TOWARDS THE DRY PRESERVATION OF FELINE SPERM: THE EFFECT OF DEHYDRATION ON DNA, CENTROSOMAL FUNCTION, AND EMBRYO DEVELOPMENT

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ABSTRACT

JENNIFER LYNN PATRICK: Towards the dry preservation of feline sperm: the effect of dehydration on DNA, centrosomal function, and embryo development (Under the direction of DR. GLORIA ELLIOTT)

The current gold standard for gamete preservation is cryogenic storage. Dry preservation is an attractive alternative, eliminating the need for refrigeration, reducing storage maintenance costs, and providing logistical flexibility for shipping. The current study focuses on understanding the injury during dehydration of feline epididymal sperm. This animal model serves as a closer model to humans than a rodent species because of centrosomal inheritance from the sperm. Drying to end moisture levels of 0.16 gH_20/gDW did not significantly increase sperm nuclear DNA damage compared to control samples. Large sperm aster formation 5 hours post insemination (hpi) was significantly lower ($p<0.05$) in the dried group (5.6%) compared to control groups (14.6%), indicating a level of centrosomal damage during dehydration. No difference in the overall level of sperm DNA decondensation at 5hpi was noted. Analysis of embryo development post dehydration showed a significant decrease ($p<0.01$) in development to the blastocyst stage in the dehydrated group (6.4%) compared to the fresh group (15.1%). The mRNA levels, however, in developed blastocysts from dried sperm were not significantly different from the control group, suggesting equal fetal development capacity. Together, these data add to the current understanding of cellular damage during dehydration of sperm and supports the feasibility of anhydrous preservation. Future studies to evaluate specific embryonic gene activation patterns and centrosomal replacement post-dehydration could yield further advances.

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CHAPTER 1: INTRODUCTION

1.1 Cryopreservation: Gold Standard

Cryopreservation has become the gold standard for the long term preservation of spermatozoa of many species. With advancements in genetics and bio-medical fields, an increasing number of valuable transgenic and rare genetic models are available and need to be preserved (Bhowmich, 2003). However, the requirement for liquid nitrogen poses several logistical problems centered on handling, storage, and shipping difficulties, thus other approaches have been explored. The movement towards dry preservation of sperm cells has emerged in response to the need for more efficient storage and transport approaches for gametes to support the fields of reproductive biology, species conservation, and biobanking of research strains of animals (Storey, 1998; Situala, 2009). A more effective and efficient stabilization method of nucleated cells for dry storage would transform these areas of medicine and research. Dehydration and storage of biologic samples at non-freezing temperatures would eliminate the large cost of liquid nitrogen dewars for storage as well as the constant expense of liquid nitrogen to keep these dewars at cryogenic temperatures. Non-cryogenic storage would also address the shipping issues of these biologic samples, as refrigerated or cold pack shipping of a small box could be done, replacing the costly large nitrogen dewars or dry ice shippers used today.

Cryopreservation routinely involves exposing cells to a series of non-physiological conditions, including exposure to a cryoprotective agent, cooling to a

subzero temperature, storage in liquid nitrogen vapor, then thawing and cryoprotectant removal before a return to physiological conditions. Many living cells have a fairly low toxicity threshold to (1) cryoprotectants and (2) an associated transient osmotic imbalance when exposed to a hyperosmotic cryoprotectant solution. Cryoinjury (cell membrane disruption, cytoplasmic fragmentation, DNA damage) can occur during cooling due to both 'solution effects' and lethal intracellular ice formation (Hammerstedt, 1990).

Regardless of the specific cryopreservation protocol used, it has been well documented that cryopreservation of sperm reduces motility and fertilization ability (Martins, 2007; Pukazenthi, 2002; Hammadeh, 1999; Rybar, 2012). Decreases in fertilization rate can be attributed to the effects on one or more aspects of sperm cell function. Cryopreservation decreases sperm motility due to decreased ATP production within the mitochondria in the sperm mid-piece. It also has detrimental effects on acrosomal activity and irreversibly changes membrane permeability and protein distribution. Chromatin decondensation is also problematic, leaving the nuclear DNA exposed for damage (Hammadeh, 1999). The sperm plasma membrane, along the tail, midpiece, and head, is the primary site of cryopreservation induced damage through several mechanisms including exposure to low temperatures followed by warming (Bailey, 2000). Changes in salt concentrations and osmolality, due to changes in concentrations of the cryoprotectant, (Meyers, 2005;Martins, 2007; Ball, 2008) also cause membrane damage. Irreversible increases in membrane permeabilization and mechanical stress on the membrane are imposed by alterations in cell water volumes. (Bailey, 2000; Hammerstedt, 1990).

1.2 The Science and Technology of Dry Preservation

Nature has taught us that the concept of dry preservation, in contrast to cryopreservation, is possible, as seeds and many anhydrobiotic organisms are able to survive extended periods in the dry state (Crowe, 2000). Currently, many proteins, bacteria, pharmaceutical drugs, and foods are successfully preserved in the dry state (Prokopiev, 2012; Wang, 2000). Because of the interest and need for pharmaceutical and food storage, this is not a new concept, and can serve to demonstrate what may be possible for mammalian sperm cells. Mammalian sperm are however, by nature, desiccation sensitive and cannot be stabilized in the dry state without the use of biotechnology (Kanias, 2006). Effective stabilization of nucleated cells for dry storage would transform many fields of cell-based biology, medicine, and reproduction.

Many sugars are involved in the preservation of living organisms under extreme conditions. Through the *in vivo* accumulation of these sugars, these organism are able to survive an extreme loss of cellular water, up to 90% of their original water content, and desiccation for extended periods of time with a return to fully functional states with minimal cellular loss (Hengherr, 2007). Trehalose, a natural sugar composed of 2 glucose molecules joined by a 1-1 glycosidic bond, is found at particularly high concentrations in many desiccation-tolerant organisms (Crowe, 2000; Chen, 2001; Buchanan, 2005; Li, 2007). The protectant forms a protective milieu around biomolecules and chromatin (Mazzobre, 2001; McGinnis, 2005), promotes the formation of amorphous, glassy systems, inhibits crystallization, and interacts with biological structures to stabilize them during drying. The actions of anhydroprotectants can be ascribed to both kinetic and specific effects. At the kinetic level, the protectants promote

the formation of amorphous, glassy systems, inhibit crystallization, and influence the kinetics of reactions responsible for deterioration during storage. Specific effects include interaction with biological structures to stabilize them during drying. The crystallization of sugars or the formation of ice from the amorphous state leads to the loss of the protective effect of amorphous matrices and consequent deterioration of biomolecules and biological structures (Mazzobre, 2001). Buitink et al, using electron paramagnetic resonance spectroscopy to study seeds and pollen, showed that molecular mobility decreases with lowering of the moisture content and then increases again when the water content becomes very low (Buitink, 1998). Maintaining a negligible molecular mobility is critical for long term storage, and it seems as though there is a threshold, both upper and lower, of moisture content that must be obtained during drying and then maintained during storage in order to sufficiently stabilize critical cellular components.

The interaction of trehalose with biomolecules have been theorized to be 3-fold, to include the non-exclusive and complementary theories of vitrification, preferential exclusion, and water replacement (Kumar, 2008; Hengherr, 2007; Crowe 2007). The vitrification theory proposes that trehalose forms a glassy matrix around biomolecules, physically shielding them from stresses, while the preferential exclusion theory suggests that there is no direct interaction between trehalose and the biomolecules, but that trehalose sequesters the water molecules surrounding the biomolecules, decreasing the hydration radius of the molecules, therefore increasing compactness and stability during drying. The water replacement theory suggests trehalose substitutes the water molecules bound to and surrounding the biomolecules, maintaining their native structure, and therefore further maintaining their function.

To function as a desiccation protectant, trehalose hydrogen bonds with head groups of phospholipid bilayers under dehydration conditions, depressing the gel to liquid crystalline phase transition (T_m), preventing damaging phase changes during rehydration (Oliver, 2012; Crowe, 2007; Hengherr, 2007; Jain, 2009). Trehalose also vitrifies at lower water contents, and is characterized by an extremely high glass transition temperature (T_g), allowing it to remain in the glass state even under conditions of elevated temperature and humidity. And trehalose is capable of transforming to the crystalline dihydrate in the presence of water vapor, allowing it to sequester more water, avoiding plasticization, thus maintaining a high T_g in the non-crystallized fraction. Trehalose also has a highly stable glycosidic bond, unable to interact with amino acids and proteins, so as a "non-reducing" sugar, prevents damaging browning reactions that other sugars are susceptible to (Oliver, 2012; Crowe, 2007; Jain, 2009; Li, 2007).

Much of the protective capabilities of this disaccharide come from its T_g, the reversible transition from a viscous, rubbery state below T_g to a fluid state above T_g. Below the T_g, in the viscous state, the viscosity is so high that there is negligible molecular mobility and metabolic function. Trehalose has a high T_g at all water contents compared to other molecules, and therefore can form a stable glass over a wide range of temperatures. This unique characteristic could allow for long term non-freezing storage of biomolecules such as sperm cells if the glassy state could be achieved and then maintained during storage. The Gordon Taylor equation has been routinely used to predict glass transition temperatures of binary mixtures of water and trehalose (Chen, 2000). Using the estimated T_g of pure trehalose to be 100 °C (Roos, 1997), the T_g of pure water to be -135 °C (Green, 1998), and k=5.2, the T_g of trehalose at water contents of

0.200, 0.133, 0.101, and 0.090 gH2O/gDW, is estimated to be -20 °C, 4 °C, 20 °C, and 25 °C respectively. The glass transition temperature is one of the key parameters to determine storage stability in the dried state and the only way to achieve a stable glass is to maintain the dried sample below its T_g: $T_{storage} < T_g$ (Crowe, 2001; Katkov, 2004; Li, 2007).

1.3 Dry Preservation Technologies

Several anhydrous technologies have been explored for cell preservation, including lyophilization/freeze drying, convective drying, spin-drying, desiccation, and evaporative drying (Wang, 2000; DePaoli 2005). In general, these dry preservation technologies involve the removal of water from cells so that degradative biochemical processes are slowed to an extent to allow extended storage in this state (Chakraborty, 2007). Drying to an extreme level is defined as desiccation. Freeze drying or lyophilization, a dehydration process typically used to preserve a perishable material or make the material more convenient for transport, works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase. Convective drying involves drying in an environment (usually nitrogen gas) that has a lower water vapor pressure than that of the sample so that drying occurs due to the difference in the water vapor pressure between the sample and the environment (Kanias, 2006). Convective drying is the main technique used in food drying, and freeze drying is the most common process for drying protein pharmaceuticals, biological standards, and preserving specimens in biological banks. Convective drying has several drawbacks, including long processing time, understanding vapor pressures at the surface of concentrated solutes, and the inability to control final

moisture content (Kanias, 2006). Forced convection, using a gas flow over the sample, can increase drying rates, but such rates are often not uniform, making them unsuitable for biological samples (Kanias, 2006).

Our lab has previously worked on a unique temperature modulated microwave processing method as a method of drying for long term storage (Elliott, 2013). This process delivers microwave energy to moderately heat samples while drying, resulting in faster drying rates and altered drying physics to yield a more uniformly dried sample where cells are homogenously dispersed within the dried matrix (Chakraborty et al,2004). With this controlled process, we can more accurately prescribe the end moisture content of the samples post drying, and be assured that the microscopic environment is similar to the measured macroscopic environment.

1.4 Intracellular Loading of Trehalose

Bacterial cells and platelets have been successfully lyophilized to survive dry storage, however nucleated cells pose additional difficulties, as their high level of intracellular complexity makes survival more challenging (Oliver, 2012). Cells have membranes, organelles such as mitochondria and nuclei, proteins, and morphologies that need to be maintained to retain proper function (Kanias, 2006; Crowe 2007). Success with the dry preservation of mammalian cells has been quite varied depending on the cell type.

Previous work suggests that the presence of the sugar on both sides of the plasma membrane is required in order to confer this desiccation tolerance (Acker, 2002; Chen, 2001; Crowe, 2001; Elliott, 2006; Eroglu, 2000; Buchanan, 2005). Mammalian cells lack the enzymatic pathways to produce trehalose, and therefore trehalose loading is a

common first step in processing cells for dehydration. Several methods have been explored to accomplish this, including thermal poration (He, 2006), electroporation (Situala, 2009), ATP poration (Elliott, 2006), α-hemolysin poration (Eroglu, 2002; Acker, 2002), genetic engineering, and fluid phase endocytosis (Oliver, 2012).

Introduction of trehalose into sperm cells has been accomplished through poration of the cellular membranes with α-hemolysin, a genetically engineered bacterial protein added exogenously. This protein causes pore formation in the plasma membranes, which allows trehalose to diffuse into the cytosol through concentration gradients. Pores created by α-hemolysin can be closed through the addition of zinc, preventing loss of any important cellular components (Oliver, 2012).

Significant progress has been made toward the goal of dry preservation, including loading cells with protective disaccharide trehalose, and removing intracellular and extracellular water to achieve vitrification of the intra- and extracellular trehalose. However, despite the efficient introduction of protective solutes into the cell, the ability to maintain cellular viability is hampered by the low viabilities at low water contents. Regardless of the loading technique, reports of survival are diminished at water contents below 0.2-0.5 g H_20/g DW (Oliver, 2012). A low level of residual water is required, and must be maintained throughout the dry storage, to achieve viability post rehydration.

1.5 Dry Preservation of Rodent Sperm

1.5.1 Advances with Rodent Sperm

Most of the sperm dry preservation research has been done using a mouse sperm model, with only a small number of studies in non-rodent mammalian species (Table 1).

Bhowmick et al (2003), reported fetal mice production using a forced convective drying of mouse sperm with nitrogen gas at ambient temperatures (Bhowmick, 2003). During these studies, it was reported that optimization of the drying rates was critical to successful fertilization and blastocyst formation. Rapid drying rates (10 L/min for 10 min with a velocity of 2.187 m/sec) of a 20ul droplet at ambient temperature, storage for 18-24 hrs at 4°C, followed by ICSI, yielded blastocyst formation at a rate of 64%. Moderate forced convective drying rates (4 L/min for 30 min with a velocity of 0.87 m/sec) yielded a lower blastocyst formation rate of 58%, indicating the slower drying rate was detrimental to DNA integrity. Both rapid and moderate forced convective drying rates yielded higher blastocyst development rates compared to the 35% achieved using a slow natural convective drying for 60 min with negligible convective velocity. Even with a 64% blastocyst development rate using the rapid drying rate, day 15 fetal development using this dried sperm (13%) was significantly less than using fresh sperm (40%). The goal for all dried samples was to achieve a less than 5% residual final moisture content, determined by gravimetric analysis. The resulting mean moisture contents achieved, as a percent of the original moisture content, were variable at 3.6% +/- 1.1 (rapid), 1.5% +/- 0.6 (moderate), and 1.2% +/- 0.3 (slow), with the sample spread the highest for the rapid drying indicating a less controlled drying, even though the blastocyst formation was the highest. In this study, it was reported that the final moisture content (5-7%) did not affect the rate of blastocyst formation as much as the drying rate. In most reports of protein, bacteria, and plant dehydration, stable storage was achieved when the final moisture content was reported as < 5% (Leino, 2009; Prokopiev, 2012; Sharma, 2007; Wang, 2000).

McGinnis et al (2005) reported mouse sperm cells convectively dried with nitrogen gas post trehalose loading through α-hemolysin pores. Drying time varied from 5.0 - 7.5 min, with storage from 1 week to 3 months at 22 °C or 4 °C. Their findings showed that the percent of blastocysts produced post ICSI were significantly higher at all drying times (51%, 31%, 20%) than those without trehalose (10%, 3%, 5%), indicating that desiccation of sperm cells with trehalose did increase their developmental potential. A drying time of 5 minutes produced the highest percentage of blastocysts in this group, and this dried sperm stored at 4 °C produced more blastocysts at each storage length (73%, 84%, 63%, 39%) than those stored at 22 °C (53%, 17%, 6%). Implantation rates from this trehalose loaded, 5 min convectively dried sperm stored for 1 and 3 months at 4 °C were 81% and 48%, with liveborn rates of 26% and 5%, respectively.

Similar results were found in several studies of freeze dried mouse sperm (Kaneko, 2005; Kawase, 2005). Gravimetric analysis of pre- and post- dry weights were obtained to determine moisture levels in the samples both after drying and after storage. This data shows that as drying times increased, both samples, with and without trehalose in the drying medium, exponentially decreased in residual moisture, but the trehalose medium reached a steady state residual moisture level of 3-4% higher than the medium without trehalose, showing the ability of trehalose to hold on to water, while the medium without trehalose dried to an undetectable moisture level after 10 minutes. These findings show that developmental potential (protection of genetic material and proteins required for oocyte activation and embryonic development) of mouse sperm dried and stored in this manner can be maintained at some level, and that drying time, storage

temperature, and length of storage play a large role in the level of developmental potential that can be preserved.

Li et al (2007) used a similar system of drying murine sperm with trehalose for a period of 5 minutes (10 L/min, nitrogen gas), with storage lengths of 1 week to 5 months at 4 °C, -20 °C, and -80 °C. Fertilization rates were not significantly different for any combination of storage length or temperature. Blastocyst formation rates were not significantly different for any storage temperature for a storage length of 1 week or 1 month. For a storage length of 3 months, the blastocyst formation rates decreased as storage temperature increased (74.4%, 54.3%, 35.1%). Storage for 5 months at 4 °C had a significant drop in blastocyst formation rates (10.2%) where storage at -20 °C and -80 °C was not significantly different compared to 3 month storage rates. Linear regression fits of all three storage temperatures revealed a highly significant negative regression for storage time at 4 °C, a significant but steeper, negative regression at -20 °C, and a statistically insignificant negative regression at -80 °C, indicating significant deterioration at 4 °C, slightly less at -20 °C , and no degeneration of sperm fetal production ability at -80 °C. Taken together, these studies suggest that trehalose is conferring protection to the rodent sperm cell components required for fertilization, embryo development, and liveborn pup production.

