 1.6 ± 0.24 kg) and nanopurified (12.7 ± 7.3 and 1.5 ± 0.24 kg) were observed (P=0.8). A total of 35 piglets out of 38 were weaned alive (92%) compared to 40/45 (89%) in the control group. All neonate pigs grew at normal and comparable pace, up to weaning at day 28 post-natal (8.2 ± 0.24 kg vs. 7.7 ± 0.24 kg for control and nanopurified groups, respectively; P=0.20), with interaction effects due to trial and gender found statistically insignificant (P=0.60). Similar observations were shown with other developmental parameters (body length, head circumference, heart girth, etc.), except

head length where pigs born from nanopurified semen litters appeared to have significantly shorter heads at weaning compared to the control (15.0 ± 0.2 cm and 16.2 ± 0.2 cm; $P < 0.01$). Glucose (56 ± 8.2 vs 48 ± 8.4 mg/dL), hematocrit (22 ± 1.5 vs 22 ± 1.6 %), or immunoglobulin type G (IgG: 342 ± 108.1 vs 568 ± 111.6 ng/ml) analyses from neonatal pigs in trial 2, did not show any further differences between pigs born from control and nanopurified spermatozoa, with insignificant interaction effects between treatment and gender ($P > 0.1$).

3.5 Discussion

Influences on male fertility due to the presence of non-viable spermatozoa have been widely studied [2-4]. Industry standards for boar semen (> 70% motility and < 15-30% abnormal sperm) allow for effective artificial insemination and fertility predictions [20, 23, 29]. Previous studies have found various influential proteins and ligands exposed upon sperm damages, with emphasis on premature acrosome reacted and apoptotic sperm, which are thought to make up the majority of the non-viable sperm population within an ejaculate [11, 30-33]. In spite of their accuracy, results provided by the routine sperm analytical tools remain useless information as the entire ejaculate is discarded when quality standards are not met. This is a great concern in commercial studs, particularly during hot seasons with numerous semen ejaculates not meeting production standards leading to substantial productivity losses [20, 23]. As an attempt to address this important problem, we examined the possibility of a non-destructive nanotechnology-based approach to specifically target and remove damaged spermatozoa from ejaculates and assessed the quality, viability, and fertility performance of residual nanopurified spermatozoa.

3.5.1 Characterization of MNP conjugates.

Apoptosis and acrosome reaction are clearly established as the main causes of poor semen quality, and excellent and viable technical are widely available for the detection of such damage spermatozoa [10, 11]. To target such cells, annexin V and lectin have been used as the biomarkers which could also serve to develop novel nanotechnology-based approaches for non-destructive removal of targeted cells from ejaculates, while preserving the fertility of remaining spermatozoa. In the current study, we used magnetic nanoparticles conjugated with each biomarker (annexin V or lectin). Synthesis were done under a disclosed intellectual property which led us to perform some characterization of the assembled MNP conjugates. The MNP core (iron oxide) was measured by TEM at approximately 14 nm in diameter, and the conjugate preparations showed various size distributions under DLS and AFM/MFM. We believe that the solvents used for measurements may have caused inconsistencies in diameter variations between DLS vs. AFM/MFM. Indeed, aliquots of MNP conjugates stored in a phosphate stock solution were directly spread onto microscope histology slides for AFM/MFM while milliQ water-diluted aliquots were used for DLS hydrodynamic measurements. On the other hand, it could be argued that the various aggregate formations may effect total magnetic strength allowing for effective elimination of damaged spermatozoa. Most importantly, the AFM/MFM confirmed the maintenance of the MNP magnetism property during conjugation.

3.5.2 Evaluation of sperm-MNP interactions.

In the current study, the decision to target 20% of spermatozoa within the semen was based upon the current threshold for quality controls in ART, having 15 to 30% as the maximum acceptable proportion of apoptotic sperm cells per usable ejaculates. A single amount of each MNP conjugates (1mg/2 X 10^9 spermatozoa), highly enriched in biomarkers was used to avoid potential toxicity. Nonetheless, previous studies have used higher amounts in boar (1mg/33 X 10^6 spermatozoa [19]) and bull (1mg/1 X 10^6 spermatozoa [18]) without observed negative effects on dams and born offspring. Both MNP conjugates were able to interact with spermatozoa as observed with a variety of technical imaging (TEM, AFM/MFM, and hyperspectral fluorescence). All images showed MNP conjugates in the sperm head area, indicative of the targeting specificity given that annexin V binds the membrane translocated phosphatidylserine to label apoptotic cells while carbohydrate receptors of lectins are located in the sperm acrosome area. Also, the single and multi-point attachments of MNP conjugates to spermatozoa may reflect the extend of the damage and therefore implying that harshly damaged cells inducing MNP aggregations create a stronger magnetic signal and allows for easier and effective removal of damaged sperm using magnets. As such aggregated free MNP conjugates also provide effective removal (in case of single-point attachments), which contributes to limiting the amount of residual MNP within the purified doses (nanopurified semen), ultimately reducing downstream occurrences of nano-toxicities.

3.5.3 Motility characteristics of nanopurified spermatozoa.

The double nanopurification applied in this study did not impair, but rather improved the motility characteristics of nanopurified spermatozoa. These findings were

consistent with a previous work using single nanopurification with lectin-MNP [19] and which positive fertility outcomes may be supported by early studies in humans showing positive correlations between straightness (STR), straight-line velocity (VSL), and fertilization rates [33]. The successful removal of defective sperm cells in this study was evidenced by the significant decreased proportions of static spermatozoa following nanopurification, which removal likely permitted increased VSL, STR, and LIN that are valuable predictors of effective and smooth displacement of spermatozoa within the utero-oviduct tube [34-35].

The present work proposed the combination assessment of two MNP conjugates, which motility data raised questions regarding the 1) orderly application of annexin V- then lectin-MNP conjugates and 2) need for a double nanopurification process. Indeed, the beneficial effects of nanopurification on sperm motility (total, progressive, rapid, and static) and velocity (VAP, VSL, and VCL) parameters were mainly limited to the first (or single) removal using annexin V-MNP conjugate, while second (or double) removal had lesser or no effects on the same parameters. It is worth mentioning that the use of lectin-MPN conjugates in a single nanopurification process had generated similar effects [19], which prompted us to the suggestion that majority of damaged spermatozoa may possess both apoptotic and acrosome damage features leading to their nearly full removal during the first nanopurification. Interestingly, the second (or double) nanopurification showed beneficial effects on ALH, BCF, STR, and LIN instead. Altogether, generated data indicate the synergistic effects of the double nanopurification process, which further perfection through optimized sperm-to-MNP conjugate ratios will massively generate high performance spermatozoa for enhanced ART outcomes.

3.5.4 Viability assessment of nanopurified spermatozoa.

Interestingly, the proposed nanopurification did not add further impairments of spermatozoa. In comparison to the control (non-purified), the proportion of viable spermatozoa was not affected, and nanopurified spermatozoa exhibited more stable membranes, lower ROS accumulation and stronger mitochondrial membrane potential as seen with the fluorescence intensities. These sperm features are all beneficial for successful fertilization [36].

3.5.5 Reproductive outcomes of nanopurified spermatozoa.

The field fertility test was conducted on a small-scale. Overall, comparable fertility (pregnancy and farrow) and prolificacy (litter size at birth and weaning) rates were obtained between control and nanopurified spermatozoa, and no detrimental effects were observed on sows and offspring (litter weights, morphometry, and developmental rates). Furthermore, the assessment of blood parameters (glucose, hematocrit, and IgG) did not reveal any health issue in pigs born from nanopurified spermatozoa, as compared to the control group. These data are first reports to validate the use of magnetic nanoparticle conjugates to improve assisted reproduction in livestock.

3.6 Conclusion

The present study proposes a double nanopurification process using annexin V and lectin to target the main causes of non-viable sperm within the semen ejaculate. Nanopurification led to improved sperm motility and velocity performance with no observed interferences regarding viability and fertility performance. Evaluation of offspring indicated no impairment in growth and development, implying no obvious signs

of cytotoxicity due to utilized nanoparticles. Although further studies of a likely latent long-term toxicity are required during post-natal life, the present findings already provide greater insight towards the influential role magnetic nanoparticles that become a routine tool for post-collection semen handling in both clinic and agriculture.

3.7 Future perspectives from objective 1

Transmission electron microscopy, atomic force microscopy, and hyperspectral imaging techniques confirmed attachments of specifically designed magnetic nanoparticle conjugates to damaged spermatozoa. The computer assisted-sperm analyzer (CASA) revealed a significant increase in sperm motility characteristics after purification, while flow cytometry analyses indicated comparable viability parameters (reactive oxygen species accumulation and acrosome, plasma, and mitochondrial membrane intactness) between purified and non-purified (control) spermatozoa. Furthermore, sow fertility and performance (growth and health) of pigs born from purified spermatozoa were unaffected compared to their non-purified counterparts. The current findings indicate that the designed magnetic nanoparticle conjugates can be used for high-throughput targeting, rapid, and low cost purification of mammalian semen prior to insemination. The absence of negative effects on dams and resulting offspring makes magnetic sperm purification a promising tool for improving male fertility. However, further studies regarding offspring potential and viability produced from nanopurified semen is required.

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CHAPTER IV

OBJECTIVE 2: GROWTH DEVELOPMENT AND HEALTH ASSESSMENT OF OFFSPRING BORN FROM MAGNETIC NANOPARTICLE PURIFIED BOAR SPERMATOZOA

Durfey, C[1]., Burnett, D. D[1]., Liao, S. F[1]., Crenshaw, M.A[1]., Clemente, H[4].,

Willard, S. T[1,2]., Ryan, P[3]. L., Feugang, J.M[1]

1.Departments of Animal and Dairy Sciences, Mississippi State University, Mississippi State, MS
2. Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS
3. Pathobiology and Population Medicine, Mississippi State University, Mississippi State, MS
4. Clemente Associates, Madison, CT

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Figure 4.1 Image of neonatal pigs under a heat lamp

4.1 Summary

Disproportionate populations of viable and non-viable spermatozoa in semen ejaculates influence sire fertility, due to non-viable spermatozoa generating toxic waste products (reactive oxygen species). Advancements in nanotechnology have allowed the development of magnetic-based technique for semen purification through a process called "nanopurification". This technique allows for selective removal of non-viable spermatozoa, however, the safe application and potential toxicity concerns have yet to be explored. Here, we applied the nanopurification procedure to discard acrosome damaged and apoptotic spermatozoa from semen before inseminations and to evaluate the growth and health performance of resulting offspring.

Analyses performed from weaning to market (164 days post-natal) indicated that various growth (weigh), developmental (reproductive tract), and health (blood) parameters were not significantly impaired in pigs born from treated (nanopurified) semen versus the controls. Both groups of pigs showed similar growth rates and reached market at comparable sizes. Other parameters such as the hepatic cytochrome P450 enzyme activities, blood glucose and immunoglobulin G concentrations, hematocrit, and white blood cell counts were comparable between both groups of pigs ($P>0.10$). Furthermore, female reproductive tract analysis showed a marked shortening in uterine horn length in treated female offspring when compared to the control. ($P=0.04$).

4.2 Background

Spermatozoa are highly specialized and haploid which fertility acquisition and maintenance depend upon a variety of intrinsic (genetic, morphology, physiology, etc.) and extrinsic (environment, nutrition, health, etc.) factors to the sires. Under such

influences, spermatozoa can undergo a multitude of damages, creating a heterogeneous population of viable and non-viable spermatozoa within the ejaculate that interfere with their migration, ability to fertilize the oocyte, and initiate embryo development [1, 2]. Efficient methods to eliminate such damage spermatozoa from ejaculates are still unavailable for semen fertility and productivity improvements in commercial farms.

