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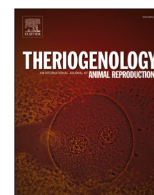

Original Research Article

Voltage-dependent anion channels are involved in the maintenance of pig sperm quality during liquid preservation

Ferran Garriga [a,b], Jesús Martínez-Hernández [a,b,c], Núria Gener-Velasco [b], Joan E. Rodríguez-Gil [d], Marc Yeste [a,b,e,*]

[a] Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003, Girona, Spain
[b] Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003, Girona, Spain
[c] Department of Cell Biology and Histology, Faculty of Medicine, IMIB-Arrixaca, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, ES-30120 Murcia, Spain
[d] Unit of Animal Reproduction, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, ES-08193, Cerdanyola Del Vallès, Barcelona, Spain
[e] Catalan Institution for Research and Advanced Studies (ICREA), ES-08010, Barcelona, Spain

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ABSTRACT

Pigs are usually bred through artificial insemination with liquid semen preserved at 15–20 °C. While this method of preservation brings many benefits, including a greater reproductive performance compared to frozen-thawed sperm, the period of storage is a limiting factor. As the mitochondrion regulates many facets of sperm physiology, modulating its activity could have an impact on their lifespan. Aligned with this hypothesis, the present study sought to investigate whether inhibition of voltage-dependent anion channels (VDACs), which reside in the outer mitochondrial membrane and regulate the flux of ions between mitochondria and the cytosol in somatic cells, influences the resilience of pig sperm to liquid preservation at 17 °C. For this purpose, semen samples (N = 7) were treated with two different concentrations of TRO19622 (5 μM and 50 μM), an inhibitor of VDACs, and stored at 17 °C for 10 days. At days 0, 4 and 10, sperm quality and functionality parameters were evaluated by flow cytometry and computer-assisted sperm analysis (CASA). The effects of inhibiting VDACs depended on the concentration of the inhibitor. On the one hand, the greatest concentration of TRO19622 (50 μM) led to a decrease in sperm motility, viability and mitochondrial membrane potential, which could be related to the observed intracellular Ca^{2+} increase. In contrast, total sperm motility was higher in samples treated with 5 μM TRO19622 than in the control, suggesting that when VDACs channels are inhibited by the lowest concentration of the blocking agent the resilience of pig sperm to liquid storage increases. In conclusion, the current research indicates that mitochondrial function, as regulated by ion channels in the outer mitochondrial membrane like VDACs, is related to the sperm resilience to liquid preservation and may influence cell lifespan.

1. Introduction

It is well known that artificial insemination (AI) is gaining prevalence worldwide as the most efficient method for breeding and genetic improvement in pigs [1]. Artificial insemination has to be combined with methods that effectively preserve sperm quality and fertilizing ability until the moment of servicing females [2]. While sperm cryopreservation allows for long periods of storage, which is of great interest for genetic selection [3], it may impair sperm quality, decreasing sperm viability, motility and mitochondrial activity, and inducing DNA fragmentation [4,5]. For this reason, and because AI with frozen-thawed sperm is associated to a lower reproductive performance, it is not employed routinely. Alternatively, most of the AI conducted utilize liquid semen [6], which can be preserved for 5–7 days - and even for a greater period - without showing a significant decline in sperm quality and fertilizing ability [7,8]. The basis of this approach is that the reduction of storage temperature decreases sperm metabolism, which contributes to extend the lifespan. Because of the high sensitivity of pig sperm to low temperatures, which can impair plasma membrane integrity and thus cell survival [9], they are usually stored between

* Corresponding author. Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003, Girona, Spain.
E-mail address: marc.yeste@udg.edu (M. Yeste).

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15 °C and 20 °C rather than at 4–5 °C as in other species [10]. In addition to be less capable of preventing bacterial growth [10,11], one of the issues of preserving sperm at 15–20 °C is that these temperatures can only reduce mitochondrial activity partially, thus limiting the period of storage [10].

Although the existence of proper apoptosis in sperm is still under debate, mounting evidence supports that apoptotic-like changes occur in these cells, and that they are tightly related to mitochondrial activity [12,13]. In somatic cells, it is known that the activation of intrinsic mitochondria-related pathways involves the release of some pro-apoptotic factors, such as cytochrome *c* [14], which activates the caspases pathway. High levels of reactive oxygen species (ROS) and calcium are known to induce apoptosis [15,16]. This indicates that, among other factors, a proper regulation of calcium homeostasis in mitochondria is needed for keeping sperm alive. In frozen-thawed sperm, some authors described the concept of spermptosis which refers to the apoptotic-like changes induced by ROS [17,18]. In this context, Voltage-Dependent Anion Channels (VDACs), which reside in the outer mitochondrial membrane and are involved in the flow of ions (including calcium) and molecules between the cytosol and mitochondria could play a role in the regulation of mitochondria [19]. Three different isoforms of VDAC channels have been described. Voltage-Dependent Anion Channel Isoform 2 (VDAC2) has been identified in pig sperm [20], where it appears to be implicated in spermatogenesis, sperm maturation, regulation of motility and fertilization [21–23]. On the other hand, while the presence of the VDAC1 protein is yet to be reported in sperm, VDAC3 has been identified in cattle and mouse [24,25] but not in pig sperm. Interaction of VDACs with pro-apoptotic proteins such as BAK and BAX is known to cause changes in the mitochondrial membrane, which becomes permeable to cytochrome *c* [26,27].

