THESIS

IMPACT OF EQUINE SPERM PHOSPHOLIPASE C ZETA CONTENT AND SPERM TAIL COMPONENTS ON CLEAVAGE RATES AFTER INTRACYTOPLASMIC SPERM INJECTIONS OF EQUINE AND BOVINE OOCYTES

Submitted by

Fabio Amoroso G. Sanches

Department of Biomedical Sciences

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Master's committee:

Advisor: Elaine Carnevale
Co-Advisor: Jerry Black

Jennifer Hatzel
James K. Graham
Rao Veeramachaneni

ABSTRACT

IMPACT OF EQUINE SPERM PHOSPHOLIPASE C ZETA CONTENT ON CLEAVAGE RATES AFTER ICSI

Intracytoplasmic sperm injection (ICSI) is used in equine assisted reproductive medicine to generate offspring when other procedures fail. However, for some stallions, ICSI is not successful in producing embryos. This could be caused by multiple factors associated with stallion or mare gametes, which result in low cleavage and embryo development rates. Phospholipase C zeta (PLCz) has been identified as a sperm-associated factor that contributes to oocyte activation and is correlated to ICSI success in other species. We hypothesized that sperm population content of PLCz is associated with cleavage after ICSI and that components of the sperm tail, by virtue of containing oocyte activation factors can activate oocytes and induce cleavage.

For the experiments, ICSI was performed using equine sperm with “High” or “Low” PLCz content on bovine oocytes (heterologous model) and equine oocytes to confirm results in the same species. More bovine oocytes ($P=0.04$) and equine oocytes ($P=0.01$) cleaved after injections with sperm having High PLCz content than Low PLCz content (bovine: High, 33/62, 53% and Low, 19/56, 34%; and equine: High, 9/10, 90% and Low 4/12, 33%). The addition of equine sperm tail components to the injection of sperm from a stallion with low PLCz population during ICSI improved ($P<0.05$) cleavage rates of bovine oocytes.

In conclusion, PLCz content of equine sperm population was associated with cleavage rates following ICSI. Bovine oocytes provided a heterologous model to estimate the ICSI potential of an equine sperm population before use with equine oocytes, providing a more feasible and less costly system to evaluate sperm. Components of the equine sperm tail appear to assist activation for sperm from stallions with low PLCz content.

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DEDICATION

This work is dedicated to:

My family for their love, encouragement, and endless support.

To the horses, which inspire me to improve my craft and give me the opportunity to do what I love.

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LITERATURE REVIEW

INTRODUCTION

In horses, assisted reproduction techniques have been used for decades to help produce animals with superior genetics and desired characteristics (Squires *et al.* 1999). Intracytoplasmic sperm injection (ICSI) is an accepted technique in equine reproductive medicine, which is employed in cases when other techniques fail to produce offspring (Hinrichs, 2005).

In equids, as in other farm animal species, oocyte activation is higher when the sperm tail is damaged before injection. This indicates that damage of sperm membranes before injection may increase the exposure of sperm-borne, oocyte activation factors (Keskintepe *et al.* 1997). However, in cases where ICSI fails to produce a zygote, failure of oocyte activation is a potential cause. Chemical oocyte activation has been described in multiple species as a valid technique to increase ooplasm levels of calcium and trigger oocyte activation (Wani, 2008; Borges *et al.* 2020; Che *et al.* 2007). However, there are still concerns to the future health of the offspring when chemical oocyte activation is used (Miller *et al.* 2016). Reagents such as ionomycin, ethanol, and calcium ionophore induce a single large increase in ooplasm calcium levels, which is different from the wave-like increases that occur during regular fertilization (Ferrer-Buitrago *et al.* 2019). Studies in other mammalian species have shown that oocytes that fail to activate retain their capacity to generate and maintain calcium signaling in response to stimuli (Swann *et al.* 2012). This suggests that sperm factors are a cause of failed oocyte activation.

Most information about mammalian oocyte activation, including sperm oocyte activation factors and sperm interactions, are derived from species other than the equid. Although there is the assumption that similar sperm factors are associated with equine oocyte activation, this has not been ascertained. In the present studies, we evaluated the importance of the most widely accepted mammalian sperm-born activation factor, phospholipase C zeta (PLCz), on ICSI success and the importance of sperm-tail content on oocyte activation, as determined by cleavage rates.

EQUINE INTRACYTOPLASMIC SPERM INJECTION

Horses are selected for breeding based on their athletic performance or pedigree in contrast to production animals that are also subjected to selection pressure based on their reproductive traits. The constant practice of breeding selections based on those characteristics and the use of assisted reproductive technologies to overcome poor fertility fitness, result in animals that inherit subfertility traits and equine populations that present low conception rates (Nath *et al.* 2010). Assisted reproductive techniques, such as artificial insemination and embryo transfer, have been used for many decades in horses to overcome those fertility problems; but every technique has limitations. The success of more widely used techniques such as artificial insemination, which is responsible for production of 90% of Standardbred foals (Nath *et al.* 2010), can be limited by problems of the tubular genitalia. Equine intracytoplasmic sperm injection (ICSI) was first performed in the mid-1990s but was not commercially available until the early 2000s (Squires *et al.* 2003). With the availability of ICSI, other fertility problems commonly associated with some mares and stallions could be bypassed and embryos

produced (Carnevale, 2008). Intracytoplasmic sperm injection has become a useful tool to preserve valuable genetics, to produce foals when sperm availability is limited, or to use when a subfertile mare fails to generate embryos in vivo. Although ICSI can be used when sperm numbers or quality is limited (Choi *et al.* 2016), the success of ICSI is still dependent on sperm factors (Stout *et al.* 2018). In clinical ICSI programs, variability among stallions and mares make studying causes of ICSI failure difficult. A stallions' breed, in vivo fertility, method of sperm preparation, and sperm differences among collections influence cleavage rates and blastocyst formation (Galli *et al.* 2016). However, specific factors associated with equine sperm that are critical for ICSI success have not yet been determined.

