FRESH AND CRYOPRESERVED SEMEN CHARACTERISTICS OF BUFFALO BULLS AS INFLUENCED BY MELATONIN IMPLANTATION

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ABSTRACT

This study explored the effect of melatonin implantation on the fresh and cryopreserved semen quality of Iraqi buffalo bulls (*Bubalus bubalis*). Nine adult bulls were used and divided equally into three groups. The first group was left without treatment and regarded as control (T1), whereas the second (T2) and third (T3) groups were subcutaneously implanted with 72 and 90 mg of melatonin respectively on the left ear base and repeated one month post the first implantation. Semen was collected for 12 weeks and evaluated weekly for fresh, cooling, and post-cryopreserved (PC) protocols. The T3 group exhibited a higher ($P \leq 0.05$) sperm cell individual motility percentage, whereas, the T1 and T2 groups recorded better ($P \leq 0.05$) sperm acrosome integrity percentage for fresh semen. A lesser ($P \leq 0.05$) total sperm abnormality (TSA) percentage in the T2 and T3 groups and a greater ($P \leq 0.05$) sperm acrosome integrity (SAI) percentage in the T3 group were noticed during the cooling preservation. Moreover, the T3 group exhibited a lesser ($P \leq 0.05$) TSA percentage whereas, the T2 and T3 groups revealed a leaser ($P \leq 0.05$) SAI percentage during the PC period. Concomitantly, lower ($P \leq 0.05$) PC malondialdehyde concentration in seminal plasma was observed in the T2 and T3 groups than control group. In conclusion, the melatonin implantation (72 and 90 mg) ameliorated some fresh and PC semen attributes of the Iraqi buffalo bulls.

Keywords: Sperm quality, Hormonal treatment, *Bubalus bubalis*.

خصائص السائل المنوي الطازج والمحفوظ بالتجميد لثيران الجاموس بتأثير غرز هرمون الميلاتونين

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المستخلص

أجريت هذه الدراسة لمعرفة تأثير غرز هرمون الميلاتونين في نوعية السائل المنوي الطازج والمحفوظ بالتجميد لثيران الجاموس العراقي (*Bubalus bubalis*). تم استعمال تسعة ثيران بالغة قسمت الى ثلاثة مجاميع متساوية. تركت المجموعة الأولى بدون معاملة هرمونية وعدت بمثابة مجموعة سيطرة (T1)، في الوقت الذي عوملت فيه المجموعتين الثانية (T2) والثالثة (T3) عن طريق الغرز تحت الجلد عند قاعدة الاذن اليسرى بهرمون الميلاتونين بمقدار 72 و90 ملغم وتم اعادتها بعد شهر من الجرعة الأولى. تم جمع السائل المنوي لمدة 12 أسبوع وتقييمه أسبوعيا لكل من الطازج والمبرد والمحفوظ بالتجميد. أظهرت المجموعة الثالثة (T3) أعلى ($P \leq 0.05$) نسبة مئوية لحركة النطف الفردية، في الوقت الذي سجلت فيه المجموعتين T1 وT2 أفضل نسبة مئوية لسلامة أكروسوم النطف للسائل المنوي الطازج. لوحظت أقل ($P \leq 0.05$) نسبة مئوية لتشوهات النطف الكلية في المجموعتين T2 وT3 وأعلى ($P \leq 0.05$) نسبة مئوية لسلامة أكروسوم النطف لدى المجموعة T3 خلال مرحلة الحفظ بالتبريد. كما أظهرت المجموعة T3 أقل ($P \leq 0.05$) نسبة مئوية لتشوهات النطف الكلية، في الوقت الذي سجلت فيه المجموعتين T2 وT3 أقل ($P \leq 0.05$) نسبة مئوية لسلامة أكروسوم النطف بعد الحفظ بالتجميد. من جانب آخر، لوحظ أقل ($P \leq 0.05$) تركيز للمركب مالون ثنائي الألديهايد في البلازما المنوية بعد الحفظ بالتجميد لدى المجموعة T2 و T3 مقارنةً مع مجموعة السيطرة. يمكن الاستنتاج بان الغرز بهرمون الميلاتونين (72 و90 ملغم) حسن من بعض خصائص النطف لدى ثيران الجاموس العراقي.

