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Effect of adding butylated hydroxytoluene on post-thaw semen quality of Saanen bucks

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ABSTRACT

This study aimed to evaluate the beneficial effect of different concentrations of butylated hydroxytoluene (BHT) as an antioxidant at 0.6, 2.0, and 5.0 mM in Tris-citric acid-glucose (TCG) extender on semen quality of Saanen bucks. During mating season (November-January), four Saanen bucks were used in this study. Following collection, semen was divided into two parts. The first part was diluted with a ratio of 1:5 in TCG solution and then washed twice by centrifugation at 3000 rpm/10 minutes. While, the second part was left unwashed. Semen parts were re-suspended in TCG medium as follows: washed semen, washed semen +0.6 mM BHT, washed semen +2.0 mM BHT, washed semen +5.0 mM BHT, unwashed semen and unwashed semen +5.0 mM BHT. Semen straws were chilled at 4°C for 2 hours and then stored in liquid nitrogen. After thawing, total and progressive sperm motility (%), and dead spermatozoa (%) were recorded by computer-assisted semen analysis (CASA). The obtained results showed that addition of 2.0 mM BHT in washed semen recorded the highest percentages of total and progressive sperm motility and the lowest percentage of dead spermatozoa. The washed semen was significantly ($P<0.05$) higher compared to unwashed semen. In conclusion, BHT increased significantly ($P<0.05$) both sperm motility and viability percentages in Saanen buck semen. Also, it could be concluded that, addition of 2.0 mM of BHT was the best concentration to improve semen quality and sperm cryosurvival of Saanen buck semen, by acting as an antioxidant thereby reducing the lipid peroxidation of the sperm.

Keywords: Saanen buck, semen, cryopreservation, butylated hydroxytoluene.

INTRODUCTION

Goats are resilient animals that can adapt to extreme temperature and humidity conditions. Less input, simple management, adaptability, and the capacity to live and produce on limited fodder supplies make them popular animals in tropical and subtropical areas. As a result, Asia (particularly developing countries) may account for 60% of the goat population in the world **(FAO, 2007)**. Caprines, in this way, play a vital role in improving the socio-economic standards of low and middle-income areas **(Şengonca and Koşum, 2015)**.

In terms of assisted reproduction technologies, semen cryopreservation is a valuable treatment tool. Although post-thawed semen offers various advantages in terms of reproduction, it is recognized that cryopreservation processes including cooling, freezing, and thawing cause major unfavorable alterations in sperm functions. **(Colas *et al.*, 2009 and Raheja *et al.*, 2018)**. Due to reactive oxygen species (ROS) formation and thermal shock occurring to spermatozoa, the processes mentioned above make oxidative damage to the sperm membrane. It is known that sperm freezing and thawing not only cause unfavorable alterations in the composition of membrane lipids, the status of the acrosome, motile and viable spermatozoa but also make damage to sperm DNA **(Zribi *et al.*, 2010)**.

To decrease the formation of ROS and its impacts, the inclusion of antioxidants in semen extenders has emerged as a new trend in sperm cryopreservation procedures. Among several antioxidants, butylated hydroxytoluene (BHT)

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has been tested in different concentrations with success towards sperm preservation. BHT extremely reduces the sperm cryo-injury during freezing by protecting the sperm membrane for different species such as sheep **(Farshad *et al.*, 2010)**, cattle **(Patel *et al.*, 2015)**, goat **(Alcay *et al.*, 2016; Dewry *et al.*, 2020)** and buffalo **(Wadood *et al.*, 2016)**.

BHT is a synthetic, non-enzymatic counterpart of vitamin E. BHT has a variety of functions, including; antioxidant preservative, antiviral and antimicrobial agent. BHT aids in inhibiting lipid peroxidation in biological membranes and improves sperm quality **(Alcay *et al.*, 2016)**. The use of BHT as an antioxidant has been reported for improving the quality of frozen semen **(Lalramdintluanga, 2012; Nain *et al.*, 2023)**, but the effect of antioxidant depends on its concentration **(Priyadharsini *et al.*, 2011)**. To date, there are no previous studies about use of the BHT in the cryopreservation of Saanen buck semen. Subsequently, the present study purposed to assess the effect of several concentrations of BHT (0.6 mM, 2.0 mM, and 5.0 mM) in TCG based extender on cryopreservation of Saanen buck semen.

