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Research article

Green tea extract increases the quality and reduced DNA mutation of post-thawed Kacang buck sperm

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ABSTRACT

The study aimed to determine the addition of green tea extract (GTE) in extender on the quality and DNA mutation of post-thawed Kacang buck sperm. The sperm DNA mutation was observed on nicotinamide adenine dinucleotide hydride (NADH) dehydrogenase 1 (ND1) of mitochondrial Deoxyribonucleic Acid (mtDNA). A pool of 12 Kacang buck ejaculates was diluted in skim milk-egg yolk extender contained 0, 0.05, 0.10, and 0.15 mg of GTE/100 mL for T0, T1, T2, and T3 group, respectively. Each of the aliquot groups was packaged in 0.25 mL French mini straw contained 60 million alive sperm and froze according to the protocol. The ND1 mtDNA amplification of samples was carried out Polymerase Chain Reaction machine, followed by DNA sequencing using the Sanger method. Meanwhile, the phylogenetic tree was constructed using the neighbor-joining (NJ) method with MEGA 7.0 software. The results showed that the T2 group maintained the highest quality for Kacang buck post-thawed semen. There was the highest percentages of sperms viability, motility, intact plasma membrane (IPM), the lowest of malondialdehyde (MDA) concentration, sperm DNA fragmentation (SDF), the total and types of ND1 mtDNA mutation frequency. The phylogenetic tree analysis revealed that the clade of the T2 group was most closely related to the sequence reference. However, there was no correlation between the semen quality parameters (sperm viability, motility, IPM, MDA concentration, and SDF) with ND1 mtDNA mutation of post-thawed Kacang buck semen. It could be concluded that GTE was useful as an antioxidant for Kacang buck semen extender for frozen sperm.

1. Introduction

Kacang goats (*Capra hircus*) are an indigenous Indonesian livestock species that can be reared by rural communities as an additional source of income to prevent poverty and to provide animal protein. Breeding of Kacang goat through artificial insemination (AI) technique expected to accelerate the population. Unfortunately, the frozen semen of Kacang buck semen has not available yet. Kacang buck sperm's main problem is that goat sperm is more susceptible to cold shock stress than the other species [1, 2]. Kacang buck's sperm is more sensitive to cryo-damaged than Ettawa goat, ram, and Simmental bull's semen. Post-thawed of Kacang buck semen, which extended without any antioxidant, about 60% of the sperm die, leaving less than 40% of the motile sperm [3]. This post-thawed motility has not met the artificial insemination (AI) requirement, which must more than 40% [4]. It was caused by the freezing process of semen leads to the overproduction of reactive oxygen species (ROS). The plasma membranes contain large quantities of polyunsaturated fatty acids (PUFA), susceptible to oxidation. The cytoplasm contains antioxidant enzymes but low concentrations due to the low volume of the cytoplasm itself. Seminal plasma also contains both enzymatic and nonenzymatic antioxidants. The enzymatic antioxidant includes superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and catalase (CAT). Meanwhile, the nonenzymatic antioxidant covers glutathione (GSH), vitamins, minerals, amino acids, and proteins. Those antioxidants protect sperm from oxidative stress; however, their concentration decreases and becomes insufficient to prevent oxidation after the extender's addition [5].

Oxidative stress may result in an imbalance between ROS generation and endogenous antioxidant activities. The higher ROS causes peroxidation of PUFAs, especially docosahexaenoic acid, which has six double bonds per molecule in sperm cell membranes. One of the products of lipid peroxidation is MDA [6]. The MDA level is an indicator of plasma

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membrane damage followed by reduced sperm viability and motility [5]. The DNA bases and phosphodiester backbones of sperm are also susceptible to oxidative stress [7]. Free radicals may cause lipid peroxidation on nuclear DNA base modifications. The change of 8-OH-guanine or 8-OH-20-deoxyguanosine destabilizes DNA structure and causes sperm DNA fragmentation (SDF) [8]. Additionally, cold sock causes the opening of interchain disulfide bridges in protamines that may interfere with sperm DNA [9]. DNA integrity is necessary for embryo development [10]. Higher sperm SDF resulted in lower fertilization rates [11], lower pregnancy rate [12], and finally, lower pregnancy rate [3].

The extender was essential to maintaining semen quality during freeze-thawing semen. The skim milk-egg yok (SM–EY) extender was chosen for this study. The proteins in skim milk functioned as a pH buffer. The sulfhydryl groups, β-lactoglobulin, and phosphocaseinase of skim milk prevent sperm from the harmful effects of sperm metabolism byproducts themselves. The casein micelle of skim milk maintains plasma membrane stability and protects sperm from the ROS action [13]. Cholesterol derived from egg yolk maintains plasma membrane stability [14] and inhibits DNA damage [15]. The addition of cholesterol in extenders before cryopreservation will increment the cholesterol membrane content, protecting sperm against cryodamage [5]. Skim milk and egg yolk's combination proved better protection during the freezing process in ram semen [16]. The antioxidants addition is needed to balance the excessive ROS during the freeze-thawing process and enhance sperm function by neutralizing ROS and reducing oxidative stress [17, 18] and improves sperm cryo survival [5]. Green tea extract (GTE) is a plant-derived antioxidant that contains epigallocatechin-3 gallate (EGCG). The EGCG has been reported to have a potent antioxidant and to be able to decrease lipid peroxidation and protein carbonylation [19]. The EGCG has been shown to improve the quality of post-thawed ram sperm [20], lowering oxidative stress, increases post-thaw microscopic parameters of sperm buffalo [21], and decrease DNA damage [19].

The mitochondrial DNA (mtDNA) is maternal inheritance [22]. This means the mtDNA in the Kacang buck sperm also comes from the dam. The mtDNA encodes respiratory chain components required for oxidative energy metabolism [23]. Genetic variants of mtDNA were associated with male infertility [24]. Mutation of mtDNA is correlated to reproductive capacity [22]. The mtDNA genetic variants in the NADH dehydrogenase 1 (ND1) genes may cause decreasing activities of mitochondrial respiratory chain complexes [25]. Best to our knowledge, there has not yet revealed mtDNA mutation on post-thawed Kacang buck sperm, even in other livestock animals. The present study aimed to determine whether the GTE addition in SM-EY extender increased the semen quality (sperms viability, motility, IPM, MDA levels, SDF) and reduced the ND1 mtDNA mutation of post-thawed Kacang buck sperm.

2. Material and methods

This study was conducted at The Regional Artificial Insemination Center, the Faculty of Veterinary Medicine, Universitas Airlangga, located in the village of Tanjung, Kedamean, Gresik District, East Java, Indonesia. Furthermore, DNA analysis laboratory at the Animal Disease Diagnostic Laboratory, Faculty of Veterinary Medicine, Brawijaya University, Lowokwaru, Malang, East Java, Indonesia.

