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Research Article **Araştırma Makalesi**

Protective Effects of Different Egg Yolk Sources on Cryopreservation of Scaly Carp (*Cyprinus carpio*) Sperm

Farklı Yumurta Sarısı Kaynaklarının Pullu Sazan (*Cyprinus carpio*) Spermasının Kriyoprezervasyonu Üzerine Koruyucu Etkileri

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Abstract: Egg yolk is one of the most widely used cryoprotective components of extenders, especially for the cryopreservation of mammalian species' sperm cells. However, there is a lack of information regarding their efficacy in cryopreservation of fish sperm cells. Thus, the objective of this experiment was to compare the effectiveness of egg yolk from different avian species (duck, goose, and chicken) on post-thaw quality and fertilization ability of scaly carp (*Cyprinus carpio*) semen following cryopreservation. Sperm samples diluted with the sucrose-based extender at the ratio of 1:10 were supplemented with 10, 15, and 20% ratios of different avian egg yolks. In the control group, sperm samples were diluted with the sucrose-based extender, without egg yolk. Following dilution, sperm samples were equilibrated at +4ºC for 10 min and aspirated into 0.25-ml straws. Then, sperm samples were frozen 3 cm above the liquid nitrogen (LN_2) surface and plunged directly into the LN_2. The frozen sperm cells were thawed in a water bath at 35 ºC for 30 s and fertilization was carried out using a $1x10^5$ spermatozoa/egg ratio. Based on the results, supplementation of sperm cells with 20 % duck egg yolk in a sucrose-based extender exhibited the best post-thaw progressive motility (67.8 ±1.24%), progressive motility duration (32.6 ±1.45 s), viability (82.4 ±1.36%), and fertility (92.6 ±1.28%) compared to the control group ($P<0.05$). The results of the experiment showed that duck egg yolk could be used as an alternative instead of chicken egg yolk in a sucrose-based extender for the cryopreservation of scaly carp sperm.

Keywords

- Cryopreservation
- Egg yolk
- Fertility
- Extender
- Sperm

Özet: Yumurta sarısı, özellikle memeli türlerine ait sperm hücrelerinin dondurularak saklanmasında kullanılan sulandırıcıların en yaygın kriyoprotektif bileşenlerinden biridir. Dolayısıyla, balık sperm hücrelerinin dondurularak saklanmasındaki etkinlikleri hakkında bilgi eksikliği bulunmaktadır. Bu nedenle bu çalışmanın amacı, farklı kanatlı türlerine (ördek, kaz ve tavuk) ait yumurta sarılarının, kriyoprezervasyonu takiben pullu sazan (*Cyprinus carpio*) spermasının çözüm sonu kalite ve fertilizasyon yeteneği üzerindeki etkinliklerinin karşılaştırılmasıydı. Sukroz bazlı sulandırıcı ile 1:10 oranında dilüe edilen sperm örneklerine %10, 15 ve %20 oranlarında farklı kanatlı yumurtası sarıları ilave edildi. Kontrol grubunda ise sperm örnekleri, yumurta sarısı içermeyen sukroz bazlı sulandırıcı ile dilüe edildi. Dilüsyon işlemini takiben, sperm örnekleri +4ºC'de 10 dakika ekilibere edilerek 0,25 ml'lik payetlere çekildi. Daha sonra sperm hücreleri sıvı nitrojen (LN_2) yüzeyinin 3 cm üzerinde dondurularak doğrudan sıvı azota

Anahtar kelimeler

- Kriyoprezervasyon
- Yumurta sarısı
- Fertilite
- Sulandırıcı
- Sperm

(LN_2) aktarıldı. Dondurulan sperm hücreleri 35 ºC su banyosunda 30 sn süre ile çözdürüldü ve $1x10^5$ spermatozoa/yumurta oranı kullanılarak fertilizasyon işlemi gerçekleştirildi. Sonuçlara göre, sperm hücreleri sukroz bazlı sulandırıcıda %20 ördek yumurtası sarısı ile takviye edildiğinde, kontrol grubu ile karşılaştırıldığında en iyi çözüm sonu progresif motilite (%67,8 ±1,24), progresif motilite süresi (32,6 ±1,45 s), canlılık (%82,4 ±1,36) ve fertilite (%92,6 ±1,28) değerleri elde edilmiştir ($P<0.05$). Çalışmanın sonuçları, pullu sazan sperminin dondurularak saklanmasında sukroz bazlı sulandırıcı kullanıldığında, tavuk yumurtası sarısı yerine ördek yumurtası sarısının alternatif olarak kullanılabileceğini göstermiştir.

