

Effects of different concentrations of honeysuckle (*Lonicera iberica* M. Bieb.) and barberry (*Berberis vulgaris* L.) extracts in cryopreserving of duck semen on sperm quality

Auswirkungen unterschiedlicher Konzentrationen von Extrakten aus Geißblatt (*Lonicera iberica* M. Bieb.) und Berberitze (*Berberis vulgaris* L.) bei der Kryokonservierung von Entensperma auf die Spermienqualität

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Abstract

The current study examines the effects of honeysuckle (*Lonicera iberica* M. Bieb.) (Li) and barberry (*Berberis vulgaris* L.) (Bv) extracts in cryopreserved duck semen on sperm quality due to their proposed antioxidant effects. Of both extracts, 0.1% and 0.2% were added to the duck semen extender and the sperm quality was evaluated after thawing. For this purpose, semen was collected from six 1-year-old drakes by abdominal massage (for 4 weeks, twice a week, 48 ejaculates). Semen volume, sperm density, motility, and viability were determined. An extender containing 6% dimethylformamide (DMF) cryoprotectant (sodium lactate 3 g; sodium chloride 6 g; potassium chloride 0.4 g; calcium chloride dihydrate 0.3 g; dextrose monohydrate (D-glucose) 50 g) and Li and Bv extracts were used. After pooling, collected duck semen samples were randomly divided into 5 equal parts in amount. Then, using an extender at a 1:3 ratio, control (without Li- or Bv-additive), 0.1% Li, 0.2% Li, 0.1% Bv, and 0.2% Bv groups were formed. Semen samples divided into groups were subjected to freezing procedure and preserved in liquid nitrogen. Then, the sperm quality parameters of the groups were assessed after the semen samples were thawed at 37°C. Our findings revealed that the use of plant extracts in different concentrations in the cryopreservation of duck semen can positively affect sperm quality after thawing. Consequently, the extender with 0.1% Bv extract proved to be potentially useful for the long-term preservation of duck semen.

Key words

duck; avian sperm; sperm freezing; sperm thawing; antioxidant

Zusammenfassung

In der vorliegenden Studie wurde der Effekt von Geißblatt- (*Lonicera iberica* M. Bieb.) (Li) und Berberitzen- (*Berberis vulgaris* L.) (Bv) Extrakten in der Kryokonservierung von Entensperma auf die Spermienqualität hinsichtlich ihrer antioxidativen Wirkung untersucht. Von beiden Extrakten wurden 0,1% bzw. 0,2% dem Entenspermaverdünner zugesetzt, und die Spermienqualität wurde nach dem Auftauen bewertet. Zu diesem Zweck wurde das Sperma von sechs einjährigen Erpeln durch Bauchmassage gewonnen (4 Wochen lang, zweimal pro Woche, 48 Ejakulate). Spermavolumen, Spermiedichte, Motilität und Lebensfähigkeit wurden bestimmt. Es wurden ein Verdünner mit 6% Dimethylformamid (DMF) als Kälteschutzmittel (Natriumlactat 3 g; Natriumchlorid 6 g; Kaliumchlorid 0,4 g; Kalziumchlorid-Dihydrat 0,3 g; Dextrose-Monohydrat (D-Glucose) 50 g) sowie Li und Bv-Extrakte verwendet. Nach dem Pooling wurden die gesammelten Entensperma-Proben nach dem Zufallsprinzip in 5 gleiche Teile aufgeteilt. Dann wurden unter Verwendung eines Verdünners im Verhältnis 1:3 fünf Gruppen gebildet: Kontrolle (ohne Li- oder Bv-Zusatz), 0,1% Li, 0,2% Li, 0,1% Bv und 0,2% Bv. Die in die Gruppen eingeteilten Spermaproben wurden eingefroren und in flüssigem Stickstoff aufbewahrt. Anschließend wurden die Spermienqualitätsparameter der Gruppen nach dem Auftauen der Spermaproben bei 37°C bewertet. Unsere Ergebnisse zeigten, dass die Verwendung von Pflanzenextrakten in unterschiedlichen Konzentrationen bei der Kryokonservierung von Entensperma die Spermienqualität nach dem Auftauen positiv beeinflussen kann. Letztendlich erwies sich ein Verdünner mit 0,1% Bv-Extrakt für die langfristige Konservierung von Entensperma als besonders geeignet.

