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Investigation of the Effectiveness of Quercetin on Acrosome Integrity of Merino Ram Sperm

Ali Doğan Ömür[1,3,4,*], Oğuzhan Uluyol[2]

[1]Atatürk University Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Erzurum, Turkey

[2]Uluyol-Ak Veterinary Clinic, Inegol, Bursa, Turkey

[3]Atatürk University Faculty of Veterinary Medicine, Department of Biochemistry, Erzurum, Turkey

[4]Ataturk University, Faculty of Science, Department of Molecular Biology and Genetics, Erzurum, Turkey

[*]**Corresponding author:** Ali Doğan Ömür, Atatürk University Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Erzurum, Turkey

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Abstract

To achieving high efficiency in sheep, ram sperm must have high fertilization ability. Quercetin, a potent antioxidant, has been shown to have a positive effect on semen in different species. This study aims to improve the efficiency of the Merino ram sperm using quercetin. The ejaculates collected from Merino rams were used for the dilution of the sperm (sperm density ≥ 3×109 spermatozoa/ml, motility ≥ 75%). Tris diluent was used to dilute the semen. The ejaculates were divided into 4 equal volumes at 32 °C. Three groups were formed by adding quercetin (15, 30, 45 µg/mL) at different doses. Antioxidant (quercetin) did not participate in the 4th group (the control group). Spermatozoa were diluted and frozen to approximately 4x108 spermatozoa per ml with Tris diluent. After thawing, the master mix was prepared with 60µl semen, 10µl FITC-PNA and 2.5µl PI and allowed to incubate at 37 °C for 20 minutes. The reaction was then terminated after treatment with 10µL Hancock solution. An amount of 2.5µl of the sample was taken and 200 sperm were counted at 400 magnifications on a fluorescent microscope. Sperm cells with a green color were evaluated as damaged acrosome, and no green colour was regarded as undamaged acrosome. In terms of the undamaged percentage of acrosomal integrity was assessed in the control group and quercetin groups (15, 30, and 45µg/mL). Findings: 39.16 ± 2.65b in the 15µg/mL group, 40.83 ± 0.30b in the 30µ/mL group, 20.83 ± 2.95a in the 45µg/mL group and 54.83 ± 3.38c in the control group. The percentage of the three groups formed with the quercetin substance added at different doses (15, 30, 45µg/mL) were lower than the control group. Quercetin has been shown to toxicize acrosome integrity at these doses.

The study consisted of 6 replicates.

Keywords: Acrosome İntegrity; Quercetin; Ram; Semen.

Introduction

Sheep breeding has an important place in animal production, therefore in agricultural production and the economies of countries. Products such as meat, milk and dairy products, fleece and leather obtained from sheep meet the important needs of people. These animals can utilize feed sources that

many livestock species cannot utilize. For these reasons, sheep breeding is carried out all over the world. While sheep breeding is so important, it is aimed to increase productivity in sheep breeding. In order to increase production in sheep breeding, it is necessary to get more yield from a unit animal. For this purpose, it is necessary to improve the breeding characteristics of sheep. It is obvious that a high-fertile offspring cannot be obtained from a female whose fertility is not good, samely, a good reproductive yield cannot be obtained from a male animal whose semen quality are not suitable. For these reasons, it is important that the fertilization ability of the ram sperm is high in the efficient continuation of sheep breeding. In order to increase the fertilization ability of ram semen, antioxidant substances are used especially to reduce the damaged sperm cells after freezing-thawing **[1]**.

After freezing-thawing, the antioxidant capacity of semen decreases and the formation of reactive oxygen radicals increases, causing adverse effects on spermatological characteristics and fertility. Freeze-thawed semen is more sensitive to peroxidation than fresh semen. Recent studies point to the peroxidation of spermatozoa membrane lipids as the most important factor causing infertility during semen preservation. For this reason, antioxidant substances are added to semen extenders due to the thought that they may have positive effects on spermatological properties after freezing-thawing **[2]**.

Antioxidant substances, which are present in high amounts in body fluids and reproductive tissues of mammals, when added to semen extender, reduce the peroxidation of fatty acids and phospholipids in the cell membranes caused by free radicals in the semen **[3]**.

Quercetin is an antioxidant with flavonoid structure, consisting of 3 rings and 5 hydroxyl groups and is widely found in different plant materials such as fruits, vegetables, and grains **[4-6]**.

Besides, quercetin is a powerful antioxidant with broad biological effects such as anticarcinogenic, antiasthmatic, antiviral, antiallergenic, antihypertensive, hepatoprotective, antidiabetic, anti-inflammatory, cardioprotective and antibacterial activities. It is found in vegetables and fruits such as apples, onions and red wine, grapefruit, black tea, raspberries, blueberries, cherries, broccoli, and it is a type of flavonoid that daily consumption of 25-30 mg is sufficient for humans **[6-9]**.

