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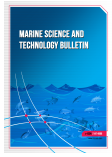

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RESEARCH ARTICLE

# The comparative effect of hesperidin and date palm pollen added to rainbow trout (*Oncorhynchus mykiss*) sperm diluent on sperm membrane integrity and fertilization

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## ABSTRACT

Hesperidin is an important flavonoid compound found at high levels in citrus fruits. In addition to its protective effects on vital organs, numerous studies have highlighted its beneficial impact on the reproductive system. Date palm pollen (DPP) is the male reproductive cell of the date palm flower, known for its successful results in the treatment of infertility. The present study aimed to comparatively investigate the effects of hesperidin and date pollen on fertilization and sperm membrane integrity in rainbow trout sperm. Four male rainbow trout were used in the study. Sperm was collected from the trout by abdominal massage method and divided into 5 equal groups: 0.024 DPP, 0.5 DPP, 0.009 hesperidin, normal diluent and native sperm. Each group was diluted with the previously prepared diluent. Sperm was collected from female fish by the abdominal massage method. Egg fluid was obtained and diluted sperm was added. They were left to incubate in the incubator, and the total number of fertilized eggs was determined after 28-30 days. Smear samples were taken from the groups with eosin-nigrosin staining and evaluated for membrane integrity. As a result, fertilization was observed only in the groups reconstituted with normal diluent and hesperidin-added groups. When sperm membrane integrity was evaluated, it was found that the highest membrane damage was observed in the two groups to which date pollen was added. Date palm pollen-added diluent caused an increase in sperm membrane damage in rainbow trout sperm. The addition of hesperidin to the rainbow trout sperm diluent caused an increase in fertilization potential and a positive effect on the preservation of seminal membrane integrity.

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## Introduction

Rainbow trout (*Oncorhynchus mykiss*) is one of the important fish species due to its high breeding potential, economic value, and high demand by consumers (Aksu et al., 2018). This commercial value makes rainbow trout important in aquaculture, and studies on the reproductive system were carried out to investigate and improve sperm quality (Bozkurt et al., 2005; Aral et al., 2007; Canyurt & Akhan, 2008; Pourkazemi et al., 2013; Aguilar-Juárez et al., 2014; Ubilla et al., 2015; Risopatrón et al., 2018).

Flavonoids are one of the groups of phenolic compounds found in plants (Parhiz et al., 2015). Due to the many effects of flavonoids, such as antioxidant, anti-inflammatory, and anticarcinogenic, they are finding use in the pharmaceutical industry. Hesperidin is a flavonoid compound found in high concentrations in citrus fruits such as oranges, lemons, and grapefruits (Aksu et al., 2021a). Hesperidin was first isolated from citrus peel by Lebreton, a French chemist (Parhiz et al., 2015). Hesperidin can affect many tissues and organs in the body thanks to its antioxidant, anti-apoptotic, anti-inflammatory, anti-allergic, anticarcinogenic, and anti-edematous effects (Aksu et al., 2021a). In previous studies, hesperidin has been shown to be protective against toxicity to vital organs in the body, especially the kidneys, liver, and heart, as a result of various chemicals (Caglayan et al., 2019; Turk et al., 2019).

Positive outcomes have been reported in many studies investigating the effects of hesperidin on the reproductive system. For instance, Tekin & Çelebi (2022) examined the protective role of hesperidin against Bisphenol A (BPA)-induced testicular toxicity in rats. Their findings demonstrated that hesperidin supplementation significantly reduced oxidative stress, testicular inflammation, and apoptosis caused by BPA (Tekin & Çelebi, 2022). Similarly, Aksu et al. (2021b) evaluated the effects of hesperidin on diabetes mellitus–induced testicular damage and reported that hesperidin supplementation markedly alleviated oxidative stress, prevented DNA damage, and decreased the incidence of abnormal and dead spermatozoa associated with diabetes.

