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Received: 11 June, 2019
Accepted: 09 July, 2019
Published: 10 July, 2019

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Abstract

Evaluation of goat semen, extended in Cornell University 16 extender, was used to study the effects of melatonin of various concentrations on sperm motility (SM) %, alive sperm (AS) % and sperm abnormalities (SA) % of extended goat semen stored at 5°C for seven days were studied. Melatonin at the doses of 0.0, 10.0, 15.0 and 20.0 μg/100 x 10^6 sperm was added to the extended semen and the samples were examined daily for the previous parameters. Additionally, the influence of melatonin on post-thawing motility was assessed.

The results of the present investigation elaborated that melatonin particularly the high concentration (20.0 μg) significantly improved SM%, reduced dead sperm % and improved post-thaw sperm motility. In conclusion, the addition of melatonin (particularly 20.0 μg) induced remarkable and profound physiological actions on goat semen quality during storage for long time at 5°C and improved its freezability during preservation.

Introduction

The success of an artificial insemination (AI) program depends on the proper management of semen collection, storage and use. Goat semen has been stored at temperatures ranging from 2 to 15°C and mostly at 5°C using various diluents such as sodium citrate-yolk, sodium citrate-fructose-yolk, milk (whole, skimmed or reconstituted) with or without egg yolk, Spermasol, Neoseminan, saline [1]. It was found that tris-buffer extenders were effective for dilution and storage of goat, sheep and rabbit semen [2-4].

Nishikawa et al., [5], stored goat spermatozoa in Neoseminan diluent at 4°C for 8-15 days and maintained their fertilizing capacity for 6 days. Moreover, Eppleston et al., [6], demonstrated that goat spermatozoa have retained their fertilizing capacity for at least 8 days of storage at 5°C in Tris- Fructose-citric acid-yolk diluent. Unfortunately, there is no available literature regarding the use of glycine containing extenders to keep the viability of goat semen. On the other hand, the replacement of citrate buffer with glycine was reported to improve the survival of ram spermatozoa. Also, El-Chahidi [7-9], elaborated that the use of different combinations of electrolytes mixture and glycine improved the motility and viability of ram spermatozoa for long time. Moreover, the use of glycine and glucose containing extenders maintain high fertility over a period of several days in bull [10].

Melatonin (N-acetylle-5-Methoxytryptamine) is a hormone produced mainly by the pineal gland besides other tissues like retina [11]. It neutralizes free radicals thus it prevents cell damage. Mammdouh et al., [12,13], recorded that the addition of melatonin to liquid bull semen improved its storage and preservation for 6 days. Unfortunately, no available literature was found concerning the addition of melatonin to goat semen. Thus the objective of the present study is to:

1. Study the effect of some extenders on viability of goat semen during preservation at 5°C.
2. Study the physiological influence of melatonin on the quality of goat semen preserved in liquid or frozen condition.

Materials and Methods

This investigation was carried out at private goat farm at Alexandria desert road.

A-experimental animals

Six male Zaraibi goats aged 19 month approximately and weighed 30-40 kg were used. Each buck was fed one Kg balanced concentrate and berseem hayad libidium. Water was offered to those animals ad libidium all the day using manual water trough system.

Citation: El-Battawy KA (2019) Preservation of goat semen at 5°C with emphasis on its freezability and the impact of melatonin. Int J Vet Sci Res 5(2): 035-038.
DOI: http://dx.doi.org/10.17352/ijvsr.000039
B-experimental materials

Melatonin was imported from Twinlab Specialty Corporation, Ronkon Koma, New York, USA. While, the other chemical reagents used for the preparation of extenders were purchased from Sigma–Aldrich Co., Deisenhofen, Germany.

C-semen collection and evaluation. At 1000 rpm for ten minutes

Semen was collected twice weekly by means of an artificial vagina using an anestrous doe as a teaser for a period of two months. After collections, the ejaculates were transferred to the laboratory of the farm within 2-3 minutes where they were centrifuged to remove the seminal plasma and consequently to avoid the harmful effect of seminal goat enzyme. Semen was kept in water bath at 30°C for evaluation by means of conventional methods.

D-Semen extender

Five types of extenders were used for preserving the goat semen:

1. TGGY was prepared according to Roca et al., [4], for rabbit semen. It consisted of Tris–hydroxymethyl amino methane (3.801 g), glucose (0.6 g), citric acid monohydrate (2.166 g), glycerol (6.7 ml), the egg yolk and antibiotics were added as previously mentioned.

