

DISSERTATION

CHARACTERIZATION OF EQUINE SPERM ATTRIBUTES
AND SELECTION FOR INTRACYTOPLASMIC SPERM INJECTION

Submitted by

Raul A. Gonzalez-Castro

Department of Biomedical Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2018

Doctoral Committee:

Advisor: Elaine Carnevale

Co-Advisor: James Graham

George Seidel

Gerrit Bouma

Ann Hess

Copyright by Raul A. Gonzalez-Castro 2018

All Rights Reserved

ABSTRACT

CHARACTERIZATION OF EQUINE SPERM ATTRIBUTES AND SELECTION FOR INTRACYTOPLASMIC SPERM INJECTION

When performing intracytoplasmic sperm injection (ICSI), many in vivo mechanisms for sperm selection are bypassed; however, sperm must still be capable of activating the oocyte for successful fertilization. Limited information is available for horses on the effect of sperm preparation method and sperm characteristics that affect ICSI outcome. The overall objectives of this dissertation were to: 1) study the association between sperm sorting methods, sperm population characteristics, and equine ICSI outcome, and 2) characterize sperm oocyte activating factors in stallion sperm, such as phospholipase C zeta (PLCz) and postacrosomal WW binding protein (PAWP).

In Experiment 1, a microfluidic device was used to sort frozen-thawed sperm from stallions (n=19), which resulted in a sperm subpopulation with improved motility, morphology, viability and DNA integrity ($P<0.05$) compared to the original sample. Then, microfluidic sorting was compared with the swim-up procedure and density gradient centrifugation. Swim-up was the least effective method to separate equine sperm. Microfluidic sorting and density gradient centrifugation sorted a sperm subpopulation with similar parameters, improving motility, viability and DNA integrity. After ICSI (n=45), no differences ($P>0.3$) were observed for cleavage and embryo development among sorting methods. In Experiment 2, sperm population parameters from which individual sperm were selected for injection were analyzed immediately after ICSI and correlated with the outcome. Sperm morphology, viability,

membrane integrity measurement of hypoosmotic swelling and DNA integrity were evaluated in frozen-thawed sperm (n=114) used for ICSI in a program. Among sperm parameters, viability correlated positively with normal morphology and membrane integrity ($P<0.05$). Normal sperm morphology and DNA integrity were not predictive of ICSI outcome. Viability was predictive of cleavage and blastocyst formation, and membrane integrity was predictive of early pregnancy ($P<0.05$). In Experiment 3, PLCz and PAWP were identified, localized and quantified in stallion sperm, and the relationship with other sperm parameters was investigated. PLCz was identified as a 71 kDa protein and located in the acrosomal and postacrosomal region, midpiece and principal piece of the tail. PAWP was identified by two bands of ~28 and ~32 kDa, located in the postacrosomal region, midpiece and principal piece of the tail. The expression of PLCz and PAWP correlated positively ($P=0.04$) when analyzed for sperm of 14 stallions. Flow cytometric assessment was feasible for PLCz, but not for PAWP. Expression and percentages of positive labeled sperm for PLCz varied among stallions (n=21). Expression of PLCz was higher in live than dead sperm ($P<0.005$), and DNA fragmentation correlated negatively with PLCz expression ($P<0.04$).

In conclusion, microfluidic sorting and density gradient centrifugation resulted in a subpopulation of sperm with high quality parameters for ICSI. The probability of sperm-injected oocytes to develop into an embryo and to establish pregnancy improved when sperm were selected from a sample population with higher viability and membrane integrity. This is the first report that describes PAWP in equine sperm, which displayed a novel localization in the midpiece and principal piece of sperm tail in addition to the expected postacrosomal region. Protein levels of PLCz and PAWP were correlated in sperm heads. The expression of PLCz in sperm varied widely among stallions and was associated with DNA integrity. Sperm membrane

integrity is indicative of well-maintained plasma membrane architecture, conserving sperm quality and membrane components that are required for oocyte activation and early embryo development. Assessment of PLCz in stallion sperm represents a potent feature to investigate sperm quality for equine ICSI, and potentially can serve as a prognostic biomarker for oocyte activation ability and male infertility. Further studies are needed to determine the relationship between PLCz and PAWP with fertility in horses.

ACKNOWLEDGEMENTS

I want to deeply and sincerely thank to Dr. Elaine M. Carnevale for giving me the incredible chance to do my PhD program at Colorado State University and to conduct my academic development and research under her guidance in the Equine Assisted Reproduction Program at Colorado State University. And to my committee for giving me the right pushes in the right moments to uncover what I am able to do and to reach the goal in this adventure.

Dr. Jacobo Rodriguez and Vista Equine Colorado, LLC provided stallion semen samples to perform experiments in Chapter II and IV. DxNow Inc. provided FERTILE PLUS™ Sperm Sorting Chips to implement experiments in Chapter II. JoAnne Stokes performed all the ICSI procedures, and students and staff of Assisted Reproduction Program participated in the collection of oocytes for experiments in Chapter II and III.

The present study was funded by The Cecil and Irene Hylton Foundation and the Equine Assisted Reproduction Program at Colorado State University. Doctoral scholarship was granted to Raul Gonzalez-Castro by Becas Chile (CONICYT, Ministry of Education of Chile).

And most importantly, I want to deeply thank to my family: Carolina, my wife, for her endless patience and understanding in this challenge; Raul Alexander “The Thunder”, my son, for feeding me every day with his energy and love; my Mom –Olombi–, my Dad –Raul– and brothers -Pablo and Felipe-, for their unconditional love and backing me up from my homeland and Heaven.

DEDICATION

This work is dedicated to my family.

For making me who I am.

Everyone is import in this journey.

TABLES OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	v
DEDICATION	vi
LIST OF TABLES	x
LIST OF FIGURES	xii
CHAPTER 1: REVIEW OF LITERATURE	1
INTRODUCTION	1
INTRACYTOPLASMIC SPERM INJECTION	2
SPERM PROCESSING FOR ASSISTED REPRODUCTIVE TECHNOLOGIES	4
Sperm sorting techniques	5
<i>Swim-up</i>	5
<i>Density gradient centrifugation</i>	6
<i>Zeta potential</i>	8
<i>Annexin-V binding</i>	8
<i>Electrophoresis-based sperm sorting</i>	9
<i>Microfluidic sperm sorting</i>	10
Individual sperm selection	11
<i>Motile sperm organelle morphology examination</i>	11
<i>Polarized light microscopy</i>	13
<i>Hypoosmotic swelling test</i>	14
<i>Hyaluronic acid binding assay</i>	15
<i>Zona pellucida binding assay</i>	17
<i>Methylxanthines</i>	17
<i>Laser assisted immotile sperm selection</i>	18
OOCYTE ACTIVATION.....	18
Artificial Oocyte activation	20
Sperm-mediated oocyte activation	22
<i>Phospholipase C zeta 1</i>	24
<i>Postacrosomal WW binding protein</i>	28
<i>Sperm oocyte activating factors and fertility</i>	30
DISCUSSION	31
REFERENCES	35
CHAPTER II: SELECTION OF SPERM FOR INTRACYTOPLASMIC SPERM	
INJECTION	69
SUMMARY	69

INTRODUCTION	70
MATERIAL AND METHODS	71
Chemical and reagents	71
Experiment 1: Sorting of equine sperm using a microfluidics	72
<i>Sperm samples and microfluidic sorting</i>	72
<i>Sperm analysis</i>	72
Experiment 2: Comparison of sperm population sorting using microfluidics, single layer colloidal centrifugation, and swim-up procedure.....	74
<i>Sperm samples and microfluidic sorting</i>	74
<i>Sperm analysis</i>	75
Experiment 3: Use of microfluidics for sorting sperm prior to ICSI and comparison with single layer colloidal centrifugation.....	76
<i>Sperm samples and sperm sorting for oocyte injection</i>	76
<i>Sperm analysis</i>	77
<i>Oocyte collection and sperm injection</i>	77
Statistical analysis	78
RESULTS	79
Experiment 1: Sorting of equine sperm using microfluidics	79
Experiment 2: Comparison of sperm population sorting using microfluidics, single layer colloidal centrifugation, and swim-up procedure.....	80
Experiment 3: Use of microfluidics for sorting sperm prior to ICSI and comparison with single layer colloidal centrifugation.....	81
DISCUSSION	83
REFERENCES	90
CHAPTER III: ASSOCIATION OF EQUINE SPERM POPULATION PARAMETERS WITH OUTCOME OF INTRACYTOPLASMIC SPERM INJECTION	98
SUMMARY	98
INTRODUCTION	99
MATERIAL AND METHODS	102
Chemical and reagents	102
Sperm samples and oocyte injections	102
Morphology and live sperm analysis	105
Hypoosmotic swelling test	105
Sperm Chromatin dispersion	106
Statistical analysis	107
RESULTS	108
DISCUSSION	112
REFERENCES	118

CHAPTER IV: PHOSPHOLIPASE C ZETA AND POSTACROSOMAL WW BINDING PROTEIN: LOCALIZATION AND QUANTIFICATION IN EQUINE SPERM	130
SUMMARY	130
INTRODUCTION	131
MATERIAL AND METHODS	135
Chemical, reagents and antibodies	135
Immunofluorescence	135
Western blotting analysis	137
Quantitative immunofluorescent analyses of PLCz and PAWP in equine sperm head	138
Flow cytometric assessments.....	139
<i>General information about flow cytometric assessment</i>	139
<i>Flow cytometric assessment of PLCz</i>	140
<i>Flow cytometric assessment of sperm parameters</i>	141
Statistical analysis.....	143
RESULTS	144
Identification and localization of PLCz and PAWP in equine sperm	144
Quantitative immunofluorescent analyses of PLCz and PAWP in equine sperm head	145
Flow cytometric assessment of PLCz and association with sperm parameters	147
DISCUSSION	150
REFERENCES	156
 CHAPTER V: CONCLUDING REMARKS	 167
REFERENCES	170

LIST OF TABLES

<p>Table 2.1: Mean \pm SEM percentages (range) from 20 stallions of sperm parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA-: percentage of sperm with fragmented DNA) between unsorted and sorted sperm populations using microfluidics</p>	80
<p>Table 2.2: Mean \pm SEM percentages (range) of individual sperm population parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA-: percentage of sperm with fragmented DNA) between the original samples (Unsorted, n=19) and sperm sorted using microfluidics (MF), single layer colloidal centrifugation (SLC) and swim-up procedure (SU)</p>	81
<p>Table 2.3: Mean \pm SEM percentages (range) of individual sperm population parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA-: percentage of sperm with fragmented DNA) between the unsorted original sample and sorted sperm using microfluidics (MF, n=22) and single layer colloidal centrifugation (SLC, n=18)</p>	82
<p>Table 2.4: Correlations among sperm population parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA-: percentage of sperm with fragmented DNA) after sorting by microfluidics (MF) and single layer colloidal centrifugation (SLC) (n=40 samples).....</p>	82
<p>Table 2.5: Cleavage and Blastocyst (development rates per sperm injected oocyte) after sorting by microfluidics (MF) and single layer colloidal centrifugation (SLC) and mean \pm SEM percentages of sperm population parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA-: percentage of sperm with fragmented DNA) in which ICSI resulted in positive (Yes) or negative (No) outcomes.....</p>	83
<p>Table 3.1: Mean percentages \pm SEM and number of samples (n) for sperm parameters (normal morphology, MORPH+; live sperm, LIVE+; membrane integrity assessed by hypoosmotic swelling, HOS+; DNA fragmentation, DNA-) associated with samples from which sperm were removed for ICSI and resulted in positive (Yes) and negative (No) ICSI outcomes for Cleavage, Embryo, and Pregnancy per injected oocyte</p>	110

Table 3.2: Logistic regressions of individual sperm population parameters as predictors and stepwise logistic regression for Cleavage, Embryo and Pregnancy after ICSI. Stepwise regression and P-value for the resulting model (P-model) for Cleavage and Embryo did not include MORPH+, and model for Pregnancy did not include MORPH+ and LIVE+	110
Table 3.3: Means \pm SEM for sperm parameter groupings (percentages of normal morphology, MORPH+; live sperm, LIVE+; functional membrane assessed by hypoosmotic swelling, HOS+; DNA fragmentation, DNA-) and rates for Cleavage, Embryo or Pregnancy per sperm-injected oocyte and odds ratios (OR)	111
Table 4.1: Means \pm SEM of florescence intensity (MFI) and percentages of sperm labeled with antiPLCz H50 (H50+) and M163 (M163+) for whole sperm, live sperm, and dead sperm populations)	149
Table 4.2: Mean percentages \pm SEM of sperm parameters and correlations among parameters assessed for 21 stallions	150

LIST OF FIGURES

Figure 1.1: Schematic illustration of PLCz domain structure.....	25
Figure 4.1: Immunoblots for PLCz, using antiPLCz of human (H50, A) and mouse (M163, B) origin, and PAWP proteins in separated pooled heads and tails from four stallions and whole equine sperm from two stallions (St1 and St2) (C) and sperm from 2 bulls (Bull1 and Bull2) (D).....	145
Figure 4.2: Immunofluorescence for PLCz for PAWP in equine sperm	146
Figure 4.3: Mean fluorescence intensity (MFI) (A) and percentage of positive labeled sperm (B) for PLCz (PLCz+) using antiPLCz H50 and M163 in sperm from 21 stallions	148

CHAPTER 1: REVIEW OF LITERATURE

INTRODUCTION

Since the introduction of intracytoplasmic sperm injection (ICSI) in reproductive medicine in humans (Palermo et al., 1992) and horses (Meintjes et al., 1996; Squires et al., 1996), ICSI has become in the elective assisted reproductive technique (ART) used to produce in vitro equine embryos (Carnevale, 2008; Choi et al., 2016; Galli et al., 2016). This is currently the only repeatable and effective method for in vitro fertilization in the horse, because efficient methods for standard in vitro fertilization, placing sperm and a mature oocytes together in media to allow sperm penetration, have not yet been identified (Hinrichs, 2013; Rader et al., 2016).

Clinical use of ICSI has initially focused on producing offspring from mares with compromised fertility (Carnevale, 2008). The increasing commercial demand for multiple valuable offspring or potential to avoid interfering with sporting activities are currently reasons to perform ICSI in fertile mares (Galli et al., 2016). In stallions, indications for ICSI include poor sperm quality for standard insemination or limited availability of frozen sperm stores (Carnevale, 2008). (Carnevale, 2008) The advantage of ICSI for these cases is that only a small quantity of sperm is required for injection; therefore, sperm injection could theoretically be used to produce multiple in vitro embryos from a single straw of frozen sperm, maximizing its use (Carnevale, 2008). Additionally, unused frozen-thawed sperm can be refrozen, maintaining sufficient sperm quality and fertility potential for later microinjections (Choi et al., 2006; Gonzalez-Castro et al., 2016; Gonzalez-Castro and Carnevale, 2016; McCue et al., 2004).

INTRACYTOPLASMIC SPERM INJECTION

During ICSI, a micromanipulator is used to manually inject a selected sperm into an oocyte, resulting in assisted fertilization (Carnevale, 2008). The first reports of ICSI using equine gametes were in mid 1990s. Immature oocytes were collected by ultrasound-guided transvaginal procedure, then matured in vitro and injected with a single sperm. Early 4-cell embryos (n=2) and embryos at morula stage (n=3) were surgically transferred to the oviduct and nonsurgically transferred to recipient mares, respectively. From the all embryo transfers, one of the surgical transfer resulted in early pregnancy, but lost at 16 days post transfer (Meintjes et al., 1996). In the other study, four in vitro matured oocytes collected from excised ovaries were injected with a single sperm. One oocyte cleaved and the embryo at 10-12 cell stage was surgically transferred to the oviduct recipient of a mare, resulting in pregnancy and live birth (Squires et al, 1996). At present, ICSI is the only repeatable and effective method to produce equine embryo in vitro, since standard in vitro fertilization (IVF) technique has not been repeated successfully in horses (Palmer et al., 1991; Zhang et al., 1990).

Equine ICSI requires the collection and selection of viable oocytes, obtained from excised ovaries or follicles from live mares (Carnevale, 2008). Mature in vivo oocytes can be collected from maturing dominant follicles that have started the follicular and oocyte maturation, initiated and timed through the administration of ovulation-inducing compounds or naturally through endogenous luteinizing hormone (Carnevale, 2016; Carnevale et al., 2005). Immature oocytes from small follicles non-stimulated to mature or follicles incapable of responding to ovulatory stimuli can be collected and matured in vitro. For sperm injection, in vivo or in vitro matured oocytes need to reach metaphase II to be competent and successfully fertilized, which are identifiable by extrusion of the first polar body (Carnevale, 2016).

The selection of equine sperm is essential for ICSI, as it influences the success outcome (Choi et al., 2016; Herrera et al., 2012). In humans, a positive relationship is observed between sperm quality and embryo development before or after embryonic genome activation, suggesting that sperm affect embryogenesis during a very early stage during fertilization and later during embryo development (Loutradi et al., 2006; Miller and Smith, 2001). Evidence in human suggests that failure of fertilization or early cleavage events is the result of an early paternal effect that include an abnormal release of a sperm oocyte activating factor as phospholipase C zeta (PLCz), which is responsible for inducing and regulating calcium oscillations necessary for oocyte activation (Swann and Lai, 2016). After oocyte activation, sperm derived centrosome was shown to be the only functionally active centrosome in the early stages of embryogenesis which is responsible for microtubule organization. A centrosomal dysfunction at fertilization can result in spindle disturbances with abnormal or arrested cleavage (Tesarik et al., 2002a). At later stages during embryo development, developmental abnormalities, implantation failure or early pregnancy loss are associated with a late paternal effect related to nuclear or chromatin defects in the sperm as aneuploidy, genetic anomalies and DNA fragmentation (Barroso et al., 2009). In equine ICSI, limited information have linked male factor and ICSI outcome. Under experimental and clinical conditions, cleavage and blastocyst rates are significantly different among stallions after ICSI (Choi et al., 2016; Colleoni et al., 2012; Herrera et al., 2012; Lazzari et al., 2002). These findings suggest that there is a strong relationship between male and sperm quality with ICSI success.

Cleavage rate in clinical programs is reported between 63 and 73% for equine embryo production in vitro (Carnevale, 2008; Colleoni et al., 2007; Rader et al., 2016), exhibiting no difference in cleavage between injected oocytes provided by fertile (69%) or infertile mares

(63%) in field conditions (Carnevale, 2008). However, blastocyst development rates range between 12 and 38% per injected oocyte (Carnevale, 2008; Colleoni et al., 2007; Rader et al., 2016). This limited blastocyst rate could be derived from species-specific biological limitations for in vitro embryo production, potential fertility of the donors, heterogeneous gamete quality, and laboratory procedures differences (Galli et al., 2016).

SPERM PROCESSING FOR ASSISTED REPRODUCTIVE TECHNOLOGIES

Under in vivo conditions, sperm must migrate through a complex environment with several barriers and anatomical compartments with a variety of physiological properties in the female reproductive tract, before reaching the oocyte (Suarez and Pacey, 2006). Motile sperm are separated by active migration from immotile sperm and debris (Mortimer, 1989), excluding immature and aneuploid sperm from participating in fertilization (Suarez and Pacey, 2006). Selected progressively motile sperm undergo capacitation, which is a fundamental requisite for functional competence (Bedford, 1983; Yanagimachi, 1988). In contrast, under in vitro conditions, the arbitrary selection of sperm for ICSI is routinely based on sperm motility and gross morphology (Boitrelle et al., 2014; Herbemont and Sifer, 2015; Lazzari et al., 2002; Palermo et al., 1992; Sessions-Bresnahan et al., 2014). This clinical approach involves multistep and manual screening processes, which are prone to human error (Intra et al., 2016). Studies in men reveal that sperm morphology and motility characteristics do not exclude sperm with DNA damage, mostly in individuals with male factor infertility and impacts negatively embryo quality and pregnancy in ICSI cycles (Avendaño et al., 2010, 2009; Celik-Ozenci et al., 2004). This suggests that ICSI may provide an opportunity for damaged sperm to participate in the fertilization, resulting in failed fertilization and embryo development.

During ICSI, there is a higher need to select an ideal sperm. Techniques for sperm processing prior ICSI can be classified as sorting methods that sort the sperm sample in a sperm subpopulation with increased quality parameters, or methods that identify individual sperm cell based on specific attributes. Sperm sorting method for ART needs 1) to be a quick, easy and cost-effective technique; 2) to have high sorting asset and recovery rate to separate motile from immotile sperm; 3) not to be detrimental for sperm physiology; and 4) to be able to eliminate toxic or bioactive substances, such as reactive oxygen species (ROS) (Henkel and Schill, 2003). Several methods for sperm separation or individual sperm selection with potential use in ICSI has been reported based on motility, morphology, density, charge, membrane integrity and functional binding to specific elements (Henkel, 2012; Herbemont and Sifer, 2015; Rappa et al., 2016; Simopoulou et al., 2016). In equine ICSI, limited information has been reported linking the use of sperm sorting techniques and ICSI outcome, and no information is available for methods to select an individual sperm for oocyte injection.

Sperm sorting techniques

Swim-up

Sperm separation by swim-up (SU) technique is based on the active movement of sperm from the prewashed sperm pellet into an overlaying medium (Mahadevan and Baker, 1984). Swim-up is a method easy to perform and cost-effective. However, the recovery of motile sperm is limited as efficiency is based on the surface of the sperm pellet and the initial sperm motility. The layered cells in the pellet prevent motile sperm in the lower levels of the pellet from reaching the interface with the culture medium (Henkel and Schill, 2003). Additionally, functional sperm are pelleted by centrifugation and come into close cell-to-cell contact with

defective sperm, which produces high levels of ROS (Aitken and Clarkson, 1988). The generation of ROS is caused by the high amount of polyunsaturated fatty acids in sperm plasma membranes (Aitken and Clarkson, 1987), which results in lipid peroxidation that induce a reduction of sperm motility and DNA integrity (Barroso et al., 2000; Mortimer, 1991).

The use of SU with human sperm provide a sorted sperm subpopulation with increased viability, motility, morphology, DNA integrity and reduced percentage of apoptotic sperm (Kim et al., 2015; Luppi et al., 2015; Ricci et al., 2009; Sakkas et al., 2000; Zini et al., 2000). In stallions, the use of SU also improves sperm quality when using raw semen (Morrell et al., 2009, 2010; Sieme et al., 2003).

Density gradient centrifugation

Density gradient centrifugation (DGC) is based on sperm motility and density (Oshio et al., 1987; Pousette et al., 1986). A mature morphologically normal human sperm has a slightly higher density (1.10 g/mL) compared to an immature and morphologically abnormal sperm (1.06 and 1.09 g/mL) (Oshio et al., 1987). In a continuous gradient, sperm sample is layered on top of a single colloidal medium with uniform density from the top to the bottom and then centrifuged at a defined relative centrifugal force for a determined time. Highly motile sperm move actively in the direction of the sedimentation gradient and can reach the bottom quicker than poorly motile or immotile sperm, consequently highly motile sperm are increased in the pellet at the bottom (Bolton and Braude, 1984). In a discontinuous gradient, a colloidal media with different density are layered, resulting in distinctive boundaries between layers (Pousette et al., 1986). During centrifugation, specific sperm fractions are situated at the gradient level that matches their density. As a result, the first interphase between the sample and lower density layer

contains cell debris and immotile sperm; the second interphase between both low and high density layers contains morphologically abnormal sperm with poor motility; and at the bottom of the tube, morphologically normal, motile and viable sperm are pelleted (Henkel and Schill, 2003).

Commercially density gradients for sperm separation are compound by a colloidal suspension of silica particles stabilized with covalently bonded hydrophilic silane supplied with HEPES (Malvezzi et al., 2014), adjusted for osmolarity with polysucrose (Henkel and Schill, 2003).

The first report using DGC as sorting methods in samples from fertile and subfertile men, resulted in a sorted sperm subpopulation with increased motility, normal morphology and viability, but the results were more variable in subfertile individuals with abnormal semen profiles (Pousette et al., 1986). Later, several studies in men confirmed that DGC improve sperm population parameters, such as viability, motility, morphology, DNA integrity and reduction of apoptotic sperm (Kim et al., 2015; Luppi et al., 2015; Ricci et al., 2009; Sakkas et al., 2000; Zini et al., 2000). Likewise, stallion sperm subpopulation sorted by DGC from raw or thawed-frozen samples have improved motility, normal morphology, viability, mitochondrial membrane potential (MMP), plasma membrane permeability, DNA integrity and reduced activated caspase (Brum et al., 2008; Macpherson et al., 2002; Morrell et al., 2009, 2010; Sieme et al., 2003; Stoll et al., 2013; Stuhtmann et al., 2012).

The combination of single or double layer density gradient centrifugation and SU results in improved normal morphology and DNA integrity in human sperm samples when compare with a only one method (Jayaraman et al., 2012; Yamanaka et al., 2016). In stallion sperm , the combination of single layer density gradient centrifugation and SU for sperm sorting was

associated with higher cleavage and blastocyst rates after ICSI when compared to single layer density gradient centrifugation alone (Choi et al., 2016).

Zeta potential

The zeta potential or electrokinetic potential is the charge across the plasma membrane of mature sperm and is about -16 mV to -20 mV for human sperm (Henkel, 2012). The negative charge is used to separate mature sperm that adhere to a positively charged centrifuge tube. After a few minutes, the tube is centrifuged to remove sperm and particles that do not adhere to the tube surface (Said and Land, 2011). Human sperm sorted by zeta selection show larger percentages of mature sperm exhibiting hyperactivation and improved progressive motility, morphology and DNA integrity with normal protamine content (Chan et al., 2006; Razavi et al., 2010). However, this method is not beneficial in cases with low sperm counts due to the poor (<10%) sperm recovery rate (Chan et al., 2006).

Annexin-V binding

Annexin-V is a calcium-dependent phospholipid binding protein with high affinity for phosphatidylserine, an early marker of apoptosis (Martin et al., 1995). Apoptotic sperm bind annexin-V, allowing separation from annexin-V negative sperm which are associated with high MMP, DNA integrity and fertilization potential (Hoogendijk et al., 2009; Said et al., 2008).

Annexin-V-activated glass wool filtration is based on the principles of glass wool filtration and the affinity of annexin-V to phosphatidylserine (Grunewald et al., 2007; Paulson and Polakoski, 1977). During sperm glass wool filtration, motile sperm are separated from immotile sperm by self-propelled sperm movement and the filtration effect of the densely packed

glass wool fibers (Henkel and Schill, 2003). Sperm sorting efficiency depends on the chemical nature of the glass (i.e. borate glass, silicate glass or quartz glass), the surface structure and charge of the glass wool, thickness of the glass wool fibers or the pore size of the filter (Henkel and Schill, 2003). Annexin-V, linked chemically to glass fibers, deselects annexin-V positive sperm. This method resulted in the selection of Annexin-V negative sperm associated to high motility, normal morphology, high MMP, and DNA integrity (Grunewald et al., 2007; Hoogendijk et al., 2009).

Similarly, magnetic-activated cell sorting uses Annexin-V-conjugated paramagnetic microbeads to bind sperm that expose phosphatidylserine. Apoptotic sperm bound annexin-V-microbeads are separate through a magnetic field, deselecting sperm with signs of early apoptosis. Sperm that are not bound to the microbeads elute freely through the column (Said and Land, 2011). Apoptotic sperm deselected by this method display a disrupted mitochondrial membrane potential and high DNA fragmentation (Said et al., 2008).

The use of annexin-V-conjugated fluorochrome facilitate to recognize apoptotic sperm by flow cytometric cell sorting and separate apoptotic sperm from unlabeled sperm. Unlabeled sperm exhibit better normal morphology and reduced DNA fragmentation compared to labeled annexin-V sperm (Hoogendijk et al., 2009).

Electrophoresis-based sperm sorting

Normal and mature human sperm have a negative charge that is created by a highly sialylated glycosylphosphatidylinositol-anchored protein (CD52) expressed on the surface (Fleming et al., 2008). Electrophoresis technology sorts sperm based on their surface charge. When a voltage is applied, morphologically normal and negatively charged sperm move through

a 5- μm polycarbonate membrane toward the positive electrode, leaving behind immature sperm (Fleming et al., 2008). This sperm sorting method results in a sperm population with improved motility, morphology and DNA integrity when using human sperm. Sperm recovery and sorting efficiency are similar to DGC, but with less oxidative DNA damage (Fleming et al., 2008) due to decreased exposure to ROS by eliminating the centrifugation steps (Aitken and Clarkson, 1988). No significant differences in fertilization or cleavage rate and embryo quality have been observed after IVF or ICSI in human when the selected sperm were sorted by electrophoresis or DGC (Fleming et al., 2008).

Microfluidic sperm sorting

Microfluidics is the science of manipulating small amounts of fluids (Whitesides, 2006). Microfluidic devices can be used to mimic the geometry of microconfined regions, fluidic, and chemical environments within the female reproductive tract, imitating the in vivo sperm selection approach. In addition, microfluidics and dynamic imaging provided new insights into how sperm motility is affected by environmental cues, improving the understanding on diagnosis and treatment of infertility (Nosrati et al., 2017).

Microfluidic technology uses particular features of sperm motility to study their processing: 1) sperm cross the border that separates the parallel streams of the sample and the fresh medium (Swain et al, 2013); 2) sperm achieve an optimal swimming pattern near surfaces allowing sperm accumulation due to a combination of hydrodynamic forces and steric repulsion (El-Sherry et al., 2015; Kantsler et al., 2014; Smith et al., 2009); 3) sperm in presence of a nearby wall can increase swimming speed with 2D flagellar movement due to the hydrodynamic influence of the no-slip boundary (Ishimoto et al., 2017); 4) sperm are influenced by viscoelastic

property of the mucus in the reproductive tract, increasing the swimming velocity by forming regions of highly strained fluid behind the tail (Teran et al., 2010); and 5) sperm exhibit positive rheotaxis migration by orienting to and swimming against the flow (Miki and Clapham, 2013).

Microfluidic systems for sperm analysis and sorting can be categorized as active, using external source to impose selection forces as fluid flow or chemical gradients, or passive, based only on sperm motility (Knowlton et al., 2015; Rappa et al., 2016). As a consequence, microfluidic sperm sorting does not require centrifugation, which reduces sperm exposure to ROS and potential DNA damage (Aitken and Clarkson, 1988; Barroso et al., 2000). Experimentally, microfluidic devices have been used for the human, bull, pig and mouse to sort sperm with improved motility, viability, morphology and DNA integrity with the potential to be used in ART (Asghar et al., 2014; Cho et al., 2003; Matsuura et al., 2013; Nosrati et al., 2014; Schuster et al., 2003; Seo et al., 2007; Shirota et al., 2016; Tasoglu et al., 2013).

Individual sperm selection

Motile sperm organelle morphology examination

Advances in microscopy have allowed the introduction of motile sperm organelle morphology examination (MSOME), which allows the selection of individual motile sperm under high magnification ($> X 6000$) in real time (Bartoov et al., 2002). This microscopic assessment allows a determination of individual sperm for normal nucleus (shape, chromatin content and absence of nuclear vacuoles), acrosome size, post-acrosomal lamina, neck, mitochondria and tail (Bartoov et al., 2002). Human sperm selected for ICSI by routine procedures and after analyzed through MSOME, 65% of those previous selected sperm would not have been suitable for ICSI, associated with an increase of nuclear abnormalities and DNA

fragmentation (Wilding et al., 2011). Individual morphologically normal sperm with absence of nuclear vacuoles analyzed by MSOME, have higher mitochondrial function and better DNA integrity and chromatin condensation, when compared to sperm morphologically normal but with vacuoles (Boitrelle et al., 2013, 2011; Garolla et al., 2008; Gosálvez et al., 2013). It has been observed that human sperm with large nuclear vacuoles show high levels of single- and double-stranded DNA fragmentation (Franco et al., 2008). In human sperm, an extensive disulfide cross-linking of protamines is a normal process of sperm maturation, resulting in the stability of the nucleus which is important for normal fertilization (Kosower et al., 1992). An abnormal nuclear structure of sperm chromatin influences negatively the initiation and regulation of paternal gene activity during preimplantation development (Haaf and Ward, 1995). A reduction of disulfide bond in protamines leads an increase of sperm chromatin decondensation (Ménézo et al., 2007) (Hutchison et al., 2017), that potentially increases the level of sperm DNA fragmentation (Kosower et al., 1992). In human clinical practice, high level of sperm chromatin decondensation (>28%) is associated to failure of pregnancy after IVF and ICSI (Ménézo et al., 2007). These findings suggest that the presence of large nuclear vacuoles in sperm heads links with high DNA fragmentation and chromatin decondensation that can result in asynchronous chromosome condensation after fertilization, and lead to cytoplasmic fragments in the embryo (Ménézo et al., 2007).

The positive relationship is observed between morphologically normal sperm selected by MSOME and fertilization rates, embryo quality, implantation and pregnancy rates (Vanderzwalmen et al., 2008; Wilding et al., 2011). However, the improvement in ICSI outcome after sperm selection by MSOME is still debated for general application (Nadalini et al., 2009; Nasr-Esfahani et al., 2012; Simopoulou et al., 2016), and should be limited strictly in cases of

severe male factor infertility and repeated implantation failures following ICSI (Boitrelle et al., 2014; Ebner et al., 2014).

Polarized light microscopy

The cellular characteristic of the sperm can be assessed by the particular birefringence patterns of sperm head. In the case of an isotropic structure, no birefringence effect is observed because the light propagates at a single velocity without being polarized. When crossing an anisotropic structure as sperm head, the incident light beam is refracted into two rays traveling at different velocities. The “retardance” of the slow ray relative to the fast ray generates the birefringence effect (Gianaroli et al., 2010). The presence of birefringence in a mature sperm head is the expression of an organized and very compact texture that characterizes normal sperm nuclei, acrosomes, and motile tails (Baccetti, 2004), depending on the presence of nucleoprotein filaments that are oriented longitudinally (Gianaroli et al., 2008). A normal human sperm with a nonpyknotic nuclei and normal acrosome produce strong negative birefringence. Most of the sperm tail is birefringent due to the organization of axoneme and mitochondria in the midpiece (Gianaroli et al., 2008). The proportion of birefringent sperm in the sperm samples is positively correlated with progressive motility, viability and normal morphology (Gianaroli et al., 2008). Deviation or loss of the birefringent pattern identify dead and morphological abnormal sperm due to the absence of conventional sperm texture (Collodel et al., 2010; Gianaroli et al., 2008).

In human, sperm with completed acrosomal reaction and absence of nuclear vacuoles detected by birefringence are related with higher DNA integrity and fertilization potential (Gianaroli et al., 2010; Petersen et al., 2011). Higher fertilization, embryo quality and implantation rates after ICSI have been observed when human sperm were selected based on

their individual birefringence (Gianaroli et al., 2008; Vermey et al., 2015). The improvement in clinical outcome after human ICSI is more favorable when the individual sperm is selected based in birefringent pattern in cases of severe male factor as oligoasthenoteratozoospermia [semen with abnormally low sperm concentration, motility and normal morphology (Cavallini, 2006)], or samples compromised as testicular sperm obtained by testicular sperm extraction (Gianaroli et al., 2010).

Hypoosmotic swelling test

Hypoosmotic swelling (HOS) test was developed to assess the functional integrity of plasmatic membrane in human sperm (Jeyendran et al., 1984). A sperm with functional membrane expands, especially in the tail, when it is exposed to hypoosmotic conditions, keeping fully competence for oocyte fertilization after swelling (Rossato et al., 1996). HOS is real time and not destructive test for sperm viability and fertility potential, and it has been recommended to select individual healthy sperm for ICSI (Sallam et al., 2001), even from immotile sperm samples (Casper et al., 1996; Sallam et al., 2005; Westlander et al., 2003). Human sperm samples with high HOS scores have lower incidences of abnormal head morphology, apoptotic markers, protamine deficiency, membrane damage, and DNA fragmentation, all of which relate to fertility potential (Bassiri et al., 2012; Charehjooy et al., 2014; Herbemont and Sifer, 2015; Miciński et al., 2011; Nasr-Esfahani et al., 2002; Stanger et al., 2010; Tartagni et al., 2002; Zhang et al., 2015). Individual human sperm identified through HOS exhibits low rates of DNA fragmentation (Stanger et al., 2010). It has been suggested that the hypoosmotic stress that induces sperm swelling has potential negative effects such as decreased tyrosine phosphorylation, reduction of sperm-zona pellucida binding and K^+/Cl^- channels opening (Stanger et al., 2010). However, the

HOS procedure was found not to cause degenerative changes in sperm ultrastructure evaluated by electron microscopy (Tutsi et al., 2015).

The selection of individual immotile sperm using HOS from infertile men with primary ciliary dyskinesia as Kartagener's syndrome, resulted in successful pregnancies and live birth after ICSI (Kordus et al., 2008; Westlander et al., 2003). Immotile testicular or epididymal human sperm has also been selected using HOS to perform ICSI, resulting in higher fertilization and pregnancy rates compared with morphological-based selection (Casper et al., 1996; Sallam et al., 2001, 2005). These results suggest that sperm HOS selection for ICSI is not detrimental to the fertilization and embryo development.

In equine sperm, high HOS values are positively correlated with normal morphology and viability (Dogan et al., 2009; Neild et al., 2000), and HOS is indicative of high fertility potential (Neild et al., 2000). HOS has not been used as a method to select individual stallion sperm for ICSI. In field fertility, stallion potential fertility declined when HOS was <40%. Otherwise, fertile stallions have higher percentages of morphologically normal and HOS positive sperm, which are associated with higher pregnancy rates and a lower number of services per pregnancy (Neild et al., 2000).

Hyaluronic acid binding assay

Hyaluronic acid (HA) is a glycosaminoglycan present in the oocyte cumulus. During fertilization, sperm bind HA in the extracellular matrix of cumulus cells, and by releasing hyaluronidase, the sperm can interact with zona pellucida (ZP). Sperm-HA binding is mediated via a specific protein located in human sperm head (PH-20) which is anchored protein to the sperm head plasma membrane via glycosylphosphatidylinositol. The PH-20 protein exhibits

hyaluronidase activity and induces acrosome reaction in human sperm (Cherr et al., 2001; Sabeur et al., 1997). The HA-binding assay is described as a physiologic selector for human sperm because mature sperm with a completed plasma membrane remodeling, cytoplasmic protrusion and nuclear maturation can bind firmly HA (Cayli et al., 2003; Huszar et al., 2007). Also HA-bound sperm exhibit normal nuclear morphology with low level of chromosomal aneuploidy and DNA fragmentation (Jakab et al., 2005; Parmegiani et al., 2010a). In contrast, sperm with reduced HA-binding display abnormal morphology, low zona pellucida-binding and high retention of cytoplasmic enzymes. Sperm with poor HA-binding have high level of lipid peroxidation, that potentially induces DNA damage (Huszar et al., 2007). When individual sperm were selected by HA-binding for human ICSI, resulted in higher embryo quality, development and implantation rates and reduced abortion rate when compared to sperm selected in polyvinylpyrrolidone (Huszar et al., 2007; Parmegiani et al., 2010a, 2010b).

In fresh stallion sperm, membrane-associated hyaluronidase localizes in the postacrosomal region and inner acrosomal membrane after acrosomal exocytosis (Meyers, 2001), sharing numerous attributes with PH-20 protein from mouse, monkey, and human sperm (Cherr et al., 2001; Meyers, 2001). Sperm from fertile stallions are able to bind HA through membrane-associated hyaluronidase (Colleoni et al., 2011; Meyers, 2001), but it is unknown if the equine sperm hyaluronidase is involved in cumulus penetration, sperm-oocyte recognition and membrane fusion (Meyers, 2001). In a clinical study, equine cooled or frozen-thawed sperm bound effectively to HA, but in lower proportion compared to human sperm. Potentially, cooling, cryopreservation and the use of milk-based extender can block or inactivate HA-binding sites in equine sperm. When the initial sperm samples were used for artificial insemination, the percentage of HA-bound sperm did not relate with pregnancy outcome

(Colleoni et al., 2011). The potential use and benefit of the HA-binding assay has not been reported as an individual sperm selection method for equine ICSI.

Zona pellucida binding assay

During the processes of human fertilization in vivo or in standard IVF, sperm bind to the zona pellucida (ZP), undergo the acrosome reaction, penetrate the ZP, and finally fuse with the oolemma (Yanagimachi, 1988). In the ZP-binding assay, donor's immature sibling oocyte is incubated with previously processed motile sperm. Sperm that bind to the ZP of the immature oocyte are removed by repeated aspiration using a fine pipette. The isolated ZP-bound sperm are used for ICSI. The sperm removing procedure from the surface of ZP has no detrimental effect on sperm motility, morphology and DNA integrity (Black et al., 2010). In human, no difference has been observed in fertilization, embryo development, implantation, and pregnancy rates after ICSI using ZP-bound sperm or embryologist-selected sperm (Black et al., 2010). However, sperm selected by ZP-binding appear to be more competent, improving embryo quality and implantation rates, when compared to sperm selected based on embryologist experience (Braga et al., 2018; Liu, 2011).

Methylxanthines

Viable sperm can be identified among immotile sperm adding pentoxifylline or theophylline to the medium (Ebner et al., 2011; Kovačič et al., 2006). Pentoxifylline and theophylline are methylxanthines and phosphodiesterase inhibitors that downregulate the breakdown of cyclic adenosine monophosphate cAMP (Kovačič et al., 2006). The increase of intracellular levels of cAMP activates cAMP-dependent protein kinase and catalyzes protein

phosphorylation, stimulating sperm flagellar motility (Tash and Means, 1983). In human, pentoxifylline and theophylline help to identify individual viable sperm for ICSI within a immotile sperm population from testicular samples, resulting in higher fertilization rates, development potential and blastocyst morphology when compared to untreated sperm samples (Ebner et al., 2011; Kovačič et al., 2006).

Laser assisted immotile sperm selection (LAISS)

A viable sperm can be identified in an immotile sample by LAISS. A viable sperm tail starts curling immediately after a single laser shot (laser beam of 1.48 μm diode) is applied near the tip of the tail, while a nonviable sperm does not react. This method can be used in any culture medium and the selected sperm can be injected directly (Aktan et al., 2004). Sperm selected by LAISS do not exhibit membrane damage or increasing of DNA fragmentation (Ebner et al., 2005; Montag et al., 2000).

OOCYTE ACTIVATION

Activation of the mammalian oocyte involves a series of collective events. After sperm-oocyte membrane fusion, oocyte activation is triggered by a series of pulsatile calcium (Ca^{2+}) oscillations that increase the intracellular Ca^{2+} concentration. High intracellular Ca^{2+} concentration activates pathways that results in the resumption and completion of meiosis II, extrusion of the second polar body, and initiation of preimplantation development (Kline and Kline, 1992). Calcium oscillations in fertilized oocytes regulate short- and long-term developmental events (Ducibella et al., 2002; Stricker, 1999).