الكلمات المفتاحية: نوعية النطف، المعاملة الهرمونية، *Bubalus bubalis*.

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INTRODUCTION

Buffalo bull's semen is characterized by low concentration, motility, viability, maturation within the epididymis, and a weak sperm mitochondrial membrane (4, 11,24,25,30,31,32,35). Reactive oxygen species (ROS) are harmful to sperm during their processing inside the testicle and the cryopreservation of semen (9,19,22,33,37,41). It is worth mentioning that sperm damage resulting from freezing-thawing processes can be treated by adding enzymatic, non-enzymatic, natural, and synthetic antioxidants to semen extenders (2,3,5,7,8,10,11,34). It has been found that the most significant obstacle facing adding antioxidants to semen extenders for buffalo bulls is the lack of treatment of damaged sperm with ROS during the spermatogenesis in the testicle, so there is an urgent need to give powerful antioxidants by treating the animals directly (4,5,10,25,35). Melatonin is a neurohormone involving various biological processes (1). The melatonin hormone is secreted from the pineal gland, and its receptors exist in many body cells, including sperm (13). It is powerful in regulating the daily and seasonal circadian rhythm of vital activities in the body and is considered to enhance the body's immune status(29). It also works directly in scavenging free radicals of various types and indirectly as an antioxidant by activating antioxidants (20,26,44). Moreover, it has a role in inhibiting enzymes that catalyze oxidation (14), which reduces oxidative damage, in cells (28). Many studies have indicated that melatonin is more efficient than other antioxidants because it is lipophilic and water-loving, which enables it to easily pass through membranes (16, 17,18,39, 40). It is two times more efficient as an antioxidant than vitamin E. In addition, it can cross the blood-testis barrier, thus protecting most cells from oxidative damage (6, 18,21). Treatment with melatonin protects the seminiferous tubules from deterioration and sperm loss, partly by inhibiting sperm apoptosis and improving their ability to grow when the tubules are exposed to radiation or poisoning (12). Eidan et al., (17,18) explained that injecting Holstein bulls with the 72 mg melatonin hormone significantly improved the concentration of sperm/ ejaculate, sperm cell individual motility, and viability, and the acrosomal and plasma membrane integrity compared to the untreated bulls. Ramadan et al., (38) indicated that implanting the buffalo bulls with 18 mg/50 kg live weight of melatonin outside the reproductive season led to an improvement in the quality of the bulls' semen, the concentration of total protein, albumin, cholesterol, and the activity of the SOD enzyme in seminal plasma compared to untreated bulls. Nsaif and Eidan (35) reported that 90 mg melatonin implantation improves the semen quality of Iraqi buffalo bulls . Due to the lack of studies on the effect of melatonin implantation during the reproductive season of Iraqi buffaloes, this study was conducted to investigate the influence of melatonin implantation on the characteristics of fresh and post-cryopreserved (PC) buffalo bull's semen.

MATERIALS AND METHODS

Experimental animals and design

This study was carried out at the Department of Artificial Insemination, that belonging to the Directorate of Animal Resources, Ministry of Agriculture, Iraq. Nine Iraqi buffalo bulls (5–7 years old and 550–800 kg weighting) were trained for semen collection using an artificial vagina. Bulls were divided into three groups. The first group was considered a control group (T1). The second (T2) and third (T3) groups were implanted subcutaneously on the left base ear with 72 and 90 mg of melatonin hormone and repeated one month after the first implantation. Semen was collected using a previously prepared artificial vagina. The semen was collected for 12 weeks. Fresh, cooled, and PC semen were evaluated regarding the sperm cell individual motility, live sperm, TSA, sperm plasma membrane integrity, SAI, sperm freezability, sperm DNA damage, malondialdehyde (MDA) and total antioxidants concentrations (TCA) in seminal plasma.