1. INTRODUCTION

The cryopreservation technique, which is an important biotechnological tool for the conservation of aquatic genetic resources, has been successfully utilized for the long-term storage of sperm cells in the aquaculture industry. The benefits of cryogenic preservation of sperm cells in the aquaculture industry can be summarized as follows: year-round supplying of sperm, artificial hybridization between species, transportation of sperm cells among fish farms, reduction in the nursing cost of male broodstock, and establishment of cryobanks (Bozkurt, 2019; Yavaş et al., 2014).

Although cryopreservation of sperm cells offers many advantages mentioned above, it is also a complex process leading to several forms of cellular damage (Purdy, 2006). The main cryodamage of the cryopreservation process on sperm cells is associated with cold shock and intercellular ice crystal formation, which may lead to a decrease in motility and fertilizing ability of sperm following thawing (Matsuoka et al., 2006; Bozkurt et al., 2019).

Even though cryoprotectants can inhibit cryodamages during the cryopreservation process, they can become toxic to the sperm cells at high levels (Tekin et al., 2007). Therefore, egg yolks from different avian species in an extender have been used to protect sperm cells against cold shock damages during cryopreservation in domestic animals recently (Aboagla and Terada, 2004). The useful effect of avian egg yolks in the cryopreservation process can be ascribed to a resistance factor, which is necessary to protect sperm cells from the cold shock and to maintain viability as well (Webb et al., 2011).

Chicken egg yolk traditionally has been used as a complementary for the cryopreservation of sperm cells due to its easy availability (Bathgate et al., 2006). It ensures protection to sperm membranes against the cryodamages, which occur due to the significant temperature variations during cryopreservation (Andrabi, 2009). However, it has been reported that extenders including egg yolks from different avian species other than that of chicken significantly improved post-thaw quality parameters of bovine (Su et al., 2008), equine (Trimeche et al., 1997; Webb et al., 2011; Burris and Webb, 2009), and ovine (Ali et al., 2013) sperm. It is supposed that the post-thaw quality improvement in sperm cells is based on the variations in the biochemical composition of different avian egg yolks (Bathgate et al., 2006).

As far as we know, there is limited knowledge in terms of the protective roles of avian egg yolk sources on cryopreservation of fish sperm. Thus, the present study was performed to explore the protective effect of egg yolks of different avian species (duck, goose, and chicken) on post-thaw quality and fertilization ability of cryopreserved scaly carp sperm.

2. MATERIAL and METHOD

2.1. Broodstock

Mature male (2478.3±3.2 g, n=13) and female (3628.4±2.7 g, n=3) scaly carp broodstock (2- to 3 years old) were provided by a state aquaculture production station located in Şanlıurfa (Turkey) in June 2021. The broodstock was held in wintering ponds under a natural photoperiod regime. For gamete collection, male and female broodstock were transferred into the hatchery and were held separately in shadowed tanks supplied with continuously (4.0 l/min) well-aerated water at 22°C.

2.2. Collection of gametes

Each brood fish was taken out from the water, and its abdomen was dried. Before injections and stripping, individuals were anesthetized separately in a 50-L tank with 0.7 ml/l diethyl ether (Sigma-Aldrich, Germany) for a few minutes. The urogenital papillae of all broodstock were dried to avoid contamination of gametes with water, urine, or feces.

Carp pituitary extract (CPE), which was suspended in 0.65% NaCl solution, was injected intramuscularly into the brood fishes. Adult males were injected with 1 mg/kg of body weight of CPE 12 h before stripping. Females were injected at 4 mg/kg body weight of the same hormone in two doses, of which 10% of the total dose was administered 24 h before stripping while the remaining 90% was injected 12 h later.

Sperm was stripped by gentle abdominal massage directly into 10-ml glass tubes, which were covered with a parafilm and stored in a styrofoam box holding crushed ice (4±2°C). The sperm quality parameters were evaluated following stripping in 10 minutes at the laboratory. Eggs were also collected by gentle abdominal massage in a dry metal bowl. The eggs were checked visually and only transparent, and well-rounded eggs were used for the fertilization experiments.

