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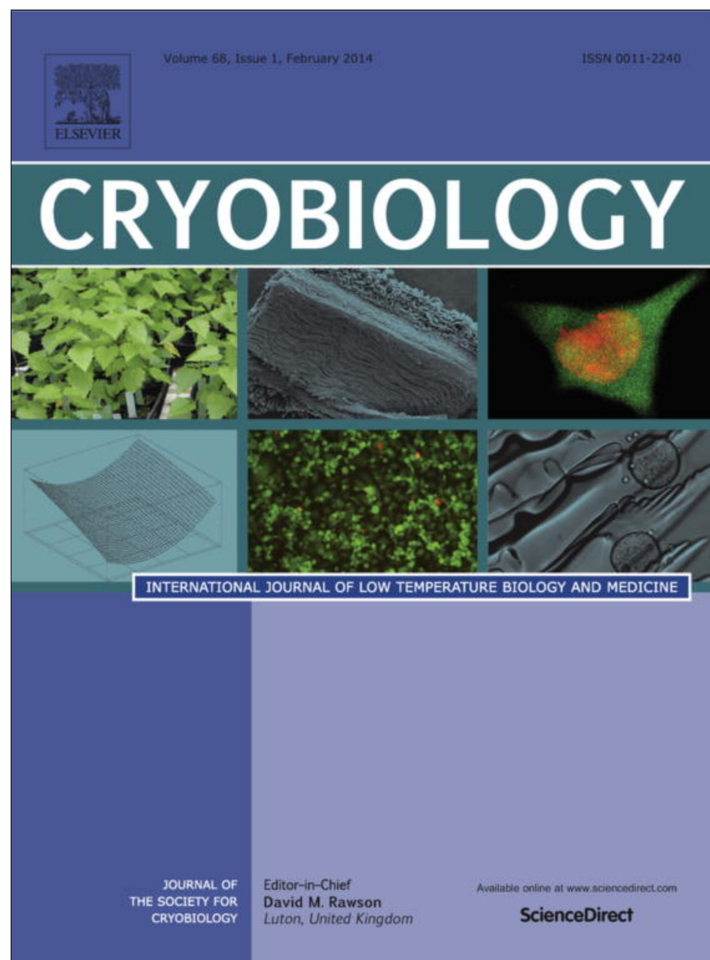

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Protective effects of propolis on cryopreservation of common carp (*Cyprinus carpio*) sperm ☆

Fatih Öğretmen [a,*], Burak E. İnanan [b], Mehmet Öztürk [c]

[a] *Muğla SıtkıKoçman University, Faculty of Fisheries and Aquaculture, Muğla, Turkey*
[b] *Muğla SıtkıKoçman University, Faculty of Science, Department of Biology, Muğla, Turkey*
[c] *Muğla SıtkıKoçman University, Faculty of Science, Department of Chemistry, Muğla, Turkey*

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ABSTRACT

Cryopreservation of sperm is common procedures in aquaculture, particularly used for routine in artificial insemination. However, these application cause damages and adversely affected sperm motility, viability and consequently lower hatching rates. The objective of this study is to determine whether propolis has an effect on cryopreservation and fertilization ability and to investigate the potential protective effect of propolis on spermatozoa of *Cyprinus carpio*. Many studies have been done in cryopreservation offish spermatozoa, but none of them contain propolis in extender composition. The extenders were prepared by using modified Kurokura Solution to which 10% Me_2SO added with different levels of propolis (0.2, 0.4, 0.6, 0.8 and 1 mg ml^{-1}) and 10% egg yolk (as a control without propolis). The pooled semen samples diluted at the ratio of 1:9 by the extenders were subjected to cryopreservation. The percentage and duration of motility and fertilization tests of cryopreserved sperm samples have been done immediately after thawing and compared with control and fresh semen. The extenders containing propolis exhibited higher percentage motility and motility duration than control group ($P < 0.05$). Especially the group IV (0.8 mg ml^{-1} propolis) and the group V (1 mg ml^{-1} propolis) showed significant positive effects on both post thaw motility and hatching ability. The propolis maintained the integrity of the spermatozoa during the cryopreservation process. Evaluating with its contents, it has been shown that propolis is an appropriate cryoprotective agent in fish semen.