Stichworte

Ente; Vogelsperma; Einfrieren von Sperma; Auftauen von Sperma; Antioxidationsmittel

List of Abbreviations

BI:	0.1% <i>Berberis vulgaris</i> L. extract
BII:	0.2% <i>Berberis vulgaris</i> L. extract
Bv:	<i>Berberis vulgaris</i> L.
C:	control group without plant extracts
D-glucose:	dextrose monohydrate
DMF:	dimethylformamide
DPPH:	2,2-diphenyl-1-picrylhydrazil
DTNB:	5,5'-dithiobis-(2-nitrobenzoic acid)
FS:	physiological saline
GAE:	gallic acid equivalent
GSH:	glutathione
IC ₅₀ :	half maximum inhibitory concentration
Li:	<i>Lonicera iberica</i> M. Bieb.
LI:	0.1% <i>Lonicera iberica</i> M. Bieb. extract
LII:	0.2% <i>Lonicera iberica</i> M. Bieb. Extract
MDA:	malondialdehyde
ME:	metabolisable energy
MJ/kg:	megajoule per kilogram
nm:	nanometre
ppm:	parts per million
QE:	quercetin equivalents
ROS:	reactive oxygen species
SM:	sperm motility
SV:	sperm viability
TBA:	thiobarbituric acid

TCA: trichloroacetic acid
 µg/ml: microgram per millilitre

Introduction

Semen cryopreservation is a practical and critical method for the long-term preservation of genetic material. Factors such as the recent growth in breeding activities, global warming, environmental pollution, epidemics, and unconscious hunting have raised awareness of the necessity of preserving genetic material and spurred interest in semen cryopreservation. The primary goal of the semen cryopreservation is to preserve the genetic material for an extended period without deterioration. In this procedure, the optimal atmosphere is liquid nitrogen at -196°C . In this atmosphere, the amount of time that spermatozoa spend in preservation is no longer important (TARVIS, 2013).

The body structures of poultry spermatozoa differ from those of mammals. Their membrane surfaces are wider, their cytoplasm is less, and their flagellum is longer (SANTIAGO-MORENO et al., 2016). Their nuclei are very dense, and the only cytoplasmic organelles they have are mitochondria. Cryopreservation of poultry spermatozoa is difficult since they have these structural features, as well as limited energy reserves and low resistance to crystallization (BLESBOIS et al., 2005). Cryopreservation also differs between species compared to structural similarities. Therefore, the cryoprotectants should diffuse rapidly across the membrane, heat transfer during heat treatment should be slow and the density difference and phase change during freezing/thawing should be minimal (VARADI et al., 2013).

Ducks with webbed feet that belong to the subfamily *Anatinae* and live in wetlands, play a major role among the poultry, which account for a significant proportion of the biodiversity of the world. After the *Anas platyrhynchos* ducks get used to the environment, the sperm is easily collected through the abdomen massage method. During the semen collection process, it is necessary to pay attention to avoid contamination such as by lymphatic fluid, blood and faeces.

Since the frequency of semen collection influences the quality of the semen, studies recommend limiting the frequency of semen collection to twice a week (GHONIM et al., 2009). The intracellular energy reserves of duck spermatozoa are low and they are not able to endure the *in vitro* environment for a prolonged period of time; their needs must be met externally. Specially prepared extenders satisfy the energy and metabolic needs of spermatozoa in *in vitro* setting. Also, during the cryopreservation process, certain ratios of cryoprotectants are mixed with such extenders to avoid crystallization (SEXTON, 1974). For this reason, the extenders must be able to provide the energy and metabolites required by spermatozoa in general. Cryoprotectants, on the other hand, should have a non-toxic and low-molecular structure.

The cell membranes of avian spermatozoa contain high amounts of polyunsaturated fatty acids (CEROLINI et al., 2006). This structure, which maintains the integrity of the cell, is destroyed when it reacts with the reactive oxygen species (ROS) in the setting (SHAHVERDI et al., 2015). This leads to the destruction of the cell membrane and disruption of cell integrity. This destructive effect of ROS on spermatozoa occurs both during and after cryopreservation. Consequently, the ability to fertilize is weakened (MASOUDI et al., 2016). Spermatozoa can neutralize the effects of ROS through their systems. However, this system is sometimes insufficient. One of these circumstances is the cryopreservation process. To protect spermatozoa from the effect of ROS, antioxidants must be added along with semen extenders throughout this process. This is because antioxidants protect spermatozoa from such effects both *in vivo* and *in vitro*. This feature has led to a rise in the use of antioxidants in long-term preservation technologies of genetic materials such as semen cryopreservation (THANANURAK et al., 2020; MELIANI et al., 2011).

Antioxidants are divided into two groups: natural and synthetic. However, because of undesirable side effects, the use of synthetic antioxidants, which have a broad area of usage, has been limited (GUNDOGDU, 2013). This has increased the need for natural antioxidants. For this purpose, some plants are increasingly used due to their natural antioxidant potential.

Lonicera iberica M. B. and *Berberis vulgaris* L. are perennial forestry plants. Studies on these species reported that the antioxidant capacity of extracts obtained from their fruits was quite high (GUNDOGDU, 2013; JURIKOVA et al., 2012). The antioxidants obtained from these plants are gallic acid, p-coumaric acid, caffeic acid, chlorogenic acid, cinnamic acid, rutin, apigenin and quercetin (GHOLIZADEH-MOGHADAM et al., 2019).

