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Follicular dynamics in antioxidants supplemented heat stressed goats

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ABSTRACT

The aim of the study was to know the effect of antioxidants supplementation on reproductive status in heat stressed goats. Goats of similar age groups were divided into 5 groups of 5 goats in each group i.e. control, heat stress and treatment groups 1, 2 and 3 with antioxidant supplementation. Except control, all groups were exposed to a temperature of 40±1°C with a relative humidity of 30% for 5 h/day for one estrus cycle in psychrometric chamber. Rectal temperature and respiratory rates were recorded daily post exposure. Follicular dynamics was studied through real time B-mode transrectal ultrasonography at every third day for 2 consecutive cycles. Blood samples were collected on the same day of ultrasonography for estimation of plasma estrogen and progesterone levels. Mean estrous cycle length and variation in the population of small, medium and large follicles did not differ significantly among the groups either in exposure or post exposure cycle. There was significant difference in diameter of CL, progesterone and oestradiol levels between control and heat stress and also between control and treatment groups during both exposure and post-exposure cycle. Among the treatment groups the CL diameter, progesterone and oestradiol levels were higher in treatment groups 1 and 3 as compared to treatment group 2.

Key words: Antioxidants, Follicular dynamics, Goats, Heat stress

High environmental temperature is one the major concerns in tropical and arid areas (Silannikove 1992) that affects reproduction by altering hypothalamo-pituitary-gonadal axis and also stimulates excessive production of free radicals (super oxide anion radicals, hydroxyl radical, hydrogen peroxide and singlet oxygen), which are continuously produced in the course of normal aerobic metabolism (Bernabucchi *et al.* 2002) leading to reduced intensity of estrous signs, ovulation abnormalities, reduced fertilization rate and decreased embryo survival rate (Soto *et al.* 1998). The major strategies to reduce the effect of heat stress on reproduction have been to alter the environment through the use of sheds, fans or evaporative cooling (Bucklin *et al.* 1991). Such practices are not possible in India because the goats are mainly reared on semi-intensive system as they are left for grazing for most of the day time where ambient temperature is high. This necessitates other strategies to counteract the adverse effects of heat stress such as supplementation of antioxidants to enhance reproductive function. These antioxidants are free radical scavengers which protect the body defense system against excessively produced free radicals and stabilize animal health status. The understanding of follicular dynamics and their regulation in goats with use of ultrasonography has gained importance in recent years. The present study was envisaged as there is dearth of information pertaining to the role of antioxidants on follicular dynamics during heat stress in goats.

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MATERIALS AND METHODS

Healthy cyclic female goats (25) of similar age group (1.5 years) and reproductive status were selected from the experimental animal shed of the physiology and climatology division IVRI, Izatnagar. The animals were synchronized with 2 doses of prostaglandin $F_2\alpha$ @ 10 mg injected intra-muscularly at 10 day interval. Estrus behavior was checked twice daily with mature breeding buck. The goats were maintained under uniform management and husbandry conditions. The animals were fed with concentrate mixture in addition to green fodder and water *ad lib.*

All animals were divided into 5 groups of 5 animals in each group. Group 1 (n=5): Control; heat stress group (n=5): heat stressed; treatment group 1 (n=5): heat stress with vitamin C (2 g); treatment group 2 (n=5): heat stress with vitamin E (250 mg) and selenium (0.1 mg); treatment group 3 (n=5): heat stress with vitamin C (1g) and vitamin E (125 mg) with selenium (0.05 mg).

Vitamin C was given as chewable tablets; vitamin E was supplemented as α tocopherol acetate, and selenium as sodium selenite. Vitamin C and E supplemented to the animals were over and above the NRC recommendations orally for 21 days. All groups except control group were exposed to a temperature of 40±1°C for 5 h with a relative humidity of 30% per day for 1 estrus cycle in psychrometric chamber. Control group animals were maintained in natural indoor environment. Rectal temperature was recorded by digital thermometer and respiration rate by noticing the flank movements from a distance prior to examining the rectal temperature to avoid any disturbance to the animals daily post exposure. Animals were subjected to transrectal ultrasonographic examination of ovaries using real time B-mode scanner equipped with 6.0 MHz linear array transducer at every third day for 2 consecutive estrus cycles. Follicles were counted, measured and classified according to Khan (2001) as small (< 3 mm), medium (3–4 mm) and large (>4 mm). The diameter of corpus lutem (CL) was also measured in similar way. Both the ovaries were scanned one by one. Blood samples of all groups were collected from jugular vein by venipuncture in sterile vials containing anti-coagulant heparin on the same day of ultrasonic examination. The plasma samples were separated by centrifugation at 3 000 rpm for 15 min and samples were frozen and stored at –20°C till estimations were over. Plasma estrogen and progesterone concentrations were estimated by radioimmunoassay (RIA) using the diagnostic I 125 kits supplied by Immunotech, France. Statistical analysis was done by ANOVA as per Snedechor and Cochran (1989).

