Polish Journal of Veterinary Sciences Vol. 26, No. 1 (2023), 91–98

DOI 10.24425/pjvs.2023.145010

Original article

The effect of luteolin on spermatological parameters, apoptosis, oxidative stress rate in freezing rabbit semen

S.A. Akarsu[1], T.C. Acısu[2], İ.H. Güngör[2], A. Çakır Cihangiroğlu[2], R.H. Koca[3], G. Türk[2], M. Sönmez[2], S. Gür[2], F. Fırat[2], H.E. Esmer Duruel[4]

[1] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Atatürk University, 25240 Yakutiye /Erzurum, Turkey
[2] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Fırat University, 23200, Elazığ, Turkey
[3] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Bingöl University, Selahaddin-i Eyyübi Neighbourhood, University Street No: 1, 12000, Bingöl, Turkey
[4] Elbistan Vocational School, Kahramanmaraş İstiklal University, Şehit Astsubay Ömer Halisdemir Street No:5, Doğan Neighbourhooh, 46340, Elbistan, Kahramanmaraş, Turkey

Abstract

The aim of the present study was to determine the effects of Luteolin (LUT) on semen quality, oxidative stress, apoptosis, acrosomal integrity, mitochondrial membrane potential and dead sperm ratio in rabbits. Ejaculates from six New Zealand rabbits were collected, evaluated and pooled. The pooling was divided into five groups as control (no additive) LUT 25 µM, LUT 50 µM, LUT 100 µM and LUT 200 µM and LUT added. It was then filled into a falcon tube with Tris-based extender at a final concentration of approximately 35 x 10^6 spermatozoa. Diluated rabbit semen samples were drawn into frozen and thawed. Frozen semen straws were thawed at 37°C in 30 seconds. According to our findings, no statistical difference was found between all doses of luteolin and the control group in the CASA (computer assisted sperm analysis) analysis performed at 4°C. However, total motility, progressive motility and rapid sperm percentage were found to be higher in the frozen and thawed rabbit semen at a dose of LUT 50 µM compared to the other groups ($p<0.05$). While amplitude of lateral head displacement (ALH) and beat cross-frequency (BCF) values were found at the lowest dose of LUT 200 µM, a statistically significant difference was observed between the other groups. When the flow cytometry results were examined, no statistical difference was found between the rate of dead sperm, acrosomal integrity, mitochondrial membrane potential and apoptosis rate. Morever, the H_2O_2 percentage was found to be lower in all experimental groups compared to the control group ($p<0.001$). In conclusion, the addition of LUT in long-term storage of rabbit semen provided a protective effect for spermatozoa with its antioxidative properties against damage caused by cryopreservation.

Key words: rabbit, semen, cryopreservation, luteolin, oxidative stress, apoptosis

Correspondence to: S.A. Akarsu, e-mail; serkan.akarsu@atauni.edu.tr

Introduction

In order to meet the high demands rates created by of the rabbit industry, methods of cryopreservation procedures of semen need to be developed and improved (Rosato and Iaffaldano 2013). Semen cryopreservation enables the development of reproductive biotechnology with applications such as artificial insemination and in vitro fertilization (IVF) (Sarıözkan et al. 2013). Rabbit semen has low water permeability and high activation energy (Curry et al.1995). Cryopreservation of the rabbit sperm leads to a decrease in the percentage of motility and survival of spermatozoa, and an increase in the level of oxidative stress and therefore a decrease in the fertilization rate. Cryopreservation of rabbit semen leads to a decrease in the percentage of motility and survival of spermatozoa, and an increase in the level of oxidative stress, thus reducing the fertilization rate (Mocé and Vicente 2009). Since the sperm membrane is rich in long-chain polyunsaturated fatty acids, freezing and thawing of semen produces high concentrations of reactive oxygen species (ROS) (Chatterjee and Gagnon 2001), which are harmful to spermatozoa (Alvarez et al. 1984). Although spermatozoa can remove some of the ROS from the cytosol by enzymatic reactions, this enzyme activity is not reduced as mammalian spermatozoa discard most of the cytoplasm during their differentiation (Paál et al. 2017). Excessive production of ROS leads to lipid peroxidation (LPO), which causes changes in the plasma membrane (Bucak et al. 2007). Therefore, the addition of an exogenous antioxidant to the semen freezing diluent protects rabbit spermatozoa against the detrimental effect of ROS during the cryopreservation process (Fadl et al. 2022).

