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Effects of Porcine Relaxin Hormone on Motility Characteristics of Boar Spermatozoa during Storage

Juan Camilo Rodríguez Muñoz

EFFECTS OF PORCINE RELAXIN HORMONE ON MOTILITY CHARACTERISTICS OF BOAR SPERMATOZOA

By

Juan Camilo Rodríguez Muñoz

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EFFECTS OF PORCINE RELAXIN HORMONE ON MOTILITY

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By

Juan Camilo Rodríguez Muñoz

Approved:

Peter L. Ryan Professor Animal and Dairy Sciences, and Pathobiology and Population Medicine Graduate Coordinator of Animal Physiology Interim Associate of the Provost office (Major Professor and Director of Thesis)	Dean Jousan Assistant Extension Professor Animal and Dairy Sciences (Committee Member)
Mark Crenshaw Associate Extension Professor Animal and Dairy Sciences (Committee Member)	Jean M. Feugang Assistant Research Professor Animal and Dairy Sciences (Committee Member)
David Christiansen Assistant Clinical Professor Pathobiology and Population Medicine (Committee Member)	George Hopper Interim Dean College of Agriculture/Life Sciences

Name: Juan Camilo Rodríguez Muñoz

Date of Degree: April 29, 2011

Institution: Mississippi State University

Major Field: Animal Physiology

Major Professor: Peter L. Ryan

Title of Study: EFFECTS OF PORCINE RELAXIN HORMONE ON MOTILITY CHARACTERISTICS OF BOAR SPERMATOZOA

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Candidate for Degree of Master of Science

First, a preliminary study was conducted looking for the optimum sperm concentration to be used for analysis with the Computer-Assisted Sperm Analysis (CASA). Results showed that $75x10^6$ sperm cells/mL is the optimum one. Then, the actions of relaxin on sperm motility were evaluated by determining the effect of relaxin on full motility characteristics of spermatozoa during storage, using CASA; then identifying the relaxin receptors on spermatozoa, and finally establishing actions of relaxin in intraspermatic cAMP content. Motile spermatozoa were selected through percoll gradient and incubated for 1 hour with 4 relaxin concentrations at 37^oC, during four days. Relaxin affected sperm motility ($P<0.05$). This action appears associated with the presence of relaxin receptors RXFP1 and RXFP2 that were found in spermatozoa. However, the cAMP levels were not affected by relaxin ($P<0.05$). This study indicates a beneficial action of relaxin on sperm motility; however, its mechanism of action requires further research.

Key words: relaxin hormone, boar semen, sperm motility, CASA system, storage

DEDICATION

Quisiera dedicar ésta tesis y todo mi trabajo realizado acá en la Universidad del Estado de Mississippi (MSU) (Starkville, Mississippi - Estados Unidos de América) a mis Padres Blaca Elvia Muñoz Suárez y Óscar de Jesús Rodríguez Castañeda, mis hermanas Maria Fernanda Rodríguez Muñoz y Estefanía Rodríguez Muñoz, mi abuela Betsabé Suárez Berrío, y en general a toda mi familia por su immenso apoyo, estímulo y oraciones en éste proceso lejos de casa. Gracias por hacer de mí quien soy, y por ser la inspiración de cada día para luchar.

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LIST OF ABBREVIATIONS

A1 Differentiated A1 spermatogonia

A2 Differentiated A2 spermatogonia

A3 Differentiated A3 spermatogonia

A4 Differentiated A4 spermatogonia

Aal Undifferentiated A aligned spermatogonia

AI Artificial insemination

ALH Amplitude of lateral head displacement

ANOVA Analysis of variance

Apr Undifferentiated A paired spermatogonia

As Undifferentiated A single spermatogonia

ATP Adenosine 5'-triphosphate

B B spermatogonia

BCF Beat cross frequency

BTS Beltsville thawing solution

cAMP Cyclic adenosine 3',5'-monophosphate

CASA Computer-Assisted Sperm Analysis

Casp Cysteine-rich regions

DAPI 4', 6-diamidino-2-phenylindole

ESC Primary human endometrial stromal cells from the cycle

FITC Fluorescein isothiocyanate

g gravity

GLM General linear model

GPCR G-protein-coupled receptor

GPCR 135 Somatostatin and angiotensin-like peptide receptor

GPCR 142 G-protein-coupled receptor 142

GPR 100 G-protein-coupled receptor 142

$G_{\alpha i}$ G-proteins inhibiting α subunits

$G_{\alpha s}$ G-proteins stimulating α subunits

HCl Hydrochloric acid

HEK Human embryonic kidney 293T

IGF-I Insulin-like growth factor I

IGF-II Insulin-like growth factor II

IgG Immunoglobulin G

In Intermediate spermatogonia

INSL 3 Leydig cell insulin-like peptide or relaxin-like factor

INSL 4 Novel insulin-like peptide 4

INSL 5 Novel insulin-like peptide 5

INSL 6 Novel insulin-like peptide 6

INSL 7 Relaxin-3

IUPHAR International Union of Pharmacology LVII

LDLa Low density lipoprotein class A

LGRs Leucine-rich repeat-containing guanine nucleotide-binding (G-protein)-coupled receptors

LGR 7 Leucine-rich repeat-containing G-protein-coupled receptor 7

LGR 8 Leucine-rich repeat-containing G-protein-coupled receptor 8

LIN Linearity

LSD Least significant difference

min Minutes

PBS Phosphate buffered saline solution

PKA Protein Kinase A

PVP Polyvinylpyrrolidone

R3 Relaxin-3

RLF Relaxin-like factor

rpm revolutions per minute

RXFP 1 Relaxin family peptide receptor 1

RXFP 2 Relaxin family peptide receptor 2

RXFP 3 Relaxin family peptide receptor 3

RXFP 4 Relaxin family peptide receptor 4

SALPR Somatostatin and angiotensin-like peptide receptor

SAS Statistical analysis system

SEM Standard error of the mean

STR Straightness

THP-1 Human monocyte cell line

VAP Average path velocity

VCL Curvilinear velocity

VSL Straight line velocity

CHAPTER I

INTRODUCTION

1.1 Swine Industry

Pork has accompanied civilizations for several generations and it has become an important source of protein and food for humans. According to the Food and Agriculture Organization of the United Nations (FAO, 2002), the amount of pork consumed annually worldwide to that date was 94 million metric tons, corresponding to 40% of the total red meat consumed. The consumption of pork has increased parallel to the increasing world population and swine numbers have risen too. Ten years back from 2002, the consumption of pork has increased from 73 to 94 million metric tons (FAO, 2002). In the same way, estimations have projected that the demand of pork will increased to 125 million metric tons by 2020. Surprisingly, most of this increase is estimated for countries in development (Delgado *et al.,* 1999). In the same way, there has been an increasing interest in swine to be used as research models in biomedicine.

Research and swine producer management has become important in increasing the numbers of swine worldwide. In fact, during the last decades, efficiency of production and carcass composition has been improved by nutrition and genetic selection, respectively. Similarly, there have been some other biotechnological techniques employed such as artificial insemination (AI) that have constituted a major impact in the industry. Likewise, the development of semen extenders has improved the success of AI, allowing the possibility to use fresh semen for more than 5 days post-collection, further

optimizing the utilization of semen. In order to have a sustainable supply of pigs for human consumption and research the swine industry must continue to improve their standards at a reproductive level to meet these expectations. The development of a long-term boar semen storage system that would allow the transport of semen around the world would be of great importance for the commercial swine industry.

The use of AI has facilitated and maximized the transfer of the best genetic material over the last several decades. Conversely, when using frozen-thawed boar semen (long-term semen storage) this goal has not been achieved completely. In addition, AI also abolishes physical contact between animals, thus reducing the incidence of sexually transmitted diseases. The utilization of frozen-thawed (cryopreserved) boar semen in the swine industry for reproduction is low compared to other species. Reasons for that are the reduced conception rates, smaller litter size and reduced farrowing rate (Jonhson *et. al.,* 1981) after AI with frozen-thawed boar semen compared to inseminations with fresh semen (Almlid *et al.,* 1987), despite the use of a higher number of spermatozoa (Pursel and Johnson, 1975). Among the factors responsible for such reduced conception rates when using frozen semen are: cooling of semen, methodology employed (Cochran *et al.,* 1984), composition of cryoprotectants (Wilmut and Polge, 1977), sperm concentration and thawing temperature (Pace et al, 1981). It has been proposed that cryopreservation of semen destroys the boar sperm head plasma membrane, promoting molecular and biochemical changes that will later on affect the fertilization process (Buhr *et al.,* 1989, 1994; Bailey *et al.,* 2000). There is still a crucial need to maximize the use of spermatozoa derived from high genetic merit boars. Therefore, the swine industry routinely employs freshly diluted-semen whose short lifespan significantly reduces its long-term application, despite the use of semen extenders. Consequently, the

identification and characterization of specific factors present in the seminal plasma of swine semen, such as relaxin are of great importance (Kohsaka *et al.,* 2001).

Relaxin is a small polypeptide (~6 Kda) discovered almost a century ago and is well known as a hormone of pregnancy in many mammals (Hisaw, 1926; Sherwood, 2004). However, relaxin is detected in various reproductive and non-reproductive tissues of both females and males. Relaxin family consists of several members having pleiotropic roles in a variety of tissues and cells, including spermatozoa (Sherwood, 2004). Numerous *in vivo* (Sasaki *et al.,* 2001) and *in vitro* (Juang *et al.,* 1989) studies have highlighted the beneficial role of relaxin on motility of boar spermatozoa. Nonetheless, it is not known whether relaxin can extend the lifetime of spermatozoa motility during storage, while maintaining acceptable fertility rates.

A preliminary study was conducted to determine the optimal boar sperm concentration that would provide an adequate number of spermatozoa/sample (between 200 to 400 cells) to be analyzed by CASA. Consequently, results from this preliminary study were employed in the study involving the evaluation of porcine relaxin hormone on full motility characteristics of boar spermatozoa using CASA. Second, to assess relaxin mode of action, a study was conducted to establish if relaxin receptors (RXFP1 and RXFP2) were present on boar sperm membrane employing an immunofluorescent assay. A final study measured the intracellular cAMP content in boar spermatozoa that have been treated with relaxin and stored for up to 5 days at room temperature (18-21°C).

CHAPTER II

REVIEW OF PERTINENT LITERATURE

2.1 Spermatogenesis

The process of producing male gametes (spermatozoa) is called spermatogenesis. The main goal of it is to supply males with a continuous source of gametes based on a stem cell renovation (up to several years). Spermatogenesis is a complex and highly coordinated process that takes place within the seminiferous tubules, and is comprised by the combination of several cellular transformations in producing germ cells in the seminiferous epithelium. In pigs as in mammals, this process is divided into three main phases. The first one denominated as mitotic (spermatogonial or proliferative) phase; the second one as the meiotic one, and the third one as the differentiation (spermiogenic) phase (Russell *et al.,* 1993). In pigs, during the first phase (proliferative phase), several mitotic divisions take place, that at the end will generate a bigger number of B spermatogonia. Four types of undifferentiated A spermatogonia are present [A single (As), A paired (Apr), A aligned (Aal)], four types of differentiated A spermatogonia (A1, A2, A3, and A4), intermidiate (In) and finally B spermatogonia; at this point spermatogonia are diploid cells (Frankenhuis *et al.,* 1982). The second phase (meiotic) consists of a couple of meiotic divisions that gives rise to primary and secondary spermatocytes. As soon as B spermatogonia (from the first phase) reach the stage of primary spermatocytes they will have to move from interphase (immediately after they divide) to enter the first meiotic prophase which is composed of five stages: preleptotene,

leptotene, zygotene, pachitene and diplotene. Progression of DNA synthesis and replication is guaranteed by all the prophase stages. Later on, at the end of the second meiotic division, haploid spermatids have been generated (Senger, 2003). Knowing that the goal of a spermatozoon is to deliver the genetic material of males to an oocyte during the process of fertilization, the third phase is in charge of making sperm cells that are capable of achieve their main objective. During this phase, spermatids undergo a series of changes, including the formation of the acrosome, condensation of the nucleus and acquisition of motility. In making a spermatozoon motile, it is necessary to form a flagellum and an energy supply that is achieved by the mitochondrial helix. During the reshaping of sperm cells there is a significant decrease in cell size as well, that ultimately gives rise to a sophisticated, self-propelled spermatozoon carrying a package of DNA and enzyme (Eddy and O'Brien, 1994). All the changes observed during this third phase are known as germ cell differentiation. Mature spermatozoa are released into the lumen of seminiferous tubules, where spermatozoa start their transit through the different genital tract structures until ejaculation occurs. Throughout this transit, sperm cells are exposed to different seminal plasma molecules having specific effects on the motility of spermatozoa. Relaxin is one of these molecules that have been found to affect sperm motility; however, relaxin actions on sperm motility are not fully characterized.

2.2 Relaxin

Relaxin is a small polypeptide hormone (~6 Kda) that belongs to the structurally related insulin-like hormones superfamily, which includes, insulin, insulin-like growth factor-I (IGF-I), IGF-II, the Leydig cell insulin-like peptide (INSL3), which is also referred to as relaxin-like factor (RLF), novel insulin-like peptides (INSL4, INSL5,

INSL6), and relaxin-3 (R3)/INSL7 (Bell *et al.,* 1980, 1984; Rinderknecht and Humbel 1978; Hudson *et al.,* 1983, 1984; Adham *et al.,* 1993; Koman *et al.,* 1996; Conklin *et al.,* 1999; Lok *et al.,* 2000; Bathgate *et al.,* 2002). The total sequence similarity between relaxin and insulin is only about 25%, whereas the structural conformation of relaxin is identical to that of insulin (Bathgate *et al.,* 2006). The hormone was first identified by Hisaw in 1926, when he realized that injecting virgin guinea pigs shortly after estrous with serum from pregnant guinea pigs or rabbits promoted a relaxation of the pubic ligament similar to that naturally occurring in pregnancy (Hisaw, 1926); however, the hormone structure was not established until the 1970s (Schwabe and Harmon, 1978). The source of relaxin is variable among species, and it is produced in the reproductive tract of several mammals during pregnancy. While circulating relaxin is produced in the uterus of guinea pigs and in the placenta of hamsters and rabbits. In pigs, mice and rats it is produced in the corpus luteum (Sherwood, 1994).

