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Abstract

The critical situation of the European eel (*Anguilla anguilla*) has urged the development of sperm cryopreservation protocols for reproduction in captivity and cryobanking. In the last years, two research groups have developed their own protocols in Spain and Hungary with positive results, but difficult to compare.

Here, a series of experiments were conducted to test the quality of thawed sperm after using both protocols, determining which of them produce the best results and aiming for standardization. The quality of thawed sperm was assessed by studying the motility and kinetic values of thawed sperm from both cryopreservation protocols using a computer-assisted sperm analysis (CASA-Mot) system. In addition, a viability analysis was performed using flow cytometry to test if the cryoprotectants or the freezing-thawing process led to a reduction in spermatozoa survival. Furthermore, since during cryopreservation the sperm was treated with methylated cryoprotectants (DMSO or methanol) that may induce epigenetic changes in the sperm DNA (cytosine methylation) and could affect the offspring, we conducted a luminometric methylation assay (LUMA) to study the DNA methylation levels induced by both protocols.

In this work, all the above-mentioned parameters were analyzed in fresh and frozen-thawed sperm samples. Our results showed that thawed sperm samples from both protocols presented lower sperm motility and velocity, and lower percentage of live cells than those shown in fresh sperm samples. Furthermore, sperm samples from the methanol based protocol showed significantly higher motility, velocity and percentage of live spermatozoa than the same sperm samples treated with the DMSO based protocol. In addition, the DMSO based protocol induced a hypomethylation of sperm DNA compared to fresh samples whereas the methanol based protocol did not alter sperm DNA methylation level. Our results indicate that the methanol based protocol is a more suitable protocol that preserves better the motility and genetic qualities of the European eel sperm.

Keywords: *Anguilla anguilla;* Methanol; DMSO; DNA methylation; Epigenetics

Abbreviations: DMSO, dimethyl sulfoxide; ROS, reactive oxygen species; rhCG, recombinant human chorionic gonadotropin; CASA-Mot, computer assisted sperm analysis motility module; FBS, foetal bovine serum; HBSS, Hank´s balanced salt solution.

1. Introduction

During the last years, a drastic decrease has been observed in the number of European eels (*Anguilla anguilla*) returning from Europe and North Africa to the spawning sites in the Atlantic Ocean (Dekker 2000; Jacoby & Gollock, 2014). Several impacts such as water pollution, overfishing or habitat fragmentation, have led the European eel to be included on the IUCN red list as critically endangered (Jacoby & Gollock, 2014). Consequently, the development of techniques and protocols for reproduction in captivity are necessary to reverse this situation.

The maturation of the European eel in captivity is only achieved by costly and long hormonal treatments (Asturiano et al., 2006; Gallego et al., 2012; Pérez et al., 2000), and still the production of gametes in both sexes can be unsynchronized (Asturiano et al., 2016). During the last years, several researchers have worked in the development of new maturation protocols such as alternative hormonal treatments with recombinant hormones (Peñaranda et al., 2018) or androgen implants (Di Biase et al., 2017; Mordenti et al., 2018), but the timing of final maturation in females is still highly variable and difficult to control (Mylonas et al., 2017). Therefore, the development of cryopreservation protocols for European eel sperm has been considered important for reproduction management, by guaranteeing the availability of both types of gametes when female spawns (Asturiano et al., 2017), besides its application for cryobanking and future broodstock management.

Cryopreservation of European eel sperm has been faced by different groups since early 2000s. Mainly two groups of research established successfully their own cryopreservation protocols in Spain (Asturiano et al., 2003; 2004; Peñaranda et al., 2009) and Hungary (Müller et al., 2004; Szabó et al., 2005). These protocols differ greatly in many aspects such as the composition of the extenders, the cryoprotectants used, the volume of the straws or the cooling rates within others, evidencing the need for standardization (Asturiano et al., 2017; Rosenthal et al., 2010).