RESULTS AND DISCUSSION

The rectal temperatures and respiratory rates are presented in Table 1. The rectal temperature and respiratory rates are recognized as important measures of physiological status (Lefcourt *et al.* 1986) as well as ideal indicators for assessment of stress in animals. Hence, the increased rectal temperature and respiratory rates after exposing them to 40±1°C in the climatic chamber suggested that the goats were under stressful environment in the chamber. The decreased rectal temperature and respiratory rates in treatment groups indicated that supplementation of vitamin E with selenium and vitamin C had ameliorated the heat stress in goats. Similar decrease in rectal temperature and respiratory rates by vitamin E and C was reported by Shenglins *et al.* (2003) in pigs, Kobeisy (1997) in goats, Sedki *et al.* (2002) in rabbits and Kutlu and Forbes (1993) in broilers under heat stressed conditions. In the post exposure cycle, all groups of animals, i.e. heat stress group and treatment groups 1, 2 and 3 were kept along with control group in natural indoor environment and there were no significant differences in rectal temperature and respiratory rates among different groups (hence their values are not depicted in Table 1) indicating that the heat stress of previous exposed cycle did not have any carry-over effect on rectal temperature and respiratory rates.

Among domesticated animals, the goat exhibits very prominent signs of heat (Maurico and Michael 2003). All animals showed prominent estrus signs i.e. change in attitude, increased activity, frequent urination, restlessness, moaning, vulval discharge, redness and swelling of vulva, standing while mounted by buck, vocalization-yelling were noticed in exposure cycle. In a subsequent cycle, i.e. post exposure cycle heat stress group and treatment group 2 showed comparatively mild signs of estrus as compared to treatment groups 1 and 3. These mild signs of estrus could be due to reduction of oestradiol during heat stress as oestradiol mediates the estrus signs and behaviour (Badinga *et al.* 1993, Wolfenson *et al.* 1997, Willson *et al.* 1998).

The control animals exhibited an estrous cycle length varying from 20 to 22 days with an average cycle length of 20.8 days. In treatment group 2 and heat stress group the estrus cycle length varied from 18.5 to 22.5 days averaging 21.1 days. Estrus cycle length in treatment groups 1 and 3 ranged between 20.62 and 22 days with an average of 21.6 days. Mean estrus cycle length did not differ significantly among the groups neither in exposed cycle nor in post-exposed cycle. Similar findings were reported by Roth *et al.* (2000) in heat stressed cows.

The number of small follicles (< 3 mm), medium follicles (3–4 mm) and large follicles (> 4 mm) ranged from 5.25 to 7.2, 6.5 to 8.5, and 1.75 to 3 respectively. No variation in the population of small, medium and large follicles were found among control, heat stress and treatment groups either in exposed cycle or in post-exposure cycle. The diameter of largest follicle in the waves differs according to characteristics of wave pattern in estrus cycle. Studies in the cattle showed that heat stress resulted in an increase in the number of large size follicle and decrease in medium size follicle (Wolfenson *et al.* 1997, Wilson *et al.* 1998, Roth *et al.* 2000). The authors attributed to a greater reduction in

Table 1. Physiological responses in control, heat stress and treatment groups

Parameter *	Control group	Heat stress group	Treatment group 1	Treatment group 2	Treatment group 3
Rectal temperature (°F)	101.80±0.04^a	103.92±0.06^d	102.65±0.06^b	103.06±0.06^c	102.67±0.07^b
Respiratory rate/min	20.30±0.10^a	122.22±0.63^d	73.04±2.13^b	81.82±1.91^c	72.85±2.13^b

Means with different superscripts in rows differ significantly ($P<0.05$), *represents average of 21 days.

