Immunofluorescence and Biochemical Investigation of the Protective Effects of Naringin and Diosmin on the Freezability of Merino Ram Semen

Ali Doğan Ömür
Ataturk University: Ataturk Universitesi
Özgür Kaynar
Kastamonu Üniversitesi: Kastamonu Universitesi
Mustafa İleritürk
Ataturk University: Ataturk Universitesi
Mustafa Özkaraca
Cumhuriyet Üniversitesi: Sivas Cumhuriyet Universitesi
Ali Erdem Öztürk
Erciyes Üniversitesi: Erciyes Universitesi
Mustafa Bodu
Selçuk Üniversitesi: Selcuk Universitesi
İpek Nur Şahin
Türkiye Cumhuriyeti Tarım ve Orman Bakanlığı: Turkiye Cumhuriyeti Tarim ve Orman Bakanligi
serkan akarsu (serkanaliakarsu@gmail.com)
Kahramanmaras Istiklal Universitesi https://orcid.org/0000-0003-4450-6540

Research Article

Keywords: Diosmin, Naringin, Cryopreservation, Sperm, Lipid / Protein Profile Analysis

Posted Date: April 25th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1424939/v2

Abstract

In this study, the effects of diosmin and naringin on freezability of ram semen were investigated. For this purpose, 6 Merino rams were used during the breeding season. The ejaculates were pooled after being collected from the rams. Pooled ejaculates were divided into 6 groups as control (C; no additive) Naringin (1, 2, and 4 mM) and Diosmin (2 and 4 mM) groups, and then reconstituted with TRIS-based diluent. Pooled semen was equilibrated, placed in 0,25 mL straws with 10×10^7 sperm cells in each straw and frozen in liquid nitrogen vapor. After waiting for 24 hours, the straws were thawed at 37 ^{o}C for 25 s and analyzes were made. There was no statistical difference in total motility between the groups. D2 and D4 preserved the integrity of the plasma membrane better than the other groups. D4 showed better effect than other groups in terms of acrosome integrity, there was no difference between the groups in terms of mitochondrial activity. In the analysis of the sperm membrane lipid profile, it was observed that the diosmin added group had the highest lipid-phospholipid ratio. In the sperm membrane protein profile analysis, it was observed that both additives had protective effects at different levels. The highest total protein amount was seen in D4 and N4 groups. 8-OhDG positivity was more common in the control group than in the diosmin and naringin groups. Cu-Zn SOD positivity was less in the control group but more intense in all other groups. The positives were especially in the acrosome parts of the sperm cells.

1. Introduction

Reproductive biotechnology includes areas such as the cryopreservation of semen [1]. The semen taken from the male animal only survives for a few hours in the external environment [2]. Freezing and long-term storage of semen in rams is one of the achievements of reproductive biotechnology. However, pregnancy success obtained from long-term storage of ram semen is low [3].

Ram sperm is very sensitive against cryopreservation. The main reason for this is that unsaturated fatty acids (phospholipids) constitute a significant part of the sperm membrane. Therefore, the cooling processes performed during the freezing of the spermatozoa cause the spermatozoa membrane to change from the liquid phase to the gel phase, and changes in the kinetics of intra-membrane enzymes cause post-thawing viability parameters decreases [4]. Due to the membrane lipid phase change, osmotic-mechanical stress and free oxygen radicals that develop during the freezing-thawing of the ram sperm, structural deformations occur in the membrane structures, cell organelles and DNA. These negative effects can be reduced by the addition of antioxidants to sperm diluents and post-thawing sperm functions can be improved [5-7]. Damages due to freezing in spermatozoa can be physical, biochemical or functional. Physical damage is more common in ram spermatozoa. Physical damage occurs in the plasma and acrosome membranes or in the mitochondrial sheath. The outer membrane of the acrosome is more easily damaged and injured than the inner membrane [8,9]. The easy lipid peroxidation of frozen / thawed spermatozoa and the fact that the membranes contain a large amount of lipids led to the investigation of effective antioxidant systems against developing peroxidative damage and sperm dysfunctions [7,10].

Naringin(N), a flavanone glycoside, is the major flavonoid that is abundant in grapefruit and gives the bitter taste of grapefruit [11]. Studies have indicated that naringin has hepatoprotective [12], antidiabetic [13], anti-atherosclerotic [14], anti-hyperlipidemic[15], reno-protective [16], cardio-protective effects [17]. The protective effect of naringin on spermatological parameters has been shown in rat studies [18,19]. Diosmin(D) is a flavonoid and has antihyperglycemic, anti lipid peroxidative [20], anti-inflammatory, antioxidant, antimutagenic [21], antihypertensive properties [22]. Therefore, the aim of this study is to investigate the effect of different doses of naringin and diosmin on ram semen after freezing thawing.

2. Materials And Methods

Animal Material, Ethical Permission and Chemicals

The approval of Atatürk University Animal Experiments Local Ethics Committee was obtained (No: 2015/191). All chemicals were purchased from Sigma (Sigma-Aldrich Co.) unless stated otherwise.

The study was carried out by obtaining ejaculates from 6 adult Merino rams aged 2-5 years with the help of an artificial vagina twice a week for 3 weeks. Those with suitable characteristics (sperm density ≥ 3×10^9 spermatozoa / ml; motility ≥ 80%) among the ejaculates obtained were used in the study.

Dilution and Evaluation of Semen

Tris extender (297.58 mM tris, 96.32 mM citric acid, 82.66 mM fructose, 15% egg yolk, glycerol 5%, gentamicin 0.1 ml / 100ml: pH 6.8-7.0) was used for dilution of the semen [23]. Diosmin and naringin were dissolved in DMSO and stock solutions were prepared. The ejaculates were divided into 6 equal volumes at 37 °C and diluted with Tris diluent containing naringin (1, 2 and 4 mM), diosmin (2 and 4 mM) and and containing no additive (control) to approximately 4×10^8 spermatozoa per ml. After dilution, the semen samples were kept at room temperature for 10 minutes, then they were filled into 0.25 ml straws and left to equilibrate at +5^{o}C for approximately 3 hours. In the period following the equilibration, samples were frozen in liquid nitrogen vapor (~ -100^{o}C) for 10 minutes and stored in liquid nitrogen at -196^{o}C. The study consisted of 6 replications.

In the study, semen samples with and without antioxidants were evaluated in terms of freeze thawing motility. Besides, samples were evaluated in terms of membrane integrity, acrosome integrity and mitochondrial activity using fluorescent staining techniques. The straws kept in liquid nitrogen for at least 24 hours were thawed at 37^{o}C for 25 seconds. Spermatozoa motility was examined in at least 3 microscope areas in a drop of semen (5 µl) placed directly on a microscope slide and covered by a cover slip at 400x magnification of the phase contrast microscope with heating apparatus at 37 ^{o}C. The average of the motility values in the fields as % motility ratio was recorded.

