

Impact of Curcumin Supplementation on Sperm Characteristics and Gene Expression in Fish Sperm Freezing Medium

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Abstract

This study aimed to determine whether curcumin addition in freezing media affected the ability of trout semen to resist cryodamage. Fish semen was collected and cryopreserved in a curcumin-supplemented freezing medium at 20 µM. Sperm motility parameters, and nuclear factor 2 gene expressions (NRF-2 and Keap-1) were assessed following freezing and thawing. Adding 20 µM curcumin to the sperm extender increased the rate and duration of motility, as well as the percentage of live sperm at 277 mOsm with a 1:3 dilution. However, the sperm quality decreased at 135 and 365 mOsm with 1:2, 1:3, and 1:4 dilutions. Following cryopreservation, curcumin-supplemented frozen-thawed semen exhibited significantly elevated levels of NRF-2 and Keap-1 gene expressions. The findings demonstrated that adding curcumin to freezing media protected fish and motility parameters against damage induced by the freeze-thawing process.

Introduction

Sperm, delicate and essential for reproduction, possess a remarkable ability to adapt to changing environmental conditions. As they are cooled, complex cellular mechanisms come into play, responding to variations in temperature and osmotic conditions. These adaptations include changes in water permeability, plasma membrane lipids, and cytoskeletal elements, all crucial for maintaining cellular volume. In recent years, extensive research has been conducted to determine the osmotic tolerance limits of sperm in various species, such as boar, mouse, fish, and humans (Willoughby et al., 1996; Gilmore et al., 1997, 1998; Şahinöz et al., 2020). The determination of osmotic tolerance limits is a critical step in comprehending the characteristics of

sperm and aiding in the development of reproductive technologies. Most fish species' motility can be triggered by variations in osmotic pressure (0–300 Mosmol/l)(Morisawa and Suzuki, 1980, Aral et. al., 2007).

The importance of using a suitable extender in the process of semen dilution cannot be overstated. Not only must the chosen solution be non-damaging to the spermatozoa during the freezing process, but it must also closely resemble the salt concentrations found in seminal plasma. Extenders, which are composed of salts and organic compounds, serve to maintain the viability of cells during refrigeration (Besty et. al., 2019). There are two main types of extenders, namely ionic extenders or seminal plasma-mimicking media, and sugar-based extenders. While the former utilizes a combination of

salts, the latter is solely composed of various sugars, such as glucose and sucrose (Graybill and Horton, 1969; Kurokura et al., 1984; Cognie et al., 1989). Dilution of semen has been a widely used technique to maximize the potential for fertilization in limited quantities of sperm. Specifically, in fish spermatozoa cryopreservation, dilution has been linked to improved fertilization rates compared to undiluted milt. In the case of *Cyprinus carpio*, observations have shown that dilution ratios of 1:10, 1:20, and 1:50 lead to longer sperm motility durations, with a decrease in motility being observed with higher dilution ratios. However, for trout spermatozoa, a standardized dilution ratio has yet to be established to enhance the effectiveness of artificial fertilization after cryopreservation (Poon and Johnson, 1970; Rieniets and Millard, 1987; Rana and McAndrew, 1989; Gwo et al., 1991; Lahnsteiner et al., 2003).

The cytoplasm in spermatozoa is limited. The amount of seminal plasma containing sperm is also restricted and decreases as sperm are diluted for freezing. Freezing pig semen has been found to cause a 32% decrease in reduced glutathione (GSH) levels (Gadea et al., 2004). Bovine semen cryopreservation has been shown to drastically lower sperm GSH levels by 78% and SOD activity by 50%. (Bilodeau et al., 2000). These factors reduce the preservation time of fish semen's antioxidant ability after cryopreservation. Cryopreservation considerably impacted the viability, acrosomal integrity, and proteins involved in spermatozoa motility (Valcarce et al., 2013).

