

Application of Glucose-Methanol Extender to Cryopreservation of Mozambique Tilapia (*Oreochromis mossambicus*) Sperm

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Abstract

Cryopreservation of fish sperm offers key advantages in genetic improvement and can be used to preserve threatened and endangered fish species or strains. The use of simple cryoprotective media facilitates cryopreservation procedures. In this study, we used a glucose-methanol extender for cryopreservation of *Oreochromis mossambicus* sperm. Optimal cryopreservation conditions were found to be 175 mM glucose and 12.5% methanol at 1:8 dilutions (sperm: extender), 60 min equilibration after dilution and pre-freezing at 8 cm above liquid nitrogen. Cryopreservation at these conditions resulted in ~ 50% decrease in post-thaw sperm motility (27 - 28% post-thaw motility compared to the 62% fresh control motility). However, fertilization rate of cryopreserved sperm reached 49% for sperm diluted 10 times, and 52% for 8 times diluted sperm. These rates were comparable to fertilization rate (56%) obtained using fresh sperm. Our results, therefore, reveal that even though the progressively motile sperm and motility percentage decreased significantly after cryopreservation, the fertilizing ability of cryopreserved sperm was not significantly affected. Our results show that glucose-methanol mixture is not only suitable for cryopreservation of *O. mossambicus* sperm but also allows cryopreservation in small volumes as low as 10 µl.

Introduction

Tilapia is widely distributed and cultured in many tropical and subtropical countries. In addition to their commercial value, tilapias are also regarded as one of the most important sources of nutrition, especially in developing countries (Ahmed and Lorica, 2002; Tacon, 1999). Fast growth and adaptability to a wide range of culture conditions are characteristics of tilapias that encourage their farming. Mozambique tilapia (*Oreochromis mossambicus*), is one of the tilapia species farmed on a large scale worldwide (El-Sayed, 1999; Romano, 2013).

Freshwater fishes play a crucial role in global food security yet recognized as one of the most threatened groups of vertebrates (Reid Contreras, & Csatadi, 2013). The threats due to overfishing, pollution, habitat loss and climate change have been of a serious concern to conservation biologists. Since the first report of successful cryopreservation of fish sperm about 5 decades ago (Blaxter, 1953), cryopreservation of fish sperm has been incorporated in conservation programs to preserve fish gametes for use in aquaculture.

Cryopreservation of fish sperm has been used to preserve threatened and endangered fish species or strains (Sarder, Saha, & Sarker, 2012; 2013). Sperm cryopreservation offers key advantages in genetic improvement. The availability of frozen sperm on demand accelerates breeding programs, such as the production and dissemination of improved and better-conditioned breeds (Lende *et al.*, 1998). Furthermore, the sperm of desirable specific genotypes can be preserved to create monosex stocks, as they grow to a larger size (Mair & Little, 1991).

Among other factors, the type of cryoprotective media is one of the factors to be considered for a successful cryopreservation of sperm (Kopeika, Kopeika, & Zhang, 2007). In Mozambique tilapia, extender containing 15% skim milk and 5% methanol was previously used for sperm cryopreservation (Harvey, 1983). In this study, freezing was carried out immediately after diluting the sperm with the cryoprotectants to avoid the detrimental effects imposed on spermatozoa by the cryoprotectants. However, in most cases, there may be delays between sperm collection/dilution and cryopreservation. This

may be challenging, especially when several batches of semen should be cryopreserved. A recent study on the use of glucose and methanol for sperm cryopreservation showed that equilibration time (15 min) might improve cryopreservation success (Ciereszko, Dietrich, Nynca, Dobosz, & Zalewski, 2014). This indicates that specific time might be necessary for cryoprotectant to penetrate the cells in order to provide adequate protection during freezing. Therefore, an alternative cryopreservation procedure for *O. mossambicus* sperm enabling longer time of manipulation prior to cooling which might also ensure better penetration of cells by cryoprotectant should be investigated.