Relaxin was initially named as a hormone of pregnancy in many mammals, due to its pleiotropic actions on reproductive tissues during pregnancy such as inhibition of uterine contractility, relaxation of the pelvic ligaments, development and growth of the mammary gland, softening and enlargement of the cervix, vasodilation of the circulatory vessels, and regulation of cardiovascular function (Sherwood, 1994). However, it has been shown that the actions of relaxin go beyond its effects on the reproductive system. Relaxin exerts several other functions in non-reproductive tissues such as kidney, lungs and heart (Siebold, 1997). For instance, relaxin and its receptors are involved in allergic responses (Bani *et al.,* 1997), protection of the cardiovascular system, wound healing and fibrosis (Halls *et al.,* 2007; Sherwood, 2004) among others. Interestingly, relaxin is also present in males. Loumaye *et al.* (1980) identified a relaxin-like substance in seminal

plasma of humans; Steinetz *et al*. (1959) recognized its bioactivity in the testes of roosters, while Dubois and Dacheux, (1978) described relaxin in testes of boars. Kohsaka *et al*. (2003) measured the content of immunoreactive relaxin in seminal plasma in both bulls and boars; it was found to be 41.92 ng /mL and 2.35 ng /mL, respectively. According to Kohsaka *et al*. (1992) and Lessing *et al.* (1986), the male accessory glands appeared to be the source of relaxin, which is then secreted into the seminal plasma (Kohsaka *et al*., 1988; Lessing *et al*., 1986; Juang *et al*., 1990; Sasaki *et al*., 2001), and finally joining sperm cells after ejaculation (Kohsaka *et al*., 2001; Sasaki *et al*., 2001). Nonetheless, some researchers have reported the prostate gland as the source of relaxin (Yki-jarvinen *et al.,* 1983; Hansell *et al.,* 1991). Some research supports this hypothesis, knowing that relaxin has been found in semen of men after surgical vasectomy, which eliminates testicular and epididymal components from the ejaculate. In the same way, the peptide hormone was found in the ejaculate of a pair of men with congenital absence of the seminal vesicles and the vas deferens (so the ejaculate contains only components from the prostate gland and distal structures). It was found that the content of relaxin was still higher than in most samples from normal patients (Loumaye *et al.,* 1980; Essig *et al.,* 1982b). De Cooman *et al.* (1983) also found that the first part of a human split ejaculate possesses higher amounts of relaxin, confirming the previous findings, and giving support to the idea of an extra-testes source of relaxin.

2.2.1 Effects of Relaxin on Sperm Motility

Several *in vivo* (Sasaki *et al.,* 2001) and *in vitro* (Juang *et al.,* 1989) studies have reported the beneficial action of relaxin on boar sperm motility. Similar effects of relaxin have been noticed in human sperm (Colon *et al*., 1986; Lessing *et al*., 1986). Even more,

other researchers have reported that the action of relaxin is not just limited to the enhancement of sperm motility, but also promoted acrosome reaction in cryopreserved and fresh swine spermatozoa (Miah *et al.,* 2006). On the other hand, boar sperm motility is inhibited by incubation with antirelaxin antiserum (Juang *et al*., 1987). The same author also demonstrated that relaxin can preserve porcine sperm motility. According to Juang *et al*. (1990) and Sasaki *et al*. (2001), immunoreactive relaxin in boar seminal plasma positively correlates with sperm motility, although in human semen samples such correlations were not found (Schieferstein *et al*., 1989; Brenner *et al*., 1987), suggesting that the biological action of immunoreactive relaxin in humans may be different. Conversely, in an *in vitro* study, the addition of anti-porcine relaxin antiserum, but not normal rabbit serum, promotes a rapid decrease in the motility of washed human sperm, but this effect is eliminated by preincubating the antiserum with excess of relaxin (Sarosi *et al.,* 1983). Lessing *et al.* (1986) evaluated effects of relaxin when added to whole semen and washed spermatozoa from normal samples, normal samples aged for 5 h, and initial low motility samples and evaluated the percentage of motility and forward progressively motile sperm. Relaxin was observed to have no effect on the motility of whole unwashed normal semen. The sperm-washing practice decreased motility of normal samples, but supplementation with the porcine relaxin (10 to 100 ng/mL) increased motility. Aging whole semen samples for 5 h at 37º C decreased motility, but adding relaxin at a physiological range improved motility. When the washing procedure was applied to aged spermatozoa motility was further reduced; however, relaxin did not have a significant effect. When relaxin was added in the physiological range to samples of initial low motility there was a positive improvement. Nevertheless, the washing practice further reduced the motility of sample with initial low motility, but relaxin did

not have any effect, suggesting that there is a certain damage level of sperm in terms of motility from which recovery is no longer possible. Knowing that aging or storage reduce motility of normal sperm, it led Lessing *et al.* (1986) to hypothesize that immunoreactive relaxin in seminal plasma loses biological action with storage. To prove this previous hypothesis, normal sperm samples were washed, stored for 5 h at 37º C and finally treated with their original seminal plasma, original seminal plasma with added relaxin, or fresh seminal plasma alone. As a result, the fresh seminal plasma or the original seminal plasma plus added relaxin significantly increased sperm motility, while the original seminal plasma did not show any significant effect, therefore supporting the hypothesis. Some other studies have reported the beneficial effect of relaxin on aged (5 h at 37º C) human sperm (Colon *et al.,* 1986). In the same way, Park *et al*. (1988) also confirmed such effects on aged human sperm using porcine relaxin. Chan and Tang (1984) used washed normal human sperm stored with relaxin and did not find any effect on sperm motility. In contrast to these previous findings, relaxin in boar seminal plasma has a positive correlation with motility of sperm (Juang *et al.,* 1989).

Thus, it has been proposed that the physiological actions of relaxin on fertility and sperm motility are driven by specific cell-surface receptors for relaxin on spermatozoa (Kohsaka *et. al.,* 2001)

2.2.2 Relaxin receptors - Structure

Although relaxin hormone was discovered almost 85 years ago, the identification of its receptors was not until the beginning of this decade (Bathgate *et al.,* 2006). Four receptors have been identified that are the physiological targets for the relaxin family peptides: LGR7, LGR8, GPCR135 (SALPR or somatostatin and angiotensin-like peptide

receptor) and GPCR142 (GPR100). All four receptors are class I (rhodopsin like) G-protein-coupled relaxin family peptide receptors (GPCRs). Recently, the International Union of Pharmacology LVII (IUPHAR) has renamed the relaxin family receptors as follows: LGR7 as RXFP1; LGR8 as RXFP2, GPCR135 as RXFP3; and GPCR142 as RXFP4 (Bathgate *et al.,* 2006).

2.2.3 RXFP1 and RXFP2

Due to the advances in genome sequencing, the identification of novel genes has been possible through their sequence similarities with respect to known genes in the hormonal signaling pathway (Hsu *et al.,* 2000). The determination of LGRs (leucine-rich repeat-containing G-protein-coupled receptors), a group of GPCRs was due to the research developed in paralogs of the known gonadotropin and thyrotropin. Research carried out in different species on LGRs, suggest that three different forms of LGRs named (A, B, and C) emerged during early metazoans evolution (Hsu, 2003) (Table 2.1). LGRs are mosaic proteins similar in structure to the glycoprotein hormone receptors (Hsu *et al.,* 2000); containing an ectodomain or extracellular domain with several leucine-rich repeats (LRRs), that are considered to play an important role in ligand binding, due to their seven-transmembrane spanning GPCRs. Similarly, each subtype of LGRs shares a singular hinge region, located between the transmembrane region and the LRR (Hsu, 2003). RXFP1 (LGR7) consists of 757 amino acids (Hsu, 2002), while RXFP2 (LGR8) is made of 737 amino acids in length, which contain 10 leucine-rich repeats in their large N-terminal extracellular domain, and they both share an amino acid sequence homology of about 60% (Overbeek *et al.,* 2001).

RXFP1 and RXFP2 belong to the type C LGRs (Hsu *et al.,* 2005) (Table 2.1). Type C LGRs are characterized by a low-density lipoprotein class A (LDLa) module at the N-terminus, which is followed by an alternatively spliced flanking region, and ultimately the LRRs. At the end of each LRRs, there is a structure that has been denominated as "caps" (cysteine-rich regions). In several proteins, these caps have been shown to be an essential part of the LRR (Kobe and Kajava, 2001). The signaling pathway of type C LGR is considered to be one of the earliest pathways of GPCR signaling. The seven-transmembrane-spanning GPCR region is the structure located in the middle of the C-terminal tail and the ectodomain (Hsu *et al.,* 2002).

Table 2.1 Multiple GPCR signaling pathways with coupling of relaxin family peptides. Re-drawn from Hsu *et al.* (2005)

	RECEPTORS	LIGANDS
Type A	FSHR	FSH
	LHR	LH
	TSHR	TSH
Type B	LGR 4	-----
	LGR 5	-----
	LGR 6	-----
Type C	RXFP 1	Relaxin 1, 2, INSL 7
	RXFP 2	Relaxin 1, 2, INSL 3
N-C	RXFP 3	INSL 7
	RXFP 4	INSL 5; INSL 7

(----) Orphan receptors; (*N-C*) Non-Classified receptors.

Conservation of these two receptors (RXFP1 and RXFP2) is high across species. For instance, between humans and rodents, the sequence similarity observed was more than 90% (Hsu *et al*., 1998; 2002; 2003). According to Hsu (2003), both receptors contain N-linked glycosylation sites and promote cAMP accumulation through activation of adenylate cyclase, as well as stimulate PKA signaling mechanism.

2.2.4 RXFP3 and RXFP4

Much less is known about these two receptors in comparison to RXFP1 and RXFP2. The RXFP3, which is also known as somatostatin and angiotensin-like peptide receptor and RXFP4 are also G-protein-coupled receptors (Bathgate *et al.,* 2006) and have been proposed to be the putative receptors for relaxin-3 (Liu *et al.* 2003a, b). However, later experiments have demonstrated that RXFP4 is perhaps the receptor for INSL5 (Liu *et al.* 2005). In contrast to RXFP1 and RXFP2, the two following receptors RXFP3 and RXFP4 belong to the type I family of GPCRs, which possess a short N-terminal extracellular domain. Apparently, these receptors interact exclusively with the inhibitory subunit G protein α ($G_{\alpha i}$) to inhibit cAMP, while RXFP1 and RXFP2 act through the stimulatory G protein α ($G_{\alpha s}$) to increase cAMP through activation of adenylate cyclase (Liu *et al.* 2003a, b). RXFP3 has 469 amino acid residues, while RXFP4 possesses 374 (Bathgate *et al.,* 2006). These two receptors (RXFP3 and RXFP4) share an amino acid sequence identity of about 43% (Liu *et al.* 2003a).

2.3 Analysis of sperm motility: Use of the Computer Assisted Sperm-Analysis (CASA) approach

Sperm motility is considered as one of the most important parameters for determining the fertility capacity of an individual male. However, the use of microscopic equipment to assess such characteristic is not well correlated with either *in vitro* or *in vivo* fertilization rates (Zaini *et al.* 1985; Liu *et al.,* 1988a). This limited correlation is not only due to human errors while employing a subjective method for assessment of sperm movement, where high coefficient of variations have been reported between technicians (44%), but also within technicians (33%) (Dunphy *et al.* 1989), which is confounded by the equipment limitations, used for determination of detailed sperm movement patterns.

Improvement in this field had been impeded by the dearth of an objective system for motility assessment (Mack *et al*., 1988). Sperm motility was usually determined employing semi-objective or highly subjective techniques that establish motility on a routine basis. On the other hand, several studies have reported that technical variability can be diminished when employing an objective method (Katz and Overstreet, 1981), besides several sperm movement characteristics can be determined as well (individual cell kinematics), that could be potentially of great importance (Mack *et al*., 1988).

Thus, new systems such as the Computer Assisted Sperm-Analysis (CASA) have been developed seeking for solution of the previous inconveniences just mentioned. The CASA equipments were initially available during the mid-1980s (Aitken *et al.,* 1985; Katz *et al.,* 1986; Jeulin *et al.,* 1986). Nevertheless, the consensus about the role of CASA is divided. Although there has been an optimistic and determined action to establish the role of CASA in both the research and clinical andrology laboratory (Oehninger *et al*., 2000), others have said that because of the higher number of sperm motility parameters assessed by CASA, it does imply more accuracy in the prediction of fertility (Hirano *et al.,* 2001).

Several CASA systems have been developed, and the technique employed for the tracking and identification of sperm cells varies among them. For instance, the Hamilton Thorne CASA instrument identifies sperm cells through the location of the brightest region of every bright object (Yeung *et al.,* 1992); while some other systems (e.g. CellSoft) determine the head of the sperm cells as a group of pixels, and then estimate the center of this series of pixels, that is considered as the centroid (Berns and Berns, 1982). The CellTrak system (Motion Analysis) determines the centroid of the sperm head as well based on the pixels which delineate the edge of an object (Boyers *et al*., 1989).

Besides the different methods employed by each CASA system to track and identify sperm cells, the sperm motility parameters given by the systems can vary as well. For instance, there are some parameters that are not universally acquired and obviously not reported by all CASA equipments. However, there are some other sperm movement parameters that are determined by all CASA apparatus (Mortimer, 1997), as follow (see also Figure 2.1):

2.3.1 General sperm motility parameters

1. Total number of sperm cells.
2. Percentage of motile spermatozoa (%).
3. Percentage of progressive spermatozoa (%).
4. Number of rapid spermatozoa (≥ 45 μm/s) (speed's threshold determined by researchers).