One of the main controllers of the cellular oxidative stress response is the transcription factor nuclear factor erythroid 2-associated factor 2 (NRF2) (He et al., 2020). Nuclear factor 2 (NRF2), which is encoded by the nuclear factor gene, is a member of the Cap'n'Collar (CNC) subfamily of essential leucine zipper (bZIP) transcription factors, along with nuclear factor erythroid-derived 2 (NFE2), erythroid 2-like 2 (NFE2L2), NRF1, NRF2, and NRF3 (Jaramillo and Zhang, 2013; He et al., 2020). Genes containing antioxidant response elements (AREs) in their promoters are transcribed under the control of the transcription factor NRF2, also known as Cap-N-Collar basic leucine zipper (CNC-bZip) (Wild et al., 1999). Cells are protected from endogenous and external stressors via Keap1-NRF2-ARE signaling. When cells are dormant, the actin-associated protein Keap1, which is mostly located in the cytoplasm, interacts with the NRF2 transcription factor. NRF2 is released from Keap1 in response to chemical cues provided by oxidative and electrophilic chemicals. It increases cell viability by transactivating the expression of several dozen cytoprotective genes (Kensler et al., 2007).

During sperm freezing, environmental stress is unavoidable and might interfere with cell processes. Due to reduced sperm motility and decreased sperm viability at advanced levels, oxidative stress in spermatozoa is linked to decreased male fertility (de Lamirande et al., 1997; Baker and Aitken, 2005; Tekin et

al., 2007). Because of the high level of polyunsaturated fatty acids in spermatozoa's cell membranes, which may be oxidized in the presence of ROS (Griveau and Le Lannou, 1997; de Lamirande et al., 1997; Baker and Aitken, 2005), spermatozoa are vulnerable to oxidative damage. Spermatozoa utilize regulatory systems to react to and adapt to stressors.

A yellow phenolic pigment called curcumin, a kind of antioxidant is a part of the *Curcuma longa rhizome* and has a variety of biological and pharmacological properties, including anti-inflammatory, antineoplastic, antioxidant, and anti-mutagenic activity (Şahin et al., 2012). As an antioxidant, curcumin has two different modes of action: one is based on the chemical composition of the compound, and the other is based on its capacity to promote the synthesis of antioxidant enzymes. Curcumin comprises a variety of functional antioxidant groups, including phenyl rings, carbon-carbon double bonds, and dike to groups (Ak and Gülçin, 2008).

The assumption that ROS is created during such a cycle supports the idea that it is harmful to sperm function (Bilodeau et al., 2000). Curcumin dramatically reduces the production of reactive oxygen species (ROS) by activated macrophages, including superanions, H₂O₂, and nitrite radicals, which are key players in inflammation (Mathuria and Verma, 2008). Studies on some species found that semen treated with antioxidant compounds was more resistant to freezing and thawing processes. Antioxidants enhance the genetic material's susceptibility to freezing and eliminate free oxygen products that are harmful to spermatozoa (Chanapiwat and Kaeoket, 2015; Shah et al., 2016).

According to several studies (Bucak et al., 2010; Chanapiwat and Kaeoket, 2015; Shah et al., 2016), the addition of curcumin to sperm freezing extenders was found to have a positive effect on post-thaw sperm parameters in different species such as bulls, pigs, rams and buffaloes. The effect of curcumin on the motility and vitality of spermatozoa under different conditions of osmotic pressure and dilution rates is not well documented even in these studies. To date, there has been limited research on these characteristics of fish spermatozoa.

The study aimed to examine the effect of curcumin on sperm motility parameters and live/dead spermatozoa rates in frozen rainbow trout sperm. The effect of osmotic pressures and dilution ratios on sperm characteristics was investigated by the addition of curcumin to the extender.

Materials and Methods

Workplace and Semen Collection

The rainbow trout (*Oncorhynchus mykiss* W., 1792) used in the study are the samples caught from the Atatürk Dam Lake fed by the Euphrates (Euphrates) River in January 2018 and were purchased from

fishermen in Şanlıurfa. The Fisheries Department of Harran University's Bozova Vocational School evaluated the trout semen samples that were collected. The weights of the fish were determined using an electronic scale that had an accuracy of 0.1 g. A specific measuring board, on which a ruler with mm intervals was fixed, was used to take height measurements. For height measurements, total height (TL) was applied. Semen was collected in 5 ml glass tubes by gently massaging the abdomen after the fish's urogenital papilla was well dried and contact with urea or feces was avoided. As soon as the sperm are collected, they will be immediately transferred to the lab in styrofoam with ice (4°C).