1.5.2 Advances with Non-Rodent Sperm

There is a significant difference between sperm function in rodent and non-rodent mammalian species with respect to centrosomal inheritance. Rodent mammalian species inherit the centrosome from the oocyte, whereas non-rodent mammalian species inherit the centrosome from the spermatozoa, underlining the importance of preserving this

structural element during preservation. Most of the dry sperm preservation work that has been done in non-rodent mammalian species to date has utilized freeze-drying, with examples in the rabbit, horse, bovine, pig, monkey, and cat (Liu, 2004; Choi, 2011; Lee, 2006; Kwon, 2004; Sanchez-Partida, 2008; Ringleb, 2011).

Liu, et al (2004) generated blastocyst embryos using freeze dried rabbit sperm at a similar rate to those created using fresh sperm, and was able to generate liveborn offspring from embryos created with freeze dried sperm that had been stored at room temperature for more than two years. Using horse sperm, Choi, et all, 2011, created blastocysts from freeze dried sperm that underwent several freeze-thaw cycles followed by storage at 4°C for 3.5 months. A pregnancy rate of 71% (5/7 embryos implanting) occurred with this group, also resulting in 2 live births from the first 3 pregnancies.

Lee, et all, (2006) convectively dried bovine sperm at 50°C for varying lengths of time (8-16 hrs), followed by storage at 4°C for 7 days – 12 months. Embryo development success was varied over the different combinations of drying time and storage length, with limited development to the blastocyst stage in all categories. The longer lengths of storage time did not affect pronuclear development, however, drying for 8 hours and storage for 7-10 days gave the best blastocyst development rates. Post drying moisture contents were analyzed in these experiments using gravimetric analysis and bake out data to obtain the gH_20/gDW. Drying for 8 hours gave an average dry weight of 0.3 gH_20/gDW. Post storage water content was not evaluated.

Using freeze-dried pig sperm, successful fertilization and cleavage was obtained, however a significant decrease in development to the morula or blastocyst stage was noted (Kwon, 2004). Blastocyst stage embryos were only achieved after 4 hours of

storage at 4°C, with no development observed after storage for 1 month. Freeze-dried monkey sperm, with storage at room temperature for 1-2 months did not yield any embryo development past the cellular stage (Sanchez-Partida, 2008). In the domestic feline model, freeze drying of sperm impeded embryo cleavage at 36 hours post insemination (Ringleb, 2011).

1.5.3 Opportunities and Considerations for Dry Preservation of Feline Sperm

Even though there has been some research in the specific area of dry preservation of non-rodent sperm, it has been limited, and much remains to be understood. In nature, slow drying allows organisms to go through a series of complex molecular changes and physical adaptations, including the accumulation of sugars (Crowe, 1998). However, unlike plants and anhydrobiotic organisms, mammalian cells lack natural mechanisms to cope with desiccation injury. Understanding the damage to the specific cellular components incurred during the dry preservation process is critical to and the next step towards development of a functional dry preservation protocol.

There are several factors that need to be considered for the dry preservation and storage of sperm cells. Obtaining a low enough final moisture content to decrease molecular mobility/cellular aging to near zero and obtaining this low moisture content in a manner/time frame that is not detrimental to the cellular components that need preserving is a first step. Equally important is determining the proper drying medium to preserve functionality of the critical cellular components post dehydration. Determination of the environment the dried samples must be stored in to maintain the achieved moisture level without significant gain or loss of moisture over the storage period is critical to long term storage success. Little has been done to date with respect

to these factors, and with feline sperm cells in particular, therefore there is much knowledge to be gained. The state-of-the-art with dry preservation of other species will be used as the starting point for understanding the mechanisms of injury associated with dehydration processing in the feline sperm model.

Fertilization Attempts Post Sperm Desiccation

Species	Desiccation Method	Moisture Content	Storage Temperature (°C)	Storage Length	Outcome
mouse	conective drying	<5%	4	18-24 hrs.	birth
mouse	conective drying	unknown	4 / 22	1 wk - 3 mo	birth
mouse	conective drying	0.2–0.3 g H2O/g dry weight	4 / 22	1 yr	birth
mouse	convective drying	0.18 - 0.27 g H2O/g dry weight	4 / 25	3 mo - 2 yr	birth
mouse	convective drying	unknown	4 / -20 / -80	1 wk / 5 mo	birth
mouse	convective drying	unknown	4 / 22	3 mo	birth
mouse	convective drying	unknown	-80	5 mo	birth
mouse	freeze drying	unknown	-70 / -20 / 4	5 mo	birth
mouse	freeze drying	unknown	4 / -80	3mo / 6 mo	blastocyst formation
mouse	freeze drying	unknown	4	1 yr	ongoing gestation
rat	freeze drying	unknown	4 / 25 / -196	1 yr	birth
rat	freeze drying	unknown	4	2 days	birth
horse	freeze drying	unknown	4	3.5 mo	birth
bovine	convenctive dried (heated)	0.3 g H2O/g dry weight	4	3 mo	blastocyst formation
pig	freeze drying	unknown	4	4 hr	blastocyst formation
monkey	freeze drying	unknown	25	1-2 months	8-16 cell embryo
cat	freeze drying	unknown	4	72 hrs	cleavage stage

Table 1: Comparative table of fertilization attempts following sperm dehydration.

CHAPTER 2: OBJECTIVES

2.1 Hypothesis

In order to determine whether preservation in a dry state can be achieved by mitigating against injury, we first must understand the damage that occurs during the dehydration process. The goal of this research is thus to gain an understanding of the molecular DNA and centrosomal damage that occurs during dry preservation processing via microwave assisted drying, and to determine the role that each plays in preservation success. Felid sperm serves as a closer model to humans than other rodent species because in both felines and humans, the centrosome is paternally derived during fertilization, whereas the centrosome is maternally derived in all non-mammalian rodent species.

We hypothesize that through damage assays, the use of advanced molecular techniques, and observation of embryo development, we will be able to better understand the damage incurred to the nuclear DNA and centrosome during processing into the dry state. With this information it may be possible to develop an improved dry preservation technique that better preserves DNA and centrosomal integrity and function, thus better enabling preservation of the reproductive potential of the gamete. We further propose to use a novel technique of microwave drying to optimize processing conditions for feline sperm samples, in order to control the end moisture content of the samples during drying. The final moisture content impacts the ability to store samples in the dry state, as extended storage in the dry state requires a storage temperature below the glass transition temperature of the sample to ensure that molecular mobility is constrained. We expect that there will be a critical moisture content below which damage is

irreversibly compromised. In summary the overall goal of understanding the DNA and centrosomal damage accumulated within the feline sperm cell during dry storage microwave processing will be achieved by completing three specific aims.

2.2 Aim 1: Optimization of Environmental and Substrate Processing Conditions

The concept of microwave drying of a biologic sample in a droplet requires optimization that includes adjusting the environmental conditions that allow the removal of sufficient water from the sample in a timely manner and selection of an appropriate drying surface that allows the wetting out of the solution on the surface for even and fast drying and maintenance of normophysiologic temperatures during the drying process. The interaction with and adhesion to the drying surface must also be considered as the sample must be able to be released for use during rehydration. A consistent water content of the samples must also be able to be achieved, while keeping the temperature of the sample within a safe range for bioactive molecules. AIM 1 of this work will thus be to optimize the processing conditions for drying epididymal felid spermatozoa by identifying a) the environmental conditions (% RH) that enables drying to moisture contents below 0.2 gH_20/gDW and b) a drying surface that maximizes sperm recovery.

2.3 Aim 2: Determination of Damage as a Function of Moisture Loss

The sperm head is a unique cellular structure comprised almost entirely of a nucleus with very little cytoplasm. The nucleus is made up of two components, the chromatin, a densely packed DNA in an almost crystalline state, and the nuclear matrix which is made up of proteins (McGinnis, 2005). The stability of the nuclear matrix is influenced by the drying medium (Bhowmick, 2003). To achieve the highest viability post preservation, the drying solution must be optimized to protect the nuclear DNA integrity of the spermatozoa. Successful fertilization depends on both proper DNA content and centrosomal contribution/function from the male

gamete in order to provide the molecular machinery which organizes and forms the sperm aster for proper pronuclear migration, pronuclear apposition, and proper cellular division. We hypothesize that there will be a limit to the extent of moisture loss the feline sperm cells can tolerate during processing without DNA and centrosomal damage. In order to test this hypothesis, in AIM 2 the TUNEL assay will be used to determine DNA damage as a function of moisture loss. Tubulin staining will be used to assess centrosomal function, and hoechst staining for DNA decondensation post dehydration. Fertilization ability and embryo development will also be examined in parallel to determine this limit.

2.4 Aim 3: Examination of Changes in Embryonic Genome Activation Post Dehydration

We know that embryonic gene activation (EGA) is essential for embryonic development, and also reflects the quality of the produced embryos (Waurich, 2010; Haribal, 2012). Stage specific morphology and cleavage timing have been commonly accepted criteria for assessing and comparing embryo development. Improvements in molecular methods have added to that list to include mRNA levels and gene expression patterns (Waurich, 2010; Haribal, 2012). In bovines, mRNA abundances during early embryonic development is an accepted quality marker (Wrenzychi, 2007). The comparison of mRNA levels between groups of embryos has shown differences between *in vivo* and *in vitro* produced embryos (Lee, 2001; Corcoran, 2006), between alterations in culture medias (Warzych, 2007), and between fresh and cryopreserved sperm sources (Waurich, 2010). Proceeding on the assumption that the only source of mRNA in the oocytes is of maternal origin, we can assume any increase in mRNA level within the feline embryo is due to EGA and embryonic RNA transcription. With this understanding, in Aim 3 we hypothesize that by microwave dry processing feline sperm to the tolerance limit for DNA and centrosomal damage determined in Aim 2, there will not be any significant changes to

embryonic genome activation and mRNA levels in the resulting embryos compared to those created with fresh sperm.

CHAPTER 3: UNDERSTANDING NORMO-PHYSIOLOGIC FUNCTION AND DAMAGE

3.1 Spermatogenesis and Sperm Morphology

In order to understand the molecular damage incurred during the dehydration process, we must first understand the basics of normal spermatogenesis. The fully functional sperm is formed during the process of spermatogenesis, during which each spermatid is differentiated into a mature sperm cell. Spermatogenesis can be divided into two successive sections, spermacytogenesis and spermiogenesis. Spermatocytogenesis includes the generations of the cells of the spermatogonium up to and including the secondary spermatocytes. The approximately 1 billion Type A spermatogonia in the testicles form the basal layer of the germinal epithelium of the seminiferous tubules. These Type A Spermatogonia undergo mitosis over an approximate 16 day period to form Type B Spermatogonia. During this process, one of the daughter cells renew the stock of Type A Spermatogonia, while the other becomes a Type B Spermatogonia. The Type B Spermatogonia then undergo a clonal expansion where the daughter cells continue to divide and form the Primary Spermatocytes. The Primary Spermatocytes are then involved in a period of maturation where they undergo the first meiosis over an approximate 24 day period to form the Secondary Spermatocytes, which then undergo the second meiosis, over a period of only several hours, to form the Spermatids. Neither DNA replication nor a recombination of genetic material occurs during the second meiosis, which allows the process to occur quickly. Through the division of the chromatids of a Secondary Spermatocyte, two haploid

spermatids arise that contain only half of the original DNA content. During all of the cellular divisions of spermatocytogenesis, the separation of the cytoplasm is not complete, and a network of connected cells bound between thin cytoplasmic bridges arise from this process. This connection assures that all of the processes in each generation occur in step with each other.

Spermatid formation denotes the beginning of the process of spermiogenesis, which is a period of approximately 24 days, during which time the connected spermatids are transformed into sperm cells. There are four distinct phases of this process; nuclear condensation, acrosome formation, flagellum formation, and cytoplasmic reduction. During nuclear condensation, there is a thickening and reduction of the nuclear size by a condensation of the nuclear contents in the nucleus and takes on a flattened, oval/pear shape. Acrosome formation is the formation of a cap at the front region of the sperm head that functionally acts as a lysosome and contains enzymes, specifically hyaluronidase, which is used to penetrate through the zona pellucida of the oocyte. The Golgi complex brings together the many small acrosome vesicles to give rise to the full acrosome. A rotation of the nucleus causes repositioning of the acrosome. The nucleus and the acrosome form the sperm cell's head that is bound to the mid-piece by a short neck region.

Sperm tail formation occurs during the process of flagellum formation, where the axonemal structure grows out of the distal centriole, the latter consisting of a bundle of nine peripheral double microtubules and two single ones in the center. This flagellar primordium is positioned on the opposite side of the acrosome through the rotation of the nucleus and the acrosome. The mitochondria are packed thickly and tightly together

around the beginning part of the flagellum. There are four distinct regions of the finished flagellum – the neck, mid piece, principle piece, and tail. The neck contains the proximal and distal centrioles; the midpiece consists of sheath of ring shaped mitochondria grouped around the axoneme that provide energy for movement; the principle piece is a sheath of ring fibers arounds the axoneome; the tail consists of the 9+2 structure of the axoneme.

During cytoplasmic reduction, there is an elimination of all unnecessary cytoplasm. The cytoplasm cell components that are no longer needed are phagocytosed by Sertoli cells and are discarded into the lumen of the seminiferous tubules. The end result of Spermiogenesis is an immature sperm, denoted by a small amount of residual cytoplasm around its neck region. Through the processes of Spermatogenesis, the developing sperm cells are moved in succession from the basal layer of the epithelium to the lumen of the seminiferous tubules. The mature sperm cell, approximately 60um long and 5um wide and completely enveloped by the plasma membrane, is developed through final stages of maturation from the seminiferous tubules through the epididymis.

Centrosomal reduction also occurs during spermatogenesis. In the primary spermatocyte, the two centrioles duplicate as the DNA replicates. After Meiosis I, the secondary spermatocyte with two centrioles duplicate, but the DNA does not, resulting in two centrioles in each of the haploid spermatids. The spermatid then undergoes spermatogenesis, at which time the proximal centriole is retained intact in the sperm neck, while the distal centriole progressively degenerates and is partially reduced, eventually merging with the sperm axoneme in the midpiece and tail (Sathananthan, 2001; Schatten 2009; Palermo, 1997). The functional proximal centriole displays the pin-

wheel structure of nine triplet microtubules surrounded by pericentriolar components. The mature spermatozoa is the motile sperm cell that is the haploid male gamete cell containing one functional proximal centriole, whose function is to join the ovum to form a zygote, a single cell that is complete with a full set of chromosomes, half gained from the spermatozoa and half from the ovum, that develops into an embryo (Figure 1).

Development of a preservation process for a particular cell type requires knowledge of its development, function, and specific cellular compartments in order to preserve the critical components of the cell. Through understanding the maturation and development of the sperm cell, we are able to better make decision on which developmental stages of sperm are best for preservation, and what the specific challenges are at each stage.

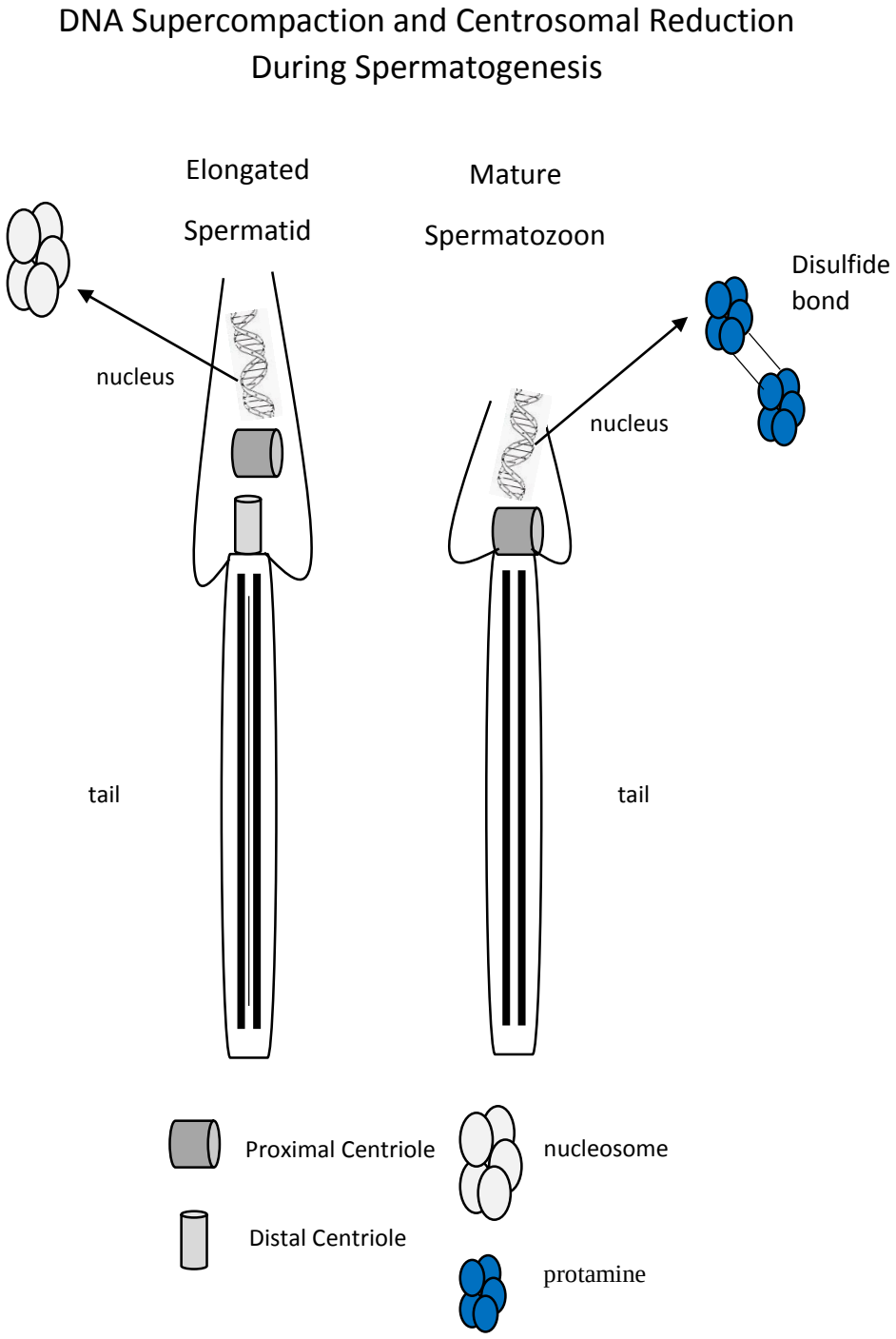

Figure1: DNA supercompaction and centrosomal reduction during spermatogenesis.

