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CHANGES IN THE LEVEL OF MELATONIN IN EGYPTIAN BUFFALOES DURING ESTROUS CYCLE AND TREATED INACTIVE OVARIES

(With 5 Tables and 2 Figures)

By

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التغيرات فى مستوى الميلاتونين فى الجاموس المصرى خلال فترة الشبق وبعد علاج خمول المبايض

ممدوح أنور ، جابر مجاهد

تم فى هذه الدراسة إيجاد العلاقة بين التغيرات فى مستوى هرمون الميلاتونين والهرمونات الأنثوية وكذلك هرمونات الغدة الدرقية أثناء دورة الشبق بالاضافة الى مدى تأثير الفيتامينات والفوسفات على إعادة حيوية ونشاط المبايض الخاملة وتأثير هذا العلاج على مستويات الهرمونات السالفة الذكر. وأثبتت الدراسة أن هناك علاقة بين هذه الهرمونات والفترات المختلفة لدورة الشبق فى الجاموس المصرى . ووجد أن هناك علاقة إرتباط إيجابية ومعنوية بين الميلاتونين والبروجستيرون بينما العلاقة بين الميلاتونين والاستروجين كانت ارتباطيه سالبة ومعنوية بالإضافة الى علاقة ارتباطيه سالبة ولكنها غير معنوية مع هرمونات الغدة الدرقية. وفى حالة علاج خمول المبايض وجد أن نسبة الحيوانات التى أظهرت علامات الشياع هى ٧٣,٣٣٪ وكانت نسبة الإخصاب بعد التلقيح لهذه الحيوانات هى ٧٧,٢٧٪ ، أما فى حالة التغيرات فى مستويات الهرمونات بعد العلاج فكان هناك انخفاض معنوى لهرمون الميلاتونين وكذلك ارتفاع معنوى لهرمونات الغدة الدرقية.

SUMMARY

The changes in melatonin concentration in relation to sexual steroid and thyroid hormones in cyclic Egyptian buffaloes were studied. In addition, the beneficial effect of vitamins (AD_3E) plus tonophosphan on the subsequent reproductive performance of buffaloes with inactive ovaries as well as the effect upon melatonin in relation to sexual steriod and thyroid hormones were investigated. The present study revealed that, there was a relationship between levels of melatonin and thyroid hormones with sexual steroid hormones during estrous phases. The melatonin was significantly positive correlation with progesterone and significantly negative correlation with estradiol. Moreover, melatonin has a non-significantly negative correlation with thyroid hormones. The percentage of Egyptian buffaloes that exhibited heat after treatment was 73.33% and conception rate was 77.27%. The melatonin was significantly decreased after treatment as well as thyroid hormones arise. In conclusion, there is a role for melatonin in the regulation of estrous cycle. Ovarian inactivity in Egyptian buffaloes seems to be due to poor nutrition which considered a primary factor limiting net reproductive rate, as well as vitamins (AD_3E) plus phosphorus (tonophosphan) appears to be the most appropriate treatment.

Key words: Egyptian Buffaloes - Estrus - Melatonin level

INTRODUCTION

In Egypt, buffaloes play an important role in farmer's economy as well as used as an important source of milk, meat and draught power by the individual farmer breeder. There are many problems which are common in buffalo production (Ranjhan and Pathak, 1993). The physiological view in buffaloes reproduction is less known inspite of its highly economic importance.

In domestic animals, anestrus has an economic importance because it prolongs the intercalving period resulting in great economic losses (Boyd, 1977). The incidence of anestrus was higher in buffaloes than in cattle (Purbey and Agarwal, 1982). The dramatically

greater incidence of anestrus, in the field occurs due to dysfunction of ovaries (Megahed, et al.,1995 and Singh and Sahni, 1995).