2. TFGY formulated according to Foote [14], for bull semen. It consisted of Tris–hydroxymethyl amino methane (3.028 g), fructose (1.25 g), citric acid monohydrate (1.675 g), glycerol (6.7 ml), the egg yolk and antibiotics were added as previously mentioned.

3. Cornell University (CU–16) extender was prepared according to Shannon [10], for bull semen. It consisted of trisodium citrate dihydrate (1.45 g), glucose (1.25 g), glycine (0.94 g) the egg yolk and antibiotics were added as previously mentioned.

4. Extender 14 was prepared according to Shannon [10], for bull semen. It consisted of trisodium citrate dihydrate (2.00 g), glucose (0.30 g), glycine (1.00 g) the egg yolk and antibiotics were added as previously mentioned.

E-semen processing and experimental design

Only ejaculates of >70% initial motility and 2000 x 106 sperm cells/ ml were used in the following experiments:

Experiment 1: This experiment was designed to find out the impact of melatonin on the viability of chilled goat semen in CU–16 extender as it achieved the best results in comparison with the rest of used extenders. Semen samples were processed as previously mentioned in experiment I, then melatonin was added to the extended semen at the rate of 0.0 μg (control sample), 10, 15 and 20 μg/100x106 sperm [13]. After daily examination, the samples were centrifuged at 3000 rpm for 20 minutes to get supernatant for immediate daily determination of acid phosphatase (ACP) according to Moss [16].

Experiment 2: This experiment was designed to find out the impact of melatonin on the viability of chilled goat semen in CU–16 extender as it achieved the best results in comparison with the rest of used extenders. Semen samples were processed as previously mentioned in experiment I, then melatonin was added to the extended semen at the rate of 0.0 μg (control sample), 10, 15 and 20 μg/100x106 sperm [13]. After daily examination, the samples were centrifuged at 3000 rpm for 20 minutes to get supernatant for immediate daily determination of acid phosphatase (ACP) according to Moss [16].

Experiment 3: This experiment was designed to investigate the influence of melatonin (0.0, 10, 15 and 20 μg) on freezability of goat semen extended in TGGY, TFGY, CU–16 and 14 extenders. Semen samples were split and diluted 1 : 4 at 30°C. The diluted semen was cooled and loaded into 0.25 ml straws at 5°C. The straws were placed horizontally on freezing racks and lowered into liquid nitrogen vapour inside small tank containing 10 liters of liquid nitrogen at a height of 2.0 cm above the level of liquid nitrogen, for 15 minutes. The straws were then immersed gradually (within 2-3 minutes) in liquid nitrogen and transferred into liquid nitrogen storage container. After few weeks, frozen goat semen was thawed in a water bath at 40°C for 30 seconds. The thawed semen was emptied in pre-warmed tubes and incubated in water bath at 30°C for assessment of sperm motility [17].

F-statistical analysis

Data were transformed from percentage to absolute figures using arcsin tables. The ANOVA test was used at a confidence not less than limit 95% using SAS program (1988). LSD test was used to evaluate the significant difference between means at P<0.05.

Results

Table 1 declared the significant (P<0.0001) effect of melatonin on sperm motility %. Furthermore, both Table 2 and Table 3 showed the influence of melatonin on both AS % and SA %.

The high concentration of melatonin (20 μg) had significantly (P<0.0001) increased the motility and the live sperm in comparison with the other concentrations, during

| Storage time (days) | Control | Treatment with different melatonin concentrations | Overall mean |
|--------------------|---------|-----------------------------------------------|-------------|
|                    | 10 μg   | 15 μg | 20 μg |                |
| 1                  | 67.63 ± 2.75 | 69.53 ± 2.03 | 74.61 ± 2.47 | 75.70 ± 1.38 | 71.86A |
| 2                  | 60.92 ± 1.60 | 64.47 ± 1.73 | 68.44 ± 1.96 | 70.47 ± 1.09 | 66.07B |
| 3                  | 56.06 ± 1.50 | 60.86 ± 0.86 | 62.66 ± 1.72 | 66.41 ± 1.93 | 61.50C |
| 4                  | 53.02 ± 1.44 | 54.80 ± 1.93 | 60.86 ± 0.86 | 63.52 ± 1.47 | 58.95D |
| 5                  | 49.34 ± 1.88 | 56.06 ± 1.50 | 58.40 ± 1.93 | 60.92 ± 1.60 | 56.18E |
| 6                  | 47.16 ± 1.38 | 54.52 ± 1.44 | 56.83 ± 1.28 | 58.40 ± 0.93 | 54.23F |
| 7                  | 43.56 ± 1.44 | 52.25 ± 0.85 | 54.52 ± 1.44 | 57.63 ± 1.50 | 51.49G |
| Overall mean       | 53.96D | 59.44C | 62.33B | 64.72A | 57.46B |

Mean ± SE.; LSD for days 2.201 (P<0.05); LSD for treatments 1.664 (P<0.05).
the storage duration. Moreover, melatonin (20 μg) resulted in a significant (P<0.0001) decrease in the sperm abnormalities.

