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Original Article

Hesperetin Impacts during Cryopreservation on Rooster Semen Fertility and Hatchability

Rozhgar Abd Hussein[1], Sahar Abdalkarim Salih[1,4], Shorsh Hussein Bapir[*2,3], Kareem Maulud Hamad[4], Imad Majeed Noori[5], Saber Wasman Hamad[6,7], Abdulrahman Mahmoud Dogara[7]

[1]*Department of Medical Laboratory Science, Technical College of Applied Science, Sulaimani Polytechnic University, Sulaymaniyah, Kurdistan Region, Iraq.*
[2]*Department of Pharmacy, Raparin Technical and Vocational Institute, Rania, Sulaymaniyah, Kurdistan Region, Iraq.*
[3]*Department of Horticulture, College of Agricultural Engineering Sciences, University of Raparin, Rania, Kurdistan Region, Iraq.*
[4]*Department of Animal Resource, College of Agricultural Engineering Sciences, University of Raparin, Rania, Kurdistan Region, Iraq.*
[5]*Department of Nursing, Technical College of Health and Medical, Sulaimani Polytechnic University, Sulaymaniyah, Kurdistan Region, Iraq.*
[6]*Department of Field Crops and Medicinal Plants, College of Agricultural Engineering Sciences, Salahaddin University-Erbil, Erbil, Kurdistan Region, Iraq.*
[7]*Biology Education Department, Faculty of Education, Tishk International University, Erbil, Kurdistan Region, Iraq.*

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ABSTRACT

This study looked into how varied hesperetin concentrations affected the rooster sperm cryopreservation. A Lake Extender diluted sperm samples and supplemented with hesperetin at 0, 15, 20, 25, and 30 micromolar (µM) concentrations. Key parameters such as glutathione peroxidase (GPx), superoxide dismutase (SOD), total antioxidant capacity (TAC), reactive oxygen species (ROS), adenosine triphosphate (ATP), fertilization rate, hatch rate, and hatchability ratio were evaluated. The 25 µM hesperetin concentration yielded the highest values for SOD (126.78 U/mg), TAC (2.43 mmol/L), GPx (69.35 U/mg), ATP (108.36 pmol/10^6 sperm), fertilization rate (71.79%), hatch rate (56.92%), and hatchability ratio (79.28%), while the control (0 µM hesperetin) recorded the lowest values for these parameters (102.57, 1.30, 51.11, 89.07, 37.43, 23.07, and 61.64, respectively, $P < 0.01$). Conversely, ROS levels were highest in the control group (4.24 10^3cpm/10^6sperm) and lowest at 25 µM hesperetin (2.4510^{3}cpm/10^6sperm). These findings suggest that adding 25 µM hesperetin to cryopreserved rooster sperm effectively enhances SOD, TAC, GPx, ATP levels, fertilization, hatching, and hatchability rates while reducing ROS. This approach may offer significant benefits for addressing hunger, poverty, and environmental sustainability concerns.

Keywords: Spermatozoa, Cryopreservation, Hesperetin, Rooster, Hatchability.

1 Introduction

The reproductive health preservation of the birds is crucial to the viability of the poultry industry. [1]. Cryopreservation is a well-established technique for preserving genetic diversity in bird populations, especially in essential or endangered species. However, despite its potential, cryopreservation is not commonly applied in poultry breeding because the sperm quality in preserved samples tends to decline significantly [2].

Sperm viability and motility are crucial for successful fertility. Factors such as environment, nutrition, and physiology influence sperm motility [3, 4], and oxidative stress is also a key contributor to reduced reproductive performance. Sperm are vulnerable to oxidative stress, which negatively affecting motility and viability [5]. Oxidative stress occurs when the production of reactive oxygen species (ROS) increases, leading to enhanced cell death and reduced motility [6, 7]. An effective antioxidant defense system is therefore required to protect sperm membranes from oxidative damage [8].

Traditional Chinese medicine uses hesperetin [9]. And is widely found in citrus plants as hesperidin, a glycoside-prodrug. [10]. Citrus fruits of the Rutaceae family are highly consumed worldwide, and Industrial processing of these fruits generates significant waste due to their thick peels, inedible peels and seeds. Recently, citrus byproducts have been repurposed for the extraction of biofunctional compounds such as hesperetin, essential oils, and pectin, as well as for use as animal feed [11]. Citrus peel, which constitutes 40%–50% of the fruit's mass, is a

* Corresponding author
E-mail address shorsh.husein@gmail.com (Instructor).
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rich source of bioactive compounds, particularly hesperetin, a potent dietary antioxidant [12]. Among citrus fruits, mandarins and hybrids contain notably high levels of hesperetin [13]. Hesperetin in citrus peel is recognized for its antioxidant properties, protecting cells [14]. Additionally, hesperetin may help manage type 2 diabetes and metabolic syndrome [15, 16].