In the current study, the loss of spermatozoa during the cryopreservation process should be reduced by using extracts from the fruits of the plants *Lonicera iberica* M. Bieb. (Li) and *Berberis vulgaris* L. (Bv) due to their high antioxidant

properties. In this context, 0.1% and 0.2% Li and Bv fruit extracts were added to the semen extender to examine their effects on duck sperm quality during cryopreservation, and afterwards.

Material and Methods

Birds, experimental design, and management

This study was conducted in the Animal Physiology and Histology Laboratory of the Department of Animal Science, Faculty of Agriculture, Kirsehir Ahi Evran University in accordance with the approval of the Animal Experiments Local Ethics Committee of Kirsehir Ahi Evran University (Date: 31/03/2021 and decision no 06/1). Six 24/32-week-old male *Anas platyrhynchos* ducks were used as animal material in this study. Ducks were assessed as a single group for sperm collection, and study groups were formed under laboratory conditions.

During the study period, ducks were kept in an unheated pen (150 cm height × 250 cm width × 300 cm length) with sawdust as the bedding material, with gravity ventilation and natural photoperiod. The ambient temperature and humidity in the pen were measured with a data logger (Onset Inc.) as $20.11^{\circ}\text{C} \pm 1.45$ and $62.34 \pm 1.34\%$. Ducks were fed *ad libitum* (free access) on a diet based on corn and soybean meal, containing 18% crude protein and metabolisable energy (ME) 10.878 MJ/kg.

Preparation of the plant extracts

Dried plants obtained from a local licensed herbal product vendor were first washed with tap water and then with distilled water to eliminate physical impurities. The moisture in the dried plants was evaporated at room temperature and the plants were kept at $+4^{\circ}\text{C}$. Plant extracts were prepared using methanol as a solvent. After grinding separately in the mill, 15 g each of dried Li- and Bv-sample were weighed and placed in one liter covered erlenmeyer flask. 300 ml of methanol was added, mixed in a magnetic stirrer and the resulting methanol extract was filtered. Methanol was removed from the filtered extracts using an evaporator set at 45°C . The total amount of phenolic and flavonoid substances as well as the radical scavenging activity were determined by preparing a 1000 ppm solution. The antioxidant activity of the plant extracts was determined by the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging activity method. Spectrophotometric measurements (517 nm) were performed at different concentrations in the range of 20–100 g/ml. Based on the absorbance values obtained, the percentage DPPH removal activities were determined, and the half maximum inhibitory concentration (IC_{50}) value was calculated. The antioxidant activity and content of plant extracts were examined. Total phenols were calculated as milligrams of gallic acid equivalent (GAE). It was determined to be 19.87 ± 0.99 mg GAE/g in the Li-extract and 91.87 ± 3.87 mg GAE/g in the Bv-extract. The total flavonoid content was expressed in mg quercetin equivalents (QE). The amounts of flavonoid substances were calculated to be 35.52 ± 4.03 mg QE/g in Li and 77.63 ± 1.53 mg QE/g in Bv. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) removal activities of the plant extracts were determined to be 24.37% in Li and 55.62% in Bv at 100 mg/ml. Additionally, IC_{50} value of the Li-extract was determined to be 183.32 $\mu\text{g}/\text{ml}$ and the Bv-extract to be 89.02 $\mu\text{g}/\text{ml}$. The extracted samples were stored at $+4^{\circ}\text{C}$ until used in the study (ERGUN, 2021a; ERGUN, 2021b).

Semen collection

All drakes were accustomed to the experimental semen collection one week before semen collection. The experimental group consisted only of males and no other restrictions such as sexual abstinence were imposed on them. Semen was collected from ducks by the dorso-abdominal massage method. This method was used to avoid the faecal contamination to the semen before the ducks were fed in the morning (7–8 a.m.). Semen was collected from each bird twice a week for four consecutive weeks by the same collector. A total of forty-eight semen samples were collected from six ducks.

The volume of each ejaculate was recorded using a pipette with an accuracy of 0.01 ml. pH values were determined instantly using a pH paper (Universal indicator pH 0–14 Merck, Merck KgaA, 64271 Darmstadt, Germany) (TASKIN et al., 2020a). Semen samples were carefully transferred to the laboratory in a water bath (37°C) without being exposed to any heat shocks and sunlight. In the laboratory the liquefied semen was pooled. Pooled semen samples were stored at $+37.0^{\circ}\text{C}$ until dilution after determination of spermatological characteristics.

Preparation of sperm extender and division into groups

DMF (6%, Sigma-Aldrich, St. Louis, MO, USA) was used as a cryoprotectant in the current study. The semen extender was prepared in the laboratory using the appropriate sterilization procedure. Its chemical components are given in Table 1. The semen extender was stored at 4°C until use. The pooled semen was filled into closed tubes in five equal amounts and the prepared semen extender was added in a ratio of 1:3. Throughout this process, attention was taken to ensure that the extenders and semen were at the same temperature (+37.0°C) and that the extender was added slowly to the semen. The study was divided into 5 groups. The LI, LII, BI, and BII groups were formed by adding 0.1% Li, 0.2% Li, 0.1% Bv, and 0.2% Bv plant extracts, respectively, to the extender (see Table 1), to which 6% DMF was added. No plant extracts were added to the control group.