An effective cryopreservation procedure that involves the use of simple media, such as glucose-methanol extender has attracted much attention recently. Glucose-methanol extender has been used to successfully cryopreserve sperm of salmonids, sturgeons, cyprinids and percid species (Aramli, Golshahi, Nazari, Aramli, & Banan, 2015; Ciereszko *et al.*, 2014; Dietrich, Nynca, Dobosz, Zalewski, & Ciereszko, 2014; Nynca, Dietrich, Dobosz, Grudniewska, & Ciereszko, 2014; Judycka, Szczepkowski, Ciereszko, & Dietrich, 2015; Lujić, Benarh, Marinović, Radojković, Simić, Ćirković, ... & Hovarth, 2017; Marinović, Lujić, Kása, Bernáth, Urbányi, & Horváth, 2017; Bernath, Bokor, Žarski, Várkonyi, Hegyi, Staszny, ... & Horváth, 2016). However, in Mozambique tilapia, sperm cryopreservation using glucose-methanol extender has not been reported. Moreover, despite the low volume of individual sperm samples usually obtained from the males, sperm cryopreservation in low volumes has not yet been investigated for *O. mossambicus*. Therefore, the aim of this study is to test the suitability of glucose-methanol mixture as a simple extender for cryopreservation of *O. mossambicus* sperm in small volumes.

Material and Methods

Collection of Sperm and Measurement of Sperm Concentration

Males (more than 2 years old, body weight, BW = 78 ± 19.5 g, total length, TL = 19.0 ± 1.6 cm, $n=10$) and females (more than 2 years old, BW = 55 ± 15.8 g, TL = 15.5 ± 1.2 cm, $n=10$) were raised at the Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. The fish were stocked in a concrete tank (0.73 m^3) and supplied with fresh water. The temperature of the water ranged from 28 to 30°C and the pH was 7.2 to 7.5. The fish were fed twice daily with a commercial diet (PIA Gold EP2; Marubeni Nisshin, Japan). Semen collection was carried out by gentle abdominal massage and care was taken to avoid contamination with blood, urine, or fecal materials. The semen was carefully collected with a pipette and

transferred to a 1.5 ml Eppendorf tube. Contaminated samples were discarded. The sperm concentration was determined by using a Thoma hemocytometer (Erma Inc., Tokyo, Japan). Average sperm concentration used for the experiment was 1.81×10^9 spermatozoa mL⁻¹.

Cryopreservation

The cryopreservation procedure described by Judycka *et al.* (2016) was used. Prior to dilution and freezing, contaminated samples were discarded. Only semen with viable spermatozoa (over 40 % motility) was cryopreserved. Sperm samples obtained from mature males were diluted individually and were loaded into 250 µl straws (IMV Technologies, L'Agile, France). The straws containing the sperm samples were placed on a floating rack adjusted to heights 3, 5 and 8 cm. The floating rack containing the straws was carefully lowered inside liquid nitrogen in a Polystyrene box and allowed to float on the surface of the liquid nitrogen. The system was covered with the lid of Polystyrene box, allowed to cool for 10 min and the straws were transferred to the liquid nitrogen. During post-thaw sperm motility analyses and fertilization, the straws were thawed in a water bath at a temperature of 40°C for 5 sec. Cryopreservation trials were duplicated for each sample.

Cryopreserved Sperm and Motility

The motility parameters of the cryopreserved sperm samples were measured immediately after thawing using a computer-assisted sperm analysis system. Analyses were duplicated for each sample.

Experimental Design

The experiments were conducted sequentially so that in each experiment one cryopreservation parameter was modified, and that in the each following step the best (optimal) parameter from the previous trial was used.

Experiment 1. Effect of Methanol Concentration on Sperm Cryopreservation

Sperm samples ($n=4$) were cryopreserved using an extender consisting of 10, 12.5, and 15% of methanol and 175 mM glucose. Semen was diluted with the extender at a ratio of 1:7 (sperm: extender). Floating rack height was 5 cm and equilibration time was 15 min.

Experiment 2. Effect of Glucose Concentration

Sperm samples ($n=4$) were cryopreserved using extenders consisting of 150, 175, and 200 mM of glucose and 12.5% methanol. The ratio of sperm to extender dilution was 1:7. The final concentrations of glucose

were 131, 153, and 175 mM and the final concentration of methanol was 10.9%. The height of floating rack was 5 cm and equilibration time was 15 min.

