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Original Research Article

The ability of donkey sperm to induce oocyte activation and mule embryo development after ICSI

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ABSTRACT

Members of the *Equus* genus exhibit a fascinating capacity for hybridization, giving rise to healthy offspring. Mules, resulting from the mating of a mare with a jack, represent the most prevalent equid hybrid, serving diverse roles in our society. While *in vitro* embryo production, particularly through Intracytoplasmic Sperm Injection (ICSI), has rapidly gained significance in domestic horses, the *in vitro* production in other equids remains largely unexplored. Utilizing donkey sperm for fertilizing horse oocytes not only addresses this gap but also provides an opportunity to investigate donkey sperm's fertilization capability *in vitro* to further improve donkey ICSI. In this work, we initially studied the localization of donkey sperm Phospholipase C zeta (PLCζ) and assessed the sperm's capacity to induce pronuclear formation and maternal SMARCA4 recruitment upon injection into pig oocytes through ICSI. Subsequently, we investigated the injection of donkey sperm into horse oocytes, evaluating *in vitro* production up to the blastocyst stage using sperm from different jacks, including frozen and refrigerated samples. Distinct patterns of PLCζ localization were observed for donkey sperm cells compared to their horse counterparts. Additionally, donkey sperm exhibits a reduced ability to induce porcine oocyte activation. However, when injected into horse oocytes, donkey sperm demonstrated sufficient capability to induce oocyte activation as no discernible differences in cleavage or blastocyst rates are observed between *in vitro* produced mules and horse ICSI embryos. Our study not only delineates PLCζ localization in donkey sperm but also suggests potential differences in the ability to induce oocyte activation in pigs compared to horses while observing no distinctions in pronuclear recruitment of SMARCA4. Interestingly, donkey sperm remains sufficiently capable of inducing horse oocyte activation for *in vitro* mule blastocyst production.

1. Introduction

The existence of the horse–donkey hybrids is documented back to at least 3000 years ago [1]. The mule, the hybrid progeny resulting from the mating of a female horse (mare) with a male donkey (jack), occupies a distinctive niche within the *Equidae* family. Renowned for its hybrid vigour and resilience, the mule exemplifies the valuable convergence of the distinct attributes contributed by its parental species. In agricultural

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and industrial domains, mules have historically proven indispensable, serving as efficient draught animals due to their endurance, strength, resistance to certain equine diseases and adaptability to diverse terrains [2]. Mules have also found utility in recreational and competitive spheres, notably in equestrian activities and specialized competitions. Beyond their indispensable societal role, mules stand out as a compelling model for the study of hybridization. This unique interspecies hybrid provides valuable insights into the broader impacts of crossbreeding, for example, in understanding cellular processes such as the reprogramming of fibroblast cells [3].

While well-established *in vivo*, the early embryonic stages of equid hybrids have received limited attention in research. *In vitro* embryo production techniques, including somatic cell nuclear transfer (SCNT), intracytoplasmic sperm injection (ICSI), and *in vitro* fertilization (IVF), offer valuable tools for investigating the intricacies of early preimplantation stages. The application of these *in vitro* methods has notably gained significance in recent years within the horse breeding industry [4], providing a platform for a more detailed understanding of early embryonic development in horses. Interestingly, the first equid clone born was a mule [5], but mule embryos produced by fertilization in laboratory settings have yet not been reported. The pursuit of *in vitro* mule embryo production holds promises not only for its potential commercial implications but also for advancing our comprehension of the fertilization capacity of donkey sperm. Currently, there are six extant species of donkeys, with Somali wild asses (*Equus africanus somaliensis*) considered critically endangered [6]. This emphasizes the urgency of leveraging reproductive technologies to aid conservation efforts, as ICSI in donkeys is not yet fully established [7]. Furthermore, one notable advantage of ICSI is its capability to facilitate the production of interspecific embryos, thereby emerging as a valuable technique for evaluating sperm's ability to induce oocyte activation and as a means to investigate essential early events, such as DNA damage/repair [8,9], DNA methylation [8] and recruitment of chromatin remodelling proteins, including SMARCA4 [10], during pronuclear formation.

The relevance of the sperm for successful embryo production has been largely studied [11]. The phospholipase C (PLCZeta1), PLCζ, is one of the molecules responsible for initiating fundamental events associated with oocyte activation and embryo development [12], and has been characterized in several mammalian species, but not in the donkey. Interestingly, sperm cryopreservation can impact on PLCζ availability [13,14]. Studies suggest that equine PLCζ has one of the highest activities among the mammalian species studied to date [15] and the production of mouse offspring using horse PLCζ mRNA has been recently reported [16]. The question of whether various members within the *Equidae* family exhibit conserved PLCζ localization or activity is a compelling inquiry. The exploration of such protein may hold the key to enhancing our understanding of assisted reproductive technologies in these species.