Melatonin is one of the most important indole secreted from pineal gland. It has an important role in reproduction in both male and female. Injection of melatonin produced infertility in both male and female (Reiter, 1984 and Anwar, 1987). The role of melatonin in ovarian inactivity was investigated in rodents. Alonso et al. (1993) reported that both pineal (NAT) enzyme activity and melatonin levels were reduced during the first hours of proestrus night as compared with day-time proestrus levels. Moreover, Joshi et al. (1994) reported that melatonin control estrous cycle in Indian desert gerbil and increase of melatonin level would be interpreted in the estrous cycle. Increased level of melatonin was accompaned with anestrus, this fact was postulated by El-Miligy (1990) who reported that daily injection of melatonin in female mice produced an inactive ovaries.

Borderline nutrient deficiencies may be manifested as impaired fertility before other clinical symptoms are apparent (Hurley and Doane, 1989). One of this nutrient is vitamins, particularly vitamin A (Ferrell, 1991) as well as phosphorus (Singh and Sahni, 1995). Vitamin A has important roles in ovarian follicular development and normal reproductive function (Ferrell, 1991). Its deficiency can reduce reproductive efficiency and result in deleterious effects on pituitary and ovarian function which lead to anestrus.

The present study focuses on two objects, the first on the levels of melatonin in relations with sexual steriod hormones as well as thyroid hormones in different stages of estrous cycle. The second object, is the effect of treatment with vitamins (AD_3E) and phosphorus on the subsequent reproductive performance of buffaloes suffered from inactive ovaries. Moreover, all hormones mentioned above were determined before and after treatment.

MATERIALS and METHODS

The study was undertaken in Assiut Governorate, Egypt and conducted on Egyptian buffalo-cows. There were two phases to the study. The first phase was designed to determine the changes in melatonin, progesterone, estradiol, triiodothyronine (T_3) and tetraiodothyroxin (T_4) in serum of slaughtered animals during estrous

cycle. The second phase of this study was carried out in field on buffalo-cows suffering from inactive ovaries. The hormones mentioned above were also estimated before and after treatment with vitamins AD_3E (HURON,Germany) and tonophosphan (Hoechst, Germany). All animals in this study were approximately 3-6 years old as determined by their dentition (Kartha, 1975).

Animals and Sampling:

Sixty nocturnal blood samples were obtained from slaughtered animals through jugular veinpuncture in clean sterile centrifuge tubes without anticoagulant. Serum were separated after centrifuged the blood samples at 3000 rpm for 20 minutes, then stored at -20°C until hormonal assays. Immediately after slaughter and evisceration, the female reproductive tracts were excised from each animals and kept in cool box then transferred to the laboratory. The genitalia were examined especially the status of the ovaries where determine the stage of the estrous cycle according to Eissa (1996).

Thirty buffalo-cows suffering from anestrus were studied. These animals did not show any signs of heat since 6-8 months. Rectal examination of these animals revealed smooth ovaries without any palpated structures in their surface. These animals received 5 ml AD_3E and 20 ml tonophosphan intramuscular twice weekly for two weeks. The nocturnal blood samples were collected each week after treatment. The animals were closely observed for heat signs and were inseminated naturally 12-16 hours after heat detection (Ranjhan and Pathak, 1993). Pregnancy status was confirmed by rectal palpation 75-90 days of post-insemination.

Hormonal assay:

Melatonin levels were assayed by using ELISA kit and automatic reader (Anthos, 2000). Before using of the serum, extraction of samples was carried as the following:

a) Column preparation and conditioning:

- One extraction column for each sample and placed in polypropylene tube.
- Two ml of methanol was added into each column, then centrifuged for one minute at 200 x g.
- Two ml of water was added to each column, then centrifuged for one minute at 200 x g.

b) Sample application:

- Added 1 ml of sample to the corresponding column then centrifuged for one minute at 200 x g.

c) Washing:

- 1 ml of 10% methanol in water (v/v) was added to each column and centrifuged for one minute at 500 x g.
- 1 ml of hexane was added to column and centrifuged for one minute at 500 x g.

d) Elution of extract:

- Columns were placed into clean marked borosilicate glass tubes.
- 1 ml of methanol was added to all columns and centrifuged for one minute at 200 x g.
- Stored columns at 30°C.

e) Evaporation and Reconstitution of extract:

- Evaporate the methanol to dryness using a vacuum concentrator.
- Reconstitute the samples with 0.125 ml to 1 ml zero standard, then vortex.
- The extracts were equilibrated at least 30 minutes at 30°C then, stored at -20°C until assay.