Oocyte activation is characterized by a two-step pattern of rises in intracellular Ca^{2+} concentrations, termed “Trigger” and “Oscillator”, which initiates and maintains oocyte activation respectively (Tesarik et al., 2002b). The “Trigger” is the first Ca^{2+} rise, which is released from internal stores and initiated by a receptor-mediated interaction between the sperm and oocyte plasma membrane. The “Trigger” is followed 30 min later by the “Oscillator”, which is a series of shorter Ca^{2+} transient oscillations of high amplitude that continue for 3 to 4 h. The “Oscillator” function is dependent on release of a soluble sperm factor into the oocyte cytoplasm that conditions the oocyte to sustain repetitive Ca^{2+} releases from intracellular stores, supporting the ongoing and autonomous series of Ca^{2+} oscillations for several hours (Ben-Yosef and Shalgi, 2001; Tesarik et al., 2000; Tesatik and Mendoza, 1999).

In human oocytes activated by sperm injection, the “Trigger” is replaced by a so-called “Pseudotrigger”; a massive influx of Ca^{2+} into the oocyte that is provoked by the injection procedure itself. This first and single transient increase in Ca^{2+} starts 20–30 min after microinjection, and is originated from the oocyte cortex rather than from the vicinity of the injected sperm head. This Ca^{2+} rise alone is insufficient to fully activate the oocyte (Tesarik et al., 1994; Tesarik and Sousa, 1995, 1994).

To sustain the oscillation function, sperm demembranization is necessary to facilitate the liberation of the sperm factor responsible for the “Oscillator” function (Dozortsev et al., 1997). Evidence in human oocytes show that any reduction in the frequency of Ca^{2+} peaks can be compensated by higher amplitude and longer total duration, increasing the total amount of Ca^{2+} released (Nikiforaki et al., 2014). Also oocyte activation is tolerant to perturbations in the Ca^{2+} oscillation pattern as long as the total amount of Ca^{2+} release is uncompromised and passes a

critical threshold (Ozil et al., 2005; Tóth et al., 2006), responding to a wide range of intracellular Ca^{2+} signaling parameters (Ducibella et al., 2006).

Artificial Oocyte activation

The normal pattern of oocyte activation events, including Ca^{2+} oscillations, appears to be restored by artificially increasing the oocyte Ca^{2+} load at the time of ICSI (Tesarik et al., 2002b). In ART, artificial oocyte activation (AOA) using Ca^{2+} ionophores such as A23187 or ionomycin, is a applied technique in human laboratories (Vanden Meerschaut et al., 2012). Ca^{2+} ionophore has been used clinically in cases of failed fertilization after ICSI, resulting in completion of normal fertilization and live births (Nasr-Esfahani et al., 2010).

Alternatively, strontium chloride (SrCl_2) binds and activates the Ca^{2+} -binding site on the IP_3 receptor in the endoplasmic reticulum, inducing Ca^{2+} oscillations, cortical reaction, and completion of meiosis. Mouse oocytes artificially activated by SrCl_2 exhibit a similar first Ca^{2+} transient peak, but lower in frequency, and identical cortical reaction when compared to oocytes activated by sperm. SrCl_2 is an effective parthenogenetic inducer of Ca^{2+} oscillations (Tomashov-Matar et al., 2005).

An artificially sustained increase in intracellular Ca^{2+} concentration can have effects on downstream molecular events as epigenetic reprogramming of the genome which could result in an altered gene expression and/or impaired protein synthesis and degradation during the first cell cycle (Kurokawa and Fissore, 2003; Ozil and Huneau, 2001). In this scenario, AOA could affect gene expression or chromosome segregation; however, there is no evidence that AOA causes an increase of chromosome segregation errors at meiosis II when compared with standard IVF in humans (Capalbo et al., 2016). The gene expression pattern of oocyte artificially activated are

closer to IVF than to ICSI alone, suggesting that AOA effectively mimics the genetic events initiated by sperm entrance (Bridges et al., 2011).

In human reproductive laboratories, AOA protocols diverge in the ionophore concentration (5 to 10 μ M or unknown), duration of exposure (10 to 30 min), and timing of ionophore exposure (immediately or 30 min after ICSI) (Vanden Meerschaut et al., 2012). Clinical data indicate that fertilization rates after AOA are restored to a normal level, resulting in successful pregnancies to term (Heindryckx et al., 2008). In selected patients with cleavage failure, ICSI-AOA can be indicated to help zygotes reach more advanced developmental stages (Darwish and Magdi, 2015). In infertile men with globozoospermia, semen with round-headed sperm with absence of acrosome (Singh, 1992), and oligoasthenoteratozoospermia, semen with abnormally low sperm concentration, motility and normal morphology (Cavallini, 2006); ICSI-AOA resulted in >70% fertilization rates and pregnancies, suggesting that ICSI-AOA seems not affect preimplantation development (Heindryckx et al., 2005). The obstetric evaluations, neonatal exams and neurodevelopmental outcome of children are within expected ranges for ICSI-AOA (Heindryckx et al., 2008; Vanden Meerschaut et al., 2014).

In patients with oocyte activation failure or low fertilization rates even after AOA by Ca^{2+} ionophore, successful pregnancy and birth were reported after ICSI-AOA using SrCl_2 (Kyono et al., 2010; Yanagida et al., 2006). When ICSI-AOA using SrCl_2 was compared with conventional ICSI or ICSI-AOA using Ca^{2+} ionophore, fertilization, pronucleus formation, embryo quality, blastocyst formation, pregnancy and miscarriage rates were similar or improved. After AOA using SrCl_2 , the mental or physical development of the offspring were not affected adversely (Kim et al., 2014; Kyono et al., 2010). The AOA using SrCl_2 can be considered as alternative for Ca^{2+} ionophore-oocyte activation (Kim et al., 2014; Kyono et al., 2010; Yanagida

et al., 2006). Overall, AOA should be considered as a legitimate indication for cases of failed oocyte activation or low fertilization by ICSI and not as a routine adjuvant to ICSI (Miller et al., 2016; van Blerkom et al., 2015).

In bovine oocytes, parthenogenetic artificial activation is efficient using roscovitine [maturing promoting factor (MPF) inhibitor], 6-dimethylaminopurine (6-DMAP, protein phosphorylation inhibitor), or a combination of ionomycin and cycloheximide (protein synthesis inhibitor), with a high Ca^{2+} concentrations. In contrast, high Ca^{2+} concentrations are not beneficial for parthenogenetic activation in equine oocytes; the combination of ionomycin with 6-DMAP or roscovitine is more effective (Fernandes et al., 2014). Experimentally, parthenogenetic activation with ionomycin and 6-DMAP in equine oocytes resulted in 91% of cleavage and 40% blastocysts formation. However, using an immotile, frozen–thawed sperm for ICSI-AOA, the cleavage rate was similar to control (88%), but no cleaved oocyte developed to blastocyst (Choi et al., 2016). Similarly, equine oocytes parthenogenetic activated by sequential incubation in ionomycin, cytochalasin B and cycloheximide resulted in cleavage, (Li et al., 2004).

Sperm-mediated oocyte activation

After gamete-membrane fusion, the whole sperm, except the plasma membrane, is engulfed by the oocyte cytoplasm, triggering oocyte activation with the associated series of Ca^{2+} oscillations. The “sperm factor theory” was proposed to explain how Ca^{2+} is released, in which a hypothesized catalytic substance in the sperm head triggers Ca^{2+} release in the oocyte following gamete fusion (Dale et al., 1985). The specific identity of the sperm protein responsible for activating the oocyte has been the source of debate over recent years (Yeste et

al., 2016). The “sperm factor theory” was established when microinjection of sperm soluble extract into mouse oocytes induced Ca^{2+} oscillations (Jones et al., 1998). Oscillin was the first protein initially proposed as the sperm-borne oocyte activation factor (SOAF), but microinjections of recombinant oscillin into mouse oocytes failed to induce Ca^{2+} release (Parrington et al., 1996).

Several factors have been considered as candidate for SOAF over the recent years, including phospholipase C zeta 1 (PLCz) (Saunders et al., 2002) and postacrosomal WW binding protein (PAWP) (Wu et al., 2007a). Mounting evidence supports the role of PLCz as the physiologic trigger of Ca^{2+} oscillations after membrane-gamete fusion (Escoffier et al., 2016; Hachem et al., 2017; Heytens et al., 2009; Ito et al., 2011; Kashir et al., 2012; Nozawa et al., 2018; Swann and Lai, 2016). However, the relevance of other proteins remains unclear, particularly with regard to inconsistencies in the literature relating to PAWP (Amdani et al., 2016; Yeste et al., 2017) as studies from different laboratories report contradictory results. Some groups report PAWP is able to trigger Ca^{2+} oscillations in mammalian and nonmammalian oocytes (Aarabi et al., 2014a; Wu et al., 2007a, 2007b), but other research groups have not observed the same results (Nomikos et al., 2015, 2014). Sperm expression of PAWP is positively related to ICSI outcome in human and bovine artificial insemination (Aarabi et al., 2014b; Azad et al., 2018; Kennedy et al., 2014). However, others clinical studies in human reported that PAWP expression does not related to sperm quality or ICSI outcome (Freour et al., 2018, 2017). Using a knockout (KO) model in mouse, PAWP-KO sperm are able to trigger Ca^{2+} oscillations and promote embryo development after IVF (Satouh et al., 2015). PLCz-KO sperm exhibit no apparent differences in the level of PAWP protein compared to wild type sperm (Hachem et al., 2017), but they are not able to induce Ca^{2+} oscillations after ICSI (Hachem et al.,

2017; Nozawa et al., 2018). However, PLCz-KO sperm induce pronuclei formation after conventional IVF, although most of the zygotes are arrested due to polyspermy, few zygotes develop to term. When used for natural mating, PLCz-KO mouse males are subfertile but not sterile. This suggests an alternative mechanism or others potential factors are involved in oocyte activation at least in mice (Hachem et al., 2017; Nozawa et al., 2018).

Phospholipase C zeta 1

Sperm-borne PLCz is the smallest PLC isoform (Nomikos et al., 2013a; Saunders et al., 2002). The size of PLCz is similar across species at 70 to 75 kDa (Bedford-Guaus et al., 2011; Cox et al., 2002; Ito et al., 2008; Nomikos et al., 2013b; Saunders et al., 2002; Villaverde et al., 2013), indicating that the role is conserved in mammals (Yeste et al., 2017). PLCz is formed by tandem pair of N-terminal EF hand-like domains (domain of twelve residue loop flanked on both sides by a twelve residue alpha-helical exhibiting a helix-loop-helix topology, involved in binding intracellular Ca^{2+}), the characteristic X and Y catalytic domains for all PLCs which are connected by and X-Y linker region, and a single C-terminal C2 domain (Figure 1.1). The major difference to other PLCs is the absence of N-terminal pleckstrin (PH) and Src homology (SH) domains (Saunders et al., 2002).

During the spermatogenesis in the mouse, PLCz mRNA is present in spermatocyte, round and elongated spermatids (Saunders et al., 2002). The protein expression of PLCz in human sperm is initially detected in the acrosomic vesicles in round spermatids at step 2 of the Golgi phase, and is concentrated in spermatids at step 4 with a fully formed acrosome in the cap phase. Minimal PLCz reactivity is found in the head of the elongated spermatid during the elongation phase (Aarabi et al., 2012). In a mature human sperm, PLCz is primarily localized in acrosomal,

equatorial and postacrosomal regions (Grasa et al., 2008), and in subcellular regions as in the perinuclear theca of the equatorial and postacrosomal regions (Escoffier et al., 2014).

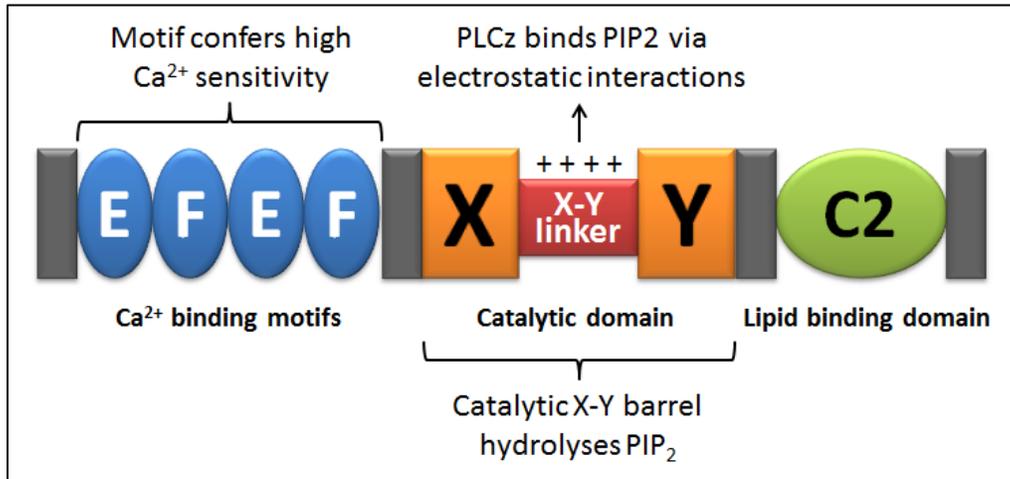


Figure 1.1: Schematic illustration of PLCz domain structure

Permeabilization of sperm with an ionic detergent reduces PLCz presence, suggesting that the protein is on the sperm surface (Aarabi et al., 2012). Equine PLCz localizes initially in round spermatids, and in the heads and tails of elongating spermatids and developing sperm (Bedford-Guaus et al., 2011). In equine mature sperm, PLCz is a 73 kDa protein with 638 amino acids, located in acrosomal and equatorial regions, connecting piece, and principal piece of the tail (Bedford-Guaus et al., 2011; Sato et al., 2013). Equine PLCz presents significant homology with porcine and human (82%) and partial homology with bovine (79%) and murine (72%) PLCz (Bedford-Guaus et al., 2011).

After sperm-oocyte membrane fusion, PLCz's target substrate is phosphatidylinositol 4,5-bisphosphate (PIP₂), which is contained in vesicles distributed across the ooplasm instead targeting PIP₂ present in the plasma membrane as others PLCs (Swann and Lai, 2013). The X

and Y domains are conserved across the entire PLC family that contains active site residues for hydrolysis of PIP₂. The X-Y linker region is composed of positively charged residues that interact electrostatically with PIP₂, which is negatively charged, regulating the enzymatic activity (Nomikos et al., 2011). The hydrolysis of PIP₂ results in inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). After which, IP₃ binds to IP₃ receptors in the endoplasmic reticulum, resulting in Ca²⁺ release and the oocyte activation cascade, and DAG activates protein kinase C (PKC) (Swann and Lai, 2013). The increase of intracellular Ca²⁺ activates Ca²⁺-calmodulin-dependent protein kinase II gamma (CaMKII γ) and in turn inhibits cytosolic factor (CSF). This inhibition liberates anaphase-promoting complex (APC) and subsequently degrades the levels of cyclin B1 from the maturation promoting factor (MPF) complex, composed of cyclin-dependent kinase I (CDK1) and cyclin B1. Reduction of cyclin B1 inactivates MPF causing the release of the oocyte from meiotic arrest. Simultaneously, the Ca²⁺ released also inactivates mitogen-activated protein kinase (MAPK), allowing pronucleus formation. Diacylglycerol activates the PKC pathway and translates Ca²⁺ signals into cellular responses, such as the exocytosis of cortical granules and the hardening of the ZP (Li et al., 2013).

The initiation of Ca²⁺ oscillations in oocytes by PLC ζ appears to be consistent among species. The microinjection of mouse and human oocytes with PLC ζ complementary RNA (cRNA) and recombinant protein initiates Ca²⁺ oscillations (Kashir et al., 2011; Nomikos et al., 2013b; Sanusi et al., 2015; Saunders et al., 2002). Correspondingly, microinjection of a recombinant PLC ζ with an infertility-linked mutation or sperm extracts depleted of PLC ζ are not able to trigger Ca²⁺ oscillations in mouse and human oocytes (Kashir et al., 2011; Saunders et al., 2002). Supplemental PLC ζ mRNA or recombinant protein rescues oocyte activation in mouse and human oocytes after ICSI when an infertile sperm is used, promoting resumption of meiosis,

pronucleus formation, and blastocyst development (Nozawa et al., 2018; Sanusi et al., 2015; Yoon et al., 2008). Using knockout mice, microinjection of PLCz-KO sperm fail to induce Ca^{2+} changes in mouse oocytes (Hachem et al., 2017; Nozawa et al., 2018), with very few injected oocytes reaching the 2-cell and blastocyst stages (Hachem et al., 2017). In contrast to ICSI, PLCz-KO sperm induced atypical patterns of Ca^{2+} oscillations after IVF, and most cleaved oocytes ceased development at the 1–2-cell stage due to failure of oocyte activation or polyspermy; very few cleaved oocytes develop to term (Hachem et al., 2017; Nozawa et al., 2018). These IVF fertilized oocytes with PLCz-KO sperm exhibit delayed ZP and plasma membrane block to polyspermy, as the plasma membrane block plays a more critical role than ZP block in vivo, suggesting that PLCz has a role in ensuring monospermic fertilization in vivo (Nozawa et al., 2018). For natural mating, PLCz-KO males are subfertile but not sterile, suggesting that oocyte activation in absence of PLCz can occur via an alternative route or mechanism that is PLCz-independent (Hachem et al., 2017; Nozawa et al., 2018).

After fertilization in mouse, PLCz localizes in the pronucleus during the interphase due to a nuclear localization signal, resulting in cessation of Ca^{2+} oscillation. This cessation of Ca^{2+} oscillation resumes following pronuclear envelope breakdown when the zygote entry into mitosis (Swann et al., 2006). PLCz translocates to the pronucleus 6 h after mouse PLCz cRNA injection (Yoda et al., 2004). In contrast, PLCz does not translocate to the pronucleus, remaining in cytoplasm and secondary polar body in human, rat, bovine and equine oocytes (Cooney et al., 2010; Ito et al., 2008; Sato et al., 2013).

The specific activity of PLCz from various species was compared, using the same cRNA concentration to inject mouse oocytes. Calcium oscillations started earlier with the highest frequency for PLCz of equine origin, followed by PLCz of human and mouse origin. However,

PLCz cRNA of bovine origin did not induce Ca^{2+} oscillations in mouse oocytes (Sato et al., 2013), confirming the low specific activity of bovine PLCz for this heterologous model (Cooney et al. 2010). In equine oocytes, injection of mouse PLCz cRNA triggers Ca^{2+} oscillations, inducing parthenogenetic oocyte activation with high cleavage rates in mare oocytes. The Ca^{2+} oscillations began within 40 min after injection and are relatively short in duration and highly variable in frequency among oocytes. The Ca^{2+} oscillations pattern induced by cRNA varies in a dose-dependent manner. However, the interval observed between Ca^{2+} spikes are similar for those produced by injection of PLCz cRNA or a single equine sperm (Bedford-Guaus et al., 2008). Likewise using mouse oocytes, injection of equine PLCz cRNA induces Ca^{2+} oscillations and PN formation (Sato et al., 2013). Altogether, these findings suggest that the injection in equine oocytes of PLCz cRNA mimics the oocyte activation induced by a sperm (Bedford-Guaus et al., 2008). Limited information suggests that subfertile stallions under field conditions have reduced PLCz expression (Bedford-Guaus et al., 2012; Gradil et al., 2006). No information is available that relates PLCz in stallion sperm with ICSI outcome and in vitro equine embryo production.

Postacrosomal WW binding protein

Although not previously described for stallion sperm, PAWP is a 32 kDa protein with 313 amino acids, localized in the postacrosomal sheath of the perinuclear theca of sperm from the man, monkey, pig, rabbit and bull (Aarabi et al., 2014b; Kennedy et al., 2014; Wu et al., 2007a). During spermatogenesis in bulls, PAWP is detectable in elongated spermatids, firstly in spermatids at step 9 in the early elongating phase, becoming prominent in the cytoplasmic lobe at step 11 during the elongation phase (Wu et al., 2007a).

It is proposed that PAWP interacts with WW-domain-Src family tyrosine kinase-protein complex PAWP-WW I pathway that targets tyrosine kinase regulation of the oocyte meiotic spindle and/or male remodeling factors. PAWP binds to oocyte-borne YAP proteins and interacts with the SH3 domain of PLC, resulting in activation of the phosphoinositide signaling pathway (Wu et al., 2007a). During fertilization with ICSI and IVF in porcine oocytes, PAWP localizes with similar distribution inside of the ooplasm. Initially, PAWP appeared as a distinct band covering the postacrosomal region below the equatorial region of the acrosome in the intact sperm. During the initial swelling, PAWP migrates to the anterior pole of the sperm nucleus, marking the onset of sperm nucleus decondensation and male PN development (Wu et al., 2007a).

Injection of recombinant PAWP induces Ca^{2+} oscillations and promotes formation of a single pronucleus in mature (metaphase II) human, mouse, porcine, bovine, and monkey oocytes (Aarabi et al., 2014a; Wu et al., 2007a). Oocytes fail to activate when recombinant PAWP is coinjected with anti-PAWP antibodies or competitive-inhibiting peptides derived from the PAWP-signaling domain (Wu et al., 2007a). Injection of mouse recombinant PAWP results in Ca^{2+} oscillations similar to those observed when a mouse sperm is microinjected into mouse oocyte. Likewise, injection of PAWP cRNA into human oocytes induces multiple Ca^{2+} oscillations, which are temporally and spatially similar to human sperm-induced oscillations (Aarabi et al., 2014a). These findings suggest that PAWP is sufficient to initiate zygotic development (Aarabi et al., 2014a).

Sperm oocyte activating factors and fertility

In various species, a relationship between male infertility and deficiencies of PLCz and/or PAWP is apparent. Reduced levels, abnormal localization or genetic mutations of PLCz are associated with ICSI failure or low fertilization rates in the human, mouse and feline (Chithiwala et al., 2015; Durban et al., 2015; Escoffier et al., 2016, 2014, Heytens et al., 2010, 2009, Kashir et al., 2012, 2010; Lee et al., 2014; Tavalae and Nasr-Esfahani, 2016; Villaverde et al., 2013; Yoon et al., 2008). In some cases, sperm with no or reduced PLCz occur with other major sperm defects that cause infertility, such as globozoospermia (Tavalae et al., 2018; Taylor et al., 2010; Yassine et al., 2014). Overall, total levels and proper localization patterns of PLCz in sperm are positively linked to fertilization rates and ICSI success (Tavalae et al., 2016; Yelumalai et al., 2015). High human sperm PAWP expression is positively related to higher fertilization rates and embryo development after ICSI, independent of age and other sperm parameters (Aarabi et al., 2014b). Likewise, PAWP is associated positively with bull semen quality and fertility parameters (Kennedy et al., 2014).

In human reproductive practice, PLCz and PAWP level are often correlated in sperm samples (Azad et al., 2018; Tavalae et al., 2016). Patients with fertilization failure following ICSI have lower protein levels and positive sperm for PLCz and PAWP when compared to fertile men (Azad et al., 2018). After ICSI, percentages of PLCz and PAWP positive sperm relate positively with fertilization, but not with embryo quality or pregnancy (Tavalae et al., 2016). Additionally, in cases of infertility with severe male factor, such as globozoospermia, the expression of PLCz and PAWP are reduced at the RNA and protein levels (Kamali-Dolat Abadi et al., 2016). However, the presence, localization and importance of PLCz and PAWP in stallion sperm and their association with fertility are not definitively known.

DISCUSSION

Intracytoplasmic sperm injection is the only repeatable and effective method to produce in vitro equine embryo (Carnevale, 2008; Choi et al., 2016; Galli et al., 2016). During ICSI procedure, one of the major concerns is the time consumed in trying to choose the ideal sperm and to determine what sperm attributes are the appropriate criteria for the individual sperm selection (Simopoulou et al., 2016). The diversity and development of techniques to improve ICSI from the sperm perspective is worth pursuing and the most of these techniques are based in human. However, limited information is available for sperm sorting and individual sperm selection techniques and the relationship with equine ICSI outcome.

Sperm separation techniques should be low-cost, quick, easy, and have the ability to select motile and morphologically normal sperm without the production of ROS. However, methods involving multiple centrifugation steps produce higher ROS and damage sperm DNA (Lewis and Aitken, 2005), impacting negatively ART outcomes in human (Lewis et al., 2013). Thus, centrifugation steps should be avoided during sperm sorting procedures by using alternative methods (Rappa et al., 2016). Motility-based sperm sorting approaches for sperm selection, such as DGC and swim-up, have enabled throughput selection of motile sperm that also have improved morphology (Mahadevan and Baker, 1984; Oshio et al., 1987; Pousette et al., 1986). However, both methods include centrifugation steps. Alternatively, microfluidic sperm sorting systems are noninvasive, fast and centrifugation-free that can protect the DNA integrity of the selected sperm population by preventing formation of ROS and DNA fragmentation (Nosrati et al., 2017). Motility-based methods are not applicable in instances of male infertility or poor quality sperm samples in which motility is low or absent (Nosrati et al.,

2017). Therefore, the efficiency of sorting and improvement in sperm parameters for any particular sperm sample for ICSI can be confounded by the sperm quality of the original sample.

In equine ICSI, the use of limited sperm samples and sperm samples with poor quality is recurrent (Carnevale, 2008); consequently there is higher need to use sperm sorting techniques that provide a sperm population with increased quality parameters. Current sperm sorting methods for equine ICSI, such as DGC and swim-up, provide efficient means of sperm quality (Choi et al., 2016; Colleoni et al., 2011; Galli et al., 2016). Stallion sperm sorted by DGC and swim-up have better motility, viability, morphology, mitochondrial membrane potential, and membrane and DNA integrity (Brum et al., 2008; Choi et al., 2016; Colleoni et al., 2011; Macpherson et al., 2002; Morrell et al., 2009, 2010; Sieme et al., 2003; Stoll et al., 2013; Stuhtmann et al., 2012). After ICSI, stallion sperm sorted by combined DGC and swim-up procedure compared to DGC, resulted in higher cleavage and blastocyst rates, but similar to sperm sorted by washing or swim-up (Choi et al., 2016). However, sperm motility and morphology are used as the final individual sperm selection criteria for ICSI; this arbitrary sperm selection could have negated some of the influence of sorting techniques.

There is no available information for methods to select an individual sperm for equine ICSI. Techniques used in human ICSI could be applied for individual stallion sperm selection, such as use sperm organelle morphology examination, birefringence microscopy, HOS test, hyaluronic acid-binding assay, methylxanthines supplementation and laser assisted immotile sperm selection (Aktan et al., 2004; Baccetti, 2004; Bartoov et al., 2002; Cayli et al., 2003; Ebner et al., 2011; Huszar et al., 2007; Jeyendran et al., 1984; Kovačič et al., 2006). However, further research should focus if these methods are suitable to evaluate specific attributes in individual stallion sperm desirable for ICSI, how these methods influence the ICSI outcome and

how they can fit in the equine ICSI laboratories. Potentially, the use of individual sperm selection methods should be limited in cases of severe male factor infertility, repeated implantation failures and poor quality sperm samples.

In ICSI procedure, many *in vivo* mechanisms for sperm selection are bypassed; however, sperm must still be capable of activating the oocyte for successful fertilization. A number of proteins have been suggested to be involved in oocyte activation in mammalian. Phospholipase C zeta and PAWP have been proposed as sperm activating factors (Saunders et al., 2002; Wu et al., 2007a). However, while mounting evidence supports the role of PLCz as oocyte activating factors (Kashir et al., 2011; Nomikos et al., 2013b; Nozawa et al., 2018; Sanusi et al., 2015; Saunders et al., 2002; Yoon et al., 2008); the relevance of some of the other proteins remains unclear. Particularly with regard to inconsistencies in the literature relating to PAWP with studies from separate laboratories reporting conflicting results (Aarabi et al., 2012; Amdani et al., 2016, 2015; Escoffier et al., 2016; Satouh et al., 2015; Wu et al., 2007a).

In human, reduced expression, abnormal localization or genetic mutations of PLCz are associated with ICSI failure or low fertilization rates (Chithiwala et al., 2015; Durban et al., 2015; Heytens et al., 2010; Lee et al., 2014; Tavalae et al., 2018; Taylor et al., 2010; Yassine et al., 2014). On the other hand, total levels and proper localization patterns of PLCz in sperm are positively related to fertilization rates and ICSI success (Tavalae et al., 2016; Yelumalai et al., 2015). Sperm expression of PAWP is positively related to higher fertilization rates and embryo development after ICSI (Aarabi et al., 2014b). Patients with fertilization failure after ICSI, sperm expression of PLCz and PAWP are reduced compared to fertile men (Azad et al., 2018). After ICSI, PLCz and PAWP expression relate positively with fertilization, but not with embryo quality or pregnancy (Tavalae et al., 2016). However, the importance of PLCz and PAWP in

stallion sperm and their association with ICSI outcome are not definitively known. Two studies using a very few stallions found that subfertile stallions in field conditions exhibit reduced PLCz expression (Bedford-Guaus et al., 2012; Gradil et al., 2006). Based in the human evidence, proper localization and expression of sperm activating factors in stallion sperm potentially impact positively ICSI outcome.

Based on limited information available which sperm factors affect ICSI outcome, we hypothesized that improving sperm characteristic using sorting methods and characterizing specific sperm attributes involved in fertilization in the sperm subpopulation which sperm are selected for oocyte injection, will result in improvement in ICSI outcomes. We aimed to investigate the effect of sorting methods and sample sperm characteristics on ICSI outcome, and to quantify sperm proteins involved in oocyte activation.

REFERENCES

- Aarabi, M., Balakier, H., Bashar, S., Moskovtsev, S.I., Sutovsky, P., Librach, C.L., Oko, R., 2014a. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *FASEB J* 28, 4434–4440.
<https://doi.org/10.1096/fj.14-256495>
- Aarabi, M., Balakier, H., Bashar, S., Moskovtsev, S.I., Sutovsky, P., Librach, C.L., Oko, R., 2014b. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. *Fertil Steril* 102, 440–447. <https://doi.org/10.1016/j.fertnstert.2014.05.003>
- Aarabi, M., Yu, Y., Xu, W., Tse, M.Y., Pang, S.C., Yi, Y.J., Sutovsky, P., Oko, R., 2012. The testicular and epididymal expression profile of PLC ζ in mouse and human does not support its role as a sperm-borne oocyte activating factor. *PLoS One* 7, 1–12.
<https://doi.org/10.1371/journal.pone.0033496>
- Aitken, R.J., Clarkson, J., 1988. Significance and Antioxidants of Reactive Oxygen Species in Defining the Efficacy of Sperm Preparation Techniques. *J Androl* 9, 367–76.
- Aitken, R.J., Clarkson, J.S., 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 81, 459–469. <https://doi.org/10.1530/jrf.0.0810459>
- Aktan, T.M., Montag, M., Duman, S., Gorkemli, H., Rink, K., Yurdakul, T., 2004. Use of a laser to detect viable but immotile spermatozoa. *Andrologia* 36, 366–369.
<https://doi.org/10.1111/j.1439-0272.2004.00636.x>
- Amdani, S.N., Yeste, M., Jones, C., Coward, K., 2016. Phospholipase C zeta (PLC ζ) and male

- infertility: Clinical update and topical developments. *Adv Biol Regul* 61, 58–67.
<https://doi.org/10.1016/j.jbior.2015.11.009>
- Amdani, S.N., Yeste, M., Jones, C., Coward, K., 2015. Sperm Factors and Oocyte Activation: Current Controversies and Considerations. *Biol Reprod* 93, 1–8.
<https://doi.org/10.1095/biolreprod.115.130609>
- Asghar, W., Velasco, V., Kingsley, J.L., Shoukat, M.S., Shafiee, H., Anchan, R.M., Mutter, G.L., Tüzel, E., Demirci, U., 2014. Selection of Functional Human Sperm with Higher DNA Integrity and Fewer Reactive Oxygen Species. *Adv Healthc Mater* 3, 1671–1679.
<https://doi.org/10.1002/adhm.201400058>
- Avendaño, C., Franchi, A., Duran, H., Oehninger, S., 2010. DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. *Fertil Steril* 94, 549–557. <https://doi.org/10.1016/j.fertnstert.2009.02.050>
- Avendaño, C., Franchi, A., Taylor, S., Morshedi, M., Bocca, S., Oehninger, S., 2009. Fragmentation of DNA in morphologically normal human spermatozoa. *Fertil Steril* 91, 1077–1084. <https://doi.org/10.1016/j.fertnstert.2008.01.015>
- Azad, N., Nazarian, H., Nazari, L., Novin, M.G., Piryaee, A., Hassan, M., Farahani, R.M., Sadjadpour, S.S., 2018. Evaluation of PAWP and PLC ζ Expression in Infertile Men with Previous ICSI Fertilization Failure. *Urol J*. <https://doi.org/10.22037/uj.v0i0.3966>
- Baccetti, B., 2004. Microscopical advances in assisted reproduction. *J Submicrosc Cytol Pathol* 36, 333–339.
- Barroso, G., Morshedi, M., Oehninger, S., 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 15, 1338–1344. <https://doi.org/10.1093/humrep/15.6.1338>

- Barroso, G., Valdespin, C., Vega, E., Kershenovich, R., Avila, R., Avendaño, C., Oehninger, S., 2009. Developmental sperm contributions: fertilization and beyond. *Fertil Steril* 92, 835–848. <https://doi.org/10.1016/j.fertnstert.2009.06.030>
- Bartoov, B., Berkovitz, A., Eltes, F., Kogosowski, A., Menezes, Y., Barak, Y., 2002. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. *J Androl* 23, 1–8. <https://doi.org/10.1002/j.1939-4640.2002.tb02595.x>
- Bassiri, F., Tavalaei, M., Shiravi, A.H., Mansouri, S., Nasr-Esfahani, M.H., 2012. Is there an association between HOST grades and sperm quality? *Hum Reprod* 27, 2277–2284. <https://doi.org/10.1093/humrep/des155>
- Bedford-Guaus, S., Yoon, S.Y., Fissore, R.A., Choi, Y.O., Hinrichs, K., 2008. Microinjection of mouse phospholipase C ζ complementary RNA into mare oocytes induces long-lasting intracellular calcium oscillations and embryonic development. *Reprod Fertil Dev* 20, 875–883. <https://doi.org/10.1071/RD08115>
- Bedford-Guaus, S.J., McPartlin, L.A., Varner, D.D., 2012. Characterization of Equine Phospholipase C Zeta: A Review and Preliminary Results on Expression Defects in Subfertile Stallions. *J Equine Vet Sci* 32, 445–450. <https://doi.org/10.1016/j.jevs.2012.06.002>
- Bedford-Guaus, S.J., McPartlin, L.A., Xie, J., Westmiller, S.L., Buffone, M.G., Roberson, M.S., 2011. Molecular Cloning and Characterization of Phospholipase C Zeta in Equine Sperm and Testis Reveals Species-Specific Differences in Expression of Catalytically Active Protein1. *Biol Reprod* 85, 78–88. <https://doi.org/10.1095/biolreprod.110.089466>
- Bedford, J., 1983. Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol Reprod* 28, 108–120.

- Ben-Yosef, D., Shalgi, R., 2001. Oocyte activation: Lessons from human infertility. *Trends Mol Med* 7, 163–168. [https://doi.org/10.1016/S1471-4914\(01\)01957-8](https://doi.org/10.1016/S1471-4914(01)01957-8)
- Black, M., Liu, D.Y., Bourne, H., Baker, H.W.G., 2010. Comparison of outcomes of conventional intracytoplasmic sperm injection and intracytoplasmic sperm injection using sperm bound to the zona pellucida of immature oocytes. *Fertil Steril* 93, 672–674. <https://doi.org/https://doi.org/10.1016/j.fertnstert.2009.08.063>
- Boitrelle, F., Albert, M., Petit, J.M., Ferfour, F., Wainer, R., Bergere, M., Bailly, M., Vialard, F., Selva, J., 2013. Small human sperm vacuoles observed under high magnification are pocket-like nuclear concavities linked to chromatin condensation failure. *Reprod Biomed Online* 27, 201–211. <https://doi.org/10.1016/j.rbmo.2013.05.004>
- Boitrelle, F., Ferfour, F., Petit, J.M., Segretain, D., Tourain, C., Bergere, M., Bailly, M., Vialard, F., Albert, M., Selva, J., 2011. Large human sperm vacuoles observed in motile spermatozoa under high magnification: Nuclear thumbprints linked to failure of chromatin condensation. *Hum Reprod* 26, 1650–1658. <https://doi.org/10.1093/humrep/der129>
- Boitrelle, F., Guthauser, B., Alter, L., Bailly, M., Bergere, M., Wainer, R., Vialard, F., Albert, M., Selva, J., 2014. High-magnification selection of spermatozoa prior to oocyte injection: Confirmed and potential indications. *Reprod Biomed Online* 28, 6–13. <https://doi.org/10.1016/j.rbmo.2013.09.019>
- Bolton, V.N., Braude, P.R., 1984. Preparation of human spermatozoa for in vitro fertilization by isopycnic centrifugation on self-generating density gradients. *Syst Biol Reprod Med* 13, 167–176. <https://doi.org/10.3109/01485018408987515>
- Braga, D.P. de A.F., Iaconelli Jr, A., Figueira, R. de C.S., Madaschi, C., Semião-Francisco, L., Borges Jr, E., 2018. Outcome of ICSI using zona pellucida-bound spermatozoa and

conventionally selected spermatozoa. *Reprod Biomed Online* 19, 802–807.

<https://doi.org/10.1016/j.rbmo.2009.09.020>

Bridges, P.J., Jeung, M., Kim, H., Kim, J.H., Lee, D.R., Ko, C., Baker, D.J., 2011.

Methodology matters: IVF versus ICSI and embryonic gene expression. *Reprod Biomed Online* 23, 234–244. <https://doi.org/10.1016/j.rbmo.2011.04.007>

Brum, A.M., Sabeur, K., Ball, B.A., 2008. Apoptotic-like changes in equine spermatozoa separated by density-gradient centrifugation or after cryopreservation. *Theriogenology* 69, 1041–1055. <https://doi.org/10.1016/j.theriogenology.2008.01.014>

Capalbo, A., Ottolini, C.S., Griffin, D.K., Ubaldi, F.M., Handyside, A.H., Rienzi, L., 2016.

Artificial oocyte activation with calcium ionophore does not cause a widespread increase in chromosome segregation errors in the second meiotic division of the oocyte. *Fertil Steril* 105, 807–814.e2. <https://doi.org/10.1016/j.fertnstert.2015.11.017>

Carnevale, E.M., 2016. Advances in Collection, Transport and Maturation of Equine Oocytes for Assisted Reproductive Techniques. *Vet Clin North Am - Equine Pract* 32, 379–399.

<https://doi.org/10.1016/j.cveq.2016.07.002>

Carnevale, E.M., 2008. Clinical Considerations Regarding Assisted Reproductive Procedures in Horses. *J Equine Vet Sci* 28, 686–690. <https://doi.org/10.1016/j.jevs.2008.10.005>

Carnevale, E.M., Coutinho Da Silva, M.A., Panzani, D., Stokes, J.E., Squires, E.L., 2005.

Factors affecting the success of oocyte transfer in a clinical program for subfertile mares. *Theriogenology* 64, 519–527. <https://doi.org/10.1016/j.theriogenology.2005.05.008>

Casper, R.F., Meriano, J.S., Jarvi, K.A., Cowan, L., Lucato, M.L., 1996. The hypo-osmotic swelling test for selection of viable sperm for intracytoplasmic sperm injection in men with complete asthenozoospermia. *Fertil Steril* 65, 972–976. <https://doi.org/10.1016/S0015->

0282(16)58271-5

Cavallini, G., 2006. Male idiopathic oligoasthenoteratozoospermia. *Asian J Androl* 8, 143–157.

<https://doi.org/10.1111/j.1745-7262.2006.00123.x>

Cayli, S., Jakab, A., Ovari, L., Delpiano, E., Celik-Ozenci, C., Sakkas, D., Ward, D., Huszar, G.,

2003. Biochemical markers of sperm function: Male fertility and sperm selection for ICSI.

Reprod Biomed Online 7, 462–468. [https://doi.org/10.1016/S1472-6483\(10\)61891-3](https://doi.org/10.1016/S1472-6483(10)61891-3)

Celik-Ozenci, C., Jakab, A., Kovacs, T., Catalanotti, J., Demir, R., Bray-Ward, P., Ward, D.,

Huszar, G., 2004. Sperm selection for ICSI: Shape properties do not predict the absence or presence of numerical chromosomal aberrations. *Hum Reprod* 19, 2052–2059.

<https://doi.org/10.1093/humrep/deh361>

Chan, P.J., Jacobson, J.D., Corselli, J.U., Patton, W.C., 2006. A simple zeta method for sperm selection based on membrane charge. *Fertil Steril* 85, 481–486.

<https://doi.org/10.1016/j.fertnstert.2005.07.1302>

Charehjooy, N., Najafi, M.H., Tavalae, M., Deemeh, M.R., Azadi, L., Shiravi, A.H., Nasr-

Esfahani, M.H., 2014. Selection of sperm based on hypo-osmotic swelling may improve ICSI outcome: A preliminary prospective clinical trial. *Int J Fertil Steril* 8, 21–28.

Cherr, G.N., Yudin, A.I., Overstreet, J.W., 2001. The dual functions of GPI-anchored PH-20:

hyaluronidase and intracellular signaling. *Matrix Biol* 20, 515–525.

[https://doi.org/https://doi.org/10.1016/S0945-053X\(01\)00171-8](https://doi.org/https://doi.org/10.1016/S0945-053X(01)00171-8)

Chithiwala, Z.H., Lee, H.C., Hill, D.L., Jellerette-Nolan, T., Fissore, R., Grow, D., Dumesic,

D.A., 2015. Phospholipase C-zeta deficiency as a cause for repetitive oocyte fertilization failure during ovarian stimulation for in vitro fertilization with ICSI: a case report. *J Assist*

Reprod Genet 32, 1415–1419. <https://doi.org/10.1007/s10815-015-0531-1>

- Cho, B.S., Schuster, T.G., Zhu, X., Chang, D., Smith, G.D., Takayama, S., 2003. Passively driven integrated microfluidic system for separation of motile sperm. *Anal Chem* 75, 1671–1675. <https://doi.org/10.1021/ac020579e>
- Choi, Y.H., Love, C.C., Varner, D.D., Hinrichs, K., 2006. Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. *Theriogenology* 65, 808–819. <https://doi.org/10.1016/j.theriogenology.2005.04.035>
- Choi, Y.H., Velez, I.C., Macías-García, B., Riera, F.L., Ballard, C.S., Hinrichs, K., 2016. Effect of clinically-related factors on in vitro blastocyst development after equine ICSI. *Theriogenology* 85, 1289–1296. <https://doi.org/10.1016/j.theriogenology.2015.12.015>
- Colleoni, S., Barbacini, S., Nechi, D., Duchi, R., Lazzari, G., Galli, C., 2007. Application of ovum pick-up, intracytoplasmic sperm injection and embryo culture in equine practice, in: *53rd Ann Conf Am Assoc Equine Pract.* p. 554.
- Colleoni, S., Lagutina, I., Lazzari, G., Rodriguez-Martinez, H., Galli, C., Morrell, J.M., 2011. New Methods for Selecting Stallion Spermatozoa for Assisted Reproduction. *J Equine Vet Sci* 31, 536–541. <https://doi.org/10.1016/j.jevs.2011.03.009>
- Colleoni, S., Lazzari, G., Duchi, R., Baca Castex, C., Mari, G., Lagutina, I., Galli, C., 2012. Fertilization and development of oocytes after ICSI with semen of stallions with different in vivo fertility. *J Equine Vet Sci* 32, 408–409. <https://doi.org/10.1016/j.jevs.2012.05.030>
- Collodel, G., Serena, S., Federico, M.G., Pascarelli, N.A., Geminiani, M., Moretti, E., 2010. Natural sperm birefringence can be used to estimate sperm viability and morphology. *Syst Biol Reprod Med* 56, 465–472. <https://doi.org/10.3109/19396368.2010.503360>
- Cooney, M.A., Malcuit, C., Cheon, B., Holland, M.K., Fissore, R.A., D’Cruz, N.T., 2010. Species-specific differences in the activity and nuclear localization of murine and bovine

phospholipase C zeta 1. *Biol Reprod* 83, 92–101.