Nanotechnology is a novel, growing multidisciplinary field incorporating nanoparticles at a nanometer level scale. Biomedical innovations involving nanotechnology have dramatically increased due to the diverse array of potential applications, allowing nanotechnology to become a "key technology" for current biomedical innovations [3]. Magnetic nanoparticles (MNP) have become of particular interest for agriculture and biomedical applications due their controllable sizes (1-100 nm), cell-specific targeting mechanisms, and magnetic non-invasive characteristics [4]. MNP offer a variety of new applications within agriculture and biomedical fields, including reproductive health with exposures to mature spermatozoa [3, 5-8].

Recent developments in nanotechnology have allowed for harmless purification of livestock (cattle and pig) semen ejaculates through the use of magnetic nanoparticles (MNP), in a process termed sperm "nanopurification" [9, 10]. The sperm nanopurification methodology is still in its exploratory phase and its current application has focused primarily on the targeting of non-viable prematurely acrosome reacted spermatozoa through lectin proteins, disregarding the apoptotic spermatozoa that constitute a significant part of non-viable spermatozoa. Therefore, the use of specifically designed MNPs to simultaneously or separately trap both sperm sub-populations will likely boost semen fertility [11].

Yet, the use of MNP for biomedical and reproductive medicine have triggered an increasing concern for potential genotoxic and cytotoxic affects. Common to all nanoparticles, the small nanometer scale and large surface-to-volume ratio of MNP allow for enhanced reactivity for efficient cell targeting, while potentially allowing for the diffusion of nanoparticles through biological membranes and tissue barriers that raises potential cytotoxicity worries [12, 13]. Induction of cytotoxicity from MNP could lead to impairments in critical components of cellular function resulting in membrane damage, formation of apoptotic bodies, or impairments in mitochondrial function [14]. More concerning, nanoparticles often undergo aggregation, potentially increasing MNP concentrations on imposed cells, further increasing the risk of potential cytotoxicity [12]. For their use in sperm nanopurification, MNP have yet to show toxicological potentials with many studies showing an enhanced spermatozoal functionality due to the removal of MNP-targeted sperm [9, 15]. However, sperm nanopurification has yet to conclude in a (quasi-) complete removal of sperm-bound and unbound MNP nanoparticles from semen doses intended for artificial inseminations, as residual MNP may cause delayed damages of the maternal utero-tubal epithelium impeding sperm migration and fertilization or within the exposed spermatozoa, leading to genotoxicity affecting embryo-fetal viability.

Developmental and cytotoxic concerns are widely expressed when using nanoparticles for in vivo techniques. Although magnetic nanoparticles have shown no indication of altered spermatozoal functionality upon exposure in previous studies [9, 10, 15], limited knowledge has been shown to assess or validate the viability of resulting offspring. As the first attempt to explore this issue, the current study aims at evaluating basic growth (weight and female reproductive tract) and various health (glucose, IgG,

white blood cell composition, hematocrit, and hepatic cytochrome P450 activity) parameters, to explore potential offspring viability in rotational and terminal breeding systems.

4.3 Methods

4.3.1 Magnetic nanoparticle synthesis

Iron oxide (Fe_3O_4) magnetic nanoparticles (MNP) were coated with lectins and apoptotic proteins (PNA/ PSA; Sigma Aldrich, St Louis, MO, USA). Coating of lectin and apoptotic proteins allow particles to selectively bind to glycolipid molecules exposed on spermatozoa undergoing acrosome reaction and membrane impairment. Functionalized MNP were synthesized under an intellectual property (Clemente Associates; Madison, CT, USA) and used for sperm labeling [9].

4.3.2 Sperm labeling and purification with nanoparticles (nanopurification)

Boar (n=3) semen doses (n=4/boar) were obtained from a local boar stud (Prestage Farms, West Point MS) and aliquoted in artificial insemination doses (Beltsville awing Solution). Each dose was mixed with or without 0.3mg of apoptotic (annexin V) followed by lectin nanoparticles. Upon each individual mixture, doses were incubated at 37°C with a gentle rotation for 30 min to allow sperm-nanoparticle interactions. Subsequent doses were then placed against an external magnetic field consisting of magnets fixed in polycarbonate for 10 min at room temperature. Free and unbound spermatozoa were eluted from nanoparticle-bound spermatozoa through magnetic separation into a new identified tubes. This magnetic elution process was completed twice for each MNP conjugate (apoptotic and lectin) for a total of three

purification cycles. Upon purification semen were placed into artificial insemination tubes at room temperature for immediate artificial insemination.

4.3.3 Animals and artificial inseminations

All animals were maintained in the swine facility at the Animal Physiology Unit, H.H. Leveck Animal Research Center, Mississippi Agricultural and Forestry Experiment Station, Mississippi State University, and had ad libitum access to water and feed. Prepared semen doses purified with or without (control) magnetic nanoparticles were immediately transported approximately one mile to the farm for artificial insemination. Females (gilts; n=6) were bred with control (n=3) or nanopurified (n=3) semen as previously described [9]. Pregnancies were confirmed by ultrasonography 30 days post-breeding and pregnant gilts were maintained until farrowing and weaning. Ten offspring pigs of equal genders (5 females and 5 males) born from control and purified (treated) semen were selected based on weight and average daily gain, from control (n=2) and nanopurified semen-born (n=1) litters and grown to market size. All pigs were administered iron (intramuscular) at 3 days of age with males castrated at approximately two weeks. All offspring were initially dewormed (Ivomec; Merial, Duluth, GA) and vaccinated for Erysipelas (Erysipelothrix Rhnsiopathiae Bacterian; Novartis, Larchwood, IA) at weaning with a booster injection approximately one month after. Animals were harvested at approximately 165 days of age upon reaching market size (≈ 120kg). Animal care and use were performed according to protocols approved by the Institutional Animal Care and Use Committee of Mississippi State University (14-026).

4.3.4 Growth and Developmental Parameters

Each pig (n=20) was weighed approximately every 30 days from weaning (day 28) until market (day 164). At harvest, reproductive tracts of all females (5 control and 5 treated) were placed in bags containing PBS and maintained in ice. Liver specimens of all pigs were harvested, immediately snap-frozen in liquid-nitrogen, and stored at -80^{o}C. All samples were used for developmental analyses.

4.3.4.1 Female reproductive tract

Ovary weights and visible antral follicles were evaluated, and morphometric measurements were done on oviduct, cervix, uterine horn, and body sections. Subsets of each tissue were fixed in 4% paraformaldehyde for epithelia histology analysis of samples stained with Hematoxylin-Eosin and observed under an optical microscope.

4.3.4.2 Liver function

Hepatic activity of cytochrome P450 (an essential enzyme for toxin and drug metabolism) was evaluated through the CYP1A, CYP2C, and CYP3A assay kits and NADPH regeneration system (Promega Corporation; Madison, WI) as previously described (Hart et al., 2014). Briefly, approximately 100 mg liver samples were homogenized in potassium phosphate (KPO4) buffer (400 mM; pH 7.4). Cellular protein was extracted and quantified (Coomassie Plus; Bradford protein assay), with analysis of all enzymatic assays (CYP1A, 2C, and 3A) using a Promega Multi-Plus plate reader with luminescence detection. A linear relationship between rate of luminescence in relative light units (RLU) vs. concentration of hepatic protein was determined. Enzyme activities

were expressed relative to the individual pig's liver protein (RLU/min/mg of protein) and relative to the pig's individual liver weight (RLU/min/liver).

4.3.5 Assessment of Health Parameters

Approximately every 30 days from weaning (day 28) until market (day 164), blood samples were collected intravenously (jugular vein) from each pig (n=20) into EDTA-coated tubes and placed on ice for various analyses.

4.3.5.1 Glucose measurements (mg/dL)

Whole blood samples were immediately tested to prevent glucose degradation (Glucometer; Agamatrix Inc, Salem, NH) of ±15 mg/dL sensitivity [16].

4.3.5.2 Hematocrit (pact cell volume)

Measurements were conducted on whole blood samples using a standard procedure, allowing for accurate evaluation of the ratio of red blood cells to the total volume of blood.

4.3.5.3 White blood cell count

Aliquots of whole blood samples were smeared onto histology slides, air-dried, and stained with a Wright stain solution (QuickLink I™ Wright; HealthLink, Jacksonville, FL, USA). The staining allows to differentiate between white blood cell types (neutrophil, basophil, eosinophil, lymphocyte, and monocytes) that were evaluated by double-blind counting and each cell type was expressed as percentage of total white cell count.

4.3.5.4 Immunoglobulin G (mg/ml) or IgG concentrations

Whole blood samples collected within EDTA-coated tubes were centrifuged at 15 rpm for 15 min at 4°C. Supernatant corresponding to plasma were used for IgG determination in pigs at weaning and market size (porcine ELIZA kits; Bethyl Laboratories, Inc., Montgomery, TX, USA).

4.3.6 Statistical Analysis

All statistical analysis was performed using the Statistical Analysis Software (SAS) 9.4 (SAS Institute, Inc., Cary, NC). To analyze the effects of nanopurified semen on offspring glucose and IgG concentrations, weight, and white blood cell composition, a linear mixed model (Proc MIXED) was used. Treatment, gender, and day were included as fixed effects with two-way (treatment*gender, and treatment*day) and three-way (treatment*gender*day) interactions measured. Repeated measures of growth and health performance over time were analyzed using an autoregressive one covariance method. Liver enzyme concentrations were analyzed similarly using a linear mixed model (Proc MIXED), with treatment and gender as fixed effects, and a two-way interaction (treatment*gender) measured. Additionally, offspring female reproductive tract evaluation were analyzed for comparisons through a general linear model (Proc GLM) and students t-test. Litter was considered a random effect in all statistical analyses when applicable. All data are expressed as mean ± standard error mean (sem), with significant differences set as $P \leq 0.05$.

4.4 Results

4.4.1 Growth and Developmental Parameters

The overall growth of offspring was comparable between both control and treated groups of pigs from weaning to market size, with no significant gender interactions (P>0.10; Figure 4.2). No significant differences between offspring were found regarding female reproductive tract parameters: ovary weight, follicle number per ovary, and oviduct, cervix, and uterine body lengths (P>0.05). However, uterine horn lengths appeared significantly shorter in treated females when compared to the control (P=0.04; Table 4.1). Additionally, uterine and oviduct epithelia did not show distinct differences between both groups (Data not shown; P>0.05).

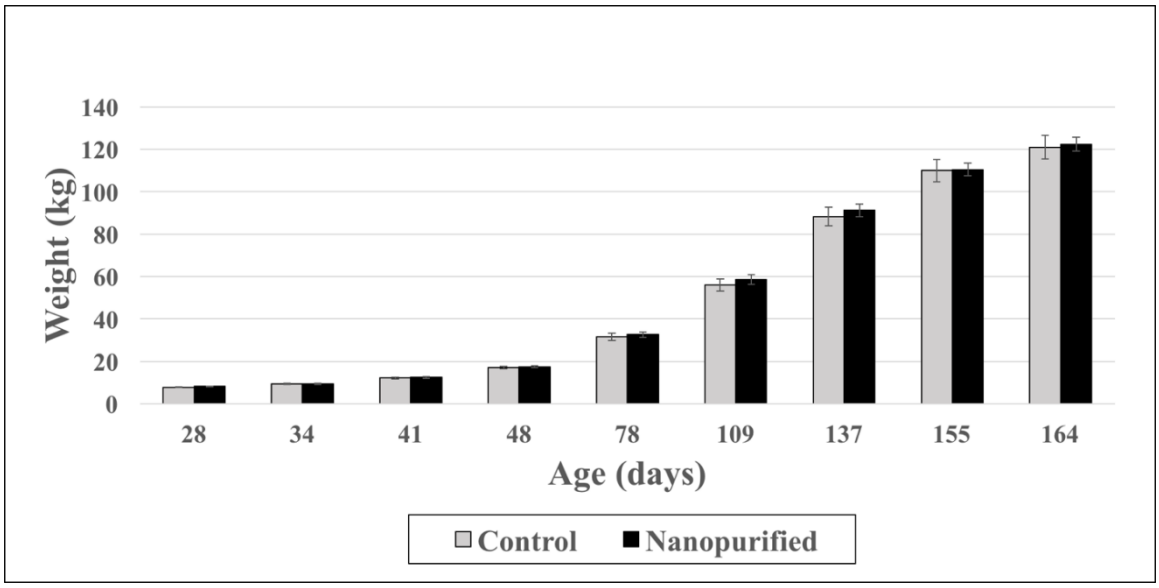

Figure 4.2 Offspring Growth.

