

# **Influence of Vitamin B2, Ascorbic Acid and Melatonin on Ovarian Activities, Reproductive Performance of Ewes during the Summer Season under Subtropical Conditions**

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## **Research Article**

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## **Abstract**

The present study was conducted to investigate the effect of different treatments (vitamin B2, ascorbic acid and melatonin) on ewes' fertility rates during summer under the local environmental conditions. The study was carried out on 42 mature cross ewes (3/8 Finnish X 5/8 Rahamani). Ewes were allocated into 4 groups: the first group (G1; n=11) injected with 2mL saline for 3 wk., served as control. The second (G2; n=10) and third group (G3; n=11) were injected intramuscular daily for 3 wk with 2 mL (15mg) of vitamin B2 and ascorbic acid, respectively. Ewes of the fourth group (G4; n=10) received twice daily subcutaneous injections of 0.5 mg melatonin at 8AM and 4PM at the dorsal surface of the ear for 3 wk. All treated groups (G2, G3, and G4) received 1g zinc chloride and 2.5mg chromium (Merck Millipore, USA) in form of 6 ml oral drench once per week for 3 weeks. All animals were checked for the onset of estrus 2 times daily using an intact ram and ewes were bred via natural mating using 3 rams. Laparoscopic examinations were carried out throughout the study for detection of ovulation rates. Results revealed that ascorbic acid resulted in a significantly ( $P < 0.05$ ) decreased mean interval (days) from treatment to estrus as compared with the control group ( $15.73 \pm 1.83$  vs.  $22.64 \pm 2.50$ , respectively). A remarkable improvement in the lambing rate in G2, G3 and G4 treated groups than in the G1 ( $80.0$ ,  $90.9$ , and  $90.0$  vs.  $72.7\%$ , respectively) was also observed. The ovulation rate was significantly ( $P < 0.05$ ) elevated in G4 than that in G2 and G1 ( $1.60 \pm 0.16$  vs.  $1.20 \pm 0.13$  and  $1.14 \pm 0.00$ , respectively). The follicular development did not

vary among groups. In conclusion, vitamin B2, ascorbic acid and melatonin have a beneficial effect on the reproductive performance of ewes during summer under subtropical conditions.

**Keywords:** Ewes; Vitamin B2; Ascorbic acid; Melatonin

## Introduction

Sheep and goats are considered as prolific species, despite the fact that most breeds of both species show annual reproductive cycles [1]. During each annual reproductive cycle, there is a season of low or absent (anestrous season) and a season of high (breeding season) reproductive activity. Seasonal reproductive patterns of sheep are dependent largely on latitude [2,3], breed and are influenced to a lesser extent by other factors such as strain, age and nutrition [4]. However, the molecular mechanisms through which nutrition is involved in the control of reproductive activity are not completely known [5]. The photoperiod and the annual environmental temperature cycle, are the main modulators of seasonal reproduction; whereas, in tropical regions, the annual reproductive cycle in sheep and goats is more likely regulated by annual rainfall and food availability. Ewes are typically anestrous in spring after lambing and lactating and remain so until late August or early September. Seasonal anestrous reduces reproductive efficiency and continues to hinder productivity [6,7]. Therefore, the present study was conducted to investigate the effect of different treatments (vitamin B2, ascorbic acid and melatonin) on ewes' fertility rates during summer under the local environmental conditions.

## Material and Methods

This study was approved ethically by the Ministry of Agriculture, Arab Republic of Egypt.  
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### Animals and Ration

The study was conducted at the experimental sheep station of in Sakha belonging to animal production research institute. The station is located in the north part of Nile Delta (latitude 30' 01" N; longitude 31'21" E). Twenty mature cross ewes (3/8 Finnish x 5/8 Rahamani) aged between 2 to 3 years old weighting 40 kg average body weight were used in the present work. All ewes were fed Egyptian clover (*Trifolium alexandrinum*),

besides the green maize (Darawa) and wheat straw as a source of roughage. The concentrate mixture was comprised of Yellow corn (30%), wheat bran (29%), Anti corticated cotton seed meal (25%), soya bean meal (6%), Rice bran (4%), Molasses (3%), Limestone (2%) and common salts (1%) as illustrated in Table 1. Diet was formulated to meet the nutrient requirements of NRC [8] for sheep. All ewes were healthy and clinically free of external and internal parasites. Animals were housed in semi open yards.