Experiment 3. The effect of Height above the Surface of Liquid Nitrogen

The floating rack was adjusted to three different heights, 3, 5 and 8 cm and the effects on sperm motility were measured. Equilibration time was 15 min.

Experiment 4. The Effect of Dilution Ratio and Equilibration Time

Sperm samples were collected as described above. Sperm was diluted immediately after collection with the extender containing 175mM of glucose supplemented with 12.5% of methanol. The ratio of sperm to extender dilution was 1:7, 1:9 or 1:11 (sperm: extender). The final concentrations of glucose were 153 mM for 1:7 ratio, 158 mM for 1:9 ratio, 160 mM for 1:11 ratio. The final concentrations of methanol were 10.9, 11.3 and 11.6% (for the ratios 1:7, 1:9 and 1:11, respectively). Prior to cryopreservation, diluted sperm was allowed to equilibrate for 15 and 60 min on ice (about +4°C).

Experiment 5. Post-Thaw Fertilization

For the fertilization trial, we used samples incubated 60 min prior to cryopreservation from the experiment 4. The eggs were collected in July (28 °C ± 2, 13 h 30 min light and 10 h 30 min darkness). The genital papilla of the fish was checked at 2-3 pm every day for a sign of spawning. Females and males ready for spawning released oocytes and sperm respectively, upon gentle abdominal massage. Due to the low fecundity of females used (approximately 200-300 eggs), each batch of eggs was carefully divided into small portions (22 eggs approximately). Duplicate batches for all fertilization variants were used. An adequate volume of sperm was diluted with 2 ml of hatchery water containing the eggs, to obtain the final sperm concentration of 2×10^6 spermatozoa mL⁻¹ in activation media. Post-thaw fertilization was carried out immediately (~ 3 – 5 min) after egg collection. After 5 minutes from the addition of sperm, the fertilized eggs were rinsed with fresh water from the fish tank and incubated in a 12-well glass. Oocyte development was observed 2 hours after fertilization using the light microscope (Nikon, Tokyo, Japan). Fertilization rate was evaluated by determining the number of fertilized and unfertilized eggs. Fertilized egg was defined as the egg at early embryonic stage (1-4) according to developmental staging system of Nile tilapia sperm by Fujimura and Okada (2007). Fertilization trials were duplicated for each sample.

Sperm Motility Parameters of Fresh, Diluted in Extender, and Frozen/Thawed Sperm

The CASA system was used to analyze the motility parameters of fresh sperm and frozen/thawed sperm. The parameters assessed were the percentage of motile sperm (MOT, %), the progressive motility (PRG, %), the curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), straight-line velocity (VSL, $\mu\text{m s}^{-1}$), movement linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz). Sperm activating solution (50 mM NaCl, 10 mM CaCl₂, 0.5% albumin, 10 mM HEPES-NaOH, pH 8.0) was mixed with one microliter of the sperm mixture, placed into a well of a multi-test glass slide (Tekdon, Inc., Myakka City, USA) and covered with a cover-slip. The recordings (200 frames each, 50 fps) were analyzed for a 4 s period, 6-10 s post-activation using the CRISMAS program (Image House Ltd., Denmark) integrated with a microscope (Nikon OptiPhoto, Tokyo, Japan) and a Basler a202K digital camera (Basler, Germany). All analyses were duplicated.

Statistical Analysis

Statistical analyses were performed at a significance level of $P < 0.05$ using GraphPad Prism software v. 6.02 (GraphPad Software Inc., San Diego, CA, USA) and the results presented as mean ± SD. The percentage data for motility and fertilization were normalized using an arcsine square root transformation. This was followed by data analyses using repeated measures one-way ANOVA, Tukey's post hoc test or repeated measures two-way analysis of variance (ANOVA), and Sidak's post hoc test.

Results

Experiment 1. Effect of Methanol Concentration on Sperm Cryopreservation

Cryopreserved samples had a lower motility ($P < 0.05$) than the fresh control (Figure 1). Although the motility percentage of sperm cryopreserved with 12.5% methanol in 175 mM glucose had a higher mean value than with 10 or 15% methanol in 175 mM glucose, the difference was not significant ($P > 0.05$).

