Video Article

Bottlenose Dolphin (*Tursiops truncatus*) Spermatozoa: Collection, Cryopreservation, and Heterologous *In Vitro* Fertilization

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Abstract

The use of cryopreserved dolphin spermatozoa facilitates the exchange of genetic material between aquatic parks and makes spermatozoa accessible to laboratories for studies to further our understanding of marine mammal reproduction. Heterologous IVF, a replacement for homologous IVF, could provide a means to test the sperm fertility potential; to study gamete physiology and early embryo development; and to avoid the use of valuable dolphin oocytes, which are difficult to obtain. Here, we present protocols that have been successfully used to collect and cryopreserve dolphin spermatozoa. The collection of semen is performed by manual stimulation on trained dolphins. Cryopreservation is accomplished using a TRIS egg-yolk based extender with glycerol. In addition, we present a protocol that describes heterologous IVF using dolphin spermatozoa and bovine oocytes and that verifies the hybrid nature of the resulting embryo using PCR. Heterologous fertilization raises questions on fertilization and can be used as a tool to study gamete physiology and early embryo development. In addition, the success of heterologous IVF demonstrates the potential of this technique to test dolphin sperm fertilizing capacity, which is worth further examination.

Video Link

The video component of this article can be found at https://www.jove.com/video/55237/

Introduction

Assisted reproductive technologies are poorly developed in wild animals, including marine mammals. The lack of sensitive methods to assess sperm fertilizing success contributes to the slow development of reproductive technologies in species such as dolphin. It was not until recently that the basic seminal parameters of the bottlenose dolphin (*Tursiops truncatus*) were reported[1,2]. However, variables such as motility and morphology, although widely used, give limited information on reproductive efficiency. The best indicator of sperm quality is the evaluation of the fertilizing potential.

Recently, our group used a method to assess dolphin sperm fertilizing potential by assessing male pronuclear formation and/or hybrid embryo formation after heterologous IVF using zona intact bovine oocytes[3]. The use of dolphin-bovine heterologous IVF has important advantages over homologous IVF, as it overcomes the difficulty of obtaining dolphin oocytes and facilitates the use of well-tested *in vitro* bovine oocyte maturation systems. In order to avoid species specificity, heterologous fertilization is generally performed in the absence of ZP. Although it allows for the evaluation of the ability of the acrosome-reacted spermatozoa to fuse with the vitelline membrane, it impairs the evaluation of other features related to fertilization. The procedure described uses zona intact oocytes and permits the evaluation of the following parameters: sperm zona binding and attachment, penetration, polyspermy, pronuclear formation, and hybrid embryo cleavage.

Here, we present several protocols for sperm collection, basic sperm analysis, spermatozoa freezing as well as the evaluation of the dolphin sperm functionality by assessing male pronuclear and/or hybrid embryo formation after heterologous IVF using zona intact bovine oocytes.

Protocol

Ethics statement: All the experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). All the experiments were performed in accordance to the Guide for

the Care and Use of Laboratory Animals, as adopted by the Society for the Study of Reproduction, and to the Animal Welfare Act for the care of Marine Mammals.

1. Dolphin Sperm Collection and Cryopreservation

1. **Preparation**
    1. Make FERT medium (heparin- and hypotaurine-free). Prepare FERT-TALP medium supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6-mg/mL fatty acid-free BSA, and 10 mg/mL heparin (pH 7.4).
    NOTE: Prepare this medium on the day of use and keep it at 38.5 °C, under an atmosphere of 5% CO_2, and in air with maximum humidity for at least 2 h before use.
    2. Prepare TRIS egg yolk-based buffer. Dissolve 30.28 g/L Tris, 16.75 g/L citric acid, 12.5 g/L fructose, and 0.5 g/L streptomycin in ultrapure water (pH 7.3, osmolality = 310 mOsm/kg). Add 20% egg yolk (v/v).