TRO19622, also known as olesoxime, inhibits VDACs and translocator protein 18 kDa (TSPO), which both form a complex in the outer mitochondrial membrane [28]. TRO19622 has been previously found to exert a neuroprotective effect [29], prevent *in vitro* sperm capacitation, and maintain sperm survival better than the standard capacitation medium [30]. Based on these findings, the hypothesis of this study was that inhibition of VDACs with TRO19622 could reduce mitochondrial function, which would have a positive impact on the preservation of sperm function and lifespan at 17 °C. For this purpose, two different concentrations of TRO19622 (5 μM and 50 μM) were added to conventional pig semen doses stored at 17 °C for 10 days. The main result was that blocking VDACs with TRO19622 reduced mitochondrial activity. When these channels were inhibited with 5 μM TRO19622, a positive impact on sperm motility was also observed.

2. Materials and methods

2.1. Semen samples

A total of seven semen samples (N = 7), each coming from a separate boar, were used in the present study. All ejaculates were purchased from an artificial insemination center (Grup Gepork SL, Masies de Roda, Spain). According to the information provided by the farm, all animals were sexually mature, healthy and kept under controlled conditions. Samples were diluted in a commercial extender (Vitasem LD, Magapor SL, Zaragoza, Spain) to a final concentration of 33×10^6 sperm/mL in doses of 90 mL, then cooled to 17 °C, and finally transported within the following 12 h to the University of Girona. Authors did not manipulate any boar as samples were purchased from the farm; thus, no permission from an Ethics committee was required.

Upon arrival, sperm quality and functionality parameters were evaluated (0 days), and each seminal dose was split into three aliquots of 5 mL that were incubated with 0 μM, 5 μM (TRO5) or 50 μM TRO19622 (TRO50). These two concentrations of TRO19622 were chosen on the basis of preliminary experiments, where concentrations greater than 50 μM were found to be cytotoxic (Suppl. Table 1). Samples were stored at 17 °C for 10 days, and evaluated at 4 and 10 days.

2.2. Evaluation of sperm motility

Sperm motility was assessed with a CASA system, consisting of a phase-contrast microscope (Olympus BX41; Olympus, Tokyo, Japan) equipped with a camera and connected to a computer where the Integrated Sperm Analysis System (ISAS; V1.0) software was installed (Proiser SL, Valencia, Spain). Briefly, 3 μL of the sample were placed into a Leja chamber (Leja Products BV; Nieuw-Vennep, The Netherlands), and observed under the phase-contrast microscope. Images were captured and analyzed using the ISAS software, and each capture consisted of 25 images taken in 1 s. Two technical replicates of at least 1000 sperm per replicate were analyzed for each sample. The kinematic parameters evaluated in each analysis were the following: average pathway velocity (VAP, μm/s); straight-line velocity (VSL, μm/s); curvilinear velocity (VCL, μm/s); linearity index (LIN, %), which is the quotient between VSL and VCL and multiplied per 100; straightness index (STR, %), which is the quotient between VSL and VAP and multiplied per 100; oscillation index (WOB, %), which is the quotient between VAP and VCL and multiplied per 100; amplitude of lateral head displacement (ALH, μm); and beat-cross frequency (BCF, Hz). Moreover, percentages of total motile sperm and progressively motile sperm were also determined in each replicate. Sperm were considered motile when their VAP was equal to or greater than 10 μm/s, and progressively motile when their STR was equal to or greater than 45 %. Connectivity was set at 11, and at least ten images were required to calculate the ALH. The area of captured particles ranged between 10 and 80 μm^2.

2.3. Flow cytometry

A CytoFlex flow cytometer (Beckman Coulter; Brea, CA, USA) was used to determine sperm viability (SYBR-14/propidium iodide [PI]), mitochondrial membrane potential (JC-1/[LIVE/DEAD far red]), and the intracellular levels of Ca^{2+} (Fluo4-AM/PI), total ROS (dichlorodihyodrofluorescin diacetate [H_2DCFDA]/PI), and superoxides (dihydroethidium [HE]/Yo-Pro-1). Information on flow cytometry analyses is provided following the MIFlowCyt guidelines [31].

