EFFECTS OF A WALNUT-ENRICHED DIET ON MURINE SPERM AND EPIDIDYMIS: ITS IMPACT ON PLASMA MEMBRANE CALCIUM ATPASE-4 (*Pmca4*) NULL MICE

by

Lauren Susan Coffua

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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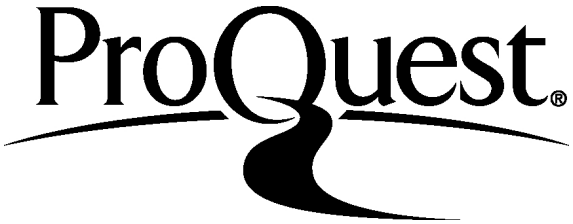

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ABSTRACT

Male factors account for 50% of infertile couples, with 25% of these cases being idiopathic. Sperm defects are the single most common cause of male infertility and it is believed that genetic factors play a major role in idiopathic cases. Recently, lifestyle choices have also been shown to impact sperm function, both positively and negatively. Positive effects have resulted from a diet enriched in walnuts, which are high in polyunsaturated fatty acids (PUFAs) and antioxidants: when this diet was administered to men on a Western diet for 12 weeks there was a significant increase in sperm motility, vitality, and normal morphology. To date, a study of the effects of a walnut-enriched diet on sperm quality has not been conducted in an animal model and was one of the goals of the present study. Here it is shown that a walnut-enriched diet (accounting for 19.6% caloric intake) given to male mice for 9-11 weeks, significantly improved sperm motility and morphology. Thus, the beneficial effects of a walnut enriched diet on sperm quality were confirmed in the murine model.

To determine if this diet could have a beneficial effect on sperm with a motility defect due to a genetic mutation, *Pmca4* null male mice, in which the Plasma Membrane Calcium ATPase 4 (PMCA4) Ca^{2+} efflux pump is missing, were subjected to the diet. Deletion of PMCA4, the major Ca^{2+} efflux mechanism in murine sperm, results in infertility due to loss of hyperactivated motility by a mechanism that remains unclear. To explore the possible underlying mechanism, various properties of *Pmca4* null sperm were examined and attempts were made to modulate them by exposure to

the diet. Although motility rates were not affected by the administration of the diet in *Pmca4* nulls, the potential impacts of *Pmca4* deletion on membrane integrity were determined by studying three sperm characteristics (vitality, morphology, and acrosomal reaction rates). Membrane integrity, as assessed by vitality staining, and the ability to acrosome react showed no significant difference between control and KO or between diet and no-diet. On the other hand, morphologically normal sperm were significantly decreased in the *Pmca4* KO control, compared to control WT; and in diet-treated animals normal morphology was significantly increased for both WT and KO sperm. The levels of lipid peroxidation showed a trending increase in *Pmca4* null, compared to WT control sperm, and significantly decreased in both WT and KO sperm following administration of the diet. Thus lipid peroxidation was determined to be a potential mechanism resulting in the loss of motility and the increase in morphologically abnormal sperm, the majority of which were tail defects, in *Pmca4* nulls.

Testicular germ cells were also affected by *Pmca4* deletion, as shown by the TUNEL assay, for which there was a significant increase in spermatogonia ($p<0.005$) and spermatocytes ($p<0.05$) in nulls, compared to WT males. This was confirmed by hematoxylin and eosin (H&E) staining, which revealed increased disorganization of the seminiferous tubule structure. *Pmca4* KO epididymides were analyzed via H&E staining and revealed cellular damage associated with apoptosis. There was a significant increase in vacuolization of epithelial cells in the cauda in the *Pmca4* KO males, as well as an increased number of pyknotic-rounded cells. These results support a mechanism in which *Pmca4* deletion results in loss of motility, increased tail defects, and apoptosis via oxidative stress and lipid peroxidation. These findings give support

for the addition of dietary supplements for the maintenance of reproductive health, as a potential lifestyle modification to increase reproductive success.

Chapter 1

INTRODUCTION

1.1 Male Factor Infertility

Infertility affects 10-15% of couples in the United States and around 70 million couples worldwide (Boivin et al., 2007; Mayo Clinic, 2014). Its prevalence has increased in recent decades and research has expanded our understanding of issues, both genetic and environmental, that result in infertility. Such factors are shared evenly between the genders, with 40% of the cases having factors attributed to the female, 40% to the male, and 20% to a combination of both. This work focuses on male factor infertility.

Approximately 1 in 20 men will experience issues relating to fertility in their lifetime (Aitken et al., 2014). Of these individuals, 75% of male factor infertility cases can be attributed to known causes such as trauma, endocrine abnormalities, or injury, etc. The remaining 25%, however, are of idiopathic or unknown origin (Kantartzi, 2007). In such cases, sperm defects are the single most important source with low motility or low sperm count resulting in low fertilization success. It has been reported that genetic defects may play a significant role in these idiopathic cases (Matzuk and Lamb, 2008).

Over the past three decades, methods to combat infertility have become available to the population. These methods, Assisted Reproductive Technologies (ART), account for >1% of total US births and since implementation in 1978, have

been responsible for ~5 million babies (Schieve et al., 1999; Bauquis, 2012). Techniques employed in ART include in vitro fertilization or IVF and ICSI (intracytoplasmic sperm injection), the most common procedure. However, despite ART's increasing demand and intense laboratory research, there is only a 32% success rate. In addition, the average cost of these procedures is $12,400/cycle with, on average, three cycles/couple before success (Asch and Marmor, 2008; American Society of Reproductive Medicine). The low success rate and high cost of ART have facilitated the need for continued research to create techniques and procedures that are not only innovative but also more efficient and cost-effective.

It is the hope that continued research on the causes attributing to male infertility will lead to the development of improved methods to increase the success rate and/or decrease the need for ART. In particular, this project focuses on the use of dietary therapy to improve male reproductive health and its specific effects on sperm physiology and function.

1.2 Primary Functions of Sperm

A specialized reproductive cell, sperm is characterized by the presence of a head and tail region (Fig. 1.1). The head, rounded in human sperm and hooked in murine sperm, consists of a tightly compacted nucleus surrounded by the acrosome. The acrosome region contains many of the vital degradation enzymes necessary for fertilization to occur and becomes activated when exposed to elevated levels of calcium (Breitbart, 2002). The tail or flagellum of sperm is separated into 3 sections: the midpiece, containing the mitochondrial sheath which is responsible for providing energy for movement, the principal piece, and finally the end piece. The entire cell is surrounded by a plasma membrane that is enriched in polyunsaturated fatty acids

(PUFA), which play major roles in membrane fluidity, flexibility, and oocyte fusion (Fleming and Yanagimachi, 1981; Israelachvili et al., 1990). A diet low in PUFAs delivered to rats revealed degeneration of the seminiferous tubules of the testes, decreases in germ cells and an absence of spermatozoa in the lumen of the testes and epididymis suggesting a pivotal role of PUFAs in fertility (Leath et al., 1983).

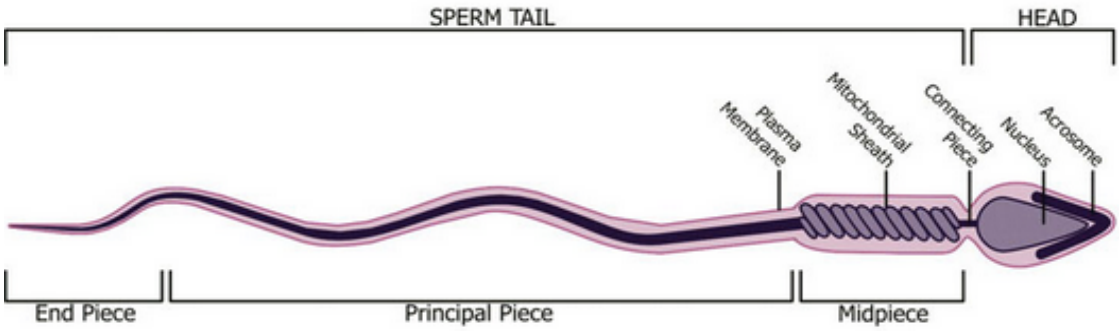


Figure 1.1. **Diagram of sperm.** Areas of interest include the head and tail regions as well as the plasma membrane, acrosome, and midpiece. (Adapted from Borg et al., 2010)

1.3 Epididymal Sperm Maturation

Upon completion of spermatogenesis in the testes, sperm are morphologically mature but functionally incompetent and transcriptionally inactive (Reid et al. 2011). At this stage, they lack the necessary mechanisms that allow for progressive motility and oocyte fusion (Jones, 1989; Cooper, 1995; Jones, 1998; Reid et al., 2011). Sperm gain these functions, and reach maturity in the epididymis (Yeung and Cooper, 2002). Secretory mechanisms of the epididymal epithelium are responsible for providing sperm with molecules such as proteins that enable them to attain functional maturity

(Cooper, 1998; Jones, 1998). As the sperm traverse through the epididymis, they are immersed in luminal fluids that provide these necessary components.

Anatomically, the epididymis is divided into three separate regions: the caput (head), corpus (body), and cauda (tail) (Robaire et al., 2006; Shum, 2011) all connected via a single convoluted tubule (Fig. 1.2). The ability to distinguish between the various regions comes primarily from the thickness of the epithelium, with the thickest portion found in the caput and the thinnest in the cauda as well as the diameter of the lumen, which enlarges as the epithelium thins (Lasserre et al., 2001). Current research supports the notion that each of these regions plays an independent and vital role in sperm maturation through the release of specific cellular components via specialized cell types. In addition to these properties, the chemical nature of the epididymis, particularly its acidic state and low bicarbonate concentration, are necessary for maintaining sperm dormancy in order to preserve pH-sensitive sperm motor proteins such as dynein ATPases (Christen et al., 1983; Giroux-Widemann et al., 1991).

Various cell types present in the epididymal tissue, specifically principal, basal, apical (not pictured), narrow, and clear cells, control the regulation of these chemical processes (Fig. 1.2). The location of these cells is indicative of their primary functions. Principal cells as well as basal cells are found in every region of the epididymis and form a majority of the tubule. Principal cells play a major role in the secretion of proteins in the epididymal lumen, which will be elaborated on in following sections, and in turn function as the regulator of protein concentration in the lumen (Hermo et al., 1995; Robaire et al., 2006). Following principal cells, basal cells are the second most populous cell type found in the epididymis. These small, flat,

triangular cells rest along the basal layer of the tubule, hence their name, and have no contact with the luminal environment. The function of this cell type has not been fully developed; however, it is believed they play a role in electrolyte control of the principal cells (Robaire et al., 2006).

Apical cells (not pictured) are found in smaller numbers in the epithelium and primarily reside in the proximal region near the lumen (Adamali and Hermo, 1996). The location of this cell type relates strongly to its function, which is pH regulation of the lumen and control of sperm dormancy (Hermo et al., 2005). Narrow cells, are present in the initial portion and into the corpus and are responsible for intracellular transport, protein degradation, and sperm protection (Adamali and Hermo, 1996; Robaire et al., 2006). The final epididymal cell type of interest is clear cells. While found in all regions of the epididymis, clear cells play major roles in the cauda where they function as endocytic cells; clearing the lumen of proteins, cellular debris and cytoplasmic droplets from sperm (Hermo et al., 1994; Robaire et al., 2006). Together, these cell types work in the protection and maturation of spermatozoa as they progress through the epididymis.

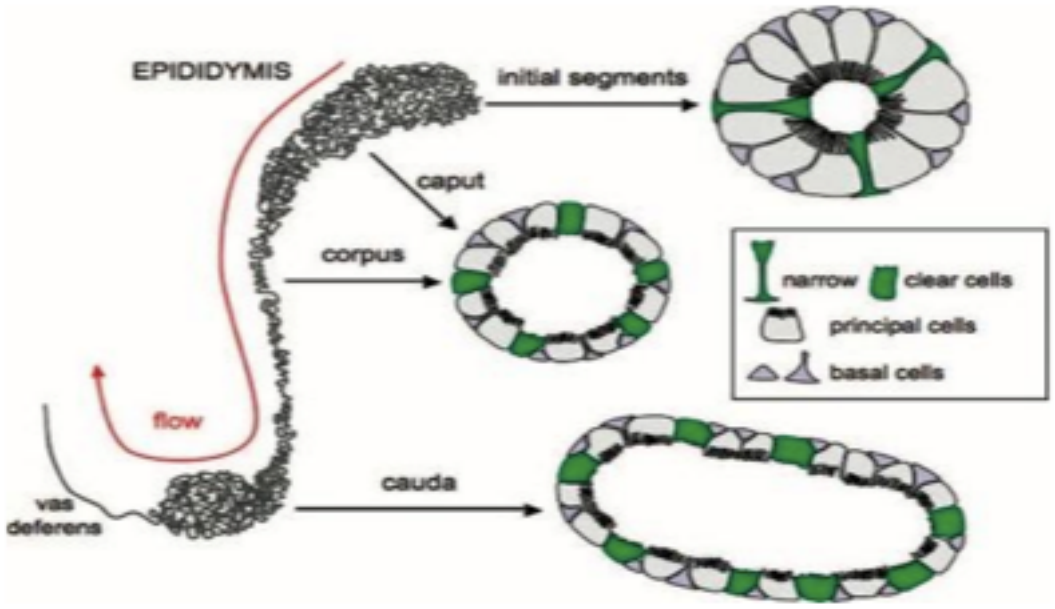


Figure 1.2. **Diagram of epididymal regions and cell types.** (Adapted from Shum, 2011).

1.3.1 Epididymosomes

In recent years, the mechanism by which sperm receive and acquire biological molecules from the epididymal environment has been elucidated. Membrane vesicles, vehicles of transmission, termed epididymosomes, are exosomal carriers responsible for the transfer of proteins and other cellular cargoes to the sperm membrane (Frenette and Sullivan, 2001; Saez et al., 2003). The DeLeon Lab has shown for the first-time that epididymosomes carry PMCA4a, Plasma Membrane Calcium ATP-ase 4a, suggesting that it is delivered to sperm and plays a vital role in sperm maturation and function (Patel et al., 2013). The Lab has also shown the mechanism by which epididymosomes and other reproductive exosomes deliver their cargo to sperm (Al-Dossary et al., 2015).

Recently, it has been shown that in addition to proteins, small RNAs are transferred to maturing sperm in the epididymis (Sharma et al., 2016). These investigators have also revealed a major connection between parental diet and the sperm epigenome and the role that the epididymis plays in these changes.

1.4 Sperm Motility

The structure of sperm is indicative of its primary functions, which are to 1) travel to the fertilization site and 2) deliver the genome to the oocyte. Thus, motility is of vital importance in order for the sperm to accomplish its role. The flagellum of a sperm exhibits the characteristic 9+ 2 microtubule pattern in which two central singlet microtubules are encircled by nine outer doublet microtubules. The bending or movement of the flagella is a result of dynein arms, which enable the sliding between pairs of doublet microtubules. Dynein is powered by ATP hydrolysis via dynein ATP-ase. There are two sources of the ATP in sperm, oxidative phosphorylation in the mitochondria of the midpiece and glycolysis on the principal piece (Tourmente et al., 2015).

Sperm cells exhibit two types of motility: progressive and hyperactivated motility (Figure 1.3). Progressive motility, observed in freshly ejaculated sperm, is defined by a low amplitude waveform that drives the sperm in a forward direction. Hyperactivated motility is most commonly seen in sperm exposed to the female environment and activated by high $[Ca^{2+}]_i$ (Ho et al., 2002; Ho and Suarez, 2003). It is characterized by high-energy requirements and high amplitude movement, which results in irregular beating that aids not only in the propulsion of sperm, but also their

release from the crevasses in the lining of the epithelium throughout the female tract. Hyperactivated motility is therefore a vital component of the fertilization process.

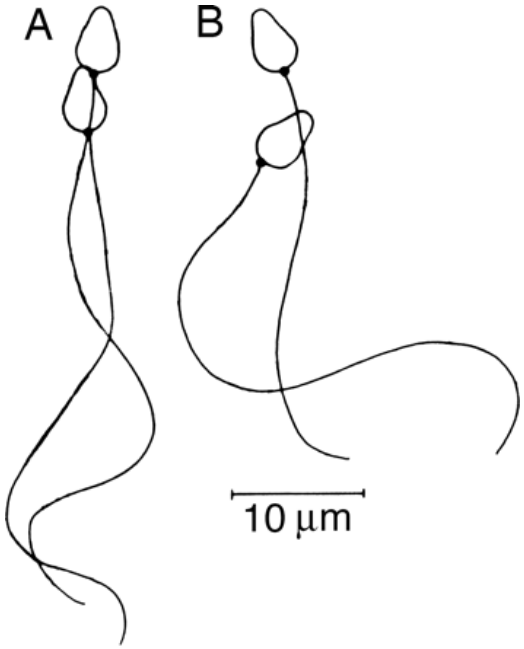


Figure 1.3 **Schematic representation of the two types of sperm motility.** (A) Progressive motility moves sperm in a straight line and (B) hyperactivated motility is high powered to allow sperm to traverse the female tract. (Adapted from Morales et al., 1988).