This paper characterizes and compares the localization of PLCζ in donkey and horse sperm while also reporting, for the first time, mule blastocyst production *in vitro*. Moreover, we assessed the ability of donkey sperm to induce oocyte activation in a heterospecific model, utilizing porcine oocytes to determine pronuclear formation and SMARCA4 incorporation. Following this, horse oocytes were employed to evaluate *in vitro* mule production up to the blastocyst stage, utilizing sperm from various male donkeys in two different laboratories, including frozen and refrigerated samples.

2. Material and methods

Unless otherwise stated, all chemicals were obtained from Merck KGaA (Darmstadt, Germany).

2.1. Experimental design

2.1.1. Experiment 1

PLCζ expression *and localization in horses and donkey sperm cells.* Cryopreserved semen samples were fixed and subjected to immunofluorescence to compare the expression pattern of PLCζ between equids. Semen samples from three stallions and three jacks were used in this experiment.

2.1.2. Experiment 2

Pronuclear formation and recruitment of SMARCA4 after interspecific ICSI in porcine oocytes. In vitro matured porcine oocytes were injected with horse or donkey sperm cells. Presumptive zygotes were fixed, stained and subjected to immunofluorescent to determine the levels of SMARCA4 associated with DNA. The proportion of embryos with one pronucleus (1-PN) or two pronuclei (2-PN) was registered. Three biological replicates were performed using the semen from one stallion and one jack.

2.1.3. Experiment 3

In vitro development of horse and mule ICSI embryos. The *in vitro* development up to the blastocyst stage of ICSI embryos was compared between horse oocytes injected with horse (horse-horse) or donkey sperm (mule). Three replicates were performed using the semen from two stallions (#1 and #2) and two jacks (#1 and #2).

2.1.4. Experiment 4

In vitro development of mule ICSI embryos produced with refrigerated or frozen donkey semen. The *in vitro* development up to the blastocyst stage of ICSI embryos was compared between horse oocytes injected with refrigerated or frozen donkey sperm from one jack. Three replicates were performed using the semen from one jack (#3).

2.2. Ethics and animal welfare statement

For all animals used in this study guidelines from the National Research Council's Guide for the care and use of laboratory animals have been followed. Semen collection procedures were also conducted in accordance with the Facultad de Ciencias Veterinarias, Universidad de Buenos Aires and Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto regarding the use of animals in research. This research protocol (# 04/2020) was approved by the Ethics and Animal Use Committee of the Rio Cuarto State University, Córdoba, Argentina. The use of animals for semen collection and preservation of donkey and horses at the Universidad de Buenos Aires, Facultad de CianciasVeterinarias was approved by the Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL) # 2017/68. Animals were kept in paddocks, fed with grain forage, straw, hay, and water ad libitum, and housed at the mentioned institutes, which works under strict health control and animal welfare protocols.

2.3. Phospholipase C (PLCζ) characterization

For experiment 1, frozen ejaculated sperm cells from three jacks and three stallions were used. PLCζ localization patterns were evaluated by immunocytochemistry. Frozen-thawed sperm were fixed (4 % paraformaldehyde, 10 min at room temperature) and washed twice with Dulbecco's phosphate-buffered saline (DPBS) by centrifugation (300×*g* for 5 min). Then, sperm were dried on glass slides and permeabilized (0.5 % Triton X-100, 2 min), washed with DPBS-0.1 % Tween 20 (DPBS-T) (3 times, 5 min), and incubated in blocking solution (3 % w/v BSA in DPBS-T; 60 min). After blocking, sperm were incubated with *anti*-PLCζ polyclonal antibody made in rabbits against the *N*-terminus of porcine PLCζ, 1:50 [17] in blocking solution overnight and with the secondary antibody (Donkey anti-rabbit IgG, Alexa Fluor™ 488, cat#A-21206, ThermoFisher) for 1 h. Finally, sperm were mounted with

Vectashield® mounting medium and examined at 1000*x* under a fluorescence microscope (Nikon Eclipse E200, Japan). The percentage of the different localization patterns observed was calculated in a minimum of 200 sperm cells.