### Experimental Design

Relaying on laproscopic examination and evaluation of blood progesterone as described by Restall, et al. [9], all animals were confirmed as acyclic (true anestrum). No corpora lutea were found on both ovaries by laparoscope. Ewes were randomly allocated into four groups: the first group (G1; n=11) injected with 2mL saline for 3 wk., served as control. The second (G2; n = 10) and the third group (G3; n = 11) were injected intramuscular daily for 3 wk. with 2 mL (15mg) of vitamin B2 and ascorbic acid dissolved in distilled water, respectively. Ewes in the fourth group (G4; n=10) received twice daily subcutaneous injections of 0.5 mg melatonin dissolved in 20 ml of a mixture (4:1 v / v) of water and ethanol at 8AM and 4PM at the dorsal surface of the ear for 3 wk. All treated groups (G2, G3, and G4) received 1g zinc chloride and 2.5mg chromium (Merck Millipore, USA, Cat #. 1088161000 and 1120970500, respectively) in form of 6 ml oral drench once per week for 3 wk.

### Reproductive Performance

All the experimental ewes were detected for the onset of estrus. Estrus was detected every day by visual observation between 0900-1200 and 1400-1700 h using intact ram. Ewes were considered to be in estrus when standing to be mounted. Ewes came in heat were naturally bred by a proven fertile ram. Fertility measures including estrus response, time interval between the treatment to onset of estrus, lambing rates, number of lambs, sex ratio (male/ female) and frequency of single, twin and triplet were recorded for each group.

### Laparoscopic Examination

Follicular population, size and ovulation rates were determined by Laparoscopies of 7mm diameter, previous infiltration with lidocaine 2% in the puncture site as previously reported by Kelly, et al. [10] Abdel Monem, et al. [11]. Laparoscopic examination was carried out pre and post-treatment (after 3 weeks post treatment). Ovarian follicles were counted and classified according to their diameter into small (>2mm), medium (2-4mm) and large follicles (>4mm) as well as number of corpora lutea on both ovaries were recorded.

### Hormonal Assay

Individual blood samples were collected prior to the beginning of the study (pretreatment or 0 blood samples); thereafter, blood samples were daily obtained till the end of the study period. Blood samples were allowed to clot and sera were separated by centrifugation at 3000 rpm for 15 minutes. Sera were divided into aliquots and frozen at -20°C until assayed for progesterone. Serum progesterone was measured by competitive ELISA according to Wisdom, et al. [12] using kits obtained from Dima, Germany.

### Statistical Analysis

Data were expressed as mean  $\pm$  SEM. The data were analyzed statistically by ANOVA method and Duncan's test was used to detect differences among means using SPSS® Statistical Software (SPSS ®11.01 for Windows, 2001). Reproductive performance percentages were analyzed by the Chi-square test.

### Results

#### Effect on Incidence of Estrous Expression and Time Interval between the Treatment to Onset of Estrus

Table 1 showed that the incidence of estrous expression among different treatments was statistically non-significant. Meanwhile, the time elapsed from treatment to 1<sup>st</sup> estrus was shorter in G3 than that in the other groups. Although this elapsed time was non-significant, there was a tendency of increase in the lambing rate in G2, G3 and G4 on comparison to G1 (Table 1).

Groups	Reproductive traits			
	Number of ewes	Estrus response (%)	Onset of estrus(days)	Lambing rate (%)
G1 (Control)	11	11 (100.0) <sup>b</sup>	22.6 $\pm$ 2.5 <sup>a</sup>	8 (72.7)
G2 (Vitamin B2)	10	10 (100.0) <sup>b</sup>	19.5 <sup>ab</sup> $\pm$ 1.5	8 (80.9)
G3 (Ascorbic acid)	11	11	15.7 $\pm$ 1.8 <sup>b</sup>	10 (90.9)
G4 (Melatonin)	11	10	19.8 <sup>ab</sup> $\pm$ 1.4	9 (90.0)

Values within the same column with different superscripts (a, b) are different ( $p < 0.05$ ).

**Table 1:** Incidence of estrus (%), onset of estrus (Mean  $\pm$  S.E) and lambing rate (%) among different treatments.

#### Effect on Lambing Rate Lambing Rates, Number of Lambs, Sex Ratio (Male/ Female) and Frequency Of Single, Twin And Triplet

Table 2 declared that the mean number of lambs per ewe was not altered significantly among the different treatments. The higher percentage of ewes having one lamb was observed in G3 (100.00%) and the lower one

(75.00%) was recorded in G2. The proportion of twinning among different groups (G1, G2, and G4) was not varied. Incidence of lamb mortality (3 out of 11) was observed only in a vitamin B2 treated ewe bearing a triplet (Table 2). The sex ratio (male/female) was found to be higher in G4 than in the other groups.