2.1. Ethical approval

This study's proposal was approved by The Animal Care and Use Committee, Airlangga University, Surabaya, Indonesia, No 520/HRECC.FODM/VII/2019. The assessment was based on the animal welfare principles, including the UK Animals (Scientific Procedures) Act 1986 and its associated guidelines (EU Directive 2010/63/EU) for animal experiments. Buck semen was collected according to the protocol of Chapter 4.7 ("The collection and processing of bovine, small ruminant, and porcine semen") the Terrestrial Animal Health Code of the World Organization for Animal Health [26].

2.2. GTE preparation

A total of 1.5 kg of green tea leaves (*Camellia sinensis* L. Kuntze, herbarium accession number TRI-2025, Indonesia Research Institute for Tea and Cinchona, Indonesia) were dried and then ground to a particle size of 0.75 μm. The ground leaves were soaked in 96% ethanol for three days in containers covered with aluminum foil to prevent evaporation. The green tea solution was then filtered through standard cellulose extraction filter paper and thimbles (Whatman) that had 0.5 μm nominal pore size. The filtrate was evaporated at 50 °C in a rotary evaporator at 45 RPM to separate the solvent's green tea extract. The extract was then evaporated in an acid chamber until a solid was formed and then freeze-dried to obtain ready-to-use dried GTE. The extract was stored at −20 °C [27].

2.3. Experimental animals

Four head of Kacang bucks (2–3 years; 35–45 kg) were kept in individual stalls. All of those animals were fed 5 kg forage and 3.5 kg concentrate (16%–18% crude protein) daily and *ad libitum* drinking water.

2.4. Semen collection

An artificial vagina was used for semen collection twice a week. A total of 12 ejaculate samples were examined for progressive individual movements. If the sperm motility percentage of fresh semen was reached at least 70% [4], the ejaculate was further processed as frozen semen.

2.5. Skim milk–egg yolk extender

Ten grams of skim milk powder (Merck 115338) were dissolved in 100 mL of distilled water, heated to 92–95 °C for 10 min, and subsequently cooled to 37 °C. The skim milk solution was added to 5 mL of egg yolk derived from laboratory chicken eggs to obtain 100 mL of the skim milk-egg yolk mix. Next, penicillin (1.000 IU/mL) and streptomycin (1 mg/mL) were added to the mixture [27]. The aliquot was divided into four equal portions, for the control group (T0, without GTE) and the treatment groups T1 (0.05 mg GTE), T2 (0.1 mg GTE), and T3 (0.15 mg GTE) added per 100 mL extender.

2.6. Frozen semen

Each group of aliquots (as mentioned in section 2.5) was further divided into two equal amounts. The first portion was added to the semen samples to obtain a final concentration of 480 million spermatozoa/mL. The second was added with glycerol to reach 16% concentration, then slowly added into the first mixture equally to obtain a concentration of 240 million spermatozoa/mL extended semen, then equilibrated at 5 °C for one hour. The extended semen was packaged in 0.25 mL French straws at 60 million sperm/straw. The filled straws were placed on the Cold Handling Cabinet (Minitube), held in liquid nitrogen vapor (−140 °C) for 10 min, immersed, and stored immediately in liquid nitrogen (−196 °C) for two weeks before it was assessed [3].

2.7. Post-thawed sperm quality assessment

Straw samples from each group were thawed in sterile water at 37 °C for 30 s to evaluate six replicates' post-thawed semen quality. Sperm viability, progressive motility, IPM, MDA, and SDF were evaluated. Eosin-nigrosin stained of the dry-smeared sample was used for sperm viability. The live sperm were marked by their bright transparent heads; meanwhile, the head of the dead sperm was a reddish color. The Computer-assisted Sperm Analyzer (CASA) method was used to measure sperm motility. Only sperm with progressive motility was counted as motile sperm. The hypoosmotic swelling test was used to evaluate the

IPM. The intact plasma membranes were marked by circular tails; meanwhile, the damaged plasma membranes were characterized by straight tails. The thiobarbituric acid method was used for MDA concentration measurement [3]. Toluidine blue staining was used for SDF assessment. Bright blue spermatozoa heads indicated intact chromatin integrity (normal DNA), whereas dark blue heads indicated reduced chromatin integrity (fragmented DNA) [27]. The sperm viability, motility, IPM, and SDF measurement was conducted in 400 spermatozoa under the light microscope (Olympus BX-53, Japan) at 400× magnification.

2.8. Amplification and sequencing of the subunit 1 NADH dehydrogenase gene

Amplification of the subunit 1 NADH dehydrogenase (ND1) mtDNA housekeeping gene was performed before sequencing. The primer used for this process were AB 736120.1 F: 5′ CCCATACCCTACCCCCTCAT 3′, R: 5′ GGGGTAGGATGCTCGGATTC 3'. The primer was designed using the Primer3plus program based on goat (*Capra hircus*) sperm ND1 Capra mtDNA (ID number: 1485856) from the NCBI Gene Bank (www.ncbi.nlm.nih.gov). The amplification was carried out with a 35-cycle Polymerase Chain Reaction (PCR) machine, using the QIAamp DNA Mini Kit (Qiagen). Initial denaturation was performed at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing 60 °C for 45 sec (gradient: ± 4 °C), extension at 72 °C for 1 min, and post extension at 72 °C for 1 min. The cycle begins with a hot start at 94 °C for 2 min and ends with incubation at 72 °C for 5 min. The measurement of DNA quantity was carried out by the spectrophotometric method at a wavelength of 260 and 280 nm. DNA purity was determined based on the absorbance (A) ratio A260 to A280 [28]. The amplification was observed on the 2% agarose gel electrophoresis stained with red nucleic acid gel dye. The 2 μl of PCR product was put into a 2% gel agarose well placed in the electrophoresis device, then Tris-borate-Ethylene diamine tetra-acetic acid (TBE 10x, Promega Corporation) was added to the limit covering the surface of the agarose gel. The DNA electrophoresis tool was set with 400 V 100mA for 35 min [29]. The Sanger method was used for DNA sequencing; meanwhile, the MEGA 7.0 software was used to read the sequence of nucleotide bases and the neighbor-joining phylogenetic tree construction with 1,000 bootstrap replicates [30].

2.9. Data analysis

The sperm quality was evaluated based on its viability, motility, IPM, MDA concentrations, SDFs, and ND1 mtDNA fragment mutation frequency. Sperm quality among the group was evaluated with analysis of variance (ANOVA) and followed by the Tukey Honestly Significant Difference (HSD) test. Pearson's Product-Moment Correlation use for analyzing the correlation of semen quality with ND1 mt DNA sperm mutation. All statistical analysis was conducted to a 95% level of significance using Statistical Product and Service Solutions (SPSS) Version 23. Meanwhile, the type of ND1 mtDNA sperm mutation was presented as descriptive statistics.