<https://doi.org/10.1095/biolreprod.109.079814>

Cox, L.J., Larman, M.G., Saunders, C.M., Hashimoto, K., Swann, K., Lai, F.A., 2002. Sperm phospholipase C ζ from humans and cynomolgus monkeys triggers Ca²⁺ oscillations, activation and development of mouse oocytes. *Reproduction* 124, 611–623.

<https://doi.org/10.1530/reprod/124.5.611>

Dale, B., DeFelice, L.J., Ehrenstein, G., 1985. Injection of a soluble sperm fraction into sea-urchin eggs triggers the cortical reaction. *Experientia* 41, 1068–1070.

<https://doi.org/10.1007/BF01952148>

Darwish, E., Magdi, Y., 2015. A preliminary report of successful cleavage after calcium ionophore activation at ICSI in cases with previous arrest at the pronuclear stage. *Reprod Biomed Online* 31, 799–804. <https://doi.org/https://doi.org/10.1016/j.rbmo.2015.08.012>

Dogan, I., Polat, U., Nur, Z., 2009. Correlations between seminal plasma enzyme activities and semen parameters in seminal fluid of Arabian horses. *Iran J Vet Res* 10, 119–124.

Dozortsev, D., Qian, C., Ermilov, A., Rybouchkin, A., De Sutter, P., Dhont, M., 1997. Sperm-associated oocyte-activating factor is released from the spermatozoon within 30 minutes after injection as a result of the sperm-oocyte interaction. *Hum Reprod* 12, 2792–2796.

<https://doi.org/10.1093/humrep/12.12.2792>

Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R.M., Kopf, G.S., Fissore, R., Madoux, S., Ozil, J.-P., 2002. Egg-to-Embryo Transition Is Driven by Differential Responses to Ca²⁺ Oscillation Number. *Dev Biol* 250, 280–291.

<https://doi.org/https://doi.org/10.1006/dbio.2002.0788>

Ducibella, T., Schultz, R.M., Ozil, J.-P., 2006. Role of calcium signals in early development.

Semin Cell Dev Biol 17, 324–332.

<https://doi.org/https://doi.org/10.1016/j.semcd.2006.02.010>

- Durban, M., Barragán, M., Colodron, M., Ferrer-Buitrago, M., De Sutter, P., Heindryckx, B., Vernaev, V., Vassena, R., 2015. PLC ζ disruption with complete fertilization failure in normozoospermia. *J Assist Reprod Genet* 32, 879–886. <https://doi.org/10.1007/s10815-015-0496-0>
- Ebner, T., Moser, M., Tews, G., 2005. Possible applications of a non-contact 1.48 μ m wavelength diode laser in assisted reproduction technologies. *Hum Reprod Update* 11, 425–435. <https://doi.org/10.1093/humupd/dmi009>
- Ebner, T., Shebl, O., Oppelt, P., Mayer, R.B., 2014. Some reflections on intracytoplasmic morphologically selected sperm injection. *Int J Fertil Steril* 8, 105–112.
- Ebner, T., Tews, G., Mayer, R.B., Ziehr, S., Arzt, W., Costamoling, W., Shebl, O., 2011. Pharmacological stimulation of sperm motility in frozen and thawed testicular sperm using the dimethylxanthine theophylline. *Fertil Steril* 96, 1331–1336. <https://doi.org/10.1016/j.fertnstert.2011.08.041>
- El-Sherry, T.M., Elsayed, M., Abdelhafez, H.K., Abdelgawad, M., 2015. Characterization of rheotaxis of bull sperm using microfluidics. *Proc 7th Cairo Int Biomed Eng Conf CIBEC 2014* 6, 181–184. <https://doi.org/10.1109/CIBEC.2014.7020951>
- Escoffier, J., Lee, H.C., Yassine, S., Zouari, R., Martinez, G., Karaouz??ne, T., Coutton, C., Kherraf, Z.E., Halouani, L., Triki, C., Nef, S., Thierry-Mieg, N., Savinov, S.N., Fissore, R., Ray, P.F., Arnoult, C., 2016. Homozygous mutation of PLCZ1 leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. *Hum Mol Genet* 25, 878–891. <https://doi.org/10.1093/hmg/ddv617>

- Escoffier, J., Yassine, S., Lee, H.C., Martinez, G., Delaroche, J., Coutton, C., Karaouze, T., Zouari, R., Metzler-Guillemain, C., Pernet-Gallay, K., Hennebicq, S., Ray, P.F., Fissore, R., Arnoult, C., 2014. Subcellular localization of phospholipase Cz in human sperm and its absence in DPY19L2-deficient sperm are consistent with its role in oocyte activation. *Mol Hum Reprod* 21, 157–168. <https://doi.org/10.1093/molehr/gau098>
- Fernandes, C.B., Devito, L.G., Martins, L.R., Blanco, I.D.P., de Lima Neto, J.F., Tsuribe, P.M., Gonçalves, C.G.P., da Cruz Landim-Alvarenga, F., 2014. Artificial activation of bovine and equine oocytes with cycloheximide, roscovitine, strontium, or 6-dimethylaminopurine in low or high calcium concentrations. *Zygote* 22, 387–394. [https://doi.org/DOI: 10.1017/S0967199412000627](https://doi.org/DOI:10.1017/S0967199412000627)
- Fleming, S.D., Ilad, R.S., Griffin, A.-M.G., Wu, Y., Ong, K.J., Smith, H.C., Aitken, R.J., 2008. Prospective controlled trial of an electrophoretic method of sperm preparation for assisted reproduction: comparison with density gradient centrifugation. *Hum Reprod* 23, 2646–2651.
- Franco, J.G., Baruffi, R.L.R., Mauri, A.L., Petersen, C.G., Oliveira, J.B.A., Vagnini, L., 2008. Significance of large nuclear vacuoles in human spermatozoa: implications for ICSI. *Reprod Biomed Online* 17, 42–45. [https://doi.org/10.1016/S1472-6483\(10\)60291-X](https://doi.org/10.1016/S1472-6483(10)60291-X)
- Freour, T., Barragan, M., Ferrer-Vaquer, A., Rodríguez, A., Vassena, R., 2017. WBP2NL/PAWP mRNA and protein expression in sperm cells are not related to semen parameters, fertilization rate, or reproductive outcome. *J Assist Reprod Genet* 34, 803–810. <https://doi.org/10.1007/s10815-017-0902-x>
- Freour, T., Barragan, M., Torra-Massana, M., Ferrer-Vaquer, A., Vassena, R., 2018. Is there an association between PAWP/WBP2NL sequence, expression, and distribution in sperm cells

and fertilization failures in ICSI cycles? *Mol Reprod Dev* 1–8.

<https://doi.org/10.1002/mrd.22950>

Galli, C., Colleoni, S., Duchi, R., Lazzari, G., 2016. Male Factors Affecting the Success of Equine In Vitro Embryo Production by Ovum Pickup-Intracytoplasmic Sperm Injection in a Clinical Setting. *J Equine Vet Sci* 43, S6–S10. <https://doi.org/10.1016/j.jevs.2016.05.014>

Garolla, A., Fortini, D., Menegazzo, M., De Toni, L., Nicoletti, V., Moretti, A., Selice, R., Engl, B., Foresta, C., 2008. High-power microscopy for selecting spermatozoa for ICSI by physiological status. *Reprod Biomed Online* 17, 610–6. [https://doi.org/10.1016/S1472-6483\(10\)60307-0](https://doi.org/10.1016/S1472-6483(10)60307-0)

Gianaroli, L., Magli, M.C., Collodel, G., Moretti, E., Ferraretti, A.P., Baccetti, B., 2008. Sperm head's birefringence: a new criterion for sperm selection. *Fertil Steril* 90, 104–112. <https://doi.org/10.1016/j.fertnstert.2007.05.078>

Gianaroli, L., Magli, M.C., Ferraretti, A.P., Crippa, A., Lappi, M., Capitani, S., Baccetti, B., 2010. Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection. *Fertil Steril* 93, 807–813. <https://doi.org/10.1016/j.fertnstert.2008.10.024>

Gonzalez-Castro, R., Carnevale, E., 2016. Association between equine sperm parameters and ICSI outcome. *J Equine Vet Sci* 41, 62. <https://doi.org/10.1016/j.jevs.2016.04.041>

Gonzalez-Castro, R., Sanches, F.A., Graham, J., Carnevale, E., 2016. Comparison of extenders for equine semen refreezing for intracytoplasmic sperm injection. *Anim Reprod Sci* 169, 131. <https://doi.org/10.1016/j.anireprosci.2016.03.084>

Gosálvez, J., Migueles, B., López-Fernández, C., Sánchez-Martín, F., Sáchez-Martín, P., 2013. Single sperm selection and DNA fragmentation analysis: The case of MSOME/IMSI. *Nat*

Sci 05, 7–14. <https://doi.org/10.4236/ns.2013.57A002>

Gradil, C., Yoon, S., Brown, J., He, C., Visconti, P., Fissore, R., 2006. PLC zeta: a marker of fertility for stallions? *Anim Reprod Sci* 94, 23–25.

<https://doi.org/10.1016/j.anireprosci.2006.03.095>

Grasa, P., Coward, K., Young, C., Parrington, J., 2008. The pattern of localization of the putative oocyte activation factor, phospholipase C ζ , in uncapacitated, capacitated, and ionophore-treated human spermatozoa. *Hum Reprod* 23, 2513–2522.

<https://doi.org/10.1093/humrep/den280>

Grunewald, S., Miska, W., Miska, G., Rasch, M., Reinhardt, M., Glander, H.J., Paasch, U., 2007. Molecular glass wool filtration as a new tool for sperm preparation. *Hum Reprod* 22, 1405–1412. <https://doi.org/10.1093/humrep/dem015>

Haaf, T., Ward, D.C., 1995. Higher order nuclear structure in mammalian sperm revealed by in situ hybridization and extended chromatin fibers. *Exp Cell Res.*

<https://doi.org/10.1006/excr.1995.1270>

Hachem, A., Godwin, J., Ruas, M., Lee, H.C., Ferrer Buitrago, M., Ardestani, G., Bassett, A., Fox, S., Navarrete, F., de Sutter, P., Heindryckx, B., Fissore, R., Parrington, J., 2017. PLC ζ is the physiological trigger of the Ca²⁺ oscillations that induce embryogenesis in mammals but conception can occur in its absence. *Development* 144, 2914–2924.

<https://doi.org/10.1242/dev.150227>

Heindryckx, B., De Gheselle, S., Gerris, J., Dhont, M., De Sutter, P., 2008. Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. *Reprod Biomed Online* 17, 662–668. [https://doi.org/https://doi.org/10.1016/S1472-6483\(10\)60313-6](https://doi.org/https://doi.org/10.1016/S1472-6483(10)60313-6)

Heindryckx, B., Van der Elst, J., De Sutter, P., Dhont, M., 2005. Treatment option for sperm- or

- oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. *Hum Reprod* 20, 2237–2241.
- Henkel, R., 2012. Sperm preparation: State-of-the-art physiological aspects and application of advanced sperm preparation methods. *Asian J Androl* 14, 260–269.
<https://doi.org/10.1038/aja.2011.133>
- Henkel, R.R., Schill, W.B., 2003. Sperm preparation for ART. *Reprod Biol Endocrinol* 1, 1–22.
<https://doi.org/10.1186/1477-7827-1-108>
- Herbement, C., Sifer, C., 2015. How to select the spermatozoon for intracytoplasmic sperm injection in 2015? *Minerva Ginecol* 67, 185–193.
- Herrera, C., Dufourq, P., Freije, M., Morikawa, I., Centeno, J.E., Aristi, V., Menghini, L., Sporleder, C., 2012. Selection of stallions for in vitro embryo production by ICSI in a commercial program. *J Equine Vet Sci* 32, 409. <https://doi.org/10.1016/j.jevs.2012.05.031>
- Heytens, E., Parrington, J., Coward, K., Young, C., Lambrecht, S., Yoon, S.Y., Fissore, R.A., Hamer, R., Deane, C.M., Ruas, M., Grasa, P., Soleimani, R., Cuvelier, C.A., Gerris, J., Dhont, M., Deforce, D., Leybaert, L., De Sutter, P., 2009. Reduced amounts and abnormal forms of phospholipase C zeta (PLC ζ) in spermatozoa from infertile men. *Hum Reprod* 24, 2417–2428. <https://doi.org/10.1093/humrep/dep207>
- Heytens, E., Schmitt-John, T., Moser, J.M., Jensen, N.M., Soleimani, R., Young, C., Coward, K., Parrington, J., De Sutter, P., 2010. Reduced fertilization after ICSI and abnormal phospholipase C zeta presence in spermatozoa from the wobbler mouse. *Reprod Biomed Online* 21, 742–749. <https://doi.org/10.1016/j.rbmo.2010.07.006>
- Hinrichs, K., 2013. Assisted reproduction techniques in the horse. *Reprod Fertil Dev* 25, 80–93.
<https://doi.org/10.1071/RD12263>

- Hoogendijk, C.F., Kruger, T.F., Bouic, P.J.D., Henkel, R.R., 2009. A novel approach for the selection of human sperm using annexin V-binding and flow cytometry. *Fertil Steril* 91, 1285–1292. <https://doi.org/10.1016/j.fertnstert.2008.01.042>
- Huszar, G., Jakab, A., Sakkas, D., Ozenci, C.-C., Cayli, S., Delpiano, E., Ozkavukcu, S., 2007. Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. *Reprod Biomed Online* 14, 650–663. [https://doi.org/10.1016/S1472-6483\(10\)61060-7](https://doi.org/10.1016/S1472-6483(10)61060-7)
- Hutchison, J.M., Rau, D.C., DeRouchey, J.E., 2017. Role of Disulfide Bonds on DNA Packaging Forces in Bull Sperm Chromatin. *Biophys J* 113, 1925–1933. <https://doi.org/10.1016/j.bpj.2017.08.050>
- Intra, G., Alteri, A., Corti, L., Rabellotti, E., Papaleo, E., Restelli, L., Biondo, S., Garancini, M.P., Candiani, M., Viganò, P., 2016. Application of failure mode and effect analysis in an assisted reproduction technology laboratory. *Reprod Biomed Online* 33, 132–139. <https://doi.org/10.1016/j.rbmo.2016.05.008>
- Ishimoto, K., Gadêlha, H., Gaffney, E.A., Smith, D.J., Kirkman-Brown, J., 2017. Coarse-Graining the Fluid Flow around a Human Sperm. *Phys Rev Lett* 118, 124501.
- Ito, J., Parrington, J., Fissore, R.A., 2011. PLC?? and its role as a trigger of development in vertebrates. *Mol Reprod Dev* 78, 846–853. <https://doi.org/10.1002/mrd.21359>
- Ito, M., Shikano, T., Oda, S., Horiguchi, T., Tanimoto, S., Awaji, T., Mitani, H., Miyazaki, S., 2008. Difference in Ca²⁺ Oscillation-Inducing Activity and Nuclear Translocation Ability of PLCZ1, an Egg-Activating Sperm Factor Candidate, Between Mouse, Rat, Human, and Medaka Fish1. *Biol Reprod* 78, 1081–1090. <https://doi.org/10.1095/biolreprod.108.067801>
- Jakab, A., Sakkas, D., Delpiano, E., Cayli, S., Kovanci, E., Ward, D., Ravelli, A., Huszar, G.,

2005. Intracytoplasmic sperm injection: A novel selection method for sperm with normal frequency of chromosomal aneuploidies. *Fertil Steril* 84, 1665–1673.
<https://doi.org/10.1016/j.fertnstert.2005.05.068>
- Jayaraman, V., Upadhyaya, D., Narayan, P.K., Adiga, S.K., 2012. Sperm processing by swim-up and density gradient is effective in elimination of sperm with DNA damage. *J Assist Reprod Genet* 29, 557–563. <https://doi.org/10.1007/s10815-012-9742-x>
- Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G., Zaneveld, L.J., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 70, 219–228.
<https://doi.org/10.1530/jrf.0.0700219>
- Jones, K.T., Soeller, C., Cannell, M.B., 1998. The passage of Ca²⁺ and fluorescent markers between the sperm and egg after fusion in the mouse. *Development* 125, 4627 LP-4635.
- Kamali-Dolat Abadi, M., Tavalae, M., Shahverdi, A., Nasr-Esfahani, M.H., 2016. Evaluation of PLC ζ and PAWP expression in globozoospermic individuals. *Cell J* 18, 438–445.
- Kantsler, V., Dunkel, J., Blayney, M., Goldstein, R.E., 2014. Rheotaxis facilitates upstream navigation of mammalian sperm cells. *Elife* 3, e02403. <https://doi.org/10.7554/eLife.02403>
- Kashir, J., Heindryckx, B., Jones, C., de Sutter, P., Parrington, J., Coward, K., 2010. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update* 16, 690–703.
<https://doi.org/10.1093/humupd/dmq018>
- Kashir, J., Jones, C., Lee, H.C., Rietdorf, K., Nikiforaki, D., Durrans, C., Ruas, M., Tee, S.T., Heindryckx, B., Galione, A., De Sutter, P., Fissore, R.A., Parrington, J., Coward, K., 2011. Loss of activity mutations in phospholipase C zeta (PLC ζ) abolishes calcium oscillatory ability of human recombinant protein in mouse oocytes. *Hum Reprod* 26, 3372–3387.

<https://doi.org/10.1093/humrep/der336>

Kashir, J., Konstantinidis, M., Jones, C., Heindryckx, B., De Sutter, P., Parrington, J., Wells, D., Coward, K., 2012. Characterization of two heterozygous mutations of the oocyte activation factor phospholipase C zeta (PLC ζ) from an infertile man by use of minisequencing of individual sperm and expression in somatic cells. *Fertil Steril* 98, 423–431.

<https://doi.org/10.1016/j.fertnstert.2012.05.002>

Kennedy, C.E., Krieger, K.B., Sutovsky, M., Xu, W., Vargovič, P., Didion, B.A., Ellersieck, M.R., Hennessy, M.E., Verstegen, J., Oko, R., Sutovsky, P., 2014. Protein expression pattern of PAWP in bull spermatozoa is associated with sperm quality and fertility following artificial insemination. *Mol Reprod Dev* 81, 436–449.

<https://doi.org/10.1002/mrd.22309>

Kim, E.K., Kim, E.H., Kim, E.A., Lee, K.A., Shin, J.E., Kwon, H., 2015. Comparison of the effect of different media on the clinical outcomes of the density-gradient centrifugation/swim-up and swim-up methods. *Clin Exp Reprod Med* 42, 22–29.

<https://doi.org/10.5653/cerm.2015.42.1.22>

Kim, J.-W., Kim, S.-D., Yang, S.-H., Yoon, S.-H., Jung, J.-H., Lim, J.-H., 2014. Successful pregnancy after SrCl₂ oocyte activation in couples with repeated low fertilization rates following calcium ionophore treatment. *Syst Biol Reprod Med* 60, 177–182.

<https://doi.org/10.3109/19396368.2014.900832>

Kline, D., Kline, J.T., 1992. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev Biol* 149, 80–89.

[https://doi.org/https://doi.org/10.1016/0012-1606\(92\)90265-I](https://doi.org/https://doi.org/10.1016/0012-1606(92)90265-I)

Knowlton, S.M., Sadasivam, M., Tasoglu, S., 2015. Microfluidics for sperm research. *Trends*

Biotechnol 33, 221–229. <https://doi.org/10.1016/j.tibtech.2015.01.005>

Kordus, R.J., Price, R.L., Davis, J.M., Whitman-Elia, G.F., 2008. Successful twin birth following blastocyst culture of embryos derived from the immotile ejaculated spermatozoa from a patient with primary ciliary dyskinesia: A case report. *J Assist Reprod Genet* 25, 437–443. <https://doi.org/10.1007/s10815-008-9254-x>

Kosower, S., Katayose, H., Yanagimachi, R., 1992. Thiol-disulfide status and acridine orange fluorescence of mammalian sperm nuclei. *J Androl* 13, 342–348.

Kovačić, B., Vlaisavljević, V., Reljić, M., 2006. Clinical use of pentoxifylline for activation of immotile testicular sperm before ICSI in patients with azoospermia. *J Androl* 27, 45–52. <https://doi.org/10.2164/jandrol.05079>

Kurokawa, M., Fissore, R.A., 2003. ICSI-generated mouse zygotes exhibit altered calcium oscillations, inositol 1,4,5-trisphosphate receptor-1 down-regulation, and embryo development. *MHR Basic Sci Reprod Med* 9, 523–533.

Kyono, K., Nakajo, Y., Takisawa, T., Kyoya, T., Hattori, H., Sato, Y., Doshida, M., Toya, M., 2010. 187 COMPARISON BETWEEN TWO OOCYTE ACTIVATION METHODS (CALCIUM IONOPHORE A23187 VS STRONTIUM CHLORIDE). *Reprod Biomed Online* 20, S85. [https://doi.org/https://doi.org/10.1016/S1472-6483\(10\)62605-3](https://doi.org/https://doi.org/10.1016/S1472-6483(10)62605-3)

Lazzari, G., Crotti, G., Turini, P., Duchi, R., Mari, G., Zavaglia, G., Barbacini, S., Galli, C., 2002. Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of in vitro matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. *Theriogenology* 58, 709–712. [https://doi.org/10.1016/S0093-691X\(02\)00777-X](https://doi.org/10.1016/S0093-691X(02)00777-X)

Lee, H.C., Arny, M., Grow, D., Dumesic, D., Fissore, R.A., Jellerette-Nolan, T., 2014. Protein

- phospholipase C Zeta1 expression in patients with failed ICSI but with normal sperm parameters. *J Assist Reprod Genet* 31, 749–756. <https://doi.org/10.1007/s10815-014-0229-9>
- Lewis, S.E.M., Aitken, R.J., 2005. DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell Tissue Res* 322, 33–41. <https://doi.org/10.1007/s00441-005-1097-5>
- Lewis, S.E.M., Aitken, R.J., Conner, S.J., De Iuliis, G., Evenson, D.P., Henkel, R., Giwercman G, A., Gharagozloo, P., 2013. The impact of sperm DNA damage in assisted conception and beyond: Recent advances in diagnosis and treatment. *Reprod Biomed Online* 27, 325–337. <https://doi.org/10.1016/j.rbmo.2013.06.014>
- Li, L., Lu, X., Dean, J., 2013. The maternal to zygotic transition in mammals. *Mol Aspects Med* 34, 919–938. <https://doi.org/10.1016/j.mam.2013.01.003>
- Li, X., Dai, Y., Allen, W.R., 2004. Influence of Insulin-Like Growth Factor-I on Cytoplasmic Maturation of Horse Oocytes In Vitro and Organization of the First Cell Cycle Following Nuclear Transfer and Parthenogenesis1. *Biol Reprod* 71, 1391–1396.
- Liu, D.-Y., 2011. Could using the zona pellucida bound sperm for intracytoplasmic sperm injection (ICSI) enhance the outcome of ICSI? *Asian J Androl* 13, 197–198. <https://doi.org/10.1038/aja.2010.179>
- Loutradi, K.E., Tarlatzis, B.C., Goulis, D.G., Zepiridis, L., Pagou, T., Chatziioannou, E., Grimbizis, G.F., Papadimas, I., Bontis, I., 2006. The effects of sperm quality on embryo development after intracytoplasmic sperm injection. *J Assist Reprod Genet* 23, 69–74. <https://doi.org/10.1007/s10815-006-9022-8>
- Luppi, S., Martinelli, M., Giacomini, E., Giolo, E., Zito, G., Garcia, R.C., Ricci, G., 2015. Comparative proteomic analysis of spermatozoa isolated by swim-up or density gradient centrifugation. *Reprod Biol Endocrinol* 13, 1–7. <https://doi.org/10.1186/s12958-015-0027-y>

- Macpherson, M.L., Blanchard, T.L., Love, C.C., Brinsko, S.P., Varner, D.D., 2002. Use of a silane-coated silica particle solution to enhance the quality of ejaculated semen in stallions. *Theriogenology* 58, 317–320. [https://doi.org/10.1016/S0093-691X\(02\)00736-7](https://doi.org/10.1016/S0093-691X(02)00736-7)
- Mahadevan, M., Baker, G., 1984. Assessment and preparation of semen for in vitro fertilization, in: Wood, C., Trounson, A. (Eds.), *Clinical In Vitro Fertilization*. Springer-Verlag, Berlin, Germany., pp. 83–97.
- Malvezzi, H., Sharma, R., Agarwal, A., Abuzenadah, A.M., Abu-Elmagd, M., 2014. Sperm quality after density gradient centrifugation with three commercially available media: a controlled trial. *Reprod Biol Endocrinol* 12, 121. <https://doi.org/10.1186/1477-7827-12-121>
- Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., Green, D.R., 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 182, 1545 LP-1556.
- Matsuura, K., Uozumi, T., Furuichi, T., Sugimoto, I., Kodama, M., Funahashi, H., 2013. A microfluidic device to reduce treatment time of intracytoplasmic sperm injection. *Fertil Steril* 99, 400–407. <https://doi.org/10.1016/j.fertnstert.2012.10.022>
- McCue, P., Moore, A., Bruemmer, J., 2004. Refreezing stallion spermatozoa for assisted reproduction. *Reprod Fertil Dev* 16, 176–177.
- Meintjes, M., Grati, K.J., Paccamonti, D., Eilts, B.E., Co, R., Sullivan, M., Fall, H., 1996. In vitro development and embryo transfer of sperm-injected oocytes derived from pregnant mares. *Theriogenology* 45, 304.
- Ménézo, Y.J.R., Hazout, A., Panteix, G., Robert, F., Rollet, J., Cohen-Bacrie, P., Chapuis, F., Clément, P., Benkhalifa, M., 2007. Antioxidants to reduce sperm DNA fragmentation: An

- unexpected adverse effect. *Reprod Biomed Online* 14, 418–421.
[https://doi.org/10.1016/S1472-6483\(10\)60887-5](https://doi.org/10.1016/S1472-6483(10)60887-5)
- Meyers, S.A., 2001. Equine sperm-oocyte interaction: The role of sperm surface hyaluronidase. *Anim Reprod Sci* 68, 291–303. [https://doi.org/10.1016/S0378-4320\(01\)00166-X](https://doi.org/10.1016/S0378-4320(01)00166-X)
- Miciński, P., Pawlicki, K., Wielgus, E., Bochenek, M., Gogol, P., Ficek, B., 2011. Total reactive antioxidant potential and DNA fragmentation index as fertility sperm parameters. *Reprod Biol* 11, 135–144. [https://doi.org/10.1016/S1642-431X\(12\)60050-3](https://doi.org/10.1016/S1642-431X(12)60050-3)
- Miki, K., Clapham, D.E., 2013. Rheotaxis Guides Mammalian Sperm. *Curr Biol* 23, 443–452.
<https://doi.org/https://doi.org/10.1016/j.cub.2013.02.007>
- Miller, J.E., Smith, T.T., 2001. The effect of intracytoplasmic sperm injection and semen parameters on blastocyst development in vitro. *Hum Reprod* 16, 918–924.
<https://doi.org/10.1093/humrep/16.5.918>
- Miller, N., Biron-Shental, T., Sukenik-Halevy, R., Klement, A.H., Sharony, R., Berkovitz, A., 2016. Oocyte activation by calcium ionophore and congenital birth defects: a retrospective cohort study. *Fertil Steril* 106, 590–596.e2.
<https://doi.org/https://doi.org/10.1016/j.fertnstert.2016.04.025>
- Montag, M., Rink, K., Delacrétaiz, G., van der Ven, H., 2000. Laser-induced immobilization and plasma membrane permeabilization in human spermatozoa. *Hum Reprod* 15, 846–852.
<https://doi.org/10.1093/HUMREP/15.4.846>
- Morrell, J., Johannisson, A., Dalin, A.M., Rodriguez-Martinez, H., 2009. Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. *Reprod Domest Anim* 44, 512–517.
<https://doi.org/10.1111/j.1439-0531.2008.01265.x>

- Morrell, J.M., Rodriguez-Martinez, H., Johannisson, A., 2010. Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size. *Equine Vet J* 42, 579–585. <https://doi.org/10.1111/j.2042-3306.2010.00101.x>
- Mortimer, D., 1991. Sperm preparation techniques and iatrogenic failures of in-vitro fertilization. *Hum Reprod* 6, 173–176.
- Mortimer, D., 1989. Sperm Transport in the Human Reproductive Female Tract, in: Finn, C. (Ed.), *Oxford Reviews in Reproductive Biology Volume 5*. Oxford University Press, Oxford, p. 30.
- Nadalini, M., Tarozzi, N., Distratis, V., Scaravelli, G., Borini, A., 2009. Impact of intracytoplasmic morphologically selected sperm injection on assisted reproduction outcome: A review. *Reprod Biomed Online* 19, 45–55. [https://doi.org/10.1016/S1472-6483\(10\)60283-0](https://doi.org/10.1016/S1472-6483(10)60283-0)
- Nasr-Esfahani, M.H., Aboutorabi, R., Esfandiari, E., Mardani, M., 2002. Sperm MTT viability assay: A new method for evaluation of human sperm viability. *J Assist Reprod Genet* 19, 477–482. <https://doi.org/10.1023/A:1020310503143>
- Nasr-Esfahani, M.H., Deemeh, M.R., Tavalae, M., 2012. New era in sperm selection for ICSI. *Int J Androl* 35, 475–484. <https://doi.org/10.1111/j.1365-2605.2011.01227.x>
- Nasr-Esfahani, M.H., Deemeh, M.R., Tavalae, M., 2010. Artificial oocyte activation and intracytoplasmic sperm injection. *Fertil Steril* 94, 520–526. <https://doi.org/https://doi.org/10.1016/j.fertnstert.2009.03.061>
- Neild, D.M., Chaves, M.G., Flores, M., Miragaya, M.H., Gonzalez, E., Agüero, A., 2000. The HOS test and its relationship to fertility in the stallion. *Andrologia* 32, 351–355.

<https://doi.org/10.1046/j.1439-0272.2000.00357.x>

- Nikiforaki, D., Vanden Meerschaut, F., Qian, C., De Croo, I., Lu, Y., Deroo, T., Van den Abbeel, E., Heindryckx, B., De Sutter, P., 2014. Oocyte cryopreservation and in vitro culture affect calcium signalling during human fertilization. *Hum Reprod* 29, 29–40.
- Nomikos, M., Elgmati, K., Theodoridou, M., Calver, B.L., Cumbes, B., Nounesis, G., Nounesis, G., Swann, K., Lai, F.A., 2011. Male infertility-linked point mutation disrupts the Ca²⁺ oscillation-inducing and PIP(2) hydrolysis activity of sperm PLC ζ . *Biochem J* 434, 211–7. <https://doi.org/10.1042/BJ20101772>
- Nomikos, M., Kashir, J., Swann, K., Lai, F.A., 2013a. Sperm PLC ζ : From structure to Ca²⁺ oscillations, egg activation and therapeutic potential. *FEBS Lett* 587, 3609–3616. <https://doi.org/10.1016/j.febslet.2013.10.008>
- Nomikos, M., Sanders, J.R., Kashir, J., Sanusi, R., Buntwal, L., Love, D., Ashley, P., Sanders, D., Knaggs, P., Bunkheila, A., Swann, K., Anthony Lai, F., 2015. Functional disparity between human pawpand plc ζ in the generation of Ca²⁺ oscillations for oocyte activation. *Mol Hum Reprod* 21, 702–710. <https://doi.org/10.1093/molehr/gav034>
- Nomikos, M., Sanders, J.R., Theodoridou, M., Kashir, J., Matthews, E., Nounesis, G., Lai, F.A., Swann, K., 2014. Sperm-specific post-acrosomal WW-domain binding protein (PAWP) does not cause Ca²⁺ release in mouse oocytes. *Mol Hum Reprod* 20, 938–947. <https://doi.org/10.1093/molehr/gau056>
- Nomikos, M., Yu, Y., Elgmati, K., Theodoridou, M., Campbell, K., Vassilakopoulou, V., Zikos, C., Livaniou, E., Amso, N., Nounesis, G., Swann, K., Lai, F.A., 2013b. Phospholipase C ζ rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril* 99, 76–85. <https://doi.org/10.1016/j.fertnstert.2012.08.035>

- Nosrati, R., Graham, P.J., Zhang, B., Riordon, J., Lagunov, A., Hannam, T.G., Escobedo, C., Jarvi, K., Sinton, D., 2017. Microfluidics for sperm analysis and selection. *Nat Rev Urol* 14, 707–730. <https://doi.org/10.1038/nrurol.2017.175>
- Nosrati, R., Vollmer, M., Eamer, L., San Gabriel, M.C., Zeidan, K., Zini, A., Sinton, D., 2014. Rapid selection of sperm with high DNA integrity. *Lab Chip* 14, 1142. <https://doi.org/10.1039/c3lc51254a>
- Nozawa, K., Satouh, Y., Fujimoto, T., Oji, A., Ikawa, M., 2018. Sperm-borne phospholipase C zeta-1 ensures monospermic fertilization in mice. *Sci Rep* 8, 1315. <https://doi.org/10.1038/s41598-018-19497-6>
- Oshio, S., Kaneko, S., Iizuka, R., Mohri, H., 1987. Effects of gradient centrifugation on human sperm. *Syst Biol Reprod Med* 19, 85–93. <https://doi.org/10.3109/01485018708986804>
- Ozil, J.-P., Markoulaki, S., Toth, S., Matson, S., Banrezes, B., Knott, J.G., Schultz, R.M., Huneau, D., Ducibella, T., 2005. Egg activation events are regulated by the duration of a sustained $[Ca^{2+}]_{cyt}$ signal in the mouse. *Dev Biol* 282, 39–54. <https://doi.org/https://doi.org/10.1016/j.ydbio.2005.02.035>
- Ozil, J.P., Huneau, D., 2001. Activation of rabbit oocytes: the impact of the Ca^{2+} signal regime on development. *Development* 128, 917 LP-928.
- Palermo, G., Joris, H., Devroey, P., Van Steirteghem, A.C., 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 340, 17–18. [https://doi.org/10.1016/0140-6736\(92\)92425-F](https://doi.org/10.1016/0140-6736(92)92425-F)
- Palmer, E., Bezard, J., Magistrini, M., Duchamp, G., 1991. In vitro fertilization in the horse. A retrospective study. *J Reprod Fertil Suppl* 44, 375–384.
- Parmegiani, L., Cognigni, G.E., Bernardi, S., Troilo, E., Ciampaglia, W., Filicori, M., 2010a.

- “Physiologic ICSI”: Hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. *Fertil Steril* 93, 598–604. <https://doi.org/10.1016/j.fertnstert.2009.03.033>
- Parmegiani, L., Cognigni, G.E., Ciampaglia, W., Pocognoli, P., Marchi, F., Filicori, M., 2010b. Efficiency of hyaluronic acid (HA) sperm selection. *J Assist Reprod Genet* 27, 13–16. <https://doi.org/10.1007/s10815-009-9380-0>
- Parrington, J., Swann, K., Shevchenko, V.I., Sesay, A.K., Lai, F.A., 1996. Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. *Nature* 379, 364.
- Paulson, J.D., Polakoski, K.L., 1977. A Glass Wool Column Procedure for Removing Extraneous Material from the Human Ejaculate**Supported in part by a grant from The Rockefeller Foundation. *Fertil Steril* 28, 178–181. [https://doi.org/https://doi.org/10.1016/S0015-0282\(16\)42379-4](https://doi.org/https://doi.org/10.1016/S0015-0282(16)42379-4)
- Petersen, C.G., Vagnini, L.D., Mauri, A.L., Massaro, F.C., Cavagna, M., Baruffi, R.L.R., Oliveira, J.B.A., Franco, J.G., 2011. Relationship between DNA damage and sperm head birefringence. *Reprod Biomed Online* 22, 583–589. <https://doi.org/10.1016/j.rbmo.2011.03.017>
- Pousette, Å., Åkerlöf, E., Rosenborg, L., Fredricsson, B., 1986. Increase in progressive motility and improved morphology of human spermatozoa following their migration through Percoll gradients. *Int J Androl* 9, 1–13. <https://doi.org/10.1111/j.1365-2605.1986.tb00862.x>
- Rader, K., Choi, Y.H., Hinrichs, K., 2016. Intracytoplasmic Sperm Injection, Embryo Culture, and Transfer of In Vitro–Produced Blastocysts. *Vet Clin North Am - Equine Pract* 32, 401–413. <https://doi.org/10.1016/j.cveq.2016.07.003>
- Rappa, K.L., Rodriguez, H.F., Hakkarainen, G.C., Anchan, R.M., Mutter, G.L., Asghar, W.,

2016. Sperm processing for advanced reproductive technologies: Where are we today? *Biotechnol Adv* 34, 578–587. <https://doi.org/10.1016/j.biotechadv.2016.01.007>
- Razavi, S.H., Nasr-Esfahani, M.H., Deemeh, M.R., Shayesteh, M., Tavalae, M., 2010. Evaluation of zeta and HA-binding methods for selection of spermatozoa with normal morphology, protamine content and DNA integrity. *Andrologia* 42, 13–19. <https://doi.org/10.1111/j.1439-0272.2009.00948.x>
- Ricci, G., Perticarari, S., Boscolo, R., Montico, M., Guaschino, S., Presani, G., 2009. Semen preparation methods and sperm apoptosis: swim-up versus gradient-density centrifugation technique. *Fertil Steril* 91, 632–638. <https://doi.org/10.1016/j.fertnstert.2007.11.068>
- Rossato, M., Di Virgilio, F., Foresta, C., 1996. Involvement of osmo-sensitive calcium influx in human sperm activation. *Mol Hum Reprod* 2, 903–909. <https://doi.org/10.1093/molehr/2.12.903>
- Sabeur, K., Cherr, G., Yudin, A., Primakoff, P., Li, M., Overstreet, J., 1997. The PH-20 protein in human spermatozoa. *J Androl* 18, 151–158.
- Said, T.M., Agarwal, A., Zborowski, M., Grunewald, S., Glander, H.J., Paasch, U., 2008. Utility of magnetic cell separation as a molecular sperm preparation technique. *J Androl* 29, 134–142. <https://doi.org/10.2164/jandrol.107.003632>
- Said, T.M., Land, J.A., 2011. Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. *Hum Reprod Update* 17, 719–733.
- Sakkas, D., Manicardi, G.C., Tomlinson, M., Mandrioli, M., Bizzaro, D., Bianchi, P.G., Bianchi, U., 2000. The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. *Hum Reprod* 15, 1112–1116. <https://doi.org/10.1093/humrep/15.5.1112>

- Sallam, H., Farrag, a, Agameya, a, Ezzeldin, F., Eid, a, Sallam, a, 2001. The use of a modified hypo-osmotic swelling test for the selection of viable ejaculated and testicular immotile spermatozoa in ICSI. *Hum Reprod* 16, 272–6.
- Sallam, H.N., Farrag, A., Agameya, A.F., El-Garem, Y., Ezzeldin, F., 2005. The use of the modified hypo-osmotic swelling test for the selection of immotile testicular spermatozoa in patients treated with ICSI: A randomized controlled study. *Hum Reprod* 20, 3435–3440. <https://doi.org/10.1093/humrep/dei249>
- Sanusi, R., Yu, Y., Nomikos, M., Lai, F.A., Swann, K., 2015. Rescue of failed oocyte activation after ICSI in a mouse model of male factor infertility by recombinant phospholipase C ζ . *Mol Hum Reprod* 21, 783–791. <https://doi.org/10.1093/molehr/gav042>
- Sato, K., Wakai, T., Seita, Y., Takizawa, A., Fissore, R.A., Ito, J., Kashiwazaki, N., 2013. Molecular characteristics of horse phospholipase C zeta (PLC ζ). *Anim Sci J* 84, 359–368. <https://doi.org/10.1111/asj.12044>
- Satouh, Y., Nozawa, K., Ikawa, M., 2015. Sperm Postacrosomal WW Domain-Binding Protein Is Not Required for Mouse Egg Activation. *Biol Reprod* 93, 94–94. <https://doi.org/10.1095/biolreprod.115.131441>
- Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., Lai, F.A., 2002. PLC zeta: a sperm-specific trigger of Ca⁽²⁺⁾ oscillations in eggs and embryo development. *Development* 129, 3533–3544.
- Schuster, T.G., Cho, B., Keller, L.M., Takayama, S., Smith, G.D., 2003. Isolation of motile spermatozoa from semen samples using microfluidics. *Reprod Biomed Online* 7, 75–81. [https://doi.org/10.1016/S1472-6483\(10\)61732-4](https://doi.org/10.1016/S1472-6483(10)61732-4)
- Seo, D., Agca, Y., Feng, Z.C., Critser, J.K., 2007. Development of sorting, aligning, and

- orienting motile sperm using microfluidic device operated by hydrostatic pressure. *Microfluid Nanofluidics* 3, 561–570. <https://doi.org/10.1007/s10404-006-0142-3>
- Sessions-Bresnahan, D.R., Graham, J.K., Carnevale, E.M., 2014. Validation of a heterologous fertilization assay and comparison of fertilization rates of equine oocytes using invitro fertilization, perivitelline, and intracytoplasmic sperm injections. *Theriogenology* 82, 274–282. <https://doi.org/10.1016/j.theriogenology.2014.04.002>
- Shirota, K., Yotsumoto, F., Itoh, H., Obama, H., Hidaka, N., Nakajima, K., Miyamoto, S., 2016. Separation efficiency of a microfluidic sperm sorter to minimize sperm DNA damage. *Fertil Steril* 105, 315–321. <https://doi.org/10.1016/j.fertnstert.2015.10.023>
- Sieme, H., Martinsson, G., Rauterberg, H., Walter, K., Aurich, C., Petzoldt, R., Klug, E., 2003. Application of techniques for sperm selection in fresh and frozen-thawed stallion semen. *Reprod Domest Anim* 38, 134–140. <https://doi.org/10.1046/j.1439-0531.2003.00416.x>
- Simopoulou, M., Gkoles, L., Bakas, P., Giannelou, P., Kalampokas, T., Pantos, K., Koutsilieris, M., 2016. Improving ICSI: A review from the spermatozoon perspective. *Syst Biol Reprod Med* 62, 359–371. <https://doi.org/10.1080/19396368.2016.1229365>
- Singh, G., 1992. Ultrastructural features of round-headed human spermatozoa. *Int J Fertil* 37, 99–102.
- Smith, D.J., Gaffney, E.A., Blake, J.R., Kirkman-Brown, J.C., 2009. Human sperm accumulation near surfaces: A simulation study. *J Fluid Mech* 621, 289–320. <https://doi.org/10.1017/S0022112008004953>
- Squires, E., Wislon, J., Kato, H., Blaszczyk, A., 1996. a Pregnancy After Intracytoplasmic Sperm Injection. *Theriogenology* 45, 306.
- Stanger, J.D., Vo, L., Yovich, J.L., Almahbobi, G., 2010. Hypo-osmotic swelling test identifies

- individual spermatozoa with minimal DNA fragmentation. *Reprod Biomed Online* 21, 474–484. <https://doi.org/10.1016/j.rbmo.2010.06.026>
- Stoll, A., Love, C.C., Ball, B.A., 2013. Use of a single-layer density centrifugation method enhances sperm quality in cryopreserved-thawed equine spermatozoa. *J Equine Vet Sci* 33, 547–551. <https://doi.org/10.1016/j.jevs.2012.08.009>
- Stricker, S.A., 1999. Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol* 211, 157–176. <https://doi.org/10.1006/dbio.1999.9340>
- Stuhtmann, G., Oldenhof, H., Peters, P., Klewitz, J., Martinsson, G., Sieme, H., 2012. Iodixanol density gradient centrifugation for selecting stallion sperm for cold storage and cryopreservation. *Anim Reprod Sci* 133, 184–190. <https://doi.org/10.1016/j.anireprosci.2012.06.017>
- Suarez, S.S., Pacey, A.A., 2006. Sperm transport in the female reproductive tract. *Hum Reprod Update* 12, 23–37. <https://doi.org/10.1093/humupd/dmi047>
- Swann, K., Lai, F.A., 2016. The sperm phospholipase C- and Ca²⁺ signalling at fertilization in mammals. *Biochem Soc Trans* 44, 267–272. <https://doi.org/10.1042/BST20150221>
- Swann, K., Lai, F.A., 2013. PLC ζ and the initiation of Ca²⁺ oscillations in fertilizing mammalian eggs. *Cell Calcium* 53, 55–62. <https://doi.org/10.1016/j.ceca.2012.11.001>
- Swann, K., Saunders, C.M., Rogers, N.T., Lai, F.A., 2006. PLC ζ (zeta): A sperm protein that triggers Ca²⁺oscillations and egg activation in mammals. *Semin Cell Dev Biol* 17, 264–273. <https://doi.org/10.1016/j.semcdb.2006.03.009>
- Tartagni, M., Schonauer, M.M., Cicinelli, E., Selman, H., De Ziegler, D., Petruzzelli, F., D’Addario, V., 2002. Usefulness of the hypo-osmotic swelling test in predicting pregnancy rate and outcome in couples undergoing intrauterine insemination. *J Androl* 23, 498–502.