Sperm were specifically selected from other particles/cells by using the Forward Scatter Detector (FSD), which analyzes the size of particles; and the Side Scatter Detector (SSD), which detects their roughness. The combination of both detectors allowed for the discernment of sperm from other particles, such as debris and cell aggregates. All samples were excited with the blue laser (488 nm), except the ones labeled with the LIVE/DEAD fixable far red dead cell stain, which were excited with the red laser (638 nm). The blue fluorescence emitted by the LIVE/DEAD fixable far red fluorochrome was detected through the APC channel (660/20); the green fluorescence emitted by SYBR-14, M540, Yo-Pro-1, PNA-FITC, DCF, Fluo4 and JC-1 monomers was collected through the FITC channel (524/40); the orange fluorescence emitted by JC-1 aggregates was detected via PE channel (585/42); and the red fluorescence of PI and E was measured with the PC5.5 channel (690/50). For each sample, two technical replicates of at least 5000 sperm per replicate were evaluated.

2.3.1. Evaluation of sperm viability

Sperm viability was determined with the LIVE/DEAD viability kit (Molecular Probes; Eugene, OR, United States), following the protocol of Garner and Johnson [32]. Briefly, samples were stained with both SYBR-14 (final concentration: 31.5 nM) and PI (final concentration: 7.6 μM) at 38 °C in the dark for 10 min. Staining with both fluorochromes resulted in four different populations: (i) viable sperm (SYBR-14$^+$/PI$^-$); (ii) moribund sperm (SYBR-14$^+$/PI$^+$); (iii) non-viable sperm (SYBR-14$^-$/PI$^+$); and (iv) debris particles (SYBR-14$^-$/PI$^-$). The percentage of debris particles was used to correct and recalculate the percentage of

viable sperm.

2.3.2. Evaluation of mitochondrial membrane potential

Mitochondrial membrane potential was evaluated after staining with JC-1 (final concentration: 750 nM) and the LIVE/DEAD fixable far red dead cell fluorochrome (Molecular Probes; Eugene, OR, United States), diluted at 1:8000 (v:v) in PBS, following the protocol of Llavanera et al. [33] with minor modifications. Samples were stained at 38 °C in the dark for 30 min. When sperm have high mitochondrial membrane potential JC-1 forms aggregates, which emit orange fluorescence. On the contrary, low mitochondrial membrane potential maintains JC-1 molecules in the monomeric form, emitting green fluorescence. Four populations were determined: (i) viable sperm with high MMP; (ii) viable sperm with low MMP; (iii) non-viable sperm with high MMP; and (iv) non-viable sperm with low MMP. Mitochondrial membrane potential was expressed as the ratio between the intensity of fluorescence of JC-1_{agg} and JC-1_{mon} in viable sperm.

2.3.3. Evaluation of Ca^{2+} levels

For the assessment of intracellular Ca^{2+} levels, double staining with Fluo4-AM and PI was performed, following the protocol of Harrison et al. [34] with some minor modifications. Fluo4-AM penetrates the cell and emits fluorescence after de-esterification and binding to Ca^{2+}; thus, the greater the fluorescence of Fluo4, the greater the intracellular levels of Ca^{2+}. In brief, samples were stained with both Fluo4-AM (final concentration: 1.17 μM) and PI (final concentration: 5.6 μM) at 38 °C in the dark for 10 min. Results were analyzed by normalizing the Fluo4 intensity in viable sperm (PI^-) with the respective control. Samples stained with Fluo4 were also observed under a confocal microscope, as described in Suppl. File 1.

2.3.4. Evaluation of total ROS levels

Total ROS levels in sperm were determined through staining with H_2DCFDA and PI [35]. ROS oxidizes and de-esterifies H_2DCFDA, which is not fluorescent, to DCF that, in contrast, emits green fluorescence. First, samples were incubated with H_2DCFDA (final concentration: 0.35 μM) at 38 °C in the dark for 20 min. Subsequently, PI was added to samples (final concentration: 6 μM), which were incubated under the same conditions for further 5 min. For each sample, total ROS levels were expressed as the intensity of DCF in live sperm (PI^-), normalized with the respective control.

2.3.5. Evaluation of superoxide levels

Superoxide (O_2^-) levels in sperm were assessed following double staining with HE (5 μM) and Yo-Pro-1 (25 nM), as described by Guthrie and Welch [35]. O_2^- oxidizes HE to ethidium (E), which emits red fluorescence. Samples were incubated at 38 °C in the dark for 20 min. In each sample, the intensity of E in viable sperm (Yo-Pro-1^-) in each sample was normalized with its respective control, in order to express intracellular O_2^- levels.

2.4. Statistical analyses

Statistical analyses were conducted using IBM SPSS Statistics 27.0 (IBM Corp., Armonk, NY, USA), whereas data were plotted with GraphPad Prism v.8 (GraphPad Software, La Jolla, CA, USA). First, normal distribution was tested with Shapiro-Wilk and homogeneity of variances (homoscedasticity) was checked with the Levene test. Subsequently, a linear mixed model (i.e., with repeated measures) was performed to determine the effects of blocking VDACs with TRO19622 on sperm quality and functionality parameters during storage of semen at 17 °C. The different time points when samples were analyzed (0, 4 and 10 days) were considered as an intra-subject factor, and the different treatments (control, 5 μM TRO19622 and 50 μM TRO19622) were considered as an inter-subject factor. Pair-wise comparisons were made using the Bonferroni post-hoc test.