2.3.2 Velocities

1. VCL (Curvilinear velocity, μm/s) = is the velocity of progression following the entire sperm trajectory (Figure 2.1).
2. VAP (Average path velocity, μm/s) = is determined from an average path smoothed trajectory by five-point, running average of the centers of brightness (Figure 2.1).
3. VSL (Straight line velocity, μm/s) = is determined from the distance among the first and last track points (Figure 2.1).

2.3.3 Progression ratios

$$\text{LIN (Linearity, \%)} = \text{VSL/VCL} \times 100. \quad (2.1)$$

$$\text{STR (Straightness, \%)} = \text{VSL/VAP} \times 100. \quad (2.2)$$

2.3.4 Head movements

1. BCF (Beat cross frequency, Hz) = frequency that the sperm head crosses the smoothed trajectory.
2. ALH (Amplitude of lateral head displacement, μm) = mean lateral sperm head displacement throughout the smoothed trajectory (Figure 2.1).

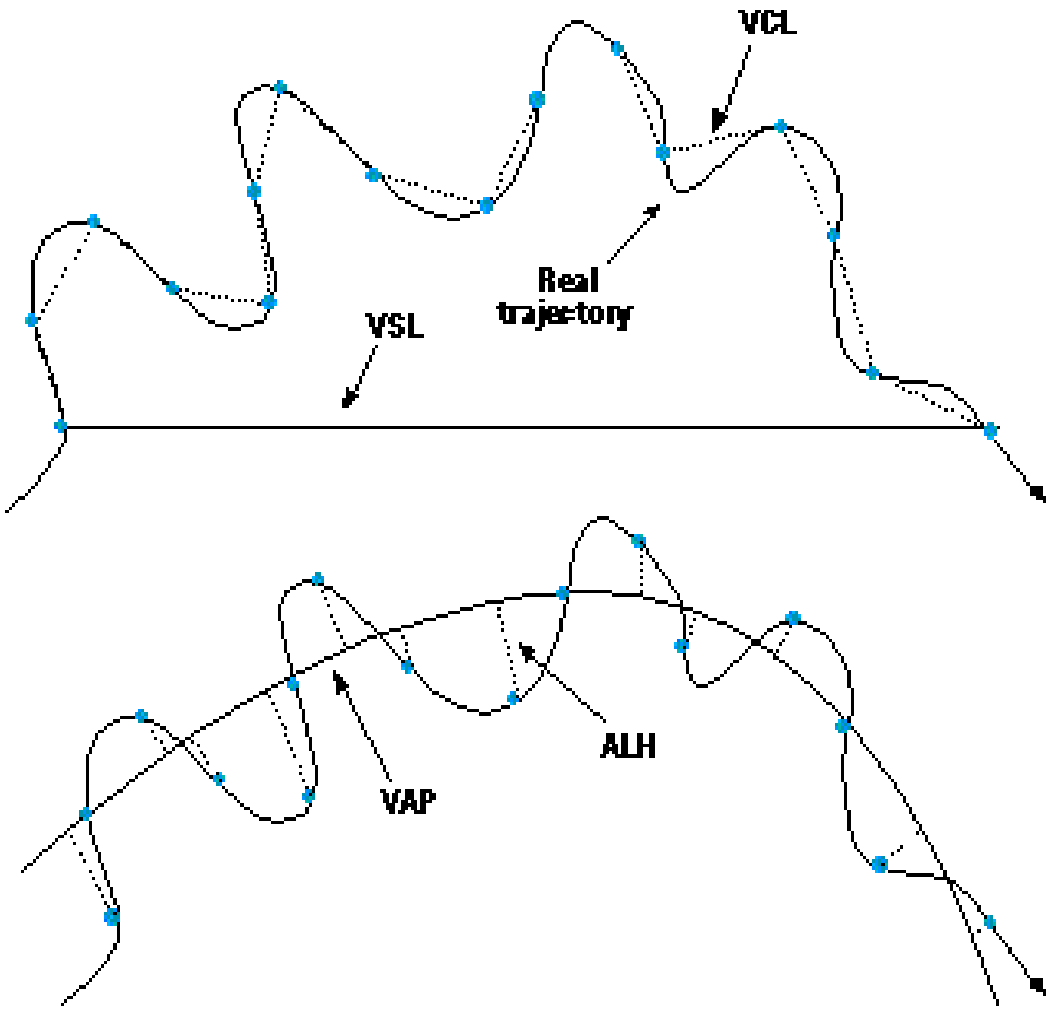

Figure 2.1 Different track speed and movement of spermatozoa measured by the Hamilton-Thorn Motility Analyzer. Velocities (μm/s): Curvilinear velocity

(VCL); average path velocity (VAP); straight line velocity (VSL). Progression ratios (%): linearity (LIN = VSL/VCL x 100); straightness (STR = VSL/VAP x 100). Head movements (μm): amplitude of lateral head displacement (ALH). Re-drawn from Liu *et al.* (1991).

2.4 Relaxin hormone and the second messenger cyclic adenosine 3', 5'-monophosphate in sperm motility

Some of the physiological answers to several biologically relevant or important compounds are carried out through "second messengers". This term was introduced and described by Sutherland *et al.* (1968) for the type of molecules able to exert intracellularly, the biological response of substances or compounds that are not allowed to enter the target cells by them. Cyclic adenosine 3', 5'-monophosphate (cAMP or cyclic AMP) is considered as one of the most important 'second messengers' involved as a mediator of several physiological processes, and signaling cascades through the action of several neurotransmitter and hormone receptor complexes. The binding of neurotransmitters and hormones occur throughout specific receptors that are coupled with G-proteins. Therefore, the already activated G-proteins regulate the action of the complex membrane-bound adenylyl cyclase that promotes the conversion of cytoplasmic ATP to cAMP (Beavo and Brunton, 2002).

In the same way, the actions of relaxin hormone after stimulation of RXFP1 receptor involve accumulation of cAMP (Halls *et al.,* 2006). Although relaxin was discovered almost a century ago, the identification of its cognate receptor RXFP1 was not possible until the past 10 years. Since then, research has increased its understanding of the different paths on how relaxin and its receptors are acting. In some cell culture-based bioassay systems the actions of the peptide hormone-receptor compound have been determined. This is the case of the human monocyte cell line THP-1 and the primary human endometrial stromal cells from the cycle (ESC), where a rapid increase in cAMP

accumulation was evidence after activation with relaxin (Bartsch *et al.,* 2001). The same cAMP increase was observed when using the human embryonic kidney (HEK) 293T cells (Halls *et al.,* 2006). On the other hand, as far as we know, there is not any study showing any cAMP increase and/or modification in sperm cells after treatment with relaxin. However, Miah *et al*. (2006) have hypothesized that, although the mechanism of relaxin actions for inducing acrosome reaction and increasing sperm motility are not well understood, it might be thought that relaxin is acting by increasing the intracellular cAMP levels. These increased levels in cAMP might be responsible for inducing acrosome reaction and enhancing sperm motility.

CHAPTER III

PRELIMINARY STUDY

3.1 Abstract

Three boar sperm concentrations (50, 75 and 100x10^6 boar sperm cells) were used in a preliminary study to establish the optimal sperm concentration that would provide a number of spermatozoa between 200 to 400 per sample for analysis with CASA. Motile spermatozoa were selected through a discontinuous percoll. Then, 3µL of Boviextend-suspended motile spermatozoa were loaded in caffeine-free Leja chamber slides, and then placed into CASA. Data were analyzed using the GLM procedure in SAS. Then, Tukey test was used to establish significant differences between means ($P < 0.05$). The 75 x 10^6 boar sperm concentration provided the required amount of cells for CASA analysis. We also obtained the percentage of motile and progressive spermatozoa, as well as VSL and VCL (µm/s). Having determined the optimal concentration, all settings (for equipment) and conditions (for sperm cells) were ready to be used for the study of relaxin effects on sperm motility.

Key words: boar semen, CASA system, kinematic parameters, sperm concentration

3.2 Introduction

Analysis of spermatozoa is commonly carried out employing subjective methods in which a trained technician judges the sample under his/her own criteria using a microscope under which sperm cells are placed for analysis. When employing these

methods, sperm motility (%) can be determined based on the speed and straightness of sperm on a routine basis, as well as a motility index estimated with reasonable accuracy among technicians (Liu *et al.,* 1988a). Nevertheless, according to the World Health Organization (1987), the categories can not be identified with exactitude, as well as this type of analysis does not correlate well with either *in vitro* or *in vivo* fertilization (Zaini *et al.,* 1985; Liu *et al.,* 1988a).

On the other hand, several objective methods have been developed, such as, semi-automatic image analysis (Holt *et al.,* 1985), time-exposure photomicrography (Aiken *et al.,* 1982), cinemicrography (David *et al.,* 1981), and videomicrography (Katz and Overstreet, 1980). Even though, the used of these objective systems have not been widely accepted in the clinical field (Liu *et al.,* 1991), it has been proven that they are able to provide objective measurements of sperm behavior in terms of motility, plus detailed movement characteristics of semen that are not provided by subjective methods. Several computer-assisted sperm motility analysis (CASA) systems have been developed, and ample variety of sperm movement patterns can now be determined. Nonetheless, there are some difficulties associated with the use of CASA, such as the lack of agreement with regards to accuracy, precision and measurements provided by the system, which are generally associated with the semen sample (specie, volume, etc.). High or low number of sperm cells during each trial can contribute to misleading results. Thus, the aim of this preliminary study was to determine the best starting sperm concentration that would provide an adequate number of spermatozoa per sample (between 200 to 400 cells) to be analyzed by CASA. These results would serve as a standard for further studies involving the addition of porcine relaxin hormone to boar spermatozoa during storage.

3.3 Materials and Methods

3.3.1 Boar sperm cells preparation

Four batches of pooled boar semen diluted in Beltsville Thawing Solution (BTS) were employed. Semen was obtained at Prestage Farm (West Point, MS) and kept at room temperature (18-21°C) during experimentation. Separation of sperm cells from BTS was carried out by centrifugation at 1000 rpm (253 g) for 5 min at room temperature. Then, selection of motile sperm cells was achieved by using a percoll gradient after a 30 min centrifugation at 600 g. Spermatozoa were resuspended in Boviwash medium and recentrifuged at 253 g for 5 min. Finally, washed motile sperm were brought to 1 mL of pre-warmed Boviextend medium and counted on a Hemacytometer (Improved Neubaver, Buffalo, NY) chamber. Three different sperm concentration were used as follow: 50 x 10^6; 75 x 10^6 and 100 x 10^6 boar sperm cells/mL. Analysis were performed during three consecutive days (1[day of boar sperm collection], 2, and 3), and BTS-diluted sperm cells were kept at room temperature. Motile spermatozoa were freshly prepared each day.

3.3.2 Analysis of total boar spermatozoa cells and motility parameters

The number of sperm cells and motility was objectively analyzed using CASA (HTM-IVOS Hamilton-Thorne Biosciences, Version 12.3. Beverly, MA). Briefly, analyses of 3µl of Boviextend-suspended motile spermatozoa were loaded in caffeine-free Leja counting chamber slides (Standard Count 4 Chamber Slide Leja®, 20 micron, Nieuw Vennep, The Netherlands) in triplicates, and placed into the CASA system set at 37ºC. Three separate fields were analyzed for a minimum of 200 spermatozoa per sample. Experiments were repeated four times with different pools of semen (biological replicates).

3.3.3 Statistical Analysis

Data were analyzed using the GLM procedure. Then, Tukey's test was used to established significant differences between means, and $P \leq 0.05$ was fixed as threshold of significance (SAS® 9.2, Copyright (c) 2002-2008 by SAS Institute Inc., Cary, NC).

3.4 Results

With a CASA set up of three fields (number of sperm cells counted at three different spots in the slide chamber), the two highest sperm concentrations (75 and 100 x 10^6 /mL) provided with the number of sperm cells in between the expected range (200-400) (Figure 3.1). However, when using 100 x 10^6 sperm cells/mL, the number of spermatozoa obtained was relatively close to the maximum limit range (400 spermatozoa), especially on days 1 and 3 of analysis (Figure 3.1). While using 50 x 10^6 sperm cells/mL, only on day 3 of experimentation the number of spermatozoa obtained were in between the range, while during the first two days those values were below 200 sperm cells/sample (Figure 3.1).