Macroscopic and Microscopic Examination of Sperm

The semen obtained was visually analyzed. Any sperm with abnormal color, viscosity, or contamination were removed from the sample. All samples were found to be immobile after 5 µl of each sample was taken and examined under a light microscope at 400X magnification. Following that, all samples' motility was assessed using activation solutions (125 mM NaCl, 20 mM Tris-HCl, and 30 mM glycine, pH 9) (Billard, 1992).

The samples with the highest motility, 80 % or higher, were chosen. After the activation solution was added to the semen, the time it took for the spermatozoa to become immobile was measured in seconds (s). Spermatozoa densities were determined using a hemocytometric counting technique on Thoma slides (Tvedt et al., 2001). Each sample's 1.5 ml of semen was centrifuged at 2000 g for 30 minutes to assess the seminal plasma osmotic pressure. The sperm samples were pooled. The pooled samples (n=20) were divided into six aliquots.

Extender Composition

The final diluent concentration was obtained using three different osmotic pressures and three different dilution ratios. Before freezing, the rate and duration of motility were assessed. The glucose diluent's osmotic pressures are 135 mOsm, 277 mOsm, and 365 mOsm. 20 µM curcumin was used. Curcumin stock solution (0.1 M) was prepared in 96% ethanol (as solvent). Curcumin powder was supplied by Sigma-Aldrich (CAS number 458-37-7).

Sperm dilution rates;

1: 2 (semen: extender; 650 µl semen + 1297,4 µl 0.3 M glucose extender (12% egg yolk, 10% DMSO, and 78% glucose) + 20 µM curcumin group was created by adding 2.6 µL of curcumin stock solution to 1.297 µL extender), total 1950 µl),

1: 3 (semen: extender); 433 µl semen + 1297,4 µl glucose extender (12% egg yolk, 10% DMSO, and 78% glucose) + (20 µM curcumin group was created by adding 2.6 µL of curcumin stock solution to 1.297 µL extender), total 1733 µl),

1:4 (semen: extender); 325 µl semen + 1297,4 µl glucose extender (12% egg yolk, 10% DMSO, and 78% glucose) + (20 µM curcumin group was created by adding 2.6 µL of curcumin stock solution to 1.297 µL extender), total 1625 µl).

In the vehicle control group (0), ethanol was added at the same concentration used to dissolve curcumin in each group. 1: 3 (semen: extender); 433 µl semen + 1297,4 µl glucose extender (12% egg yolk, 10% DMSO, and 78% glucose) + adding 2.6 µL of 96% ethanol to 1.297 µL extender), total 1733 µl),

The ratio was adjusted to after dilution, and sperm motility rate, and motility duration were measured.

Sperm Cryopreservation

Sperm samples (n=6) were cryopreserved. The final concentrations of curcumin were 20 µM, whereas the final osmolarities of glucose were 135, 277, and 365 mOsm. Sperm was diluted with the extender at a ratio of 1:2, 1:3, and 1:4 (sperm: extender) (Table 1).

Pooled sperm samples were cryopreserved. Glucose was added to three final osmolarities: 135, 277, and 365 mOsm, with curcumin added to a final concentration of 20 µM. Sperm was diluted with an extender at 1:2, 1:3, and 1:4 (sperm: extender) ratios (Table 1). The final osmolarities of the vehicle control group (0) treated with glucose were 280 mOsm.

The extended semen was loaded into 0.25 French straws and cooled to 4°C. It was allowed to equilibrate for 15 minutes. Sperm was frozen on a floating rack that was elevated to a height of 8 cm above liquid nitrogen in the vapor of the liquid nitrogen. After a 4-week storage period within a portable nitrogen tank, the frozen sperm was thawed and examined. Frozen sperm were thawed and examined after being kept for 4 weeks in a portable nitrogen tank (Maria et al., 2006).

Sperm Thawing

For each group, thawing occurred in a water bath at 40°C for 5 seconds. After thawing, the motility rate and the motility duration were visually determined under the light microscope. 50 mM of NaCl (20 mM Tris-HCl, pH 8.0) was used as the activation solution.