The success of a sperm cryopreservation protocol is commonly assessed using parameters such as sperm viability and motility, fertilizing capacity and the quality of the offspring

(Cabrita et al., 2010). However, in the case of the eel both protocols have yielded high post-thaw sperm viability (58 to 63%) and motility values ranging between 18 and 38% (Asturiano et al., 2017). Furthermore, the fertilizing capacity of the Spanish protocol (from now on referred as DMSO protocol) was successfully tested by producing European eel larvae after fertilization with thawed sperm (Asturiano et al., 2016), and following the Hungarian protocol (from now on referred as methanol protocol), hybrid larvae were successfully produced using thawed sperm from European eel and eggs from Japanese eel (*Anguilla japonica*) (Müller et al., 2012; 2018). In this last study, Müller and collaborators showed that the malformation rate of larvae was higher when using cryopreserved sperm than in the control groups using fresh sperm, suggesting that the cryopreservation methodology needs further refinement.

Aditionally, a growing concern is that the epigenetic effects of cryopreservation on the sperm DNA might be altered by the freezing, cryobanking and thawing process (Labbé et al., 2017; Pérez-Cerezales et al., 2010). The use of methylated cryoprotectants such as methanol or dimethyl sulfoxide (DMSO) has been proven to produce reactive oxygen species (ROS) that can induce cytosine methylation in fish sperm (De Mello et al., 2017; Kawai et al., 2010). Methylation of cytosine residues in DNA is considered to be one of the major epigenetic mechanisms stabilizing gene silencing (Schaefer et al., 2007). Furthermore, cytosine methylation can be altered by cryopreservation, inducing hypo- and hypermethylation profiles in sperm DNA (Labbé et al., 2017). Therefore, the study of epigenetic effects of cryopreservation may be a good indicator of the success of a cryopreservation protocol, since damaged DNA or abnormal DNA regulation have been observed to have a negative effect on the generated embryos (Herráez et al., 2017).

The main objective of this work was to compare the main protocols previously developed for European eel sperm cryopreservation, aiming for standardization. The comparison was made considering sperm quality after thawing, when sperm motilities, sperm velocities, and sperm viability were analyzed. Furthermore, epigenetic effects of sperm cryopreservation were studied by analyzing whether DNA methylation patterns were affected by the different cryopreservation protocols.

2. Material and methods

2.1. Ethics statement

The protocol was approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV) and final permission was given by the local government (Generalitat Valenciana, Permit Number: 2015/VSC/PEA/00064).

2.2. Fish handling

For this experiment, 28 immature male European eels from the fish farm Valenciana de Acuicultura S.A. (Puzol, Valencia) were brought to our facilities in the Universitat Politècnica de València. Fish were distributed in two 200 L aquaria with recirculation systems, and thermostats and coolers to maintain water temperature at 20 ºC. They were gradually acclimated to seawater (salinity 37 ± 0.2 g/L) increasing the salinity 10 ppt each 2 days for 8 days, and 2 days more of resting at 37 ppt. The aquaria were covered to maintain a constant shadow and reduce fish stress.

After 10 days of acclimation, male fish anesthetized with benzocaine (60 ppm) were weekly treated with injections of recombinant human chorionic gonadotropin (rhCG; Ovitrelle, Madrid, Spain, 1.5 IU/g fish) to induce maturation and spermiation (Gallego et al., 2012; Pérez et al., 2000). From the sixth week of hormonal treatment, sperm samples were collected weekly, 24h after the hormone injections.

For sperm collection, fish were anesthetized with benzocaine. Thereafter, the genital area was carefully cleaned with distilled water and thoroughly dried with paper to avoid contamination with feces, urine or seawater to avoid accidental sperm activation. Then, sperm was collected by applying a ventral massage from the pectoral fins to the genital opening and collected in graduated Falcon tubes using a vacuum pump.

Sperm samples were collected after 11-14 weeks of hormonal treatment. The samples were diluted 1:9 (sperm:extender) in P1 medium (in mM: NaCl 125, $NaHCO_3$ 20, $MgCl_2$ 2.5, $CaCl_2$

1, KCl 30; pH adjusted to 8.5, described by Peñaranda *et al.*, 2010) , kept in plastic tubes at 4 ºC and evaluated for motility.