1.5 Role of Calcium in Sperm Function

Calcium is a major regulator in sperm function and plays a critical role in various processes that are necessary for fertilization: particularly capacitation, hyperactivated motility and acrosome reaction (de Lamirande et al., 1997). Capacitation is the final maturation stage that sperm undergo in the female reproductive tract in order to gain the ability to fertilize the oocyte (Jimenez-Gonzalez et al., 2006). This process results in modifications to the sperm structure including the

head, membrane, cytosol, and cytoskeleton. Changes in the biochemical gradients are responsible for these modifications that occur in the cell. Ion fluxes, particularly that of calcium, are responsible for the switch in motility seen in sperm in the female tract as well as the acrosome reaction (Breitbart, 2002). This results in the release of the acrosomal degradation enzymes necessary for breakdown of the zona pellucida surrounding the oocyte (Jimenez-Gonzalez et al., 2006).

The regulation of calcium in sperm is of utmost importance and optimal levels are controlled via homeostatic mechanisms to return cytosolic Ca^{2+} to its resting level (50-100 nM)(Herrick et al., 2005). In order for sperm to regulate calcium entry and removal there is a need for specific calcium influx and efflux channels. In these cells, the influx of calcium is controlled by cyclic nucleotide-gated channels, CatSpers [voltage operated calcium channels (VOCC)] and store-operated channels (SOCs)(Jimenez-Gonzalez et al., 2006; Qi et al., 2007). While calcium influx is important to sperm function, efflux is of equal importance, yet less studied in sperm.

Calcium clearance is regulated via sodium-calcium exchange (NCX), mitochondrial calcium uniporter (MCU) and Plasma membrane calcium ATPase 4 (PMCA4), with PMCA4 being the most efficient calcium extrusion pump found in sperm (Wennemuth et al., 2003; Jimenez-Gonzalez et al., 2006).

1.6 PMCA4 and Its Role in Fertility

PMCAs, P-type ATPases, have been a primary focus in the DeLeon Lab in recent times. PMCA4 is a major calcium efflux pump and intracellular pH regulator in murine sperm (Wennemuth et al., 2003; Di Leva et al., 2008). This pump is expressed in all eukaryotic cells and plays vital roles in cardiac and neuronal tissues. The PMCA family (PMCAs 1-4) consists of varying pumps each encoded by one of 4 genes, with

PMCA1 and 4 being ubiquitously expressed (Strehler et al., 2007; Di Leva et al., 2008). PMCA4 (variants "a" and "b") has been found to be the major PMCA isoform in the testis, accounting for more than 90% of the PMCAs (Okunade et al. 2004).

The DeLeon Lab has also identified PMCA4 variants in the three regions of the murine epididymis: caput, corpus, and cauda where they are secreted in the luminal fluid and transferred to sperm during epididymal maturation (Patel et al., 2013). PMCA4 has been localized in murine sperm to the proximal principal piece and acrosomal cap (Aravindan et al., 2012) and in human sperm over the acrosome, the midpiece and the proximal principal piece (Andrews et al., 2015). In the absence of PMCA4 in murine sperm there is loss of motility and consequently, infertility (Okunade et al., 2004; Schuh et al., 2004), revealing that PMCA4 is an absolute requirement for male fertility.

Structurally, PMCA4 is a ten-pass transmembrane protein (Figure 1.4), with two cytosolic loops and a regulatory C-terminal domain that allows for interaction with various partners (Strehler et al., 2007). Functionally, PMCA4 works to maintain cytosolic Ca^{2+} below 300 nm, between 50- 100 nM, and has a three times higher affinity for Ca^{2+} than NCX or MCU (Wennemuth et al., 2003; Herrick et al., 2005). During periods of resting cytoplasmic Ca^{2+}, PMCA exists in an auto-inhibited state in which the calmodulin-binding domain interacts with the catalytic domain on the cytosolic loops, rendering the active site inaccessible to calcium or ATP. During periods of elevated Ca^{2+}, such as capacitation, calcium will bind to calmodulin, displacing it from the catalytic domain and activating the pump. This activation is responsible for controlling intracellular Ca^{2+} ($[Ca^{2+}]_i$) as well as intracellular pH, via the counter-transport of protons with Ca^{2+} (Di Leva et al., 2008).

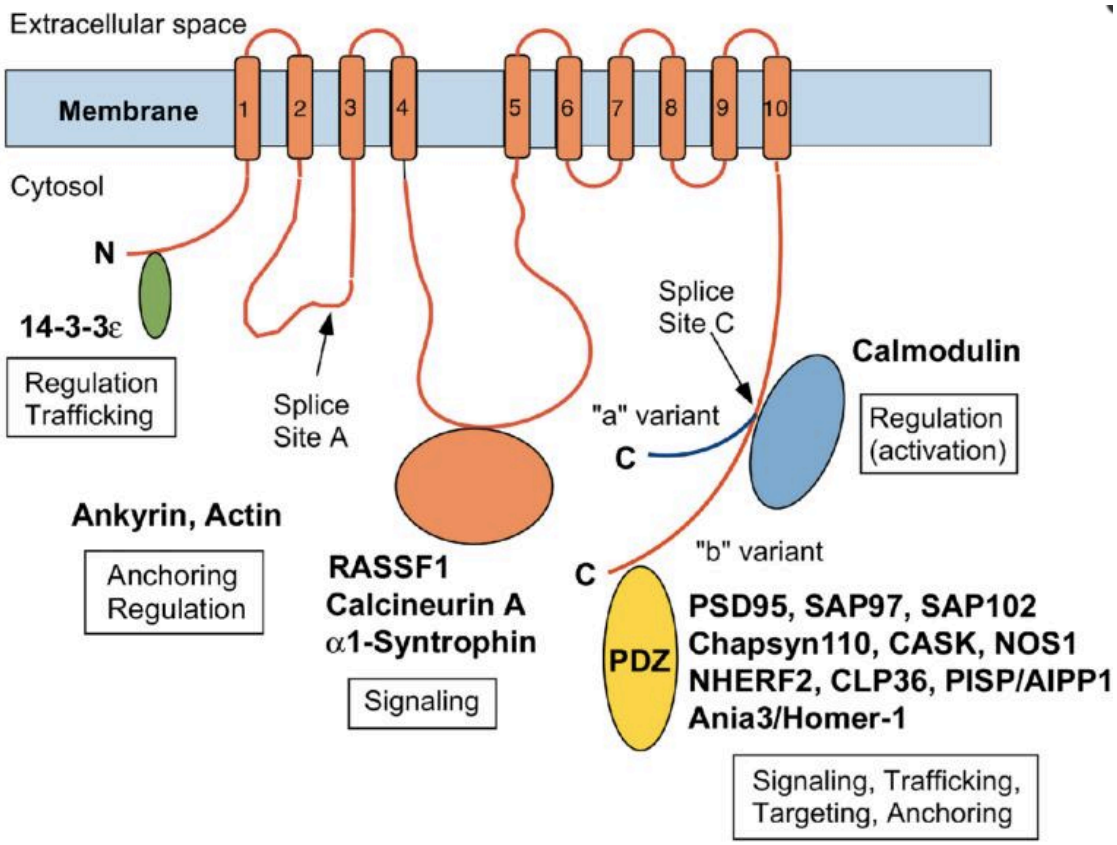


Figure 1.4 **Structure of Plasma Membrane Calcium ATPase4.** Interacting partners and their associated domains are pictured included the catalytic site (found in the large cytoplasmic loop between membrane passes 4 and 5), necessary for regulating ATP generation and the PDZ domain, necessary for signaling. (Adapted from Shrehler, 2013).

1.6.1 PMCA4's Absence and the Mechanism of Motility Loss-Oxidative Stress

It has been shown that when PMCA4 is deleted, male mice are infertile due to loss of hyperactivated sperm motility (Okunade et al., 2004). However, the mechanism by which this loss occurs has yet to be confirmed. By studying PMCA4's interactions with various partners, the DeLeon Lab has begun to piece together a mechanism for the loss of sperm motility in *Pmca4* KOs. Major interacting partners of PMCAs are the nitric oxide synthases (neuronal NOS, nNOS and endothelial NO, eNOS), both of

which are activated by calcium (Knowles and Moncada, 1994) and CASK (Ca^{2+}/calmodulin-dependent serine kinase). PMCA4 is known to regulate nitric oxide levels in cells by interacting with eNOS via the proximal part of the PMCA4b catalytic domain (Holton et al., 2010) and with nNOS via its PDZ-binging ligand (Schuh et al., 2001). Through these interactions, PMCA4 has been shown to negatively regulate NO activity (Holton et al., 2010).

In sperm, NO plays major roles in multiple processes necessary for fertilization including motility, capacitation, acrosome reaction, and binding to the zona pellucida (Herrero and Gagnon, 2001). In a similar manner, peroxynitrite [($OONO^-$), which is formed by the reaction between NO and superoxide (O_2^-)], at optimal levels, performs a role in capacitation and motility (Herrero et al., 2001). The DeLeon Lab has shown that *Pmca4* KO sperm have elevated levels of nitric oxide and peroxynitrite, as well as increased germ cell apoptosis resulting from oxidative stress (Olli et al., *in revision*)

Based on the findings above, the DeLeon Lab has proposed a mechanism where PMCA4's absence leads to cellular injury via oxidative stress (Fig. 1.5). When PMCA4 is absent there is a lack of Ca^{2+} extrusion from the sperm membrane. As PMCA4 negatively regulates the activity of the NOSs, its absence leads to upregulation of nitric oxide synthases, which in turn release more nitric oxide.

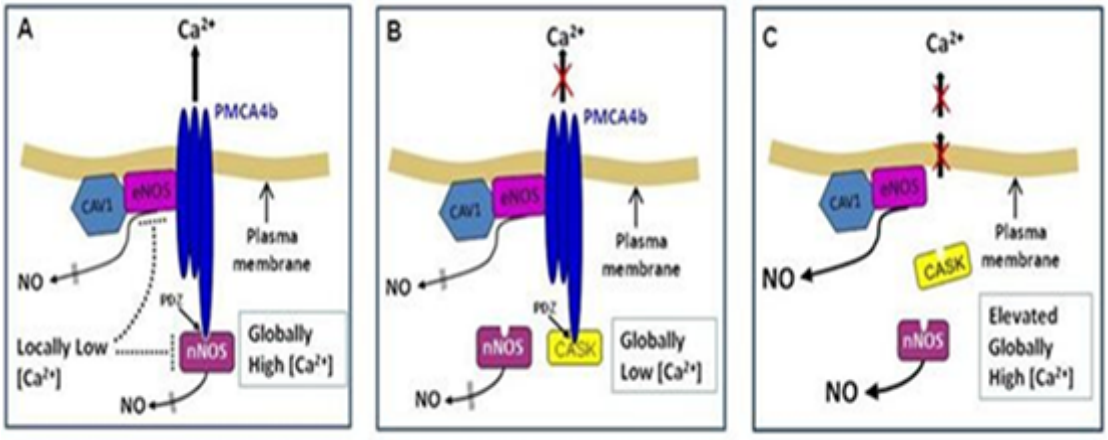


Figure 1.5. **Proposed DeLeon Lab model of PMCA4's regulation of interacting partners.** (A) When bound to PMCA4, eNOS and nNOS are in a microenvironment with locally low $[Ca^{2+}]_i$ and thus are inhibited when $[Ca^{2+}]_i$ is globally high. (B) In globally low $[Ca^{2+}]_i$ environments, CASK maintains the inactivation of PMCA4 (Aravindan et al., 2012). (C) When PMCA4 is absent, Ca^{2+} is not extruded from the membrane, in turn, NOS activity is not regulated and NO production is elevated.

Oxidation of polyunsaturated fatty acids (PUFA), a key component of the sperm plasma membrane, occurs via elevated levels of NO and $ONOO^-$ in the absence of NOS regulation by PMCA4 and, in turn, lipid peroxidation is initiated (Aitken, 1995; Zalata et al., 1998). Lipid peroxidation is a central mechanism in sperm motility loss (Hellstrom et al., 1994; Weinberg et al., 1995). This increase in reactive oxygen species overwhelms the antioxidant protection mechanisms and propagates a lipid peroxidation cascade in the sperm membrane.

1.7 Epigenetics and Its Role in Fertility

In addition to genetic factors, there is growing evidence that lifestyle choices and environment play a role in fertility. This has generated a high degree of interest in the reproductive field. Epigenetics, or the study of factors 'above the genome' and their effects on offspring health has revealed the potential dangers of lifestyle decisions for good health. Epigenetic changes and the processes that cause these changes demonstrate how modifications to the gamete or embryo can be made independently of its genetic sequence. Briefly, the biological factors responsible for these changes include, and are not limited to, DNA methylation, histone modification and RNA-associated silencing (Egger et al., 2004). In regions of high DNA methylation, a particular gene is inactivated due to its inaccessibility to DNA machinery (Egger et al., 2004). Histone modification includes the addition of either a methyl or acetyl group to the histone itself. Whether a methyl or an acetyl group is added to the histone determines the expression of the DNA coiled around the protein. Finally, RNA-associated silencing involves the inactivation of genes via RNA interference (RNAi), which leads to the formation of highly condensed chromatin (Egger et al., 2004). Disorders stemming from epigenetic changes have been seen in cancer, mental retardation, and anemia (Egger et al., 2004).

Despite its prevalence in concerns related to health, epigenetic changes play pivotal roles in organismal development. Epigenetic reprogramming is a natural process that involves the remodeling of certain epigenetic features like methylation of DNA that creates a blank epigenetic state of the embryo so that it is free to develop into various cell types (Bintu et al., 2016). However, in cases where erasure is interrupted, epigenetic reprogramming of these germ cells has been shown to result in low sperm motility, concentration, and morphology (Houshdaran et al., 2007). It is

important to understand both the physiological and environmentally-derived causes of epigenetic changes and their effects on germ cells. This project aims to shed some light on environmentally-induced epigenetic changes.

1.7.1 Epigenetics and Diet

The interaction of diet and overall health has been of increasing concern in the past decades. A western style diet, one that is heavy in processed food, sugar, and refined grains, has become a major focus (Hu, 2002), and has been shown to be associated with reduced sperm function, particularly motility, vitality, and morphology in both humans and mice (Bakos, et al., 2010; Palmer, et al., 2012; Robbins et al., 2012). In addition, in males, this diet is known to lead to obesity and subsequently DNA damage in germ cells, which lowers rates of fertilization (Bakos, et al., 2010; Palmer, et al., 2012) and impairs the health of the fetus and offspring (Bakos, et al., 2011; Fullerton et al., 2012). Increased oxidative stress has been identified as the major cause of reduced sperm function in mice consuming a high-fat western style diet (Bakos et al., 2010). Therefore, methods to counteract the negative effects of a western style diet are currently being implemented.

In 2012, a randomized control trial in 117 healthy men, ages 21-35, found that the addition of 75 g of walnuts to their regular diet for a period of 12 weeks significantly improved sperm vitality, motility, and morphology (Robbins et al., 2012). Walnuts, a balanced whole food, are high in polyunsaturated fatty acids (PUFA), much like the sperm plasma membrane, as well as antioxidants. PUFA play key roles in steroid metabolism and membrane fluidity necessary for sperm motility and oocyte fusion. However, high levels of PUFA in sperm render them susceptible to ROS-induced damage (Alvarez and Storey, 1995; Griveau and LeLannou, 1997). In

turn, sperm, which are low in scavenging enzymes (de Laminarde et al., 1995), rely on antioxidants found in seminal fluid for protection from oxidative stress that they encounter in the uterus. Therefore, as previously reported, the addition of walnuts to healthy men positively impacted sperm health via the delivery of PUFA and antioxidants (Robbins et al., 2012).

It is important to confirm these findings in a controlled animal model where the effects of a walnut supplement can be directly studied in relation to male fertility. Further, the major contents of a walnut-enriched diet, abundance of PUFA and antioxidants, could be particularly useful to an animal model in which sperm quality is defective. As *Pmca4* KO males are infertile due to poor sperm quality, specifically motility loss, it is meaningful to determine how their phenotype could be impacted by a walnut-enriched diet. It is also important to determine the impact of the diet on the epididymis where sperm mature and are stored, as this organ has recently been shown to transfer RNA to sperm and to mediate dietary effects on the sperm epigenome (Sharma et al., 2016).

1.8 Project Hypothesis and Aims

Based on the evidence provided above, the goals of this project are to 1) investigate the effects of a diet enriched in PUFAs and antioxidants on murine sperm parameters and on the epididymis where sperm mature and 2) determine if this diet is capable of rescuing sperm with defective function resulting from *Pmca4* deletion while exploring the potential mechanism of their loss of motility. The overall hypothesis is that a walnut supplement enriched in PUFAs and antioxidants could work to replenish sperm and epididymis damage resulting from a genetic deletion that causes increased oxidative stress and low motility.

1.8.1 **Aim 1:** A diet enriched in PUFAs and antioxidants administered for 9-11 weeks will positively impact sperm parameters in the murine model.

1.8.1.1 **Sub-Aim 1:** Determine motility characteristics of sperm in both uncapacitated and capacitated conditions.

1.8.1.2 **Sub-Aim 2:** Analyze effects of diet on sperm membrane quality through Eosin-Nigrosin staining and Coomassie Blue staining of morphology.

1.8.2 **Aim 2:** The diet will improve parameters of sperm damaged by the loss of *Pmca4*.

1.8.2.1 **Sub-Aim 1:** Determine the effects of this diet on sperm vitality, motility, acrosome reaction, morphology, and ATP levels.

1.8.2.2 **Sub-Aim 2:** Determine if lipid peroxidation is involved in the mechanism for motility loss in *Pmca4* sperm using thiobarbituric acid reactive substances (TBARS) and analyze the effects of oxidative stress on germ cell apoptosis in testes.

1.8.3 **Aim 3:** Increased oxidative stress initiated by *Pmca4* deletion and deregulation of the NOSs will be reflected in altered epididymal cell morphology.