2.4. Cumulus-oocytes complexes collection and in vitro maturation

For experiment 2, porcine cumulus-oocytes complexes (COCs) were aspirated from ovaries derived from the local slaughterhouse using an 18-gauge needle attached to a 10 mL disposable syringe. Compact COCs were selected, washed twice in Tyrode's Album Lactate Pyruvate Hepes (TALP-H) [18], and matured for 44 h in 100 μL drops of bicarbonate-buffered Tissue Culture Medium (TCM-199, 11150–059, Thermo Fisher Scientific, Waltham, USA) under mineral oil (Fisher Scientific, 0121–1, Pennsylvania, USA), supplemented with 0.3 mM sodium pyruvate (P2256), 100 mM cysteamine (M9768), 5 μg/mL myo-inositol (I5125), 1 μL/mL Insulin-Transferrin-Selenium (51300044, Thermo Fisher Scientific, Waltham, USA), and 1 % v/v penicillin-streptomycin antibiotic (P4458), 10 μg/mL follicle stimulating hormone (FSH, NIH-FSH-P1, Folltropin®, Bioniche, Ontario, Canada), 5 ng/mL Epidermal Growth Factor (PHG0315, Gibco, Carlsbad, USA) and 10 % v/v porcine follicular fluid. Between 20 and 30 COCs were placed in each drop. Porcine follicular fluid was obtained from follicles of 3–6 mm of diameter, centrifuged at 400 g for 30 min at 5 °C, filtered and then aliquoted and stored at −20 °C. The cumulus cells of porcine COCs were removed by vortexing for 1 min in TALP-H containing 3 mg/mL hyaluronidase (H3506). Porcine oocytes exhibiting the first polar body were subjected to ICSI with donkey sperm (Experiment 2).

For experiments 3 and 4, post-mortem ovaries from mares were collected within 2 h after slaughter at a local abattoir. COCs were immediately recovered *in situ* by a combination of scraping and washing of all visible follicles using an 18-gauge needle and a syringe filled with flushing medium following the procedure described by Gambini et al., [19]. The collected fluid was filtered through a sterile 70 mm embryo filter (EmCon®; IMV Technologies, France). The filtered contents were emptied into a sterile 120 mm Petri dish, and COCs were identified under a stereomicroscope (SMZ800 N, Nikon Corporation, Tokyo, Japan), washed three times with embryo holding medium (EquiHold ®, Minitüb, Tiefenbach, Germany), transferred into a 2 mL cryovial containing embryo holding media and shipped overnight at 18 to 20 °C in a highly neopor insulating box (17229/0002, Minitüb, Tiefenbach, Germany). Transportation time to the ICSI laboratory varied from 24 to 28 h.

In vitro maturation of horse oocytes was performed for 30–32 h in 100 μL drops of TCM-199 supplemented with 10 % v/v fetal bovine serum (FBS, 10091148, Thermo Fisher Scientific, Waltham, USA). Media was also supplemented with 1 μL/mL insulin-transferrin-selenium, 1 mM sodium pyruvate, 100 mM cysteamine, 10 μg/mL follicle stimulating hormone, and 25 μg/mL gentamycin (15710064, Thermo Fisher Scientific, Waltham, USA) under mineral oil (ART-4008PA, Origio Oil for Tissue Culture, CooperSurgical Fertility Companies, Målov, Denmark) in 5 % CO_2 in humidified air at 38.2 °C. After *in vitro* maturation, cumulus cells were removed mechanically and oocytes with a visible first polar body were considered as matured oocytes. Oocytes exhibiting an intact cytoplasmic membrane and lacking any discernible polar body were categorized as immature. Conversely, oocytes with any form of membrane damage were considered degenerate.

2.5. Equid semen collection, refrigeration and cryopreservation

Ejaculates from three donkeys (15 years old) were collected using a Missouri-model artificial vagina (Minitüb, Tiefenbach, Germany). Donkey semen refrigeration was performed as described by Alonso et al., [20]. The cryopreservation protocol of donkey sperm was based on a previously published procedure [21]. Briefly, semen samples were diluted in a milk-based extender, and then centrifuged at 800 g for 15 min. The sperm pellets were re-suspended in a Kenney extender supplemented with 20 % egg yolk and 7 % dimethylformamide to reach a concentration of about 200 × 10^6 spermatozoa/mL and maintained at room temperature for 30 min. The straws were placed in plastic goblets and submerged in a mixture of ethanol and acetone (1:1) in the bronze canister. The temperature was rapidly decreased inside a 9–10 L liquid nitrogen tank, according to Miragaya et al., [22]. Ejaculates from three stallions (8–15 years old) were frozen using the following methodology. Raw semen was diluted 1:1 in INRA 96 (IMV Technologies, L'Aigle, France) and centrifuged at 600×*g* for 10 min at RT. Sperm pellets were resuspended in Botucrio (Nidacon, Sweden) to a final concentration of 100 × 10^6 sperm/mL. Samples were cooled and stored in straws, positioned 4 cm above the surface of liquid nitrogen for 10 min, and finally stored in LN_2 tanks.