2. Train a proven breeding male bottlenose dolphin to lie in dorsal recumbence near the edge of the pool for voluntary semen collection.
NOTE: The male dolphin should be trained over 6 months by positive reinforcement, as described previously[4].
3. Perform various tactile stimulations by gently pressing on the cranial area of the genital groove of the dolphin to elicit voluntary extrusion of the penis from the genital groove.
4. After achieving a complete erection, direct the penis tip into a sterile container to obtain the ejaculate.
5. Maintain the sperm sample at 37 °C until the analysis. Repeat steps 1.3 and 1.4 for successive ejaculate collections, using a new propylene glass each time.
6. Immediately after collection, determine the volume using a graduated glass cylinder previously warmed to 37 °C. Evaluate the color, pH, and osmolarity (using a micro-osmometer) of the ejaculate.
7. To evaluate the concentration of spermatozoa in the ejaculate, add 10 µL of the sperm suspension to a eppendorf tube containing 90 µL of distilled water. Determine the sperm concentration using a sperm counting chamber.
NOTE: During the evaluation, keep the rest of the ejaculate at 37 °C.
8. **Evaluate the motility parameters.**
    1. Take 10 µL of the sperm sample and place it in a pre-warmed sperm chamber.
    2. Evaluate the motility using a computer-assisted sperm analysis system, previously validated for the bottlenose dolphin, to objectively assess the sperm motility[2].
    NOTE: If necessary, dilute the sperm sample with FERT medium (heparin- and hypotaurine-free) for the adequate visualization of the sperm motility. During the evaluation, maintain the sample at 37 °C on a heated microscope stage.

9. Take 20 µL of sperm sample and evaluate the viability and sperm morphology as previously described[5].
10. **Transfer the sperm sample to a centrifuge tube. Centrifuge the sperm sample at 250 x g for 5 min. After determine the sperm concentration using a counting chamber, adjust the sperm concentration to 400 x 10^6 spermatozoa/mL.**
NOTE: If necessary, dilute the pellet from the concentrated ejaculates with isolated seminal plasma from an excess volume of the same ejaculate by centrifugation (250 x g, 10 min).
    1. Over the course of 5 min, slowly dilute the sperm sample 1:1 (v/v) with a TRIS egg yolk-based buffer containing 1.5% glycerol. Place the tube containing the sperm suspension at 5 °C for 1 h 30 min.
    2. Perform a second dilution of the sperm suspension with a TRIS egg yolk-based buffer containing glycerol to obtain a 3% final glycerol concentration and a final concentration of 200 x 10^6 sperm/mL. Maintain the sperm suspension at 5 °C for 10 min.

11. Load the sperm suspension into 0.25 mL straws. Heat-seal the straws. Place the straws in a rack 4.5 cm above liquid nitrogen for 10 min. Plunge the straws into the liquid nitrogen and store until evaluation.

2. Heterologous *In Vitro* Fertilization Using Zona Intact Bovine Oocytes

1. **Preparation**
    1. Prepare the staining solution and the mounting medium.
        1. Prepare Hoechst stock solution by dissolving 1 mg of Hoechst in 1 mL of distilled water. Make 30 µL aliquots and keep them in the dark at -20 °C until use. Prepare staining solution by adding 25 µL of Hoechst stock solution to 5 mL of phosphate-buffered saline (PBS) supplemented with 1 g/L PVA. Mix well and keep in the dark at 4 °C until use.
        2. Prepare mounting medium by mixing 6.25 mL of PBS with 6.25 mL of glycerol and 6.25 µL of Hoechst stock solution. Mix well and keep in the dark at 4 °C until use.

    2. Prepare the medium for oocyte collection and *in vitro* fertilization.
        1. Prepare saline solution by adding 0.5 mL of gentamycin to 0.9% NaCl distilled water.
        2. Prepare the stock maturation medium by adding 10 ng/mL epidermal growth factor (EGF) and 10% fetal calf serum (FCS) to 10 mL of TCM-199. Maintain both media at 38.5 °C, under an atmosphere of 5% CO_2, and in air with maximum humidity for at least 2 h before use.

2. Collect the ovaries at the slaughterhouse in saline solution at 33-35 °C and transport them to the laboratory within 4 h from collection.
3. Once in the laboratory, wash the ovaries three times to remove the debris and blood using saline solution previously warmed to 37 °C. Maintain the ovaries in saline solution at 37 °C during the process.
4. Aspirate 2 to 8 mm follicles with an 18G needle and a 10-mL syringe. Collect the aspirated follicular fluid in 50 mL tubes.
5. Allow the follicular fluid containing the oocytes to stand for approximately 15 min at 37 °C, until the cells sediment. Remove the supernatant of the tube with a Pasteur pipette and re-suspend the pellet in PBS.
6. Recover the cumulus-oocyte complexes (COCs) in 90 mm dishes under a stereomicroscope.