Table 1. Chemical composition of the semen extender

Chemische Zusammensetzung des Spermaverdünners

Chemical composition	Amount
Distilled water, ml	1000
Sodium lactate, g	3
Sodium chloride, g	6
Potassium chloride, g	0.4
Calcium chloride, g	0.3
Glucose (dextrose monohydrate), g	50

Preparation of straws and the freeze-thaw process

Semen straws with a volume of 0.25 ml were used. The semen was filled into 0.25 ml straws per group, then equilibrated at +5°C for 90 min and then subjected to the freeze-thaw procedure (Figure 1).

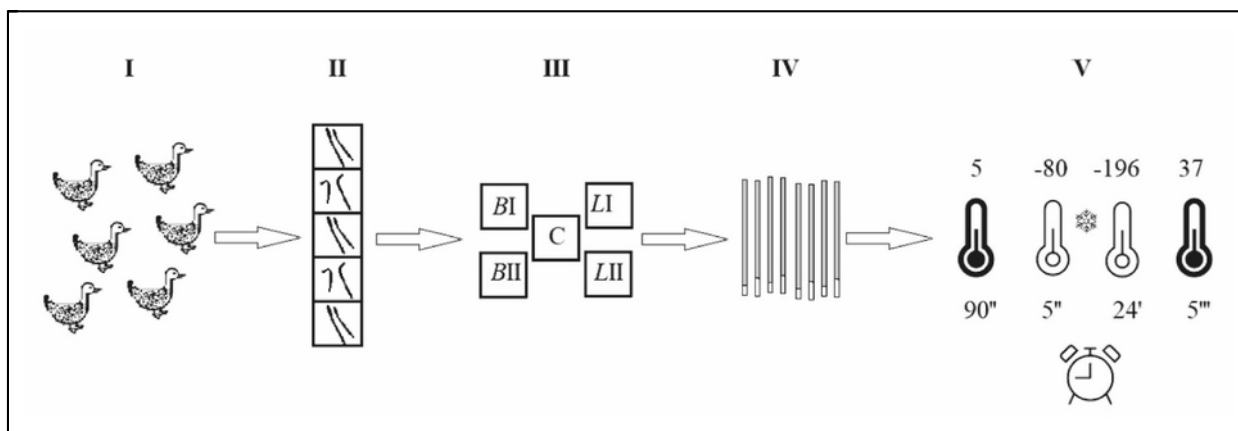


Figure 1. Workflow of semen sample assessment (I. collecting ejaculate from ducks in the early morning hours, II. pooled semen, III. extending and creating groups, IV. straws (containing extended duck semen), V. order of the process: equilibration (5°C, 90 min), freezing of the semen (5 cm above the surface of liquid nitrogen (LN) and in a horizontal position. 5 min in LN vapour at -80°C), preservation of the semen (24 hr in LN at -196°C) and thawing of the semen (5 sec. in a water bath at 37°C). C: control group that contains no plant extracts; LI: 0.1% *Lonicera iberica* M. Bieb. extract; LII: 0.2% *Lonicera iberica* M. Bieb. extract; BI: 0.1% *Berberis vulgaris* L. extract; BII: 0.2% *Berberis vulgaris* L. extract.

Arbeitsablauf bei der Beurteilung von Spermaproben (I. Sammeln des Ejakulats von Enten in den frühen Morgenstunden, II. gepooltes Sperma, III. Verdünnung und Bildung von Gruppen, IV. Sammelröhrchen (mit verdünntem Entensperma), V. Reihenfolge des Verfahrens: Äquilibriumierung (5°C, 90 min), Einfrieren des Spermas (5 cm über der Oberfläche von flüssigem Stickstoff (LN) und in horizontaler Position. 5 Min. in LN-Dampf bei -80°C), Konservierung des Spermas (24 Std. in LN bei -196°C) und Auftauen des Spermas (5 Sek. in einem Wasserbad bei 37°C). C: Kontrollgruppe, die keine Pflanzenextrakte enthält; LI: 0,1% *Lonicera iberica* M. Bieb. Extrakt; LII: 0,2% *Lonicera iberica* M. Bieb. Extrakt; BI: 0,1% *Berberis vulgaris* L. Extrakt; BII: 0,2% *Berberis vulgaris* L. Extrakt.

Data collection and analyses

Sperm concentration refers to the total number of active spermatozoa per unit volume. Determination of sperm concentration is significant in deciding the rate at which the semen is extended and how many insemination doses can be produced from a single ejaculate.

The determination of sperm concentration is significant to determine the final extension rate (dilution), which determines how many insemination doses can be produced from a single ejaculate. In this study, sperm concentration was determined using the hemocytometric method (TASKIN et al., 2020a).