2.6. Intracytoplasmic sperm injection and embryo culture

Matured zona-intact horse oocytes were subjected to ICSI with donkey sperm. For experiment 3, a portion of one frozen sperm straw was cut under LN2 and thawed by submerging in 1 mL of TALP-H at 37 °C, placed in a 1.5 mL tube, and centrifuged once at 400×*g* for 5 min. Supernatant was removed, and 400 μL of TALP-H were gently layered above the pellet, the tube was inclined at a 45° angle and incubated for 30 min at 38.2 °C. A 1 μL aliquot of semen was taken from the supernatant and placed in a 4 μL drop of 7 % v/v polyvinylpyrrolidone (ART-4006-A, Origio, CooperSurgical Fertility Companies, Målov, Denmark). ICSI was performed using a 7 μm glass sharp micropipette (MIC-50-30, Origio, CooperSurgical Fertility Companies, Målov, Denmark) in an inverted microscope (Nikon Eclipse Ti2-A, Nikon Corporation, Tokyo, Japan) using micromanipulators (Narishige, Medical Systems, Great Neck, NY, USA). Embryo culture was performed in commercial media (Eq-IVC-1 and Eq-IVC-2 IVF bioscience, United Kingdom) supplemented with 10 % of FBS for 10 days. Blastocysts were identified daily from day 7 up to day 11.

For experiment 4, ICSI was performed as described by Clerico et al. [23], using frozen and refrigerated donkey semen. A portion of one frozen semen straw was thawed, and 100 μL of refrigerated semen was layered into separated centrifuge conical tubes with 3 mL of TALP-H. After centrifugation at 300*g* for 5 min, the supernatant was discarded, and 300 μL of TALP-H was gently layered above the pellet, following swim-up at 38.2 °C for at least 30 min. The ICSI procedure was performed as described in Experiment 3. Mule ICSI zygotes were cultured in a mixture of DMEM/F12-Global Total medium (54–40; LGGG, Life-Global, Guilford, CT, USA) supplemented with 6 % v/v FBS and 0.1 mM sodium pyruvate for up to 11 days. Cleavage was assessed on day 5 when media was renewed, and the blastocyst rate was recorded from day 7 daily until day 11. Cleavage and blastocyst rates were determined considering the number of injected matured oocytes in all experiments. Blastocysts for all experimental groups were vitrified for future studies.

2.7. Pronuclear staining and detection of SMARCA4 levels

For experiment 2, presumptive ICSI zygotes were fixed 18–20 h after ICSI for 20 min in 4 % formaldehyde (47608) in DPBS (14190136, Thermo Fisher Scientific), rinsed in DPBS with 0.4 % w/v bovine serum albumin (BSA, A6003), and stored at 4 °C in 96-well plates. Embryos were treated with permeabilization solution (DPBS containing 0.2 % v/v Triton X-100, 21123) for 15 min and washed in blocking buffer (DPBS containing 0.1 % v/v Tween 20 (P9416) and 0.4 % w/v BSA). Presumptive zygotes were then incubated with SMARCA4 antibody (Brg-1 (G-7), 1:100, SC-17796, mouse monoclonal, Santa Cruz Biotechnology, Dallas, TX, USA. AB_626762) overnight at 4 °C in blocking buffer. A negative control group without primary antibody was included for all assays. After washing, all embryos were incubated with secondary antibody (Alexa Fluor® Plus 594,1:1000, donkey anti-mouse, #A32744,

Thermo Fisher Scientific) in blocking buffer for 1 h in the dark. Finally, presumptive zygotes were mounted in Vectashield® containing 1.5 μg/mL DAPI (Vector Laboratories, Burlingame, CA), and slides were scanned using an inverted confocal microscope (Olympus IX83 Spinning Disk Confocal System). The intensity of SMARCA4 protein in the PN was only analysed in ICSI zygotes with the presence of 2-PN. A region of interest was drawn around each PN and the average pixel intensity was determined with FIJI image processing software [24].

2.8. Statistical analyses

All statistical analyses were completed using GraphPad 7 Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Shapiro-Wilks and Levene tests were performed to assess the normality of data distribution and variance homogeneity. An independent samples *t*-test was used to analyse the difference in PLCζ localization patterns between the two species. Embryo preimplantation development rates were compared using two-tailed Fisher's exact test. Differences were considered statistically significant with a value of $P \leq 0.05$.

3. Results

3.1. Experiment 1: PLC characterization in donkey sperm cells

Differences in the subcellular localization or expression levels of the PLCζ protein within a sperm may bear a direct influence on its capacity to induce oocyte activation and subsequent embryo development. Consequently, we first performed a comparison of PLCζ expression and localization in horses and donkey sperm cells by immunofluorescence. Four different main patterns of PLCζ expression were described after analyzing a total of 590 sperm cells: A, acrosomal; PA + A: postacrosomal and acrosomal; F, flagellum only and PA, postacrosomal. Surprisingly, compared to horses, a significantly higher presence of A PLCζ localization pattern ($P = 0.0099$) and a lower PA PLCζ localization pattern ($P = 0.0386$) was found in donkey sperm cells. All sperm cells showed flagellum localization of PLCζ (Fig. 1).