Statistical analysis

The statistical computations were performed using SAS program based on study on completely randomized deign to study the effect of different factors on the studies characteristics.

$$\mathbf{Y_{ij} = \mu + T_i + e_{ij}}$$

Y_{ij} = dependent variable pertaining to the j observation of the i treatment
μ = overall mean
T_i = effect of ith treatment (i- T1, T2 and T3 groups).
e_{ij} = error term
Means with significant differences were compared using the Duncan multiple range test.

RESULTS AND DISCUSSION

Sperm cell individual motility percentage (SCIM): The T3 group exhibited higher (P≤0.05) SCIM percentage as compared to the T2 (Fresh semen) and T1 [Post-cryopreservation (PC)] groups of Iraqi buffalo bulls (Table 1). On the other hand, non-significant differences were observed among groups in the SCIM percentage during cooling and PC periods (Table 1). Higher (P≤0.05) SCIM percentage was noticed for fresh semen compared to the cooling and PC periods within each group (T1, T2 and T3). However, lesser (P≤0.05) SIM was shown for PC than for cooling periods (Table 1).

Live sperm percentage (LS): The differences among groups in LS percentage during fresh, cooling, and PC periods lacked significance (Table 2). Moreover, a higher (P≤0.05) LS percentage was shown in fresh semen than for cooling and PC periods for the T1 group (Table 2). Similarly, the fresh semen exhibited higher (P≤0.05) LS percentages than cooling and PC periods for the T2 and T3 groups (Table 2). For all groups, lesser (P≤0.05) LS were noticed during PC than in other preservation periods (Table 2).

Total sperm abnormality (TSA %)

Non-significant differences were observed among groups in TSA percentage for fresh semen ranging from 3.69 to 4.20% for the three included groups (Table 3). Furthermore, a lesser (P≤0.05) TSA percentage was noticed in T3 (9.68 ± 0.29%) and T2 (10.10 ± 1.94%) groups than T1 group (11.74± 0.26%) during the cooling preservation period (Table 3). At PC period, a lesser (P≤0.05) TSA percentage was shown in the T2 group than T3 group but did not differ from those in the T1 group(Table 3).

Sperm acrosome integrity percentage (SAI)

Table 1. Effect of melatonin implantation on sperm individual motility (SIM%) of Iraqi buffalo bulls during different preservation periods (Mean ± SE).

Groups	SIM (%)			Significance level
	Fresh	Cooling (5ºC)	Post-cryopreservation	
T1	45.38±0.92 ab A	36.46±1.01a B	25.98±1.19 b C	P<0.05
T2	42.90±0.92 b A	35.17±0.96 a B	26.00±1.25 ab C	P<0.05
T3	46.29±1.27 a A	38.23±1.44 a B	27.09±1.52 a C	P<0.05
Significance level	P<0.05	NS*	P<0.05	--

Means with different small superscripts within similar columns indicated significant differences among groups, and large superscripts within each row indicated significant differences among periods. T1= Control group; T2, T3= Implanted subcutaneously on the left base ear with 72 and 90 mg of melatonin hormone and repeated one month after the first implantation

Table 2. Effect of melatonin implantation on live sperm (LS%) of Iraqi buffalo bulls during different preservation periods (Mean ± SE).

Groups	LS (%)			Significance Level
	Fresh	Cooling (5ºC)	post-cryopreservation	
T1	83.73±1.57 a A	67.15±1.76 a B	44.73±1.69 a C	P<0.05
T2	82.30±1.46 a A	61.55±2.51a B	43.13±1.82 a C	P<0.05
T3	83.43±2.09 a A	66.90±2.04 a B	44.84±1.91 a C	P<0.05
Significance Level	N.S	N.S	N.S	--

Means with different small superscripts within similar columns indicated significant differences among groups, and large superscripts within each row indicated significant differences among periods. T1= Control group; T2, T3= Implanted subcutaneously on the left base ear with 72 and 90 mg of melatonin hormone and repeated one month after the first implantation

Table 3. Effect of melatonin implantation on total sperm abnormality percentage of Iraqi buffalo bulls during different preservation periods (Mean ± SE).

