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Impacts of vitamin C and E injections on ovarian structures and fertility in Holstein cows under heat stress conditions

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Abstract: This study evaluated the effect of injecting vitamin C (VC) and E (VE) on size of the preovulatory follicle, volume of the corpus luteum, and pregnancy rates in Holstein cows under heat stress conditions (temperature humidity index > 74). Sixty-two cows were randomly assigned to one of four treatments: 1, control, n = 15: cows were not supplemented with vitamins; 2, VCG, n = 15: cows were simultaneously injected i.v. with 500 and s.c. with 2500 mg of VC before and after estrus; 3, VEG, n = 15: cows received an i.m. injection of 3000 IU of VE before estrus; 4, VCEG, n = 17: cows were injected with VC and VE in the same way and doses as in treatments 2 and 3. Treatment did not significantly affect any of the measured variables, despite a numerical increase in pregnancy rates in cows injected with vitamins (7.5 ± 7.3%, 9.6 ± 9.4%, 15.1 ± 10.0%, and 18.34 ± 9.9% for control, VCG, VEG, and VCEG, respectively). In conclusion, injections of vitamin C and E did not affect either the development of the preovulatory follicle and the corpus luteum or pregnancy rates in Holstein dairy cattle under heat stress conditions.

Key words: Corpus luteum, dairy cattle, heat stress, pregnancy rate, vitamin C, vitamin E

1. Introduction

Heat stress induces behavioral, metabolic, and hormonal changes in dairy cattle, resulting in poor reproductive performance (1). To date, there is no treatment that can fully restore the fertility of dairy cattle under heat stress. However, supplementation of antioxidants could be a feasible way to improve fertility in cows under such conditions (2). This seems logical, since blood concentrations of antioxidants, such as vitamins C and E, are diminished by heat stress (3,4).

Vitamin C and E are necessary for normal reproduction in cattle (5,6) and required for follicle and corpus luteum (CL) development (7,8). Low fertility in dairy cattle may be a consequence of smaller preovulatory follicle and CL size compared to animals without heat stress (9–11). We speculated that cows exposed to heat stress conditions and supplemented with vitamin C and E have larger preovulatory follicles and CL, resulting in higher pregnancy rates compared to cows that did not receive vitamin supplementation.

2. Materials and methods

2.1. Animal welfare

All technical and management procedures were performed based on the guidelines set by the Canadian Council on Animal Care (12).

2.2. Location

The experiment was conducted at the dairy farm "18 de Julio" of the Universidad Autónoma Chapingo. The farm is near Tlahualilo, Durango, México, located at 25°54′N and 103°35′W, 1137 m above sea level. The climate of the region is semiarid, with a mean annual temperature of 21.1 °C and 239 mm of rainfall per year (13). The experiment was conducted during the third week of August and the first week of September 2014.

2.3. Animals, treatments, and experimental design

Multiparous Holstein dairy cows (n = 62) with an average of 188.75 ± 15.90 days in milk and producing 37.50 ± 1.13 liters of milk per day, were randomly assigned to one of four treatments: 1, control, n = 15: cows were not injected with vitamins; 2, VCG, n = 15: cows received a total dose of 3000 mg of vitamin C (ascorbic acid, Q.P., Reasol; 500 mg via i.v. and 2500 mg via s.c.) at night on day –5 (day 0 was the day of progesterone release device (CIDR) removal), immediately after estrus detection and 2 days after artificial insemination; 3, VEG, n = 15: cows received a single i.m. injection of 3000 IU of vitamin E ((±)α-tocopherol, Sigma-Aldrich) at night on day –5; 4, VCEG, n = 17: cows were injected with both vitamins on the same days and doses

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as mentioned in treatments 2 and 3. The experimental design was completely randomized, with one cow as an experimental unit.

2.4. Estrus synchronization and breeding

The follicular wave of the cows was synchronized with a CIDR containing 1.9 g of progesterone (CIDR 1900 CATTLE INSERT, Zoetis), inserted intravaginally for 8 days, and an i.m. injection of 250 µg of GnRH analogue (GnRH, Sanfer). Estrus behavior was induced by an i.m. injection of 500 µg of cloprostenol (Celosil, MSD Animal Health) at CIDR removal. Once the CIDR was withdrawn, the animals were constantly monitored by direct observation for signs of standing estrus. The cows were artificially inseminated 12 h after standing estrus with a single dose (approximately 2×10^6 spermatozoa) of semen from a single bull of proven fertility. To induce ovulation, an injection of 250 µg of GnRH analogue was given to each cow immediately after AI.