2.3. Evaluation of sperm quality

The motility of selected sperm samples was evaluated with the aid of an activation solution (AS) (45mM NaCl, 5mM KCl, and 30mM Tris-HCl, pH 8.2). For this aim, each 1 µl of sperm sample was placed on a glass slide and activated by adding a 10 µl activation solution (AS). Sperm motility was determined using a phase-contrast microscope at 100x magnification (BX43; Olympus, Tokyo, Japan). The percentages (%) and duration (s) of motility were evaluated nine times for each sample. Samples showing below 80% motility were discarded. Sperm motility (%) was evaluated as the percentage of cells exhibiting progressive forward movement, whereas the duration of motility (s) was evaluated until forward movement stopped.

For the purpose of spermatozoa density evaluation, sperm samples were diluted at a ratio of 1:1000 with Hayem solution (35.2 mM Na_2SO_4, 17.1 mM NaCl, 1.8 mM $HgCl_2$, 200-ml bicine). In this way, spermatozoa density was evaluated using a 100 µm deep Thoma hemocytometer (TH-100; Hecht-Assistent, Sondheim, Germany) at 400x magnification with an Olympus BX50 phase contrast microscope (Olympus) and expressed as spermatozoa $x10^9$/ml (three replicates). While indicator papers (Merck, 5.5–9) were used to measure sperm pH, whereas semen colour was evaluated visually within 30 minutes following sperm collection.

Sperm viability was evaluated according to Bjorndahl et al. (2003) using eosin-nigrosin stain (0.67 g eosin Y, 0.9 g of sodium chloride, and 10 g nigrosin dissolved in 100 ml of distilled water). For this aim, a mixture of 5 µl of sperm with 5 µl of the stain was spread on a clean slide and remained to air dry in a dust-free environment. The percentage of live sperm cells was calculated from a total of 300 sperm cells examined under ×100 oil immersion with a phase-contrast microscope (Olympus). In this way, unstained sperm cells were considered alive, while stained sperm cells were considered as dead (Bozkurt and Yavaş, 2021).

2.4. Sperm cryopreservation

Sperm samples (n=13) exhibiting high progressive motility (>80%) and having approximately 12×10^9 spermatozoa/ml sperm density were used in this study. Sperm samples individually were split into four subsamples, and each sample was diluted at a ratio of 1:10 (v:v) with the base extender, which was composed of 3.4314 g sucrose, 0.3427 g NaCl, 21 µl NaOH, 0.5 ml antibiotic (10,000 Unit/ml penicillin and 10,000 µg/ml streptomycin), 100 ml distilled water, pH: 7.7, 325 mmol/kg Osm (Irawan et al. 2010) containing 0 (control), 10, 15 and 20% egg yolk from each of the three avian species such as duck, goose, and domestic chicken. Diluted sperm samples were drawn into 0.25-ml straws by sealing with polyvinyl alcohol (PVA) and were equilibrated in a cool chamber at +4°C for 15 min to obtain isothermal conditions before freezing. Sperm samples were frozen 3 cm

above the liquid nitrogen (LN_2) surface inside a polystyrene box for 10 min. Then, in each experiment, the frozen samples were plunged into the LN_2 for 1 min and finally, nine straws per sperm sample were frozen. Subsequently, the straws were plunged into the LN_2 (-196°C) storage tank. For thawing, the straws were removed from the LN_2 tank and immersed in a 35°C water bath for 30 s, meanwhile the straws were kindly agitated. Thawed sperm samples were activated using an activation solution (AS) and examined under a phase-contrast microscope (Olympus) for the post-thaw sperm characteristics.

2.5. Fertilization experiments

For fertilization, pooled eggs from mature four females were used. The fertilization process was performed at spermatozoa to egg ratio of $1x10^5$ in dry Petri dishes (containing about 500 eggs) using fresh or thawed sperm. Thawed sperm was added over the eggs and kindly mixed before activation with 20 ml of fertilization solution (3 g urea and 4 g NaCl in 1-L distilled water). Following fertilization, the eggs were stirred for 30 min and then, the eggs were washed with the tannic acid solution (0.5 g/l) to eliminate adhesiveness for 10 min. Following, the eggs were rinsed with hatchery water and kindly transferred to Zuger glass incubators with running water (22°C) and kept until eyeing (14-16 h) and hatching (3-4 d). Dead eggs were removed from each incubator during incubation. Fertilization ratios were evaluated in the 4-cell stage under a stereo-microscope at 20-fold magnification. Fertilizing experiments were replicated three times.