Experiment 2. Effect of Glucose Concentration

Sperm cryopreserved in 175 mM glucose and 12.5% methanol resulted in motility percentage significantly higher ($P < 0.05$) than that cryopreserved in 150 mM glucose and 12.5% methanol or 200 mM glucose and 12.5% methanol (Figure 2). Motility percentage in control (fresh sperm) was significantly higher ($P < 0.05$) than that of sperm cryopreserved in 150, 175 or 200 mM glucose with 12.5% methanol.

Experiment 3. The Effect of Height Above the Surface of Liquid Nitrogen

The effect of height above the surface of liquid nitrogen on sperm motility is shown in Figure 3. The motility percentage of sperm cryopreserved in 175 mM glucose with 12.5 % methanol and placed 8 cm above the surface of liquid nitrogen was higher than that placed 3 and 5 cm above the surface of liquid nitrogen. The motility percentage in the treatments was significantly lower than that of the control.

Experiment 4. The Effect of Dilution Ratio and Equilibration Time

Sperm motility percentage was higher when the sperm-extender mixture was allowed to equilibrate for 60 min before freezing than that of 15 min before freezing (Figure 4 A). However, this difference was significant ($P < 0.05$) only between 60 min equilibration (8 times diluted samples), 15 min after equilibration (10 times diluted samples) and 15 min after equilibration (12 times diluted samples). The percentage of progressive movement (PRG) was not

significantly different ($P > 0.05$) between 15 and 60 min for all the dilutions (Figure 4 B). However, the PGR of the control was significantly different ($P < 0.05$) from that of the treatments. Among all the dilutions, only the sperm equilibrated for 15 min and diluted 1:9 (sperm: extender) before cryopreservation differed significantly ($P < 0.05$) in straight-line velocity (VSL) from the control (Figure 4 C). There was no significant difference ($P > 0.05$) in curvilinear velocity (VCL) between the sperm equilibrated for 15 and 60 min for all the dilutions (Figure 4 D). The linearity (LIN) percentage was not significantly different ($P > 0.05$) between the fresh sperm and the sperm equilibrated for 15 and 60 min before cryopreservation for all dilutions (Figure 4 E). Among all the dilutions, only the sperm diluted 1:9 (sperm: extender) and equilibrated for 15 min before cryopreservation was significantly different in linearity percentage from the control. Neither the amplitude of lateral head displacement (ALH) nor the beat cross frequency (BCF) (Figure 4 F and G respectively) differed significantly ($P < 0.05$) from the control at 15 or 60 min before freezing for all the dilutions.

The fertilization rate was significantly higher in control than in 1:11 (sperm: extender) dilution.

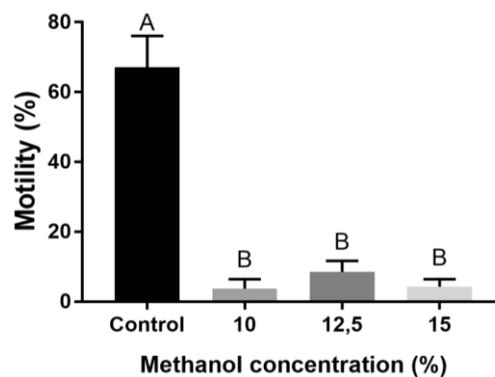


Figure 1. Effect of methanol concentration on post-thaw sperm motility ($n=5$). Results are displayed as mean \pm SD. Different letters above the SD bars indicate significant differences ($P < 0.05$).

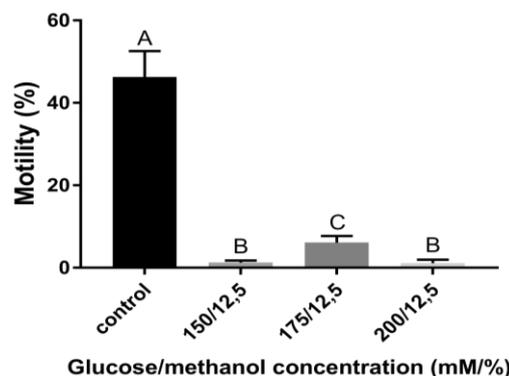


Figure 2. Effect of glucose concentration on post-thaw sperm motility ($n=4$). Results are displayed as mean \pm SD. Different letters above the SD bars indicate significant differences ($P < 0.05$).

