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The effectiveness on addition of vitamins as an antioxidant in sperm cryopreservation of catfish *Clarias gariepinus*

Efektivitas penambahan vitamin sebagai antioksidan pada kriopreservasi sperma ikan lele *Clarias gariepinus*

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ABSTRACT

One of the problems faced in catfish hatcheries is that the reproductive cycle does not occur throughout the year, so that available male broodstocks with mature gonads are rare. Cryopreservation is a method of storing spermatozoa with the help of diluents and cryoprotectants in frozen conditions to maintain the quality of spermatozoa. Cryopreservation has a negative effect due to the influence of reactive oxygen species (ROS) which causes a decrease in the quality of spermatozoa. An alternative that can be done is using antioxidants in the form of vitamin C and cryoprotectants such as vitamin E. This research aims to test the effectiveness of adding vitamin E and vitamin C to catfish (*Clarias gariepinus*) sperm on post-cryopreservation sperm quality. The test fish were catfish that had mature gonads and were injected with the ovaprim hormone for final approval. This study consisted of eight treatments and three replications, namely control (fresh sperm), DMSO 10%, DC1 (vitamin C dose 1 mg/mL), DC2 (vitamin C dose 2.5 mg/mL), and DC3 (vitamin C dose 4 mg/mL), DMSO 10%, DE 1 (vitamin E 2 mg/mL), DE2 (vitamin E 4 mg/mL), DE3 (vitamin E 6 mg/mL). The parameters observed include sperm motility, sperm viability, degree of egg fertilization, and degree of egg hatching. Sperm cryopreservation is carried out for one hour. The results showed that the motility, viability, and degree of egg fertilization in the vitamin C and vitamin E treatments were lower ($P<0.05$) than the control and 10% DMSO as the basic control.

Keywords: *Clarias gariepinus*, cryopreservation, vitamin C, vitamin E

ABSTRAK

Salah satu permasalahan yang dihadapi dalam pembenihan ikan lele adalah siklus reproduksi tidak terjadi sepanjang tahun sehingga ketersediaan induk jantan dengan gonad matang menjadi langka. Kriopreservasi merupakan salah satu metode penyimpanan spermatozoa dengan bantuan bahan pengencer dan krioprotektan dalam kondisi beku untuk mempertahankan kualitas spermatozoa. Kriopreservasi memiliki dampak negatif akibat adanya pengaruh *reactive oxygen species* (ROS) yang menyebabkan penurunan kualitas spermatozoa. Alternatif yang dapat dilakukan adalah dengan menggunakan antioksidan berupa vitamin C dan krioprotektan seperti vitamin E. Penelitian ini bertujuan untuk menguji efektivitas penambahan vitamin E dan vitamin C pada sperma ikan lele (*Clarias gariepinus*) terhadap kualitas sperma pascakriopreservasi. Ikan uji adalah ikan lele yang telah memiliki gonad matang dan disuntik hormon ovaprim untuk persetujuan akhir. Penelitian ini terdiri dari delapan perlakuan dan tiga kali ulangan, yaitu kontrol (sperma segar), DMSO 10%, DC1 (dosis vitamin C 1 mg/mL), DC2 (dosis vitamin C 2,5 mg/mL), dan DC3 (dosis vitamin C 4 mg/mL), DMSO 10%, DE 1 (vitamin E 2 mg/mL), DE2 (vitamin E 4 mg/mL), DE3 (vitamin E 6 mg/mL). Parameter yang diamati meliputi motilitas sperma, viabilitas sperma, derajat fertilisasi sel telur dan derajat penetasan sel telur. Kriopreservasi sperma dilakukan selama satu jam. Hasil penelitian menunjukkan bahwa motilitas, viabilitas dan derajat fertilisasi sel telur pada perlakuan vitamin C dan vitamin E lebih rendah ($P<0,05$) dibandingkan kontrol dan DMSO 10% sebagai kontrol dasar.

Kata kunci: *Clarias gariepinus*, kriopreservasi, vitamin C, vitamin E

INTRODUCTION

African catfish (*Clarias* sp.) is a freshwater fish that is very popular with most Indonesian people. This is because catfish has a relatively affordable price, has high nutritional value, and tastes good meat (Wulansari *et al.,* 2022). Apart from that, catfish are widely cultivated because they have several advantages, such as rapid growth, efficient feed conversion, strong resistance to infectious diseases, and adaptability to a wide range of environmental conditions (Say *et al.,* 2023). Catfish production in Indonesia tends to increase every year, so catfish has quite good market potential. Between 2018 and 2022, catfish production demonstrated solid performance, with an average annual growth rate of 10.9% (DJPB, 2022).