MATERIALS AND METHODS

1. Experimental animals

Four healthy Saanen bucks with 1-2 years old and the average 50-60 kg body weight (BW) were used in this study. This study was carried out at the Animal Husbandry Unit, Department of Animal Science, Faculty of Agriculture, Ege University, Izmir province, Türkiye. The study was executed with the approval of Ege University, Faculty of Agriculture, Farm Animal Experiments Local Ethics Committee (Ethics committee decision with a date of 09.08.2021 and number of 2021/005).

2. Feeding of animals

The bucks were fed on 2.0 kg corn silage, 0.7 kg alfalfa hay, 0.3 kg rice straw and 1.0 kg of goat milk feed (18% crude protein, 2600 kcal/kg ME) given daily to the bucks according to a feeding program applied to meet the nutritional requirements for the breeding of bucks.

3. Collection of semen

Semen samples of the bucks were collected regularly twice a week during the mating season (November-January 2020/2021) by using an artificial vagina in the presence of a female goat. Semen samples were transferred to the laboratory within one minute after collection and were placed in a water bath at 37°C. Semen samples with normal morphology and concentration characteristics were instantly mixed and then divided into two equal amounts.

4. Extension of semen

A Tris-citric acid-glucose (TCG) extender consisting of 0.3M of Tris, 94.7 mM of anhydrous citric acid and 27.75 mM of D (+)-glucose was used in this study, as described by **Salamon and Maxwell (2000)**. A pH of 7.25 and an osmolarity of 333 mOsm were adjusted to this solution. A final concentration of 5% glycerol, 15% egg yolk, and 1000 IU/mL of sodium penicillin with 1.0 mg/mL of streptomycin sulfate as antibiotics were mixed with the solution. In this way, the solution was adjusted to 7.0-7.17 of pH and 1327 mOsm of osmolarity as a final concentration. Following collection, semen ejaculates were instantly mixed and then divided into two equal parts. One part was washed twice (1:5) with a solution of TCG before being centrifuged at 3000 rpm for 10 minutes. The supernatant was accurately removed to separate the sediment into four equal aliquots, whereas the other part (unwashed semen) was separated into two equal aliquots.

Meanwhile, a different TCG media (5% glycerol and 15% egg yolk as a final concentration) supplemented or not with different concentrations of BHT, was used to re-suspend the 6 aliquots according to the following treatments:

1) Washed semen;
2) Washed semen + 0.6 mM of BHT;
3) Washed semen + 2.0 mM of BHT;

4) Washed semen + 5.0 mM of BHT;
5) Unwashed semen and
6) Unwashed semen + 5.0 mM of BHT.

5. **Freezing of semen**

Semen aliquots were chilled at 4°C for 2 hours before freezing and then were packaged in 0.25 ml straws with 400×10^6 sperm/ml as a final concentration. Before being immersed in the liquid nitrogen for storage, straws were frozen in liquid nitrogen vapors for 15 minutes at 4 cm above the liquid nitrogen level. As mentioned by **Khalifa *et al.*, (2008)**, the different BHT concentrations (0.6, 2.0, and 5.0 mM) were dissolved in dimethyl sulfoxide (DMSO) and mixed to the extender. The final concentration of DMSO was determined at 0.25% (35.2 mM) in the extender.

6. Thawing of semen

Semen straws (0.25 ml) were kept in the liquid nitrogen container until the time of spermatological examination and liquid nitrogen was added to the container periodically. For semen analysis, a water bath was used for thawing the straws at 37ºC for 30 seconds as described by **Üstüner *et al.*, (2015)** and then the contents were put into a dry and clean tube that was stored at the same temperatures for semen analysis.