3. Results

The sperm motility is the main indicator of semen quality as a requirement for AI. The minimum sperm motility of ejaculates that qualify for froze was 70% [4]. The result showed that the average sperm motility of Kacang buck ejaculate was 89.02 ± 1.77% (Table 2); this was higher than the standard.

3.1. The quality of post-thawed semen

There was a sharp decrease in the quality of fresh semen to the post-thawed semen without adding GTE to the extender. The sperm viability, motility, and IPM were decreased from 91.86 ± 1.49, 89.02 ± 1.77, and 85.75 ± 1.78% in fresh semen (Table 1) to 33.55 ± 0.15, 29.63 ± 0.45, and 28.80 ± 0.29% in the control group (Table 2). The addition of GTE 0.1 mg/100 mL extender showed the best semen quality ($p < 0.05$), with the highest sperms viability, motility, and IPM and the lowest MDA concentration (Table 2), and the lowest SDF (Table 3).

3.2. Mutation of ND1 mtDNA of post-thawed semen

The ND1 mtDNA amplification target was 550–575 bp band, and reach 560 bp band (Figure 1). Meanwhile, the electrophoresis results of PCR products were presented in Figure 2. The lowest ($p < 0.05$) mutation frequency of ND1 mtDNA fragments was revealed in the T2 group (addition of GTE 0.1 mg/100 mL extender, Table 3). The characteristics of ND1 mtDNA mutation of post-thawed buck semen were based on groups presented in Table 4. There was no correlation between the semen quality parameters (sperm viability, motility, IPM, MDA concentration, and SDF) with ND1 mtDNA mutation of post-thawed Kacang buck semen (Table 5).

Based on characteristic joining in the phylogenic tree, T1 was an outgroup (pleisiomorphic character), T0, T2, and T3 as a synapomorphic character, while T2 a monophyletic character (Figure 3). Bootstrap confidence values of the phylogenic tree were moderate (T0) and moderate to good (T1, T2, and T3). The T2 genetic distance value was 0.008, which was nearest to the reference sequence compared to 0.34, 0.35, and 0.66 of T0, T3, and T1, respectively (Figure 3, Table 6).

4. Discussion

Kacang buck ejaculate's average motility meets for AI requirement (more than 70%) [4]. This percentage was better than another study's findings of 75.2% [31] but lower than the 94.8% [32]. In this study, Kacang buck ejaculates diluted in SM-EY extender with glycerol cryoprotectant. Sperm need cryoprotectants to survive the freezing process. The viability, motility, IPM, and DNA integrity of the sperm were susceptible to the freezing-thawing process [33] resulting from the extreme temperatures and osmolarities marked by ROS production [34]. The cryoprotectants should ideally have a low molecular weight. Glycerol is an intracellular cryoprotectant that can penetrate the cell, thereby preventing the formation of ice crystals that could otherwise result in membrane rupture [35].

4.1. The quality of post-thawed semen

During the freeze-thawing process, there was a reorganization of sperm membrane phospholipids, altering lipid-protein, lipid–carbohydrate, and protein-carbohydrate interactions, which causes alteration of membrane activity [5]. Normally, the plasma membrane protects the sperm's organelles and acts as a filter for transmembrane transport. Therefore, the integrity of the plasma membrane vital for sperm viability and motility [36]. Excessive production of ROS leads to protein, lipid, and carbohydrate changes in the sperm membrane [37]. The ROS will reduce the disulfide bonds between membrane proteins and membrane phospholipids' peroxidation and modifications of the sperm glycocalyx. As a result, the sperm membrane becomes fragile and lost of semi permeability. Higher ROS impair several axonemal and mitochondrial proteins, which results in the loss of sperm motility [5].

The T0 group had the lowest quality of post-thawed semen. There was the lowest sperm viability, motility, IPM, and highest MDA levels and SDF. This is not surprising since the freezing process of semen leads to the cold sock of sperm. The cold sock changes the ratio of unsaturated fatty acids and lowers cholesterol content [38], resulting in a less stable sperm membrane [39]. Increased ROS causes peroxidation of PUFAs, especially docosahexaenoic acid, which has six double bonds per molecule in sperm cell membranes. One of the products of lipid peroxidation is MDA [6]. Free radicals of ROS may cause lipid peroxidation and mitochondrial and nuclear DNA base modifications [40]. Modification of 8-OH-guanine or

Table 1. The qualification of Kacang buck ejaculates.

Parameters	Values
Volume (mL)	2.43 ± 0.40
Concentration (million/mL)	3820.04 ± 324.59
Viability (%)	91.86 ± 1.49
Progressive motility (%)	89.02 ± 1.77
Intact plasma membrane (%)	85.75 ± 1.78

8-OH-20-deoxyguanosine will destabilize the DNA structure and causes SDF [8]. The opening of interchain disulfide bridges in protamines during the freezing process may interfere with sperm DNA [41].

Various antioxidants have been supplemented in semen extenders, including GTE. The EGCG contained in GTE can decrease lipid peroxidation, protein carbonylation, and DNA damage, which improves sperm motility and acrosome integrity [9]. The post-thawed semen quality increased until a certain dose of GTE addition in the extender. The T1 group had better semen quality than had the control group, although the number of antioxidants did not yet compensate for the excess ROS created during the freeze-thawing process. Sperm produce its antioxidant enzymes, such as glutathione peroxidase, catalase, and superoxide dismutase. However, the transport of enzymes into another part of sperm was challenged by a small volume of sperm cytoplasm [42]. Oxidative stress has numerous effects, including reduced motility and plasma sperm integrity. Green tea polyphenols may increase sperm quality through catechin's capability to reduce ROS production [43].