Regarding the effect of melatonin on the post-thawed sperm motility, only there was a significant (P<0.0001) difference between the different concentrations of melatonin, while no significant differences were found in case of extenders or the interaction between the melatonin concentrations and the extenders factor (Table 4).

**Discussion**

This investigation elaborates to effect of various extenders on preservation of extended goat semen, impact of melatonin on sperm motility %, alive sperm %, sperm abnormalities % as well as freezability of that semen. It was noticed that the use of TGGY, TFGY, CU-16 and 14 extenders improved significantly the storage of extended semen. These results are compatible with Evans and Maxwell [3], Roca et al., [4] and Chehadeh et al., [2], who reported that Tris-buffer extenders were the best diluents for sheep, rabbit and goat respectively. Regarding the storage of goat semen in glycine containing extenders (CU-16 and 14), our results are in agreement with Shannon [10] and El-Chahidi [9], who reported that the inclusion of glycine in extender improved and allowed long time storage of bull and sheep semen respectively. On the contrary Dessouky et al., [18], found that the glycine containing extenders were less efficient in storage of rams semen Paleg et al., [19], attributed the beneficial effect of glycine to its ability to retard thermal denaturation of enzymes thus it maintains the enzyme structure and function via its protective action.

It was observed that addition of melatonin resulted in a significant increase in sperm motility and alive sperm percentages in all types of extenders. The effects of melatonin as an additive were profound and clear in high concentrations and at the last four days of incubation. These results are in accord with Mamdouh et al., [13], who reported the same results on addition of melatonin to liquid bull semen. On the other hand, our results were in disagreement with Bornmann et al., [20], who concluded that seminal plasma melatonin play no important role in sperm motility. The influence of melatonin on sperm motility and alive sperm percentages may be ascribed to one and/or all of the following physiological mechanisms:

I- Melatonin increases ATPase levels [21]. The increase of ATPase is correlated with an increase in ATP which is the main energy source used by the sperm flagellum to initiate and activate forward motility [22].

II- Melatonin stimulates cellular influx of Ca+2 into sperm cells enhancing their motility [23].

III- Melatoni could be a potent cyclic AMP (cAMP) stimulator [24]. cAMP stimulates sperm motility via its direct action on the axoneme of the tail [25] or indirectly through acting on the cell membrane as secondary messenger [26].

Also, it was obvious that melatonin induced a significant decrease in the sperm abnormalities percentages and a significant reduction in seminal acid phosphastase. These results were in accord with Abdine [27], who recorded that melatonin treatment of Cambridge rams, in vivo, lowered the sperm dead percentage and decreased the abnormal sperms. Additionally, our results were coincided with Poeggeler et al., [12], who reported that the number of abnormal and dead sperms was reduced after addition of melatonin. Moreover, the current results were in a harmony with those results obtained by Mamdouh et al., [13], who concluded that melatonin decreased significantly sperm abnormalities and acid phosphastase level. The impact of melatonin on sperm abnormalities and acid phosphastase could be accredited to one and/or the following physiological actions:

1- Melatonin can pass the cell membrane and protects DNA from free radical damage effect through its potent antioxidant and anti-aging effects on the cells [12].

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**Table 2:** The effect of melatonin on the alive sperm % stored at 5°C for 7 days using CU-16 extender.