7. Evaluation of semen

7.1. Sperm motility (%)

The percentage of motile spermatozoa was determined by computer-assisted semen analysis (CASA). CASA is software used to analysis the semen quality in an automatic way. For motility determination, a drop of semen samples was put on a slide then a coverslip was covered on the semen drop and examined by an examination microscope with a heating table at X200 magnification. In order to determine motility, evaluation was made in at least three microscope fields and motility was determined as the percentage of spermatozoa moving in a straight line in one direction to all spermatozoa in the same area.

7.2. Sperm viability (%)

The percentage of viable spermatozoa was determined by CASA. For dead spermatozoa (%) determination, a warm slide was used to place a drop of semen then a coverslip was covered on the semen drop and examined by an examination microscope with a heating table at X200 magnification. To determine the dead spermatozoa percentage, evaluation was made in at least three microscope fields, and the percentage of all spermatozoa was determined and recorded as dead spermatozoa.

8. Statistical analysis

The mean values and standard errors of the obtained data in this study were calculated. Statistical analysis was made by the **SAS (2000)** program. Two-way analysis of variance (ANOVA) was applied to understand whether there was a difference between the groups. When a difference was found as a result of the analysis, **Duncan's test (1955)** was used to determine the groups that created the difference.

The statistical model applied in the experimental study was as follows:

$Y_{ijk} = \mu + B_i + W_j + (B_i \times W_j) + Y_{ijk}$

Where,

Y_{ijk} = the observed value of the dependent variable that is determined from a sample of spermatozoa.

μ = the overall mean.

B_i = the fixed effect of BHT (mM) as an antioxidant, i = 0.0, 0.6, 2.0, and 5.0.

W_j = the fixed effect of washing the semen, j = Washed and unwashed semen.

$B_i \times W_j$ = the interaction between BHT (mM) and washing of semen.

e_{ijk} = the residual error.

Since there were no significant differences among months in temperature and humidity values as obtained from the meteorological data of Izmir province, where semen collection process was carried out, the effect of the month and season was not taken into account in the statistical model used in the experimental study.

RESULTS AND DISCUSSION

1. Total sperm motility (%)

The percentage of total sperm motility after thawing is presented in Table 1. A significant (P<0.05) difference between BHT groups and the control group in both washed and unwashed semen treatments was observed. In washed semen treatment, the highest percentage of total motile spermatozoa was observed in the BHT group of 2.0 mM with an average of 71.65±2.86, while the lowest percentage was recorded in the control group with an average of 48.74±1.45. Moreover, the difference between the washed and unwashed semen treatments was significant (P<0.05). The highest total sperm motility (%) was recorded in washed semen treatment with an average of 60.72±2.17 and the lowest percentage was recoded in unwashed semen treatment with an average of 47.13±1.91. The total sperm motility (%) recorded in a sample of buck semen post thawing with 0.0, 0.6, 2.0 and 5.0 mM of BHT groups were 45%, 78%, 85%, and 65%, respectively (Fig. 1-4).

Table 1. Percentage of total motile spermatozoa post thawing (mean±SE) in Saanen bucks, supplemented with different concentrations (mM) of butylated hydroxytoluene in tris-citric acid-glucose extender.

Treatment	Group	Number of ejaculates	Total sperm motility (%)
Washed semen	Control (0 mM BHT)	20	48.74±1.45[a]
	+ 0.6 mM BHT	20	65.43±1.53[b]
	+ 2.0 mM BHT	20	71.65±2.86[b]
	+ 5.0 mM BHT	20	57.04±2.60[c]
General mean			60.72±2.17
Unwashed semen	Control (0 mM BHT)	20	40.97±2.05[a]
	+ 5.0 mM BHT	20	53.28±3.44[b]
General mean			47.13±1.91

[a-c] *Values with different superscript letters in the same column in each treatment differ significantly (P<0.05).*