The T2 group had the best semen quality, as it had the highest post-thawed viability, motility, and IPM and the lowest MDA concentration and SDF. This result was consistent with findings that GTE's addition to an extender maintained the motility, viability, IPM, and DNA integrity of Simmental bull sperm [27]. In boar semen, GTE supplementation also reduced ROS production during freezing, as evidenced by higher motility and viability of the GTE-supplemented group compared with that of the control group [44]. The beneficial effects of EGCG are most likely due to the antioxidative activity of polyphenol groups of catechin, which perform as chelating agents, whereas flavonoids act as catalysts on the lipid bilayers and membrane function [45]. At the molecular level, green tea polyphenol action occurs via the signaling pathway of adenosine monophosphate-activated protein kinase, cyclic adenosine monophosphate, calcium ion [43], Fe2+, ferric iron, and low-density lipoproteins [19]. The T3 group showed a decrease in post-thawed semen

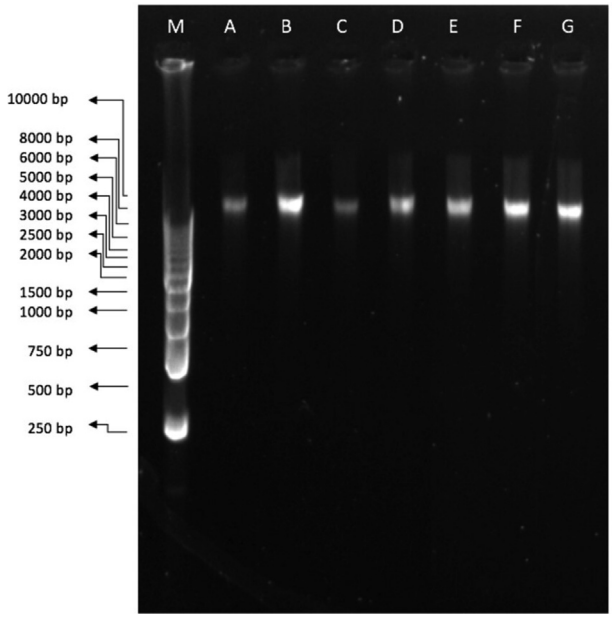

Figure 1. Total DNA electrophoresis results at 1% agarose gel. Description: M: 1 kb marker, and seven samples were taken to represent each treatment group. K = control group, P1A, P1B = T1, P2A, P2B- T2, P3B, P3A = T3 samples group.

quality. The higher antioxidant level in the T3 group disrupted the balance state needed for the physiologic function of sperm [16]. This finding was in consonance with an earlier report that higher exposure of antioxidants results in a paradox of antioxidants causes decreased male fertility [47, 48].

4.2. Mutation of ND1 mtDNA of post-thawed semen

The cryopreservation of sperm causes water movement, dehydration by intra and extracellular ice formation, ROS, apoptotic stimulation, lipid peroxidation, cytochrome C release, activation of caspases, and activation of endonucleases and induced SDF [7]. During the cryopreservation process, ruminant sperm suffer several structural and functional damages. The structural changes are followed by functional changes, cover the membrane integrity/permeability changes, acrosome damage,

Table 2. Effect of green tea extract (GTE) in skim milk-egg yolk (SM-EY) extender on viability (%), motility (%), intactness of the plasma membrane (%), and MDA level (nmol/mL) of Kacang buck sperm (six replicate respectively).

Group	Sperm viability	Sperm motility	Sperm IPM	MDA
T0	33.55 ± 0.15c	29.63 ± 0.45d	28.80 ± 0.297b	2563.83 ± 0.77a
T1	37.18 ± 0.15b	37.15 ± 0.98b	29.44 ± 0.34b	2232.30 ± 0.19c
T2	**45.28 ± 0.21a**	**45.25 ± 0.35a**	**34.04 ± 0.20a**	**1876.20 ± 0.11d**
T3	33.23 ± 1.12c	32.23 ± 0.24c	27.66 ± 0.26d	2356.32 ± 0.25b

Values with different superscripts in the same column are significantly different ($p < 0.05$). T0 denotes SM-EY extender without GTE; and T1, T2, and T3 denote SM-EY extender with the addition of 0.05, 0.1, and 0.15 mg GTE/100 mL extender, respectively. Values in bold were the best quality parameters of semen.

Table 3. SDF (%, six replicate) and the frequency of DNA mutation of post-thawed of Kacang buck semen.

Group	SDF	Total mutation	Mutation type			
			Transition	Transversion	Deletion	Insertion
T0	7.69 ± 0.14a	51.11 ± 8.39^{b}	42.78 ± 5.85^{b}	8.33 ± 2.89^{b}	-	-
T1	6.80 ± 0.14c	64.60 ± 0.89^{a}	50.40 ± 1.14^{a}	12.40 ± 0.55^{a}	1.00 ± 0.00	1.20 ± 0.45
T2	**4.32 ± 0.11d**	**11.25 ± 5.86^{c}**	**9.06 ± 4.83^{c}**	**1.88 ± 0.72^{c}**	**1.25 ± 0.00**	**-**
T3	7.15 ± 0.11b	54.44 ± 35.37^{b}	46.67 ± 23.33^{b}	7.50 ± 4.95^{b}	-	8.33 ± 0.00

Bold signifies ($p < 0.05$).

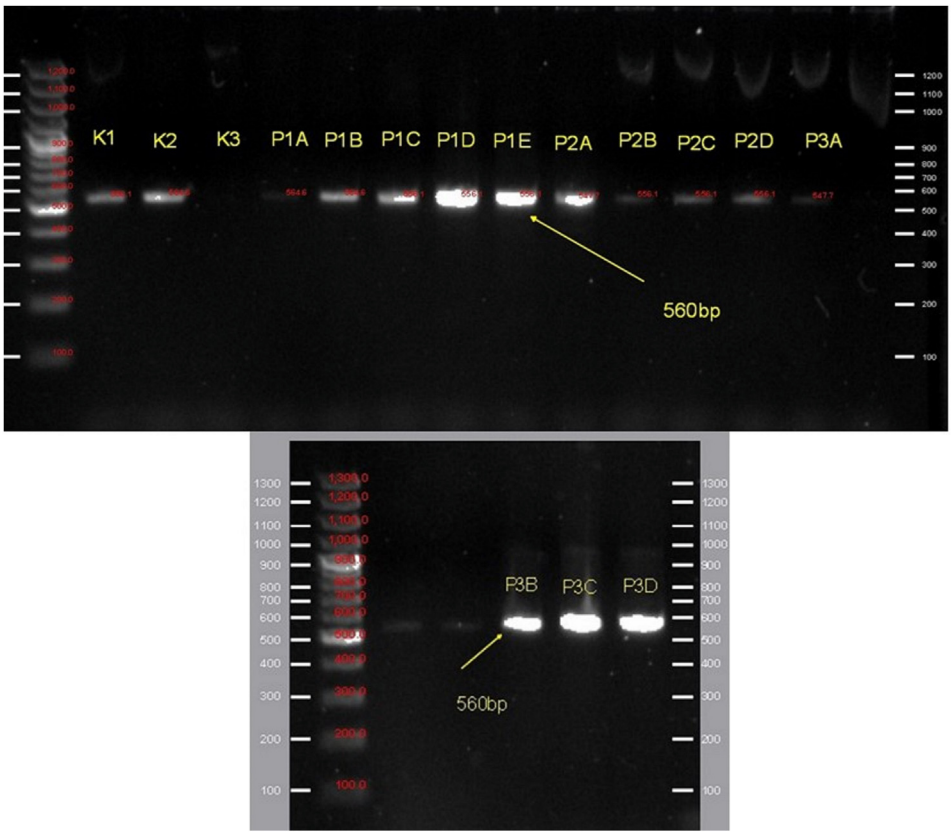

Figure 2. Electrophoresis results of 2% agarose concentration PCR product. M: Marker 100 bp. P3B, P3C, P3D: samples of T3 group.