3.1.1 Ejaculated Versus Non-Ejaculated Sperm

The type of sperm utilized for preservation studies deserves some attention. We know that there is a level of DNA fragmentation damage that is initiated in the sperm cell during the necessary processes of chromatin repackaging during spermatogenesis, and that these levels of damage are typically repaired during epididymal transit and before ejaculation, and we know that unrepaired damage leads to DNA fragmentation in the resulting sperm (Mengual, 2003). The next logical question would be why use and attempt to store sperm from non-ejaculated samples. In order to achieve the best reproductive outcome, we have to consider several aspects that are not exclusive of each other; which set of sperm have the least amount of DNA damage, which have the highest embryo growth potential, and which set of sperm are more easily available, especially when we are considering wild or endangered species.

It has been shown in domestic felines, that testicular sperm, ICSI injected into *in vitro* matured feline oocytes, have proper embryo growth potential (11%), at the rate of about half that of ejaculated sperm (21%), showing that feline domestic cat testicular sperm are capable of supporting proper embryo development and that even though there are complex mechanisms involving epididymal maturation that are beneficial for conventional fertilization, these processes are not absolutely necessary to produce blastocyst embryos in the feline model (Comizzoli, 2006). Studies assessing feline chromatin stability and resistance of the DNA to acidic denaturation has been evaluated in different regions of the epididymis – Caput/Head receiving spermatozoa from the testis via the efferent ducts, moving to the Corpus/Body, then to the Cauda/Tail. This data showed that as sperm progressed from the Caput to the Cauda regions, the percentage of

sperm heads with persistent histones via aniline blue staining decreased from 31.8 to 7.8%, indicating a further compaction of the chromatin via protamine replacement, increasing stability and decreasing exposure to damage. This data also showed chromatin stability in the form of dsDNA via acridine orange staining increased significantly from the Caput (51.1%) to the Cauda (86.5%) regions of the epididymis (Hingst, 1995). With this data, we can come to the conclusion that epididymal sperm collected from the caudal epididymis has the best embryo growth potential outside of ejaculated spermatozoa.

3.2 Oogenesis

Oogenesis, the process of the female gamete development, starts following the migration of the primordial germ cells into the gonadal ridge during fetal development. At birth, the female germ cell is called the primary oocytes, where it arrests in prophase until puberty. Post puberty, the primary oocyte goes through a sequence of ordered matuations starting with finishing the first unequal meiotic division, resulting in a secondary oocyte and a polar body containing half of its genetic material. The secondary oocyte then arrests in Metaphase II until ovulation, where it travels through the oviduct towards the uterus. It will compete meiosis II, and produce a second polar body, only if successfully fertilized by a mature sperm cell. During oogenesis, the egg centrosome is reduced and inactivated since there should be only one functional centrosome to ensure normal development.

3.3 Mammalian Fertilization

Mammalian fertilization encompasses a series of ordered steps, involving distinct regions of the sperm and oocyte that culminates in zygote formation (Wassarman, 2001, Wasserman, 1999). Sperm motility depends on ATP production by the mitochondria

located in the midpiece. Sperm mitochondria must produce ample energy in the form of ATP to power the flagellar motion of the tail region to propel the sperm to the ova. Once at the site of fertilization, the roles of the sperm head come into play. The sperm head is comprised mainly of an acrosome, a large secretory vesicle that overlies the apical region of the sperm head, and a densely packed region of chromatin safeguarding the sperm's genetic material (Wasserman, 1999). Sperm with an intact acrosome must first bind in a species-specific manner to the thick extracellular membrane, or zona pellucida, of the egg. Once bound to the zona pellucida, sperm must undergo the acrosome reaction, or cellular exocytosis, and then penetrate the extracellular membrane. Having reached the perivitelline space between the egg zona pellucida and plasma membrane, sperm must bind to the plasma membrane and then fuse with it. Fusion with a single sperm prevents the oocyte plasma membrane from fusing with further sperm that have penetrated the zona pellucida. At this point, the egg has been fertilized and becomes a zygote. (Wassarman, 2001).

Once the spermatozoa has bound to and penetrated the plasma membrane of the oocyte, several events involving the DNA within the sperm head must take place, including decondensation of the sperm nucleus, development of the male and female pronuclei, and pronuclear migration to the center of the oocyte (Plachot, 2000). Chromatin decondensation must take place to ensure proper pronuclear formation. It is well documented that sperm nuclear material is greater than 10-fold more compacted than somatic cell chromatin (Miller, 2010). It has been speculated that this super compaction may help optimize nuclear shape to increase swimming ability and/or to confer additional protection from genotoxic factors (Miller, 2010; Balhorn, 2007). The super compaction

is achieved through the replacement of their nucleosomes with smaller arginine rich protamines. Nucleosomes are octomers consisting of two copies of each of the four replication-dependent DNA-binding histones (H2A, H2B, H3, H4) (Miller, 2010). During the spermiogenic phase of spermatogenesis, the nucleosomes are destabilized by the hyperacetylation of histones, which reduces the charge difference between the histone and the DNA, decreasing their affinity for DNA (Kurtz, 2007; Miller, 2010). The histones are temporarily replaced by transition proteins, then by protamines - a diverse family of proteins synthesized in late-stage spermatids that bind to DNA, condensing the spermatid genome into a genetically inactive state (Balhorn, 2007; Chapman 2003). During the continued maturation process through the epididymis, the chromatin packaging continues to stabilize as inter- and intra-protamine disulphide bonds are further established, maintaining the chromatin in a crystalline state (Golan, 1996). Once in the oocyte, this super condensed chromatin must decondense to form a pronucleus. Maternal factors begin replacing the protamines with maternal histones. In contrast to spermatogenesis, the process of fertilization requires that the disulfide bonds between protamines be broken (Chapman, 2003). There are many hypotheses on the exact mechanism for the reduction of the disulfide bonds formed during spermatogenesis, but there is no question that the oxidation, and reduction of the sulfhydryl groups is critical to sperm chromatin decondensation (Chapman, 2003; Zirkin, 1989).

Considering that success of fertilization depends on proper DNA content and centrosomal contributions, simultaneous to chromatin decondensation, sperm aster formation must start to take place. In non-rodent mammalian species, with the exception of the meiotic spindle, the oocyte lacks any centriolar structure until penetration by the

sperm, and the sperm centrosome contributes the dominant nucleating seed consisting of the proximal centriole surrounded by pericentriolar components onto which the oocyte's centrosomal material is assembled (Schatten, 2009). The sperm centrosome is primarily responsible for nucleating and organizing the sperm aster, which pushes the sperm head towards the oocyte center and guides migration of the female pronucleus for union with the male pronucleus, completing the fertilization process (Schatten, 2009; Desai, 2009; Moomjy, 1999).

The sperm aster is a formation and organization of microtubule arrays originating from the sperm basal body and extending virtually to the egg periphery. The highly dynamic sperm aster ensures the appropriate positioning of the male and female pronuclei to form the zygote aster, which surrounds the fused pronuclei. The sperm centriole duplicates during the pronuclear stage and separates after syngamy, migrating around the zygote nucleus to form opposite poles of the first, and all subsequent, cell divisions (Schatten, 2009; Moomjy, 1999).

3.4 Critical Components

3.4.1 Assisted Reproductive Technologies

The advent of Intra Cytoplasmic Sperm Injection (ICSI), or the manual injection of a single sperm into an oocyte for in vitro fertilization, has allowed immotile sperm to fertilize an oocyte, with the first successful fertilization in 1998, showing that an immotile sperm can retain genetic integrity. With the use of assisted reproductive technologies, neither the plasma membrane nor motility of the spermatozoa are aspects of the sperm cell that must be preserved in order to maintain function. ICSI bypasses the need for sperm motility to bind to cells of the female reproductive tract, and the need for

sperm to undergo hyperactivation, zona binding, and the acrosome reaction (Desai, 2009; Martins, 2007). Sperm cells remain viable and capable of fertilization via ICSI based on the fact that the sperm nuclear integrity is maintained (Martins, 2007) for proper chromatin decondensation and centrosomal integrity is maintained for proper sperm aster formation in the zygote. In order to determine safeguards and assays necessary to assess dry preservation processing and storage, it is important to understand the damage that can occur to the critical components of the sperm cell. Damage can render these critical components incapable of performing their necessary tasks for proper embryo development.

3.4.2 Molecular Damage

The two characteristics that differentiate sperm cells from somatic cells are protamination and DNA repair mechanisms (Gonzalez-Marin, 2012). DNA fragmentation damage can be caused by a wide range of intrinsic (apoptosis, deficiencies in recombination, protamine imbalance, oxidative stress) or extrinsic factors (storage temperature, extenders, handling conditions, physiologic stresses pre-ejaculation) (Gonzalez-Marin, 2012). The dense compaction of sperm DNA provides protection to the DNA from a wide range of assailants that might impart fragmentation. The normal and necessary processes of meiotic crossing-over and the replacement of histones with protamines to super-coil the sperm DNA impart levels of temporary DNA fragmentation to the cells, which if unfixed, evolve into DNA fragmentation of mature sperm. Meiotic crossing-over is associated with genetically programmed introduction of DNA breaks by specific nucleases, whereas chromatin packaging includes endogenous nuclease activity necessary for the transient relief of torsional stress during the loosening of chromatin-

histone bonds and the introduction of chromatin-protamine bonds (Gonzalez-Marin, 2012; Mengual 2003).

These levels of damage are typically repaired during epididymal transit and before ejaculation, but unrepaired damage leads to DNA fragmentation (Mengual, 2003). DNA repair in sperm is terminated as transcription and translation stops post spermatogenesis, therefore sperm cells have no mean of repairing DNA damage that occurs post epididymal transit and ejaculation (Gonzalez-Marin, 2012; Comizzoli, 2006). It has been clinically documented that sperm DNA damage is positively correlated with lower fertilization rates in both natural and assisted reproduction technologies (ART) (Evenson, 1980; Larson, 2000; Fernandez-Gonzalez, 2008). Successful embryo development depends on both proper DNA content and centrosomal function of the male gamete.

ICSI can overcome membrane damage and loss of motility, but cannot overcome DNA damage, one of the most important components of the reproductive outcome in the spermatozoa. It has been shown that oocytes and early embryos can help repair sperm DNA damage, so the effect of sperm damage on the developing embryo depends on both sperm chromatin damage and the oocyte's ability to repair some of this damage (Aitken, 2001). Fertilization of an oocyte by a spermatozoa damaged by extensive double stranded DNA fragmentation can be incompatible with complete fertilization and embryo development. Activation checkpoints slow down cell cycle progression until DNA damage is resolved, and if remain unrepaired, cellular senescence and apoptosis are initiated (Gonzalez-Marin, 2012). It has been well clinically documented that sperm DNA damage is positively correlated with lower fertilization rates and embryo

development in both natural and assisted reproduction technologies (ART) (Evenson, 1980; Larson, 2000; Fernandez-Gonzalez, 2008).

Not much is known about oxidative damage in feline sperm, with most studies focusing on retention of plasma membrane, motility, and acrosome integrity post cryopreservation (Pukhazenthi, 1999). Work in some mammalian species, rodent, non-rodent, and human, have found that sperm exposure to high oxidant environments causes greater levels of DNA damage; this damage did not necessarily minimize the sperm's ability to fertilize an oocyte, but showed a detrimental effect on embryo development (O, 2006; Chen, 2002; Aitken, 1998, 2001,2004; Morris, 2002).

Some work specific to feline DNA has been done looking at antioxidant capacity to ameliorate Reactive Oxygen Species (ROS) DNA damage generated during cryopreservation, and it was found that the addition of 5 mM cysteine to the resuspension medium increased DNA integrity at 6 hours post thaw (Thuwanut, 2008). These are bases to start from in delving into quantifying and assessing feline sperm DNA damage during dry preservation processing and storage. The majority of felines have low sperm concentrations, and a high proportion of abnormal spermatozoa (Mota, 2006). When this DNA damage in the sperm cell is not completely repaired by the oocyte and resulting zygote, and embryo progression continues, the damage gets passed on to the resulting embryo, transitioning any pathology associated with the unrepaired damage to the offspring.

The sperm centrosome is the second critical component of the sperm cell necessary for function and fertilization. Defects in proper centrosomal function have been documented to have serious consequences on fertilization and embryo development

(Comizzoli, 2006; Schatten, 2011). The mature MII oocyte supplies the molecular machinery that separates oocytes chromosomes post fertilization, allowing the extrusion of half of the chromosomes into the second polar body and retain the other half to form the female pronucleus (Schatten, 2011). The sperm contributes the centriole-centrosome complex, which has been reduced during spermatogenesis but has retained the functional proximal centriole required for sperm aster formation, for zygote nuclear formation, and for the bipolar mitotic apparatus that forms after centriolar duplication during the pronuclear stage, as well as critical centrosomal proteins (tubulin) required for the recruitment of additional centrosomal proteins (tubulin) from the oocyte (Schatten, 2011).

The sperm's centriolar complex consists of a proximal centriole surrounded by centrosomal proteins tubulin, pericentrin, and centrin, and a distal centriole that is degraded post fertilization. Much research is ongoing concerning the complexity, functionality, and pathologies associated with the centrosome and surrounding centrosomal components. It has been reported that insufficient tubulin or centrin proteins in the sperm centriolar complex results in abnormal sperm aster formation, and decreased fertilization (Hinduj, 2010; Schatten 2011; Comizzoli, 2006). In the domestic cat, defects in proper centrosomal function have been documented to have serious consequences on fertilization and embryo cleavage - it has been shown that poor patterns of aster formation can contribute to delayed first cell division and decreased developmental rates, demonstrating that sperm centrosome maturity, obtained during epididymal maturation, contribute to fertilizing ability and embryo quality (Comizzoli, 2006). These poor developmental patterns can be reversed by replacing the centrosome, at the time of ICSI

fertilization, with a more mature one, indicating centrosomal maturation and function is essential to proper development of the embryo (Comizzoli, 2006).

CHAPTER 4: MATERIALS AND METHODS

4.1 Moisture Content Metrics

4.1.1 Determination of Dry Weight

A glass culture dish was weighed and the mass recorded. A piece of Whatman conjugate release filter paper (GE life Sciences) was added to the glass culture dish, and the mass of the filter paper recorded. A volume of 400ul of 0.3M trehalose in 1x TE buffer was pipetted onto the filter paper inside of the glass culture dish, and the mass of the solution was recorded. Five replicates were performed using the same procedure. All samples were dried in an oven at 125°C for 48 hours. Post drying, the mass of each culture dish with the dried sample on the Whatman paper was recorded. The dry weight (DW) was calculated for each sample by subtracting the mass of the glass culture dish and the mass of the Whatman paper from the total mass post drying. Dry weights for the 5 replicates were used to determine an average dry weight for 400ul of 0.3M trehalose in 1xTE buffer.

4.1.2 Reporting Moisture Content

The sample volume used for all drying experiments was 40ul. With knowledge of the average dry weight for 400 ul of 0.3M trehalose in 1xTE, the dry weight for 40ul was determined by scaling. Sample water content post dehydration was determined by Karl Fisher titration. End moisture content was reported as gH_20/gDW, calculated as the mass of water (g) as determined by Karl Fisher divided by the DW of 40ul of 0.3M trehalose in 1xTE buffer.

4.2 Optimization of Drying Surface

4.2.1 Sperm Preparation

Testes from domestic cats were harvested from a local spay and neuter clinic and transported at 4°C in 1x PBS to the laboratory within 4 hours post routine orchiectomy. Testes (6 testes per replicate) were dissected to remove the cauda epididymis in F10 Hams Hepes medium (Irvine Scientific, Santa Ana, Ca) supplemented with 1.0 mM pyruvate, 1.0 mM glutamate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum. The cauda epididymis were sliced through several times and after 15 minutes of incubation at room temperature in supplemented F10 Hams Hepes medium, testicular cellular suspensions were centrifuged at 300g for 8 minutes and the pellet was suspended in complete Hams-Hepes medium and stored at room temperature, using methodology as described by Comizzoli et al, 2006. A volume of 10ul of epididymal sperm suspension was analyzed for basic semen parameters, including concentration, percent motility, forward progression (1-4), and activity (1-4).

4.2.2 Substrate Wetting – Visual Observations

In general, samples that spread out and uniformly wet a surface will dry more quickly and reproducibly, therefore the wettability of surfaces was evaluated in order to characterize the suitability as a substrate for sperm drying. A volume of 40ul of 0.3M trehalose in 1x TE buffer was added to various potential drying substrates, loading vertically onto the middle of the surface. Visual degree of wetting out across the sample was categorized as good, fair, or poor, based on the degree of spread across the surface of the substrate. Substrates assessed include PCR cap tubes (Fisher), 1oz. plastic coverslips (Fisher), poly-lysine coated 1oz. glass coverslips (Fisher), 13mm Thermanox plastic

coverslips (ThermoScientific), 35mm plastic culture dishes (Sarsdedt), Whatman conjugate release filter paper (GE Life Sciences), Whatman 3 Chr Chromatography paper (GE Life Sciences), Whatman 1 Chr Chromatography paper (GE Life Sciences), Whatman 20 Chr Chromatography paper (GE Life Sciences), and Whatman 31 ET Chromatography paper (GE Life Sciences). To coat glass coverslips with poly-lysine, each glass coverslip was placed in the lid of a 50ml conical tube. A volume of 1ml of poly-lysine (Sigma) was added to each lid, and incubated for 30 min at room temperature. Coverslips were removed using tweezers, taking care to keep the poly-lysine coated side up, and placed on an absorbant surface to dry. Once dry, coated coverslips were stored at room temperature for periods up to several months.