Date palm pollen (DPP: pollen grain of date palm) is the male reproductive cells of date palm flowers (Tahvilzadeh et al., 2016). DPP, which was first discovered by Chinese and Egyptian scientists, contains many compounds such as amino acids, flavonoids, fatty acids, and sterols. DPP was originally used as a rejuvenating agent, but today it is mostly used as a libido enhancer in males and a fertility enhancer in females (Tahvilzadeh et al., 2016; Abdi et al., 2017). Thanks to its estrogen- and estrone-like compounds and sterols, it is claimed that it increases fertility in females and is effective in the treatment of infertility in males (Moshfegh et al., 2015). The palmitic acid and stearic acid found in DPP have the ability to inhibit the 5-alpha reductase enzyme. As a result of inhibition, the conversion of testosterone to dihydrotestosterone is reduced, and thus blood testosterone concentration is increased (Alvarez et al., 1987). Previous studies have shown that DPP increases motility, viability, and acrosome reaction in spermatozoa (Fallahi et al., 2015). Studies in rats and rabbits have consistently shown that DPP-treated groups increase sperm density and overall sperm quality (Bahmanpour et al., 2006; Faleh & Sawad, 2006; Mehraban et al., 2014).

The present study aimed to determine the changes in sperm membrane integrity and fertilization ability in rainbow trout sperm treated with different doses of DPP and hesperidin.

## Material and Methods

### *Sperm Collection*

In this study, four 3-year-old male rainbow trout (*O. mykiss*) raised at Atatürk University Faculty of Fisheries were used. Before the spawning period, female fish and male fish were kept in separate ponds, and male fish were deprived of feed for 48 h before sperm collection. Approval for the study was obtained from Atatürk University Animal Experiments Local Ethics Committee (Protocol no: 2025/02–38). The abdomens of the male fish were dried, and then sperm was collected by abdominal massage. Sperm was preserved by preventing contamination with blood, urine, feces, water, or mucus.

### *Evaluation of Motility and Formation of Experimental Groups*

To evaluate the motility of spermatozoa, 10 µl of a sperm sample was taken and diluted with 100 µl of 0.3% NaCl. The diluted sperm was placed on a slide, and at least three fields were examined at 400× magnification using a microscope (Primo Star; Carl Zeiss). Motile spermatozoa were expressed as a percentage. Ejaculates with sperm motility of 50% and above were pooled in a collection container. The combined sperm was divided into 5 equal parts. The study consisted of 5 groups: 0.024 DPP, 0.5 DPP, 0.009 hesperidin, a normal diluent group, and a native sperm group. The first group was diluted with 0.024 mg, and the second group with 0.5 mg DPP added

diluent. In the third group, 0.009 mg of hesperidin-supplemented diluent was used for the dilution of sperm. The fourth group was diluted with diluent (6.52 mg/ml NaCl, 0.8 mg/ml KCl, 2 mg/ml $NaHCO_3$, 2 mg/ml glucose, 4 mg/ml bovine serum albumin, 7.5% egg yolk, and 10% DMSO) (Kutluyer et al., 2014). The fifth group was used as native, without dilution. Sperm samples were kept at room temperature (24°C) and spermatological analyses were performed.

### *Collection of Eggs and Fertilization*

The abdominal regions of the female fish were dried, and then the eggs were collected by abdominal massage. Of the collected eggs, those that were round, transparent, unwrinkled, and non-sticky were selected for use in the study.

The ovarian fluid of the eggs is drained into a homogeneous egg pool. The egg fluids were then diluted 1:2 with fertilization solution. Sperm groups were added to diluted egg fluids (0.2–0.3 µl sperm/egg). After fertilization, the eggs were washed in hatchery water and placed in an incubator for incubation. The group-based outcomes were assessed by counting the total number of eggs 28-30 days post-fertilization and calculating the proportion of embryos exhibiting ocular development. Sperm collection and fertilization occurred four times, with a two-day interval between each session.