In recent years, plant-derived bioactive compounds have been the subject of research in the field of medicine (Ahmadi et al. 2015). More than 5000 flavonoids have been identified in these plants and are found in a wide variety of plants (Kocic et al. 2013). Luteolin (LUT) is a natural flavonoid substance found in many plants. It is abundant in agricultural products such as celery, chrysanthemum flowers, sweet peppers, carrots, onion leaves, broccoli and parsley (Chen et al. 2012b, Lim et al. 2013). Luteolin has anti-inflammation, anti-tumor, anti-viral activity and exhibits antioxidant, anti-inflammatory, autophagic regulatory, anti-apoptotic and anti-neoplastic effects (Luo et al. 2019, Yu et al. 2019, Kim et al. 2020, Tan et al. 2020). A study showed that LUT treatment increases testicular weight, number of Leydig cells, primary spermatocyte, spermatids, and level of serum testosterone in rats with a specific absorption rate of 2 W/kg and an average power density of 1±0.4 mW/cm^2 in the electromagnetic field for 28 days. Similarly, serum testosterone level and testicular weight increased. Moreover LUT improved histological changes in the testicular tissue (Yahyazadeh and Altunkaynak 2019). An another study was reported that luteolin treatment minimized testicular damage and prevented oxidative stress, inflammation and apoptosis in lead acetate administered rats (AL-Megrin et al. 2020).

Materials and Methods

Chemicals

Luteolin Cas No: 491-70-3 was purchased from Cayman Chemical Company (Cayman Chemical, Michigan, USA). Annexin V FITC assay kit and and JC-1 were purchased from Cayman Chemical Company (Cayman Chemical, Michigan, USA). Lectin PNA and DFCDA were purchased from Thermo Fisher (Thermo Fisher Scientific, Massachusetts, ABD).

Animals, semen collection, cryopreservation process and sperm analyses

A total of seven New Zealand rabbits, six male and one female, were used in the study. The animals were obtained from the Fırat University Experimental Research Center. The rabbits were kept for adaptation for a week. They were housed in separate cages under standard laboratory conditions (55-60% RH at 22-24°C and 12 h/12 h light/dark cycle). The rabbits were given a commercial pellet diet and fresh drinking water. Before the study, a certificate of approval (Protocol no:2021/14) was obtained from the Fırat University Animal Experiment Local Ethics Committee. Applications to the animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. The rabbits were first acclimated to the artificial vagina. Semen samples were taken from animals once a week between April and May, for a total of 6 repetitions. The gel part was removed from the semen samples. Semen quality was examined with a heating plate light microscope (Celestron, Torrance, California, USA) at 100x magnification. Semen samples with a motility value below 70 % were not used in the study. Semen dilution was performed with modification of the method described by Muhammed et al. (2022). Samples of semen were diluted 1/1 with Tris-egg yolk extender (250 mmol/L TRIS-hydroxymethyl-aminomethane, 88 mmol/L citric acid and 47 mmol/L glucose, 15% egg yolk) for pooling. Then semen was diluted with TCG egg yolk (250 mM tris (hydroxymethyl amino methane), 88 mM citric acid, 47 mM glucose, gentamycin (125 µg/dL) and ciprofloxacin (62.5 µg/dL), % 15 egg yolk and % 5 DMSO (final concentration) so that the semen density was 1 mL of 170x10^6.