Statistical cases were the different biological replicates, and data used for analyses were the mean of the two technical replicates. Differences were considered to be significant when $P \leq 0.05$. Data are represented in plots that show the mean ± the standard error of the mean (SEM).

3. Results

3.1. Inhibition of VDACs with 50 μM TRO19622 impairs sperm viability during preservation at 17 °C

Sperm viability was evaluated on the basis of plasma membrane integrity after co-staining with SYBR-14 and PI, and data are shown in Fig. 1A. While sperm viability did not vary when VDACs were blocked with a low concentration of TRO19622 (5 μM TRO19622), a significant decrease in the percentage of viable sperm was observed after 4 and 10 days of storage at 17 °C when these channels were inhibited with 50 μM TRO19622 ($P < 0.05$).

3.2. Blocking VDACs during preservation of sperm at 17 °C increases Ca^{2+} levels in a dose-dependent manner

As depicted in Fig. 1B, inhibition of VDACs with TRO19622 led to an increase of intracellular Ca^{2+} levels in viable sperm ($Fluo4^+/PI^-$). The extent of this increase was dose-dependent, as the inhibition of VDACs with 50 μM TRO19622 induced a greater rise in Ca^{2+} levels than the inhibition with 5 μM TRO19622. Moreover, confocal microscopy confirmed that the green fluorescence emitted by Fluo4 distributed throughout the whole cell (especially, the midpiece and post-acrosomal region) (Suppl. Fig. 1).

3.3. Inhibition of VDACs diminishes mitochondrial activity after 4 days of preservation at 17 °C

Fig. 2A shows the ratio between JC-1_{agg} and JC-1_{mon}, which represents the mitochondrial membrane potential during preservation at 17 °C. Liquid storage for 10 days led to a decrease of MMP in the control and VDAC-inhibited samples. Yet, after 4 days of preservation at 17 °C, there were differences between the control and treatments containing TRO19622, so that the greater the concentration (50 μM TRO19622) the lower the MMP, again showing that the effects of blocking VDACs were dose-dependent.

3.4. Inhibition of VDACs with TRO19622 alters the levels of superoxide and total ROS in pig sperm during liquid preservation at 17 °C

To evaluate how inhibiting VDACs with TRO19622 affects ROS levels during sperm storage, double staining with HE and Yo-Pro-1 was used to assess intracellular superoxide levels, whereas incubation with H_2DCFDA and PI allowed for the determination of total ROS levels. In both cases, the results were expressed as the relative intensity of E or DCF in the different treatments normalized against the control at each time point (Fig. 2B–C). Regarding superoxide levels, inhibition of VDACs with the highest concentration of TRO19622 led to a significant decrease in the E intensity at days 4 and 10 compared to both the control and 5 μM TRO19622. Nevertheless, the lowest concentration of TRO19622 significantly increased ($P < 0.05$) these levels at day 4, notwithstanding they were significantly reduced at day 10. On the other hand, whereas the two concentrations of TRO19622 significantly decreased ($P < 0.05$) total ROS levels of sperm after 4 days of preservation at 17 °C, inhibition of VDACs with 50 μM TRO19622 led to a significant increase ($P > 0.05$) of ROS levels at day 10.

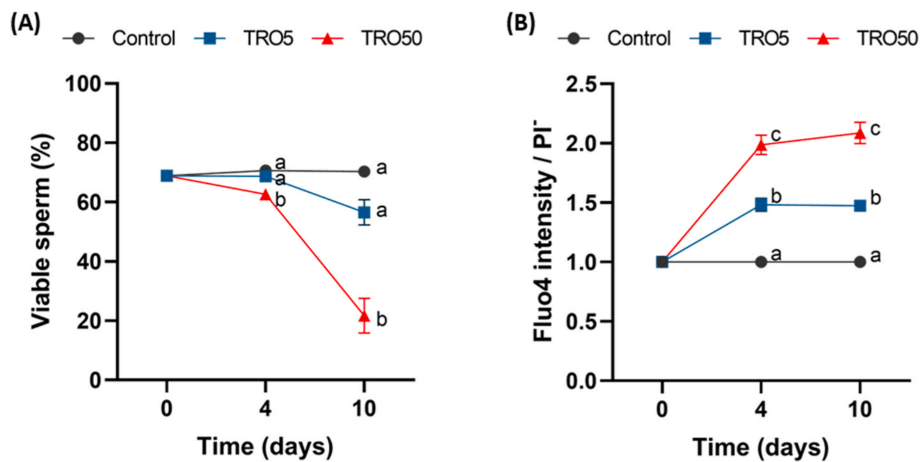

Fig. 1. Effects of 5 μM and 50 μM TRO19622 on sperm viability and whole-cell Ca^{2+} levels during liquid preservation of pig sperm at 17 °C. Lines represent (A) sperm viability; and (B) the ratio of $Fluo4^+$ intensity of viable (PI^-) sperm between the treatment and the respective control at day 0, and after 4 and 10 days of incubation with 0 μM (control), 5 μM or 50 μM TRO19622 at 17 °C. Different letters indicate significant differences ($P \leq 0.05$) between experimental groups at a given time point. Results are expressed as the mean ± SEM (N = 7).