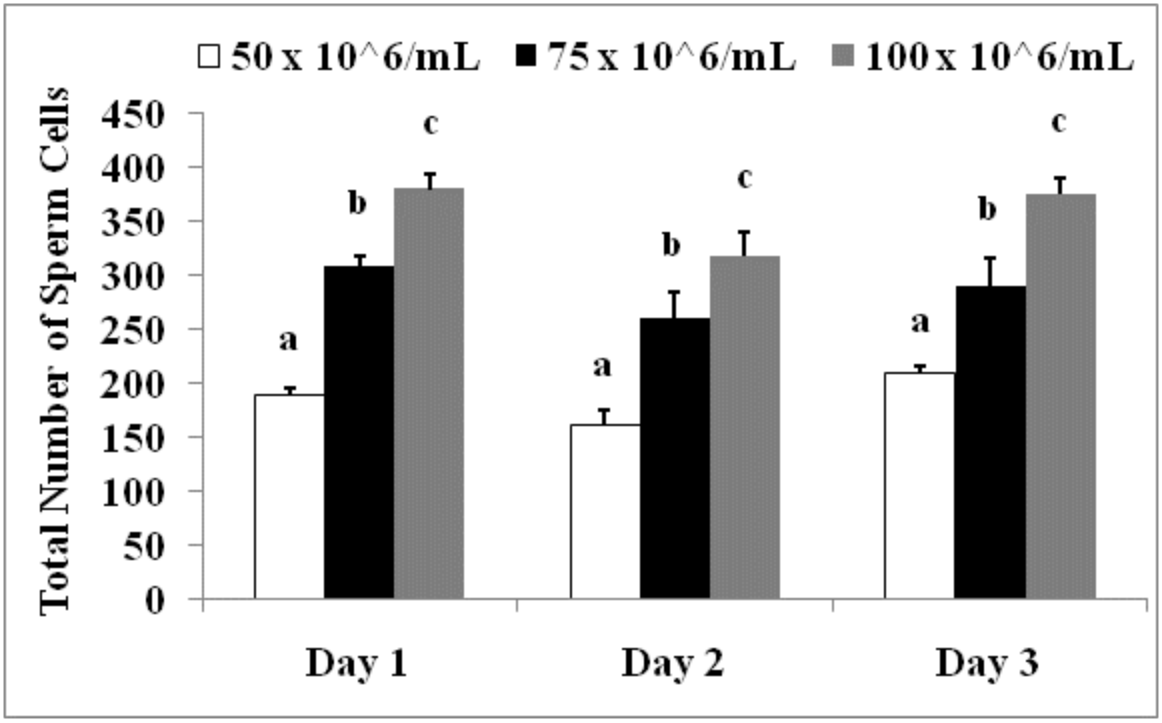

Figure 3.1 Total number of sperm cells obtained by CASA when using three different sperm concentrations (50, 75 and 100 x 10^6 boar sperm cells/mL) during three consecutive days (1, 2, and 3) of storage. Data are shown as mean values ± SEM. Significant differences ($P < 0.05$) within the same day are indicated by different letters ($^{a, b, c}$).

We also measured other kinematic parameters such as motility, progressiveness of sperm, VSL and VCL.

The mean percentage of motile spermatozoa (%, obtained from the total number of sperm cells) are shown in figure 3.2. As expected, the higher the sperm concentration, the higher the percentage of motile spermatozoa. However, on day three of analysis the percentage of motile spermatozoa obtained when using a concentration of 75 x 10^6 sperm cells/mL was lower compared to the one obtained using 50 x 10^6 sperm cells/mL, although there was not significant difference between sperm concentration. Conversely, on days 1 and 2 of analysis there was a significant difference between the lowest sperm concentration (50 x 10^6/mL) and the highest relaxin dose (100 x 10^6 /mL).

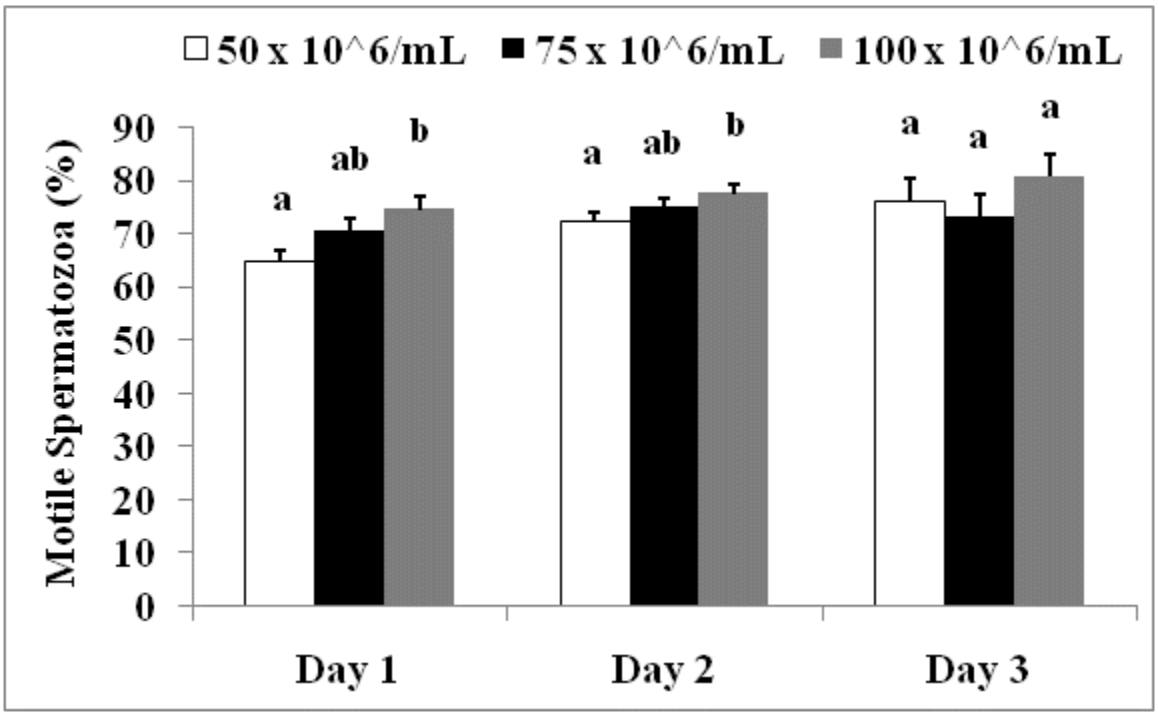

Figure 3.2 Motile spermatozoa obtained by CASA when using three different sperm concentrations (50, 75 and 100 x 10^6 boar sperm cells/mL) during three consecutive days (1, 2, and 3) of storage. Data are shown as mean percentage (%) ± SEM from the total number of sperm cells. Significant differences ($P < 0.05$) within the same day are indicated by different letters ($^{a, b, c}$).

With regards to the percentage of progressive spermatozoa, it was observed that the higher the sperm concentration, the higher the percentage of progressive spermatozoa. There were no significant differences between sperm concentrations in none of the three different days of analysis (Figure 3.3)

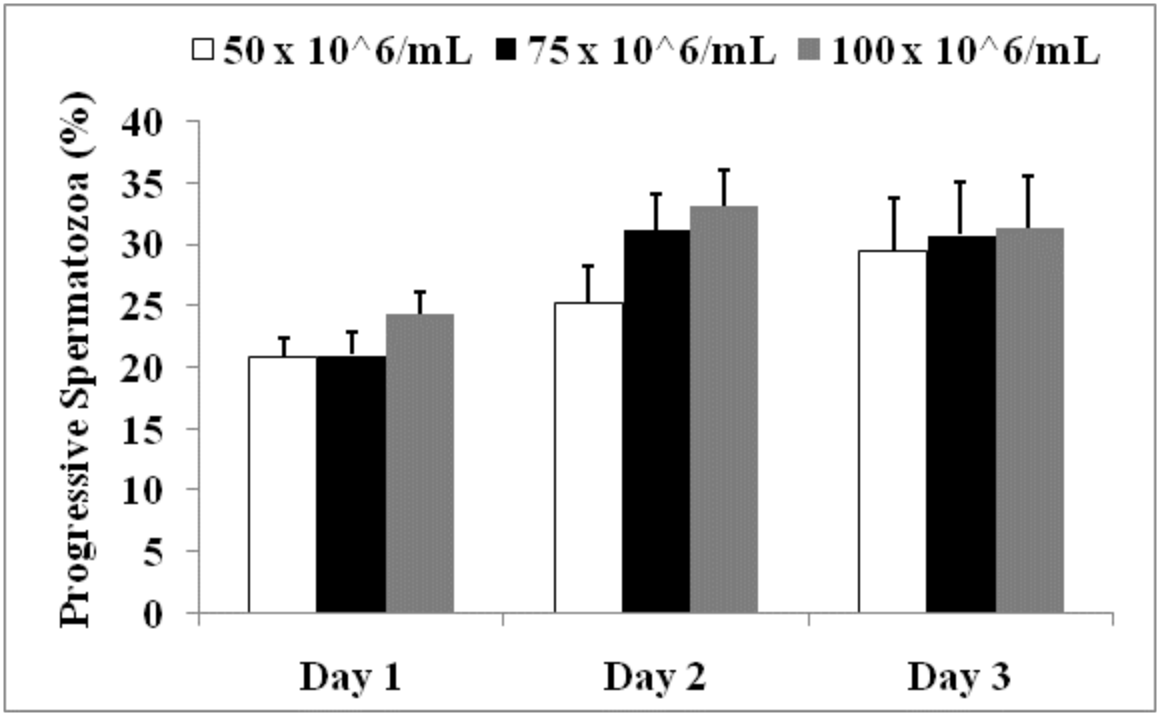

Figure 3.3 Progressive spermatozoa obtained by CASA when using three different sperm concentrations (50, 75 and 100 x 10^6 boar sperm cells/mL) during three consecutive days (1, 2, and 3) of storage. Data are shown as mean percentage (%) ± SEM from the total number of sperm cells. There were not significant differences ($P < 0.05$) within the same day between sperm concentrations.

The straight line velocity (VSL, µm/s) (Figure 3.4) of spermatozoa was similar between all three sperm concentrations on days 1 and 3, but VSL was significantly ($P < 0.05$) lower when using the lowest sperm concentration (50 x 10^6) on day 2.

With regard to the curvilinear velocity (VCL, µm/s) (Figure 3.5) of spermatozoa, there were no significant differences ($P < 0.05$) across days of experimentations among sperm concentrations.

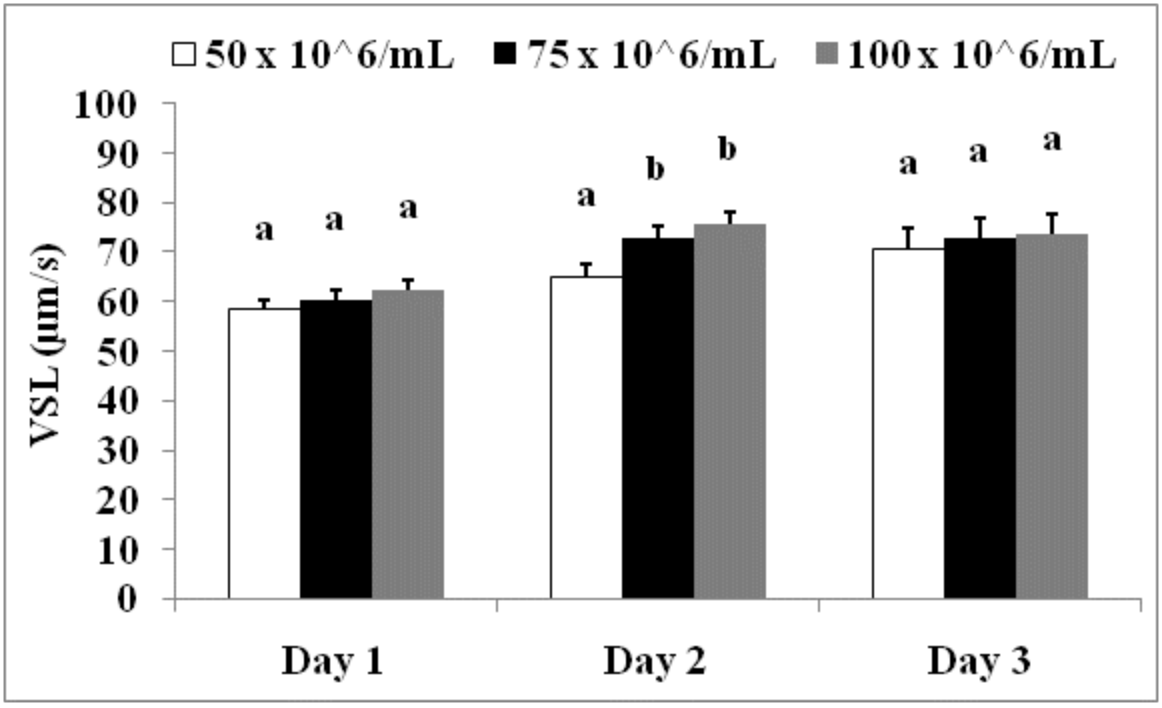

Figure 3.4 Straight line velocity (VSL, µm/s) of spermatozoa obtained by CASA when using three different sperm concentrations (50, 75 and 100 x 10^6 boar sperm cells/mL) during three consecutive days (1, 2, and 3) of storage. Data are shown as mean values ± SEM. Significant differences ($P < 0.05$) within the same day are indicated by different letters ($^{a, b, c}$)

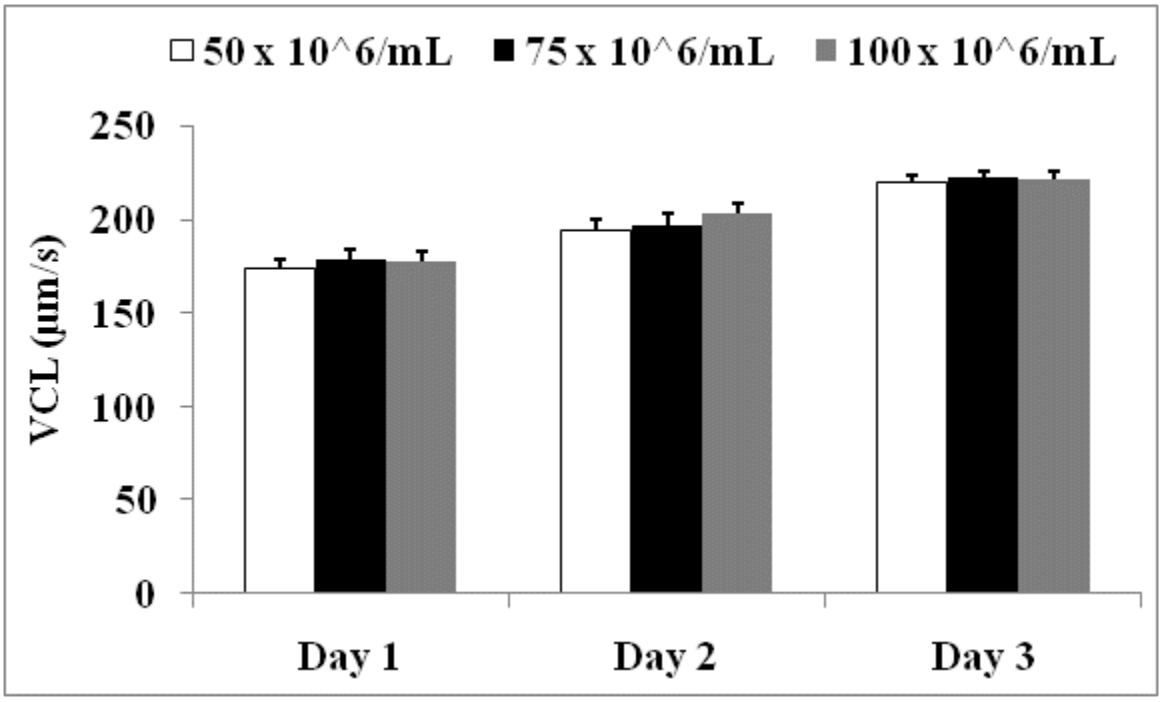

Figure 3.5 Curvilinear velocity (VCL, µm/s) of spermatozoa obtained by CASA when using three different sperm concentrations (50, 75 and 100 x 10^6 boar sperm cells) during three consecutive days (1, 2, and 3) of storage. Data are shown as mean values ± SEM ($P < 0.05$). There were not significant differences within the same day between sperm concentrations.