Determination of Sperm Membrane Integrity

Sperm membrane integrity was performed using the SYBR-14/PI (L 7011 Invitrogen) kit as described by Garner and Johnson [24]. Thawed straws were diluted with Tris and then mixed with 30 µl of semen, 6 µl of SYBR-14 and 2.5 µl of PI and incubated at 37°C for 20 minutes in a light-free environment. Then, 10 µl of Hancock solution was added to prevent the active movement of sperm. A total of 200 spermatozoa were examined at 400X magnification with the help of a fluorescent microscope, covered with a coverslip, and 2.5 µl of the mix was taken and dripped onto the slide. Sperm displaying green–red or red colourisation were considered as having damaged membranes, while green colourisation was considered as indicating an intact membrane.

Determination of Sperm Acrosome Integrity

Sperm acrosome integrity was measured using the method used by Nagy et al.,[25] using FITC-PNA, (L7381Invitrogen) and PI staining technique. Thawed straws were diluted 1:3 with Tris solution and then mixed with 60 µl diluted semen, 10 µl FITC-PNA and 2.5 µl PI. Then, 10 µl of Hancock solution was added to prevent the active movement of sperm. A total of 200 spermatozoa were examined at 400X magnification with the help of a fluorescent microscope, covered with a coverslip, and 2.5 µl of the mix was taken and dripped onto the slide. Sperm with bright green or patchy green fluorescence were considered as damaged acrosome, while cells without green color were considered intact.

Determination of Mitochondrial Activity

Sperm mitochondrial membrane potential was measured with the JC-1/PI staining technique using the method used by Garner et al. [26]. The thawed straws were diluted 1:3 with Tris, and then 300 µl of diluted semen was mixed with 2.5 µl of JC-1 and 2.5 µl of PI, incubated at 37 °C in the dark for 20 minutes, and then 10 µl of Hancock's solution was added to inhibit sperm motility. A total of 200 spermatozoa were examined at 400X magnification with the help of a fluorescent microscope, covered with a coverslip, and 2.5 µl of the mix was taken and dripped onto the slide. Yellow/orange in the mitochondrial part of the spermatozoon indicates high mitochondrial membrane potential, while green color was evaluated as low mitochondrial membrane potential.

Homogenization of Sperm Samples

1 ml of 10% SDS (sodium dodecyl sulfate) was added onto 1 ml of frozen-thawed semen. It was homogenized in the homogenizer (Qiagen Tissue-Lyser LT) for 2 minutes and stored at -86 ^{o}C until the analyzes were performed.

Determination of Sperm Protein Profile

Protein profile analysis of sperm samples was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method [27]. The flat glass plate (10x8.5 cm) and the plate with 1.5 mm thick plastic strips (spacers) were assembled to form a sandwich. First, the resolving gel was filled with a 10 ml injector until it was 2.5 cm thick to the upper edge without creating air bubbles between the plates. After

the gel was poured, a flat polymerization line was formed by forming a thin layer with butanol on the surface of the gel with a 1 ml injector. After waiting for polymerization for at least 2 hours, butanol was removed with filter paper and the stacking gel was prepared and poured with the help of an injector without creating air bubbles. Afterwards, combs 1.5 mm thick were placed and the process continued for at least 3 hours for polymerization. At the end of the period, the combs were removed and the wells where the samples will be applied were washed 3 times with tris-glycine electrode buffer (pH 8.3) to remove the polymerization residues, and after washing the wells were filled with the same buffer and the samples were applied to the wells in the amount of 15 µl. Electrophoresis was performed for approximately 60 minutes in 20 mA / gel constant current mode. Then, gels were stained in oriole for 2 hours and then analyzed with a 1D gel analysis program by photographing them on a gel imaging system.

The molecular weights of the sperm proteins photographed were carried out automatically according to the above principle, with the ImageLab program obtained from BioRad and result of the analysis, total protein amounts were determined by using the sum of "individual volume" values in the total protein concentration of each protein band (ImageLab Software User Guide, Version 6.0, USA, 2017).

Determination of Sperm Lipid Profile

To determine the sperm lipid profile; the homogenized sperm samples were taken out of the freezer one day before the analysis and left to thaw. Subsequently, sperm lipid profile analysis was performed using the High Performance Liquid Chromatography (HPLC) method. This process was carried out using a 20 x 10 cm Silica Gel 60 F254 HPLC plate. After adding 1 ml of n-hexane / iso-propanol (2: 1 (v / v) mixture to sperm homogenates (1 ml each), the caps of the tubes were closed and vortexed vigorously and after waiting for ten minutes, vortexed again [28]. This process was repeated 2 more times. Vortexed tubes were centrifuged at 5000 xg for 10 minutes and the upper phase (hexane phase) was loaded on HPLC plates (5 µl). The lipid classes loaded on the plates were run 7 cm in a mixture of hexane: diethylether: formic acid (80: 20: 2 (v / v / v)) and dried at room temperature. 3% $CuSO_4$ in 8% H_3PO_4 was sprayed on these dried plates and burned in an incubator at 180^0C for about 10 minutes to make lipid bands visible.

After the HPLC plates were photographed in a photo scanner and lipid bands of each sample was determined using Phoretix 1D (TL120) software and expressed as % in the total mixture [29].

Seven different lipid classes were detected in sperm samples; Cholesterol esters (ChoE), triacyl glycerol (TAG), free fatty acids (FFA), cholesterol (Chol), diacyl glycerol (DAG), monoacyl glycerol (MAG) and phospholipids (PL).

Immunfluorescent Method

The smear samples taken were fixed in acetone for 10 minutes. After the fixation process, the samples were kept in 1% BSA for 10 minutes. The samples washed with PBS were incubated with 8-OhDG (Santa Cruz, catalog no. Sc-66036) and Cu-Zn SOD (Lifespan, catalog no. LS-B9346) antibodies for 45 min at 37 °C. Samples were treated after washing with PBS, using secondary antibodies [Goat Anti-Mouse IgG

(FITC) (Abcam, catalog no. Ab6785) for 8-OhDG and Goat Anti-Rabbit IgG (FITC) for Cu-Zn SOD (Abcam, catalog no.ab6717)] at 37 ° C for 45 min. All antibodies were applied at 1/50 dilution rate. In the last stage, gylcerol was dropped onto the sections washed with PBS and after covered with coverslip, Zeiss AxioCam Icc5 (Japan) was examined under fluorescence microscope. The intensity of the irradiation was evaluated as no (0), mild (1), moderate (2) and severe (3) [30].