Table 2. CL diameter (mm) in control, heat stress and treatment groups

Day	Control group	Heat stress group	Treatment group 1	Treatment group 2	Treatment group 3
0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
6	7.6±0.04^{c}	7.2±0.04^{a}	7.3±0.04^{b}	7.2±0.02^{a}	7.3±0.04^{b}
9	8.7±0.04^{c}	8.1±0.02^{a}	8.4±0.04^{b}	8.1±0.04^{a}	8.4±0.04^{b}
12	10.5±0.04^{c}	9.4±0.04^{a}	10.1±0.07^{b}	9.5±0.02^{a}	10.0±0.10^{b}
15	11.2±0.03^{c}	9.9±0.04a	10.9±0.06^{b}	10.1±0.02^{a}	10.8±0.04^{b}
18	7.7±0.04^{c}	7.1±0.03^{a}	7.5±0.02^{b}	7.2±0.05^{a}	7.5±0.04^{b}
21/0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
6	7.7±0.06^{c}	6.9±0.02^{a}	7.6±0.04^{b}	7.0±0.02^{a}	7.5±0.04^{b}
9	9.3±0.08^{c}	8.1±0.02^{a}	8.9±0.04^{b}	8.3±0.02^{a}	8.8±0.02^{b}
12	10.8±0.04^{c}	9.3±0.03^{a}	10.4±0.04^{b}	9.8±0.02^{a}	10.3±0.04^{b}
15	11.4±0.04^{c}	10.3±0.04^{a}	10.9±0.02^{b}	10.4±0.02^{a}	10.97±0.04^{b}
18	8.0±0.06^{c}	7.1±0.02^{a}	7.7±0.02^{b}	7.3±0.02^{a}	7.7±0.07^{b}
21	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Means with different superscripts in rows within a particular day differ Significantly ($P<0.05$).

follicular dominance phenomenon of dominant follicle. This decrease in follicular dominance of dominant follicle due to heat stress in cattle could not inhibit the growth of subordinate follicles and allowed them to grow and attain the diameter of large follicles. But goat is a multiovulatory species and occurrence of 2 or more dominant follicles per wave is more common (Ginther and Kot 1994, Medan *et al.* 2003), and dominance phenomenon is also not clear in goats hence concept of co-dominance was suggested in goats by Rubianes and Menchaca (2003) to explain the development of two or more large follicles in each wave. Even in the present study co-dominance phenomenon seemed to be existing, which might be the reason for not varying in the population of different size follicles.

The diameter of CL in different groups is presented in Table 2. The CL was detected on sixth day of estrus cycle by ultrasonography and it started increasing in diameter from day 6 to 15 and then declined on 18th day indicating regression of CL of estrus cycle in all groups. There was significant ($P<0.05$) difference in diameter of CL between control and heat stress and also between control and treatment groups during both exposure and post-exposure cycle. This decrease in CL diameter in heat exposed animals might be due to either reduced size of largest follicle of ovulatory wave or decrease in substrate required for remodeling and growth of CL (Castro *et al.* 1999, Ginther and Kot 1994, Medan *et al.* 2003).