3.2. Experiment 2: the ability of donkey sperm to induce porcine oocyte activation and recruit SMARCA4

Given the differences observed in PLCζ localization between equids in our first experiment, we then proceeded to assess the capability of horse and donkey sperm to induce pronuclear formation after injection into matured pig oocytes. Furthermore, we examined whether the observed pronuclear formation was accompanied by the recruitment of maternal SMARCA4 as a marker for chromatin remodelling. Interestingly, pig oocytes were significantly less activated after injection with donkey sperm than horse sperm ($P = 0.0213$, Table 1), although SMARCA4 pronuclear localization levels in the zygotes were similar among groups ($P > 0.05$, Fig. 2).

3.3. Experiment 3: In vitro preimplantation development of horse and mule ICSI embryos

To further understand the potential of donkey sperm to initiate embryo development, we injected jack sperm into horse-matured oocytes to generate *in vitro* mule embryos. A control group involved the injection of horse sperm into horse oocytes, resulting in horse ICSI embryos. The assessment of development up to the blastocyst stage revealed no significant differences between the two groups ($P > 0.05$). This suggests that, when injected into a horse oocyte, both donkey and

Table 1
Pronuclear evaluation of horse and donkey sperm cells injected into matured porcine oocytes.

Group	No. Injected oocytes	No. 2-PN (%)	No. 1-PN (%)	No. Non-activated (%)	No. Others (%)
Horse-Porcine	34	26 (76.47)[a]	2 (5.88)	4 (11.76)[a]	2 (5.88)
Donkey-Porcine	32	11 (34.38)[b]	4 (12.50)	12 (37.50)[b]	5 (15.62)

a,b. Different superscript letters indicate statistical significance, n = 3, Fisher's exact test, $P \leq 0.05$.

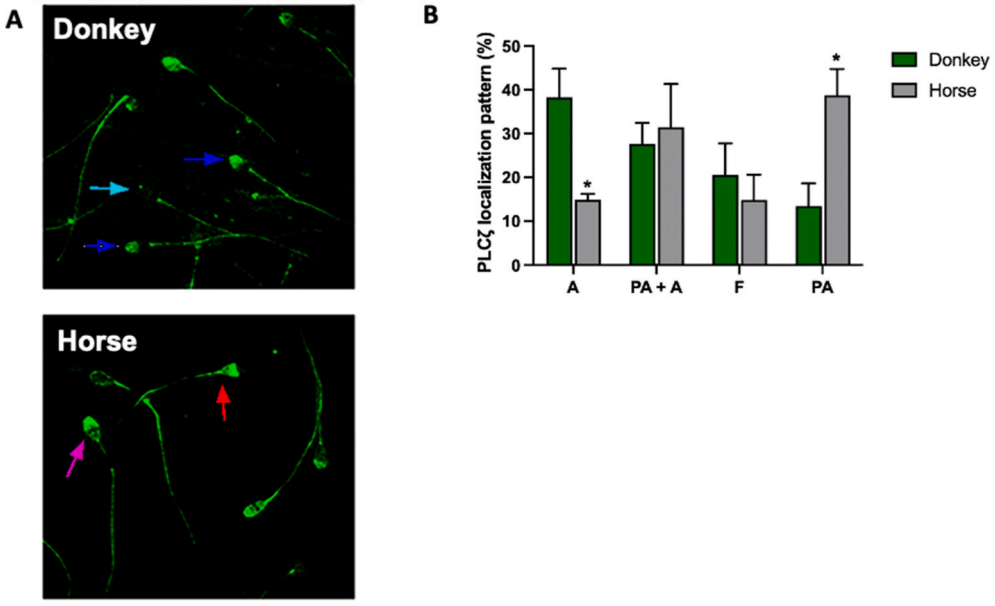

Fig. 1. PLCζ Localization Patterns in Donkey and Horse Sperm. (A) Immunofluorescence images of frozen/thawed donkey and horse sperm for PLCZ1 localization. Arrows: blue, Acrosomal; cyan, flagellum only; magenta, Postacrosomal + Acrosomal; red, Postacrosomal. (B) The percentage of sperm labelled with *anti*-PLCζ antibody in the acrosomal (A), postacrosomal + acrosomal (PA + A), flagellum only (F), and postacrosomal (PA) regions is shown. Green bars represent donkey sperm, while grey bars represent horse sperm. An asterisk (*) indicates significant differences between the two species. Mean ± SEM, n = 3, *t*-test, $P \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