Statistical Analysis

In statistical analysis, one-way analysis of variance (ANOVA) Duncan test was used to compare different groups in sperm analysis. In immunofluorescent analyis Kruskal Wallis test was used to determine the difference between groups, and Mann Whitney U test was used to determine the group that made the difference (SPSS 26. 0). The significance level was accepted as $p<0.05$.

3. Results

Sperm assessment

The Table 1 reflects the data in terms of the spermatological parameters after freezing-thawing (motility, plasma membrane integrity, acrosome integrity and mitochondrial activity) values of of the additives the antioxidants used in the study.

Considering the motility findings, there was a statistical difference between the control group and the D4 group ($p<0,05$), but there was no difference between the control group and the other groups. In addition, it was observed that the diosmin group (2, 4 mM) provided protection statistically, compared to the naringin group (1, 2, 4 mM) and the control group in terms of plasma membrane integrity during the freezing of semen. In terms of acrosome integrity, the diosmin 4 mM group had a statistically positive effect compared to the other groups, while a statistical difference was observed between the control group and the N4, D2 and D4 groups in terms of mitochondrial activity.

Figure 1 shows a sample photograph in terms of membrane integrity. Sperm cells with green-red and red colors were evaluated as membrane damaged, and only green-colored sperm cells were evaluated as not having membrane damage. In Figure 2, in terms of acrosome integrity, sperm cells that give bright green and fragmented green color are considered as those with damaged acrosomes, and sperm cells that do not give green color as those with undamaged acrosomes. In the sample photo in Figure 3, yellow-orange areas in the middle regions of the cells in terms of mitochondrial activity have high activity, while the areas that give green color are considered to have low activity.

Immunfluorescent Evaluations

There was no difference in 8-OhDG positivity between groups, statistically. However, a statistically significant difference was found in Cu-Zn SOD positivity ($p <0.05$).

8-OhDG positivity was more common in the control group compared to the diosmin and naringin groups. (Figure 4, 5). Cu-Zn SOD positivity was less common in the control group, while it was more common in all other groups (Figure 6, 7). Positive expressions were especially in the acrosome parts of sperm cells.

Biochemical Evaluations

Sperm Lipid Profile

As a result of HPLC analysis of sperm lipids, 7 different lipid classes were detected; CholE, TAG, FFA, Chol, DAG, MAG and PL (Figure 8, Table 2). Among these lipid classes, sperm phospholipid ratio was taken into consideration, especially since it is the main component of the membrane structure and its destruction during storage damages cell integrity.

While the highest CholE level was seen in the control group, it was found to be lower in the D2, D4, N1 and N2 groups ($p<0.05$). No difference was found in the N4 group compared to the control group. There is a statistical difference between the control group and the other experimental groups in terms of TAG, FFA and Chol ($p<0.05$). DAG level did not make a statistical difference between the groups. A statistical difference was observed between the control and experimental groups in MAG and PL levels ($p<0.05$).

Sperm Protein Profile

30 protein bands between 287 kDa and 19.96 kDa were detected (Figure 9). Since there may be a decrease in both individual protein bands and total protein levels due to oxidative degradation during the storage of sperm cells, two different additives (naringin and diosmin) and a total of 5 different doses were compared for this purpose. For the total protein amount, the sum of the "band volume" values obtained directly from the densitometric analysis of the same sample, and the ratio of the "band volume" values of each individual protein band to the total "band volume" values were used for the% values (Table 3).

In general, it was observed that different antioxidants exhibited different levels of protective effect on different sperm proteins (Table 3). As a result, total protein amounts at different levels were determined (Table 3). The highest amount of total protein was seen in the N4 and D4 groups. Although the total amount of protein between the groups was statistically significant, quite large numbers were not seen. However, the total protein amounts - protein protective effects of all sperm samples with added antioxidants were higher than the control group (Table 3).

4. Discussion

In the presented study, the effects of naringin added to diluent at doses of 1, 2 and 4 mM and diosmin at doses of 2 and 4 mM on motility, membrane integrity, acrosome integrity and mitochondrial activity rates were investigated using fluorescent staining techniques regarding to post-freeze-thawing ram spermatological parameters. In addition, sperm membrane lipid profile analysis was performed with the High Performance Layer Chromatography (HPLC) method and sperm membrane protein profiles analysis

with SDS-PAGE method were performed. In terms of immunofluorescence, 8-OhDG antibody was used for DNA damage, and Cu - Zn SOD antibody was used for cell membrane integrity.

Motility, which is one of the success criteria of spermatozoa, is expressed as the ratio of all spermatozoon moving in any direction[31,32]. Motility is one of the markers of viability and structural integrity of spermatozoa [33]. Motility is controlled from the flagella and main part. While the flagellar part provides motility, the main part is responsible for hyperactivation [34-36]. In our study, a statistical difference was found between the D4 group and the control group in terms of motility findings ($p<0,05$). No difference was observed between the other groups. Similarly, it has been stated that 200 nM to 5 µM fullerene added to ram semen increases the total and progressive motility value [37]. In another study, gallic acid and carnosic acid added to ram semen were found to have the highest total motility value in gallic acid [38]. These differences are thought to be due to the added antioxidants and their doses.

Ram spermatozoon is extremely sensitive to lipid peroxidation caused by reactive oxygen species, as its plasma membrane is rich in unsaturated fatty acids. Freezing of semen causes cold shock that develops during the freezing process, damage due to phase change in membrane structures and oxidative stress. Developing oxidative stress and cytotoxic aldehydes (malondialdehyde, etc.) that develop cause damage to spermatozoon functions [39]. During the freezing of sperm cells, the formation of reactive oxygen species (ROS) is observed as a result of oxidative stress. These oxidative changes cause changes in sperm integrity and behavior. As a result, lipid, protein, DNA degradation, cell death, and ultimately a decrease in fertilization occur [40-44]. For this reason, the cold shock damage that develops in the environment can be minimized with some additives with cryoprotective and antioxidative properties, which are added to semen extender. In addition, antioxidants such as GPX, CAT and SOD that neutralize these species such as produced O_2 and H_2O_2 are found in both the mitochondria and the secretions of the reproductive system [45,46]. The fact that antioxidant compounds also have cryoprotectant properties provides better results from semen frozen with these substances [47-49]. Superoxide dismutase (SOD), which is in the family of metalloproteins, has two forms: Cu-Zn SOD, which is cytosolic, and Mn SOD, which is mitochondrial. SOD is common in mammalian tissues and has a significant protective effect against the harmful effects of superoxide anions [50,51]. In the present study, Cu Zn SOD positivity was observed less frequently in the control group, while it was observed more intensely in the experimental groups, and it was thought that the applied antioxidants activate the protective mechanism in the sperm cells. In a study, 5 mM methionine, 5 mM cysteamine and 1 mM cysteine were added to ram semen and a lower MDA level was observed compared to the control group [52]. Similarly, in our study, the Cu-Zn SOD level, which is a sign of oxidative stress, was found to be decreased in the antioxidant added groups. This suggests that it is necessary to add antioxidants to semen extenders in rams. In other study with naringin, [18] applied naringin to rats with diabetes, and it was observed that sperm parameters improved and oxidative stress decreased compared to the r diabetes group. This situation is similar to the low level of Cu-Zn SOD seen in naringin groups, and it is thought to be due to the antioxidant property of naringin.