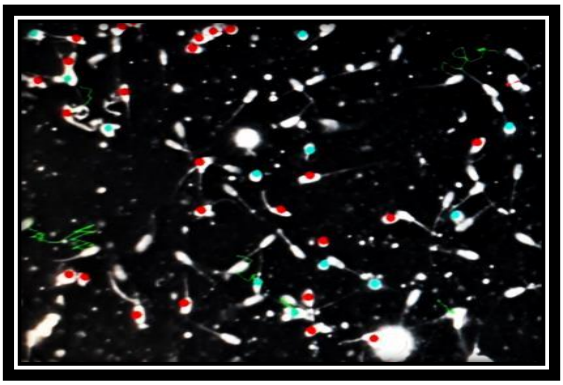

Fig 1. A microscope field was recorded by CASA showing a total motility (%) of buck sperm with a value of 45% with supplementation of 0.0 mM of butylated hydroxytoluene post thawing.

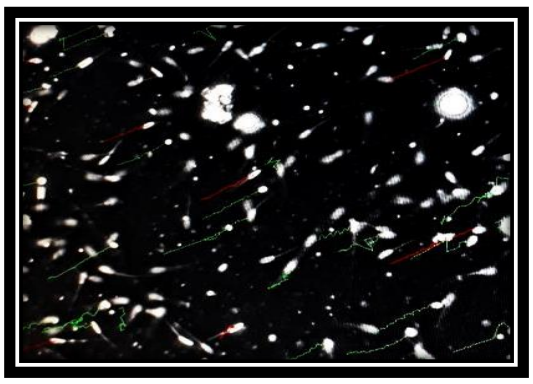

Fig 2. A microscope field was recorded by CASA showing a total motility (%) of buck sperm with a value of 78% with supplementation of 0.6 mM of butylated hydroxytoluene post thawing.

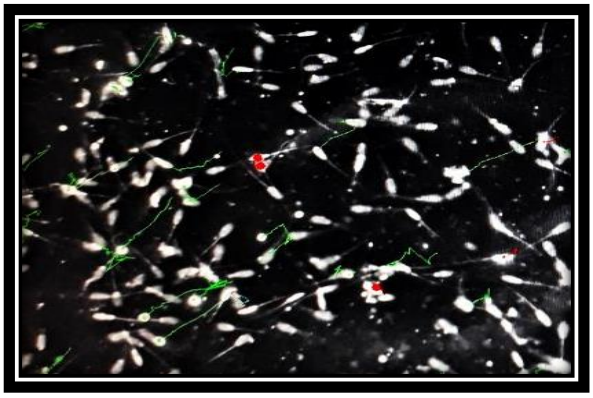

Fig 3. A microscope field was recorded by CASA showing a total motility (%) of buck sperm with a value of 85% with supplementation of 2.0 mM of butylated hydroxytoluene post thawing.

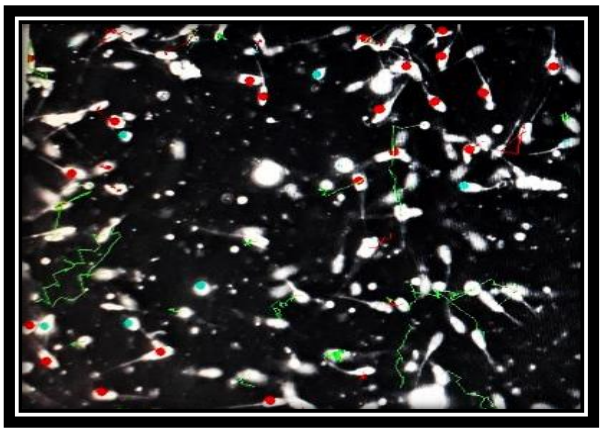

Fig 4. A microscope field was recorded by CASA showing a total motility (%) of buck sperm with a value of 65% with supplementation of 5.0 mM of butylated hydroxytoluene post thawing.

The obtained results agree with the results of **Dewry *et al.* (2015)** where sperm motility (%) was extremely higher in Tris extender that containing a concentration of 2.0 mM of vitamin E than that containing 0.0, 5.0 and 7.0 mM of vitamin E after equilibration and freezing of buck semen. While, it was lower than control in both of 5.0 and 7.0 mM of vitamin E. A similar trend was reported by **Dewry *et al.* (2020)** in buck semen. A significant improvement in sperm motility was also observed by **Saraswat *et al.* (2012)** in post-thawed semen of buck with 3.5 and 4.5 mM of vitamin E. Otherwise, **Iqbal *et al.* (2015)** stated that BHT inclusions negatively affected sperm motility where the highest sperm motility was reached in 0.0 mM BHT group. However, this difference was insignificant in progressive motility after thawing.

