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Effect of resveratrol on post-thaw motility, kinematics, structural parameters and antioxidant/oxidant status of Kamori buck spermatozoa

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ABSTRACT

Resveratrol is a polyphenol compound showing strong antioxidant properties. It is believed that semen cryopreservation causes significant sperm losses which eventually affects sperm quality. Improving antioxidant status of semen may reduce this damage and enhance sperm fertilizing potential. This study investigated the resveratrol treated freeze-thawing sperm motion, kinetics, structural parameters and antioxidant/oxidant status of Kamori buck spermatozoa. Thirty-two ejaculates from 4 fertile Kamori bucks were processed in tris-citric-glyercol-egg yolk (TCG-EY) based with varying concentrations of resveratrol (0, 10, 20, 40 and 50 μM). Over 75 % sperm motility were pooled and frozen in liquid nitrogen. The results unveiled that adding resveratrol at 40 and 50 μM concentration significantly enhanced ($P < 0.05$) total motility (progressive motility, rapid velocity, and average path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head displacement, beat cross frequency, straightness and linearity in contrast with control and other groups. Supplementing resveratrol at 40 and 50 μM concentration significantly improved ($P < 0.05$) functional plasma membrane integrity, acrosome integrity, whereas, all resveratrol groups had same significant ($P < 0.05$) effect on DNA integrity in response to control group. The 40 and 50 μM resveratrol significantly promoted ($P < 0.05$) superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), and total antioxidant capacity (TAC) levels while significantly reducing ($P < 0.05$) the total oxidant status (TOS) and malondialdehyde (MDA) contents as compared to control and other groups, respectively. The principal component analysis (PCA) exhibited samples were present in different clusters except two groups which had partially overlapped. Using hierarchical clustering analysis, two clusters were constructed showing the relationship within the treated groups. The results of spearman correlation coefficient showed that yellow color showed highly positive correlation while turquoise color exhibited highly negative association between sperm variables. Overall, the results concluded that resveratrol at 50 μM performed slightly better results than 40 μM in terms of improving sperm quality parameters.

1. Introduction

Kamori is a goat breed, localized in Sindh province, Pakistan. It is known for its distinct color and features [1] and is reared by keen goat keepers. This milk and meat type of breed is found in Hyderabad, Bhit Shah, Saeed Abad, Matiari and others districts of Sindh province, Pakistan [2]. The adult male weighed 25 % heavier than female goat. The female goat produces 2–3 L milk/day and thus called as livelihood for poor goat keepers [1]. The rare presence of pure Kamori goats makes it highly worth able and is very expensive. According to local farmers, more than 10 strains are present within Kamori breed. They are bred through a selective strain of Kamori goat which is decided by goat farmers. So far, the genetic potential of this breed is still unexploited. It is a dire need to explore the semen quality index and its possible

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propagation via artificial insemination program in the near future.

Semen cryopreservation and artificial insemination are reproductive biotechnologies potentially implied to maximize the animal fertility [3]. Cryopreservation methods are utilized to maintain the quality and fertility of sperm cells for extended periods [4,5]. The cryopreservation and thawing methods result in half of the sperm cells dying during this process [6]. Sperm cells undergo cryopreservation, which can lead to ice crystal formation, oxidation production, and cold shock, which can negatively impact their quality [7,8]. The excessive production of free radicals can lead to oxidative stress, which can potentially impact sperm motility and motion parameters [9]. In caprine species, the LPO triggering process starts the generation of ROS that binds to PUFAs, causing lipid radicals and cellular damage [10]. Oxidative stress triggers a chain reaction in the sperm plasma membrane, leading to a decline in overall sperm quality parameters [11].

Sperm cells are embarked with antioxidant defense system which governs them from sperm cryo-damage [12]. However, full exploitation of sperm antioxidant potential has not been observed due to absence of sperm cytoplasm part [13]. Sperm freezing resistance varies among different species and is influenced by the size, shape, protein, and lipid composition of the sperm [14]. Improved techniques and media in sperm freezing have reduced its harmful effects, but cryopreservation still causes various forms of sperm damage in ruminants [15]. Addition of antioxidants in semen may protect sperm cells from vulnerable effect of oxidant production [9]. Exceeding production of oxidants may reduce antioxidant potential of both sperm cells as well as seminal plasma resulted in poor survival of sperms [16]. Egg yolk (EY) is a crucial additive in semen cryopreservation, acting as a reservoir of phospholipids and cholesterol, and protecting the plasma membrane and acrosome from temperature-related damage [17]. Spermatozoa are protected from freezing via sequestration of lipid-binding proteins from low-density lipoproteins in the EY [18]. Tris is a base diluent in which egg yolk is added to preserve sperm quality characteristics [19]. Egg-yolk contains a coagulating enzyme that interacts with goat seminal plasma, can potentially impact the viability of spermatozoa [14]. It is utmost important to remove seminal plasma from goat semen to secure the sperm viability [20].

Resveratrol is a natural polyphenol compound derived from fruits and has potent antioxidant activities involved in free radical scavenging, suppressing ROS and lipid peroxidation [21,22]. Resveratrol at 20 μg/mL in semen extender resulted in reduction of mitochondria membrane activity but failed to provide effect on motility in ram sperms [23]. Moreover, semen extender supplemented with different levels of resveratrol caused higher total and progressive motility, membrane and acrosome integrity and mitochondrial activity of goat sperm [24]. Use of resveratrol at 10 μM and 50 μM enhanced progressive motility, viable spermatozoa, membrane and acrosome integrity, and mitochondrial activity of frozen-thawed sperms [24]. It is thought that resveratrol promotes energetic metabolism of spermatozoa through mitigation of oxidative products and thus enhance freeze-thaw sperm fertility [25,26]. However, the addition of resveratrol in goat's semen needs further investigation. This study aimed to investigate the effect of different levels of resveratrol on sperm kinematics, structural parameters and antioxidant/antioxidant status of cryopreserved Kamori buck spermatozoa.