The increase in consumption fish production is in line with the increase in demand for fish seeds. One of the problems experienced in hatchery segmentation is that naturally the catfish reproductive cycle does not occur throughout the year so the availability of high-quality broodstock becomes scarce. Repeated handling of male broodstocks during artificial spawning can result in a decrease in sperm volume and quality. This can occur due to the spawning cycle and the lack of male fish (Mylonas *et al.,* 2010). In addition, for male broodstocks, the quality and quantity of spermatozoa will decrease outside the spawning season (Momin & Memiş, 2018). This decrease in sperm volume or quality can be overcome by storing sperm (Hastuti & Riviani, 2020). Therefore, sperm cryopreservation techniques are needed so that they can be preserved at very low temperatures for future use (Tang, 2024).

Cryopreservation is essential not only for fish farming but also for the conservation and enhancement of genetic resources. It plays a crucial role in preserving genetic material for extended periods, contributing to long-term genetic storage and management (Maulida *et al.,* 2024). Cryopreservation is a method of storing spermatozoa with the help of diluents (extenders) and cryoprotectants in frozen conditions to maintain the quality of spermatozoa (Novianto *et al.,* 2014). The aims and benefits of sperm cryopreservation are maintaining sperm viability for a long period of time, providing a supply of male gametes, increasing selective breeding in maintaining existing stock so that it is more economical and effective, and reducing the cost of maintaining male broodstocks (Maulida *et al.,* 2020). The diluent should possess high solubility in water while maintaining minimal solubility in other solvents. It should also exhibit low salt effects, low buffer concentrations, minimal sensitivity to temperature changes, stable interactions with cations, high ionic strength, and strong chemical stability.

The composition of the diluent plays a crucial role in preserving sperm quality and maintaining fertility for an extended period (Saha *et al.,* 2022). Apart from that, according to Barozha (2015), diluent ingredients play a role in inhibiting sperm activation and preventing sperm from moving, so sperm will save energy and will affect sperm survival. Cryoprotectants are non-electrolyte chemical substances that play a role in maintaining sperm stability from external influences such as temperature, osmotic pressure, and minimizing the formation of ice so that sperm can survive (Do *et al.,* 2016). The use of cryoprotectants must have special criteria that are able to defend sperm from low temperatures and have nutrients that can supply sperm's energy needs during the storage process (Parihar *et al.,* 2023).

The ingredients that have been widely used are dimethyl sulfoxide (DMSO) and glycerol which were tested on Batak fish (*Tor Soro*) and climbing perch (*Anabas testudineus*) (Zairin, 2005; Maulida *et al.,* 2023). Furthermore, it was also stated that DMSO and glycerol are ingredients that are able to maintain Batak fish sperm during the storage process. Natural ingredients that have been used include coconut water and honey. According to Dewi *et al.* (2023), using coconut water with the addition of glycerol in a combination of 60%:40% got the best results in storing comet fish sperm. In addition, honey tested on snakehead fish can retain sperm for seven days (Mangkunegara *et al.,* 2019).

Apart from the positive things about cryopreservation, this process also has an impact on sperm quality. The freezing process in sperm results in physiological and structural changes, increased lipids, decreased enzymatic activity, motility, sperm integrity and viability (Xavier *et al.,* 2021). Small amounts of reactive oxygen species (ROS) are necessary for key sperm processes like capacitation, hyperactivation, and the acrosome reaction. However, excessive ROS production or an imbalance in antioxidant defenses (such as in cryopreserved sperm) can lead to oxidative stress (OS), which negatively affects sperm function by harming proteins, lipids, and DNA (Bollwein & Bittner, 2018).

ROS are considered a major factor contributing to the loss of structural integrity and function of fish spermatozoa during cryopreservation. This vulnerability arises from the high levels of unsaturated fatty acids in sperm cells combined with the limited antioxidant defense present in diluted semen (Sandoval-Vargas *et al.,* 2021).

The addition of antioxidants to the cryopreservation process can be done to improve sperm quality. Antioxidants have a role as protectors for cells from oxidation processes which can be detrimental and reduce the activity of free radicals (Muthmainnah *et al.,* 2018). Vitamin E is a potent radical-scavenging antioxidant that inhibits lipid peroxidation mediated by free radicals but not enzymatic oxidation by lipoxygenase and cyclooxygenase, thereby preserving cell structure (Niki, 2015). In addition, vitamin E can prevent lipid peroxidation by scavenging free hydroxyl radicals and superoxide. This research was conducted to test the effect of adding vitamin E to sperm on post-cryopreservation sperm quality. There are several things that influence the success of the sperm cryopreservation process, such as the type of extender or diluent solution, cryoprotectant material, freezing process and re-thawing of sperm (Maulida *et al.,* 2020).

An extender is a material used to make sperm remain immotile so that sperm is maintained during the cryopreservation process (Barozha, 2015). A good extender possesses ionic characterictics and a pH level that closely matches of the fish's seminal plasma (Cejko *et al.,* 2018). Various extenders frequently used in artificial fish breeding include Ringer's solution, which has been tested on depik (*Rasbora tawarensis*) sperm (Eriani *et al.,* 2021), artificial seminal plasma (ASP) applied to grouper (*Epinephelus bruneus*) and seurukan fish (*Osteochilus vittatus*) sperm (Lim & Le, 2013; Adami *et al.,* 2016), as well as glucose-based extenders used for climbing perch sperm (Maulida *et al.,* 2022). In addition, Handayani *et al.* (2024) also used several extenders such as tap water, Ringer's solution, physiological solution, Alsever's solution, urea solution, and glucose base solution for the *Clarias batrachus* species.