Live Sperm Percentage

In our study, Acridine Orange (AO-Sigma A8014 St. Louis, MO., USA), which dyes the cell DNA, and Ethidium Bromide (EBSigma E7637 St. Louis, MO., USA), which only dyes apoptotic or necrotic cells of which membrane integrity has deteriorated. For this purpose, 10 µg ml⁻¹ of AO and EB stock solutions were prepared and diluted at the rate of 1/9 in PBS (Phosphate Buffered Saline, Cellgro 21-040-CM Manassas, VA, USA). 50 µl of AO and 50 µl of EB mixture solution were added to 10 µl of the cell suspension (120x10⁶ spermatozoa ml⁻¹) and incubated at room temperature for 5 minutes.

Subsequently, it was placed on a lame, and the changes of necrotic in the cells were assessed by imaging under a fluorescent microscope (40×) (Olympus cfx 53-EM480 X350). With this method, three cell groups can be detected using double dyeing. Live cells appeared green under the nucleus due to the absence of membrane degradation. Necrotic cells appeared red due to complete membrane degradation. At least 200 cells per sample were counted for the determination of the dead/live spermatozoa ratio.

Real-Time PCR

Fish semen was frozen by adding 20 μ M of curcumin (2.6 μ L of curcumin stock solution) and vehicle control (0 curcumin; 2.6 μ L of 96% ethanol), and then the thawing procedure for qPCR was performed in the following groups and temperatures. They were thawed in a water bath for 5 seconds at 40°C and performed RNA isolation. Total RNA was isolated using the miRNeasy mini kit (Qiagen Hilden, Germany) and reverse transcribed using the Ipsogen RT Kit (Qiagen Hilden, Germany) according to the kit procedure. RT-qPCR was performed on the Rotor-Gene Q Real-Time PCR System (Qiagen Hilden, Germany) using a QuantiTect SYBR Green PCR kit (Qiagen Hilden, Germany). Each sample was run in triplicate using the primer sequences (Table 2) and the B-actin housekeeping gene. Gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method relative to the control group. Three replicates were performed for sperm analysis (Table 2).

Statistical Analysis

Normality and homogeneity of variance of the data were checked using the Kolmogorov–Smirnow test before analyses. The data that did not yield a normal distribution underwent a logarithmic transformation. Percent motility and apoptotic cell data were arcsine transformed and analyzed with a one-way analysis of variance (ANOVA). The SPSS statistics program version 20 used the one-way ANOVA analysis in conjunction with Tukey's multiple comparison post-test to identify significant differences between various findings (IBM Corp., Armonk, NY, USA). All sperm parameters are expressed as the mean \pm standard errors (SE). Statistics were considered significant at $p < 0.05$.

Results

Sperm Motility, Duration, and Viability

According to the planned experimental protocol, the results for the prepared sperm freezing media are given in Table 1.

Only high motility rates of fresh sperm spermatozoa (over 80 %) were used for the experiment. The fresh sperm and 277 mOsm at 1:3 dilution rates groups were similar but higher than the other groups when the post-thaw motility percentage of the 20 μ M curcumin groups was compared with the vehicle control group ($p < 0.001$). Motility percentages at 365 mOsm 1:2, 1:3, and 1:4 remained below 7%.

Post-thaw motility of the 20 μ M curcumin groups was found to be significantly higher in the 277 mOsm at

Table 1. Some spermatological examination results of rainbow trout (*O. mykiss*) semen at different dilution ratios with different amounts of antioxidants added at different osmotic pressures

Exp. No	Factors			Three Recurrence Mean (\pm SEM)		
	Diluent (Glucose) (mOsm)	Antioxidant (Curcumin) (μ M)	Dilution rate (%)	Spermatological Characteristics		
				Motility Percentage (%)	Mot Dur (s)	Live sperm Percentage (%)
1	135	20	1/2	18.33 \pm 2.36 ^c	94.00 \pm 14.51 ^e	18.33 \pm 2.36 ^f
2	135	20	1/3	23.33 \pm 4.71 ^c	108.33 \pm 14.34 ^e	21.67 \pm 2.36 ^e
3	135	20	1/4	30.00 \pm 4.08 ^{bc}	126.67 \pm 18.86 ^d	25.00 \pm 4.08 ^d
4	277	20	1/2	16.67 \pm 4.71 ^c	72.00 \pm 10.23 ^f	8.33 \pm 2.36 ^g
5	277	20	1/3	70.00 \pm 8.16 ^a	266.67 \pm 23.57 ^a	66.67 \pm 4.71 ^b
6	277	20	1/4	6.67 \pm 2.36 ^d	40.00 \pm 8.16 ^g	3.33 \pm 2.36 ^g
7	365	20	1/2	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
8	365	20	1/3	5.00 \pm 0.00 ^d	63.33 \pm 9.43 ^f	5.00 \pm 0.00 ^g
9	365	20	1/3	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
Vehicle Control Group	-	-	1/3	47.64 \pm 2.07 ^b	158.84 \pm 19.68 ^c	52.48 \pm 4.90 ^c
Fresh Sperm Control Group	315	-	-	85.00 \pm 4.08 ^a	184.67 \pm 2.05 ^b	96.67 \pm 2.36 ^a

