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Research Article

In Vitro Effects of Melatonin on Hyaluronidase Activity and Sperm Motility in Bull Semen

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Abstract: The effects of melatonin on hyaluronidase activity of semen and spermatozoa motility of Holstein bulls were investigation in vitro. Semen samples were divided into 5 equal parts and incubated (v/v) with melatonin at the doses of 0.5, 1, 2 and 4 mM for 60 min. Percentages of spermatozoa motility, morphological abnormality and hyaluronidase activity of semen were determined at 5, 10, 15, 20, 30 and 60 min during incubation. Results show that melatonin caused a significant ($P < 0.001$) decrease in the spermatozoa motility, while causing a significant increase in the hyaluronidase activities of the semen. However, there was no significant difference in the percentages of abnormal spermatozoa when compared with control samples. Doses of 0.5, 1, 2 and 4 mM of melatonin in bull semen decreased the percentage of spermatozoa motility in a time- and dose-dependent manner. The mean percentages of abnormal spermatozoa were not affected by melatonin treatment. However, it caused an increased hyaluronidase activity of bull semen. In conclusion, the existence of melatonin in bull semen has a significant effect on fertilization ability by affecting spermatozoa motility and hyaluronidase activity.

Key Words: Bull spermatozoa, semen, melatonin, hyaluronidase

Melatoninin Boğa Semeninde Sperm Motilitesi ve Hiyaluronidaz Aktivitesi Üzerine In Vitro Etkileri

Özet: Bu çalışmada, melatoninin Holştayn boğalara ait semen hyaluronidaz aktivitesi ve sperm motilitesi üzerine olan in vitro etkileri araştırıldı. Semen numuneleri 0,5, 1, 2 ve 4 mM düzeylerindeki melatoninle 60 dk. boyunca inkube edildi ve 5, 10, 15, 20, 30 ile 60. dakikalardaki sperm motilitesi, morfolojik anormallik ve semen hyaluronidaz aktiviteleri belirlendi. Elde edilen sonuçlar; melatoninin semen hyaluronidaz aktivitesinde önemli ($P < 0,001$) bir artışa sebep olurken sperm motilitesinde ise önemli ($P < 0,001$) bir azalmaya neden olduğunu göstermektedir. Bununla birlikte anormal sperm yüzdelerinde kontrol gruplarına göre önemli bir değişikliğin olmadığı belirlendi. Yapılan bu çalışmada boğa semeninde bulunan 0,5, 1, 2 ve 4 mM dozlarındaki melatoninin varlığının hyaluronidaz aktivitesini arttırırken sperm motilitelerinde ise zamana ve doza bağlı olarak azalmalara sebep olduğunu göstermektedir. Sonuç olarak; boğa semeninde bulunan melatonin, sperm motilitesi ve hyaluronidaz aktivitesi düzeylerini etkilediğinden fertilize yeteneği üzerine önemli bir etkiye sahiptir.

Anahtar Sözcükler: Boğa spermatozoası, semen, melatonin, hyaluronidaz

Introduction

Melatonin, a pineal hormone, regulates the dynamic physiological adaptations that occur in seasonally breeding mammals in response to changes in day length (1). However, the exact sites of melatonin action in the reproductive system are not clear (2). It has been reported that sites of melatonin action on the reproductive system can be hypothalamus, pituitary, gonads, reproductive tract and male-accessory genital organs. Since melatonin is a lipid soluble molecule, it has no barrier and reaches every part of the body (3). Additionally, melatonin regulates the pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus (4).

Hyaluronic acid is a component of the cumulus oophorus matrix that holds the follicular cells together and is degraded by sperm hyaluronidase located on the surface of the sperm membrane and on the inner acrosomal membrane (5,6). This enzyme has an important role in the passage of spermatozoa through the zona pellicuda (7). Luboshitzky et al. (8) suggested that the melatonin level of seminal plasma in humans was

between 0.1 and 0.9 ng/ml. It was reported that semen hyaluronidase activity is an index of fertilizing ability and its deficiency causes a decrease in fertilizing ability of sperm in in vitro fertilization (9). It was reported that melatonin had negative effects on sperm forward progression and the quality of sperm motility in rats (10). However, there are no published data about the effects of melatonin on hyaluronidase activity. The effects of in vitro melatonin on hyaluronidase activity of semen, sperm motility and morphological abnormality in bull sperm were investigated in this study.