Sperm for ICSI

Fresh, Cooled, Frozen and Refrozen Sperm

On the final phases of the spermatogenesis, spermatozoa lose biosynthesis ability which simplify the metabolic function of those cells. This make the natural demise of those cells easily delayed or prevented by low temperatures (Hammerstedt, 1993). Intracytoplasmic sperm injection can be performed using fresh, cooled, frozen, or refrozen semen. When available, fresh or cooled semen may be preferred for ICSI. Because of stallion sperm variability, standard freezing protocols that efficiently work for different stallions have not been developed (Rodríguez *et al.* 2011; Squires at al., 2004), and the success of freezing protocols varies among stallions (Graham, 1996). The success of ICSI seems to be higher when fresh or cooled semen is used (Carnevale, personal communication). However, frozen semen for ART use has the advantage of

easy storage and ready availability, making its use more convenient when compared to fresh or cooled semen that has to be collected and often shipped to the ICSI laboratory in synchrony with the mare oocyte collections. Furthermore, in many cases, only frozen semen is available for clinical use. Semen that is of high value, but low availability, can be preserved by either refreezing previously thawed sperm that were not selected for injection or by diluting and refreezing a single straw into ICSI-dose straws. This process maximizes the use of sperm and raises the number of possible offspring that can be produced (Choi *et al.* 2006). However, the freezing process damages sperm and, when repeated, sperm fertility parameters can decrease substantially (Gonzalez-Castro *et al.* 2016; Leisinger *et al.* 2017). When frozen or refrozen sperm does not achieve desirable results, failure of oocyte activation is the probable cause (Ruggeri *et al.* 2015) with oocytes not cleaving after ICSI (Tremoleda *et al.* 2003). The failure of an oocyte to cleave is most probably caused by the loss of sperm activation factors during the cryopreservation process (Choi *et al.* 2006). In humans, sperm cryopreservation has been shown to decrease PLCz content by 20 – 56% in sperm of some donors (Kashir *et al.* 2011). Therefore, new methods are needed to either optimize equine sperm freezing for ICSI or to improve oocyte activation when using frozen or refrozen sperm.

Stallion variability

Reproductive work on subfertile stallions can be challenging, and ICSI can be the only technique for successful outcomes (Lazzari *et al.* 2002). Stallion variability in ICSI is recognized in clinical programs, although results from studies are mixed, perhaps because of the low number of stallions compared in most published studies. Stallions

with normal sperm motility and morphology can have poor ICSI results (Choi *et al.* 2016; Galli *et al.* 2016). When post-thaw motility and field fertility were correlated with cleavage, morula and blastocyst formation, only sperm with less than 10% of post-thaw motility and no field fertility resulted in poor cleavage rates after ICSI (Lazzari *et al.* 2002). An analysis of 564 ICSI sessions was performed in a commercial program, with semen from 18 stallions; for 16 stallions, there was no significant difference on blastocyst formation per oocyte injected. Blastocyst rates were clustered from 6 to 19% for the stallions, although a range of 4% to 27% was observed for the whole group (Stout *et al.* 2018). When fresh semen was collected at the time of ICSI, one stallion in a group of seven had lower cleavage rates on oocytes from slaughter ovaries (Herrera *et al.* 2012). In another study, frozen-thawed sperm population parameters were compared with ICSI outcome. Sperm population morphology and DNA integrity did not correlate with oocyte cleavage, embryo development, or pregnancy. However, the live/dead percentage and hypoosmotic swelling test were good predictors for cleavage (Gonzalez-Castro and Carnevale, 2018). In another study, levels of PLCz on human sperm were compared before and after cryopreservation. Sperm that were frozen had PLCz levels decreased, particularly at the post-acrosomal region (Moreau, 2019). This indicates that plasma membrane integrity may be related to the ability of sperm to induce oocyte activation after ICSI.

Selection criteria

For each ICSI injection, a single sperm is selected. The initial sperm selection will influence ICSI outcomes (Choi *et al.* 2016). Techniques can be used which help select

sperm with good morphology, viability, membrane integrity, and low DNA fragmentation (Gonzalez-Castro and Carnevale, 2019). Semen selection for equine ICSI has been done using a swim-up procedure, density gradient centrifugation, microfluidic devices, or a combination of the methods, depending on preference and semen quality. The swim-up method consists of placing sperm in the bottom of a conical tube with a layer of medium on top into which motile sperm will swim upwards. The supernatant that contain only sperm capable of swimming is collected for ICSI (Choi *et al.* 2004). Density gradient centrifugation consists of layering the initial sperm sample over a layer of colloidal solution in a conical tube. The tube is centrifuged, and the pellet of sperm from the bottom of the tube, which most has the capability of swimming forward, is washed to remove the colloidal solution and used for ICSI (Stoll *et al.* 2013). Sperm sorting by microfluidic separation involves using a microfluidic chip for isolation of healthy sperm by laminar flow, creating gradients through microchannels and chambers (Gonzalez-Castro and Carnevale, 2019). All the techniques commonly used to select ICSI sperm are primarily based on motility, although they can result in the co-selection of other desirable characteristics within the selected subpopulation of sperm. For instance, sperm motility is associated with normal membrane function. The initial selection of sperm does not differentiate many parameters that are known to influence ICSI outcomes and does not eliminate unviable sperm.

After the initial selection, the sperm will be imaged, typically at 200x magnification for equine ICSI, in a drop of viscous medium often containing polyvinylpyrrolidinone (PVP) to decrease motility and facilitate selection. Individual sperm cells are selected for ICSI by the technician based on normal morphology and

progressive motility (Carnevale and Sessions, 2012). Although sperm selection techniques are efficient to select subpopulations with better sperm parameters, the individual sperm picked up for ICSI may still not be perfect. This final selection based on motility and morphology can fail to select sperm with necessary factors and, in some cases, lead to failed ICSI results.