Table 4. The type of ND1 mtDNA mutation of post-thawed of Kacang buck semen based on groups.

Group	Transition	Transversion	Deletion	Insertion
T0	T-C, C-T, A-G, G-A (4)	A-C, A-T, T-A, G-C (4)	-(0)	-(0)
T1	T-C, C-T, A-G, G-A (4)	A-C, C-A, A-T, G-C, G-T (5)	T, A (2)	A (1)
T2	T-C, G-A, T-C (3)	A-C, C-A, A-T (3)	T (1)	-(0)
T3	T-C, C-T, A-G, G-A (4)	A-C, C-A, G-C, T-A, G-T (5)	-(0)	T, A, G (3)

Table 5. Pearson's correlation coefficient of the semen quality parameters with ND1 mtDNA mutation of post-thawed of Kacang buck semen.

Parameters	Correlation coefficient (r)	p-value
Viability	-0.29	0.71
Motility	-0.22	0.78
IPM	-0.05	0.95
MDA	-0.02	0.98
DNA	0.24	0.76

enzyme leakage, impairment of mitochondrial activity, increased ROS resulting in oxidative stress, causes reduced viability, lower sperm motility, and apoptosis. Meanwhile, the molecular change consists of lipid peroxidation, DNA damage, epigenetic change, disruption of a disulfide bond, change in mRNAs expression, tyrosine phosphorylation increment, and protein/carbohydrate changes [5].

The total obtaining of isolation DNA based on the 1% agarose electrophoregram (Figure 1) was more than 10,000 bp fragment. The electrophoresis results at 1% agarose gel was an excellent yielding of DNA isolation based on the quantity of DNA obtained [49]. The partial DNA sequencing results indicate that there were fragments of nucleotide bases with the same nucleotide bases as the NCBI database. However, conserved regions have a mutation in their nucleotide bases [50]. Without any antioxidant (control group, Table 3), there was high SDF, total mutation, transition, and transversion mutation of ND1 mtDNA fragments mutation frequency. The mitochondrial membrane properties were cryosensitive and caused increasing in ROS production [51]. High ROS results in the oxidation of DNA, producing high frequencies of single and double-strand DNA breaks. The SDF also causes defects in DNA repair enzymes during the freeze-thawing process. The cryopreservation was affected by the stability of sperm DNA. Oxidative stress cause disruption of nucleoprotein structure, disulfide bonds, and the DNA–-protamine complex of DNA [52].

Antioxidants improved sperm viability, motility, and reduced sperm SDF [53]. The addition of GTE 0.1 mg/100 mL extender (T2 group, Table 3) revealed the lowest SDF, total, and types of ND1 mtDNA fragments mutation frequency. Only T2 has three of four probability transitions (change from a purine to a purine or a pyrimidine to a pyrimidine) of the ND1 mtDNA mutation. Meanwhile, the other group (T0, T1, and T3) has a complete transition type. Transversion mutations (a purine replaced by a pyrimidine, or vice versa) occur 4, 5, 3, and 5 in T0, T1, T2, and T3, respectively, of eight the probability. Overall, nothing changes of C to G, T to G of transversion ND1 mtDNA mutation. The deletion and insertion mutation of ND1 mtDNA did not follow a specific pattern. Overall, there was no deletion in G, C, and no insertion of C (Table 4).

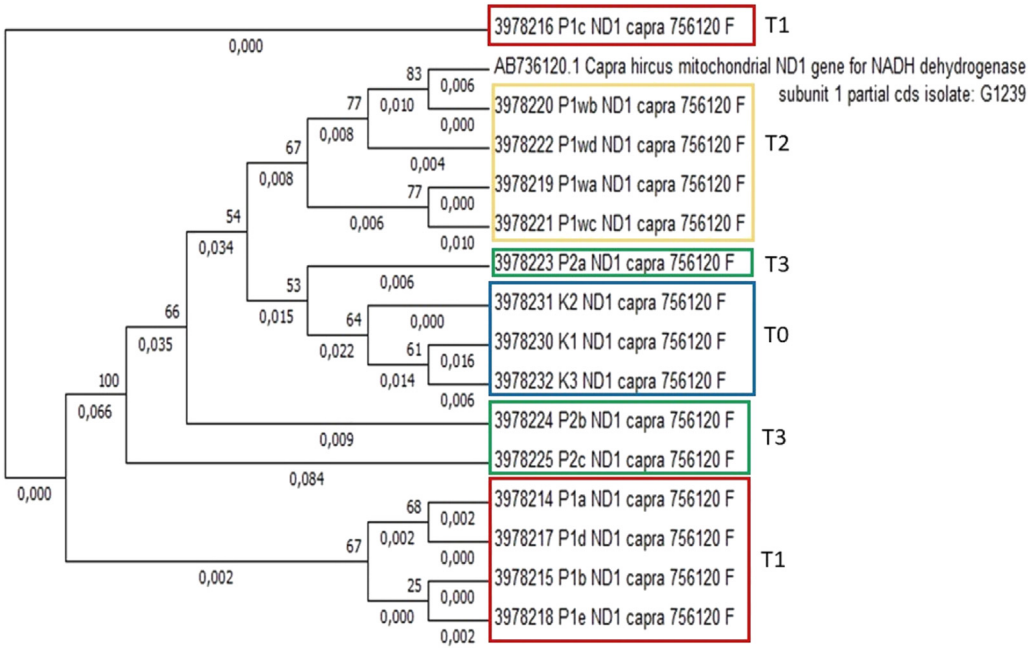

Figure 3. Phylogenic tree analysis of ND1 mtDNA mutation of post-thawed of Kacang buck semen. T0 denotes SM-EY extender without GTE; and T1, T2, and T3 denote SM-EY extender with the addition of 0.05, 0.1, and 0.15 mg GTE/100 mL extender, respectively. Values in bold were the best quality parameters of semen.

Disruptions of mitochondrial mtDNA may affect male reproductive function [54]. Higher sperm mitochondrial DNA copy numbers (mtDNAcn) and mitochondrial DNA deletions (mtDNAdel) were associated with lower sperm concentration, count, motility, and morphology [55] and lower sperm fertilizing ability [56]. Sperm with mitochondrial DNA (mtDNA) mutations was associated with lower semen quality and fertility [7]. The damage of the mitochondrial genome and chromatin integrity of sperm impairs male reproductive potential [57]. The mtDNA copy number alterations and impaired chromatin integrity could affect reproductive function [58]. In this study, there was no correlation between the semen quality parameters (sperm viability, motility, IPM, MDA concentration, and SDF) with ND1 mtDNA mutation of post-thawed Kacang buck semen (Table 5). It was linear to the finding that no correlation was found between SDF and mtDNA copy number [58]. However, the post-thaw sperm with SDF was altered gene expression in surviving embryos [52]. The blastulation rates were higher in the lower of SDF [59]. The normal embryonic development is dependent on the intactness and completeness of genetic material of sperm [60]. There was a threshold of SDF to retain its fertilizing ability [61]. Sperm DNA integrity is essential for embryo development [62]. Lower SDF of sperm, followed by a higher pregnancy rate [3]. Sperm with SDF can complete the fertilization process. However, embryo development can be interrupted due to the transcription of damaged paternal genes [5].

