Extenders with vitamins C and E applied to Rhamdia quelen sperm cryopreservation

Extensores com vitaminas C e E aplicados à criopreservação de esperma de Rhamdia quelen

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ABSTRACT
We performed this experiment to evaluate the effects of adding vitamins C and E on extenders for sperm cryopreservation of Rhamdia quelen over spermatic mobility after thawing. At cryopreservation, sperm samples were diluted in a proportion of 1:3 (v/v), following pre-freezing in nitrogen steam and subsequent immersion in liquid nitrogen. The diluents were composed by 5% milk powder, 5% glucose, 10% methanol and different levels of vitamin. Three sperm cryopreservation tests were carried out with (1) diluent containing 0.0; 4.0; 6.5; 9.0 and 11.5 mg of vitamin C mL⁻¹, (2) diluent containing 0.0; 2.0; 4.0; 6.0 and 8.0 mg of vitamin E mL⁻¹; (3) diluent containing 0.0; 4.0 + 2.0; 6.5 + 4.0; 9.0 + 6.0 and 11.5 + 8.0 mg of vitamin C mL⁻¹ plus vitamin E mL⁻¹, respectively. The spermatic motility rate, spermatic curvilinear velocity, average path and straight line velocities were measured in thawed semen by CASA. Data were submitted to ANOVA and Duncan’s test at 5% of significance. After thawing the effect (P<0.05) of vitamin C was observed only for sperm motility, with higher values (38.2±20.7%) on solution containing 4.0 mg of vitamin C mL⁻¹. The concomitant addition of both vitamins influenced (P<0.05) only the curvilinear velocity, reducing the velocity at any concentration. In conclusion, diluents with 4.0 mg vitamin C mL⁻¹ to cryopreservation of the silver catfish semen improve the sperm quality after thawing, and the use of diluents with vitamin E or both vitamins are not recommended because do not ensure the cells protection.

Keywords: antioxidant, CASA, motility, reproduction, silver catfish, velocity.

ABSTRACT
Realizámos esta experiência para avaliar os efeitos da adição de vitaminas C e E em extensores para criopreservação do esperma de Rhamdia quelen sobre a mobilidade espermática após o descongelamento. Na criopreservação, as amostras de esperma foram diluídas numa proporção de 1:3 (v/v), após a pré-congelação em vapor de azoto e subsequente imersão em azoto líquido. Os diluents foram compostos por 5% de leite em pó, 5% de glicose, 10% de metanol e diferentes níveis de vitaminas. Foram efectuados três testes de criopreservação de esperma com (1) diluente contendo 0,0; 4,0; 6,5; 9,0 e 11,5 mg de vitamina C mL⁻¹, (2) diluente contendo 0,0; 2,0; 4,0; 6,0 e 8,0 mg de vitamina E mL⁻¹; (3) diluente contendo 0,0; 4,0 + 2,0; 6,5 + 4,0; 9,0 + 6,0 e 11,5 + 8,0 mg de vitamina C mL⁻¹ mais vitamina E mL⁻¹, respectivamente. A taxa de motilidade espermática, a velocidade curvílinea espermática, a trajetória média e as velocidades em linha recta foram medidas em sêmen descongelado pela CASA. Os dados foram submetidos ao teste da ANOVA e Duncan a 5% de significância. Após o descongelamento foi observado o efeito (P<0,05) da vitamina C apenas na motilidade espermática, com valores mais elevados (38,2±20,7%) na solução contendo 4,0 mg de vitamina C mL⁻¹. A adição concomitante de ambas as vitaminas influenciou (P<0,05) apenas a velocidade curvílinea, reduzindo a velocidade a qualquer concentração. Em conclusão, os diluentes com 4,0 mg de vitamina C mL⁻¹ para criopreservação do sêmen de peixe-gato de prata melhoram a qualidade do esperma após o descongelamento, e a utilização de diluentes com vitamina E ou ambas as vitaminas não são recomendados porque não garantem a protecção das células.