2. Material and methods

Ethical statement

The study adhered to the guidelines set by the Institutional Animal Care and Use Committee of the Animal Sciences Division, Nuclear Institute for Agriculture and Biology in Faisalabad, Pakistan (NIAB-C-PIEAS-0024).

2.1. Chemicals

All the chemicals were purchased from Sigma–Aldrich Chemical Co., USA and Merck, Darmstadt, Germany.

2.2. Animal Management

Four healthy and sexually active Kamori bucks, aged 8–12 months; weighing about 38 ± 0.8 were purchased and gathered at Panj Moro, Sindh Province, Pakistan. After that bucks were transported approximately 879 km from Panj Moro to Faisalabad, Pakistan. Then bucks were subjected to quarantine for 30 days to adopt and appearances of any disease. Drenching and vaccination were practiced according to the schedule of Livestock and Dairy Development Department, Punjab, Pakistan. Green fodder mixed with wheat bran and two times grazing posture were routinely followed. The concentrates were provided according to their body weight. Vitamins and minerals supplementation were also given. Bucks had free access to fresh drinking water.

2.3. Semen collection and cryopreservation

Thirty-two ejaculates (Eight ejaculations/buck) from four fertile Kamori bucks were obtained at Animal Farm, Nuclear institute for Agriculture and Biology (NIAB), Faisalabad. The collections were performed using caprine artificial vagina method (IMV, France) at 45°C during the month of October and November 2023. Preparation of artificial vagina was done with the technique [27] and semen samples were collected for consecutive eight weeks. After collection, the semen was kept in water bath at 37 °C for basic semen parameter analysis. The sperm motility (%) was examined under magnification 40× through phase-contrast microscope (CX 43, Olympus, Japan) and over 75 % sperm motility were subjected for further experimentation. After that semen samples were centrifuged at 315×*g* for 3 min in order to remove the seminal plasma [28]. Semen samples having $2.5 - 4 \times 10^9$ sperms/ml were counted by (Neubauer Counting Chamber, Germany). The tris-citric acid-glycerol egg yolk (TCG-EY) based extender was prepared (tris 3.07g, citric acid 1.64 g, fructose 1.26 g, streptomycin 100 mg, fresh egg yolk 15 % (v/v) and glycerol 5 mL [29,30], pH 6.8–7.2. Five aliquots according to the group-wise were prepared, adding tris-citric acid-glycerol egg yolk extender in it and adjusted the sperm concentration 100×10^6 sperms/mL. Once this ratio was prepared in five aliquots, then removed 0, 10, 20, 40, 50 μM from already prepared TCG-EY semen. And then added the varying levels of resveratrol such as control (0), 10, 20, 40, 50 μM in five aliquots separately. The volume was maintained (10 mL) in each group. Then different levels of resveratrol-supplemented five semen aliquots were kept in a single step at 37 °C with the same adjusted concentration up to 100×10^6 motile sperm/mL. After that tris-citric acid-glycerol egg yolk (TCG-EY) based resveratrol supplemented semen were cooled at 4 °C and then equilibrated for 3 h. After that 0.5 mL French straws was used for aspiration of semen under refrigeration temp and then sealed with straw sealer. After this step, semen straws were subjected in vapor freezing (above 4 cm in liquid nitrogen) for 8 min [24,31] and then deep frozen in liquid nitrogen for storage Since then, frozen straws were subjected to thawing at 37 °C for 30 s (digital thawing jug) for freeze-thaw assessment.

2.4. Freeze-thaw evaluation

2.4.1. Sperm kinematics

Sperm kinematic parameters were performed in earlier studies by Ref. [32] using computer-assisted sperm analyzer (CEROS, version 12.3; Hamilton Thorne Biosciences, Beverly, MA, USA) at Animal Reproduction Laboratory, Animal Sciences Institute, National Agricultural Research Centre, Islamabad, Pakistan. The CASA configuration such as frame rate 60 Hz, minimum cell size 5 pixels, minimum contrast 56,

motile, VAP cut off 20 μm/s, progressive motile VAP 80 μm/s, STR 80 %, Rapid (velocity) VAP & 80 μm/s, medium (velocity) VAP >20 μm/s < 80 μm/s, magnification 1.89 and temperature, set at 37 °C were applied to analyze sperm kinematics. A 5 μl of frozen–thawed sperm suspension was placed using clean pre-warmed glass slide under cover-slip and positioned in a portable MiniTherm stage (37 °C) of microscope. Approximately, 200 spermatozoa were observed under magnification, × 10 in two to five fields [33,34].

2.4.2. Plasma membrane integrity (PMI)

The integrity of sperm-vital plasma membrane was examined using the methodology described in Ref. [35]. Using a phase-contrast microscope (magnification x400; BH2 Olympus, Japan), about 200 spermatozoa were measured. The heads of the intact spermatozoa were white, and their tails were coiled while the stained spermatozoa had pink in appearance.

2.4.3. Viable sperm with intact acrosome

Trypan blue-Geimsa staining assay was conducted to examine the sperm viability and acrosome intactness [36,37]. Each sample (200 spermatozoa) was subjected to a phase-contrast microscope examination with a magnification of ×400. Spermatozoa with a blue head area and a pale acrosomal region (non-viable and non-intact acrosome) as well as those with an unstained head and a purple acrosomal region (viable with intact acrosome) were observed.