Animals (n=20/group) were weighted from weaning (day 28) until market size (day 164). Data are expressed as mean±sem; no significant differences were noted at each specific weighing day (P>0.1).

Table 4.2 Female reproductive tract analysis.

Analyzed parameters	Control	Treated	P-value
Ovary (g)	5.3 ± 0.3 g	4.5 ± 0.3 g	0.07
Follicle per ovary ratio*	16 ± 3	17 ± 3	0.82
Oviduct length (cm)	23.2 ± 2.2	19 ± 2.1	0.20
Cervix length (cm)	7.7 ± 1.5	7.5 ± 2.9	0.95
Uterine horn length (cm)	64.4 ± 2.2^{a}	56.3 ± 2.6^{b}	0.04
Uterine body length (cm)	6.2 ± 0.3	6.3 ± 0.5	0.9

Reproductive tracts harvested from control (n=5) and treated (n=5) female offspring with various parameters indicative of potential reproductive performance analyzed. Data are expressed as mean±sem. *Follicle per ovary ratio is representative of the average number of follicles present on each ovary. Values that share the same letters (^{a,b}) are not statistically different ($P < 0.05$).

Hepatic CYP 1A, 2C, and 3A enzyme activities were not influenced by the nanopurification procedure ($P>0.05$). Nonetheless, CYP 3A showed a numerical trend in decreased activity in females born from nanopurified vs. control spermatozoa, which respectively corresponded to 383.6 ± 422.6 vs. 1,704.0 ± 422.7 RLU/min per mg of liver protein or 16,742.1 ± 18,263.2 vs. 74,152.9 ± 18,263.2 RLU/min per liver (mean ± sem; $P=0.07$).

4.4.2 Assessment of Health Parameters

Microscopic analysis of blood smears indicated comparable neutrophils, monocytes, and lymphocytes values between treated and control offspring, with no significant gender interactions (Figure 4.3). Basophil cells significantly increased in treated offspring on day

137 (1.3 ± 0.24 % vs. 0.2 ± 0.24 %; P<0.05). Similarly, eosinophils were dramatically increased at market (day 164) compared to previous time points, with the treated group maintaining higher levels than the control (6.9 ± 0.61 vs. 4.6 ± 0.61; P<0.05).

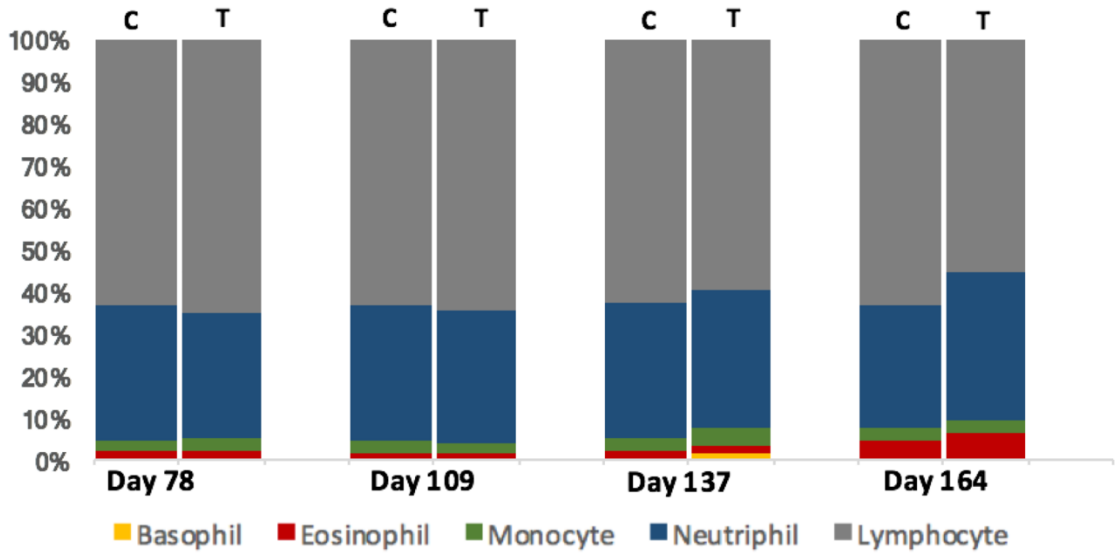

Figure 4.3 Distribution of white blood cell (WBC) in growing offspring.

White blood cell proportions in growing offspring. Data are mean proportions (±sem) of each WBC class harvested from each control or C (n=10) and treated or T (n=10) pigs, at different growing stages (Day 78, Day 109, Day 137, Day 164). All values are consistent within a normal physiological range, regardless of variations between treatments and time points.

Glucose levels were significantly increased in both control and treated offspring from day 78 but stabilized from day 109 to day 164 (market). No significant differences were found between both groups of pigs at any time-point (Figure 4.4A; P>0.05).

Hematocrit values followed a similar pattern between all offspring, but remained significantly elevated in treated offspring on day 78 (33.5 ± 1.1 vs. 29.8 ± 1.1; P=0.02).

Immunoglobulin (IgG) concentration was significantly reduced in treated offspring at weaning ($P<0.01$; Figure 4.4B), while no difference was found between both groups at market ($P>0.05$). In comparison to weaning, the IgG levels were significantly increased at market regardless of the group ($P<0.05$; Figure 4.4B).

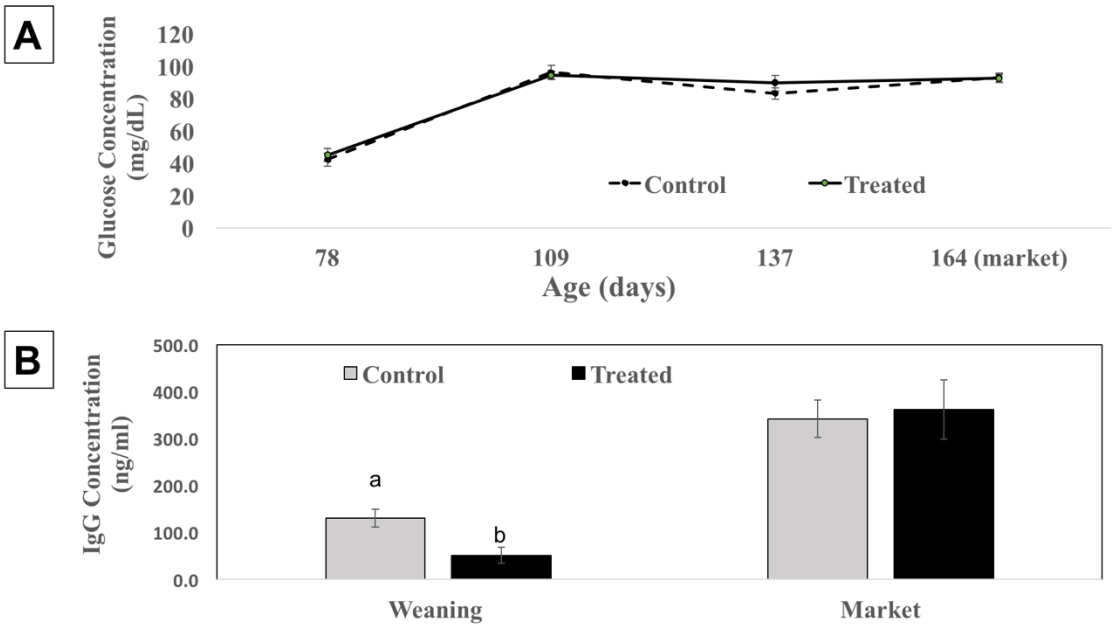

Figure 4.4 Offspring glucose and Immunoglobulin G concentrations

Glucose (A) and Immunoglobulin G or IgG (B) levels in growing offspring born from Control (n=10) and Treated (n=10) spermatozoa. The weaning and market days correspond to day 28 and 164, respectively. Values that share the same letters (^{a,b}) are not statistically different ($P < 0.05$). Data are expressed as mean±sem.

4.5 Discussion

Sperm nanopurification with MNP have been increasingly studied to improve male fertility [17]. Although hindered sow fecundity in females exposed to MNP nanopurified spermatozoa have yet to be noted [9], observations regarding offspring development and health are unavailable or scarce. Thus, this study focused on wean to

finish time period to identify signs of impaired or weakened growth, development, or basic health parameters which may hinder offspring performance and quality.

To meet current pork market demands, efficient swine growth for desired lean pork is critical for successful economic output [18]. Continuous measurements of offspring weight from weaning to market size remain a discernible way to distinguish normal swine development for pork production. Thus, results from this study show that the growth of offspring born from double-nanopurified (acrosome reaction and apoptosis) spermatozoa was not impaired, but instead showed subtle improvements from weaning to market compared to the control.

Although uncommon, female congenital abnormalities can have a profound effect on fertility and fecundity potentials. Quantitative measurements of female reproductive tracts can provide essential insight to the reproductive and conceivability potential of females [19]. Here we found that female offspring born from nanopurified semen did not significantly differ from control females regarding various parameters of the reproductive tract pertaining to ovulatory (follicle to ovary ratio, and ovary weight) potential, and indicating no obvious impairments for sperm transport (oviduct, cervix, and uterine body length) and successful fertilization. Nonetheless, the current study suggests that sperm nanopurification may not affect the fertility potential of offspring, but rather their fecundity, given the short length of the uterine horn in the treated group. Uterine horn supports embryo implantation and intra-uterine development, and marked shortening may likely indicate a reduced fecundity in female offspring in the treated group, through the inability to support large litters or sizable offspring as previously reported [20]. This being said, the shorter oviduct length may not necessarily translate into smaller litter

sizes, as an accommodation for more piglets could be achieved with lighter baby pigs during in utero and post-natal development. Further breeding of gilts born from nanopurified spermatozoa are needed to address and validate this potential concern as a consequence of using nanopurified semen in rotational breeding systems, but may not be of interest for terminal line producers.

Comparable cytochrome (CYP) P450 enzyme activities were found between offspring born from treated and control spermatozoa. The CYP family comprises a large number of enzymes related to oxidative metabolism. Among these, CYP 1A, 2C, and 3A have shown to be common markers for steroid and drug metabolism, critical for efficient growth and maturity [21]. These enzymes are found to catalyze the oxidative metabolism of a variety of lipogenetic compounds and play an important role in steroid metabolism [22]. No effect of sperm nanopurification was found on both CYP1A and 2C family members, suggesting a normal hepatic physiology and function. Indeed, both CYP1A and 2C have been shown to play important roles in the formation of epoxygenases, critical for the metabolism of polyunsaturated fatty acids; while their increased activities may be indicative of cardiovascular function [23]. On the other hand, although gender interactions showed a decreased trend in the activity of CYP3A in treated female offspring, further large scale studies are required to validate its correlation with the sperm nanopurification. Even so, gender differences in CYP3A expression have been reported in various studies and species (swine, cattle, and mice) [24-26].