2.6. Statistical analysis

Mean values (±SD) regarding freezing and fertilizing experiments were used for statistical analysis. Motility values were normalized through arcsine transformation and differences among the parameters were analyzed using one-way ANOVA. Duncan's post-hoc test was implemented for all comparisons among the treatments at a level of $P<0.05$. All statistical analyses were performed using SPSS 17 for Windows statistical software package.

3. RESULTS

3.1. Sperm quality parameters

In fresh sperm, the mean percentage (%) and duration (s) of motile spermatozoa were 87.30±7.80% and 75.07±14.25 s, respectively. Mean spermatological properties of fresh sperm are given in Table 1.

Table 1. Mean spermatological properties of fresh scaly carp (*Cyprinus carpio*) sperm(n=13).

Volume (ml)	Motility (%)	Motility Duration (s)	Density ($x10^9$/mL)	Total Density ($x10^9$)	pH	Colour
2.96 ±0.48	87.30 ±7.80	75.07 ±14.25	12.11 ±2.50	35.84 ±2.91	7.65 ±0.46	Milky white

3.2. Chemical composition of avian egg yolks

The protein, total fat, dry matter, and raw ash contents of duck, goose, and chicken egg yolks are summarized in Table 2. Duck egg yolk contained more protein than the other two types of egg yolk ($P<0.05$), and goose egg yolk contained more total fat, dry matter, and raw ash than the others ($P<0.05$).

Table 2. Content of egg yolks from different avian species.

Egg Origin	Protein	Total Fat	Dry Matter	Raw Ash
Duck	18.4^{b}	27.0^{a}	53.8^{ab}	2.1^{ab}
Goose	15.6^{a}	34.7^{b}	56.4^{b}	2.4^{b}
Chicken	16.8^{ab}	29.6^{a}	51.0^{a}	1.6^{a}

Different superscripts indicate significant differences within columns ($P<0.05$).

The fatty acid and cholesterol contents of duck, goose, and chicken egg yolks are summarized in Table 3. There are some variations among the avian species in terms of fatty acid levels ($P<0.05$). Chicken egg yolk contains more cholesterol than duck and gooses egg yolks ($P<0.05$).

Table 3. Fatty acid (% w/w of total lipids) and cholesterol (mg/g of yolks) content of different avian egg yolk types.

Component	**Avian Egg Yolk Types**			**Reference**
Fatty acid	**Duck**	**Goose**	**Chicken**	**-**
14 : 0	0.5 ± 0.1^{a}	0.7 ± 0.2^{a}	0.4 ± 0.1^{a}	Surai et. al. 1999
16 : 0	26.4 ± 1.1^{a}	31.2 ± 0.9^{b}	25.8 ± 0.8^{a}	Surai et. al. 1999
16 : 1n7	2.7 ± 0.2^{a}	3.8 ± 0.1^{a}	2.1 ± 0.2^{a}	Surai et. al. 1999
18 : 0	6.4 ± 0.2^{a}	7.0 ± 0.5^{a}	8.6 ± 0.3^{a}	Surai et. al. 1999
18 : 1n-9	47 ± 1.2^{b}	41.9 ± 1.3^{a}	40.5 ± 1.1^{a}	Surai et. al. 1999
18 : 1n-7	1.9 ± 0.1^{a}	2.0 ± 0.2^{a}	1.6 ± 0.2^{a}	Surai et. al. 1999
18 : 2n-6	5.6 ± 0.3^{a}	9.3 ± 0.4^{ab}	14.7 ± 0.5^{b}	Surai et. al. 1999
18 : 3n-3	0.3 ± 0.0^{a}	0.4 ± 0.1^{a}	0.4 ± 0.0^{a}	Surai et. al. 1999
20 : 1n-9	0.5 ± 0.1^{a}	0.4 ± 0.1^{a}	0.3 ± 0.1^{a}	Surai et. al. 1999
20 : 4n-6	4.0 ± 0.1^{b}	2.3 ± 0.1^{ab}	1.7 ± 0.0^{a}	Surai et. al. 1999
20 : 6n-3	0.6 ± 0.1^{a}	0.3 ± 0.2^{a}	1.6 ± 0.2^{a}	Surai et. al. 1999
Cholesterol	Duck	Goose	Chicken	Reference
	10.6 ± 0.01^{a}	-	22.9 ± 0.02^{b}	Surai et. al. 2013
		15.81 ± 0.1^{ab}		Golzar Adabi et al. 2013

Different superscripts indicate significant differences within columns ($P<0.05$).