  Progesterone (P_4), estradiol -17ß (E_2), triiodothyronine (T_3) and tetraiodothyroxin (T_4) were determined by using DELFIA kits and folurometer LBK.

Statistical analyses:

Data were expressed as the mean ± S.D for all phases of estrous cycle, then analysed by using Analysis of Variance (ANOVA) and means ± S.D were tested at least significant difference (LSD). Multiple correlation analysis was tested between parameter. The inactive ovaries group and after treatment (one and two weeks), (mean ± S.D) were analysed according to student's (t) test. All testes were done by using PC-Stat computer programme. Results were considered significant at $P< 0.05$ or less.

RESULTS

Hormonal levels in estrous cycle stages:

Table (1) and Fig. (1) shows that melatonin and progesterone levels were significantly ($P< 0.01$) increased in diestrus phase when compared with estrus and metestrus phases, while estradiol was highly significant ($P< 0.01$) decreased in diestrus phase from the levels in

proestrus and estrus phases. On the other hand, T_3 levels were significant ($P< 0.01$) increased in estrus phase only in comparison with all phases and T_4 levels were significant ($P< 0.01$) increased in proestrus and estrus phases when compared with other phases.

Table (2) shows that melatonin was significant ($P< 0.05$) negative and positive correlation with estradiol and progesterone respectively. A positive correlation was observed between estradiol and progesterone, T_3 and T_4, but it was significant ($P< 0.01$) only with T_4.

Hormonal changes in inactive ovaries and post treated:

Table (3) and Fig. (2) shows the changes in hormonal levels before and after one and two weeks of treatment. After treatment serum levels of melatonin were decreased significantly ($P< 0.05$ after one week and $P< 0.01$ after two weeks). The serum levels of estradiol after treatment were increased significantly ($P< 0.01$) while no significant change in progesterone serum levels. After two weeks of treatment, serum levels of melatonin and estradiol when compared with pretreated animals. No significant difference was observed at serum levels of T_3 and T_4 after one week of treatment, while they increased significantly ($P< 0.05$ and $P< 0.01$) after two weeks of treatment.

Table (4) presented the percentage of animals which response to treatment and exhibited estrous cycle. This table shows that, 30 anestrus buffaloes with inactive ovaries were treated with AD_3E plus tonophosphan, of 22 (73.33%) cases showed the signs of estrus. Of these 22 estrus buffaloes, 17 (77.27%) buffaloes conceived after insemination.

Table (5) revealed the fertile estrus and pregnancy after treatment. All animals were pregnant after the first service except animals No. (5, 7 and 16) become pregnant after the second service.

DISCUSSION

The results obtained on the circulating profile of the various estimated hormones in Egyptian buffaloes have revealed relationship between levels of these hormones and different stages of estrus cycle. For some time, it has been suspected that gonadal steroids may have an inhibitory feedback effect on pineal melatonin production (Cardinali, 1981). In views of this, it was surmised that perhaps

circulating melatonin levels would be differentially modified during the course of the estrous cycle.