3.5 Discussion

Following the completion of preliminary studies evaluating three different sperm concentrations, it was determined that the most optimal boar sperm starting concentration was 75 x 10^6 sperm/mL. This sperm concentration provided the required number of sperm cells (between 200 and 400) to be analyzed per trial while employing CASA. Although, results obtained using the highest sperm concentrations (100 x 10^6) also fell down in between the established sperm cell range (between 200 and 400), this results were slightly below the maximum range limit (400 sperm cells), especially on days 1 and 3 of analysis. For that reason, the risk of having a sample with more than 400

spermatozoa while using a concentration 100 x 10^6 sperm cells/mL was high. So, when analyzing a high number of sperm cells (above 400) with CASA, it might conduct to misleading results. According to Davis and Katz (1993), the minimum number of motile sperm cells to be analyzed should be at least 200 to obtain accurate estimates of population means, and at least 300 motile spermatozoa should be analyzed to acquire precise estimates of population distribution. For that reason, the 75 x 10^6 sperm/mL concentration was employed in the following study involving the addition of porcine relaxin hormone.

With resgard to the percentage of motile spermatozoa, it is one of the most common parameters obtained by both subjective and/or objective methods. It has been used as a parameter to evaluate quality of semen and fertility of males. In fact, motility of sperm is considered as one of the main factors that correlates with fertility (Bostofte et al., 1990). In addition, some studies have reported that motility of spermatozoa acquired by CASA could be predictive of fertility (Aitken *et al.,* 1985; Katz *et al.,* 1986; Jeulin *et al.,* 1986; Liu *et al.,* 1991). However, the information regarding the role of sperm motility during fertilization is contradictory. Conversely, it has been shown that there is not a high correlations between the fertilizing capacity of spermatozoa and its motility. One reason by which sperm motiltiy estimates does not correlate well with fertilization is that motility is not the only factor that spermatozoa must possess in order to fertilize an oocyte (Graham, 2001). Motility is just one factor among the many that might be influencing the fertilization process.

Besides of sperm motility, the percentage of progressive spermatozoa was also determined. This is not only the sperm that is presenting any motility, but it is also the sperm that is travelling in a forward progression manner. According to Björndahl (2010),

inflamatory reactions and acute infections in the male reproductive tract can be evidence through sperm cells with a reduced progressive (forward) motility. In the same way, the importance of progressive spermatozoa go beyond this point. For instance, in either *in vivo* (Aitken *et al.,* 1985) or *in vitro* (Hirano *et al.,* 2001) studies, it has been evidenced that spermatozoa presenting a progressive (forward) pattern of travel is required for successful results, and has been considered as a useful indicator with *in vivo* fertilization.

One of the advantages of using CASA, besides of being an objective system, is that it can break down motility in different parameters. For instance, it can track the the different sperm movements and velocities in an ejaculate. This preliminary study also shows information about different velocities such as VSL and VCL (μm/s). Knowing that VSL, VCL, and VAP are parameters that explain spermatozoa velocities in diverse ways, strong correlations have been found among them (Wijchman *et al.,* 2001). VSL is determined from the distance among the first and last track points of a sperm trajectory (Figure 2.1). Some studies have found strong correlations in humans between VSL and fertilization rates *in vitro*, concluding that sperm cells travelling in a straight line manner conjugated with speed (faster sperm) have a higher probability of achieving its goal, fertilization of the female gamete (Liu *et al.,* 1991). With respect to the VCL, defined as the total trajectory travelled by the sperm head, some studies have found this velocity in human samples related to in vitro fertilization (IVF) rates (Holt *et al.,* 1985).

3.6 Conclusions

Our preliminary study indicated that a concentration of 75 x 10^6 boar sperm cells/mL provided the required amount of sperm cells by CASA to obtain accurate results. This sperm concentration (75 x 10^6 boar sperm cells/mL) was employed in the

following experiments involving addition of different porcine relaxin hormone concentrations to boar spermatozoa during storage to evaluate its effects on motility using CASA.

On the other hand, information reported about the different CASA estimates vary greatly. Some studies have found correlations between sperm motility parameters with either *in vitro* or *in vivo* fertility rates, while others have not. In the same way, research has shown that some of these estimates can be considered as good predictors of fertilizing abilities; however, some other research haven not. Although information and results vary greatly, one might think that reasons for that are the different approaches, techniques, experimental designs and obviously study purposes used to obtain such CASA estimates, independently of the CASA equipment employed. Nonetheless, the usefulness of CASA in determining motility sperm parameters is of great value. Accuracy of this system for determining detailed information about sperm movement patterns (kinematics), as well as establishing the paths calculated in different ways and speed of sperm cells is greater compared to subjective methods. Further research is needed in order to determine the functionality and implications of the different sperm movements with regards to the fertilization process.

CHAPTER IV

EFFECTS OF RELAXIN HORMONE ON MOTILITY CHARACTERISTICS OF BOAR SPERMATOZOA DURING STORAGE

4.1 Abstract

The objectives of this study were to (i) determine the effect of relaxin on full motility characteristics of boar spermatozoa during storage, using the Computer-Assisted Sperm Analysis (CASA), (ii) identify the presence of RXFP1 and RXFP2 receptors on boar spermatozoa and (iii) establish if relaxin promotes an intracytoplasmic cAMP increase in boar spermatozoa. Motile spermatozoa were selected through a discontinuous percoll gradient. Semen was incubated for 60 minutes at 37^{o}C with 0, 50, 100 and 500 ng/mL pig relaxin during four different days (1, 2, 3, and 5). Relaxin positively affected boar sperm motility parameters ($P < 0.05$). Relaxin receptors RXFP1 and RXFP2 were found in boar sperm cells when employing an immunofluorescent assay. However, intraspermatic cAMP levels were not affected by relaxin ($P < 0.05$). This study indicates a beneficial action of relaxin on sperm motility; however its mechanism of action requires further research.

Key words: relaxin hormone, semen, cAMP, sperm motility, storage.

4.2 Introduction

Relaxin is a small polypeptide of about 6 Kda discovered almost a century ago and is well known as a hormone of pregnancy in many mammals (Hisaw, 1926; Sherwood, 2004). Renowned by its role in parturition and other aspects of female

reproduction, relaxin is also present in various reproductive and non-reproductive tissues of both females and males (Sherwood, 2004). Relaxin family consists of several members having pleiotropic roles in a variety of tissues and cells, including spermatozoa (Sherwood, 2004).

The prostate gland appears as the major source of relaxin synthesis in males (Ivell *et al.,* 1989), and its secretions are found in seminal plasma (Lessing *et al.,* 1986). Even though, the physiological action of relaxin in spermatozoa is not well understood, *in vivo* studies have shown positive correlations between the immunoreactive relaxin content of boar seminal plasma and sperm motility (Juang *et al.,* 1990), suggesting that relaxin might have a role during sperm motility. This beneficial effect has been confirmed with *in vitro* studies showing that addition of relaxin to boar and human spermatozoa stimulates sperm motility (Juang *et al.,* 1989; Colon *et al.,* 1986), whilst addition of anti-relaxin antiserum restrained this effect (Juang *et al.,* 1989). It has been proposed that relaxin actions are accomplished through specific receptors. In fact, relaxin is the endogenous ligand of the G-protein coupled receptor LGR7 that has been renamed as RXFP1. In some species such as the human, relaxin can bind and activate, but with a lower affinity, the closely associated receptor RXFP2 (previously known as LGR8) for the insulin-like peptide from Leydig cells (INSL3) (Filonzi *et al.,* 2007).

Subjective methods are generally employed for the determination of sperm motility (Liu and Baker, 1988). The Computer-Assisted Sperm Analysis (CASA) systems however, offer the possibility of determining several sperm movement characteristics in an objective manner (Mack *et al.,* 1988). The use of CASA has allowed for an important advancement in the research of sperm biology, especially with regard to sperm hyperactivation (Lamirande and Gagnon, 1993). Given the beneficial role of relaxin

hormone on sperm motility, the potential of relaxin to extend motility lifetime of spermatozoa during storage would be of great interest for the swine breeding industry and pork producers.

Therefore, the objective of this study was to determine the effect of relaxin on full motility characteristics of boar spermatozoa during storage. Firstly, we analyzed the boar sperm motility using (CASA). Secondly, we assessed the presence of relaxin receptors (RXFP1 and RXFP2) on boar sperm cells using an immunofluorescense approach. Thirdly, the relaxin effect on intraspermatic cyclic AMP was evaluated.

4.3 Materials and Methods

4.3.1 Semen Samples

Four batches of (pooled of 2-4 boars each batch) boar semen diluted in Beltsville Thawing Solution (BTS) were obtained at Prestage Farm (West Point, Mississippi) and kept at room temperature (18-21°C). Sperm cells were separated from the BTS by centrifugation at 1000 rpm (253 g) for 5 min at room temperature. Motile sperm were purified using a discontinuous percoll gradient after a 30 min centrifugation at 600 g. Sperm were resuspended in Boviwash (Nidacon products, Mölndal Sweden) medium and recentrifuged at 253 g for 5 min. Washed motile sperm were resuspended in 1 mL of pre-warmed Boviextend medium and counted on a Hemacytometer (Improved Neubaver, Buffalo, NY). Spermatozoa were brought to a final concentration of 75 x 10^6 /mL (as determined in the preliminary study) for treatments.

4.3.2 Semen Treatment

The action of porcine relaxin (pRLX), purified from pregnant sow ovaries (Kohsaka *et al.,* 1993) was evaluated on boar spermatozoa. The relaxin concentrations

employed in this study (0, 50, 100, and 500 ng/mL) were selected based on preliminary studies conducted by Miah *et al.* (2006) and Kohsaka *et al.* (2001), respectively. According to Miah *et al.* (2006), the highest increases in progressive motility of boar spermatozoa was observed when using 20 ng/mL and 40 ng/mL relaxin in comparison to other relaxin concentrations as follow, 0, 10, 60, 80, and 100 ng/mL. However, Kohsaka *et al.* (2001) in a dose response curve study found that the highest significant improvements of relaxin in boar sperm motility was observed from 100 ng/mL to 1000 ng/mL, and picking at 100 ng/mL, among different relaxin concentrations as follow, 0, 5, 10, 25, 50, 100, 250, 500, 1000, 2500, 5000, and 10000 ng/mL. BTS-diluted sperm was stored for up to 5 days at room temperature (18-21 ºC). In experiment 1, boar sperm was daily prepared during 3 consecutive days (days 1 [day of sperm collection], 2 and 3). Purified motile spermatozoa were incubated in the presence of various relaxin concentrations: 0, 50, and 100 ng/mL. In experiment 2, boar sperm was prepared on day 5 of storage. Purified motile spermatozoa were incubated in the presence of relaxin at 500 ng/mL. In both experiments, incubation with relaxin lasted for 1 hour at 37^{o}C. The time of exposure of boar sperm cells to relaxin was determined based on the study conducted by Miah *et al.* (2006). They evidenced that the highest impact of relaxin on progressive motility of boar sperm was around 1 hour of incubation compared to other time periods, 0, 1, 2, 3, 4, 5, 6 hours. Treated spermatozoa were then used for analysis of sperm motility.

4.3.3 Analysis of Sperm Motility

Sperm movement characteristics were objectively analyzed using CASA (HTM-IVOS Hamilton-Thorne Biosciences, Version 12.3. Beverly, MA). Briefly, 3µl aliquot of

treated sperm samples were placed in caffeine-free Leja counting chamber slides (Standard Count 4 Chamber Slide Leja®, 20 micron, Nieuw Vennep, The Netherlands) in triplicates and placed into the CASA equipment set at 37ºC. Three separate fields were analyzed for a minimum of 200 spermatozoa per sample.

The following parameters were considered: total cell count (counted cells), percent of motile cells (%), percent of progressive cells (%), percent of rapid cells (%, ≥ 45 μm/s), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), percentage of straightness (STR; VSL/VAP), and the percentage of linearity (LIN; VSL/ VCL). The CASA settings were adjusted according to manufacturer's instructions.