3.2. Post-thaw quality parameters

The effect of supplementation of egg yolks of different avian species at different concentrations to the sucrose-based extender on post-thaw progressive motility, motility duration, and viability parameters of frozen–thawed scaly carp sperm are presented in Figures 1-3.

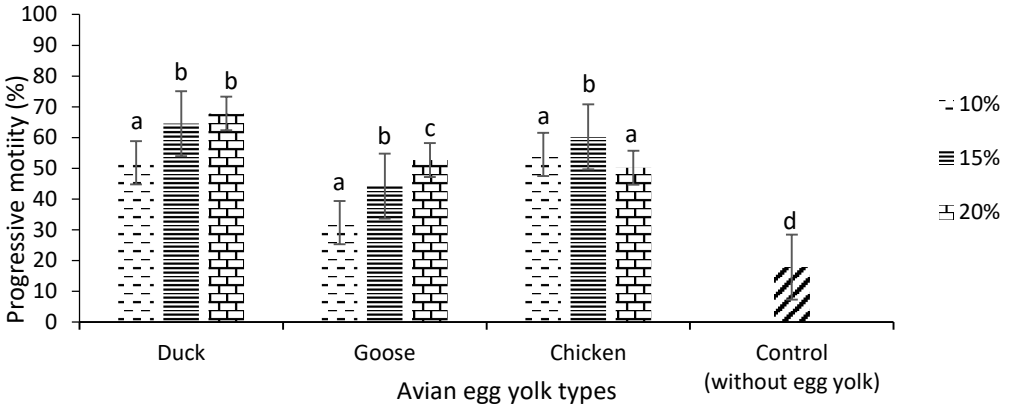

Figure 1. The mean post-thaw progressive motility (%) of frozen-thawed scaly carp (*Cyprinus carpio*) sperm. Different letters indicate differences among treatments (ANOVA, $P<0.05$, n=9).

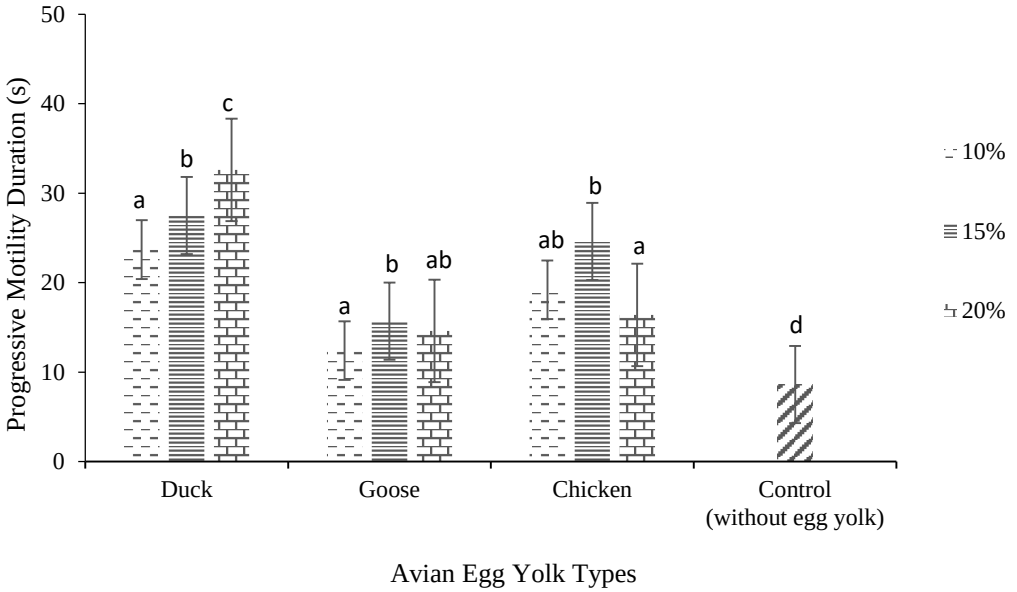

Figure 2. The mean post-thaw progressive motility duration (s) of frozen-thawed scaly carp (*Cyprinus carpio*) sperm. Different letters indicate differences among treatments (ANOVA, P<0.05, n=9).