OOCYTE ACTIVATION

During fertilization, oocytes must undergo a series of intracellular events that initiate further development, such as a block to polyspermy and a signal to initiate development (Boron and Boulpaep, 2009). The fertilizing sperm is responsible for delivering some of these signals. Sea urchin, fish, and frog oocytes have a single large calcium increase in the ooplasm, at the point of sperm entry which then spreads through the ooplasm (Stricker, 1999). The main change observed in the mammalian oocyte at fertilization is a rise of calcium followed by multiple wave-like oscillations of calcium concentration in the oocyte cytoplasm (Miyazaki, 1988). This calcium release lasts several hours, and calcium oscillation patterns vary, depending on species. The calcium concentration and the intensity of the oscillations are modulated by calcium channels located in the endoplasmic reticulum. (Boulware and Marchant, 2005). The frequency of calcium oscillations tends to be lower in species with larger oocytes, such as cows and pigs (Fissore *et al.* 1992; Macháty *et al.* 1997). In mice and hamster oocytes, the initial calcium rise originates at the site of sperm-oocyte fusion, but calcium oscillations increase in frequency and potency as more waves are generated throughout the ooplasm (Swann and Ozil, 1994). Changes in wave profiles potentially result from a

change in the cytoplasm from ‘non-excitable’ to ‘excitable’ upon fertilization. Calcium injected into unfertilized hamster oocytes does not promote new calcium waves; but calcium injection, even in low doses, triggers substantial waves when the oocytes have been fertilized (Ozil and Swann, 1995). These calcium waves are identified as a major component of oocyte activation, and researchers have started to study which sperm components are responsible for triggering this calcium release in the fertilized oocyte.

Oocyte activation concepts

Mechanisms

Two hypotheses have been proposed to explain how sperm initiate calcium oscillations during fertilization. One hypothesis is that sperm binding to an oolemmal surface receptor mediates the ooplasm response, the other hypothesis is that sperm introduce molecule(s) after gamete fusion that act within the ooplasm to trigger the calcium response (Stricker, 1999). The oolemmal receptor hypotheses was initially accepted, prior to the discovery of sperm-associated molecules that can trigger calcium release in the ooplasm. Recently, successful ICSI demonstrates that sperm interaction with the oolemma is not required for oocyte activation and embryo development (White *et al.* 2010).

The receptor-mediated hypothesis proposes that a transduction signal of a receptor located on the oocyte plasma membrane binds to a protein tyrosine kinase or G-protein complex. This activates phospholipase C and induces hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) into diacylglycerol and inositol triphosphate (IP3). IP3 then binds to its receptors on the endoplasmic reticulum to promote the transit

of calcium into the cytoplasm (Fissore and Robl, 1994). Potentially, integrins are involved in the sperm-oocyte binding process; however, no specific receptors have been identified (Almeida *et al.* 1995).

Alternatively, the sperm-factor hypothesis has gained support due to the relatively long latent period from the initial sperm-oolemma contact to the start of calcium oscillations. This period would be compatible with the time necessary for the diffusion of a soluble sperm-supplied molecule into the ooplasm.

Oocyte activation is fundamental for proper embryo development. The most broadly studied mechanism in oocyte activation following fertilization is calcium signaling. It has been shown that insufficient or inconsistent calcium waves lead to failure of oocyte activation. Experimentally blocking calcium inhibits cortical granule exocytosis, calcium transients, second polar body formation, and initiation of oocyte development (Kline and Kline, 1992).

Potential candidates

Researchers have tried for decades to identify a molecule responsible for oocyte activation. While the results from earlier studies showed that calcium rise is fundamental for proper oocyte activation and embryonic development, what triggers the calcium release in the oocyte was not clear (Jaffe, 1983). Initial studies on receptor-mediated sperm activation were done in model species to identify sperm ligands and oocyte receptors that would be responsible for the initiation of calcium modulations. The identification process was done by trying to discover sperm ligands and oolemal receptors that would generate calcium transients via signaling cascades involving G-

proteins, integrins, and/or tyrosine kinases. The external application of P23, a sperm acrosomal peptide, resulted in increased cytoplasmic calcium levels in *Urechis caupo* oocytes (Stephano and Gould, 1997). A similar increase in intracellular calcium was observed by the external use of metalloprotease/disintegrin/cysteine-rich sperm protein to frog eggs (Shilling *et al.* 1998); but the effect was not replicated in mammalian oocytes, indicating that a different mechanism is involved for mammalian oocyte activation.

An “oscillin” protein was proposed as an oscillogen candidate for a mammalian sperm-factor oocyte activator (Parrington *et al.* 1996), but further research demonstrated that oscillins are not responsible for mobilizing calcium (Wolosker *et al.* 1998). Other molecules historically investigated as possible sperm-born oocyte activators included different forms of phospholipase C (PLC), calcilin and C-kit. However, although calcium waves were observed, cleavage did not occur. This happens when candidate molecule concentrations necessary to induce oocyte activation do not match those found in a single sperm, ruling them out as a sperm-born activation factor. However, the mechanism by which sperm causes the release of calcium was determined to be through stimulating IP3 production (Jones *et al.* 2000), indicating that a PI-specific PLC is likely involved in the mechanism of signal transduction (Rice *et al.* 2000). This led a group of researchers to propose a newly discovered PLC isoform named phospholipase C zeta (PLCz), as a sperm factor responsible for triggering the cytosolic calcium oscillations in mammal oocytes (Saunders *et al.* 2002). PLCz at concentrations associated with a single sperm were shown to promote calcium oscillations and normal embryo development to the

blastocyst stage in mouse oocytes. Moreover, sperm treated with an anti-PLCz antibody failed to trigger calcium release and induce oocyte activation (Saunders *et al.* 2002). However, some studies suggest while PLCz importance for oocyte activation is clearly high, it may not be the only sperm oocyte activation factor. This conclusion is based on the fact that knock out PLCz sperm is not capable of inducing calcium release in the oocyte after ICSI but triggers a small calcium release after IVF (Jones, 2018). Recent studies have confirmed the importance of this molecule to oocyte activation in multiple species, but very little has been done in horses.