The plasma membrane envelops the spermatozoon and holds the organelles and intracellular contents, and with its semi-permeable feature, it allows the passage of some soluble substances and ions. Some

plasma membrane proteins facilitate the transition of glucose and fructose from the extracellular environment into the spermatozoon [53-55]. In cases where the sperm plasma membrane is damaged, spermatozoon are considered dead and lose the ability to fertilize in vivo [56-58]. Therefore, it is essential to determine the plasma membrane integrity before the use of semen for assisted reproduction techniques such as in vitro fertilization (IVF) and artificial insemination (AI) [59]. In our study, plasma membrane integrity was found to be lower in N1 and N2 groups compared to other groups ($p<0.05$). However, plasma membrane integrity was found to be better in N4, D2 and D4 groups compared to other groups ($p<0.05$). Gungor et al. [38] in their study, observed that carnosic acid's plasma membrane and acrosome integrity were better in ram semen compared to other groups. In the same study, it was determined that gallic acid did not sufficiently protect the integrity of the plasma membrane and acrosome. In a study determining the effect of 7-dehydrocholesterol in ram semen, it was observed that it provided acrosome integrity compared to the control group [60]. This situation is similar to our study. It was observed that plasma membrane integrity from N1 and N2 groups was lower compared to N4 group. It can be interpreted that the dose increase preserves the integrity of the plasma membrane in the naringin groups. When the acrosome integrity was examined, it was observed that there was a statistical difference between the D4 group and the other experimental groups ($p<0.05$). Diosmin groups were observed to preserve acrosome integrity compared to naringin groups. The dose used in the D4 group was thought to be more effective in providing acrosomal integrity in ram semen.

Mitochondria in the spermatozoon are localized in the middle part above the main part of the flagellum. Mitochondria produce ATP through oxidative phosphorylation [61]. Mitochondria are located in the neck of the spermatozoon, and the mitochondria in the sperm are morphologically and biochemically different from somatic cell organelles. These differences are related to the finding of specific enzymes in organelles. Spermatozoa use different energy sources and thus their metabolic and physicochemical states change [62]. It is important to evaluate spermatozoon before fertilization process with assisted reproduction techniques in terms of mitochondrial functional integrity. If a sperm cell has a functional mitochondria, after staining with a fluorescent dye, the entire middle part is stained; if it does not have a functional mitochondria, none of the middle part is stained [63]. It has been reported that antioxidant substances used in ram semen increase the mitochondrial membrane potential [38]. In our study, N4, D2 and D4 groups were found to have higher mitochondrial activity ($p<0,05$). However, no difference was observed in the N1 and N2 groups compared to the other groups. In this case, it can be thought that diosmin is an exogenous energy source for spermatozoa. However, low-dose naringin appears to be ineffective. In a similar study with different antioxidants (methionine, curcumin, ellagic acid; 1, 2, 4 mM; each), the antioxidant-containing groups showed higher motility value and acrosome integrity ratio than the control group in ram semen. The doses of methionine (1 mM), curcumin (1, 2 mM) and ellagic acid (1, 2 mM) gave a higher rate of sperm plasma membrane integrity than the control group. In addition, in terms of mitochondrial activity, it was determined that 1 mM doses of antioxidant groups provided higher protection compared to other groups [64].

Lipids are important components of the cell membrane. Membrane lipid composition has been associated with the different functions of spermatozoa. Many researchers associate membrane lipids

with the survival success of sperm cells after cryopreservation or cold shock. Because while sperm cell maturation or acrosome reaction is a natural phenomenon, cryopreservation or cold shock is not. Sperm cells are unnatural for such an alteration and are not programmed in such a situation and are directly under stress. Therefore, the change in membrane lipid composition due to cold shock or cryopreservation can be seen as the response of sperm cells to a certain stressful situation [65]. The greatest damage during cryopreservation is the peroxidation of lipids, especially phospholipid-bound polyunsaturated fatty acids (PUFAs) [66,67]. Increased free cholesterol, free fatty acids, triacylglycerol and cholesterol ester concentrations cause negative effects on sperm functions [68]. The biggest problem associated with the cryopreservation of sperm cells is the loss of viability as a result of freezing- thawing process [69-71]. Loss of viability is associated with membrane damage induced by peroxidation of sperm phospholipids [72-73]. In the study, a statistical difference was observed in CholE, TAG, FFA, CHOL, MAG, PL levels compared to the control group. This can be interpreted as naringin and diosmin change the lipid composition of semen and make it resistant to oxidative stress. However, there was no difference between the groups in terms of DAG level.

Cryopreservation changes sperm protein composition and sperm quality. In a study, they observed that the protein content of sperm membrane and seminal fluid changed in the freezing process of semen taken from Murrah buffaloes [73]. In our study, the highest protein amounts were obtained from the N4 and D4 groups. However, sperm total protein amounts of all groups with antioxidant added were higher than the control group. When the ratios of 30 individual proteins in the total protein content were examined to control why this situation occurred, it was observed that each antioxidant prevented oxidative protein degradation during cryopreservation by exerting different levels of protective effect on different proteins (Table 3).

It has been reported that 8-OhDG is a marker showing DNA degradation in sperm [74]. In the present study, the detection of 8-OhDG positivity in the control group and the other experimental groups in immunofluorescence staining at a similar and very low level was interpreted as no statistically significant DNA damage occurred in the sperm cells during the dilution and freezing of the semen samples.