Because quercetin is a powerful antioxidant, it can improve sperm quality by reducing the damage caused by reactive oxygen species **[10,11]**.

It is aimed to investigation of the effectiveness of quercetin on sperm acrosome integrity of merino ram sperm.

Material And Method

Prior to the project application, the approval of Atatürk University Animal Experiments Local Ethics Committee was obtained (No: 2016/145).

Animal Material

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 Sperma

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. The ejaculates were divided into 6 equal volumes at 32 °C and diluted with Tris diluent containing quercetin (15, 30, and 45µg/mL) and containing no antioxidant (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 °C for approximately 3 hours. In the period following the equilibration, samples were frozen in liquid nitrogen vapor (~ -100 °C) for 10 minutes and stored in liquid nitrogen at -196 °C. The study consisted of 6 replications.

Determination of Sperm Acrosome Integrity

In the study, semen samples with and without antioxidants were evaluated in terms of acrosome integrity using fluorescent staining techniques.

Sperm acrosome status was assessed using fluorescein isothiocyanate conjugated to Arachis hypogea (peanut) (L7381 FITC-PNA, Invitrogen) and PI staining as described by Nagy et al. (2003). About 120 µg of FITC-PNA was added to 1 ml of PBS (15630056, Invitrogen) to make staining

solution, and then divided into equal aliquots (100 µl) after filtering and stored at –20 °C. The thawed straw was (at 37 °C for 30 seconds) diluted 1: 3 with Tris stock solution without glycerol and egg yolk, and then 60 µl of diluted semen were mixed with 10 µl of FITC-PNA and 2.5 µl of PI. The sample was gently mixed, incubated at 37 °C in the dark for 20 min, and then 10 µl of Hancock solution were added to stop sperm movement. A wet mount was made using a 2.5 µl drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 sperm cells were examined at 400 × magnification under a fluorescence microscope (Leica DM 3000; excitation at 450–490 nm, emission at 520 nm) to assess the sperm acrosome integrity. Spermatozoa displaying bright green or patchy green fluorescence were considered as acrosome non-intact or damaged, whereas cells that did not stain green in the acrosome cap were regarded as acrosome intact.

Statistical Analysis

In the study, in order to compare four different groups with statistical analysis, analysis of variance and Post Hoc Tukey HSD test were used to compare more than two groups with significant differences between them. SPSS 15.0 for Windows Evaluation Version statistical analysis program was used. As a result, the difference at the $P < 0.001$ level was accepted as very significant.

Results

Table 1. Effect of quercetin on acrosome integrity (x ± SEM)

Groups	Rate of undamaged acrosomes (%)
Control	54, 83 ± 3, 38^{c}
Quercetin 15 µg/mL	39, 16 ± 2, 65^{b}
Quercetin 30 µg/mL	40, 83 ± 0, 30^{b}
Quercetin 45 µg/mL	20, 83 ± 2, 95^{a}
P	*

a, b, c: Differences between means with different letters in the same column are significant (*: $p < 0.001$).

The effect of quercetin at different doses on the acrosome integrity of ram semen is shown in **Table 1**. In the control group without quercetin, the percentage of intact acrosome integrity was found to be 54.83 ± 3.38^{c}. When the effects of different doses of quercetin substances on acrosome integrity are examined.

The percentage of undamaged acrosomes was found to be 39.16 ± 2.65^{b} in the semen group that added 15µg/mL quercetin. When this result is compared with the control group. It was determined that the ratio of undamaged acrosomes was lower than the control group and this difference was statistically significant. It was evaluated that the addition of 15µg/mL quercetin had a negative effect on the acrosome integrity.

The percentage of acrosomes that were not damaged in the sperm group that added 30µg/mL quercetin was found to be 40.83 ± 0.30b. When this result is compared with the control group. It was determined that the ratio of undamaged acrosomes was lower than the control group and this difference was statistically significant. The difference between the group that added 30µg/mL quercetin and the group that added 15µg/mL quercetin was not significant but was similar.

The percentage of undamaged acrosomes in the semen group with 45µg/mL quercetin was found to be 20.83 ± 2.95^{a}. When this result is compared with the control group. It was determined that the rate of undamaged acrosomes was much lower than the control group. The difference between the control group and the other two (15µg/mL quercetin, 30µg/mL quercetin) groups was statistically significant. It

was determined that the addition of 45µg/mL quercetin had a toxic effect on the acrosome integrity. Below are the microscopic images of the undamaged acrosome (Red Colored Sperm Cell) integrity of the control group and the sperm groups with different doses of quercetin.