Palavras-chave: antioxidante, CASA, motilidade, reprodução, peixe-gato prateado, velocidade.
1 INTRODUCTION

The silver catfish, *Rhamdia quelen*, is a neotropical fish species (Peil *et al.*, 2007) and occurring in southern of Mexico to central Argentina (Townsend & Baldisserotto 2001). This is a potential species for aquaculture in South America (Koakoski *et al.*, 2012) due its good response to hormonal induction (Carneiro & Mikos 2008) and its marketable as a food source for humans. Due its increasing importance, fishes from the genus *Rhamdia*, especially the *Rhamdia quelen*, has been focused on research endeavors since 1990 with different focus as biochemistry and physiology, health and welfare, management, nutrition and and breeding (Adames *et al.*, 2015). So, recently many efforts have been devoted to developing technologies to support *Rhamdia* aquaculture, where the next barrier to be overcome are the efficient reproductive techniques and the establishment of genetic improvement programs along with the establishment of in vitro gene banks. So, the sperm cryopreservation plays a major role in the storage of biological material for undetermined periods (Linhart *et al.*, 2005; Mariante *et al.*, 2011) and can be applied to biodiversity conservation programs (Godinho 2007) or genetic improvement programs of commercial lineages (Butts *et al.*, 2011).

Despite the positive points, cryopreservation generates changes in the structural and physiological components of cells (Figueroa *et al.*, 2020) increase in lipid peroxidation and decrease in certain enzymatic actions, resulting in decreased sperm motility, integrity and viability (Figueroa *et al.*, 2018, 2020; Magnotti *et al.*, 2018). Among the damage caused by cryopreservation, oxidative stress is common and entails in the imbalance between the presence of the reactive oxygen species (ROS) and the antioxidant activity in spermatozoa during cryopreservation (Bansal *et al.*, 2010). In addition, these damages can also occur during the activation of fresh and/or cryopreserved spermatozoa, because this process also produces reactive oxygen species (Cabrita *et al.*, 2010).

So, the development of extenders with antioxidants have been researched in effort to produce solutions that reduces the cellular damages provoked by ROS during the sperm cryopreservation or sperm activation (Viveiros *et al.*, 2009; Cabrita *et al.*, 2011), thereby preserving the sperm movement, the integrity of DNA and the spermatocidal membranes integrity (Silva *et al.*, 2007). Studies with vitamins C and E have been used as antioxidants for sperm cryopreservation and have been showing protective activity in the cell, resulting in sperm quality (Kowalski, 2019), its use is restrict to fishes possibly due to the lack of information about the mechanisms involved on maintenance of sperm
viability and/or the antioxidant system in semen (Liu et al., 2015). In this way, vitamin C and E are common antioxidants with great potential to be used in extenders.

Ascorbic acid (vitamin C) prevents the action of hydrogen peroxide, released during cryopreservation, inhibiting its toxic effect, making sperm viable for a long time (Mittal et al., 2014; Patel et al., 2016) and the α-tocopherol (vitamin E) acts as an antioxidant substance, preventing cells from oxidative stress (Satoh et al., 1997), mainly by protecting biological membranes and DNA integrity (Agarwal et al., 2004).

Therefore, this work was carried out to evaluate the effects of adding vitamins C and E in extenders used to sperm cryopreservation from Rhamdia queilen over spermatic motility and spermatic velocity after thawing.

2 MATERIALS AND METHODS

2.1 THE FISHES AND FACILITIES

The experiment comprised three semen cryopreservation tests using extenders with vitamin C, vitamin E and the combining both. One-year old males of silver catfish (Rhamdia queilen) (n=15) were selected from earth ponds in the research facility from Universidade Estadual do Oeste do Paraná. Previously to the experiment the fishes were fed with commercial ration with 32% of crude protein and 3.200kcal of crude energy/kg, twice a day at 3% of body weight. These fishes were selected based on semen flow after slight coelomatic pressure as described by Bombardelli et al. (2006). Afterwards, the specimens were placed in 2.500 L tanks equipped with water recirculation system, water aeration, water filtration and water heating.