4.2.3 Microwave Drying of Sperm on Selected Substrate for Sperm Recovery Determination

A volume of 40ul of 0.3M trehalose in 1x TE buffer was microwave dried on selected substrates demonstrating fair to good visual wetting ability in order to determine sperm recovery post drying. A commercial CEM SAM 225 microwave (Matthews, NC), set to 20% power, was used to deliver low power setting microwave energy in an 11% relative humidity environment for a period of 30 minutes, as previously reported in Cellemme et al, 2013.

4.2.4 Sperm Recovery Determination

Immediately post microwave drying, dried sperm samples were rehydrated in 1 ml Hams Hepes media at 37°C for 1 hour with mild shaking. Sperm concentration was determined using a Makler counting chamber with a volume of 10 ul of sample. Sperm concentration in 1 ml was determined, and then calculated for the original 40ul sample.

Percent sperm recovery was calculated as ((sperm concentration post-drying in 40ul) / (sperm concentration pre-drying in 40ul)) * 100.

4.3 Optimization of Processing Conditions

4.3.1 Relative Humidity (RH) and End Moisture Content

With the goal or drying to a low end moisture content, less than 0.2 gH_20/gDW, drying at different relative humidity levels was assessed. With decreasing relative humidity, the chemical potential of water above the sample is decreased , leading to more rapid drying and lower end moisture contents. Microwave drying at various relative humidities was performed to assess end moisture content of the dried sample as a function of relative humidity. A volume of 40ul of 0.3M trehalose in 1x TE buffer was dried on 13mm Thermanox coverslips using a commercial CEM SAM 225 microwave, set to 20% power, for a period of 30 minutes, in 45, 23, and 11% relative humidity environments. A total of 8 samples were processed per microwave run. Immediately post drying, one sample was titrated and end moisture content reported as described in section 4.1.2.. A total of 4 replicates were performed per selected RH.

4.3.2 End Moisture Content as a Function of Microwave Time at 11% Relative Humidity

To assess end moisture content as a function of microwave drying time at 11% RH, 40 ul volume samples of 0.3M trehalose in 1x TE buffer were dried for 0-50 minutes and end moisture content reported as previously described in section 4.3.1. A total of 8 replicates were performed at each 10 minute interval, and 5 replicates performed at each intermediate 5 minute interval.

4.3.3 Evaluation of Temperatures Post Dehydration

Increases in sample temperature during the microwave dehydration process is an important aspect to evaluate, as increases above the normo-physiological temperatures of the dried sample can have deleterious effects on function post rehydration. For each of the microwave runs described in the previous section, the temperature of the titrated sample was measured immediately post dehydration using a hand held infra-red thermometer (General Tools, IRT207).

4.4 DNA Damage Evaluation Post Dehydration

4.4.1 Sperm Sample Poration

Mammalian cells do not have the ability to produce trehalose *in vivo*, nor are they naturally permeable to trehalose. In attempts to confer dehydration tolerance to the sperm cells, trehalose within the dry preservation medium must be introduced into the sperm cell through poration of the plasma membranes. Epididymal sperm suspensions were permeabilized by incubating with 0.5 mg/mL α-hemolysin (Sigma) for 15 minutes at 37.5°C. Incubated samples were centrifuged at 300g for 8 minutes, and the pellet resuspended in 0.3M trehalose (Sigma) for 30 minutes at room temperature.

4.4.2 TUNEL Assay

Levels of sperm nuclear DNA fragmentation damage were assessed in both fresh samples and dehydrated samples using the TUNEL assay (Roche Diagnostics, Indianapolis, IN). A volume of 10ul of each sperm preparation were smeared onto microscope slides (positive control, negative control, and experimental samples) and fixed in 4% paraformaldehyde for 30 min at room temperature. Slides were washed in methanol-free Ethanol for 5 min at room temperature, followed by 3 x 5 min washes at

room temperature in a 1mg/mL PVP-PBS solution. Cells were permeabilized with 0.5% Triton-X-100 in PBS for 5 min at RT, followed by 3 x 5 min washes at room temperature in 1mg/mL PVP-PBS. Positive controls were incubated with 100ul RQ1 DNAse 10X Reaction Buffer for 5 min at room temperature. Following reaction buffer incubation, 6ul of RQ1 RNase-Free DNase was added to the mixture and incubated for an additional 30 min at room temperature. Positive control samples were washed 3 x with 1 ml of deionized water for 5 min. All positive controls, negative controls and experimental samples were incubated in 100ul Equilibration Buffer for 5 min at room temperature. The negative control was incubated in Equilibration Buffer with 5% FCS to decrease false staining. Positive (47.25ul Equilibration Buffer, 3ul Nucleotides, and 0.75ul rTDT enzyme), Experimental (47.25ul Equilibration Buffer, 3ul Nucleotides, and 0.75ul rTDT enzyme) and Negative control (48ul Equilibration Buffer with 5% FCS and 3ul Nucleotides) samples were covered with a cover slip and incubated for 1 hour at 37°C in the dark. After 1 hour, 1ml of 2X SSC was added to each sample and followed by incubation for 15 min at room temperature. All samples were washed in 1ml of 0.2% Triton-X-100 for 5 min at room temperature, followed by 3 x 5 min washes at room temperature in 1ml of deionized water, and thenby 3 x 5 min washes at room temperature in 1ml of 1mg/mL PVP in PBS. Excess liquid was blotted away, and a 30ul volume of Vectoshield/DAPI Flouromount-G (Vector Laboratories, Burlingame, CA) was added to each sample. Samples were then covered with a coverslip and allowed to dry for 15 minutes at room temperature before fluorescence imaging. A minimum of 400 spermatozoon were imaged per sample, selected equally over 4 quadrants, to determine the level of nuclear DNA fragmentation damage within each sample.

4.5 Embryo Creation

4.5.1 Oocyte Collection and Preparation

Ovaries from domestic cats were harvested from a local spay and neuter clinic and transported at 4°C in 1X PBS to the laboratory within 4 hours post routine ovario-hysterectomy. The ovaries were sliced in H-MEM (Hepes-buffered Minimal Essential Medium, supplemented with 0.4mM cysteine, 0.4 mM glutamate, 4.0 mM pyruvate, 100 ul/mL penicillin, 100 ul/mL streptomycin) to recover the immature oocytes. Grade I immature oocytes, characterized by dark, homogenous cytoplasm surrounded by several layers of compacted cumulous cells, were selected for *in vitro*-maturation, using methodology as previously described by Pukazhenthi, 2004.

4.5.2 Oocyte *In Vitro* Maturation

Grade 1 oocytes were cultured *in vitro* in Protein Plus Blastocyst Embryo Culture Medium (Sage, Trumbull, CT) supplemented with 0.2 ug/mL FSH (Sigma), 0.1 ug/ml LH (Sigma), and 0.1 ug/ml estradiol (Sigma). Oocytes were cultured for 24-28 hours at 37.5°C, 6.0% CO_2 and 5.0% O_2 in groups of 5 oocytes per 50 ul drops of *in vitro* culture medium. To determine the maturation stage of individual oocytes, oocytes were denuded following *in vitro* culture by gentle pipetting in 0.2% cumulase (Origio). Only mature oocytes at the MII stage, indicated by the presence of a polar body, were selected for Intracytoplasmic Sperm Injection (ICSI) fertilization.

4.5.3 Intracytoplasmic Sperm Injection (ICSI) Fertilization and Embryo Culture

To identify differences in embryo development between embryos created from fresh epididymal sperm and dehydrated epididymal sperm, embryos were created via Intracytoplasmic Sperm Injection (ICSI) fertilization of *in vitro* matured feline oocytes with either unprocessed or dehydrated epididymal sperm. A volume of 2ul of sperm suspension was added to 10ul of 10% polyvinylpyrrolidone (PVP, Irvine Scientific) in the center drop of a 50x9 mm ICSI dish (NUNC) surrounded by 8 x 10ul drops of complete Hams Hepes medium and overlayed with oil. Due to the poor mixing of the trehalose in PVP, for dehydrated sperm suspensions, spermatozoon were first added to a drop of Hams Hepes medium in the ICSI dish, collected, and moved to the middle PVP drop for immobilization, then aspirated into the injection pipette, and injected into a mature oocyte. Mature *in vitro* matured oocytes were added to the top 3 Hams Hepes drops of the ICSI dish. The dish was maintained on a heated stage (38.5°C) of an inverted microscope (Olympus IX73) equipped with holding pipette and micromanipulators (Narishige, Sterling, VA). A single morphologically normal spermatozoon was selected, immobilized by drawing the injection pipette across the midpiece aspirated into the injection pipette, and injected into a mature oocyte (polar body at 12 or 6 o-clock). Motile fresh spermatozoon as well as immotile dehydrated spermatozoon were immobilized across the midpiece prior to injection, Injected mature oocytes were cultured in groups of 5 oocytes in 50ul drops in Protein Plus Blastocyst Medium (Sage, Trumbull, CT) at 37.5°C 6% CO_2 and 5% O_2 for 7 days, as previously reported in Comizzoli, 2006. Additional 10 mature oocytes were sham injected (no sperm) for each replicate to serve as parthenogenic controls.

4.6 Assessment of Developmental Processing Damage

4.6.1 Comparison of Embryo Development

Embryos were assessed on Day 3 and Day 7 post fertilization for embryo cell number and/or embryonic stage. On Day 3, embryos were imaged on an inverted microscope (Olympus IX 73) with a heated stage (38.5°C), and the cell number recorded for each embryo within 4 categories (1-cell; 2-4 cell; 5-8 cell; 9-16 cell). Following embryo assessment, embryos were returned to the incubator for further culture. Embryos on Day 7 were imaged on an inverted microscope (Olympus IX 73) with a heated stage (38.5°C). Day 7 embryos were fixed onto microscope slides using 2.5% paraformaldehyde incubation for 30 min at 38.5°C followed by chromatin staining with 1ug/ml Hoechst 33342 in PBS for 5 min at 38.5°C. Embryos were mounted with Vectoshield/DAPI/Flouromount, covered with a coverslip, and allowed to dry for 15 min at room temperature prior to imaging with epifluorescence microscopy. Embryonic cell number or stage was determined for each embryo within 6 categories (1-cell; 2-4 cell; 5-8 cell; 9-16 cell; Morula; Blastocyst).

4.6.2 Sperm Aster Assessment: Centrosomal Function

To assess the preservation of centrosomal function post dehydration processing, sperm aster formation was evaluated in embryos created from dehydrated and unprocessed sperm samples. Presumptive zygotes from ICSI of *in vitro* matured oocytes with fresh or microwave dehydrated sperm, and parthenogenic controls from SHAM ICSI injection, were created as described in section 4.5. Presumptive zygotes were fixed at 5 hour post insemination (hpi) after *in vitro* cultured at 38°C in 6% CO_2 and 5% O_2. Zygotes were fixed in 2.5% paraformaldehyde for 30 min at 38.5°C, followed by 3 x 5

min washes in 1X PBS at room temperature. Nonspecific antigenic sites were saturated with 0.5% Triton-X-100 and 20% FCS in PBS for 30 min at 38.5°C. Presumptive zygotes were incubated overnight with anti-α-tubulin (Sigma) and anti-β-tubulin (Sigma) monoclonal antibodies (1/1000 in PBS with 0.5% Triton-X-100 and 2% FCS) at 4°C. Embryos were washed in PBS (3 x 15 min at room temperature), and incubated with FITC-labeled anti-mouse IgG (Sigma), diluted 1/150 in PBS, and held for 1 hour at 38.5°C. Chromatin were stained with 1ug/ml Hoechst 33342 in PBS for 5 min at 38.5°C, followed by mounting on microscope slides with a 30ul volume of Vectoshield/DAPI/Flouromount-G and covering with a coverslip. Presumptive zygotes were imaged under epifluorescence with a confocal microscope (Olympus Fluoview Laser Scanning Microscope) to assess the degree of sperm aster formation in each zygote.

4.6.3 Evaluation of Levels of DNA Decondensation

In addition to proper centrosomal function, sperm DNA decondensation is a critical step is continued embryo development. Presumptive zygotes were created and stained as described in section 4.6.2, to compare the level of sperm DNA decondensation between embryos created from unprocessed and microwave dehydrated sperm. Presumptive zygotes were imaged under epifluorescence with a confocal microscope (Olympus Fluoview Laser Scanning Microscope) to assess the level of sperm DNA decondensation in each zygote.

4.7 Determination of Embryonic Genome Activation

Embryos were created via ICSI insemination of in-vitro matured oocytes with fresh and microwave dehydrated spermatozoon as previously described, and *in vitro* cultured at 38°C in 6% CO_2 and 5% O_2 for up to 7 days. Oocytes and embryos at all stages of development from ICSI with fresh and microwave dried spermatozoon (immature oocyte through blastocyst stages) were collected and frozen at -80°C in 10ul of RNAse Later until sufficient quantities of each stage for each sperm source could be obtained. Embryos and oocytes were collected and frozen until 3 replicates from each sperm source for each batch (40 x MII oocytes, 20 x 2-4cell embryos, 10 x 5-8 cell embryos, 5 x 9-16 cell embryos, 2 x morula, and 1 x blastocyst) were frozen. Total RNA was isolated from each pooled group (RNeasy Micro kit (Qiagen) following the manufacturers' instructions (RNeasy Micro Handbook, second edition, December 2007) and as previously described in Waurich, 2010. Oocytes and embryos were pooled under microscopic analysis and transferred onto ice just prior to RNA preparation. Cells were lysed and homogenized using needle aspiration (50 x per sample) in buffer/β-mercaptoethanol (10ul/ml). After adding 70% ethanol, total RNA was bound to the MinElute spin column by centrifugation, followed by DNA digestion with DNAse1. After washing with buffer, and 80% ethanol, total RNA was eluted in 14ul RNAse-free water. The RNA concentration (ng/ml) per pooled replicate was determined using a nanodrop spectrophotometer (Thermofisher) reading at absorbance 260 (A260), blanking between samples with the final RNAse free water eludent.

CHAPTER 5: RESULTS

5.1 Trehalose Dry Weight Determination

A 48 hour bake out of 5 replicates of 400ul volumes of 0.3M trehalose on Whatman conjugate release paper yielded an average dry weight of 0.0412 g for a 400ul sample (Table 2). . Dry Weight (DW) was calculated as total mass post drying (g) – mass of culture dish (g) – mass of Whatman paper (g).

Table 2. Dry weight determination of trehalose in 1x TE buffer after 48 hours of bake out in a dry over at 125°C. Dry Weight (DW) was calculated as total mass post drying (g) – mass of culture dish (g) – mass of Whatman paper (g).

Mass Culture Dish (g)	Mass Whatman Paper (g)	Mass Soln (400ul) (g)	Total Mass Post Drying (g)	Dry Weight (DW) (g)	Average DW (g) of 400ul
15.6390	0.0447	15.6803	15.7250	0.0413	
20.5480	0.0442	20.5888	20.6330	0.0408	
19.1270	0.0431	19.1689	19.2120	0.0419	
16.5890	0.0443	16.6297	16.6740	0.0407	
16.0100	0.0465	16.0515	16.0980	0.0415	0.0412

5.2 Visual Substrate Wetting and Sperm Recovery

Visual wetting ability was determined to be poor for PCR cap tubes, 1oz. Fisher brand plastic coverslips, and poly-l-lysine coated glass coverslip drying substrates (Table 3). Visual wetting ability was assessed as fair for 13mm Thermanox coverslips, and good for Whatman Conjugate Release filter paper, Whatman 3Chr Chromatography paper (0.36mm thick), Whatman 1Chr chromatography paper (0.18mm thick), Whatman 20Chr Chromatography paper (0.17mm thick), and Whatman 31ET Chr Chromatography paper (0.50mm thick).

Whatman conjugate release filter paper had an average sperm recovery, post 30 min microwave dehydration of 6.12% (n = 6) (Table 3). Sperm recovery averaged 5.6% using Whatman 3Chr Chromatography paper (0.36mm thick), 3.6% on Whatman 1Chr chromatography paper (0.18mm thick), 4.8% with Whatman 20 Chr Chromatography paper (0.17mm thick), and 7.4% using Whatman 31ET Chr Chromatography paper (0.50mm thick) (n = 6 for each substrate). Average sperm recovery on 35mm plastic culture dishes was 2.67% (n = 6). 13mm Thermanox coverslips averaged a percent sperm recovery of 87.1 (n = 6).

Table 3. Comparison of visual wetting ability and sperm recovery post microwave drying. Percent sperm recovery was determined as ((sperm concentration post-drying in 40ul) / (sperm concentration pre-drying in 40ul)) * 100.