*Exp.: Experiment; Mot: Motility; Dur.: Duration; s:second; The values were expressed as mean \pm SD. * $p < 0.05$

Table 2. List of primers used for real-time PCR

Primer	Forward(5'-3')	Reverse(5'-3')
NRF-2	GGAGGCAAGTCCACATGCTAG	CACTCTCAGGGTACTGTCCATC
Keap-1	GTGCTACAACGACCTGGAGG	GTTGGTGAACATGGCTCTGAAG
B-actin	AGCCACACCCGACTACCA	CCATGCCAATGAGACTGAGAAG

1:3 dilution group than in the vehicle control and fresh sperm groups ($p < 0.001$). In the vehicle control and fresh sperm groups, the 1:2, 1:3, and 1:4 dilutions were higher than the dilution groups at 135 and 365 mOsm. The vehicle control and fresh sperm groups also had higher motility durations than the other groups at 135 and 365 mOsm in the 1:2, 1:3, and 1:4 dilutions, and 277 mOsm in the 1:2 and 1:4 dilutions ($p < 0.05$).

The assay results indicate a significantly higher percentage of live sperm in the fresh sperm group as compared to the vehicle control and curcumin-treated groups ($p < 0.001$). Comparing the curcumin and vehicle groups, a significantly higher percentage of live sperm was observed at 277 mOsm in the 1:2 dilution ($p < 0.05$). The vehicle control group had a higher percentage of viable sperm than the other groups (at 135 and 365 mOsm in the 1:2, 1:3, and 1:4 dilutions and 277 mOsm in the 1:2 and 1:4 dilutions) ($p < 0.05$). The proportions of viable sperm at 365 mOsm, 1:2, 1:3, and 1:4 and at 277 mOsm in the 1:4 dilution were all less than 7%.

Sperm NRF-2 and Keap-1 gene expression

NRF-2 and Keap-1 gene expression levels in frozen and thawed semen samples treated with curcumin (20 μ M curcumin; 2.6 μ L of curcumin stock solution) and vehicle control (0 curcumin; 2.6 μ L of 96% ethanol) varied from -2.84 ± 0.06 to $+3.18 \pm 1.17$ and -1.61 ± 0.38 to $+2.92 \pm 0.42$, respectively (Table 3). NRF-2 gene expression levels showed a significant increase in sperm frozen with the freezing extender containing 20 μ M curcumin compared to those frozen without curcumin ($p < 0.001$). Keap-1 gene expression levels showed a significant increase in sperm frozen with the curcumin-containing extender compared to vehicle control sperm ($p < 0.001$).

As a result, a substantial rise in the expression of the NRF-2 $+3.18 \pm 1.17$ and Keap-1 $+2.92 \pm 0.42$ genes was seen in the vehicle control group that had a 5-second thaw at 40°C. NRF-2 and Keap-1 gene expression levels in frozen and thawed semen samples treated with curcumin were -2.77 and -2.65 , respectively (Table 3).

Discussion

During the sperm freezing process, the internal parameters of the sperm are influenced by external factors such as the composition of the diluent, the type and concentration of cryoprotectants, the rate of

dilution and cooling, and the procedures of equilibration, freezing and thawing. It is therefore important that these factors are carefully considered to ensure successful results (Pontbriand et al., 1989). Rapid temperature changes, including cold shock and ice formation and thawing during the freeze-thaw process, affect the acrosome, mitochondria, axoneme, and plasma membrane (O'Connell et al., 2002; Chelucci et al., 2015). Some of these changes lead to increased levels of lipid peroxidation, cytoplasmic reactive oxygen species, and DNA damage (Agarwal et al., 2005). Cryopreservation attempts to optimally dehydrate cells to prevent the production of intracellular ice crystals (Ozkavukcu et al., 2008). However, both intracellular and extracellular ice crystals can form during the freezing and thawing process. Osmotic shock and temperature shock are also thought to be harmful to sperm (Stanic et al., 2000). Sperm motility depends on the thawing process, which must occur quickly to avoid recrystallization (Lahnsteiner, 2000). During freezing and thawing, rapid osmolarity changes frequently take place and deform the membrane structures (Escalier and Bisson, 1980).