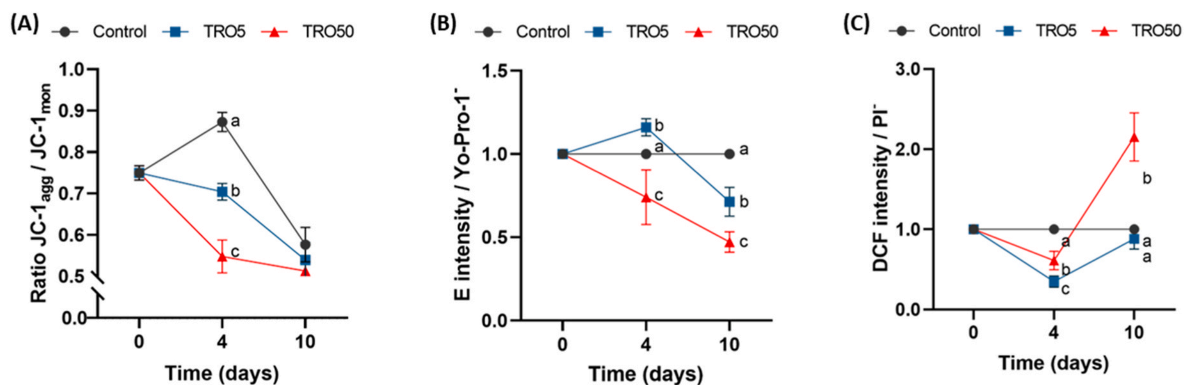

Fig. 2. Effects of 5 μM and 50 μM TRO19622 on MMP, total ROS levels and superoxide levels during liquid preservation of pig sperm at 17 °C. Lines represent (A) the ratio between JC-1_{agg} and JC-1_{mon}; (B) the ratio of E^+ intensity in viable (Yo-Pro-1^-) sperm between the treatment and the respective control; and (C) the ratio of DCF^+ intensity in viable sperm (PI^-) between the treatment and the control at day 0, and after 4 and 10 days of incubation with 0 μM (control), 5 μM or 50 μM TRO19622 at 17 °C. Different letters indicate significant differences ($P \leq 0.05$) between experimental groups at a given time point. Results are expressed as the mean ± SEM (N = 7).

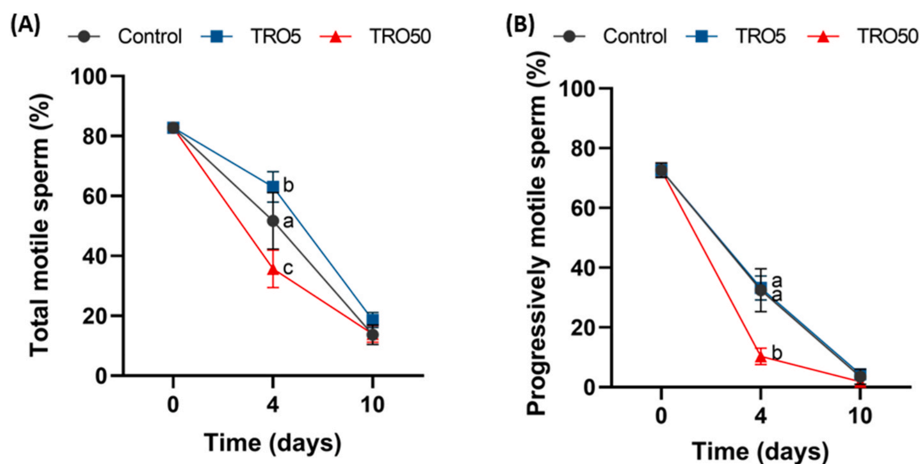

Fig. 3. Effects of 5 μM and 50 μM TRO19622 on sperm motility during liquid preservation of pig sperm at 17 °C. Lines represent the percentages of (A) total motile sperm; and (B) progressively motile sperm at day 0, and after 4 and 10 days of incubation with 0 μM (control), 5 μM or 50 μM TRO19622 at 17 °C. Different letters indicate significant differences ($P \leq 0.05$) between experimental groups at a given time point. Results are expressed as the mean ± SEM (N = 7).

3.5. Treating sperm with 5 μM TRO19622 improves total motility after 4 days of preservation at 17 °C

Blockage of VDACs with the highest concentration of TRO19622 caused a significant reduction ($P < 0.05$) in total and progressively motile sperm at day 4, as shown in Fig. 3A and B, respectively. On the other hand, inhibition of these channels with 5 μM TRO19622 significantly ($P < 0.05$) increased total motility at day 4. At day 10, however, no effect was observed from any of the concentrations of the inhibitor.