2.2 HORMONAL MANIPULATION, SEMEN COLLECTION AND ANALYSIS

The fishes were submitted to hormonal manipulation by treatment with carp pituitary extract (CPE) (Woynarovich & Horváth, 1983; Tessaro et al., 2012), comprising a single dose of 2.5 mg CPE kg male⁻¹. The injections were applied into the back muscle, close to dorsal fin (Sanches et al., 2011). Gametes were collected after 240 hour-degree (10 hours; water at 24°C), estimated from the moment of hormonal manipulation (Bombardelli et al., 2006).

To semen collect, the fishes were individually restrained without anaesthesia and subject to an abdominal massaged in the coelomatic region from head-to-tail. The semen was collected into graduate assay tubes (accuracy ± 0.1 mL) to measure total volume of
released semen per male. Then, the semen was kept under refrigeration (±12°C) (Sanches et al., 2013) up to semen processing and analysis.

To evaluate the breeding condition and the semen quality used in this study, fresh semen samples from each male were analyzed to determine the sperm motility rate and spermatic velocities by Computer Assisted Sperm Analysis (CASA), during 1s-image intervals, 10 seconds after spermatic activation (Adames et al., 2015). To accomplish that, semen was diluted in a proportion of 1:50 (semen: distilled water) (Sanches et al., 2013) up to semen processing and analysis.

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2.3 CRYOPRESERVATION EXPERIMENTS: VITAMIN C

Were used semen samples from five males (136.7 ± 60.7 g) that released 5.4 ± 1.9 mL of semen with 87.2 ± 6.1% of motile spermatozoa with curvilinear, average path and straight line spermatozoa velocities corresponding to 68.8 ± 4.0; 45.3 ± 3.9 and 34.3 ± 3.0 µm/s, respectively. The semen cryopreservation procedure was subsequently performed using an entirely randomized experimental design, with five treatments and five repetitions. The semen from each male was diluted in extenders containing 0.0; 4.0; 6.5; 9.0 and 11.5 mg of vitamin C (BRAVET – Monovin C®)/mL and the spermatic movement was evaluated after thawing. The vitamin C levels were determined by previous pilot experiments. The semen from one male was considered as one experimental unit.

In this experiment, the fresh semen was diluted in a proportion of 1:3 (v/v) in extender composed by 5% milk powder (Molico brand®), 5% anhydro glucose and 10% methanol (adapted from Carolsfeld et al., 2003) and the different levels of vitamin described above. Right away after dilution, the semen was filled into 0.25-mL straws (15 straws per treatment; total of 75 straws per experiment) and subjected to pre-freezing in a nitrogen vapor vessel (MVE®, mod. MVE SC4/2E) for a period of 18 hours (adapted from Carolsfeld et al., 2003). This procedure allow achieve -170ºC at a freezing rate of approximately -35.6ºC/min (Maria et al., 2006). Then, the straws were transferred and stored in liquid nitrogen at -196ºC. After 24 hours, cryopreserved semen was subjected to the thawing protocol by immersing straws in water at 25ºC for 10 seconds. After thawing, the same spermatic parameters described to fresh semen samples were evaluated.
2.4 CRYOPRESERVATION EXPERIMENTS: VITAMIN E

This experiment was carried out with semen samples from other five males (69.8 ± 28.7 g) that released 4.9 ± 0.9 mL of semen with 62.0 ± 21.7% of motile spermatozoa and curvilinear, average path and straight line spermatozoa velocities corresponding to 58.7 ± 7.3; 39.0 ± 5.2 and 32.3 ± 6.1 µm/s, respectively. In an identical experimental design how described above, were evaluate effects of extenders containing 0.0; 2.0; 4.0; 6.0 and 8.0 mg of vitamin E (BRAVET – Monovin E®)/mL. The levels of vitamin E were determined by pilot experiments and the procedures to semen packing, cryopreservation, thawing and sperm analysis were performed as above mentioned.