An inhibitory role between immune system and meat carcass quality have been well established, indicating its importance in livestock systems [27]. Neutrophils, lymphocytes, and monocytes form an essential part of the immune system, playing

primary roles in innate and adaptive immune responses. Thus, absence of alteration in white blood cell composition and growth (weight) between treated and control offspring in this study is indicative of unaffected immune health, and growth efficiency. Although elevated basophils in the treated offspring were found on day 137, indicating a slight immune inflammatory response, values still remained within a normal physiologic range for swine (0-2%) [28]. Additionally, increased eosinophil percentages were found across control and treated offspring upon market size (day 164) compared to previous time points throughout the study, and were still maintained within a normal physiological range (0.5-11%) despite statistical differences between treatments [28]. This suggests all pigs were subjected to a temporary immune irritant (i.e. allergies) at this time point, rather than an overwhelming immune response (i.e., infection).

Glucose is an important energy source for growth and health maintenance, labeling it a critical health parameter for evaluation of energy production for efficient swine production [29]. This study revealed a large variation between glucose values on day 78 and the remainder of the experimental timeline across both control and treated offspring, with concentrations well below the normal range (85-150mg/dL) documented for swine [30]. Even so, although environmental conditions show pigs were briefly subjected to hypoglycemic conditions during this time, normal physiologic values were steadily obtained in both control and treated offspring throughout the remainder of the study. This demonstrated all offspring had the ability to regulate glucose energy metabolism at an efficient rate, regardless of being born from nanopurified semen.

Hematocrit (pact cell volume) percentage is an important indication of the proportion of blood cells within a whole blood sample, commonly measured to determine

anemia or hydration status. Hematocrit percentages were comparable between groups and time points throughout the study, with values remaining stable within or in close proximity to a normal physiologic range of 32-46% [28]. Significant variations from this range would indicate anemia with an extremely low percentage or dehydration with a high percentage value, however, results indicated no such physiological impediments in offspring born from semen nanopurification.

Immunoglobulin G (IgG) is a common antibody for the maintenance of humoral immunity. It is well known that IgG consumed from colostrum during the first day of birth plays a critical role in the transfer of maternal immunoglobulins to the neonatal pigs, normally falling to low concentrations during nursing as the neonatal pig intestinal tract matures [31]. In a previous study (personal communication), we found that IgG concentrations of treated offspring obtained at birth before colostrum intake, had higher IgG levels compared to control offspring. Therefore, the lower level obtained in offspring born from nanopurified (vs. control) at weaning was a surprise, and further large-scale analysis are required to identify it as a direct consequence of sperm nanopurification versus genetic variation. Even so, regardless of the lower IgG concentrations at weaning, treated offspring were shown to have comparable proportions at market when compared to the control, indicating no impairment in the overall development of IgG antibodies as a consequence of being born from nanopurified semen. Moreover, significant increases of IgG levels across all offspring between weaning and market time points were expected and can be attributed to various environmental, nutritional, and physiological factors during this transitional phase of development [32].

4.6 Conclusion

The present study offers an insight towards offspring developmental and cytotoxicity concerns when using MNP purified semen. Through numerous evaluations of developmental and health parameters were conducted, treated offspring were consistently shown to have no impairments regarding growth or health development from weaning to market size, as a consequence of being born from nanopurified semen. Thus it is likely that sperm nanopurification will ultimately lead to successful and typical offspring performance, for efficient use in the swine industry. Additionally, female offspring showed no signs of infertility, though decreased fecundity remains for investigation. Overall this study concluded there were no obvious indications of cytotoxicity or developmental challenges in offspring born from magnetically nanopurified semen. Although further large-scale studies are required to insure resulted offspring are not exposed to any unobserved detrimental performance, this study allows us to be one-step further towards indicating a reliable and safe use of high-throughput semen nanopurification in commercial studs.

4.7 Future perspectives from objective 2

No obvious developmental impairments or cytotoxicity in pigs born from nanopurified semen were observed. Furthermore, results from this study indicate offspring born from nanopurified semen are able to grow and reach market size at a comparable rate with the control. Although no overall health parameters indicated an impairment in the overall biological and physiological function of offspring born from nanopurified semen the decreased uterine body length found in female offspring indicate the potential for decreased fecundity. Overall, the study indicates that the proposed

nanotechnology-based purification is safe for post-collection semen handling for terminal line production in commercial livestock operations. However, further studies indicating pork characteristics is required to identify any impairments associated with the market quality of offspring.

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CHAPTER V

OBJECTIVE 3: ASSESSMENT OF MARKET CARCASS AND MEAT QUALITY OF PIGS BORN FROM MAGNETIC NANOPARTICLE PURIFIED SPERMATOZOA

Durfey, C. L.[1], Counsell, K. R.[2], Moorhead, W. A.[3], Clemente, H.[4], Vance, C. R.[2], Willard, S. T.[1,2], Ryan, P. L.[4], Feugang, J. M.[1]

1. Departments of Animal and Dairy Sciences, Mississippi State University, Mississippi State, MS
2. Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS
3. College of Veterinary Medicine, Mississippi State University, Mississippi State, MS
4. Pathobiology and Population Medicine, Mississippi State University, Mississippi State, MS
5. Clemente Associates, Madison, CT

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Figure 5.1 Feeder pig

5.1 Summary

Sperm ejaculates contain a mixed population of nonviable and viable spermatozoal cells. Ejaculates with high concentrations of poor quality or damaged spermatozoa can greatly impair the overall fertility of males. Advancements in nanotechnology have allowed for the non-invasive targeting and removal of poor quality semen, in a process termed “nanopurification”. Such selective removal of damaged spermatozoa is critical to enrich insemination doses with high quality spermatozoa as a means to enhance fertility successes. However, effects associated with offspring born from nanopurified semen (treated) and possible meat quality assurance have yet to be extensively studied. Here, we measured the growth performance, market characteristics, and molecular analysis of pork from pigs born from standard or nanopurified spermatozoa.

Near infrared spectroscopy (NIRS) analysis revealed no indication of differences in blood chemistry composition in offspring produced from control and treated sperm. Standard pork quality parameters revealed no significant differences between groups, however, dressing percentage was found to be significantly increased in treated offspring. Western immunoblot and in situ immunofluorescence detected both myoglobin (MYO) and fatty acid synthase (FAS) proteins in muscle and fat tissues, indicating the physiological performance of pork quality potential. Fat tissue had significantly decreased signal activity of both MYO and FAS in treated female offspring Whereas, muscle tissue revealed no differences in MYO across pigs. However, significantly lower FAS signals were detected in both treated females and males. Furthermore, data indicated no impairments in offspring pork quality when born from nanopurified semen.

5.2 Background

Efficient and sustainable livestock production are necessary to feed the rapidly growing world human population that is projected to surpass 9 billion by 2050 (FAO, 2015). Pork in particular is the most widely consumed meat product in the world, and is the fastest growing livestock sector (FAO, 2016). Major advancements in swine breeding and management technologies have allowed producers to increase their economic output gain, creating a more efficient production system. Increasing genetic progress within the swine industry for more efficiently growing offspring and pork production, continues to be an immense advantage for artificial insemination techniques. However, there are still demands for technological advancements within the breeding industry for better reproductive efficiencies and offspring performance.

Nanobiotechnology is a novel, growing multidisciplinary field incorporating nanoparticles into biological sciences. During the past decade, biomedical innovations involving nanotechnology have shown a dramatic increase due to the diverse array of applications, making nanotechnology a “key technology” for current biomedical innovations [1]. Magnetic nanoparticles (MNP) have become of particular interest for use in agriculture and biomedical applications because of their controllable size production (1-100nm), cell-specific targeting potential, and magnetic characteristics [2]. These specific properties of MNP offer a variety of novel approaches for their applications in the current reproductive techniques.

Recent studies have successfully used MNP to target specific features of spermatozoa in human and livestock [3-6]. The introduction of MNP technology in assisted reproduction is aimed to target non-viable or moribund spermatozoa that

interfere with fertilization potential of semen ejaculates [7]. The use of MNP has been successful for purification of fish [8], bull [9], and boar [10] semen in a process termed as "nanopurification". The current nanopurification technique in the bull and boar allows for high-throughput removal of non-viable from semen ejaculates before artificial insemination in livestock, and therefore may become a useful tool in commercial livestock operations as an attempt to combat male infertility or subfertility [11]. Prior studies have shown that semen nanopurification does not affect sperm function, fertility and fecundity of inseminated gilts, without impeding post-natal development of offspring [9, 10]. However, this innovative technique in livestock has yet to be studied for influences in offspring performance and quality.

Previous studies have shown sperm age and surrounding environment affect offspring performance and likely market outcomes [12, 13]. For instance, pork carcass quality characteristics are dependent upon a variety of genetic and environmental factors, and genetic correlations between sire-based meat quality characteristics (i.e., leanness and fat content) and pork flavor and quality have been reported in prior studies [14, 15]. With the presence of both viable and non-viable sperm in semen ejaculates, it could be speculated that viable spermatozoa possess genetically superior traits for transmission to offspring. Meanwhile, non-viable spermatozoa possess acrosome reaction, chromatin abnormalities, and DNA fragmentation, playing a vital role in male fertility [16-18].

The incidence of these abnormalities in semen ejaculates of boars are complex and multifactorial [19], which can jeopardize swine production. The current study proposes the use of a semen nanopurification as a reliable breeding technique, through

the exploration of overall pork carcass and market quality of offspring born from standard or nanopurified semen.

5.3 Methods

5.3.1 Magnetic nanoparticle synthesis

Iron oxide (Fe_3O_4) magnetic nanoparticles (MNP) were coated with lectins and apoptotic proteins (PNA/ PSA; Sigma Aldrich, St Louis, MO, USA). Coating of lectin and apoptotic proteins allow particles to selectively bind to glycogen molecules exposed on cells undergoing an acrosome reaction and membrane impairment. Functionalized MNP were synthesized in accordance with previous experiments (Clemente Associates; Madison, CT, USA) and provided for sperm labeling [10].

5.3.2 Sperm nanopurification and artificial insemination

Boar (n=3) semen doses (n=4/boar) were obtained from a local boar stud (Prestage Farms, West Point MS) and aliquoted in artificial insemination doses (Beltsville awing Solution). Each bag was mixed with or without 0.3 mg of each apoptotic and lectin nanoparticles. Following nanoparticle exposure, doses were placed against an external magnetic field for 10 min at room temperature. Free and unbound spermatozoa from nanoparticles were eluted from nanoparticle-bound spermatozoa through magnetic separation, and prepared MNP purified and control semen doses were immediately transported to the farm for artificial insemination.

5.3.3 Animals

Six gilts were bred with control (n=3) or nanopurified (n=3) semen. Following successful farrowing and weaning of viable offspring, ten offspring pigs of equal ages

and genders (5 females and 5 males) were selected based on weight and average daily gain, and grown to market size. Selected pig offspring originated from two control litters (n=10) and one nanopurified or treated litter (n=10), and were all (n=20) maintained in a partially outdoor facility at the Animal Physiology Unit, H.H. Leveck Animal Research Center, Mississippi Agricultural and Forestry Experiment Station, Mississippi State University, and had ad libitum access to water and feed. Animals were fasted approximately 12 hours before harvesting at market size (approximately 120 kg) at 164 days of age. All pigs were harvested at the Mississippi State University Meat Science and Muscle Biology Laboratory. Animal care and use were performed according to protocols approved by the Institutional Animal Care and Use Committee of Mississippi State University (14-026).