It was observed in this study that melatonin level was reduced significantly at the estrus phase where ovulation was expected. This result was coincided with the elevation of estradiol level at estrus phase. Moreover, a significant negative correlation between estradiol level and melatonin level was observed. Progesterone reduced significantly at estrus phase and increased significantly at diestrus, thereby a positive correlation was observed between progesterone and melatonin. The thyroid hormones, triiodothyroning (T_3) and tetraiodothyroxin (T_4) were correlated negatively with the levels of melatonin during the stages of estrus cycle. These results were coincided with Hariharasubramanian et al. (1986) who reported that at the middle of menstrual cycle, the nocturnal levels of melatonin reduced significantly. It was well known that, melatonin reduced the levels of gonadotropins (Reiter, 1986). Miyauchi et al. (1992) reported that there were a negative correlations between melatonin and FSH and LH, where melatonin increased while LH and FSH were reduced. The author reported also, that melatonin hormone could be regulated menstrual cyclicity. Furthermore, estrous cycle reflects the expression of a cyclical process involving the interaction between the hypothalamic-pituitary axis and the ovaries. This complex process requires an integrated neural and humoral control mechanism. It is now, well established that a hypothalamic "transducer" located in the medial basal hypothalamus integrates neural and humoral information and translates it into an oscillatory signal which eventually results in the release of gonadotropin releasing hormone (Gn RH), triggering the secretion of gonadotropins. Sandy (1992) reported that melatonin influences the functions of the hypothalamic pituitary-gonadal axis by modifying the firing frequency of the hypothalamic GnRH pulse generator. Consequently, the pineal gland, through the action of melatonin may exert an important modulatory effect on the mechanisms controlling estrous cyclicity.

The relations between thyroid hormones and the phases of estrous cycle were studies. The concentration of T_3 revealed a significantly increased at estrus phase, while T_4 increased significantly at both proestrus and estrus phases. These results were in agreement with Kumar et al. (1991), Baruah et al. (1994), Megahed et al. (1995) and

Chede et al. (1996), they reported that normal estrous cycle was correlated with the normal status of the thyroid gland. The activity of thyroid gland during estrous cycle was controlled by two hormones, estradiol and melatonin. The high level of estradiol at proestrus and estrus phases stimulated the activity of thyroid gland. Kumar et al. (1991) reported that estradiol stimulated the activity of thyroid gland directly rather than its action on pituitary gland while Shi and Barrell (1994) found that estrogen increased the sensitivity of TRH-receptors in pituitary gland which elevated levels of TSH in blood and inturn stimulate thyroid gland. Bugalia et al. (1995) reported that serum levels of T_3 and T_4 were elevated in cycling cows, especially at proestrus and estrus phases, and these were suggestive of normal thyroid activity regulating functional relationship of hypothalamic - pituitary - gonadal axis for synthesis and release of cyclic gonadotropins and steroids. Furthermore, melatonin hormone play a role in controlling thyroid gland. The negative correlation between melatonin and T_3 and T_4 in this study revealed the relation between melatonin and thyroid activity during stages of estrous cycle. Vriend et al. (1982) reported that melatonin administration inhibited both TRH and TSH which reflect on the thyroid activity.

Treatment of ovarian dysfunction with vitamins and phosphorous led to significant decrease in serum levels of melatonin particularly after two weeks of treatment, and significant increase in serum levels of estradiol, T_3 and T_4. These results indicated that the treatment correct the hypogonadal condition and stimulate follicular growth in ovaries. These finding were somewhat in agreement with that reported by Zaabel et al. (1997) who mentioned that there were highly significant increase in the levels of serum estradiol, T_3, T_4 and progesterone after all medical regimes which used in dysfunction or smooth inactive ovaries.