Cryoprotectants are non-electrolyte chemicals added in the sperm cryopreservation process. Cryoprotectants function to protect sperm from cold and heat shock, as well as toxicity during cryopreservation (Sieme *et al.,* 2016). In addition, cryoprotectants function to maintain cell membranes intact thereby avoiding cell dehydration by preventing the release of fluids in the cells (Do *et al.,* 2016). Cryoprotectants are divided into two, namely intracellular cryoprotectants and extracellular cryoprotectants (Magnotti *et al.,* 2016). The most commonly reported cryoprotectants include dimethyl sulfoxide (DMSO), methanol, and glycerol, while non-permeable cryoprotectants like sucrose or glucose are rarely used (Ha *et al.* 2019; Yee & Yang 2023), while extracellular cryoprotectants include milk, egg yolk, vegetable oil (Handayani *et al.* 2024), and honey (Muchlisin *et al.,* 2015).

The cryopreservation process has a negative effect because it causes the spermatozoa plasma membrane to be damaged (Kumar *et al.,* 2019). An increase in lipid peroxidation and a decrease in certain enzymatic activities also occur after the cryopreservation process. This results in a decrease in sperm motility, integrity, and viability (Magnotti *et al.,* 2016). Exposure of sperm cells to low temperatures during the freezing process causes destabilization of the cell membrane, loss of surface proteins, reduced motility and viability, decreased mitochondrial activity, and increased production of reactive oxygen species (ROS) (Benko *et al.,* 2021). Reactive oxygen species (ROS) are naturally generated as a byproduct of cellular aerobic metabolism (Juan *et al.,* 2021).

Excessive production of reactive oxygen species (ROS) harms the sperm membrane, proteins, and DNA, leading to reduced motility, decreased viability, and diminished fertilization potential. Additionally, oxidative stress is associated with DNA fragmentation, which has been linked to higher infertility rates, poor pregnancy outcomes, and an increased risk of genetic abnormalities in offspring (Wang *et al.,* 2025). Antioxidants are nucleophilic compounds that have the ability to reduce and suppress free radical reactions, slow down, and prevent lipid oxidation. The inclusion of antioxidants, specifically BHT and taurine, offered improved protection for spermatozoa against oxidative damage during storage for up to 72 hours at 4°C (Rather *et al.,* 2016).

Antioxidants help safeguard sperm from reactive oxygen species (ROS) generated by defective sperm or leukocytes, prevent DNA fragmentation and early sperm maturation, minimize cryodamage, and enhance overall sperm quality (Qamar *et al.,* 2023). One antioxidant that can be used is vitamin C. Ascorbic acid (vitamin C) is among the most

extensively studied antioxidants in fish sperm cryopreservation, including in sturgeon (Shaluei *et al.,* 2015). It is characterized by low toxicity, high water solubility, and, most importantly, its oxidized form does not harm cells (Kolyada *et al.,* 2023). Research on the addition of vitamin C as an antioxidant for sperm cryopreservation was carried out by Xavier *et al.* (2021). The use of cryoprotectant with the addition of 4.0 mg/mL vitamin C for cryopreservation of silver catfish (*Rhamdia quelen*) sperm can improve sperm quality after thawing with a spermatozoa motility value of 38.2% (Xavier *et al.,* 2021). Therefore, this research was conducted to evaluate the effect of adding vitamin C and vitamin E to cryoprotectants on the reproductive performance of catfish (*Clarias* sp.) after the cryopreservation process.

MATERIALS AND METHODS

Materials

The catfish broodstocks were obtained from farmers in Bogor, West Java. One pair of catfish broodstock were used that had mature gonads with the male weighing 1.6 kg and the female weighing 0.8 kg.

Experimental design

A completely randomized factorial design was used in this study, and the factors tested were the types and concentrations of antioxidant, namely control (fresh sperm), DMSO 10%, DC 1 (vitamin C dose 1 mg/mL), DC 2.5 (vitamin C dose 2.5 mg/mL), and DC 4 (vitamin C dose 4 mg/mL), DMSO 10%, DE 1 (vitamin E 2 mg/mL), DE 2 (vitamin E 4 mg/mL), DE 3 (vitamin E 6 mg/mL). The parameters observed include sperm motility, sperm viability, degree of egg fertilization, and degree of egg hatching. Each treatment was performed in three replications.