Luteolin was dissolved in DMSO added to the diluent. Semen was divided into 5 equal volumes of control (without LUT), 200 µM, 100 µM, 50 µM and 25 µM LUT. The semen temperature was gradually reduced to 5°C in 1 hour and equilibrated for 2 hours. Equilibrated semen samples were analyzed with computer assisted sperm analysis (CASA) program (ISAS, Proiser V1, Spain) at 5°C. The CASA system used was equipped with a Sony video camera. 3 µL of diluted semen was placed in a SpermTrack 20 chamber heated at 37°C. Three different fields (approximately 300 spermatozoa) were analyzed from each sample. The particle area was determined between 10 and 80 μ^2. Sperm velocity was between 10 and 50 micron/sc. Then, it was drawn into 0.25 mL straws (Mohammed et al. 2022) and frozen in a programmable semen freezing device (Mini Digitcool, IMV Technologies, France) (Castellini et al. 2011). Frozen semen was stored in a liquid nitrogen tank for 2 months (Salisbury et al. 1978). Then, frozen semen samples were thawed at 37°C for 30 s (Küçük et al. 2021). Sperm analyzes were performed with CASA in thawed semen samples (Castellini et al. 2011). 3 µL of semen samples placed on a SpermTrack 20 slide were analyzed at 37°C. Examined CASA parameters included total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), velocity of straight line (VSL, µm/s), velocity of average path (VAP, µm/s), linearity (LIN, %), straightness (STR, %), wobbel balancing (WOB, %), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), rapid (%), medium (%), slow (%) and static (%) sperm ratio.

Flow cytometric analysis

Acrosomal status

Acrosomal integrity was achieved by modification of the method described by Jimenez et al. (2015). Briefly, 30 µL of semen sample was diluted in 860 µL of PBS. It was incubated with 5 µL of PNA and 2.5 µL of PI for 15 minutes. Measurements were made with a 488 nm argon ion laser in flow cytometry (Beckman Coulter, Brea, USA) with $10x10^3$ spermatozoa in each sample. Data were expressed as %.

Sperm apoptosis rate

Sperm apoptosis rate was determined using annexin V staining method with modification of the method described by Chaveiro et al. (2007). Annexin V (5 µl) and propidium iodide (PI; 5 µl) were placed on 100 µl of semen samples taken into the binding buffer. It was incubated for 15 minutes at room temperature in the dark and analyzed by flow cytometry. Apoptosis rate was calculated by collecting early apoptotic sperm (A+/PI−), early necrotic sperm (A+/PI+) using the method described by Pena et al. (2003). Sperm apoptosis rates were expressed as a percentage (%).

Mitochondrial membrane potential (MMP) assay

Semen samples were washed with a phosphate buffer solution (PBS) and made following the steps included in the JC-1 commercial kit. Sperm samples were incubated with JC-1 for 10 minutes at 37°C in the dark and examined under a flow cytometer (Abdelnour et al. 2022). MMP results were expressed as a percentage (%).

Production of intracellular H_20_2 analysis

This analysis was performed using the method described by Kim et al. (2011) with 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen). Double staining with PI (Rosato and Iaffaldano, 2013) was used to evaluate sperm viability. Reconstituted sperm samples were mixed with 200 µm H2DCFDA and incubated at 37°C for 55 minutes in the dark. PI was added at a final concentration of 3 µM and incubated for an additional 5 minutes. H_2O_2 analysis results were expressed as a percentage.

The rate of viable sperm

The proportion of dead-viable sperm was determined by dual fluorescent staining with SYBR-14/PI (Viudes-De-Castro et al. 2014). 980 µL of PBS was diluted with 10µL of semen sample transferred to tubes containing 5 µL of SYBR-14 and 5 µL of PI incubated at 22°C for 10 minutes and analyzed by flow cytometry. The results were expressed as a percentage (%).

Statistical Analysis

Statistical analysis of the data in all experiments was performed with the SPSS (Version 26, SPSS, Chicago, IL) program. One-Way ANOVA with Tukey's post hoc was chosen for statistical evaluations. Values according to the results ($p<0.05$) were used for statistical significance. The results were interpreted as mean and finally expressed as standard deviation.