In conclusion in order to increase the fertility ability of frozen semen in rams, some antioxidant substances are added to the extenders. Although they have positive effects in general, the effectiveness of antioxidants varies according to animal species, breed, season, diluent components and freezing protocols. In addition, the findings obtained in spermatological parameters after freezing-thawing may also vary depending on the techniques used in reconstitution and freezing of semen, changes in solution time and temperature, or the person performing the analysis. In our presented project study, it was observed that the protective effect of diosmin on plasma membrane integrity (2. 4 mM), acrosome integrity (4 mM), lipid profile (4 mM) and protein profile (4 mM) came to the fore. It was observed that naringin, applied at a dose of 1 mM, increased mitochondrial activity compared to the control group. It is thought that it would be meaningful to support in vitro examination parameters with in vitro/vivo fertility parameters in further studies to be carried out.

Declarations

CRediT authorship contribution statement

Ali Doğan Ömür: Conceptualization, investigation, resources, writing and editing. **Özgür Kaynar:** Investigation, formal analysis. **Mustafa İleritürk:** Investigation, formal analysis. **Ali Erdem Öztürk:** Semen collection, dilution, freezing and also spermatological analysis. **Mustafa Bodu:** Semen collection, dilution, freezing and also spermatological analysis.

İpek Nur Şahin: Semen collection, dilution, freezing and also spermatological analysis. **Serkan Ali Akarsu:** Investigation, formal analysis, supervision.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The project work entitled "Immunofluorescence and Biochemical Investigation of the Protective Effects of Naringin and Diosmine on the Freezability of Merino Ram Semen" was financially supported by TUBİTAK (116O604 -3001).

References

[1]Ömür AD. Effect of Antioxidants on Cryopreservation of Ram Semen in and out of Breeding Season, doctoral thesis, Selcuk Unıversıty Health Scıences Instıtute, Konya. 2011.

[2]Baıley JL, Bılodeau JF, Cormıer N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. Journal of andrology, 2000; 21(1): 1-7.

[3]Maxwell WM, Evans G, Rhodes SL, Hillard MA, Bindon BM. Fertility of superovulated ewes after intrauterine or oviducal insemination with low numbers of fresh or frozen-thawed spermatozoa. Reproduction, fertility and development, 1993; 5(1): 57-63.

[4]Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post- thawing function. Reprod Fertil Dev 1995; 7: 871-91.

[5]Chen Y, Foote RH, Brockett CC. Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. Cryobiology 1993; 30(4): 423-31.

[6] Purdy PH. A review on goat sperm cryopreservation. Small Ruminat Research 2006; 63: 215-25.

[7]Bucak MN, Ateşşahin A, Varışlı Ö, Yüce A, Tekin N, Akçay A. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: Microscopic and oxidative stress parameters after freeze-thawing process. Theriogenology 2007; 60: 1060-7.

[8]Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. J Androl 2000; 21(1): 1-7.

[9]Salamon S, Maxwell WMC. Storage of ram semen. Anim Reprod Sci 2000; 62: 77-111.

[10]Alvarez JG, Storey BT. Role of superoxide dismutase in protecting rabbit spermatozoa from O2 toxicity due to lipid peroxidation. Biol Reprod 1983;28(5): 1129-36.

[11]Akpınar D, Özyürek M. Synthesis, characterization and analysis of antioxidant capacities of some flavanon-oxime compounds. Istanbul University Institute of Science Thesis, 2013.

[12]Rathi VK, Das S. Parampalli Raghavendra, A, Rao BSS. Naringin abates adverse effects of cadmium-mediated hepatotoxicity: An experimental study using HepG2 cells. Journal of biochemical and molecular toxicology 2017; 31(8): e21915.

[13]Murunga AN, Miruka DO, Driver C, Nkomo FS, Cobongela SZ, Owira PM. Grapefruit derived flavonoid naringin improves ketoacidosis and lipid peroxidation in type 1 diabetes rat model. PLoS One 2016; 11(4): e0153241.

[14]Xulu S, Owira PMO. Naringin ameliorates atherogenic dyslipidemia but not hyperglycemia in rats with type 1 diabetes. Journal of Cardiovascular Pharmacology 2012; 59(2): 133-41.

[15]Pu P, Gao DM, Mohamed S, Chen J, Zhang J, Zhou XY, Jiang H. Naringin ameliorates metabolic syndrome by activating AMP-activated protein kinase in mice fed a high-fat diet. Archives of biochemistry and biophysics 2012; 518(1): 61-70.

[16]Chandramohan Y, Parameswari CS. Therapeutic efficacy of naringin on cyclosporine (A) induced nephrotoxicity in rats. Involvement of hemeoxygenase-1. Pharmacological Reports 2013; 65(5): 1336-44.

[17]Kwatra M, Kumar V, Jangra A, Mishra M, Ahmed S, Ghosh P, Khanam R. Ameliorative effect of naringin against doxorubicin-induced acute cardiac toxicity in rats. Pharmaceutical biology 2016; 54(4): 637-47.

[18]Akondi RB, Kumar P, Annapurna A, Pujari M. Protective Effect of Rutin and Naringin on Sperm Quality in Streptozotocin (STZ) Induced Type 1 Diabetic Rats. Iran J Pharm Res 2011; 10(3): 585–96.

[19]Adil M, Visnagri A, Kumar VS, Kandhare AD, Ghosh P,Bodhankar SL. Protective Effect of Naringin on Sodium Arsenite Induced Testicular Toxicity via Modulation of Biochemical Perturbations in Experimental Rats. Pharmacologia, 2014; 5: 222-34.

[20]Srinivasan S, Pari L. Ameliorative effect of diosmin, a citrus flavonoid against streptozotocin-nicotinamide generated oxidative stress induced diabetic rats. Chem. Biol. Interact 2012; 195: 43-51.

[21]Camarda L, Di Stefano V, Del Bosco SF, Schillaci D. Antiproliferative activity of citrus juices and HPLC evaluation of their flavonoid composition. Fitoterapia 2007; 78: 426-29.

[22]Silambarasan T, Raja B. Diosmin, a bioflavonoid reverses alterations in blood pressure, nitric oxide, lipid peroxides and antioxidant status in DOCA-salt induced hypertensive rats. Eur J Pharm 2012; 679: 81-9.

[23]Aisen EG, Medina VH, Venturino A. Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. Theriogenology 2002; 57(7): 1801-08.

[24]Garner DL, Johnson LA Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biology of Reproduction 1995; 53: 276-84.

[25]Nagy S, Jansen J, Topper EK, Gadella BM. A triplestain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biology of Reproduction 2003; 68: 1828-35.

[26]Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa.

Biology of Reproduction 1997; 57: 1401-6.

[27]Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227(5259): 680-5.

[28]Hara A, Radin NS. Lipid extraction of tissues with a low-toxicity solvent. Analytical Biochemistry 1978; 90(1): 420-6.