2.4.4. Viability

Using a micropipette tip, 5 μl of freezing-thawed semen samples were mixed with 5 μl of eosin-nigrosin stain. Smear was made, let it dry and observed under the phase-contrast microscope 40 X (CX 43, Olympus, Japan). Sperm with pink heads were dead, and sperms with white heads were live.

2.4.5. DNA integrity

Acridine orange (AO) fluorescence assay was performed to assess the DNA integrity of spermatozoa [38]. Over 350 spermatozoa per smear were observed under fluorescent microscope (magnification × 400; Nikon Optiphot 2, Japan; 480/550 nm excitation/barrier filter). The head of the spermatozoa emitting green fluorescence had intact DNA, whereas the head of the spermatozoa emitting yellowish-orange-red fluorescence had fragmented DNA.

2.4.6. Detection of oxidant/antioxidant enzymes

Four straws per group, each have 0.5 mL (50 × 10^6 sperm concentration) were subjected to thawing at 37 °C for 30 s and centrifugation was done at 1000×*g* at 4 °C for 10 min. The pellet was centrifuged again at 1000×*g* for 10 min after being cleaned in 50 mM potassium phosphate buffer (pH 7.0) [39]. After that, samples were sonicated and subjected for following parameters.

2.4.7. Superoxide dismutase

The assay solution contains the inhibition of NBT (57 μM) with 50 mM phosphate buffer using the protocol of Beyer and Fridovich [6] of pH 7.8, 13-mM L-methionine, 0.004 % riboflavin, with 0.025 % Triton and 50 μL sample to make up volume of 3 mL. The photoreaction was conceded out under the 15 W and took absorbance at 560 nm in spectrophotometer. SOD activity was measured in one unit as the amount of enzyme required to inhibit a 50 % reduction of NBT.

2.4.8. Peroxidase activity

The guaiacol peroxidase (POD) measurement was done from sperm cells homogenized in 50 mM potassium phosphate buffer with pH 7.0, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT). The POD activity was estimated referring to Hameed et al. [40] with minor modification. The 3 mL solution consisted 50 mM phosphate buffer (7.0 pH, 20 mM guaiacol, 40 mM H2O2 and 0.1 mL) enzyme extract. After that reaction was initiated by inclusion of enzyme extract. Enhanced absorbance of reaction mixture at 470 nm was measured every 20 s for 1 min. POD activity in one unit was referred as an absorbance change of 0.01 units/min.

2.4.9. Catalase

The catalase activity of samples was estimated using 50 mM potassium phosphate buffer (pH 7.0) and dithiothreitol (1 mM) in earlier protocols by Beers and Sizer, [41]. The reaction mixture comprised 59 mM H2O2, 50 mM phosphate buffer (pH 7), and a 100-μl sample. The reduction pattern of absorbance was assessed after every 20 s for 1 min at 240 nm and a unit of CAT activity was described as a change in absorbance in 0.01 min.

2.4.10. Malondialdehyde

The MDA levels in semen samples were estimated calorimetrically using MDA as standard by Dhindsa et al. [42]. The ample (25 μl) was homogenized in 0.1 % trichloroacetic acid and centrifuged for 5 min at 14,000×*g*. Then, trichloroacetic acid (20 %) consisting thiobarbituric acid (0.05 %) was added in 1 ml aliquot of supernatants and heated for 30 min at water bath. Then reaction mixture was cooled and centrifuged for 10 min at 14,000×*g*. The absorbance of supernatants was computed at 535 nm and the value of non-specific absorbance (600 nm) was subtracted from it. The MDA contents were measured by a coefficient of extinction 155 per mM per cm.

2.4.11. Total oxidant status

The procedure of total oxidant status (TOS) is based on the oxidation of iron and iron complex to an acidic medium by Dhindsa et al. [42]. Ferric ions interact with xylenol orange and form a particular colored complex. The intensity of the color is linked with quantity of oxidant molecule and can be measured using spectrophotometer. The mixture for analysis of TOS is consisted of reagentR1; xylenol orange solution (0.38 g in 500 μL of 25 Mm H2SO4), reagent R2; (o-dianisidine 0.0317 g, ferrous ammonium sulfate (II) 0.0196 g, NaCl 0.4 g, glycerol 500 μL); and the sample. The absorbance can be recorded at 520 nm after 5 min. The standard used were 5, 10, 15, 20, 25, 30 μM/ml hydrogen peroxide equivalents. The calibration curve of H_2O_2 was used to calculate TOS and is denoted in μmol H_2O_2 equivalent mL^{-1}.

2.4.12. Total antioxidant capacity

The TAC samples were measured based on the reduction of a blue radical cation (ABTS•+) to its original ABTS colorless form by antioxidants by Dikilitas et al. [43]. The solution to detect TAC consisted of reagent R1 (CH3COONa buffer and glacial acetic acid), R2 (H2O2, Na3PO4 buffer, glacial acetic acid, and ABTS), and sample. The reacted solution was incubated at 6 min on room temperature and absorbance was taken at 734 nm. The standards used were 0, 0.075, 0.125, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2.0). The TAC was calculated using ascorbic acid as a calibration curve and represented as ascorbic acid (μM) equivalent per milliliter of sample.

3. Statistical analysis

To check whether the data is normal, Shapiro–Wilk test was applied. The data were analyzed using general linear model procedure (ANOVA) and Dunn's multiple comparisons test was performed to compare the means of different groups using SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA). The lineal model included the effects of the four concentrations of resveratrol and control group). Results are expressed as mean ± standard error. Probability values < 0.05 were taken to indicate statistical significance. Prism GraphPad (8.4.3) was used to make graphs. PCA, dendogram and spearman correlation coefficient analysis were performed using MetaboAnalyst and SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA). The spearman correlation coefficient (rho) and *p*-values were made manually in Microsoft word.

