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EVALUATION OF CRYOPROTECTIVE EFFECT OFTURKISH PINE HONEY ON COMMON CARP (*CYPRINUS CARPIO*) SPERMATOZOA

Fatih Ögretmen* and Burak E. İnanan[2]

[1]Muğla Sıtkı Koçman University, Faculty of Fisheries Department of Aquaculture, 48000 Kötekli, Muğla, Turkey
[2]Muğla Sıtkı Koçman University, Faculty of Science, Department of Biology, Muğla, Turkey
tel:+90 252 2113179 fax: +90 252 2113179.
*Corresponding author: fatihogretmen@mu.edu.tr

Abstract

BACKGROUND: The cryopreservation procedures that allow preserving sperm cells have been applied for sperm of many species. A sugar like glucose, fructose and sucrose were frequently used in cryomedia but up to the present pine honey was not used for cryopreservation of sperm cells. **OBJECTIVE:** The objective of present study is to investigate the effect of pine honey in various concentrations of 100, 200, 300, 400 and 500 mg ml^{-1} solutions on cryopreservation and fertilization ability of spermatozoa of common carp (*Cyprinus carpio*).**MATERIALS AND METHODS:** Totally 12.5% (v/v) Me2SO as a cryoprotectant and 10% (v/v) egg yolk added all extenders. Pine honey also compared with sugars as glucose, fructose (monosaccharide) and sucrose (disaccharide). Collected semen samples were diluted at the ratio of 1:9 with the extenders. After dilutions, the sperm motility was assessed for each group and then the diluted semen samples were cryopreserved. **RESULTS:** The extenders containing 300 mg ml-1 pine honey group showed both highest post thaw motility 75.3±5.1%, motility duration (s) 47.3±2.5% and hatching ratio 42.6±4.2% than other cryopreserved groups ($P<0.05$). **CONCLUSION:** Using the pine honey in cryomedia is effective for cryopreservation especially about hatching success of egg fertilized by frozen-thawed sperm of common carp.

Keywords: Pine honey, sperm cryopreservation, *C. carpio* spermatozoa

INTRODUCTION

Sperm cryopreservation is the method to preserve male gametes. It has been applied in many fish species. Cryopreservation of freshwater fish species especially common carp sperm has been studied by many researchers (13, 21, 16, 29). These methods have several benefits such as efficient utilizing of sperm, synchronizing of artificial reproduction, maintaining genetic variability of broodstock and and laboratory experiments. However, the procedures of sperm cryopreservation encountered some problems, such as considerable low after thawing motility, fertility and hataching rates (48). The cryoinjuries also occur in relation to freezing and thawing process during cryopreservation (29). Besides the multiplicity of cryopreservation procedures, variability in diluents, cryoprotectants and also other constituents affect the consistency of fertilization results. The suitability of the same extenders and cryoprotectants differs from

fish to another fish species (28). Common carp spermatozoa is a model cells that frequently used to investigating basic cryobiological parameters and also improving cryopreservation procedure for aquacultural practise.

Pine honey is produced only in Turkey and Greece. The major chemical ingredients of pine honey have been reported by many researcher (32, 39, 2). The content of honey varies widely according to the floral sources and geographical origin of honey (41). Pine honey is produced by honey bees from secretion of *Marchalina hellenica*. It comprises sugars (70–80%) mainly the monosaccharides. Fructose and glucose are the main components found in honey. Other minor constituents, however, are the organic acids, minerals, proteins, phenolic compounds and free amino acids (2). Pine honey also has minerals such as magnesium, potassium, calcium, sodium chloride, sulphur, iron and phosphates; as well as vitamins B1, B2, C, B6, B5 and B3 (8). Honey have several beneficial properties which demonstrates antibacterial, antioxidant, anticarcinogenic, antiinflammatory, antiatherogenic, antithrombotic, immunemodulating and analgesic activities (45, 43). The pine honey may protect storage stability without losing its consistency and crystallisation facilitates (24). Akbulut et al. showed that pine honey collected from Western Anatolia involve high levels of phenolics and antioxidant activity, as well as minerals such as calcium, potassium and phosphorous (2). Eraslan et al. reported that the administration of pine honey alleviated the adverse effects of trichlorfon, an organophophorous insecticide due to its high content of phenolics and amino acids (7).