Motility was assessed subjectively by visual observation using a microscope (Leica DM 750, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) with phase contrast optics and a heating plate (type D, Leica Mats). It was expressed as the percentage of motile spermatozoa with moderate to rapid progressive movement. For this, 5 µl of the semen sample (diluted 1:1 with physiological saline (FS) solution) was placed on a slide heated to +37.5°C and covered with a cover slip at the same temperature. It was placed on a heating plate and examined by two observers in at least three different microscope fields at 400x magnification, with the determined ratio of motile sperm to total sperm recorded as a percentage (TASKIN et al., 2020b). No diluent was added for repeated measurements (TASKIN et al., 2022).

Viability was determined with 2% eosin blue and the addition of 3% sodium citrate. After the eosin stain had been applied and mixed onto a drop of semen on the slide, the smear was pulled, dried and 400 sperm cells were counted at 400x magnification. Sperm viability (SV) was defined as the percentage of alive sperm in a sperm sample. Sperm cells were assessed as dead (stained) or alive (unstained) in relation to the staining condition of the heads (TASKIN et al., 2020b).

Malondialdehyde (MDA) analysis was performed using the spectrophotometric method, which is a lipid peroxidation measurement method. The principle of this method is based on the spectrophotometric measurement of the pink pigment generated by the reaction of thiobarbituric acid (TBA) with MDA in an acidic pH and hot environment (ASKARIANZADEH et al., 2018). For analysis, 250 µl of the samples were placed in screw-capped glass tubes. Then 1.25 ml of a 10% solution of trichloroacetic acid (TCA) was added and mixed, and then 0.5 ml of 0.67% TBA was added. The blank tube was filled with 1.5-ml TCA and 0.5-ml TBA. The openings of the tubes were tightly closed, placed in a boiling water bath for 30 min, cooled under a tap and then centrifuged at 3000 rpm for 10 min with the addition of 2 ml of n-butanol. A standard curve was constructed by reading the upper phase at 535 nm and using 1,1,3,3-tetraethoxypropane, and the amount of MDA was calculated using the curve equation (BADR et al., 2021).

In the glutathione (GSH) assay method, reducing glutathione reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form a pale yellow colour that can be measured at 412 nm, and the amount of GSH in the tissue can be determined from the GSH standard calibration curve. A 0.25 ml homogenate was mixed with 0.75 ml Tris buffer (0.2 M Tris, 0.02 M EDTA, pH: 8.2), 0.05 ml DTNB (0.01 M) and 3.95 ml methanol and incubated for 30 min, at room temperature. Subsequently, the proteins were removed by centrifugation at 3000 rpm for 15 minutes and the absorbance values at 412 nm were read against the blank tube. The standard plot was created with GSH (BADR et al., 2021).

Statistical analyses

The data obtained were evaluated using the statistics program SPSS. First, the normal distribution of the values was tested with the Kolmogorov-Smirnov test, and then the differences between the groups were determined with a one-way analysis of variance (ANOVA). All results are expressed as mean ± SD. If the difference was significant, a multiple comparison test was performed to determine which application(s) caused the difference (DUGGAN et al., 2017). The significance of the differences between the groups was assessed with the Duncan test at $P < 0.05$.

Results

The quantitative and qualitative characteristics of the semen samples obtained by the dorsal abdominal massage method from ducks are summarised in Table 2. Thus, the average ejaculate volume was 0.68 ± 0.50 ml, semen pH was 7.50 ± 0.05 and sperm concentration ($\times 10^9$) was 3.52 ± 0.24 ml.

Table 2. Characteristics of fresh pooled sperm

Merkmale von gepooltem Frischsperma

Spermatological characteristics	Mean ± SD
Ejaculate volume (ml)	0.68 ± 0.50
pH	7.50 ± 0.05
Concentration (x10 ⁹ /ml)	3.52 ± 0.24
Viability (%)	75.88 ± 7.07
Motility (%)	69.00 ± 5.35

Mean: arithmetic mean; SD: standard deviation

Sperm viability (SV) was determined in all three stages of the study (Figure 2). These stages corresponded to the state of the sperm as pooled, equilibrated and post-thawing. The SV of the pooled semen was determined to be 75.88% ± 7.07%. There was a difference between the SV of the groups (P < 0.05). The BI group had higher SV in the equilibration and post-thaw phases than the control group and the other groups. SV was lowest at 64.00 ± 1.73% (control) and highest at 72.00 ± 6.55% (BI) when equilibrated at +5°C for 90 min. The difference between the groups in terms of post-thawing sperm viability was significant (P < 0.05). Sperm viability of the groups was determined according to in order high to low: 42.00 ± 2.16% in BI, 33.00 ± 6.37% in BII, 27.75 ± 4.27% in LI, 24.50 ± 4.12% in LII and 23.75 ± 7.54% in the control group.