7. Wash the COCs three times in PBS and once in maturation medium.
8. Transfer morphologically normal COCs to 4-well dishes, each well containing 500 mL of maturation medium, in groups of 50 COCs per well.
   NOTE: Here, three wells – one well for heterologous IVF, one control well for homologous IVF, and one well containing only matured COCs as a parthenogenetic control – were used.
9. Incubate the COCs for 24 h at 38.5 °C, under an atmosphere of 5% CO_2 and in air with maximum humidity.
10. **Prepare the medium and the density gradient.**
    1. To prepare fertilization medium (FERT), supplement FERT-TALP medium with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL fatty acid-free BSA, and 10 mg/mL heparin. Maintain at 38.5 °C, under an atmosphere of 5% CO_2, and in air with maximum humidity for at least 2 h before use.
    2. Prepare the density gradient by adding 1 mL of density gradient medium to a 15-mL tube and 3 mL of washing solution to a 15-mL tube. Maintain them at 38.5 °C for at least 1 h.

11. Wash the *in vitro* matured oocytes twice in FERT medium.
12. Transfer the oocytes to 4-well dishes, each well containing 50 COCs in 250 mL of FERT. Maintain the 4-well dishes at 38.5 °C, under an atmosphere of 5% CO_2, and in air with maximum humidity while preparing the sperm for fertilization.
13. Thaw a frozen straw containing dolphin spermatozoa in a water bath at 37 °C for 50 s.
    NOTE: Bovine semen for homologous IVF must be processed in parallel, following the standard protocol for homologous bovine IVF.
14. Take 10 µL of the sperm sample and place it in a pre-warmed sperm counting chamber. Evaluate the motility using a computer-assisted sperm analysis system, previously validated for the species, to objectively assess the sperm motility. During this time, maintain the sperm sample at 38.5 °C.
15. Add the sperm sample to the density gradient tube (step 2.10.2). Centrifuge for 10 min at 250 x g.
16. Carefully remove the supernatant using a Pasteur pipette. Add 3 mL of the washing solution to the tube containing the pellet. Re-suspend the pellet by gently mixing.
17. Centrifuge for 5 min at 250 x g. Remove the supernatant with a Pasteur pipette to a sperm volume of 300 µL.
18. Add 5 µL of the sperm suspension to a microcentrifuge containing 95 µL of distilled water. Maintain the sperm suspension at 38.5 °C. Fill the cell counting chamber with 10 µL of the 1:20 sperm dilution and measure the concentration. Calculate the amount of FERT medium that must be added to 100 µL of the sperm to obtain 2 x 10^6 spermatozoa/mL.
19. Add the calculated FERT and sperm volume to a 15-mL tube and gently mix using a Pasteur pipette.
20. Add 250 µL of the sperm-FERT suspension to each well of the 4-well dish containing the FERT medium with the matured oocytes to obtain a final concentration of 1 x 10^6 spermatozoa/mL.
21. Co-incubate the gametes for 18 h at 38.5 °C, under an atmosphere of 5% CO_2, and in air with maximum humidity.
22. Prepare the culture medium based on the synthetic oviductal fluid supplemented with 5% FCS. Prepare 25 µL of synthetic oviductal fluid culture droplets in 35-mm dishes covered with 3 mL of mineral oil. Maintain at 38.5 °C, under an atmosphere of 5% CO_2, and in air with maximum humidity for at least 2 h before use.
23. **At 2.5 h post co-incubation, remove 20 oocytes from the dish; maintain the remaining oocytes in the incubator under the same conditions.**
    1. Remove the loosely attached spermatozoa by vigorously pipetting half of the oocytes through a narrow-bore Pasteur pipette 10 times.
    2. Fix the 20 oocytes in 50 µL of 0.5% glutaraldehyde for 30 min. Wash the oocytes in PBS for 5 min. Transfer the oocytes to 50-µL of Hoechst staining solution for 15 min and wash the oocytes in PBS for 5 min.
    3. Transfer the oocytes individually to 2 µL droplets of mounting medium at a rate of 10 oocytes per slide. Gently cover with a coverslip and seal with nail polish.
    4. Count the number of spermatozoa attached to the bovine oocytes using a phase-contrast microscope equipped with epifluorescent optics and a 361 nm excitation filter at 40x magnification.