Elevation of estradiol in blood indicated that animals become periodically and these finding interpreted that vitamins has very important roles in ovarian follicular development. Nolan et al. (1988) reported that delayed return to estrus or anestrus in animals with malnutrient might be due to reduced gonadotropin release and decreased responsiveness of pituitary gland to GnRH. Furthermore, Hurley and Doane (1989), Ferrell (1991), Mariana et al. (1991) and Adams and Martin (1992) observed that nutritional status can be

considered an impact on the reproduction through the endocrine system particularly FSH which stimulate follicular growth on ovaries. The correction of gonadotropins may be through reduction of melatonin level. After treatment, especially after two weeks, melatonin level was reduced with increased levels of estradiol, T_3 and T_4. These results were in agreement with Alonso et al. (1993) and Ashturkar et al. (1995) who reported that increased serum level of estradiol reduced melatonin level, and abnormal melatonin levels may be involved in the pathogenesis of several disorders of the menstrual cycle inducing some forms of hypothalamic amenorrhea (ovarian dysfunction), such as malnutrition induced amenorrhea. Moreover, the treatment would be corrected the thyroid gland activity, and the elevation of serum levels of T_3 and T_4 may be resulted from treatment or/and it may be through effects of estradiol on thyroid gland as reported by Shi and Barrell (1994). Bugalia et al. (1995) found a correlation between thyroid gland activity and the anestrus in cows. They reported that anestrus cows were suffered from hypofunction in thyroid gland. Furthermore, reduction of melatonin levels as result of increased estradiol level may be led to increase T_3 and T_4 which inturn play a role in ovarian activation.

The use of vitamins (AD_3E) and tonophosphan increased the percentage of buffaloes that either came into estrus or conceived following treatment. These finding was in agreement with Osman (1984), Singal and Arora (1992) and Samad et al. (1994) who reported that, the injection of tonophosphan resulted the highest incidence of heat appearance and conception rate.

In conclusion, it may be indicated that melatonin kept the cyclicity of buffaloes and the nutritional deficiency could be an important factor for inactive ovaries, and the correction by vitamins (AD_3E) plus tonophosphan could be used in treatment of infertile buffaloes. These results may be due to increase gonadotropins which inturn stimulate follicular growth. The elevated estradiol from grown follicles consequently reduced melatonin, which reflected on stimulate gonadotropin release. Moreover, the thyroid gland activity was stimulated by reduction of melatonin levels and increased estradiol levels, this would be stimulated ovarian activity.

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Table (1): Serum levels (means±S.D) of melatonin, estradiol (E_2), progesterone (P_4), T_3 and T_4 during estrous cycle in Egyptian buffaloes.

	Melatonin Pg/ml	E_2 Pg/ml	P_4 ng/ml	T_3 ng/ml	T_4 ng/ml
Diestrus	11.67±1.65^{a}	12.03±1.37^{a}	1.96±0.69^{a}	0.93±0.35^{a}	26.8±12.65^{a}
Proestrus	10.03±1.84^{ab}	25.03±2.52^{b}	0.58±0.15^{b}	1.05±0.48^{a}	41.75±15.12^{b}
Estrus	9.11±1.36^{b}	31.84±2.23^{c}	0.19±0.01^{c}	1.57±0.49^{b}	45.86±17.62^{b}
Metestrus	9.86±1.54^{b}	15.69±1.79^{b}	0.69±0.13^{b}	0.98±0.28^{a}	28.29±13.15^{a}
L.S.D at 5%	1.1976	1.511	0.267	0.304	11.01
L.S.D at 1%	1.6129	2.034	0.359	0.409	14.83

- Means with the same letter in the same column are not significantly different.
- Significant levels are calculated according to the value of L.S.D.

Table (2): Correlation coefficients (r) among hormones during estrous cycle in Egyptian buffaloes.

	Melatonin	Estradiol	Progesterone	T_3	T_4
Melatonin	1	- 0.84*	+ 0.97*	- 0.80	- 0.75
Estradiol		1	+ 0.84	+ 0.88	+ 0.98**
Progesterone			1	- 0.68	- 0.77
T_3				1	+ 0.81
T_4					1

* : Significant at the level of 0.05
** : Significant at the level of 0.01

Table (3): Serum levels (means±S.D) of melatonin, estradiol, progesterone, T_3 and T_4 in inactive ovaries and post-treated Egyptian buffaloes.