Honey is used orally to enhance sperm quality and fertility of male rats and it was enhanced spermatogenesis, increased sperm count, percentage of normal sperm and was reduced the percentage of sperm head and tail abnormalities (38, 23). Glucose and fructose are also available in the fish seminal plasma combination, but the glucose and fructose concentrations in the seminal plasma of freshwater salmonids are more than the other freshwater species (30). Simple freezing protocols are available for the cryopreservation of freshwater and marine fish semen that consists of DMSO containing saline or sugar solutions (37) (Table 4).

The honey with its rich nutrient content also reported to be suitable for cryopreservation media of *Osteochiius hasseltii* sperma. Simple dose of honey (500 mg/ml) was combined with %15 DMSO and was reached 63.33% post tahaw motility rates (36). Honey was also found to be effective as an extender (0.5% in the Ringer solution) for sperm cryopreservation of marine fishes like milkfish (*Chanos chanos*) and black porgy (*Acanthopagrus schlegeli*) (4).

Many researchers have been developed different artificial extenders for different species which succesfully prolonged the sort time storage or cryopreservation of spermatozoa. The principle of cryopreservation is to cause cell dehydration and eventually concentrate the cytosol for prevent injury so that ice crystallization in the plasma is minimized during freezing. Sugars as sucrose and glucose were used in extender for their chemical dehydration properties before freezing (9). Sugars have been used with many exstenders mainly glucose (3, 31, 14,) fructose (46) or sucrose (42, 12, 5, 1) for short term storage and long term cryopreservation of fish sperm. It is reported for salmonids like *Oncorhynchus mykiss, Salmo salar* and *Salmo trutta* simple cryomedia consisting of 0,3 M glucose and 10% DMSO were used successfully (40,35). According to our experinece 0.3 M glucose was not succesful cryomedia for rainbow trout *Oncorhynchus mykiss* (Unpublished data). However, no studies have been conducted to examine the influence of pine honey

supplemented to extender. The aim of the present study was to analyse the influence of extender that supplemented different doses of pine honey and different sugars on cryopreservation, post-thaw sperm motility, fertilization and hatching success of common carp spermatozoa. The experiment was also conducted to investigate the best combination of honey in extender combination with (DMSO) and egg yolk as cryoprotectants.

METHOD

Sperm collection

This study conducted at the hatchery of General Directorate of State Hydraulic Works, Fish Production Station (Adana, Turkey) during May. Common Carp males used in this study were 3–4 years of age. Ten mature common carp males (2.77±0.52 kg, 42.5±3.3 cm as mean±SD) were randomly selected from the stock pond for sperm collection. Fish were held in 5000 lt. tanks under a natural photoperiod regime. Water temperature and oxygen ranged from 22±1ºC to 8.1±0.4 mg l^{-1} respectively. Males and females were anesthetized in 1:3000 aqueous solution of 2-phenoxyethanol and given a single injection of 1 pellet kg^{-1} of ovopel before 24 hours stripping. The sperm was collected by manual abdominal stripping and 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 2-4 ^{0}C until analysis and cryopreservation. After collection, some physical and chemical characteristics were also observed, such as semen volume, osmolarity and pH.

Preparation of the cryomedia

Pine honey samples were collected during the autumn from local dealer Muğla city (Southwestern Anatolia) of Turkey. Sperm from 3 of 10 carp males were used to cryopreservation individually with the following cryomedia. Control group were diluted (1:9) in an modified Kurokura (MK) 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 365 mOsm, 12.5% Me_2SO as permeating cryoprotectant, 10% egg yolk used non-permeating cryoprotectant (29). The pine honey concentration in cryomedia more than 75-80 mg ml^{-1} can inactivate common carp spermatozoa. The collected semen was diluted with different doses of pine honey (100, 200, 300, 400 and 500 mg ml-1) which used in cryomedia. The pine honey was prepared with distilled water at different concentrations. The pine honey groups also compared with sugars; glucose, fructose and sucrose at 300 mM concentration. Osmolarity was measured with Gonotec Osmomat 30 cryoscopic osmometer. Constituents of cryomedia and pine honey doses are presented in Table 1.