Substrate	**Visual Wetting Ability**	**Average Sperm Recovery (%) n = 6**	**SD**
PCR tube caps	poor	n/a	n/a
1oz. Fisher brand plastic coverslips	poor	n/a	n/a
Poly-lysine coated glass coverslips	poor	n/a	n/a
13mm Thermanox plastic coverslips	fair	87.1	3.6
35mm plastic culture dishes	fair	2.7	0.4
Whatman Conjugate Release Filter Paper	good	6.1	5.5
Whatman 3 Chr Chromatography paper (0.36mm thick)	Good	5.6	1.9
Whatman 1 Chr Chromatography paper (0.18mm thick)	Good	3.6	1.7
Whatman 20 Chr Chromatography paper (0.17mm thick)	Good	4.8	1.6
Whatman 31 ET Chromatography paper (0.50 mm thick)	Good	7.4	0.8

5.3 Relative Humidity (RH), End Moisture Content, and Sample Temperature

Final moisture content post microwave drying of 0.3M trehalose on 13mm Thermanox coverslips for 30 minutes at 45% ambient RH averaged 0.35 gH_20/gDW, with a SD of 0.02 among the 4 replicates (Table 4). Final moisture content of the same solution achieved post drying on Thermanox coverslips for 30 minutes at 23% ambient RH averaged 0.27 gH_20/gDW, with a SD of 0.03 among the 4 replicates (Table 4). With final moisture contents, we can estimate the Tg associated with them. A final moisture content of less than 0.2 gH_20/gDW is the goal of this research, as the estimated T_g associated with that is -20°C, and out of the liquid nitrogen cryogenic storage range. Estimated sample storage temperature must be below the T_g to ensure the sample does not transition from a viscous, rubbery state below T_g to a fluid state above T_g. Below the T_g, In the viscous state, the viscosity is so high that there is negligible molecular mobility and metabolic function, however above the T_g,molecular mobility and molecular ageing increase. Average final moisture content at 45% RH was 0.35 gH_20/gDW, with estimated T_g of ~ -50°C, and average final moisture content at 23% RH was 0.27 gH_20/gDW, with estimated T_g of ~ -36°C. Drying under RH above 23% does not achieve the goal of less than 0.2 gH_20/gDW, and estimated Tg of -20°C. To reach this goal, the drying must be done at a lower RH.

Table 4. Average end moisture content post microwave dehydration at 23 and 45% relative humidity.

Ambient RH (%)	Average gH_20/gDW n = 4	SD
45	0.35	0.02
23	0.27	0.03

The lowest RH maintainable in the drying chamber was 11%. RH was measured every 1 minute for a 4 hour period using an Omega HH314A Handheld Data Logger, and was consistent with an average of 10.96 +/- 0.7% RH (Figure 2). The average end moisture content and estimated T_g after 30 minutes of microwave drying 40ul of 0.3M trehalose on 13mm Thermanox coverslips at 11% RH, was 0.16 gH_2O/gDW and ~ -7°C. This end moisture content is below the goal of 0.2 gH_2O/gDW, and provides an estimated Tg below cryogenic storage. The storage temperature to maintain the sample in this dried state would have to be below -7°C. No further drying was noted after 30 minutes of drying. With increased drying time, end sample temperature increased from 27.9°C after 5 minutes of drying to 37.3°C after 50 minutes. End sample temperature after 30 minutes of drying was measured as 33.2°C, and within the normophysiological temperature range for feline sperm. Microwave drying for 30 minutes under these conditions produced the lowest end moisture content, and therefore the lowest estimated storage temperature.

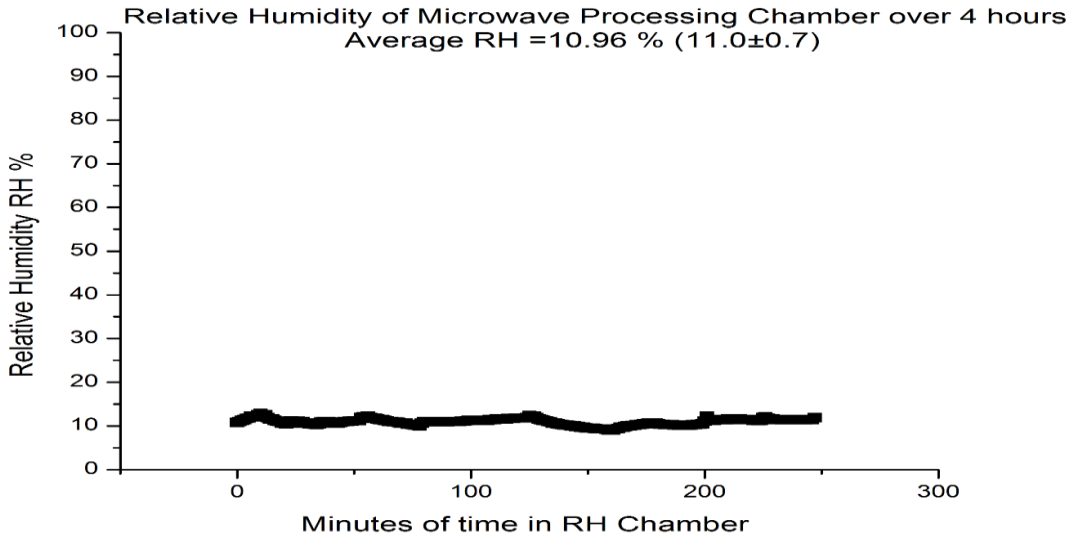

Figure 2. Relative humidity of microwave processing chamber over 4 hours.

Table 5. Final moisture content and sample temperature post microwave drying. Estimated storage temperature determined from T_g calculations based on Gordon Taylor (T_g Tre: 100C; T_g H_20: -135C; k: 5.2).

Microwave Time (min)	**n**	**Average Titration Water Content (g)**	**g H_2O/g DW**	**SD**	**Estimated T_g (°C)**
0	8	0.035402	8.59	0.37	~ -130
5	4	0.029142	7.07	0.76	~ -128
10	8	0.023673	5.74	0.13	~ -126
15	4	0.017180	4.17	1.58	~ -124
20	8	0.010096	2.45	0.40	~ -117
25	4	0.000683	0.17	0.03	~ -10
30	8	0.000650	0.16	0.02	~ -7
35	4	0.000733	0.18	0.05	~ -13
40	8	0.000788	0.19	0.04	~ - 20
45	4	0.000697	0.17	0.03	~ - 10
50	8	0.000738	0.18	0.02	~ - 13

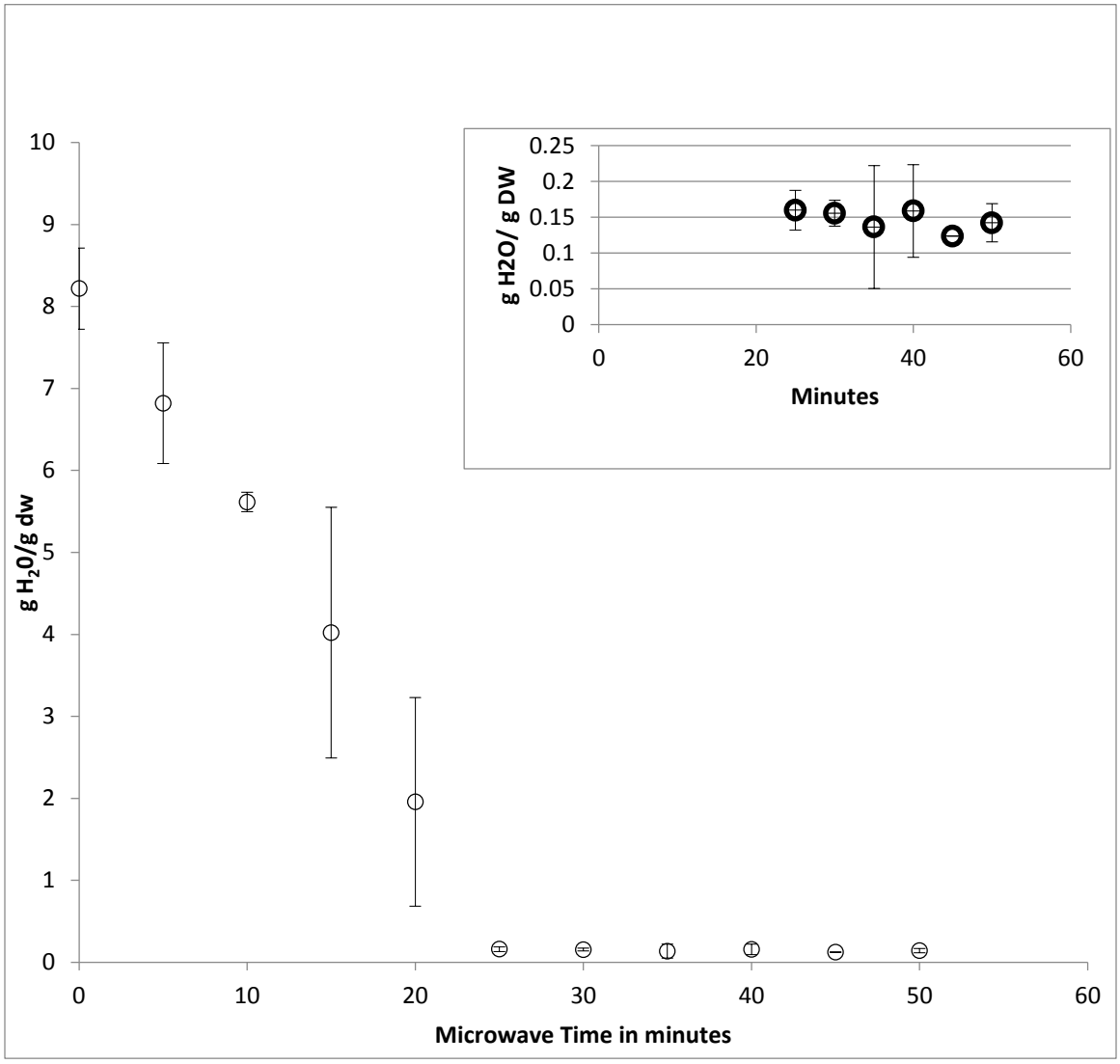

Figure 3. Final moisture contents post microwave drying.

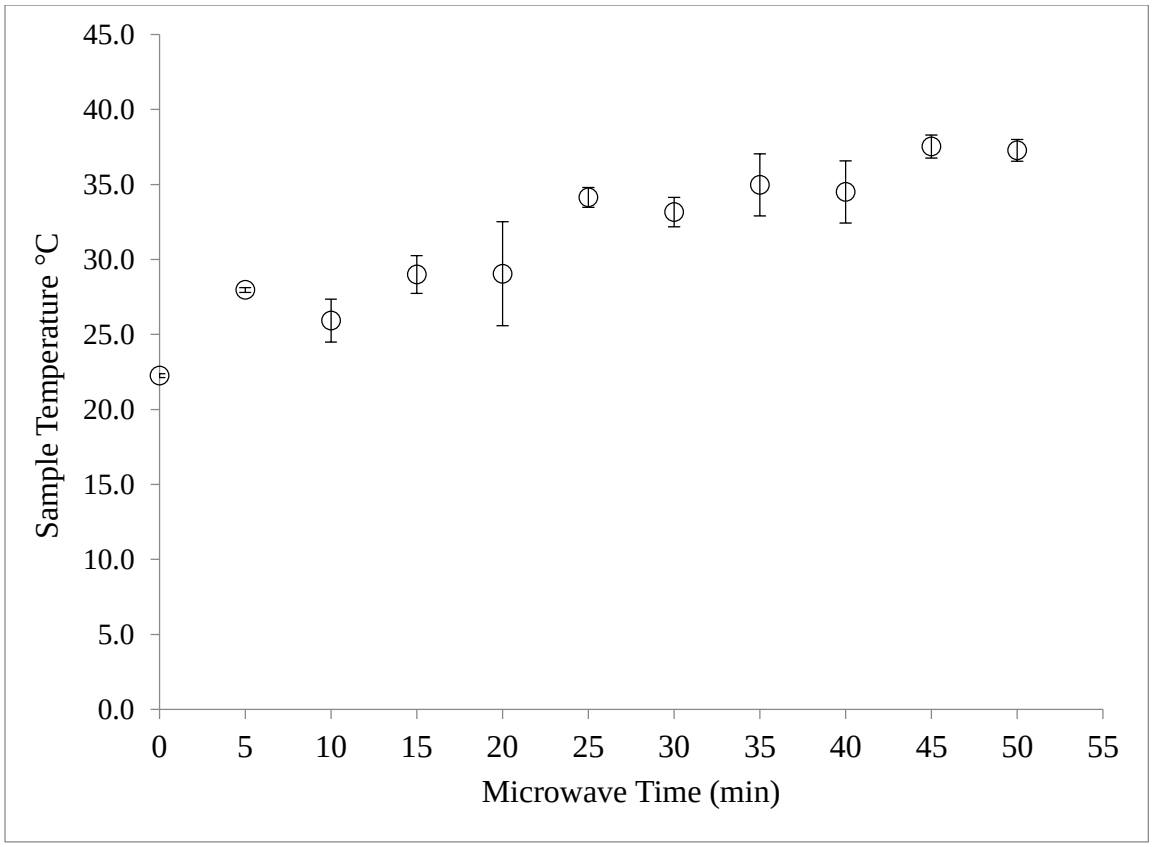

Figure 4. Sample temperature post drying.

5.4 DNA Damage Assessed Using TUNEL Assay

The level of nuclear DNA damage, assessed via TUNEL assay, in unprocessed epididymal sperm was not significantly different than the negative control (Figure 5). The average level of nuclear DNA damage, measured as FITC fluorescence, in the unprocessed sample was 385ADU, with a SD of 12.1 , compared to the negative control FITC fluorescence of 381 ADU, with a SD of 12.6 (Figure 6). The average positive control fluorescence was 2474 ADU with a SD of 110.

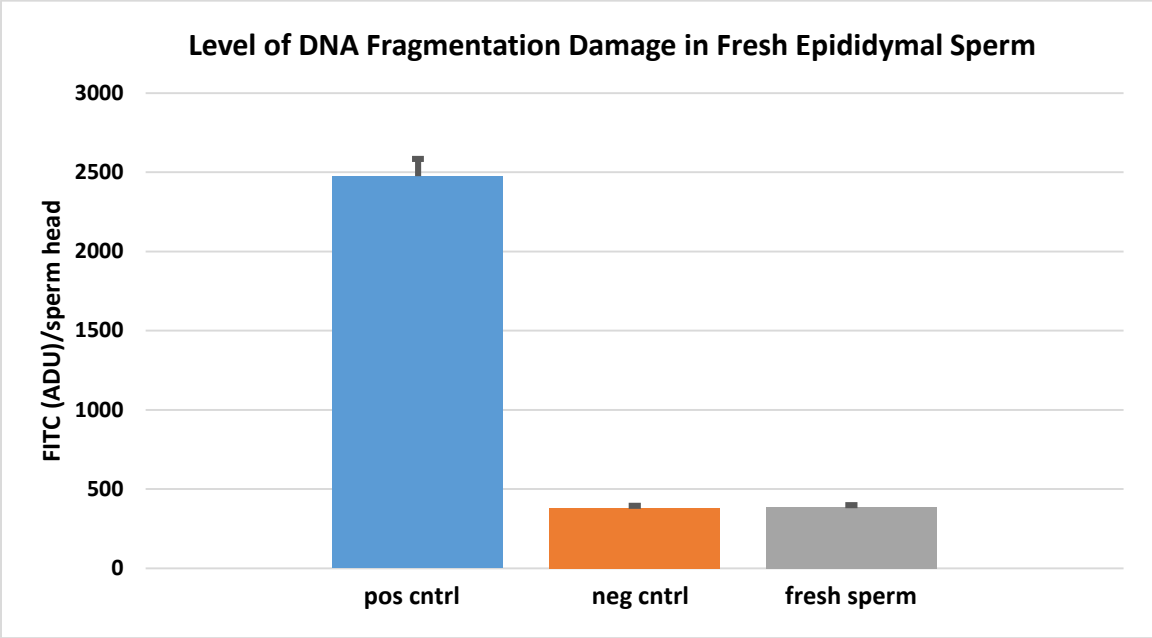

Figure 5. Level of Nuclear DNA Fragmentation Damage in Unprocessed Epididymal Sperm. Fresh, unprocessed sample and negative control samples not significantly different by unpaired T-test.

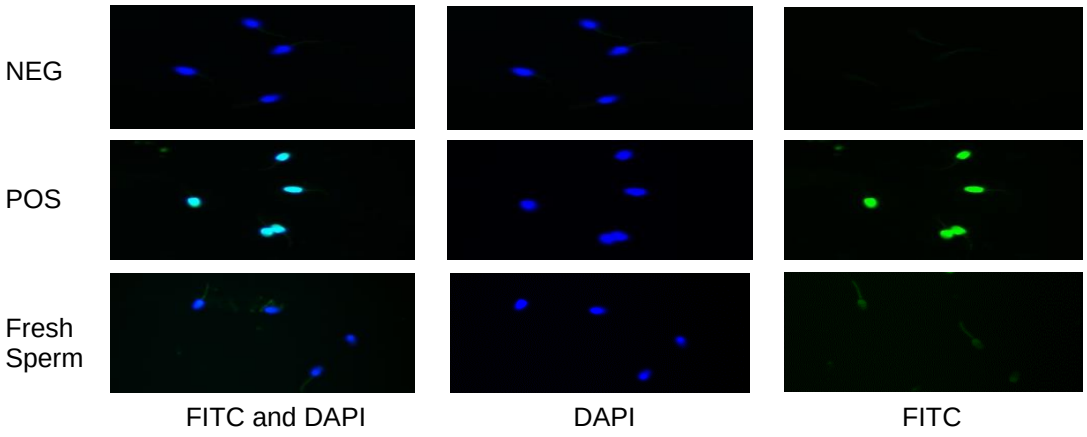

Figure 6. FITC fluorescence (ADU) in fresh, unprocessed epididymal sperm via TUNEL assay.

The level of nuclear DNA damage in unprocessed epididymal sperm and dehydrated epididymal sperm was determined using TUNEL assay with 4 replicates in each group per timepoint (10, 20 30 minutes), More than 400 sperm were analyzed per replicate (Table 6). The average level of nuclear DNA damage for the unprocessed and 30 minute microwave dried sperm samples was 385 ADU and 395 ADU, respectively, which were not significantly different levels by unpaired T-test analysis.