1.8.3.1 **Sub-Aim 1:** Perform histological analysis of epididymal regions, using Hematoxylin & Eosin staining in sexually mature WT and *Pmca4* KO mice.

1.8.3.2 **Sub-Aim 2:** Determine if the diet impacts epididymal histology.

Chapter 2

MATERIALS AND METHODS

2.1 Animals and Reagents

Sexually mature male mice of the FVBN background were used in the investigations. *Pmca4* KO males were generated from matings of *Pmca4* $^{+/-}$ donated by the Shull laboratory (Okunade et al., 2004). The breeding and genotyping of these mice were described previously (Okunade et al., 2004). Studies were approved by the Institutional Animal Care and Use Committee at the University of Delaware and agree with the Guide for the Care and Use of Laboratory Animals published by the National Research Council of the National Academies, 8th ed., Washington, D.C. (pub. 85-23, revised 2011). All chemicals were purchased from Fisher Scientific Co. (Malvern, PA), Sigma (St. Louis, MO) or Invitrogen (Carlsbad, CA), unless otherwise specified.

2.2 Dietary Intervention

Groups of *Pmca4* WT and KO males were randomly assigned a control diet or a parallel diet to Robbins et al. (2012), in which walnuts consisted of 19.6% caloric intake (PMI Nutrition, Shoreview, MN) over a 9-11 week period. The choice of this percentage of walnuts for this study was based on a proportional scale where the amount of walnuts is equivalent to that used in the human trial, 75 g/day. Robbins et al., from previous studies, determined that this value exists within a range where blood lipid levels are affected but weight is not.

2.3 Caudal Sperm Retrieval and Evaluation

Following the 9-11 week period, males were sacrificed via CO_2 asphyxiation and the caudal epididymides were removed and placed in 400 µl pre-warmed PBS. The tissue was minced and incubated at 37°C for 10 min to release the sperm. The sperm were separated from the tissue by gravity settling. An aliquot of the sperm suspension was used to assess motility and concentration, using a hemocytometer. The remaining sperm were washed twice in PBS and recovered by centrifugation at 500 x *g* for 15 min. The pellet was reconstituted in Human Tubal Fluid (HTF, Invitrocare, Frederick, MD), a calcium-enriched capacitating medium and incubated at 37°C for 90 min to affect capacitation (Cap) or used as uncapacitated (Uncap) sperm for further experimentation.

Sperm motility was determined by visual estimation using AmScope (Irvine, CA) and expressed as a percentage of motile sperm in a given sample for uncap and cap sperm. A drop of 2 µl of sperm was loaded into 20 µm deep disposable chambers (Microtool-20-4, Cytonix, Beltsville, MD), which prevent sperm adhesion to the glass by a crystal clear coating (Armant and Ellis, 1995), that was pre-warmed at 37°C. The prepared slide was examined using a camera (5.0MP)[model #A3550U, Microscopenet], attached to a phase-contrast microscope. Approximately ~200 spermatozoa and a minimum of five fields were recorded from each specimen. The mean number of total motility (progressive+ no progression) as well as progressively motile sperm were recorded and expressed as a percentage of the total number of sperm.

Vitality of the caudal sperm was determined using eosin-nigrosin dye exclusion test as described (World Health Organization, 2010). A 50 µl sperm suspension was mixed with an equal volume of eosin-nigrosin staining solution (0.67 g eosin Y and 0.9 g NaCl were mixed in 100 ml of purified water with gentle heating, and 10 g of nigrosin added, mixed, boiled, cooled and filtered) and allowed to stand for 30 sec. The suspension was quickly smeared onto a clean glass slides and allowed to air dry before examination using brightfield optics on a Zeiss microscope. The number of stained (dead) and unstained (live) sperm of at least 200 cells were counted for each of duplicate slides from each sample and percent vitality calculated.

2.4 Systemic Analysis

Body weight for individual males was measured weekly at the same time. Testes weight was also recorded and averaged between the left and right testicle.

2.5 Hyaluronic Acid-Enhanced Progesterone-Induced Acrosome Reactions

Sperm to be capacitated were collected in pre-warmed Human Tubal Fluid (HTF, Invitrocare, Frederick, MD) similar to above. Following separation by gravity settling and two washes in HTF, the sperm/HTF suspension was incubated at 37^{o}C in a water bath for 1 h. The suspension was then centrifuged at 500 x *g* for 15 min and the supernatant discarded. The sperm were treated with hyaluronic acid (HA; 100 µg/ml) in a 60x dilution of a 6 mg/ml HA solution with PBS for 30 min at 37^{o}C. Following incubation, progesterone (3.18 uM) in a 20x dilution of 6.359 x 10^{-5} mM solution in ETOH with PBS was added for 5 min at 37^{o}C. The treated sperm were centrifuged again at 500 x *g* for 15 min and the supernatant discarded. Sperm were fixed in 1 ml of 4% paraformaldehyde and stored overnight at 4^{o}C.

The fixative was removed by centrifugation and sperm were washed with 500 µl PBS, suspended, and spun at 500 x *g* for 15 min. The process was repeated two more times after which the supernatant was removed. Approximately 200 µl of sperm were dragged across a clean glass slide and allowed to air dry. Slides were stained with 0.44% Coomassie Brilliant Blue G-250 in 60% methanol-acetic acid for 10 min, rinsed in ddH_20 and sealed with Permount. Coded slides (to ensure objectivity) were analyzed microscopically for the presence or absence of the acrosomal cap in 400 sperm/sample.

To assess sperm morphology, an aliquot of sperm suspension was fixed (4% paraformaldehyde) mounted on slides and stained with Coomassie Brilliant Blue G-250 in 60% methanol-acetic acid for 10 min, rinsed in ddH_20 and sealed as above. Sperm (~400 sperm/sample) were analyzed blindly for morphological abnormalities.

2.6 Thiobarbituric Acid Reactive Substances

Lipid peroxidation levels were assessed using TBARS Assay Kit (Cayman Chemical Company, Ann Arbor, MI) and fluorometry. Briefly, caudal sperm was pelleted at 500 x *g* for 15 min after which the supernatant was removed and discarded. Approximately 180 µl RIPA buffer was added to the pellet, which was homogenized and stored overnight at 4^oC. The suspension was centrifuged at 500 x *g* for 15 min, and the supernatant collected for analysis. Lipid levels were assessed as a function of protein levels in the lysate. The protein concentration was determined using the Pierce bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockland, IL). Malondialydehyde (MDA) levels, indicative of lipid peroxidation, were analyzed according to the manufacturer's protocol and the fluorescence read at an excitation wavelength of 530 nm and an emission wavelength of 550 nm and analyzed by the

FelixGx program. A standard curve was generated using malondialdehyde. Lipid levels were normalized to 100 μg for analysis.

2.7 TUNEL Assay for the Detection of Apoptosis in Testicular Tissues

Testes from WT and *Pmca4* KO control and diet treated mice were placed into Tissue-Tek Cyromold (Sakura, Torrance, CA) with O.C.T. compound (Sakura, Torrance, CA), and placed on dry ice for 15 min before freezing at -80^{o}C. Frozen sections were prepared at -20^{o}C at 25 μm thickness. TUNEL assays were conducted on prepared frozen sections with the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Cat. # S7110, Millipore) according to the manufacturer's instructions.

Briefly, prepared slides were fixed in 1% paraformaldehyde for 10 min at RT before post-fixing in pre-cooled ethanol: acetic acid (2:1) for 5 min at -20^{o}C in a coplin jar followed by two washes in PBS for 5 min each. Following processing, slides were incubated in Tdt (terminal deoxynucleotidyl transferase) enzyme in a humidified chamber at 37^{o}C for 1 h. After washing in stop/wash buffer, slides were incubated in anti-digoxigenic conjugate for 30 min, washed in PBS, counter-stained with fluorescein and mounted with fluoro-Gel II with DAPI. Slides were stored at -20^{o}C until analyzed and imaged using a Zeiss LSM 780 confocal microscope. The number of apoptotic cells in 100 tubules total and their location were recorded blindly for each group.

2.8 Determination of Sperm ATP Levels

Adenosine 5' –triphosphate (ATP) estimation was performed for uncap and cap sperm, using a luciferase bioluminescence assay, as previously described in our lab (Aravindan et al., 2010). Samples were either assayed immediately (uncap) after

retrieval or after 90 min incubation at 37^{o}C in HTF medium (cap). ATP levels were determined by the ATP Bioluminescent Somatic Cell Assay Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Reactions were assembled in a shielded 96-well microtiter plate and read using a Glomax Multi-Modal Plate Reader with Injectors (Promega, Madison, WI).

2.9 Histological Analyses

Regions of the epididymis (caput, corpus, cauda) and testes were fixed in 10% Buffered Formalin solution. Samples were sent to the Histology Lab at the School of Agriculture where they were dehydrated, and embedded in paraffin. After processing, sections were stained with hematoxylin and eosin for histological analysis.

2.10 Mating of *Pmca4* KO Males Treated with Diet to WT Females to Assess Fertility

Two *Pmca4* KO males treated for 9-weeks with the diet high in PUFAs and antioxidants were set up in separate cages with 2 virgin WT females each. The duration of the experiment was dependent on the production of offspring. During the duration of the experiment the entire cage received the walnut-enhanced diet. If, after 5 months, no progeny were produced, the experiment was ended.

2.11 Statistical Analysis

Two-way ANOVA and Student's t-tests were performed on the means ± SEM for replicates. P-values were calculated and *$P<0.05$ was considered significant.

Chapter 3

RESULTS

3.1 Body Mass was Unaltered with Administration of the Diet

Body mass was determined by weighing the animals at precisely the same time and day for 9-weeks. The data showed that mice on the diet did not differ significantly in their body mass compared to control mice (**Fig. 3.1**). This lack of change in body mass was seen for both WT and *Pmca4* KO males. A similar lack of change in body mass was also seen in the human study (Robbins et al., 2012); and the data suggest that, with respect to calorific intake and walnut composition, the diets are parallel.

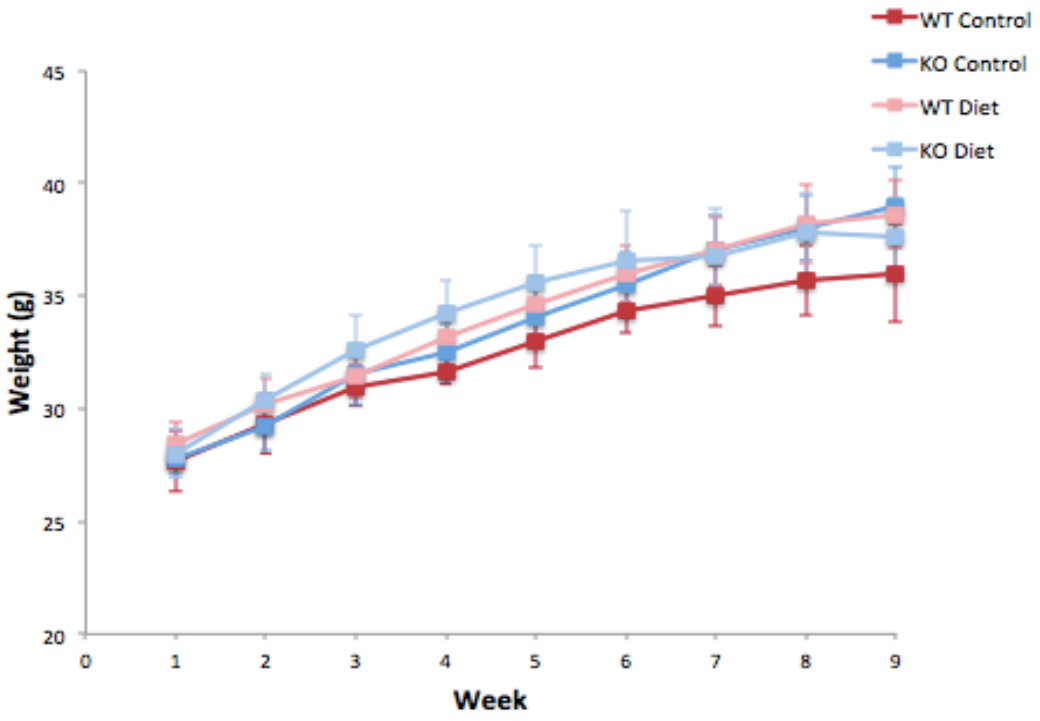


Figure 3.1. **Body weight analysis over 9-week period.** Weight (g) of WT control (N=3) and diet-treated (N=5) and *Pmca4* KO control (N=4) and diet-treated (N=5) mice over a 9-week period. No significant difference in body mass is evident between genotype or diet-treatment (Two-way ANOVA: $p>0.05$, ±SEM).

3.2 Motility Rates were Positively Impacted in WT Sperm from Males in which the Diet was Administered

When males were treated for 9-11 weeks with a walnut-enriched diet, there was a significant increase in total motility in both uncap (low Ca^{2+}) and cap (high Ca^{2+}) sperm (**Fig. 3.2A**), compared to control sperm from animals without the diet (*$p<0.05$). Total motility for cap, compared to uncap, sperm decreased significantly (***$p<0.005$), reflecting the passage of time from recovery of sperm from the animals.

Figure 3.2B illustrates that while not significant, there is an~ 2-fold increase in progressive motility between control cap and diet cap sperm as well as a slight

increase in uncap sperm. Similar to total motility, there is a significant decrease in progressive motility between uncap and cap sperm (***p<0.005), as expected for the reason mentioned above. Thus, while total motility was positively affected in WT sperm by the diet, there was no significant increase seen in progressive motility.

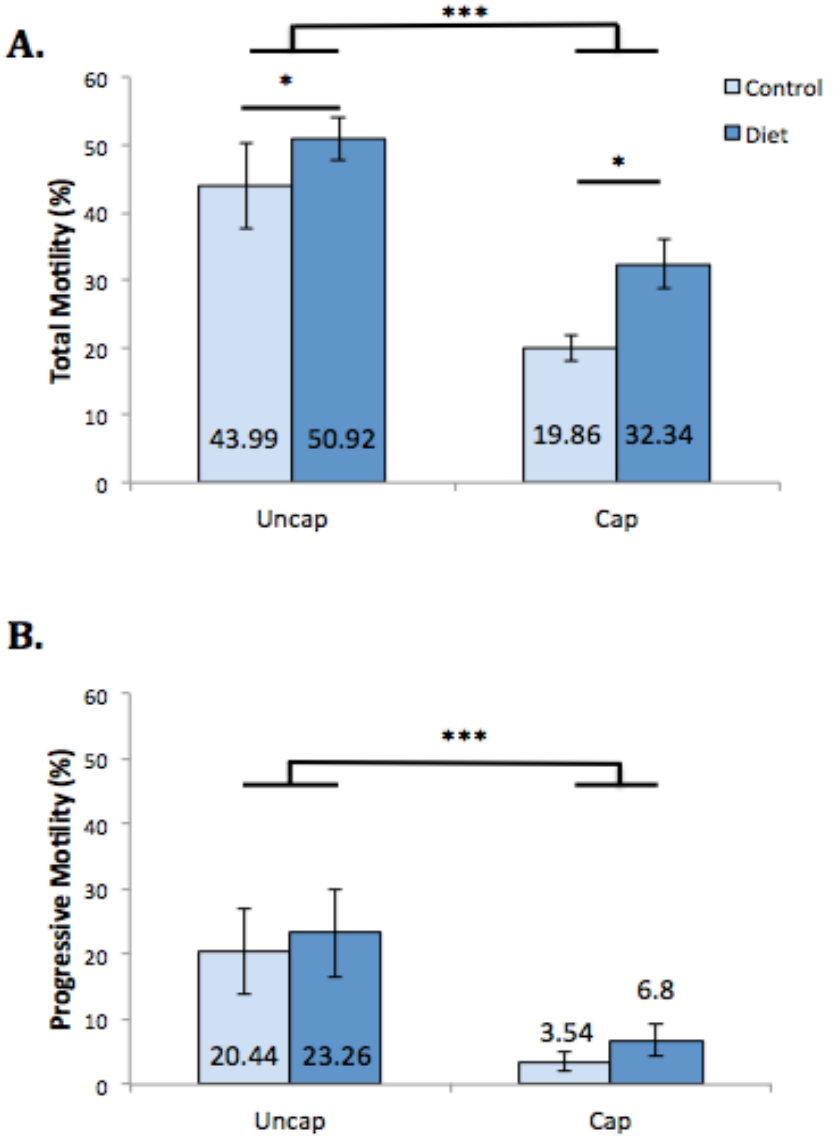


Figure 3.2. **Motility levels of WT control and diet-treated sperm.** (**A)** Average percentage total motility (non-progression + progressive) (±SEM) for WT sperm in uncap (low Ca^{2+}) and cap (high Ca^{2+}) conditions. Total motility is significantly increased in both uncap and cap conditions in sperm treated with the diet. **(B)** Average progressive motility (±SEM) in control and diet-treated WT sperm in uncap and cap conditions. A ~2-fold increase in progressive motility between control cap and diet-treated cap sperm is seen, although it is not significant. (Two-way ANOVA: *p<0.05, ***p<0.005, N=5).

3.3 Eosin-Nigrosin Staining Reveals No Change in Vitality of WT Sperm Treated with Diet

As sperm vitality levels were shown to increase when treated with a walnut-enriched diet in humans (Robbins et al., 2012), we asked if a similar increase is seen in murine sperm (**Fig. 3.3A**). Using 1X Eosin Nigrosin (EN) staining which identifies live (high membrane integrity) versus dead (low membrane integrity) sperm, it was revealed that in the murine model there was no significant difference between sperm treated with the walnut diet versus the control (**Fig. 3.3B**). While this suggests that there is no difference in membrane integrity, it is possible that low sampling numbers compared to those of Robbins et al., are responsible for the lack of effect.