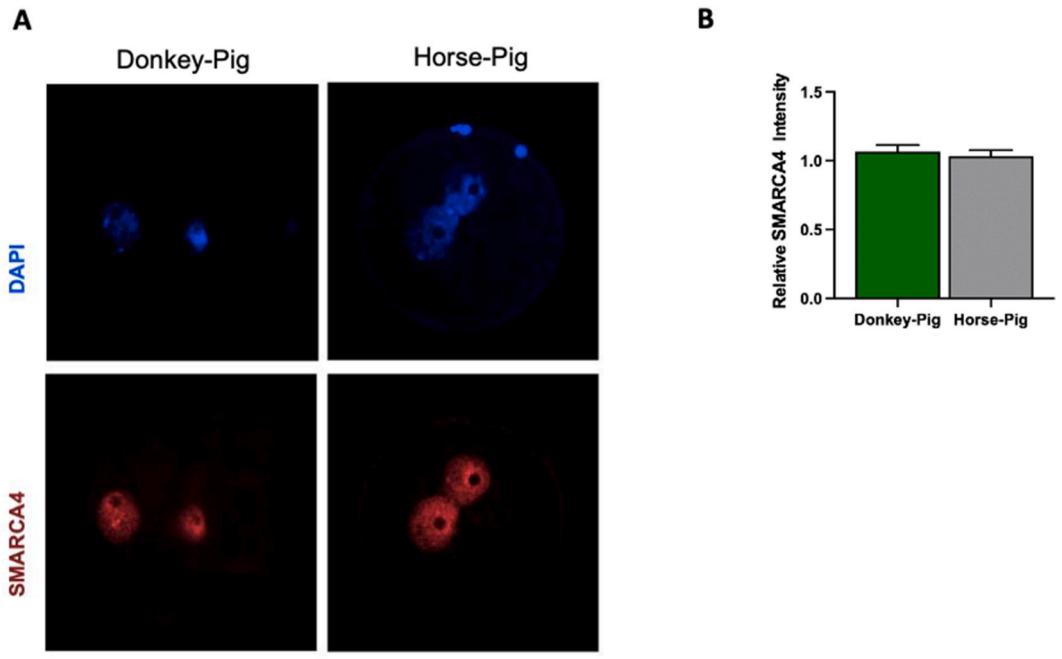

Fig. 2. Pronuclear formation and SMARCA4 characterization after heterospecific ICSI of donkey sperm into pig oocytes (Donkey-Pig) and horse sperm into pig oocytes (Horse-Pig). (A) Representative immunofluorescent staining of ICSI zygotes of the indicated groups. (B) Quantification of pronuclei levels of SMARCA4 of the indicated groups. Green bars represent donkey sperm, while grey bars represent horse sperm. No significant differences were found between groups. Mean ± SEM, n = 3, *t*-test, Mann-Whitney test, $P \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

horse sperm exhibit a similar ability to induce preimplantation development (Tables 2 and 3). Fig. 3 reveals day 8 blastocysts of horse and mule embryos.

3.4. Experiment 4: refrigerated or cryopreserved donkey sperm for in vitro mule embryo production

To enhance developmental rates in mule ICSI *in vitro*, we then tested the hypothesis that donkey refrigerated sperm might be more efficient than cryopreserved sperm, as recently demonstrated in horses [25]. Frozen/thawed or refrigerated donkey sperm cells were injected into horse-matured oocytes to generate *in vitro* mule embryos. The assessment of development up to the blastocyst stage revealed no significant differences between the two groups ($P > 0.05$, Table 4). Fig. 4 reveals day 8 mule blastocysts from frozen/thawed or refrigerated experimental groups.

4. Discussion

Our investigation reveals distinct PLCζ expression patterns in donkey sperm compared to horses, potentially associated with a lower porcine oocyte activation efficiency after ICSI. Interestingly, when introduced into mare oocytes, horse and donkey sperm exhibit similar preimplantation embryo development rates. Notably, our study indicates a lack of male-specific effects in the tested donkey, irrespective of the sperm preservation method employed. These findings represent a noteworthy stride in comprehending the genetic plasticity within the *Equus* genus *in vitro*. They underscore the potential of *in vitro* techniques as tools for genetic enhancement and conservation in donkeys, indicating that the efficiency of *in vitro* mule production is comparable to horses.

Despite the relatively close phylogenetic relationship between horses and donkeys, there are distinct differences in their sperm biology. This has been demonstrated by several authors across various facets of sperm function, including chromatin structure [26], kinetic parameters [27], responses to capacitating agents [28] and resistance to cryopreservation protocols [29]. However, to the best of our knowledge, PLCζ has not been previously described or characterized in donkey sperm. Our investigation reveals a different proportion of expression patterns of PLCζ in donkey sperm cells compared to horses. Species-specific differences have been reported at the level of PLCζ expression and localization in several mammals, including the bovine [30], hamster and rat [31], and humans [32] among others. The expression of PLCζ in horses is associated with the acrosome, equatorial segment, and head-midpiece junction, and PLCζ was also localized to the principal piece of the flagellum [33,34]. These findings agree with our observations. The significance of the distinct pattern expressions observed between equids in our study is yet to be fully elucidated. One plausible explanation could be linked to the differential ability of sperm to initiate oocyte activation

Table 2
In vitro preimplantation development of horse and mule ICSI embryos produced with frozen horse or donkey semen respectively.