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Introduction

The cryopreservation procedures that allow preserving sperm cells have been applied for sperm of many fish species. Cryopreservation of common carp sperm has also been well established by many researchers [14,18,24,31]. The improvement of semen cryopreservation technologies requires an extensive knowledge of the gamete physiology and the biochemical processes occurring during sperm collection, processing and freeze–thawing [48]. These cryopreservation methods offer several benefits such as stock protection from being totally eliminated species, supplying of sperm for optimal utilization in hatchery production and laboratory experiments. However, the methods of sperm cryopreservation induced some problems, such as considerable low motility after thawing, fertilizing and hatching rates as a result of cryoinjuries [19]. The cryoinjuries occur at the defined temperature ranges by the procedures in relation to process during cryopreservation, particularly in cold and hot shocks throughout freezing and thawing [14]. Propolis used as a folk medicine from ancient times. It is adhesive, dark yellow to brown colored balsam that smells like resin. Bees collect propolis from the parts of many trees and plants and mixed with their wax in order to use as a sealant and sterilant in honeybee nests [44]. Especially dermatoplastic, anti-inflammatory, anticancer, detoxicant, antioxidative, immunomodulatory, antimutagenic, and antimicrobial activities of propolis are based on its rich, flavonoid, phenolic acid, and terpenoid contents [6,34,36,42,43,46,50]. Rather than chemical studies, its ethanolic extract have been used to determine the biological activities. In fact propolis could contain more than 300 components, including phenolic aldehydes, polyphenols, sesquiterpene quinines, coumarins, steroids, amino acids, and inorganic compounds [34]. The investigations have show that among them, phenolic compounds such as flavonoids are evaluated as mainly responsible for the biological activity of propolis.

To the best of our knowledge propolis protects reproductive system from toxicity, especially flavonoids and phenolic compounds are also responsible for antioxidant activity and shows

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* Corresponding author. Address: MuğlaSıtkıKocman University, Faculty of Fisheries, 121 Kotekli, Mugla, Turkey. Fax: +9 0252211887.

E-mail address: fatihogretmen@mu.edu.tr (F. Öğretmen).

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protective effects against aluminium chloride which caused testicular dysfunction, and deterioration in semen quality and testosterone levels [50]. Also the studies in mammals reported that propolis significantly increases testosterone level, body weight, relative weight of testis, relative weight of epididymis, the percentage of motile sperm, morphological abnormalities of sperm, and seminal plasma enzymes and decreases the levels of free radicals and lactate dehydrogenase in rabbits [49], and also decreases dead and abnormal sperm and increases testosterone levels in rats [37]. In fish, however, no data have been available regarding the effect of propolis on fish sperm cryopreservation. Castilho et al. [13] studied the effect of propolis and ascorbic acid on plasmatic membrane integrity of goat spermatozoa and revealed the benefit of these antioxidants in the use of goat spermatozoa cryopreservation. Several studies have been conducted about the role of antioxidants on protecting spermatozoa from the loss of motility that occurs following cryopreservation and thawing. Most studies have reported on the use of vitamins, enzymes and amino acids as antioxidants have been used to enhance post-thaw motility; however, the results have been modest [2,10,39]. There are also studies that altered cell membrane integrity and damages to cellular organs [11,12] others concentrate on the loss of DNA integrity in sperm cells in the process of cryopreservation [5,25]. It is demonstrated that sperm in vitro incubation with antioxidant enzymes, such as catalase, increased in motility and integrity in brown trout (*Salmo trutta*) sperm during short storage [29].

Cryopreservation procedures could induce oxidative stress on sperm structure reducing sperm motility and fertilizing ability due to significant reactive oxygen species (ROS) generation [4] and consequently causes DNA damage associated with damage at oxidative levels in fish sperm [40]. Seminal plasma protects sperm against oxidative stress, which contains several antioxidant components. However during cryopreservation, high dilution rates in the extender reduces the seminal plasma antioxidant concentrations and sperm to be more exposed to oxidative stress. In some cases, antioxidants supplementation in vitro (e.g. combination of vitamins C and E) reduce sperm DNA damage [17,16]. Not only because of its antioxidative effect but because of its other effects, propolis has been considered being useable for fish sperm cryopreservation. The aim of the present study was to analyse the effect of extender supplementation with several doses of propolis extract on post-thawed sperm motility, fertilization, eyeing and hatching rates of common carp (*Cyprinus carpio*), considering with propolis content.