Groups	Total sperm abnormalities (%)			Significance Level
	Fresh	Cooling (5ºC)	post-cryopreservation	
T1	3.69±0.18 a C	11.74±0.26 a B	16.3±0.17 ab A	P≤0.05
T2	4.20±0.21 a C	10.10±1.94 bB	15.98±1.47 b A	P≤0.05
T3	3.93±0.21 a C	9.68±0.29 b B	16.68±1.07 a A	P≤0.05
Significance level	N.S	P≤0.05	P≤0.05	--

Means with different small superscripts within similar columns indicated significant differences among groups, and large superscripts within each row indicated significant differences among periods. T1= Control group; T2, T3= Implanted subcutaneously on the left base ear with 72 and 90 mg of melatonin hormone and repeated one month after the first implantation.

The T1 and T2 groups exhibited better (P≤0.05) SAI percentages than the T3 group for fresh semen of Iraqi buffalo bulls (Table 4). A similar manner was noticed during the PC period, being higher (P≤0.05) SAI percentages shown for T1 and T2 than in the T3 groups (Table 4). In the cooling preservation period, a higher (P≤0.05) SAI percentage was observed for the T3 group compared to the T1 and T2 groups which are not different from each other at cooling preservation (Table 4). For all groups, better (P≤0.05) SAI percentages were noticed for fresh than for cooling and PC preservation periods. However, these percentages were exceeded for cooling than for PC periods (Table 4).=

Sperm freezability and DNA (%): The differences among groups in sperm freezability percentage lacked significance. However, it tended to be higher in the T3 group than the others (Table 6). On the other hand, non-significant differences were observed among groups in sperm DNA damage percentage, despite its tendency to be lesser in the T3 group than the others (Table 6).

Malondialdehyde (MDA) and total antioxidant concentrations (TAC) in seminal plasma: Lesser (P≤0.05) MDA concentrations in seminal plasma (µmole/10^9 sperm) were noticed in the T3 group (20.27 ± 3.73 and T2 group (36.14 ±4.31) than in the T1 group (66.58 ± 8.19; Table 6). Moreover, the differences among groups in TAC concentration in seminal plasma lacked significance (Table 6). Buffalo bulls implanted with melatonin (90 mg, T3) outperformed the control treatment in individual sperm motility post-cryopreservation. Our results agreed with Ramadan et al., (37) results, who found that when Murrah buffalo bulls were treated with 18 mg of the hormone melatonin/50 kg body weight for two months during the non-breeding season led to an increase in individual sperm motility. Perhaps the significant superiority of our current study is due to the effect of the melatonin dose (90 mg), which may have been sufficient to stimulate the hypothalamus to secrete more amounts of the GnRH hormone and, consequently, FSH and LH from the anterior lobe of the pituitary gland and increase the secretion of testosterone from the Leydig cells (44). It is well known that semen contains concentrations of reactive oxygen species (ROS) necessary for sperm capacitation (36). However, high concentrations of ROS cause oxidative stress that harms motility, membrane integrity, and DNA sperm integrity (36). One study indicated that melatonin works to increase the percentages of individual motility in men's sperm, increase mitochondrial activity, improve spermatogenesis, and protect the blood-testis barrier (43). Melatonin showed a protective effect on the integrity of

Table 4. Effect of melatonin implantation on sperm acrosome integrity (%) of Iraqi buffalo bulls during different preservation periods (Mean ± SE).

Groups	Sperm acrosome integrity (%)			Significance level
	Fresh	Cooling(5ºC)	Post-Cryopreservation	
T1	96.72±0.13 aA	75.65±1.55 bB	49.24±0.57 aC	P<0.05
T2	96.50±0.24 aA	72.59±2.65bB	45.90±1.59 bC	P<0.05
T3	95.65±0.32 bA	77.96±0.77 aB	46.03±1.07 bC	P<0.05
Significance level	P<0.05	P<0.05	P<0.05	

Means with different small superscripts within similar columns indicated significant differences among groups, and large superscripts within each row indicated significant differences among periods. T1= Control group; T2, T3= Implanted subcutaneously on the left base ear with 72 and 90 mg of melatonin hormone and repeated one month after the first implantation

Table 5. Effect of melatonin implantation on sperm plasma membrane integrity of Iraqi buffalo bulls at different preservation periods (Mean ± SE).