PHOSPHOLIPASE C ZETA: STRUCTURE AND LOCALIZATION

Phospholipase C zeta is the smallest PLC enzyme found in mammals and is the only PLC that does not have a Pleckstrin homology domain (Amdani *et al.* 2013). Phospholipase C zeta is a testis-specific PLC that is expressed in sperm and depending on the species, PLCz mRNA can be present in a range of spermatogenic phases (Aarabi *et al.* 2012). In the equine species PLCz protein is expressed during spermatogenesis to the round spermatids (Bedford-Guaus *et al.* 2011). The capacity of PLCz to release calcium in the ooplasm is 100 times greater than PLC delta (Everett and Katan, 2016). In human sperm, PLCz is located primarily in the equatorial region of the sperm head, although it is also found in the acrosomal and post-acrosomal regions in some sperm (Grasa *et al.* 2008). After capacitation, human sperm tend to have more post-acrosomal PLCz immunoreactivity (Grasa *et al.* 2008). Mouse and hamster sperm share the same PLCz localization pre- and post-capacitation as does human sperm (Young *et al.* 2009). In stallion sperm, PLCz contains 1914 base pairs that translate to a

~73 kDa protein and is located in the equatorial segment, head-midpiece junction, and principal piece of the flagellum (Bedford-Guaus *et al.* 2011). Despite current and past efforts to validate PLCz as a sperm-oocyte activation factor, some researchers don't agree that PLCz meets the requirements to be considered one (Aarabi *et al.* 2012). Further research is necessary to understand better how PLCz impacts oocyte activation mechanisms and identify other sperm molecules that may be involved in this process.

Mechanism of action

The proposed mechanism by which PLCz induces oocyte activation is that the sperm PLCz is released into the ooplasm immediately after the sperm-oolema fusion. PLCz hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2), generating diacylglycerol (DAG), and inositol 1,4,5-triphosphate (IP3), which then binds to its own receptor (IP3R) in the membrane of the endoplasmic reticulum. When IP3 binds to IP3R, calcium is released from the endoplasmic reticulum, generating the initial calcium rise in the ooplasm. After the initial calcium rise, wave-like calcium oscillations are generated and continue until IP3 receptors are down-regulated by increased calcium concentrations (Anifandis *et al.* 2016).

Relevance to male fertility

After its discovery, multiple studies were done to investigate the possible impact of PLCz on male fertility, most studies were performed on human or mouse sperm as a model. Sperm from men with repeated ICSI cycle failures were analyzed and found to have mutated PLCz. Abnormal PLCz was linked to infertility conditions caused by

abnormal or a complete absence of PLCz in morphologically normal or abnormal sperm (Heytens *et al.* 2009; Kashir *et al.* 2012). A comparison of PLCz at RNA and protein levels in sperm from couples going under ICSI trials suggested that low levels of PLCz in sperm with globozoospermia may be the cause of low fertilization rates when this condition is present (Tavalaee *et al.* 2018). In pigs, percentage of sperm positively labeled for PLCz and cleavage rate after ICSI were higher after Percoll gradient centrifugation, indicating PLCz levels are correlated to ICSI outcomes for this species (Nakai *et al.* 2016). In horses, the concentration of PLCz was shown to be lower in sperm from subfertile stallions with morphologically "normal" sperm; and PLCz level has been suggested as a possible stallion fertility indicator (Gradil *et al.* 2006). When examined using flow cytometry, the percentage of sperm cells with intact membranes was positively correlated with the percentage of sperm positively labelled for PLCz in stallion sperm (Gonzales-Castro *et al.* 2017), suggesting that PLCz presence is correlated to the presence of viable sperm. The percentage of equine sperm positively labeled for PLCz can vary widely among samples designated for ICSI, suggesting that PLCz levels could impact success rates in this technique (Gonzalez-Castro and Carnevale, 2018).

ICSI

Intracytoplasmic sperm injection is widely used in human reproductive medicine (Boulet *et al.* 2015). For couples with fertility problems, ICSI can higher their chances of obtaining a pregnancy depending on which fertility problems are involved. In vitro fertilization methods are used for many cases of male infertility, such as when sperm

count is low (Johnson *et al.* 2013). However, 1 to 5% of oocytes fail to activate after ICSI (Liu *et al.* 1995). Typically, ten human oocytes are available for an ICSI attempt on each cycle, which make cases of total fertilization failure relatively rare. In such cases, options are limited and may include methods such as chemical activation of oocytes using a calcium ionophore; however, this is a concern for potential health side effects (Deemeh *et al.* 2015). Financial restrictions from the couple undergoing an ICSI program may also play a role to determinate how many unsuccessful cycles can be done before an approach of oocyte chemical activation is taken in consideration. A model used to research methods to rescue oocytes that fail to activate, is to perform ICSI in human and mouse oocytes with heat-treated human sperm. Heat causes the loss of sperm capacity to generate calcium oscillations. Previous research has shown that calcium ionophores or recombinant human PLCz can be used to promote activation of murine oocytes with similar cleavage rates, although the development to blastocyst is higher after using the recombinant protein (Sanusi *et al.* 2015). PLCz injection offers an alternative to calcium ionophores and are a more physiological stimulus to rescue oocytes that fail to activate. A similar study, also using human recombinant PLCz but no sperm, showed that both human and murine oocytes expressed calcium waves in a dose-dependent manner after PLCz injections. When U73122, a PLC inhibitor was used in conjunction, oocytes failed to activate (Yoon *et al.* 2012). This indicates that PLCz can, by itself, trigger the physiological mechanisms that lead to oocyte activation.