Grup	Cryomedia	Osmolarity	pH
Kontrol	Modified Kurokura*+ 12.5%Me2SO + 10% egg yolk	365	8.2
I	100 mg ml-1 pine honey + 12.5%Me_2SO + 10% egg yolk	539	8.2
II	200 mg ml-1 pine honey + 12.5%Me_2SO +10% egg yolk	889	8.2
III	300 mg ml-1 pine honey + 12.5%Me_2SO +10% egg yolk	1273	8.2
IV	400 mg ml-1 pine honey + 12.5%Me_2SO + 10% egg yolk	1770	8.2
V	500 mg ml-1 pine honey + 12.5%Me_2SO + 10% egg yolk	2366	8.2

VI	300 mMGlucose + 12.5%Me_2SO + 10% egg yolk	310	8.2
VII	300 mM Sucrose + 12.5%Me_2SO + 10% egg yolk	307	8.2
VIII	300 mM Frucose + 12.5%Me_2SO + 10% egg yolk	317	8.2

Table 1. Constituents of cryomedia and pine honey dose in cryomedia at final concentration, osmolarity and pH. *Modified Kurokura Solution as described in Ogretmen et al 2014 (29).

The total components of the pine honey obtained from Muğla city of Tukey were listed as in percentages in Table 2.

Main content of pine honey	**Average values (Mean±SD)**	**Mineral content of pine honey (mg/kg)**	**Average values (Mean±SD)**
Water %	16.51±1.12	Aluminum	64.73±27.98
Fructose %	35.38±3.48	Boron	17.49±7.41
Glucose %	27.97±4.53	Calcium	2,665±2,517
Sucrose %	2.91±1.27	Copper	3.61± 1.17
Fructose/glucose ratio %	1.21±0.23	Iron	235.2±65.0
Fructose+glucose %	62.31±3.95	Potassium	3,802±866
Saccarose %	0.64±0.17	Magnesium	198.8±24.9
Maltose %	1.32±0.43	Manganese	12.07 ±1.08
Total ash %	0.45±0.19	Sodium	473.5 ±81.5
Protein mg/kg	3,57±0.65	Nickel	48.85 ±5.20
Conductivity mS/cm	1.33±0.37	Phosphorus	903.7 ±139.8
pH	4.53±0.42	Zinc	45.2±59.1

Table 2. Chemical composition of pine honey (2).

Freezing-thawing methods

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 extender as control group and also diluted with eight different cryomedia (Table 1). After sperm dilution, samples were packaged in 0.5 ml straws (IMV, France). Afterwards sealed with polyvinyl alcohol and equilibrated at temperature 2-4ºC for 5 minutes, vaporized at a height of 3 cm above liquid nirogen surface for 10 min and plunged into liquid nitrogen. At least ten straws per sperm sample were frozen. After 7 days of storage in liquid nitrogen, the samples were thawed in a water bath 20ºC for a periodof 30 s. After thawing each sample was evaluated for the motility parameters using a light microscope with a software connected to the computer (SCA sperm class analyser) to evaluate the percentage of spermatozoa motility and viability. Ogretmen et al 2014 (29).

Evaluation of motility parameters

An activation solution (45 mMNaCl, 5 mMKCl, 30 mM Tris–HCl, pH 8.2) was used to evaluate freshly collected and cryopreserved samples motility (29). For fresh semen a 1 µl drop of sperm was placed on a microscope slide and 50µl of activation solution was added thereafter the sperm suspensions were thoroughly mixed

for 2-3s. The percent of motile spermatozoa and motility duration was immediately recorded for 1 min post-activation using a CCD video camera (Basler 542) mounted on a phase-contrast microscope (Nikon Ci-S, x400 magnification) at room temperature (20°C). Three aliquots of each milt sample were inspected to calculate an average motility. The percentage of sperm motility was estimated as described by Öğretmen et al. 2014 (29).