Groups	Sperm plasma membrane integrity(%)			Significance Level
	Fresh	Cooling(5ºC)	post-cryopreservation	
T1	96.52±0.22 aA	77.41±0.74aB	47.80±0.55aC	P≤0.05
T2	96.45±0.29 aA	78.52±0.52 aB	48.13±0.70 aC	P≤0.05
T3	96.22±0.26 aA	77.00±0.92 aB	47.74±0.39 aC	P≤0.05
Significance level	N.S	N.S	N.S	

Means with different small superscripts within similar columns indicated significant differences among groups, and large superscripts within each row indicated significant differences among periods. T1= Control group; T2, T3= Implanted subcutaneously on the left base ear with 72 and 90 mg of melatonin hormone and repeated one month after the first implantation

Table 6. Effect of melatonin implantation on the sperm freezability, DNA damage, malondialdehyde (MDA), total antioxidant (TAC) concentrations in seminal plasma of Iraqi buffalo bulls (Mean ± SE).

Group	Sperm freezability(%)	MDA concentration (µmole/10^9 sperm)	Sperm DNA damage (%)	TAC concentration(mg/dl)
T1	56.23±2.95A	66.58±8.19A	3.12±0.22A	0.03±0.01A
T2	56.64±4.19A	36.14±4.31B	3.13±0.21A	0.03±0.01A
T3	57.01±3.28A	20.27±3.73B	2.88±0.21A	0.04±0.01A
Significance level	NS	P≤0.05	NS	NS

Means with different small superscripts within similar columns indicated significant differences among groups, and large superscripts within each row indicated significant differences among periods. T1= Control group; T2, T3= Implanted subcutaneously on the left base ear with 72 and 90 mg of melatonin hormone and repeated one month after the first implantation.

sperm mitochondrial function when preserved by freezing. This effect was important because the mitochondria are responsible for generating ATP within the cells, thus maintaining or improving sperm motility after thawing (14). The improvement of motility in the T3 group may be because melatonin receptors (MT1 and MT2) in sperm and Sertoli cells contribute to energy metabolism, which leads to an increase in glucose consumption and lactate metabolism. Rocha et al., (40). The increasing motility (T3,Table 1) may be due to the role of melatonin in reducing sperm abnormalities and the level of the acid phosphatase enzyme (27). Our current study revealed a decrease in the total abnormalities in the T3 group post-cooling (Table). The concentration of MDA decreased significantly (P<0.05) in T2 and T3 compared to the T1 treatment (Table 6). Melatonin reduces oxidative stress in fresh and cryopreserved semen due to its ability to remove ROS. The decrease of malondialdehyde is due to the role of the melatonin hormone as a powerful antioxidant that works to protect sperm from oxidative stress, reduce lipid peroxidation of the sperm membrane, and malondialdehyde formation, especially after the Iraqi buffalo bulls passed through a hot summer (above 45°C) and a hot fall (30°C until mid-November 2021, Iraqi weather forecasts). This temperature is above the optimum temperature for spermatogenesis and causes thermal and oxidative stress. Heat stress continues to affect bulls for a long time until they regain their activity in producing good-quality sperm. Myerhoeffer et al., (33) have shown that the mass activity and individual motility of sperm do not return to their normal state after 8–10 weeks of exposure to heat stress. The lack of significant differences in the live sperm, plasma membrane integrity, and acrosome post-cooling and cryopreservation among the three treatments may be the sperms have been exposed to cold shock upon cooling. This leads to a change in the distribution of calcium ions between the sperm cell and seminal plasma, which leads to a decrease in the seminal plasma's calcium ion contents and an increase in calcium concentration associated with protein in the sperm (23). As well as changes in the attention of positive ions and enzymes in the sperm during cooling, thus decreasing sperm motility, live sperm, membrane integrity, and metabolic activity (42). In conclusion, the results that it is possible to raise the hormone melatonin dose to improve the characteristics of the semen of

Iraqi buffaloes, which will reflect positively on the fertility of Iraqi buffaloes and the economic return for breeders and artificial insemination centers.

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