24. **After 12 h of co-incubation, remove 10 presumptive zygotes from the 4-well dishes; maintain the remaining presumptive zygotes in the incubator under the same conditions.**
    1. Transfer the 10 presumptive zygotes to a 15-mL centrifuge tube containing 2 mL of PBS and vortex gently for 2 min to remove the cumulus cells. Wash twice in PBS, fix, and stain, as previously described (step 2.23.2.)

25. **After 18, 20, 22, and 24 h of co-incubation, remove 10 presumptive zygotes from the 4-well dishes and vortex, fix, and stain them as previously described (step 2.23.2). Maintain the rest of presumptive zygotes in the incubator under the same conditions.**
    1. Evaluate pronuclear formation and polyspermy under a phase-contrast/epifluorescent microscope in both the homologous and heterologous IVF groups by staining with Hoechst.
    2. Evaluate pronuclear formation in 10 randomly chosen presumptive hybrid zygotes per sample. Confirm pronuclear formation under a confocal laser scanning microscope at 488-nm argon laser excitation and 515 to 530 nm detection.
    3. Optically section presumptive zygotes sequentially (2 mm) and collect images using an imaging software. Visualize using a 3D analysis software package.
    4. After 24 h of co-incubation, wash 50 presumptive zygotes four times in PBS and twice in culture medium.

26. Transfer them in groups of 25 to microdroplets of culture medium previously prepared and equilibrated.
27. Maintain at 38.5 °C under an atmosphere of 5% O_2/5% CO_2 and in air with maximum humidity.
28. After 26 and 28 h of co-incubation, remove 10 presumptive zygotes from the dishes and vortex, fix, and stain them, as previously described (step 2.23.2).
29. After 48 h of culture, observe the cleavage under a stereomicroscope.
30. Remove the zona pellucida (ZP) by transferring the embryos to a 5 mg/mL protease solution.
31. Individually wash each embryo three times in PBS. Snap-freeze them in liquid nitrogen in 0.2 mL microcentrifuge tubes. Store them at -80 °C until analysis.

3. PCR

1. **Materials and Reagents**
   NOTE: The reagents, materials, and equipment used to perform the PCR protocol are detailed in the Table of Materials and include a PCR tube rack and a set of micropipettes (P2, P20, P200, P1000), 1.5 mL microcentrifuge tubes, PCR tubes and caps, a thermal cycler, a UV illuminator, agarose gel, and buffer (Tris Borate EDTA (TBE)).
   1. Gently thaw all reagents on ice. Maintain them in ice throughout the experiment. Use deoxynucleotides (dNTPs; dATP, dCTP, dTTP and dGTP), target primers on the reference gene coding for the dolphin 18 S ribosomal protein (DQ404537.1); R1: TTGGACACACCCACGGTGCGG and F2: CAGAAGGACGTGAAGGATGGA), DNA polymerase, 5X buffer for the DNA polymerase, DNA template, sterile water, and magnesium chloride ($MgCl_2$ at a final concentration of 5.0 mM).
   2. Agarose gel and sample preparation.
      1. Prepare dolphin and bovine sperm DNA samples.
         1. Thaw a frozen straw of each species in a water bath at 37 °C for 50 s. Add 3 mL of washing solution. Centrifuge at 250 x g for 10 min. Remove the supernatant. Add 30 µL of STES lysis buffer (50 mM Tris-HCl, pH 8; 20 mM NaCl; and 1 mM 0.1% SDS), 2 µL of proteinase K (20 mg/mL), and 5 µL of dithiothreitol (DTT); final concentration: 150 mM. Maintain overnight at 55 °C. Add 200 µL of ultrapure water. Centrifuge at 250 x g for 5 min. Keep the supernatant. Measure the DNA concentration using a spectrophotometer.
      2. Perform serial dilutions of DNA from dolphin spermatozoa (*i.e.,* 40, 4, and 0.4 ng/mL) with ultrapure water to check that the PCR conditions are capable of detecting a small amount of dolphin DNA.
      3. Perform serial dilutions of bovine sperm DNA (*i.e.,* 4,000, 400, 40, and 4 ng/mL) with ultrapure water to check that the PCR conditions are capable of detecting a small amount of bovine DNA.
      4. Prepare the dolphin and bovine embryos. Thaw the embryos on ice. Add 8 µL of 100 µg/mL proteinase K and maintain overnight at 55 °C. To stop the reaction, place the samples at 96 °C for 5 min.
      5. Prepare 2% agarose gel TBE buffer and add 20 µL of nucleic acid stain using standard techniques.
   3. PCR preparation.
      1. Label PCR tube(s): dolphin serial dilutions (40, 4, and 0.4 ng/mL), bovine serial dilutions (4,000, 400, 40, and 4 ng/mL), homologous bovine two-cell embryo, two-cell presumptive heterologous embryos, and negative control.
      2. Prepare a master mixture in a sterile 1.5-mL microcentrifuge tube to a final volume of 25 µL per reaction.
         1. Add 5 µL of 5X DNA polymerase flexi buffer, 0.5 µL of dNTPs (10 mM) 1.5 µL of $MgCl_2$ (25 mM), 0.5 µL of forward and reverse primer (10 µM), 1.5 µL of DNA template (sperm) or 8 µL of DNA template (two-cell embryos), 0.07 µL of Taq polymerase, and sterile distilled water up to a 25 µL final volume. Mix well.