After consumption, dietary hesperetins are metabolized to hesperetin by intestinal microbes before absorption [17]. Hesperetin has anti-inflammatory, antioxidant, and antiallergic effects [18], effectively in preventing cadmium-induced testicular damage [19]. It also exhibits protective effects against doxorubicin-induced cardiotoxicity [20], it prevents testicular toxicity in cases of doxorubicin exposure and reduces testicular damage in diabetic mice by lowering inflammation, apoptosis, and oxidative stress [21].

In avian spermatozoa, adding antioxidant to freeze extenders has been explored in various studies to mitigate oxidative stress during sperm thawing [22]. The evaluation of different hesperetin concentrations on SOD, TAC, GPx, ROS, and ATP levels in rooster semen during cryopreservation, as well as on fertilized eggs, hatched eggs, and hatchability rate, are the aims of this study.

2 Materials and methods

2.1 Hesperetin Preparation from Citrus Peel

Sweet orange peels were air-dried, ground into powder, and processed through a 12-cycle extraction, yielding 960 g of powdered material. For extraction, 80 g of this powder was placed in a reflux condenser and 600 mL of petroleum ether was refluxed for 1.5 hours. After filtering the hot mixture through a Buchner funnel, the powder was dried, and reintroduced into the flask, and 600 mL of methanol was added. The contents were heated under reflux for 2 hours, then filtered, and the filtrate was concentrated using a distillation column. This process produced a syrupy residue that crystallized from 6% acetic acid, forming orange needle-shaped crystals.

A sample of the crude hesperidin was dissolved in dimethylformamide (7 mL gram^{-1} of syrup), followed by the addition of a warmed acetic acid solution at approximately 60°C. After filtering the solution, add 1:1 water via left for 4 hours, allowing the hesperidin to crystallize. The crystals were then filtered off.

To produce hesperetin, methanol (250) mL via 9 mL of concentrated sulfuric acid was added to 9 g of hesperidin, then stirred and refluxed for 8 hours. The resulting solution was cooled, concentrated, and mixed with 500 mL of ethyl acetate. The organic solution was washed four times. This solution was mixed with a vigorously stirred 200 mL of water with 3 mL of acetic acid in an ice bath. The resulting precipitate was washed, cooled, and yielded pure yellow hesperetin powder with a melting point of 220-221°C [23].

2.2 Sample and Animal Preparation

Individual cages measuring 70 x 95 x 85 cm were used to keep ten broiler-roosters 6.5 months old at 18 to 20 °C, exposed to a photoperiod of 15 hours light and 9 hours dark. The roosters were fed commercial breeding flock feed, with water available *ad libitum* at all times.

The dorso-abdominal massage technique was used for semen collection, placed in a water bath (37) °C, 2 times/week [5, 22]. For semen analysis, five ejaculates were collected per rooster. Samples meeting the criteria of 80% sperm motility, 3 x 10^9 sperm/mL, 0.2–0.6 mL, and no more than 10% morphological abnormalities were pooled. For D-fructose-dilution given six portions, the extender used contained 8 g/L D-fructose, 3 g/L polyvinylpyrrolidone, 19.2 g/L sodium glutamate, 3.74 g/L glycine, 5 g/L potassium-citrate, 0.7 g/L magnesium-acetate, a pH of 7.1, and 310 mOsm/kg osmolarity [24].

In Lake Extender, the sperm samples were diluted at 37°C via varying (control (0), 15, 20, 25, and 30 µM) hesperetin concentrations. After dilution, 0.25 mL straws of each sample were equilibrated at 4°C for 3 hours, then placed on the surface of liquid nitrogen for 7 minutes before being submerged. After one week, the storage samples were thawed individually in a 37.8°C water bath for 30 seconds before analysis [24].

2.3 TAC, SOD and GPx Determination

The activities of glutathione peroxidase (GPx), superoxide dismutase (SOD), and the measurement of total antioxidant capacity (TAC) were used to evaluate the antioxidant system [25]. These measurements were performed spectrophotometrically using an automatic biochemistry analyzer via Randox-kits.

