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Effects of Carnosic and Gallic Acid on Ram Sperm Parameters and Seminal Plasma Homocysteine-Nesfatin Levels after Thawing

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Abstract

In the presented study, the protective role of carnosic and gallic acid on post-thaw ram sperm acrosome and membrane integrity was examined, homocysteine (Hcy) and nesfatin levels were determined.
In study semen samples from 6 Merino rams were used. Each ejaculate, split into five equal aliquots was diluted with extenders including 0.05 mM gallic acid (GA), 2 mM gallic acid, 0.05 mM carnosic acid (CA), 0.2 mM carnosic acid and no additive (control) at 37 °C cooled to 5 °C then frozen at nitrogen vapor.
Freeze-thawed ram semen viability was achieved in gallic acid 2mM (57.13± 2.38%) group and statistical difference was found with control group (45.08±2.98%) ($p<0.05$). There was no statistical difference between the groups in HOS test analysis ($p>0.05$). The lowest level of homocysteine was obtained in the gallic acid groups (0.67±0.11 and 0.61±0.26 µmol/L) and was found statistically different with the control group (1.36±0.9 µmol/L) ($p<0.05$). No significant difference was found in nesfatin levels among groups ($p>0.05$).
GA supplementation in ram semen extender has been determined to protect viability and HOS test parameters also to decrease homocysteine level.

Key Words: Carnosic acid, gallic acid, homocysteine, nesfatin, sperm

Karnosik ve Gallik Asidin Çözdürme Sonrası Koç Sperm Parametreleri ve Seminal Plazma Homosistein-Nesfatin Düzeyleri Üzerindeki Etkileri

Öz

Sunulan çalışmada koç spermasının çözüm sonu akrozom ve membran bütünlüğü üzerine karnosik ve gallik asidin koruyucu rolü incelendi, homosistein ve nesfatin düzeyleri belirlendi.
Çalışmada 6 Merinos koçundan sperma örnekleri kullanıldı. Beş eşit parçaya bölünen ejakülatlardan, 0.05 mM gallik asit (GA), 2 mM gallik asit, 0.05 mM karnosik asit (CA), 0.2 mM karnosik asit ve katkı maddesi içermeyen (kontrol) gruplar oluşturuldu. Sulandırma işlemi 37 °C'de yapıldı ve 5 °C de soğutulduktan sonra azot buharında dondurulduk.
Çözüm sonu koç sperması canlılığı gallik asit 2mM (%57.13±2.38) grubunda, kontrol grubundan (45.08 ±% 2.98) istatistiksel olarak farklı bulundu ($p<0.05$). HOS test parametreleri değerlendirmesinde gruplar arasında istatistiksel fark bulunmadı ($p> 0.05$). En düşük homosistein seviyesi gallik asit gruplarında (0.67±0.11 ve 0.61±0.26 µmol/L) elde edildi ve kontrol grubu (1.36 ± 0.9 µmol/L) ile istatistiksel olarak farklı bulundu ($p <0.05$). Nesfatin düzeylerinde gruplar arasında anlamlı fark bulunmadı ($p> 0.05$).
Sperma sulandırıcısına GA ilavesinin koç spermasının çözüm sonu canlılığı ve HOS test değerleri üzerine koruyucu etkisi ile birlikte homosistein seviyesini düşürücü etkisi olduğu belirlendi. Nesfatin düzeyinin değerlendirilmesinde gruplar arasında anlamlı fark bulunmadı.

Anahtar Kelimeler: Karnosik asit, gallik asit, homosistein, nesfatin, sperma

INTRODUCTION

Improvement of animal genetics and reproductive parameters through ram semen cryopreservation are practiced in many countries. The worldwide availability of frozen ram sperm ensures conservation of endangered species and biodiversity (1). Freezing of spermatozoa induces various forms of cellular lesions. Cryoprotectants are used to reduce harmful effects of the process (2). Thus, cold damage has been attributed to extreme osmotic changes, intracellular ice crystals and reactive oxygen species (3).

Homocysteine (Hcy) is an essential amino acid containing thiol and is involved in the growth of cells and tissues. Structural modifications of Hcy protein are involved in various pathological conditions by taking part in induction of oxidative stress and neurotoxicity (4). Oxidative stress is

generated during the oxidation of free thiol group in Hcy by plasma proteins, other low molecular weight plasma thiols, or via a disulfide bond. By this way, Hcy increases reactive oxygen production. This reaction produces hydroxyl radicals that removes electrons from other molecules and then induces oxidation of lipids, proteins, carbohydrates and nucleic acids (5, 6).

Nesfatin-1 a Hypothalamic 82-amino acid neuropeptide, which is retained from its precursor protein, nucleobindin 2 (NUCB2), is an anorexigenic peptide. Nesfatin-1 is distributed to brain regions involved in nutrition and metabolic regulation and has been reported to be expressed in peripheral organs, such as reproductive organs (7, 8).