Groups	No. of lambs born	Litter size at Birth	Sex of lamb			No. of ewe bearing (%)		
			M	F	M/F ratio	Single	Twin	Triplet
G1 (Control)	9	1.13 $\pm$ 0.13	4	5	0.8	7(87.5)	1(12.5)	0
G2 (Vitamin B2)	11	1.38 $\pm$ 0.26	7	4	1.7	6(75.0)	1(10.0)	1(12.5)*
G3 (Ascorbic acid)	10	1.00 $\pm$ 0.00	5	5	1	10(100)	0	0
G4 (Melatonin)	10	1.11 $\pm$ 0.11	8	2	4	8(88.8)	1(11.1)	0

\*The born lambs died

**Table 2:** Survey of born lamb between groups (Mean  $\pm$  S.E).

### Effect on Follicular Population, Size and Ovulation Rates

According to laparoscopic examination of ewes after 3 week post-treatment (Table 3), there was a significant ( $P < 0.05$ ) higher percentage of ovulated ewes in G1, G2 and G4 as compared to G3. On other hand, G4 ewes had a

significant ( $P < 0.05$ ) increase in ovulation rate than those in G1 and G2 groups (Table 3). Table 4 showed that the follicular development was similar without any significant difference in ewes among different groups after 3 weeks post treatment.

Groups	Number of examined ewes	Ovulated ewes (%)	Number of corpora lutea			Ovulation rate (mean $\pm$ SEM)
			Right	Left	Total	
G1 (Control)	9	7(100.0) <sup>a</sup>	4	4	8	1.14 <sup>a</sup> $\pm$ 0.00
G2 (Vitamin B2)	11	10(100.0) <sup>a</sup>	5	7	12	1.20 <sup>a</sup> $\pm$ 0.13
G3 (Ascorbic acid)	10	4(66.6) <sup>b</sup>	5	2	7	1.75 <sup>ab</sup> $\pm$ 0.25
G4 (Melatonin)	10	10(100.0) <sup>a</sup>	5	11	16	1.60 <sup>b</sup> $\pm$ 0.16

Values with different superscript in the same column are significantly different at  $P < 0.05$ .

**Table 3:** Laparoscopy of ovarian activity in ewes three weeks post-treatment (Mean  $\pm$  S.E).

Groups	Number of examined ewes	Number of follicles		
		Small follicles (<2mm)	Medium follicles (2-4mm)	Large follicles (>4mm)
G1 (Control)	7	4.67 $\pm$ 1.05	1.33 $\pm$ 0.61	0.33 $\pm$ 0.21
G2 (Vitamin B2)	10	4.71 $\pm$ 1.21	1.29 $\pm$ 0.36	0.57 $\pm$ 0.30
G3 (Ascorbic acid)	6	2.83 $\pm$ 0.98	0.67 $\pm$ 0.33	0.50 $\pm$ 0.34
G4 (Melatonin)	10	5.00 $\pm$ 2.48	0.50 $\pm$ 0.29	0.25 $\pm$ 0.25

Values with different superscript in the same column are significantly different at  $P < 0.05$ .

**Table 4:** Ovarian follicular development in different groups (Mean  $\pm$  S.E).

### Effect on Serum Progesterone Concentration

At the beginning of dietary supplementation, there were no significant differences between groups in the concentration of serum progesterone. The circulating

serum progesterone was ranged from  $0.36 \pm 0.41$  to  $0.52 \pm 0.43$  ng/ml. Three weeks later, serum progesterone concentrations started to increase from day 15 in G3 and from day 20 in the other groups as shown in Table 5.

Groups	weeks of study		
	0 <sup>th</sup> -week	2 <sup>nd</sup> -week	3 <sup>th</sup> -week
G1 (Control)	0.36 $\pm$ 0.41 <sup>a</sup>	0.52 $\pm$ 0.43 <sup>a</sup>	1.5 $\pm$ 0.43 <sup>a</sup>
G2 (Vitamin B2)	0.51 $\pm$ 0.50 <sup>a</sup>	0.6 $\pm$ 0.43 <sup>a</sup>	1.6 $\pm$ 0.43 <sup>a</sup>
G3 (Ascorbic acid)	0.52 $\pm$ 0.33 <sup>a</sup>	1.5 $\pm$ 0.17 <sup>b</sup>	25 $\pm$ 0.17 <sup>b</sup>
G4 (Melatonin)	0.42 $\pm$ 0.40 <sup>a</sup>	0.6 $\pm$ 0.43 <sup>a</sup>	1.6 $\pm$ 0.43 <sup>a</sup>

Means with different superscripts within the same row were significantly different at  $p < 0.05$ .