2.3. Evaluation of sperm motility

In a maximum of 2 h after the sperm extraction, sperm samples were evaluated in triplicates following the method described by Gallego et al. (2013). Briefly, each sperm sample was activated by mixing 0.5 µL of P1-diluted sperm sample with 4.5 µL of artificial seawater (in mM: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4 28.2, KCl 9.4, in distilled water) with 2% (w:v) bovine serum albumin (BSA), pH adjusted to 8.2 and osmolality of 1100 mOsm/kg. The activation was performed in an ISAS Spermtrack 10 counting chamber (Proiser R+D, S.L., Spain) on a microscope in negative phase with a 10X magnification (Nikon Eclipse 80i) connected to a computer with an ISAS 782M camera (Proiser R+D, S.L., Spain), recording 60 frames per second (fps). All samples were analyzed 15 s after activation, using the CASA module ISAS v1 software (Proiser R+D, S.L., Spain). Several kinetic parameters such as percentage of motile spermatozoa (MOT, %), progressive motility (pMOT, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), and average path velocity (VAP, µm/s), as well as percentage of slow (average path velocity (VAP) = 10-50 µm/s), medium (VAP = 50-100 µm/s) and fast (VAP >100 µm/s) spermatozoa were recorded for further analysis (Gallego and Asturiano 2018a for details). Samples with motility values higher than 65% were selected for cryopreservation.

2.4. Experimental design

A total number of 18 sperm samples were selected for cryopreservation. Each sample was first evaluated for motility and then frozen and thawed following both protocols. In addition, before freezing, each sample was evaluated for motility approximately 10 minutes after diluted with the freezing media corresponding to each protocol. Then, four straws (IMV Technologies, l'Aigle, France) of 250 µL for the DMSO protocol and four straws of 500 µL for the methanol protocol were frozen. Therefrom, three straws per protocol were thawed and

immediately analyzed with CASA-Mot for sperm quality. Moreover, 50 µL of fresh and thawed sperm from each sample were used for the viability analysis using the flow cytometer (see down). The left straw per protocol was maintained frozen in liquid nitrogen and was sent to INRA's lab in Rennes (France) for sperm epigenetic analysis, by studying the DNA methylation level. In addition, 100 µL of fresh sperm from each sample were frozen as well by directly throwing the tube with the sperm into the liquid nitrogen and then storing it at -80 ºC for DNA methylation analysis of the sperm control. We demonstrated previously that such snap freezing allows that the DNA methylation level of the fresh sperm is preserved (unpublished data).

2.5. Cryopreservation protocols

Every selected sample was frozen and thawed following both protocols. For the DMSO protocol, a freezing medium was prepared in advance by mixing a modified P1 extender solution (in mM: NaCl 50, $NaHCO_3$ 100, $MgCl_2$ 2.5, $CaCl_2$ 1, KCl 30; described by Peñaranda *et al.*, 2009; and named M5 in that paper), 25% (v/v) of fetal bovine serum (FBS) and 10% (v/v) of DMSO. The freezing medium was adjusted to a pH of 6.5, an osmolality of 330 mOsm/kg and maintained at 4 ºC. Thereafter, a dilution 1:2 of sperm: freezing medium, was prepared and immediately packed in 250 µL straws, sealed with modeling clay and frozen for 5 min in liquid nitrogen vapor 1 cm above the surface using a floating structure. Following, the straws were thrown into the liquid nitrogen where the sperm was preserved as long as needed. The thawing consisted in a water bath at 30 ºC for 8 s.

For the methanol protocol, modified Tanaka´s extender (in mM: NaCl 137, $NaHCO_3$ 76.2) was prepared in advance and maintained at 4 ºC. Then, a dilution consisting in sperm:Tanaka's extender:methanol (1:8:1) was prepared and packed in 500 µL straws, and frozen for 3 min in liquid nitrogen vapor 3 cm above the liquid nitrogen before throwing the straws into the liquid nitrogen. For thawing, the straws were immersed in a water bath at 40 ºC for 13 s.

2.6. Thawed sperm evaluation

The quality of thawed sperm samples was assessed by analyzing several sperm motility parameters with CASA-Mot, sperm viability (membrane integrity) with a flow cytometer and epigenetic effects with an analysis of sperm DNA methylation pattern.