Natural polysaccharides are important biopolymers with valuable biological activities. Polysaccharides are transferred to polysaccharides by a variety of chemical and enzymatic methods to extend gallic acid applications (9). Gallic acid (GA), 3,4,5-trihydroxybenzoic acid, is one of the phenolic acids found in fruit and medicinal plants. It is a colorless or slightly yellow crystalline compound with successful therapeutic applications (10). GA and its derivatives (eg: propyl gallate and octyl gallate) have been found to have antioxidant, antimicrobial, anti-tumor, anti-inflammatory, antimelanogenic, and anti-viral effects (11, 12).

Carnosic acid (CA) is a commonly used phenolic diterperne compound abundant, in sage and rosemary. CA has been reported to have multiple bioactive properties including antioxidant, anti-inflammatory and anticancer activities (13). In plants exposed to intense solar radiation, CA can be converted into methyl derivatives. In this way, it captures free radicals in chloroplasts and maintains the stability of cell membrane (14). Tomato lycopene (5 mM) and CA (25 mM) acted synergistically as an effective antioxidant against low density lipoprotein (LDL) oxidation (15).

This study aimed to determine the effects of diluents containing different doses of gallic and carnosic acid on ram sperm viability, plasma membrane integrity, homocysteine and nesfatin levels.

MATERIALS AND METHODS

Sperm Collection and Dilution

Ejaculates from 6 adult Merino rams were used in the study. The ejaculates were taken twice a week with an artificial vagina out of breeding season. The semen was collected in 5 replicates. The ejaculates with more than 80% motility and $1x10^9$ spermatozoa ml^{-1} were mixed. The main diluent, that was used to dilute sperm, was tris diluent (297.58 mM tris, 82.66 mM fructose, 96.32 mM citric acid, 15% egg yolk, 6% glycerol; pH 6.8) The ejaculate was divided into five equal parts and diluted at 37 °C and the groups were formed. The study was approved by Bahri Dağdaş International Agricultural Research Institute Local Animal Research Ethics Committee (No 2016/51).

Semen Process

The samples were filled into straws (0.25 ml) and sealed with polyvinyl alcohol powder then equilibrated at 5 ° C for 2 hours. The straws were frozen 4 cm above the liquid nitrogen vapor in 15 minutes and immersed in liquid nitrogen. Frozen straws were thawed at 38±2 °C for 25 seconds.

Determination of Sperm Viability

Eosin-nigrosin staining method was used to determine the sperm viability rate (16). The sample was prepared by mixing a drop of sperm sample with 2 drops of dye on a hot slide. The viability of the cells was evaluated by counting 200 cells on a phase-contrast microscope. Sperm cells which absorbed Eosin were considered dead.

Sperm Membrane Integrity (The Hypo-Osmotic Swelling Test)

Sperm membrane functional integrity was assessed by Hypo-osmotic swelling test (Hos-Test). For the test, 30 ml semen was incubated in 300 ml hypo-osmotic solution (9 g fructose + 4.9 g sodium citrate, 100 mOsm) were prepared and incubated at 37°C for 1 hour then twisted tails were listed.

Homocysteine and Nesfatin Assays

Thawed straws were centrifuged at 4 °C and 800 rpm for 15 minutes to separate the cells. After centrifugation the samples were washed with phosphate buffer saline. The supernatant was discarded and made up to 500 µL with phosphate buffered saline. The sperm suspension was then sonicated for 10 seconds on ice.

Homocysteine Assays

Homocysteine peptides were blindly measured and read (450 nm) ELISA plate reader (ELx800 Absorbance Microplate Reader) by the Biotin double-antibody sandwich technology, blindly measured by the Hcy enzyme-linked immunosorbent assay (ELISA) Kit (Shangai Sunred, Biological Tech., China-Biotek) using. Homocysteine concentrations were calculated from standard curves.

Nesfatin Assays

Nesfatin measurement was performed by commercial enzyme-dependent immune sorbent assay (ELISA) based on biotin double antibody sandwich technology (Shangai Sunred, Biological Tech., China). After the procedure of the ELISA kit, plates were read at 450 nm by the ELISA plate reader (ELx800 Absorbance Microplate Reader-Biotek) and Nesfatin levels were calculated from standard curves.

Data Analysis

Data were examined for normality with Shapiro Wilks and Kurtosis test and homogeneity of variance with Levene test. The data obtained was presented as mean ± standard error of the mean (mean ± SEM). All results were analyzed by Variance and then by Duncan's differences between the groups. $p < 0.05$ value was considered significant from the results. Analyzes were made with SPSS 21 package program.