2. **Amplification Protocol**
   1. Place the tubes in the thermal cycler. Select the following program: 94 °C for 3 min; 40 cycles of 94 °C for 15 s, 59 °C for 25 s, 72 °C for 20 s; and 72 °C for 5 min. Start the program.
   2. Store the tubes at 4 °C. Add 2.5 µL of loading buffer to each PCR product at 4 °C and load 15 µL of the mixture into the wells of the 2% agarose gel. Use a DNA ladder marker for the accurate size estimation of PCR fragments.
   3. Perform electrophoresis for 15 min to allow DNA migration. Visualized bands using a UV illuminator.

Representative Results

All the results, tables, and figures (1 and 2) presented here were reproduced with permission[3].

Dolphin spermatozoa have high motility after freezing and thawing

The frozen-thawed dolphin ejaculates showed percentages (% ± SD) of 84.5 ± 5.3 total motile and 69.1 ± 5.1 progressive motile spermatozoa. In motility terms, these numbers are representative of high-quality cryopreserved spermatozoa.

Dolphin spermatozoa are capable of penetrating zona intact bovine oocytes and producing hybrid embryos

Figure 1A and **Table 1** show dolphin spermatozoa attached to the bovine ZP after 2.5 h of co-incubation. Dolphin spermatozoa were attached at a higher percentage than bovine spermatozoa (**Figure 1B** and **Table 1**). Indeed, fluorescence microscopy shows that dolphin spermatozoa are able to penetrate zona intact bovine spermatozoa (**Figure 1C**), leading to pronuclear formation and cleavage (**Figure 1E** and **Table 1**). This was confirmed by confocal microscopy (**Figure 1D** and **Figure 1F**). No polyspermy was observed in oocytes from the heterologous IVF group after 12 h of co-incubation (**Table 1**). Pronuclear formation in the heterologous IVF reached the highest percentage at 24 h, a longer time than in the homologous group, which occurred at 18 h of co-incubation (**Table 1**). The cleavage rate at 48 h after incubation was lower in the heterologous than the homologous IVF (**Table 1**). A spontaneous parthenogenetic activation rate of 8.0% was observed in bovine matured unfertilized oocytes at 48 h (**Table 1**).

Finally, the PCR results confirmed the presence of dolphin genetic material in the hybrid embryos (**Figure 3**) by evaluating the presence of a dolphin reference gene that encodes for the ribosomal 18 S protein (**Figure 2**).