Preparation of broodstock

The male catfish broodstock whose gonads have matured can be seen from the physical body, namely the slender abdomen, dark body color, red and pointed genitals, and active movements. The female catfish mother can be seen from her physical appearance, namely her abdominal body is bulging and large, her body color is bright, her head bones are convex, her genitals are rounded, and her movements are slow (Sudrajat & Rasid, 2020). Next, the selected broodstock is placed in a fiber tub with a diameter of 1.5 meters with a water level of 20 cm. The female and male broodstock are put into different containers.

Preparation of extender solution

Before catfish sperm is collected, an extender solution which functions as a cryoprotectant is prepared first according to the treatment dose. The extender is made by mixing Ringer's lactate, DMSO, vitamin C, vitamin E, and antibiotics according to the concentration of each treatment. The vitamin C used is in the form of powder with a vitamin C content of 500 mg/g. The antibiotic used is kanamycin. Antibiotic stock was made by dissolving 0.001 grams of kanamycin in Ringer's lactate.

For the vitamin C treatment, 1 mg/mL was made by mixing 0.5 mL of DMSO and 0.01 gram of vitamin C. Then the mixture was added with Ringer's lactate until the volume reached 5 mL. After that, 10 µL kanamycin was added. The addition of antibiotics is intended to inhibit the growth of bacteria and germs in diluted sperm.

Sperm collection and observation of fresh catfish sperm

Male catfish broodstock were injected with the ovaprim hormone at a dose of 0.3 mL/kg body weight. After that, wait for 10 hours to maximize gonad maturation and increase the number of sperm produced (Maulana *et al.,* 2014). The broodstock is then dissected with the help of surgical tools. The catfish gonads were then rinsed with Ringer's solution and then chopped in a petri dish. The sperm fluid is then transferred into the microtube with the help of a micropipette. The gonad handling process is carried out in an icebox to keep sperm alive.

The sperm is then put into an icebox and distributed to the laboratory for observation. Before observing sperm, the male catfish broodstock is first injected with the next process: the male broodstock is dissected using sterile scissors to remove the gonads, then the male gonads are rinsed with Ringer's lactate to remove any blood that may still be attached. After that, the fish gonads were chopped with sterile scissors on a petri dish. The enumeration of male gonads is carried out on a coolbox so that sperm can survive longer.

The sperm fluid obtained was transferred with a micropipette into a microtube. Sperm is brought to the laboratory at a low temperature and stored in a cool box to then observe sperm quality including sperm movement or motility

and the ratio between live and dead sperm cells or sperm viability (Maulana *et al.,* 2014). Motility was observed for movement and scored according to Guest *et al.* (1976).

Catfish sperm cryopreservation

The cryopreservation process for catfish sperm begins with dilution sperm into the extender solution that has been made previously according to the concentration of each treatment. The ratio between extender solution and sperm is 1:10. The diluted sperm was placed in a 2 mL cryotube. The next process is that the sperm in the cryotube is equilibrated for 15 minutes at a temperature of -80°C. After that, the cryotube is put into liquid nitrogen for the sperm preservation process at a temperature of -196°C for one hour.

Post-cryopreservation sperm observations

Observations of catfish sperm include motility and viability, the examination was conducted using an Olympus CX23 microscope connected to an Indomicro HD resolution camera, which was interfaced with a computer equipped with OptiLab Image Raster software version 1.3.2. Measurements were performed at magnifications of 20× and 40×. Sperm removed from the liquid nitrogen tube and then carried out a thawing process at a temperature of 27°C for five minutes. The sperm that has been observed is then fertilized into the egg.

Catfish fertility test

Sperm that has gone through the cryopreservation process is then tested fertilization ability of eggs. Catfish female broodstocks who are ready to spawn are prepared to get good eggs to be fertilized. The female broodstock's ovulation is stimulated by injecting ovaprim at 0.3 mL/kg of the female broodstock. The fertility test also uses fresh sperm collected from the same male broodstock as a comparison with the cryopreservation treatment that has been carried out. Once the female is ready, the stripping process is carried out to obtain the eggs. The eggs obtained were divided into petri dishes with each egg weighing 0.2 grams. 100 µL of cryopreserved sperm for each treatment was diluted again using Ringer's lactate with a ratio of 1:20, while fresh sperm as a control was diluted using Ringer's lactate with a ratio of 1:200.

Next, the eggs and sperm are stirred using a chicken feather, then the sperm is activated by adding 1 ml of water so that the sperm is motile and can fertilize the egg. Then the eggs are spread evenly on a filter, then the filter is placed in a hatching container in the form of an aquarium measuring 40×30×30 cm^3 which is equipped with aeration. Egg incubation is carried out in water that has been treated with methylene blue to prevent mold on the eggs. Eggs were incubated at 29–30°C for 24 hours.

RESULTS AND DISCUSSION

Result

The African catfish sperm observed included fresh sperm and semen which has gone through the cryopreservation process. The results of motility observations are presented in Table 1 below. Based on the results in Table 1, the treatment with the addition of vitamin C with a greater concentration had an effect on the motility scores obtained during the observations. The best motility was the control which was fresh sperm with a score of 5, while the motility for the 10% DMSO treatment obtained a score of 2. Meanwhile, for the treatment with the addition of vitamin C, namely the DC1 and DC2 treatments, the motility score was the same, namely 0.25.