RESULTS

After freeze-thaw process, semen viability and Hos-Test results were presented in Table 1. Gallic acid (2mM) was found to be more effective in viability than other groups ($p<0.05$). In the Hos-Test results, there was no statistical difference between the groups ($p>0.05$). In biochemical analysis (Table 2), homocysteine level was lower and statistically different in gallic acid (2mM) group than other groups ($p<0.05$), and no statistical difference was observed between groups in nesfatin levels ($p>0.05$).

Table 1. Semen Viability and Hos-Test (Mean±SEM)

Groups	Viability (%)	Hos-Test (%)
Control	45.08±2.98^{b}	30.30±5.30
GA 0.05 mM	50.30±2.66^{b}	34.05±3.50
GA 2 mM	57.13±2.38^{a}	37.08±7.30
CA 0.2 mM	48.33±5.54^{b}	33.60±3.65
CA 0.05 mM	47.55±1.21^{b}	34.03±2.64
p	*	-

* $p < 0.05$
a,b; Different superscripts within the same column demonstrate significant differences.

Table 2. Hcy and Nesfatin Assays assays (Mean±SEM)

Groups	Hcy(µmol/L)	Nesfatin (ng/ml)
Control	1.36±0.9^{a}	1.56±0.39
GA 0.05 mM	0.67±0.11^{b}	2.19±0.67
GA 2 mM	0.61±0.26^{b}	2.31±0.34
CA 0.2 mM	0.85±0.40^{ab}	1.86±0.69
CA 0.05 mM	1.26±0.51^{a}	1.67±0.34
p	*	-

* $p < 0.05$
a,b; Different superscripts within the same column demonstrate significant differences.

DISCUSSION AND CONCLUSION

Cryopreservation of cells causes permanent chemical and physical destruction in mammalian sperm cells (17). This is due to the lipid peroxidation increase (LPO) of the membrane induced by reactive oxygen species (ROS), caused by changes in the lipid phase transition (18).The results of our study showed that 2 mM GA additive to semen extender preserved sperm viability, decreased the level of Hcy and increased nesfatin.

Hcy, an important product of methionine metabolism, has many toxic effects (19). Reactive thiol group of Hcy is easily oxidized in plasma and causes ROS formation in metabolism (20). Studies have shown that low quality spermatozoa are characterized by accumulation of homocysteine. It is also characterized by multiple oxidative stress symptoms as a result of increased ROS production (21). Rezk et al. (22) stated, Hcy causes a decrease in sperm motility in normal and subfertile subjects and this decrease is due to the increase in increased mitochondrial superoxide anions. In our study, GA 2 mM groups homocysteine level was found statistically different from the other groups.

Nesfatin-1 is hypothalamic 82-amino acid neuropeptide and synthesized from the apical areas of the seminiferous tubules, sertoli cells and spermatocytes in the testicle, as well as from the ovaries (8, 23). It has been determined that nesfatin plays a positive role in the regulation of the reproductive system in male rats (24). In the study presented, the highest nesfatin level was found in the 2 mM GA group, but no statistical difference was observed between the groups.

Gallic acid is commonly found in grapes, tea leaves, strawberries, pineapples, bananas, and lemons as part of the tannin molecule. It has antioxidant and anti-inflammatory properties. (25, 26). A positive effect was observed on the reproductive performance of rats fed with GA extract (27). Similar to the study presented, Güngör et al. (28) determined 2 mM GA dose added to the ram sperm diluent had a positive effect on membrane integrity rates. GA has a powerful antioxidant effect that can prevent the harmful effect of CP on the apoptosis of Mouse sperm (29). In our study it was determined that GA 2mM had a protective effect on plasma membrane integrity and plasma membrane functionality. While statistical difference was observed in membrane integrity, there was no difference in Hos-Test results.

Carnosic components and rosmaric acids are the main components of rosemary (30). The supplement of bull semen with rosmarinic acid containing CA was significantly beneficial on spermotological parameters, similar to our study (31). Zanganeh et al. (32) showed that rose-mary aqueous extract in the buck semen extender has a positive effect on post-thaw motility, plasma membrane integrity, and plasma membrane functionality (HOS test). In their studies in different animal species, the researchers stated that semen freezing supplement with rosemary juicy extract achieved success in the spermatological parameters of the dog (33) and ovine (34). In our study among the parameters examined, CA did not produce a statistical difference with the control group in two different doses used.

In conclusion, Gallic acid added to semen diluent showed a statistical difference compared to the control group by showing a protective effect on viability and plasma membrane integrity. In gallic acid groups, homocysteine level was lower and nesfatin level was higher than other groups and also statistical difference was found between the 2 mM GA group and the control. In CA groups no significant difference was observed with control group. In the study, the use of diluent containing gallic acid in cryopreservation of ram sperm succeeded in freeze-thawing evaluations.

CONFLICTS OF INTEREST

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper. The authors have nothing to disclose. There are no conflicts of interest exist.

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