<https://doi.org/10.1002/j.1939-4640.2002.tb02271.x>

Tash, J.S., Means, A.R., 1983. Cyclic Adenosine 3',5' Monophosphate, Calcium and Protein Phosphorylation in Flagellar Motility. *Biol Reprod* 28, 75–104.

Tasoglu, S., Safaee, H., Zhang, X., Kingsley, J.L., Catalano, P.N., Gurkan, U.A., Nureddin, A., Kayaalp, E., Anchan, R.M., Maas, R.L., Tüzel, E., Demirci, U., 2013. Exhaustion of racing sperm in nature-mimicking microfluidic channels during sorting. *Small* 9, 3374–3384.

<https://doi.org/10.1002/smll.201300020>

Tavalaee, M., Kiani-Esfahani, A., Nasr-Esfahani, M.H., 2016. Relationship between potential sperm factors involved in oocyte activation and sperm DNA fragmentation with intra-cytoplasmic sperm injection clinical outcomes. *Cell J* 18, 588–596.

Tavalaee, M., Nasr-Esfahani, M.H., 2016. Expression profile of PLC ζ , PAWP, and TR-KIT in association with fertilization potential, embryo development, and pregnancy outcomes in globozoospermic candidates for intra-cytoplasmic sperm injection and artificial oocyte activation. *Andrology* 4, 850–856. <https://doi.org/10.1111/andr.12179>

Tavalaee, M., Nomikos, M., Lai, F.A., Nasr-Esfahani, M.H., 2018. Expression of sperm PLC ζ and clinical outcomes of ICSI-AOA in men affected by globozoospermia due to DPY19L2 deletion. *Reprod Biomed Online*. <https://doi.org/10.1016/j.rbmo.2017.12.013>

Taylor, S.L., Yoon, S.Y., Morshedi, M.S., Lacey, D.R., Jellerette, T., Fissore, R.A., Oehninger, S., 2010. Complete globozoospermia associated with PLC ζ deficiency treated with calcium ionophore and ICSI results in pregnancy. *Reprod Biomed Online* 20, 559–564.

<https://doi.org/10.1016/j.rbmo.2009.12.024>

Teran, J., Fauci, L., Shelley, M., 2010. Viscoelastic Fluid Response Can Increase the Speed and Efficiency of a Free Swimmer. *Phys Rev Lett* 104, 38101.

- Tesarik, J., Mendoza, C., Greco, E., 2002a. Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. *Hum Reprod* 17, 184–189.
<https://doi.org/10.1093/humrep/17.1.184>
- Tesarik, J., Mendoza, C., Greco, E., 2000. The activity (calcium oscillator?) responsible for human oocyte activation after injection with round spermatids is associated with spermatid nuclei. *Fertil Steril* 74, 1245–1247. [https://doi.org/https://doi.org/10.1016/S0015-0282\(00\)01598-3](https://doi.org/https://doi.org/10.1016/S0015-0282(00)01598-3)
- Tesarik, J., Rienzi, L., Ubaldi, F., Mendoza, C., Greco, E., 2002b. Use of a modified intracytoplasmic sperm injection technique to overcome sperm-borne and oocyte-borne oocyte activation failures. *Fertil Steril* 78, 619–624.
[https://doi.org/https://doi.org/10.1016/S0015-0282\(02\)03291-0](https://doi.org/https://doi.org/10.1016/S0015-0282(02)03291-0)
- Tesarik, J., Sousa, M., 1995. More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore**Supported in part by a grant from the “Junta de Andalucía” reseach group 3137 (J.T.). *Fertil Steril* 63, 343–349. [https://doi.org/https://doi.org/10.1016/S0015-0282\(16\)57366-X](https://doi.org/https://doi.org/10.1016/S0015-0282(16)57366-X)
- Tesarik, J., Sousa, M., 1994. Comparison of Ca²⁺ responses in human oocytes fertilized by subzonal insemination and by intracytoplasmic sperm injection. *Fertil Steril* 62, 1197–1204.
[https://doi.org/https://doi.org/10.1016/S0015-0282\(16\)57185-4](https://doi.org/https://doi.org/10.1016/S0015-0282(16)57185-4)
- Tesarik, J., Sousa, M., Testart, J., 1994. Human oocyte activation after intracytoplasmic sperm injection. *Hum Reprod* 9, 511–518.
- Tesarik, J., Mendoza, C., 1999. In vitro fertilization by intracytoplasmic sperm injection. *BioEssays* 21, 791–801. [https://doi.org/10.1002/\(SICI\)1521-1878\(199909\)21:9<791::AID-BIES11>3.0.CO;2-Z](https://doi.org/10.1002/(SICI)1521-1878(199909)21:9<791::AID-BIES11>3.0.CO;2-Z)

- Tomashov-Matar, R., Tchetchik, D., Eldar, A., Kaplan-Kraicer, R., Oron, Y., Shalgi, R., 2005. Strontium-induced rat egg activation. *Reprod* 130, 467–474.
<https://doi.org/10.1530/rep.1.00746>
- Tóth, S., Huneau, D., Banrezes, B., Ozil, J.-P., 2006. Egg activation is the result of calcium signal summation in the mouse. *Reprod* 131, 27–34. <https://doi.org/10.1530/rep.1.00764>
- Tutsi, Y., Akkus, M., Tunik, S., Nergiz, Y., Kaplanoglu, I., Nasir, Y., 2015. Investigation of functional sperm membrane integrity after consequence of anti-hypoosmotic swelling test technique: an electron microscopic study. *J Reprod Med* 60, 141–148.
- van Blerkom, J., Cohen, J., Johnson, M., 2015. A plea for caution and more research in the ‘experimental’ use of ionophores in ICSI. *Reprod Biomed Online* 30, 323–324.
<https://doi.org/https://doi.org/10.1016/j.rbmo.2015.02.002>
- Vanden Meerschaut, F., Nikiforaki, D., De Gheselle, S., Dullaerts, V., Van den Abbeel, E., Gerris, J., Heindryckx, B., De Sutter, P., 2012. Assisted oocyte activation is not beneficial for all patients with a suspected oocyte-related activation deficiency. *Hum Reprod* 27, 1977–1984.
- Vanden Meerschaut, F., Nikiforaki, D., Heindryckx, B., De Sutter, P., 2014. Assisted oocyte activation following ICSI fertilization failure. *Reprod Biomed Online* 28, 560–571.
<https://doi.org/https://doi.org/10.1016/j.rbmo.2014.01.008>
- Vanderzwalmen, P., Hiemer, A., Rubner, P., Bach, M., Neyer, A., Stecher, A., Uher, P., Zintz, M., Lejeune, B., Vanderzwalmen, S., Cassuto, G., Zech, N.H., 2008. Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles. *Reprod Biomed Online* 17, 617–627. [https://doi.org/10.1016/S1472-6483\(10\)60308-2](https://doi.org/10.1016/S1472-6483(10)60308-2)

- Vermeij, B.G., Chapman, M.G., Cooke, S., Kilani, S., 2015. The relationship between sperm head retardance using polarized light microscopy and clinical outcomes. *Reprod Biomed Online* 30, 67–73. <https://doi.org/10.1016/j.rbmo.2014.09.011>
- Villaverde, A.I.S.B., Fioratti, E.G., Fissore, R.A., He, C., Lee, H.C., Souza, F.F., Landim-Alvarenga, F.C., Lopes, M.D., 2013. Identification of phospholipase C zeta in normospermic and teratospermic domestic cat sperm. *Theriogenology* 80, 722–729. <https://doi.org/10.1016/j.theriogenology.2013.06.005>
- Westlander, G., Barry, M., Petrucco, O., Norman, R., 2003. Different fertilization rates between immotile testicular spermatozoa and immotile ejaculated spermatozoa for ICSI in men Kartagener's syndrome: Case report. *Hum Reprod* 18, 1286–1288. <https://doi.org/10.1093/humrep/deg240>
- Whitesides, G.M., 2006. The origins and the future of microfluidics. *Nature* 442, 368–373. <https://doi.org/10.1038/nature05058>
- Wilding, M., Coppola, G., Di Matteo, L., Palagiano, A., Fusco, E., Dale, B., 2011. Intracytoplasmic injection of morphologically selected spermatozoa (IMSI) improves outcome after assisted reproduction by deselecting physiologically poor quality spermatozoa. *J Assist Reprod Genet* 28, 253–262. <https://doi.org/10.1007/s10815-010-9505-5>
- Wu, A., Sutovsky, P., Manandhar, G., Xu, W., Katayama, M., Day, B., Park, K., Yi, Y., Yan, W., Prather, R., Oko, R., 2007a. PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem* 282, 12164–12175. <https://doi.org/10.1074/jbc.M609132200>
- Wu, A., Sutovsky, P., Xu, W., van der Spoel, A.C., Platt, M., Oko, R., 2007b. The postacrosomal

assembly of sperm head protein, PAWP, is independent of acrosome formation and dependent on microtubular manchette transport. *Dev Biol* 312, 471–483.

<https://doi.org/10.1016/j.ydbio.2007.08.051>

Yamanaka, M., Tomita, K., Hashimoto, S., Matsumoto, H., Satoh, M., Kato, H., Hosoi, Y., Inoue, M., Nakaoka, Y., Morimoto, Y., 2016. effectively decreases morphologically abnormal sperms 62, 16–18.

Yanagida, K., Morozumi, K., Katayose, H., Hayashi, S., Sato, A., 2006. Successful pregnancy after ICSI with strontium oocyte activation in low rates of fertilization. *Reprod Biomed Online* 13, 801–806. [https://doi.org/https://doi.org/10.1016/S1472-6483\(10\)61027-9](https://doi.org/10.1016/S1472-6483(10)61027-9)

Yanagimachi, R., 1988. Mammalian fertilization, in: Knobil, E., Neil, J., Ewing, L., Markert, C., Greenwald, G., Pfaff, D. (Eds.), *The Physiology of Reproduction Volume 1*. Raven Press, New York, pp. 135–185.

Yassine, S., Escoffier, J., Martinez, G., Coutton, C., Karaouzène, T., Zouari, R., Ravanat, J.L., Metzler-Guillemain, C., Lee, H.C., Fissore, R., Hennebicq, S., Ray, P.F., Arnoult, C., 2014. Dpy19l2-deficient globozoospermic sperm display altered genome packaging and DNA damage that compromises the initiation of embryo development. *Mol Hum Reprod* 21, 169–185. <https://doi.org/10.1093/molehr/gau099>

Yelumalai, S., Yeste, M., Jones, C., Amdani, S.N., Kashir, J., Mounce, G., Da Silva, S.J.M., Barratt, C.L., McVeigh, E., Coward, K., 2015. Total levels, localization patterns, and proportions of sperm exhibiting phospholipase C zeta are significantly correlated with fertilization rates after intracytoplasmic sperm injection. *Fertil Steril* 104, 561–568.e4. <https://doi.org/10.1016/j.fertnstert.2015.05.018>

Yeste, M., Jones, C., Amdani, S.N., Coward, K., 2017. Oocyte Activation and Fertilisation:

- Crucial Contributors from the Sperm and Oocyte BT - Signaling-Mediated Control of Cell Division : From Oogenesis to Oocyte-to-Embryo Development, in: Arur, S. (Ed.), . Springer International Publishing, Cham, pp. 213–239. https://doi.org/10.1007/978-3-319-44820-6_8
- Yeste, M., Jones, C., Amdani, S.N., Patel, S., Coward, K., 2016. Oocyte activation deficiency: A role for an oocyte contribution? *Hum Reprod Update* 22, 23–47. <https://doi.org/10.1093/humupd/dmv040>
- Yoda, A., Oda, S., Shikano, T., Kouchi, Z., Awaji, T., Shirakawa, H., Kinoshita, K., Miyazaki, S., 2004. Ca²⁺ oscillation-inducing phospholipase C zeta expressed in mouse eggs is accumulated to the pronucleus during egg activation. *Dev Biol* 268, 245–257. <https://doi.org/10.1016/j.ydbio.2003.12.028>
- Yoon, S.Y., Jellerette, T., Salicioni, A.M., Lee, H.C., Yoo, M., Coward, K., Parrington, J., Grow, D., Cibelli, J., Visconti, P., Mager, J., Fissore, R.A., 2008. Human sperm devoid of PLC, zeta 1 fail to induce Ca²⁺ release and are unable to initiate the first step of embryo development. *J Clin Invest* 118, 3671–3681. <https://doi.org/10.1172/JCI36942>
- Zhang, J.J., Muzs, L.Z., Boyle, M.S., 1990. In vitro fertilization of horse follicular oocytes matured in vitro. *Mol Reprod Dev* 26, 361–365. <https://doi.org/10.1002/mrd.1080260411>
- Zhang, M.H., Shi, Z. Da, Yu, J.C., Zhang, Y.P., Wang, L.G., Qiu, Y., 2015. Scrotal heat stress causes sperm chromatin damage and cysteinyl aspartate-specific proteinases 3 changes in fertile men. *J Assist Reprod Genet* 32, 747–755. <https://doi.org/10.1007/s10815-015-0451-0>
- Zini, A., Finelli, a, Phang, D., Jarvi, K., 2000. Influence of semen processing technique on human sperm DNA integrity. *Urology* 56, 1081–1084. [https://doi.org/10.1016/S0090-4295\(00\)00770-6](https://doi.org/10.1016/S0090-4295(00)00770-6)

CHAPTER II: USE OF MICROFLUIDICS TO SORT EQUINE SPERM FOR INTRACYTOPLASMIC SPERM INJECTION

SUMMARY

The overall aim of the study was to determine if microfluidic sorting of equine sperm would improve sperm population parameters for intracytoplasmic sperm injection (ICSI). Specific aims were to: 1) evaluate efficiency of microfluidic sorting of equine sperm on population quality parameters, 2) compare sperm population quality parameters after sorting equine sperm using microfluidics (MF) and more established methods, e.g., single-layer colloidal centrifugation (SLC) and swim-up procedure (SU), and 3) assess use of MF to sort sperm in an ICSI program. After MF sorting of frozen-thawed equine sperm in Experiment 1, sorted sperm had significant improvements in motility, morphology, viability, membrane integrity, and DNA integrity. In Experiment 2, frozen-thawed sperm were sorted using MF, SLC and SU. SU was the least effective sorting method. Post-sorting sperm population parameters were similar between MF and SLC for motility, viability, and membrane integrity; however, morphology and DNA integrity was higher in sperm sorted by MF when compared to SLC. In Experiment 3, sperm samples of variable quality were processed by MF and SLC prior to ICSI. After sorting, motility, morphology and DNA integrity were similar for sperm populations sorted by either method; but viability was significantly higher in sperm sorted by MF than SLC. The sorting method did not affect membrane integrity. After ICSI, no significant differences were observed between sorting methods for establishment of cleavage or a blastocyst. We concluded that separation of stallion sperm by MF was effective in yielding a subpopulation of sperm with improved quality parameters for ICSI, comparable or better than SLC and SU. Embryos were produced after sperm sorting by MF, confirming the potential for use in ICSI programs.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is the only repeatable and effective assisted reproductive technique (ART) to produce equine embryos in vitro (Carnevale, 2008). The arbitrary selection of individual sperm for ICSI is dependent on the embryologist's experience, primarily based on sperm motility and morphology (Sessions-Bresnahan et al., 2014), while bypassing in vivo mechanisms for sperm selection. However, when sperm are selected by morphology and motility in men with male factor infertility, sperm with DNA damage are not excluded (Avendaño et al., 2009; Celik-Ozenci et al., 2004), which can negatively impact embryo quality and pregnancy (Barroso et al., 2009).

Optimal methods to evaluate and sort stallion sperm for ICSI have not been identified. Potentially, sperm from any stallion could produce embryos after ICSI. However, differences in cleavage and embryo development rates have been observed among stallions under experimental and clinical conditions (Choi et al., 2016; Colleoni et al., 2012; Herrera et al., 2012; Hinrichs, 2013; Lazzari et al., 2002). Stallions with low in vivo fertility also have low in vitro fertilizing potential (Colleoni et al., 2012). The type of sperm that is used for ICSI has an impact on outcome. Fresh, frozen and refrozen sperm can result in cleavage and embryo development after ICSI, but at different rates (Choi et al., 2002, 2006). As the individual stallion and his sperm quality affect ICSI success, there is a need for selecting ideal sperm to improve outcomes (Carnevale, 2008). Therefore, sperm samples are usually sorted prior to ICSI to obtain a subpopulation of better quality sperm, increasing the probability of selecting an optimal sperm for injection into the oocyte.

Different methods have been used to sort stallion sperm prior to ICSI, with the most common being a swim-up procedure or density gradient centrifugation (Choi et al., 2016;

Colleoni et al., 2011). For men and stallions, both methods provide a sperm subpopulation with improved motility, viability, morphology, mitochondrial membrane potential, and membrane and DNA integrity (Colleoni et al., 2011; Kim et al., 2015; Luppi et al., 2015a; Macpherson et al., 2002; Morrell et al., 2009, 2010; Sakkas et al., 2000; Sieme et al., 2003). Microfluidic technology for sperm sorting offers an alternative method to obtain a subpopulation with improved motility, viability, morphology and DNA integrity (Asghar et al., 2014; Matsuura et al., 2013; Nosrati et al., 2014; Shirota et al., 2016). Because centrifugation is not used, microfluidic technology can reduce sperm exposure to reactive oxygen species (ROS) and the potential to induce DNA damage (Aitken and Clarkson, 1988; Barroso et al., 2000). For equine ICSI, no information is available on the use of microfluidic sperm separation methods.

We hypothesized that microfluidic sorting of equine sperm would result in improved sperm population quality parameters when compared to unsorted sperm and to other sperm separation methods currently used for ICSI. We endeavored to use microfluidic sperm sorting in an ICSI program for embryo production. Aims of the studies were to: 1) determine the extent that microfluidic sperm sorting would improve sperm population quality parameters, 2) compare sperm population quality parameters for frozen-thawed sperm after sorting using microfluidics, single-layer colloidal centrifugation, and a swim-up procedure, and 3) determine if microfluidic sperm sorting would result in cleavage and embryo production after ICSI.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Experiment 1: Sorting of equine sperm using a microfluidics

Sperm samples and microfluidic sorting

Frozen-thawed sperm samples were obtained from stallions (n = 20). Samples were cryopreserved in-house or in another facility as previously described (Kirk et al., 2005) using one of two extenders (E-Z Freezin™ -"LE" Equine Semen Extender, Animal Reproduction System Inc., Chino, CA, USA; or BotuCrio™, BetsLab, Lexington, KY, USA). Straws (0.5 mL) were loaded with 200×10^6 motile sperm/mL and frozen. Animal care and semen collection procedures were performed in accordance with the Animal Care and Use Committee at Colorado State University.

For thawing, a small section of each straw (approximately a tenth of the length) was cut under liquid nitrogen to mimic clinical procedures. The cut sections were thawed at 37°C in G-BSA [G-MOPS™ (Vitrolife Sweden AB, Frölunda, Sweden) plus 0.4% BSA]. Frozen-thawed sperm were processed using microfluidics (MF) with the use of a commercial device (FERTILE PLUS™ Sperm Sorting Chip, DxNow Inc., MA, USA) previously used for human sperm. The manufacturer's guidelines were followed with modifications for equine sperm. Thawed sperm were suspended in $\leq 800 \mu\text{L}$ G-BSA and placed into the loading chamber. The collection chamber had 500 μL of G-BSA added, and the device was incubated in room air at 37°C for 20 min, allowing sperm to swim up and through a membrane into the collection chamber. From the collection chamber, 300 μL of sorted sperm were removed for evaluation.

Sperm analysis

Frozen-thawed sperm were analyzed before and after MF sorting for the following parameters (percentage per total sperm) of: 1) total motile sperm, MOT+, 2) normal morphology,

MORPH+, 3) live sperm, as a measurement of viability and sperm head membrane integrity, LIVE+, 4) positive hypoosmotic swelling, indicative of membrane integrity in the principal piece of the tail, HOS+, and 5) DNA fragmentation, DNA-.

For MOT+, visual assessment by a single observer was performed, using phase-contrast microscopy at X 200 magnification. For MORPH+ and LIVE+, an aliquot of the sperm sample (20 μ L) was mixed on a slide with 20 μ L of an eosin-nigrosin stain (Hancock Stain®, Animal Reproduction Systems Inc.). The mixture was smeared on a slide and dried on a warming plate at 37°C to avoid hypotonic artifacts. Sperm (n=100 per sample) were examined under X 1000 magnification using bright field microscopy. Morphological abnormalities included defects of the head (altered head size and shape head, acrosome defects, nuclear vacuoles and deformities), midpiece (proximal or distal cytoplasmic droplets, swollen or rough midpiece, and fractured axonemal fibers), and principal piece (cytoplasmic retention, bending and coiling). For LIVE+, sperm with an intact postacrosomal plasma membrane that did not uptake eosin in the head region were considered to be live, and sperm with any detectable eosin in the head region were counted as dead (Brito, 2007; Mocé and Graham, 2008). LIVE+ was determined for each slide as the percentage of unstained sperm per 200 total sperm (Brito, 2007).

For HOS+, 10 μ L of the sperm sample was mixed with 100 μ L of hypoosmotic sucrose solution (100 mOsm/Kg) in a 1.5 mL microtube. The mixture was incubated at 37°C for 30 min. Sperm (n=300 per sample) were examined under X 400 magnification with contrast field microscopy. Sperm with unaltered tail morphology were considered HOS- and sperm with a distinctive curling of the tail were considered HOS+. Results are expressed as percentages of HOS+ sperm per total sperm (Jeyendran et al., 1984).

Sperm chromatin dispersion was used to determine DNA-. Briefly, 50 μ L of 1% low-melting point agarose in 1.5 mL microtube was placed in a water bath at 90°C for 3 min and then moved to a slide warmer at 37° C for 5 min. An aliquot (10 μ L) of the sperm sample was mixed with the melted agarose. The mixture was pipetted onto a 0.65% agarose-precoated slide, covered with cover glass, and cooled at 4°C for 5 min. After removing the cover glass, the slide was incubated in the dark with an acid denaturation solution (0.08 N HCl) for 7 min at room temperature. The slide was then immersed in Lysing Solution I (0.4 M 2-Amino-2-(hydroxymethyl)-1,3-propanediol, 0.4 M 2-mercaptophenol, 1% sodium dodecyl sulfate, and 50 mM ethylenediaminetetraacetic acid, pH 7.5) for 10 min and Lysing Solution II (0.4 M 2-Amino-2-(hydroxymethyl)-1,3-propanediol, 2 M NaCl, and 1% sodium dodecyl sulfate, pH 7.5) for 5 min. After washing with distilled water for 5 min, the slide was consecutively immersed for 2 min in 70, 90 and 100% ethanol. The slide was stained with Dip Quick Stain (Jorgensen Laboratories Inc., Loveland, CO, USA), and 300 sperm were counted under X 1000 magnification using bright field microscopy. Sperm exhibiting a large or medium halo of DNA dispersion were considered to have intact DNA, and sperm with small or no halo of DNA dispersion were considered to have fragmented DNA (DNA-). DNA- was determined for each slide as the percentage of sperm exhibiting fragmented DNA per total sperm (Fernandez et al., 2005, 2003).

Experiment 2: Comparison of sperm population sorting using microfluidics, single layer colloidal centrifugation, and swim-up procedure

Sperm samples and sperm sorting

Frozen sperm from 19 stallions were frozen in-house or in another facility as described in Experiment 1, using one of three extenders (E-Z Freezin™ - “LE”, E-Z Freezin™ - “MFR5”, Animal Reproduction System Inc; or BotuCrio™, Betlabs). Before freezing, 0.5-mL straws were loaded with 200×10^6 motile sperm/mL. A single straw from each stallion was thawed in a water bath at 37°C for 30 sec before being divided into three aliquots for sorting by MF, single-layer colloidal centrifugation (SLC) and swim-up (SU). For MF, thawed sperm samples were processed as described for Experiment 1. For SLC, thawed samples were suspended in $\leq 200 \mu\text{L}$ of G-BSA and layered onto 500 μL of Equipure™ (Nidacon, International AB, Gothenburg, Sweden) diluted with G-BSA (3:1) in a 1.5-mL microtube and centrifuged at 200g for 8 min. Supernatant was discarded, and 50 μL of remaining sediment was washed in 300 μL G-BSA at 400g for 3 min for analysis. For SU, thawed sperm were overlaid by 1 mL of G-IVF (Vitrolife, plus 0.4% BSA) at 38.2°C. The tube was positioned at $\sim 45^\circ$ angle and incubated in 6% CO₂ and air at 38.2°C for 15 min. Approximately 0.5 ml of supernatant was removed and centrifuged at 300 g for 3 min. The supernatant was discarded and the remaining sediment (100 μL) was used for evaluation.

Sperm analysis

Frozen-thawed sperm were analyzed before and after sorting for MOT+, MORPH+, LIVE+, HOS+ and DNA-. Assessment of MORPH+, LIVE+ and HOS+ were performed as described for Experiment 1. For MOT+ and DNA-, sufficient numbers of sperm were available to evaluate larger numbers of sperm by different methods. For MOT+ evaluation, thawed sperm were diluted in prewarmed E-Z Mixin® “BF” (Animal Reproduction System Inc.) to a final sperm concentration of $50 \times 10^6/\text{mL}$. Total motility was analyzed using a computer-assisted

motility analyzer (Sperm Vision® Therio, MOFA Global, Verona, WI, USA). For DNA–, samples were evaluated following the protocol for Sperm Chromatin Structure Assay (Evenson and Melamed, 1983). Briefly, an aliquot of frozen-thawed sperm was layered onto 500 µL of Equipure™ in a 1.5 mL microtube and centrifuged for 10 min at 200g to remove extender. For samples sorted by SLC, this step was not needed, and the SLC samples were not exposed to another centrifugation step with Equipure™ prior to the DNA assay. The supernatant was removed, and the pellet was resuspended in TNE-Tris (1.0 mM 2-Amino-2-(hydroxymethyl)-1,3-propanediol, 15.0 mM NaCl and 1.0 mM ethylenediaminetetraacetic acid, pH 7.4). Sperm concentration was adjusted to 2×10^6 /mL. A 200 µL sperm-TNE-Tris suspension was mixed with 400 µL of a detergent solution (0.08 N HCl, 15 mM NaCl and 0.1% Triton X-100). Thirty seconds later, 1.2 mL of acridine orange solution (20 mM Na₂HPO₄, 10 mM citric acid, 1 mM ethylenediaminetetraacetic acid, 15 mM NaCl and 6 µg acridine orange/mL) was added. A minimum of 10,000 events per sample were analysed within 3-5 min using an Accuri™ C6 Flow cytometer and CFlow Plus (Becton Dickinson, Franklin Lakes, NJ, USA). Percentage of DNA fragmentation (DNA–) was assessed by selecting sperm to the right of the main population on a dot plot of red (FL3, >670 nm/long pass band) versus green (FL1, 530/30 nm band pass) fluorescence.

Experiment 3: Use of microfluidics for sorting sperm prior to ICSI and comparison with single layer colloidal centrifugation

Sperm samples and sperm sorting for oocyte injection

Sperm frozen with various extenders and under unknown conditions in 0.25-, 0.5- or 5-mL straws (n=40 analyzed samples) from 21 stallions were included. Individual straws were cut

under liquid nitrogen to use a minimal number of sperm. The cut sections were thawed at 37°C in G-BSA. Sperm were sorted by MF or SLC as described in Experiments 1 and 2. Samples were incorporated into the program without specific preconditions and rotating days for different sample processing. In samples processed by MF (n = 22), sperm from the collection chamber were used for final selection. The SLC samples (n = 18) were processed, and sperm from the sediment were washed prior to final selection. After processing and just prior to ICSI, the final selection of sperm for oocyte injection was based on motility and morphology at X 200 magnification by the ICSI technician.

Sperm analysis

Samples were analyzed before and after sorting for MOT+, MORPH+, LIVE+, HOS+ and DNA- as described in Experiment 1.

Oocyte collection and sperm microinjection

Oocyte donors were mares 3 to 25 yr (n = 20, mean \pm SEM of 12.2 ± 4.7 yr). Oocytes were collected by transvaginal, ultrasound-guided follicle aspirations during the follicular phase of the cycle from dominant follicles, yielding maturing oocytes (Carnevale, 2016). Maturing oocytes completed maturation in Medium 199 with Earle's salts, L-glutamine, and 2.2 g/L sodium bicarbonate (GIBCO BRL Life Technologies, Grand Island, NY, USA with additions of 10% fetal calf serum, 0.2 mM pyruvate and 25 mg/mL gentamicin) for approximately 20 h at 38°C in 6% CO₂ and air. During the study, matured oocytes in metaphase II with extrusion of a polar body were used for ICSI. Sperm injections were performed by a single technician into 50 oocytes (Day 0 = day of ICSI) using a piezo drill. After ICSI, potential zygotes were placed into

30- μ l drops of culture medium (Dulbecco Modified Eagle medium/F12, GIBCO BRL Life Technologies, with 10% fetal calf serum) under oil (Ovoil™, Vitrolife, Göteborg, Sweden) at 38 °C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. The day after ICSI, potential zygotes were observed under X 200 magnification for cleavage; if complete cell division was not observed, but signs of imminent cleavage were noted, such as change in shape or fragmentation of the oolemma, the potential zygote was reassessed for cleavage on the following day. Cleaved embryos were evaluated on Days 5 and daily thereafter until development into a blastocyst or confirmation of embryo degeneration. Development after ICSI was defined per injected oocyte as Cleavage, ≥ 2 cells by Day 2, and Blastocyst, development of a blastocyst.

Statistical analysis

Statistical analyses were performed using R Core Team Software 3.4.2017 (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). Data obtained from sperm parameters analysis were tested for normality and homogeneity of variances using the Shapiro-Wilk and Levene test. In Experiment 1, percentages of sperm population quality parameters before and after MF sorting were compared for each sperm parameter separately by Wilcoxon Rank Sum test for paired samples. In order to determine the influence of the originating sperm sample on the effectiveness of microfluidic sperm sorting, a two-factor repeated measures design was performed, with unsorted sperm quality and improvement after sorting used as variables and stallion as a random effect. A mixed model was fit to each sperm parameter separately. Association between percentages of unsorted sperm and improvement after sorting for each parameter was performed by Spearman's rank correlation (r_s). In Experiment 2, sperm population parameter percentages before and after

sorting among methods were analyzed using one-factor repeated measures design with sorting methods as the within-factor and stallion as a random effect for repeated measures. A mixed model was fit to each sperm parameter separately. Tukey adjusted pairwise comparisons were considered among sorting methods. In Experiment 3, comparisons of percentages of sperm parameters before and after sorting within each method and between methods were performed by Wilcoxon Rank Sum test for paired and independent samples, respectively. Associations among sperm parameters after sorting were performed by Spearman's rank correlation (r_s). Percentages of sperm parameters after sorting that resulted in positive (Yes) or negative (No) ICSI outcome for Cleavage and Blastocyst were compared by Wilcoxon Rank Sum test for independent samples. Logistic regressions and Tukey adjusted pairwise comparisons were conducted to investigate the association between sorting methods and ICSI outcomes for Cleavage and Blastocyst. Odd ratios (OR) and 2.5-97.5% confidence interval (CI) were calculated. Sperm parameters are presented as mean \pm SEM. Statistical differences were considered significant at $P < 0.05$.

RESULTS

Experiment 1: Sorting of equine sperm using microfluidics

When compared to the original sperm samples, MOT+, MORPH+, LIVE+ and HOS+ were higher and DNA- was lower ($P < 0.003$) after MF sorting (Table 2.1). No interaction ($P > 0.2$) was observed between unsorted sperm population parameters and the percentage improvement (difference between original sample and sorted sample) after sorting for MOT+, MORPH+, LIVE+ and DNA-, with a linear correlation ($P < 0.008$) between individual sperm parameter percentages of the original sample and improvement after MF sorting (MOT+, $r_s = -$

0.6; MORPH+, $r_s = -0.7$; LIVE+, $r_s = -0.8$ and DNA-, $r_s = -0.8$). There was a tendency ($P=0.09$) for an interaction and no correlation ($r_s = 0.4$, $P>0.1$) between percentages of HOS+ in the original sample and in the level of improvement that was observed after sorting. No significant improvement was observed in the sperm population HOS+ after sorting if the unsorted sperm population had HOS+ $\leq 30\%$ ($P>0.08$).

Table 2.1: Mean \pm SEM percentages (range) from 20 stallions of sperm parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA-: percentage of sperm with fragmented DNA) between unsorted and sorted sperm populations using microfluidics. ^{a,b}Values with different superscripts between unsorted and sorted sperm for individual sperm parameters differ at $P<0.05$.

MOT+		MORPH+		LIVE+		HOS+		DNA-	
Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted
29 \pm 3 ^a	59 \pm 3 ^b	59 \pm 2 ^a	75 \pm 2 ^b	56 \pm 3 ^a	71 \pm 3 ^b	27 \pm 2 ^a	36 \pm 4 ^b	17 \pm 3 ^a	7 \pm 1 ^b
(10-60)	(40-90)	(42-85)	(52-89)	(25-80)	(47-98)	(14-44)	(7-67)	(6-52)	(1-26)

Experiment 2: Comparison of sperm population sorting using microfluidics, single layer colloidal centrifugation, and swim-up procedure

When the original sperm population was compared to sorted sperm, MF was the only sorting method to significantly improve MORPH+, LIVE+ and DNA- (Table 2.2). Significantly higher MOT+ was observed after MF and SLC than in the original sperm population. No sorting method significantly increased HOS+ when compared to the originating samples, although the percentage of HOS+ was significantly higher after sorting with SLC than SU (Table 2.2). Sperm population parameters were not significantly different after SU when compared to original samples (Table 2.2).

Table 2.2: Mean \pm SEM percentages (range) of individual sperm population parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA–: percentage of sperm with fragmented DNA) between the original samples (Unsorted, n=19) and sperm sorted using microfluidics (MF), single layer colloidal centrifugation (SLC) and swim-up procedure (SU). ^{a,b}Values with different superscripts within a row differ at P<0.05.

	Unsorted	Sorting Methods		
		MF	SLC	SU
MOT+	44 \pm 3 ^a	54 \pm 3 ^{bc}	62 \pm 4 ^c	49 \pm 4 ^{ab}
MORPH+	57 \pm 5 ^a	71 \pm 4 ^b	62 \pm 5 ^a	63 \pm 5 ^a
LIVE+	66 \pm 2 ^a	74 \pm 2 ^b	68 \pm 3 ^{ab}	64 \pm 3 ^a
HOS+	34 \pm 2 ^{ab}	30 \pm 2 ^{ab}	35 \pm 2 ^b	30 \pm 2 ^a
DNA–	13 \pm 2 ^a	6 \pm 1 ^b	12 \pm 2 ^a	11 \pm 1 ^a

Experiment 3: Use of microfluidics for sorting sperm prior to ICSI and comparison with single layer colloidal centrifugation

Sorting of sperm using MF was successful in an ICSI program using sperm samples with variable and limited quality sperm. Sperm sorted using MF was compared to a standard sorting method, SLC, for sperm samples of limited quality. Prior to sorting by either method, sperm population parameters (MOT+, MORPH+, LIVE+, HOS+ and DNA–) were not different in the originating samples (Table 2.3). With the exception of HOS+, all other sperm population parameters significantly improved after sorting with MF or SLC (Table 2.3). Only LIVE+ was significantly higher for sperm sorted by MF than SLC; no significant differences were observed for other sperm parameters after sorting (Table 2.3). Some sperm population parameters were correlated after sorting by MF and SLC (Table 2.4), although correlations were not significantly different between sorting methods (MF and SLC).

Table 2.3: Mean \pm SEM percentages (range) of individual sperm population parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA–: percentage of sperm with fragmented DNA) between the unsorted original sample and sorted sperm using microfluidics (MF, n=22) and single layer colloidal centrifugation (SLC, n=18). ^{a,b}Values with different superscripts for Unsorted and Sorted within a row for sperm parameters differ at $P < 0.05$. ^{A,B}Values within a column differ at $P < 0.05$ between MF and SLC.

	MOT+		MORPH+		LIVE+		HOS+		DNA–	
	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted
MF	24 \pm 3 ^a (10-60)	55 \pm 3 ^b (40-90)	55 \pm 3 ^a (18-74)	74 \pm 2 ^b (49-95)	51 \pm 3 ^a (22-80)	67 \pm 3 ^{bA} (39-91)	21 \pm 3 (11-34)	21 \pm 2 (4-45)	22 \pm 3 ^a (7-52)	9 \pm 2 ^b (1-26)
SLC	25 \pm 3 ^a (10-50)	53 \pm 4 ^b (25-80)	54 \pm 4 ^a (25-76)	66 \pm 4 ^b (19-88)	46 \pm 3 ^a (22-68)	58 \pm 4 ^{bB} (19-86)	21 \pm 2 (12-51)	21 \pm 2 (9-36)	17 \pm 2 ^a (7-36)	12 \pm 2 ^b (4-30)
Total	25 \pm 2 ^a (10-60)	54 \pm 2 ^b (25-90)	55 \pm 2 ^a (18-76)	71 \pm 2 ^b (19-95)	49 \pm 2 ^a (22-80)	63 \pm 3 ^b (19-91)	21 \pm 1 (11-51)	21 \pm 1 (4-45)	20 \pm 2 ^a (7-52)	11 \pm 1 ^b (1-30)

Table 2.4: Correlations among sperm population parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA–: percentage of sperm with fragmented DNA) after sorting by microfluidics (MF) and single layer colloidal centrifugation (SLC) (n=40 samples). No differences in correlations were observed between MF and SLC. Spearman's correlation coefficients (r_s) noted with asterisk (*) were significant at $P < 0.05$.

	MORPH+	LIVE+	HOS+	DNA–
MOT+	$r_s = 0.52^*$ P = 0.0001	$r_s = 0.53^*$ P = 0.0001	$r_s = 0.07$ P = 0.7	$r_s = -0.56^*$ P < 0.0001
MORPH+		$r_s = 0.40^*$ P = 0.004	$r_s = -0.03$ P = 0.8	$r_s = -0.44^*$ P = 0.001
LIVE+			$r_s = 0.32^*$ P = 0.04	$r_s = -0.68^*$ P < 0.0001
HOS+				$r_s = 0.001$ P = 0.9

After ICSI, 54% (27/50) of sperm-injected oocytes cleaved, and 46% (23/50) of sperm-injected oocytes resulted in blastocyst by Day 7; no blastocysts developed after Day 7. No association was observed between sorting methods and ICSI outcome for Cleavage [P>0.3; MF: OR=1 (CI, 0.4-1.9) and SLC: OR=1.74 (CI, 0.6-5.6)] or Blastocyst [P>0.4; MF: OR=1 (CI, 0.3-1.50) and SLC: OR=1.55 (CI 0.5-4.9)]. Sperm population parameters that resulted in positive or negative Cleavage or Blastocyst were not different within sorting methods, with the exception that MORPH+ was higher (P<0.02) in sperm populations that did not result in blastocyst formation when sperm were selected using SLC (Table 2.5).

Table 2.5: Cleavage and Blastocyst (development rates per sperm injected oocyte) after sorting by microfluidics (MF) and single layer colloidal centrifugation (SLC) and mean \pm SEM percentages of sperm population parameters (MOT+: percentage of total motile sperm; MORPH+: percentage of morphologically normal sperm; LIVE+: percentage of live sperm; HOS+: percentage of swollen sperm by HOS test; DNA-: percentage of sperm with fragmented DNA) in which ICSI resulted in positive (Yes) or negative (No) outcomes. ^{a,b}Values with different superscripts for individual sperm parameters differ at P<0.05.