Table 1. The Results of observations of catfish sperm motility with addition the concentrations of vitamin C and vitamin E were different after cryopreservation.

Treatment	Explanation	Motility Score
Control	All spermatozoa move very quickly with varying tail movements	5
DMSO 10%	Many sperm move with a small amount shows fast movement	2
DC1	Many sperm do not move. Sometimes looks like it's shaking weakly	0.25
DC2	Many sperm do not move. Sometimes looks like it's shaking weakly	0.25
DC3	All spermatozoa are motionless and vibrate	0
DE1	Many sperm do not move. Sometimes looks like it's shaking weakly	0.25
DE2	Many sperm do not move. Sometimes looks like it's shaking weakly	0.25
DE3	Many sperm do not move. Sometimes looks like it's shaking weakly	0.25

The DC3 treatment had the lowest motility, namely 0. The addition of a higher concentration of vitamin C showed that sperm motility tended to decrease. While the vitamin E treatment had the same score, namely 0.25, which means that the sperm observed did not move, had little movement or vibrated. In the 10% DMSO treatment it has a score of 2, that is, movement can be seen even though it is slight and moves in a forward direction. The best results were obtained in the control treatment which had a score of 5, meaning that all sperm moved quickly and moved in a forward direction.

Observations of control viability were carried out after the sperm was collected, while observations of the viability of the 10% DMSO, DC1, DC2, DC3, DE1, DE2, and DE3 treatments were carried out after the sperm had gone through the thawing process. The results of the viability calculation are in Figure 1. Based on the research results, it was found that the viability value decreased at higher vitamin C concentrations. The viability value of each treatment had significantly different results at P<0.05. The highest viability value was obtained by the control treatment, namely 89.91 ± 1.07%. The treatment with the addition of vitamins C and E showed lower results than the 10% DMSO treatment.

The value of the degree of egg fertilization is obtained from observing the egg after 10 hours post fertilization. The percentage value of the degree of egg fertilization can be seen in Figure 2. Based on the research results, the value of the degree of egg fertilization shows significantly different results. The percentage value of the degree of fertilization of post-cryopreservation catfish eggs tends to decrease compared to the control. The percentage value of the degree of

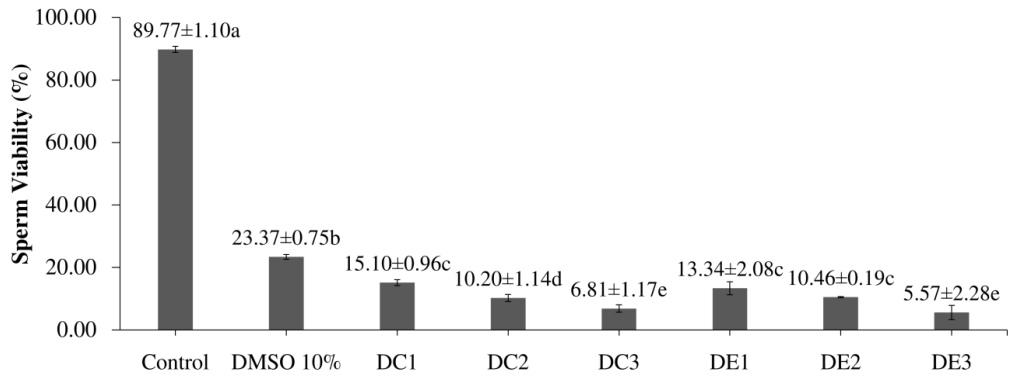

Figure 1. Sperm viability value with increasing concentration Vitamin C and Vitamin E are different. Control (fresh sperm), DMSO 10%, DC 1 (vitamin C dose 1 mg/mL), DC 2.5 (vitamin C dose 2.5 mg/mL), and DC 4 (vitamin C dose 4 mg/mL), DMSO 10%, DE 1 (vitamin E 2 mg/mL), DE 2 (vitamin E 4 mg/mL), DE 3 (vitamin E 6 mg/mL). Different letters above the bar diagram indicate significantly different results (Duncan P<0.05).