Table 3. Progesterone (ng/ml) in control, heat stress and treatment groups

Day	Control group	Heat stress group	Treatment group 1	Treatment group 2	Treatment group 3
0	0.175±0.006	0.16±0.008	0.18±0.007	0.17±0.008	0.15±0.016
3	0.87±0.007^{a}	0.81±0.002^{b}	0.82±0.005^{b}	0.82±0.005^{b}	0.86±0.014^{a}
6	3.7±0.06^{a}	3.35±0.02^{b}	3.48±0.012^{b}	3.34±0.023^{b}	3.49±0.013^{b}
9	6.07±0.018^{a}	5.17±0.015^{b}	5.95±0.02^{c}	5.19±0.007^{b}	5.86±0.02^{c}
12	7.20±0.01^{a}	6.06±0.011^{b}	6.37±0.02^{c}	6.13±0.006^{b}	6.39±0.05^{c}
15	7.79±0.04^{a}	6.40±0.015^{b}	6.83±0.02^{c}	6.46±0.02^{b}	6.73±0.06^{c}
18	0.80±0.015^{a}	0.67±0.019^{b}	0.73±0.001^{c}	0.69±0.008^{b}	0.74±0.01^{c}
21/0	0.18±0.003^{a}	0.12±0.001^{b}	0.15±0.001^{c}	0.12±0.006^{b}	0.14±0.005^{c}
3	0.87±0.008^{a}	0.75±0.02^{b}	0.85±0.009^{a}	0.80±0.002^{b}	0.84±0.01^{a}
6	3.81±0.05^{a}	3.17±0.02^{b}	3.50±0.03^{c}	3.28±0.02^{b}	3.46±0.01^{c}
9	6.09±0.03^{a}	5.18±0.007^{b}	5.93±0.21^{c}	5.41±0.02^{d}	5.83±0.03^{c}
12	7.19±0.02^{a}	5.88±0.04^{b}	6.92±0.01^{c}	6.13±0.01^{d}	6.97±0.01^{c}
15	7.81±0.04^{a}	6.12±0.004^{b}	7.32±0.09^{c}	6.56±0.03^{d}	7.44±0.11^{c}
18	0.81±0.02^{a}	0.53±0.01^{b}	0.80±0.01^{a}	0.71±0.01^{c}	0.79±0.007^{a}
21	0.18±0.002	0.16±0.002	0.18±0.003	0.17±0.001	0.18±0.001

Means with different superscripts in rows within a particular day differ significantly ($P<0.05$).

Table 4. Oestradiol 17-ß (Pg/ml) in control, heat stress and treatment groups

Day	Control group	Heat stress group	Treatment group 1	Treatment group 2	Treatment group 3
0	26.40±0.03	26.41±0.002	26.48±0.03	26.52±0.01	26.47±0.006
3	3.58±0.01^{c}	3.25±0.006^{a}	3.29±0.04^{b}	3.24±0.004^{a}	3.30±0006^{b}
6	13.71±0.06^{c}	10.97±0.006^{a}	11.97±0.02^{b}	11.0±0.08^{a}	11.97±0.006^{b}
9	3.08±0.01^{c}	2.85±0.004^{a}	2.94±0.004^{b}	2.84±0.006^{a}	2.93±0.006^{b}
12	3.07±0.006^{c}	2.86±0.008^{a}	2.96±0.008^{b}	2.89±0.01^{a}	2.96±0.018^{b}
15	3.14±0.009^{c}	2.88±0.01^{a}	3.00±0.005^{b}	2.87±0.006^{a}	2.97±0.01^{b}
18	3.26±0.02^{c}	3.13±0.006^{a}	3.22±0.011^{b}	3.14±0.008^{a}	3.20±0.006^{b}
21/0	26.35±0.03^{d}	22.18±0.02^{a}	24.21±0.02^{c}	23.22±0.014^{b}	24.26±0.03^{c}
3	3.49±0.006^{b}	3.30±0.004^{a}	3.33±0.02^{a}	3.29±0.004^{a}	3.30±0.005^{a}
6	13.88±0.02^{c}	12.54±0.015^{a}	13.09±0.008^{b}	13.06±0.02^{b}	13.08±0.008^{b}
9	3.05±0.002^{c}	2.91±0.004^{a}	2.97±0.006^{b}	2.93±0.018^{a}	2.96±0.006^{b}
12	3.08±0.002^{c}	2.96±0.006^{a}	2.98±0.002^{b}	2.96±0.009^{a}	2.99±0.006^{b}
15	3.16±0.015^{c}	3.04±0.006^{a}	3.11±0.009^{b}	3.07±0.022^{a}	3.09±0.011^{b}
18	3.29±0.006^{b}	3.20±0.005^{a}	3.21±0.002^{a}	3.20±0.007^{a}	3.21±0.007^{a}
21	26.26±0.011^{c}	24.28±0.031^{a}	25.27±0.03^{b}	25.01±0.07^{b}	25.27±0.032^{b}

Means with different superscripts in rows within a particular day differ significantly (P<0.05).