HYPOTHESIS

Oocyte activation is a necessary event for fertilization, which is initiated by an increase in ooplasmic calcium levels. This event is triggered by sperm oocyte activation factors, including PLCz, and is followed by characteristic ooplasmic calcium oscillations in a species-specific manner. Recent studies have linked low cleavage rates and infertility with abnormal or absent PLCz on mammalian sperm (Heytens *et al.* 2010; Saunders *et al.* 2002), we hypothesized that the concentration of sperm positively labeled for PLCz in a population of equine sperm can be a predictor of cleavage rate after equine ICSI. For this study, we used equine sperm populations with high or low concentrations of PLCz for ICSI of equine oocytes and bovine oocytes, as a heterologous model, to assess stallion fertility for ICSI. Finally, we hypothesized that components of the sperm tail contain factors that can be used to improve oocyte activation after ICSI.

MATERIALS AND METHODS

Experimental design

In Experiment 1, bovine and equine oocytes were injected with stallion sperm from populations with “High” or “Low” PLCz content to determine the impact on cleavage rates. In Experiment 2, bovine oocytes were injected with sperm considered “Low” PLCz, as in Experiment 1, with the addition of sperm tail components to determine if cleavage rates would be improved.

Media for bovine oocytes

Unless stated otherwise, all chemicals for bovine media were obtained from Millipore Sigma (St. Louis, MO, USA). Chemically defined media (CDM) was prepared at Colorado State University (CSU, Fort Collins, CO). Media used for bovine in vitro maturation (IVM), oocyte handling (H-CDM-M), in vitro maturation (IVM), embryo handling (H-CDM-1), and embryo culture (CDM-1) were made as published (Olson and Seidel, 2000), with modifications (Torre-Sanchez *et al.* 2006).

Collection of bovine and equine oocytes

Bovine ovaries were obtained from a local abattoir, rinsed with saline and transported to the laboratory within 1 to 2 h in an insulated container with saline at 24 ± 1$_{o}$C. Cumulus oocyte complexes (COCs) were aspirated from 2 - 8 mm follicles using

an 18-G needle attached to a vacuum pump (Rocket® Craft Suction Pump; Rocket Medical, Washington, Tyne and Wear, UK) set at -175 mm Hg. Cumulus oocyte complexes were identified and graded according to cumulus cell and ooplasm morphology. Only oocytes with compact cumulus cells and normal morphology were used for the project. The COCs were rinsed twice in H-CDM-M and transferred into four-well dishes (Nunc, Thermo Scientific, Waltham, MA) containing 500 μL of bovine IVM medium per well. The COCs were incubated at 5% CO_2 in air at 38.5^{o}C for 23 ± 1 h. After maturation, COCs were denuded by vortexing for 60 seconds in H-CDM-1, and oocytes with normal morphology and extruded polar body were used for intracytoplasmic sperm injection (ICSI) with equine sperm.

Equine oocytes were obtained from light-horse mares that were housed in dry lot paddocks with access to covered shelters and provided grass-alfalfa hay, mineral blocks, and water ad libitum. All procedures with animals were performed in accordance with the University's IACUC protocols. Mares' reproductive tracts were monitored by transrectal ultrasonography to determine the phase of their reproductive cycle and to determine follicular growth. Mares that were in the follicular phase and had one or more follicles ≥ 35 mm in diameter were administered human chorionic gonadotropin (hCG; 2000 IU, intravenous; Chorulon, Merck Animal Health, Madison, NJ) and deslorelin acetate (0.75mg, intramuscular; Precision Pharmacy, Bakersfield, CA). Oocytes were collected from the mares approximately 24 h after administration of induction drugs by ultrasound-guided, transvaginal follicular aspirations (Carnevale, 2016). Collected oocytes, with expanded cumulus, were cultured in medium (TCM-199; Gibco BRL Life Technologies, Grand Island, NY, USA) containing 10% fetal calf serum (FCS; Cell

Generation, Fort Collins, CO, USA), 0.2 mM pyruvate, and 25 µg mL^{-1} gentamicin (Sigma) at 38.2°C in an atmosphere of 7% CO_2 and air. If cumulus expansion was not observed at collection indicating the oocyte was not mature, oocytes were cultured in the same maturation medium, but with the addition of 1 µg/ml luteinizing hormone, 15 ng/ml follicle stimulating hormone (NHPP; UCLA Medical Center, Torrance, CA) 50 ng/ml epidermal growth factor, 100 ng/ml insulin-like growth factor 1, 1 µg/ml estradiol, 500 ng/ml progesterone (Sigma). Oocytes were denuded from cumulus cells 44 ± 1h after administration of deslorelin and hCG using hyaluronidase at 200 IU/ml. Oocytes with a polar body were injected with equine sperm.

Selection of equine sperm

Prior to this experiment, sperm from stallions (n=21) were assessed by flow cytometry for PLCz content based on mean fluorescence intensity (MFI) and percentage of sperm positive labeling for PLCz. Frozen-thawed samples were washed in phosphate buffered saline (PBS) by centrifugation, antiPLCz H50 was added as primary antibody (1:100) and incubated overnight at 4°C. Samples were then washed in PBS and incubated for 60 min with secondary antibody Alexa Fluor®488 at 1:200 in room temperature (Gonzalez-Castro *et al.* 2017). For this experiment, four stallions were selected from the larger group. These stallions' sperm had similar fertility indicators (>70% normal morphology, <8% DNA fragmentation as based on flow cytometry using the Sperm Chromatin Structure Assay, and 41 ± 15% of viability assessed using SYBR14+/PI–). Sperm from two stallions were considered to have high PLCz (High; Stallion 1, 87% of sperm positively labeled for PLCz with 10,384 of Mean

Fluorescence Intensity and Stallion 2, 84% with 10,784 MFI). Sperm from an additional two stallions had low PLCz (Low; Stallion 3, 56% of sperm positively labeled for PLCz with 4,789 MFI and Stallion 4, 59% with 5,360 MFI). Prior to flow cytometry, western blotting and immunofluorescence were performed to validate antibody binding and to identify PLCz as a 71 kDa protein in stallion sperm, located in the acrosomal and post-acrosomal region, and the tail (Gonzalez-Castro *et al.* 2017).