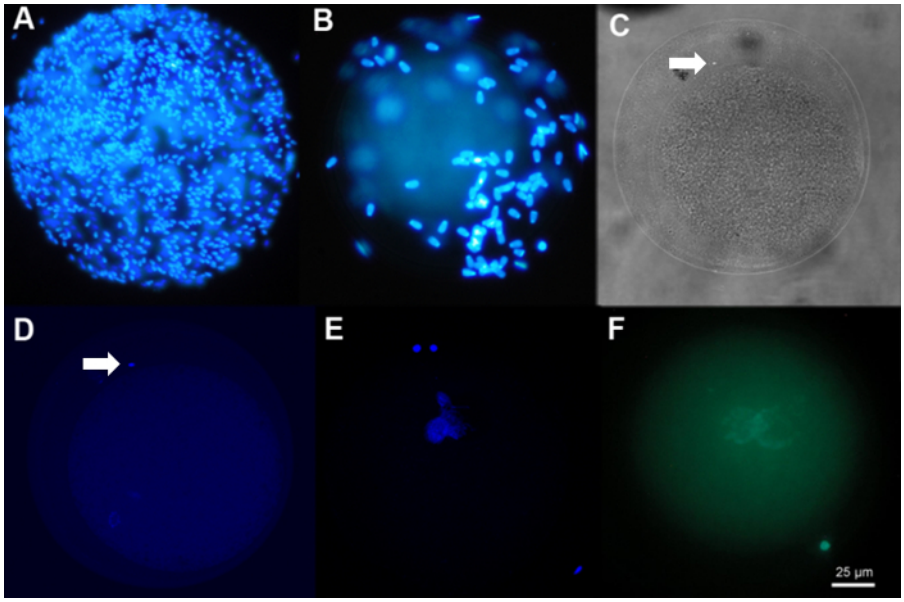

Figure 1: Dolphin spermatozoa attached to the bovine zona pellucida (ZP). Attached dolphin (**A**) and bovine (**B**) spermatozoa after 2.5 h of co-incubation with zona intact bovine oocytes. Gametes were stained with Hoechst and visualized under phase-contrast microscope (40X magnification). A dolphin spermatozoa in the perivitelline space of a bovine oocyte (**C** and **D**) and a decondensing dolphin sperm head in the bovine oocyte cytoplasm (**E**). Images were captured under a confocal microscope and correspond to the equatorial plane of the presumptive zygote (Hoechst staining; 40X magnification). Two pronuclei (**F**) observed under a phase contrast microscope after 24 h of heterologous IVF consisting of the co-incubation of dolphin spermatozoa with zona intact bovine oocytes (Hoechst staining; 40X magnification; Scale bar = 25 µm). The figure was reproduced from reference[3] with permission. Please click here to view a larger version of this figure.

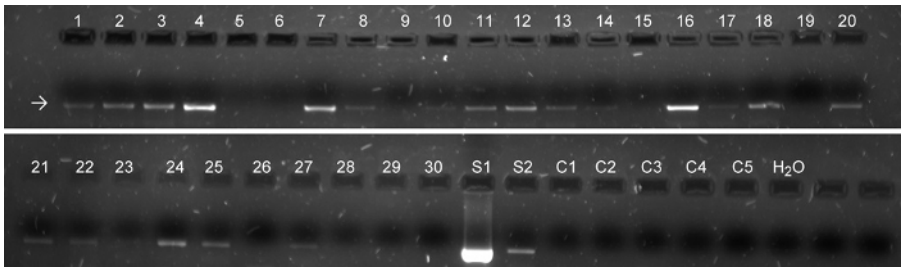

Figure 2: Representative electrophoresis (2% agarose gel ethidium bromide stained) showing the PCR amplification results for the 18 S dolphin gene. Lanes 1 to 30 show presumptive dolphin/bovine hybrid embryos; lanes S1 and S2 feature 4 ng/mL and 0.4 ng/mL dolphin sperm DNA, respectively; and lanes C1 to C5 feature two-cell bovine embryos and H_2O (water) (the blank control). The arrow indicates the 190-bp band corresponding to the amplified 18 S rDNA. The figure was reproduced from reference[3] with permission. Please click here to view a larger version of this figure.