Materials and Methods

Chemicals

Hyaluronic acid was purchased from Merck Co. Melatonin (M-5250, mol. wt. 232.3) and the other chemicals were purchased from Sigma Co. (St. Louis, MO, USA).

Animals and Semen Collection

Semen was obtained from 6 fertile Holstein bulls in August, aged between 2 and 3 years, housed at the Research Barn of Veterinary Medicine at the University of Fırat, Elazığ. The bulls were maintained on a diet of mixed grass and alfalfa hay, with fresh water ad libitum. Semen was collected using an artificial vagina. All samples were divided into 5 equal parts and 1 of them was used as a control sample. Semen samples were obtained from all bulls prior to initiation of treatment and hyaluronidase activity and sperm characteristics were determined.

Melatonin incubations

Melatonin was dissolved in a small volume (0.04 ml) of absolute ethanol and diluted with isotonic sodium citrate solution (3% in distilled water) up to concentrations of 0.5, 1, 2 and 4 mM. Control sperm contained only diluted solution of ethanol. Both control and melatonin solutions were mixed with bull sperm at the rate of 1:1 and incubated at 37 ºC in an incubator and then spermatozoa motilities at 5, 10, 15, 20, 30 and 60 min. Hyaluronidase activities of semen were determined at 30 min.

Sperm Motility Assay

Sperm samples were diluted with isotonic sodium citrate solution (3%, dissolved in distilled water) at the rate of 1 to 10. A slide was placed on the phase contrast microscope stage and allowed to warm to 37 ºC and then a small droplet of diluted semen was placed on the slide and percent motility was evaluated visually at a magnification of x400 (11). Motility estimations were performed from 5 different fields in each sample. The mean value averaged from 5 successive estimations was used as the final motility score. The percentage of morphologically abnormal sperm was determined from slides prepared with Indian ink. A total of 300 sperm cells were counted on each slide under a phase-contrast microscope at x400 magnification (11).

Hyaluronidase Assay

Hyaluronidase activity of semen samples was measured using the methods described by Tanyıldızı and Bozkurt (5) and Joyce et al. (12). The semen samples of the control and test groups were diluted 1 in 5 with 0.15 mol/l sodium chloride before assay. One milliliter of diluted samples were added to 0.1 ml of acetate buffer (0.3 mol/l, containing 0.45 mol/l sodium chloride) and 0.1 ml of hyaluronic acid substrate (4 mg hyaluronic acid was dissolved in 1 l of water) was added to this mixtures and then incubated for 24 h at 37 °C in a thermostatically controlled room. After the reaction mixtures were taken, 60 µl of potassium tetraborate (0.8 mol/l in water, pH 10) was added and the reaction was terminated by heating block for 5 min. Then the mixtures were cooled in an ice-water bath before adding 2 ml of p-dimethylaminobenzaldhyde (Stock DMAB reagent-10% w/v in 12.5% v/v concentrated hydrochloric acid in glacial acetic acid: Stock reagent diluted 1 in 10 with glacial acetic acid before use) and then incubated for 20 min at 37 °C in a water bath. The reaction mixtures were centrifuged immediately at 1500 x g for 10 min and the absorbance of the supernatant read at 582 nm using a spectrophotometer within 30 min. N-acetylglucosamine was used as a standard and was reacted with p-dimethylaminobenzaldehyde as describe above. Hyaluronidase activity was expressed as mean µmol NAG/min/l.

Statistical Analyses

Results are expressed as mean ± SEM. Chi-square analysis was used to determine differences in the sperm motilities and morphological abnormality between the control and treatment groups. The non-parametric Mann-Whitney U-test was applied to determine statistically significant differences between the control and treatment

groups. Spearman rank correlation test was used to establish the relationship between the hyaluronidase activity of semen and sperm motility. All analyses were carried out using SPSS 6.0.