| Storage time (days) | Control | Treatment with different melatonin concentrations Overall mean |
|---------------------|---------|-------------------------------------------------------------|
|                     |         | 10 μg  | 15 μg  | 20 μg  | |
| 1                   | 87.25 ± 3.79 | 90.25 ± 2.75 | 95.00 ± 3.00 | 96.75 ± 1.25 | 92.31A |
| 2                   | 78.25 ± 2.39 | 82.75 ± 2.14 | 88.50 ± 2.72 | 91.25 ± 1.75 | 85.19B |
| 3                   | 71.25 ± 2.14 | 78.25 ± 1.25 | 80.50 ± 2.18 | 85.75 ± 2.59 | 78.94C |
| 4                   | 66.25 ± 2.32 | 74.50 ± 1.44 | 78.25 ± 1.25 | 81.75 ± 1.84 | 75.19D |
| 5                   | 63.25 ± 3.30 | 71.25 ± 2.14 | 74.50 ± 1.44 | 78.25 ± 2.39 | 71.81E |
| 6                   | 57.25 ± 2.14 | 68.75 ± 2.14 | 72.25 ± 1.84 | 74.50 ± 1.44 | 68.19F |
| 7                   | 51.25 ± 2.75 | 65.50 ± 1.44 | 68.75 ± 2.14 | 73.50 ± 2.18 | 64.75G |
| Overall mean        | 67.82D   | 75.89C  | 79.68B  | 83.11A  | |

Mean ± SE.; LSD for days 3.146 (P<0.05); LSD for treatments 2.378 (P<0.05).

**Table 3:** The effect of melatonin on the sperm abnormalities % stored at 5°C for 7 days using CU-16 extender.

| Storage time (days) | Control | Treatment with different melatonin concentrations Overall mean |
|---------------------|---------|-------------------------------------------------------------|
|                     |         | 10 μg  | 15 μg  | 20 μg  | |
| 1                   | 10.00 ± 0.71 | 8.25 ± 0.25 | 7.25 ± 0.25 | 6.00 ± 0.41 | 7.88B |
| 2                   | 11.75 ± 0.48 | 9.75 ± 0.48 | 8.75 ± 0.48 | 7.00 ± 0.41 | 9.31E |
| 3                   | 13.00 ± 0.56 | 10.75 ± 0.25 | 10.50 ± 0.65 | 9.50 ± 0.65 | 10.94D |
| 4                   | 13.25 ± 0.61 | 11.75 ± 0.25 | 11.00 ± 0.41 | 10.75 ± 0.63 | 11.69C |
| 5                   | 14.00 ± 0.41 | 12.25 ± 0.25 | 11.50 ± 0.65 | 11.50 ± 0.29 | 12.31B |
| 6                   | 14.75 ± 0.48 | 12.75 ± 0.25 | 12.25 ± 0.48 | 12.00 ± 0.41 | 12.94A |
| 7                   | 15.50 ± 0.29 | 13.25 ± 0.25 | 12.75 ± 0.48 | 12.25 ± 0.48 | 13.44B |
| Overall mean        | 13.18A   | 11.25B  | 10.57C  | 9.86D   | |

Mean ± SE.; LSD for days 0.637 (P<0.05); LSD for treatments 0.482 (P<0.05).

**Table 4:** The effect of melatonin on the sperm motility % of goat spermatozoa after freezing and thawing for various extenders.

| Melatonin concentrations | TGYY | TFGY | CU-16 | 14 | Overall mean |
|--------------------------|------|------|-------|----|-------------|
| 0.0 μg (Control)         | 36.99 ± 1.44 | 34.69 ± 1.99 | 36.73 ± 4.22 | 39.09 ± 3.25 | 36.87A |
| 10 μg                    | 34.74 ± 0.88 | 38.43 ± 2.51 | 39.15 ± 3.16 | 40.66 ± 1.88 | 38.24A |
| 15 μg                    | 42.12 ± 1.18 | 43.56 ± 1.86 | 42.12 ± 1.18 | 44.28 ± 1.37 | 43.02B |
| 20 μg                    | 45.72 ± 1.37 | 46.44 ± 0.83 | 46.44 ± 0.83 | 47.15 ± 0.72 | 46.44C |

Mean ± SE.; LSD for melatonin concentration 2.906 (P<0.05).

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2- Melatonin lowers the levels of acid phosphatase enzyme which is considered as an indicator of cellular death or damage (Moss and Henderson, 1993).

At last, it was clear that melatonin produced significant increase in post-thawing motility. These results were in agreement with Kaya et al., [28], who concluded that melatonin administration to rams, in vivo, improved post-thawed sperm viability as well as the intact acrosome rates. The beneficial effect of melatonin on post-thawed motility could be attributed to its decreasing effect on the phosphatase enzyme release (leakage) from sperm cells during cryopreservation [29].

In conclusion, the addition of melatonin (particularly 20.0 μg/100 x 106 goat sperm) induced remarkable and profound physiological actions that improved the extended goat semen quality, its storage for long time at 5°C and improve its freezability.

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