4. Results

4.1. Frozen-thawed sperm kinematics

The varying levels of resveratrol treated post-thaw sperm parameters are displayed in Table 1. The resveratrol had found significant effect in all parameters ($P < 0.05$). The results revealed adding 10, 20, 40 and 50 μM resveratrol significantly enhanced TM ($P < 0.001$), PM ($P < 0.001$), RV ($P < 0.001$), VAP ($P < 0.002$), VSL ($P < 0.001$), VCL ($P < 0.001$), ALH ($P < 0.001$), BCF ($P < 0.001$), STR ($P < 0.001$) and LIN ($P < 0.005$) in response to control group respectively. Overall, 50 μM and 40 μM resveratrol were found significantly ($P < 0.05$) efficient in comparison with other groups.

4.2. Permeable membrane integrity, acrosome integrity, viability and DNA integrity

The plasma membrane and acrosome integrity, viability and the DNA integrity of frozen-thawed resveratrol treated spermatozoa are shown in Table 2. The resveratrol had shown positive impact ($P < 0.001$) on post-thawed membrane integrity at 10, 20, 40 and 50 μM in contrast with control group. Resveratrol had also observed significantly higher impact ($P < 0.001$) on viable sperm with intact acrosome and DNA integrity of spermatozoa ($P < 0.001$) in response to control group respectively. Resveratrol has failed to provide any effect on viability of spermatozoa ($P > 0.121$) in all treated groups. Overall, 40 and 50 μM resveratrol were found efficient in improving sperm structural parameters than control and other groups (see Table 3).

4.3. Antioxidant/oxidant status

The results of incorporating resveratrol in semen extender on post-thawed antioxidant/oxidant indices are shown in (Fig. 1-A to Fig. 1-F). Adding resveratrol significantly increased ($P < 0.001$) SOD, CAT, POD and TAC levels, while TOS and MDA levels were reduced ($P < 0.001$) in treated groups as compared to control group respectively. Altogether, 50 μM resveratrol had observed slightly better performance than 40 μM resveratrol in improving antioxidant status and reducing oxidant levels.

Table 1
Effect of inclusion resveratrol in cyro-diluent on post-thawed spermatozoa quality index in Kamori buck spermatozoa.

Parameters	Groups					*P value*
	Control	10 μM	20 μM	40 μM	50 μM	
TM (%)	50.0 ± 2.94^{c}	63.33 ± 0.33^{b}	66.67 ± 0.33^{b}	73.33 ± 1.76^{a}	74.66 ± 1.79^{a}	<0.001
PM (%)	17.67 ± 0.66^{c}	20.0 ± 0.57^{bc}	23.0 ± 1.73^{b}	30.33 ± 1.20^{a}	30.33 ± 0.33^{a}	<0.001
RV (%)	31.33 ± 0.89^{b}	32.33 ± 0.89^{b}	34.0 ± 2.04^{b}	39.0 ± 0.68^{a}	39.33 ± 0.66^{a}	<0.001
VAP (μm/s)	62.07 ± 0.88^{c}	67.17 ± 1.53^{b}	71.67 ± 1.75^{ab}	72.63 ± 1.61^{a}	70.1 ± 1.06^{ab}	<0.002
VSL (μm/s)	41.53 ± 0.89^{d}	45.5 ± 0.45^{c}	48.13 ± 1.26^{b}	49.7 ± 0.27^{ab}	51.57 ± 0.65^{a}	<0.001
VCL (μm/s)	98.06 ± 0.09^{c}	99.6 ± 0.36^{c}	100.73 ± 0.55^{c}	106.83 ± 1.66^{b}	113.6 ± 3.46^{a}	<0.001
ALH (μm)	4.33 ± 0.15^{c}	4.47 ± 0.09^{c}	4.7 ± 0.10^{bc}	5.07 ± 0.12^{b}	5.6 ± 0.21^{a}	<0.001
BCF (Hz)	17.3 ± 0.36^{c}	18.67 ± 0.26^{b}	18.77 ± 0.12^{b}	19.9 ± 0.20^{a}	20.53 ± 0.48^{a}	<0.001
STR (%)	68.0 ± 0.59^{b}	66.67 ± 0.33^{b}	67.67 ± 0.33^{b}	71.67 ± 0.68^{a}	71.33 ± 0.89^{a}	<0.001
LIN (%)	47.67 ± 0.33^{b}	48.0 ± 0.58^{b}	50.67 ± 0.33^{a}	49.67 ± 0.67^{a}	49.67 ± 0.33^{a}	<0.005

Data are presented as mean ± Standard error. (n = 3 replications of each sample). Abbreviations. Total motility (TM), Progressive motility (PM), Rapid velocity (RV), Average path velocity (VAP), Straight line velocity (VSL), Curvilinear velocity (VCL), Amplitude of lateral head displacement (ALH), Beat cross frequency (BCF), Straightness (STR), Linearity (LIN), $^{a\text{-}c}$Mean values sharing different superscripts differ significantly (P < 0.05).

4.4. Principal component analysis (PCA)

To analyze the variation in resveratrol supplemented sperm parameters, the data was subjected to principal component analysis to examine the interrelationship between different variables. From two principal component analyses, i.e, PC-1 and PC-2 showed 83.2 % total accumulative variability across the treated samples (Fig. 2). The contribution of PC-1 had greater variability (62.6 %), followed by PC2 (20.6 %) respectively.

4.5. Agglomerative hierarchical clustering (AHC) dendogram

In this study, the results of clustering analysis of resveratrol supplemented freeze-thawing sperm parameters are shown in Fig. 3. Cluster analysis assembled five treated groups into two clusters. The control and Res-10 groups were constructed in one cluster, while Res-20, 40 and 50 were gathered in second cluster. In cluster, 1 and 2, the similarity and distance of treated groups were exhibited and are displayed in Fig. 2.