2.5 CRYOPRESERVATION EXPERIMENTS: VITAMIN C+E

This experiment was carried out with semen samples from other five males (96.8 ± 18.3 g) that released 4.1 ± 2.1 mL of semen with 60.0 ± 3.1% of motile spermatozoa and curvilinear, average path and straight line spermatozoa velocities corresponding to 70.1 ± 2.3; 51.5 ± 11.4 and 36.9 ± 9.2 µm/s, respectively. Were evaluated the effects of extenders contending both vitamins on concentrations of 0.0; 4.0 + 2.0; 6.5 + 4.0; 9.0 + 6.0 and 11.5 + 8.0 mg of vitamin C (BRAVET – Monovin C®) plus vitamin E (BRAVET – Monovin E®)/mL, respectively. Similarly, was used the same experimental design described above. The packing, cryopreservation, thawing and sperm analysis procedures were performed as above mentioned.

2.6 STATISTICAL ANALYSIS

Finally, the results of spermatic motility, spermatic curvilinear velocity, spermatic average path velocity and spermatic straight line velocity form thawed sperm were submitted to variance analysis (ANOVA) at 5% significance level. The assumptions of normality and homoscedasticity were checked and, when necessary, data transformations were accomplished. When effects of treatments over spermatic parameters were detected, the Duncan’s multiple mean comparison test was applied using the same significance level. The software Statistica 7.0© was used in statistical analysis (StatSoft 2005).

3 RESULTS

The cryopreservation of Rhamdia quelen semen with extenders containing vitamin C, influenced (P<0.05) the sperm movement on thawed semen. The higher sperm motility (38.2 ± 12.7%) was verified on solution containing 4.0 mg of vitamin C/mL
On the other hand, the use of extenders containing vitamin C levels above than 4.0 mg/mL reduced (P<0.05) the spermatic motility rate (Table 1). The spermatic velocities after thawing were not influenced (P > 0.05) by vitamin C levels on extender (Table 1). The spermatic curvilinear velocity, spermatic average path velocity and spermatic straight line velocity remained between 52.1 ± 14.0 and 61.4 ± 10.8 µm/s, between 34.7 ± 4.9 and 41.0 ± 9.18 µm/s, between 28.6 ± 7.9 and 36.7 ± 9.58 µm/s, respectively (Table 1).

The *Rhamdia quelen* semen cryopreservation with extenders containing different levels of vitamin E did not influence (P > 0.05) the spermatic motility (between 9.5 ± 7.4 and 18.1 ± 0.8) or the spermatic velocity on thawed semen (Table 1). The spermatic curvilinear velocity, spermatic average path velocity and spermatic straight line velocity remained between 49.8 ± 4.7 and 56.5 ± 7.5 µm/s, between 31.7 ± 5.7 and 40.2 ± 11.5 µm/s, between 22.5 ± 6.2 and 35.7 ± 13.0 µm/s, respectively (Table 1).

On the other hand, the semen cryopreservation with extenders combining vitamin C and vitamin E decreased (P<0.05) the spermatic curvilinear velocity on any vitamins concentration, once the other spermatic movement parameters were not affected (P>0.05) by the extenders (Table 1). The spermatic motility rate was kept between 12.0 ± 4.8 and 18.6 ± 7.3%, while the spermatic average path velocity and spermatic straight line velocity were kept between 29.9 ± 0.9 and 34.5 ± 2.6 µm/s and 23.4 ± 8.9 and 28.5 ± 4.3 µm/s, respectively (Table 1).
Table 1. Spermatc parameters of cryopreserved semen of silver catfish (*Rhamdia quelen*) diluted at a proportion of 1:3 (v/v), in extender containing 5% milk powder, 5% anhydro glucose, 10% methanol and different levels of vitamins