Group	No. Injected oocytes	No. Cleaved (%)	No. Day 8 blastocyst (%)	No. Day 11 blastocyst (%)
Horse #1	42	25 (59.54)	3 (7.14)	6 (14.28)
Mule #1	82	54 (65.85)	4 (4.8)	16 (19.51)

n = 3, Fisher's exact test, $P < 0.05$. No significant differences were found.

Table 3
In vitro preimplantation development of horse and mule ICSI embryos produced with frozen horse or donkey semen.

Group	No. Injected oocytes	No. Cleaved (%)	No. Day 8 blastocyst (%)	No. Day 11 blastocyst (%)
Horse #2	20	14 (70.00)	2 (10.00)	5 (25.00)
Mule #2	21	13 (61.90)	3 (14.28)	7 (33.33)

n = 3, Fisher's exact test, $P \leq 0.05$. No significant differences were found.

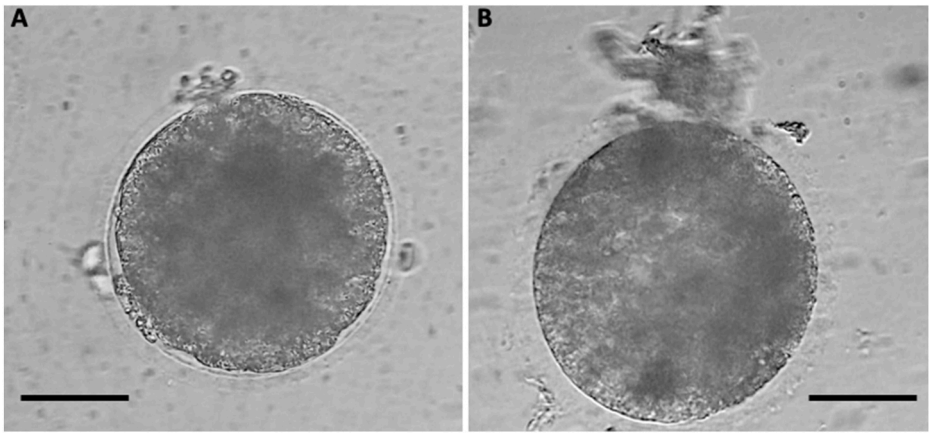

Fig. 3. Day 8 ICSI horse and mule blastocysts. (A) Horse ICSI blastocyst. (B) Mule ICSI blastocyst. Scale bar = 50 μm.

Table 4
In vitro preimplantation development of mule ICSI embryos produced with frozen/thawed or refrigerated donkey semen.

Group	No. Injected oocytes	No. Cleaved (%)	No. Day 8 blastocyst (%)	No. Day 11 blastocyst (%)
Mule #3 Frozen	75	53 (70.67)	7 (9.33)	12 (16.00)
Mule #3 Refrigerated	87	58 (66.67)	10 (11.49)	13 (14.94)

n = 3, Fisher's exact test, $P \leq 0.05$. No significant differences were found.

[35]. This hypothesis gains support from the notably lower efficiency in pronuclear formation observed in zygotes produced by interspecific ICSI using donkey sperm in our study, even though SMARCA4 levels were similar. Interestingly, donkey sperm appears to activate horse oocytes in a manner akin to horse sperm, as evidenced by our *in vitro* mule embryo development rates. This intriguing observation underscores discernible species-specific differences in the oocyte response elicited by the same sperm from the same species. The chromatin remodelers and transcription machinery factor translocation into the PN are essential for embryonic development [36,37], and it could be potentially used as an approach to identify chromatin remodelling failures during heterospecific reprogramming as asymmetry localization of SMARCA4 in PN could be associated with lower developmental rates in pigs [10]. Further insights into potential differences in the activity of PLCζ in donkeys could be gained through cloning and the injection of donkey PLCζ into oocytes, as has been done in horses [15].