4.6. Spearman correlation coefficient analysis

The results of spearman correlation coefficient of resveratrol supplemented freeze-thaw sperm parameters are illustrated in Table 1. The different color patterns showed the different levels of highly positive and highly negative correlations between the treated sperm parameters. Specifically, the parameter values marked with yellow color showed perfect significant association while, parameter values ed with turquoise color exhibited perfect negative associations.

5. Discussions

Sperm kinematic and antioxidant parameters are the potential predictors of sperm quality indices [24]. Our study showed that inclusion of resveratrol in semen extender significantly improved sperm motility, velocity and kinematics parameters in all treated groups as compared to control group respectively. Amidst all resveratrol treated groups, 50 μM and 40 μM was found best concentration in response to sperm quality parameters but somewhere 50 μM performed a better response than 40 μM. Resveratrol has ability to inhibit lipid peroxidation and free radical scavenging activities through employing various pathways [44]. In previous studies, resveratrol was found effective in preservation of sperm fertility in several species using different concentrations of resveratrol at 10 μM, 25 μM, 50 μM [45,24,46,47].

The fertilizing potential of the spermatozoa are assessed by intact membrane and acrosomal integrity. Our results exhibited that 50 μM of resveratrol significantly improved membrane and acrosomal integrity than other groups respectively. Hence, our results match with Zhu et al. [48], who observed 50 μM resveratrol enhanced acrosome and plasma membrane integrity in frozen-thawed ram spermatozoa. During freeze-thawing process, ROS induced lipid peroxidation and DNA damage that alter sperm function and ultimately affect fertilizing ability of spermatozoa [17]. Addition of resveratrol improved plasma membrane integrity and acrosome intactness in cryopreserved goat semen [24] and inclusion of resveratrol in buck semen reduced lipid peroxidation, increased motility and declined apoptosis [49]. Overwhelming oxidant production leads to cause spermatozoa DNA damage during freeze-thawing process [50]. We observed that 50 μM of resveratrol reported higher DNA integrity in comparison with other groups. The resveratrol gives protection to spermatozoa structural components against free radical's damage [51,52]. In a previous study, resveratrol has found protective effect on DNA damage in human sperm [51]. The positive effect is likely occurred due to the enhanced antioxidant

Table 2
Effect of resveratrol on freeze-thawing plasma membrane integrity, viable sperm with intact acrosome and DNA integrity of buck spermatozoa.

Parameter (%)	Groups					
	Control	10 μM	20 μM	40 μM	50 μM	*P-value*
PMI	64.5 ± 1.07^{c}	67.93 ± 0.30^{b}	69.00 ± 0.33^{b}	72.53 ± 0.98^{a}	72.76 ± 0.50^{a}	<0.001
ACR	63.13 ± 0.83^{d}	66.63 ± 0.54^{c}	67.96 ± 0.41^{bc}	70.23 ± 0.53^{ab}	71.20 ± 0.25^{a}	<0.001
Viability	66.44 ± 0.79	68.51 ± 0.92	70.60 ± 0.41	70.35 ± 3.60	73.44 ± 0.39	0.121
DNA integrity	94.13 ± 1.00^{b}	97.13 ± 0.50^{a}	97.33 ± 0.23^{a}	98.90 ± 0.37^{a}	99.27 ± 0.21^{a}	<0.001

Mean ± Standard error. Values of three independent replicates for Plasma membrane integrity (PMI), Viable sperm with intact acrosome (V/IACR) and percentage of live spermatozoa (viability) and DNA integrity. (n = 3 replications of each sample). $^{a-d}$Mean values sharing different superscripts differ significantly (P < 0.05).

Table 3
Spearman correlation coefficient of resveratrol treated Kamori buck spermatozoa parameters.