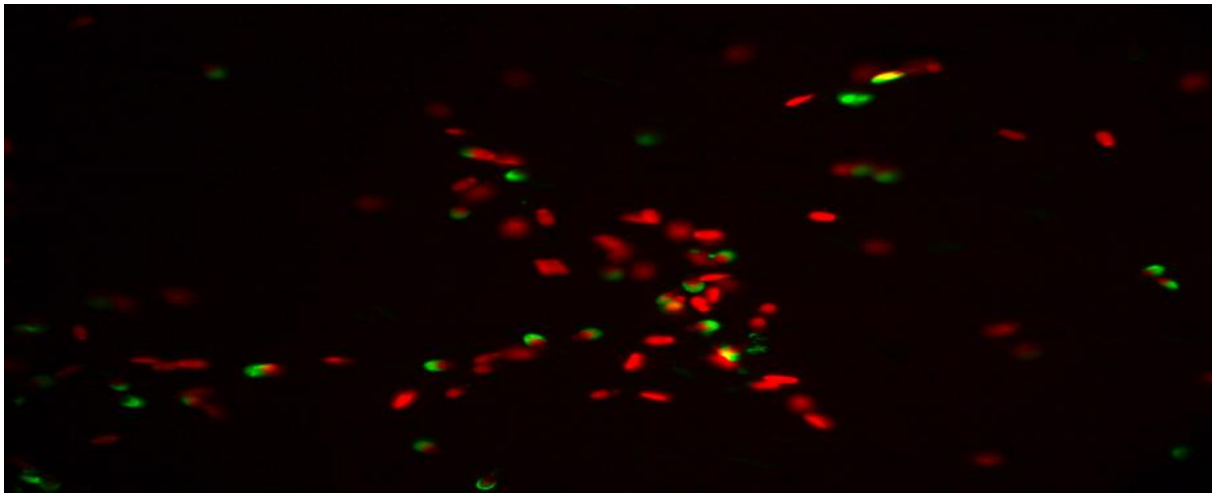


Figure 1.: Microscopic image of undamaged acrosome integrity in the control group.

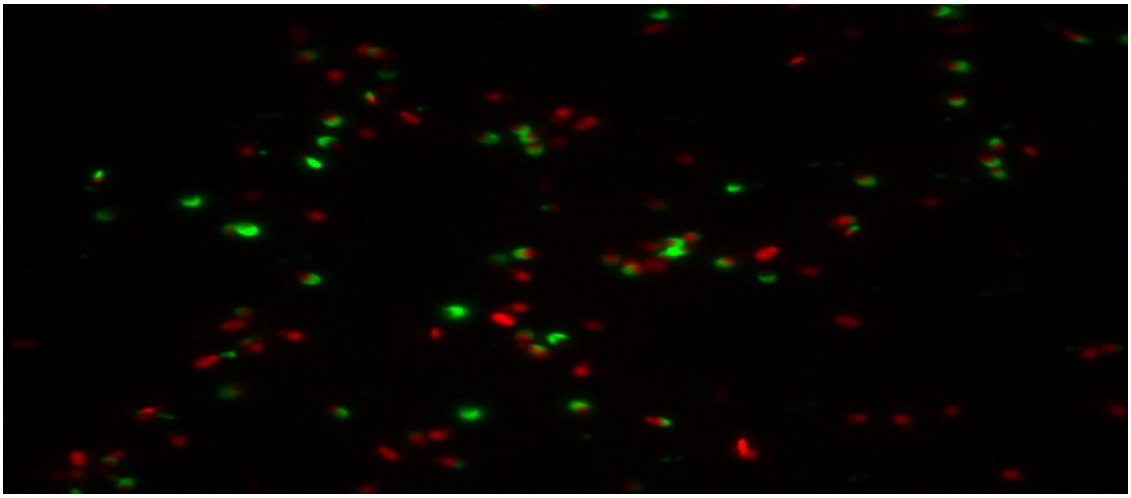


Figure 2.: Microscopic image of undamaged acrosome integrity in the 15 µg/mL quercetin group.