5.3.4 Sample collection and preparation for analyses

Upon harvest, blood was collected from all pigs during exsanguination in EDTA-coated tubes and set on ice for immediate centrifugation at 3,000 rpm for 15 min at 4^{o}C. Blood plasma were collected and stored at -80^{o}C for Near Infrared Spectroscopy (NIRS). Subcutaneous fat and muscle from the longissimus dorsi were collected post-mortem, immediately snap-frozen in liquid nitrogen and stored at -80^{o}C (for western immunoblotting) or fixed in 4% methanol-free paraformaldehyde (for *in situ* immunofluorescence).

5.3.5 Near infrared spectroscopy (NIRS)

Aliquots of plasma (200 µl) were held frozen-thawed at room temperature and absorbance spectra analyses were performed at 22^{o}C, between 350 and 2,500 nm using an

ASD FieldSpec®3 spectrophotometer (ASD Inc., Boulder, CO, USA), to assess differences in overall blood chemistry. The focal region for data evaluation was the 1st water overtone interval (1,300-1,600 nm). Each blood plasma sample was analyzed in triplicate.

5.3.6 Carcass preparation and measurement evaluation

Upon harvest, carcass characteristics were determined as described in Wang et al., 2015 [20]. Live and hot carcass (without head, feet, or organs) weights were recorded. Briefly, hot carcasses (without head, feet, or organs) were split longitudinally into two halves and weighed prior to cooling at 4ºC. Dressing percentage was calculated by dividing the hot carcass weight by the live weight at slaughter and multiplying by 100.

Approximately two days post-mortem after chilling, the following carcass measurements were taken by a single trained personal through a double-blind method: chilled carcass weight, carcass length, average back fat thickness (first rib, last rib, and last lumbar vertebrae), ham fat thickness, 10th rib back fat thickness, untrimmed ham weight, trimmed ham weight, loin weight, picnic weight, boson butt weight, belly weight, rib weight, and marbling score. Total weight of chilled carcass was recorded to calculate total shrinkage percent ([hot carcass weight-chilled carcass weight]/hot carcass weight X 100). Total lean cuts and percentages were calculated from the average of all lean cuts (trimmed ham, loin, picnic, and boston butt weights) times two (for each carcass half).

Color and firmness scores were obtained on the ham and longissimus dorsi (loin) muscles subjectively using a 5-point scale from 1-5 (1=pale, soft, and lacking marbling, 5= dark, firm, greater amount of marbling), with a score of 3 being ideal for quality pork. Objective color measurements of the loin muscle were obtained with a HunterLab

MiniScan 4500L, Spectrophotometer (Hunter Associates Laboratory, Inc., Reston, VA), to analyze meat lightness (L), redness(A), yellowness (B), chroma, and hue.

The pH measurements were taken in center of the loin muscle using a Multiparameter pH Meter HI2020 (Hanna Instruments, Woonsocket, RI) and a pH Electrode FC2320 (Hanna Instruments, Woonsocket, RI). Carcass dressing percentage was calculated by the following formula: hot carcass weight divided by final live weight multiplied by 100. Shrinkage percentage was calculated by dividing the chilled carcass weight by the hot carcass weight multiplied by 100. The loin eye area (10th rib) was calculated using a Plastic Grid (Product number AS 0235E, Extension and Outreach, Ames, IA, USA).

5.3.7 Pork molecular analyses

5.3.7.1 Section of antibodies

Fatty acid synthase (FAS) is a multifunctional enzymatic complex which plays a critical role in fat accumulation. Similarly, myoglobin (MYO) has shown to have a pronounced correlation with pork quality. Therefore, for the context of this study we focused on the expression levels of fatty acid synthase (Santa Cruz Biotechnology Antibody (C-20), goat polyclonal IgG; 200 µg/ml) and myoglobin (Santa Cruz Biotechnology Antibody (FL-154) # sc-25607, rabbit polyclonal IgG; 200 µg/ml) within muscle and fat tissue collected from swine offspring to evaluate differences in pork leanness quality.

5.3.7.2 Protein isolation and Western-immunoblot

Immunoblotting was performed as previously described Feugang et al., 2015 [21]. Samples were thawed at room temperature, mechanically grinded, and total proteins were extracted using the complete RIPA buffer (Santa Cruz Biotech Inc., Santa Clara, CA, USA), as previously described. After centrifugation total proteins were quantified (Coomassie Plus Bradford protein assay kit; Thermo Fisher Scientific, Rockford, IL, USA) and equal amounts of proteins were resolved onto 4-12.5% SDS-PAGE NuPage gels. Proteins were electrophoresed, transferred onto a PVDF membrane (Millipore Corp, Bedford, USA), and immunoblotted (Novex® HRP Chromogenic Western Blot Immunodetection kit; Life Technology; Grans Island, NY. Membranes were exposed for one hour (or 24h - 4 ^{o}C) to primary antibodies (myoglobin or MYO, sc-25607 and fatty acid synthase or FAS, sc-16147 diluted 1:500), then 30 minutes to the secondary antibody (diluted 1:500). Gel membranes were submitted for quantification of detected bands (iBox® Scientia, UVP-LLC, Upland, CA, USA)

5.3.7.3 Immunohistochemistry

Samples were processed as previously described [21]. Previously fixed samples (muscle and fat; in 4% methanol-free paraformaldehyde) were processed for histology slides, deparafinized, permeabilized in 1% (v/v) Triton X-100, and blocked with 0.5% (v/v) normal goat serum. Samples were incubated one hour with or without antibodies (anti-FAS or anti-MYO; diluted 1:100), followed by one hour incubation with the FITC-conjugated secondary antibody (diluted 1:200). Samples mounted with a DAPI mounting medium (UltraCruz; Santa Cruz) and sealed for fluorescence imaging. All procedures were performed at room temperature, PBS was used for washes between steps, and

samples incubated with only the secondary antibody used as negative controls. Antibodies were purchased from Santa Cruz, Santa Clara, CA, USA). All samples were visualized (EVOS-FL Auto inverted microscope) and images (5 images/sample/animal) were documented for digital quantification (ImageJ software; NIH Image).

5.3.8 Statistical analysis

NIR spectra were analyzed using The Unscrambler X 10.4 (CAMO Software Inc., Woodbridge, NJ, USA) chemometric software. Full spectral pretreatment consisted of a 7 point Savitsky-Golay smoothing, with further treatment using a 2nd derivative 7 point Savitsky-Golay and averaging of sample triplicates and treatments (control and treated offspring; n=20). Variations between treatments were examined by Principal component analysis (PCA) using a random selection leave-one-out cross validation. A partial least squares (PLS) 2-block discriminant analysis was implemented to test defined group recognition.

To analyze the effects of nanopurified semen on offspring pork carcass and market quality a linear mixed model (Proc MIXED) was performed using the Statistical Analysis Software (SAS) 9.4 (SAS Institute, Inc., Cary, NC). Treatment and gender were included as fixed effects with a two-way (treatment*gender) interaction measured. Additionally, litter was considered a random effect. All data are expressed as mean ± standard error mean (sem), with significant differences set as $p \leq 0.05$.

5.4 Results

5.4.1 Near infrared spectroscopy (NIRS)

Figure 5.2 shows the principal component analysis (PCA) of consecutive spectra of each animal describing random distributions between treatments (Figure 5.2A), with cross validation confirmed with a partial least squares 2-block discriminant analysis describing variations between animals in the same treatment (PLS; Figure 5.2B). The PCA revealed an evenly distributed cluster of samples from each treatment (control vs. nanopurified). The PLS model showed calibration statistical parameters (slope = 0.043, R2 = 0.043, and SEC = 0.493) and cross-validation parameters (slope = 0.026, R2 = n/a and SECV = 0.516), indicating a lack of variation between offspring in the same treatment. Meanwhile, NIR spectrum profiles generated by the blood plasma collected from each group of pigs showed a perfect absorbance match across the entire wavelength range, indicating a lack of variation between control and treated offspring blood plasma chemistry (Figure 5.3).

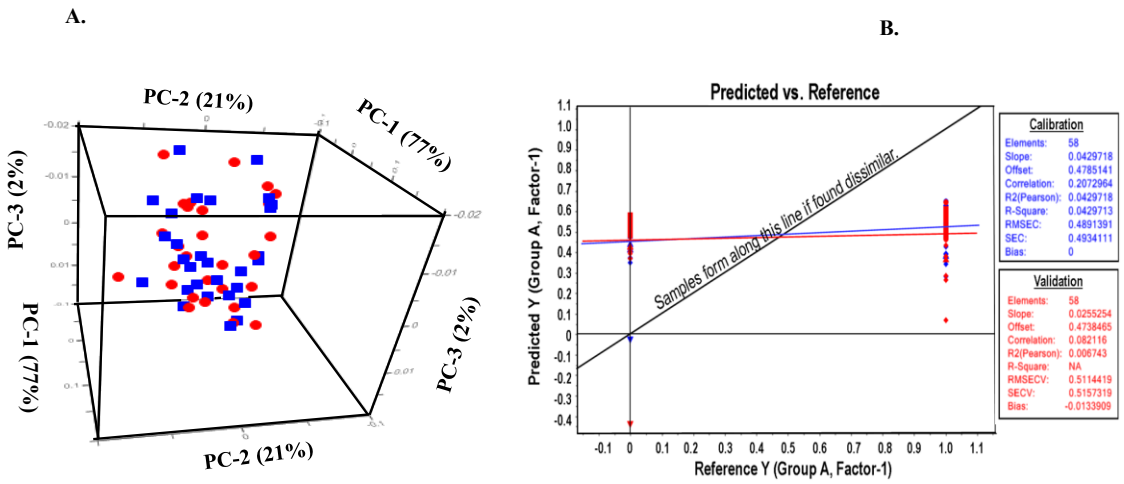

Figure 5.3 Blood plasma near infrared spectroscopy analysis with a principal component and partial least squares discriminant plot

(A) Principal Component (PCA) analysis showing an even distribution of blood plasma samples across control (blue) and treated (red) offspring, and (B) Partial Least Squares 2-block discriminant (PLS) analysis plots, showing variation between offspring within a single treatment (red) compared to the calibrated control (blue) of offspring blood plasma.

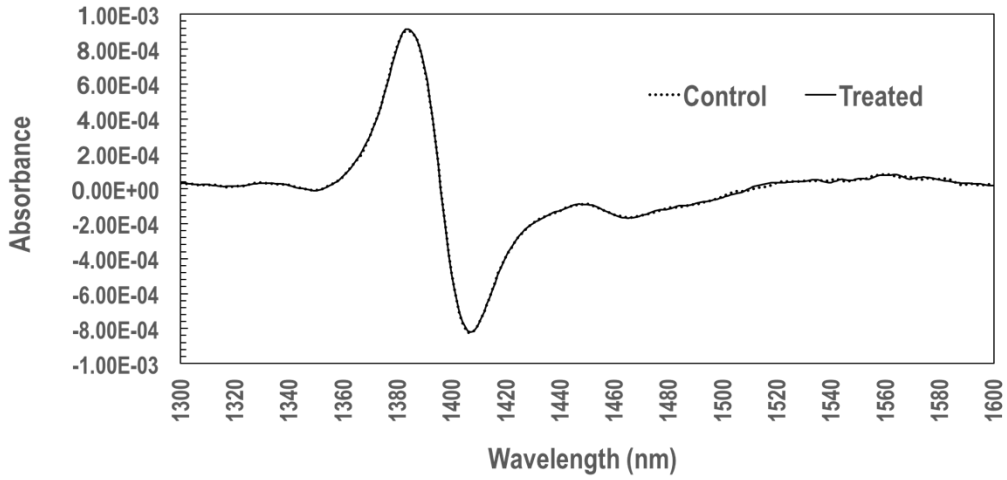

Figure 5.4 Near Infrared Spectroscopy (NIRS) of Blood Serum Composition.