The levels of nuclear DNA damage in each microwave dried group (0, 10, 20, 30 min) was broken down into percentage of damage with respect to the unprocessed sample (1-10%, 11-20%, 21-30%, and 50+%), and graphed as a normalized percent to the positive TUNEL control (Figure 7). The percent of damage 1-10% above the unprocessed sample after 30 minutes of microwave drying was significantly different than that in the unprocessed sample ($p<0.05$). Levels of damage between 11-50% above the unprocessed sample were not significantly different than those in the unprocessed sample. The percent of nuclear damage in unporated and trehalose exposed sperm heads was significantly greater (unpaired T-test, $p<0.01$) than that in porated and trehalose exposed sperm heads after 10 minutes of microwave dehydration (Figure 8).

Table 6. Nuclear DNA damage FITC fluorescence (ADU) in unprocessed and microwave dried sperm.

	n	average FITC (ADU) /sperm head	Average FITC (ADU)	SD
Positive Control	410	2556		
	422	2400	2474	110
Negative Control	408	371		
	487	389	381	12.6
Fresh, Unprocessed Sperm	430	376		
	437	375		
	443	401		
	403	388	385	12.1
Dried Sperm 10 min	481	397		
	491	407		
	477	412		
	452	410	407	6.5
Dried Sperm 20 min	408	382		
	414	373		
	406	410		
	418	417	395	21.5
Dried Sperm 30 min	428	386		
	429	382		
	449	401		
	469	408	395	12.6

Figure 7. Levels of nuclear DNA damage above the unprocessed sample in microwave dried sperm, graphed as normalized percents to the positive TUNEL control. * $p<0.05$ with unpaired T-test.

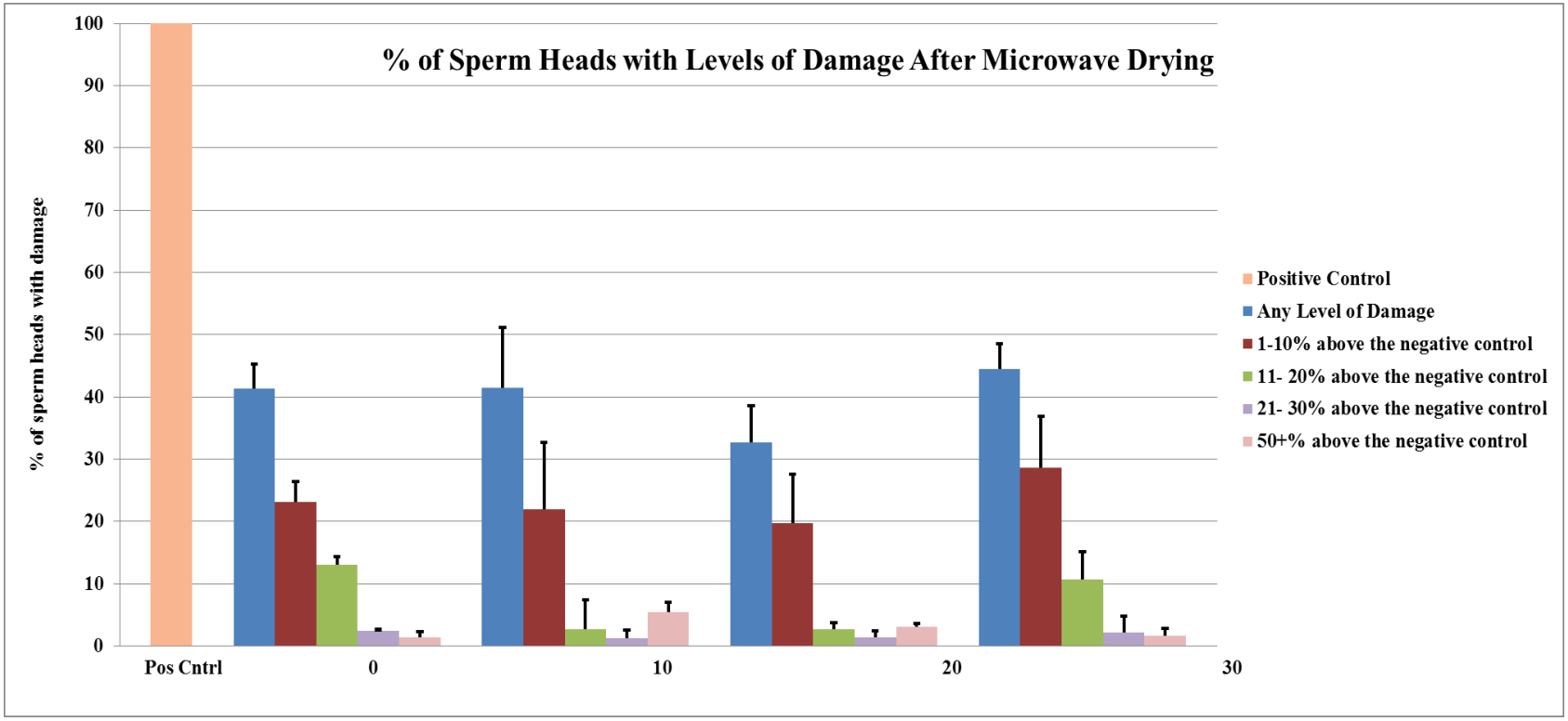

Figure 8. Percentage of nuclear DNA damage in microwave dried and trehalose exposed sperm heads, with and without poration. Level of damage was normalized to the positive control. * $p<0.01$ with unpaired T-test compared to fresh sample.

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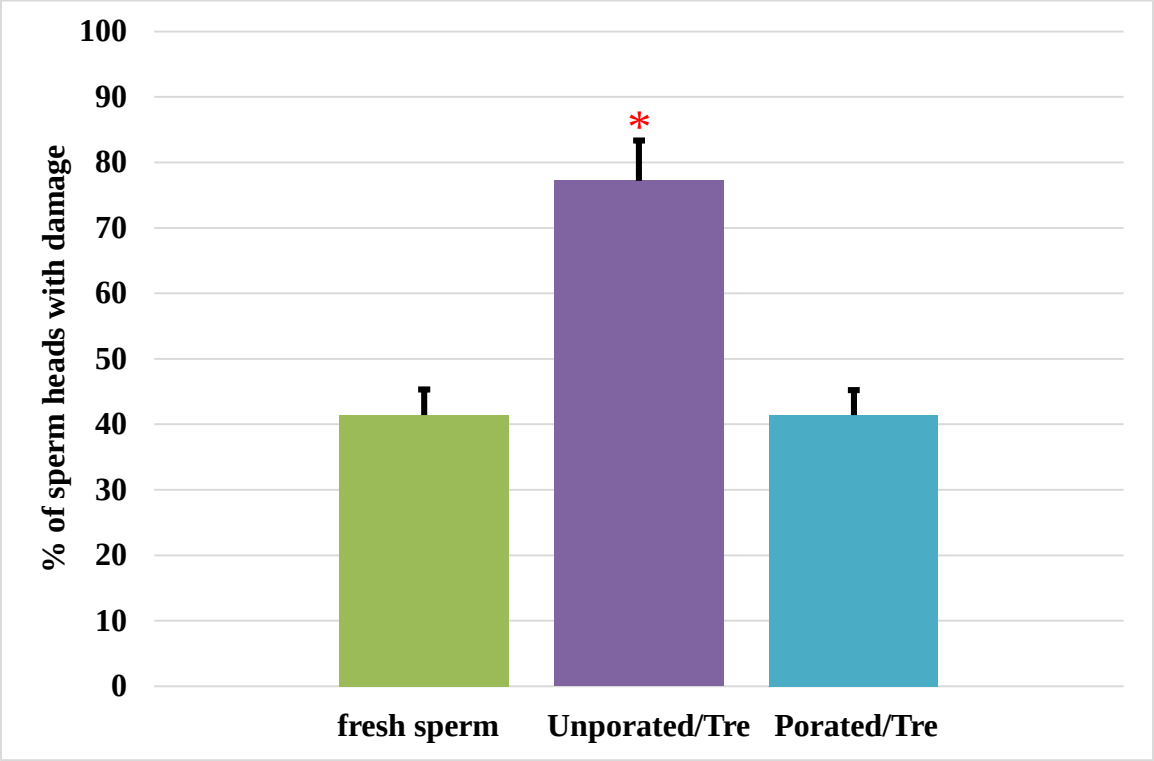

5.5 Embryo Development

Table 7 shows comparisons of embryo growth through day 7 between embryos created from fresh, unprocessed epididymal sperm and microwave dehydrated epididymal sperm, including data on oocyte maturation rates, cleavage rates, 2-4 cell / 5-8 cell / and 9-16 cell embryos on day 3 and day 7, as well as morula and blastocyst

formation rates on day 7 for each of 4 replicates. No significant difference was seen among *in-vitro* maturation rate between any of the replicates. On day 3 of *in vitro* culture there was a significant decrease (unpaired t-test, $p<0.01$) in the percent of cleaved oocytes from the dehydrated sperm group (75.8%) compared to the fresh sperm group (88.9%) (Table 7). There was also a significant increase (unpaired t-test, $p<0.05$) in the percent of embryos at the 2-4 cell stage on Day 3 in the dried group (32.0%) compared to the fresh group (24.0%). There was not a significant difference between the percent of embryos at the 5-8 cell stage on day 3 between the fresh and dehydrated groups. There were significantly fewer (unpaired t-test, $p<0.01$) embryos that reached the 9-16 cell stage on day 3 in the dehydrated group (16.7%) compared to the fresh group (27.1%). On day 7 of *in vitro* culture there continued to be a significant increase (unpaired t-test, $p<0.05$) in the percent of uncleaved embryos (22.2%) in the dehydrated group compared to the fresh group (10.9%), as well as a significant (unpaired t-test, $p<0.01$) decrease in the percent of embryos at the 2-4 cell stage in the dehydrated group (23.2%) compared to the fresh group (8.9%). There was not a significant difference between the percent of embryos at the 5-8 cell or 9-16 cell stage on day 7 between the fresh and dehydrated groups. For embryos that continued to progress past the cellular stage by Day 7, there was a significant difference (unpaired t-test, $p<0.01$) between the percent that progressed to the morula stage in the fresh (26.6%) and dehydrated (12.3%) groups, as well as a significant difference (unpaired t-test, $p<0.01$) in the percent that progressed to the blastocyst stage in the fresh (15.1%) and dehydrated (6.4%) groups. A total of 40 *in-vitro* matured oocytes were SHAM injected (without a sperm, 10 per replicate) to serve as parthenogenic controls, with a 0% cleavage rate by Day 7.

Blastomere numbers within the blastocycts were not significantly different between the fresh (92) and dried (87) groups (Table 8). Figure 9 shows representative images of unstained and hoechst stained day 3 and morula embryos created from fresh and dried epididymal sperm. Figure 10 shows representative image of unstained and hoechst stained blastocysts created from fresh and dried epididymal sperm.

Oocyte Collection	Treatment Group	# G1 eggs	# G1 MII	% maturation	# ICSI	Day 3 Assessment	Cleavage	1-cell	2-4 cell	5-8 cell	9-16cell		Day 7 Assessment	1-cell	2-4 cell	5-8 cell	9-16cell	Morula	Blastocyst	
		650	415	63.85																
total of 4 replicates	Fresh Epididymal				192	n	170	22	46	72	52	192	n	21	17	35	39	51	29	192
						%	* 88.9	** 11.5	** 24.0	37.5	* 27.1	100.0	%	** 10.9	* 8.9	18.2	20.3	* 26.6	* 15.1	100.0
						SD	0.0	1.2	5.1	10.2	4.9		SD	1.5	3.2	3.5	2.8	3.3	3.3	
total of 4 replicates	Dried Epididymal				203	n	158	45	65	59	34	203	n	45	47	35	38	25	13	203
						%	* 75.8	** 22.2	** 32.0	29.1	* 16.7	100.0	%	** 22.2	* 23.2	17.2	18.7	* 12.3	* 6.4	100.0
						SD	0.1	7.9	3.4	6.3	4.3		SD	7.9	5.6	6.4	8.0	5.7	3.7	
total of 4 replicates	SHAM				40	n	0	40				40	n	40						40
						%	0.0	100.0				100.0	%	100.0						100.0
						SD	0.0	0.0					SD	0.0						

Table 7. Comparison of embryo growth from *in-vitro* matured oocytes ICSI inseminated with unprocessed and microwave dried epididymal sperm. ** $p<0.05$ * $p<0.01$ unpaired T-test

Table 8. Blastomere numbers in blastocysts created from fresh and dried epididymal sperm.

	Average	SD
Fresh Epididymal Sperm (n = 8)	92	15.0
Dried Epididymal Sperm (n = 8)	87	10.9

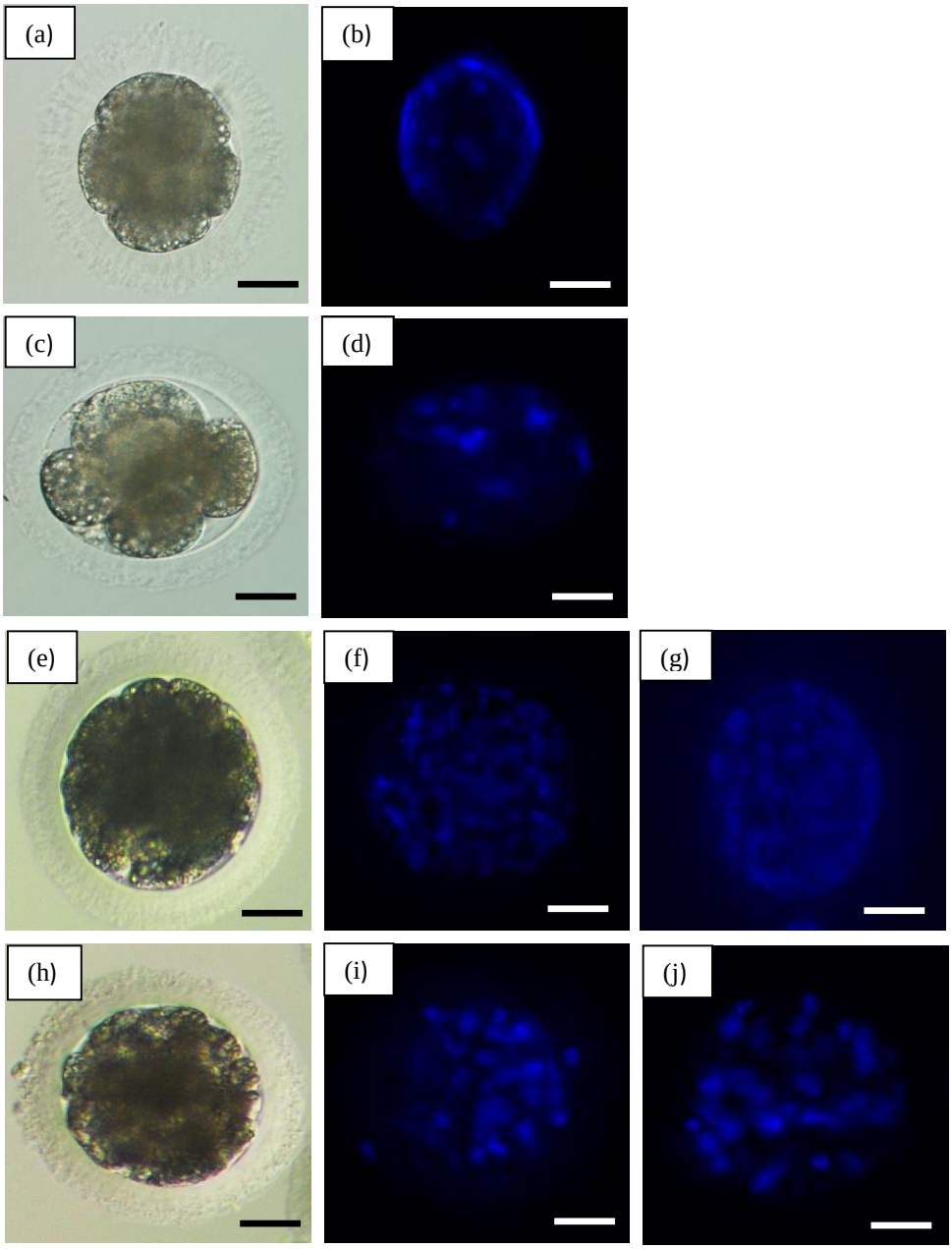

Figure 9. Unstained and hoechst stained day 3 and morula embryos created from fresh and dried epididymal sperm. Fresh day 3 unstained (a) and hoechst stained (b); dried day 3 unstained (c) and hoechst stained (d); fresh morula unstained (e) and hoechst stained (f, g); dried morula unstained (h) and hoechst stained (i, j). bar = 25 µm