Fertilization and hatching of embryos

For fertilization assay three females were stripped and mixed eggs were used for fertilization. 3 g of eggs (aproximately 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 10^5:1 sperm/egg with activation solution. Dishes were placed on an experimental stand that supplied with hatchery water flow rates, 10 l $hour^{-1}$ for each experimental group. Fertilization ratios were recorded three hours 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±1°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).

1. RESULTS

Effects of pine honey on post-thaw motility charecteristics, fertilization, eyeing and hatching rates

Compared to fresh milt, cryopreserved spermatozoa especially control group showed a significant decrease in sperm motility (p>0.05). All treatments were motile after thawing and agglutination of sperm after thawing was not observed in experimental groups except glucose group (Group VI).

The influence of the pine honey varied significantly based on the evaluation criteria in post-thaw motiliry of this study. Cryopreserved sperm groups that extenders contain pine honey howed a higher percentage of motile cells than the control group (p<0.05). Group III. (containing 300 mg ml-1 pine honey) exhibiting the highest post-thaw motility (75.3±5.1%). The longest duration of post-thaw sperm motility (47.3±2.5 s) was also observed from Group III. The groups VI. and VIII. (containing glucose and fructose) showed the high motility ratio (60.1±5% and 68.4±2.8%) than the control group (43.4±2.7%). Although high motility rates of these groups (VI and VIII), hatching rates were (21.3±2.1% and 17.5±2.5%) significantly lower than the group III. and fresh semen. The overall results of motility and fertilization rates are shown in Table 3. Average motility of fresh common carp sperm was 95.4 ± 4.3% in the experiments. In conrol group percentage of eyed eggs were observed 32.4±4%. Similarly, in tests on the Cammon Carp that 5 g of eggs inseminated with 1 ml of cryopreserved semen and percentages of eyed eggs were obtained between 31.5% and 25.5% (16). Hatching rates of carp eggs fertilized with either cryopreserved or control sperm are shown in Table 3 as percentage of ayed eggs and hatched embriyos. In terms of percentage hatching, all of the sugars (glucose, sucrose and fructose) resulted near control group (p<0.05). Even if group III observed best result in hatching ratio (42.6±.4.2) it was significantly lower than fresh sperm (70.6±2.8). Fertilization rates were generally high and not significantly different among fresh and cryopreserved groups II, III, VI and control. Hatching rates of eggs that sperma frozen without supplemented pine honey groups VI, VII,

VIII and control resulted in 14.6±2.5%, 24.4±1.5%, 17.5±2.5%, and 21.1±2.6 respectively.

With increasing in pine honey concentration in cryomedia up to 300 mg ml-1, motility, fertility and hatching rates were increased. The results of treatments with pine honey especially at concentration of 300 mg ml^{-1} were remarkable. The use of frozen-thawed semen preserved with pine honey yielded in hatching rates between 21.3±1.5% and 42.6±4.2%. However, no significant difference was observed among hatching rates with the group except the group III. (42.6±4.2) and fresh semen (66.6±2.8%) (Table 3).