### *Spermatological Analyses*

In order to measure the plasma membrane integrity ratio, a 20 µl sperm sample was combined with equal amounts of eosin-nigrosin stain (1.67% eosin, 10% nigrosin, and 0.1 M sodium citrate) according to Türk et al (2008). Smears were prepared from the sperm mixture with a coverslip and allowed to dry. Then sperm smears of groups were examined under a light microscope at 400× magnification. The sperm cell membrane damage rate was determined based on the staining of the sperm cell head. A total of 300 sperm cells were analyzed for each sample, and the percentage of sperm cells with damaged membranes was determined.

### *Statistical Analysis*

Statistical analysis of the obtained data was performed with the package program SPSS 26.0 (IBM SPSS Corp., Armonk, NY, USA). Data were analyzed by the Shapiro-Wilk test for normality and Levene's test for homogeneity of variances. Statistical control of the difference between variables was analyzed with One-Way ANOVA and post-hoc Tukey test was used for significant variables. Data are presented as mean ± standard error of the mean (mean ± SEM). $p < 0.05$ was considered significant.

## Results

In the fertilization analysis, 660 eggs were observed in each group. However, fertilization was observed only in the group 3, which was diluted with 0.009 mg of Hesperidin, and in group 4, which was diluted with normal extender. In the third group, 72% fertilized eggs were detected. In the fourth group, 7% fertilized eggs were detected.

When the membrane integrity of spermatozoa was examined, the highest membrane damage was found in the 2nd group, which 0.5 mg DPP was added. The lowest membrane damage rate was observed in the native sperm group (Figure 1). It was also revealed that the least membrane damage was observed in the group to which 0.009 mg of hesperidin was added after the native sperm (Table 1).

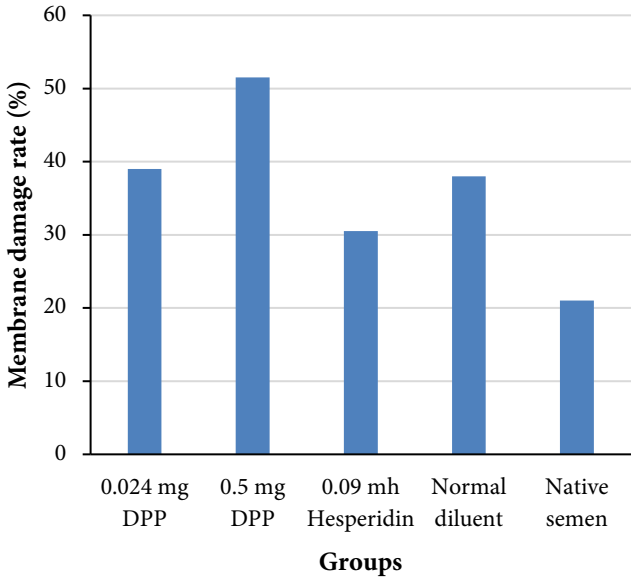


**Figure 1.** Membrane damage rate

**Table 1.** Reproductive parameters

| Group | Motility (%) | Membrane Damage Rate (%) |
|---|---|---|
| 0.024 mg DPP | 46.25±2.28 | 39.00±2.85$^{c}$ |
| 0.5 mg DPP | 46.75±2.78 | 51.50±1.44$^{d}$ |
| 0.009 mg Hesperidin | 47.50±1.84 | 30.50±2.10$^{b}$ |
| Normal diluent | 46.00±2.79 | 38.00±1.87$^{c}$ |
| Native semen | 45.25±1.88 | 21.00±1.58$^{a}$ |

***Note:*** Superscript letters (a, b, c, d) indicate the difference between groups. $p < 0.05$. The motility values in the table represent the results evaluated after reconstitution of the respective group.