		Rates	Outcome	MOT+	MORPH+	LIVE+	HOS+	DNA-
Cleavage	MF	48%	YES	53 \pm 3	74 \pm 3	67 \pm 4	21 \pm 4	12 \pm 2
		(14/29)	NO	57 \pm 3	75 \pm 3	68 \pm 4	20 \pm 9	8 \pm 2
	SLC	62%	YES	53 \pm 4	62 \pm 6	59 \pm 5	22 \pm 2	12 \pm 2
		(13/21)	NO	53 \pm 9	75 \pm 6	57 \pm 5	19 \pm 1	12 \pm 2
Blastocyst	MF	41%	YES	54 \pm 4	74 \pm 4	66 \pm 5	21 \pm 4	12 \pm 2
		(12/29)	NO	56 \pm 3	75 \pm 2	69 \pm 3	20 \pm 9	9 \pm 8
	SLC	52%	YES	53 \pm 4	57 \pm 6 ^a	62 \pm 5	21 \pm 3	11 \pm 2
		(11/21)	NO	54 \pm 7	77 \pm 2 ^b	54 \pm 5	20 \pm 3	13 \pm 2

DISCUSSION

Methods for sperm selection are required for sperm preparation before the individual sperm selection for microinjection; since most of sperm samples require improvement for the

motile fraction and removal of the immotile sperm, extender medium and debris. Several studies in different species have produced robust evidence that the use of microfluidic sperm sorting devices provided sperm with improved motility, morphology and DNA integrity (Chen et al., 2013, 2011; Cho et al., 2003; Matsuura et al., 2013; Nosrati et al., 2014; Schulte et al., 2007; Schuster et al., 2003; Shirota et al., 2016; Tasoglu et al., 2013). Potentially, microfluidic devices sort sperm with better characteristics to have clinical utility in ART (Nosrati et al., 2017; Smith and Takayama, 2017; Swain et al., 2013). In this study, our results demonstrated that use of the microfluidic sorting device FERTILE PLUS™ Sperm Sorting Chip in equine sperm, resulted in a sorted sperm population with improved motility, morphology, viability and DNA integrity, independent of the starting point values for individual sperm parameters. However, in sperm samples with poor motility and membrane integrity as measured of HOS, the microfluidic device improved significantly motility, but not sperm membrane integrity. Congruently, using a prototype of the commercial device, human sorted sperm exhibited increased motility, viability, normal morphology, nuclear maturity and DNA integrity (Asghar et al., 2014). Current sperm processing methods such as DGC and swim-up provide efficient means of sperm quality for IVF or ICSI in humans and horses (Choi et al., 2016; Galli et al., 2016; Smith and Takayama, 2017); microfluidic devices do not required centrifugation. Centrifugation during sorting can cause sub-lethal damage to sperm. DNA exposure to high levels of ROS (Aitken and Clarkson, 1988; Alvarez et al., 1993; Hughes et al., 1998), subsequently can produce DNA damage (Barroso et al., 2000). Our findings support the potential use of microfluidic devices to sort a sperm population with high quality parameters from an individual sperm can be selected for oocyte injections to produce equine embryos in vitro.

Numbers of studies in human have evaluated and compared the efficiency of DGC and SU, but results are not conclusive (Simopoulou et al., 2016). The efficiency of sorting a particular sperm sample by DGC or swim-up can be cofounded by the sperm quality of the original sample (Simopoulou et al., 2016; Xue et al., 2014). A proteomic comparison concluded that DGC offered a better capacitation potential (Luppi et al., 2015b), but in most studies both methods provide sperm of equal quality for motility, morphology, viability or DNA integrity (Kim et al., 2015, 2017; Luppi et al., 2015b; Sakkas et al., 2000; Zini et al., 2000). In our study, swim-up was the least effective method to separate equine sperm. The microfluidic device and colloidal centrifugation were the most successful sorting methods to provide a sperm population with better normal morphology, motility, viability and DNA integrity, significantly higher than those sperm sorted by swim-up. In agreement with our results, microfluidic systems provided sperm with higher normal morphology and DNA integrity compared to colloidal centrifugation and swim-up in sperm samples from fertile men (de Martin et al., 2017; Shirota et al., 2016). Sperm sorted by microfluidic devices also produced significantly less ROS compared to basic washing or swim-up (Asghar et al., 2014), indicative that the increased generation of ROS in swim-up came from centrifugation steps (Aitken and Clarkson, 1988; Hughes et al., 1998). Likewise, in sperm samples from men with male infertile factor, microfluidic sperm processing provided sperm with high motility, normal morphology and DNA integrity in comparison with basic washing and DGC (Schulte et al., 2007). In line with our results, fresh sperm from stallions processed by one- or double-layer DGC had improved motility, normal morphology and DNA integrity compared to unprocessed sperm (Morrell et al., 2009, 2010). In other study using fresh stallion sperm, DGC increased viability, but not motility or morphology compared to unsorted sample; and swim-up improved motility, but not normal morphology or viability. DGC and

swim-up resulted in similar viability, but swim-up had higher motility and lower normal morphology than DGC (Sieme et al., 2003). In equine ICSI, the use of limited quality in sperm samples is frequent; thus there is higher need to select sperm sorting techniques that provide a sperm population with increased quality parameters. Our findings suggest that swim-up is a low efficient method to sort poor quality sperm samples, and colloidal centrifugation and microfluidic device are more efficient techniques and less sensitive to the confounding effect derived from the original sample.

In clinical practice, the male effect is confounded by mare selection and can require more ICSI cycles to demonstrate differences (Galli et al., 2016). We did not observe differences between ICSI outcome and sorting methods, with relatively low sample numbers. Likewise in human, fertilization and pregnancy outcomes after in vitro fertilization were similar for both DGC and swim-up (Kim et al., 2015). In equine ICSI, higher cleavage and blastocyst rates were observed when sperm were selected after a combined DGC and swim-up procedure compared to DGC alone, but results were similar to sperm sorted by basic washing or swim-up (Choi et al., 2016). In ICSI procedure, as sperm motility and morphology were used as the final sperm selection criteria for ICSI, this could have negated some of the impact of sorting method. Overall, the microfluidic device and colloidal centrifugation resulted in a subpopulation of sperm with high quality parameters for ICSI.

Several studies in different species have produced robust evidence that the use of microfluidic sperm sorting devices provide sperm with improved characteristics for clinical assisted reproductive technologies (Asghar et al., 2014; Matsuura et al., 2013; Nosrati et al., 2014; Shirota et al., 2016). In this study, we sorted stallion sperm using a MF approach. The device that was used was designed with two chambers separated by a membrane filter with 8- μ m

micropores. Sperm are loaded into the first chamber, allowing motile stallion, approximately 3 μm (2.79- 3.26 μm) in width (Brito, 2007), to swim through the membrane and into the collection chamber. The most motile and functional sperm move selectively against gravity and through the micropores, with less motile or nonmotile sperm and debris remaining behind (Asghar et al., 2014). Advantageously, microfluidic sorting does not require centrifugation, which can cause sublethal damage to sperm and expose DNA to high levels of ROS that can subsequently produce DNA fragmentation (Aitken and Clarkson, 1988; Barroso et al., 2000; Hughes et al., 1998). Our results demonstrated that MF can be used with stallion sperm and result in a sperm subpopulation with improved motility, morphology, viability and DNA integrity, independent of the starting point values for most individual sperm parameters. However, in sperm samples with poor membrane integrity as measured by HOS+ at $\leq 30\%$, MF did not significantly improve membrane integrity. Human sperm sorted with similar microfluidic devices yield a sperm subpopulation with increased motility, viability, normal morphology, nuclear maturity, and DNA integrity (Asghar et al., 2014). Our findings support the use of microfluidics to sort a sperm subpopulation with high sperm quality parameters from which an individual sperm can be selected for equine ICSI.

Current sperm sorting methods, such as density gradient centrifugation or swim-up procedures, can provide a means to select a more desirable population of sperm prior to use for ART in horses and humans (Choi et al., 2016; Galli et al., 2016; Smith and Takayama, 2017). A proteomic comparison of human sperm concluded that density gradient centrifugation resulted in sperm with better capacitation potential than SU (Luppi et al., 2015a); but in most studies, both methods provide sperm of similar quality for motility, morphology, viability or DNA integrity (Kim et al., 2015, 2017; Sakkas et al., 2000; Zini et al., 2000). In our study, SU was the least

effective method to separate small quantities for sperm with improved quality parameters, with MF and SLC resulting in sperm subpopulations with better normal morphology, motility, viability and DNA integrity. In agreement with our results, microfluidic systems sort sperm with higher normal morphology and DNA integrity when compared to density gradient centrifugation and SU in sperm samples from infertile and fertile men (de Martin et al., 2017; Schulte et al., 2007; Shirota et al., 2016). Human sperm sorted by microfluidics also produces significantly less ROS when compared to basic washing or SU (Asghar et al., 2014). The findings indicate that the increased ROS generation in swim-up came from the centrifugation steps (Aitken and Clarkson, 1988; Hughes et al., 1998), which are avoided using MF. In agreement with our results, fresh sperm from stallions processed by single- or double-layer colloidal centrifugation have improved motility, normal morphology, and DNA integrity when compared to unprocessed sperm (Morrell et al., 2009, 2010). Density gradient centrifugation increases viability, but not motility or morphology, for fresh stallion sperm when compared to unsorted samples, and swim-up improves motility, but not normal morphology or viability. Density gradient centrifugation and swim-up result in similar viability, but swim-up has higher motility and lower normal morphology (Sieme et al., 2003). The efficiency of sperm sorting based on motility can be confounded by the sperm quality of the originating sample (Simopoulou et al., 2016; Xue et al., 2014). Our findings suggest that SU is not an efficient method to sort low numbers of limited quality stallion sperm, and SLC and MF are more efficient techniques that are less sensitive to the confounding effects of quality of the original sample.

In clinical practice, the stallion effect on ICSI is confounded by mare selection and can require additional ICSI cycles to demonstrate differences (Galli et al., 2016). In the present study, we did not observe differences between ICSI outcome and sorting methods, with

relatively low sample numbers. Human fertilization and pregnancy outcomes after standard in vitro fertilization are similar for density gradient and swim-up (Kim et al., 2015). For equine ICSI, higher cleavage and blastocyst rates were observed when sperm were selected after a combination of density gradient centrifugation and swim-up procedures when compared to density gradient centrifugation alone, but the results were similar to sperm sorted by basic washing or swim-up (Choi et al., 2016). In our study, sperm motility and morphology were used as the final criteria for sperm selection, and this could have negated some of the impact of sorting method. Selection of a sperm sorting method in a clinical program, to some extent, is going to depend on particular aspects of the originating sperm sample. In our studies, we were able to use MF sorting with relatively low numbers of sperm and using sperm with compromised quality parameters. In addition, MF was the only sorting method that significantly resulted in a subpopulation of sperm with improved DNA integrity when compared to SLC and SU. Additional use of MF sorting for equine sperm is needed before we know if it will improve clinical ICSI outcomes.

REFERENCES

- Aitken, R.J., Clarkson, J., 1988. Significance and Antioxidants of Reactive Oxygen Species in Defining the Efficacy of Sperm Preparation Techniques. *J Androl* 9, 367–76.
- Alvarez, J., Lasso, J., Blasco, L., Nunez, R., Heyner, S., Caballero, P., Storey, B., 1993. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. *Hum Reprod* 8, 1087–1092.
- Asghar, W., Velasco, V., Kingsley, J.L., Shoukat, M.S., Shafiee, H., Anchan, R.M., Mutter, G.L., Tüzel, E., Demirci, U., 2014. Selection of Functional Human Sperm with Higher DNA Integrity and Fewer Reactive Oxygen Species. *Adv Healthc Mater* 3, 1671–1679. <https://doi.org/10.1002/adhm.201400058>
- Avendaño, C., Franchi, A., Taylor, S., Morshedi, M., Bocca, S., Oehninger, S., 2009. Fragmentation of DNA in morphologically normal human spermatozoa. *Fertil Steril* 91, 1077–1084. <https://doi.org/10.1016/j.fertnstert.2008.01.015>
- Barroso, G., Morshedi, M., Oehninger, S., 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 15, 1338–1344. <https://doi.org/10.1093/humrep/15.6.1338>
- Brito, L.F.C., 2007. Evaluation of Stallion Sperm Morphology. *Clin Tech Equine Pract* 6, 249–264. <https://doi.org/10.1053/j.ctep.2007.09.004>
- Carnevale, E.M., 2016. Advances in Collection, Transport and Maturation of Equine Oocytes for Assisted Reproductive Techniques. *Vet Clin North Am - Equine Pract* 32, 379–399. <https://doi.org/10.1016/j.cveq.2016.07.002>

- Carnevale, E.M., 2008. Clinical Considerations Regarding Assisted Reproductive Procedures in Horses. *J Equine Vet Sci* 28, 686–690. <https://doi.org/10.1016/j.jevs.2008.10.005>
- Celik-Ozenci, C., Jakab, A., Kovacs, T., Catalanotti, J., Demir, R., Bray-Ward, P., Ward, D., Huszar, G., 2004. Sperm selection for ICSI: Shape properties do not predict the absence or presence of numerical chromosomal aberrations. *Hum Reprod* 19, 2052–2059. <https://doi.org/10.1093/humrep/deh361>
- Chen, C.-Y., Chiang, T.-C., Lin, C.-M., Lin, S.-S., Jong, D.-S., Tsai, V.F.-S., Hsieh, J.-T., Wo, A.M., 2013. Sperm quality assessment via separation and sedimentation in a microfluidic device. *Analyst* 138, 4967. <https://doi.org/10.1039/c3an00900a>
- Chen, Y.A., Huang, Z.W., Tsai, F.S., Chen, C.Y., Lin, C.M., Wo, A.M., 2011. Analysis of sperm concentration and motility in a microfluidic device. *Microfluid Nanofluidics* 10, 59–67. <https://doi.org/10.1007/s10404-010-0646-8>
- Cho, B.S., Schuster, T.G., Zhu, X., Chang, D., Smith, G.D., Takayama, S., 2003. Passively driven integrated microfluidic system for separation of motile sperm. *Anal Chem* 75, 1671–1675. <https://doi.org/10.1021/ac020579e>
- Choi, Y.H., Love, C.C., Love, L.B., Varner, D.D., Brinsko, S., Hinrichs, K., 2002. Developmental competence in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. *Reproduction* 123, 455–465. <https://doi.org/http://dx.doi.org/10.1530/rep.0.1230455>
- Choi, Y.H., Love, C.C., Varner, D.D., Hinrichs, K., 2006. Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. *Theriogenology* 65, 808–819. <https://doi.org/10.1016/j.theriogenology.2005.04.035>
- Choi, Y.H., Velez, I.C., Macías-García, B., Riera, F.L., Ballard, C.S., Hinrichs, K., 2016. Effect

- of clinically-related factors on in vitro blastocyst development after equine ICSI. *Theriogenology* 85, 1289–1296. <https://doi.org/10.1016/j.theriogenology.2015.12.015>
- Colleoni, S., Lagutina, I., Lazzari, G., Rodriguez-Martinez, H., Galli, C., Morrell, J.M., 2011. New Methods for Selecting Stallion Spermatozoa for Assisted Reproduction. *J Equine Vet Sci* 31, 536–541. <https://doi.org/10.1016/j.jevs.2011.03.009>
- Colleoni, S., Lazzari, G., Duchi, R., Baca Castex, C., Mari, G., Lagutina, I., Galli, C., 2012. Fertilization and development of oocytes after ICSI with semen of stallions with different in vivo fertility. *J Equine Vet Sci* 32, 408–409. <https://doi.org/10.1016/j.jevs.2012.05.030>
- de Martin, H., Cocuzza, M.S., Tiseo, B.C., Wood, G.J.A., Miranda, E.P., Monteleone, P.A.A., Soares, J.M., Serafini, P.C., Srougi, M., Baracat, E.C., 2017. Positive rheotaxis extended drop: a one-step procedure to select and recover sperm with mature chromatin for intracytoplasmic sperm injection. *J Assist Reprod Genet* 1–10. <https://doi.org/10.1007/s10815-017-1024-1>
- Evenson, D.P., Melamed, M.R., 1983. Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. *J Histochem Cytochem* 31, 248–53.
- Fernandez, J.L., Muriel, L., Goyanes, V., Segrelles, E., Gosallvez, J., Enciso, M., LaFromboise, M., De Jonge, C., 2005. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* 84, 833–842. <https://doi.org/10.1016/j.fertnstert.2004.11.089>
- Fernandez, J.L., Muriel, L., Rivero, M.T., Goyanes, V., Vazquez, R., Alvarez, J.G., 2003. The Sperm Chromatin Dispersion Test: A Simple Method for the Determination of Sperm DNA Fragmentation. *J Androl* 24, 59–66. <https://doi.org/10.1002/j.1939-4640.2003.tb02641.x>
- Galli, C., Colleoni, S., Duchi, R., Lazzari, G., 2016. Male Factors Affecting the Success of

- Equine In Vitro Embryo Production by Ovum Pickup-Intracytoplasmic Sperm Injection in a Clinical Setting. *J Equine Vet Sci* 43, S6–S10. <https://doi.org/10.1016/j.jevs.2016.05.014>
- Herrera, C., Dufourq, P., Freije, M., Morikawa, I., Centeno, J.E., Aristi, V., Menghini, L., Sporleder, C., 2012. Selection of stallions for in vitro embryo production by ICSI in a commercial program. *J Equine Vet Sci* 32, 409. <https://doi.org/10.1016/j.jevs.2012.05.031>
- Hinrichs, K., 2013. Assisted reproduction techniques in the horse. *Reprod Fertil Dev* 25, 80–93. <https://doi.org/10.1071/RD12263>
- Hughes, C.M., Lewis, S.E., McKelvey-Martin, V.J., Thompson, W., 1998. The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. *Hum Reprod* 13, 1240–7. <https://doi.org/10.1093/humrep/13.5.1240>
- Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G., Zaneveld, L.J., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 70, 219–228. <https://doi.org/10.1530/jrf.0.0700219>
- Kim, E.K., Kim, E.H., Kim, E.A., Lee, K.A., Shin, J.E., Kwon, H., 2015. Comparison of the effect of different media on the clinical outcomes of the density-gradient centrifugation/swim-up and swim-up methods. *Clin Exp Reprod Med* 42, 22–29. <https://doi.org/10.5653/cerm.2015.42.1.22>
- Kim, S.W., Jee, B.C., Kim, S.K., Kim, S.H., 2017. Sperm DNA fragmentation and sex chromosome aneuploidy after swim-up versus density gradient centrifugation. *Clin Exp Reprod Med* 44, 201–206. <https://doi.org/10.5653/cerm.2017.44.4.201>
- Kirk, E.S., Squires, E.L., Graham, J.K., 2005. Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. *Theriogenology* 64, 1422–1439.

<https://doi.org/10.1016/j.theriogenology.2005.03.006>

Lazzari, G., Crotti, G., Turini, P., Duchi, R., Mari, G., Zavaglia, G., Barbacini, S., Galli, C., 2002. Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of in vitro matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. *Theriogenology* 58, 709–712.

[https://doi.org/10.1016/S0093-691X\(02\)00777-X](https://doi.org/10.1016/S0093-691X(02)00777-X)

Luppi, S., Martinelli, M., Giacomini, E., Giolo, E., Zito, G., Garcia, R.C., Ricci, G., 2015a. Comparative proteomic analysis of spermatozoa isolated by swim-up or density gradient centrifugation. *Reprod Biol Endocrinol* 13, 1–7. <https://doi.org/10.1186/s12958-015-0027-y>

Luppi, S., Martinelli, M., Giacomini, E., Giolo, E., Zito, G., Garcia, R.C., Ricci, G., 2015b. Comparative proteomic analysis of spermatozoa isolated by swim-up or density gradient centrifugation. *Reprod Biol Endocrinol* 13, 1–7. <https://doi.org/10.1186/s12958-015-0027-y>

Macpherson, M.L., Blanchard, T.L., Love, C.C., Brinsko, S.P., Varner, D.D., 2002. Use of a silane-coated silica particle solution to enhance the quality of ejaculated semen in stallions. *Theriogenology* 58, 317–320. [https://doi.org/10.1016/S0093-691X\(02\)00736-7](https://doi.org/10.1016/S0093-691X(02)00736-7)

Matsuura, K., Uozumi, T., Furuichi, T., Sugimoto, I., Kodama, M., Funahashi, H., 2013. A microfluidic device to reduce treatment time of intracytoplasmic sperm injection. *Fertil Steril* 99, 400–407. <https://doi.org/10.1016/j.fertnstert.2012.10.022>

Mocé, E., Graham, J.K., 2008. In vitro evaluation of sperm quality. *Anim Reprod Sci* 105, 104–118. <https://doi.org/10.1016/j.anireprosci.2007.11.016>

Morrell, J., Johannisson, A., Dalin, A.M., Rodriguez-Martinez, H., 2009. Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. *Reprod Domest Anim* 44, 512–517.

<https://doi.org/10.1111/j.1439-0531.2008.01265.x>

- Morrell, J.M., Rodriguez-Martinez, H., Johannisson, A., 2010. Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size. *Equine Vet J* 42, 579–585. <https://doi.org/10.1111/j.2042-3306.2010.00101.x>
- Nosrati, R., Graham, P.J., Zhang, B., Riordon, J., Lagunov, A., Hannam, T.G., Escobedo, C., Jarvi, K., Sinton, D., 2017. Microfluidics for sperm analysis and selection. *Nat Rev Urol* 14, 707–730. <https://doi.org/10.1038/nrurol.2017.175>
- Nosrati, R., Vollmer, M., Eamer, L., San Gabriel, M.C., Zeidan, K., Zini, A., Sinton, D., 2014. Rapid selection of sperm with high DNA integrity. *Lab Chip* 14, 1142. <https://doi.org/10.1039/c3lc51254a>
- Sakkas, D., Manicardi, G.C., Tomlinson, M., Mandrioli, M., Bizzaro, D., Bianchi, P.G., Bianchi, U., 2000. The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. *Hum Reprod* 15, 1112–1116. <https://doi.org/10.1093/humrep/15.5.1112>
- Schulte, R., Chung, Y., Ohl, S., Takayama, S., Smith, G.D., 2007. Microfluidic sperm sorting device provides a novel method for selecting motile sperm with higher DNA integrity. *Fertil Steril* 88, S76.
- Schuster, T.G., Cho, B., Keller, L.M., Takayama, S., Smith, G.D., 2003. Isolation of motile spermatozoa from semen samples using microfluidics. *Reprod Biomed Online* 7, 75–81. [https://doi.org/10.1016/S1472-6483\(10\)61732-4](https://doi.org/10.1016/S1472-6483(10)61732-4)
- Sessions-Bresnahan, D.R., Graham, J.K., Carnevale, E.M., 2014. Validation of a heterologous fertilization assay and comparison of fertilization rates of equine oocytes using invitro

- fertilization, perivitelline, and intracytoplasmic sperm injections. *Theriogenology* 82, 274–282. <https://doi.org/10.1016/j.theriogenology.2014.04.002>
- Shirota, K., Yotsumoto, F., Itoh, H., Obama, H., Hidaka, N., Nakajima, K., Miyamoto, S., 2016. Separation efficiency of a microfluidic sperm sorter to minimize sperm DNA damage. *Fertil Steril* 105, 315–321. <https://doi.org/10.1016/j.fertnstert.2015.10.023>
- Sieme, H., Martinsson, G., Rauterberg, H., Walter, K., Aurich, C., Petzoldt, R., Klug, E., 2003. Application of techniques for sperm selection in fresh and frozen-thawed stallion semen. *Reprod Domest Anim* 38, 134–140. <https://doi.org/10.1046/j.1439-0531.2003.00416.x>
- Simopoulou, M., Gkoles, L., Bakas, P., Giannelou, P., Kalampokas, T., Pantos, K., Koutsilieris, M., 2016. Improving ICSI: A review from the spermatozoon perspective. *Syst Biol Reprod Med* 62, 359–371. <https://doi.org/10.1080/19396368.2016.1229365>
- Smith, G.D., Takayama, S., 2017. Application of microfluidic technologies to human assisted reproduction. *Mol Hum Reprod* 23, 257–268. <https://doi.org/10.1093/molehr/gaw076>
- Swain, J.E., Lai, D., Takayama, S., Smith, G.D., 2013. Thinking big by thinking small: application of microfluidic technology to improve ART. *Lab Chip* 13, 1213. <https://doi.org/10.1039/c3lc41290c>
- Tasoglu, S., Safaee, H., Zhang, X., Kingsley, J.L., Catalano, P.N., Gurkan, U.A., Nureddin, A., Kayaalp, E., Anchan, R.M., Maas, R.L., Tüzel, E., Demirci, U., 2013. Exhaustion of racing sperm in nature-mimicking microfluidic channels during sorting. *Small* 9, 3374–3384. <https://doi.org/10.1002/sml.201300020>
- Xue, X., Wang, W.S., Shi, J.Z., Zhang, S.L., Zhao, W.Q., Shi, W.H., Guo, B.Z., Qin, Z., 2014. Efficacy of swim-up versus density gradient centrifugation in improving sperm deformity rate and DNA fragmentation index in semen samples from teratozoospermic patients. *J*

Assist Reprod Genet 31, 1161–1166. <https://doi.org/10.1007/s10815-014-0287-z>

Zini, A., Finelli, a, Phang, D., Jarvi, K., 2000. Influence of semen processing technique on human sperm DNA integrity. *Urology* 56, 1081–1084. [https://doi.org/10.1016/S0090-4295\(00\)00770-6](https://doi.org/10.1016/S0090-4295(00)00770-6)

CHAPTER III: ASSOCIATION OF EQUINE SPERM POPULATION PARAMETERS WITH OUTCOME OF INTRACYTOPLASMIC SPERM INJECTIONS¹

SUMMARY

Limited clinical information is available regarding sperm population parameters that are important for use with equine intracytoplasmic sperm injection (ICSI). Therefore, the appropriateness of a sample of sperm is typically not known before ICSI. The aim of our study was to determine which sperm population characteristics were predictive of ICSI outcome. Frozen-thawed sperm samples (n=114) from 37 stallions in a clinical program were analyzed after ICSI for percentages of normal morphology (MORPH+), live as assessed by eosin/nigrosin stain (LIVE+), membrane intact as assessed by hypoosmotic swelling test (HOS+), and DNA fragmentation determined by sperm chromatin dispersion (DNA-). ICSI was performed on 147 oocytes, and cleavage (≥ 2 cells), embryo development (morula or blastocyst), and pregnancy status after embryo transfer were determined. Among the examined sperm parameters, LIVE+ correlated positively with MORPH+ and HOS+, and MORPH+ negatively with DNA-; no other significant correlations were observed. When used for ICSI, sperm population percentages for MORPH+ and DNA- were not predictive of ICSI outcome, including cleavage, embryo development, and establishment of a pregnancy. Sperm population percentages significantly affecting ICSI outcomes were LIVE+ and HOS+ for oocyte cleavage, LIVE+ for embryo development, and HOS+ for establishment of a pregnancy. The probability of a pregnancy was

¹ Manuscript accepted for publication in *Theriogenology* on June 28, 2018. Article title: “Association of equine sperm population parameters with outcome of intracytoplasmic sperm injection”. Authors: Raul A. Gonzalez-Castro and Elaine M. Carnevale. Department of Biomedical Sciences, Colorado State University, Equine Reproduction Laboratory, 3101 Rampart Road, Fort Collins, Colorado, 80521, USA

significantly higher for sperm populations having HOS+ $\geq 40\%$ than populations having HOS+ $\leq 20\%$. The mean age of the donor mare per sperm-injected oocyte did not differ for oocyte cleavage, embryo production, or establishment of pregnancy. In our study, the probability of sperm-injected oocytes to develop into an embryo (morula or blastocyst) improved when sperm were selected from a population with higher indicators of membrane integrity (LIVE+ and HOS+).

Equine, sperm, stallion, intracytoplasmic sperm injection, hypoosmotic swelling

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) has predominantly been used in human reproductive medicine. In the last 1.5 decades, ICSI has been developed for use in the equine industry for a diversity of reasons – including fertility status, gamete availability, and scheduling around performance activities. Frozen-thawed sperm are frequently used for equine ICSI (Carnevale, 2008). Cryopreservation reduces sperm viability, motility, and fertility, although the detrimental effects of cryopreservation on sperm quality vary widely among stallions (Bedford et al., 1995). In men, sperm fertilizing potential and subsequent embryo development and pregnancy establishment are positively associated with normal sperm morphology, motility parameters, and DNA integrity (Morris et al., 2002; Zini et al., 2008). Minimal information is available on the relationship with the population parameters of normal sperm morphology and ICSI outcome in horses. The final step of sperm selection for equine and human ICSI is often performed under X 200 or 400 magnification and based on gross morphology and motility (Boitrelle et al., 2014; Lazzari et al., 2002; Sessions-Bresnahan et al., 2014). However, these two sperm characteristics may be insufficient to select an ideal sperm, especially if morphological

and functional changes have occurred after cryopreservation. Consequently, use of a compromised sperm for ICSI can result in failure of fertilization or embryo development in horses (Choi et al., 2016), and it has been associated with an increased abortion rate in women (Nasr-Esfahani et al., 2012).

Associations of sperm population quality parameters with the success of ICSI are limited in horses, although they have been more thoroughly investigated in other species. Selection of human sperm by morphology and motility does not exclude sperm with DNA damage from being selected for ICSI, primarily for men with male factor infertility (Celik-Ozenci et al., 2004). A higher percentage of sperm with normal morphology has damaged DNA in infertile when compared to fertile men (Avendaño et al., 2009), which negatively impacts embryo quality and the probability of pregnancy after ICSI (Avendaño et al., 2010). In cattle, sperm population with more morphologically abnormal sperm are negatively associated with oocyte cleavage, embryo development, and blastocyst formation after IVF (Walters et al., 2005). Sperm DNA fragmentation has also been associated with reduced fertility. In men, sperm DNA fragmentation has a detrimental effect on conception in vivo and pregnancy outcomes after fertilization in vitro (Khadem et al., 2014; Zini et al., 2008), and it is negatively associated with sperm concentration, motility, and normal morphology (Moskovtsev et al., 2009; Zini et al., 2001). The sperm's plasma membrane has essential functions for fertilization in vivo; however, characteristics of the plasma membrane also appear to be important for assisted fertilization. Sperm that display a functional plasma membrane under hypoosmotic conditions have lower incidences of abnormal head morphology, apoptotic markers, protamine deficiency, membrane damage, and DNA fragmentation, all of which relate to fertility potential (Bassiri et al., 2012; Herbemont and Sifer, 2015; Stanger et al., 2010; Tartagni et al., 2002). Pregnancies and live births have resulted from

nonmotile, human sperm populations when individual sperm are selected for ICSI based on membrane swelling under hypoosmotic conditions (Casper et al., 1996; Kordus et al., 2008; Sallam et al., 2001, 2005; Westlander et al., 2003), suggesting that hypoosmotic swelling of the sperm membrane is indicative of factors beyond those required for motility. The associations between sperm characteristics and clinical success in other species suggest that sperm population parameters could be valuable considerations for equine ICSI. Although limited clinical information is available regarding which sperm factors affect equine ICSI outcome (Galli et al., 2016), the individual stallion does affect cleavage and embryo development rates (Choi et al., 2016; Herrera et al., 2012; Sanchez et al., 2016). Stallions with low or no field fertility have lower cleavage and blastocysts rates after ICSI (Colleoni et al., 2012; Galli et al., 2016, 2015). The association between specific sperm characteristics and equine ICSI outcome has not been adequately studied.

The aim of our study was to determine sperm population parameters associated with equine ICSI outcome. To do so, we first identified assays to be used with the low numbers of sperm that were available after ICSI. The first objective was to determine correlations among different assessment parameters in samples from which sperm were selected for clinical ICSI. Sperm remaining after the ICSI procedure was completed were assessed for percentages of: 1) normal morphology, 2) live sperm, 3) membrane integrity based on hypoosmotic swelling, and 4) DNA fragmentation. Our second objective was to relate sperm population parameters from which a sperm was selected for ICSI to: 1) cleavage, 2) development of a morula or blastocyst acceptable for embryo transfer, and 3) establishment of early pregnancy.

MATERIALS AND METHODS

Chemicals and reagents

Unless noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sperm samples and oocyte injections

Frozen-thawed, sperm samples (n=114 from 37 stallions) were obtained after sperm processing for clinical ICSI procedures. Sperm samples were analyzed for the following parameters: 1) percentage of sperm with normal morphology, MORPH+, 2) percentage of live sperm, as a measurement of viability and sperm head membrane integrity, LIVE+, 3) percentage of sperm with positive hypoosmotic swelling, indicative of intact principal piece membrane, HOS+, and 4) percentage of sperm with chromatin dispersion, consistent with fragmentation of DNA, DNA-. Sperm were obtained for analyses from samples processed by swim-up or direct dilution procedure for ICSI at approximately 5 min after ICSI was performed.

Frozen-thawed sperm samples were obtained from partial sections of straws (0.25, 0.5 or 5 mL) that were cut under liquid nitrogen. The swim-up procedure was used when frozen sperm had sufficient motility and sperm numbers to complete the procedure. For swim-up, the cut section of straw was placed directly into 1 mL of G-IVF (G-IVF™, Vitrolife Sweden AB, V, Frölunda, Sweden; with the addition of 0.4% BSA) at 38.2°C, and the tube was placed at an approximately 45° angle in an atmosphere of 6% CO₂ and air for 15 min at 38.2°C. The supernatant (500 µL) was removed and placed into 2 ml of gas equilibrated G-IVF and centrifuged for 5 min at 308 X g. The supernatant was removed, and sperm from the pellet was selected for ICSI. Frozen-thawed samples with poor quality or limited sperm motility were used for ICSI after direct dilution. Sperm were thawed by placing a section of cut straw into 0.25 to 1

mL of a commercial bench-top medium, G-MOPS™ (Vitrolife Sweden AB) with 0.4% BSA at 38.2°C, and a sperm from the diluted sample was selected for ICSI. Sperm for oocyte injections were selected by a single technician based on microscopic evaluation for motility and normal morphology at X 200 magnification.

Oocyte donors were light-horse mares, 7 to 26 y (n=42, mean \pm SEM of 17.6 \pm 0.50 y), in a clinical ART program. Mare reproductive tracts were examined using transrectal ultrasonography. When a growing follicle(s) with a mean diameter of approximately 35 mm and uterine edema was observed during the follicular phase, an ovulation inducing compound (human chorionic gonadotropin and/or deslorelin acetate) was administered to induce follicle maturation.

The following day, between 18 and 24 h after maturation induction, oocytes were collected by ultrasound-guided, transvaginal aspirations. Oocyte collection attempts were made from the dominant follicle to obtain maturing oocytes, probably in metaphase I; in some mares, one to three of the larger subordinate follicle(s) were aspirated to obtain immature oocytes, probably in the germinal vesicle stage. Maturing oocytes from dominant follicles completed maturation in Medium 199 with Earle's salts, L-glutamine, and 2.2 g/L sodium bicarbonate (GIBCO BRL Life Technologies, Grand Island, NY, USA; with additions of 10% fetal calf serum, 0.2 mM pyruvate and 25 mg/mL gentamicin) for approximately 20 h at 38°C and in 6% CO₂ and air. Oocytes from subordinate follicles were cultured under the same conditions in Medium 199 with the added supplementation of hormones and growth factors [15 ng/ml FSH (National Hormone & Peptide Program, NHPP, Torrance, CA USA), 1 ug/ml LH (NHPP), 50 ng/ml EGF, 100 ng/ml IGF, 1 ug/ml E₂, and 500 ng/ml P₄] for approximately 28 h (Sessions-Bresnahan et al., 2014). Oocytes that matured to metaphase II, as determined by the extrusion of

a polar body, were used for ICSI. During the study, ICSI was performed using a piezo drill by a single technician on 147 oocytes, with 103 oocytes from dominant follicles and 44 oocytes from subordinate follicles. In some cases, oocytes from dominant and subordinate follicles of the same mare underwent ICSI at different times. In these cases, two sperm samples were prepared and analyzed individually.

After ICSI, potential zygotes were placed into 30- μ l drops of culture medium (Dulbecco Modified Eagle medium/F12 with 10% fetal calf serum) under oil (Ovoil™, Vitrolife, Göteborg, Sweden) at 38 °C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. The day after ICSI, potential zygotes were observed at X 200 magnification for cleavage; if cell division had not occurred but signs of imminent cleavage, such as change in shape or fragmentation of the oolemma, were observed, the zygote was reassessed for cleavage on the following day. Cleaved embryos were evaluated on Days 5, 6 and 7 (Day 0 = day of ICSI) for development into a morula or blastocyst. Embryos were considered to be at the morula stage and acceptable for transfer when a compact mass of cells was observed. Embryos were considered to be at the blastocyst stage of development when a defined ring of trophoblast was observed. Embryos (Day 6 or 7) were transferred into the uteri of recipient mares by transcervical embryo transfer.

Development after ICSI was defined per injected oocyte as: 1) Cleavage, \geq 2 cells by Day 2, 2) Embryo, development of a morula or blastocyst by Day 7 that was acceptable for embryo transfer, and 3) Pregnancy, detection by transrectal ultrasonography of an embryonic vesicle in the recipient's uterus to Day 25, with detection of an embryonic heartbeat. Recipients determined not to be pregnant were examined until 11 days after transfer or 17 to 18 days after ICSI.

Morphology and live sperm analysis

An aliquot of the sperm sample (20 μ L), remaining after completion of the ICSI procedure, was mixed on a slide with 20 μ L of an eosin-nigrosin stain (Hancock Stain®, Animal Reproduction Systems Inc., Chino, CA) to evaluate MORPH+ and LIVE+. The mixture was smeared with another slide and dried on a warming plate at 37°C to avoid hypotonic artifacts. Sperm (n=100 per sample) were examined and classified under X 1000 magnification using bright field microscopy. Morphological abnormalities were classified as: 1) head defect, with alterations in size (microcephalic head), shape (pyriform, narrowed or tapered), or other major abnormalities (acrosome defect, nuclear vacuoles, and deformities); 2) midpiece defects, with proximal or distal cytoplasmic droplets, swollen and rough midpiece, or fractured axonemal fibers; and 3) principal piece defects, such as cytoplasmic retention, J-shape, dag-like defects, simple- or double-bends, or coiling. Sperm that did not uptake the stain in the head region were counted as live sperm, whereas sperm with any detectable eosin in the head region were counted as dead sperm. The percentage of LIVE+ was determined for each slide as the percentage of unstained sperm per the 100 sperm counted (Brito, 2007). For statistical analyses, sperm samples for MORPH+ or LIVE+ were grouped as low ($\leq 24\%$), medium (25 to 49%) and high ($\geq 50\%$), based on previous classifications for stallion field fertility (Love, 2011).

Hypoosmotic swelling test (HOS)

An aliquot of the sperm sample (10 μ L) was mixed with 100 μ L of hypoosmotic sucrose solution (100 mOsm/Kg) in an Eppendorf tube. The mixture was incubated at 37°C for 30 min. Sperm (n=300 per sample) were examined and classified under X 400 magnification with contrast field microscopy. Swelling of the sperm membrane was evaluated and expressed in

percentages (Neild et al., 1999). For statistical analyses, sperm samples for HOS+ were grouped in low ($\leq 20\%$), medium (21 to 39%) and high ($\geq 40\%$), based on previous classification for stallion field fertility (Neild et al., 2000).

Sperm chromatin dispersion (SCD)

An Eppendorf tube containing 30 μL of 1% low-melting point agarose was placed in a water bath at 90°C for 5 min to fuse the agarose, and then in a water bath at 37°C for 3 min. An aliquot (10 μL) of the semen sample was mixed with the fused agarose. The mixture was pipetted onto slides precoated with 0.65% agarose and covered with a 22 X 22 mm cover glass. The slides were refrigerated for 5 min at 4°C . The cover glass was removed, and the slide was immersed horizontally in an Acid Denaturation Solution (0.08 N HCl) and incubated for 7 min in the dark at room temperature. The slide was then immersed in Lysing Solution I (0.4 M Tris, 0.4 M 2-Mercaptophenol, 1% SDS, and 50mM EDTA, pH 7.5) for 10 min and Lysing Solution II (0.4 M Tris, 2 M NaCl, and 1% SDS, pH 7.5) for 5 min. The slide was washed with distilled water for 5 min and dehydrated in sequential ethanol immersion (70, 90 and 100% for 2 min each), and stained (Dip Quick Stain, Jorgensen Laboratories Inc., Loveland, CO). To determine, the percentage of sperm with fragmented DNA (DNA-), the dispersion pattern was counted in 300 sperm per sample under X 1000 magnification using bright field microscopy. Sperm with large or medium halos were determined to have intact DNA. Sperm with small halos or no halos and sperm determined to be degraded (weakly or irregular staining) were classified as having fragmented DNA, as previously described (Fernandez et al., 2005, 2003). For statistical evaluations, sperm sample for DNA- was grouped as low ($\leq 9\%$), medium (10 to 19%) and high ($\geq 20\%$).

Statistical Analyses

Analyses were performed using R Core Team Software 3.4.2017 (R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>). Percentages of sperm population sample parameters (MORPH+, LIVE+, HOS+ and DNA-) and mare age as continuous variables were tested for normality and homogeneity of variances using Shapiro-Wilk and Levene tests, respectively. Sperm parameters and mare age are presented as percentage (means \pm SEM). Correlations among sperm population sample parameters were calculated using Spearman's rank correlation. Comparisons for Cleavage, Embryo and Pregnancy rates for oocytes from dominant and subordinate follicles were performed using chi-square test. The association between sperm population parameters, from which sperm were selected for injection, and ICSI outcome (Cleavage, Embryo, and Pregnancy per injected oocyte) was examined using three different analyses. First, sperm population parameters as continuous variable that resulted in positive and negative ICSI outcomes for Cleavage, Embryo and Pregnancy were compared using Wilcoxon signed-rank test for independent samples. Second, ICSI outcome was used as a response (Yes or No) for logistic regression with each sperm population parameter as predictor. Finally, a multivariable stepwise logistic regression model was selected using Akaike information criterion (AIC) and backward elimination approach (Yamashita et al., 2007; Zhang, 2016). For grouped samples, logistic regressions were performed using assigned levels (low, medium and high as categorical variable) to sperm population parameters as predictors with Tukey-adjusted pairwise comparison. Odds ratios (OR) with a confidence interval (CI) of 2.5-97.5% were calculated. For multiple comparisons among sperm parameters, original P-values were corrected using Bonferroni adjustment. A Bonferroni-corrected P-value <0.008 was considered significant. For

all analyses, $P < 0.05$ was considered statically significant. For odds ratios, a confidence interval that does not include “1” was considered statistically significant.