There was no difference in CL diameter between treatment group 2 and heat stress group and also between treatment groups 1 and 3. In the present study vitamin E with selenium in treatment group 2 did not increase the diameter of CL whereas vitamin C in treatment groups 1 and 3 could increase the diameter of CL because ascorbic acid was found to be involved in the process of remodeling and growth of CL as CL is made up of collagen, and vitamin C is required for collagen synthesis (Luck and Zaho 1993) and this might be the reason for increased CL diameter in the treatment groups 1 and 3.

The plasma progesterone and estrogen levels are presented in Tables 3 and 4. The basal levels of plasma oestradiol 17 ß (pg/ml) ranged from 3.1 to 3.5, 2.8 to 3, 2.9 to 3.2, 2.8 to 3.2 and 2.9 to 3.3, in control, heat stress groups and treatment groups 1, 2 and 3, respectively. There was an increase in plasma oestradiol 17 ß concentration on sixth day of estrus cycle in all groups. The peak values of oestradiol 17 ß were found on the day of estrus after estrus synchronization and these values did not differ among the different groups. Significant (P<0.05) decrease in the levels of oestradiol 17 ß concentration was found from day 3 onwards in heat stress and treatment groups as compared to control group. There was no variation in oestradiol concentration between heat stress group and treatment group 2 and also between treatment groups 1 and 3. In the post exposure cycle the peak oestradiol 17 ß values on the day of estrus were significantly (P<0.01) lower in heat stress and treatment groups as compared to control group. In treatment groups 1 and 3, where vitamin C was supplemented there was significant (P<0.01) increase in oestradiol 17 ß level as compared to heat stress and treatment group 2. The initial raise in oestradiol 17 ß levels on the day of estrus coincided with the decline in plasma progesterone (luteolysis). Increased oestradiol 17 ß concentration on sixth day (early luteal phase) was associated with development of wave 1 and another increase on the day of estrus was related with ovulatory wave (Castro *et al.* 1999).

This increase in oestradiol 17 ß concentration is produced mainly by the largest follicle of wave, whereas other follicles contribution is less than 10% of the ovarian oestradiol concentration (Mann *et al.* 1992). Except on the day of estrus and sixth day, the oestradiol 17 ß concentration remained low at the basal levels and this could be due to high concentration of progesterone which is inhibitory to pulsatile LH secretion leading to lower levels of stimulation to the developing follicles, which in turn do not produce and contribute to high levels of oestradiol 17 ß. In heat stress group and treatment group 2 lower levels of oestradiol 17 ß were noticed when compared to control as well as treatment groups 1 and 3. Roth *et al.* (2000), Badinga *et al.* (1993), Wilson *et al.* (1998a) reported reduced levels of oestradiol 17 ß in heat exposed cows. This reduction in oestradiol 17 ß levels could be due to decreased steroidogenic capacity of follicle during heat stress. This decreased steroidogenic capacity of follicle during heat stress might be due to (i) lower production of androstenedione by theca cells, (ii) lower viability of theca cells or granulosa cells (Wolfenson *et al.* 1997), (iii) lower aromatase activity in granulosa cells (Badinga *et al.* 1993), (iv) decrease in secretion rate of oestradiol 17 ß into circulation, (v) increase levels of nitric oxide which in turn inhibits the synthesis of oestradiol (Adams *et al.* 1992, Masuda *et al.* 2001).

An increase in oestradiol 17 ß concentration in treatment groups 1 and 3 by vitamin C supplementation could be due to (i) improved steroidogenesis by vitamin C (Luck *et al.*

1995) as steroidogenesis is ascorbic acid dependent particularly at hydroxylation stage (Tsuji *et al.* 1989), (ii) deposition of high concentration of ascorbic acid in theca interna, granulosa and luteal compartments of ovary (Deane 1952), (iii) protecting from reactive oxygen radicals which inhibits steroidogenesis by blocking intracellular transport of cholesterol to mitochondria or translocation of cholesterol across the outer mitochondrial membrane (Behrman and Aten 1991).

The plasma progesterone concentration was lower on the day of estrus in control, heat stress and treatment groups after synchronization. The concentration of progesterone started increasing from day 3 onwards and reached a peak level on 15th day and then declined on 18th day onwards in all groups. Similar trend of progesterone was reported by Castro *et al.* (1999) and Medan *et al.* (2003) in goats. From day 3 onwards plasma levels of progesterone were decreased significantly ($P<0.01$) in heat stress and treatment groups as compared to control group but decrease was less in treatment groups 1and 3 than heat stress and treatment group 2. In post-exposure cycle the progesterone concentration showed similar trend as observed in exposure cycle. The progesterone levels positively correlated to the size of CL in all groups as observed by ultrasonography. Kastelic *et al.* (1990) also reported a parallel change in plasma progesterone concentration with diameter of CL by ultrasonography.