4.3.4 Immunofluorescence Detection of RXFP1 and RXFP2 on Boar Spermatozoa

After treatments, sperm cells were pelleted through centrifugation at 253 g for 5 min, and resuspended in 1 mL of cold PBS for relaxin receptors detection. Resuspended sperm cells were spread out on Poly-lysine microscope slides (Erie Scientific Company, Portsmouth, NH), and air-dried. Slides were incubated in 4% formaldehyde solution for 30 min at 37ºC, followed by two washes in PBS for 5 min each at room temperature (~21°C). Slides were incubated for 1 hour at room temperature with 10% (v/v) normal goat blocking serum (PBS-PVP), followed by incubation with RXFP1 (H-160; sc-50328) or RXFP2 (H-150; sc-50327) (Santa Cruz Biotech Inc. Santa Cruz, CA) antibodies [1:100 dilution in PBS-PVP + 0.1 % Tween 20]. After a 4 h incubation period at room temperature, slides were washed 3 times in PBS-PVP + 0.1% Tween 20 for 5 min each time, and incubated with the secondary antibody (goat anti-rabbit IgG-FITC; sc-2012; 1/200 dilution in PBS-PVP) for 1 h at room temperature. Slides were washed 3 times

with PBS-PVP + 0.1% Tween 20 for 5 min each time. Then, boar sperm cells were counterstained with DAPI dye and placed under a confocal microscope for analysis. Fluorescent images were acquired using a Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss Microimaging, Inc) with an Inverted Zeiss Axiovert 200 M Light microscope and a plan apochromat 100 X/1.4 NA objective lens. A (DAPI/Fluorescing/Transmission) filter set was used in Single channel mode imaging. Excitation wavelengths of 405nm/488nm and Band Pass Emission wavelengths of 420-480 nm (Blue) and Long Pass wavelengths of 505nm (Green) were acquired at 1024x1024 pixel format for imaging purposes. Sperm without primary antibody were used as a negative control. All procedures were performed at room temperature, unless otherwise indicated.

4.3.5 Measurement of cAMP Content in Boar Spermatozoa

After treatments, sperm cells (75 x 10^6/mL) were pelleted through centrifugation at 253 g for 5 min, and stored at -20ºC until further processing. The assay was performed as recommended by the manufacturer (Assay Designs Inc., cyclic AMP (DIRECT), Ann Arbor, M). Briefly, frozen-thawed spermatozoa were lysed in 0.1 M HCl during 30 min at room temperature (~21°C), and cell debris were eliminated after centrifugation (600 g for 15 min at room temperature). Supernatant was recovered and used immediately for acetylation. Intraspermatic cAMP concentrations were determined in 100 µL of sample, corresponding to 7 x 10^6 sperm cells.

4.3.6 Statistical Analysis

Data were analyzed using a Two-way ANOVA, followed by Fisher's LSD test, and a $P \leq 0.05$ was fixed as threshold of significance (SAS® 9.2, Copyright (c) 2002-2008 by SAS Institute Inc., Cary, NC).

4.4 Results

4.4.1 Sperm motility

The effects of 0, 50, and 100 ng/mL relaxin concentrations on boar sperm motility characteristics are summarized in Table 4.1. Data are mean percentages of motile cells from total sperm cells ± SEM assessed by CASA. As expected, sperm motility significantly decreased with storage from day 1 through 3 in the control group ($P < 0.05$). Both 50 and 100 ng/mL relaxin doses significantly increased the percentage of motile spermatozoa on day 2 of storage compared to the control groups. However, the presence of relaxin did not affect sperm motility on day 1 or day 3 of storage. Across time, 50 and 100 ng/mL relaxin doses kept sperm motility (days 1 and 2), although on day 3 motility significantly decreased.

Table 4.1 Effect of increasing relaxin concentration on boar sperm motility during storage.

Relaxin treatment groups (ng/ml)	motile spermatozoa (mean %age of total cells ± SEM) on:		
	Day1	Day2	Day3
0	$62.7 \pm 2.7^{a¥}$	$58.5 \pm 2.5^{a§}$	$54.1 \pm 3.2^{a€}$
50	$65.3 \pm 2.9^{a¥}$	$62.3 \pm 2.5^{b¥}$	$57.1 \pm 2.9^{a§}$
100	$65.4 \pm 2.3^{a¥}$	$66.1 \pm 1.9^{c¥}$	$56.8 \pm 3.1^{a§}$

Significant differences ($P < 0.05$) within the same column and row are indicated by different letters ($^{a, b, c}$) and symbols ($^{¥, §, €}$), respectively. Data are mean values ± SEM of 4 independent experiments.

Analysis of the relationship between the addition of relaxin and boar progressive spermatozoa is shown in Figure 4.1. Addition of 50 and 100 ng/mL relaxin increased the percentage of progressive spermatozoa on each day of the experiment as compared to the control treatments.

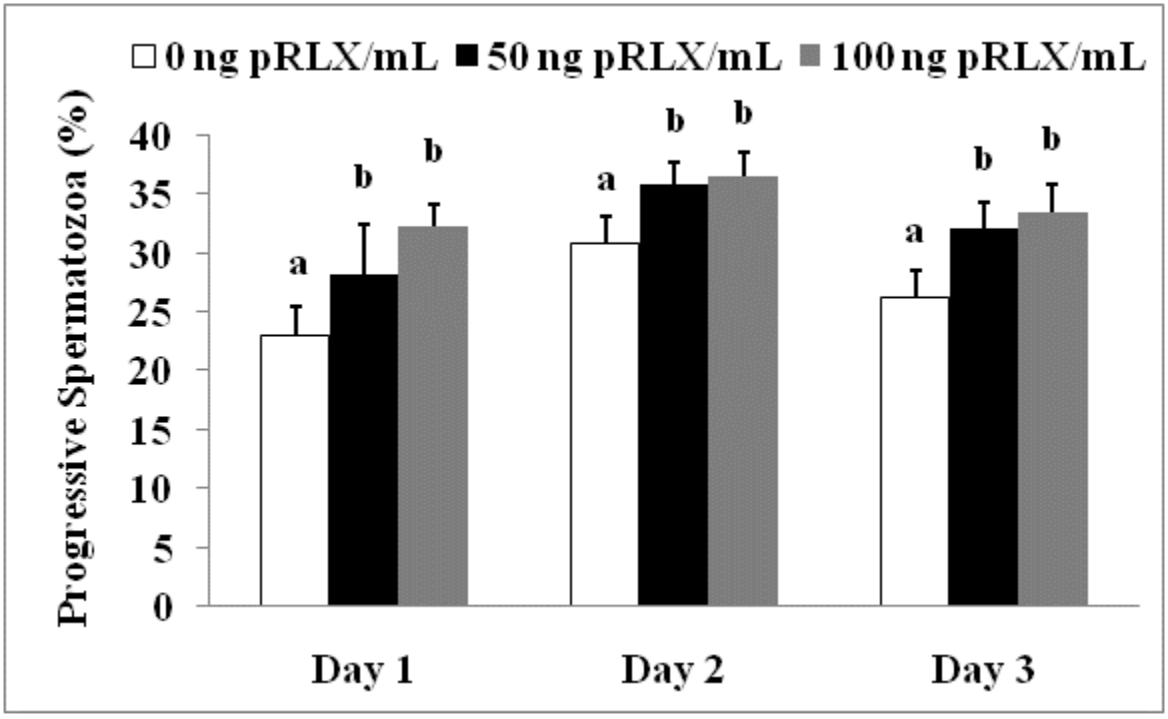

Figure 4.1 Effect of porcine relaxin on proportion of progressive sperm. Data are shown as mean percentage (%) ± SEM from the total number of sperm cells. Significant differences ($P < 0.05$) within the same day are indicated by different letters ($^{a, b}$).

The proportion of rapidly moving spermatozoa (sperm traveling at 45 µm/sec or more) is shown in Figure 4.2. The addition of 100 ng/mL relaxin on day 1 increased the percentage of rapidly moving spermatozoa ($P < 0.05$). On day 3 of storage, 50 ng/mL relaxin increased rapid sperm. On the other hand, there was not effect with any of the relaxin doses used during the second day of storage.

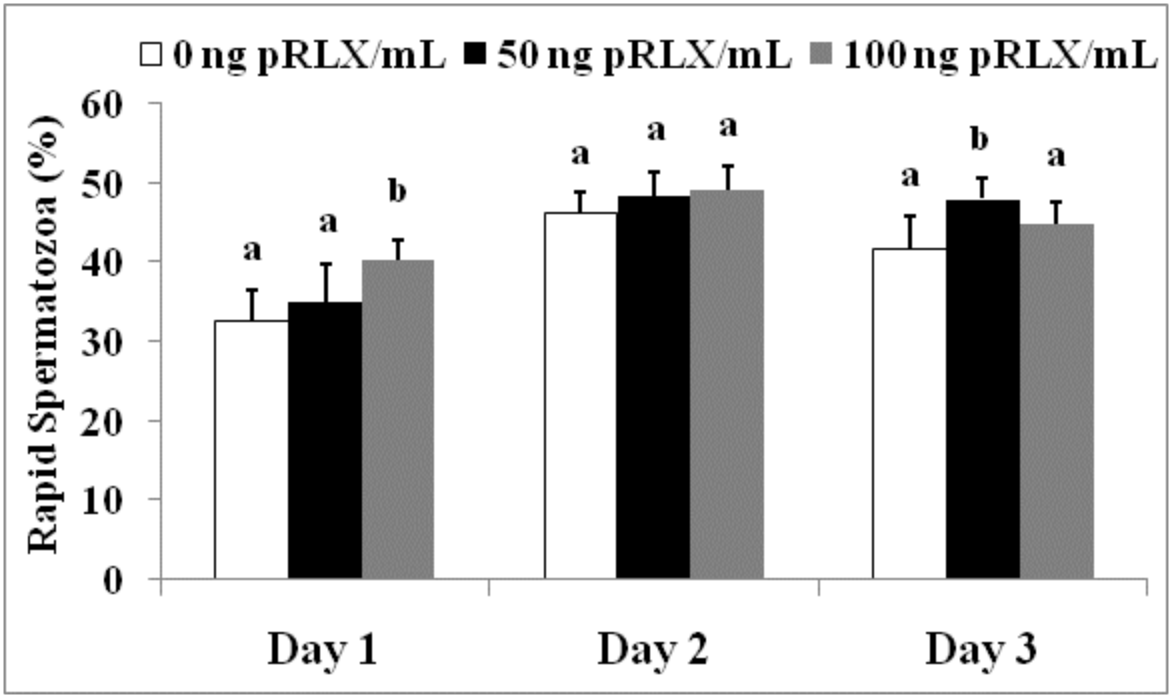

Figure 4.2 Effects of relaxin on rapidly moving spermatozoa (≥ 45 μm/sec) during storage. Data are shown as mean percentage (%) ± SEM from the total number of sperm cells. Significant differences ($P < 0.05$) within the same day are indicated by different letters ($^{a, b}$).

With regard to the straight line velocity (VSL), the action of both the two highest relaxin doses (50 & 100 ng/mL) had a significant effect ($P < 0.05$) during the first two days of storage, while there was no effect on day 3 (Table 4.2). Across time, there was an increased of VSL of sperm when using 50 ng/mL relaxin as well as in the control group compared to day 1 of storage.

Table 4.2 Effect of an increasing relaxin concentration on the straight line velocity (VSL) of boar spermatozoa during storage.

Relaxin treatment groups (ng/ml)	Spermatozoa VSL (mean μm/s ±SE) on:		
	Day1	Day2	Day3
0	$48.0 \pm 1.9^{a¥}$	$52.7 \pm 1.7^{a§}$	$53.7 \pm 2.5^{a§}$
50	$53.2 \pm 3.0^{b¥}$	$57.5 \pm 2.0^{b§}$	$57.0 \pm 2.4^{a§}$
100	$57.1 \pm 2.1^{b¥}$	$58.6 \pm 1.2^{b¥}$	$56.9 \pm 2.3^{a¥}$

Significant differences ($P < 0.05$) within the same column and row are indicated by different letters ($^{a, b, c}$) and symbols ($^{¥, §}$), respectively. Data are mean values ± SEM of 4 independent experiments.

The straightness (VSL/VAP x 100) of sperm is shown in Figures 4.3. Both 50 & 100 ng/mL relaxin significantly increased straightness of spermatozoa since the very first day of analysis. It also was evidence that across time, there was a general decrease of straightness. However, the presence of porcine relaxin reduced ($P < 0.05$) the decrease in percentage of sperm straightness on the last two days of storage in comparison with the control group.

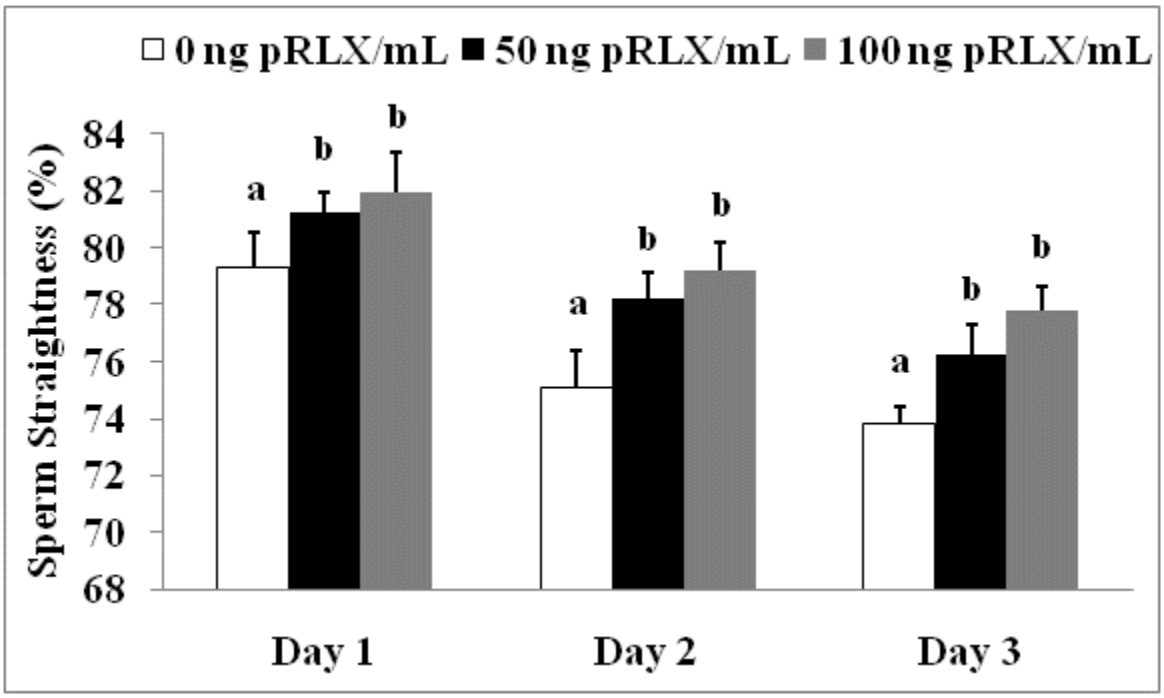

Figure 4.3 Effects of porcine relaxin on the straightness (STR, % = VSL/VAP x 100) of spermatozoa. Data are shown as mean (%) values ± SEM. Significant differences ($P < 0.05$) within the same day are indicated by different letters ($^{a, b}$).