RESULTS

The means (\pm SEM) for population parameters assessed in 114 sperm samples were: MORPH+ (38.4 ± 1.3), LIVE+ (39.8 ± 1.4), HOS+ (29.4 ± 1.0) and DNA- (8.9 ± 0.7). In some cases, more than one oocyte was injected with the same sperm sample. Not all sperm samples could be evaluated completely, as sperm numbers were too low in some samples to run all assays. Among sperm population parameters, MORPH+ correlated positively with LIVE+ ($\rho = 0.45$, $P < 0.001$) and negatively with DNA- ($\rho = -0.30$, $P = 0.007$). LIVE+ positively correlated with HOS+ ($\rho = 0.43$, $P < 0.001$) and tended to associated negatively to DNA- ($\rho = -0.21$, $P = 0.06$). No other significant correlations were observed among sperm population parameters.

The collection of donor oocytes from dominant versus subordinate follicles, respectively, did not significantly affect Cleavage (63/103, 61% and 28/44, 64%), Embryo (41/103, 40% and 15/44, 34%) and Pregnancy (20/103, 19% and 6/44, 14%), and oocyte source was not used as a factor in further analyses. Overall after ICSI, 62% (91/147) of injected oocytes cleaved, 38% (56/147) of injected oocytes resulted in a transferable embryo by Day 7, and 18% (26/147) of injected oocytes resulted in a pregnancy at Day 25. All but one embryo was a blastocyst at the time of transfer; the single morula did not result in a pregnancy. In this study, no embryo development was observed after Day 7, although all potential embryos were observed until they were determined to be nonviable. Pregnancy losses did not occur from first detection of an embryonic vesicle to 25 days of gestation, and heartbeats were noted in all pregnancies. Three

pregnancy losses were observed between 25 and 35 days; however, recipients started departing after Day 25, and complete pregnancy information was not available after Day 25.

Not all sperm population parameters were associated with ICSI outcome. Percentages of MORPH+ and DNA- did not differ among sperm populations from which ICSI resulted in a positive or negative outcome for Cleavage, Embryo, and Pregnancy. However, percentages of LIVE+, indicative of an intact sperm head plasma membrane, and HOS+, indicative of functional plasma membrane in the tail, were higher ($P < 0.01$) in sperm populations in which oocyte injections resulted in Cleavage. Percentages of LIVE+ were higher ($P = 0.03$) in sperm populations in which sperm-injected oocytes resulted in embryo development. Only a higher ($P = 0.02$) percentage of HOS+ in the evaluated sperm populations was associated with establishment of pregnancy (Table 3.1).

In the logistic regression using each sperm population parameter as predictor for ICSI outcome, Cleavage ($P < 0.002$) and Embryo ($P < 0.04$) increased with higher LIVE+, but Pregnancy was not significantly influenced. Cleavage ($P = 0.02$) and Pregnancy ($P < 0.05$) increased with higher HOS+, but Embryo was not significantly influenced. No significant associations were noted between Cleavage, Embryo or Pregnancy with MORPH+ or DNA-, and these two parameters were determined not to have predictive value (Table 3.2).

In the stepwise multiple logistic regression analysis, LIVE+, HOS+ and DNA- were predictive and retained in the stepwise models for Cleavage ($P < 0.0003$) and Embryo ($P = 0.05$), while MORPH+ was not retained in the models. For Pregnancy, HOS+ and DNA- were predictive and retained in the stepwise model ($P < 0.03$), while MORPH+ and LIVE+ were excluded (Table 3.2).

Table 3.1: Mean percentages \pm SEM and number of samples (n) for sperm parameters (normal morphology, MORPH+; live sperm, LIVE+; membrane integrity assessed by hypoosmotic swelling, HOS+; DNA fragmentation, DNA-) associated with samples from which sperm were removed for ICSI and resulted in positive (Yes) and negative (No) ICSI outcomes for Cleavage, Embryo, and Pregnancy per injected oocyte. Percentages for Yes and No for Cleavage, Embryo or Pregnancy for a sperm population parameters differ at $P < 0.05$ (^{ab}).

Sperm Parameter	Cleavage		Embryo		Pregnancy	
	Yes	No	Yes	No	Yes	No
MORPH+ (n = 147)	40.5 \pm 1.4 (91)	36.6 \pm 1.5 (56)	40.8 \pm 1.9 (56)	37.9 \pm 1.3 (91)	42.3 \pm 2.9 (26)	38.4 \pm 1.1 (121)
LIVE+ (n = 147)	44.2 \pm 1.4 ^a (91)	35.5 \pm 1.1 ^b (56)	44.5 \pm 1.9 ^a (56)	38.8 \pm 1.6 ^b (91)	43.8 \pm 2.6 (26)	40.4 \pm 1.2 (121)
HOS+ (n = 147)	31.5 \pm 1.1 ^a (91)	26.9 \pm 1.2 ^b (56)	30.7 \pm 1.5 (56)	29.2 \pm 1.2 (91)	33.8 \pm 2.0 ^a (26)	28.9 \pm 0.9 ^b (121)
DNA- (n = 102)	8.3 \pm 0.8 (69)	9.4 \pm 1.0 (33)	7.67 \pm 1.1 (40)	9.3 \pm 0.8 (62)	6.4 \pm 1.9 (18)	9.1 \pm 0.6 (84)

Table 3.2: Logistic regressions of individual sperm population parameters as predictors and stepwise logistic regression for Cleavage, Embryo and Pregnancy after ICSI. Stepwise regression and P-value for the resulting model (P-model) for Cleavage and Embryo did not include MORPH+, and model for Pregnancy did not include MORPH+ and LIVE+. Statistically significant odd ratios (OR) with 2.5-97.5% confidence interval (CI) are marked by asterisk (*).

		Logistic regression			Stepwise logistic regression			
		P-value	OR	CI	P-value	OR	CI	P-model
Cleavage	MORPH+	<0.09	1.02	0.99-1.05				
	LIVE+	<0.002	1.04*	1.01-1.08	0.01	1.05*	1.01-1.09	<0.0003
	HOS+	<0.04	1.03*	1.01-1.07	>0.1	1.03	0.98-1.08	
	DNA-	>0.4	0.97	0.90-1.04	>0.5	0.97	0.91-1.05	
Embryo	MORPH+	>0.2	1.02	0.99-1.04				
	LIVE+	<0.04	1.03*	1.01-1.05	0.06	1.03	0.99-1.07	0.05
	HOS+	>0.4	1.01	0.98-1.05	>0.6	1.01	0.97-1.05	
	DNA-	>0.2	0.96	0.88-1.03	>0.3	0.96	0.89-1.03	
Pregnancy	MORPH+	>0.1	1.02	0.98-1.06				
	LIVE+	>0.3	1.02	0.98-1.04				
	HOS+	<0.05	1.03*	1.01-1.07	0.05	1.04*	1.01-1.09	<0.03
	DNA-	>0.09	0.91	0.80-1.01	0.09	0.91	0.80-1.01	

When sperm sample population parameters were divided into groups, consistent with previous associations with fertility in vivo, no differences were observed among groups for MORPH+ or DNA- (Table 3.3).

Table 3.3: Means \pm SEM for sperm parameter groupings (percentages of normal morphology, MORPH+; live sperm, LIVE+; functional membrane assessed by hypoosmotic swelling, HOS+; DNA fragmentation, DNA-) and rates for Cleavage, Embryo or Pregnancy per sperm-injected oocyte and odds ratios (OR). Within a column for a specific sperm population parameters, ICSI outcome differed at $P < 0.05$ (^{ab}) or $P < 0.08$ (^{AB}); statistically significant OR were marked by an asterisk (*).

Sperm Parameter	Mean \pm SEM	Cleavage		Embryo		Pregnancy		
		Rate (%)	OR (CI)	Rate (%)	OR (CI)	Rate (%)	OR (CI)	
MORPH+	$\leq 24\%$	18.8 \pm 0.8	50 (14/28)	1	29 (8/28)	1	14 (4/28)	1
	25-49%	38.1 \pm 0.4	61 (51/83)	1.6 (0.7-3.8)	36 (30/83)	1.4 (0.6-3.8)	16 (13/83)	1.1 (0.4-4.2)
	$\geq 50\%$	56.4 \pm 0.4	72 (26/37)	2.6 (0.9-7.5)	50 (18/36)	2.5 (0.9-7.4)	25 (9/36)	2.0 (0.6-8.1)
LIVE+	$\leq 24\%$	17.7 \pm 0.3	38 ^a (9/24)	1	25 ^A (6/24)	1	13 (3/24)	1
	25-49%	38.7 \pm 0.3	55 ^a (43/78)	2.1 (0.8-5.4)	33 ^A (26/78)	1.5 (0.5-4.5)	15 (12/78)	1.3 (0.4-6.0)
	$\geq 50\%$	57.6 \pm 0.3	87 ^b (39/45)	10.8* (3.4-38)	53% ^B (24/45)	3.4* (1.2-10)	24 (11/45)	2.3 (0.6-11)
HOS+	$\leq 20\%$	14.8 \pm 0.9	53 (16/30)	1	30 (9/30)	1	7 ^a (2/30)	1
	21-39%	29.5 \pm 0.5	63 (57/91)	1.5 (0.6-3.4)	40 (36/91)	1.5 (0.6-3.9)	16 ^{ab} (15/91)	2.8 (0.7-18)
	$\geq 40\%$	47.5 \pm 1.7	69 (18/26)	2.0 (0.7-6.1)	42 (11/26)	1.7 (0.6-5.3)	35 ^b (9/26)	7.4* (1.7-52)
DNA-	$\leq 9\%$	5.1 \pm 0.3	69 (47/68)	1	40 (27/68)	1	21 (14/68)	1
	10-20%	13.5 \pm 0.5	67 (18/27)	0.9 (0.3-2.4)	48 (13/27)	1.4 (0.6-3.5)	15 (4/27)	0.67 (0.2-2.3)
	$\geq 21\%$	24.4 \pm 1.7	57 (4/7)	0.6 (0.1-3.2)	0 (0/7)	<0.1 (0-Inf)	8 (0/7)	<0.1 (0-Inf)

However, LIVE+ $\geq 50\%$ resulted in higher Cleavage ($P < 0.002$) and Embryo ($P < 0.08$) compared to LIVE+ $\leq 24\%$ and 25-49%. The odds of LIVE+ $\geq 50\%$ resulting in Cleavage and Embryo were 10.8 and 3.4 times larger than LIVE+ $\leq 24\%$, respectively. Pregnancy was not significantly affected by LIVE+ groupings. The only sperm parameter grouping to affect Pregnancy was HOS+, with HOS+ $\leq 20\%$ and 21-39% resulting in fewer ($P < 0.02$) pregnancies than HOS+ $\geq 40\%$. The odds of a pregnancy were 7.4 times larger for HOS+ $\geq 40\%$ compared to HOS+ $\leq 20\%$ (Table 3.3).

The mean \pm SEM age of the donor mare per sperm-injected oocyte did not significantly differ for oocytes that did or did not result in Cleavage (Yes, 18.0 ± 5.3 and No, 17.3 ± 5.8 y), Embryo (Yes, 18.3 ± 5.0 and No, 17.4 ± 5.8 y) and Pregnancy (Yes, 18.9 ± 5.0 and No, 17.5 ± 5.6 y).

DISCUSSION

In the present study, equine sperm populations from which individual sperm were selected for ICSI were evaluated using techniques appropriate for low sperm numbers to assess morphology, viability, membrane integrity, and DNA fragmentation. We attempted to associate specific sperm population parameters to ICSI outcomes (cleavage, embryo development, and early pregnancy) to determine if they could be used to assess the suitability of samples for ICSI and to predict outcomes. In our study, percentages of live sperm (viable), determined by eosin-nigrosin stain, were positively correlated with normal morphology and hypoosmotic swelling (HOS+). This is consistent with other species, with normal sperm morphology, viability, and hypoosmotic swelling being strongly correlated in sperm from men (Jeyendran et al., 1984; Miciński et al., 2011), buffalo and bulls (Lodhi et al., 2008), boars (Samardžija et al., 2008), and

stallions (Dogan et al., 2009; Neild et al., 2000). No other statistically significant correlations among sperm population parameters were observed in this study. However, in fresh semen samples from men, low HOS+ values are associated with increased DNA damage, and the HOS value is highly predictive of an abnormal DNA fragmentation index (Miciński et al., 2011; Stanger et al., 2010). Similarly in cooled equine semen, a negative relationship was reported between normal sperm morphology and the DNA fragmentation index; more specifically, pear-shaped sperm heads and the presence of nuclear vacuoles were positively correlated with sperm DNA fragmentation (Morrell et al., 2008). Associations among equine sperm population parameters have not been previously studied in association with equine ICSI.

Under field conditions, good sperm motility and normal morphology are associated with good fertility in stallions (Gravance et al., 1996; Heckenbichler et al., 2011; Jasko et al., 1992; Love, 2011; Morrell et al., 2008). The extent that these sperm population characteristics impact success of ICSI had not been determined for the horse, as it has in some other species. In men, sperm selected individually based on normal morphology for assisted fertilization are associated with low DNA damage (Franco et al., 2008; Gosálvez et al., 2013; Nadalini et al., 2009; Wilding et al., 2011) and related positively with fertilization, high quality embryos, implantation and pregnancy rates, and low miscarriage rates (Balaban et al., 2011; Bartoov et al., 2002; Garolla et al., 2008; Souza Setti et al., 2010; Wilding et al., 2011), especially in cases of severe male factor infertility and repeated implantation failures after ICSI (Boitrelle et al., 2014). In our study, the percentage of sperm with normal morphology, in population from which a sperm was selected for ICSI, was not associated with outcome (Cleavage, Embryo or Pregnancy). However, because morphology and motility were part of the final selection criteria for an individual sperm just prior to oocyte injection, this could have negated the impact of morphology within the

originating sperm population. In human ICSI, the association between the initial semen morphology and outcome after ICSI is eliminated when sperm are selected individually based on morphology with high magnification (greater than X 6000); however, using standard magnification for ICSI procedures (X 200), sperm quality selection was less effective (Wilding et al., 2011). In our study, sperm were selected at X 200 magnification, which would have allowed for selection based on more general sperm morphology, but it would not have allowed for the exclusion of more specific morphological defects, such as nuclear vacuoles.

In our study, LIVE+ and HOS+ were correlated, as observed in other species (Dogan et al., 2009; Jeyendran et al., 1984; Samardžija et al., 2008). Membrane integrity, as measured by HOS test, was the only sperm parameter that was positively associated with Pregnancy. Under hypoosmotic conditions, a functional tail membrane results in cytoplasmic swelling and tail curling, recognizable at low magnification (X 200-400) (Casper et al., 1996; Jeyendran et al., 1984). Vital stains, such as eosin nigrosin, evaluate physical sperm plasma membrane damage, while HOS evaluates plasma membrane functional ability. A physically intact plasma membrane does not ensure that it is functional (Brito et al., 2003; Jeyendran et al., 1984).-Aquaporins (AQPs) are integral membrane proteins that control the selective passage of water molecules and small solutes in and out of cells (King and Agre, 1996; Verkman, 2002). Expression of AQP mRNA in bull sperm relates to the increase in sperm volume during hypoosmotic incubation (Kasimanickam et al., 2017). In human sperm, presence of normal sperm membranes with no phospholipid scrambling relates positively with the percentage of swollen sperm under hypoosmotic conditions (Fraczek et al., 2014). These findings suggest that AQPs expression and lipid membrane status contribute to the regulation of sperm volume under hypoosmotic conditions and potentially have a role in conserving sperm quality (Bonilla-Correal et al., 2017;

Kasimanickam et al., 2017). In our study, oocytes injected with sperm from samples with HOS >40% had higher probability of resulting in cleavage and early pregnancy. Similarly, human sperm samples with HOS>50% have a higher likelihood for fertilization and pregnancy after intrauterine insemination (Tartagni et al., 2002). For horses, HOS+ is associated with normal sperm morphology, and stallion field fertility declines when HOS+ is <40% (Neild et al., 2000). Individual human sperm selected by HOS have low DNA fragmentation (Stanger et al., 2010) and frequency of aneuploidy (Pang et al., 2010). The HOS test has been used to select human sperm for ICSI from a nonmotile population, resulting in pregnancies and live births (Casper et al., 1996; Kordus et al., 2008; Sallam et al., 2001, 2005; Westlander et al., 2003). In our study, the percentage of live sperm was correlated with morphologically normal and HOS+ sperm; therefore, selection of a live sperm at the time of ICSI could increase our selection of HOS+ sperm. However, the association between HOS+ and ICSI success suggests that this parameter could be associated with other factors that improve ICSI success. For example, it has been hypothesized that PLCz, a soluble sperm-borne oocyte activating factor, could be reduced or lost in sperm with altered plasma membrane permeability (Bedford-Guaus et al., 2012; Kashir et al., 2011). In this regard, Cleavage and HOS+ were significantly associated in this study. We have been able to select HOS+ sperm for equine ICSI; however, the extent that the HOS selection process will improve ICSI success has not yet been determined.

In our study, the percentage of sperm with DNA fragmentation did not impact ICSI outcome, although the mean percentage of sperm with DNA fragmentation was low (< 10%) in the overall population. Therefore, the limited chance of selecting a sperm with fragmented DNA could have negated any observable impact on ICSI success with the available number of samples. Under field conditions in horses, sperm DNA fragmentation has a negative relationship

with pregnancy rates (Kenney et al., 1995; Love et al., 2002; Morrell et al., 2008). No consensus has been reached to determine the impact of sperm DNA fragmentation on pregnancy rates after IVF or ICSI in humans (Cissen et al., 2016; Collins et al., 2008). In several studies, a negative association was observed between sperm DNA fragmentation and pregnancy for IVF and/or ICSI (Avendaño et al., 2010; Bakos et al., 2008; Borini et al., 2006; Collins et al., 2008; Dar et al., 2013; Gu et al., 2009; López et al., 2013; Tavares et al., 2013; Zhao et al., 2014). Pregnancy rates for IVF were negatively affected when sperm had >30% DNA fragmentation, but a similar association was not observed for ICSI (Bungum et al., 2006; Li et al., 2006). Congruently, when sperm DNA fragmentation was <30%, there was no significant difference in pregnancy rates for IVF or ICSI (Evenson and Wixon, 2006). The selection of sperm by motility and morphology characteristics from a population with high DNA fragmentation appears to result in a similar pregnancy rate when compared to controls with low DNA fragmentation (Dar et al., 2013). This is consistent with our study in which most of oocytes were injected with sperm from samples with less than 21% DNA fragmentation. As a sperm parameter, DNA fragmentation is sensitive; but it has low specificity for predictive power, suggesting that a sperm population with low DNA fragmentation is not predictive of higher pregnancy rates (Cissen et al., 2016). The impact of DNA fragmentation on embryo or pregnancy loss could not be determined in our study.

In the present study, oocytes were collected from mares aging from 7 to 26 y. Mare age has been shown to impact oocyte quality and later developmental potential after oocyte transfer and ICSI (Carnevale and Ginther, 1995; Carnevale and Stokes, 2010; Frank-Guest et al., 2010). Therefore, we evaluated the impact of mare age on ICSI success to assess if this was a confounding factor in our study. However, we found no consistent effect of mare age on the ICSI outcomes that we evaluated.

In conclusion, limited numbers of sperm were available after ICSI to assess sperm populations for percentages of sperm with normal morphology, live (viable), membrane integrity, and DNA integrity. The percentage of sperm with normal morphology or good DNA integrity did not influence ICSI outcome. The probability of sperm-injected oocytes to develop into an embryo (morula or blastocyst) improved when sperm were selected from a sample population with higher membrane integrity in the head (LIVE+) and principal piece of the tail (HOS+). Membrane integrity, as evaluated through the hypoosmotic swelling test, was the factor that was most closely associated with improved ICSI outcome. Equine sperm samples for ICSI with >40% HOS+ and > 50% live sperm increases the chances for a successful ICSI outcome. The model most predictive of ICSI outcome included LIVE+, HOS+ and DNA-. The percentage of morphologically normal sperm in the population did not have a predictive impact, but this could have been impacted by use of morphology as a final selection criterion of individual sperm for ICSI.

REFERENCES

- Avendaño, C., Franchi, A., Duran, H., Oehninger, S., 2010. DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. *Fertil Steril* 94, 549–557. <https://doi.org/10.1016/j.fertnstert.2009.02.050>
- Avendaño, C., Franchi, A., Taylor, S., Morshedi, M., Bocca, S., Oehninger, S., 2009. Fragmentation of DNA in morphologically normal human spermatozoa. *Fertil Steril* 91, 1077–1084. <https://doi.org/10.1016/j.fertnstert.2008.01.015>
- Bakos, H.W., Thompson, J.G., Feil, D., Lane, M., 2008. Sperm DNA damage is associated with assisted reproductive technology pregnancy. *Int J Androl* 31, 518–526. <https://doi.org/10.1111/j.1365-2605.2007.00803.x>
- Balaban, B., Yakin, K., Alatas, C., Oktem, O., Isiklar, A., Urman, B., 2011. Clinical outcome of intracytoplasmic injection of spermatozoa morphologically selected under high magnification: A prospective randomized study. *Reprod Biomed Online* 22, 472–476. <https://doi.org/10.1016/j.rbmo.2010.11.003>
- Bartoov, B., Berkovitz, A., Eltes, F., Kogosowski, A., Menezes, Y., Barak, Y., 2002. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. *J Androl* 23, 1–8. <https://doi.org/10.1002/j.1939-4640.2002.tb02595.x>
- Bassiri, F., Tavalae, M., Shiravi, A.H., Mansouri, S., Nasr-Esfahani, M.H., 2012. Is there an association between HOST grades and sperm quality? *Hum Reprod* 27, 2277–2284. <https://doi.org/10.1093/humrep/des155>
- Bedford-Guaus, S.J., McPartlin, L.A., Varner, D.D., 2012. Characterization of Equine Phospholipase C Zeta: A Review and Preliminary Results on Expression Defects in

- Subfertile Stallions. *J Equine Vet Sci* 32, 445–450.
<https://doi.org/10.1016/j.jevs.2012.06.002>
- Bedford, S., Jasko, D.J., Graham, J.K., Amann, R.P., Squires, E.L., Pickett, B.W., 1995. Effect of seminal extenders containing egg yolk and glycerol on motion characteristics and fertility of stallion spermatozoa. *Theriogenology* 43, 955–967. [https://doi.org/10.1016/0093-691X\(95\)00045-A](https://doi.org/10.1016/0093-691X(95)00045-A)
- Boitrelle, F., Guthauser, B., Alter, L., Bailly, M., Bergere, M., Wainer, R., Vialard, F., Albert, M., Selva, J., 2014. High-magnification selection of spermatozoa prior to oocyte injection: Confirmed and potential indications. *Reprod Biomed Online* 28, 6–13.
<https://doi.org/10.1016/j.rbmo.2013.09.019>
- Bonilla-Correal, S., Noto, F., Garcia-Bonavila, E., Rodríguez-Gil, J.E., Yeste, M., Miro, J., 2017. First evidence for the presence of aquaporins in stallion sperm. *Reprod Domest Anim* 52, 61–64. <https://doi.org/10.1111/rda.13059>
- Borini, A., Tarozzi, N., Bizzaro, D., Bonu, M.A., Fava, L., Flamigni, C., Coticchio, G., 2006. Sperm DNA fragmentation: Paternal effect on early post-implantation embryo development in ART. *Hum Reprod* 21, 2876–2881. <https://doi.org/10.1093/humrep/del251>
- Brito, L.F.C., 2007. Evaluation of Stallion Sperm Morphology. *Clin Tech Equine Pract* 6, 249–264. <https://doi.org/10.1053/j.ctep.2007.09.004>
- Brito, L.F.C., Barth, A.D., Bilodeau-Goeseels, S., Panich, P.L., Kastelic, J.P., 2003. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. *Theriogenology* 60, 1539–1551. [https://doi.org/10.1016/S0093-691X\(03\)00174-2](https://doi.org/10.1016/S0093-691X(03)00174-2)
- Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J., Giwercman, A.,

2006. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum Reprod* 22, 174–179. <https://doi.org/10.1093/humrep/del326>
- Carnevale, E.M., 2008. Clinical Considerations Regarding Assisted Reproductive Procedures in Horses. *J Equine Vet Sci* 28, 686–690. <https://doi.org/10.1016/j.jevs.2008.10.005>
- Carnevale, E.M., Ginther, O., 1995. Defective oocytes as a cause of subfertility in old mares. *Biol Reprod Monogr* 1, 209–214.
- Carnevale, E.M., Stokes, J.E., 2010. Effect of equine oocyte donor age on success of oocyte transfer and intracytoplasmic sperm injection. *Anim Reprod Sci* 121, 258–259. <https://doi.org/10.1016/j.anireprosci.2010.04.036>
- Casper, R.F., Meriano, J.S., Jarvi, K.A., Cowan, L., Lucato, M.L., 1996. The hypo-osmotic swelling test for selection of viable sperm for intracytoplasmic sperm injection in men with complete asthenozoospermia. *Fertil Steril* 65, 972–976. [https://doi.org/10.1016/S0015-0282\(16\)58271-5](https://doi.org/10.1016/S0015-0282(16)58271-5)
- Celik-Ozenci, C., Jakab, A., Kovacs, T., Catalanotti, J., Demir, R., Bray-Ward, P., Ward, D., Huszar, G., 2004. Sperm selection for ICSI: Shape properties do not predict the absence or presence of numerical chromosomal aberrations. *Hum Reprod* 19, 2052–2059. <https://doi.org/10.1093/humrep/deh361>
- Choi, Y.H., Velez, I.C., Macías-García, B., Riera, F.L., Ballard, C.S., Hinrichs, K., 2016. Effect of clinically-related factors on in vitro blastocyst development after equine ICSI. *Theriogenology* 85, 1289–1296. <https://doi.org/10.1016/j.theriogenology.2015.12.015>
- Cissen, M., Wely, M. van, Scholten, I., Mansell, S., Bruin, J.P. de, Mol, B.W., Braat, D., Repping, S., Hamer, G., 2016. Measuring Sperm DNA Fragmentation and Clinical Outcomes of Medically Assisted Reproduction: A Systematic Review and Meta-Analysis.

PLoS One 11, e0165125. <https://doi.org/10.1371/journal.pone.0165125>

Colleoni, S., Lazzari, G., Duchi, R., Baca Castex, C., Mari, G., Lagutina, I., Galli, C., 2012.

Fertilization and development of oocytes after ICSI with semen of stallions with different in vivo fertility. *J Equine Vet Sci* 32, 408–409. <https://doi.org/10.1016/j.jevs.2012.05.030>

Collins, J.A., Barnhart, K.T., Schlegel, P.N., 2008. Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? *Fertil Steril* 89, 823–831.

<https://doi.org/10.1016/j.fertnstert.2007.04.055>

Dar, S., Grover, S.A., Moskovtsev, S.I., Swanson, S., Baratz, A., Librach, C.L., 2013. In vitro fertilization-intracytoplasmic sperm injection outcome in patients with a markedly high DNA fragmentation index (>50%). *Fertil Steril* 100, 75–80.

<https://doi.org/10.1016/j.fertnstert.2013.03.011>

Dogan, I., Polat, U., Nur, Z., 2009. Correlations between seminal plasma enzyme activities and semen parameters in seminal fluid of Arabian horses. *Iran J Vet Res* 10, 119–124.

Evenson, D., Wixon, R., 2006. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. *Reprod Biomed Online* 12, 466–72.

[https://doi.org/10.1016/S1472-6483\(10\)62000-7](https://doi.org/10.1016/S1472-6483(10)62000-7)

Fernandez, J.L., Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Enciso, M., LaFromboise, M., De Jonge, C., 2005. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* 84, 833–842.

<https://doi.org/10.1016/j.fertnstert.2004.11.089>

Fernandez, J.L., Muriel, L., Rivero, M.T., Goyanes, V., Vazquez, R., Alvarez, J.G., 2003. The Sperm Chromatin Dispersion Test: A Simple Method for the Determination of Sperm DNA Fragmentation. *J Androl* 24, 59–66. <https://doi.org/10.1002/j.1939-4640.2003.tb02641.x>

- Fraczek, M., Wiland, E., Piasecka, M., Boksa, M., Gaczarzewicz, D., Szumala-Kakol, A., Kolanowski, T., Beutin, L., Kurpisz, M., 2014. Fertilizing potential of ejaculated human spermatozoa during in vitro semen bacterial infection. *Fertil Steril* 102, 711–720. <https://doi.org/10.1016/j.fertnstert.2014.06.002>
- Franco, J.G., Baruffi, R.L.R., Mauri, A.L., Petersen, C.G., Oliveira, J.B.A., Vagnini, L., 2008. Significance of large nuclear vacuoles in human spermatozoa: implications for ICSI. *Reprod Biomed Online* 17, 42–45. [https://doi.org/10.1016/S1472-6483\(10\)60291-X](https://doi.org/10.1016/S1472-6483(10)60291-X)
- Frank-Guest, B.L., Stokes, J.E., Carnevale, E.M., 2010. Association of oocyte morphology and mare age with pregnancy rates after intracytoplasmic sperm injection. *Anim Reprod Sci* 121, 241–243. <https://doi.org/10.1016/j.anireprosci.2010.04.037>
- Galli, C., Colleoni, S., Duchi, R., Lazzari, G., 2016. Male Factors Affecting the Success of Equine In Vitro Embryo Production by Ovum Pickup-Intracytoplasmic Sperm Injection in a Clinical Setting. *J Equine Vet Sci* 43, S6–S10. <https://doi.org/10.1016/j.jevs.2016.05.014>
- Galli, C., Duchi, R., Crotti, G., Turini, P., Colleoni, S., Lazzari, G., 2015. Sperm quality for ICSI, in: *Proceedings of the AAF IETS Equine Reproduction Symposium, Paris, France*. pp. 18–19.
- Garolla, A., Fortini, D., Menegazzo, M., De Toni, L., Nicoletti, V., Moretti, A., Selice, R., Engl, B., Foresta, C., 2008. High-power microscopy for selecting spermatozoa for ICSI by physiological status. *Reprod Biomed Online* 17, 610–6. [https://doi.org/10.1016/S1472-6483\(10\)60307-0](https://doi.org/10.1016/S1472-6483(10)60307-0)
- Gosálvez, J., Migueles, B., López-Fernández, C., Sánchez-Martín, F., Sáchez-Martín, P., 2013. Single sperm selection and DNA fragmentation analysis: The case of MSOME/IMSI. *Nat Sci* 05, 7–14. <https://doi.org/10.4236/ns.2013.57A002>

- Gravance, C.G., Liu, I.K., Davis, R.O., Hughes, J.P., Casey, P.J., 1996. Quantification of normal head morphometry of stallion spermatozoa. *J Reprod Fertil* 108, 41–46.
<https://doi.org/10.1530/jrf.0.1080041>
- Gu, L.J., Chen, Z.W., Chen, Z.J., Xu, J.F., Li, M., 2009. Sperm chromatin anomalies have an adverse effect on the outcome of conventional in vitro fertilization: a study with strictly controlled external factors. *Fertil Steril* 92, 1344–1346.
<https://doi.org/10.1016/j.fertnstert.2009.03.031>
- Heckenbichler, S., Deichsel, K., Peters, P., Aurich, C., 2011. Quality and fertility of cooled-shipped stallion semen at the time of insemination. *Theriogenology* 75, 849–856.
<https://doi.org/10.1016/j.theriogenology.2010.10.027>
- Herbement, C., Sifer, C., 2015. How to select the spermatozoon for intracytoplasmic sperm injection in 2015? *Minerva Ginecol* 67, 185–193.
- Herrera, C., Dufourq, P., Freije, M., Morikawa, I., Centeno, J.E., Aristi, V., Menghini, L., Sporleder, C., 2012. Selection of stallions for in vitro embryo production by ICSI in a commercial program. *J Equine Vet Sci* 32, 409. <https://doi.org/10.1016/j.jevs.2012.05.031>
- Jasko, D., Little, T., Lein, D., Foote, R., 1992. Comparison of spermatozoal movement and semen characteristics with fertility in stallions : 64 cases (1987-1988). *J Am Vet Med Assoc* 200, 975–985.
- Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G., Zaneveld, L.J., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 70, 219–228.
<https://doi.org/10.1530/jrf.0.0700219>
- Kashir, J., Heynen, A., Jones, C., Durrans, C., Craig, J., Gadea, J., Turner, K., Parrington, J.,

- Coward, K., 2011. Effects of cryopreservation and density-gradient washing on phospholipase C zeta concentrations in human spermatozoa. *Reprod Biomed Online* 23, 263–267. <https://doi.org/10.1016/j.rbmo.2011.04.006>
- Kasimanickam, R.K., Kasimanickam, V.R., Arangasamy, A., Kastelic, J.P., 2017. Associations of hypoosmotic swelling test, relative sperm volume shift, aquaporin7 mRNA abundance and bull fertility estimates. *Theriogenology* 89, 162–168. <https://doi.org/10.1016/j.theriogenology.2016.11.011>
- Kenney, R.M., Evenson, D.P., Garcia, M.C., Love, C.C., 1995. Relationships between sperm chromatin structure, motility, and morphology of ejaculated sperm, and seasonal pregnancy rate. *Biol Reprod*.
- Khadem, N., Poorhoseyni, A., Jalali, M., Akbary, A., Heydari, S.T., 2014. Sperm DNA fragmentation in couples with unexplained recurrent spontaneous abortions. *Andrologia* 46, 126–130. <https://doi.org/10.1111/and.12056>
- King, L.S., Agre, P., 1996. Pathophysiology of the Aquaporin Water Channels. *Annu Rev Physiol* 58, 619–648. <https://doi.org/10.1146/annurev.ph.58.030196.003155>
- Kordus, R.J., Price, R.L., Davis, J.M., Whitman-Elia, G.F., 2008. Successful twin birth following blastocyst culture of embryos derived from the immotile ejaculated spermatozoa from a patient with primary ciliary dyskinesia: A case report. *J Assist Reprod Genet* 25, 437–443. <https://doi.org/10.1007/s10815-008-9254-x>
- Lazzari, G., Crotti, G., Turini, P., Duchi, R., Mari, G., Zavaglia, G., Barbacini, S., Galli, C., 2002. Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of in vitro matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. *Theriogenology* 58, 709–712.

[https://doi.org/10.1016/S0093-691X\(02\)00777-X](https://doi.org/10.1016/S0093-691X(02)00777-X)

Li, Z., Wang, L., Cai, J., Huang, H., 2006. Correlation of sperm DNA damage with IVF and ICSI outcomes: A systematic review and meta-analysis. *J Assist Reprod Genet* 23, 367–376.

<https://doi.org/10.1007/s10815-006-9066-9>

Lodhi, L.A., Zubair, M., Qureshi, Z.I., Ahmad, I., Jamil, H., 2008. Conventional Semen Evaluation Parameters in Fresh Nili-Ravi 28, 186–188.

López, G., Lafuente, R., Checa, M.A., Carreras, R., Brassesco, M., 2013. Diagnostic value of sperm DNA fragmentation and sperm high-magnification for predicting outcome of assisted reproduction treatment. *Asian J Androl* 15, 790–794. <https://doi.org/10.1038/aja.2013.81>

Love, C.C., 2011. Relationship between sperm motility, morphology and the fertility of stallions. *Theriogenology* 76, 547–557. <https://doi.org/10.1016/j.theriogenology.2011.03.007>

Love, C.C., Thompson, J.A., Lowry, V.K., Varner, D.D., 2002. Effect of storage time and temperature on stallion sperm DNA and fertility. *Theriogenology* 57, 1135–42.

[https://doi.org/10.1016/S0093-691X\(01\)00689-6](https://doi.org/10.1016/S0093-691X(01)00689-6)

Miciński, P., Pawlicki, K., Wielgus, E., Bochenek, M., Gogol, P., Ficek, B., 2011. Total reactive antioxidant potential and DNA fragmentation index as fertility sperm parameters. *Reprod Biol* 11, 135–144. [https://doi.org/10.1016/S1642-431X\(12\)60050-3](https://doi.org/10.1016/S1642-431X(12)60050-3)

Morrell, J.M., Johannisson, A., Dalin, A.-M., Hammar, L., Sandebert, T., Rodriguez-Martinez, H., 2008. Sperm morphology and chromatin integrity in Swedish warmblood stallions and their relationship to pregnancy rates. *Acta Vet Scand* 50, 2. <https://doi.org/10.1186/1751-0147-50-2>

Morris, I.D., Ilott, S., Dixon, L., Brison, D.R., 2002. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to

fertilization and embryo development. *Hum Reprod* 17, 990–998.

<https://doi.org/10.1093/humrep/17.4.990>

Moskovtsev, S.I., Willis, J., White, J., Mullen, J.B.M., 2009. Sperm DNA Damage: Correlation to Severity of Semen Abnormalities. *Urology* 74, 789–793.

<https://doi.org/10.1016/j.urology.2009.05.043>

Nadalini, M., Tarozzi, N., Distratis, V., Scaravelli, G., Borini, A., 2009. Impact of intracytoplasmic morphologically selected sperm injection on assisted reproduction outcome: A review. *Reprod Biomed Online* 19, 45–55. [https://doi.org/10.1016/S1472-6483\(10\)60283-0](https://doi.org/10.1016/S1472-6483(10)60283-0)

Nasr-Esfahani, M.H., Deemeh, M.R., Tavalae, M., 2012. New era in sperm selection for ICSI. *Int J Androl* 35, 475–484. <https://doi.org/10.1111/j.1365-2605.2011.01227.x>

Neild, D., Chaves, G., Flores, M., Mora, N., Beconi, M., Agüero, A., 1999. Hypoosmotic test in equine spermatozoa. *Theriogenology* 51, 721–727. [https://doi.org/10.1016/S0093-691X\(99\)00021-7](https://doi.org/10.1016/S0093-691X(99)00021-7)

Neild, D.M., Chaves, M.G., Flores, M., Miragaya, M.H., Gonzalez, E., Agüero, A., 2000. The HOS test and its relationship to fertility in the stallion. *Andrologia* 32, 351–355. <https://doi.org/10.1046/j.1439-0272.2000.00357.x>

Pang, M.G., You, Y.A., Park, Y.J., Oh, S.A., Kim, D.S., Kim, Y.J., 2010. Numerical chromosome abnormalities are associated with sperm tail swelling patterns. *Fertil Steril* 94, 1012–1020. <https://doi.org/10.1016/j.fertnstert.2009.04.043>

Sallam, H., Farrag, a, Agameya, a, Ezzeldin, F., Eid, a, Sallam, a, 2001. The use of a modified hypo-osmotic swelling test for the selection of viable ejaculated and testicular immotile spermatozoa in ICSI. *Hum Reprod* 16, 272–6.

- Sallam, H.N., Farrag, A., Agameya, A.F., El-Garem, Y., Ezzeldin, F., 2005. The use of the modified hypo-osmotic swelling test for the selection of immotile testicular spermatozoa in patients treated with ICSI: A randomized controlled study. *Hum Reprod* 20, 3435–3440. <https://doi.org/10.1093/humrep/dei249>
- Samardžija, M., Dobranić, T., Krušlin, S., Cergolj, M., Karadjole, M., Prvanović, N., Grizelj, J., 2008. The use of the hypoosmotic swelling test and supravital staining in evaluation of sperm quality in boars. *Vet Arch* 78, 279–287.
- Sanchez, R., Herrera, C., Blanco, M., Rosati, I., Lazzari, G., Colleoni, S., Sieme, H., Galli, C., 2016. In Vitro Production of Equine Embryos by Ovum Pick Up and Intracytoplasmic Sperm Injection in Young Mares. *J Equine Vet Sci* 41, 77. <https://doi.org/10.1016/j.jevs.2016.04.074>
- Sessions-Bresnahan, D.R., Graham, J.K., Carnevale, E.M., 2014. Validation of a heterologous fertilization assay and comparison of fertilization rates of equine oocytes using invitro fertilization, perivitelline, and intracytoplasmic sperm injections. *Theriogenology* 82, 274–282. <https://doi.org/10.1016/j.theriogenology.2014.04.002>
- Souza Setti, A., Ferreira, R.C., Paes De Almeida Ferreira Braga, D., De Cássia Sávio Figueira, R., Iaconelli, A., Borges, E., 2010. Intracytoplasmic sperm injection outcome versus intracytoplasmic morphologically selected sperm injection outcome: A meta-analysis. *Reprod Biomed Online* 21, 450–455. <https://doi.org/10.1016/j.rbmo.2010.05.017>
- Stanger, J.D., Vo, L., Yovich, J.L., Almahbobi, G., 2010. Hypo-osmotic swelling test identifies individual spermatozoa with minimal DNA fragmentation. *Reprod Biomed Online* 21, 474–484. <https://doi.org/10.1016/j.rbmo.2010.06.026>
- Tartagni, M., Schonauer, M.M., Cicinelli, E., Selman, H., De Ziegler, D., Petruzzelli, F.,

- D'Addario, V., 2002. Usefulness of the hypo-osmotic swelling test in predicting pregnancy rate and outcome in couples undergoing intrauterine insemination. *J Androl* 23, 498–502. <https://doi.org/10.1002/j.1939-4640.2002.tb02271.x>
- Tavares, R.S., Silva, A.F., Lourenço, B., Almeida-Santos, T., Sousa, A.P., Ramalho-Santos, J., 2013. Evaluation of human sperm chromatin status after selection using a modified Diff-Quik stain indicates embryo quality and pregnancy outcomes following in vitro fertilization. *Andrology* 1, 830–837. <https://doi.org/10.1111/j.2047-2927.2013.00127.x>
- Verkman, A., 2002. Aquaporin water channels and endothelial cell function*. *J Anat* 200, 617–627. <https://doi.org/10.1046/j.1469-7580.2002.00058.x>
- Walters, A.H., Eyestone, W.E., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C., 2005. Bovine embryo development after IVF with spermatozoa having abnormal morphology. *Theriogenology* 63, 1925–1937. <https://doi.org/10.1016/j.theriogenology.2004.09.001>
- Westlander, G., Barry, M., Petrucco, O., Norman, R., 2003. Different fertilization rates between immotile testicular spermatozoa and immotile ejaculated spermatozoa for ICSI in men Kartagener's syndrome: Case report. *Hum Reprod* 18, 1286–1288. <https://doi.org/10.1093/humrep/deg240>
- Wilding, M., Coppola, G., Di Matteo, L., Palagiano, A., Fusco, E., Dale, B., 2011. Intracytoplasmic injection of morphologically selected spermatozoa (IMSI) improves outcome after assisted reproduction by deselecting physiologically poor quality spermatozoa. *J Assist Reprod Genet* 28, 253–262. <https://doi.org/10.1007/s10815-010-9505-5>
- Yamashita, T., Yamashita, K., Kamimura, R., 2007. A Stepwise AIC Method for Variable Selection in Linear Regression. *Commun Stat - Theory Methods* 36, 2395–2403.