Results

Sperm characteristics were determined prior to the initiation of treatment. The bull ejaculates had an average (mean ± SEM) sperm concentration of 1.5 ± 0.1 x 10^9 spermatozoa/ml; the percentage of abnormal spermatozoa 5.82 ± 0.15%, volume of 6.7 ± 0.3 ml, and the percentage of spermatozoa motility 75 ± 1.84% (n = 6). The mean (± SEM) values of sperm pH is 6.8 ± 0.3.

The treatment of semen samples with melatonin at all doses caused a significant ($P < 0.001$) increase in the hyaluronidase activity of the whole semen compared to control samples in a dose-dependent manner (Figure 1). After the treatment of semen samples with melatonin at the concentrations of 0.5, 1, 2 and 4 mM, the spermatozoa motilities were decreased significantly ($P < 0.001$) in comparison with the control samples at all time intervals except the 5 min and then all spermatozoa to be immobile 60, 30, 20 and 15 min respectively (Figure 2). No significant correlation was found between hyaluronidase activity and sperm motility. The mean (± SEM) percentages of abnormal spermatozoa were not affected by melatonin treatment. These values were between 5.65% and 6.03%.

Discussion

Melatonin has negative effects on the mammalian reproduction physiology. It was reported that the anterior pituitary of neonatal rats has a high concentration of melatonin receptors (13). Melatonin receptors affect via these receptors to reduce gonadotropin releasing hormone (GnRH)-induced release of luteinizing hormone (LH) (14). Melatonin treatment induced regression of the prostate and atrophy of secretory cell organelles in the accessory sex organs (15). The inhibitory effect of melatonin on the prostate may be mediated through other endocrine system. Melatonin alters morphology, steriodogenesis or cyclicGMP production of testicular tissues and leydig cells (16). Although the inhibitory effects of melatonin on reproductive physiology are known, its role on sperm functions is unknown. Melatonin may affect every subcellular compartment in every cell since it is both lipophilic and hydrophilic. In this study, the decrease in sperm motility caused by melatonin may be explained by

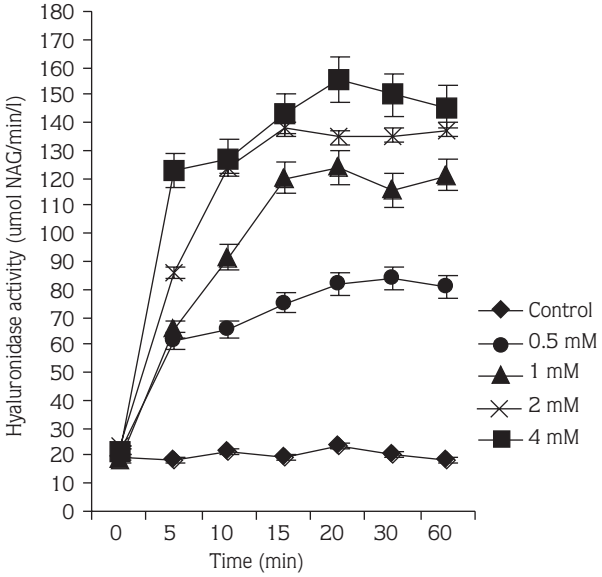

Figure 1. Hyaluronidase activity (Mean ± SEM) of semen samples, after incubation of melatonin. Significant ($P < 0.001$) increases were observed in the hyaluronidase activities when compared with the control group (n = 6).