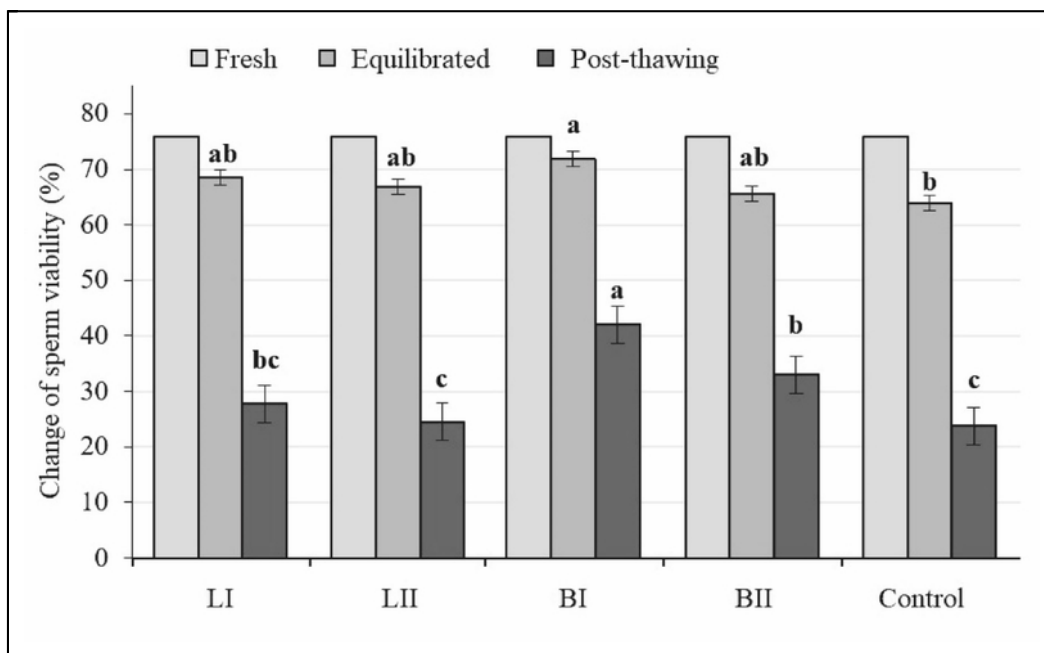


Figure 2. Sperm viability change (fresh pooled semen, equilibrated semen, post-thawing semen). The LI, LII, BI and BII groups were formed by adding 0.1% Li, 0.2% Li, 0.1% Bv and 0.2% Bv plant extracts, respectively, to the extender. No plant extract was added to the control group. Li: *Lonicera iberica* M. Bieb.; Bv: *Berberis vulgaris* L. The differences between the means, indicated by different letters, are significant at the P < 0.05 level.

Veränderung der Lebensfähigkeit der Spermien (frisches gepooltes Sperma, äquilibriertes Sperma, Sperma nach dem Auftauen). Die Gruppen LI, LII, BI und BII wurden durch Zugabe von 0,1% Li, 0,2% Li, 0,1% Bv bzw. 0,2% Bv Pflanzenextrakten zum Verdünner gebildet. Der Kontrollgruppe wurde kein Pflanzenextrakt zugesetzt. Li: *Lonicera iberica* M. Bieb.; Bv: *Berberis vulgaris* L. Mittelwerte, die durch unterschiedliche Buchstaben gekennzeichnet sind, unterscheiden sich signifikant (P < 0,05).

In all three stages of our study, sperm motility (SM) was calculated (Figure 3). The motility of pooled semen was 69.00 ± 5.35%. A significant difference was found between the groups in the equilibrated phase (P < 0.05). The sperm motility was 67.20 ± 8.07% in BI, 60.40 ± 4.97% in LI, 58.20 ± 2.77% in LII, 58.00 ± 5.87% in BII and

56.60 ± 3.91% in the control group. Post-thawing sperm motility was significant between the groups (P < 0.05). The highest value was determined as 40.18 ± 2.16% in BI while the lowest value was obtained as 18.75 ± 2.21% in the control group.