The reactive agent provided was added to TAC, and then absorbance was measured (600 antioxidant nm), with results expressed in mmol/L. So, GPx activity was assessed in NADPH with glutathione presence. GPx reduces the glutathione while oxidizing NADPH and changing to NADP+, and GPx activity was measured by recording the drop in absorbance at 340 nm. The ability of SOD to prevent INT from oxidizing to red formazan in a xanthine/xanthine oxidase system that produces superoxide radicals was used to measure its activity. The SOD (1 unit) is the amount that inhibits the INT by 50% under assay conditions with absorbance measured (505 nm).

2.4 Determination of ATP in Sperm

Initially, in Lake Buffer (750 µL), each sample (5 µL) was diluted. Then, to initiate the reaction, perchloric acid (190 mL) was mixed with this diluted sample (5 mL). At room temperature with 12,000 rcf, then centrifuged/2 min.

It neutralized by mixing it with KCl (10.7 mL), KOH (58.7) mL, red-phenol (1mg/mL), and 10.7 mL Tris. Following this, 100 µL of reconstituted luciferin luciferase (pH 7.4, containing 20 mM $MgSO_4$, 100 mM glycine) was added for ATP measurement. Bioluminescence was recorded using a

luminometer, and ATP levels in the sperm were expressed as ATP/10^6 sperm [26, 27].

2.5 Determination of ROS

In brief, at 37°C in PBS (250 µL/20 min), semen samples were incubated, followed by then centrifuged (300 x g/7 min). The supernatant was discarded, and 3 mL of PBS was added to the pellet, followed by centrifugation at 300 x g for 7 minutes. The sperm were then diluted with PBS to adjust the concentration to 20 x 10^6 mL^{-1}. Next, the sample (400 µL) was added to luminol (10 µL), and the Orion II Microplate Luminometer was used to place the tubes for analysis. Results were reported as 10^3 cpm per 10^6 spermatozoa [26, 27].

2.6 Analysis of Reproductive Performance

Artificial insemination (AI) was performed on 180 hens after a month without roosters. For each treatment group, 30 hens were inseminated with 100 x 10^6 sperm per hen. For 2 weeks, AI was conducted twice weekly. Eggs were collected daily for five days following the final AI. Hatchability via fertility rates was assessed by candling eggs on day 7 to determine fertility and counting the number of chicks hatched on day 21 [26].

2.7 Statistical Analysis

The data normal distribution was assessed using the Shapiro-Wilk and UNIVARIATE technique. The SAS software (SAS NC 2002, V 9.1, and Cary) was employed, incorporating the fixed hesperetin levels effects into the statistical model and reported as LSmeans ± SEM. Tukey's test was used to determine significant differences, with differences considered significant at a P value < 0.01.

3 Results

As indicated in Table 1, the effect of hesperetin levels was significant differences for TAC, GPx, ROS and ATP of frozen sperm, but it was no significant difference for SOD. Hesperetin significantly increased SOD, TAC, GPx and ATP in comparison to the control (0). It was significantly lower for ROS compared to the (0). However, the hesperetin effect was reversed at level 30 µM for all parameters.

Table 1 shows that there was no significant difference between level 30, level 15, and (0). Also, there was no significant difference between levels 25 and 20 for all parameters except ROS.

The highest values were recorded with Level 25 µM (126.78, 2.43, 69.35 and 108.36) with SOD, TAC, GPx and ATP, respectively ($P < 0.01$). In contrast, it recorded the lowest value (2.45) with ROS. Controls (0) recorded the lowest values (102.57, 1.30, 51.11 and 89.07) with SOD, TAC, GPx and ATP, respectively. Also, it recorded the highest value (4.24) with ROS.

Table 1: Effect of hesperetin on SOD, TAC, GPX, ROS, and ATP of rooster semen after freeze-thawing.

Treatments	Parameters				
	SOD (U/mg)	TAC (mmol/l)	GPX (U/mg protein	ROS (10^3cpm/10^6sperm	ATP (pmol/10^6sperm)
Control (0)	102.57^a	1.30^b	51.11^b	4.24^a	89.07^c
15	109.74^a	1.41^b	52.92^b	4.06^a	92.93^{bc}
20	118.04^a	2.12^a	63.24^a	3.10^b	104.64^{ab}
25	126.78^a	2.43^a	69.35^a	2.45^c	108.36^a
30	103.65^a	1.30^b	52.47^b	4.14^a	90.31^c
SEM	6.25	0.14	1.54	0.13	3.46

Abbreviations: SOD, superoxide dismutase; TAC, total antioxidant capacity; GPX, glutathione peroxidase; ROS, reactive oxygen species, ATP, adenosine triphosphate, and SEM, Standard Error
$^{a-c}$ Different superscripts within the same column indicate group differences ($P < 0.01$).