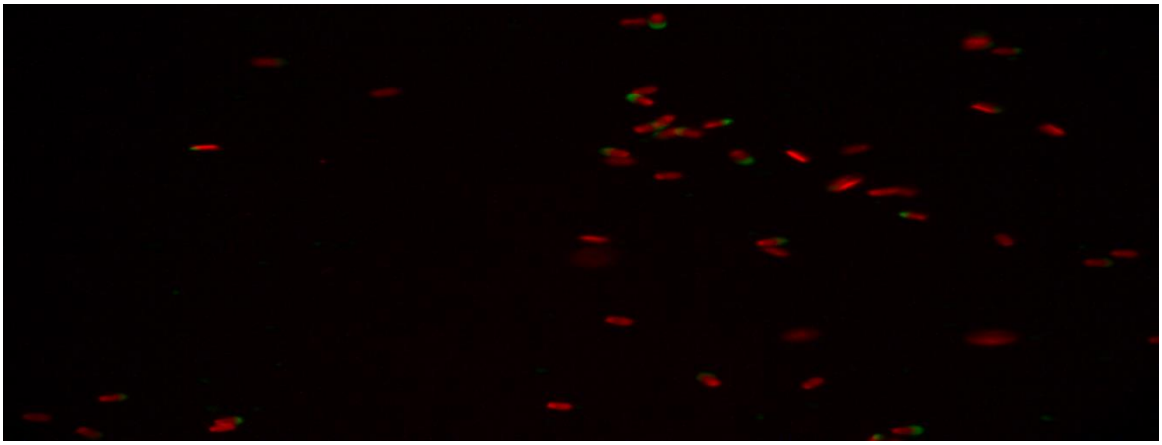


Figure 3.: Microscopic image of undamaged acrosome integrity in the 30 µg/mL quercetin group.

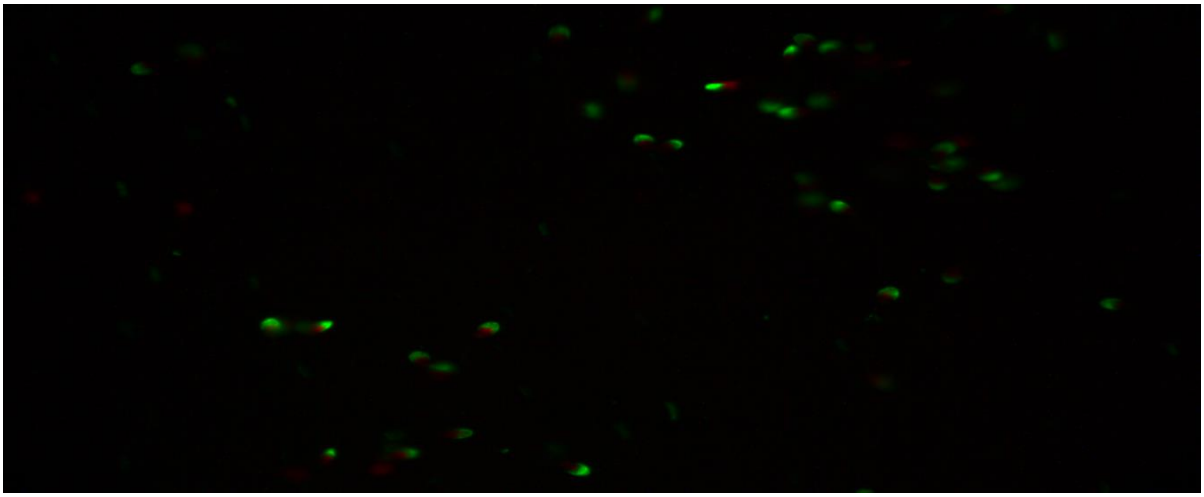


Figure 4.: Microscopic image of undamaged acrosome integrity in the 45 µg/mL quercetin group.

Discussion

Many in vivo and in vitro studies have been conducted on the antioxidant potential of quercetin. These studies showed that the antioxidant effect of quercetin protects the brain, heart, testicles, and other tissues against ischemia-reperfusion damage, toxic compounds and other factors caused by oxidative stress **[9]**. Quercetin is recognized as a powerful antioxidant due to its ability to retain Reactive oxygen species (ROS) and Reactive nitrogen species (RNS). It has also been stated that it directly destroys lipid peroxides **[12]** and indirectly increases the efficiency of enzymatic antioxidants and increases the production of nonenzymatic antioxidants **[13]**.

Quercetin (10 µmol/L) application to semen obtained from leukocytospermic patients was seen a positive effect on sperm motility and acrosome reaction. Besides, the level of H_2O_2 was significantly decreased in the supernatant of leukocytospermic patients after Quercetin treatment **[11]**.

On the other hand, 15 mM quercetin loaded nanostructured lipid carrier improved rooster sperm quality parameters **[14]**. It is well known; an intact functional sperm acrosome is an essential prerequisite for oocyte fertilization **[15]**. Sperm with damaged acrosomes or undergoing an early acrosome reaction after freezing thawing lose their ability to attach to the zona pellucida and are no longer able to fertilize. In other words, acrosome-reacting sperm have a short lifespan **[16]** and eventually an increase in the proportion of such sperm will adversely affect the fertilization capacity of semen doses **[17]**.

When it was interpreted the findings obtained from our research, it can be understanding the exceed dosage of quercetin may be harmful effect on sperm acrosome integrity.

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ORCID ID: Ali Doğan ÖMÜR https://orcidorg/0000-0002-2976-4368

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