Based on characteristic joining in the phylogenic tree, T1 was an outgroup (pleisiomorphic character) [63], T0, T2, and T3 as a synapomorphic character [64], while T2 a monophyletic character [65]. The T2 genetic distance value was 0.008, and it was nearest to the reference sequence (AB736120.1 *Capra hircus* mitochondrial ND1 gene for NADH dehydrogenase subunit 1 partial cds isolate: G1239) compared to other groups (Figure 3, Table6). It means the T2 clade was most closely related to the sequence reference [22] *Capra hircus* sperm ND1 Capra mtDNA (Figure 3). The bootstrap confidence values of the T2 group were 77–83, which means 77–83% of the same branch was observed when repeating 1000 times of the phylogenic reconstruction on the samples [66]. It showed that based on neighbor-joining to the reference sequence, the T2 group (addition of 0.1 mg GTE/100 mL SM-EY extender) was the best quality post-thawed Kacang buck semen.

Table 6. Bootstrap values and genetic distance values (p-distance) of base sequence result toward reference.

Group	Boostrap	Genetic distance values
T0	54%	0.034
T1	67–100%	0.066
T2	67–83%	0.008
T3	66%	0.035

This study was limited in extracting green tea using ethanol solvent and exploring the advanced methods. The nanoparticle extract has a large surface area to volume ratio [67] than ethanol extract, thereby improving penetration into the cell [68]. Future studies were suggested to use green tea extract nanoparticles to increase the post-thawed semen quality, measure total antioxidant capacity, and implement the post-thawed semen for artificial insemination on Kacang buck does recipients.

5. Conclusion

The addition of GTE 0.1 mg/100 mL extender resulted in the best quality of Kacang buck post-thawed semen based on the highest percentages of sperms viability, motility, the lowest MDA levels, SDF, the total amount and types of ND1 mtDNA fragments mutation frequency. The clade of the T2 group was most closely related to the sequence reference. However, there was no correlation between the semen quality parameters (sperm viability, motility, IPM, MDA concentration, and SDF) with ND1 mtDNA mutation of post-thawed Kacang buck semen.

Declarations

Author contribution statement

Imam Mustofa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Suherni Susilowati; Wurlina Wurlina: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Tatik Hernawati: Performed the experiments; Performed the experiments; Wrote the paper.

Yudit Oktanella: Performed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

[1] T. Kaewkesa, A. Sathanawongs, A. Oranratnachai, J. Sumretprasong, The goat semen quality after being frozen using albumin and cholesterol substituted for egg yolk in semen extender, Thai. J. Vet. Med. 46 (2016) 201–207. https://he01.tci-thaijo.org/index.php/tjvm/article/view/63812. (Accessed 30 December 2019).

[2] C. Lv, G. Wu, Q. Hong, G. Quan, Sperm cryopreservation: state of art and future in small ruminants, Biopreserv. Biobanking 17 (2018) 171–182.

[3] S. Susilowati, I.N. Triana, T. Sardjito, T.W. Suprayogi, W. Wurlina, I. Mustofa, Effect of Simmental bull seminal plasma protein in egg yolk-citrate extender on Kacang buck semen fertility, Cryobiology (2020).

[4] INSA (Indonesian National Standard Agency), Frozen Semen – Part 3: Goat and Sheep. Jakarta, 2014. Retrieved December 30, (2019), from, http://bibit.ditjenpkh.pertanian.go.id/sites/.

[5] P. Peris-Frau, A.J. Soler, M. Iniesta-Cuerda, A. Martín-Maestro, I. Sánchez-Ajofrín, D.A. Medina-Chávez, M.R. Fernández-Santos, O. García-Álvarez, A. Maroto-Morales, V. Montoro, J.J. Garde, Sperm cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality, Int. J. Mol. Sci. 21 (2020) 2781.

[6] T. Takeshima, S. Kuroda, Y. Yumura, Chapter 6: Reactive Oxygen Species and Sperm Cells, Intech open, 2018.

[7] K. Katepogu, D. Kurumala, T.R. Kedam, Effect of cryopreservation on rat sperm DNA and involvement of elevated oxidative stress, apoptosis – a review, IJPRT 2 (2012) 33–41.

[8] J. Ribas-Maynou, J. Benet, Single and double strand sperm DNA damage: different reproductive effects on male fertility, Genes 10 (2019) 105.

[9] D. Martin-Hidalgo, M.J. Bragado, A.R. Batista, P.F. Oliveira, M.G. Alves, Antioxidants and male fertility: from molecular studies to clinical evidence, Antioxidants 8 (2019) 89.

[10] E. L Furno, I. Busseau, C. Lorenzi, C. Saghira, S. Zuchner, D. Maiorano, Translesion DNA synthesis-driven mutagenesis in very early embryogenesis of fast cleaving embryos, bioRxiv 11 (28) (2020) 401471.

[11] L. Manente, S. PecoraroE. Picillo, U. Gargiulo, P. Gargiulo, A. De Luca, L. Politano, Molecular evidence of apoptotic pathway activation in semen samples with high DNA fragmentation, In vivo 29 (2015) 289–294. PMID: 25792659.

[12] K. Takeda, K. Uchiyama, M. Kinukawa, T. Tagami, M. Kaneda, S. Watanabe, Evaluation of sperm DNA damage in bulls by TUNEL assay as a parameter of semen quality, J. Reprod. Dev. 61 (2015) 185–190.

[13] A.M. Fadl, A.M. Ghallab, M.M. Abou-Ahmed, Comparison between Tris-buffer and INRA-82 extenders on the quality of chilled rabbit spermatozoa, World Rabbit Sci. 28 (2020) 13–18.

[14] M. Anzar, K. Rajapaksha, L. Boswall, Egg yolk-free cryopreservation of bull semen, PloS One 14 (2019), e0223977.

[15] M.A. Ezz, A.E. Montasser, M. Hussein, A. Eldesouky, M. Badr, A.E. Hegab, A. Balboula, S.M. Zaabel, The effect of cholesterol loaded cyclodextrins on the post-thawing quality of buffalo semen in relation to sperm DNA damage and ultrastructure, Reprod. Biol. 17 (2017) 42–50.