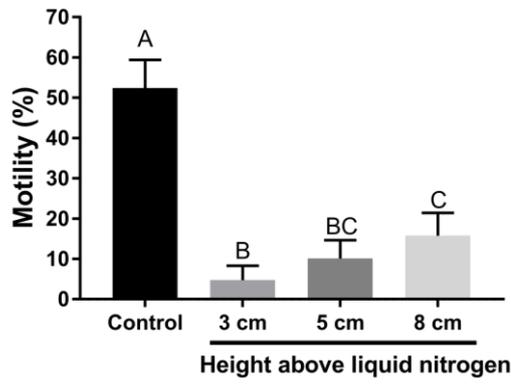


Figure 3. The effect of height above the surface of liquid nitrogen on post-thaw sperm motility (n=6). Results are displayed as mean \pm SD. Different letters above the SD bars indicate significant differences (P < 0.05).

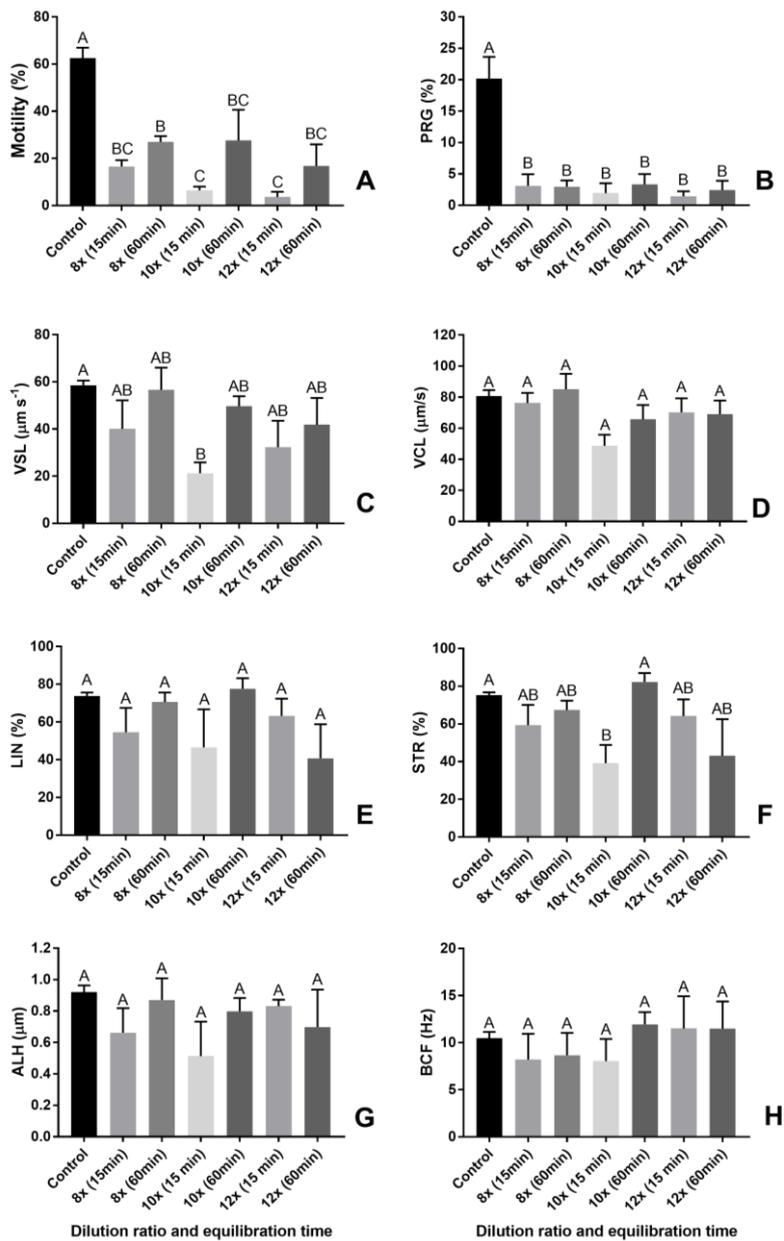


Figure 4. The effect of dilution ratio and equilibration time after dilution on sperm post thaw motility parameters (n=4). Caption represents dilution ratio and time of equilibration. Results are displayed as mean \pm SD. Different letters above the SD bars indicate significant differences (P < 0.05).