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vitamin C levels (mg/mL)</th>
<th>Vitamin E levels (mg/mL)</th>
<th>Vitamins C + E levels (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>4.0</td>
<td>6.5</td>
</tr>
<tr>
<td>MOT (%)</td>
<td>20.4 ± 3.3 (^{a})</td>
<td>38.2 ± 12.7 (^{a})</td>
<td>28.4 ± 3.0 (^{b})</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>52.1 ± 14.0</td>
<td>53.1 ± 12.9</td>
<td>61.4 ± 10.8</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>34.7 ± 4.9</td>
<td>40.4 ± 9.6</td>
<td>40.5 ± 4.2</td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>28.6 ± 7.9</td>
<td>36.7 ± 9.5</td>
<td>32.3 ± 2.9</td>
</tr>
</tbody>
</table>

Distinct letters in the same line represent significant difference by Duncan’s test at 5% of probability. MOT: Spermatc motility rate; VCL: Spermatc curvilinear velocity; VAP: Spermatc average path velocity; VSL: Spermatc straight line velocity.

4 DISCUSSION

The sperm cryopreservation necessarily causes cell damage during storage. Most of changes or damages reported in this process are irreversible and associated with nuclear instability, membrane injuries, loss of intracellular components, lipid peroxidation (Zúccari 1998) and production of oxygen reactive species (Andrade et al., 2010). Both cryopreservation and thawing are processes that increase the amount of oxygen reactive species (Andrade et al., 2010). Therefore, even though the seminal plasma contains antioxidants or substances able to remove free radicals (Cabrita et al., 2011) such as vitamin C, α-tocopherol (Mansour et al., 2006) or uric acid (Borges et al., 2005), the addition of these compounds on extenders is usually associated with the maintenance of spermatc fertility and viability after cryopreservation (Ciereszko et al., 2011).

Under normal physiological conditions, oxygen reactive species and antioxidants are in equilibrium and when unbalanced, these reactive species accumulate, thus causing oxidative stress (Andrade et al., 2010). In fish spermatozoa, such oxidative stress might be related to some environmental conditions, such as hypoxia, hyperoxia, presence of pollutants (Ciereszko 2008) and even associated with regular cell function like mitochondrial activity (Andrade et al., 2010). The main damages caused by oxidative stress affect the integrity of spermatc membranes and DNA (Ciereszko 2008).
The beneficial effect of vitamin C verified in the present experiment over spermatic motility could be related to the reduction of oxidative stress in cells, thus protecting the plasmatic membrane against lipid peroxidation, reducing the action of hydrogen peroxide (H$_2$O$_2$) and also avoiding DNA damages (Agarwal et al., 2004) during cryopreservation or thawing.

Altogether, the positive effects of the use of vitamin C on extenders applied to mammals sperm cryopreservation were reported. In the light of them, the different researches suggest the beneficial effects of vitamin C over spermatic progressive motility, integrity of acrosome and DNA integrity in humans semen (Agarwal et al., 2004) and equines semen (Ball et al., 2002). Although these perspectives and considering that the ascorbic acid is naturally present in semen of several fishes (Lahnsteine & Mansour, 2010), the vitamin C was ineffective to improve the spermatozoa movement in Perca fluviatilis L semen subjected to short-term storage (Słowińska et al., 2013) or sperm cryopreservation of Sparus aurata and Dicentrarchus labrax (Cabrita et al., 2011).