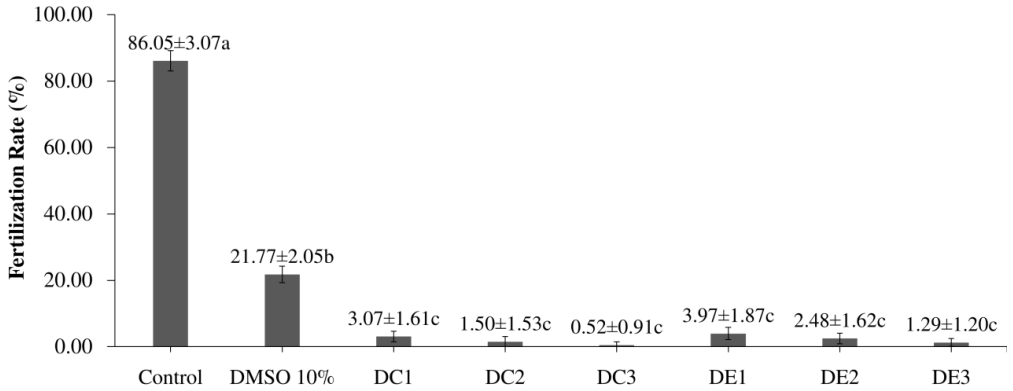

Figure 2. The fertilization rate of the embryo with increasing concentrations of Vitamin C and Vitamin E is different. Control (fresh sperm), DMSO 10%, DC 1 (vitamin C dose 1 mg/mL), DC 2.5 (vitamin C dose 2.5 mg/mL), and DC 4 (vitamin C dose 4 mg/mL), DMSO 10%, DE 1 (vitamin E 2 mg/mL), DE 2 (vitamin E 4 mg/mL), DE 3 (vitamin E 6 mg/mL). Different letters above the bar diagram indicate significantly different results (Duncan P<0.05).

fertilization of eggs treated with 10% DMSO was significantly different (P<0.05) compared to all vitamin C and vitamin E treatments.

The hatching rate (HR) value is the percentage of eggs that hatch out of the total fertilized eggs. The value of the degree of egg hatching is obtained after the embryo is 24-30 hours after fertilization. The percentage value of the degree of egg hatching can be seen in Figure 3. There are no outcome groups, where the control, DC1, and DE2 treatments showed no significant difference (P>0.05) and were significantly different from the 10% DMSO, DC2, DE1 treatment groups, and DE3 and DC3 which get no eggs hatch. The pH value and electrical conductivity value were measured on the solution used for the cryopreservation process. The measurement results are presented in Table 2 below.

Discussion

According to Taşdemir (2024), fresh semen to be cryopreserved should exhibit a minimum of 70% motility, a concentration of 1×10^9 spt/mL, and a mass activity score above 3 on a scale of 0 to 5. The quality of control sperm in this study had a motility score of 5, which means that spermatozoa motility moves rapidly in a forward direction (progressively) with varying movements. The 10% DMSO treatment had a score of 2 that was significantly different (P<0.05) from the other treatments, each of which had the same score, namely 0.25 and 0. The low motility value was caused by the cryopreservation process. According to Castro *et al.* (2025) damage caused by cold shock results in ultrastructural changes to the sperm membrane, which is evident through declines in motility, viability, and membrane integrity, ultimately lowering the sperm's ability to fertilize. In addition, the freezing process causes intracellular changes in sperm cells. The release of water associated with the formation of ice crystals results in a buildup of electrolytes in cells which can cause physical and chemical changes in spermatozoa (Zairin, 2005).

The process of forming ice crystals causes organelles such as lysosomes and mitochondria to become damaged and causes the death of spermatozoa. The antioxidant role of vitamin C

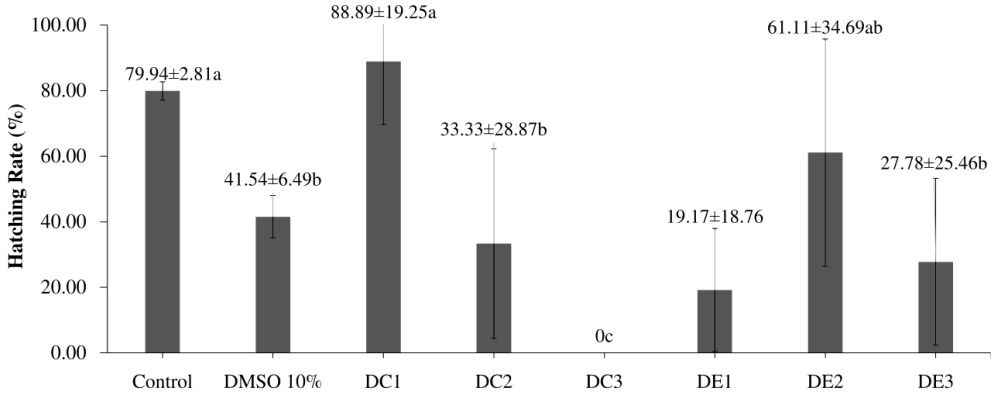

Figure 3. The hatching rate of eggs with increasing concentrations of Vitamin C and Vitamin E is different. Control (fresh sperm), DMSO 10%, DC 1 (vitamin C dose 1 mg/mL), DC 2.5 (vitamin C dose 2.5 mg/mL), and DC 4 (vitamin C dose 4 mg/mL), DMSO 10%, DE 1 (vitamin E 2 mg/mL), DE 2 (vitamin E 4 mg/mL), DE 3 (vitamin E 6 mg/mL). Different letters above the bar diagram indicate significantly different results (Duncan P<0.05).