Method

Sperm collection

This study was carried out at the hatchery of General Directorate of State Hydraulic Works (İzmir, Turkey) during May. Carp males used in this study were 3–4 years of age. Ten mature common carp males (2.65 ± 0.07 kg, 42.2 ± 1.07 cm as mean ± SEM) were randomly selected from the stock for sperm collection. Fish were held in sand ponds under a natural photoperiod regime. Water temperature and oxygen ranged from 22–24 °C to 7.1–8.3 mg l^{-1} respectively. Fish were anesthetized in 1:2000 aqueous solution of 2-phenoxyethanol and given a single injection of 1 mg kg^{-1} of carp pituitary extract (CPE) before 24 h stripping. The sperm was collected by manual abdominal stripping. Sperm was used only if it is not contaminated by water, blood, urine, or feces and percentage motilities are more than 95%. Sperm samples were not pooled and were stored under aerobic conditions at 0–4 °C until analysis and cryopreservation.

Preparation of the GC–MSD sample of the propolis

Raw propolis samples were collected during autumn from Muğla province (Southwestern Anatolia) of Turkey. Propolis (200 g) was extracted with 250 ml ethanol (Merck, 99%) for 5 times at room temperature. After filtration, the solvent was evaporated to dryness *in vacuum*. In order to silylation the ethanol extract (5 mg) was dissolved with 50 ml of dry pyridine and 75 ml bis(trimethylsilyl)trifluoroacetamide was added, and the mixture was heated at 80 °C for 20 min in an incubator. After cooling, the derivative was analysed by GC–MSD (Varian Saturn 2100T).

Procedure of gas chromatography–mass spectrometry (GC–MSD)

Varian Saturn 2100T (USA) coupled with an ion trap mass spectrometer (IT-MS) and a DB-1 MS fused silica non-polar capillary column (30 m × 0.25 mM ID, film thickness 0.25 μm) were used for the GC–MSD analyses of the sylyl-derivatives of ethanol extract of propolis. For GC–MSD detection, an electron ionization system with ionization energy of 70 eV was used. Carrier gas was helium (15 psi) at a flow rate of 1.3 ml min^{-1}. Injector and MS transfer line temperatures were set at 250 and 200 °C, respectively. The oven temperature was held at 100 °C for 5 min, then increased up to 238 °C with 3 °C/min increasements and held at this temperature for 9 min. Diluted samples (1/25, v/v, in hexane) of 0.2 μl were injected manually in the split mode. Split ratio was 20:1. Mass range was from *m/z* 50 to 650 amu. Scan time was 0.5 s with 0.1 interscan delays. Identification of components was based on GC retention indices and computer matching with the Wiley, Nists and TRLIB libraries as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature and whenever possible, by co-injection with authentic compounds. The relative percentages of separated compounds were calculated from total ion chromatography by the computerized integrator.

Evaluation of motility parameters

An activating solution composed of 45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8.2 [18] was used to evaluate freshly collected and cryopreserved samples motility. For fresh semen a 1 μl drop of sperm was placed on a microscope slide and 100 μl of activation solution was added whereafter the sperm suspensions were thoroughly mixed for 2 s. The percent of motile spermatozoa and motility duration was immediately recorded for 1 min post-activation using a CCD video camera (with Nikon, DS-U3 controller) mounted on a phase-contrast microscope (Nikon Ci-S, ×400 magnification) at room temperature (20 °C). The percentage of sperm motility was estimated as the cell performing progressive forward movement [18], while the duration of motility was determined as the time until forward movement stops. Determining the percentage of sperm motility was assessed using an arbitrary scale with 10% interval increments in which non motile represents 0% (modified from Borges et al. (2005) [7]. Three aliquots of each milt sample were inspected to calculate an average motility.

Freezing-thawing methods and cryomedia preparation

Sperm from 3 of 10 carp males were used individually to cryopreservation with the following cryomedia. Sperm were diluted (1:9) in a modified Kurokura (K) extender composed of the following: 360 mg NaCl, 1000 mg KCl, 22 mg $CaCl_2$, 8 mg $MgCl_2$, 20 mg $NaHCO_3$ for 100 ml pure water, pH 8.2, Osmolarity 378 mOsm, 10% Me_2SO, 10% egg yolk [35]. The crude propolis extract, however, was redissolved in dimethyl sulfoxide (Me_2SO) to prepare 1% (w/v) stock solution. Final solutions were stored in hermetically-sealed brown-glass bottles at room temperature.

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Constituents of cryomedia and propolis doses are presented in Table 1. After dilution sperm samples were loaded into 0.25 ml straws (IMV, France), sealed with polyvinyl alcohol and equilibrated at temperature 4–5 °C for 5 min, vaporized at a height of 2.5 cm above liquid nitrogen surface for 10 min and plunged into liquid nitrogen. At least six straws per sperm sample were frozen. After 7 days of storage in liquid nitrogen, samples were thawed in a 20 °C water bath for 30 s.