TM	TM	PM	RV	VAP	VSL	VCL	ALH	BCF	STR	LIN	PMI	ACR	LIVE	DNA integrity	POD	CAT	TAC	SOD	TOS	APX	MDA
TM	1.00																				
PM	Rho=0.98 *p*=0.0001	1.00																			
RV	Rho=0.87 *p*=0.0001	Rho=0.88 *p*=0.0001	1.00																		
VAP	-	-	-	1.00																	
VSL	Rho=0.90 *p*=0.0001	Rho=0.89 *p*=0.0001	Rho=0.82 p=0.0003	-	1.00																
VCL	Rho=0.94 *p*=0.0001	Rho=0.89 *p*=0.0001	R=0.90 *p*=0.0001	-	Rho=0.91 *p*=0.0001	1.00															
ALH	Rho=0.91 *p*=0.0001	Rho=0.93 *p*=0.0001	Rho=0.91 *p*=0.0001	-	Rho=0.88 *p*=0.0001	Rho=0.89 *p*=0.0001	1.00														
BCF	Rho=0.90 *p*=0.0001	Rho=0.85 *p*=0.0001	Rho=0.82 *p*=0.0004	-	Rho=0.81 *p*=0.0004	Rho=0.94 *p*=0.0001	-	1.00													
STR	-	-	Rho=0.82 *p*=0.0003	-	-	-	Rho=0.82 *p*=0.0003	-	1.00												
LIN	-	-	-	-	-	-	-	-	-	1.00											
PMI	Rho=0.98 *p*=0.0001	Rho=0.98 *p*=0.0001	Rho=0.87 *p*=0.0001	-	Rho=0.87 *p*=0.0001	Rho=0.92 *p*=0.0001	Rho=0.93 *p*=0.0001	Rho=0.89 *p*=0.0001	-	-	1.00										
ACR	Rho=0.93 *p*=0.0001	Rho=0.93 *p*=0.0001	-	-	Rho=0.85 *p*=0.0001	Rho=0.91 *p*=0.0001	Rho=0.91 *p*=0.0001	Rho=0.90 *p*=0.0001	-	-	Rho=0.97 *p*=0.0001	1.00									
LIVE	-	-	-	-	-	-	-	-	-	-			1.00								
DNA integrity	Rho=0.85 *p*=0.0001	Rho=0.84 *p*=0.0002	-	-	-	Rho=0.90 *p*=0.0001	Rho=0.82 *p*=0.0003	Rho=0.89 *p*=0.0001	-	-	Rho=0.88 *p*=0.0001	Rho=0.89 *p*=0.0001	-	1.00							
POD	-	-	-	-	-		-		-		-		-	-	1.00						
CAT	-	-	-	-	-		-		-	Rho=0.83 *p*=0.0003	-		-	-	-	1.00					
TAC	-	-	-	-	-		-		-	Rho=0.86 *p*=0.0001	-		-	-	-	Rho=0.94 *p*=0.0001	1.00				
SOD	-	-	-	-	-		-		-	-	-		-	-	-	Rho=0.85 *P*=0.0001		1.00			
TOS	-	-	-	-	-		-		-	-	-		-	-	-	R=-0.85 P=0.0001	r=-0.88 P=0.0001	-	1.00		
APX	-	-	-	-	-		-				-		-	-	-	-	-	-	-	1.00	
MDA	Rho=0.96 *p*=0.0001	Rho=-0.91 *p*=0.0001	Rho=-0.83 *p*=0.0001	-	Rho=-0.93 *p*=0.0001	Rho=-0.96 *p*=0.0001	Rho=-0.89 *p*=0.0001	Rho=-0.91 *p*=0.0001	-	-	Rho=-0.94 *p*=0.0001	Rho=-0.95 *p*=0.0001	-	Rho=-0.89 *p*=0.0001	-	-	-	-	-		1.00

Yellow color exhibited highly significant association, while turquoise color showed highly negative association between sperm variables. In this spearman co-efficient correlation, only values > 0.80 are considered.

Yellow color exhibited highly significant association, while turquoise color showed highly negative association between sperm variables. In this spearman co-efficient correlation, only values > 0.80 are considered.

potential of cryopreserved semen in response to inclusion of resveratrol that maintain DNA integrity improve fertilizing potential.

Spermatozoa are naturally embedded with antioxidant defense that is encompassing of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reeducates [53]. Overproduction of ROS significantly impairs sperm structural and functional intactness and eventually causes reduced fertility [29]. In our study, different levels of resveratrol significantly increased SOD, CAT and POD levels by reducing TOS and MDA contents thereby improved TAC activity, suggesting the protective effects of quercetin against ROS triggered cyro-damage. Our results are comparable with previous studies on rooster and bovine semen in which varying levels of resveratrol significantly increased SOD, CAT, GPx and reduced MDA and ROS concentration [54,55]. Enzymatic antioxidants such as SOD, CAT and GPx are naturally localized within cells to remove excess ROS [56]. Addition of resveratrol increased the levels of SOD, CAT, GSH, and GPx that can inhibit oxidative stress [57,58,59]. Resveratrol, an antioxidant compound, has been found to have beneficial effects on both fresh and frozen-thawed sperm parameters in boar, ram, buck and bull sperms [33,49,60,61]. Resveratrol can neutralize and suppress free radical production in semen, leading to a decrease in lipid peroxidation [2]. Studies have shown that resveratrol can enhance TAC contents, leading to improved semen parameters in bovine semen [62,63]. Barranco et al. [64], revealed that high contents of TAC in boar seminal plasma were related to sperm survival and fertility and can be used as a marker of predicting fertility.

Principal component analysis (PCA) is a statistical method that breakdown large data sets into smaller ones, focusing on the most significant variables [65,66]. Using PCA, resveratrol-supplemented freeze-thaw spermatozoa samples exhibited distinct clusters except Res-40 and Res-50 had partially merged, showing some similar sperm characteristics. The PC-1 and PC-2 showed total variability 83.2 %, derived from PC-1 (62.6 %), and PC-2 (20.6 %). Overall, Res-50 μM had slightly better response than Res 40 μM in improving sperm quality characteristics.

The Agglomerative hierarchical clustering (AHC) dendrogram was implied to assess the degree of similarity or dissimilarity among resveratrol supplemented freeze-thaw sperm indices. In our results, two clusters were created from five treatment groups. The control and Res-10 groups encompassed in one cluster while Res-40 and Res-50 and Res-20 were constituted in second cluster. Overall, clustering analysis revealed that Res-40 and 50 μM had almost similar effects showing the significant positive correlation.