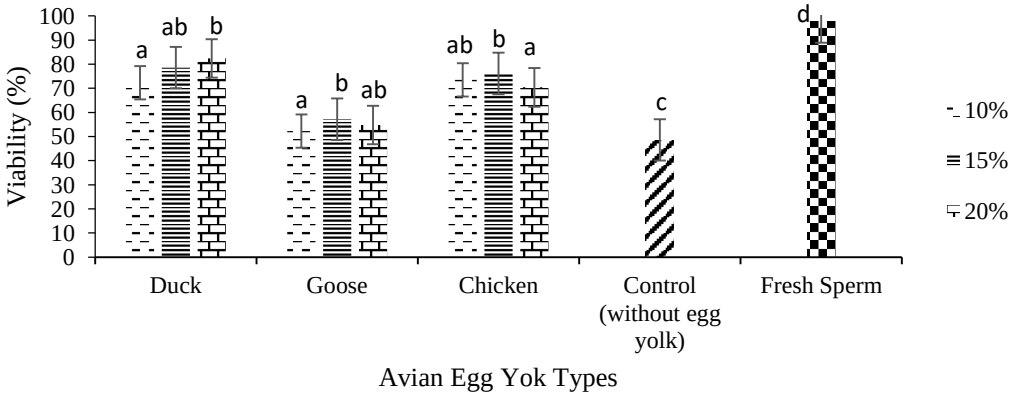

Figure 3. The mean post-thaw viability (%) of frozen-thawed scaly carp (*Cyprinus carpio*) sperm. Different letters indicate differences among treatments (ANOVA, P<0.05, n=9).

According to the results, duck egg yolk had the best cryoprotective effect in terms of the highest progressive sperm motility, and motility duration (67.8% and 32.6 s respectively) as compared to the other avian egg yolks (P<0.05) analyzed. Additionally, sperm cryopreserved in duck egg yolk recorded the highest viability rate (82.4%) than sperm cryopreserved in goose and chicken egg yolk containing extenders (P<0.05). Sperm diluted in goose egg yolk-based extender showed lower percentages in terms of progressive sperm motility, motility duration, and viability (P<0.05). Supplementation of all types of avian egg yolks in extenders caused an increase in all post-thaw quality parameters in comparison to those that did not contain egg yolks (control group) (P<0.05).

3.3. Fertilization

Supplementation of the sucrose-based extender with different avian egg yolk types caused an increase in post-thaw fertility in comparison to those that did not contain avian egg yolk (P<0.05). Fertilization rates were determined higher than 50.0% in all avian egg yolk-containing extenders. Cryopreserved sperm with an extender containing 20% duck egg yolk provided the highest fertilization result (94 %) when compared to the other tested groups (P<0.05). Sperm extended in

goose egg yolk containing extender caused lower fertility and there were no significant differences in the concentration of its (P>0.05, Figure 4).