The motility analysis was performed using CASA-Mot as explained above. In addition, a viability analysis was conducted with flow cytometry using a fluorescence kit (LIVE/DEAD Sperm Viability Kit, Thermo Fisher Scientific, MA, USA) containing the membrane-permeating dye SYBR 14, that stains the nuclei of membrane-intact cells fluorescent green and the non-permeating propidium iodide (PI), that counterstains the nuclei of cells with a damaged membrane fluorescent red. Here, 0.5 µL of SYBR 14 (100 µM) and 2 µL of PI (2.4 mM) were added to 50 µL of fresh or thawed sperm samples and incubated at room temperature in the dark for 10 min. Thereafter, samples were diluted in 500 µL of extender solution (P1 medium for the Spanish protocol or Tanaka´s medium for the Hungarian protocol) and were analyzed with a flow cytometer (Beckman Coulter FC500). The analyses were performed using the voltages: SS= 199, FS= 199, FL1= 377 and FL2= 372; for a maximum number of 5,000 events or 15 s at low flow.

Finally, a study of DNA methylation level was conducted in fresh and thawed sperm. Sperm DNA was extracted using the phenol/chloroform method: about 20×10^6 spermatozoa in 10 µL Hank´s balanced salt solution (HBSS) 300 were digested overnight at 42 °C under agitation in 1mL of TNES buffer (125 mM NaCl, 10 mM EDTA, 17 mM SDS, 4 M urea, 10 mM Tris-HCl, pH 8) with 75 µg of proteinase K (Sigma Aldrich, P6556). One mL phenol-chloroform-isoamyl alcohol (25:24:1) was added and vigorously mixed. After centrifugation for 15 min at 8,000 g at 4 °C, the upper phase (800 µL) was mixed with 200 µL NaCl 5 M and 2 mL of cold (-20 °C) 100% ethanol. After centrifugation, the dried DNA pellet was mixed with 100 µg/mL RNase in water (Promega, A7973) and incubated 1 h at 37 °C. Whole DNA methylation level was estimated using LUMA (luminometric methylation assay) (Karimi et al., 2006). Genomic DNA from each sperm (0.5-1 µg) was digested 4 h at 37 °C with 7.5 units of either HpaII and EcoRI (NEB R3101) or MspI and EcoRI in a total volume of 30 µL in duplicate. For

pyrosequencing of the digested samples, 20 µL of digested DNA were mixed with 20 µL of annealing buffer (Qiagen, 979009) and samples were placed in a Qiagen PyroMark Q96 ID. The instrument was programmed to add dNTPs in the following order: A, C+G, T, C+G, water, A, T. Peak heights (PH) were analyzed using the PyroMark Q96 software. A and T peaks refers to the amount of DNA cleaved by EcoRI (DNA content controls) whereas C + G peaks show the amount of DNA cleaved by MspI and HpaII. The percentage of methylation was calculated as 100 x (1-(PH HpaII/PH MspI)). The PH HpaII/PH MspI ratio was calculated by doing (PH HpaII/PH EcoRI) / (PH MspI/PH EcoRI).

2.7. Statistical analysis

Sperm viability and motility parameters were subjected to analysis of variance (General Linear Model, GLM). As fixed effect was chosen fresh or thawed sperm from both protocols, i.e. “fresh sperm”, “thawed DMSO” and “thawed methanol”. For all models, an examination of the residual plots verified that no systematic patterns occurred in the errors. Model results of p-values<0.05 were considered significant.

For the statistical analysis of DNA levels, a non-parametric test (paired Wilcoxon test) was performed. Differences were considered as significant if $p<0.05$.

All analyses were conducted in the R-environment (R_Development_Core_Team, 2010).