On the other hand, our results suggest an important perspective to use of the vitamin C in extenders applied to sperm cryopreservation of Rhamdia quelen, since extender containing 4.0mg vitamin C/mL improve the sperm movement. This statement can be supported by the reports of Liu et al. (2015), who find similar results on thawed sperm of Pargus major, cryopreserved in extenders containing ascorbic acid. Some authors suggest that these specie-specific variations are verified and considered dependent of the sperm features and endogenous antioxidant system, (Liu et al., 2015; Mingming et al., 2015), and the specificity and proportion/concentration of the antioxidant (Cabrita et al., 2011).

Another point which deserves attention is the reduction of the sperm movement caused by the higher levels of vitamin C in the extenders, caused probably by its proactive oxidation effect rather than protection against damage (Cabrita et al., 2011). These damages can be associated with the potential of vitamin C promote DNA damages in thawed spermatozoa of Sparus aurata and Dicentrarchus labrax (Cabrita et al., 2011), and its potential to inhibit the protective effect due its potentially metals/minerals reaction originate copper- or iron-ascorbate which induces the formation of free radicals in mammals (Norberg & Árner 2001) and fishes (Cabrita et al., 2011).

Other antioxidants like vitamin E, regarded as the main liposoluble antioxidant to protect polyunsaturated fat acids in tissues from peroxidation, could determine distinct results according to their dosage, once tocopherol can either act as antioxidant or favor
oxidation depending on the amount of hydroxyl radicals to be inactivated (Andrade et al., 2010). Therefore, the utilization of antioxidants as additives in conventional dilution media to improve the quality of spermatic cells after processing has been tested and the results are often contradictory (Câmara and Guerra 2011).

Some studies with mammals reported that the utilization of vitamin E in semen samples can lead to negative effects (Upreti et al., 1997) or cause no interference in maintaining the spermatic motility (Donnelly et al., 1999). Some reports to fishes suggest no effects or reduced effects of vitamin E on spermatozoa movement to Perca fluviatilis L semen subjected to short-term storage (Słowińska et al., 2013) or sperm cryopreserved of Sparus aurata and Dicentrarchus labrax (Cabrita et al., 2011). So, some authors suggesting that the oxidation process can undergo different pathways in spermatic cell in different species (Andrade et al., 2010) and the species-specific characteristics of vitamin E determine a more effective antioxidant activity to mammals than fishes (Liu et al., 2015).

In your review Cabrita et al. (2010) suggest that the use of vitamins (C and E) on diluents to cryopreservation procedures of fish sperm generally increase the resistance of sperm plasmatic membrane against the reactive oxygen species, reducing the lipid peroxidation. However, the reduction of spermatic curvilinear velocity reported in the present experiment when both vitamins were combined can be associated by a super dosage of these vitamins that induced the cell damages due the maximization of oxidation effects caused by the high levels of these vitamins, according discussed previously.

Particularly to fishes, the negative effects of combining both vitamin C and vitamin E over curvilinear velocity is noteworthy once the successful of the artificial breeding is directly correlated to such parameter, as reported in rainbow trout (Oncorhynchus mykiss) (Tuset et al., 2008). So, the combined addition of vitamin C and E in extenders is not recommended to cryopreservation of Rhamdia quelen semen because increase the complexity of the extenders and fails to protect the cells, once do not preserve the sperm motility as well as reduces the spermatic velocity.

The experimental evidence with Rhamdia quelen suggest that only the vitamin C on extender can protect the spermatozoa during sperm cryopreservation procedures and can improve the spermatozoa movement after thawing. On the other hand, the combined use of vitamin C and vitamin E do not ensure the success of the sperm cryopreservation and decrease the sperm velocity. In spite of these evidence, further studies should be carried out with neotropical fishes in order to elucidate the mechanisms involved on the
maintenance of cell integrity during cryopreservation and the effects of substances as vitamins focused on cell protection.

5 CONCLUSION

In conclusion, the use of diluents with 4.0 mg vitamin C mL$^{-1}$ to cryopreservation of the silver catfish semen improve the sperm quality after thawing, and the use of diluents with vitamin E or both vitamins are not recommended because do not ensure the cells protection.
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