Fertilization and hatching of embryos

For fertilization assay three females were stripped and mixed eggs were used for fertilization within 1 h post-collection. After egg stripping 3 g of eggs (approximately 2500–3000 eggs) was used to fertilize either fresh or thawed sperm in a 500 ml round bottomed dish. Eggs were fertilized at a ratio of approximately 1×10^5 spermatozoa/egg with an activating solution [33]. Dishes were placed on an experimental stand that supplied with hatchery water flow rates, 10 l h^{-1} for each experimental group. Fertilization ratios were evaluated from triplicates and recorded 3 h after insemination and dead eggs were counted and removed in each dish during incubation. Eyed embryos were counted after 3rd day and hatched fry 5th day of incubation at 22 ± 0.5 °C, respectively.

Statistical analysis

Each of the replicates was used to make six observations per replicate. Non-parametric Mann–Whitney's *U* tests following Kruskall–Wallis's test at 0.05 significant levels were used to determine whether significant variations between groups. Statistics were performed using SPSS software version 20.0. All values are presented as means and standard error of the mean (±SEM).

Results

Total contents of propolis used in the study

The components of propolis obtained from Muğla city of Turkey were listed as in percentages in Table 2. According to the results of the GC–MSD analysis, 38 compounds were identified from ethanolic extract of propolis used in the study. The studied propolis contained significant amounts of phenolic compounds and Sugars. Benzoic acid, D-Fructose, Glycerol, 5-hydroxy-7,3′,4′-dimethoxyflavone and Caffeic acid, respectively, constituted the major reaction-about 51%-of this propolis. Terpenoids have been also observed in propolis.

Effects of propolis on post-thaw motility characteristics, fertilization, eyeing and hatching rates

The obtained data are presented in Table 3. Motility percentage of fresh common carp sperm was 93.3 ± 1.7%. Sperm in all treatments have progressive motility after thawing and agglutination of sperm after thawing was not observed in experimental groups. Compared to fresh milt, cryopreserved spermatozoa especially control group showed a significant decrease in sperm motility percentage and duration ($P < 0.05$). Cryopreserved sperm groups that extenders contain propolis extract showed a higher percentage of motile cells than the control group ($P < 0.05$). Group IV (0.8 mg ml^{-1} propolis) exhibiting the highest post-thaw motility (68.7 ± 1.8%). Sperm motility duration of fresh semen was 55.7 ± 1.8 s while the longest duration of post-thaw sperm motility (42.3 ± 1.4 s) was also observed in Group IV. Post-thaw sperm motility durations in all propolis treatments were higher than the control group (28.7 ± 2.0 s). Similarly, sperm in control group had the lowest fertilization rate in comparison to other cryopreserved groups while fertilization rate of fresh sperm as the highest was 97.3 ± 0.3%. On the other hand, cryopreserved sperm with extenders containing propolis showed better results in terms of eyeing rates when compared to control group (18.3 ± 1.8%). According to the hatching rate results, availability of propolis in extenders significantly influenced hatching yield ($P < 0.05$). The hatching rates of sperm treated extenders with propolis ranged from 36.7 ± 2.0% and 62.7 ± 2.3% while 13.3 ± 1.4% in control group. The highest hatching rate of the experiments was found in fresh sperm as 69.7 ± 1.8%. However, no significant difference was observed among group IV (62.7 ± 2.3%), group V (60.3 ± 2.0%) and fresh semen with regard to hatching rates.

Table 1
Constituents of cryomedia tested. Propolis doses in cryomedia refer at final concentration.

Group	Cryomedia
Control	K[a] + %10 Me_2SO + %10 egg yolk
I	K + 0.2 mg ml^{-1} propolis + %10 Me_2SO + %10 egg yolk
II	K + 0.4 mg ml^{-1} propolis + %10 Me_2SO + %10 egg yolk
III	K + 0.6 mg ml^{-1} propolis + %10 Me_2SO + %10 egg yolk
IV	K + 0.8 mg ml^{-1} propolis + %10 Me_2SO + %10 egg yolk
V	K + 1 mg ml^{-1} propolis + %10 Me_2SO + %10 egg yolk

[a] K; modified Kurokura Solution as described in Magyary et al. (1996) [37].