In a similar study, **Akeel *et al.* (2011)** showed that sperm motility was extremely higher in all treated BHT groups (0.5, 1.0, 2.0, and 3.0 mM) where BHT addition with a concentration of 2.0 mM to the semen extender of Boer buck showed a general enhancement in semen properties than other groups. According to **Khalifa *et al.* (2008)** who stated that the mean percentages of post-thawed motile spermatozoa treated with a BHT concentration of 0.6 mM were considerably greater than that of the control and sperm treated with BHT concentrations of 0.3 or 0.9 mM in buck semen. Besides, the addition of extenders to an egg yolk-based medium with a BHT concentration of 5.0 mM caused a significant increase in sperm motility after thawing. Similar results were obtained by **Toker and Dogan (2023)** in ram semen and **Nain *et al.* (2023)** in buffalo semen. Moreover, **Palomo *et al.* (2017)** showed that preserved sperm after washing in an extender containing a BHT concentration of 5.0 mM had greater values of sperm kinetic characteristics than the control group.

2. Progressive sperm motility (%)

The percentage of progressive motility post thawing is presented in Table 2. A significant ($P<0.05$) difference between BHT groups and the control group in washed and unwashed semen treatments was observed. In washed semen treatment, the highest percentage of progressive motile spermatozoa was recorded in the BHT group of 2.0 mM with an average of 42.44±5.29, while the lowest percentage was recorded in the control group with an average of 22.45±4.41. Moreover, the difference between the washed and unwashed semen treatments was significant ($P<0.05$). The highest progressive motile spermatozoa (%) was recorded in washed semen treatment with an average of 31.62±1.34 and the lowest percentage was recoded in unwashed semen treatment with an average of 24.62±1.28.

Table 2. Percentage of progressive motile spermatozoa post thawing (mean±SE) in Saanen bucks, supplemented with different concentrations (mM) of butylated hydroxytoluene in tris-citric acid-glucose extender.

Treatment	Group	Number of ejaculates	Progressive sperm motility (%)
Washed semen	Control (0 mM BHT)	20	22.45±4.41[a]
	+ 0.6 mM BHT	20	38.12±4.92[b]
	+ 2.0 mM BHT	20	42,44±5.29[b]
	+ 5.0 mM BHT	20	23.46±2.37[a]
General mean			31.62±1.34
Unwashed semen	Control (0 mM BHT)	20	20.62±2.66[a]
	+ 5.0 mM BHT	20	28.62±2.52[b]
General mean			24.62±1.28

[a-b] *Values with different superscript letters in the same column in each treatment differ significantly (P<0.05).*

A similar result was observed by **Akhil *et al.* (2015)** in Hariana bull spermatozoa where the progressive sperm motility was considerably greater in both 0.5 and 1.0 mM of BHT groups than that in the control group. Mean while, better results were reported in the group of 1.0 mM BHT at all three stages of processing (The first ranged from 4 to -10°C at 5°C/min, the second ranged from -10 to -100°C at 40°C/min, and the third ranged from -100 to -140°C at 20°C/min). Moreover, **Mostafa *et al.*, (2019)** showed that progressive motility was extremely increased, with better results achieved using BHT concentrations of 1.0 and 1.5 mM in Tris-citrate-fructose (TCF) extender than the control group in buffalo semen.

The differences observed in sperm motility after thawing may be due to the separation of seminal plasma, the difference in the amount and proportions of the extenders used in the freezing process and the components contained in the extenders. In addition, it may depend on the differences in the technique applied in semen extension and preservation, changes in thawing time and temperature, and the person or device performing the analysis. Species, seasonal and individual differences may also cause a difference in sperm motility rates **(Kulaksız, 2009)**.