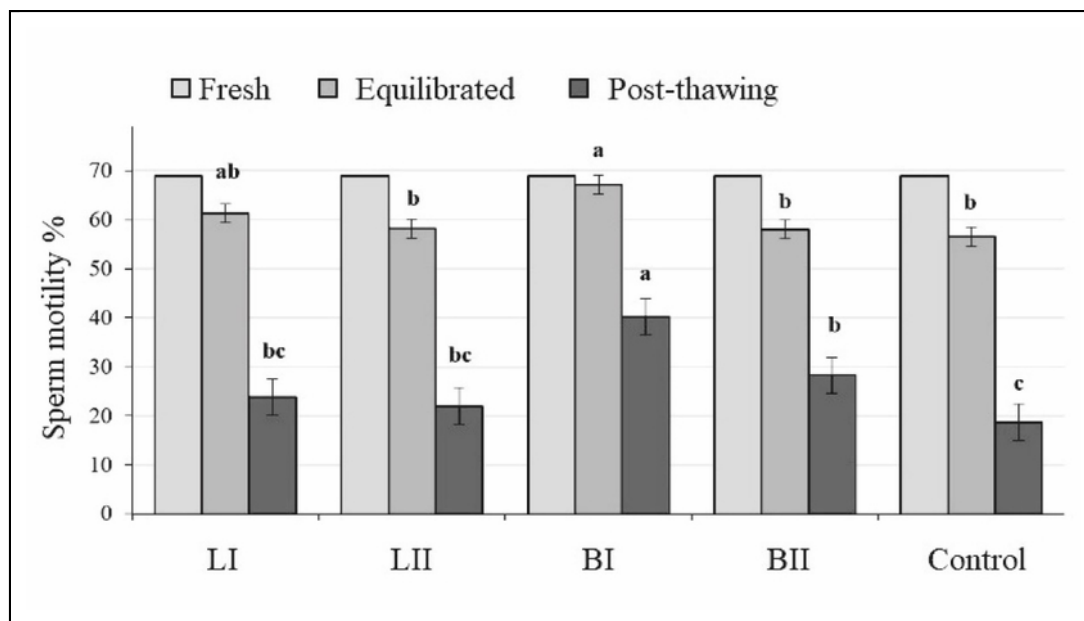


Figure 3. Diagram of sperm motility change (fresh pooled semen, equilibrated semen, post-thawing semen). The LI, LII, BI and BII groups were formed by adding 0.1% Li, 0.2% Li, 0.1% Bv and 0.2% Bv plant extracts, respectively, to the extender. No plant extract was added to the control group. Li: *Lonicera iberica* M. Bieb.; Bv: *Berberis vulgaris* L. The differences between the means, indicated by different letters, are significant at the P < 0.05 level.

Veränderung der Beweglichkeit der Spermien (frisches gepooltes Sperma, äquilibriertes Sperma, Sperma nach dem Auftauen). Die Gruppen LI, LII, BI und BII wurden durch Zugabe von 0,1% Li, 0,2% Li, 0,1% Bv bzw. 0,2% Bv Pflanzenextrakten zum Verdünner gebildet. Der Kontrollgruppe wurde kein Pflanzenextrakt zugesetzt. Li: *Lonicera iberica* M. Bieb.; Bv: *Berberis vulgaris* L. Mittelwerte, die durch unterschiedliche Buchstaben gekennzeichnet sind, unterscheiden sich signifikant (P < 0,05).

Regarding equilibration and post-thaw values, the BI group had higher motility values than the control group and the other groups (P < 0.05).

After thawing, the biochemical analyses of the semen of the groups were analysed in terms of MDA and GSH content and the results are presented in Table 3. It was found that the control group had the highest MDA level (0.73 ± 0.03 nmol/ml), while the BI group had the lowest MDA level (0.05 ± 0.01 nmol/ml) (P < 0.05). Similarly, the BI group had the lowest GSH level at 2.25 ± 0.36 mU/mg (P < 0.05).

Table 3. Malondialdehyde (MDA) and Glutathione (GSH) levels in post-thawing semen samples (mean ± SD)

Malondialdehyd- (MDA) und Glutathion- (GSH) Spiegel in den Sapermaproben nach dem Auftauen (Mittelwert ± SD)

Treatment groups	MDA nmol/ml	GSH mU/mg
LI	0.33 ± 0.04 ^{ab}	1.77 ± 0.12 ^b
LII	0.21 ± 0.02 ^b	2.10 ± 0.03 ^{ab}
BI	0.05 ± 0.01 ^c	2.25 ± 0.36 ^a
BII	0.52 ± 0.02 ^{ab}	2.11 ± 0.07 ^{ab}
Control	0.73 ± 0.03 ^a	1.83 ± 0.20 ^b

Control: no plant extracts were added; LI: 0.1% *Lonicera iberica* M. Bieb. extract; LII: 0.2% *Lonicera iberica* M. Bieb. extract; BI: 0.1% *Berberis vulgaris* L. extract; BII: 0.2% *Berberis vulgaris* L. extract; mean: arithmetic mean; SD: standard deviation. The differences between the means, indicated by different letters, are significant at the P < 0.05 level.

Discussion

One of the major determinants of reproductive performance is semen quality. Therefore, the semen of high quality is required for successful reproduction. In assessing the semen characteristics of ducks, this study focused on ejaculate volume, pH, sperm concentration, sperm viability and motility.

The ejaculate volume was reported to be 0.14 ± 0.09 ml in the study of [ANCUELO et al. \(2021\)](#) on *Anas platyrhynchos* and 0.18 ml in the study of [CYRIAC et al. \(2013\)](#) on native ducks from India. Similarly, [WAGNER and PINGEL \(1995\)](#) found a volume of 0.10–0.70 ml in Peking ducks. In our study, the ejaculate volume was determined to be 0.68 ± 0.50 ml (Table 2). It was higher than the value given by [ANCUELO et al. \(2021\)](#) and [CYRIAC et al. \(2013\)](#), but within the range given by [WAGNER and PINGEL \(1995\)](#). These differences could be related to factors such as frequency of ejaculate collection, age, season and feeding.