The exposure of the sperm to various physical and chemical conditions may be the primary cause of its regression. The creation of crystal ice outside the cell, which is affected by the environment, modifies the structure of the cell. Cryopreservation attempts to optimally dehydrate cells to prevent the production of intracellular ice crystals (Ozkavukcu et al., 2008). However, both intracellular and extracellular ice crystals can form during the freezing and thawing process. Osmotic shock and temperature shock are also thought to be harmful to sperm (Stanic et al., 2000). Sperm motility depends on the thawing process, which must occur quickly to avoid recrystallization (Lahnsteiner, 2000). During freezing and thawing, rapid osmolarity changes frequently take place and deform the membrane structures (Escalier and Bisson, 1980).

Considering the properties of curcumin and its potential to evaluate sperm quality parameters in animals exposed to oxidative stress (Bucak et al., 2010; Chanapiwat and Kaeoket, 2015; Shah et al., 2016), a study was conducted to determine the effects of the addition of 20 μ M curcumin on the quality of cryopreserved trout sperm compared to controls. The results show a significant improvement in sperm quality.

Sperm freezing success depends on internal and external factors, such as dilution, cryoprotectants, and

Table 3. NRF-2 and Keap-1 gene expression levels of rainbow trout (*O. mykiss*) sperm after thawing

Exp. No.	Factors		Gen Expressions (NRF-2)	Gen Expressions (Keap-1)
	Temp. (°C)	Dur. (s)		
1	40	5	+3,98	+2,72
2	40	30	-2,43	-1,45
3	40	5	-2,77	-2,65
4	40	30	-1,19	-1,03

*3, 4: Control groups, Exp.: Experiment, Temp.: Temperature, Dur.: Duration

procedures (Agarwal et al., 2005; Johnson et al., 2012; Hezavehei et al., 2018). Sperm quality assay results show a significant increase in sperm motility rate, motility duration, and live sperm percentage in the curcumin-treated group compared to the frozen sperm group without curcumin in the cryopreservation medium. This increase was observed in the 1:3 dilution group at 277 mOsm. Sperm quality declined in the curcumin group at dilutions of 1:2, 1:3, and 1:4 with osmolarities of 135 and 365 mOsm, as well as at a dilution of 1:3 with an osmolarity of 280 mOsm in the vehicle group. Semen is diluted with an appropriate diluent in the correct proportions to ensure that the volume of semen used for insemination contains enough sperm per dose to ensure high fertility without cell loss (Rasul et al., 2000). Hiperozmotik koşullar sperm de dehidrasyonu neden olur (Watson 2000). Whether the mechanical or the osmotic effects of freezing predominate, the result is the same; exposing cells to the cooling and thawing process without protection is a process incompatible with life (Whaley et al., 2021). On return to the isotonic state, cells swell beyond their normal isotonic volume due to the net influx of non-permeating solutes caused by hyperosmotic stress (Mazur et al., 1972). The extent of cell shrinkage or swelling depends on the membrane permeability to water and permeating solutes. Therefore, the osmotic tolerance limits are expected to vary between cell lines, similar to the cytotoxic effect of DMSO. Conversely, the decline in cell viability caused by cytotoxicity is depicted as a gradual transformation of living cells into non-living cells, triggered by DMSO accumulation inside cells during osmosis (Traversari et al., 2022). It is well known that sperm freezing and thawing reduces the number of live spermatozoa. The most prevalent cause of cellular dehydration and osmotic shock cell injury during freezing is the freezing of intracellular water and cell water due to thermal shock. It results in cell necrosis. In necrosis, dead cells have increased PI (Propidium iodide) permeability (Şahinöz et al., 2020). Due to inadequate or excessive osmosis time at 135 and 365 mOsm, sperm quality may have deteriorated in response to the DMSO-based solution. It remains unclear whether this solution penetrated the cells at dilutions of 1:2, 1:3, and 1:4. This result shows that curcumin only protects sperm against freeze-thaw damage at 277 mOsm 1:3 dilution and that this effect occurs with osmotic pressure rather than dilution rate.