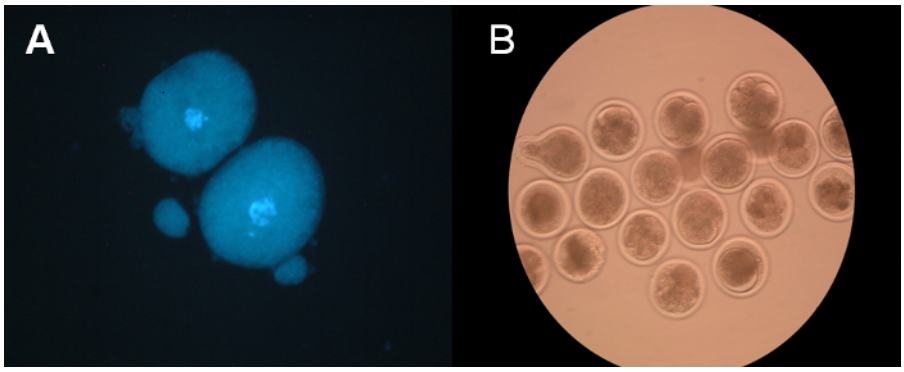

Figure 3: Representative images of hybrid embryos. Representative image of a two-cell hybrid embryo (**A**) at 48 h of incubation (Hoechst staining; 40X magnification). Unstained hybrid embryos at 48 h of incubation, at different stages of division, visualized under the stereomicroscope (**B**). Please click here to view a larger version of this figure.

	Sperm attached		Polyspermy		Pronuclear formation	Cleavage rate	
	n	Number of sperm/oocyte	n	%	%	n	%
Homologous IVF	24	$56.7 \pm 5.3^{a,f}$ (22-89)	132	$11.0 \pm 4.2^{a,g}$	$75.9 \pm 12.9^{a,g}$	371	$89.3 \pm 5.9^{e,i}$
Heterologous IVF	62	$220.7 \pm 18.1^{b,f}$ (24-489)	158	$0.0 \pm 0.0^{b,h}$	$25.2 \pm 7.4^{b,h}$	832	$34.8 \pm 18.5^{d,i}$
Parthenogenetic control group						201	$8.0^{e,i}$

Values are expressed as mean ± SEM (range) of 12 replicates; n = total number of oocytes or presumptive zygotes examined.

Within a column, values with different superscripts are significantly different, a versus b ($P<0.05$), c versus d versus e ($P<0.001$).

Superscripts f, g, h and i indicate that the evaluation was done after 2.5, 18, 24 and 48 hours of coincubation, respectively.

Table 1: Rates of attachment, polyspermy, pronuclear formation, and cleavage following homologous and heterologous co-incubation with bovine oocytes and bovine or dolphin spermatozoa, respectively. Variables were analyzed with ANOVA (Kruskal-Wallis and Mann-Whitney test). The values are expressed as the mean ± SEM (range) of twelve replicates; n = the total number of oocytes or presumptive zygotes examined. The table was reproduced from reference[3] with permission. Please click here to view a larger version of this figure.

Discussion

For many different mammalian species, there are diverse advantages to using frozen-thawed sperm. These include the ability to transfer valuable genetic material, the potential for worldwide distribution, the low risk of contamination, and the capability to preserve male gametes for decades. The use of cryopreserved sperm is essential for the bottlenose dolphin, because this species is protected under Appendix II of CITES, which limits the transport and exchange of animals between different aquatic parks. Dolphins transport is an activity that creates logistical problems and dangerous risks. However, refraining from transporting them also has consequences, such as the risk of introducing consanguinity to a captive population of animals. One solution is the cryopreservation of sperm. Some authors have cryopreserved dolphin sperm[5,7,8,9], with very encouraging results. Moreover, in 2005, the first birth of a dolphin calf conceived by artificial insemination using cryopreserved sperm was reported[10]. Some years later, this was done using sex-sorted spermatozoa[11].

Dolphin sperm have been cryopreserved using different techniques, from sophisticated methods, such as a programmable freezer[5], to common methods, such as dry ice[12] or liquid nitrogen vapors[2,9,13]. The main advantage of using dry ice or liquid nitrogen vapors is the possibility to cryopreserve at the same location where the sperm is extracted, without the use of highly specialized equipment. Sperm frozen in straws on polystyrene in liquid nitrogen vapors is a simple, quick, and inexpensive method[13]. In practice, when this methodology has been used on dolphins, sperm motility was 65%. However, it is important to consider how difficult it is to establish a standardized method: the technique may be influenced by external factors, such as temperature, humidity, polystyrene size, etc. Therefore, further studies are necessary to optimize and standardize the procedure in order to improve the success of using sperm frozen with the liquid nitrogen vapor method.