Results

Results of CASA analysis at 4°C

CASA analysis results indicating the effects of LUT on rabbit semen at 4°C are presented in Table 1. Although there was no statistical difference, total motility (%) and progressive motility (%) values were

Table 1. Results of rabbit sperm analysis with computer assisted sperm analysis (CASA) at 4°C degrees after equilibration.

	Control groups	25 µM Luteolin groups	50 µM Luteolin groups	100 µM Luteolin groups	200 µM Luteolin groups
Total Motility (%)	63.5±12.7	63.95±8.34	68.13±7.08	61.56±7.22	62.63±3.8
Progressive Motlility (%)	31.95±16.97	29.31±8.84	34.15±10.12	33.36±9.04	24.33±5.89
Rapid (%)	50.05±9.47	47.86±12.79	50.1±18.23	48.71±5.78	41.98±12.04
Medium (%)	7.31±3.66	7.98±4.51	5.63±3.17	9.98±5.05	10.0±8.15
Slow (%)	6.13±4.55	4.63±4.0	5.80±6.4	2.88±2.21	3.70±2.59
Static (%)	36.51±12.69	39.53±17.14	38.48±14.97	38.43±7.22	44.35±14.00
VCL (µm/s)	109.71±17.44	114.66±27.39	110.05±25.25	101.85±19.88	102.45±29.29
VSL (µm/s)	45.46±11.68	39.46±10.49	41.85±13.50	40.88±13.53	40.70±16.64
VAP (µm/s)	69.10±12.25	65.45±15.10	66.43±17.61	64.38±13.93	66.76±22.28
LIN (%)	42.31±12.37	36.36±8.20	39.01±11.33	40.81±12.29	40.23±12.61
STR (%)	66.43±14.79	62.11±16.06	64.28±15.87	63.95±14.67	62.16±14.19
WOB (%)	63.83±7.98	59.11±6.70	60.28±8.33	63.25±8.16	64.26±9.58
ALH (µm)	3.73±0.64	3.61±0.66	3.48±0.67	3.08±0.56	2.91±0.39
BCF (Hz)	7.71±0.79	8.03±0.38	8.08±1.13	8.10±0.90	8.30±0.40

No statistical difference was found in the data without superscript
VCL – Curvilinear velocity, VSL – Straight linear velocity, VAP – Average path velocity, LIN – Linearity, STR – sperm track straightness, WOB – Wobble, ALH – Amplitude of lateral head displacement, BCF – Beat cross-frequency.

numerically higher in the 50 µM dose group. There was no statistical difference between the kinematics and velocity findings.

Results of CASA analysis of frozen and thawed rabbit semen

The CASA analysis results of the effect of LUT on the freezing and thawing of rabbit semen are presented in Table 2. While total motility, progressive motility and rapid spermatozoa findings were found at the highest dose of 50 µM, a statistically significant difference was observed between the other groups ($p<0.05$). Static sperm ratio (%) was determined at the lowest dose of 50 µM ($p<0.05$). The VCL (µm/s) value was determined at the highest dose of 50 µM ($p<0.05$). While the highest VAP (µm/s) value was found in the 50 µM dose group, a statistical difference was observed between the 200 µM dose group ($p<0.05$). ALH (µm) and BCF (Hz) values were determined at the lowest dose of 200 µM ($p<0.05$).

Results of flow cytometry analysis

Flow cytometry results of the effects of LUT in frozen and thawed rabbit semen are presented in Table 3. There was no statistically significant difference between the groups in the rate of dead sperm (%), the rate of live and dead sperm with acrosomal damage (%), the rate of sperm with high mitochondrial membrane potential (%), and the rate of apoptosis (%). However, the rate of ROS (%) was found to be significantly lower in all doses of LUT compared to the control group ($p<0,001$)

Discussion

Semen freezing and thawing processes impair sperm function due to ice crystal formation, cold shock, oxidative damage and osmotic stress (Chen et al. 2022). This study shows the effect of LUT on cryopreservation-induced impaired sperm motility, acrosomal integrity, mitochondrial membrane potential, apoptosis and total ROS ratio in the rabbit semen.