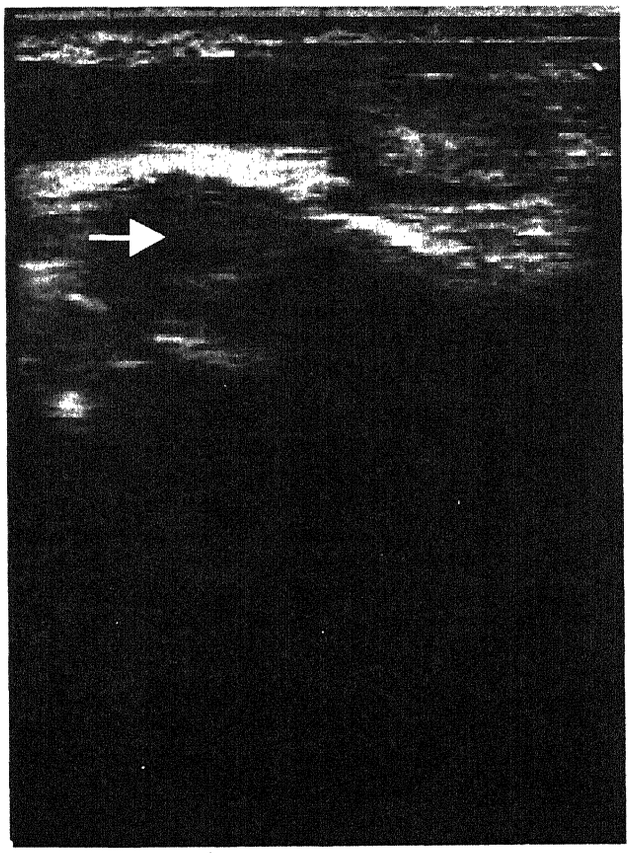

Fig. 1. Ultrasonograph depicting different size follicles of goat ovaries.

The decrease in progesterone concentration during heat exposure could be due to decrease in CL diameter and levels of plasma progesterone depend not only on its rate of production from CL but also on the rate of secretion to the circulation. Lublin and Wolfenson (1996) reported reduced ovarian blood flow during heat stress. Trout *et al.* (1998) reported that degree of hyperthermia and the type of heat exposure (acute or chronic) contributes to progesterone concentration variation in heat stress animals. Increase in concentration of progesterone in treatment groups 1 and 3 by vitamin C could be due to stimulatory effect of vitamin C on progesterone secretion (Luck and Jungclas 1988, Byrd *et al.* 1993). This might also be due to protective effect of vitamin C from reactive oxygen species, which inhibits progesterone synthesis by inhibition of cholesterol side chain cleavage of cytochrome P_{450} sec (Endo *et al.* 1993). Even though vitamin E and selenium are antioxidant and also protects from ROS but in the present study the plasma vitamin E and selenium could not affect the levels of either progesterone or estrogen during heat stress. Some beneficial effects of vitamin E and selenium on estrous cycle and fertility have been a consequence of improved uterine health as vitamin E and selenium can enhance neutrophil function and activity, which promotes removal of microorganisms and support tissue remodeling and involution of uterus (Arechiga *et al.* 1994).

The reduction in the levels of oestradiol, progesterone and CL diameter in the post exposure cycle indicated that heat stress may have long term carry over effect on quality of subsequent dominant follicles. Castro *et al.* (1999) reported that progesterone played an important role in follicular wave turn over. Low progesterone may cause aberrant follicular development, which in turn results in abnormal oocyte maturation in the ovulatory follicle. Low progesterone levels also affect steroidogenesis in the dominant follicle and subsequently formed CL and thereby altered reproductive function in the subsequent estrus cycle (Wolfenson *et al.* 1993). It is concluded from the present study that ascorbic acid has better capacity to maintain the reproductive efficiency during heat stress than vitamin E with selenium.

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