GROUP	Motility (%)	Motility duration(s)	Fertilization rates (%)	Eyeing rates (%)	Hatching rates (%)
Control	$43.4±2.7^{b}$	$27.3±2.5^{a}$	$87.4±7.5^{c}$	$32.4±4.0^{a}$	$21.1±2.6^{a}$
I	$28.3±2.9^{a}$	$21.33±2.1^{a}$	$71±5^{a}$	$24.6±1.5^{a}$	$21.3±1.5^{a}$
II	$55.6±5^{b}$	$42±2.6^{a}$	$93.3±2.8^{c}$	$32.6±2.5^{a}$	$32.3±3.1^{a}$
III	$75.3±5^{c}$	$47.3±2.5^{a}$	$94±1^{c}$	$44.6±5.6^{a}$	$42.6±4.2^{b}$
IV	$46.6±2.8^{b}$	$32.3±3.1^{a}$	$87.3±2.5^{b}$	$28.3±3.1^{a}$	$22.3±2.5^{a}$
V	$29.3±4.1^{a}$	$12.6±2.1^{a}$	$81±6.5^{a}$	$24.3±2.1^{a}$	$21.3±2.1^{a}$
VI	$60.1±5^{c}$	$45.2±2.5^{a}$	$91.2±3.5^{c}$	$17.6±2.5^{a}$	$14.6±2.5^{a}$
VII	$40.6±2.9^{a}$	$24.9±5.1^{a}$	$83.5±2.8^{b}$	$28.3±2.5^{a}$	$24.4±1.5^{a}$
VIII	$68.4±2.8^{c}$	$45.3±1.5^{a}$	$85.7±2.8^{b}$	$22.7±2.1^{a}$	$17.5±2.5^{a}$
Fresh semen	$95.4±4.3^{c}$	$57.8±2.5^{b}$	$96.1±1.5^{c}$	$75.8±4.5^{b}$	$70.6±2.8^{c}$

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

DISCUSSION

Fish sperm cryopreservation is an important technique that can give ideas for storage of all other cell types. Although effective protocols have been established for many freshwater fish but there is no standardization of procedures. Several extenders and cryoprotectants have been used for cyprinins species especially on common carp. Generally simple sugars as glucose were used for cryopreservation but results were changeable. Many of extenders have been composed of the different sugars (Table IV.)

Fish species	Method	Cryoprotectant	Extender	Post thav motility (%)	References
Cyprinus carpio	Straw- LN vapor	10% DMSO-Methanol	MK/300 mM sucrose/ 350 mM fructose / 350 mM glucose	Meth - DMSO 53 23 51 29 63 21 19 24	Horvath 2003 (49)
Cyprinus carpio	1.2 and 5 ml Straw-LN vapor	10% Methanol	350 mM glucose	1.2 ml - 69* 5 ml - 39*	Horvath 2007 (50)

Salmo salar	Straw- LN vapor	12.5% DMSO	250 mM glucose	19.6	Mounib, S. 1978 (48)
Osteochiius hasseltii	Straw- LN vapor	15% DMSO	5% Honey	87	Sunarma et al. 2007
Oncorhynchu s mykiss	Straw- LN vapor	7% DMSO	0.5% glucose	19.6	Lahnsteiner, F. 2000
A. fulvescens	Pellet -dry ice	10% DMSO	600 mM sucrose	19	Ciereszko et al. 1996
Coregonus sp.	Pellet- dry ice	20% Glycerol	300 mM glucose	86	Piironen 1994
Aspius aspius	Pellet-dry ice	10% DMSO	600 mM sucros / 300 mM glucose /300 mM sucrose	62 59 49	Babiak et al. 1998
Oncorhynchu s mykiss	Straw- LN vapor	15% DMSO	300 mM glucose	49.3	Tekin et al. 2007
E. masquinongy	Straw- LN vapor	15% DMSO	450 mM sucrose	38-40	Glogowski et al. 1999
Perca flavescens	Straw- LN vapor	15% DMSO	450 mM sucrose	70-77	Glogowski et al. 1999
Salmo salar	Straw- LN vapor	10% DMSO	300 mM glucose	65-91	Stoss & Refstie 1983
Salmo salar	Straw- LN vapor	10% Methanol	glucose extender	53-84	Jodun et al. 2006
Acipenser ruthenus	Straw- LN vapor	10% DMSO	0.5% sucrose	78.8	Lahnsteiner, F. 2004 (47)
Salvelinus alpinus	Straw- LN vapor	10% Methanol	300 mM glucose	65	Mansour et al. 2008

Table IV. Protocols which include honey and sugar based extender that used for cryopreservation of fish sperm. (*Hatching tares)

The process of semen cryopreservation which contains different phases such as temperature reduction, cellular dehydration, freezing, and thawing could produce cellular damages at different degrees (25). After release into the spawning environment, spermatozoa motility was limited with the mitecondrial ATP reserves of energy. Sugars have several functions in the extender. They provide energy substrate for sperm cells during incubation, maintain the osmotic pressure of the diluent and act as a cryoprotectant. Cryoprotective effect of sugars also may differ according to storage temperature, molecular weight of the sugar and the type of buffer (47). In viviparous fishes, spermatozoa can convert exogenous sugars to lactic acid and use the energy to prolong their motility (11).