<https://doi.org/10.1080/03610920701215639>

Zhang, Z., 2016. Variable selection with stepwise and best subset approaches. *Ann Transl Med* 4, 136. <https://doi.org/10.21037/atm.2016.03.35>

Zhao, J., Zhang, Q., Wang, Y., Li, Y., 2014. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: A systematic review and meta-analysis. *Fertil Steril* 102, 998–1005.e8. <https://doi.org/10.1016/j.fertnstert.2014.06.033>

Zini, A., Bielecki, R., Phang, D., Zenzes, M.T., 2001. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 75, 674–677. [https://doi.org/10.1016/S0015-0282\(00\)01796-9](https://doi.org/10.1016/S0015-0282(00)01796-9)

Zini, A., Boman, J.M., Belzile, E., Ciampi, A., 2008. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. *Hum Reprod* 23, 2663. <https://doi.org/10.1093/humrep/den321>

CHAPTER IV: PHOSPHOLIPASE C ZETA AND POSTACROSOMAL WW BINDING
PROTEIN: LOCALIZATION AND QUANTIFICATION IN EQUINE SPERM ²

SUMMARY

The sperm delivers activating factors into the oocyte, triggering oscillations in intracellular calcium concentrations. Candidates for oocyte activating factors are phospholipase C zeta (PLCz) and postacrosomal WW binding protein (PAWP). Limited information is available for PLCz in stallion sperm, and PAWP has not been studied. We aimed to localize and quantify PLCz and PAWP in sperm from various stallions to determine population variance and to study relationships between PLCz and sperm population quality parameters (membrane integrity, DNA integrity, reactive oxygen species, and mitochondrial activity). Equine PLCz was identified using two commercial antibodies (antihuman and antimouse) in western blots as a 71 kDa protein, and two bands of PAWP were observed at 28 and 32 kDa using a antihuman antibody produced in rabbits. Immunofluorescence localization of PLCz was noted in the acrosomal and postacrosomal region, midpiece and principal piece of the tail, as has been previously described. PAWP was localized as observed in other species in the postacrosomal region of the head; but it also had novel localization in equine sperm in the midpiece and principal piece of the tail. A positive correlation ($p=0.04$) was observed between PLCz and PAWP immunofluorescence levels in sperm heads. Flow cytometric assessment of PAWP was not successful; but PLCz displayed large variances in fluorescence intensity and percentages of

² Manuscript submitted to Andrology on July 3, 2018; under revision. Manuscript title: “Phospholipase C zeta and postacrosomal WW binding protein: Localization and quantification in equine sperm”. Authors: Raul A. Gonzalez-Castro, James K. Graham and Elaine M. Carnevale. Department of Biomedical Sciences, Colorado State University, Fort Collins, Colorado, USA

positive labeled sperm among 21 stallions and was higher ($p < 0.05$) in live than dead sperm. DNA fragmentation was negatively correlated ($p \leq 0.05$) to PLCz; no other sperm population quality parameters were associated with PLCz in sperm. This research was the first to localize PAWP in stallion sperm and to document its novel localization. Sperm population content of PLCz was successfully evaluated using flow cytometry, and substantial variation was observed in PLCz among stallions. This could be used in future studies to correlate PLCz in stallion sperm with fertility.

INTRODUCTION

Several sperm characteristics, such as motility and morphology, can be evaluated visually; others, such as sperm viability, membrane integrity, mitochondrial membrane potential, oxidative status, membrane fluidity and permeability, lipid peroxidation, and DNA integrity, can be evaluated using flow cytometry (Colenbrander *et al.*, 2003; Peña *et al.*, 2016; Varner, 2008). Although these assessments are associated with sperm fertility after artificial insemination (Barrier Battut *et al.*, 2016, 2017), they are less predictive of sperm suitability for intracytoplasmic sperm injection (ICSI) (Gonzalez-Castro & Carnevale, 2016). Many of these sperm parameters are associated with the ability of sperm to reach the oocyte in the oviduct, which are not important for ICSI. Minimal research has been conducted on equine sperm attributes that are essential for oocyte activation.

Oocyte activation is initiated when a fertilizing sperm delivers sperm-borne oocyte activating factors into the oocyte cytoplasm (Dozortsev *et al.*, 1997; J. Kashir *et al.*, 2014). The importance of oocyte activation is especially notable after ICSI, with the initial stages of embryo development being directly observed. Failure of oocyte activation after sperm injection is probably a major cause of ICSI failure in the horse and human (Ruggeri *et al.*, 2015; Souza Setti

et al., 2010; Vanden Meerschaut *et al.*, 2013). Sperm from some stallions result in consistently good embryo development rates after ICSI, while sperm from other stallions consistently result in poor or no embryo development (Galli *et al.*, 2016), suggesting a difference in oocyte activating potentials. We have studied associations between sperm morphology, membrane integrity, and DNA integrity on the success of ICSI, and membrane integrity was the sperm parameter most highly associated with embryo development and pregnancy (Gonzalez-Castro & Carnevale, 2016). This suggests that a sperm membrane component could be required for oocyte activation and initiation of early embryo development.

Prime candidates for oocyte activation are two proteins associated with the sperm membrane, phospholipase C zeta (PLCz) (Saunders *et al.*, 2002) and postacrosomal WW binding protein (PAWP) (Wu *et al.*, 2007). Equine PLCz is described as a 73 kDa protein, located in the sperm acrosomal and equatorial regions, connecting piece, and principal piece of the tail (S. J. Bedford-Guaus *et al.*, 2011; Sato *et al.*, 2013). The size of PLCz is similar across species at 70 to 75 kDa (Cox *et al.*, 2002; Ito *et al.*, 2008; Nomikos *et al.*, 2013; Saunders *et al.*, 2002; Villaverde *et al.*, 2013). Equine PLCz has significant homology with porcine (82.5%) and human (82.1%) and partial homology with bovine (79.4%) and murine (71.9%) PLCz (Bedford-Guaus *et al.*, 2011). After gamete membrane fusion, PLCz is released into the ooplasm, where it hydrolyses organelle membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). Next, IP₃ binds to IP₃ receptors in the endoplasmic reticulum, resulting in calcium (Ca²⁺) release and the oocyte activation cascade; while DAG activates protein kinase C (PKC) (Swann & Lai, 2013). The initiation of Ca²⁺ oscillations in oocytes by PLCz appears to be consistent among species. The microinjection of mouse and human oocytes with PLCz complimentary RNA (cRNA) or recombinant PLCz initiates Ca²⁺

oscillations (Nomikos *et al.*, 2013; Saunders *et al.*, 2002). Microinjection of an infertile human sperm and PLCz mRNA into mouse oocytes promotes resumption of meiosis and pronuclei formation (Yoon *et al.*, 2008). Similarly, mouse PLCz cRNA triggers Ca²⁺ oscillations in mare oocytes (S. Bedford-Guaus *et al.*, 2008), and equine PLCz cRNA induces Ca²⁺ oscillations and pronuclei formation in mouse oocytes (Sato *et al.*, 2013). A role of PLCz in oocyte activation has been widely accepted in many species, especially human and mouse.

Although not previously described for stallion sperm, PAWP is a 32 kDa protein located in the postacrosomal sheath in the perinuclear theca region of sperm from man, monkey, pig, rabbit and bull (Aarabi *et al.*, 2014; Kennedy *et al.*, 2014; Wu *et al.*, 2007). It is proposed that PAWP interacts with WW-domain-Src family tyrosine kinase-protein complex PAWP-WW I pathway that targets tyrosine kinase regulation of the oocyte meiotic spindle and/or male remodeling factors. PAWP binds to oocyte-borne YAP proteins, and interacts with the SH3 domain of phospholipase C, resulting in activation of the phosphoinositide signaling pathway (Wu *et al.*, 2007). Microinjection of recombinant PAWP induces Ca²⁺ oscillations and promotes meiotic resumption and formation of single pronuclei in porcine, bovine, and monkey oocytes (Wu *et al.*, 2007). The role of PAWP in oocyte activation has been less defined across species than PLCz.

In various species, a relationship between male infertility and deficiencies of PLCz and/or PAWP is apparent. In human and mouse, reduced levels or abnormal localization of PLCz is associated with ICSI failure and low fertilization rates (Chithiwala *et al.*, 2015; Durban *et al.*, 2015; Escoffier *et al.*, 2014; Kashir *et al.*, 2010; Lee *et al.*, 2014; Tavalae & Nasr-Esfahani, 2016; Yoon *et al.*, 2008). On the other hand, high levels and proper localization patterns of human sperm exhibiting PLCz are positively linked to fertilization rates and ICSI success

(Tavalaee *et al.*, 2016; Yelumalai *et al.*, 2015). For PAWP, high protein expression in human sperm relates to higher fertilization rates and embryo development after ICSI, independent of age and others sperm parameters (Aarabi *et al.*, 2014). Likewise, high PAWP levels in bull sperm are associated with better semen quality and improved fertility parameters after artificial insemination (Kennedy *et al.*, 2014). Specific actions of PLCz and PAWP in oocyte activation and ICSI outcome can be difficult to define, as the two proteins are often related. When PLCz and PAWP are analyzed in the same human sperm samples, the proteins are correlated in protein expression and with fertilization rates (Azad *et al.*, 2018; Tavalaee *et al.*, 2016). Limited information has been obtained for PLCz and PAWP in equine sperm, although there are suggestions that subfertile stallions have reduced PLCz expression (Bedford-Guaus *et al.*, 2012; Gradil *et al.*, 2006). However, the presence, localization and importance of PLCz and PAWP in stallion fertility are not definitively known.

In the present study, we tested the hypothesis that equine sperm populations exhibit variable levels of presumptive oocyte activating factors, PLCz and PAWP. Proof of this concept would support evaluating these sperm-associated proteins as the initial step in determining their importance in stallion fertility and ICSI success. Aims of the present studies were to: 1) validate antibodies for PLCz and PAWP in equine sperm for immunolocalization, 2) quantify expression of PLCz and PAWP in equine sperm using flow cytometry, and 3) determine associations between sperm parameters and PLCz.

MATERIAL AND METHODS

Chemicals, reagents and antibodies

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Various commercial antibodies were initially tested in equine and bovine sperm for detection of PLCz of human origin (sc-131752, sc-131753, sc-368424; Santa Cruz Biotechnology, Dallas, TX) or mouse origin (sc-131756, sc-368425; Santa Cruz Biotechnology) and PAWP of human origin (sc-86780, sc-86781; Santa Cruz Biotechnology). Bovine sperm were used as a positive control, as previous immunostaining had not been reported for equine sperm. Two of the commercial antibodies detected PLCz in equine sperm and were used to conduct further experiments – a rabbit polyclonal antibody raised against amino acids 500-549 of PLCz of human origin (sc-368424, H50) and a rabbit polyclonal antibody raised against amino acids 1-163 and mapping at the N-terminus of PLCz of mouse origin (sc-368425, M163). The commercial antibodies that were tested of human origin did not detect PAWP in equine or bovine sperm. However, a rabbit polyclonal antibody raised against human PAWP and characterized in detail in a previous study (Aarabi *et al.*, 2014) was tested and allowed detection of equine and bovine PAWP. The anti-PAWP antibody was provided by Dr. Richard J. Oko (Department of Biomedical and Molecular Sciences, School of Medicine, Queen's University, Canada), and it was used in the following experiments.

Immunofluorescence

Frozen sperm from stallions (n=5) and bulls (n=4) were used. Stallion semen was frozen in house as previously described (Kirk *et al.*, 2005). Bull sperm were obtained from a supply of frozen sperm for in vitro fertilization. Bull sperm were used as comparative controls for PLCz

and PAWP. Straws (0.5 mL) of frozen semen were thawed in a 37°C water bath for 30 seconds prior to use.

Immunofluorescence for localization of PLCz and PAWP in bull and stallion sperm was conducted as by Grasa *et al.* (2008) with some modifications. Three replicates for each sperm sample were assessed. After thawing, sperm from each 0.5-mL straw were washed [addition of up to 8 mL of phosphate buffered saline (PBS, composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.4), centrifugation for 8 min at 500 g, and removal of supernatant]. The pellet was fixed in 500 µL of 4% paraformaldehyde for 40 min. Fixed sperm were washed, and sperm in the pellet were permeabilized using 0.1% v/v Triton X-100 in PBS for 40 min. Sperm were washed and suspended in PBS at 3x10⁶ sperm/mL. An aliquot (30 µL) was pipetted onto a 0.1% poly-L-lysine-coated slide (Sigma-Aldrich) and allowed to dry for 10 min on a slide warmer at 37°C. Sperm were incubated in Blocking Solution (5% v/v normal goat serum in PBS) for 60 min in a humidified chamber. The Blocking Solution was discarded and primary antibody was pipetted (1:100 for H50 or M163; 1:50 for PAWP in Blocking Solution) onto sperm and incubated overnight at 4°C in a humidified chamber. Slides were rinsed and incubated with goat anti-rabbit IgG -H+L- Alexa Fluor®488 (Invitrogen, Eugene, OR) as the secondary antibody at 1:200 in Blocking Solution for 60 min at RT. After washing, sperm were counterstained by 0.2 µg/mL Hoechst 33258 for 10 min. Slides were washed in PBS and prepared (Fluoromount™ Aqueous Mounting Media) for analysis. The following controls were used to evaluate background and nonspecific binding: 1) permeabilized sperm incubated in Blocking Solution overnight without primary and secondary antibody and counterstained with Hoechst 33258, and 2) permeabilized sperm incubated in Blocking Solution overnight with no primary antibody and incubated only with secondary antibody. The localization of PLCz and

PAWP was examined using an Olympus BX53 epifluorescence microscope equipped with 4',6-diamidino-2-phenylindole (DAPI) and fluorescein isocyanate (FITC) filters (Olympus Scientific, Waltham, MA). Localization patterns of immunofluorescence were recorded and classified using cellSensTM Dimension 1.6 (Olympus Scientific).

Western blot analysis

Protein extracts were prepared from whole sperm and from separated heads and tails using frozen-thawed stallion (n=5) and bull (n=4) samples; protein extracts were analyzed by SDS-PAGE and immunoblotting as described with some modifications (McPartlin *et al.*, 2008). For separation of sperm heads and tails, frozen-thawed sperm samples from five stallions were pooled and washed in PBS. The pellet was suspended in PBS and sonicated for 20 sec at 4°C (30% duty cycle, 3 output; Branson Sonifier 250, Danbury CT). Heads and tails were then separated as previously described (Bedford-Guaus *et al.*, 2011) with some modifications. Sperm were centrifuged at 4°C (30 min at 100 g each). Sperm tails were collected from the supernatant after the first centrifugation. The third centrifugation produced a pellet that contained sperm heads and less than 5% of intact sperm, midpieces and tails. Whole sperm, sperm heads and tails were washed in PBS by centrifugation for 8 min at 500g to prepare a protein extract. Briefly, pellets were suspended in 300 µL of RIPA buffer (50 mM Tris HCl -pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and sonicated (30 sec, 30% duty cycle, 3 output). Lysates were centrifuged for 10 min at 14000 g; the supernatants were collected and the pellets were discarded. Total protein concentration was assessed by PierceTM BCA Protein Assay Kit (Thermo Scientific, Eugene, OR) following the manufacturer's guidelines. A total of 50 µg of protein per each sample was suspended in 6X Buffer DTT (0.375M Tris pH 6.8, 12% SDS, 60%

glycerol, 0.6M DTT, 0.06% bromophenol blue) and RIPA buffer up to 40 μ L of final volume, and then incubated for 10 min at 95°C. Samples and molecular weight marker (Precision Plus Protein™ Kaleidoscope™ Standards, Bio-Rad Laboratories Inc., Hercules, CA) were loaded into SDS-PAGE gradient gels (4-15% Mini-PROTEAN TGX™ Precast Protein Gels, Bio-Rad Laboratories Inc.) and separated at a constant 110V for 90 min at 4°C followed by transfer to nitrocellulose membrane (Nitrocellulose/Filter paper, Bio-Rad Laboratories Inc.) at a constant 100V for 90 min at 4°C. For immunodetection, membranes were blocked [5% skim milk in TBST (137 mM NaCl, 2.7 mM KCl, 19 mM Tris base, 0.1% Tween 20, pH 7.4)] for 60 min at RT and then incubated overnight with primary antibodies [H50, M163 (1:500) or PAWP (1:10)] at 4°C under continuous agitation. After incubation with primary antibodies, sperm were washed and incubated with secondary antibody [goat anti-rabbit IgG H&L-HRP conjugated (1:2000); Abcam, Cambridge, MA] for 60 min at RT. After washing, immunoreactivity was imaged using enhanced chemiluminescence detection with Super Signal West Dura (Thermo Scientific) according to the manufacturer's directions. For densitometry analysis, the immunoblots were scanned, and the relative intensity was determined within each experiment using Image Lab™ Software (Bio-Rad Laboratories Inc.). Mouse monoclonal β -tubulin antibody (Santa Cruz Biotechnology) was used as an internal control for protein expression and loading.

Quantitative immunofluorescent analyses of PLCz and PAWP in equine sperm head

For comparative analysis of quantification of PLCz and PAWP immunofluorescence in sperm heads, frozen-thawed sperm from stallions (n=14) were used. Samples were processed for immunofluorescence using H50 as primary antibody for PLCz and antiPAWP for PAWP as described above. Slides were imaged using cellSens™ Dimension 1.6 at X1000 magnification

using a bright field filter to image individual sperm cells, and DAPI and FITC filters were used to image fluorescence from sperm heads for PLCz and PAWP. Approximately 100 sperm for each stallion were analyzed using ImageJ (<http://imagej.nih.gov/ij/>) and the regions of interest (ROI) tool. The integrated density was calculated for the mean level of fluorescence and the area of each sperm head. Corrected total cell fluorescence (CTCF) was obtained after correction for background fluorescence (Liarski *et al.*, 2014).

Flow cytometric assessments

General information about flow cytometric assessment

Semen from twenty-one stallions was used; semen was frozen in different facilities in accordance with their standard procedures. Thawed samples were used to attempt flow cytometric analyses of sperm for PLCz and PAWP. For PAWP flow cytometric evaluations, fluorescence from equine sperm samples was not different from control fluorescence for the secondary antibody, potentially due to a low concentration of primary antibody or binding between primary and secondary antibodies. Therefore, flow cytometry could not identify PAWP to correlate this protein with sperm parameters. Flow cytometry was used to evaluate PLCz levels and to analyze with sperm parameters.

Analyses were conducted using an AccuriTM C6 Flow cytometer and CFlow Plus (Becton Dickinson, Franklin Lakes, NJ). This instrument was equipped with a 488-nm, solid-state blue laser and a 640-nm diode red laser. The optical characteristics of filters were: 1) FL1, 530/30 nm band pass for green fluorescence; 2) FL2, 585/40nm band pass for orange fluorescence; 3) FL3, >670 nm/long pass band for red fluorescence; 4) FL4, 675/25 nm band pass for far red fluorescence. For each sample, a minimum of 10,000 events per replicate were

analysed at forward light scatter channel (FCS) and light scatter channel (SSC). Unstained and single-stained control samples for each fluorochrome and secondary Alexa Fluor-488 conjugated antibody were used to set quadrants and compensations as well as to determine background fluorescence. Unless otherwise stated, all fluorochromes used for these analyses were purchased from Molecular Probes (Eugene, OR) and were prepared according to manufacturer's guidelines.

Flow cytometric assessment of PLCz

Frozen-thawed sperm were resuspended in 1 mL of filtered PBS at 5×10^6 sperm/mL and incubated with 1 μ L of fixable stain for viability (LIVE/DEADTM Fixable Far Red Dead Cell Stain Kit), following the manufacturer's guidelines. Samples were washed in PBS by centrifugation for 8 min at 500 g. After supernatant removal, pellets were fixed, permeabilized and blocked as described for immunofluorescence. Primary antibody (H50 or M163) was added to sample tubes (1:100), and incubated overnight at 4°C. Samples were then washed and incubated with secondary antibody (goat anti-rabbit IgG -H+L- Alexa Fluor®488) at 1:200 for 60 min at RT. After positive labeling was confirmed, flow cytometric analyses were performed. Unstained sperm incubated in blocking solution or in primary antibodies, and single-stained sperm incubated in fixable viability stain or in secondary antibody were used to set quadrants and compensations. FL1 was used to detect green fluorescence (Alexa Fluor-488) and FL4 for far red fluorescence (LIVE/DEADTM Fixable Far Red Dead Cell Stain), which was used to gate whole, live and dead sperm populations. Acquired fluorescence intensity from control and samples were plotted as histograms to measure the mean of fluorescence intensity (MFI) and percentage of positive labeled sperm. Fluorescence emitted by sperm incubated only in secondary antibody was used to assess nonspecific binding. A marker was established at a

specific intensity corresponding to the end of the nonspecific binding. Sperm exhibiting fluorescence from the primary–secondary antibody complex to the right of the marker were considered positive for each antibody (H50+ or M163+ sperm).

Flow cytometric assessment of sperm parameters

For sperm parameters, viability (live), early changes in membrane permeability, membrane lipid disorder, intracellular reactive oxygen species (ROS) levels of peroxides and superoxides, membrane mitochondrial potential (MMP), and DNA fragmentation were assessed. Samples were thawed as described, and an aliquot was diluted in 0.5 mL of HEPES-buffered saline (HBS; 20 mM HEPES, 137 mM NaCl, 10 mM glucose, 2.0 mM KOH, pH 7.4) at 37°C with the sperm concentration adjusted to 1×10^6 sperm/mL. Additionally, thawed sperm were diluted in 37°C E-Z MIXIN® “BF” (Animal Reproduction System, Chino, CA) to a final concentration of 50×10^6 sperm/mL and assessed for total and progressive motility using a computer-assisted sperm analyzer (Sperm Vision® Therio, MOFA Global, Verona, WI).

Viability was assessed using the LIVE/DEAD Sperm Viability Kit. Sperm samples were incubated at 37°C for 5 min in the dark with SYBR14 and propidium iodine (PI) at final concentrations of 100 nM and 10 μ M, respectively (Garner & Johnson, 1995). SYBR14+/ PI– sperm were considered as viable sperm.

Early changes in permeability of the plasma membrane were evaluated with YO-PRO-1 (YOPRO) and PI staining. Samples were incubated for 5 min in the dark at 37°C with YOPRO and PI at final concentrations of 40 μ M and 10 μ M, respectively (Martin *et al.*, 2004). Scatter plots were used to identify viable sperm with no permeability changes (YOPRO–/PI–) and viable sperm with early changes in membrane permeability (YOPRO+/PI–).

Sperm membrane lipid disorder was assessed using a double staining protocol with Merocyanine 540 (M540) and YOPRO. Sperm samples were incubated for 10 min at 37°C with M540 and YOPRO at final concentrations of 2.7 µM and 25 nM, respectively (Kavak *et al.*, 2003). These stains differentiated viable sperm with low (M540–/YOPRO–) and high membrane lipid disorder (M540+/YOPRO–).

Intracellular ROS levels were determined through two different oxidation-sensitive fluorochromes, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) and hydroethidine (HE), for intracellular content of peroxides (H₂O₂) and superoxide anions (O₂^{•-}), respectively (Murillo *et al.*, 2007). For intracellular content of peroxides, samples were stained with H₂DCFDA and PI at final concentrations of 100 and 10 µM, respectively, and incubated at 37°C for 15 min in the dark. H₂DCFDA is a stable cell-permeable, non-fluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2',7'-dichlorofluorescein (DCF⁺) upon oxidation in viable sperm (PI–) (Guthrie & Welch, 2006). For intracellular content of superoxide anions in viable sperm, samples were stained with HE and YOPRO (final concentration 4 µM and 40 µM, respectively) and incubated at 37°C for 15 min in the dark (Guthrie & Welch, 2006). Hydroethidine is freely permeable to cells and it is oxidised by O₂^{•-} to ethidium (E⁺) in viable sperm (PI–) (Zhao *et al.*, 2003), emitting red fluorescence.

Sperm membrane mitochondrial potential (MMP) was assessed by JC-1, a cationic carbocyanine dye that has the ability to label mitochondria with low and high membrane potential. When JC-1 is excited by blue laser (488nm), a sperm with high MMP (MMP⁺), JC-1 forms multimeric aggregates, emitting orange fluorescence. On the other hand, sperm that have low mitochondria membrane potential, JC-1 dye forms monomers, emitting in green

fluorescence. Sperm samples were stained with JC-1 at final concentration of 7.6 μM and incubated in the dark at 37°C for 30 min (Macías-García *et al.*, 2012).

Sperm DNA fragmentation was evaluated following the protocol for Sperm Chromatin Structure Assay (Evenson & Melamed, 1983). Briefly, 200 μL of frozen-thawed sperm was layered onto 500 μL of EquipureTM (Nidacon International AB, Mölndal, Sweden) in a 1.5 mL microcentrifuge tube and centrifuged for 10 min at 200g. The supernatant was removed, and the pellet was resuspended in TNE-Tris (1.0 mM Trizma base, 15.0 mM NaCl, and 1.0 mM EDTA, pH 7.4). Sperm concentration was adjusted to $2 \times 10^6/\text{ml}$. A 200 μL sperm-TNE-Tris suspension was mixed with 400 μL of a detergent solution (0.08 N HCl, 15 mM NaCl, 0.1% Triton X-100). Thirty seconds later, 1.2 ml of acridine orange solution (20 mM Na₂HPO₄, 10 mM citric acid, 1 mM EDTA, 15 mM NaCl, 6 μg acridine orange/mL) was added. The stained sample was measured within 5 min. Sperm DNA fragmentation index (DFI) was assessed by selecting sperm to the right of the main population on a dot plot of red versus green fluorescence.

Statistical Analyses

Statistical analyses were conducted using R Core Team (2013; R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). Data obtained from analyses of flow cytometric assessments and sperm parameters were tested for normality and homogeneity of variances using the Shapiro-Wilk and Levene test. Association of corrected total cell fluorescence (CTCF) between PLCz (H50 as primary antibody) and PAWP in sperm heads was calculated using Spearman's rank correlation (r_s). Measurement of PLCz MFI of controls and samples were examined by one-way ANOVA followed by Tukey adjusted pairwise comparison. Differences between live and dead sperm for MFI and percentages of

PLCz-positive sperm were analyzed using Wilcoxon Signed-Rank test for paired samples. Association between individual sperm parameters and PLCz were assessed using Spearman's rank correlation. Results of flow cytometric assessment of PLCz and sperm parameters are presented as mean \pm SEM. Differences at $p < 0.05$ were considered significant.

RESULTS

Identification and localization of PLCz and PAWP in equine sperm

Among the antibodies tested for PLCz, we found two antibodies (H50 and M163) that identified PLCz in equine sperm. Using western blotting, the expression of ePLCz protein was detected for both H50 (Fig. 4.1A) and M163 (Fig. 4.1B) antibodies in protein extracts prepared from whole sperm, heads and tails with the expected molecular weight of ~71 kDa. Protein extracts from some stallions displayed a second immune-reactive band with lower molecular weight ranging between ~60 and 68 kDa. Protein expression was also confirmed by immunostaining and fluorescence detection (Fig. 4.2 B-D and F-H). Equine PLCz was located at the acrosomal and postacrosomal region, midpiece and principal piece of the tail on equine sperm.

The presence and location of PAWP has not been previously described for stallion sperm. Commercial antibodies (sc-86780 and sc86781, Santa Cruz Biotechnology) for PAWP detection of human origin did not detect PAWP in equine sperm. However, polyclonal rabbit serum raised against human recombinant PAWP (Aarabi *et al.*, 2014) was successful for detection of PAWP in equine sperm. Western blotting analyses identified the presence of two reactive bands of ~28 and ~32 kDa in whole equine sperm and heads and tails (Fig. 4.1 C) and a single reactive band of ~32 kDa for whole bovine sperm (Fig. 4.1 D). Equine PAWP was located in the postacrosomal

region, midpiece and principal piece of tail (Fig. 4.2 J-L). In the bull, the immune labeling of PAWP demonstrated only localization at the postacrosomal region (Fig. 4.2 M and N).

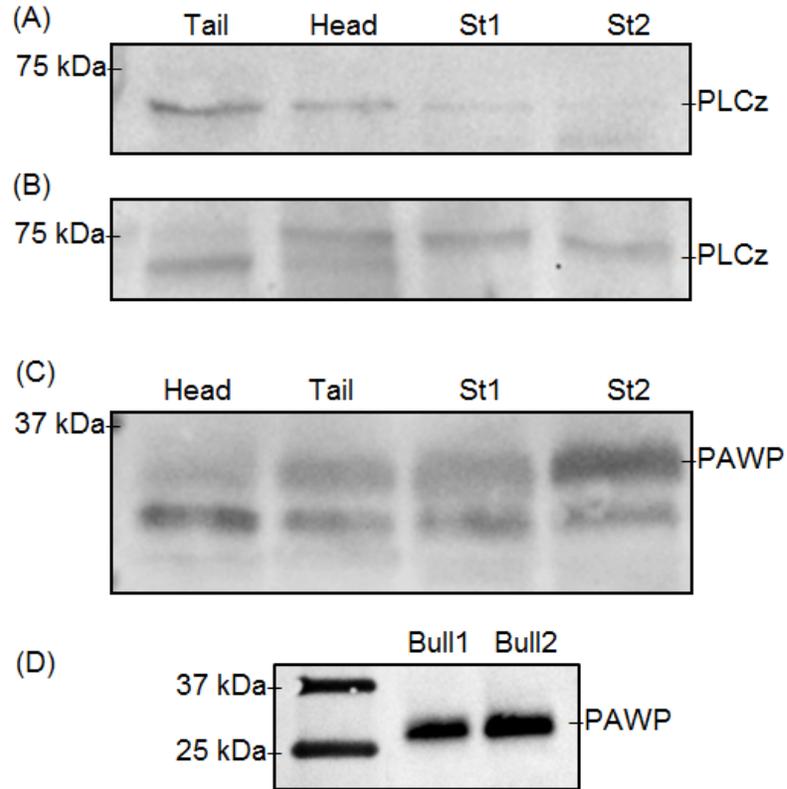


Figure 4.1: Immunoblots for PLCz, using antiPLCz of human (H50, A) and mouse (M163, B) origin, and PAWP proteins in separated pooled heads and tails from four stallions and whole equine sperm from two stallions (St1 and St2) (C) and sperm from 2 bulls (Bull1 and Bull2) (D).

Quantitative immunofluorescent analyses of PLCz and PAWP in equine sperm head

Quantitative immunofluorescence analysis of PLCz (H50 as primary antibody) and PAWP in equine sperm head indicated that the immunofluorescence level of PLCz and PAWP, measured by corrected total cell fluorescence, were correlated positively ($r_s = 0.55$, $p = 0.04$).

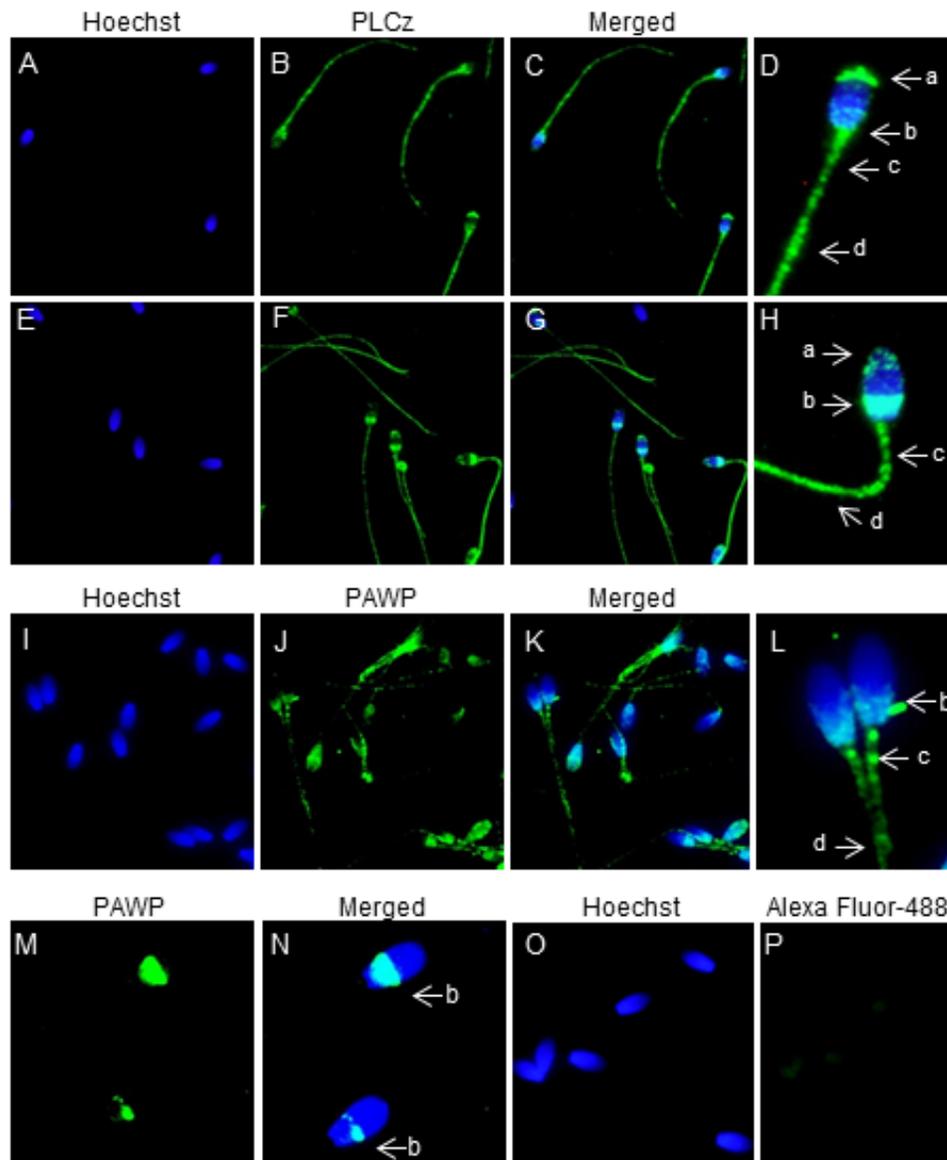


Figure 4.2: Immunofluorescence for PLCz for PAWP in equine sperm. For PLCz, two primary antibodies (H50, A-D; M163, E-H) were used. For PAWP, a primary antibody from human origin was used (I-L). Hoechst 33258 was used to counterstain DNA. Goat anti rabbit IgG -H+L- Alexa Fluor®488 was the secondary antibody, demonstrating PLCz and PAWP localization (C-D, G-H and K-L). In equine sperm, PLCz localized (D and H) on the acrosomal region (a) and postacrosomal region (b), midpiece (c) and principal piece of the tail (d) for both antiPLCz primary antibodies. Immunofluorescence labeling demonstrated PAWP localization on the postacrosomal region (b) for equine sperm (H) and bovine sperm (J) as well as the midpiece (c) and principal piece of the tail (d) in equine sperm. For negative control, equine sperm were incubated in secondary antibody Alexa Fluor-488 without antiPLCz antibody. Control sperm in the same field labeled with Hoechst 33258 (O) and secondary antibody Alexa Fluor-488 (P). Secondary antibody Alexa Fluor-488 did not exhibit significant unspecific binding (P).

Flow cytometric assessment of PLCz and association with sperm parameters

The expression of PLCz and sperm parameters was assessed by flow cytometry in frozen-thawed sperm. For PLCz assessment, controls, such as, preimmune-unstained sperm ($0.2 \pm 0.02 \times 10^3$) or sperm incubated with only primary (H50, $0.3 \pm 0.02 \times 10^3$; M163, $0.6 \pm 0.06 \times 10^3$) or secondary antibody (Alexa Fluor-488, $1.1 \pm 0.04 \times 10^3$), exhibited lower mean of fluorescence intensity (MFI) ($p < 0.0001$) when compared to sperm samples processed for PLCz analysis (H50: $35.9 \pm 4.1 \times 10^3$; M163: $37.9 \pm 4.9 \times 10^3$), independent of the primary antibody used. Sperm before processing for flow cytometric assessment were stained with fixable viability dye. Percentages (means \pm SEM) of viable sperm were not different ($p > 0.2$) between samples immunolabeled with H50 (46 ± 2) and M163 (47 ± 2). The percentage of viable sperm in fixed samples was not associated with antibody MFI ($p > 0.2$). For further analyses, the whole sperm population was gated for live and dead sperm.

The PLCz expression displayed a wide range of values and large variance for MFI and percentages of positively labeled sperm among stallions (Fig. 4.3, Table 4.1). Within each antibody, MFI correlated to percentage of positive labeled sperm (H50, $r_s = 0.71$, $p = 0.0002$; M163, $r_s = 0.81$, $p < 0.0001$). Between antibodies, the means of MFI exhibited for both primary antibodies were not significantly different in whole, live and dead sperm populations. However, the percentages of positive labeled sperm for each sperm population were higher ($p < 0.009$) for sperm incubated with M163, than sperm incubated with H50 (Table 4.1). Between antibodies, MFI and percentages of positive labeled sperm were correlated for whole (MFI: $r_s = 0.76$, $p = 0.0006$; positive labeled sperm: $r_s = 0.89$, $p < 0.0001$), live (MFI: $r_s = 0.64$, $p = 0.003$; positive labeled sperm: $r_s = 0.85$, $p < 0.0001$) and dead sperm population (MFI: $r_s = 0.73$, $p = 0.0003$, positive labeled sperm: $r_s = 0.85$, $p < 0.0001$). When live and dead sperm populations were

analyzed, live sperm population exhibited higher ($p < 0.005$) MFI compared to dead sperm population for both antibodies (Table 4.1).

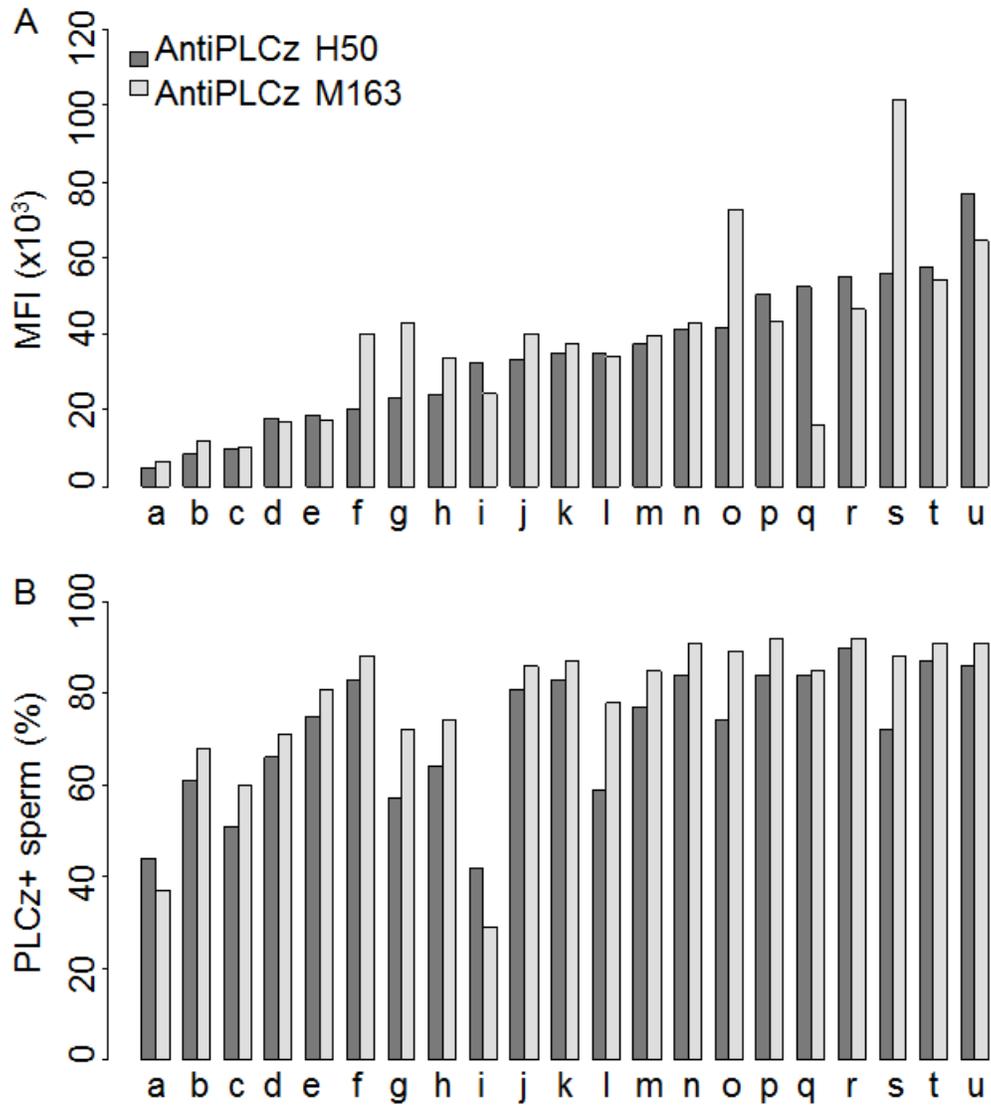


Figure 4.3: Mean fluorescence intensity (MFI) (A) and percentage of positive labeled sperm (B) for PLCz (PLCz+) using antiPLCz H50 and M163 in sperm from 21 stallions (a-u represent individual stallions).

Table 4.1: Means \pm SEM of florescence intensity (MFI) and percentages of sperm labeled with antiPLCz H50 (H50+) and M163 (M163+) for whole sperm, live sperm, and dead sperm populations. ^{a,b}Values with different superscripts for MFI or positive labeled sperm differ ($p < 0.05$) between antiPLCz H50 and M163. *, **Denotes values that differ ($p < 0.05$) for live and dead sperm within a column.

	MFI ($\times 10^3$)		Positive labeled sperm (%)	
	H50	M163	H50+ sperm	M163+ sperm
Whole sperm	35.9 \pm 4.1	37.9 \pm 4.9	72 \pm 3 ^a	78 \pm 4 ^b
Live sperm	43.1 \pm 4.7 ^{**}	45.4 \pm 6.0 ^{**}	65 \pm 3 ^a	72 \pm 4 ^b
Dead sperm	32.1 \pm 4.1 [*]	35.2 \pm 4.2 [*]	82 \pm 3 ^a	86 \pm 3 ^b

Sperm samples were assessed for several parameters (Table 4.2). Percentage of total motility (MOT+) correlated positively with percentages of live sperm (SYBR14+/PI-) ($p = 0.03$), intracellular superoxide levels (E+/YOPRO-) ($p = 0.02$), and high MMP (MMP+) ($p = 0.02$). Percentages of live sperm correlated positively with percentages of sperm without change in membrane permeability (YOPRO-/PI-) ($p < 0.001$), low lipid membrane disorder (M540-/YOPRO-) ($p < 0.001$) and peroxide levels (DCF+/PI-) ($p = 0.007$). Consistently, percentages of sperm with high MMP correlated positively with peroxide levels ($p < 0.001$). Likewise, percentages of viable sperm without changes in membrane permeability correlated positively with sperm with low membrane lipid disorder ($p < 0.001$), peroxide levels ($p < 0.001$) and high MMP ($p < 0.001$). Percentages of sperm with low lipid membrane disorder correlated positively with peroxide levels ($p = 0.005$) and high MMP ($p < 0.001$). Sperm DNA fragmentation did not correlate with other sperm parameters (Table 4.2).