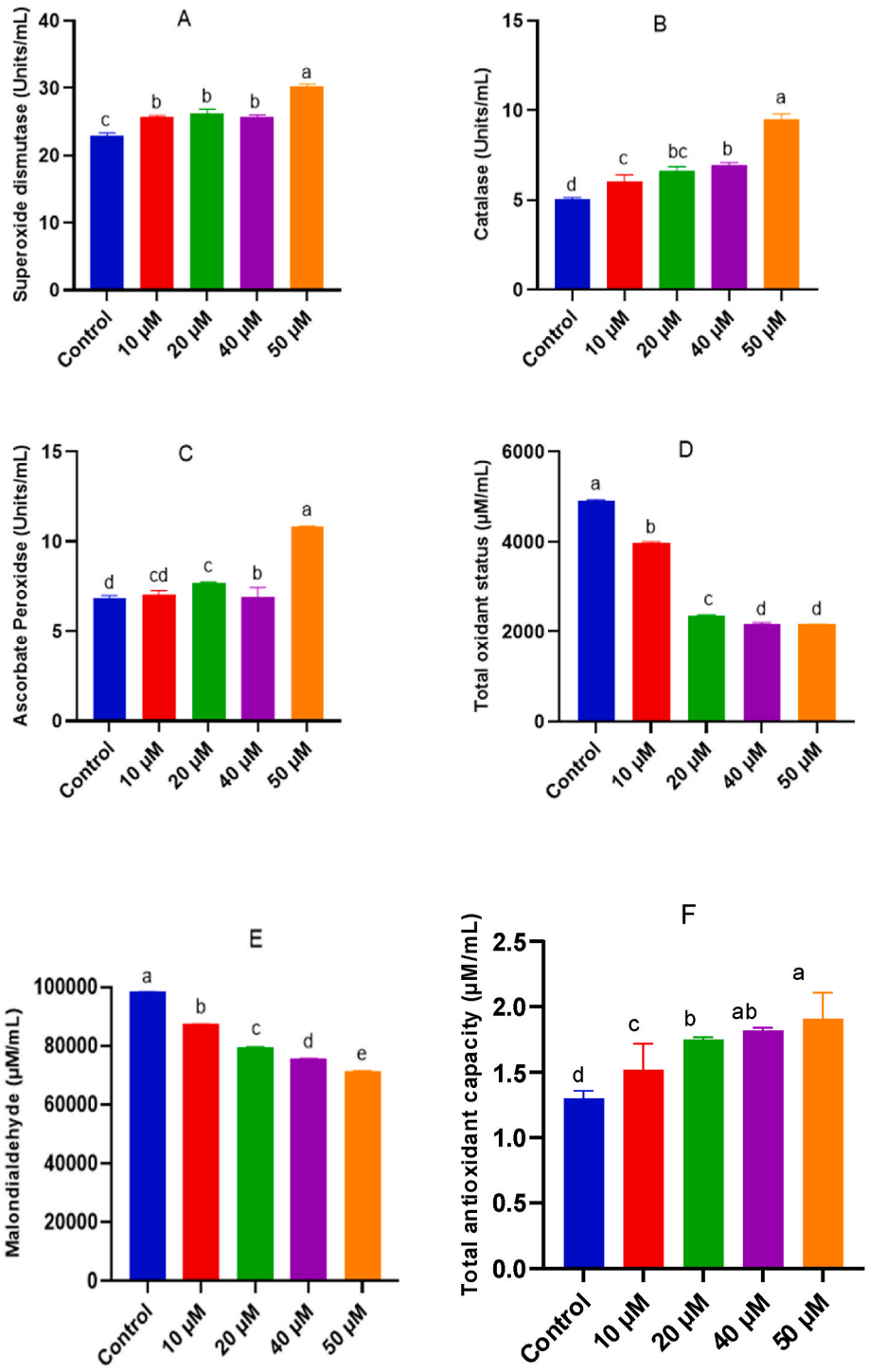


Fig. 1. Impact of adding resveratrol on freeze-thawing antioxidant/oxidant status in Kamori buck spermatozoa
The results are show in graphs. (n = replications of each sample). [a-d] Mean values sharing different superscripts differ significantly ($P < 0.05$).

Spearman correlation coefficient is a non-parametric statistical tool used to measure the correlation between different variables [67]. The correlation coefficient values ranged from +1 to −1. In our results, yellow and turquoise colors exhibited highly positive and negative correlations between various sperm parameters. In this one, values > 0.80 were considered to find out the perfect correlations between different sperm variables.

Sperm structures protect sperm cells and thus maintain the sperm quality. Overproduction of oxidants decreases antioxidant potential, leading to decline in sperm fertility [68]. Sperm motility and velocity parameters are influenced by factors such as age, collection time, ejaculation interval, energy, cell membrane particles, minerals, hormones, and prostaglandins [69]. Sperm motion parameters can be associated with individual sperm velocity index [70]. The ultra-freezing process of spermatozoa requires measurements of DNA integrity and higher mitochondrial membrane potential as essential variables [71,72]. ALH reveals sperm-oocyte interaction, while sperm velocities are categorized as VAP, VSL, STR, and LIN, which are linked to fertilizing potential [73]. Our findings are somewhat similar to Díaz Ruiz [74] which analyzed pearon correlation and other parameters to determine the relationships