3. Results

Results from this comparison experiment showed that all samples, independently of the protocol used, decreased their percentage of motile cells and cell velocity after cryopreservation (Figure 1). In addition, the motility results from thawed samples of sperm cryopreserved with the methanol protocol showed higher motility (32.4 ± 1.8%) than those from the DMSO protocol (10.8 ± 0.9%) (Figure 1). All the sperm kinetic parameters analyzed showed the same pattern, with higher motility and faster velocities in samples preserved with the methanol protocol than those preserved with the DMSO one (Figure 1). Furthermore, the proportion of fast cells (faster than 100 µm/s) was also significantly reduced after

cryopreservation (Figure 2). Nevertheless, thawed samples of sperm cryopreserved with the methanol protocol presented a higher percentage (47.9 ± 1.5%) of fast cells than using the DMSO one (29.6 ± 2.1%). Note that the sperm was instantly activated when diluted in the freezing medium of the DMSO protocol before freezing, clearly affecting the motility after thawing (Figure 3), whereas samples diluted in the freezing medium containing methanol were not activated (no differences with fresh samples) and did not affect the sperm motility prior to freezing (Figure 3).

Cell viability results (Figure 4) showed that there were more live spermatozoa in thawed sperm samples from the methanol protocol than from the DMSO one, and although survival in both cases was high (>75%), it was still lower than viability measured in fresh sperm samples.

The analysis of cysteine methylation in fresh and thawed sperm (Figure 5) showed that sperm samples treated with the DMSO protocol had lower DNA methylation than fresh samples and samples treated with the methanol protocol, whereas these two showed no differences between each other.

4. Discussion

In this work, we described and compared the two main protocols available for European eel sperm cryopreservation. Our results indicated that in every case, the sperm motility of thawed sperm was lower than in fresh sperm. The reduction in post-thawing sperm quality compared to fresh sperm is consistent with the available bibliography, although there is a great variation between fish species (Asturiano et al., 2017; Horváth et al., 2015). For instance, Dziewulska et al. (2011) used several cryoprotectants (DMSO and methanol as in the present study) to freeze fresh sperm samples of Atlantic salmon (*Salmo salar*) with a motility of 70-95%. The study showed that the sperm motility after thawing was significantly lower than in fresh samples, with post-thawing motility values in the best protocol of 8.2%, using DMSO as cryoprotectant. Oppositely, a different study with cryopreserved sperm from brown trout (*Salmo trutta*) using methanol as cryoprotectant, obtained motilities of thawed

sperm higher than 60%, which represented a reduction of only 20% of motility compared to fresh samples (Horváth et al., 2015).

In the present study, the data of sperm quality from thawed samples showed that cryopreservation using the methanol protocol, caused higher motility values than the DMSO protocol. Although the values obtained with the methanol protocol were consistent with the bibliography (Müller et al., 2004; Szabó et al., 2005), the motility results from the DMSO protocol were lower than previously reported (Asturiano et al., 2003, 2004; Peñaranda et al., 2009). Although the samples were frozen immediately after the addition of the freezing media containing DMSO to the sperm, it has been proved that the presence of DMSO in the freezing media activates the European eel sperm (Peñaranda et al., 2009), and lead to a reduced post-thawed sperm motility. Even though the DMSO protocol was improved to reduce activation by increasing the concentration of $NaHCO_3$, decreasing the pH of the media (Peñaranda et al., 2009; Vílchez et al., 2017), fast manipulation was still required. In this study, we show that the sperm was activated after diluting in the freezing media (before freezing) containing DMSO. This pre-freezing activation naturally affects the final sperm motility of thawed sperm samples.

Although DMSO is the most used cryoprotectant for fish sperm (Gallego & Asturiano, 2018b; Martínez-Páramo et al., 2017), methanol has also been widely used in freshwater species such as sturgeons, salmonids, tench or Eurasian perch within others (reviewed by Asturiano *et al.,* 2017). Furthermore, it has been recently used in cryopreservation protocols for Japanese eel sperm (Koh et al., 2017; Müller et al., 2017, 2018). As cryoprotectant, methanol has been reported to penetrate more rapidly the cells and being less toxic than DMSO (Horváth et al., 2015). In addition, methanol is osmotically inert and therefore does not activate sperm by osmotic shock (De Baulny et al., 1997; Horváth et al., 2005). In our study, the methanol was apparently less toxic than the DMSO, because thawed samples from the methanol protocol presented higher survival than samples from the DMSO protocol. Furthermore, we confirmed that since methanol is osmotically inert, it did not activate the sperm, oppositely to the DMSO that activated the sperm due to the increase of osmolality.

This difference could partially explain the higher motility and velocity of thawed samples treated with the methanol protocol. Furthermore, both protocols differ in other aspects such as extender composition, dilution rate, volume and freezing rate that could also affect the thawed sperm motility.