## Discussion

In fish culture, obtaining offspring through artificial insemination has many advantages, such as reducing the risk of spreading infections, obtaining hybrid offspring, and creating an exemplar by selecting for desired characters (Chambeyron & Zohar, 1990). Fish release their gametes into the water during reproduction, and fertilization takes place in the water (Fauvel et al., 2010). Spermatozoa, which are immobile in the testicles, acquire the ability to move when they come into contact with water under in vitro conditions. When mature spermatozoa leave the body, motility activation is induced as a result of extracellular ionic changes in the water environment (Morisawa, 1994). Activation in water continues for 30–80 seconds (Çevik, 2000). Sperm motility was measured at an average of 20 seconds. Spermatozoa that are active for a short period of time and rapidly lose their ability to move may retain their viability for longer when treated with diluents. At the same time, the antioxidants added to the diluent may increase their fertilization ability. Hesperidin is a flavonoid compound that stands out with its antioxidant, anti-inflammatory, and anti-apoptotic properties. A recent study investigated the effects of hesperidin against testicular damage caused by glyphosate. As a result of the study, it was revealed that hesperidin improved spermatological parameters and reduced histopathologic damage and lipid peroxidation (Güngör et al., 2024). In another study, it was concluded that hesperidin was effective in protecting the testes with its antioxidant properties against substances with biological and chemical toxic effects (Yan et al., 2024). DPP is the male reproductive cell of the date palm flower, which contains important amino acids and flavonoids, which are now used in the treatment of infertility and for their libido-enhancing effect. Previous studies have shown that DPP administered orally has a protective effect on the testes and supports testosterone production in testicular damage induced by carbon tetrachloride in rats (Obaid & Araak, 2024). In another study in female rats, when oxidative stress was induced by sodium nitrite exposure, DPP played an effective role in both preventing oxidative stress and preserving fertility (Jiheel, 2024). In the literature review, it was determined that the effects of DPP on spermatozoa motility, density, and acrosome reaction were determined in previous studies, but its effect on membrane integrity has not yet been investigated. In the present study, sperm collected from rainbow trout were divided into 5 equal groups and diluted. Two of the groups contain DPP at different concentrations, and one contains hesperidin. One of the last two groups was reconstituted with normal diluent, and the last group was used as a substitute. The effects of DPP and hesperidin on both sperm membrane integrity and fertilization were investigated comparatively.

In the fertilization results, fertilization was observed only in the groups diluted with normal diluent and those diluted with diluent supplemented with hesperidin. It was determined that the addition of DPP had no effect on fertilization. The use of diluted sperm, rather than as a substitute, has increased the success of fertilization.

When the sperm membrane damage results were examined, the most damage was observed in the two groups diluted with DPP. Although previous studies have shown that DPP has a positive effect on the spermatological parameters of motility and concentration, our study found that it has a negative effect on membrane integrity. Higher membrane integrity was observed in the group diluted with hesperidin compared to the group diluted with the normal diluent. This result indicates that hesperidin has a positive effect on preserving membrane integrity, similar to native sperm.

## Conclusion

It was revealed that while the addition of DPP to the diluent did not affect both the fertilization capacity of Rainbow trout sperm and membrane integrity, which is one of the spermatological parameters, the addition of hesperidin had a positive effect on both preserving membrane integrity and fertilization. In the study, hesperidin had a positive effect on rainbow trout sperm when used at a concentration of 0.009 mg. In further studies, the effects of hesperidin at different concentrations can also be investigated and added to the literature.

## Compliance With Ethical Standards

### *Authors' Contributions*

GU: Writing – original draft, Formal analysis, Writing – review & editing
ADÖ: Methodology, Data curation
SAA: Investigation, Writing – review & editing
EMK: Data curation, Methodology
AK: Data curation
AY: Formal analysis
BK: Methodology
All authors read and approved the final manuscript.

### *Conflict of Interest*

The authors declare that there is no conflict of interest.

### Ethical Approval

Approval for the study was obtained from Atatürk University Animal Experiments Local Ethics Committee (Protocol no: 2025/02–38).

### Funding

Not applicable.

### Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

### AI Disclosure

The authors confirm that no generative AI was used in writing this manuscript or creating images, tables, or graphics.

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