Intracytoplasmic sperm injection and embryo culture

Prior to ICSI, approximately one-tenth of a 0.5-ml straw of frozen semen was cut under liquid nitrogen and thawed directly in 1 ml of a medium buffered for room atmosphere (GMOPS; Vitrolife, Gothenburg, Sweden) with 0.4% bovine serum albumin (BSA) at 38.2°C. A 5-µl droplet from the sperm dilution was placed in the ICSI plate; individual sperm were selected at 200X based on the subjective evaluation of normal morphology and progressive motility. Selected sperm were placed into a second droplet of GMOPS with 3.8 mM calcium lactate and 5% polyvinylpyrrolidone (PVP) and received a short pulse at its midpiece using the Piezo drill.

For ICSI or sham injections, the oocyte was held in a 30-µl drop of GMOPS with 0.04% BSA, and the polar body was positioned at 12 o'clock. For sperm injections using High or Low with bovine and equine oocytes, stallions were rotated by ICSI session. The order of use for each stallion was randomly selected for the initial four ICSI cycles,

alternating between High and Low for subsequent injections, the same order was repeated. For bovine sham injections, oocytes were injected with GMOPS only and no sperm, to determine the rate of parthenogenetic cleavage.

Injected bovine oocytes were placed into culture wells containing 500 mL of equilibrated CDM-1. Embryo culture was performed at 38.5°C in 5% CO_2, 5% O_2, and 90% N_2.

After ICSI, presumptive equine zygotes were placed into embryo culture medium (Global®; LifeGlobal Group, Guilford, CT, USA) in 30-µL drops under oil (OVOIL™; Vitrolife) and incubated at 38.2°C in 7% CO_2, 5% O_2, and 88% N_2. Presumptive zygotes were assessed for cleavage (≥ two apparent cells) at 24 ± 2 h after sperm injection and rechecked at approximately 48 h to confirm if additional sperm-injected oocytes had cleaved.

Sperm tail component additions

Frozen semen from one ejaculate of a single, fertile stallion was used for the preparation of all samples. Sperm were frozen in 0.25-ml straws using BotuCrio® (BotuPharma, Botucatu, Brazil) at 200 million sperm/ml and thawed at 36 °C. Two types of samples were prepared for ICSI. The first was composed of a blend of refrozen sperm tail components (ST Blend) and the second was composed of a snap-frozen and filtered sperm tail blend (ST Filtrate). For the preparation of ST Blend, 2 straws of semen were diluted in 8 ml of medium (Vigro Complete Flush, Vetoquinol, Princeville,

QC, CA) and centrifuged at 500 g for 8 min. The supernatant was discarded, and the pellet was diluted with 200 μl of GMOPS in a 0.6-ml microcentrifuge tube and sonicated with an ultrasonic probe (Branson Sonifier 450, Branson Ultrasonics Corporation, Danbury, CT) for 10 cycles at power 3 and 30% of pulsar, resulting in sperm decapitation without heat or defragmentation of heads and tails. Contents of the tube were transferred into a 1.5 ml microcentrifuge tube and centrifuged (Minispin Plus Centrifuge, Eppendorf, Hamburg, Germany) for 2 min at 1000 g. The supernatant was removed and placed in a new tube containing a bottom layer of 200 ul of GMOPS with the addition of 5% PVP and centrifuged for 3 min at 1300 g. After centrifugation, the top layer was removed and aliquote into microcentrifuge tubes for storage.

For the ST Filtrate, the same procedures were used with some exceptions. Four straws of semen were used per separation. Before aliquoting and freezing, the tail blend was snap-frozen in liquid nitrogen and thawed three times. The blend was then passed through a 0.22-μl media filter (Millex-GV, Merk Millipore LTD, Tullagreen, Carringtwohill, Co. Cork, IRL) and aliquoted. All samples were stored at -80°C.

ST Blend and Filtrate were used as additions during ICSI with bovine oocytes. A single sperm from one low PLCz stallion (Stallion 3, Per first experiment), as used in the previous experiment, was injected per oocyte. For control injections (Control), ICSI was performed as previously described on bovine oocytes, with the exception that the GMOPS with 5% PVP was frozen and thawed prior to use to simulate conditions for ST Blend or Filtrate. For ST Blend injections, a single sperm was immobilized and placed within a 5-μl drop of ST Blend. The sperm was then pulled into the injection pipette, and injected into the oocyte with ST Blend, with approximately the length of the sperm plus

two sperm-heads in the injection pipette of ST Blend. A third group of sperm injections were done using similar methods with ST Filtrate.

Statistical analysis

Cleavage rates of equine oocytes were compared using Fisher's exact test. Cleavage rates of bovine oocytes were compared using chi-square test.

Results

The overall cleavage rate differed ($P<0.0001$) for bovine oocytes (n=161) after sham injections (0/43) or injections with equine sperm classified as High (33/62, 53%) or Low (19/56, 34%). More ($P=0.04$) bovine oocytes cleaved after injections with High than Low (Figure 1). For equine oocytes injected with High or Low, cleavage rates differed ($P=0.01$) between groups (High, 9/10, 90% and Low, 4/12, 33%) (Figure 1). Sham injections were not performed with equine oocytes for this experiment, because mechanical manipulation of the oocytes during sperm injection have not been reported as a cause of parthenogenetic activation for this species.