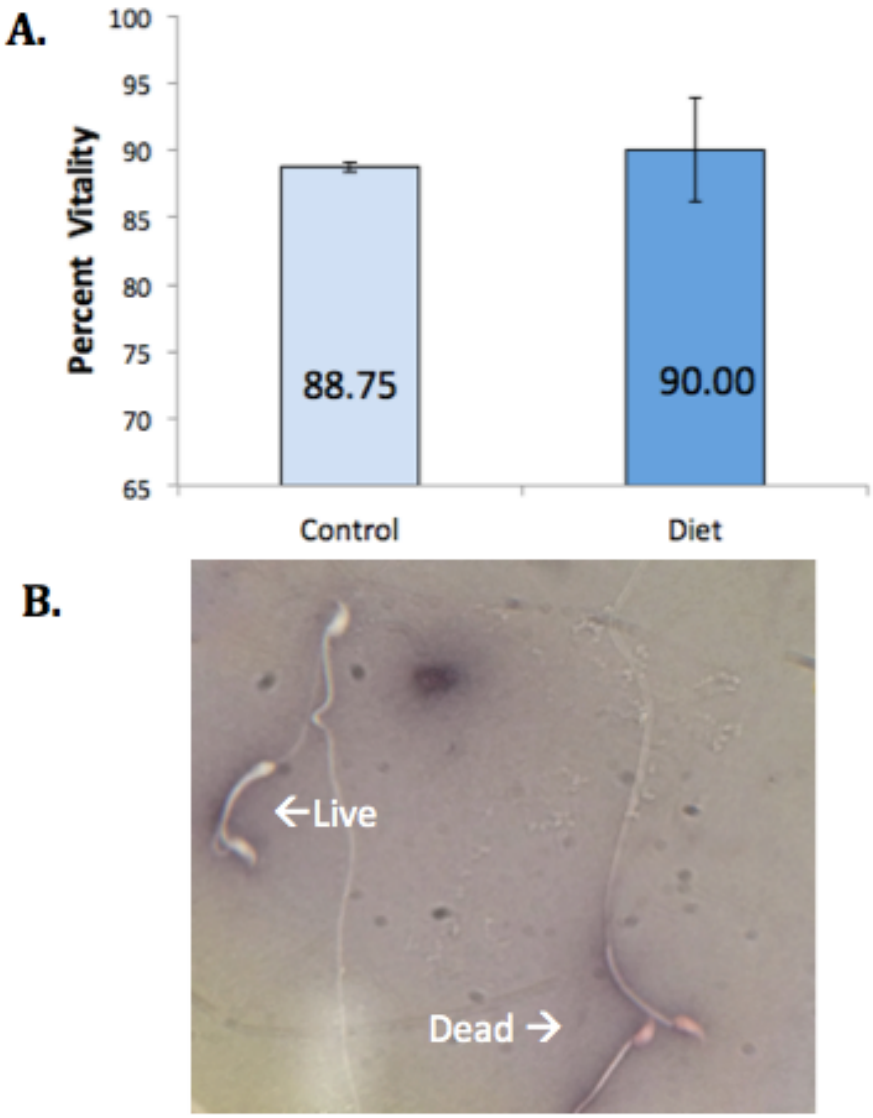


Figure 3.3. **Eosin-Nigrosin staining for vitality of WT control and diet-treated sperm**. **(A)** Average percentage of live sperm (n=400/animal) (±SEM) subjected to 1x EN staining for WT control and WT diet-treated mice. While no significant increase was seen, an increasing trend is evident. (Two-sample T-test: $p>0.05$, N=4) **(B)** Sperm were stained with 1x EN stain and identified as live or dead based on dye uptake, which is indicative of membrane quality.

3.4 Normal Sperm Morphology was Significantly Impacted by the Administration of the Diet

Sperm morphology between WT control and diet-treated sperm was studied via Coomassie Blue staining. Approximately >1600 sperm (~400/animal) were analyzed from control and diet-treated sperm on blinded slides for the presence of structural abnormalities as defined by the World Health Organization (WHO, 2010). Normal sperm morphology was significantly increased in diet-treated sperm (*$p \leq 0.05$) compared to control (**Fig. 3.4A**). Structural abnormalities detected included flagellar kinks (**Fig. 3.4B, A**), folded (**Fig. 3.4B, B-C**) and coiled (**Fig. 3.4B, D**) tails, and head deformities (**Fig. 3.4B, E**).

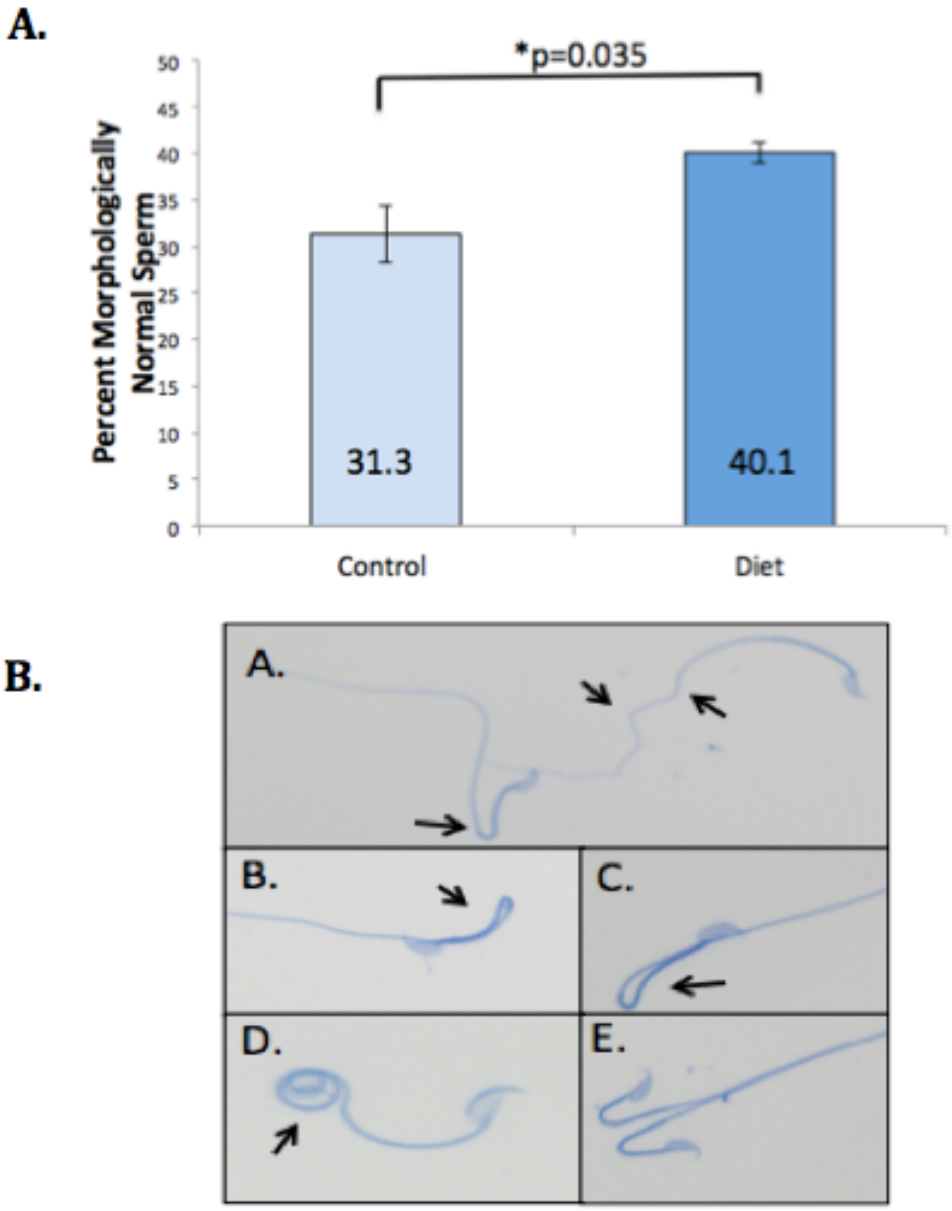


Figure 3.4. **Coomassie Blue staining for sperm morphology in WT control and diet-treated sperm (A)** Average percentage of normal sperm morphology between control and diet-treated WT sperm (n=400/animal). When treated with a diet enriched in PUFAs and antioxidants for 9-11 weeks there was a significant increase in normal sperm morphology (Two-sample T-test: *p≤0.05, SEM, N=4). **(B)** Morphological abnormalities seen via Coomassie Blue staining in control and diet-treated WT sperm. Arrows indicate kinks (A), folds (B-C), coils (D), and head deformities (E).

3.5 Characterization of *Pmca4* KO Motility and Changes Following Implementation of the Walnut-Enriched Diet

Motility levels for WT and *Pmca4* KO control sperm were characterized and were found to have no significant difference between genotypes (**Fig. 3.5A**). There was a significant decrease between capacitation conditions in total motility for both genotypes (***$p<0.005$). These data suggest that total motility is not affected by the loss of PMCA4 in null sperm, regardless of capacitating condition.

Progressive motility was analyzed separately from total motility and confirmed previous research showing loss of motility of *Pmca4* KO sperm only under capacitating conditions (Okunade et al., 2004) (**Fig. 3.5B**). A significant decrease in progressive motility is seen between capacitation conditions (***$p<0.005$).

When treated for 9-11 weeks with a walnut-enriched diet, there was no significant difference in total motility percentages between *Pmca4* KO sperm control and diet-treated sperm (**Fig. 3.6A**). Total motility decreased slightly in cap KO diet-treated sperm compared to control. Similar to that seen above, there was a significant decrease in total motility between capacitated conditions for both groups (***$p<0.005$).

Figure 3.6B shows that progressive motility in KO diet-treated sperm is unrecoverable with the diet compared to KO control sperm. There are significant decreases between capacitated states (***$p<0.005$); however, there are no increases in progressive motility in the capacitated condition for KO diet-treated sperm.

Total motility of all groups reveals significant interaction (***$p<0.005$) between genotype and diet in cap conditions (**Fig. 3.7**) and no difference in total motility levels in uncap conditions. Further analysis of cap conditions via individual two-sample T-tests reveals significant differences between WT control and diet-

treated sperm (**p<0.01) as seen previously in **Fig. 3.2A** as well as a significant difference between WT diet-treated and *Pmca4* KO diet-treated sperm (**p<0.01).

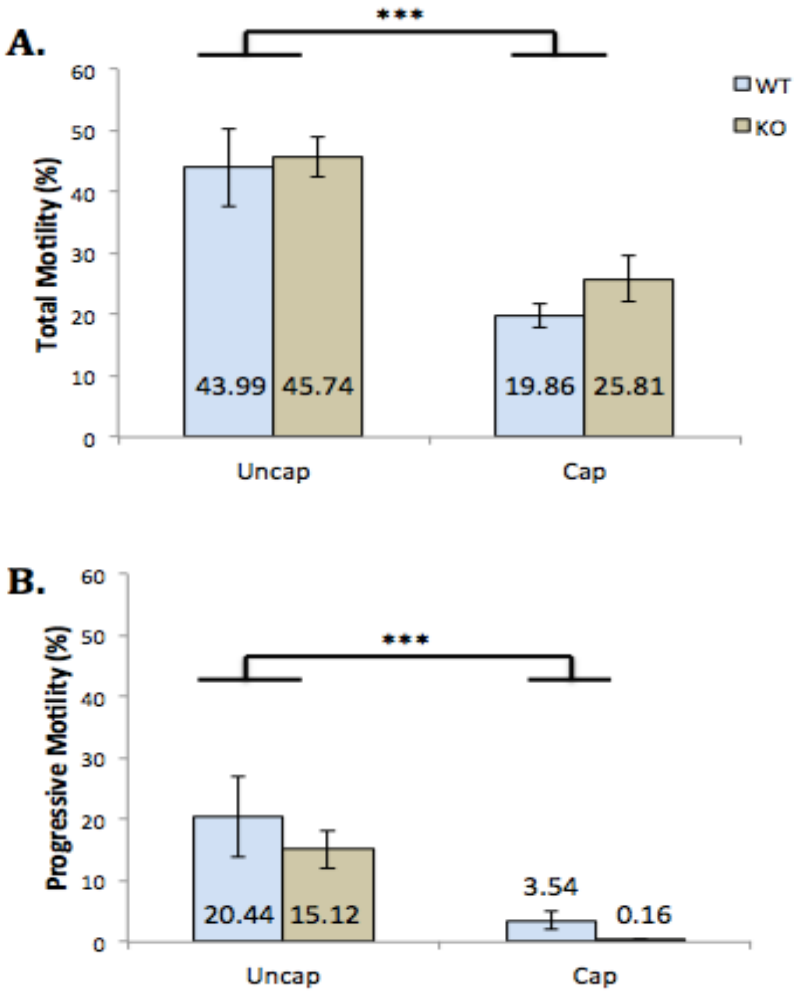


Figure 3.5. **Characterization of WT and *Pmca4* KO motility levels. (A)** Average percentage of total motility (non-progression + progressive) in WT and *Pmca4* KO sperm in uncap and cap conditions. Total motility does not vary between genotypes but is significantly decreased for both WT and KO sperm under cap conditions as expected. **(B)** Average progressive motility in WT and *Pmca4* KO sperm in uncap and cap conditions. Significant decrease between KO sperm between capacitation conditions. *Pmca4* KO sperm show limited progressive motility compared to WT in cap conditions (Two-way ANOVA: ***p<0.005, N=5, ±SEM).

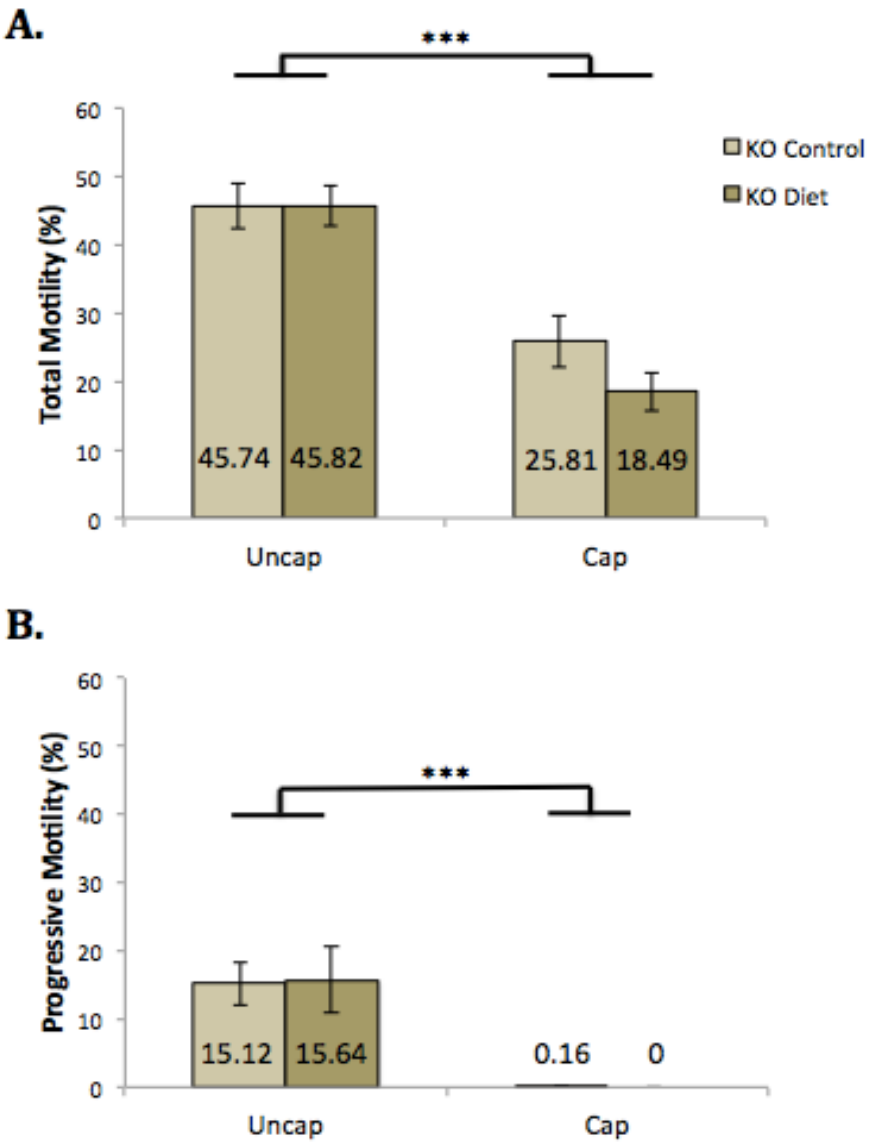


Figure 3.6. **Motility levels of *Pmca4* KO control and diet-treated sperm. (A)** Average percentage of total motility (non-progression + progressive) for *Pmca4* KO control and diet-treated sperm in uncap and cap conditions. No difference in percentage total motility between control and diet-treated KO sperm. **(B)** Average progressive motility in *Pmca4* KO control and diet-treated sperm in uncap and cap conditions. Significant differences between uncap and cap for both groups. Progressive motility in *Pmca4* KO diet-treated sperm is unrecoverable compared to KO control sperm. (Two-way ANOVA: ***$p<0.005$, N=5, ±SEM).