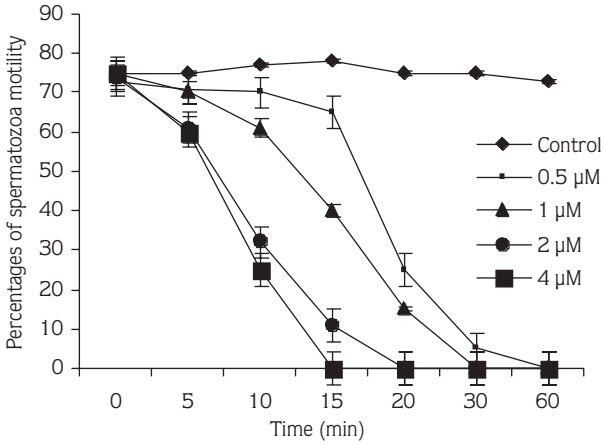

Figure 2. Percentages (Mean ± SEM) of motile spermatozoa of bulls incubated with melatonin. There were significant ($P < 0.001$) decreases between the control and treatment samples (n = 6).

the existence of melatonin receptors on spermatozoa. However, further studies are required to clarify the binding sites of melatonin on sperm cells.

Cyclic AMP (cAMP) has been proposed as the intracellular messenger in mammalian sperm motility (17). It was reported that melatonin inhibits cAMP accumulation in the rat epididymal cells (18) and pituitary cells (19). Additionally, Slanar et al. (20) suggested that melatonin inhibits the Ca^{+2} influx by decreasing cAMP concentration. The results of this study indicate that the incubation of melatonin reduced sperm motility significantly ($P < 0.001$). The inhibition of spermatozoa motility by melatonin may be explained by the prevention of Ca^{+2} influx depending on the inhibition of cAMP in sperm cells. This conclusion was supported by Bains et al. (21), who suggested that the elevation of intracellular Ca^{+2} concentration causes an increase in sperm motility. Furthermore, Bornman et al. (22) reported that melatonin has similar effects as colchicine on microtubules. Colchicine may affect in vitro sperm motility, probably by its direct effect on the microtubules rather than by causing spermatozoal death (23). In this study, the decrease in sperm motility by melatonin may be also explained by the impairment of microtubules by melatonin. Further studies are required to elaborate the mode of action for the effects of melatonin on microtubules.

Hyaluronidase activity, sperm motility and morphology are important indicators of sperm quality and these parameters may provide useful information in the determination of the fertilization potential of animals (5). It is well known that hyaluronidase enzyme is one of the acrosomal enzymes and plays an important role in gamete interaction and fertility in mammals (24). However, there are no data about the effect of melatonin on hyaluronidase activity. The findings of this study show that in vitro melatonin causes significant ($P < 0.001$) increases in the semen hyaluronidase activity of bulls in a dose-dependent manner. The elevation in semen hyaluronidase activity may indicate that melatonin causes subtle sperm damage resulting in enzyme leakage, without causing gross abnormalities.

In conclusion, the present study indicated that although in vitro melatonin causes an increase in the hyaluronidase activity of semen, it decreases spermatozoa motility in bull semen. The increase in hyaluronidase activity was independent of the decrease in spermatozoa motility due to the absence of a relationship between these parameters. This conclusion was supported by Tanyıldızı and Bozkurt (5), who suggested that there was no relationship between hyaluronidase activity and sperm motility. These results revealed that the presence of melatonin in bull semen may play an important role in fertility by affecting spermatozoa motility and hyaluronidase activity.