Sperm from the DMSO protocol presented a loss of methylation compared to fresh sperm, whereas sperm from the methanol protocol remained similar to the fresh control. Changes in cytosine methylation levels after cryopreservation have been little explored in fish. Primarily, the concern arose for the use of methylating cryoprotectants that in the presence of ROS may led to cytosine methylation (Kawai et al., 2010). Indeed, Riesco and Robles (2013) observed in zebrafish that some promoter regions were hypermethylated after genital ridge cryopreservation in DMSO. However, in tambaqui (*Colossoma macropomum*) sperm, cryopreservation with either DMSO or methanol induced in both cases a sperm DNA hypomethylation (De Mello et al., 2017), contrarily to what could have been expected from the model study from Kawai et al. (2010). It is therefore not clear if the cryoprotectant molecule is the main parameter affecting DNA methylation. It was reported that cryopreservation-induced changes in DNA methylation could be species dependent (Labbé et al., 2017), and that cryopreservation with methods which are not optimal for a given species would induce more epigenetic effect (Labbé et al., 2014). In our case, the fact that the methanol protocol did not change the overall DNA methylation level would indicate that the epigenetic risk is reduced with this method.

5. Conclusions

In conclusion, this study show that the methanol cryopreservation protocol, is nowadays the most suitable protocol for European eel sperm cryopreservation, giving the best sperm motility, sperm velocity and cell survival values. Furthermore, the methylation level of sperm DNA from thawed samples with this method are the same as in fresh sperm, indicating that there are not drastic epigenetic changes when sperm is cryopreserved in this way.

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Declaration of interest

The authors declare no conflict of interests

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Figure captions

Figure 1.

Sperm kinetic results from fresh sperm, thawed sperm from methanol cryopreservation protocol (Hungarian protocol) and thawed sperm from DMSO protocol (Spanish protocol). The motility analyses show MOT (motility) and PM (progressive motility). The velocity results presented here are VCL (curvilinear velocity), VSL (straight-line velocity) and VAP (average path velocity). Boxplots with different letters are significantly different ($p < 0.05$; $n = 16$-18).

Figure 2.

Comparison of the percentage of different velocity groups [slow (VAP = 10-50 µm/s), medium (VAP = 50-100 µm/s) and fast (VAP > 100 µm/s)] of thawed sperm samples from the DMSO and methanol protocols, and from fresh sperm. Different letters indicate significant differences between percentages of fast cells ($p < 0.05$; $n = 16$-18).

Figure 3.

Effect of freezing medium dilution on sperm motility. Percentage of motile cells after activation with artificial sea water. “Fresh” column shows motility from fresh samples. “Pre-cryo” columns shows the sperm motility of sea water-activated samples after being diluted with freezing medium containing DMSO or methanol before cryopreservation, and “Thawed” columns shows the sperm motility of thawed and sea water-activated samples from the DMSO or methanol protocol. Values are means ± SEM of sperm from 16 samples. Means with different letters are significantly different ($p < 0.05$).

Figure 4.

Comparative viability data from flow cytometry of fresh sperm and thawed sperm from methanol and DMSO cryopreservation protocols. Values represent means ± SEM ($n = 12$). Different letters indicate significant differences ($p < 0.05$) between means.

Figure 5

Global DNA methylation of eel sperm. Average percentage ± SD (n=9) of 5-methylcytosine on fresh and thawed samples. Different letters indicate significant differences ($p<0.05$).