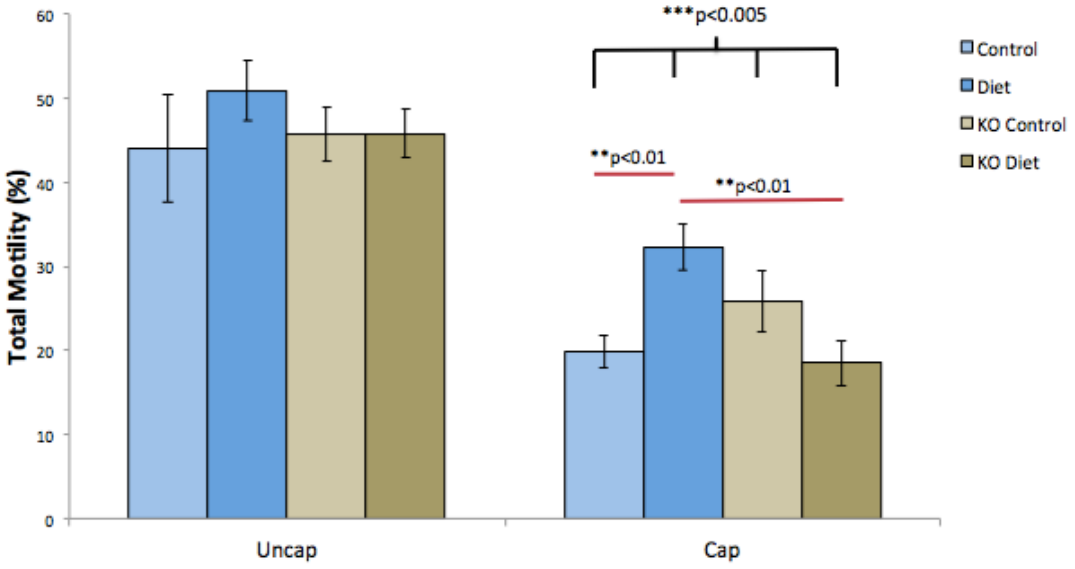


Figure 3.7. **Overall total motility levels of WT and *Pmca4* KO control and diet-treated sperm.** Average percentage of total motility (non-progression + progressive) for WT and *Pmca4* KO control and diet-treated sperm in uncap and cap conditions. No difference was seen in total motility between genotypes or diet-treatment in uncap conditions. In cap conditions, there is a significant interaction between genotype and diet (Two-way ANOVA: ***p<0.005, N=5, ±SEM). Further analysis reveals significant differences between WT control and diet-treated sperm as well as between WT diet-treated sperm and KO diet-treated sperm (Two-sample T-test: **p<0.01, N=5, ±SEM).

3.6 Eosin-Nigrosin Staining Reveals No Significant Difference in Vitality Between Sperm Genotype or Diet

As sperm vitality levels were shown to increase when treated with a walnut-enriched diet in humans (Robbins et al., 2012), we asked if a similar increase would be seen not only in WT sperm but also in *Pmca4* KO murine sperm. In **Figure 3.8** it can be seen that in caudal sperm immediately subjected to eosin-nigrosin staining (n=400/animal) and analyzed blindly, there was no significant difference in membrane quality between WT and KO in control sperm or diet-treated sperm. There is a

trending increase in both genotypes between dieting conditions; however, this increase in not significant.

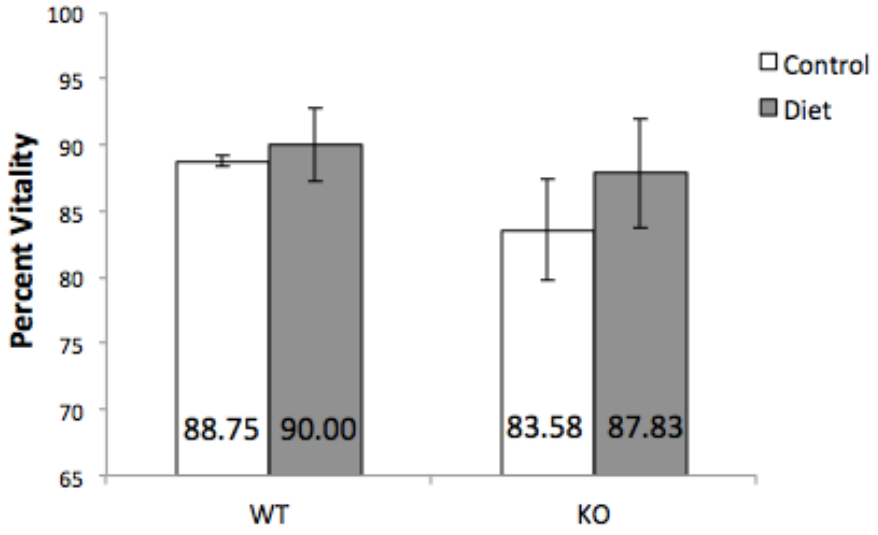


Figure 3.8. **Eosin-Nigrosin staining for vitality of WT and *Pmca4* KO control and diet-treated sperm.** Average percentage of live sperm (n=400/animal) subjected to 1x Eosin-Nigrosin staining for WT and *Pmca4* KO fed control or a diet high in PUFAs and antioxidants for a 9-11 week period. No significant difference between treatment or genotype, although a trending increase is seen between control and diet-treated sperm for both genotypes. (Two-way ANOVA: $p>0.05$, N=3, ±SEM).

3.7 The Ability to Acrosome React did not Vary between Genotypes or Treatments

An unbiased analysis of sperm on coded slides revealed that the rates of AR induced physiologically did not vary between *Pmca4* genotype or diet condition (**Fig. 3.9A**). Acrosomal status was viewed via Coomassie Blue staining for >1600 sperm (~400/animal) and determined by the absence (**Fig. 3.9B-A**.) or presence (**Fig. 3.9B-B.**) of the acrosomal cap.

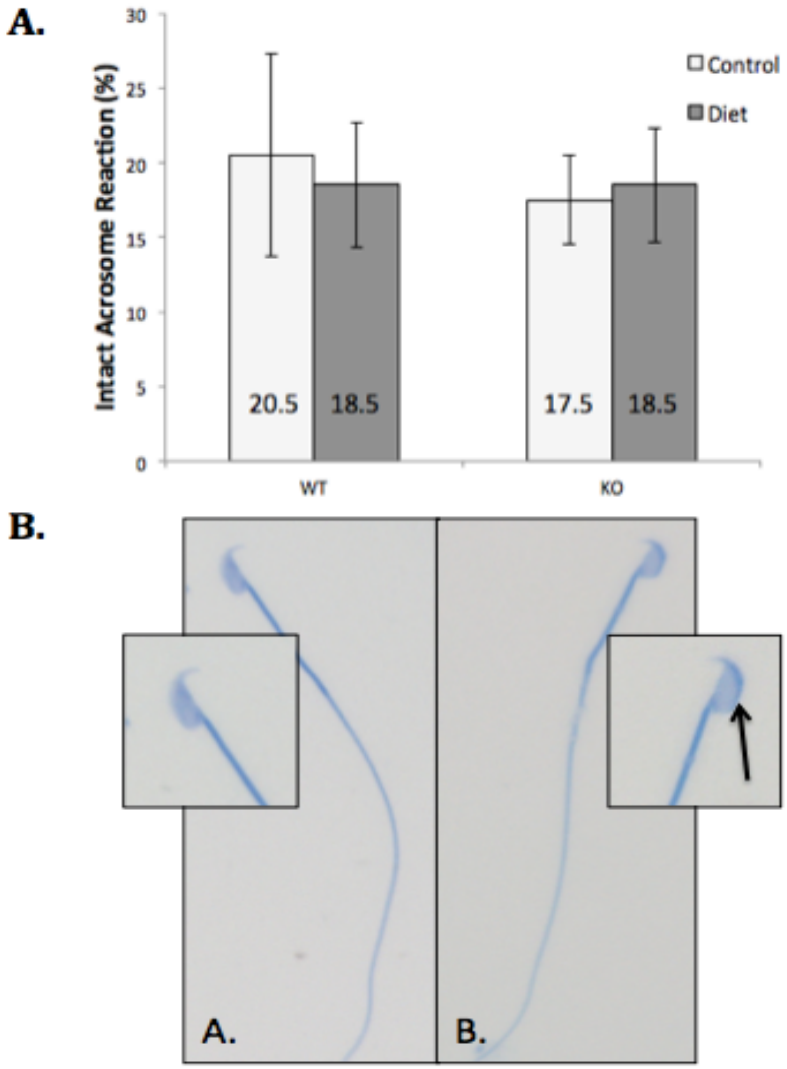


Figure 3.9. **Hyaluronic Acid-Enhanced Progesterone-Induced Acrosome Reactions in WT and *Pmca4* KO control and diet-treated sperm. (A)** Average percent of intact acrosome reacted WT and *Pmca4* KO sperm (n=400/animal) treated with a control or walnut-enriched diet for 9-11 weeks. No significant difference was seen in the ability to acrosome react between genotypes or diet type. (Two-way ANOVA: $p>0.05$, N=4, ±SEM). **(B)** Acrosomal status viewed via Coomassie Blue staining. (A) An acrosomal cap missing after AR (inset) and (B) a sperm with an intact acrosomal cap (inset-arrowed).

3.8 Normal Sperm Morphology was Significantly Impacted by the Administration of the Diet in *Pmca4* KO

The structural abnormalities described above were detected in *Pmca4* KO sperm: they included flagellar kinks (**Fig. 3.3B-A**), folded (**Fig. 3.3B-B,C**) and coiled tails (**Fig. 3.3B-D**) as well as head deformities (**Fig. 3.3B-E**). A two-way ANOVA revealed a significant relationship between genotypes (**$p<0.01$) as well as diet-types in *Pmca4* KO sperm (***$p<0.001$)(**Fig. 3.10A**). Tail abnormalities, particularly kinks, were the most common, followed by folded tails, coils, and head deformities (**Fig. 3.10B**).

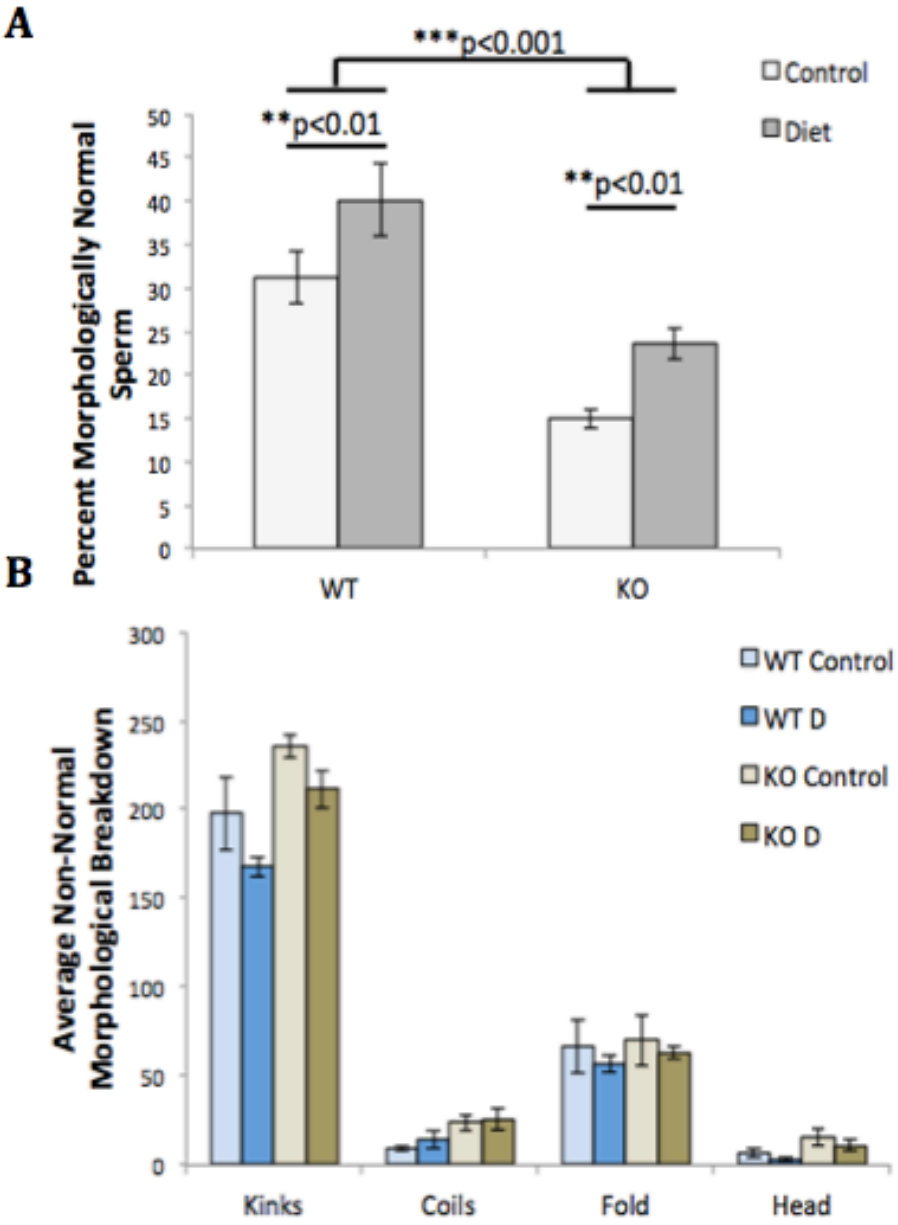


Figure 3.10. **Coomassie Blue staining for sperm morphology in WT and *Pmca4* KO control and diet-treated sperm and abnormality breakdown. (A)** Average percent of morphologically normal sperm (n=400/animal). Morphology is significantly lower in *Pmca4* KO sperm compared to WT. With the diet, morphology for both genotypes shows a significant increase. (Two-way ANOVA: **p<0.01, ***p<0.001, N=4, ±SEM**). (B)** Frequency of morphological abnormalities per genotype and diet-type. Highest occurring abnormality was sperm tail kinks.

3.9 Analysis of Sperm ATP Levels

ATP plays a major role in sperm motility as well as the activity of the *Pmca4* pump, therefore ATP levels were analyzed in both uncap and cap sperm, WT and KO, with the control or walnut-enriched diet (**Fig. 3.11**). *In situ* ATP concentrations revealed increased levels in uncap sperm versus cap for all groups, except WT control, which showed a decrease.

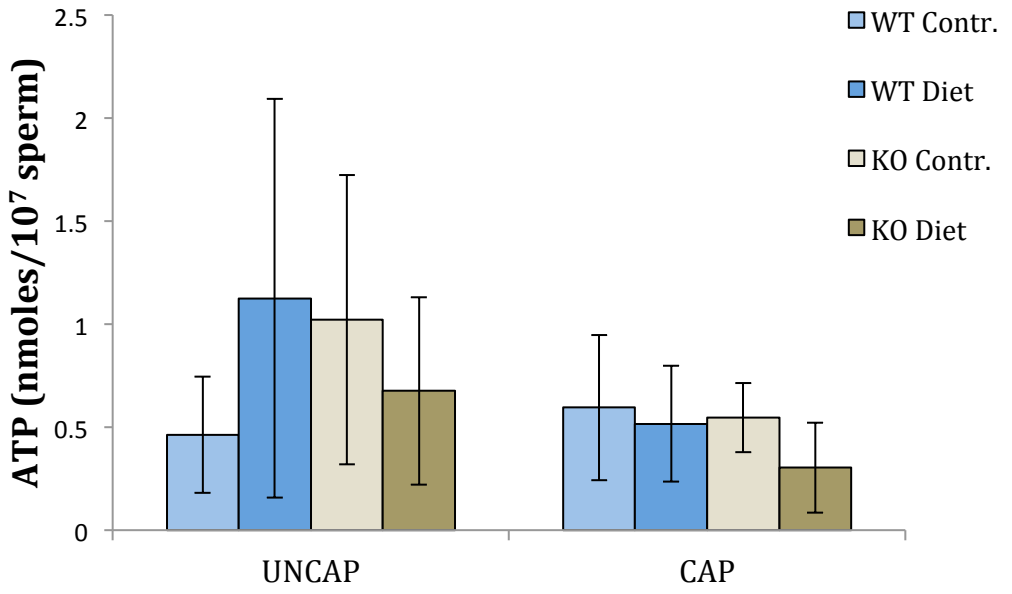


Figure 3.11. **Analysis of sperm ATP levels.** Average ATP levels (nmoles/10^7 sperm) determined via ATP Luciferase Bioluminescence Assay of *Pmca4* WT and KO control and diet-treated sperm in uncap and cap conditions. No significant difference measured between groups; however, there is a general decrease in ATP levels in cap sperm. (Two way ANOVA: N=3, ±SEM).

3.10 Assessment of Lipid Peroxidation Levels via the Thiobarbituric Acid Reactive Substances (TBARS) Assay

Lipid peroxidation levels were studied in *Pmca4* KO murine sperm under control conditions and following a 9-11 week diet enriched in PUFAs and antioxidants (**Fig. 3.12**). The TBARS assay revealed an increase in lipid peroxidation between WT and KO sperm in the control group, but this was not significant. A two-way ANOVA showed that following treatment with the diet, there was a significant decrease (~2-fold and 2.5 fold, respectively) in both WT and KO lipid peroxidation levels (*p<0.05).