Results about linearity (VSL/VCL x 100) of sperm are shown in figure 4.4. It also was noticed that across time, there was a general decrease of linearity as the one observed in the previous ratio (straightness, Figure 4.3). However, the presence of porcine relaxin reduced ($P < 0.05$) the decrease in percentage of sperm linearity on the last two days of storage in comparison with the control group; so, there was a greater percentage of sperm traveling more linear. On day 1 of analysis, relaxin did not influence any significant change on the linearity of spermatozoa.

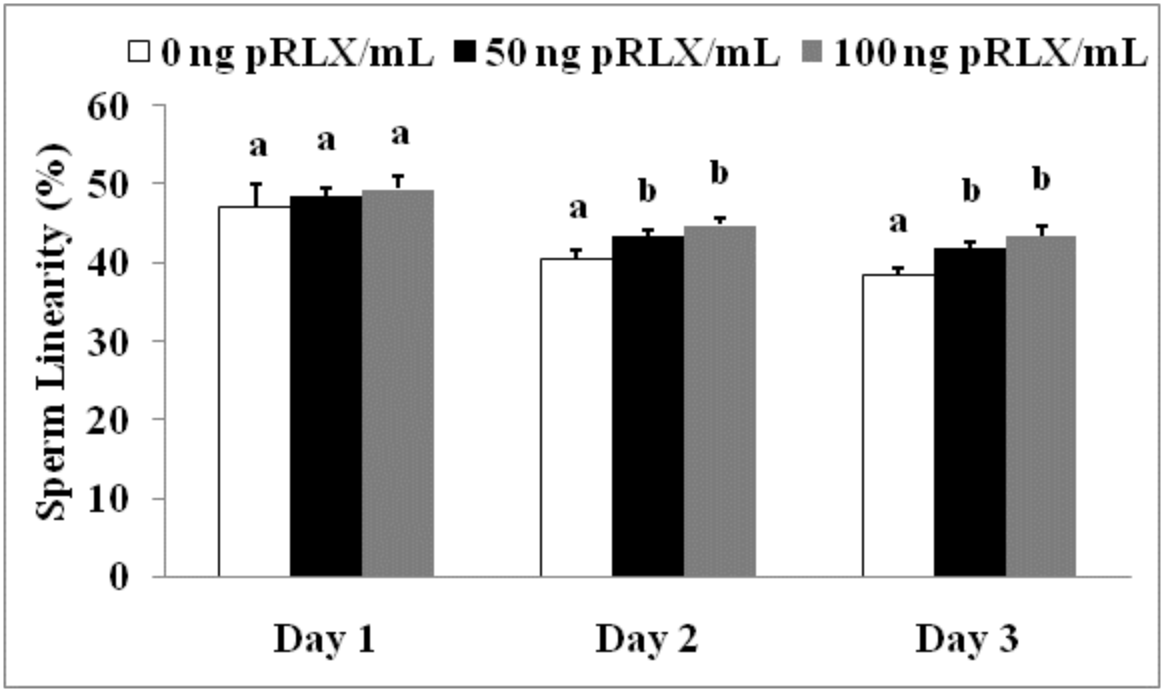

Figure 4.4 Effects of porcine relaxin on the linearity (LIN, % = VSL/VCL x 100) of spermatozoa. Data are shown as mean (%) values ± SEM. Significant differences ($P < 0.05$) within the same day are indicated by different letters ($^{a, b}$).

4.4.1.2 Effects of a high relaxin dose

Relaxin at a concentration of 100 ng/mL appears to be the concentration with higher effects on the general motility of boar sperm during storage. However, there was a particular trend when using such concentration in some of the results obtained. There was a general increased in the mean percentage of motile spermatozoa (Table 4.1) during days 1 and 2 of storage when using the highest relaxin dose (100 ng/mL). However, such increase was not evidence on day 3 of storage. Due to this lack of effect by relaxin during the last day of analysis one question arose, and it wass whether or not such lack of effect on day 3 could had been due to a minute amount of relaxin remaining in the sperm storage medium. Therefore, a higher relaxin (500 ng/mL) dose was employed to determine its effects on sperm motility parameters.

Table 4.3 summarizes the second trial carried out using a high relaxin dose (500 ng/mL) employed on day 5 of storage. Significant increases ($P < 0.05$) were evident in some of the general motility parameters such as progressive sperm cells (%), and rapid cells (%), as well as on the average path (VAP, μm/s), straight line (VSL, μm/s) and curvilinear (VCL, μm/s) velocities. With regards to the progression ratios, linearity was the only one positively affected ($P < 0.05$) by relaxin. However, the high porcine relaxin dose did not have a significant effect either on motile sperm cells (%), amplitude of the lateral head displacement (ALH, μm) or straightness (VSL/VAP, %).

Table 4.3 Effect of relaxin hormone (500 ng/mL) on full motility parameters of boar sperm on day 5th of storage.

Storage	Day 5	Day 5
Relaxin Treatment	0 ng/mL Mean ± SE	500 ng/mL Mean ± SE
Parameters		
*Motility (%)	66.8 ± 2.6^{a}	68.3 ± 1.4^{a}
**Progressive (%)	51.4 ± 1.9^{a}	58.0 ± 1.2^{b}
**Rapid (%)	68.4 ± 1.9^{a}	75.1 ± 1.2^{b}
VAP (μm/s)	80.5 ± 2.3^{a}	90.2 ± 1.5^{b}
VSL (μm/s)	51.5 ± 1.8^{a}	57.6 ± 1.4^{b}
VCL(μm/s)	175.0 ± 3.6^{a}	183.7 ± 2.3^{b}
ALH (μm)	7.5 ± 0.1^{a}	7.4 ± 0.1^{a}
STR (VSL/VAP, %)	61.0 ± 0.9^{a}	61.4 ± 0.7^{a}
LIN (VSL/VCL, %)	29.6 ± 0.6^{a}	31.7 ± 0.6^{b}

(*)Motile (mean percentage of total cells); both (**) progressive and rapid (mean percentage of motile cells). Significant differences ($P < 0.05$) between columns are indicated by different letters ($^{a, b}$). Data are mean values ± SEM of 4 independent experiments.

4.4.2 Immunofluorescense detection of RXFP1 and RXFP2

The identification of relaxin receptors (RXFP1 and RXFP2) on boar spermatozoa employing an immunofluorescense approach are shown in Figure (4.5, Panels A-F). The relaxin receptor RXFP1 (Panels C and D) was present in the head, neck, and intermediate

piece of the tail in boar sperm, where it appears with a bright light emission, compared to the negative control (Panels A and B). In the same way, RXFP2 receptor (Panels E and F) was also noticed to be present in the same regions as RXFP1, but with a higher intensity.

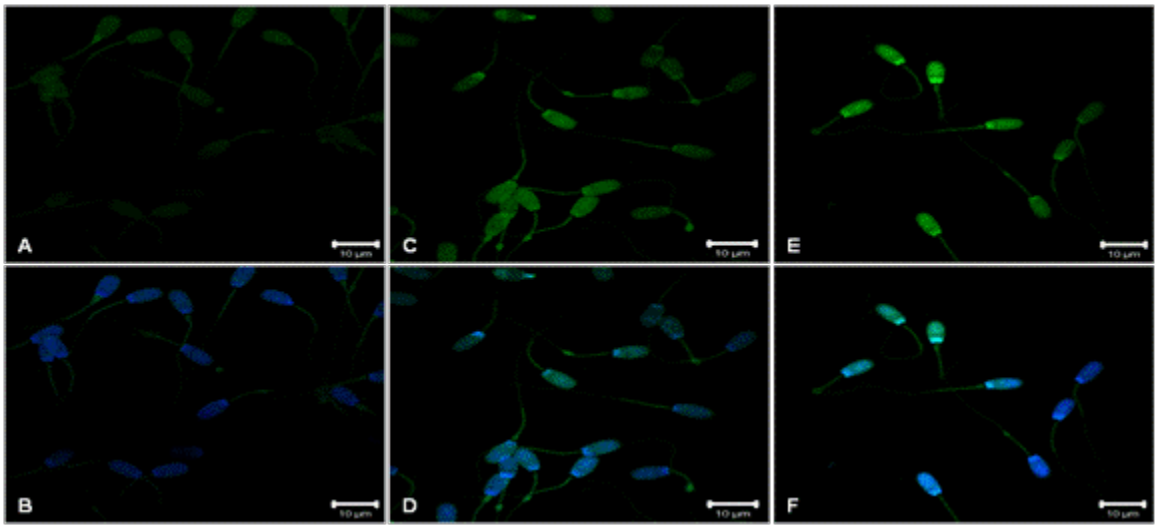

Figure 4.5 Determination of relaxin receptors RXFP1 (Panels C and D) and RXFP2 (Panels E and F) on boar spermatozoa. Negative control (Panels A and B). Pictures on top with FITC staining only (Panels A, C and E). Pictures at the bottom with FITC and DAPI stainings (Panels B, D and F).

4.4.3 Measurement of cAMP content in boar spermatozoa

As shown in Figure 4.6, relaxin did not promote any significant increase of intraspermatic cAMP content in boar spermatozoa through storage. Although, not statistically significant, when boar sperm cells were incubated with relaxin (100 and 500 ng/mL), the cAMP content was slightly higher compared to the control on day 1 and 2 of storage. On the other hand, on day 3 of storage, the content of intraspermatic cAMP was even slightly higher in the control compared to the treated groups, but not statistically significant either.

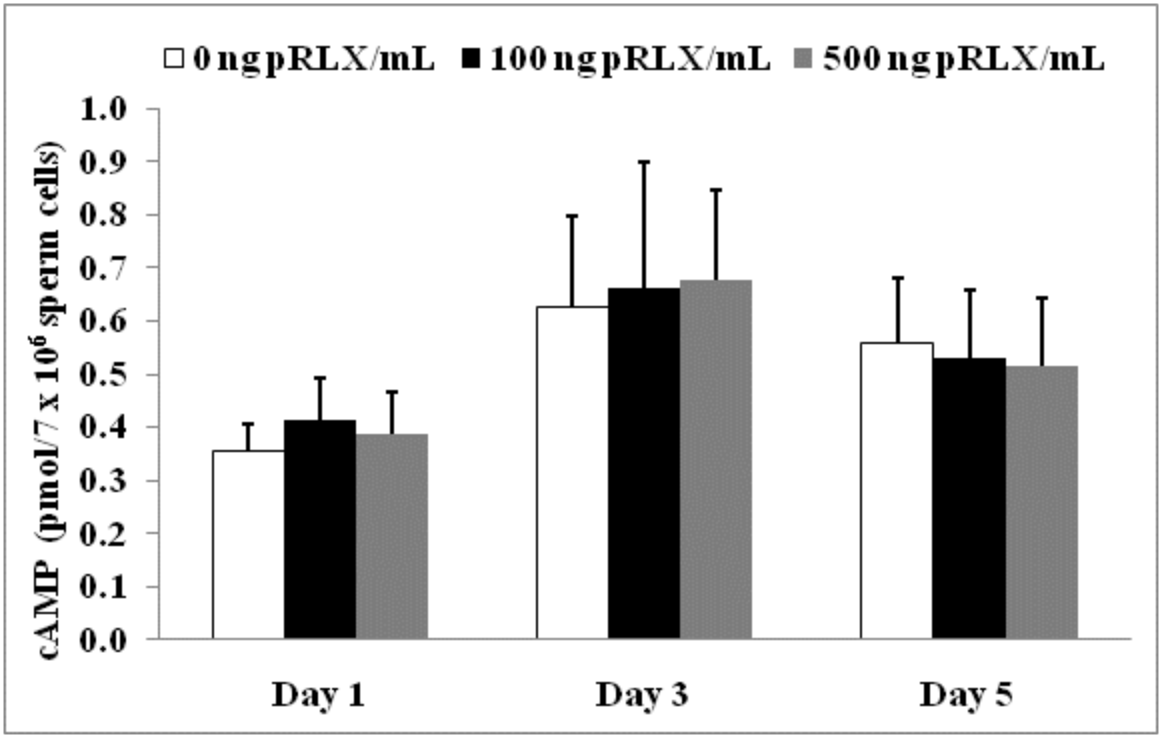

Figure 4.6 Intraspermatic cAMP content in boar sperm cells after supplementation with relaxin through storage. Data are shown as mean percentage (%) ± SEM. There were not significant differences ($P < 0.05$) within the same day of storage between relaxin treatments.