Spectral analysis of averaged blood plasma samples from control (n=10; dotted line) and treated (n=10; solid line) offspring assessed at market (164 days old).

5.4.2 Carcass quality

Data summarized in Table 5.1 indicated pork cut weights (loin, picnic, Boston butt, belly, rib, untrimmed and trimmed ham), ham, loin color, and firmness ultimately did not differ between pigs. Additionally, pH, loin eye area, shrinkage percent, hot and chilled carcass weights, were similar between both groups of pigs. Back fat and the percent (%) of lean cuts was found to be comparable across all offspring. However, there was a tendency for an increased marbling score (intramuscular fat) observed in treated offspring when compared to the control (P=0.08). All carcass characteristics displayed in table 5.1 showed no significant interactions due to gender.

Dressing percentage indicates the percent of the total carcass weight available for market use. This study showed a marked overall increase in dressing percentage in treated offspring (P=0.05; Figure 5.4). Although gender interactions were not found significant between treatments, there was a tendency for increased dressing percentage in treated females when compared to the control (P=0.09). However, male offspring derived from nanopurified semen demonstrated a comparable dressing percentage to the control (P=0.27).

Table 5.2 Evaluation of carcass quality.

Offspring	Control	Treated	p-value
Live wt. at farm (kg)	133.04 ± 2.88	132.31 ± 2.88	0.86
Shrinkage Percent	3.4 ± 0.22	3.5 ± 0.22	0.83
Carcass Length (cm)	83.52 ± 0.82	83.64 ± 0.82	0.91
Back Fat Average (cm)	3.62 ± 0.41	2.08 ± 0.41	0.36
Back Fat Thickness (10th) (cm)	2.44 ± 0.17	2.24 ± 0.17	0.40
Loin Eye Area (10th rib)	6.8 ± 0.18	6.6 ± 0.18	0.56
Marbling Score (loin)	2.3 ± 0.15	2.7 ± 0.15	0.08
Ham Color Score	3	3	-
Loin Color Score	2.8 ± 0.14	2.8 ± 0.14	0.91
Firmess Score	2.7 ± 0.16	2.6 ± 0.16	0.66
Ham Fat Thickness (cm)	2.03 ± 0.12	2.07 ± 0.12	0.88
Loin Weight (kg)	9.13 ± 0.30	9.02 ± 0.30	0.80
Picnic Weight (kg)	4.03 ± 0.33	4.61 ± 0.33	0.23
Boston Butt Weight (kg)	3.67 ± 0.14	3.93 ± 0.14	0.21
Belly Weight (kg)	4.71 ± 0.13	4.75 ± 0.13	0.86
Rib Weight (kg)	1.77 ± 0.06	1.73 ± 0.06	0.65
Lean Carcass Weight (kg)	53.72 ± 1.34	56.15 ± 1.34	0.23
% Lean Cuts (Carcass)	57.42 ± 0.99	59.06 ± 0.99	0.26
pH	5.407	5.412	0.86

Evaluation of pork carcass characteristics. Data are expressed as mean±sem, 10 pigs per control and treated (MNP offspring) group. No significant differences found ($P > 0.05$).

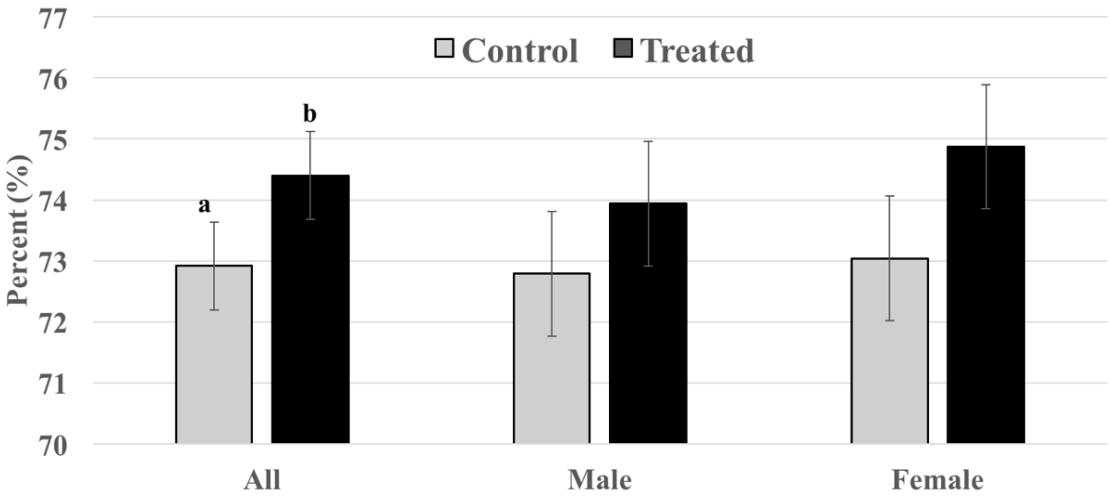

Figure 5.5 Dressing percent.

Dressing percentage represented as an average of all offspring (control, n=10; treated, n=10), and separated by gender (male and female; control n=5; treated n=5). Data are expressed as mean±sem. Values that share the same letters (^{a,b}) are not statistically different ($P < 0.05$).

5.4.3 Western Immunoblot, Immunohistochemistry and quantification

Representative westernimmunoblotting and immunofluorescence results of myoglobin (MYO) and fatty acid synthase (FAS) in tissues are shown in Figure 5.5. Westernimmunoblotting confirmed the specificity of anti-FAS and anti-MYO antibodies detecting both proteins at their expected band sizes of 270 and 17 kDa, respectively. Myoglobin and fatty acid synthase proteins were detected in both muscle and fat tissues using both immunological techniques. MYO was shown stronger in muscle (loin) tissue with FAS similarly shown strong in fat (back fat) tissue, and immunofluorescence detection of MYO appeared heterogeneously distributed in the muscle tissue.

Immunofluorescence quantification of MYO and FAS in both muscle and fat tissues was conducted with gender interactions detected (Figure 5.6). Immunofluorescence quantification of MYO in muscle revealed no impact of semen

nanopurification, with a lack of gender interactions (P>0.05; Figure 5.6). However, detection of MYO in fat showed significant gender interactions, with decreased expression in treated females compared to the control (P=0.002). Additionally, FAS expression levels in both muscle and fat tissues were significantly decreased in females born from nanopurified semen (P<0.05; Figure 5.6), while these levels remained similar among male offspring.

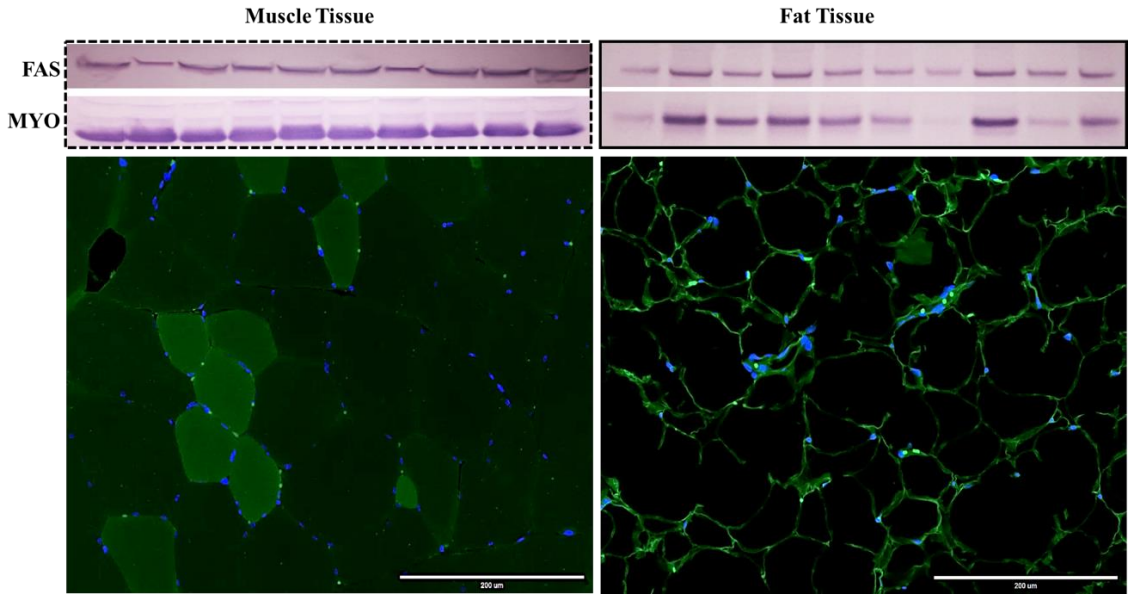

Figure 5.6 Westernimmunoblot and Immunofluorescence of muscle and fat tissue

Western Immunoblot (dashed lines) and Immunofluorescence (bold lines) detection of fatty acid synthase (FAS, 270 kDa) and myoglobin (MYO, 17 kDa) in muscle (dotted line) and fat (solid lines) tissues of offspring. MYO and FAS were used as markers of muscle and fat tissues, respectively with their detection shown in immunofluorescence (green).

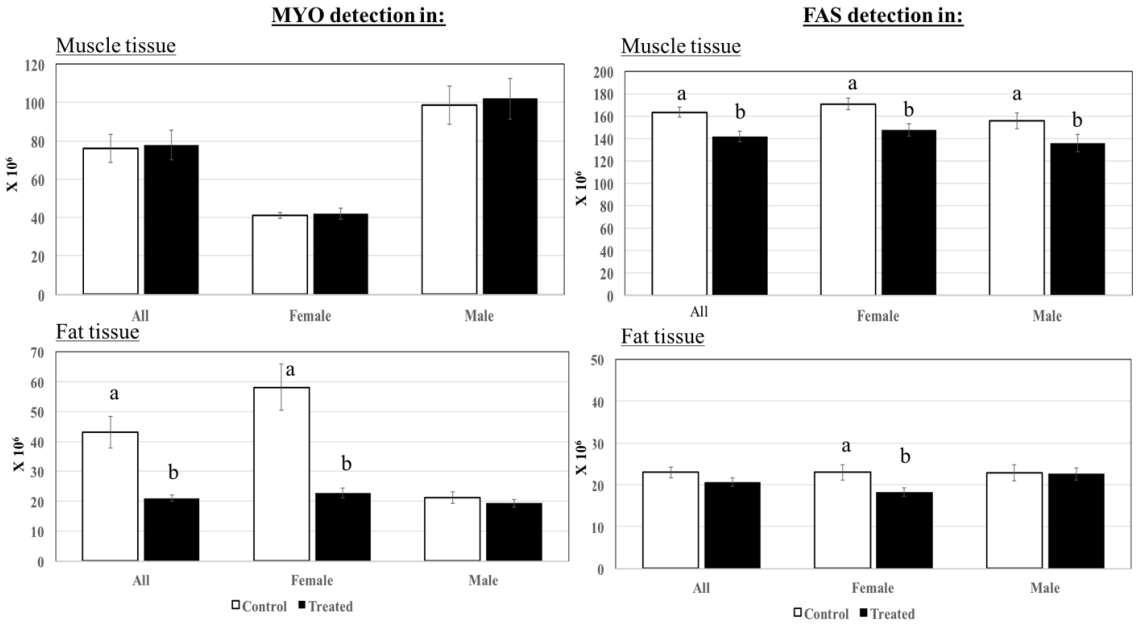

Figure 5.7 Immunofluorescence quantification for muscle and fat tissue

Immunofluorescence quantification of detected signals (minimum of 5 independent images). The Y-axes are arbitrary units represented as X 10^6. Data are means ± sem of 10 pigs per group (All) and 5 per group (Gender: female or male). Values that share the same letters (^{a,b}) are not statistically different ($P < 0.05$).