As indicated in Table 2, the treatments have significant differences for hatched, fertilized and hatched ratio. The level 25 µM of hesperetin recorded the highest values for all parameters. However, it was reversed for all parameters at 0 µM (control).

Table 2 shows that for all parameters, there was no significant difference between levels 25 and 20. There was no significant difference between level 30, level 15, and the 0 (control) group. The 25 µM showed the highest values {140 n (71.79%), 111n (56.92%) and 79.28%} ($P < 0.01$) with fertilized eggs, hatched eggs and hatched eggs ratio, respectively. In contrast, controls (0) recorded the lowest values {73 n (37.43%), 45 n (23.07%) and 61.64%, respectively.

4 Discussion

The results from flow cytometric analysis indicated that hesperetin has beneficial effects on the structure of cryopreserved sperm cells. Hesperetin treatment was shown to reduce the harmful effects of cryopreservation on spermatozoa, thereby enhancing their fertility potential after the freezing-thawing

process. Specifically, concentrations of 20 and 25 µM hesperetin were found to increase GPx, SOD, TAC, and ATP. Additionally, these concentrations improved the rates of hatched, fertilized, and the hatchability ratio of eggs while simultaneously decreasing reactive oxygen species (ROS) levels.

Table 2: Effect of hesperetin on fertility and hatchability rates of rooster semen after freeze-thawing.

Treatments	Parameters		
	Fertilized eggs (No. &%)	Hatched eggs (No. &%)	Hatched eggs ration (hatched/fertilized, %)
Control (0)	73 (37.43)^{b}	45 (23.07)^{b}	61.64^{b}
15	75 (38.46)^{b}	47 (24.10)^{b}	62.66^{b}
20	126 (64.61)^{a}	96 (49.23)^{a}	76.19^{a}
25	140 (71.79)^{a}	111 (56.92)^{a}	79.28^{a}
30	74 (37.94)^{b}	47 (24.10)^{b}	63.51^{b}

$^{a-b}$ Different superscripts within the same column indicate group differences ($P < 0.01$).

These findings align with previous research demonstrating that hesperetin can lower ROS levels and reduce the incidence of sperm apoptosis during cryopreservation. One of the bioflavonoids in citrus fruits is hesperetin, a glycoside that acts as a prodrug [9, 10].

Recent research has highlighted the significant antioxidant properties of hesperetin. Studies have shown that hesperetin can improve sperm parameters and enhance the levels of oxidative enzymes such as CAT, SOD, and GPx, while also restoring the normal histopathological structure of the testis [28]. It has been suggested that hesperetin acts as an antioxidant [29].

Cold shock in spermatozoa leads to oxidative stress and increased sperm mortality, which are believed to contribute to ROS production [30]. For sperm damage, exposure to thermal changes and free radicals makes unavoidable factors and oxidative stress particularly harmful. While in semen, antioxidant is naturally present, their levels decrease after cryopreservation and dilution. Thus, using exogenous antioxidant in semen extenders could be an effective strategy to mitigate ROS-induced damage [31].

In the freeze-thawing process, the generation of oxidants is critical, as an excess of ROS leads to oxidative stress and a loss of fertility [32]. In fatty acids, the sperms are rich, making them susceptible to lipid peroxide formation in the ROS presence [27]. It has been noted that ROS levels increase dramatically during the freezing-thawing process [33].

While low ROS concentrations are essential for maintaining fertility, sperm capacitation, and membrane fluidity [34, 35], elevated ROS levels can cause severe sperm functional impairments, including decreased motility, increased intracellular enzyme leakage, and DNA damage [36]. Low antioxidant levels are often a significant contributor to the loss of sperm function and vitality [37]. Antioxidant has a sensitive role in protecting sperm by balancing between ROS production and destruction, which helps preserve the sperm plasma membrane integrity and maintain their fertilization capability [38, 39].

Conclusion

In rooster semen cryopreservation, we have demonstrated that a 25 µM hesperetin is an effective supplement for the freezing extender. This treatment resulted in significantly higher TAC, SOD, GPx, and ATP levels. Additionally, it led to an increase in fertilized and hatched eggs, improving the hatching rate by 17.64%. Notably, this concentration also resulted in the lowest reactive oxygen species levels (ROS) compared to other treatment levels.

Furthermore, the use of 25 µM hesperetin appears to enhance fertility effectively when applied through artificial insemination (AI) in the poultry industry, making it a promising option for improving reproductive outcomes. Overall, this supplementation not only boosts SOD, TAC, GPx, and ATP to their peak levels but also significantly reduces ROS compared to the other treatments.

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Authors contribution

The authors contributed equally to all stages of the project.

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