Table 2. The Results of observations of catfish sperm motility with addition the concentrations of vitamin C and vitamin E were different after cryopreservation.

Treatment	pH	Conductivity (mS/cm)
DMSO 10%	6.4	9.31
DC1	5.2	9.18
DC2	5.0	9.34
DC3	4.7	9.58
DE1	5.2	8.64
DE2	5.0	8.60
DE3	5.0	8.56

and vitamin E does not work well. The vitamin C treatment showed a lower motility score than the treatment without vitamin C. Then the vitamin C treatment tended to show a motility score that decreased as the dose of vitamin C given increased. This indicates that the vitamin C given is unable to provide more protection during the cryopreservation process and is indicated to have side effects on spermatozoa. These results are in accordance with Gallego & Asturiano (2019) and Cabrita *et al.* (2011) who stated that if the concentration of vitamin C is excessive it will be toxic so that sperm movement will decrease. In addition, the combination of cryoprotectant materials used in this study is thought to be hyper-osmotic. This will cause excessive dehydration of the spermatozoa cells, resulting in damage to the spermatozoa cells (Mulyadi, 2017).

Excessive doses of vitamin C make the pH of the solution acidic which will cause the death of spermatozoa cells (Yahaq *et al.,* 2019). Similar to vitamin C, the addition of vitamin E in this study had no effect on increasing egg motility, viability, and fertilization rate. Martinez-Paramo *et al.* (2012) stated that this antioxidant effect is species specific. According to Xavier *et al.* (2021), the addition of vitamin E at doses of 2 mg/mL, 4 mg/mL, 6 mg/mL, and 8 mg/mL to the cryopreservation process of Rhamdia quelen (a member of the family Heptapteridae) had a negative effect and did not guarantee cell protection. However, in contrast to research conducted by Martinez-Paramo *et al.* (2012) and Bozkurt *et al.* (2021), the addition of vitamin E increased sperm motility in European sea bass (Dicentrarchus labrax, family Moronidae) and brown trout sperm (*Salmo trutta macrostigma*) compared to cryopreserved controls.

These findings suggest that the efficacy of vitamin E as a cryoprotectant or antioxidant can vary not only among species but may also show trends across taxonomic groups. For instance, within Perciformes, especially marine species such as sea bass and other Moronidae, there is evidence of a positive response to vitamin E supplementation. In contrast, several Siluriformes (catfish species), including Rhamdia quelen, have shown neutral or negative responses, suggesting that antioxidant sensitivity or metabolic handling of oxidative stress may differ across freshwater and marine species, or across evolutionary lineages. Zamani *et al.* (2022) further stated that Vitamin E, a potent antioxidant, helps preserve sperm motility and structure by preventing lipid peroxidation (LPO). The influence of vitamins E on sperm motility has been shown to vary depending on the dosage. Thus, the role of vitamin E depends on each species' physiology and its dosage, likely adjusting to the number of hydroxyl radicals that need to be inactivated.

Based on the results obtained and compared with the literature. The oxidation process in sperm cells can follow different pathways depending on the species, and the species-specific properties of vitamin E make it a more effective antioxidant in mammals compared to fish. In addition, the use of antioxidants can have positive or negative effects depending on the dose used. Martinez-Paramo *et al.* (2012) stated that this antioxidant effect is species specific. This was also mentioned by Almeida-Monteiro *et al.* (2017) that the effectiveness of antioxidants varies by species and depends on the dosage applied.

Viability parameters are parameters that show the difference between live sperm and dead sperm. The results showed that the catfish sperm viability values were significantly different ($P<0.05$). The addition of vitamin C and vitamin E in this study significantly reduced the viability value. The results of this research also show that a high viability value is directly proportional to a high motility value. This is in accordance with Adawiyah *et al.* (2019) that sperm motility with high duration and score will produce a high viability value. Based on the results of this study, the value of the degree of egg fertilization showed significant differences between treatments ($P<0.05$). These results show that the value of the degree of egg fertilization is comparable to the value of sperm motility and viability. Sperm with high motility and viability can increase the sperm's ability to fertilize eggs, and vice versa (Muthmainnah *et al.,* 2019).

The hatching rate (HR) of the control, DC1 and DE2 treatments showed no significant difference ($P>0.05$) and were significantly different from the 10% DMSO, DC2, DE1 treatment groups, and DE3 and DC3 which get no eggs hatch. The control group exhibited a high hatching rate (79.94 ± 2.81%), indicating optimal conditions for embryonic development. Similarly, the DC1 group showed the highest hatching rate (88.89 ± 9.25%), suggesting that this treatment might provide favorable conditions for embryo survival and development. According to Lam *et al.* (2025), vitamin C had a significantly positive effect on absolute fecundity and the rate of egg loss. However, it did not affect egg size or the size

of larvae three days after hatching. Nonetheless, it contributed to an increased hatching rate, improved survival of three-day post-hatch larvae, and a reduction in malformation rates.