When flow cytometric assessments of PLCz and sperm parameters were analyzed, MFI of M163 tended to be positively associated with progressive motility ($r_s = 0.42$, $p = 0.06$). Sperm DNA fragmentation correlated negatively to PLCz MFI (H50, $r_s = -0.44$, $p = 0.04$; M163, $r_s = -$

0.49, $p = 0.02$) and percentage of positive labeled sperm (H50, $r_s = -0.37$, $p = 0.09$; M163, $r_s = -0.42$, $p = 0.05$) for both antibodies.

Table 4.2: Mean percentages \pm SEM of sperm parameters and correlations among parameters assessed for 21 stallions. Correlation coefficients (r_s) with asterisk (*) were significant at $p < 0.05$. Total motility (TM+), progressive motility (PM+), viable sperm (SYBR14+/PI-), sperm without early changes in membrane permeability (YOPRO-/PI-), sperm with low lipid membrane disorder (M450-/YOPRO-), viable sperm with intracellular production of peroxide (DCF+/PI-) and superoxide (E+/YOPRO-), sperm with high mitochondrial membrane potential (MMP+), and sperm with DNA fragmentation (DNA-).

Sperm parameter	Mean \pm SEM (%)	SYBR14+/PI-	YOPRO-/PI-	M450-/YOPRO-	DCF+/PI-	E+/YOPRO-	MMP+	DNA-
TM+	38 \pm 3	$r_s = 0.48^*$	$r_s = 0.38$	$r_s = 0.44^*$	$r_s = 0.13$	$r_s = 0.49^*$	$r_s = 0.49^*$	$r_s = 0.06$
PM+	32 \pm 3	$r_s = 0.40$	$r_s = 0.33$	$r_s = 0.43$	$r_s = 0.16$	$r_s = 0.54^*$	$r_s = 0.50^*$	$r_s = 0.05$
SYBR14+/PI-	41 \pm 2		$r_s = 0.68^*$	$r_s = 0.76^*$	$r_s = 0.56^*$	$r_s = 0.23$	$r_s = 0.67^*$	$r_s = 0.11$
YOPRO-/PI-	31 \pm 2			$r_s = 0.67^*$	$r_s = 0.70^*$	$r_s = 0.04$	$r_s = 0.68^*$	$r_s = 0.01$
M450-/YOPRO-	26 \pm 2				$r_s = 0.58^*$	$r_s = 0.07$	$r_s = 0.79^*$	$r_s = -0.2$
DCF+/PI-	21 \pm 2					$r_s = 0.12$	$r_s = 0.81^*$	$r_s = -0.1$
E+/YOPRO-	4 \pm 1						$r_s = 0.31$	$r_s = 0.33$
MMP+	33 \pm 1							$r_s = 0.05$
DNA-	8 \pm 1							

DISCUSSION

Semen evaluation is an important part of assessing stallion fertility; good sperm quality is necessary, but not always indicative of fertility. In vivo fertilization is a multiparametric assessment of sperm function and the ultimate assessment of fertility (Barrier Battut *et al.*, 2016, 2017). When performing assisted fertilization by ICSI, many in vivo mechanisms for sperm selection are bypassed; however, sperm must still be capable of activating the oocyte for successful fertilization and embryo development. Under experimental and clinical conditions for ICSI, the stallion and quality of sperm have significant influences on cleavage rates and

subsequent embryo development, independent on stallion field fertility (Choi *et al.*, 2016; Colleoni *et al.*, 2012; Galli *et al.*, 2016; Herrera *et al.*, 2012). In a clinical program, membrane integrity is the sperm parameter most predictive of embryo development and pregnancy (Gonzalez-Castro & Carnevale, 2016). This suggests that a sperm membrane component(s) could be required for oocyte activation and initiation of early embryo development.

Although the use of ICSI has gained popularity in the equine industry, relatively little is known about factors that activate the oocyte. Equine PLCz has been identified as an ~73 kDa protein, localized in the acrosomal region, equatorial segment, connecting piece and principal piece of the sperm tail, with the use of an antibody raised against the N-terminus of porcine PLCz (Bedford-Guaus *et al.*, 2011; Sato *et al.*, 2013). To identify equine PLCz in our study, we tested commercially available antibodies and found two acceptable antibodies of human and mouse origin. We validated that both antibodies targeted PLCz, revealing a reactive band of ~71 kDa in extracts from whole equine sperm and separated heads and tails, with the characteristic localization for PLCz as previously described for equine sperm (Bedford-Guaus *et al.*, 2011; Sato *et al.*, 2013). In the present study, some stallions displayed a smaller second immune reactive band (~60-68 kDa). In men, a band of ~74 kDa was detected as catalytic PLCz isoform; but other PLCz-reactive smaller bands were observed, probably indicating breakdown products of PLCz or unrelated cross-reactive proteins (Aarabi *et al.*, 2012; Grasa *et al.*, 2008; Heytens *et al.*, 2009; Young *et al.*, 2009). Likewise, a band of ~74 kDa was observed in extract from intact mouse sperm, presumably including the postacrosomal PLCz population that contains the full PLCz isoform. However, the full isoform was not detected in the Triton-soluble fraction, displaying a new prominent band at ~55-60 kDa (Young *et al.*, 2009). Our findings using

commercial antibodies for PLCz in the horse are in line with the human and mouse, allowing us to use the commercial antibodies for equine PLCz evaluations.

We also used an antibody of human origin to target PAWP in equine sperm. We identified the presence of two reactive bands of ~28 and 32 kDa in the protein extract from whole stallion sperm and separated heads and tails. Previously, PAWP has not been described for the horse. However, in man, monkey, pig, rabbit and bull, PAWP has been described consistently at ~32 kDa (Aarabi *et al.*, 2014; Kennedy *et al.*, 2014; Wu *et al.*, 2007). A group of distinct SDS-insoluble proteins located in the perinuclear theca have been described in bull sperm, composed of six major proteins of 15, 25, 28, 32, 36 and 60 kDa (Oko & Maravei, 1994; Sutovsky *et al.*, 2003). The 32 kDa protein corresponds to PAWP and is less prominent but the most antigenic protein (Wu *et al.*, 2007). Possibly, the ~28 kDa PAWP-reactive band in our study is a protein of perinuclear theca origin, similar to that found in bull sperm (Oko & Maravei, 1994; Sutovsky *et al.*, 2003); possibly PAWP-breakdown products or unrelated cross-reactive protein. In the present study, PAWP was localized in the acrosomal and postacrosomal region, midpiece and principal piece of the tail in equine sperm. The midpiece and principal piece of the tail localizations of PAWP in equine sperm appear to be novel when compared to other species. When bull sperm were used as a control, PAWP localized exclusively in the post acrosomal region as previously described in others studies for the man, monkey, pig, rabbit and bull sperm (Aarabi *et al.*, 2014; Kennedy *et al.*, 2014; Wu *et al.*, 2007). Our results are in agreement with those found in other species, but PAWP in equine sperm appears to exhibit a more diffuse localization.

The importance of PLCz and PAWP in stallion fertility with fertilization in vivo or in vitro is not known. In one study, PLCz content was compared by western blots for one fertile and

two subfertile stallions, and one of the subfertile stallions had a reduced amount of PLCz (Gradil *et al.*, 2006). In another study, six subfertile stallions were evaluated by immunofluorescence and western blots and exhibited consistently low PLCz expression (Bedford-Guaus *et al.*, 2012). In the present study, the flow cytometric assessment of PLCz using human- and mouse-origin antibodies showed that mean fluorescence intensity and percentage of positive labeled sperm were strongly correlated between both antibodies. These findings indicated that both primary antibodies targeted the same protein, displaying consistent protein levels among individual stallions. We observed large variability in fluorescence levels of total PLCz in sperm from different stallions. Similarly, immunofluorescence levels of PLCz in human sperm exhibit significant variance in total levels of protein and localization patterns among men (Kashir *et al.*, 2013). The findings suggest that individual variation occurs which may be sufficient to impact fertility.

Flow cytometry provides an established method to assess quantitative endpoints for larger populations of sperm than immunofluorescence. Unfortunately, we were not able to use flow cytometry for PAWP in this study; however, flow cytometry for PLCz quantification was successful. However, gene and protein levels of PLCz are positively correlated to PAWP in sperm from fertile and infertile men (Azad *et al.*, 2018; Kamali-Dolat Abadi *et al.*, 2016; M. Tavalae & Nasr-Esfahani, 2016; Tavalae *et al.*, 2016). We had a similar finding in stallions, with a significant correlation between the two proteins in sperm heads. Consequently, although we would like to further develop methods to specifically quantify PAWP by flow cytometry, the quantification of PLCz is suggestive of the PAWP content of a stallion's sperm.

Studies of PLCz and PAWP have been focused on evaluating whole sperm populations, without differentiation of live or dead cells. We found that live sperm exhibited higher PLCz

levels than dead sperm. In our study, several sperm parameters were analyzed using flow cytometry. Live sperm were positively associated with motility, sperm membrane integrity, high mitochondrial membrane potential, and peroxide production. Sperm DNA fragmentation did not correlate with any other sperm population parameter, potentially because sperm samples displayed low values for DNA fragmentation, with 17 out of 21 stallions having DNA fragmentation of <7%. Several studies in men report a significant negative association between high sperm DNA fragmentation, reduced cleavage and implantation rates, and increased miscarriage and pregnancy loss rates after in vitro fertilization and ICSI (Avendaño *et al.*, 2010; Brahem *et al.*, 2011; Morris *et al.*, 2002; Tavalae *et al.*, 2016; Wdowiak *et al.*, 2015; Zini & Sigman, 2009). In horses, sperm DNA fragmentation has also had a negative association with pregnancy rates after in vivo fertilization (Kenney *et al.*, 1995; Love *et al.*, 2002; Morrell *et al.*, 2008). However, sperm DNA fragmentation did not correspond to early fertility outcome after equine ICSI (Gonzalez-Castro & Carnevale, 2016). In the present study, sperm DNA fragmentation was negatively correlated with PLCz fluorescence intensity and percentage of positive labeled sperm, indicating that sperm with high DNA integrity had higher PLCz expression. Consistently, sperm DNA fragmentation relates negatively with the percentage of positive labeled human sperm for PLCz and PAWP (Marziyeh Tavalae *et al.*, 2016, 2017). Conversely, using sperm from fertile men under experimental conditions, PLCz did not relate to sperm DNA fragmentation; but lower expression of PLCz was linked to high DNA oxidation status, suggesting that oxidative stress has a detrimental effect on PLCz expression (Park *et al.*, 2015). Sperm DNA integrity appears to be associated with PLCz expression when samples were assessed for potential fertility.

This is the first report that describes PAWP in equine sperm, and PAWP displayed a novel localization, with the protein located in midpiece and principal piece of sperm tail in addition to the anticipated location at the postacrosomal region. Protein levels of PLCz and PAWP were correlated in sperm. In the present study, commercial antibodies targeted PLCz on equine sperm for localization and quantification studies. The expression of PLCz in sperm exhibited a wide variance among stallions and was associated with DNA integrity. Additional studies are needed to determine the relationship between PLCz and PAWP with fertility outcome, after fertilization in vivo or in vitro.

REFERENCES

- Aarabi, M., Balakier, H., Bashar, S., Moskovtsev, S.I., Sutovsky, P., Librach, C.L., Oko, R., 2014. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. *Fertil Steril* 102, 440–447. <https://doi.org/10.1016/j.fertnstert.2014.05.003>
- Aarabi, M., Yu, Y., Xu, W., Tse, M.Y., Pang, S.C., Yi, Y.J., Sutovsky, P., Oko, R., 2012. The testicular and epididymal expression profile of PLC ζ in mouse and human does not support its role as a sperm-borne oocyte activating factor. *PLoS One* 7, 1–12. <https://doi.org/10.1371/journal.pone.0033496>
- Avendaño, C., Franchi, A., Duran, H., Oehninger, S., 2010. DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. *Fertil Steril* 94, 549–557. <https://doi.org/10.1016/j.fertnstert.2009.02.050>
- Azad, N., Azad, N., Nazarian, H., Novin, M.G., Farahani, R.M., Heidari, M.H., Alitappeh, M.A., Sciences, A., Beheshti, S., Health, R., Stem, U., Diseases, L., 2018a. Oligoasthenoteratozoospermic (OAT) men display altered phospholipase C ζ (PLC ζ) localization and a lower percentage of sperm cells expressing PLC ζ and post-acrosomal sheath WW domain-binding protein (PAWP). *Bosn J Basic Med Sci* xx, 1–7.
- Azad, N., Nazarian, H., Ghaffari Novin, M., Masteri Farahani, R., Piryaei, A., Heidari, M.H., 2018b. Phospholipase C zeta parameters in sperm from polymorphic teratozoospermic men. *Ann Anat* 215, 63–70. <https://doi.org/10.1016/j.aanat.2017.09.007>
- Barrier Battut, I., Kempfer, A., Becker, J., Lebailly, L., Camugli, S., Chevrier, L., 2016. Development of a new fertility prediction model for stallion semen, including flow

cytometry. *Theriogenology* 86, 1111–1131.

<https://doi.org/10.1016/j.theriogenology.2016.04.001>

Barrier Battut, I., Kempfer, A., Lemasson, N., Chevrier, L., Camugli, S., 2017. Prediction of the fertility of stallion frozen-thawed semen using a combination of computer-assisted motility analysis, microscopical observation and flow cytometry. *Theriogenology* 97, 186–200.

<https://doi.org/10.1016/j.theriogenology.2017.04.036>

Bedford-Guaus, S., Yoon, S.Y., Fissore, R.A., Choi, Y.O., Hinrichs, K., 2008. Microinjection of mouse phospholipase C ζ complementary RNA into mare oocytes induces long-lasting intracellular calcium oscillations and embryonic development. *Reprod Fertil Dev* 20, 875–

883. <https://doi.org/10.1071/RD08115>

Bedford-Guaus, S.J., McPartlin, L.A., Varner, D.D., 2012. Characterization of Equine Phospholipase C Zeta: A Review and Preliminary Results on Expression Defects in Subfertile Stallions. *J Equine Vet Sci* 32, 445–450.

<https://doi.org/10.1016/j.jevs.2012.06.002>

Bedford-Guaus, S.J., McPartlin, L.A., Xie, J., Westmiller, S.L., Buffone, M.G., Roberson, M.S., 2011. Molecular Cloning and Characterization of Phospholipase C Zeta in Equine Sperm and Testis Reveals Species-Specific Differences in Expression of Catalytically Active

Protein1. *Biol Reprod* 85, 78–88. <https://doi.org/10.1095/biolreprod.110.089466>

Brahem, S., Mehdi, M., Elghezal, H., Saad, A., 2011. Semen processing by density gradient centrifugation is useful in selecting sperm with higher double-strand DNA integrity.

Andrologia 43, 196–202. <https://doi.org/10.1111/j.1439-0272.2010.01050.x>

Chithiwala, Z.H., Lee, H.C., Hill, D.L., Jellerette-Nolan, T., Fissore, R., Grow, D., Dumesic, D.A., 2015. Phospholipase C-zeta deficiency as a cause for repetitive oocyte fertilization

- failure during ovarian stimulation for in vitro fertilization with ICSI: a case report. *J Assist Reprod Genet* 32, 1415–1419. <https://doi.org/10.1007/s10815-015-0531-1>
- Choi, Y.H., Velez, I.C., Macías-García, B., Riera, F.L., Ballard, C.S., Hinrichs, K., 2016. Effect of clinically-related factors on in vitro blastocyst development after equine ICSI. *Theriogenology* 85, 1289–1296. <https://doi.org/10.1016/j.theriogenology.2015.12.015>
- Colenbrander, B., Gadella, B.M., Stout, T.A.E., 2003. The Predictive Value of Semen Analysis in the Evaluation of Stallion Fertility Stallion Fertility in Practice. *Reprod Dom Anim* 311, 305–311.
- Colleoni, S., Lazzari, G., Duchi, R., Baca Castex, C., Mari, G., Lagutina, I., Galli, C., 2012. Fertilization and development of oocytes after ICSI with semen of stallions with different in vivo fertility. *J Equine Vet Sci* 32, 408–409. <https://doi.org/10.1016/j.jevs.2012.05.030>
- Cox, L.J., Larman, M.G., Saunders, C.M., Hashimoto, K., Swann, K., Lai, F.A., 2002. Sperm phospholipase C ζ from humans and cynomolgus monkeys triggers Ca²⁺ oscillations, activation and development of mouse oocytes. *Reproduction* 124, 611–623. <https://doi.org/10.1530/reprod/124.5.611>
- Dozortsev, D., Qian, C., Ermilov, A., Rybouchkin, A., De Sutter, P., Dhont, M., 1997. Sperm-associated oocyte-activating factor is released from the spermatozoon within 30 minutes after injection as a result of the sperm-oocyte interaction. *Hum Reprod* 12, 2792–2796. <https://doi.org/10.1093/humrep/12.12.2792>
- Durban, M., Barragán, M., Colodron, M., Ferrer-Buitrago, M., De Sutter, P., Heindryckx, B., Vermaeve, V., Vassena, R., 2015. PLC ζ disruption with complete fertilization failure in normozoospermia. *J Assist Reprod Genet* 32, 879–886. <https://doi.org/10.1007/s10815-015-0496-0>

- Escoffier, J., Yassine, S., Lee, H.C., Martinez, G., Delaroche, J., Coutton, C., Karaouzne, T., Zouari, R., Metzler-Guillemain, C., Pernet-Gallay, K., Hennebicq, S., Ray, P.F., Fissore, R., Arnoult, C., 2014. Subcellular localization of phospholipase C ζ in human sperm and its absence in DPY19L2-deficient sperm are consistent with its role in oocyte activation. *Mol Hum Reprod* 21, 157–168. <https://doi.org/10.1093/molehr/gau098>
- Evenson, D.P., Melamed, M.R., 1983. Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. *J Histochem Cytochem* 31, 248–53.
- Galli, C., Colleoni, S., Duchi, R., Lazzari, G., 2016. Male Factors Affecting the Success of Equine In Vitro Embryo Production by Ovum Pickup-Intracytoplasmic Sperm Injection in a Clinical Setting. *J Equine Vet Sci* 43, S6–S10. <https://doi.org/10.1016/j.jevs.2016.05.014>
- Garner, D., Johnson, L., 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol Reprod* 53, 276–284. <https://doi.org/10.1095/biolreprod53.2.276>
- Gonzalez-Castro, R., Carnevale, E., 2016. Association between equine sperm parameters and ICSI outcome. *J Equine Vet Sci* 41, 62. <https://doi.org/10.1016/j.jevs.2016.04.041>
- Gradil, C., Yoon, S., Brown, J., He, C., Visconti, P., Fissore, R., 2006. PLC zeta: a marker of fertility for stallions? *Anim Reprod Sci* 94, 23–25. <https://doi.org/10.1016/j.anireprosci.2006.03.095>
- Grasa, P., Coward, K., Young, C., Parrington, J., 2008. The pattern of localization of the putative oocyte activation factor, phospholipase C ζ , in uncapacitated, capacitated, and ionophore-treated human spermatozoa. *Hum Reprod* 23, 2513–2522. <https://doi.org/10.1093/humrep/den280>
- Guthrie, H.D., Welch, G.R., 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using

fluorescence-activated flow cytometry. *J Anim Sci* 84, 2089–2100.

<https://doi.org/10.2527/jas.2005-766>

- Herrera, C., Dufourq, P., Freije, M., Morikawa, I., Centeno, J.E., Aristi, V., Menghini, L., Sporleder, C., 2012. Selection of stallions for in vitro embryo production by ICSI in a commercial program. *J Equine Vet Sci* 32, 409. <https://doi.org/10.1016/j.jevs.2012.05.031>
- Heytens, E., Parrington, J., Coward, K., Young, C., Lambrecht, S., Yoon, S.Y., Fissore, R.A., Hamer, R., Deane, C.M., Ruas, M., Grasa, P., Soleimani, R., Cuvelier, C.A., Gerris, J., Dhont, M., Deforce, D., Leybaert, L., De Sutter, P., 2009. Reduced amounts and abnormal forms of phospholipase C zeta (PLC ζ) in spermatozoa from infertile men. *Hum Reprod* 24, 2417–2428. <https://doi.org/10.1093/humrep/dep207>
- Ito, M., Shikano, T., Oda, S., Horiguchi, T., Tanimoto, S., Awaji, T., Mitani, H., Miyazaki, S., 2008. Difference in Ca²⁺ Oscillation-Inducing Activity and Nuclear Translocation Ability of PLCZ1, an Egg-Activating Sperm Factor Candidate, Between Mouse, Rat, Human, and Medaka Fish1. *Biol Reprod* 78, 1081–1090. <https://doi.org/10.1095/biolreprod.108.067801>
- Kamali-Dolat Abadi, M., Tavalae, M., Shahverdi, A., Nasr-Esfahani, M.H., 2016. Evaluation of PLC ζ and PAWP expression in globozoospermic individuals. *Cell J* 18, 438–445.
- Kashir, J., Heindryckx, B., Jones, C., de Sutter, P., Parrington, J., Coward, K., 2010. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update* 16, 690–703. <https://doi.org/10.1093/humupd/dmq018>
- Kashir, J., Jones, C., Mounce, G., Ramadan, W.M., Lemmon, B., Heindryckx, B., De Sutter, P., Parrington, J., Turner, K., Child, T., McVeigh, E., Coward, K., 2013. Variance in total levels of phospholipase C zeta (PLC- ζ) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation

- capability. *Fertil Steril* 99, 107–117.e3. <https://doi.org/10.1016/j.fertnstert.2012.09.001>
- Kashir, J., Nomikos, M., Swann, K., Lai, F.A., 2014. PLC ζ or PAWP: Revisiting the putative mammalian sperm factor that triggers egg activation and embryogenesis. *Mol Hum Reprod* 21, 383–388. <https://doi.org/10.1093/molehr/gav009>
- Kavak, A., Johannisson, A., Lundeheim, N., Rodriguez-Martinez, H., Aidnik, M., Einarsson, S., 2003. Evaluation of cryopreserved stallion semen from Tori and Estonian breeds using CASA and flow cytometry. *Anim Reprod Sci* 76, 205–216. [https://doi.org/10.1016/S0378-4320\(02\)00247-6](https://doi.org/10.1016/S0378-4320(02)00247-6)
- Kennedy, C.E., Krieger, K.B., Sutovsky, M., Xu, W., Vargovič, P., Didion, B.A., Ellersieck, M.R., Hennessy, M.E., Verstegen, J., Oko, R., Sutovsky, P., 2014. Protein expression pattern of PAWP in bull spermatozoa is associated with sperm quality and fertility following artificial insemination. *Mol Reprod Dev* 81, 436–449. <https://doi.org/10.1002/mrd.22309>
- Kenney, R.M., Evenson, D.P., Garcia, M.C., Love, C.C., 1995. Relationships between sperm chromatin structure, motility, and morphology of ejaculated sperm, and seasonal pregnancy rate. *Biol Reprod*.
- Kirk, E.S., Squires, E.L., Graham, J.K., 2005. Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. *Theriogenology* 64, 1422–1439. <https://doi.org/10.1016/j.theriogenology.2005.03.006>
- Lee, H.C., Arny, M., Grow, D., Dumesic, D., Fissore, R.A., Jellerette-Nolan, T., 2014. Protein phospholipase C Zeta1 expression in patients with failed ICSI but with normal sperm parameters. *J Assist Reprod Genet* 31, 749–756. <https://doi.org/10.1007/s10815-014-0229-9>
- Liarski, V.M., Kaverina, N., Chang, A., Brandt, D., Yanez, D., Talasnik, L., Carlesso, G.,

- Herbst, R., Utset, T.O., Labno, C., Peng, Y., Jiang, Y., Giger, M.L., Clark, M.R., 2014. Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Transl Med* 6. <https://doi.org/10.1126/scitranslmed.3008146>
- Love, C.C., Thompson, J.A., Lowry, V.K., Varner, D.D., 2002. Effect of storage time and temperature on stallion sperm DNA and fertility. *Theriogenology* 57, 1135–42. [https://doi.org/10.1016/S0093-691X\(01\)00689-6](https://doi.org/10.1016/S0093-691X(01)00689-6)
- Macías-García, B., González-Fernández, L., Gallardo-Bolaños, J.M., Peña, F.J., Johannisson, A., Morrell, J.M., 2012. Androcoll-E large selects a subset of live stallion spermatozoa capable of producing ROS. *Anim Reprod Sci* 132, 74–82. <https://doi.org/10.1016/j.anireprosci.2012.03.017>
- Martin, G., Sabido, O., Durand, P., Levy, R., 2004. Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol Reprod* 71, 28–37. <https://doi.org/10.1095/biolreprod.103.024281>
- McPartlin, L.A., Littell, J., Mark, E., Nelson, J.L., Travis, A.J., Bedford-Guaus, S.J., 2008. A defined medium supports changes consistent with capacitation in stallion sperm, as evidenced by increases in protein tyrosine phosphorylation and high rates of acrosomal exocytosis. *Theriogenology* 69, 639–650. <https://doi.org/10.1016/j.theriogenology.2007.11.016>
- Morrell, J.M., Johannisson, A., Dalin, A.-M., Hammar, L., Sandebert, T., Rodriguez-Martinez, H., 2008. Sperm morphology and chromatin integrity in Swedish warmblood stallions and their relationship to pregnancy rates. *Acta Vet Scand* 50, 2. <https://doi.org/10.1186/1751-0147-50-2>
- Morris, I.D., Illott, S., Dixon, L., Brison, D.R., 2002. The spectrum of DNA damage in human

sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Hum Reprod* 17, 990–998.

<https://doi.org/10.1093/humrep/17.4.990>

Murillo, M.M., Carmona-Cuenca, I., del Castillo, G., Ortiz, C., Roncero, C., Sánchez, A., Fernández, M., Fabregat, I., 2007. Activation of NADPH oxidase by transforming growth factor- β in hepatocytes mediates up-regulation of epidermal growth factor receptor ligands through a nuclear factor- κ B-dependent mechanism. *Biochem J* 405, 251–259.

<https://doi.org/10.1042/BJ20061846>

Nomikos, M., Yu, Y., Elgmati, K., Theodoridou, M., Campbell, K., Vassilakopoulou, V., Zikos, C., Livaniou, E., Amso, N., Nounesis, G., Swann, K., Lai, F.A., 2013. Phospholipase C ζ rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril* 99, 76–85. <https://doi.org/10.1016/j.fertnstert.2012.08.035>

Oko, R., Maravei, D., 1994. Protein composition of the perinuclear theca of bull spermatozoa. *Biol Reprod* 50, 1000–14.

Park, J.H., Kim, S.K., Kim, J., Kim, J.H., Chang, J.H., Jee, B.C., Kim, S.H., 2015. Relationship between phospholipase C zeta immunoreactivity and DNA fragmentation and oxidation in human sperm. *Obstet Gynecol Sci* 58, 232. <https://doi.org/10.5468/ogs.2015.58.3.232>

Peña, F.J., Ortega Ferrusola, C., Martín Muñoz, P., 2016. New flow cytometry approaches in equine andrology. *Theriogenology* 86, 366–372.

<https://doi.org/10.1016/j.theriogenology.2016.04.050>

Ruggeri, E., Deluca, K.F., Galli, C., Lazzari, G., Deluca, J.G., Carnevale, E.M., 2015.

Cytoskeletal alterations associated with donor age and culture interval for equine oocytes and potential zygotes that failed to cleave after intracytoplasmic sperm injection. *Reprod*

- Fertil Dev 27, 944–956. <https://doi.org/10.1071/RD14468>
- Sato, K., Wakai, T., Seita, Y., Takizawa, A., Fissore, R.A., Ito, J., Kashiwazaki, N., 2013. Molecular characteristics of horse phospholipase C zeta (PLC ζ). *Anim Sci J* 84, 359–368. <https://doi.org/10.1111/asj.12044>
- Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., Lai, F.A., 2002. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* 129, 3533–3544.
- Souza Setti, A., Ferreira, R.C., Paes De Almeida Ferreira Braga, D., De Cássia Sávio Figueira, R., Iaconelli, A., Borges, E., 2010. Intracytoplasmic sperm injection outcome versus intracytoplasmic morphologically selected sperm injection outcome: A meta-analysis. *Reprod Biomed Online* 21, 450–455. <https://doi.org/10.1016/j.rbmo.2010.05.017>
- Sutovsky, P., Manandhar, G., Wu, A., Oko, R., 2003. Interactions of sperm perinuclear theca with the oocyte: Implications for oocyte activation, anti-polyspermy defense, and assisted reproduction. *Microsc Res Tech* 61, 362–378. <https://doi.org/10.1002/jemt.10350>
- Swann, K., Lai, F.A., 2013. PLC ζ and the initiation of Ca²⁺ oscillations in fertilizing mammalian eggs. *Cell Calcium* 53, 55–62. <https://doi.org/10.1016/j.ceca.2012.11.001>
- Tavalaee, M., Kiani-Esfahani, A., Nasr-Esfahani, M.H., 2017. Relationship between phospholipase C-zeta, semen parameters, and chromatin status. *Syst Biol Reprod Med* 63, 259–268. <https://doi.org/10.1080/19396368.2017.1298006>
- Tavalaee, M., Kiani-Esfahani, A., Nasr-Esfahani, M.H., 2016. Relationship between potential sperm factors involved in oocyte activation and sperm DNA fragmentation with intracytoplasmic sperm injection clinical outcomes. *Cell J* 18, 588–596.
- Tavalaee, M., Nasr-Esfahani, M.H., 2016. Expression profile of PLC ζ , PAWP, and TR-KIT in

- association with fertilization potential, embryo development, and pregnancy outcomes in globozoospermic candidates for intra-cytoplasmic sperm injection and artificial oocyte activation. *Andrology* 4, 850–856. <https://doi.org/10.1111/andr.12179>
- Vanden Meerschaut, F., Leybaert, L., Nikiforaki, D., Qian, C., Heindryckx, B., De Sutter, P., 2013. Diagnostic and prognostic value of calcium oscillatory pattern analysis for patients with ICSI fertilization failure. *Hum Reprod* 28, 87–98. <https://doi.org/10.1093/humrep/des368>
- Varner, D.D., 2008. Developments in stallion semen evaluation. *Theriogenology* 70, 448–462. <https://doi.org/10.1016/j.theriogenology.2008.04.023>
- Villaverde, A.I.S.B., Fioratti, E.G., Fissore, R.A., He, C., Lee, H.C., Souza, F.F., Landim-Alvarenga, F.C., Lopes, M.D., 2013. Identification of phospholipase C zeta in normospermic and teratospermic domestic cat sperm. *Theriogenology* 80, 722–729. <https://doi.org/10.1016/j.theriogenology.2013.06.005>
- Wdowiak, A., Bakalczuk, S., Bakalczuk, G., 2015. The effect of sperm DNA fragmentation on the dynamics of the embryonic development in intracytoplasmic sperm injection. *Reprod Biol* 15, 94–100. <https://doi.org/10.1016/j.repbio.2015.03.003>
- Wu, A., Sutovsky, P., Manandhar, G., Xu, W., Katayama, M., Day, B., Park, K., Yi, Y., Yan, W., Prather, R., Oko, R., 2007. PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem* 282, 12164–12175. <https://doi.org/10.1074/jbc.M609132200>
- Yelumalai, S., Yeste, M., Jones, C., Amdani, S.N., Kashir, J., Mounce, G., Da Silva, S.J.M., Barratt, C.L., McVeigh, E., Coward, K., 2015. Total levels, localization patterns, and proportions of sperm exhibiting phospholipase C zeta are significantly correlated with

fertilization rates after intracytoplasmic sperm injection. *Fertil Steril* 104, 561–568.e4.

<https://doi.org/10.1016/j.fertnstert.2015.05.018>

Yoon, S.Y., Jellertete, T., Salicioni, A.M., Lee, H.C., Yoo, M., Coward, K., Parrington, J., Grow, D., Cibelli, J., Visconti, P., Mager, J., Fissore, R.A., 2008. Human sperm devoid of PLC, zeta 1 fail to induce Ca²⁺ release and are unable to initiate the first step of embryo development. *J Clin Invest* 118, 3671–3681. <https://doi.org/10.1172/JCI36942>.the

Young, C., Grasa, P., Coward, K., Davis, L.C., Parrington, J., 2009. Phospholipase C zeta undergoes dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction. *Fertil Steril* 91, 2230–2242.

<https://doi.org/10.1016/j.fertnstert.2008.05.021>

Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vásquez-Vivar, J., Kalyanaraman, B., 2003. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: Potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med* 34, 1359–1368.

[https://doi.org/10.1016/S0891-5849\(03\)00142-4](https://doi.org/10.1016/S0891-5849(03)00142-4)

Zini, A., Sigman, M., 2009. Are Tests of Sperm DNA Damage Clinically Useful? Pros and Cons. *J Androl* 30, 219–229. <https://doi.org/10.2164/jandrol.108.006908>

CHAPTER V: CONCLUDING REMARKS

Since the introduction of intracytoplasmic sperm injection (ICSI) as an assisted reproductive technique in horses (Meintjes et al., 1996; Squires et al., 1996), ICSI has become a consistent and repeatable method to produce in vitro equine embryos (Carnevale, 2008; Choi et al., 2016; Galli et al., 2016). In stallions, indications for ICSI include poor sperm quality for standard insemination or limited availability of frozen sperm stores (Carnevale, 2008). For ICSI, the male factor is evident when sperm from some stallions result in consistently high blastocyst rates, while sperm from other stallions consistently result in low or no embryo development, independent on the stallion fertility in the field (Galli et al., 2016). When performing ICSI, many in vivo mechanisms for sperm selection are bypassed. However, the sperm must be capable of activating the oocyte for a successful fertilization and embryo development. As a consequence, specific sperm attributes, such as sperm-borne oocyte activating factors, are essential for ICSI success. The research projects described in this dissertation were designed to study the male factor impact on equine ICSI outcome. We aimed to: 1) analyze the effect of sperm selection and sperm population characteristics in samples from which individual sperm were selected for injection on ICSI outcome, and 2) characterize and quantify sperm oocyte activating factors in stallion sperm, such as phospholipase C zeta (PLCz) and postacrosomal WW binding protein (PAWP).

To date, limited information is available about the influence of sperm processing prior to ICSI and sperm characteristics of the subpopulation from which the individual sperm is selected for ICSI on cleavage, blastocyst formation and pregnancy. Routinely, sperm samples for ICSI are processed to sort a sperm subpopulation with improved characteristics, increasing the

chances of selecting an individual healthy sperm for a successful ICSI. The swim-up procedure and density gradient centrifugation are methods commonly used to sort stallion sperm for ICSI. The development of microfluidic technology offers new possibilities to sort sperm for ICSI, using a centrifugation-free method that prevents the formation of reactive oxygen species, thus protecting the sperm DNA integrity (Aitken and Clarkson, 1988; Barroso et al., 2000). Based on the findings of this present study, the swim-up procedure has a poor efficiency compared to microfluidics sorting and density gradient centrifugation. Microfluidics and density gradient centrifugation are comparable and effective methods to obtain a sperm subpopulation with improved characteristics to be used for sperm sorting prior ICSI. Microfluidics and density gradient centrifugation do not affect cleavage and blastocyst formation under clinical conditions. However, the viability and membrane integrity of sperm subpopulations from which individual sperm were selected for ICSI impacted success of cleavage and pregnancy positively. Sperm membrane integrity is indicative of well-maintained membrane architecture, which potentially conserves membrane components that are required for oocyte activation and early embryo development, such as PLCz and PAWP.

In equine sperm, the importance of PLCz and PAWP in stallion fertility is not definitively known. Based on the findings of this present study, PLCz exhibited similar size and localization as described previously (Bedford-Guaus et al., 2011; Sato et al., 2013). This is the first time that PAWP is identified in stallion sperm, with similar size and localization as described for other species (Aarabi et al., 2014; Kennedy et al., 2014; Wu et al., 2007). However, PAWP displayed a novel localization in the midpiece and principal piece of the tail. In stallion sperm, expression of PLCz and PAWP are associated positively as reported for human sperm (Azad et al., 2018; Kamali-Dolat Abadi et al., 2016; M. Tavalae & Nasr-Esfahani, 2016;

Tavalaee et al., 2016). Flow cytometry allowed quantifying PLCz in equine sperm. In stallion, PLCz expression varies widely among individual stallions and associates negatively with sperm DNA integrity. Flow cytometric assessment for PAWP was not possible. However, both proteins are expressed similarly in stallion sperm. Overall, flow cytometry was a feasible method to assess PLCz content in equine sperm. The wide variability of sperm PLCz expression among stallions, which is potentially linked to PAWP expression and sperm DNA integrity, can relate to fertility potential and sperm quality desirable for ICSI. Quantitative analysis of PLCz in clinical sperm samples for ICSI can be useful as a prognostic biomarker for oocyte activation ability and male infertility.

In ICSI, sperm motility and morphology are used as final selection criterion of individual sperm; this could have negated some of the impact of sperm processing and sperm population characteristics on ICSI outcome. However, normal morphology, viability, membrane integrity, and DNA integrity are correlated parameters. Therefore, selecting an individual sperm based on normal morphological characteristics at the time of ICSI could increase the selection of sperm with high membrane integrity. To assure fertilization after ICSI, the sperm must activate the oocyte. Sperm membrane integrity and membrane components, such as PLCz, play an essential role in sperm fertilizing potential under ICSI conditions. Quantitative assessment of PLCz can be considered as a new directive for sperm analysis to determine the oocyte activation ability in compromised sperm samples for ICSI or in cases of repetitive oocyte activation failure. Further research are needed to: 1) investigate suitable methods for individual sperm selection, 2) evaluate specific attributes in individual stallion sperm that are essential for fertilization and consequent embryo development, and 3) determine the relationship between PLCz and PAWP with sperm fertilizing ability in equine ICSI.

REFERENCES

- Aarabi, M., Balakier, H., Bashar, S., Moskovtsev, S.I., Sutovsky, P., Librach, C.L., Oko, R., 2014. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. *Fertil Steril* 102, 440–447. <https://doi.org/10.1016/j.fertnstert.2014.05.003>
- Aitken, R.J., Clarkson, J., 1988. Significance and Antioxidants of Reactive Oxygen Species in Defining the Efficacy of Sperm Preparation Techniques. *J Androl* 9, 367–76.
- Azad, N., Azad, N., Nazarian, H., Novin, M.G., Farahani, R.M., Heidari, M.H., Alitappeh, M.A., Sciences, A., Beheshti, S., Health, R., Stem, U., Diseases, L., 2018. Oligoasthenoteratozoospermic (OAT) men display altered phospholipase C ζ (PLC ζ) localization and a lower percentage of sperm cells expressing PLC ζ and post-acrosomal sheath WW domain-binding protein (PAWP). *Bosn J Basic Med Sci* xx, 1–7.
- Barroso, G., Morshedi, M., Oehninger, S., 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 15, 1338–1344. <https://doi.org/10.1093/humrep/15.6.1338>
- Bedford-Guaus, S.J., McPartlin, L.A., Xie, J., Westmiller, S.L., Buffone, M.G., Roberson, M.S., 2011. Molecular Cloning and Characterization of Phospholipase C Zeta in Equine Sperm and Testis Reveals Species-Specific Differences in Expression of Catalytically Active Protein1. *Biol Reprod* 85, 78–88. <https://doi.org/10.1095/biolreprod.110.089466>
- Carnevale, E.M., 2008. Clinical Considerations Regarding Assisted Reproductive Procedures in Horses. *J Equine Vet Sci* 28, 686–690. <https://doi.org/10.1016/j.jevs.2008.10.005>
- Choi, Y.H., Velez, I.C., Macías-García, B., Riera, F.L., Ballard, C.S., Hinrichs, K., 2016. Effect

- of clinically-related factors on in vitro blastocyst development after equine ICSI. *Theriogenology* 85, 1289–1296. <https://doi.org/10.1016/j.theriogenology.2015.12.015>
- Galli, C., Colleoni, S., Duchi, R., Lazzari, G., 2016. Male Factors Affecting the Success of Equine In Vitro Embryo Production by Ovum Pickup-Intracytoplasmic Sperm Injection in a Clinical Setting. *J Equine Vet Sci* 43, S6–S10. <https://doi.org/10.1016/j.jevs.2016.05.014>
- Kamali-Dolat Abadi, M., Tavalaeae, M., Shahverdi, A., Nasr-Esfahani, M.H., 2016. Evaluation of PLC ζ and PAWP expression in globozoospermic individuals. *Cell J* 18, 438–445.
- Kennedy, C.E., Krieger, K.B., Sutovsky, M., Xu, W., Vargovič, P., Didion, B.A., Ellersieck, M.R., Hennessy, M.E., Verstegen, J., Oko, R., Sutovsky, P., 2014. Protein expression pattern of PAWP in bull spermatozoa is associated with sperm quality and fertility following artificial insemination. *Mol Reprod Dev* 81, 436–449. <https://doi.org/10.1002/mrd.22309>
- Meintjes, M., Grati, K.J., Paccamonti, D., Eilts, B.E., Co, R., Sullivan, M., Fall, H., 1996. In vitro development and embryo transfer of sperm-injected oocytes derived from pregnant mares. *Theriogenology* 45, 304.
- Sato, K., Wakai, T., Seita, Y., Takizawa, A., Fissore, R.A., Ito, J., Kashiwazaki, N., 2013. Molecular characteristics of horse phospholipase C zeta (PLC ζ). *Anim Sci J* 84, 359–368. <https://doi.org/10.1111/asj.12044>
- Squires, E., Wislon, J., Kato, H., Blaszczyk, A., 1996. a Pregnancy After Intracytoplasmic Sperm Injection. *Theriogenology* 45, 306.
- Tavalaeae, M., Kiani-Esfahani, A., Nasr-Esfahani, M.H., 2016. Relationship between potential sperm factors involved in oocyte activation and sperm DNA fragmentation with intracytoplasmic sperm injection clinical outcomes. *Cell J* 18, 588–596.

Tavalaee, M., Nasr-Esfahani, M.H., 2016. Expression profile of PLC ζ , PAWP, and TR-KIT in association with fertilization potential, embryo development, and pregnancy outcomes in globozoospermic candidates for intra-cytoplasmic sperm injection and artificial oocyte activation. *Andrology* 4, 850–856. <https://doi.org/10.1111/andr.12179>

Wu, A., Sutovsky, P., Manandhar, G., Xu, W., Katayama, M., Day, B., Park, K., Yi, Y., Yan, W., Prather, R., Oko, R., 2007. PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem* 282, 12164–12175. <https://doi.org/10.1074/jbc.M609132200>