Semen cryopreservation reduces antioxidant levels and increases ROS production (Bansal and Bilaspuri 2011). When ROS accumulates excessively in the antioxidant defense system of spermatozoa, sperm motility decreases (Sikka 1996, Petruska et al. 2014). Rabbit semen is highly sensitive to LPO (Zhu et al. 2015). The sperm membrane destroys the membrane lipid matrix by attacks from ROS. ROS ultimately leads to damage to plasma membrane integrity and decreased sperm motility (Bucak et al. 2007). Luteolin has a strong reactive oxygen species scavenging ability and reduces lipid peroxidation (GJ et al. 2019). In our findings, the rate of ROS was found to be signifi-

Table 2. Results of rabbit sperm analysis with CASA after the freezing thawing process.

	Control groups	25 µM Luteolin groups	50 µM Luteolin groups	100 µM Luteolin groups	200 µM Luteolin groups
Total Motility (%)	23.43±0.28^{bc}	25.93±4.54^{c}	32.65±2.06^{d}	19.23±5.39^{b}	7.63±1.29^{a}
Progressive Motlility (%)	11.52±3.48^{ab}	11.05±5.78^{ab}	15.48±4.51^{b}	8.45±6.13^{ab}	3.93±2.06^{a}
Rapid (%)	14.25±6.01^{bc}	12.08±5.25^{bc}	20.73±6.74^{c}	8.15±6.15^{ab}	2.70±2.16^{a}
Medium (%)	4.83±0.89	4.91±4.21	6.0 ±4.21	3.26±2.31	2.26±0.05
Slow (%)	4.31±0.71	4.41±3.22	5.86±2.74	2.76±1.76	2.73±0.87
Static (%)	76.56±6.13^{ab}	79.06±9.06^{b}	66.73±6.17^{a}	85.76±9.99^{bc}	92.30±1.23^{c}
VCL (µm/s)	84.50±13.02^{bc}	75.98±9.43^{bc}	91.83±13.22^{c}	65.33±13.59^{ab}	50.76±20.55^{a}
VSL (µm/s)	41.88±9.20	39.06±12.05	41.55±9.35	37.10±9.20	29.10±17.04
VAP (µm/s)	56.03±10.02^{ab}	50.60±9.45^{ab}	59.83±8.41^{b}	44.35±11.16^{ab}	38.76±14.97^{a}
LIN (%)	49.28±5.56	50.98±12.35	45.18±8.79	56.31±2.76	54.26±9.24
STR (%)	74.43±5.57	76.28±12.44	68.78±9.23	83.70±2.00	70.86±13.27
WOB (%)	66.15±3.05	66.20±5.78	65.36±4.68	67.33±4.26	76.73±1.18
ALH (µm)	3.05±0.13^{b}	3.01±0.35^{b}	3.03±0.35^{b}	2.78±0.25^{b}	2.06±0.05^{a}
BCF (Hz)	7.85±1.29^{b}	7.26±1.10^{b}	7.36±1.71^{b}	7.93±0.30^{b}	4.53±1.03^{a}

Different superscript letters (a. b. c. d; $p<0.05$) in the same row display significant differences between the groups.
VCL – Curvilinear velocity, VSL – Straight linear velocity, VAP – Average path velocity, LIN – Linearity, STR – sperm track straightness, WOB – Wobble, ALH – Amplitude of lateral head displacement, BCF – Beat cross-frequency.

Table 3. Results of flow cytometry analysis of frozen thawed rabbit semen.