[16] M.S. Rahman, M.R. Gofur, M.M. Rahman, F.Y. Bari, N.S. Juyena, Effect of skim milk and tris-citrate extenders to preserve the semen of indigenous ram of Bangladesh, AJOB 5 (2018) 1–11.

[17] M.G. Al-Mutary, Use of antioxidants to augment semen efficiency during liquid storage and cryopreservation in livestock animals: a review, J. King Saud Univ. Sci. 33 (2021) 1–6.

[18] C.V. Souza, F.Z. Brandao, J.D.R. Santos, V.A.P. Alfradique, V. Santos, M. Morais, P.S.C. Rangel, A.A.D. Silva, J.M.G. Souza-Fabjan, Effect of different concentrations of l-carnitine in extender for semen cryopreservation in sheep, Cryobiology 89 (2019) 104–108.

[19] M.K.P. Selvam, A. Agarwal, A systematic review on sperm DNA fragmentation in male factor infertility: laboratory assessment, Arab J. Urol. 16 (2018) 65–76.

[20] M. Mehdipour, H.D. Kia, A. Najafi, H.V. Dodaran, O. García-Álvarez, Effect of green tea (Camellia sinensis) extract and pre-freezing equilibration periodon the post-thawing quality of ram semen cryopreserved in a soybean lecithin based extender, Cryobiology 73 (2016) 297–303.

[21] H. Ahmed, S. Jahan, M. Riaz, B.T. Khan, M.U. Ijaz, Epigallocatechin-3-gallate (EGCG) addition as an antioxidant in a cryo-diluent media improves microscopic parameters, and fertility potential, and alleviates oxidative stress parameters of buffalo spermatozoa, Cryobiology 97 (2020) 101–109.

[22] M. Tourmente, M. Hirose, S. Ibrahim, D. Dowling, D. Tompkins, E. Roldan, N. Gemmell, mtDNA polymorphism and metabolic inhibition affect sperm performance in conplastic mice, Reproduction 154 (2017) 341–354. Retrieved Dec 18, 2020, from, https://rep.bioscientifica.com/view/journals/rep/154/4/REP-17-0206.xml.

[23] D.C. Chan, E.A. Schon, Eliminating mitochondrial DNA from sperm, Dev. Cell 22 (2012) 469–470.

[24] G. Zhang, Z. Wang, X. Ling, P. Zou, H. Yang, Q. Chen, N. Zhou, L. Sun, J. Gao, Z. Zhou, J. Cao, L. Ao, Mitochondrial biomarkers reflect semen quality: results from the MARCHS study in Chongqing, China, PloS One 11 (2016), e0168823.

[25] J. Ji, M. Xu, Z. Huang, L. Li, H. Zheng, S. Yang, S. Li, L. Jin, X. Ling, Y. Xia, C. Lu, X. Wang, Mitochondrial DNA sequencing and large-scale genotyping identifies MT-ND4 gene mutation m.11696G>A associated with idiopathic oligoasthenospermia, Oncotarget 8 (2017) 52975–52982.

[26] TAH (Terrestrial Animal Health), Code. Chapter 4.7. Collection and Processing of Bovine, Small Ruminant, and Porcine Semen. World Organisation for Animal Health (OIE), OIE, Paris, 2019. Retrieved December 30, (2019), from, https://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_coll_semen.pdf.

[27] S. Susilowati, T. Sardjito, I. Mustofa, O.S. Widodo, R. Kurnijasanti, Effect of green tea extract in extender of Simmental bull semen on pregnancy rate of recipients, Anim. Biosci. (2021).

[28] T.C. Lorenz, Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies, J. Vis.Exp. JoVE 63 (2012) e3998.

[29] C. Feuillie, M.M. Merheb, B. Gillet, G. Montagnac, I. Daniel, C. Hänni, Detection of DNA sequences refractory to PCR amplification using a biophysical SERRS assay (Surface Enhanced Resonant Raman Spectroscopy), PloS One 9 (2014), e114148.

[30] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874.

[31] E.D. Kusumawati, K.N. Utomo, A.T.N. Krisnaningsih, S. Rahadi, The quality of semen of Kacang buck with different storage time at room temperature using aminomethan egg yolk extender, JITRO 4 (2017) 42–45. https://www.researchgate.net/publication/325359886.

[32] F. Setiawan, E.D. Kusumawati, The quality of fresh semen Kacang buck at 5°C storage with and without an extender, J. Anim. Sci. 95 (2017) 77–85.

[32] B. Üstüner, Z. Nur, S. Alçay, M.B. Toker, H. Sağirkaya, H.K. Soylu, Effect of freezing rate on goat sperm morphology and DNA integrity, Turk. J. Vet. Anim. Sci. 39 (2015) 110–114.

[34] H. Gürler, E. Malama, M. Heppelmann, O. Calisici, C. Leiding, J.P. Kastelic, H. Bollwein, Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm, Theriogenology 86 (2016) 562–571.

[35] A.S. Amal, R.I. Arifiantini, M.A. Setiadi, S. Said, Characteristics of the post-thawed Balinese bull semen extended in three different extenders and equilibration times, J. Indones. Trop. Anim. Agric. 44 (2019) 135–145.

[36] R. Pereira, R. Sá, A. Barros, M. Sousa, Major regulatory mechanisms involved in sperm motility, Asian J. Androl. 19 (2017) 5–14.

[37] S. Dutta, A. Majzoub, A. Agarwal, Oxidative stress and sperm function: a systematic review on evaluation and management, Arab. J. Urol. 17 (2019) 87–97.

[38] M.J. Palomo, W. García, A. Tabarez, Effect of seminal plasma and butylated hydroxytoluene (BHT) concentration on ram sperm freezability, Small Rumin. Res. 153 (2017) 66–70.

[39] A. Wusiman, Y.P. Wang, K. Ren, G.B. Zhou, X.W. Fu, L. Suo, Z.Q. Fan, L. Wang, S.E. Zhu, Semen storage at 23, 4 or -196°C and its application to artificial insemination in small-tail Han sheep, AJAVA 7 (2012) 299–308.

[40] M.T. Le, T. Nguyen, T.T. Nguyen, T.V. Nguyen, T. Nguyen, Q. Nguyen, T.N. Cao, Does conventional freezing affect sperm sperm DNA fragmentation? Clin. Exp.Reprod. Med. 46 (2019) 67–75.

[41] S.C. Esteves, D. Santi, M. Simoni, An update on clinical and surgical interventions to reduce sperm DNA fragmentation in infertile men, Andrology 8 (2020) 53–81.

[42] M.Y. Bibov, A.V. Kuzmin, A.A. Alexandrova, V.A. Chistyakov, N.M. Dobaeva, O.L. Kundupyan, Role of the reactive oxygen species induced DNA damage in human sperm dysfunction, AMJ 3 (2018) 1–12.