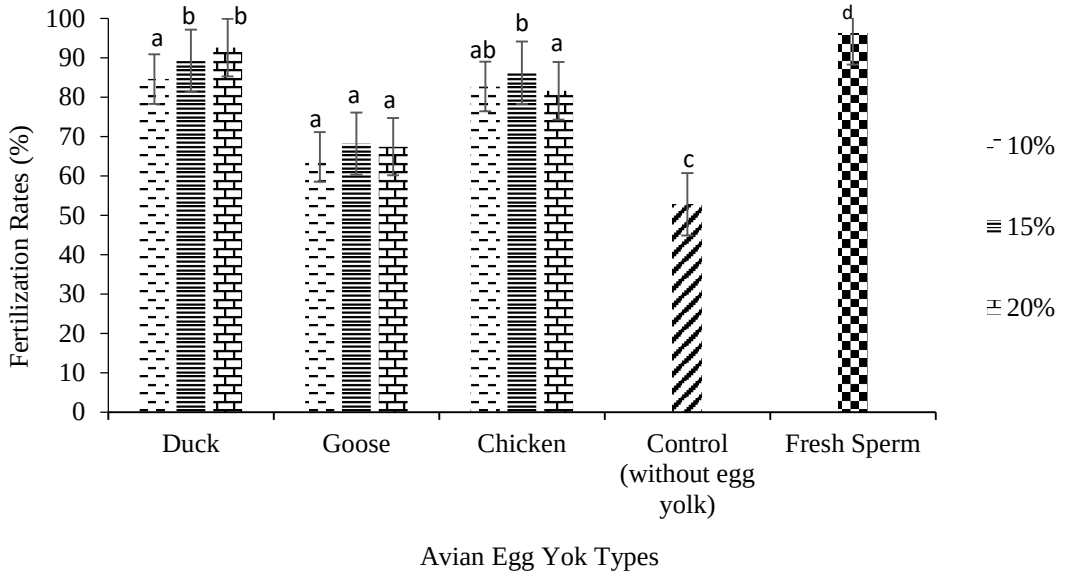

Figure 4. The mean post-thaw fertility (%) of frozen-thawed scaly carp (*Cyprinus carpio*) sperm. Different letters indicate differences among treatments (ANOVA, P<0.05, n=3).

4. DISCUSSION

Much experimental-based research has revealed the cryoprotective effect of avian egg yolks to improve post-thaw sperm quality and fertility following cryopreservation mainly in mammalian species (Aboagla and Terada, 2004; Clulow et al., 2007; Moreno et al., 2008; Akhter et al., 2017).

On the other hand, limited data are available regarding fish sperm cryopreservation using different avian egg yolk sources as a component of extenders. Additionally, most cryopreservation linking studies in aquaculture to date have not tested the role of egg yolk compositions in extender formulations.

According to previous studies regarding mammalian species, it should be also noted that the post-thaw quality of sperm may be attributed to the variations in biochemical composition of egg yolks in different avian species, especially in terms of fatty acids and cholesterol (Bathgate et al., 2006).

From this point of view, fatty acid and cholesterol contents of egg yolks belonging to different avian species are summarized in Table 3. According to Table 3, it seems that there are variations among the avian species in terms of fatty acid levels (P<0.05). On the other hand, chicken egg yolk contains more cholesterol than duck and goose egg yolks (P<0.05).

Many researchers stated that the variations in the chemical composition of the egg yolks in avian species affect their protection ability during cryopreservation (Bathgate et al., 2006; Moreno et al., 2008; Surai et al., 1999). The most important finding of this study is that sperm frozen in duck egg yolk containing extender exhibited higher post-thaw quality and fertility than sperm frozen in other avian egg yolks. The difference may be ascribed to the higher levels of protein and monounsaturated fatty acids, and lower levels of lipid and cholesterol in the duck egg yolk. The components of protein and fatty acid have been demonstrated to be effective in the protection of sperm during cryopreservation (Prasard et al., 1988; Maurice et al., 1994). From this point of view, it is clear that the levels of these components in duck egg yolk may provide better protection to the sperm resulting in higher progressive sperm motility, and fertility after thawing.

It should be noted that the results of this study are in agreement with that of other researchers. For instance, Humes and Webb (2006) reported that chucker egg yolk improved the motility of stallion sperm rather than chicken egg yolk following cryopreservation. This result may be associated with higher levels of protein present in chucker egg yolk. Additionally, the results of this study match with the findings of previous studies proving extenders containing egg yolks from the avian species other than chickens resulted in significantly high post-thawing evaluation parameters in sperm of some mammalian sperm such as boar (Bathgate et al. 2006), buffalo (Akhter et al., 2017; Waheed et al., 2012), stallion (Webb et al., 2011; Burris and Webb, 2009; Clulow et al., 2007), bulls (Su et al., 2008), and rams (Ali et al., 2013; Gholami et al., 2012).

On the other hand, Bozkurt et al. (2014) reported that common carp sperm cryopreserved in a glucose-based extender containing turkey and quail egg yolks provided high sperm quality like the sperm samples cryopreserved in the chicken egg yolk. Even though there was no report concerning the effect of sucrose-based extenders on fertilization results in fish sperm cryopreservation, the beneficial effects of egg yolk supplementations to extenders seem to be species-specific.

In conclusion, duck egg yolk improved post-thaw quality, as well as fertility in scaly carp spermatozoa. Consequently, duck egg yolk may be a promising alternative for replacing chicken egg yolk in extenders for scaly carp sperm cryopreservation.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

The authors declare that all authors contributed equally to the article.

ETHICAL STATEMENTS

There are no ethical issues with the publication of this manuscript.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

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