3.6. Blocking VDACs with 5 μM TRO19622 improves LIN, STR and WOB after 4 days of preservation at 17 °C

Fig. 4 represents the effect of TRO19622 on: (A) VSL; (B) VCL; (C) VAP; (D) LIN; (E) STR; and (F) WOB. While inhibition of VDACs with 50 μM TRO19622 impaired VSL, VCL and VAP, after 4 days of preservation at 17 °C, the lowest concentration of this inhibitor had no influence on any of these sperm velocities. In the case of LIN, STR and WOB, blocking VDACs with 50 μM TRO19622 significantly ($P < 0.05$) reduced all the three indexes after 10 days of storage. Moreover, this concentration also induced a decrease in LIN and WOB at day 4. In contrast, LIN, STR and WOB after 4 days of preservation at 17 °C were significantly greater ($P < 0.05$) in the treatment containing 5 μM TRO19622 than in the control.

3.7. Inhibition of VDACs with 5 μM TRO19622 increases both ALH and BCF

Blocking VDACs had a similar effect on ALH and BCF, as depicted in Fig. 5A and B, respectively. Whereas, at day 4, no effect was observed with any of the concentrations of the inhibitor, blocking VDACs with 5 μM TRO19622 led to a significant increase in both ALH and BCF after 10 days of preservation at 17 °C. This increase, however, was not observed when VDACs were inhibited with the highest concentration of TRO19622.

4. Discussion

Mitochondria and their ionic channels are extremely relevant in regulating sperm lifespan and functionality [36,37]. Considering that the period of storage is the main limitation of semen preservation at 17 °C, the present study aimed to determine if modulating mitochondrial function through inhibition of VDACs (and potentially TSPO) with TRO19622 could improve sperm quality during liquid storage. Data suggested that mitochondrial function via voltage-dependent anion channels plays a key role during liquid preservation of pig semen, as inhibition with the highest concentration of TRO19622 compromised both sperm motility and survival. This reduction in sperm viability and motility could result from an overload of intracellular Ca^{2+} levels, as observed in this study. Remarkably, treating sperm with 5 μM TRO19622 had the opposite effect, as it led to an increase in sperm motility and did not affect sperm viability. The effects of the two concentrations of TRO19622 on each sperm functional parameter are discussed separately. Before proceeding further, it is worth noticing that TRO19622 is known to inhibit both VDACs and TSPO [28]. To the best of the authors' knowledge, nevertheless, TSPO is yet to be identified in sperm. Thus, while the present study mainly refers to the effects of TRO19622 as the inhibition of VDACs, one cannot exclude that, if present in sperm, TSPO could also be blocked by this pharmacological agent.

Regarding sperm viability, inhibition of VDACs with 50 μM TRO19622 impaired this variable after both 4 and 10 days of preservation at 17 °C. These results suggest that VDACs are essential for the maintenance of sperm lifespan during long-term liquid storage. This is in accordance with a previous work where the incubation of sperm with