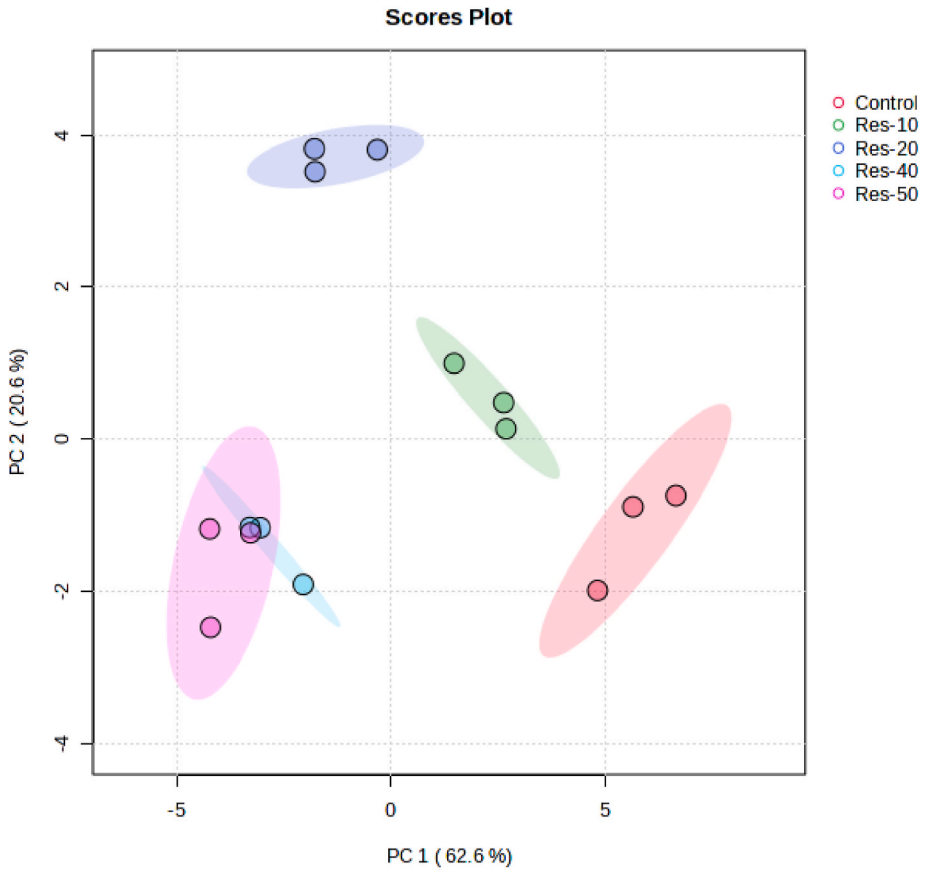


Fig. 2. Principal component analysis (PCA) of varying levels of resveratrol treated Kamori buck spermatozoa parameters.

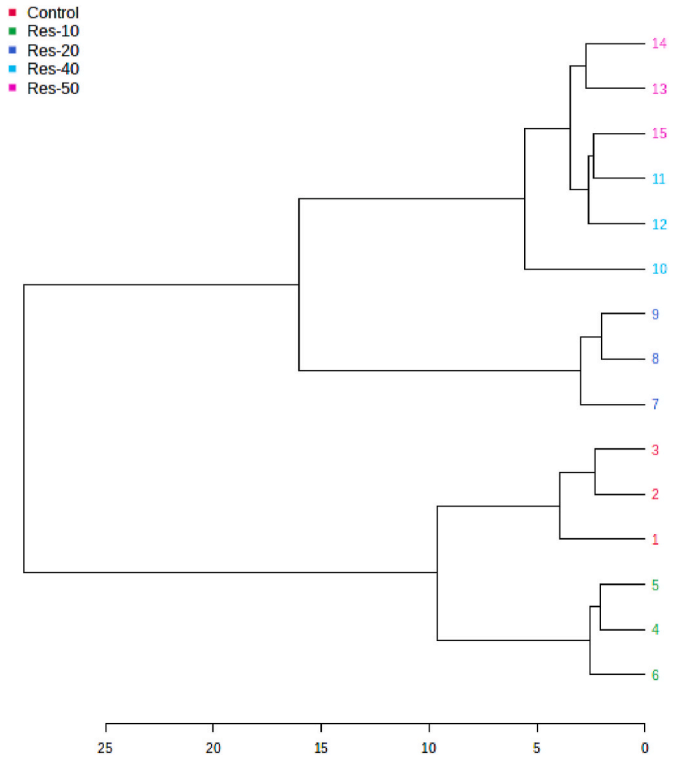


Fig. 3. Agglomerative hierarchical clustering (AHC) dendrogram of resveratrol treated Kamori buck spermatozoa parameters.

between sperm variables. Perumal et al. [75] found a positive correlation between BCF and ALH, indicating higher PFM, VAP, progressive velocity, and track speed. The plasma membrane and acrosome's integrity are crucial in the female reproductive tract, especially in facilitating ovum penetration [76]. Sperm cryopreservation can cause lipid peroxidation, DNA damage, oxidative stress and acrosome reactions leading to poor sperm quality [77]. TOS is a free radical cause's sperm damage, while MDA is implied as a biomarker of oxidative damage [78]. Enzymatic antioxidants such as SOD, CAT and TAC protect sperm cells from oxidant damages while, TOS and MDA damage sperm cells. The addition of antioxidants not only reduces oxidant production but also enhances antioxidant potential, thereby ensuring the maintenance of sperm quality [9].

6. Conclusions

In conclusion, our results revealed that resveratrol (50 μM and 40 μM) supplemented semen extender based on tris-citric acid-glycerol egg yolk (TCG-EY) mitigated oxidant production, enhanced antioxidant profile, and promoted sperm kinematics parameters. But, 50 μM resveratrol slightly performed better response in terms of enhancing sperm quality indicators. However, the response of mitigating oxidative stress and promoting antioxidant status need to be addressed at molecular level. Moreover, the fertility trials should be conducted using 50 μM and 40 μM resveratrol concentration on large scale basis.

CRediT authorship contribution statement

Tarique Hussain: Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Muhammad Hammad Fayyaz:** Validation, Software, Methodology, Formal analysis, Data

curation. **Amjad Hameed:** Validation, Software, Methodology, Formal analysis, Data curation. **Syed Murtaz Hassan Andrabi:** Supervision, Software, Methodology, Formal analysis, Data curation. **Rehana Kausar:** Visualization, Validation, Supervision, Investigation. **Yasin Mubashir:** Visualization, Supervision, Formal analysis, Data curation. **Iqra Batool:** Validation, Supervision, Investigation, Formal analysis, Data curation. **Muhammad Shahzad:** Data curation, Methodology, Supervision, Validation. **Ali Dogan Omur:** Writing – review & editing, Visualization, Software, Methodology, Formal analysis.

Conflict of interest

All authors showed no conflict of interest.

Data availability statement

The data is available on the reasonable request to corresponding author.

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