	Control groups	25 µM Luteolin groups	50 µM Luteolin groups	100 µM Luteolin groups	200 µM Luteolin groups
Viable Sperm %	19.51±4.58	19.52±8.70	20.35±6.46	17.22±5.26	15.70±2.74
Dead Sperm%	78.75±8.13	79.08±9.89	78.35±5.95	81.38±5.26	84.65±8.45
Damaged Acrosomes %	48.16±6.12	47.83±10.66	45.74±9.23	45.67±6.87	55.67±10.47
High Mitochondrial Membrane Potential Rate %	27.37±8.09	25.88±8.84	27.27±5.51	20.83±2.15	20.04±5.08
Apoptosis %	16.04±3.82	14.53±2.16	15.13±2.83	15.30±2.36	15.10±2.44
ROS Ratio %	39.77±10.59^{a}	15.75±3.83^{b}	14.63±3.04^{b}	15.39±2.21^{b}	14.80±6.27^{b}

Different superscript letters (a, b: $p<0.001$) in the same row display significant differences between the groups.

cantly lower in all doses of LUT compared to the control group. This is indicative of the LUT's ability to clear ROS. Excessive production of ROS causes a decrease in the sperm motility by reducing axonemal protein phosphorylation and sperm immobilization (De Lamirande and Gagnon 1995). It has been reported that the addition of exogenous antioxidant agents to the rabbit semen improves sperm quality in cryopreservation (Zhu et al. 2015, Domingo et al. 2018, Abdelnour et al. 2022). Antioxidants are added to semen extenders to improve sperm quality after thawing (Zhu et al. 2019). Motility is the most important indicator of fertilization capacity (İnanç et al. 2022). In our study, it was observed that LUT at a dose of 50 µM improved total motility and progressive motility values after freezing and thawing of semen in rabbits. This suggests that LUT at effective doses inhibits ROS leading to a rising in sperm motility. The obtained motility results supported the view that the addition of LUT reduced the concentrations of free radicals. However, LUT at 100 µM

and 200 µM doses appears to decrease total motility and progressive motility. In this case, LUT has a toxic effect.

In somatic cells, ROS triggers apoptosis (Fulda et al. 2010). After freezing and thawing, there is an increased rate of apoptosis in spermatozoa (Martin et al. 2004). Using the Annexin V/PI staining method, viable and apoptotic spermatozoa can be distinguished after thawing (Mohammed et al. 2021). It is stated that LUT increases Bcl-2 (antiapoptotic factor) signaling pathways and decreases cleaved caspase-3 and Bax (pro-apoptotic factor) in the liver tissue (Yan et al. 2019). Another study revealed that LUT was ineffective in antiapoptotic pathways (Choi et al. 2003). It has been reported that melatonin supplement has antiapoptotic effect in cryopreservation of the rabbit semen (Zhu et al. 2019). This suggests that LUT did not show an antiapoptotic effect in in vitro studies. Cryopreservation is mediated by mitochondrial dysfunction by inducing apoptosis in sperm cells (Kim et al. 2013). JC-1 staining method is an important marker of mitochondrial dysfunction of spermatozoa (Tvrdá et al. 2021). Luteolin has been reported to reduce mitochondrial membrane potential in lung adenocarcinoma cells and hepatoma cells in humans (Chen et al. 2012a, Ding et al. 2014). However, according to our findings, LUT did not affect mitochondrial membrane potential in spermatozoa at all doses. Acrosomal integrity is essential for sperm fertilization ability (Ugur et al. 2019). In our study, LUT at a dose of 50 µM gave the lowest result in live and dead semen with no statistical difference in acrosomal damage. These results are partially in parallel with the motility findings.

Some studies have reported that LUT protects against testicular damage with antioxidant defense pathways (Yahyazadeh and Altunkaynak 2019, AL-Megrin et al. 2020, Owumi et al. 2020, Ijaz et al. 2022). According to our findings, the fact that LUT reduced the total ROS rate at a dose of 50 µM and caused an increase in total motility and progressive motility supports the positive correlation between ROS and sperm motility.

Conclusion

In conclusion, the addition of 50 µM LUT to the rabbit semen extender improved sperm quality after thawing by increasing sperm motility and rapid sperm rate in rabbits, as well as reduced sperm ROS damage due to cryopreservation. It is stated that LUT can have beneficial results in the field of rabbit semen cryopreservation.

Acknowledgements

This study was supported by the Kahramanmaraş Istiklal University Scientific Research Projects Coordination Unit. Project Number: 2021/2-2

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