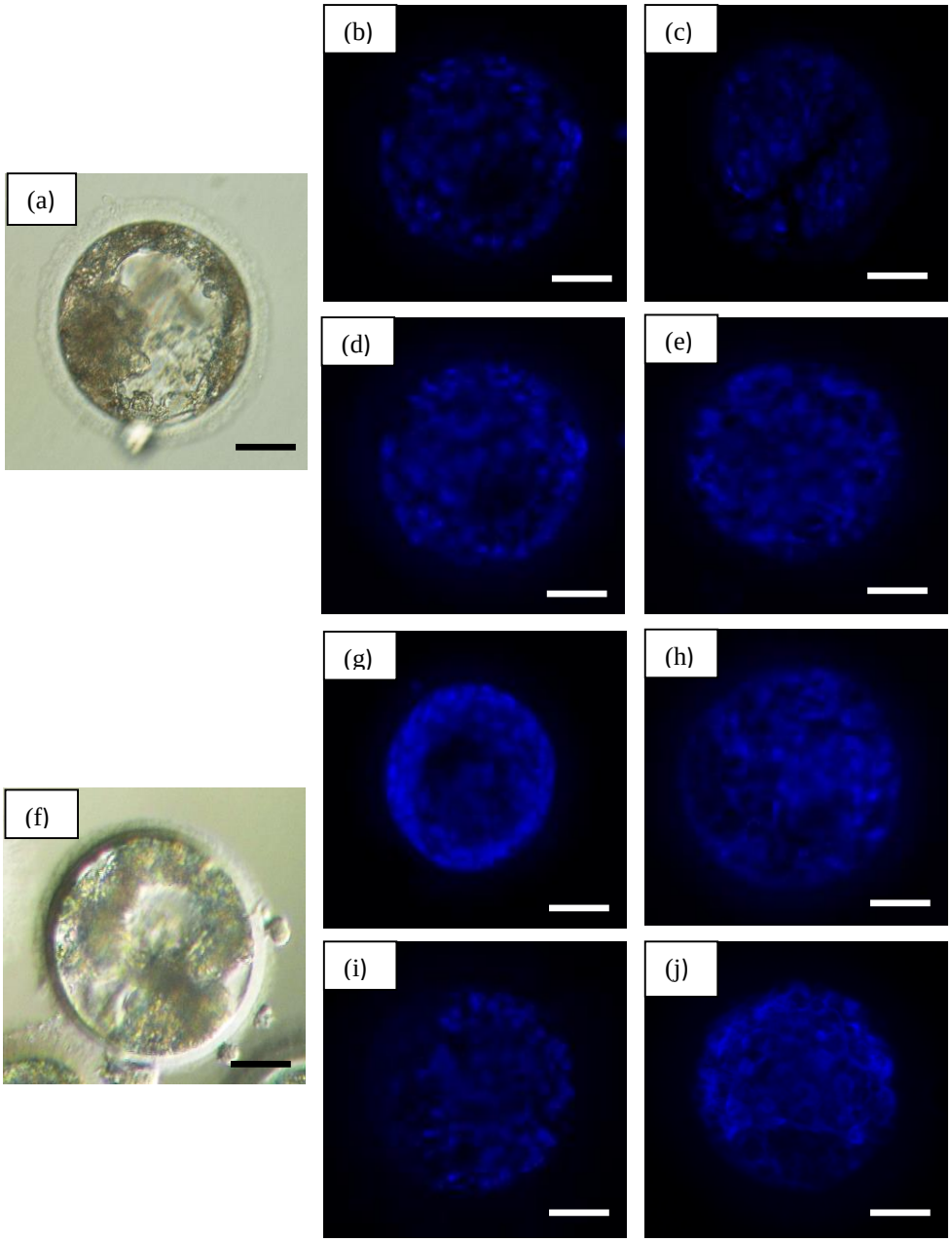

Figure 10. Unstained and Hoechst stained day 7 blastocyst embryos created from fresh and dried epididymal sperm. Fresh day 7 blastocyst unstained (a) and Hoechst stained (b, c, d, e); dried day 7 blastocyst unstained (f) and Hoechst stained (g, h, i, j). and Hoechst stained (i, j). bar = 25µm

5.6 Sperm Aster Development

Sperm aster development was assessed 5 hours post insemination (hpi) of matured oocytes with fresh and microwave dried/rehydrated epididymal sperm. Approximately 140 ICSI inseminated oocytes, in 6 replicates, were analyzed in each group (137 oocytes with fresh sperm and 144 oocytes with dried sperm) for the presence and size (absent, small or large) of the sperm aster (Table 9). Unfertilized oocytes were categorized by the absence of pronuclei. There was not a significant difference between the fresh and dried sperm groups with respect to the percent of absent sperm asters (16.8% fresh and 20.1% dried) or the percent of small sperm asters (62.0% fresh and 58.3% dried). There was a significant difference (unpaired t-test, $p<0.05$) between the percent of large sperm asters in the fresh (14.6%) compared to the dried (5.6%) groups. There was also a significant difference (unpaired t-test, $p<0.05$) between the percent of unsuccessful fertilization between the 2 groups, with 6.6% unfertilized in the fresh group and 16.0% unfertilized in the dried group. Figure 11 shows representative confocal images at 5 hip of large, small, absent sperm aster as well as unfertilized mature oocyte.

5.7 Sperm DNA Decondensation

The degree of sperm DNA decondensation was also assessed within the same set of zygotes used for the sperm aster assessment at 5 hpi. The overall degree of decondensation was reported between the groups, as well as a breakdown of the level of sperm DNA decondensation by sperm aster size between the two groups (Table 10). There was not a significant difference between the overall degree of sperm DNA decondensation between the groups, with the fresh sperm group averaging 5.43µm of

chromatin stained region, and the dried sperm group averaging 5.10µm. There was not a significant difference in the degree of sperm DNA decondensation between the two groups (5.41µm in fresh group and 5.02µm in the dried group) in the 'absent sperm aster' subset.. There was a significant difference (unpaired t-test, $p<0.01$) between the degree of sperm DNA decondensation when comparing DNA decondensation levels among subsets of small and large sperm asters. Sperm DNA decondensation in the fresh group averaged 4.91µm and 5.81µm in the dehydrated group in the small sperm aster subset. In the large sperm aster subset, sperm DNA decondensation in the fresh group averaged 6.63µm, compared to 5.31µm with the dried sperm.

Table 9. Sperm aster size at 5 hours post insemination in *in-vitro* matured oocytes inseminated with fresh or dried epididymal sperm. Unpaired t-test: ** $p<0.05$

	Fresh Sperm Aster Size			
n	absent	small	large	unfert
13	3	8	0	2
18	2	14	2	0
21	3	14	3	1
19	4	13	1	1
39	7	22	7	3
27	4	14	7	2
total n	23	85	20	9
%	16.8	62.0	**14.6	** 6.6

	Dried Sperm Aster Size			
n	absent	small	large	unfert
17	1	13	0	3
23	7	13	1	2
19	1	17	0	1
21	2	15	0	4
39	11	16	4	8
25	7	10	3	5
total n	29	84	8	23
%	20.1	58.3	** 5.6	** 16.0

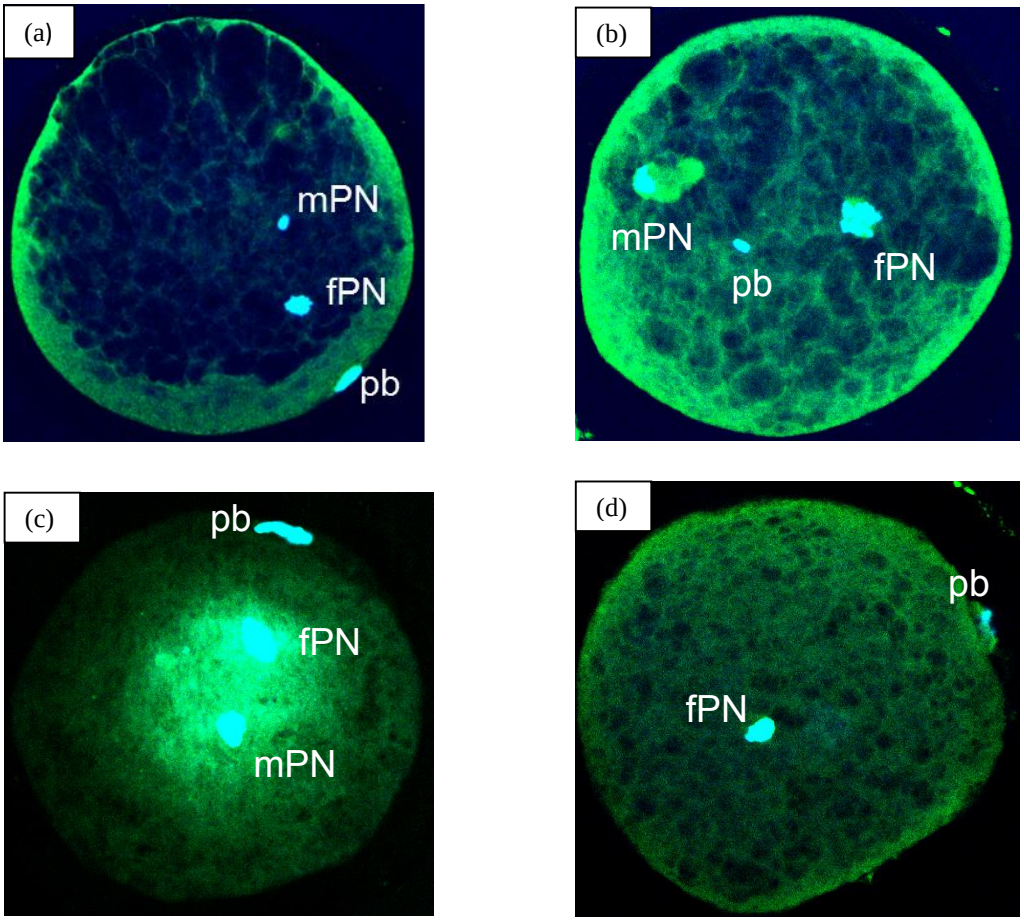

Figure 11. Confocal images of sperm asters and unfertilized mature oocyte at 5 hours post insemination. Staining with anti-α-tubulin and anti-β-tubulin monoclonal antibodies followed by FITC-labeled anti-mouse IgG, and Hoechst. Imaging with epifluorescence and confocal microscopy. (a) absent sperm aster, (b) small sperm aster, (c) large sperm aster, (d) unfertilized.

Table 10. Sperm DNA decondensation at 5 hpi in *in-vitro* matured oocytes inseminated with fresh or dried epididymal sperm. Unpaired t-test: * $p<0.01$

Degree of Fresh Sperm DNA Decondensation – avg. diameter (µm)

	absent		small		large		all size asters	
n	avg	SD	avg	SD	avg	SD	avg	SD
13	5.47	1.18	4.48	1.76	n/a	n/a	-----	-----
18	4.77	1.47	4.77	3.16	4.76	1.94	-----	-----
21	4.87	1.77	4.87	2.36	4.25	1.86	-----	-----
19	7.17	1.71	5.17	2.72	13.72	n/a	-----	-----
39	4.64	2.28	4.96	1.96	4.88	2.05	-----	-----
27	5.51	2.67	5.22	2.42	5.55	2.33	-----	-----
137	5.41	1.85	*4.91	2.40	*6.63	2.05	5.43	2.58

Degree of Dried Sperm DNA Decondensation - avg. diameter (µm)

	absent		small		large		all size asters	
n	avg	SD	avg	SD	avg	SD	avg	SD
17	4.22	n/a	6.93	2.71	n/a	n/a	-----	-----
23	5.64	2.43	5.76	2.33	6.72	n/a	-----	-----
19	3.31	n/a	5.05	1.56	n/a	n/a	-----	-----
21	8.16	1.56	8.16	1.64	n/a	n/a	-----	-----
39	3.93	1.36	4.38	1.54	4.40	1.72	-----	-----
25	4.87	2.04	4.56	1.88	4.81	2.00	-----	-----
144	5.02	1.85	*5.81	1.94	*5.31	1.86	5.10	2.69

5.8 Embryonic Genome Activation

The mRNA levels between the 6 replicates of control MII oocytes (40 oocytes per replicate) were not significantly different, with an average of 1.2 ng/µl and a SD of 0.1 (Table 11). The 2-4 cell embryos (3 replicates per group with 20 embryos per replicate) had an average mRNA concentration of 3.1 ng/µl and SD of 3.3 in the fresh group compared to an average of 2.6 ng/µl and SD of 2.5 in the dried group. The 5-8 cell embryos (3 replicates per group with 10 embryos per replicate) had an average mRNA concentration of 2.9 ng/µl and SD of 2.1 in the fresh group compared to an average of 1.5ng/µl and SD of 2.0 in the dried group. The 9-16 cell embryos (3 replicates per group with 5 embryo per replicate) had an average mRNA concentration of 1.5 ng/µl and SD of 1.9 in the fresh group compared to an average of 1.3 ng/µl and SD of 1.3 in the dried group. Embryos that reached the morula stage (3 replicates per group with 2 embryos per replicate) in the fresh group had an average mRNA concentration of 3.6 ng/µl and SD of 1.3 compared to an average of 3.0 ng/µl and SD of 2.0 in the dried group. Developed blastocysts (3 replicates per group with 1 embryo per replicate) in the fresh group had an average mRNA concentration of 4.0 ng/µl and SD of 2.6 compared to an average of 4.4 ng/µl and SD of 3.7 in the dried group. There were no significant differences between mRNA concentrations in the fresh and dried groups in any of the embryonic stages of development.

Table 11. mRNA levels in oocytes and varying stages of embryos created from ICSI insemination of *in-vitro* matured oocytes with fresh or microwave dehydrated epididymal sperm.

Stage	replicates / total n	Fresh /Dried	Average mRNA Concentration (µg/µl)	SD
MII oocyte	6 / 240	~	1.1	0.1
2-4 cell embryo	3 / 60	Fresh	3.1	3.3
2-4 cell embryo	3 / 60	Dried	2.6	2.5
5-8 cell embryo	3 / 30	Fresh	2.9	2.1
5-8 cell embryo	3 / 60	Dried	1.5	2.0
9-16 cell embryo	3 / 15	Fresh	1.5	1.9
9-16 cell embryo	3 / 15	Dried	1.3	1.3
morula embryo	3 / 6	Fresh	3.6	1.3
morula embryo	3 / 6	Dried	3.0	2.0
blastocyst embryo	3 / 3	Fresh	4.0	2.6
blastocyst embryo	3 / 3	Dried	4.4	3.7

5.9 Processing Condition Comparison Between Experiments

Several aspects of the dehydration process were monitored during each microwave dehydration run, including titration value post dehydration, percent of sperm recovery, temperature of the chamber at the beginning and end of the microwave run, humidity within the chamber at the beginning and end of the microwave run, microwave temperature at the beginning and end of the microwave run, and sample temperature at the end of the microwave run. Comparing these values between the three experiments, embryo development, sperm aster/DNA decondensation, and embryonic genome

activation, no significant difference was found among replicates in any category, confirming constant processing conditions. (Table 12).

Table 12: Titration values, percent sperm recovery, and processing temperatures during microwave dehydration of epididymal feline sperm. Titration values averaged per replicate. Average titration value per replicate, sperm recovery, start chamber temp, start micro temp, start humidity, stop chamber temp, stop micro temp, and stop humidity compared between embryo development experiments, sperm aster, or RNA experiments, showing no significant difference in any category.

Experiment	Replicate #	Titration 1	Titration 2	Titration 3	Titration 4	Avg Titration	SD	% recovery	Start Chamber Temp	Start Micro Temp	Start Humidity	Stop Chamber Temp	Stop Micro Temp	Stop Humidity
Embryo Development	1	731.561	688.242	644.87	702.419	691.773	36.1	91.1	24.0	22.8	11.2	32.0	26.0	10.6
Embryo Development	2	663.715	730.874	667.753	673.871	684.053	31.5	88.6	24.0	21.3	11.1	31.0	24.4	10.6
Embryo Development	3	723.641	698.196	675.244	702.109	699.798	19.8	89.3	23.0	21.8	11.3	30.0	25.6	10.4
Embryo Development	4	812.124	782.261	691.143	634.193	729.930	82.0	93.0	23.0	22.2	11.1	30.0	26.8	10.7
	average					701.389		90.5	23.5	22.0	11.2	30.8	25.7	10.6
	SD					20.1		1.7	0.5	0.5	0.1	0.8	0.9	0.1
Sperm Aster	1	643.196	713.243	675.239	610.916	660.649	43.8	85.2	24.0	21.1	11.2	30.0	23.3	9.9
Sperm Aster	2	693.75	753.745	713.75	712.5	718.436	25.3	91.0	24.0	21.2	11.6	29.0	23.4	10.8
Sperm Aster	3	656.25	798.75	602.11	706.035	690.786	83.6	88.5	24.0	21.2	11.4	29.0	23.4	10.8
Sperm Aster	4	632.161	701.493	742.716	610.484	671.714	61.2	86.4	24.0	22.0	10.9	30.0	23.1	10.0
Sperm Aster	5	681.416	711.152	632.779	651.238	669.146	34.4	90.5	24.0	21.2	10.9	31.0	24.1	10.2
Sperm Aster	6	609.13	722.415	640.236	731.119	675.725	60.4	82.4	24.0	22.0	11.3	31.0	24.3	10.1
	average					681.076		87.3	24.0	21.5	11.2	30.0	23.6	10.3
	SD					20.8		3.3	0.0	0.4	0.3	0.9	0.5	0.4
RNA	1	674.872	659.032	704.628	769.013	701.886	48.6	86.7	24.0	22.6	11.2	30.0	23.4	10.9
RNA	2	678.928	690.927	631.971	702.318	676.036	30.9	91.6	24.0	21.8	11.3	31.0	23.1	10.4
RNA	3	710.971	659.126	712.398	695.105	694.400	24.8	87.5	23.0	21.3	10.9	31.0	24.1	10.6
RNA	4	713.541	688.43	710.237	633.481	686.422	37.0	93.7	24.0	22.0	11.1	32.0	24.2	10.7
RNA	5	614.947	651.739	668.911	718.917	663.629	43.2	88.6	24.0	22.2	11.4	29.0	23.6	10.6
	average					684.475		89.6	23.8	22.0	11.2	30.6	23.7	10.6
	SD					15.1		2.9	0.4	0.5	0.2	1.1	0.5	0.2
	overall average					687.885		89.064	23.813	21.844	11.194	30.500	24.300	10.494
	SD					19.6		3.0	0.4	0.5	0.2	1.0	1.1	0.3

CHAPTER 6: DISCUSSION

6.1 Processing Conditions and End Moisture Content

Through our studies on optimizing the processing conditions for microwave drying feline epdidiymal sperm, we can conclude that at a stable RH of 11% sperm can be consistently dried to an average moisture content of 0.16 gH_20/gDW (SD 0.015). We also concluded that sample temperatures after these microwaving conditions averaged 33.2°C, which is well under the proposed upper limit of 40°C for proper sperm function. Microwave assisted drying at RH levels above 11% led to final moisture contents well above the 0.16 gH_20/gDW obtainable at 11% RH (0.35 gH_20/gDW at 45% RH, 0.27 gH_20/gDW at 23% RH). Statistical differences in end moisture content could not be resolved when processing beyond 25 minutes under the same conditions. We can hypothesize this stasis in end moisture content, even after increased lengths of drying, could be due to water being bound up within the matrix or slight crusting within the drying sample, impeding further drying. Sample temperatures post drying increased with increasing lengths of drying time, and reached closest to the 40°C threshold at drying times of 45-50 minutes (37.5°C, 37.3°C). Estimated T_g for aqueous trehalose solutions, based on calculations using the Gordon Taylor equation (T_g Tre: 100C; T_g H_20: -135°C; k: 5.2), with an end moisture content of 0.16 gH_20/gDW is ~ -7°C, suggesting storage would be possible at refrigerated but non-cryogenic temperatures below ~ -7°C. Further sample drying under the same processing conditions might be possible using a substrate with a better wetting ability, attempting to bring the storage temperature within the room

temperature range. Sample recovery post drying impeded this in these studies. The selected drying substrate was not the one with the best wetting ability, as sperm recovery post drying was less than 10% in substrates categorized as having good wetting ability. A substrate that met both requirements of sperm recovery post drying and end moisture contents below 0.2 gH_20/gDW was selected as both components were of equal importance in the success of the studies.