Discussion

The present study shows that supplementation of propolis to cryo media effects post-thaw motility characteristics, fertilization, eyeing, and hatching rates positively. Assuming that hatching rates are more important than fertilization rates in practice, results of propolis treatments were remarkable for not only fertilization rates, but also for hatching rates. According to our experience, propolis concentration in cryomedia more than 2 mg ml^{-1} might be toxic to spermatozoa. These might cause that spermatozoa remain quiescent or have insignificant movement when mixing by the activation solution. Castilho et al. [13] studied effect of propolis and ascorbic acid on membrane integrity and the potential of these antioxidants in the use of goat spermatozoa cryopreservation. They also reported that propolis was found toxic to spermatozoa at concentrations of 2.5 and 5 mg ml^{-1}. The use of dietary propolis treatment also reduced mortality of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) eggs in vitro [45]. On the other hand, protective and antioxidant role of propolis on chemically induced colon carcinogenesis and DNA damage have already been found in rats [6,32].

Propolis exhibits antibacterial, antifungal, anti-inflammatory, antiviral activity, and as a powerful antioxidant besides many other properties such as anticancer. These effects are obviously not just an individual substance or a particular substance class but integrated structure of propolis as indicated in its antibacterial action [22]. Besides there are some studies concerning the functions of substance classes in propolis. Antimicrobial properties of propolis are possibly attributable to its flavonoid content [15]. In cryopreservation procedure, using of antibiotics is preferable to eliminate bacterial and mycoplasma contamination originating from egg yolk [8]. In this study, 8.63% of propolis was flavonoids. Sugars which constitute around 26% of the propolis have also been used in media for fish semen. Fructose, glucose, and mannitol found also in propolis are prominent substances and frequently used in cryopreservation procedure. Presence of some sugars is often attributable to successful cryopreservation [47]. They could be used for energy supply of spermatozoa as indicated in glucose [30] or make the media isotonic to the seminal plasma as a nonelectrolyte as indicated in mannitol [38]. Additionally, glycerol formed

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Table 2
Percentage of compounds identified by GC–MSD in ethanol extract of propolis (%).

Peak No.	RT[a]		Content (%)	Identification method
Phenolic compounds				
1	2.653	Phenyl ethanolamine	0.71	MS[b]; Co-GC[c]; RI[d]
2	3.785	Benzyl alcohol	t[e]	MS; Co-GC; RI
3	8.405	Benzoic acid	13.17	MS; Co-GC; RI
4	8.836	Hydrocinnamic acid, methyl ester	t	MS
5	13.223	Hydrocinnamic acid	t	MS; Co-GC; RI
6	13.603	Cinnamic alcohol	1.66	MS
7	16.155	p-Vanilin	t	MS; Co-GC; RI
8	16.568	Cinnamic acid	t	MS; Co-GC; RI
9	18.709	m-Hydroxy benzoic acid	t	MS
10	19.110	p-Hydroxy benzoic acid	t	MS; RI
11	21.465	Benzyl benzoate	1.31	MS; RI
12	23.174	p-Metoxycinnamic acid	1.68	MS; Co-GC; RI
13	25.995	p-Coumaric acid	3.89	MS; Co-GC; RI
14	27.256	3,4-Dimethoxy cinnamic acid	2.69	MS; RI
15	28.979	3-Methoxy-4-hydroxy cinnamic acid	5.48	MS; RI
16	30.212	Caffeic acid	8.18	MS; RI
17	40.756	Propyl gallate	4.45	MS; Co-GC; RI
Terpenoids				
18	19.448	β-Eudesmol	t	MS; Co-GC; RI
19	33.532	Pimaric acid	2.33	MS; Co-GC; RI
20	34.221	Dehydroabietic acid	1.93	MS
21	34.817	Abietic acid	2.2	MS; Co-GC; RI
Flavonoid				
22	37.770	5-Hydroxy-7,3′,4′-dimethoxyflavone	8.63	MS; Co-GC; RI
Fatty acids				
23	25.659	Palmitic acid, methyl ester	0.61	MS; Co-GC; RI
24	29.107	Linoleic acid	2.27	MS; Co-GC; RI
25	31.505	Oleic acid	1.99	MS; Co-GC; RI
26	32.065	Stearic acid	t	MS; Co-GC; RI
Sugars				
27	22.634	D-Xylofuranose	0.96	MS; Co-GC; RI
28	23.896	β-D-L-Arabinopyranose	1.26	MS; Co-GC; RI
29	24.485	D-Fructose	11.67	MS; Co-GC; RI
30	24.630	2-Keto-D-gluconic acid	6.74	MS; Co-GC; RI
31	25.076	Hexopyranose	1.19	MS; Co-GC; RI
32	26.205	D-Glucose	2.93	MS; Co-GC; RI
33	27.500	D-Mannitol	t	MS; Co-GC; RI
34	28.171	β-D-Glucopyranose	2.13	MS
Other compounds				
35	3.067	2-Methyl-2-butenoic acid	0.35	MS
36	10.263	Glycerol	9.59	MS; Co-GC; RI
37	10.019	Ethyl succinate	t	MS; RI
38	16.076	Malic acid	t	MS; Co-GC; RI