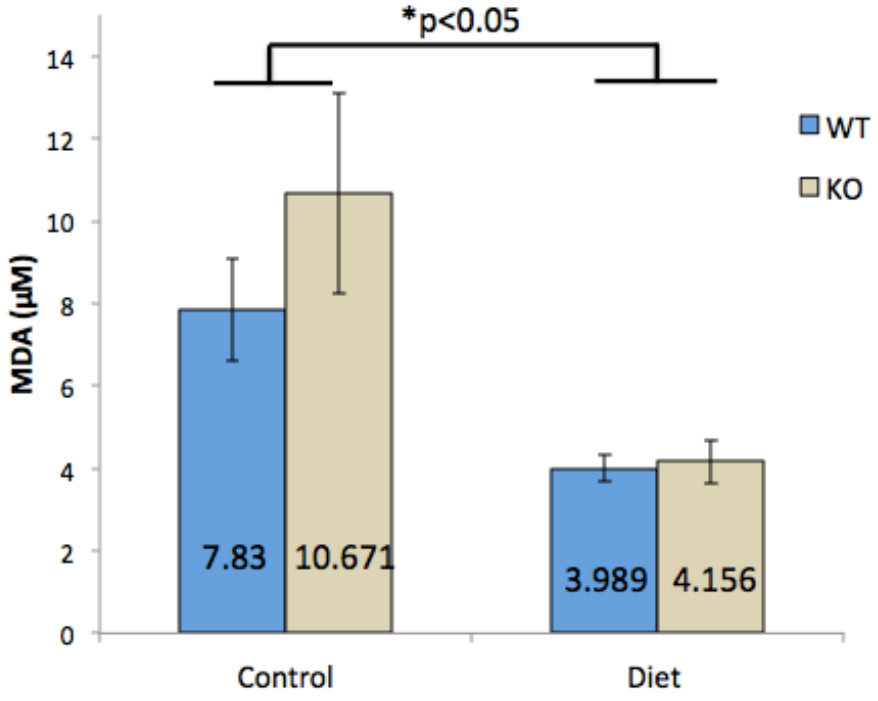


Figure 3.12. **Assessment of lipid peroxidation levels via the TBARS assay.** Average MDA (μM) levels, an indicator of lipid peroxidation, in *Pmca4* WT and KO sperm control and treated with a diet enriched in PUFAs and antioxidants for 9-11 weeks. Lipid peroxidation levels decrease in both genotypes following diet implementation. (Two-way ANOVA: *p<0.05, N=6, ±SEM).

3.11 TUNEL Assay Reveals Significant Effects of *Pmca4* Loss on Apoptosis in the Testes and a Modulation of Apoptotic Activity by the Diet

In order to determine the effects of elevated ROS, particularly $OONO^-$ that has been observed in *Pmca4* KO males (Olli et al., *in revision*), the TUNEL assay was performed on testicular sections from control WT and KO males, as well as diet-treated mice. Two-way ANOVAs were conducted separately on each cell type and revealed a significant increase in TUNEL positive cells in the *Pmca4* KO testes compared to WT, specifically in the spermatogonia (**$p<0.005$) and spermatocytes (*$p<0.05$)(**Fig. 3.13-3.14**). The increase in spermatids did not reach significance ($p=0.07$)(**Fig. 3.13-3.14**).

Once it was established that elevated levels of oxidative stress had a significant effect on germ cell apoptosis in *Pmca4* KO testes, it was necessary to determine if a diet high in PUFAs and antioxidants would work to counter this activity. The results of the two-way ANOVA revealed no significant effect of diet on the number of apoptotic cells (**Fig. 3.13**). However, the TUNEL assay did show a decrease in the amount of apoptotic cells between the KO control (KO-C) and KO diet-treated (KO-D) spermatogonia (KO-C mean=13.93, KO-D mean=11.2), spermatocytes (KO-C mean=2.63, KO-D mean=1.8) and spermatids (KO-C mean=0.96, KO-D mean=0.6).

Hematoxylin and eosin stained testes confirmed the presence of apoptotic activity in *Pmca4* KO testes (**Fig. 3.15**). Increased disorganization in the junctional complexes and darkened pyknotic cells extending to the lumen (**Fig. 3.15, B**) which, compared to controls (**Fig. 3.15, A**), were still evident in *Pmca4* KO testes treated with the diet (**Fig. 3.15, D**). However, this was not seen in WT diet-treated tubules (**Fig. 3.15, C**). Despite increased apoptosis, no difference in testis weight was evident (**Fig. 3.16**).

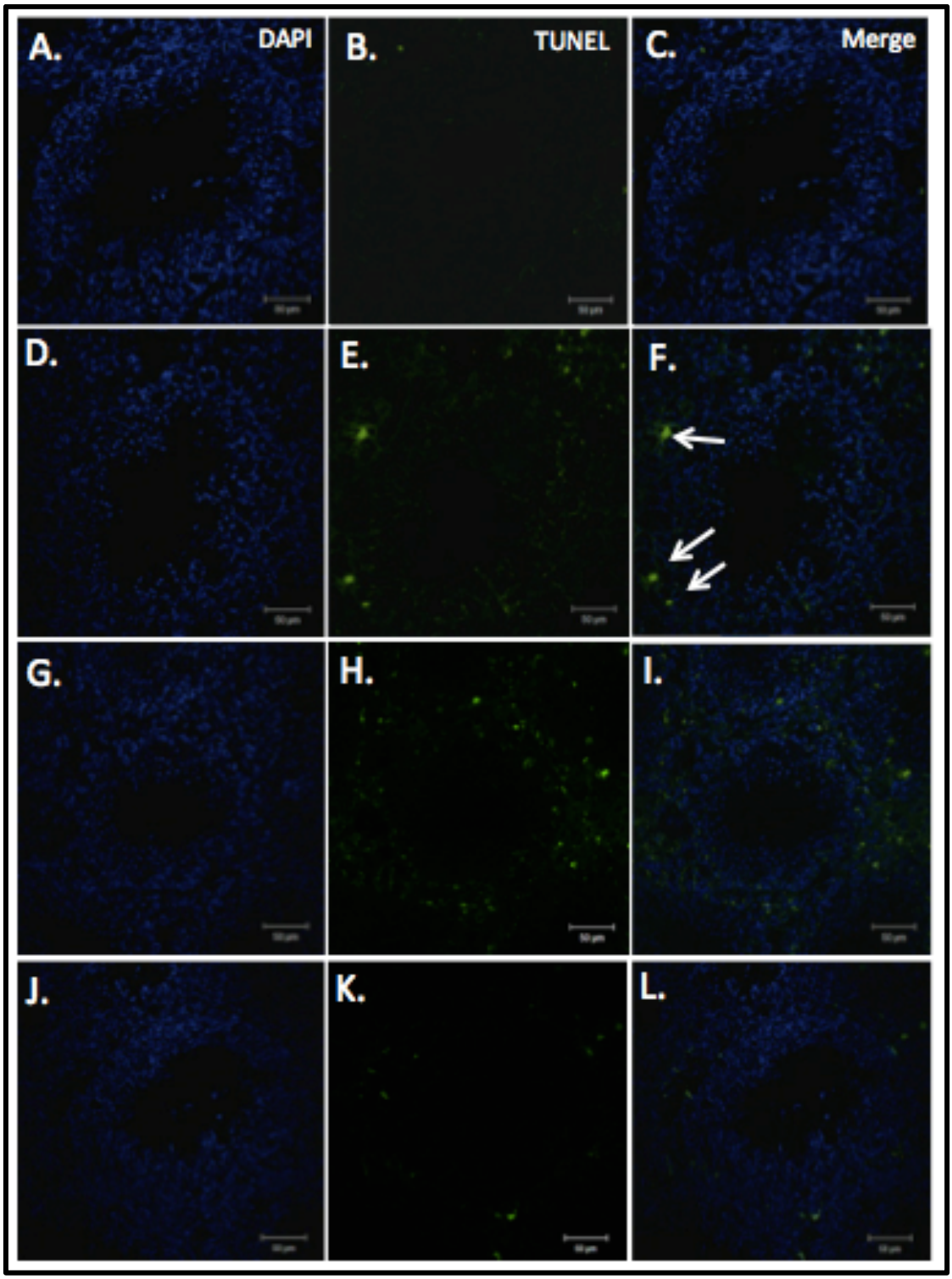

A.
DAPI
B.
TUNEL
C.
Merge
D.
E.
F.
G.
H.
I.
J.
K.
L.

Figure 3.13. **TUNEL assay reveals significant effects of *Pmca4* loss on apoptosis in testes.** TUNEL-positive cells in the testis of WT **(A-C)** and *Pmca4* KO **(D-F)** control mice. DAPI stained nuclei **(A)**, fluorescein stained apoptotic cells **(B)**, and a merge **(C)** in testes sections of WT show a low level of apoptosis. The nuclei **(D**), apoptotic cells **(E**), and merge **(F)** of testes sections of *Pmca4* KO mice show the majority of apoptotic cells in the spermatogonia (arrowed). TUNEL-positive cells in the testes of WT **(G-I)** and *Pmca4* KO **(J-L)** mice treated with a diet-enriched in PUFAs and antioxidants for a 9-11 week period. DAPI stained nuclei **(G)**, fluorescein stained apoptotic cells **(H)**, and a merge **(I)** in testes sections of WT diet-treated mice show low levels of apoptosis. The nuclei **(J)**, apoptotic cells **(K)**, and merge **(L)** of sections of *Pmca4* KO diet-treated mice show a decrease in apoptotic intensity in the germ cells. Scale bars = 50 μm.

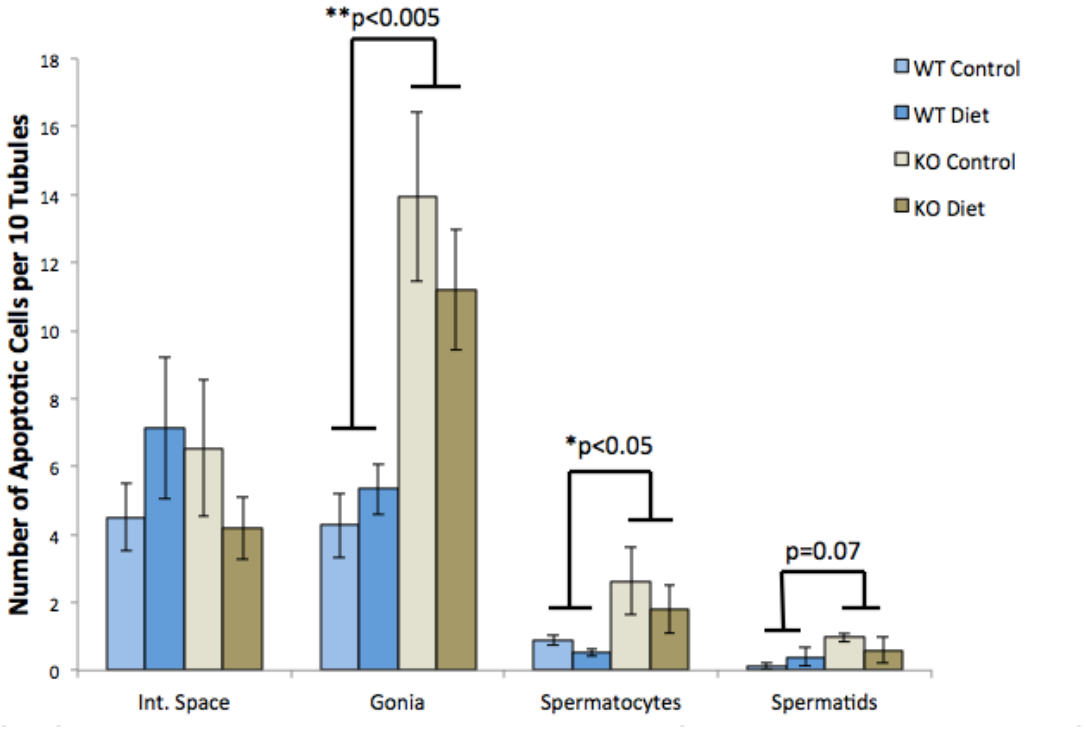


Figure 3.14. **Quantification of TUNEL results in WT and *Pmca4* control and diet-treated testes.** No significant difference is evident between genotype or diet-type in the interstitial space; however, significant differences between genotype for spermatogonia (Gonia) (**p<0.005) and spermatocytes (*p<0.05). Spermatids did not reach significance (p=0.07). Implementation of walnut-enriched diet reduced number of apoptotic cells in KO but not significantly. (Two-way ANOVA: N=3, ±SEM)

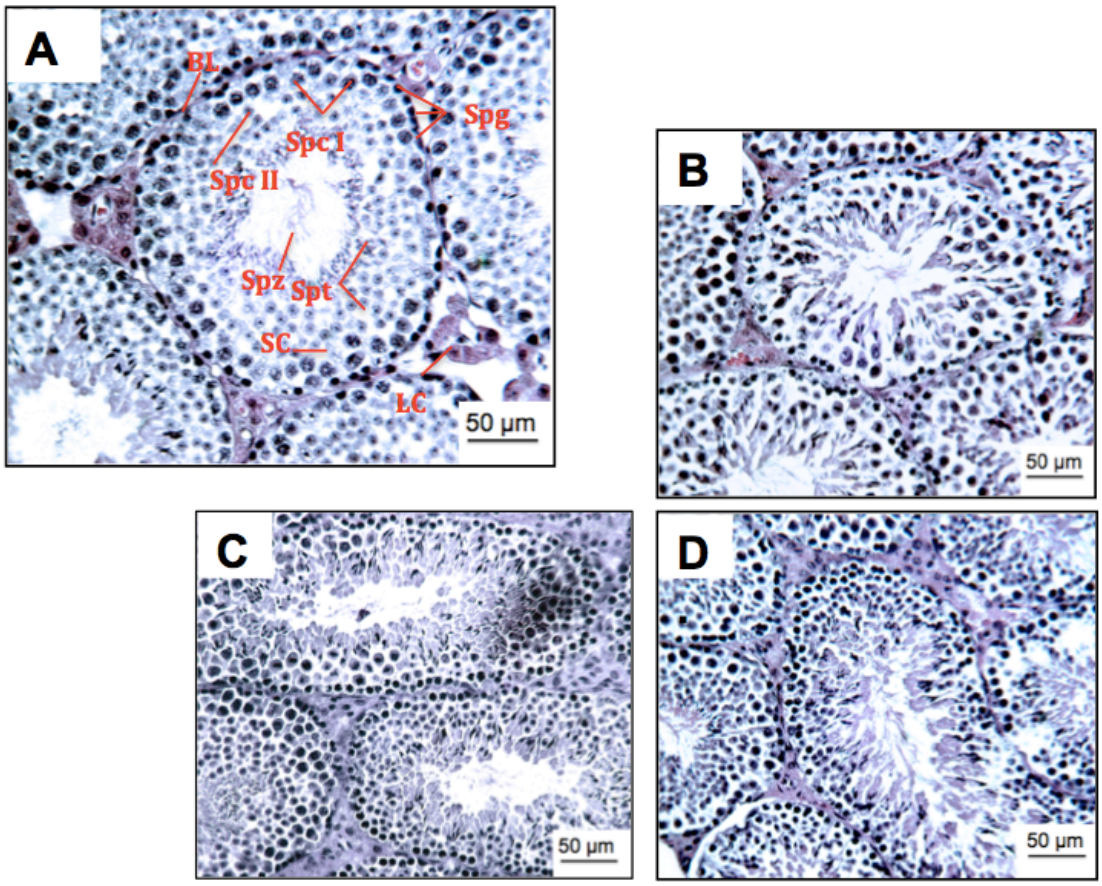


Figure 3.15. **Histological staining (H&E) of testis tissue confirms apoptotic activity in *Pmca4* KO. (A)** Tubule of *Pmca4* WT control testes noting the basal lamina (BL), spermatogonia (Spg), primary spermatocytes (Spc I), secondary spermatocytes (Spc II), spermatids (Spt), and spermatoza (Spz) in the lumen. Also labeled are leydig cells (LC) and sertoli cells (SC). **(C)** Tubules of WT diet-treated testes. Tubules of control **(B)** and diet-treated **(D)** *Pmca4* KO testes. Scale bar = 50 µm.

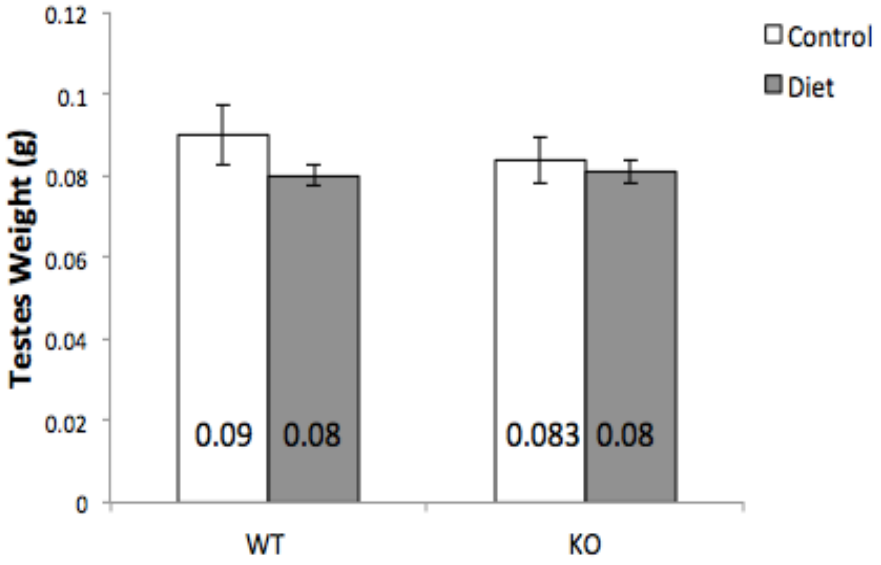


Figure 3.16. **Testicular weight does not vary between *Pmca4* genotypes or treatment** with a diet high in PUFAs for 9-11 weeks. Both testes were collected from each mouse and their weights averaged. (Two-way ANOVA: $p>0.05$, N=3, ±SEM).