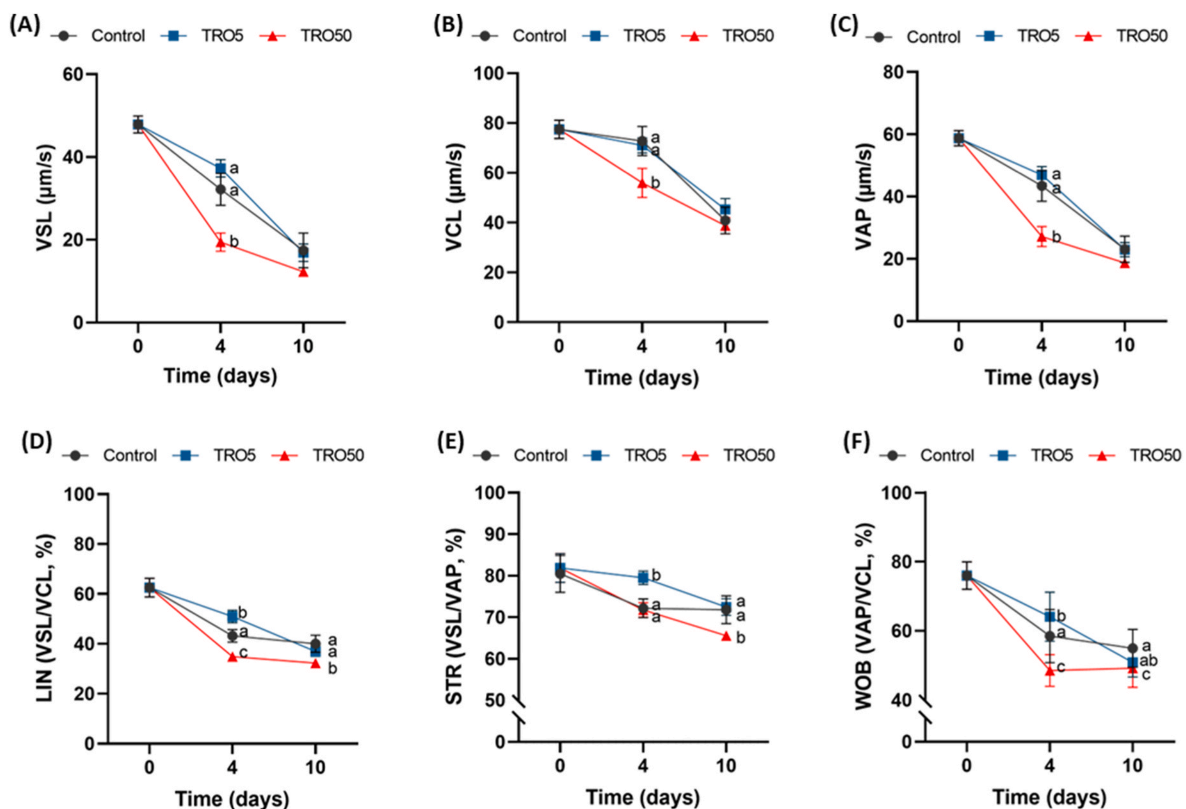

Fig. 4. Effects of 5 μM and 50 μM TRO19622 on sperm kinematics during liquid preservation of pig sperm at 17 °C. Lines represent (A) VSL; (B) VCL; (C) VAP; (D) LIN; (E) STR; and (F) WOB at day 0, and after 4 and 10 days of incubation with 0 μM (control), 5 μM or 50 μM TRO19622 at 17 °C. Different letters indicate significant differences ($P \leq 0.05$) between experimental groups at a given time point. Results are expressed as the mean ± SEM (N = 7).

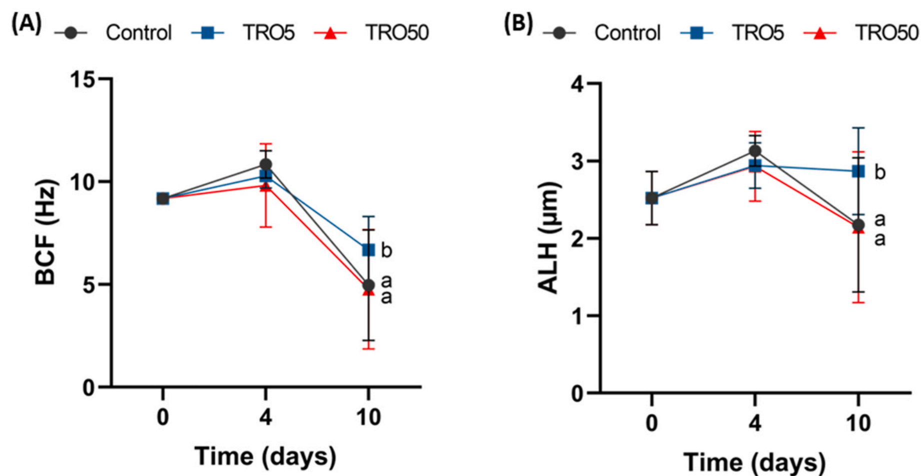

Fig. 5. Effects of 5 μM and 50 μM TRO19622 on BCF and ALH during liquid preservation of pig sperm at 17 °C. Lines represent (A) BCF; and (B) ALH at day 0, and after 4 and 10 days of incubation with 0 μM (control), 5 μM or 50 μM TRO19622 at 17 °C. Different letters indicate significant differences ($P \leq 0.05$) between experimental groups at a given time point. Results are expressed as the mean ± SEM (N = 7).

4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), a non-specific inhibitor of voltage-dependent anion channels, resulted in an impairment of sperm viability and motility [23]. Nonetheless, these findings contrast with the study of Martínez-Abad et al. [30], which found that when pig sperm were *in vitro* capacitated in the presence of TRO19622, their viability was maintained better. A possible explanation for these disparate results could be that the conditions of the current study and the previously mentioned were different, as whereas the former focused on liquid preservation, the latter investigated capacitated sperm. On the other hand, the loss of sperm viability after inhibition of VDACs could be explained by the fact that intracellular Ca^{2+} levels increased in a dose-dependent manner after incubation with TRO19622. This rise in whole-cell Ca^{2+} levels could be related to the impairment of sperm viability, as studies in human sperm revealed that an overload of Ca^{2+} induces apoptotic-like changes [16,38]. As VDACs are known to transport Ca^{2+} to mitochondria [39], one could suggest that inhibiting these channels with TRO19622 could alter this transport and thus affect intracellular Ca^{2+} levels. In neural cell types, Ca^{2+} homeostasis is known to be regulated by TSPO via its close relationship with VDACs [40]. Because Ca^{2+} is known to play an important role in regulating cell death, and the routes leading to cell death in sperm have not been fully elucidated [16,41], further research interrogating which specific cell death pathways are triggered in sperm during liquid preservation is warranted.