Experiment 5. Post Thaw Fertilization

The fertilization ratio of the eggs did not exceed 70% and their value was similar for control and cryopreserved sperm at dilution ratio 8 and 10 times (Figure 5). The samples diluted in 1:11 ratio had lower fertilization ratio than control, however not statistically different from 8 and 10 times diluted samples.

Discussion

Our results indicate that glucose and methanol are suitable cryoprotectants for preserving the sperm of *O. mossambicus*. For all investigated conditions, optimal cryopreservation conditions were found to be 175 mM glucose and 12.5% methanol at 1:7 dilutions (sperm: extender), 60 min equilibration after dilution and pre-freezing at 8 cm above liquid nitrogen. Cryopreservation at these conditions resulted in ~ 50% decrease in post-thaw sperm motility (27 - 28% post-thaw motility compared to the 62% fresh control motility). To our knowledge, this is the first study on the use of glucose-methanol extender for the cryopreservation of *O. mossambicus* sperm. Our results show that glucose-methanol mixture is not only suitable for cryopreservation of *O. mossambicus* sperm but also allows cryopreservation in small volumes as low as 10 μ l. As relatively low amounts of sperm (50-200 μ l) can be obtained from the males of *O. mossambicus*, this technique could be useful especially during breeding programs, since many cryopreservation trials may be done with a singular sperm sample.

Methanol has been used as an internal cryoprotectant to successfully cryopreserve fish sperm. However, the optimum concentration needed to obtain good results differs from species to species. In our experiment, sperm cryopreserved with 12.5% methanol in 175 mM glucose did not differ significantly ($P > 0.05$) in motility percentage than that with 10 or 15% methanol in 175 mM glucose. This concentration of methanol is similar to the concentration used by

Rana and McAndrew (1989) and higher (12.5 vs 5%) than that previously used to cryopreserve *O. mossambicus* sperm (Harvey, 1983). In addition, a similar concentration of methanol (about 10 – 11%) has been successfully used to cryopreserve *O. niloticus* sperm, when 5% of glucose was used as an external cryoprotectant (Godinho, Amorim, & Peixoto, 2003). However, in other fish species, a relatively lower concentration of methanol has been shown to be most effective for sperm cryopreservation. For instance, a 10% of methanol was successfully applied for cryopreservation of stellate sturgeon (*Acipenser stellatus*) (Sadeghi and Imanpoor, 2013), rainbow trout (*Oncorhynchus mykiss*) (Judycka, Cejko, Dryl, Dobosz, Grudniewska, & Kowalski, 2016), common carp (*Cyprinus carpio*) (Horvath *et al.*, 2003), salmonid fishes (Lahnsteiner, Weismann, & Patzner, 1997), tench sperm (Lujic *et al.*, 2017) and perch sperm (Bernath *et al.*, 2016). These findings suggest that the effective concentration of methanol in cryoprotective media may be slightly different according to the species investigated, as well as the compositions of the base solution used.

Glucose-based extenders are an ideal choice for obtaining high post-thaw sperm motility and fertilization as shown in several studies (Horvath, Miskolczi, & Urbanyi, 2003; Tekin, Secer, Ackay, & Bozkurt, 2003; Zhang, Li, Tulake, Yan, & Li, 2011). Different concentrations of glucose have been tested in other species to cryopreserve sperm. In rainbow trout, optimum results have been obtained at 150 mM (Ciereszko *et al.*, 2014), 180 mM (Dietrich, Nynca, Dobosz, Zalewski, & Ciereszko, 2014; Nynca *et al.*, 2014), 300 mM (Tekin *et al.*, 2003) and 350 mM glucose (Horvath *et al.*, 2003). Similarly, the sperm of Siberian sturgeon (*Acipenser baerii*) and European sturgeon (*Huso huso*) have been successfully cryopreserved at 100 and 200 mM glucose, respectively. In our study, a concentration above or below 175 mM led to a significant decrease in the post-thaw sperm motility percentage. These observations show that there are interspecific differences in the