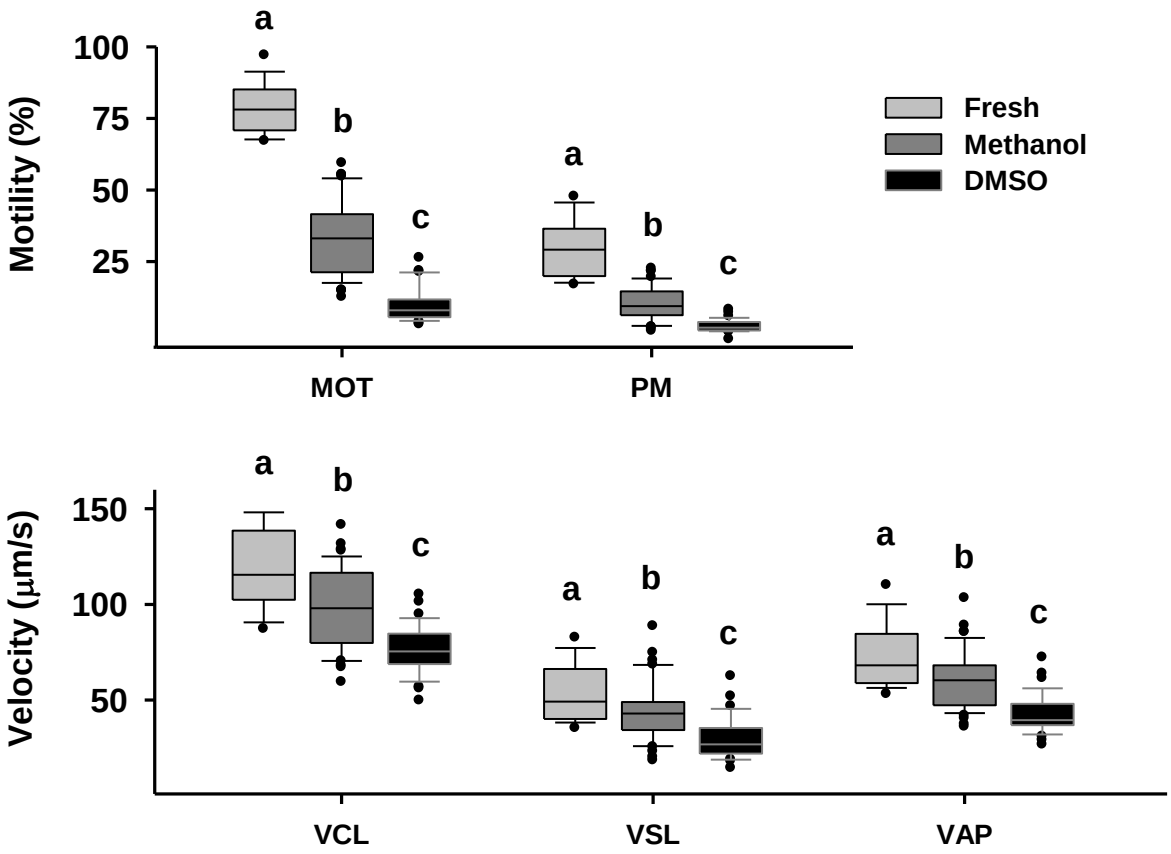
Motility (%)
100
75
50
25
a
b
c
a
b
c
MOT
PM
Fresh
Methanol
DMSO
Velocity (µm/s)
150
100
50
a
b
c
a
b
c
a
b
c
VCL
VSL
VAP

Figure 1.

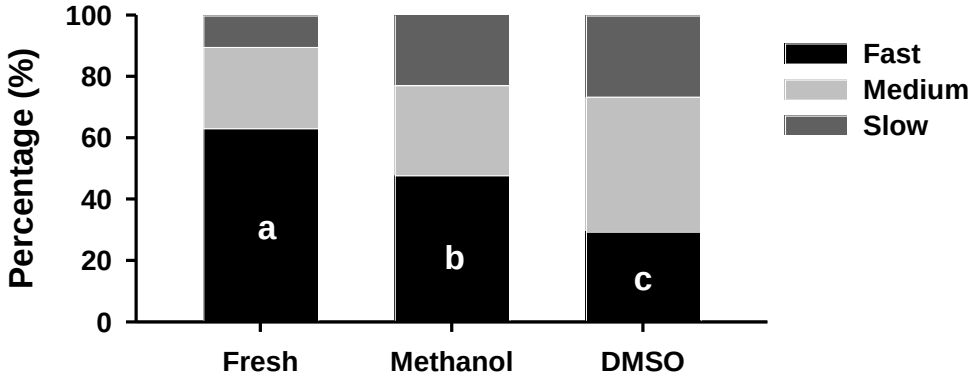
100
80
60
40
20
0
Percentage (%)
a
b
c
Fresh
Methanol
DMSO
Fast
Medium
Slow

Figure 2.