5.5 Discussion

Consumption of pork is admired due to the desired leanness of meat. Characteristic traits used to select for lean and efficiently grown pigs are difficult to improve through natural selection due to low heritability and assessment of phenotypic traits conducted primarily only after slaughter [22]. Sperm viability may have an effect on offspring performance through increased genetic merit, and the selection of viable spermatozoa prior to insemination may benefit transgenerational transmission of economical traits. Here, MNP allowed harmless nanopurification of viable spermatozoa and preservation of their fertility potential, which lead to fully grown offspring for the assessment of marketable carcass and meat quality traits.

Upon market size, blood plasma samples of all pigs were subjected to near infrared spectroscopy (NIRS) to numerically distinguish any variations between offspring composition of blood plasma chemistry before market. The NIRS termed "aquaphotomics" is a novel analytical technology relying upon variances between hydrogen bonds in water molecules, reflecting differences in the overall chemistry composition of the aqueous solution in question (i.e., plasma, serum and urine) [23]. Thus, NIRS can be used to provide non-invasive specific measurements of aqueous phase biological compounds, and as such have previously been applied in a variety of species evaluating blood lipid content and the overall health status of animals in regards to cholesterol, glucose, and immune infections [24-26]. Here, we used NIRS to analyze and compare blood plasma chemistry composition as a novel representation for the overall health status of individual pigs at the time of market. The NIRS spectra of both control and treated offspring indicated comparable physiological blood plasma components. This is further confirmed from results conducted in a previous study indicating a lack of variation in offspring health status with similarities in white blood cell composition and glucose, IgG and hematocrit concentrations between pigs born from standard and nanopurified semen. Therefore, the successful use of NIRS in this study could be considered a novel application for assessing the overall health status in swine.

Among the market criteria in swine, carcass quality traits are a clear representation of overall pork quality. Swine carcasses are commonly evaluated through various measurements of weight, cuts, factors involving leanness, palatability characteristics indicative of taste/tenderness, and growth efficiency (evenness of fat deposition distribution) to provide the best indication for quality and economic gain. One

of the major goals in the pork industry is to increase skeletal muscle gain and decrease excess fat accumulation, without reducing intramuscular fat (marbling) to lessen palatability and tenderness [27]. This study showed back fat and the percent of lean cuts were comparable among all offspring, indicating pork leanness and palatability characteristics were not impacted due to sperm nanopurification. These critical pork quality characteristics in addition to the overall lack of treatment variations between evaluated carcass parameters confirms the use of sperm nanopurification as a breeding technique could still allow for successful and viable offspring.

Dressing percentage in particular is a routine carcass evaluation method which assesses the percent of economic output possible from an individual carcass. In this study, a significantly higher dressing percentage (DP) was observed in offspring born from nanopurified semen, indicating a possible correlation between sperm nanopurification and offspring viability. Correlation between fasting pigs before slaughter leading to decreased stress during transport and higher dressing percentage and quality in pork has also been reported in previous studies [28, 29]. However, we can definitely rule out this possibility as both groups of pigs were fasted 12 hours before slaughter in this study. Still, it is important to acknowledge the small sample size and lack of genetic variation within the treated offspring in this study, limiting our assumptions for the increased dressing percentage in treated offspring, especially when regarded as an effect of the nanopurified semen treatment. However, small increases in dressing percentage as indicated in this study can have profound influences on economic gain for the producer, and should not be overlooked.

To further confirm the lack of variations between pork quality among offspring, molecular analyses of pork was conducted. Western immunoblot and immunohistochemistry were performed on samples of the longissimus dorsi muscle and back fat adipose tissue, using two common antibodies myoglobin (MYO) and fatty acid synthase (FAS) previously shown to indicate pork quality advantages. Both proteins were selected as positive makers for muscle (MYO) and fat (FAS) tissues, as well as for their relevant physiological roles. Indeed, MYO protein provides indications for the tissue color regarding type I and IA muscle fibers, through the regulation of aerobic metabolism [30], while FAS protein is an important regulator for de novo synthesis of saturated fatty acid in subcutaneous fat tissue (back fat) [31].

Western immunoblotting and immunohistochemistry results confirmed the presence of both proteins (MYO and FAS) in fat and muscle tissues, with varying intensity levels according to tissue type. These observations revealed the decreased protein levels of FAS in the fat and muscle tissues of treated offspring were influenced by gender interactions, with an overall greater effect in females in fat tissue. Although the expression of MYO was overall decreased in treated offspring due to females, MYO levels remained unchanged in muscle tissues with no notable gender interactions. Furthermore, the lack of differences in MYO detection in muscle tissue confirms the similar visual carcass coloration (ham and loin color scores), indicating offspring born from nanopurified semen were not subjected to undesirable discolored pork quality traits. However, the large presence of MYO in fat tissue was unexpected, but could be speculated as a possible indication of decreased fat deposition as similar trends were noted between MYO and FAS in fat tissue. However, it is clear further fatty acid analyses

are required to identify specific variations in fat composition between control and treated offspring.

5.6 Conclusion

In addition to our previous studies, the current work further supports the safe and harmless use of semen purification with MNP, without offspring impairments regarding overall health and pork quality at market. More specifically, we showed that pigs born from nanopurified semen have the potential for producing more efficient carcass (higher dressing percentage). Although further investigations are needed to precisely quantify the impact of semen nanopurification procedure on enhancing growth efficiency and leaner pork quality, the current data support the safe use of semen nanopurification without compromising offspring productivity within the pork industry.

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CHAPTER VI

GENERAL CONCLUSIONS

The safe assurance for the use of magnetic nanoparticles to purify boar semen is critical to establish for likely applications in commercial production settings. Our study demonstrated successful sperm-nanoparticle interactions with the non-interference of the nanopurification procedure on sperm function (motility and fertility) or offspring performance (growth, health, and market quality). Spermatozoa escaping the proposed double magnetic purification (MNP) technique exhibited enhanced motion characteristics (motility and velocity), which are essential for smooth and fast displacement of spermatozoa for interaction with the female gamete (oocyte), within the female reproductive tract.

In vitro assessments of sperm viability were successfully predictive of the fertility maintenance of nanopurified spermatozoa as observed with the birth of viable offspring, without impairment of sow fecundity. Various in vivo and in vitro techniques indicated no further perturbations on the growth, development, or health of offspring produced; of which observations implied the absence of any (or presence of insignificant amounts of) residual magnetic nanoparticles from the nanopurification process that can cause genotoxicity in spermatozoa or impede normal fertilization or embryonic development in the female reproductive tract.

Additionally, offspring produced from nanopurified spermatozoa had no impairments in growth, health, or pork carcass quality. Ultimately, the use of magnetic nanoparticles for semen purification in swine showed improvements in sperm viability and fertility with successful and typical offspring performance. The present study provides promise and a solid foundation for the continuation of double magnetic purification of semen for future use as an integrated assisted reproductive technique.

Figure 6.1 Image of a neonatal pig

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APPENDIX A

CONFERENCE PAPER

Beneficial effects of semen purification with magnetic nanoparticles

Casey Durfey[1], Sabrina Swistek[1,2], Wei Tan[3], Henry Clemente[4], Peter Ryan[1,3], Scott Willard[1,2], Jean Feugang[1]

1. Departments of Animal and Dairy Sciences, Mississippi State University
2. Biochemistry, Molecular Biology, Entomology, & Plant Pathology, Mississippi State University
3. Basic Sciences, Pathobiology and Population Medicine at Mississippi State University
4. Clemente Associates, Madison, CT

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A.1 Abstract

Semen contain both viable and non-viable spermatozoa which equilibrium affects male fertility. Current techniques for detecting non-viable spermatozoa in semen ejaculates lack specific targeting for their subtraction. Here we used magnetic nanoparticle conjugates to selectively target and remove non-viable spermatozoa, and assess the motion characteristics and viability of residual spermatozoa.

Boar semen were mixed with (nanopurified) or without (control) magnetic nanoparticle conjugates and incubated to allow specific targeting of non-viable (or moribund) spermatozoa. Afterwards, mixtures were placed against a powerful magnet trapping moribund spermatozoa and permitting elution of viable spermatozoa. Before and after incubation, sperm motion and viability parameters were respectively analyzed with a Computer-Assisted-Sperm-Analyzer and flow cytometry after specific staining to evaluate the viability status of spermatozoa. Data (mean±sem) were compared with SAS package.

The proportion of static sperm significantly decreased after purification (8.9±0.5% vs. 11.3±0.5% for the control; $P<0.05$). Motion parameters (total and progressive motility, straightness, linearity, straight line velocity or VSL, and beat cross frequency or BCF) of nanopurified spermatozoa were significantly increased, while the amplitude lateral head displacement or ALH was decreased ($P<0.05$). Sperm viability parameters (plasma and acrosome membrane integrity and mitochondrial potential) were comparable between both groups ($P>0.05$). Findings indicate the successful removal of moribund (static) spermatozoa without impairing the viability of residual spermatozoa. Beneficial effects on sperm motion has potential to improve male fertility.

A.2 Introduction

Spermatozoa provide the source for male genetic potential, serving as essential biomarkers for male fertility. Physical and physiological properties of spermatozoa are dependent upon intrinsic and extrinsic factors to the male, creating a heterozygous population of viable and non-viable (damaged or moribund) sperm cells [1]. Commonly, sperm aggregates are formed as a result of large proportions of damaged spermatozoa, impeding the migration and fertilization potential of spermatozoa both in vitro and in vivo [2, 3]. Male infertility can be a distressing issue in humans with significant genetic and economic impacts in livestock.

Various technical methodologies have been developed to improve assisted reproductive technology (ART) outcomes in livestock. Nonetheless, the elimination or substantial decrease of proportions of non-viable spermatozoa from semen ejaculates prior to artificial insemination (AI) remains an unachieved goal for maximizing semen fertility. Conversely, recent developments in nanotechnology now permit for novel sperm purification approaches. For instance, magnetic nanoparticles (MNP) have shown success for reproductive applications, through a magnetic semen purification technique, termed semen "nanopurification" [4, 5]. These studies conducted in boar and bull species eliminated (nanopurified) spermatozoa bearing a single morphologically damaging factor (acrosome reaction), disregarding the variable assorted possibilities of spermatozoal impairments. The incidence of apoptosis is another damaging factor of spermatozoa which combined with a pre-matured acrosome reaction constitutes the majority of sperm damage, and thus, the non-viable sperm population. Here, we developed MNP conjugates targeting two nanopurification steps, apoptotic and acrosome reacted spermatozoa

through respective binding to annexin V and lectins (PNA/PSA) [6] and to assess the motility and viability of nanopurified spermatozoa.

A.3 Methods

Nanoparticle synthesis Iron oxide (Fe_3O_4) magnetic nanoparticles (MNP) were synthesized and coated with annexin V (Sigma Aldrich, St Louis, MO, USA) or lectins (PNA/ PSA), allowing selective binding to early apoptotic spermatozoa and glycan's exposed by damaged acrosome membrane (or premature capacitated), respectively.

A.3.1 Sperm labeling, purification with nanoparticles, and motility characteristics

Freshly harvested and extended semen doses (~ 3 X 10^9 spermatozoa/dose) were obtained from a local boar stud. A total of 0.3 mg of annexin V- and lectin-conjugated MNP was successively mixed with each semen dose to target 0.6 X 10^9 moribund spermatozoa. Starting with the targeting of apoptotic (annexin V) and following each mixture, a co-incubation was performed at 37^{o}C with a gentle rotation to allow sperm-MNP interactions. After, semen mixtures were placed against a magnet for at room temperature for the trapping of free and sperm-bound MNP's. Free unbound, or intact spermatozoa were subsequently eluted into new tubes. This separation process was repeated twice (total of 3 purification steps) for each MNP conjugate. Aliquots of control (not purified), and double-nanopurified semen samples were obtained for motility analysis using a Computer-Assisted Sperm Analyzer (IVOS; Hamilton-Thorne Biosciences; Beverly, MA, USA).