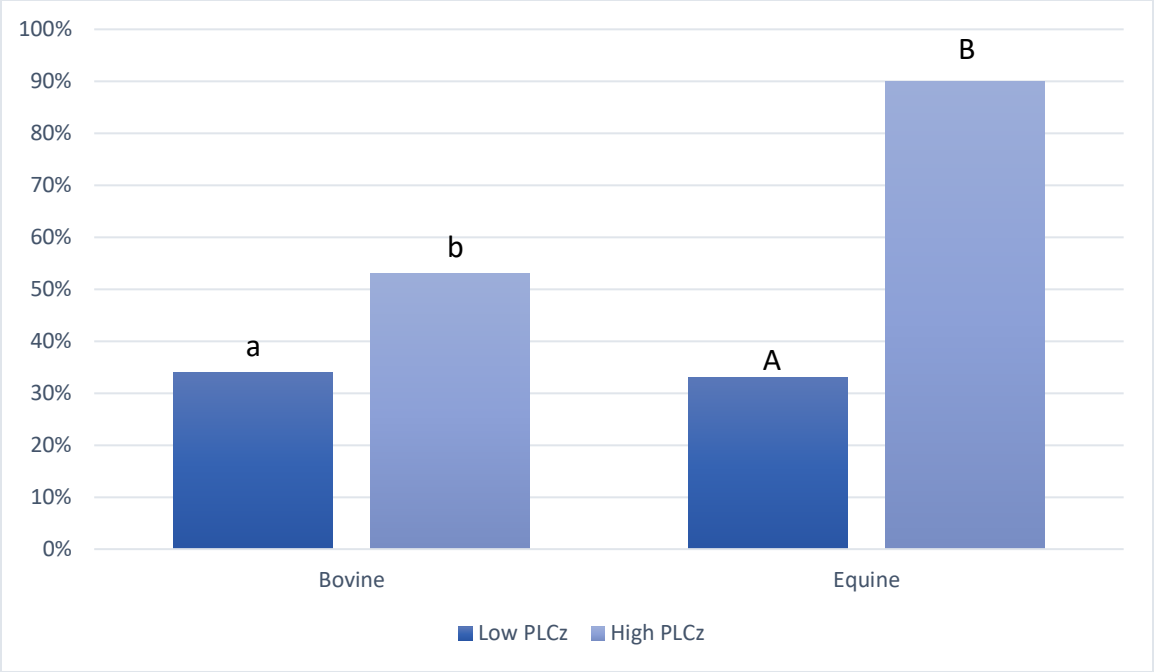

Figure 1. Percentages of bovine and equine oocytes that cleaved after injection with equine sperm with Low or High PLCz. Percentages for bovine oocytes$_{(a,b)}$ or equine oocytes$_{(A,B)}$ with different superscripts differ at $P<0.05$.

The overall cleavage rate for bovine oocytes injected with a single sperm or a sperm supplemented with ST Blend or ST Filtrate differed ($P=0.0004$), with significant differences among all groups (Control 20/68, 29%; ST Blend, 43/106, 40% and ST Filtrate, 56/94, 60%) (Figure 2). ST Blend and ST Filtrate were both used in this experiment to assure that oocyte activation molecules would be soluble and available in the medium after the processing of sperm tails.

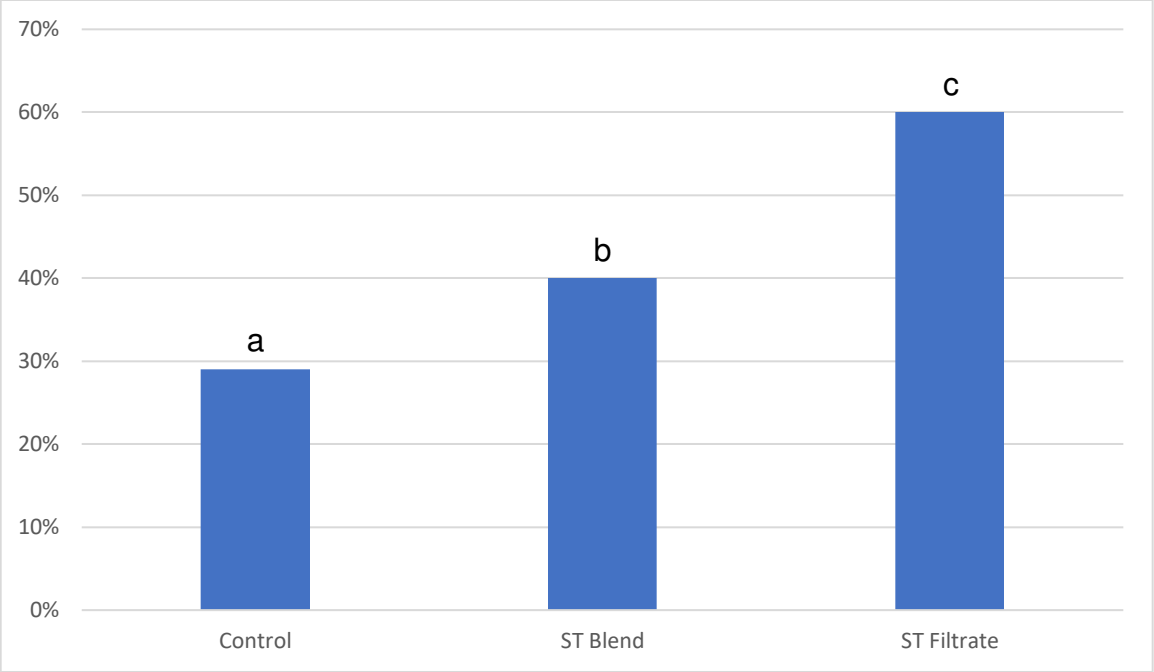

Figure 2. Cleavage rates for bovine oocytes injected with a single equine sperm (Low PLCz) as the Control or with a sperm and ST Blend or ST Filtrate. $_{abc}$Percentages with different superscripts differ ($P<0.05$).