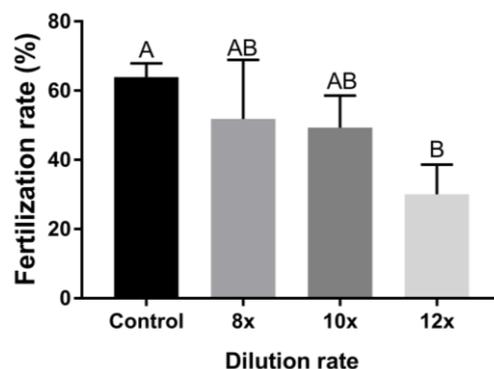


Figure 5. The effect of sperm cryopreservation on fertilization rate ($n=4$). Results are displayed as mean \pm SD. Different letters above the SD bars indicate significant differences ($P < 0.05$).

optimum concentration of glucose needed to cryopreserve fish sperm. These differences may be as a result of some factors such as the concentration of the internal cryoprotectant (e.g., methanol), dilution ratios or equilibration time. Our results show that these factors have effects on the post-thaw motility and fertilization of *O. mossambicus* sperm.

The highest post-thaw sperm motility percentage was observed when the diluted sperm was allowed to equilibrate for 60 min prior to cryopreservation. This is contrary to the result obtained by Harvey (1983), but agrees with the study by Ciereszko *et al.*, (2014), who showed that equilibration of sperm diluted in glucose-methanol extender had beneficial effects on the sperm motility parameters of cryopreserved rainbow trout sperm. An equilibration time of 60 min has also been used for the cryopreservation of Nile tilapia (*O. niloticus*) (Asmad, Khadijah, & Abdullah, 2011; Khadijah, Asmad., & Abdullah, 2008). This suggests that tilapia sperm (e.g., *O. mossambicus* and *O. niloticus*) may require more time to absorb the cryoprotectants prior to cryoprotection, when glucose-methanol extender is used. In other fish species, equilibration caused a decrease in fertilization rate for sperm cryopreserved with DMSO (dimethylsulfoxide) and ethylene glycol in rainbow trout (Babiak, Glogowski, Goryczko, Dobosz, Kuzminski, Strzezek, & Demianowicz, 2001), and low motility duration and motility rate in Persian sturgeon (*Acipenser persicus*) (Shaluei, Sadeghi, & Zadmajid, 2017). In contrast, equilibration time of not more than 10 min resulted in highest post-thaw motility in Japanese black porgy (*Acanthopagrus schlegelii*) (Chao, Chao, Liuy and Liao, 1986). Also, equilibration of diluted sperm for 15, 30, and 45 min was found to be optimum for cryopreservation of rainbow trout sperm (Bozkurt, Akcay, Tekin, & Secer, 2005; Cabrita, Alvarez, Anel, Rana, & Herraes, 1998; Ciereszko *et al.*, 2014). However, sperm motility parameters and fertilization rates were not affected after equilibration for 15 min on ice in salmonid fishes (Lahnsteiner, Patzner, & Weismann, 1996; Nynca *et al.*, 2014) and 30 min in Siberian sturgeon (Judycka, Szczepkowsld, Ciereszko, & Dietrich, 2015). These findings show that equilibration time varies according to species and the type of extender or cryoprotectant. It has been earlier suggested that these variations might be as a result of the interactions among the different components of the extender (Judycka *et al.*, 2015). Therefore, the optimum equilibration time should be empirically determined for each species because of lack of conformity in the results obtained in several studies.