[29]Kaynar O, Ileriturk M, Hayirli A. Evaluation of Computational Modifications in HPTLC with Gel Analysis Software and Flatbed Scanner for Lipid Separation. Journal of Planar Chromatography 2013; 26(3): 202-8.

[30]Soria-Meneses PJ, Jurado-Campos A, Montoro V, Soler A J, Garde JJ, Fernández-Santos MDR. Ovine sperm DNA oxidation quantification using an 8-OHdG immunodetection assay. Reproduction in Domestic Animals 2019: 54: 59-64.

[31] Hafez ESE. Semen evaluation. Reproduction in Farm Animals, 1987; 5: 455–480.

[32] Januskauskas A, Söderquist L, Håård MG, Håård MC, Lundeheim N. Influence of sperm number per straw on the post-thaw sperm viability and fertility of Swedish red and white A.I. bulls. Acta Veterinaria Scandinavica, 1996; 37 (4): 461-470. doi: 10.1186/BF03548086

[33]Kathiravan P, Kalatharan J, Karthikeya G, Rengarajan K, Kadirvel G. Objective sperm motion analysis to assess dairy bull fertility using computer-aided system–a review. Reproduction in Domestic Animals 2011; 46 (1): 165-172. doi: 10.1111/j.1439- 0531.2010.01603.x

[34]Krisfalusi M, Miki K, Magyar PL, O'Brien DA. Multiple glycolytic enzymes are tightly bound to the fibrous sheath of mouse spermatozoa. Biology of reproduction, 2006; 75(2): 270-8.

[35]Eddy EM, Toshimori K, O'Brien DA. Fibrous sheath of mammalian spermatozoa. Microscopy research and technique 2003; 61(1): 103-15.

[36]Vemuganti SA, Bell TA, Scarlett CO, Parker CE, de Villena FPM, O'Brien DA. Three male germline-specific aldolase A isozymes are generated by alternative splicing and retrotransposition. Developmental biology 2007; 309(1): 18-31.

[37]Türk, G., Koca, R. H., Güngör, İ. H., Cinkara, S. D., Acısu, T. C., Erişir, F. E., ... & Karatepe, M. (2022). Effect of hydrated C60 fullerene on lipid, vitamin and amino acid composition in frozen-thawed ram semen. Animal Reproduction Science, 106939.

[38]Gungor, S., Inanc, M. E., Ozturk, C., Korkmaz, F., Bastan, I., Cil, B., & Kastelic, J. P. (2019). Gallic and carnosic acids improve quality of frozen-thawed ram spermatozoa. Andrologia, 51(10), e13393.

[39]Aitken RJ. Pathophysiology of human spermatozoa. Curr Opin Obstet Gynecol, 1994; 6: 128-5.

[40]Twrda E, Lukac N, Jambor T, Lukacova J, Hashim F, Massanyi P. In vitro supplementation of lycopene to bovine spermatozoa: effects on motility, viability and superoxide production. Animal Science Papers and Reports 2016; 34: 319-28.

[41]Ball BA. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Animal Reproduction Science 2008; 107: 257-67.

[42]Bucak MN, Atessahin A, Yuce A. Effect of antioxidants and oxidative stress parameters on ram semen after the freeze-thawing process. Small Ruminant Research 2008; 75: 128-34.

[43]Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertility and Sterility 2003; 79: 829-43.

[44]Schieber M, Chandel NS. ROS Function in Redox Signaling and Oxidative Stress. Curr Biol. 2014; 24(10): 453-62.

[45]Vernet P, Aitken RJ, Drevet JR. Antioxidant strategies in the epididymis. Molecular and Cellular Endocrinology 2004; 216: 31-9.

[46]Starkov AA. The role of mitochondria in reactive oxygen species metabolism and signaling. Annals of the New York Academy of Sciences 2008; 1147: 37–52.

[47]Alvarez JG, Storey BT. Assessment of cell damage caused by spontaneous lipid peroxidation in rabbit spermatozoa. Biology of Reproduction 1984; 30(2): 323-31.

[48]Kobayashi T, Miyasaki T, Natori M, Nozawa S. Protective role of superoxide dismutase in human sperm motility: Superoxide dismutase activiy and lipid peroxidase in human seminal plasma and spermatozoa. Hum Reprod, 1991; 6: 987-91.

[49]Bucak MN, Tuncer PB, Sarıözkan S, Başpınar N, Taşpınar M, Çoyan K, Bilgili A, Akalın PP, Büyükleblebici S, Aydos S, Ilgaz,S, Sunguroğlu A, Öztuna D. Effects of antioxidants on post-thawed bovine sperm and oxidative stres parameters: Antioxidants protect DNA integrity against cryodamage. Cryobiology 2010; 61(3): 248-53.

[50]McCord JM, Fridovich I. Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). Journal of Biological Chemistry 1969; 244: 6049-55.

[51]Bannister JV, Bannister WH, Rotilio G. Aspects of the structure, function, and applications of superoxide dismutase. CRC. Critical Reviews in Biochemistry 1987; 22: 111-80.

[52]Alçay S, Toker M.B, Gökçe E, et al. Improvement of incubation resilience with various antioxidants in cryopreserved ram semen. Erciyes Üniv Vet Fak Derg 2017; 14; 183-190.

[53]Schurmann A, Axer H, Scheepers A, Doege H, Joost HG. The glucose transport facilitator GLUT8 is predominantly associated with the acrosomal region of mature spermatozoa. Cell and Tissue Research 2002; 307: 237–42.

[54]Angulo C, Rauch MC, Droppelmann A, Reyes AM, Slebe JC, Delgado-Lopez F, Guaiquil VH, Vera JC, Concha II. Hexose transporter expression and function in mammalian spermatozoa: cellular localization and transport of hexoses and Vitamin C. Journal of Cellular Biochemistry 1998; 71: 189-203.

[55]Burant CF, Davidson NO. GLUT3 glucose transporter isoform in rat testis: localization, effect of diabetes mellitus, and comparison to human testis. American Journal of Physiology, 1994; 267: 1488-95.

[56]Kusakabe H, Kamiguchi Y. Chromosomal integrity of freeze-dried mouse spermatozoa after 137Cs gamma-ray irradiation. Mutation Research 2004; 556: 163-8.

[57]Liu JL, Kusakabe H, Chang CC, Suzuki H, Schmidt DW, Julian M, Pfeffer R, Bormann CL, Tian XC, Yanagimachi R, Yang X. Freeze-dried sperm fertilization leads to full-term development in rabbits. Biology of Reproduction 2004; 70: 1776–81.