The pH of the poultry semen affects motility, i.e. an alkaline environment increases motility. For certain bird species, the technique of semen collection influences the pH value. In the current study, the pH of the pooled semen after collection from ducks was 7.50 ± 0.05 (Table 3). [MOSSA \(2006\)](#) determined a pH value of 6.90 ± 0.44 for ducks, which is similar to the value in the current study. Moreover, in a similar study, the pH values of duck semen were reported to be 6.90 ± 0.44 ml and 7.48 ± 0.06 ml ([ZAWADZKA et al., 2015](#)).

The sperm concentration of the pooled sperm was $3.52 \pm 0.24 \times 10^9$ sperm/ml (Table 3). [KASAI and IZUMO \(2001\)](#) reported a sperm concentration in Osaka ducks of 3.20×10^9 sperm/ml, which is in consistent with the current study. Other studies in ducks have found sperm concentrations of 1.5–8.0 $\times 10^9$ sperm/ml ([ANCUELO et al., 2021](#)), 3.03 $\times 10^9$ sperm/ml ([CYRIAC et al., 2013](#)) and 0.84–1.3 $\times 10^9$ sperm/ml ([NAHAK et al., 2015](#)), respectively. The collection method, the frequency of collection, the duck species – all these factors can influence sperm concentration. For example, one study reported that semen collected from ducks using the electroejaculation method was denser than semen collected using the back-abdominal massage method ([KASAI and IZUMO, 2001](#)).

Pooled semen had a sperm motility of $69.00 \pm 5.35\%$ and sperm viability of $75.88 \pm 7.07\%$ (Table 3). In a study on ducks ([KONTECKA, 1992](#)) these values were given as 67.90% and 72.85% respectively. In another study, the motility value was reported as 61.1% ([KASAI and IZUMO, 2001](#)). Contaminants such as lymphatic fluid, blood and faeces that flow before or after the semen during collection have a negative effect on motility and viability. In addition, the method of semen collection has been reported to influence these values, and one study found that the artificial vagina method, which is based on natural mating behaviour, positively influences sperm motility and viability ([KASAI and IZUMO, 2001](#)). In the current study, sperm viability was determined not only in pooled sperm, but also in the equilibrated state (at the end of 90 min. at $+5^\circ\text{C}$) and post-thawing (Figure 1).

The MDA levels of the groups were determined to be 0.73 ± 0.03 nmol/ml in the control group and in the groups BII 0.52 ± 0.02 , LI 0.33 ± 0.04 , LII 0.21 ± 0.02 and BI 0.05 ± 0.01 nmol/ml, respectively. The differences between the groups were statistically significant ($P < 0.05$). A similar study on ducks reported that the MDA value of post-thawing semen was 1.34 nmol/mg in the control group ([FOUDA et al., 2021](#)). Similarly, in the current study, only the control group had high MDA levels. In the current study the GSH levels of the groups were statistically significantly different ($P < 0.05$). The highest value was determined as 2.25 ± 0.36 nmol/ml in BI while the lowest value was 1.77 ± 0.12 nmol/ml in LI. This could be due to the fact that the natural plant extracts with antioxidant structures used in this study reduce the effect of stress factors that lead to spermatozoa mortality during the cryopreservation process. Furthermore, sperm motility and viability in the plant extract groups can be associated with low MDA and high GSH levels of the semen samples. In this study, the sperm motility ($40.18 \pm 2.16\%$) and viability ($42.00 \pm 2.16\%$) values of the BI group after thawing could be due to the high GSH and low MDA levels. Also, GSH-enriched bird semen extenders are known to maintain sperm viability, plasma membrane integrity, functional, kinetic, and fertility parameters, as well as prevent oxidative stress in fresh and post-thawing semen ([ANSARI et al., 2021](#); [IZANLOO et al., 2021a](#); [IZANLOO et al., 2021b](#); [PARTYKA and NIŻAŃSKI, 2021](#)).

Conclusion

The conclusion from this study is that sperm motility and viability were higher in the groups where *Lonicera iberica* M. Bieb. and *Berberis vulgaris* L. extracts were added to the sperm extender during duck semen cryopreservation than in the control group. In the BI group with 0.1% *Berberis vulgaris* L. extract the highest motility and viability of sperm ($P < 0.005$) was determined. Consequently, it was found that the addition of 0.1% *Berberis vulgaris* L. extract to semen extenders containing 6% DMF in the cryopreservation of duck sperm is beneficial and can be used for long-term preservation of duck sperm. However, further studies are needed to reduce the likely negative effects of long-term freezing of duck semen.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

Atilla Taskin planned the study, wrote the manuscript, revised the manuscript and contributed to the semen analysis, laboratory work and data analysis. Demirel Ergun performed the semen analysis, statistical analysis and laboratory work and contributed to the study design and revision of the manuscript. Fatma Ergun performed the laboratory work and contributed to the revision of the manuscript. All authors approved the final version of this article.

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