3. Dead spermatozoa (%)

The percentage of dead spermatozoa post thawing is presented in Table 3. A significant (P<0.05) difference between BHT groups and the control group in both washed and unwashed semen treatments was observed. In washed semen treatment, the lowest percentage of dead spermatozoa was recorded in the 2.0 mM of BHT group with an average of 28.34±2.86, while the highest percentage was recorded in the control group with an average of 51.25±1.45. Moreover, the difference between the washed and unwashed semen treatments was significant (P<0.05). The lowest percentage of dead spermatozoa was recorded in washed semen treatment with an average of 39.28±2.17, while the highest percentage was recoded in unwashed semen treatment with an average of 52.87±1.91.

The obtained results are in agreement with **Dewry *et al.*, (2015)** who showed that viable spermatozoa (%) were considerably higher in TCG extender which contained 2.0 mM of vitamin E than that containing 0.0, 5.0 and 7.0

mM of vitamin E after equilibration and freezing of buck semen. While, it was lower than the control group in both of 5.0 and 7.0 mM of vitamin E. Similar results were reported by **Lalramdintluanga (2012)** and **Dewry *et al.*, (2020)** in buck semen. Moreover, **Akeel *et al.*, (2011)** reported that viable spermatozoa (%) was extremely high in all treated BHT groups (0.5, 1.0, 2.0, and 3.0 mM) in Boer goat semen, where semen properties were considerably improved by addition of 2.0 mM of BHT to the semen extender when compared to the other treatments. According to **Khalifa *et al.* (2008)**, sperm viability was considerably improved by increasing BHT concentrations from 0.6 to 5.0 mM in semen extender of Damascus bucks.

Table 3. Percentage of dead spermatozoa post thawing (mean±SE) in Saanen bucks, supplemented with different concentrations (mM) of butylated hydroxytoluene in tris-citric acid-glucose extender.

Treatment	Group	Number of ejaculates	Dead spermatozoa (%)
Washed semen	Control (0 mM BHT)	20	51.25±1.45[a]
	+ 0.6 mM BHT	20	34.56±1.53[b]
	+ 2.0 mM BHT	20	28.34±2.86[b]
	+ 5.0 mM BHT	20	42.95±2.60[c]
General mean			39.28±2.17
Unwashed semen	Control (0 mM BHT)	20	59.02±2.05[a]
	+ 5.0 mM BHT	20	46.71±3.44[b]
General mean			52.87±1.91

[a-c] Values with different superscript letters in the same column in each treatment differ significantly (P<0.05).

According to **Mostafa *et al.*, (2019)** viable spermatozoa (%) was extremely high in 1.0 and 1.5 mM/mL of BHT groups than the control group in buffalo semen stored in TCF extender. A similar result was obtained by **Wadood *et al.* (2016)** who recorded greater spermatozoa viability at 2.0 mM of BHT than other groups and lower spermatozoa viability at 2.25 mM of BHT than 1.75 and 2.0 mM of BHT as a result of internal organelles damage at high levels of BHT. Furthermore, **Palomo *et al.* (2017)** showed that the lowest sperm viability was observed in unwashed semen without antioxidants in ram semen, while the highest percentage was determined in washed semen stored in an extender with 5.0 mM of BHT. A similar trend was also reported by **Bello *et al.* (2022)** in buck semen and **Nain *et al.* (2023)** in buffalo semen.

To describe the mechanism of BHT protection during cryopreservation, many authors presented a range of hypotheses and speculations.

According to **Hammerstedt *et al.* (1976)**, BHT possesses antiviral and antimicrobial activity thus it can inhibit lipid-containing viruses and microbes. In addition, as a phenolic antioxidant, BHT is absorbed into sperm membranes, reducing membrane lipid viscosity. This could suggest an increase in the fluidity of lipids at low temperatures, which would inhibit or significantly minimize the alterations of permeability in the membrane of sperm plasma during the cold-shock of the cells. **Aitken (1995)** hypothesized that BHT acts as an oxygen free radical scavenger in the extender and sperm, hence minimizing the damage occurring to the characteristics of sperm. On the other hand, this

study showed that washing semen by centrifugation had a beneficial effect on cryopreservation of buck semen. This may be due to that the technique used for washing semen by centrifugation may eliminate the percentages of damaged or dead spermatozoa **(Cebrián *et al.*, 2010)**.