In evaluating the efficacy of cryopreservation process, post-thaw motility of sperm is very important for fertilizing ability. Even if cryopreservation procedure results high fertilization rate, it is not reliable indicator of success of hatching or embryo survival rates. Likewise mortality of embryos reported insemination of eggs by other researchers (15, 33).

There have been scientific efforts on preventing cryoinjuries owing to developing extenders containing ionic contents at the value of osmolality and pH similar to investigated fish seminal plasma and generally supported by sugar (e.g.

glucose) and protein (e.g. albumin) (22). Some supplements that produced by honey bees such as propolis and honey have shown cryoprotective effect (29, 36). Fructose, glucose and K^+, Na^+, Ca^+ and Mg^{++} substances are also found prominent in pine honey and they were frequently used in cryopreservation procedure. Presence of some sugars is often attributable to successful cryopreservation (44). They could be used for energy supply for spermatozoon as indicated in glucose (19) or make the media isotonic to the seminal plasma as a nonelectrolyte as indicated in mannitol (26).

The pine honey produced in various regions of Muğla-Turkey demonstrated effective antioxidant potential in range of 70-80% at 80 mg/ml concentration, which was comparable to those of of BHA and α-tocoferol used as synthetic antioxidant. It is also reported that pine honey scavenges the free radicals moderately (6).

Succes of cryomedia can change with its components, cryopreservation procedures, sperm quality and fish species. For instance Jodun et al. (2007) found that sperm cryopreserved with glucose extender and 10% methanol supplemented with 13.3% egg yolk yielded significantly higher fertilization rates (83.5%) than did sperm cryopreserved with three other extenders in Atlantic salmon (31). Sucrose was also reported to found suitable extender components in cryopreservation of Rainbow Trout sperm using with DMSO (34). In this study, unattended sugars added to extender did not improve ayeing and hatching rates (p>0.05).

Fish semen has naturally some various antioxidants (ascorbic acid, carnitine, glutathione, methionine, tocopherol, and uric acid) and oxidant defensive enzymes (ascorbic acid, carnitine, glutathione, methionine, tocopherol, and uric acid) (29). Incubation of spermatozoa with the solution containing these antioxidants or enzymes improved the sperm motility and membrane integrity and decreases the sperm lipid peroxidation (20). However during cryopreservation, high dilution rates in the extender reduces the seminal plasma antioxidant concentrations and cells to be more exposed to oxidative stress. Low levels of antioxidants or inhibition of antioxidant enzymes causes oxidative stress and may damage or kill cells (17). In this connection, antioxidative substances in pine honey might integratedly act as an effective antioxidant for fish semen. According to thawing motility rates, sucrose as a disaccharide is not as effective as glucose and fructose as monosaccharides for the preservation of common carp sperma. Similar results found in bovine spermatozoa (10).

The present study shows that supplementation cryomedia with pine honey effects post-thaw motility characteristics, fertilization, eyeing and hatching rates positively. Adding up to 300 mg ml^{-1} pine honey to the extender increased the hatching ratios when compared with the control group. Assuming that in practice the hatching rates are more important than fertilization rates. Currently, there is a lack of established data concerning the scryoprotective use of pine honey. However, further works are needed to better understand the beneficial effects of honey on cryopreservation and also on spermatozoa cells.

According to this data it may be hypothesized that combined use of mono disaccharides, ions ant antioxidant at the proper concentration could provide better protection compared with use of this components alone. Consequently, not only its antioxidative effect but also its other components like organic and inorganic substances, pine honey has considered being usable for fish sperm cryopreservation.

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