	Pre-treatment (n = 30)	Post-treatment (n = 30)	
		One week	Two weeks
Melatonin (pg/ml)	19.88 ± 3.02	17.81 ± 3.52*	9.92 ± 2.21**
Estradiol (pg/ml)	6.59 ± 1.61	9.96 ± 2.78**	24.04 ± 8.9**
Progesterone (ng/ml)	0.08 ± 0.01	0.09 ± 0.05 N.S	0.09 ± 0.06 N.S
T_3 (ng/ml)	0.77 ± 0.17	0.93 ± 0.21 N.S	1.06 ± 0.35*
T_4 (ng/ml)	18.08 ± 0.04	22.83 ± 3.92 N.S	38.65 ± 5.83**

* : Significant at the level of 0.05
** : Significant at the level of 0.01
N.S: Non-significant

Table (4): Effect of treatment on the inactive ovaries in Egyptian buffaloes.

Items	n.	%
Number of animals	30	
Response after treat.	22	73.33
Non-response	08	26.67
Animal conceived	17*	77.27

* : Five animals were return to estrus after three services.

Table (5): Fertility after treatment.

No. of conceived animals	Fertile heat*	Pregnancy
1	+	+
2	+	+
3	+	+
4	+	+
5	+ **	+
6	+	+
7	+ **	+
8	+	+
9	+	+
10	+	+
11	+	+
12	+	+
13	+	+
14	+	+
15	+	+
16	+ **	+
17	+	+

* : Non-return to estrus after first service.
** : At next estrus after second service.

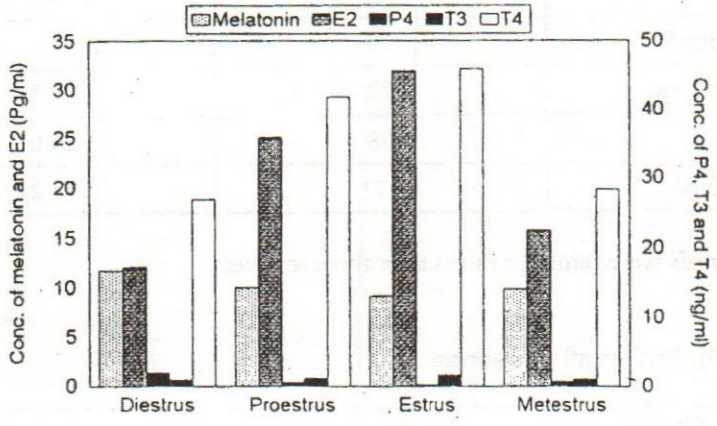

Fig (1): serum levels of melatonin, estradiol (E2), progesterone (P4), T3 and T4 in all phases of estrous cycle.

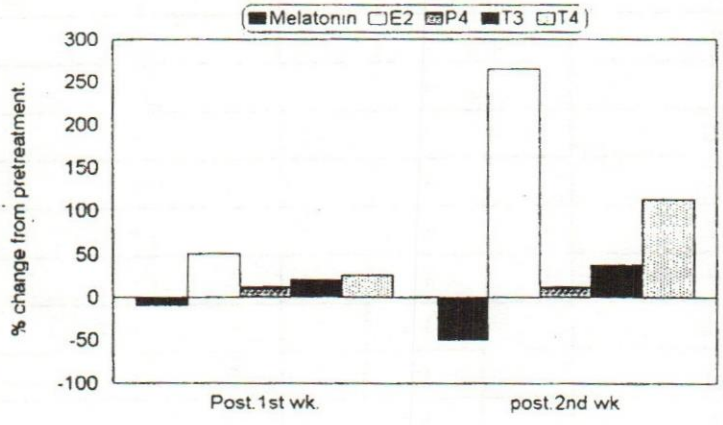

Fig (2): Percent change of melatonin, estradiol (E2), progesterone (P4), T3 and T4 after one and two weeks of treatment from pretreated group.