References

1. Chemineau, P., Malpaux, B: Melatonin and reproduction in domestic animals. Therapie, 1998; 53: 445-452.
2. Pang, S.F., Li, L., Ayre, E.A., Pang, C.S., Lee, P.P., Xu, R.K., Chow, P.H., Zu, Z.H., Shiu, S.Y.W.: Neuroendocrinology of melatonin in reproduction: recent developments. J. Chem. Neuroanat. 1998; 14: 157-166.
3. Reiter, R.J.: The melatonin rhythm: Both a clock and a calendar. Experientia, 1993; 49: 654-664.
4. Malpaux, B., Thiery, J.C., Chemineau, P.: Melatonin and the seasonal control of reproduction. Reprod. Nutr. Dev., 1999; 39: 355-366.
5. Tanyıldızı, S., Bozkurt, T.: Effects of acetylsalicylic acid and metamizol on hyaluronidase activity and sperm characteristics in rams. Anim. Reprod. Sci., 2003; 76: 195-204.
6. Li, M.W., Yudin, A.I., Robertson, K.R., Cherr, G.N., Overstreet, J.W.: Importance of glycosylation and disulfide bonds in hyaluronidase activity of macaque sperm surface PH-20. J. Androl., 2002; 23: 211-219.
7. Meyers, S.A.: Equine sperm-oocyte interaction: the role of sperm surface hyaluronidase. Anim. Reprod. Sci., 2001; 68: 291-303.
8. Luboshitzky, R., Shen-Orr, Z., Nave, R., Lavi, S., Lavie, P.: Melatonin administration alters semen quality in healthy men. J. Androl., 2002; 23: 572-578.
9. Abdul-Aziz, M., Meriano, J., Casper, R.F.: Intracytoplasmic sperm injection for treatment of infertility due to acrosomal enzyme deficiency. Fertil. Steril., 1996; 65: 977-980.
10. Gwayi, N., Bernard, R.T.: The effects of melatonin on sperm motility in vitro in Wistar rats. Andrologia, 2002; 34: 391-396.
11. Bearden, H.J., Fuquay, J.W.: Semen collection. In: Bearden, H.J. Ed. Applied animal reproduction. Prentice Hall. International, London, 1992; p. 72-73.
12. Joyce, C.L., Mack, S.R., Anderson, R.A., Zaneveld, L.J.: Effects of hyaluronidase, beta–glucuronidase and beta-N-acetylglucosaminidase inhibitors on sperm penetration of the mouse oocyte. Biology of Reproduction. 1986; 35: 336-346.

13. Vanecek, J.: The melatonin receptors in rat ontogenesis. Neuroendocrinology, 1988; 48: 201–203.

14. Martin, J.E., Sattler, C.: Selectivity of melatonin pituitary inhibition for luteinizing hormone-releasing hormone. Neuroendocrinology, 1982; 34: 112-116.

15. Chow, P.H., Pang, S.F.: The male accessory sex glands, fertility and melatonin. Adv. Pineal Res., 1989; 3: 221-224.

16. Valenti, S., Giusti, M., Guido, R., Giordano, G.: Melatonin receptors are present in adult rat Leydig cells and are coupled through a pertussis toxin-sensitive G-protein. Eur. J. Endocrinol., 1997; 136: 633-639.

17. Garbers, D.L., Kopf, G.S.: The regulation of spermatozoa by calcium and cyclic nucleotides. Adv. Cyclic Nucleotide Res., 1980; 13: 251-306.

18. Li, L, Xu, J.N., Pang, S.F, Shiu, S.Y.W.: Melatonin modulates cAMP signaling in rat epididymal epithelial cells. FASEB J., 1998; 12: A686

19. Vanecek, J., Vollrath, L.: Melatonin inhibits cyclic AMP and cyclic GMP accumulation in the rat pituitary. Brain Res., 1989; 505: 157–159.

20. Slanar, O., Pelisek, V., Vanecek, J.: Melatonin inhibits pituitary adenylyl cyclase-activating polypeptide-induced increase of cyclic AMP accumulation and [Ca^{2+}] in cultured cells of neonatal rat pituitary. Neurochem. Int., 2000; 36: 213-219.

21. Bains, R., Miles, D.M., Carson, R.J., Adeghe, J.: Hyaluronic acid increases motility/intracellular CA^{2+} concentration in human sperm in vitro. Arch. Androl., 2001; 47: 119-125.

22. Bornman, M.S., Oosthuizen, J.M., Barnard, H.C., Schulenburg, G.W., Boomker, D., Reif, S.: Melatonin and sperm motility. Andrologia, 1989; 21: 483-485.

23. Ben-Chetrit, A., Ben-Chetrit, E., Nitzan, R., Ron, M.: Colchicine inhibits spermatozoal motility in vitro. Int. J. Fertil Menopausal Stud., 1993; 38: 301-314.

24. Tanyıldızı, S., Bozkurt, T.: The investigation of in vitro fluoride effects on bovine sperm. Turk. J. Vet. Anim. Sci., 2002; 26: 325-328.