A.3.2 Viability analysis of nanopurified spermatozoa

Nanopurified spermatozoa were immediately diluted to 30 X 10^6 cells/ml with a pre-warmed phosphate-buffered saline solution. Sperm suspensions were allocated to various staining for viability assessment: propidium iodide (PI, 1 mg/ml in PBS) for plasma membrane integrity, PNA-FITC (100 mg/ml in PBS) for acrosome reaction, JC-1 (500mg/ml; Cayman Chemical Co., Ann Arbor, MI, USA) for mitochondrial integrity, and H2DCFDA (1 mM in DMSO) for reactive oxygen species (ROS) accumulation within cells. All samples were incubated and diluted with pre-warmed PBS before immediate analyses with a flow cytometer (Becton–Dickinson FACSDiva version 6.1.3) set for 10,000 total events per analysis. Sample aliquots were mounted onto microscope slides and visualized under an epifluorescence microscope (EVOS FL-Auto, Thermo Fisher Scientific, Hampton, NH, USA) to validate proper staining.

A.3.3 Statistical analysis

All data were analyzed and compared using a Statistical-Analytical-Software (SAS). Nanopurification influences on sperm motility characteristics and viability parameters were analyzed using a two-way ANOVA and a student's t-test. Data are expressed as mean ± standard error mean (sem) of 4 independent replicates. $P \leq 0.05$ were held as the threshold of significance.

A.4 Results

A.4.1 Motility characteristics of nanopurified spermatozoa

In comparison to the control, sperm nanopurification significantly increased the proportions of motile, progressive (forward-moving), and rapid (fast) spermatozoa while

decreasing the proportions of static (non-motile) spermatozoa ($P < 0.05$; Figure A.1). Nanopurified sperm showed increased sperm velocity parameters, compared to the control group (VAP, VSL and VCL, $P > 0.05$). Other spermatozoal directional parameters such as straightness (55.69 ± 0.56% vs. 60.38 ± 0.84%), and linearity (27.35 ± 0.36% vs. 29.84 ± 0.69%) were variably increased with the lateral head amplitude (ALH; 7.95 ± 0.08 vs. 7.48 ± 0.11) decreased, following nanopurification ($P < 0.05$).

A.4.2 Viability assessment of nanopurified spermatozoa through flow cytometry

Control, and nanopurified (annexin V and lectin) had statistically comparable proportions of spermatozoa, exhibiting high mitochondrial membrane potential, low reactive oxygen species (ROS) levels, and intact acrosome and plasma membrane ($P > 0.05$). Following nanopurification, the mean relative fluorescence intensities (RFI) associated with ROS production (H2DCFDA) and damaged plasma membrane (PI) were non-significantly decreased, while the mitochondrial membrane (JC-1) intensity were increased ($P > 0.05$; Table A.1). Successful staining was confirmed through fluorescence microscopy (not shown).

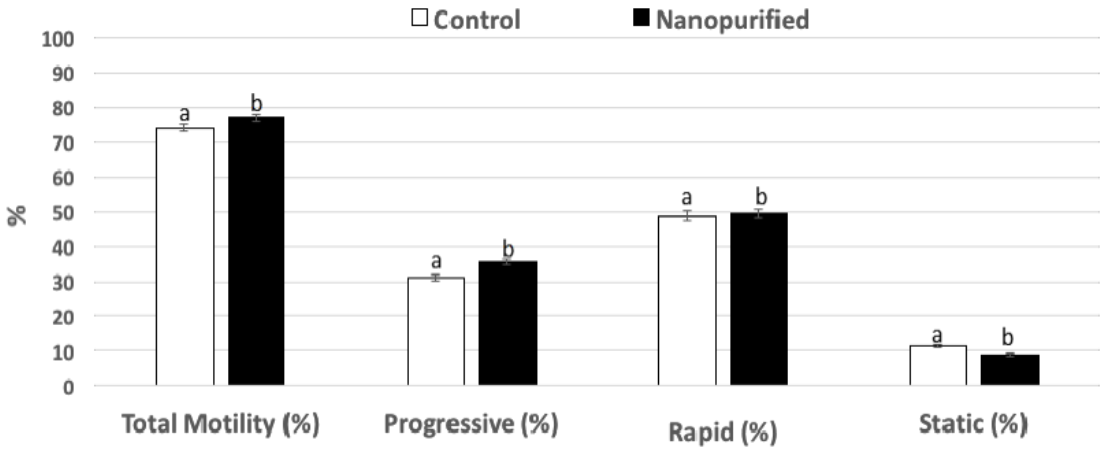

Figure A.1 Beneficial effect of sperm nanopurification on motility. (a,b; $P < 0.05$)

Table A.1 Flow cytometry florescent intensities

	Control	Nanopurified
ROS production (DCHF-DA)	1068.93 ± 200.26	864.42 ± 200.26
Plasma membrane integrity (PI)	11.892 ± 25.62	8.638 ± 25.62
Mitochondrial membrane integrity (JC-1)	425.89 ± 200.26	897.81 ± 223.9

Differential relative florescence intensities between control and nanopurified semen. ($P > 0.05$).

A.5 Discussion

The two-step nanopurification procedure conducted in this study showed there was no significant impairments but rather improvements of spermatozoa motility and velocity. The findings were consistent with previous studies using a single MNP-lectin nanopurification [5]. Significant decreased proportions of static spermatozoa succeeding nanopurification revealed successful elimination of non-motile sperm from MNP purification (nanopurification). This removal was the likely cause for improved velocity, directionality, and increased proportion of fast forward-moving (straight) spermatozoa

following nanopurification. This enhanced performance of spermatozoa suggests a better fertility potential due to greater spermatozoal activity within the female genital tract [7, 8].

Viability assessments showed no added impairments of nanopurified spermatozoa, with the proportions of viable spermatozoa remaining comparable to the control. Interestingly, nanopurified semen showed lower fluorescence intensity for ROS production that contrasted with higher plasma and mitochondrial membrane integrity, which indicate more stable plasma and mitochondrial membranes. Ultimately, results revealed better sperm robustness for improved fertility potential [8].

A.6 Conclusion

This study used a two-step MNP sperm nanopurification method to target two major defects of mature spermatozoa. Data indicate effective removal of targeted spermatozoa with improved performance and fertility potential of nanopurified semen. Although further large-scale in vivo studies are still needed, these preliminary findings indicate promising applications of this two-step nanopurification technique for improved male fertility in livestock productions.

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APPENDIX B

PRESENTED ABSTRACTS

B.1 Published Conference Abstracts

Durfey, C., Liao, S. F., Devost-Burnett, D., Dinh, T., Crenshaw, M.A., Willard, S. T., Ryan, P. L., Clemente, H., Feugang, J.M., "Growth and market quality of pigs born from magnetic nanoparticle treated boar spermatozoa". Reproduction, Fertility and Development 29(1): 141-141 (December 2016).

Moorhead, W.A., Durfey, C. L., S. Liao, S., Devost-Burnett. D., Gastal, G.D.A., Ryan, P.L., Willard, S.T., Feugang, J.M., "Effects of nanopurified boar semen for artificial insemination on protein detection in swine offspring muscle and fat tissue". Reproduction, Fertility and Development 29(1): 139-139 (December 2016).

Counsell, K.R., Durfey, C.L., Feugang, J.M., Willard, S.T., Ryan, P.L., Vance, C.K., "Developmental health assessment of offspring produced from magnetic nanoparticles using near infrared analysis of plasma". Reproduction, Fertility and Development 29(1): 140-140 (December 2016).

Myles, L. T., Durfey, C. L., Ryan, P. L., Willard, S. T., Feugang, J. M., "Exploitation of in vitro capacitation for nanoparticle incorporation within mammalian spermatozoa". Reproduction, Fertility and Development 28(2): 224-224 (December 2015).

B.2 Graduate Presentations

Durfey, C., Swistek, S., Tan, W., Clemente, H., Ryan, P., Willard, S., Feugang, J., Annual meeting of Southern Biomedical Engineering Conference (SBEC), "Beneficial effects of semen purification with magnetic nanoparticle". Gulfport, MS; March 2017. (Oral)

Durfey, C., Liao, S. F., Devost-Burnett, D., Crenshaw, M.A., Steadman, C.S., Willard, S. T., Ryan, P. L., Clemente, H., Feugang, J.M., Annual Midwest meeting of American Society of Animal Science, "Assessment of growth and health performance of pigs born from magnetically nanopurified boar spermatozoa". Omaha, NE; March 2017. (Oral)

Swistek, S. E., Durfey, C. L., Wei, T., Clemente, H., Ryan, P. L., Willard, S. T., Feugang, J. M., "Evaluation of magnetic nanoparticle exposure on boar sperm motility and viability," Mississippi Academic of Sciences, MS, Hattiesburg, MS; February 23, 2017. (*Poster*)

Durfey, C., Liao, S. F., Devost-Burnett, D., Dinh, T., Crenshaw, M.A., Willard, S. T., Ryan, P. L., Clemente, H., Feugang, J.M., Annual meeting of the International Embryo Transfer Society (IETS), "Growth and market quality of pigs born from magnetic nanoparticle treated boar spermatozoa". Austin, TX; January 2017. (Poster)

Moorhead, W.A., Durfey, C. L., S. Liao, S., Devost-Burnett. D., Gastal, G.D.A., Ryan, P.L., Willard, S.T., Feugang, J.M., Annual meeting of the International Embryo Transfer Society (IETS), "Effects of nanopurified boar semen for artificial insemination on protein detection in swine offspring muscle and fat tissue". Austin, TX; January 2017. (Poster)

Counsell, K.R., Durfey, C.L., Feugang, J.M., Willard, S.T., Ryan, P.L., Vance, C.K., Annual meeting of the International Embryo Transfer Society (IETS), "Developmental health assessment of offspring produced from magnetic nanoparticles using near infrared analysis of plasma". Austin, TX; January 2017. (Poster)

Durfey, C., Lawrence, A., Thirumalai, R., Clemente, H., Ryan, P.L., Willard, S. T., Feugang, J.M., NanoBio Summit, "Ultrastructure evaluation of boar spermatozoa following interaction with iron oxide nanoparticles". State of Alabama and Auburn University, Auburn, AL; October, 2016. (Poster)

Feugang, J. M., Durfey, C. L., Seong, P. B., Arnold, R. D., Clemente, H., Willard, S. T., Ryan, P. L., NanoBio Summit, "Nanotechnology approach to enhancing transgenic farm animal production," State of Alabama and Auburn University, Auburn, AL; October, 2016. (Oral)

Durfey, C., Liao, S. F., Crenshaw, M.A., Steadman, C.S., Clemente, H., Willard, S. T., Ryan, P. L., Feugang, J.M., Annual meeting of the Society for the Study of Reproduction (SSR), "Assessment of magnetic nanoparticle exposure on sperm motility and fertility potential", San Diego, CA; July 2016. (Poster Talk)

Feugang, J.M., Durfey, C.L., Liao, S., Devost-Burnett, D., Crenshaw, M., Clemente, H., Willard, S.T., Ryan, P.L., Annual meeting of the Society for the Study of Reproduction (SSR), "Assessment of reproductive tissues of gilts born from magnetic nanoparticles-treated boar spermatozoa", San Diego, CA; July 2016. (Poster)

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