While only one study has successfully produced *in vitro* blastocysts in donkeys [7], other attempts have failed [38,39]. The reasons for the low efficiency of *in vitro* donkey production remain unclear but could stem from factors such as oocyte maturation, sperm biology, or *in vitro* culture conditions. The production of hybrids in equids provides an opportunity to shed light on these issues. Injecting donkey sperm into horse oocytes (mules), as in our study, offers insights into sperm-related factors. Conversely, the injection of horse sperm into donkey oocytes (hinny) could help elucidate causes related to the oocyte. However, no reports of *in vitro* production of hinnies have been published yet. Surprisingly, no statistically significant differences between groups were observed in preimplantation embryo development after ICSI with different stallions and jacks. No discernible differences were observed in the timing of blastocyst stage appearance or in morphology between horse and mule ICSI embryos, suggesting that preimplantation development of mules follows a similar pattern compared to that of reported for horses and donkeys [7]. Despite the lower capacity of donkey sperm to induce oocyte activation in pig oocytes, our results imply that, when in contact with a matured horse oocyte, donkey sperm exhibits comparable efficiency to horse sperm in inducing preimplantation embryo development.

Sperm cryopreservation has revolutionized many aspects of animal reproduction and, in donkeys, is an important tool to maintain genetic diversity and preserve endangered species. However, the cryodamage generated causes structural and molecular alterations in the spermatozoa [40], including changes in PLCζ [35]. In fact, the type and concentration of the cryoprotectant can impact horse ICSI outcomes [41]. Our study demonstrated that cryopreserved donkey sperm exhibits comparable ability to induce mule preimplantation development when compared to refrigerated semen. This was achieved using semen samples from a single jack to mitigate the potential male effects of cryopreservation. Interestingly, recent reports at a commercial level suggest that in horses, ICSI might be more efficient when using refrigerated semen

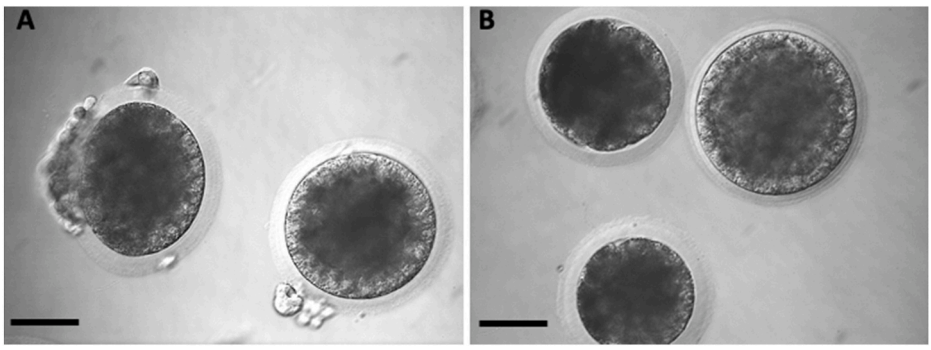

Fig. 4. Day 8 ICSI mule blastocyst. (A) ICSI blastocyst generated after injection of a donkey frozen/thawed sperm cell into a mature horse oocyte. (B) ICSI blastocyst generated after injection of a donkey refrigerated sperm cell into a mature horse oocyte. Scale bar = 50 μm.

compared to frozen samples [25]. Furthermore, the cryopreservation of semen in donkeys poses a distinct set of challenges, as pregnancy rates in jennies after artificial insemination are still unsatisfactory in part due to the particular post-breeding uterine inflammatory response [29,42–44]. Thus, although cryopreservation might impact different aspects of sperm biology and function, the process of sperm selection used in different laboratories before ICSI could mitigate or not the negative impacts of the cryopreservation process. Furthermore, ICSI has the potential to emerge as an alternative technology, enhancing the efficiency of cryopreserved semen utilization in donkeys as the uterine response commonly responsible for the low pregnancy rates in jennies can be bypassed. Additional research is required to gain a comprehensive understanding of the impact of semen preservation technologies on ICSI outcomes.

Finally, comprehensively recognizing the immunofluorescent localization of PLCζ molecules within the membrane/submembrane skeleton regions and intracellular compartments of donkey and horse spermatozoa might facilitate precise isolation of this isoenzyme and preparation of purified PLCζ extracts for the purposes of elaborating different strategies of biological activation aimed at intraooplasmic microinjection of PLCζ homogenates. These strategies used for activating the oocytes of equids and other mammalian species might provide molecular and mechanistic insights into the processes of intrapronuclear recruitment of maternal SMARCA4 chromatin remodelers in a variety of intra- and interspecies embryological models based on either microsurgical IVF by ICSI [10,35,45,46] or SCNT-mediated cloning procedures [47–50].

In conclusion, our study unveils differences in the patterns noticed for expression of PLCζ between donkeys and horses. Additionally, donkey sperm cells display a reduced ability to induce porcine oocyte activation as compared to horse sperm cells. However, this difference is not observed when donkey sperm cells are injected into horse oocytes to produce *in vitro* mules. Remarkably, mules exhibit an *in vitro* preimplantation development behavior up to the blastocyst stage, similar to horses. Our findings might contribute to the understanding of the genetic plasticity within equids and might mark the establishment of mule production by ICSI for the first time.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 3.5 to improve readability and to correct grammatical mistakes. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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