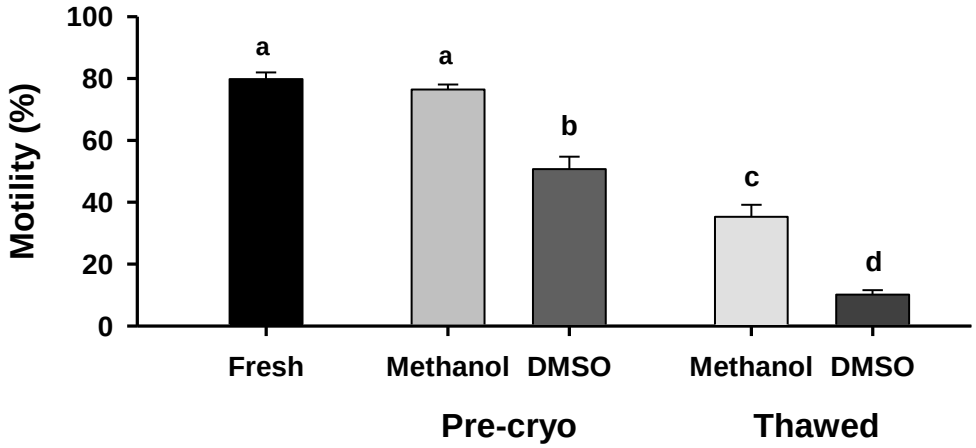
100
80
60
40
20
0
Motility (%)
a
a
b
c
d
Fresh
Methanol
DMSO
Methanol
DMSO
Pre-cryo
Thawed

Figure 3.

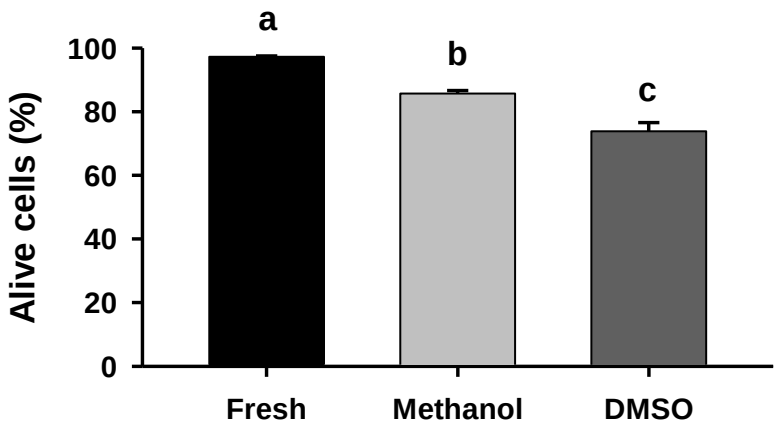

Figure 4.

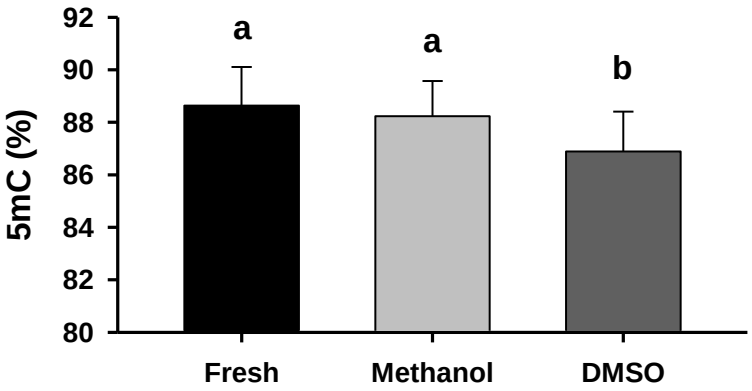

Figure 5.

Highlights

- Two previously validated protocols for the cryopreservation of European eel were compared.
- This work represent an important step in standardization of the sperm cryopreservation protocol of a farmed and endangered species such as the European eel.
- The study have shown effects on sperm motility and epigenetic changes caused by cryopreservation in eel thawed sperm.