CONCLUSION

In conclusion, BHT significantly improved the semen quality of Saanen buck. The concentration of BHT ranging from 0.6 to 2.0 mM/mL could be the optimal concentration for freezing semen in Saanen bucks. The highest percentages of motile and viable spermatozoa were recorded in washed post-thawed semen stored in Tris-citric acid-glucose extender with 2 mM of BHT than other groups. Consequently, it could be recommended to add 2.0 mM of BHT in the semen extender of Saanen buck to improve the quality of cryopreserved semen in an egg yolk-based medium. Also, washing sperm by initial centrifugation of semen could be recommended to improve sperm cryosurvival in Saanen bucks. However, further studies are needed on the use of *in-vitro* or *in-vivo* fertilization to affirm the prevailing results.

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الملخص العربى

تأثير إضافة البيوتيل هيدروكسي تولوين على جودة السائل المنوي المجمد بعد الإسالة لذكور ماعز السانين

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هدفت هذه الدراسة إلى تقييم التأثير المفيد لتركيزات مختلفة من البيوتيل هيدروكسي تولوين (BHT) كمضاد للأكسدة عند تركيزات ٠.٦ ، ٢.٠ ، ٥.٠ ملي مول في مخفف التريس (TCG) على جودة السائل المنوي لذكور ماعز السانين. تم فى هذه الدراسة إستخدام أربعة ذكور من ماعز السانين خلال موسم التزاوج (نوفمبر- يناير). عقب الجمع، تم تقسيم السائل المنوي إلى قسمين. تم تخفيف الجزء الأول بنسبة ١:٥ في محلول TCG ثم تم غسله مرتين بالطرد المركزي بسرعة ٣٠٠٠ دورة / الدقيقة لمدة ١٠ دقائق. بينما ترك الجزء الثاني بدون غسيل. تم إعادة تخفيف أجزاء السائل المنوي في وسط TCG على النحو التالي: السائل المنوي المغسول، السائل المنوي المغسول +٠.٦ ملي مول BHT، السائل المنوي المغسول +٢.٠ ملي مول BHT، السائل المنوي المغسول +٥.٠ ملي مول BHT، السائل المنوي غير المغسول، السائل المنوي غير المغسول +٥.٠ ملي مول BHT. تم تبريد قشات السائل المنوي عند ٤ درجات مئوية لمدة ساعتين ثم تخزينها في النيتروجين السائل. بعد الإسالة، تم تسجيل صفات السائل المنوى من الحركة الكلية والتقدمية للحيوانات المنوية (%) والحيوانات المنوية الميتة (%) بواسطة برنامج تحليل السائل المنوي من خلال الحاسب الآلى (CASA). أظهرت النتائج التي تم الحصول عليها أن إضافة ٢.٠ مللي مول من BHT في السائل المنوي المغسول سجلت أعلى النسب المئوية للحركة الكلية والتقدمية للحيوانات المنوية وأقل نسبة مئوية للحيوانات المنوية الميتة. كان السائل المنوي المغسول أفضل بشكل ملحوظ ($P<0.05$) مقارنة بالسائل المنوي غير المغسول. الخلاصة، أدت إضافة BHT إلى مخفف السائل المنوى إلى زيادة معنوية ($P<0.05$) في كل من نسبة حركة الحيوانات المنوية وقابليتها للحياة في السائل المنوي لذكور السانين. كذلك فإن إضافة ٢.٠ مللي مول من BHT يعتبر التركيز الأمثل لتحسين جودة السائل المنوي وزيادة بقاء الحيوانات المنوية بالتجميد لذكور ماعز السانين، وذلك من خلال العمل كمضاد للأكسدة وبالتالي تقليل بيروكسيد الدهون في الحيوانات المنوية.

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