**Table 5:** Serum progesterone concentration (ng/ml) among different groups during the study (Mean  $\pm$  S.E).

### Discussion

In the present study (under subtropical conditions), there was no difference between experimental and control group on the incidence of estrus. Similar findings were obtained by El-Maghraby, et al. [13] in mature Finn crossed ewes under subtropical conditions. However, the incidence of estrus was significantly improved in melatonin 120 implanted ewes for 40 days (75%)

compared to control ones (59%) in United States of America (subtropical) as reported by Stellflug, et al. [14]. The obtained data revealed higher incidence of estrus (100%) in untreated ewes (control group). In this respect, Aboul Naga, et al. [15] reported that about 33% of Rahmani ewes showed continuous ovarian activity throughout the year, whilst almost all ewes had periods of behavioral anestrus under subtropical conditions. The mean interval from treatment to the onset of estrus

observed in the present study was significantly shorter for ewes received ascorbic acid than controls ( $15.73 \pm 1.83$  vs.  $22.64 \pm 2.50$  respectively). Ascorbic acid possesses many biological actions that are relevant with its role as a reducing agent; it is required for the biosynthesis of collagen which is essential for follicular growth and repair of the ovulated follicle [16], for corpus luteum development [17], for biosynthesis of steroid and peptide hormones, and to prevent or reduce the oxidation of biomolecules [18]. In the current study, there was a non-significant effect of vitamin B2, ascorbic acid and melatonin on the lambing rates. However, exogenous vitamin C application prevents oxidative stress and increases the fertility rate along with body weight of pregnant ewes and newborns in Turkey (temperate; injected intramuscular; [19] and Chile (subtropical; administrated orally during mating and gestation period [20]. Moreover, vitamin C supplementation is essential for water-stress alleviation in sheep under subtropical conditions [21]. In addition, the reported lambing rate in the melatonin treated group (90.00 %) is close to that recorded (88.89%) in Egypt (subtropical) by El-Maghraby, et al. [13]. Furthermore, in Spain (temperate), Abecia, et al. [22] observed that melatonin intravenous treatment in sheep can increase both fertility and prolificacy by improving luteal function. The beneficial effect of vitamin B2 on reproductive performance of ewes observed in the present study was similar to that described early in cattle by Hurley, et al. [23] who found that all B-vitamins are required for the growth and development of fetus. In this study, ascorbic acid and melatonin had almost no effect on the occurrence of triplet fetuses. This is a desirable outcome in ewes with low fecundity under local conditions where mortality of triplet bearing ewes is very high. The same conclusions were obtained by William, et al. [24] in Merino ewes in Australia (temperate) by using melatonin subcutaneous implants. On the contrary, DeNicolo, et al. [25] reported that melatonin subcutaneous implants in conjunction with administration of progesterone and equine chorionic gonadotrophin increased the number of lambs born per ewe treated in an out-of-season breeding program in New Zealand (temperate country) sheep flocks. As shown in the present study, the sex ratio ( $\sigma/\rho$ ) in G4 (4.00) was higher than in G1, G2 and G3. However, there was no significant difference between melatonin and control groups (1.063 vs. 1.026, respectively) as also cited by Williams, et al. [24]. In this study, the ovulation rate was significantly ( $P<0.05$ ) improved in G4 than in G2 and G1 groups. These results are in accordance with the findings of Forcada, et al. [26] in Spain (temperate) in sheep during the anestrous periods. Melatonin may regulate

cyclic activity in ewes by affecting the hypothalamo-hypophyseal axis [27]. It plays a key role in the transfer of photoperiodic information to the neuro-endocrine axis [28]. Furthermore, Song, et al. [29] recorded that, melatonin at 5 mg subcutaneously injected into the neck during estrus would increase the level of melatonin and estradiol in the blood of ewe, and melatonin promoted embryo production and the pregnancy rate in the ewes naturally mated or embryo transfer. In addition, melatonin administration improved the conception rates in mice and cows [30]. Also, melatonin added during culture of mouse prokaryotic embryos significantly increased blastocyst rate, pregnancy rate after transplantation, average number of offspring and survival rate of offspring [31]. For deer, melatonin subcutaneous implantation also improved the quantity and quality of super ovulatory oocytes [32]. In the present work, follicular activity as indicated by the presence of medium and large sized follicles was observed in ewes of different groups without any significant difference. The same finding was reported by Wheaton, et al. [32] in Suffolk ewes fed 3 mg melatonin daily for 44 days in United States of America (subtropical). In the current study, serum progesterone levels started to increase between days 15 and 20 after treatment in all groups. These observations are consistent with previous studies which indicate that melatonin treatment increases serum LH and progesterone levels in goat and deer [34,35]. This increase in progesterone recorded herein indicated the cyclic activity in ewes. The serum progesterone concentration started to increase five days earlier in G3 (ascorbic acid treated) than the other groups. Vitamin C is believed to act as an antioxidant, neutralizing the oxidative by-products of cellular respiration in luteal cells, as an enzymatic cofactor in collagen synthesis and as a promotor of steroid and protein hormone synthesis [19,36]; However, no significant correlation between plasma vitamin C and progesterone levels was described in some studies [37,38]. In conclusion, vitamin B2, ascorbic acid and melatonin have a beneficial effect on the reproductive performance of ewes during summer under subtropical conditions [39-44].

### Conflict of Interest Statement

We declare that we have no conflict of interest.

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