DISCUSSION

Intracytoplasmic sperm injection in horses is relatively new when compared to other assisted reproduction techniques, such as artificial insemination and embryo transfer. However, ICSI is a proven technique for the production of equine offspring. The effectiveness of ICSI bypasses several steps that occur during in vivo fertilization and standard in vitro fertilization. Offspring can be produced by ICSI for subfertile mares and for stallions with poor quality or limited semen (Choi *et al.* 2016). However, embryos are difficult to produce by ICSI, for some mares and stallions. This is often associated with failure of the oocyte to cleave after the sperm injection. The failure to produce viable embryos is potentially associated with reduced oocyte developmental capability, including chromosomal misalignment in old mares (Carnevale and Sessions, 2012) or oocyte incompetence (Carnevale and Ginther,1995). However, although many oocyte factors are essential for proper embryo development, this is often associated with failure of embryo development and not of cleavage. This is observed with oocytes from old mares, which typically cleave at similar rates to oocytes from young mares (Altermatt *et al.* 2009), although embryo loss rates are much higher (Carnevale *et al.* 2000). Therefore, factors that are essential for oocyte cleavage may be more directly associated with the injected sperm than the oocyte.

Oocyte activation in mammals has been studied for decades, but only recently were scientists able to identify a sperm factor that can induce activation when present in the concentrations associated with a single sperm. Phospholipase C zeta (PLCz) is now considered the probable sperm factor responsible for oocyte activation and the lack thereof a possible cause for some male infertility cases. Human and mice sperm that

are deficient in PLCz fail to induce oocyte cleavage both in vivo and in vitro, and sperm from PLCz knockout mice only produce embryos in vitro after the addition of recombinant PLCz (Saunders *et al.* 2002). Recombinant mouse PLCz cRNA in different doses induced parthenogenetic activation of 97 to 100% of mare oocytes (Bedford-Guaus *et al.* 2008), confirming that PLCz is important for equine oocyte activation.

However, PLCz has been associated with male fertility in some species, such as the bovine (Kasimanickam *et al.* 2012). Little has been done in the equine. A single nucleotide polymorphism within the PLCz codifying gene was associated with the pregnancy rate per cycle for Hanoverian Warmbloods in a study with 228 stallions (Schrimpf et at., 2014), confirming an indirect assessment of sperm PLCz content and fertility. The use of anti-human and anti-mice PLCz antibodies was validated in our laboratory to assess PLCz using immunofluorescence; this was important to identify the percentage of equine sperm positively labeled for a specific population and to demonstrate variability among stallions (Gonzalez-Castro and Carnevale, 2018). Different parameters can be used to assess stallion field fertility, but there is not a gold standard for sperm that is destined for ICSI. Most parameters that would characterize an ejaculate as resulting in low fertility are overcome by the sperm preparation and selection for the ICSI process. Although for some stallions, even the selection of a morphologically normal, motile sperm for ICSI does not result in oocyte activation.

To demonstrate the association between PLCz content on sperm and zygote cleavage after ICSI, we used heterologous fertilization with bovine oocytes. Bovine oocytes are readily obtained from slaughterhouses and can be a good surrogate for equine ICSI success since equine oocytes are more difficult and expensive to obtain. In

the present study, we evaluated the extent that positive labelling with PLCz in a sperm population is an indicator of ICSI success. In our first experiment, we performed ICSI cycles using bovine oocytes and sperm from four stallions that we identify as having either high or low levels of PLCz labeled sperm. We included a sham injection group with bovine oocytes, as our laboratory has observed that some batches of bovine oocytes are susceptible to parthenogenetic activation. In addition, equine oocytes were injected with the sperm from the same stallions as bovine oocytes to confirm that differences were consistent for oocytes from the different species. For bovine and equine oocytes, sperm populations considered “High” versus “Low” for PLCz resulted in higher cleavage rates after injection into an oocyte, supporting our initial hypothesis. Consequently, the PLCz content in a sperm population appears to be a primary factor associated with the ability of sperm to initiate oocyte activation. Of course, other factors, which are associated with the injected sperm, could also play a role in oocyte activation. In this project, blastocyst rates were not determined, as embryo development after heterologous fertilization arrests at early stages of development, and equine oocyte numbers were too low for statistical comparisons.

After analyzing the results obtained from the first experiment, we hypothesized that cleavage rates could be improved by injecting a sperm tail from a stallion with known good fertility in addition to a sperm from a low PLCz subfertile stallion. This was difficult to do because of technical difficulties in injecting a single tail. Therefore, we sonicated sperm tails from a fertile stallion and injected the “tail blend” or a filtered tail blend during the ICSI process. We performed ICSI with bovine oocytes using the tail blend and filtered blend. Cleavage rates for the sperm injections supplemented with tail

components were higher than when a single “Low” PLCz sperm was injected. This suggests that compounds associated with oocyte activation are present on the sperm tail. The equine sperm tail has high concentrations of PLCz (Gonzalez-Castro *et al.* 2017), but mRNAs, WW domain-binding protein (PAWP) and other cell components that may play a role in oocyte activation are also present (Amdani *et al.* 2015). Oocyte supplementation with sperm tail components may be a more physiological way to induce activation when compared to chemical activation.

In conclusion, our experiments demonstrate that the sperm population content of PLCz is likely related to cleavage rates after ICSI. Supplementation of sperm tail extracts, which potentially contain PLCz in addition to other sperm factors, promote oocyte activation and cleavage after ICSI. Future research is needed to more accurately determine the sperm-associated factors that cause equine oocyte activation. The cloning of equine PLCz for use as an ICSI supplement would be a potential step toward determining if PLCz is the primary and essential factor needed to activate the equine oocyte. Our findings are the initial step in establishing a physiological method to promote the activation of equine oocytes after injection of sperm, which otherwise result in low cleavage rates after ICSI.

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