[58]Kwon IK, Park KE, Niwa K. Activation, pronuclear formation, and development in vitro of pig oocytes following intracytoplasmic injection of freeze-dried spermatozoa. Biology of Reproduction 2004; 71: 1430–6.

[59]Garner DL, Johnson LA, Yue ST, Roth BL, Haugland RP. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. Journal of Andrology 1994; 15: 620–9.

[60]Inanc, M. E., UYSAL, O., & Ayhan, A. T. A. (2018). Cryopreservation and evaluation of Akkaraman ram semen with 7-dehydrocholesterol. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 65(2), 187-192.

[61]Marin S, Chiang K, Bassilian S, Lee WN, Boros LG, Fernandez-Novel JM, Centelles JJ, Medrano A, Rodriguez-Gil JE, Cascante M. Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization. FEBS Letters 2003; 554: 342-6.

[62]Piomboni DJ, Foracelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. International Journal of Andrology 2012; 35(2):109-24.

[63]Mukai C, Okuno M. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biology of Reproduction 2004; 71: 540–7.

[64]Ömür AD, Çoyan K. Protective effects of the antioxidants curcumin, ellagic acid and methionine on motility, mitochondrial transmembrane potential, plasma membrane and acrosome integrity in freeze-thawed Merino ram sperm. Veterinarni Medicina 2016; 61(1): 10-16.

[65]Mandal R, Badyakar D, Chakrabarty J. Role of Membrane Lipid Fatty Acids in Sperm Cryopreservation. Advances in Andrology 2014.

[66]Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. American Journal of Reproductive Immunology 2008; 59(1): 2-11.

[67]Aitken RJ. The role of free oxygen radicals and sperm function. International Journal of Andrology 1989; 12(2): 95-7.

[68]Cerolini S, Kelso KA, Noble RC, Speake BK, Pizzi F, Cavalchini LG. Relationship between spermatozoan lipid composition and fertility during aging of chickens. Biology of Reproduction 1997; 57(5): 976-80.

[69]Overstreet JW, Drobnis EZ. Sperm transport in the female tract. In Advances in Donor Insemination, C. L. R. Barratt and I. D. Cooke, Eds., Cambridge University Press, Cambridge, UK. 1993.

[70]Centola GM, Raubertas RF, Mattox JH. Cryopreservation of human semen: comparison of cryopreservatives, sources of variability, and prediction of post-thaw survival. Journal of Andrology 1992; 13(3): 283-88.

[71]Watson PF, Kunze E, Cramer P, Hammerstedt RH. A comparison of critical osmolality and hydraulic conductivity and its activation energy in fowl and bull spermatozoa. Journal of Andrology 1992; 13(2): 131-8.

[72]Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. Journal of Andrology 1987; 8(5): 338-48.

[73]Dhanju CK, Cheema RS, Kaur SP. Effect of Freezing on Proteins and Protein Profiles of Sperm Membrane Extracts and Seminal Plasma of Buffalo Bulls. Asian-Australasian Journal of Animal Sciences 2001;14(12): 1678-82.

[74]Shen HM, Ong CN. Detection of oxidative DNA damage in human sperm and its association with sperm function and male infertility. Free Rad Biol Med, 2000; 28: 529-36.

Tables

Table 1. Evaluation of study groups in terms of spermatological parameters after freezing-thawing

	Motility %	Plasma membrane integrity %	Acrosome integrity %	Mitochondrial activity %
C	46,66±4,40^{a}	77,60 ±2,23 ^{a}	60,06±1,79 ^{a}	56,30±0,58^{abcd}
D2	48,33±4,01	78,43±3,52 ^{ab}	61,66±1,13 ^{a}	62,65±4,63 ^{ac}
D4	58,00±4,01^{a}	80,23±2.15 ^{ab}	68,50±1,09 ^{b}	64,00±0,89 ^{ad}
N1	47,50±4,42	67,85±4,32 ^{ab}	61,60±0,85 ^{a}	58,50±3,28^{bcd}
N2	44,16±,74	67,56±5,06 ^{ab}	62,53±4,16 ^{a}	55,25±9,85^{bcd}
N4	47,50±2,81	77,36±1,09 ^{b}	59,76±1,29 ^{a}	65,05±5,06 ^{ab}

a-b: Differences between means with different letters in the same column are significant ($p<0.05$).

Table 2. Lipid profiles of post freeze-thawing sperm homogenates.

Groups	CholE	TAG	FFA	CHOL	DAG	MAG	PL
C	$8,65 \pm 0,19^{a}$	$55,94 \pm 0,50^{c}$	$6,94 \pm 0,18^{a}$	$8,02 \pm 0,20^{bc}$	$2,11 \pm 0,22$	$2,53 \pm 0,46^{abc}$	$15,81 \pm 0,60^{a}$
D2	$5,34 \pm 0,11^{c}$	$58,86 \pm 0,31^{ac}$	$5,39 \pm 0,25^{b}$	$8,34 \pm 0,24^{abc}$	$1,92 \pm 0,12$	$1,46 \pm 0,11^{c}$	$18,68 \pm 0,46^{ab}$
D4	$6,13 \pm 0,22^{c}$	$57,35 \pm 0,53^{abc}$	$5,08 \pm 0,45^{b}$	$8,36 \pm 0,09^{abc}$	$1,97 \pm 0,29$	$1,75 \pm 0,26^{bc}$	$19,36 \pm 0,84^{a}$
N1	$6,18 \pm 0,06^{c}$	$58,21 \pm 0,80^{abc}$	$5,15 \pm 0,44^{b}$	$8,11 \pm 0,23^{bc}$	$2,31 \pm 0,20$	$1,91 \pm 0,27^{abc}$	$18,14 \pm 0,67^{abc}$
N2	$6,98 \pm 0,61^{b}$	$57,32 \pm 0,65^{abc}$	$5,19 \pm 0,24^{b}$	$8,58 \pm 0,05^{ab}$	$2,10 \pm 0,30$	$2,57 \pm 0,25^{ab}$	$17,27 \pm 0,71^{bc}$
N4	$8,62 \pm 0,37^{a}$	$56,24 \pm 0,77^{bc}$	$5,52 \pm 0,17^{b}$	$8,52 \pm 0,19^{ab}$	$2,13 \pm 0,23$	$2,30 \pm 0,43^{abc}$	$16,68 \pm 0,50^{ac}$
Different letters in the same line are different from each other ($p < 0.05$)							
Abbreviations:CholE, Cholesterol esters; TAG, Triacyl glycerol; FFA, Free fatty acids; CHOL, Cholesterol; DAG, Diacyl glycerol; MAG, Monoacyl glycerol; PL, Phospholipids							

Table 3
Protein profiles of post freeze-thawing sperm homogenates.