In order to optimize a freezing protocol, it is important to develop a reliable method for the functional assessment of the spermatozoa. IVF is a good means of testing the sperm potential for fertilization. Ideally, dolphin oocytes should be used for IVF, but the difficult and laborious procedures needed to obtain oocytes from females represent an important constraint. Therefore, the possibility of using heterologous IVF opens a new means to test fertilization efficiency in the dolphin. Oocytes from other species can be used for heterologous dolphin IVF. The results show that heterologous IVF between bovine oocytes and dolphin spermatozoa can be performed. Frozen and thawed dolphin spermatozoa can penetrate the zona intact bovine oocyte and generate a hybrid embryo.

The presented combination of protocols for collecting, freeze-thawing, and performing heterologous IVF with bovine oocytes have been successfully demonstrated and represents an initial step for the optimization of cryopreservation and the evaluation of dolphin sperm. It is important to note that there are still many unknowns in these procedures. However, the ability to cryopreserve dolphin semen makes it accessible and allows it to be kept in laboratories. This will facilitate scientific studies and can lead to a better understanding of the physiology, composition, and behavior of spermatozoa.

The evaluation of sperm by heterologous IVF can ease the optimization of the freezing protocols, but it may also be used in the future to test and select males for fertility efficiency. One important consideration is the relationship between real fertilization numbers and heterologous IVF values. In addition, and due to the novelty of heterologous IVF between these two phylogenetically distant species, bovine and dolphin, new biological hypotheses can be built. Altogether, these protocols open a new space for research on the reproduction of the dolphin and other marine mammals.

Disclosures

The authors have nothing to disclose.

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References

1. Montano, G. A. *et al.* Evaluation of motility, membrane status and DNA integrity of frozen-thawed bottlenose dolphin (Tursiops truncatus) spermatozoa after sex-sorting and recryopreservation. *Reproduction.* **143**(6), 799-813 (2012).
2. Sánchez-calabuig, M. J. *et al.* Validation of a field based chromatin dispersion assay to assess sperm DNA fragmentation in the bottlenose dolphin (Tursiops truncatus). *Reprod Domest Anim.* **49**(5), 761-8 (2014).
3. Sánchez-calabuig, M. J. *et al.* Heterologous murine and bovine IVF using bottlenose dolphin (Tursiops truncatus) spermatozoa. *Theriogenology.* **84**(6), 983-94 (2015).
4. Keller, K. V. Training of the Atlantic bottlenose dolphins (Tursiops truncatus) for artificial insemination. *IAAAM.* **14**(22), 22-24, (1986).
5. Robeck, T. R.; O'brien, J. K. Effect of cryopreservation methods and precryopreservation storage on bottlenose dolphin (Tursiops truncatus) spermatozoa. *Biol Reprod.* **70**(5), 1340-8 (2004).
6. Coy, P. *et al.* Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction.* **135**(1), 19-27 (2008).
7. Seager, S., G. W., Moore, L., Platz, C., Kirby, V. Semen collection (electroejaculation), evaluation and freezing in the Atlantic bottlenose dolphin (*Tursiops truncatus*). *Proceedings of the American Association of Zoo Veterinarians.* **136**, (1981).
8. Schroeder, J.P. Breeding Bottlenose Dolphins in captivity. Leatherwood, S., Reeves, R. R. (Ed.). *The Bottlenose dolphin.* San Diego: Academic Press, 447-460, (1990).
9. Sánchez-Calabuig, M. J. *et al.* Effect of cryopreservation on the sperm DNA fragmentation dynamics of the bottlenose dolphin (Tursiops truncatus). *Reprod Domest Anim.* **50(**2), 227-35 (2015).
10. Robeck, T. R. *et al.* Estrous cycle characterisation and artificial insemination using frozen-thawed spermatozoa in the bottlenose dolphin (Tursiops truncatus). *Reproduction.* **129(**5), 659-74 (2005).
11. Robeck, T. R. *et al.* Development and evaluation of deep intra-uterine artificial insemination using cryopreserved sexed spermatozoa in bottlenose dolphins (Tursiops truncatus). *Anim Reprod Sci.* **139**(1-4), 168-81 (2013).
12. Schroeder, J. P. Breeding Bottlenose Dolphins in captivity. In: Leatherwood, S., Reeves, R. R. (Ed.). *The Bottlenose dolphin.* San Diego: Academic Press, p.447-460 (1990).
13. Madeddu, M. *et al.* Effect of cooling rate on the survival of cryopreserved rooster sperm: Comparison of different distances in the vapor above the surface of the liquid nitrogen. *Anim Reprod Sci,.* (2016).