[a] Retention times (min) on DB-1 fused silica column.
[b] Mass spectroscopy technique (MS).
[c] Co-injection with authentic compounds (Co-GC).
[d] Retention index literature comparison (RI).
[e] Trace (t).

Table 3
Effects of propolis on post-thaw motility, fertilization, eyeing and hatching ratio (mean ± SEM). The same superscript letters in the same column are not significantly different at $p < 0.05$.

Group	Motility (%)	Motility duration (s)	Fertilization (%)	Eyeing (%)	Hatching (%)
Control	40.3 ± 2.9^a	28.7 ± 2.0^a	84.7 ± 1.9^a	18.3 ± 1.8^a	13.3 ± 1.4^a
I	54.3 ± 2.3^b	33.3 ± 2.0^b	92.7 ± 2.0^b	43.7 ± 2.9^b	36.7 ± 2.0^b
II	55.0 ± 2.9^b	34.3 ± 1.4^b	92.7 ± 0.9^b	46.0 ± 2.1^b	42.7 ± 1.4^b
III	61.7 ± 1.7^b	36.3 ± 0.9^b	94.3 ± 0.3^b	54.0 ± 2.7^b	48.0 ± 1.5^b
IV	68.7 ± 1.8^c	42.3 ± 1.4^c	94.7 ± 0.9^b	67.0 ± 1.5^c	62.7 ± 2.3^c
V	63.3 ± 1.7^b	39.7 ± 1.8^b	95.0 ± 0.6^b	61.3 ± 1.8^c	60.3 ± 2.0^c
Fresh semen	93.3 ± 1.7^d	55.7 ± 1.8^d	97.3 ± 0.3^c	72.7 ± 3.5^d	69.7 ± 1.8^c

9.59% of the propolis is widely used as a penetrating cryoprotectant in the cryopreservation of sperm [14,41]. The antioxidative properties of propolis also come to the fore. Especially Benzoic acid [21] and caffeic acid [23] among phenolic compounds in propolis are antioxidative compounds, constituting around 20% of the propolis.

Incubation of spermatozoa with the solution containing some antioxidants or enzymes which are naturally available in fish semen have shown that uric acid, methionine and catalase improved the sperm motility and membrane integrity and decreases the sperm lipid peroxidation [27]. In spite of all the advantages associated with the use of cryopreservation procedures, application of

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these procedures to sperm of Salmonid species (*Salvelinus fontinalis* and *Oncorhynchus mykiss*) is not recommended in routine for the reason that their effects mainly on the quality of cryopreserved semen were negative while only minor positive effects were found [28]. On the other hand, certain additives such as taurine, trehalose and cysteamine provided cryoprotective effects, improving post-thawed motility of semen as displaying antioxidative properties in ram and rabbit semen [1,9]. It seems that some antioxidants should be suitable for maintaining motility during cryopreservation. In this connection, antioxidative substances in propolis might integratedly act as an effective antioxidant for fish semen.

On the other hand, DNA damage can occur based on different factors especially during cold and frozen storage [11] and effects adversely embryonic development of offspring [20]. Cryopreservation increases DNA fragmentation but the presence of antioxidants in the seminal plasma and increased condensation level of chromatin during spermatogenesis protect spermatozoa [26]. Effect of the freezing-thawing process on the fish sperm DNA have been studied in different fish species [20,25,51]. Low levels of antioxidants or inhibition of antioxidant enzymes causes oxidative stress and may damage or kill cells. [3]. It might be assumed that the effect of propolis resulting from its antioxidative compounds is capable of protecting spermatozoa against DNA injury.

In summary, based on the present study, propolisis an appropriate cryoprotective agent in fish semen as a whole, especially at concentration of 0.8 and 1 mg ml^{-1}. The hatching ratios obtained from cryopreserved sperm treated with extenders containing propolis were higher than the control group. We strongly suggest that usage of propolis in the cryopreservation of not only fish and shellfish gametes but also embryos should be applied in further studies.

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