Inhibition of VDACs with TRO19622 significantly decreased mitochondrial membrane potential at day 4 in a dose-dependent manner. This outcome could be explained by the involvement of VDACs in the regulation of the ion flow between the cytosol and mitochondria [42]. Thus, inhibition of VDACs would, as indicated before, lead to an impairment of Ca^{2+} transport to mitochondria, which could alter MMP. In addition, and because mitochondrial activity has been widely related to sperm motility [43,44], the impairment of MMP caused by the inhibition of VDACs with the highest concentration of TRO19622 could also underlie the reduction observed in sperm motility, which is discussed in more detail below. In spite of this, and quite surprisingly, the treatment containing 5 μM TRO19622 exhibited greater total motility and lower MMP after 4 days of preservation at 17 °C.

Blockage of VDACs with 50 μM TRO19622 decreased intracellular superoxide levels in pig sperm throughout liquid preservation. While the same effect was observed on total ROS levels at day 4, a significant increase in these total ROS levels was detected at day 10. These alterations in ROS levels could be explained by the reduction of mitochondrial activity observed when VDACs were blocked with TRO19622. ROS are highly related to mitochondrial activity as this organelle appears to be one of the main sources of these chemical species in sperm [45,46]. It is thus reasonable to suggest that the reduction of MMP might lead to a lower production of ROS. The fact that, at day 10, an important increase in total ROS was observed could be related to the impairment of sperm cell functionality caused by the highest concentration of the inhibitor, which would agree with the decrease in sperm viability. In fact, high ROS levels are known to impair sperm quality - including viability - in mammalian species such as pigs and humans [47,48], which would be compatible with these results.

Inhibition of VDACs with 50 μM of TRO19622 led to a decrease in total and, especially, progressive motility after 4 days of storage. Hence, VDACs would seem to be essential for maintaining sperm motility during liquid preservation. These results agree with the fact that functional mitochondria are necessary to maintain sperm motility, as reported in a previous work where mouse sperm devoid of VDAC3 were immotile [49]. The decrease observed in mitochondrial activity could thus drive the reduction of sperm motility. In addition, a previous study inhibiting VDACs during *in vitro* capacitation of mouse sperm also reported an impairment of sperm motility [23]. Nevertheless, the current work revealed a biphasic response of inhibiting VDACs with TRO19622 as, whereas the highest concentration of the inhibitor entailed an obvious reduction of sperm motility in agreement with the aforementioned, the lowest concentration of TRO19622 appeared to have a beneficial role on total sperm motility. This positive effect of TRO19622, however, does not seem to agree with the previously mentioned literature, which found that VDACs are essential for sperm motility. These inconsistencies could be explained by the effect of the low concentration of the inhibitor, which would not completely block VDACs.

Finally, inhibition of VDACs with 50 μM of TRO19622 had a detrimental repercussion on sperm velocities (VCL, VSL and VAP) at day 4, which coincides with the findings discussed in the previous paragraph. An identical effect was observed for the separate kinetic indexes (LIN, STR and WOB) after 10 days of storage. It is worth mentioning that similar findings were reported in frozen-thawed sperm by Vilagran et al. [20], as cells with a decreased VDAC2 content also exhibited lower kinematic parameters, in addition to lower viability. When VDACs were inhibited with 5 μM TRO19622, kinetic indexes (LIN, STR and WOB) were greater after 4 days of preservation at 17 °C, suggesting that low doses of the inhibitor could be beneficial for sperm motility. Concurring with these findings, the blockage of VDACs with the lowest concentration of the inhibitor also induced an increase in ALH and BCF after 10 days of preservation. Hence, and considering the results observed in liquid-stored and cryopreserved sperm, it is reasonable to suggest that VDACs are involved in the regulation of motility in pig sperm.

5. Conclusions

In conclusion, the present work showed that mitochondrial function, and particularly VDACs, a family of outer mitochondrial membrane channels, are essential for maintaining pig sperm quality and functionality during liquid preservation at 17 °C. The effects, however, were biphasic and relied upon the concentration of the agent used to block the channel. Thus, while inhibition with 50 μM TRO19622 led to an impairment in sperm viability and mitochondrial activity, which seemed to underlie a decrease in sperm motility, the treatment with the lowest concentration of TRO19622 was beneficial for sperm motility and had no detrimental effect on sperm viability.

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Conflicts of interest

The authors have no conflict of interest to declare.

CRediT authorship contribution statement

Ferran Garriga: Writing – original draft, Methodology, Investigation. **Jesús Martínez-Hernández:** Methodology, Investigation. **Núria Gener-Velasco:** Methodology, Investigation. **Joan E. Rodríguez-Gil:** Writing – review & editing, Formal analysis, Conceptualization. **Marc Yeste:** Writing – review & editing, Supervision, Conceptualization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2024.05.003.

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