6.2 TUNEL Assay and DNA Damage

From previous research, we know that sperm from the cauda epdidiymis have the lowest percentage of sperm heads with persistent histones via aniline blue staining, decreasing from 31.8% in the caput/head to 7.8% in the caudal/tail regions, indicating more compaction of the chromatin (Hingst, 1995). This would increase stability and decrease exposure to damaging agents. The level of nuclear DNA damage, measured by TUNEL assay, in unprocessed epididymal sperm collected from the cauda epdidiymis (385 ADU) did not have a significantly increased level of damage over the TUNEL assay negative control (381 ADU), likely owing to the increased level of chromatin compaction during epididymal maturation. Standard deviations in the TUNEL assay for unprocessed and negative control samples were similar (12.08 ADU and 12.61 ADU, respectively) indicating similar dispersion within the tested sample populations.

Microwave assisted drying under the current conditions did not increase the level of nuclear DNA damage after 30 minutes. Drying without prior membrane poration with α-hemolysin showed a significant increase in damage after 10 minutes of microwave drying. With this data we can confirm that trehalose is required to be present on both sides of the membrane to confer protection. Without poration of the plasma and nuclear

membranes, trehalose is unable to enter the cell and surround the nuclear chromatin, associated proteins, and centrosome, to create the protective milieu.

Further investigation into the level of damage that was incurred after 30 minutes of microwave dehydration processing using the current processing conditions, we can subdivide the levels of damage into percents above the negative control (1-10%, 11-20, 21-30, and 50+%). This allows for a better understanding of the damage level being imparted onto the sperm cell. Comparing the unprocessed samples with the 30 minute microwave dried samples, the only significant difference in the damage levels is within the 1-10% above the negative control ($p<0.01$), which bodes well for these processing conditions, indicating that any increase in damage is occurring at a level no more than 10% above the unprocessed samples. Damage levels at intermediate points (10 and 20 minutes) in the drying process were assessed for damage levels, but not statistically compared. High variability at these intermediate drying times made it hard to prescribe moisture contents in this region. The steep changes in moisture content during these drying times could be cause for the variability.

6.3 Embryo Development

To reveal functional damage that might not be evident by the TUNEL assay, fertilization and embryo development was assessed using sperm that was dehydrated to an average of 0.16 gH_20/gDW. A total of 650 feline oocytes were *in-vitro* matured to obtain 415 mature oocytes. These oocytes were obtained in 4 separate replicates of oocyte collection, and in each replicate the oocytes were divided evenly among ICSI insemination with fresh epididymal sperm and dehydrated epididymal sperm. 10 oocytes were SHAM injected (without a sperm) in each replicate. It is important to note here

that of the 40 SHAM injected mature oocytes (10 in each of 4 replicates) a 0% cleavage rate was observed by day 7. This indicates no parthenogenic cleavage, owing all cleavage in the fresh and dried groups to zygote nuclear information. The oocyte maturation rates were not significantly different between the four replicates, indicating similar oocyte quality and *in-vitro* culture conditions for oocyte maturation. There was a significant difference (t-test, $p<0.01$) in the average cleavage rates on Day 3 between the fresh and dried groups (75.8% dried and 88.9% fresh), but there was not a significant difference in the cleavage rates on day 3 among the 4 replicates of the fresh samples, indicating consistent ICSI insemination, and culture conditions over the 4 replicates, minimizing any bias these variables may have on embryo growth. Some variation was seen in the cleavage rates on day 3 in the dried group (80, 64, 81, 78%), with the cleavage rate in one replicate significantly lower than the other three (t-test, $p<0.01$). This is most likely due to random dehydrated sperm selection in this replicate. Oocyte maturation rates and unprocessed sperm ICSI showed no variability within this replicate. This replicate also contained the smallest number of oocytes compared to the other 3 replicates.

A general trend of decreased embryo growth from embryos created with microwave dried epididymal sperm was observed, with more than half of the embryos at the 2-4 cell or uncleaved stages, compared to 35% of the embryos created with fresh sperm. After 7 days of embryo growth, the trend of delayed embryo growth continued in embryos created from dried sperm, with 81% arresting at the cellular stage on day 7, compared to 58% of the embryos created from fresh sperm. Of the 19% of embryos that did not arrest at the cellular stage on day 7 in the dried group, 12.3% were at the morula

stage on day 7 and 6.4% grew to the blastocyst stage. Morula (12.3%) and blastocyst (6.4%) developmental rates in the dried group were significantly lower ($p<0.01$) than those in the fresh group, with 26.6% morula and 15.1% blastocyst development. Feline embryo development using epididymal sperm was previously reported as 25% blastocyst development (Waurich, 2010) and 26.8% in ejaculated feline sperm, which has been noted as equivalent to epididymal sperm in terms of embryo development potential (Comizzoli, 2006). In the current studies, the age of the gamete providers is much younger than those in previous reports (Comizzoli, 2006).

The number of blastomeres in the blastocycts created from fresh and dried sperm were not significantly different from one another, indicating that even though there was a significant decrease in embryo development in the dried group, embryos that were able to develop to the blastocyst stage, appear to be of equal quality morphologically. Inner cell mass (ICM) and trophectoderm were clearly identifiable in each blastocyst from both groups. Differential staining was not performed on these embryos, but using this method in the future would provide additional data with respect to the morphologic equality of blastocysts between these two groups.

Sperm motility in the fresh samples pre-drying averaged 85%. For ICSI insemination with fresh sperm, 100% of the sperm used were visually motile. Post microwave drying, all sperm were non-motile, thus providing no basis for discrimination. Thus for every 100 dried sperm selected for ICSI insemination, 15 of those sperm were likely to be non-motile prior to drying. If we take this in to consideration, and assume those 15% were not genetically/centrosomaly capable of fertilization or embryo development as they displayed no motility pre-drying, the percentage of continued

embryo growth in the dried group could theoretically have been up to 15% higher. Applying such a correction yields an increase in cleavage rate from 75.8% to 89.5%, an increase in morula rates from 12.3% to 14.2%, and an increase in blastocyst rates from 6.4% to 7.4% (Table 13). The increase in cleavage rate is not significantly different than the fresh cleavage rate of 88.9%. The increases in both morula and blastocyst rates are still significantly lower than the fresh rates of 26.6% and 15.1%, respectively, suggesting that pre-drying sperm motility is not the only contributing factor to delays in embryo development.

Oocyte Collection	Treatment Group	Day 3 Cleavage Rate (%)	Day 7 Morula (%)	Day
total of 4 replicates	Fresh	88.9	*26.6	
	Dried (+ 15%)	89.5	*14.2	

Oocyte Collection	Treatment Group	Day 3 Cleavage Rate (%)	Day 7 Morula (%)	Day 7 Blastocyst Rate (%)
4 replicates	Fresh	88.9	*26.6	*15.1
	Dried (+ 15%)	89.5	*14.2	*7.4

Table 13: Comparison of embryo growth from *in-vitro* matured oocytes ICSI inseminated with unprocessed and microwave dried epididymal sperm.
* $p<0.01$ unpaired t-test

6.4 Sperm Aster

With DNA damage assessment via TUNEL assay indicating no significant increase in damage to the microwave dried sperm using the current processing conditions, but decreased embryo development through day 7 in the dried group, further

investigation into the cause of increased embryo arrest was needed. Sperm aster assessment was a next logical step, as it represents the functionality of the centrosome, the second critical component in the sperm cell that needs preservation in order to retain normophysiologic function post drying. No significant difference in the percent of absent or small sperm asters over 6 replicates was observed at 5hpi, however a significant ($p<0.05$) decrease in the percent of large sperm asters in the dried group (5.6%) compared to the fresh sperm group (14.6%) does indicate some centrosomal damage during the drying process.

Assuming equal oocyte quality between the groups and among replicates, as indicated by the comparable maturation rates and random division of the mature oocytes into the fresh and dried groups, and equal centrosomal protein content among the oocytes, the decreased percentage of large sperm aster formation in the dried group could be a result of the decreased ability of the proximal centriole (contributed by the sperm) to recruit and organize centrosomal proteins. This process is critical to zygote nuclear formation as well as future cleavage divisions in the embryo.

It should be noted that the pronuclear stage in feline embryos is not visible due to the increased lipid content, so a true fertilization rate based on pronuclear visibility is unobtainable. Visual cleavage rates are reported as the first stage of detectable fertilization based on embryo development. It is interesting to note the difference in the uncleaved embryo rates on day 3 and the unfertilized rates observed at 5hpi between the fresh and dried groups. The average uncleaved rate in the fresh groups on day 3, 11.5%, compares to a 6.6% unfertilized rate indicated at 5hpi by the absence of pronuclei or a second polar body. In the dried group, the day 3 uncleaved rate of 22.2% compares to

16.0% unfertilized at 5 hpi. In both groups, the physically uncleaved rates on day 3 were higher than the visual unfertilized rates at 5 hpi, reiterating that aside from the presence of pronuclei from the sperm and oocytes, proper sperm aster formation contributes to completing the fertilization process

6.5 Sperm DNA Decondensation

Disruption of the sperm chromatin DNA decondensation process was evaluated as a possible explanation for decreased embryo development within the dried group. The process of sperm chromatin decondensation occurs when a spermatozoon enters an ovum through replacement of protamines with histones. Defective chromatin decondensation prevents further development of the male pronucleus. Even though there was no statistically significant difference in the overall level of sperm decondensation between the groups (5.43µm fresh and 5.10 µm dried), when broken down into levels of DNA decondensation within subsets of sperm aster formation, significant differences ($p<0.01$) were present between the fresh and dried groups in both small (f: 4.91 µm, d: 5.81 µm) and large (f: 6.63 µm, d: 5.31 µm) sperm aster subsets. Sperm DNA from the dried group was more decondensed when there was a small sperm aster present, and sperm DNA from the fresh group was more decondensed when there was a large sperm aster. Large sperm asters and higher levels of DNA decondensation are more typical of normal function as seen in the fresh group. In light of the fact that significantly fewer large sperm asters were present in the dried group, the higher levels of DNA decondensation are noted in the small sperm aster subset.

6.6 Embryonic Genome Activation

As a final measure of success in the microwave dehydration of feline epididymal sperm, concentrations of embryonic RNA in groups of embryos created from fresh or dried sperm was the measured. Proceeding on the assumption that the only source of mRNA in the oocytes is of maternal origin, we can assume that any increase in mRNA level within the embryo is due to embryonic genome activation (EGA) and embryonic RNA transcription. EGA is essential for embryonic development and is reflected in the quality of embryos. Stage specific morphology and cleavage timing are widely accepted markers for assessing and comparing embryo development but with advancements in molecular biology, mRNA quantities and individual gene expression levels during embryonic development have become accepted quality markers as well. The average mRNA concentration of mature metaphase II stage *in-vitro* matured oocytes was 1.1 +/- 0.1 ng/ml. Batches of embryos containing 20 x 2-4 cell embryos, 10 x 5-8 cell embryos, 5 x 9-16 cell embryos, 2 x morula, and 1 blastocyst were chosen to allow for even overall cell numbers in each set. In comparing the average mRNA concentrations for the 2-4 cell, 5-8 cell, 9-16 cell, morula, and blastocyst stage embryos for the fresh and dried groups (3 experimental replicates per group), there was not a significant difference between the mRNA levels in any of the embryo stages, however, the high standard deviation within each group made it difficult to resolve any difference. Standard deviations in the groups were higher than expected, presumably owing to the random grouping of embryos in each replicate at visually undetectable and varying stages of cellular arrest. Embryos were individually frozen at various embryonic stages based on morphology. The potential for each individual embryo to continue to develop and produce mRNA or arrest at that cellular stage is visually indiscernible. It would be more

telling to have RNA levels in individual embryos at each stage, but detection levels at such low concentrations are prohibitive as the threshold for detection is 0.15ng/µl.

6.7 Conclusions

This is the first time that an in-depth assessment of individual components of cellular function have been assessed in a specific cell type post-drying. We were we able to quantify the level of nuclear DNA damage in unprocessed cauda epdidiymal sperm, and discern that microwave drying to an end moisture content of 0.16 gH_2O/gDW did not significantly increase the level of nuclear DNA damage. We followed the development of embryos created from *in-vitro* matured oocytes ICSI inseminated with unprocessed or dried sperm, monitoring growth over 7 days to the blastocyst stage, which indicated a developmental delay of a larger portion of embryos in the dried group compared to the fresh group. Sperm aster and DNA decondensation levels at 5hpi were assessed in each group as measures of centrosomal and DNA function post drying. Large sperm aster formation was present in a significantly smaller portion of the dried group compared to the fresh group, suggesting centrosomal damage during the dehydration process. Insignificant difference in the overall level of sperm DNA decondensation were noted between the fresh and dehydrated sperm groups. The mRNA levels at stages of embryonic development from 2-cell to blastocyst showed no significant difference between the fresh and dried groups. Increased standard deviations, however, made it difficult to resolve any difference. All of this data was accumulated post drying to a consistent end moisture content. Damage incurred can be related back to specific processing conditions to a specified final moisture. This is a huge step in understanding

the relationship between dehydration to a specific moisture level and cellular damage in a particular cell type. More research needs to be done in this area, opening the doors to possible future long term storage in the dry state, which could advance the fields of reproduction, biobanking, and species preservation to new levels.

CHAPTER 7: FUTURE RESEARCH

Next steps in this areas of research could include quantifying gene transcription of known developmentally important genes, using centrosomal replacement post dehydration as another method of measuring the percent of delayed embryo development that is due to the loss of centrosomal function during the dehydration process, and implantation of embryos created from dehydrated sperm into pseudopregnant females. Implantation studies could be done pre and post storage experiments as final validations of the dehydration and storage processes.

For storage experiments, the choice of storage container is a critical component of moisture control in the dried sample, especially for long term storage. Even minimal changes in moisture content of the dried sample could have a huge detrimental effect on the survival and functionality of the dried sample over a period of long term storage, especially if the sample transitions out of a glassy state. With all biological material, there is a relationship between age and DNA quality (Walters, 2006; Gomez-Campo, 2006; Leino, 2009). Increases in moisture content in the dried sample will increase the molecular mobility, which could cause aging and deterioration of the sample. Decreases in moisture content could cause the sample to dry beyond a point that can be tolerated by sperm, as described in other studies (Gomez-Campo, 2006). Traditionally, storage in glass ampoules will flame sealed glass tops has been the container method of choice for biological reference material intended for long term storage to ensure moisture content confinement in the dried sample (Patel 2011; Sacha, 2010; Perez-Garcia, 2009; Matejtschuk, 2004). The storage of biological materials in glass containers is

recommended to be in containers made of Type I glass to reduce risk of reaction with the sample (Sacha, 2010). Short term storage also has to be considered after field drying until the sample can reach a well-resourced network and be permanently stored in its final containment device and storage conditions.

Quantifying gene transcription of known developmentally important genes in the feline delves deeper into understanding the cause of increased developmental arrest in embryos created from dehydrated sperm. There are a host of genes known to be developmentally important specifically in the feline embryo as well as ones commonly used as markers for gene quality in other species. These gene products are involved in processes such as epigenetic modifications (DNA methyltransferases 1 and 3 - DNMT1, DNMT3A), intercellular communication (gap junction proteins α 1 - GJA1), transcription regulation (octamer-binding transcription factor 4 - POU5F1/OCT4), growth factor reception (insulin-like growth factors 1 and 2 receptors – IGF1R, IGF2R), and cell structure formation (housekeeping gene β-actin – ACTB) (Wrenzycki, 1996; Longeran, 2003; Warzych, 2007; Magnani, 2008).

From our research, we can conclude there is some level of centrosomal damage occurring during the dehydration process. Previous data has shown that in the domestic cat, first cell cycle kinetics are under control of the centrosome (Comizzoli, 2006). Zygotes containing small or absent sperm asters showed a delayed first cleavage and decreased development to the morula and blastocyst stages. Replacement of a less developed centrosome from testicular sperm with a mature centrosome from an ejaculated sperm prior to ICSI has been shown to improve first cell cycle kinetics and

embryo development to comparable levels with ejaculated spermatozoa (Comizzoli, 2006).

Searching deeper in to the cause of the developmental delay in embryos created from dehydrated sperm is an important aspect to further the understanding of the damage mechanisms. Implantation experiments, however, can be viewed as the final measure of a successful dehydration process. Implantation of blastocysts created from ICSI of *in-vitro* matured oocytes with dehydrated epididymal sperm into pseudopregnant females assesses the functional capability of the created blastocysts. Implantation experiments post storage assesses the validity of the storage container and environment. Liveborn kittens would be a great accomplishment in the road towards dry preservation for both species preservation and assisted reproduction.

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