[43] S.U. Rahman, Y. Huang, L. Zhu, S. Feng, I.M. Khan, J. Wu, Y. Li, X. Wang, Therapeutic role of green tea polyphenols in improving fertility: a review, Nutrients 10 (2018) 834.

[44] S.-H. Park, I.J. Yu, Effect of antioxidant supplementation in freezing extender on porcine sperm viability, motility, and reactive oxygen species, J. Anim. Reprod. Biotechnol. 32 (2017) 9–15.

[45] S. Roychoudhury, A. Agarwal, G. Virk, C.L. Cho, Potential role of green tea catechins in the management of oxidative stress-associated infertility, Reprod. Biomed. Online 34 (2017) 487–498.
[46] B. Salehi, M. Martorell, J.L. Arbiser, A. Sureda, N. Martins, P.K. Maurya, M. Sharifi-Rad, P. Kumar, J. Sharifi-Rad. Antioxidants, Positive or negative actors? Biomolecules 8 (2018) 1–11.
[47] A. Majzoub, A. Agarwal, S.C. Esteves, Antioxidants for elevated sperm DNA fragmentation: a mini review, Transl. Androl. Urol. 6 (2017) S649–S653.
[48] A. Majzoub, A. Agarwal, Systematic review of antioxidant types and doses in male infertility: benefits on semen parameters, advanced sperm function, assisted reproduction and live-birth rate, Arab J. Urol. 16 (2018) 113–124.
[49] N. Gupta, DNA extraction and Polymerase chain reaction, J. Cytol. 36 (2019) 116–117.
[50] S. Asthana, M. Roytberg, J. Stamatoyannopoulos, S. Sunyaev, Analysis of sequence conservation at nucleotide resolution, PLoS Comput. Biol. 3 (2007) e254.
[51] C. Ortega-Ferrusola, L. Anel-López, P. Martín-Muñoz, J.M. Ortíz-Rodríguez, M.C. Gil, M. Alvarez, P. de Paz, L.J. Ezquerra, A.J. Masot, E. Redondo, L. Anel, F.J. Peña, Computational flow cytometry reveals that cryopreservation induces spermatosis, but subpopulations of spermatozoa may experience capacitation-like changes, Reproduction 153 (2017) 293–304.
[52] M. Hezavehei, M. Sharafi, H.M. Kouchesfahani, R. Henkel, A. Agarwal, V. Esmaeili, A. Shahverdi, Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches, RBMO 37 (2018) 327–339.
[53] R. Bahmyari, M. Zare, R. Sharma, A. Agarwal, I. Halvaei, The efficacy of antioxidants in sperm parameters and production of reactive oxygen species levels during the freeze-thaw process: a systematic review and meta-analysis, Andrologia 52 (2020), e13514.
[54] E. Talebi, M. Karimian, H. Nikzad, Association of sperm mitochondrial DNA deletions with male infertility in an Iranian population. Mitochondrial DNA. Part A, DNA Mapp. Seq. Anal. 29 (2018) 615–623.
[55] H. Wu, A.M. Huffman, B.W. Whitcomb, S. Josyula, S. Labrie, E. Tougias, T. Rahil, C. K Sites, J.R. Pilsner, Sperm mitochondrial DNA measures and semen parameters among men undergoing fertility treatment, RBMO 38 (2019) 66–75.
[56] A.J. Rosati, B.W. Whitcomb, N. Brandon, G.M. Buck Louis, S.L. Mumford, E.F. Schisterman, J.R. Pilsner, Sperm mitochondrial DNA biomarkers and couple fecundity, Hum. Reprod. 35 (2020) 2619–2625.
[57] A. Mańkowska, P. Brym, Ł. Paukszto, J.P. Jastrzębski, L. Fraser, Gene polymorphisms in boar spermatozoa and their associations with post-thaw semen quality, Int. J. Mol. Sci. 21 (2020) 1902.
[58] F. Faja, T. Carlini, G. Coltrinari, F. Finocchi, M. Nespoli, F. Pallotti, A. Lenzi, F. Lombardo, D. Paoli, Human sperm motility: a molecular study of mitochondrial DNA, mitochondrial transcription factor A gene and DNA fragmentation, Mol. Biol. Rep. 46 (2019) 4113–4121.
[59] W. Ni, S. Xiao, X. Qiu, J. Jin, C. Pan, Y. Li, Q. Fei, X. Yang, L. Zhang, X. Huang, Effect of sperm DNA fragmentation on clinical outcome of frozen-thawed embryo transfer and on blastocyst formation, PloS One 14 (2014), e94956.
[60] C. Yildiz, P. Ottaviani, N. Law, R. Ayearst, L. Liu, C. McKerlie, Effects of cryopreservation on sperm quality, nuclear DNA integrity, in vitro fertilization, and in vitro embryo development in the mouse, Reproduction 133 (2007) 585–595. Retrieved Dec 16, 2020, from, https://rep.bioscientifica.com/view/journals/rep/133/3/1330585.xml.
[61] H. Wu, B.W. Whitcomb, A. Huffman, N. Brandon, S. Labrie, E. Tougias, K. Lynch, T. Rahil, C.K. Sites, J.R. Pilsner, Associations of sperm mitochondrial DNA copy number and deletion rate with fertilization and embryo development in a clinical setting, Hum. Reprod. 34 (2019b) 163–170.
[62] M.W. Li, K. Lloyd, DNA fragmentation index (DFI) as a measure of sperm quality and fertility in mice, Sci. Rep. 10 (2020) 3833.
[63] P. Kück, J.W. Wägele, Plesiomorphic character states cause systematic errors in molecular phylogenetic analyses: a simulation study Cladistics, Int. J. Willi Hennig Soc. 32 (2016) 461–478.
[64] E.V. Mavrodiev, Synapomorphies behind shared derived characters: examples from the great apes' genomic data, Acta Biotheor. 68 (2020) 357–365.
[65] K. Braun, F. Leubner, T. Stach, Phylogenetic analysis of phenotypic characters of Tunicata supports basal Appendicularia and monophyletic Ascidiacea, Cladistics 36 (2020) 259–300.
[66] K. Schliep, A.J. Potts, D.A. Morrison, G.W. Grimm, Intertwining phylogenetic trees and networks, Methods Ecol. Evol. 8 (2017) 1212–1220.
[67] D. Sharma, S. Kanchi, K. Bisetty, Biogenic synthesis of nanoparticles: a review, Arab. J. Chem. 12 (2019) 3576–3600.
[68] R. Zein, W. Sharrouf, K. Selting, Physical properties of nanoparticles that result in improved cancer targeting, J. Oncol. (2020), 5194780 Pubmed.