However, the DMSO 10% and DC2 groups had significantly lower hatching rates (41.54 ± 6.49% and 33.33 ± 28.87%, respectively), which may indicate potential toxic effects or suboptimal conditions affecting embryonic viability. DMSO cryoprotectant functions as a universal aprotic solvent that can penetrate biological membranes and has low toxicity to biological materials. While it does not lead to significant mortality, exposure to sublethal concentrations can result in notable biological and material abnormalities across all species (Huang *et al.,* 2018). The DC3 group displayed a 0% hatching rate, implying that this treatment may have had a lethal impact on the embryos.

The use of diluent in cryopreservation is an important factor in the success of the sperm preservation process, which is generally made according to the physiochemical structure of fish seminal plasma (Zidni *et al.,* 2020). According to Sahinoz *et al.* (2020) the diluent in cryopreservation plays an important role in regulating ionic composition, osmotic pressure and environmental pH. The combination of diluents in this study (Table 2). did not have a positive impact on sperm preservation. This can be seen from the decrease in motility values, viability and degree of fertilization of eggs when compared to controls. Muchlisin *et al.* (2015) stated that diluents that have a pH similar to sperm plasma can maintain sperm motility.

According to Hardianingsih and Yulanda (2023), catfish sperm that has matured gonads has a pH of 6.5–7.5. During freezing and thawing in the cryopreservation process. Spermatozoa experience damage due to intracellular and extracellular ice crystals, as well as osmotic shock. The sperm preservation ratio in this study used a ratio of sperm and diluent, namely 1:10. Sahinoz *et al.* (2020) said a higher diluent ratio correlates with lower sperm motility, osmotic and physical stimulation, and sperm dilution can explain the decrease in viability due to higher dilution.

The effects of different antioxidants are specific to each species and depend on the type and concentration used for a particular species (Abualreesh *et al.,* 2021). The addition of vitamin C and Vitamin E in this study had no significant effect (P>0.05) on the research results. The lack of significant effects observed from the addition of vitamin C and vitamin E in this study (P>0.05) suggests that their antioxidant properties may not universally enhance sperm quality during cryopreservation in the tested conditions. As noted by Abualreesh *et al.* (2021), the antioxidant efficacy of compounds such as vitamin C and E is species-specific and highly dependent on dosage, antioxidant formulation, and the oxidative environment. Furthermore, the use of a 1:10 sperm-to-diluent ratio may have introduced additional osmotic and mechanical stress that could mask any protective effects these antioxidants might confer, as supported by Sahinoz *et al.* (2020), who indicated that higher dilution ratios are associated with lower sperm motility and viability due to osmotic shock and excessive dilution.

The absence of improvement in motility and viability does not necessarily negate the antioxidant role of these vitamins, but rather emphasizes the importance of optimizing concentration and application protocols for each target species. For instance, some studies have demonstrated beneficial effects of vitamin E in marine Perciformes (e.g., Dicentrarchus labrax), while freshwater Siluriformes (e.g., Rhamdia quelen) have shown neutral or even adverse responses. This implies that taxonomic trends may be relevant and should be taken into account when designing antioxidant supplementation protocols. Future research should focus on testing a broader range of vitamin C and E concentrations, possibly including lower and intermediate doses that may reduce oxidative stress without inducing pro-oxidant effects. In addition, evaluating these antioxidants in combination with other compounds such as selenium, glutathione, or plant-derived antioxidants (e.g., quercetin or resveratrol) may reveal synergistic effects not seen with single-compound treatments.

Moreover, it is recommended that future studies include a comparative analysis across multiple fish species, ideally from different taxonomic groups (e.g., Cyprinidae, Cichlidae, Salmonidae), to identify patterns in antioxidant responsiveness. Incorporating biochemical markers of oxidative stress (e.g., ROS levels, lipid peroxidation, SOD/CAT enzyme activity) could provide mechanistic insights into how vitamin C and E interact with the sperm membrane and mitochondrial function under cryopreservation stress. In summary, while vitamin C and E did not show significant benefits under current experimental conditions, their potential as antioxidants remains promising.

Future studies should optimize dosage, refine protocols, and incorporate interspecies comparisons and molecular assays to clarify the conditions under which these vitamins may enhance sperm quality in fish cryopreservation.

Further research can explore more optimal variations in sperm dilution ratios to minimize negative impacts on sperm viability and motility, taking into account the species used. In addition, research can focus on more effective antioxidant combinations or formulations with doses and types tailored to the specific needs of sperm, considering that antioxidant effects are species-specific. Further studies are also needed to evaluate protective mechanisms against ice crystal damage and osmotic shock, such as modifying the dilution media or developing more innovative cryopreservation techniques.

CONCLUSION

The addition of vitamin C and vitamin E at different doses to the cryoprotectant solution has not had a positive effect on increasing the motility, viability, and fertilization rate of catfish eggs after cryopreservation for one hour.

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