Cryoprotectants protect sperm from stress, but cannot prevent loss of membrane integrity and other damage. High levels of reactive oxygen species (ROS) can be harmful, but low levels are important for sperm function (Agarwal et al., 2005; Johnson et al., 2012; Hezavehei et al., 2018). Cryopreservation methods and subsequent thawing have an impact on sperm cell survival. In general, roughly 40–50 percent of the sperm population dies after freezing and thawing (Watson, 2000). Thawing sperms quickly exposes them to a concentrated solution and cryoprotectant, restoring

equilibrium faster than slow thawing (Saha et al., 2022). Fast thawing (70°C for 5 sec) leads to higher post-thawing sperm motility and membrane integrity (67 and 50%, respectively) (Söderquist et al., 1997).

Curcumin contains chemicals that function as antioxidants. Previous research has shown that curcumin functions as a free radical scavenger. Curcumin also decreases oxidative stress and increases the viability, and integrity of the plasma membrane (Turner and Lysiak, 2008). Santonastaso et al (2021) propose that there is a dose-dependent increase in the radical scavenging activity of curcumin with increasing concentrations of curcumin, but there were no significant differences in sperm motility, ROS production and GPX4 gene expression between the control group and the groups treated with low concentrations of curcumin ranging from 2.5 μ M to 10 μ M. NRF-2 plays a crucial role in the defense against oxidative stress by stimulating the transcription of genes encoding catalase (Cat), superoxide dismutase, glutathione S-transferase (GST), and haem oxygenase-1, as well as antioxidant proteins and phase II detoxification enzymes (Deng et al., 2017). Zhou et al. (2020) showed that curcumin might improve asthenozoospermia by reducing ROS reproduction and regulating NRF-2 levels. A study by Chen et al. (2012) found that low sperm motility in human males is correlated with lower levels of NRF-2 mRNA expression, which affects specific sperm function parameters. The study found that the patients had significantly lower levels of NRF-2 mRNA expression compared to the controls ($p < 0.001$). However, there were no statistically significant differences in the levels of SOD2, CAT, or GSTM1 gene expression between the two groups. NRF-2 gene and Keap-1 expression levels showed a significant increase in sperm frozen with the freezing extender containing 20 μ M curcumin compared to those frozen without curcumin ($p < 0.001$). Our results indicate a significant increase in the expression levels of both the NRF-2 gene and Keap-1 in sperm frozen with the curcumin-containing extender compared to the vehicle control sperm ($p < 0.001$). Curcumin has been shown to dissociate the Keap-1-NRF-2 complex, resulting in the stabilization of NRF-2 and its subsequent transport to the nucleus, which then leads to the transcription of various antioxidant genes responsible for antioxidant responses, as demonstrated in numerous studies (Sreejayan and Rao, 1994; Bellezza et al., 2018).

Conclusion

Adding 20 μ M curcumin to the sperm extender increased the rate and duration of motility, as well as the percentage of live sperm at 277 mOsm with a 1:3 dilution. However, the sperm quality decreased at 135 and 365 mOsm with 1:2, 1:3, and 1:4 dilutions. To conclude, diluting osmotic pressure affects the protective effect of curcumin against freeze-thaw damage. It was observed that adding 20 μ M curcumin to

the sperm extender increased the expression levels of NRF-2 and Keap-1 genes during the rapid thawing of sperm (40°C 5 s), which protects the sperm from being damaged by freezing and thawing. Supplementing trout sperm samples with curcumin is important for artificial insemination, cryopreservation, in vitro fertilization techniques, and field studies by extending the storage and processing time of spermatozoa.

Ethical Statement

Ethics committee approval is not required for this study.

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Author Contribution

ZD: Investigation, supervision, manuscript writing, analysis. FA: Analysis, review, editing. EŞ: Supervision, conceptualization. ET: Methodology, analysis. İK: Methodology, analysis.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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