3.12 Histological Analysis Reveals Altered Epididymal Integrity in *Pmca4* KO

To investigate the effects of a potential increase in oxidative stress induced by *Pmca4* deletion in the epididymis, a vital reproductive organ, H&E staining of the three regions of the epididymis were conducted. Analysis revealed distinct segments of non-normal epididymal structure and luminal content (**Fig. 3.17)**. The WT control (**Fig. 3.17, A-C**) illustrated clear cellular organization and intact luminal spermatozoa compared to KO (**Fig. 3.17, D-F).** *Pmca4* KO tubules showed an increase in intraepithelial vacuolization in the caudal region (**Fig. 3.18A, A-B**). Infrequently, histological abnormalities, such as hyperplasia, were also found in the cauda epididymis of *Pmca4* KO (**Fig. 3.18A, C**). The *Pmca4* KO caput had a paucity of germ cells and the presence of pyknotic-rounded cells (**Fig. 3.18A, D**). The frequency

of caudal vacuolization was higher in *Pmca4* KO cauda (55.59% ± 11.13 / ~45 tubules) compared to WT cauda (20.51% ± 11.18 / ~45 tubules), although not significantly (p=0.08)(**Fig. 3.18, B**). The presence of pyknotic-rounded cells in the caput was higher in *Pmca4* KO (5.21 ± 0.16/10 tubules) compared to WT (1.07 ± 0.47/10 tubules)(**Fig 3.18, C**). A two-way ANOVA showed that there was no difference in number of pyknotic-rounded cell tubules between diet and control groups but confirmed an overall significant effect of genotype on the presence of these cells (***p<0.005)(**Fig 3.18, C**). No visual difference was noted between epididymal sections of control and diet-treated groups.

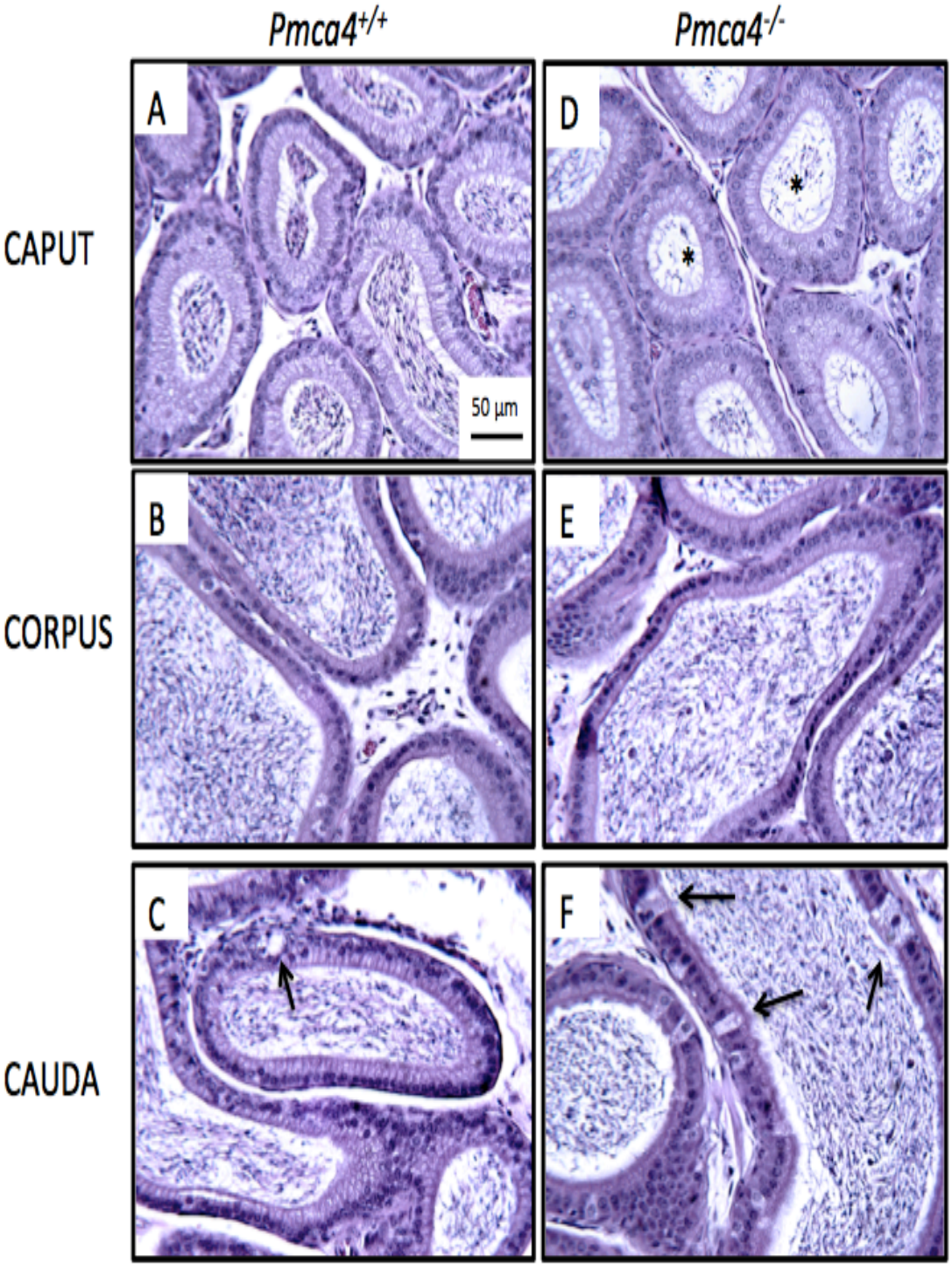
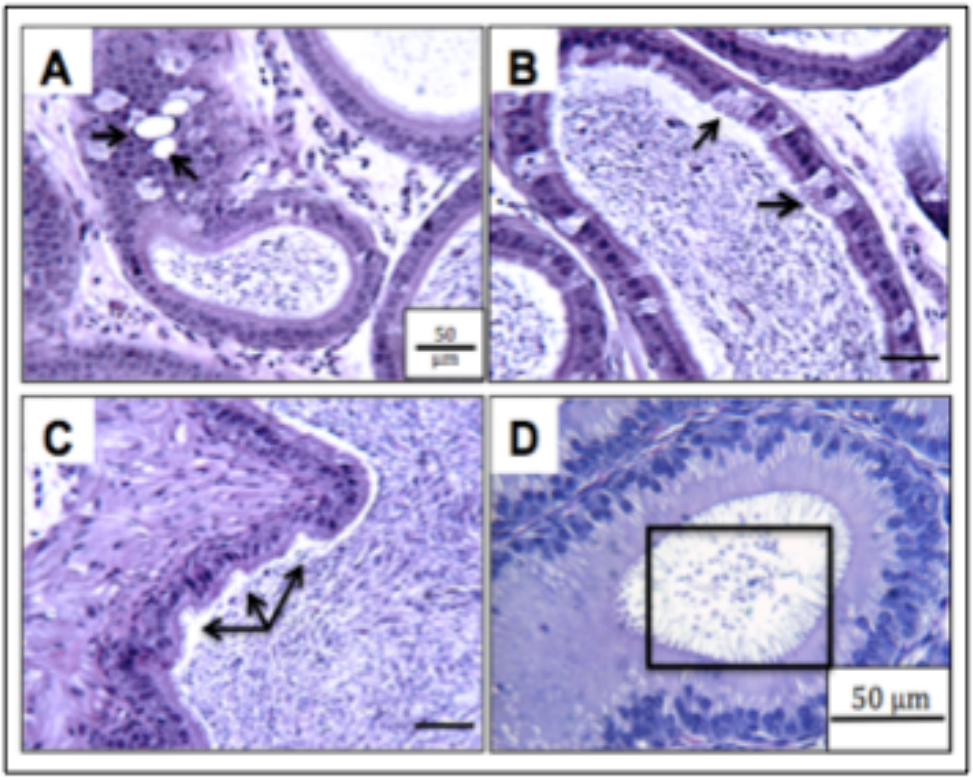
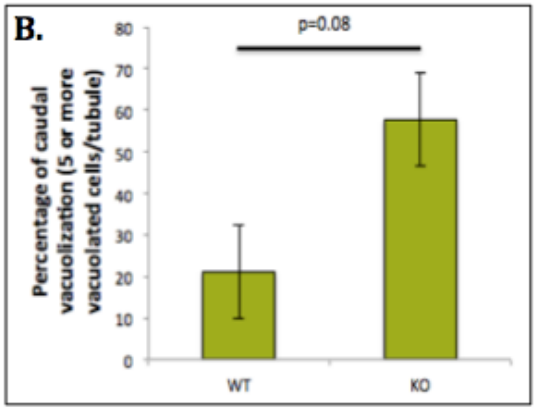

Pmca4+/+
Pmca4-/-
CAPUT
CORPUS
CAUDA
A
B
C
D
E
F
50 µm

Figure 3.17. **Histological analyses reveals variations in epididymal structure and luminal content** between control WT **(A-C)** and *Pmca4* KO **(D-F)**. Sections were prepared from caput (**A, D**), corpus (**B, E**), and cauda (**C, F**) and stained with hematoxylin and eosin. Increased paucity of germ cells and pyknotic-rounded cell (asterisk) found in caput epididymis of *Pmca4* KO as well as increased presence of intraepithelial vacuolization (arrowed) in the caudal epididymis of *Pmca4* KO. Scare bar =50 μm.

A.


A
B
C
D
50 µm


B.
p=0.08
Percentage of caudal vacuolization (5 or more vacuolated cells/tubule)
80
70
60
50
40
30
20
10
0
WT
KO

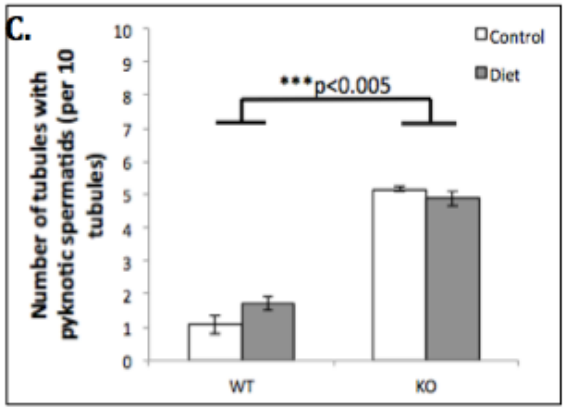

C.
Control
Diet
***p<0.005
Number of tubules with pyknotic spermatids (per 10 tubules)
10
9
8
7
6
5
4
3
2
1
0
WT
KO

Figure 3.18. **Histological abnormalities in epididymides: quantification in WT and *Pmca4* KO mice. (A)(**A-B) Intraepithelial vacuoles (arrowed), (C) occasional hyperplasia (arrowed), (D) germ cell paucity and pyknotic-rounded cells were the most prevalent findings (inset). Scale bar =50 μm. **(B)** Increased caudal vacuolization found in *Pmca4* KO epididymis; however, this increase was not significant (Two-sample T-test: p=0.08, N=3, ±SEM). **(C)** Significant increase in presence of pyknotic-rounded cells in *Pmca4* KO caput compared to WT (Two-way ANOVA: ***p<0.005, [WT-C (N=3), WT-D (N=4), KO-C (N=3), KO-D (N=5)], ±SEM).

Chapter 4

DISCUSSION

4.1 A Diet Enriched in PUFAs and Antioxidants Positively Impacts Sperm Parameters in the Murine Model

The effects of a walnut-enriched diet that is high in PUFAs and antioxidants have been documented in human sperm (Robbins et al., 2012), however, to our knowledge, a similar study has not been conducted in a model organism. In the present study, a nutritional intervention in a controlled murine model was performed where the effects of a walnut supplement were directly studied in relation to male fertility parameters. Administration of the diet where walnuts compose 19.6% of caloric intake significantly improved total sperm motility ($p<0.05$)(Fig. 3.2) and morphology ($p\leq0.05$) (normal forms) (Fig. 3.4) when treated over a 9-11 week period, compared to mice that were fed a control diet for the same period. The diet caused no change in body weight (Fig. 3.1). The stability of body weight confirms that the design of the diet accurately parallels that used by Robbins et al., (2012) where men had a supplement of 75 g of walnuts daily without weight gain. The finding of an improvement in murine sperm parameters with the diet is similar to those reported in humans (Robbins et al., 2012), and provides confirmation for the efficacy of the diet.

Progressive motility in sperm of diet-treated males showed a trending increase in uncap and an ~2-fold increase in cap conditions. While these increases were not significant, these trends suggest, similar to total motility, the benefits of the diet with respect to fertilization success. No change was seen in vitality levels (Fig. 3.3) of control and diet-treated sperm. Despite this observation, increased motility and morphology indicate the benefits of a walnut dietary supplement in reproductive

health and illustrate how the addition of PUFAs and antioxidants play vital roles in sperm function. Previous work has shown the importance of fatty acid profile changes in sperm maturation and differentiation (Rettersol et al., 2001; Oresti et al., 2010). Furthermore, these data support research showing the relationship between increased omega-3 from diet and normal sperm morphology in humans (Attaman et al., 2012). In addition to morphology, fatty acids confer increased fluidity of the sperm membrane, which is necessary for sperm motility (Wathes et al., 2007).

The finding of improved sperm characteristics with the diet is consistent with recent literature demonstrating the impact of diet on sperm maturation in the epididymis and the transfer of biological materials necessary for sperm function. Specifically, Sharma et al. (2016) demonstrated the effects of a low protein diet on small RNAs in males and how the transfer of these RNAs from the epididymis to sperm modulates embryo phenotype. Therefore, the findings in the present study not only confirm the benefits of a walnut supplement in a murine model, but also support a growing understanding of the transitory effects of the environment on gene expression.

4.2 Minor Impact of Dietary Intervention in Sperm Damaged by *Pmca4* Loss

The DeLeon Lab has proposed a mechanism in which *Pmca4* deletion results in loss of sperm motility via oxidative stress (Fig. 1.5). It is known that in the absence of *Pmca4*, NOS activity is upregulated (Olli et al., *in revision*). Our lab has confirmed the association of the NOSs and *Pmca4* in capacitated sperm as well as the presence of elevated levels of both NO and $OONO^-$ in KO sperm (Olli et al., *in revision*). Physiologically, NO and $OONO^-$ play vital roles in capacitation (Herrero et al., 2001); however, when their regulation is uncontrolled, increased NO and $OONO^-$, reactive

oxygen species (ROS), have been known to result in sperm DNA defects, low motility, and the inability for sperm and oocyte to fuse (de Lamirande and Gagnon, 1992a, 1992b, 1995; Aiken et al., 1995; Kodama et al., 1996). Downstream interactions, particularly that of NO and superoxide (O_2^-), which combine to form $OONO^-$, overwhelm the natural antioxidant protection mechanisms found in sperm and induce lipid peroxidation in the membrane (Hellstrom et al., 1994; Aitken et al., 2014).

Based on this mechanism, I proposed that a walnut supplement enriched in PUFAs and antioxidants could work to replenish sperm damaged by a genetic deletion resulting in oxidative stress and low motility. Total motility levels were characterized for WT and *Pmca4* KO control sperm and showed no difference between genotypes (Fig. 3.5A). Progressive motility results of *Pmca4* KO sperm support similar observations reported by Okunade et al. (2004) that show loss of motility in cap conditions (Fig. 3.5B).

When administered the diet, total motility of *Pmca4* KO did not increase, compared to KO control (Fig. 3.6A). These findings do not parallel the significant increases seen between WT control and diet-treated sperm (Fig. 3.2A). Progressive motility also remained unchanged between KO control and diet-treated sperm in uncap and cap conditions (Fig. 3.6B), illustrating that the supplement could not rescue motility in these nulls.

Membrane quality assays (vitality, acrosomal reaction rates, and morphology) were examined in WT and *Pmca4* KO controls and diet-treated sperm. Integrity of the membrane (Fig. 3.8), assessed by Eosin-Nigrosin staining, and acrosomal reaction rates (Fig. 3.9) showed no significant difference between genotype or diet treatment.

These findings are similar to those seen in equine sperm incubated in a ROS-generating system where motility was impacted but membrane-related parameters were not (Baumber et al., 2000). Thus, it appears that motility, specifically progressive motility in *Pmca4* KO, is more sensitive to increased ROS than is sperm membrane-related parameters.

Interestingly, normal sperm morphology was significantly affected by genotype as well as implementation of the diet. In the control, *Pmca4* KO sperm showed significantly lower morphologically normal sperm compared to WT ($p<0.005$) (Fig. 3.10A). Increased rates of abnormal sperm morphology have been shown to be associated with increased ROS and oxidative stress in our Lab (Smith et al, 2015), as well as from DNA damage; although this exact mechanism is unclear (Aziz et al., 2004; Aitken and De luliis, 2010). When treated with the walnut supplement, normal morphology of *Pmca4* KO sperm significantly ($p<0.05$) increased. This suggests that the diet may have the ability to restore membrane function damaged by oxidative stress, particularly in the tail where defects were largely found (Fig. 3.10B).