4.5 Discussion

The action of various porcine relaxin hormone concentrations on full motility parameters of boar spermatozoa was assessed during storage, using CASA. The current study indicates that porcine relaxin can keep and even increase boar sperm motility during storage. As anticipated, sperm motility was significantly decreased during storage in the control group; but a significant increase of the motility rate was evidenced on day 2 of sperm storage, when using relaxin at 50 and 100 ng/mL (Table 4.1); however, we did not find any significant ($P < 0.05$) effect of relaxin at 500 ng/mL at day 5 of storage on the motile sperm (Table 4.3). In accordance with our results, some studies have reported

beneficial effects of relaxin hormone on sperm motility (Miah *et al,.* 2006; Juang *et al.,* 1989).

Besides sperm motility, we also noticed a positive effect of relaxin with all concentrations employed in the proportion of progressive spermatozoa determined by CASA during all days of storage. Juang *et al.* (1989) in a similar study reported beneficial actions of relaxin on the progressively moving sperm when using 1000, 333, and 167 ng/mL relaxin on fresh semen after 80, 120 and 180 minutes incubation compared to the control group. Our study showed also positive effects using lower concentrations of relaxin (50 and 100 ng/mL) on fresh semen after 60 minutes of incubation with the peptide compared to the control group. Their (Juang *et al.,* 1989) initial percentage of progressively moving spermatozoa was higher than ours in the control group (around 60% vs. 23%, respectively); however, our value appears relatively lower compared to them because it was obtained from the total number of sperm cells, and not from the motile sperm cell population as they did. Their results showed that after 80, 120 and 180 minutes incubation with the peptide, it significantly attenuated the loss of motile progressively moving spermatozoa (%, between 40 and 50%) compared to the control group (around 35%). In our case, compared to the control (23%), after 60 minutes of incubation with relaxin at 50 and 100 ng/mL, it augmented the progressively moving spermatozoa to 28 and 32%, respectively. On the other hand, Juang *et al.* (1989) also studied the effect of relaxin in boar spermatozoa stored up to 5 days at 12°C. They found that when adding relaxin at 1000 ng/mL just before storage (day 0, sperm collection), it significantly attenuated the lost of progressively moving sperm (%) at 1.5, 2, 3, 3.5, 4 and 5 days compared to the control. Although, they observed a reduction in progressively moving sperm during the first 10 hours of storage in both control (57%) and treated cells

(around 64%) group compared to the initial percentage of progressive sperm (around 75%), this reduction was not significant ($P > 0.05$) between these two groups. Our study also noticed the positive effects of relaxin when using lower concentrations (50 and 100 ng/mL) on days 1 (day of sperm collection), 2, and 3 of storage compared to the control. Also, with a higher relaxin dose (500 ng/mL) on day 5 of storage we found positive effects in the progressively moving sperm compared to the control. In this trial, their (Juang *et al.,* 1989) initial percentage of progressively moving spermatozoa also was higher than ours in the control group (around 75% vs. 23%, respectively). However, on day 5 of storage their results showed that the percentage of progressively moving spermatozoa dropped to around 45%, while in their control dropped to around 35%. When compared to our higher relaxin dose (500 ng/mL) on day 5 of storage, there was a significant effect of the peptide in comparison to our control (58% vs. 51%). In comparison to their study (Juang *et al.,* 1989) on day 5 of storage, our values where higher in both controls (51% vs. 35%) and treated groups (58% vs. 45%). However, in this particular case (using 500 ng/mL relaxin on day 5 of storage) we obtained the percentage of progressively moving spermatozoa from the percentage of motile spermatozoa, and not from the total sperm cells as we did previously. In contrast to Juang *et al.* (1989) study, where they added relaxin just before the whole storage period, we daily prepared aged sperm cells and incubated them with relaxin 1 hour before motility parameters were assayed. On the other hand, we kept spermatozoa at room temperature (18-21°C) during the whole storage period, different from them that stored spermatozoa at 12°C. According to Tash and Mann (1973), slow cooling to 0°C or 5°C can promote a state of reversible inhibition of sperm motility. Based on this, we think that storage of spermatozoa at 12°C as they did (Juang *et al.,* 1989) might promote some inhibition of

sperm motility, which is translated into a slower consumption of energy reservoirs, due to the reduced sperm motility. So, we assume that relaxin utilization was slower, knowing that sperm motility was reduced due to the cooling conditions (12°C), and maybe that is why they observed the highest effects of relaxin as time passed compared to the controls even though they added the peptide just at the beginning of storage. In contrast to them, we added relaxin to boar spermatozoa stored at room temperature (18-21°C) and incubated them with the peptide for 1 hour at 37°C before motility assessment was conducted with CASA. It means Juang *et al.* (1989) required the use of a refrigerator set up at 12°C, which in not all cases is feasible to obtained one. From a production point of view, one less equipment (a refrigerator) can be a factor that can change the farmers' mind to adopt or ignore a new approach in their production system. Besides this, we used lower relaxin concentrations compared to them (Juang *et al.,* 1989) and still got positive results. Due to the high cost of relaxin, this is another factor that might influence farmers' minds before making the decision about whether or not they can use relaxin in their herds to maximize the use of boar semen. On the other hand, one more major difference between these two studies was the employment of different methods to determine the progressively moving spermatozoa. Juang *et al.* (1989) established it using a trans-membrane migration ratio (TMMR), which is basically the number of spermatozoa that are travelling across a membrane that has micropores slightly bigger that the sperm heads. Although, it is an objective system (TMMR), it just can determine motility on a routine basis. The fraction of spermatozoa making it through the membrane has been correlated with the progressively moving sperm (%) (Hong *et al.,* 1981; 1985). In our case, sperm motility was determined using another objective system, CASA, which can determine besides motility, several sperm movement parameters, velocities and ratios.

CASA can track the exact motility patter of sperm movement. Due to this variation in methods employed, we also should expect variations when comparing results.

In addition, rapid spermatozoa were also positively affected by the peptide. Several studies have shown that porcine relaxin has a stimulatory action not only on the progressive spermatozoa, but also in the rapid sperm of boar sperm (Kohsaka *et al.,* 2001; Sasaki *et al.,* 2001; Juang *et al.,* 1990; 1989). Similar actions have also been noticed in humans when porcine relaxin was added to washed ejaculated sperm (Essig *et al.,* 1982a; Colon *et al.,* 1986), as well as in thawed human sperm after cryoconservation (Lessing *et al.,* 1985), where progressive spermatozoa was positively affected, while there was a reduction in the loss of sperm motility.

We also observed the beneficial action of porcine relaxin on the straight line velocity (VSL) of sperm (defined as the velocity of sperm progression from one point to a second one in a straight line, measured in μm/s). According to Liu *et al.* (1991), there is a correlation between VSL and the fertilization rates (*in vitro*) consistent with the perception that sperm cells traveling with a faster straight line velocity have a higher chance to fertilize an oocyte. Katz *et al.* (1982) showed that morphologically normal spermatozoa swim straighter and faster than abnormal spermatozoa, therefore, explaining why morphologically normal spermatozoa are considered as one of the most significant sperm aspects associated to IVF rates (Katz *et al.,* 1982; Kruger *et al.,* 1986; 1988a, b; Liu *et al.,* 1988a; Liu *et al.,* 1988b). Liu *et al.* (1991) reported a high correlation between morphologically normal spermatozoa and the percentage of motile sperm showing straightness higher than 80%, with an average path velocity (VAP) higher than 20 μm/sec. In the current studies, straightness was over 80% during the first day of incubation when using porcine relaxin at 50 and 100 ng /mL (experiment 1); however, it

was just significantly different when using 100 ng relaxin/mL compared to the control group ($P < 0.05$) on day 1 (Figure 4.3). Additionally, Juang *et al.* (1989) also reported that supplementation of sperm cells with antirelaxin serum abruptly reduced motility. This study supports the idea that relaxin is essential as well as can improve motility of sperm.

In addition to the relaxin effects on boar sperm motility, this study also reports the presence of relaxin receptors RXFP1 and RXFP2 in boar spermatozoa. Therefore, it might be possible that relaxin binds to specific membrane receptors to exert its effects on spermatozoa. Carrell *et al.* (1995) conducted a study using ^{125}I-labled human relaxin that bound with high affinity to human spermatozoa, nevertheless, the determination of specific human relaxin binding sites were not established. Additionally, Kohsaka *et al.* (2001) reported the identification of specific relaxin binding sites on boar caudal epididymal spermatozoa using an *in situ* binding histochemical assay, and provided information that such binding has a significant action on sperm motility. Kohsaka *et al.* (2001) found that relaxin bound with specificity to the tail and midpiece of sperm. Additionally, bindings at the acrosome cap and neck of the head were also visible. Using an immunofluorescense approach, our study shows the presence of relaxin receptors (RXFP1 and RXFP2) in the head, neck and midpiece of boar sperm. It was noticed that this presence was lower in RXFP1 receptor than in RXFP2. Although, we did not quantify the amount of either RXFP1 or RXFP2 receptors in boar spermatozoa, our results are in accordance with the findings reported by Feugang *et al.* (2011). They found that after relaxin receptor proteins detection (western-immunoblotting), boar spermatozoa exhibited lower RXFP1 signal than RXFP2. It suggests that boar spermatozoa are targets

of relaxin, proposing that relaxin mechanism of action is first mediated through the binding to its specific membrane receptors.

The present study also reported the actions of relaxin in the intracytoplasmic content of cAMP of aged boar spermatozoa. After the ligand (relaxin)-receptor binding, it is believe that relaxin promotes an intracellular signaling cascade that is leaded by the second messenger cAMP. It is considered that addition of relaxin to the medium may operate on G-proteins through relaxin receptors (Nistri and Bani, 2003), that have been detected in the membrane of sperm cells by this study and others (Kohsaka *et al.,* 2003) using different means. Then, these G-proteins activate adenylate cyclase that in turn increases the intracellular content of cAMP, and avoids the breakdown of it (cAMP) by restraining the actions of cAMP phosphodiesterases (Bartsch *et al.,* 2001), that are in charge of the degradation of the second messenger. In the same way, it has been stated that the nucleotide (cAMP) has an important task in the energy production required for the sperm cells in its motility through the glycolytic pathway (Hicks *et al.,* 1972). Research has proposed that relaxin stimulates intracellular levels of cAMP in different tissues such as uterus and cervix in the rat (Judson *et al.,* 1980; Sanborn *et al.,* 1980). Although, some studies (Juang *et al.,* 1989) have suggested that relaxin is increasing sperm motility maybe through the alteration of intracellular cAMP after its binding to surface receptors in spermatozoa, we do not know about the existence of any study that has achieved this goal yet. So, this is probably the first study in doing so. However, we did not observe any significant effect by any of the different relaxin concentrations employed in the intracellular cAMP of aged boar spermatozoa. It can be that relaxin's mechanism of action on sperm motility might be driven in combination or activation of cAMP-dependent protein kinases in the cytoplasm of spermatozoa instead of just cAMP.

4.6 Conclusion

The present investigation indicates a valuable action of relaxin on several of the boar spermatozoa movement parameters (e.g. motile, progressive and rapid sperm in the general motility; VSL, and in the ratios of linearity and straightness) assessed with CASA. This study also determined the presence of relaxin receptors (RXFP1 and RXFP2) on boar spermatozoa membrane and suggests that relaxin peptide might activate sperm motility through these specific receptors. However, this study did not see relaxin is exerting any action on the intracellular cAMP content of boar sperm cells, what makes us think that the relaxin's mechanism of action may be driven in conjunction and/or activation of cAMP-dependent protein kinases in the plasma membrane of sperm. Therefore, further studies with regards to the relaxin intracellular mechanism of actions on sperm motility need to be carried out.

CHAPTER V

CONCLUSIONS

The study of sperm physiology and kinematics are of great importance in order to understand the processes that can contribute to the acquisition of the best sperm to obtain higher outcomes in terms of fertilization rates. A boar sperm concentration of 75 x 10^6/mL was determined to be as the optimal one to be employed while conducting assessment of sperm motility with CASA. The attainment of such sperm concentration was important, so accurate results can be obtained when employing CASA. Such sperm concentration was employed while conducting studies involving the addition of relaxin hormone to boar spermatozoa during storage.

The utilization of different substances such as relaxin hormone that can activate or stimulate sperm motility can be much easily now study by using CASA. Relaxin at 50 and 100 ng/mL increased several of the sperm motility parameters in boar semen during storage. For instance, the proportions of motile, progressive and rapid sperm in the general motility, the VSL, and the ratios of linearity and straightness were positively affected by the peptide. It would be of great importance if a method can be developed to study every one of these sperm populations separately and see the implications they can have in achieving higher outcomes in fertility rates. On the other hand, it would be important to see if using relaxin hormone during a longer storage period (> 5 days) can still increase further or at least maintained motility parameters close to the values observed on fresh semen. Knowing that cryopreservation of boar semen is reduced

compared to other species, this type of studies involving relaxin would be of great importance for the swine industry, knowing that it will lead to maximize the use of fresh sperm semen due to the time window widening while boar sperm can be manipulate.

Trying to understand how relaxin is improving motility, we conducted some experiments to determine whether or not boar sperm possess relaxin receptors (RXFP1 and RXFP2) in their membrane. Both receptors were found there. Although, we did not quantify the amount of these two receptors, we did notice that the presence of RXFP2 was higher than RXFP1, which is reported to be as the direct receptor for the ligand relaxin. It suggests that quantification of these receptors and their specific location within the sperm cells could be the next step, and if there is some possible implications of their localization in the spermatozoa with regard to motility.

After having determined the presence of both relaxin receptors and following the same line of thought stated previously on how relaxin might be acting, we wanted to go deeper and evaluate the intracellular cAMP concentrations in sperm cells after treatment with relaxin. We did not evidence any change in the intracellular cAMP content in sperm cells during storage as the initial pathway postulated in this study by which relaxin might be carrying out its effects on sperm motility. This study suggests that analysis of different pathways at a molecular level is of great interest in seeking out for a better understanding on how relaxin is acting.

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