MW	C		D2		D4		N4		N2		N1	
kDA	Vol	%	Vol	%	Vol	%	Vol	%	Vol	%	Vol	%
287.037	55	0,9	35	0,5	32	0,5	50	0,7	51	0,8	45	0,7
250.000	126	2	151	2,2	166	2,4	246	3,6	164	2,5	157	2,4
204.720	123	1,9	98	1,4	137	2	140	2,1	90	1,4	140	2,1
158,619	114	1,8	120	1,8	1,3	1,5	101	1,5	137	2,1	119	1,8
150.000	124	1,9	157	2,3	149	2,2	112	1,6	123	1,8	130	2,0
118.514	158	2,5	231	3,4	202	3	228	3,3	231	3,5	226	3,4
109.322	163	2,5	156	2,3	142	2,1	159	2,3	168	2,5	160	2,4
98.438	119	1,9	120	1,8	85	1,3	123	1,8	108	1,6	93	1,4
96.103	138	2,1	156	2,3	197	2,9	155	2,3	145	2,2	177	2,7
90.735	203	3,1	178	2,6	159	2,3	162	2,4	197	3,0	185	2,8
76.808	286	4,4	449	6,6	444	6,5	431	6,3	405	6,1	406	6,1
69.156	224	3,5	228	3,4	229	3,4	223	3,3	213	3,2	220	3,3
60.604	280	4,3	313	4,6	320	4,7	324	4,8	305	4,6	308	4,6
57.278	237	3,7	344	5,1	271	4,0	341	5,0	341	5,1	323	4,9
51.412	235	3,6	123	1,8	171	2,5	121	1,8	118	1,8	132	2,0
50.531	137	21	87	1,3	132	2,0	148	2,2	131	2,0	122	1,8
48.740	162	2,5	184	2,7	141	2,1	115	1,7	135	2	122	1,8
47273	125	1,9	132	1,9	116	1,7	160	2,3	134	2	127	1,9
43.941	163	2,5	170	2,5	182	2,7	162	2,4	165	2,5	140	2,1
41.763	211	3,3	190	2,8	192	2,8	177	2,6	167	2,5	171	2,6
39.851	160	2,5	135	2	132	1,9	133	1,9	143	2,2	225	3,4
36.288	474	7,4	538	7,9	528	7,8	510	7,5	505	7,6	499	7,5
31.343	248	3,9	253	3,7	312	4,6	265	3,9	201	3	219	3,3
30.954	144	2,2	154	2,3	145	2,1	145	2,1	204	3,1	189	2,8
29.064	254	3,9	245	3,6	249	3,7	281	4,1	272	4,1	241	3,6
27.536	437	6,8	431	6,4	412	5,1	380	5,6	416	6,3	392	5,9
25.000	428	6,7	496	7,3	498	7,3	535	7,8	470	7,1	508	7,6
20.541	266	4,1	253	3,7	287	4,2	267	3,9	239	3,6	207	3,1
19.963	252	3,9	278	4,1	242	3,6	249	3,6	260	3,9	290	4,4
19.963	396	6,2	372	5,5	410	6	379	5,6	401	6	373	5,6
Total	**6440^{c}**	**100**	**6775^{ab}**	**100**	**6786^{ab}**	**100**	**6820^{a}**	**100**	**6636^{b}**	**100**	**6650^{b}**	**100**
Different letters in the same line are different from each other ($p < 0.05$)												

Figures

Figure 1

Legend not included with this version.

Figure 2

Legend not included with this version.

Figure 3

Legend not included with this version.

Figure 4

Legend not included with this version.

Figure 5

Legend not included with this version.

Figure 6

Legend not included with this version.

Figure 7

Legend not included with this version.

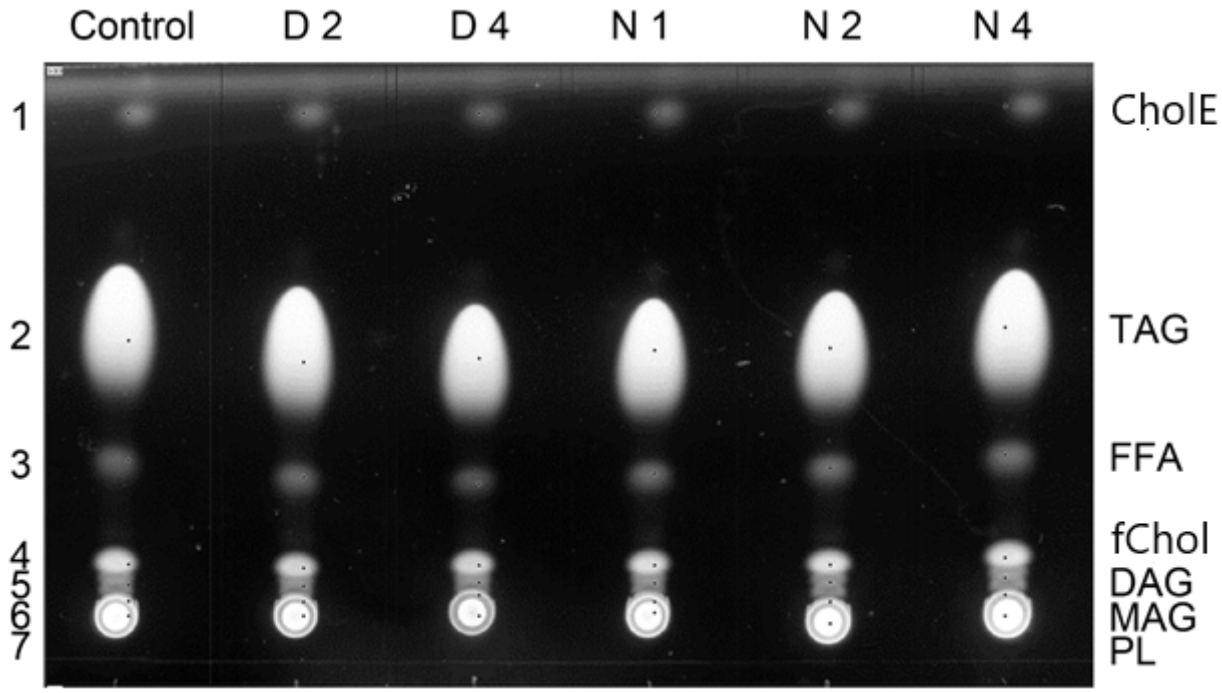

Figure 8

Legend not included with this version.

Figure 9

Legend not included with this version.