Despite recovery of flagellar morphology in *Pmca4* KO sperm, progressive motility in cap conditions remained unaffected, by the diet. Energy, in the form of ATP, is necessary for powering the motility machinery; therefore, we asked if ATP levels were affected by *Pmca4* loss. Increased NO has been shown to result in reduced cellular ATP levels via the inhibition of enzymes involved in the glycolytic pathway (Dimmeler et al., 1992) as well as the mitochondrial electron transport system (Nathan, 1992; Weinberg et al., 1995). Thus sperm motility, which is driven by dynein ATPase, is expected to be reduced in the presence of elevated NO (Gibbons, 1996; Inaba, 2003), as well as increased $[Ca^{2+}]$ and low pH (Peralta-Arias et al., 2015).

Based on these findings, I proposed that sperm damaged by *Pmca4* deletion would have decreased ATP levels due to inhibition of various pathways that mediate ATP production. Our data show that *Pmca4* KO sperm expressed higher ATP levels than the WT under noncapacitating conditions; while there were similar levels in cap conditions (Fig. 3.11). However, these results vary markedly from previous work conducted in our lab on a KO model with a low motility phenotype. ATP levels for *Jam-A* KO sperm, which exhibit milder motility defects that do not lead to infertility, revealed a significant decrease in both uncap and cap conditions compared to the WT, suggesting that low ATP levels are the cause of motility loss in these nulls (Aravindan et al., 2012). It is our belief that due to environmental disruptions from ongoing construction in the animal facility during the execution of the project, that the data obtained in the present study are not representative of the physiological ATP levels in these animals.

4.3 Lipid Peroxidation is a Mechanism of Motility Loss in *Pmca4* KO Sperm

In order to investigate if lipid peroxidation is indeed a mechanism in which *Pmca4* deletion results in increased oxidative stress and motility loss, a thiobarbituric acid reactive substances assay was performed. As mentioned previously, increased levels of [$OONO^-$] resulting from upregulation of the NOSs in *Pmca4* KO sperm is likely to overwhelm the natural antioxidant protection mechanisms in sperm, resulting in destabilization of the plasma membrane and peroxidation of the numerous PUFAs found therein. This particular type of damage to the membrane results in a decrease in the number of functioning PUFAs and an increase in radical byproducts such as malondialdehyde (MDA) and 4-hydroxynonenol (Aiken et al., 1995; Ayala et al.,

2014). Thus MDA, as well as various other decomposition products, can be measured as identifiers of lipid peroxidation in sperm.

In this study, *Pmca4* KO sperm exhibited increased levels of lipid peroxidation, although not significantly, compared to WT (Fig. 3.12). We further asked if a diet enriched in PUFAs and antioxidants could decrease lipid peroxidation levels in sperm. Analysis via a two-way ANOVA demonstrated the positive effects of this diet on both WT and KO sperm, as lipid peroxidation levels were significantly ($p<0.05$) lowered. This finding suggests that the walnut supplement is working at the membrane level to restore stability and interrupt the lipid peroxidation cascade by providing added antioxidant benefits as well as replacing damaged PUFAs.

4.4 The Effects of Oxidative Stress on Apoptotic Activity in Testicular Germ Cells in *Pmca4* KO Males

The effects of oxidative stress not only impact sperm motility in functionally mature sperm, but also sperm genome quality (Aitken et al. 2014). The TUNEL assay provided data (Fig. 3.14) showing significant increases in apoptosis in *Pmca4* KO control spermatogonia ($p<0.005$) and spermatocytes ($p<0.05$). This finding is consistent with the report from our Lab that *Pmca4* transcripts are expressed in these germ cells, but not in the interstitial space (Patel et al., 2013). Testes from *Pmca4* KO mice treated with the diet showed a reduction in apoptotic activity in all germ cell types, although it did not reach a significant level. In general, our findings are in agreement with previous findings of apoptosis in these KO's in the smooth muscle cells of the hepatic portal vein (Okunade et al., 2004).

Our TUNEL results were corroborated by H&E staining of the testes (Fig. 3.15). The latter revealed a lack of organization in the KO tubules, in addition to

increased spaces between the germ cells. Interestingly, similar histological observations, in particular, atrophy and tubule degeneration, were seen in rats given a diet lacking essential fatty acids or a supplement of linolenic acid (omega-3) only, suggesting the importance of fatty acids in testis organization (Leath et al., 1983). This finding is indicative of a disruption by reactive oxygen species in Sertoli cell integrity and, therefore, the Sertoli-Sertoli junctional complexes responsible for tubule shape (Krishnamoorthy et al., 2013). In our study, this phenotype reveals that *Pmca4* KO seminiferous tubule structure appears to be hindered by reactive oxygen species at the tissue level, an observation not noted in previous literature (Okunade et al., 2004). Despite increased apoptotic activity, no difference in testis weight was evident. This may reflect a compensatory increase in spermatogenesis in *Pmca4* nulls where sperm numbers are not different from those of WT.

4.5 Variations in Epididymal Structure and Luminal Content Suggestive of Increased Apoptosis in the Epididymis of *Pmca4* KO

In addition to the testes, epididymal histology was analyzed for the first time in *Pmca4* KO mice and revealed an increase in damage associated with apoptosis from oxidative stress. In this study, alterations in the epididymis of *Pmca4* KO animals were most prominent in the caput and caudal regions: specific types of abnormalities were typical of each region (Fig. 3.17). In the *Pmca4* KO caput, a paucity of germ cells is evident in the lumen of tubules where there is also the presence of pyknotic round immature sperm. Quantification of this observation revealed a 5-fold higher incidence of these tubules in *Pmca4* KO, compared to WT, caput epididymides ($p<0.005$) (Fig. 3.18C).

Additionally, an increased level of intraepithelial vacuoles was seen in the caudal epididymis. This is the site where sperm are stored and also the region where a majority of luminal debris is endocytosed and degraded (Robaire et al., 2006). An approximate 45% increase in caudal vacuolization is seen in *Pmca4* KO compared to WT, although a non-significant increase (Fig. 3.18B). There was no difference, visually, in the epididymal structure between control and diet-treated groups, suggesting that the diet does not function at a level capable of altering epididymal epithelial integrity. This conclusion was further confirmed by quantification of diet-treated tubules for the presence of pyknotic-rounded cells, which revealed no difference between control and diet-treated groups (Fig. 3.18C).

Normally, clear cells work to remove cellular debris from the epididymal lumen. However, it is possible that due to the large quantity of debris in the form of pyknotic-rounded cells, clear cells endocytic mechanisms are overwhelmed and they induce apoptosis, visualized as vacuolization. Increased $[Ca^{2+}]_i$ levels induced by *Pmca4* loss has been shown to result in mitochondria condensation (Okunade et al., 2004), which can initiate Ca^{2+} -dependent pathways (IP_3) that induce apoptosis by caspases and cause further damage via the release of free radicals from the mitochondria (Rizzuto et al., 2003; Okunade et al., 2004; Aitken and Koppers, 2011). As evidenced previously by TUNEL and H&E staining, testicular histology is perturbed in *Pmca4* KO. Therefore, an increase in oxidative stress will result in sperm that are immature both in function and morphology, as shown above, and may induce apoptosis. These immature sperm eventually leave the testes and enter the caput epididymis.

4.6 Overview of the Potential Mechanistic Factors Involved in the Phenotype of Reproductive Systems of *Pmca4* Males

While there are several potential pathways for the pathogenesis of male infertility in *Pmca4* nulls, the results from this study provide support for the mechanism involving increased lipid peroxidation levels as a result of oxidative stress. Below is a summary of the potential pathways where PMCA4's absence triggers a multi-pronged response that eventually leads to increased lipid peroxidation, loss of motility, and apoptosis (Fig. 4.1).

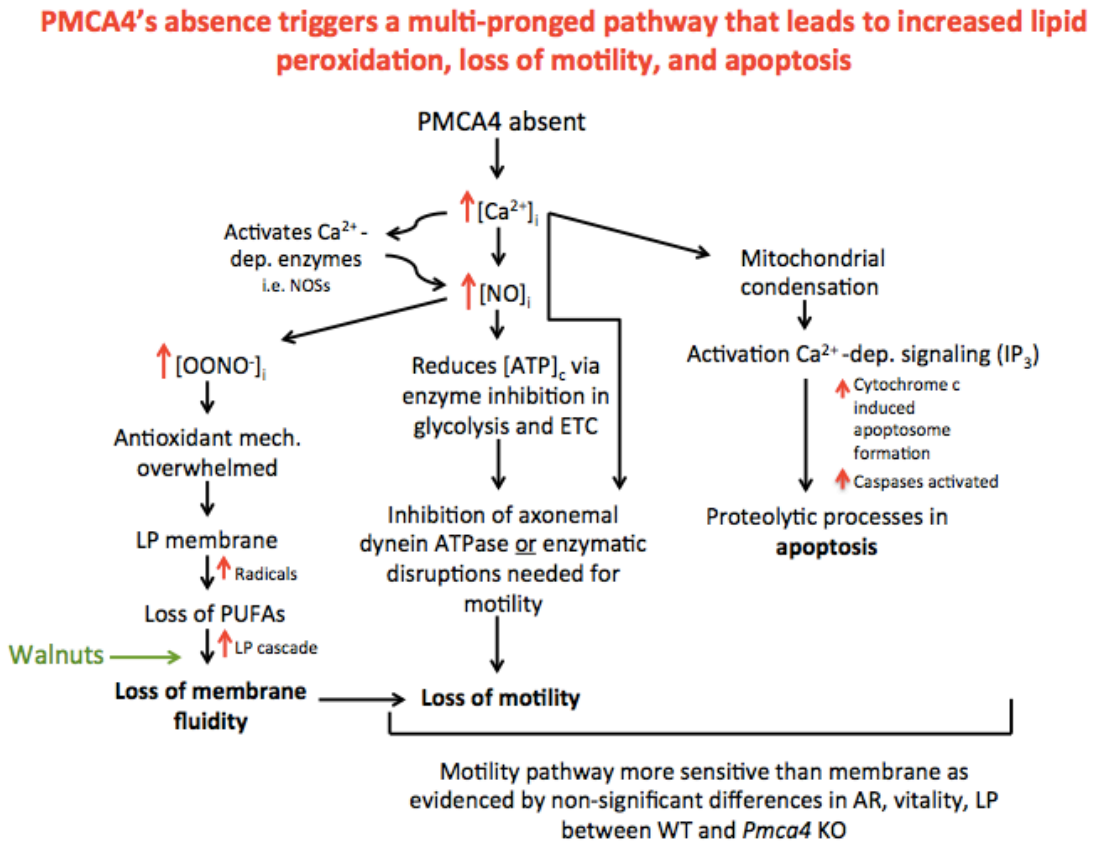


Figure 4.1. **Overview of PMCA4's absence and its downstream effects.** PMCA4's absence triggers three pathways that lead to increased lipid peroxidation, loss of motility, and apoptosis. **1)** Lipid peroxidation (LP) is initiated by PMCA4's absence leading to an increase in $[Ca^{2+}]_i$ which activates Ca^{2+} -dependent enzymes such as the NOSs to stimulate increased NO production. [NO] interacts with superoxide $[O_2^-]$ to produce elevated levels of $[OONO^-]$ which overwhelm the antioxidant protection mechanisms resulting in LP of the sperm membrane. LP cascades increase the number of free radicals and decrease the number of healthy PUFAs resulting in destabilization of the membrane and loss of motility **2)** Loss of motility is initiated via two sub-mechanisms **a)** Increased [NO] inhibits enzymes necessary for the glycolytic and ETC pathways thereby decreasing $[ATP]_c$. This decrease in ATP results in inhibition of motility machinery, such as dynein ATPase, and motility is impaired. **b)** Motility is also lost via the initial increases in $[Ca^{2+}]_i$ as well as an acidic pH, which directly impairs dynein ATPase. **3)** The apoptotic pathway is initiated by increased $[Ca^{2+}]_i$ leading to mitochondrial condensation from calcium overloading. Activation of Ca^{2+} -dependent signaling pathways, particularly IP_3, increases caspase activity and apoptotic machinery, resulting in cell death. These mechanisms, coupled with results seen in AR, vitality, and TBARS, support the idea that the motility pathway is the more sensitive of the pathways and is greatly affected by oxidative stress.

4.7 Conclusions

The results acquired from this study have led to the following conclusions:

1. Data in the murine model confirm a study that showed a significant positive effect of a walnut-enriched diet on sperm morphology and motility in humans.
2. I have examined the effects of this diet on defective sperm damaged by *Pmca4* deletion: the data revealed that total and progressive motility were unaffected by the administration of the diet.
3. I found no difference in membrane integrity via EN vitality staining as well as acrosome reaction ability in *Pmca4* KO, suggesting that the effects

of oxidative stress in the membrane are less sensitive than pathways involved in motility.

4. I have identified lipid peroxidation as a potential mechanism of motility loss in *Pmca4* KO sperm, and for the first time, that a diet enriched in PUFAs and antioxidants significantly ($p<0.05$) reduced the levels, likely due to oxidative stress in both WT and KO *Pmca4* sperm.

5. I have illustrated the effects of oxidative stress in *Pmca4* KO epididymis, the site of sperm maturation, where PMCA4 transcripts are normally found as well as in the testes and found no difference between epididymides of the control versus diet-treated mice.

6. I identified the presence of pyknotic-rounded cells in the caput region of the epididymis as well as increased intraepithelial vacuolization in the cauda, which confirms increased apoptotic activity in this organ as well as in immature sperm function.

7. These findings are vital to the understanding of the environmental impact on reproductive function and how changes in diet may induce beneficial modifications in sperm quality, and ultimately impact individuals affected by subfertility.

4.8 Future Directions

While this study answers vital questions regarding the role of a walnut enriched diet in the murine model as well as the effects of this diet on sperm damaged by a genetic mutation, it opens the door to further investigation. Histological analysis in *Pmca4* KO revealed increased disorganization and inter-germ cell space in the testes. Future studies could characterize the testicular expression of the Sertoli-Sertoli junctional complexes that maintain tubule integrity. In addition to this, it would be important to perform a fatty acid profile of serum and sperm to determine levels of omega-6 and omega-3 between the control and diet groups. Fatty acid profiles would be used as another systemic factor for analysis and support results found in sperm parameters. Finally, to determine if the mice were experiencing higher than normal levels of stress due to environmental disturbances from construction, it would be interesting to look at corticosterone levels in the blood. A limitation of this study is the differentiation of effects between the two variables: PUFAs and antioxidants. It would be pertinent to determine the benefits of each individually to better understand and separate the source of certain results.

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Appendix

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

Title of Protocol: Mechanism of Sperm motility in *Pmca4* null mice and he Role of Oviductosomes

AUP Number: 1181-2015-2 ← **(4 digits only)**

Principal Investigator: Patricia A. DeLeon

Common Name: Mouse

Genus Species: *Mus Musculus*

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: *(Note change of categories from previous form)*	
Category	**Description**
☐ **B**	Breeding or holding where NO research is conducted
x ☐ **C**	Procedure involving momentary or no pain or distress
☐ **D**	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ **E**	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: Glenn Tolbert, DVM

Date of Approval: 12/1/15

SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Patricia A. DeLeon	Patricia A. DeLeon
2. Lauren Coffua	Lauren Coffua
3. Pradeepthi Bathala	Pradeepthi
4. Tori Mallardi	Tori Mallardi
5. Click here to enter text.	
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IACUC approval of animal protocols must be renewed on an annual basis.

1. **Previous Approval Date:** 12/1/2014

Is Funding Source the same as on original, approved AUP?

xYes ☐ No

If no, please state Funding Source and Award Number: Click here to enter text.

2. **Record of Animal Use:**

Common Name	Genus Species	Total Number Previously Approved	Number Used To Date
1. Mice	Mus musculus	1920	1099
2. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
3. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
4. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. **Protocol Status:** *(Please indicate by check mark the status of project.)*

Request for Protocol Continuance:

x☐ A. Active: Project ongoing

☐ B. Currently inactive: Project was initiated but is presently inactive

☐ C. Inactive: Project never initiated but anticipated starting date is:

Click here to enter text.

Request for Protocol Termination:

☐ D. Inactive: Project never initiated

☐ E. Completed: No further activities with animals will be done.

4. **Project Personnel:** Have there been any personnel changes since the last IACUC approval?

x ☐ Yes ☐ No

If Yes, fill out the Amendment to Add/Delete Personnel form to "Add" Personnel.

4. **Project Personnel:** Have there been any personnel changes since the last IACUC approval?

x ☐ Yes ☐ No

If Yes, fill out the Amendment to Add/Delete Personnel form to "Add" Personnel.

Project Personnel Deletions:

Name	Effective Date
1. Amal Al Dossary	Click here to enter text.
2. Kathie Wu	Click here to enter text.
3. Rebecca Pollak	Click here to enter text.
4. Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.

5. **Progress Report:** If the status of this project is 3.A or 3.B, please provide a brief update on the progress made in achieving the aims of the protocol.

Preliminary data were used for the submission of an RO1 NIH grant application
We made a major discovery of oviductosomes and a manuscript has been submitted for publication.

6. **Problems or Adverse Effects:** If the status of this project is 3.A or 3.B, please describe any unanticipated adverse events, morbidity, or mortality, the cause if known, and how these problems were resolved. If there were none, this should be indicated.

An adverse effect has been seen with the mating of the animals—due to the MRI building construction we have detected a cessation in mating of the animals all summer. Within the last two weeks they have begun to mate again. But we lost precious time and money during the summer.