The highest post-thaw motility was recorded when straws were placed 8 cm above the surface of the liquid nitrogen. Lahnsteiner *et al.*, (1996) successfully cryopreserved sperm of salmonid fishes using freezing heights of as low as 1 to 2.5 cm, and showed that changes in freezing heights significantly affected post-

thaw fertilization. Freezing height of 3 cm has also been used for cryopreservation of rainbow trout semen (Bozkurt *et al.*, 2005; Nynca *et al.*, 2014), common carp sperm (Horvath *et al.*, 2003), tench sperm (Lujić *et al.*, 2017) and perch sperm (Bernath *et al.*, 2016). The high freezing height of 8 cm for *O. mossambicus* indicates that a low cooling speed is needed for sperm cryopreservation in this species. This corroborates earlier findings by Chao *et al.*, (1986) that low cooling rate is required for cryopreservation of tilapia sperm. In their study, the initial cooling rate was quite fast ($1.54^{\circ}\text{C s}^{-1}$), but only until a temperature of -35°C was reached. Further freezing was conducted using very low speed ($5^{\circ}\text{C min}^{-1}$). Our approach shows the usefulness of relatively high position (8 cm) of straws above liquid nitrogen. At this height, the effective cooling rate was equal to $20^{\circ}\text{C min}^{-1}$. This cooling speed is relatively low compared to the rate (35 up to $160^{\circ}\text{C min}^{-1}$) used for salmonid fish sperm (Scott and Baynes, 1980), but is in agreement with the results of Harvey (1983), where cooling rate of $15 - 45^{\circ}\text{C min}^{-1}$ was most effective for cryopreservation of *O. mossambicus* sperm.

Sperm-extender dilution has been shown to influence post-thaw sperm motility and fertilization rate. In this present study, the optimum dilution that gave highest post-thaw motility was 1:7. At the highest sperm dilution rate (1:11), the fertilization rate decreased as compared to control. In contrast, dilution rates between 1:2 and 1:20 had no influence on fertilization of tilapias (Rana and McAndrew, 1989). In a previous study by Harvey (1983), *O. mossambicus* sperm was cryopreserved at 1:5 dilutions. In *O. niloticus* a dilution of 1:9 was used for sperm cryopreservation (Asmad *et al.*, 2011). This indicates that sperm-extender dilution effects vary among tilapias. Similar variations are also found in salmonid fishes. Threefold dilution was previously reported to be the optimum dilution for cryopreservation of sperm of salmonid fishes; fertilization rates decreased at dilutions less than threefold and did not differ significantly at dilutions exceeding threefold (Legendre and Billard, 1980; Tekin *et al.*, 2003). However, results from other studies showed higher post-thaw motility percentage and fertilization rate from five to eleven-fold dilutions (Ciereszko *et al.*, 2014; Judycka *et al.*, 2016; Lahnsteiner *et al.*, 1996). As suggested in other studies (Dziewulska and Domagala, 2013 Judycka *et al.*, 2016), these differences might be as a result of the differences in the initial sperm concentrations and the final sperm counts in the cryopreservation buffers.

Fertilization rates of eggs using cryopreserved sperm did not differ significantly from those of fresh sperm except at the highest dilution of 1:11. This fertilization success may be attributed to the fact that the velocity parameters (VSL and VCL) of the sperm did not change significantly following cryopreservation. This is in agreement with the findings of Gage,

Macfarlane, Yeates, Ward, Searle, and Parker (2004) that showed a direct link between relative sperm velocity and fertilization success. Chao, Chao, Liu, & Liao (1986) also achieved fertilization success comparable to controls after post-thaw fertilization tests. Moreover, in a study by Rana, Muiruri, McAndrew and Gilmour (1990), high fertilization rate (approximately 50%) of eggs was obtained with the non-motile cryopreserved sperm of Nile tilapia. Our results, therefore, reveal, under our experimental conditions, that even though the progressively motile sperm and motility percentage decreased significantly after cryopreservation, the fertilizing ability of cryopreserved sperm was not significantly affected. However, it should be stressed that although fertilization rate might remain high, the overall hatching rate could be lower as a result of cryoinjuries, such as DNA fragmentation, which may affect the hatchability (Pérez-Cerezales, Martínez-Páramo, Beirão, & Herráez, 2010).

This study demonstrates that low volumes of Mozambique tilapia sperm samples can be successfully cryopreserved using a simple glucose-methanol extender. The post-thaw motility of cryopreserved sperm was reduced to about 50%; however, their fertilization rate did not change significantly when compared to the fresh sperm samples. The presented technique can be used in tilapia hatcheries for broodstock production as it could help to preserve individual sperm samples of desirable males.

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