2.5. Nutrition and feeding

The animals received a diet formulated to provide 1600 IU of vitamin E (56.5 kg day^{-1} cow^{-1}) of a total mixed ratio: alfalfa, 15.0; corn silage, 26.5; steam corn, 6.0; and concentrate, 9.0 kg as fed. The food composition of the concentrate mixture was as follows: cottonseed, 1.099; walnut, 0.895; soybean, 2.049; wheat bran, 1.911; molasses, 1.431; soy plus (cooker-expeller soybean, Soyplus), 1.135; magnesium oxide, 0.0162, sodium bicarbonate, 0.135; lactomil (bypass fat, Lactomil), 0.054; calcium carbonate, 0.189, microminerals, 0.030; vitamins, 0.030; Ganadero plus (*Saccharomyces cerevisiae*, Biotecap), 0.019; and maxifolipol (Flavofosfolipol, Pisa), 0.002 kg as fed).

2.6. Response variables

To assess the effects of vitamin C and E injections on follicle and CL development and, subsequently, on the fertility of Holstein dairy cattle, the following variables were evaluated: diameter of the largest follicle at CIDR removal (DPF0, mm) and at estrus detection (DFP1, mm), time of estrus after CIDR removal (h), growth rate of the largest follicle (mm day^{-1}), volume of the CL (cm^3), and pregnancy rate (%). Temperature and relative humidity in the stable were recorded each day from day –4 to 4 days after AI. Daily temperature humidity index (THI) values were calculated using the equation given by Mader et al. (14): THI = 0.8 × ambient temperature + [(% relative humidity ÷ 100) × (ambient temperature – 14.4)] + 46.4.

The cows were considered to be exposed to heat stress when THI values exceeded 74. In addition, body temperature was recorded in the morning and afternoon for the same period of time as in THI. The parameters DPF0, DFP1, and volume of CL were measured by real-time ultrasonography (Medison SonoVet 2000, 7.5 MHz, linear-array transducer; Universal Medical Systems Inc., Bedford Hills, NY, USA). The diameter of the largest follicle was calculated by the average of horizontal and vertical measurements, while the volume of CL was calculated directly via ultrasound. The location of the largest follicle at CIDR removal was recorded and its diameter was measured once again at estrus detection. The growth rate of the largest follicle was calculated taking into consideration the difference in size between DFP0 and DFP1 and the time from CIDR removal to estrus detection. The pregnancy test was performed 33 days after AI by ultrasonography.

2.7. Statistical analysis

Of the total initial 62 cows, only 52 showed estrus and were therefore considered in the analysis. The number of cows that completed the study for each of the treatments was control = 13, VCG =10, VCE = 12, and VCEG = 17. All measured variables were subjected to analysis of variance under the following mixed linear model:

$$Y_{ijk}=\mu+T_i+C_j+b_1(X_{ijk}-\overline{X}_{ijk})+e_{ijk}$$

where μ is the overall mean, T_i is the effect of the ith treatment (i = control, VCG, VEG, VCEG); C_j is the random effect of the jth cow ($j = 1,\ldots,52$) ~ NI $(0,\sigma_e^2)$, $b1$ is the respective coefficient of linear effect of milk production level (X); < 35, 35–40, and >40 L day^{-1} for low, medium, and high level, respectively, and e_{ijk} is the residual ~ NI $(0, \sigma_e^2)$.

Covariates that did not show significant effects ($P > 0.05$) were deleted from the final model. Means for the main effect were calculated by least squares. The variables were analyzed using the Glimmix procedure of the statistical package SAS (Statistical Analysis System, version 9.3). In the case of variables considered with normal distribution, such as DFP0, DFP1, time to estrus after CIDR removal, growth rate of the largest follicle, and CL volume, the function link used was identity. The variable pregnancy rate was analyzed considering a binary distribution and using logit as the function link. In all cases, $P \leq 0.05$ was considered significant. Means of all variables were compared using Tukey's test.

3. Results

The study evaluated the effect of vitamin C and E injections on ovarian follicle structure development during synchronized estrus as well as pregnancy rates in Holstein cows under heat stress conditions. Table 1 shows calculated THI and measured body temperature values. In general, the parameters DFPO, DFP1, time of estrus after CIDR removal, growth rate of the largest follicle, and CL volume were 14.6 ± 0.57 mm, 17.8 ± 0.54 mm, 52.2 ± 2.59 h, 1.8 ± 0.24 mm day^{-1}, and 9.3 ± 0.79 cm^3, respectively. Overall pregnancy rate was 12.8 ± 0.04%. The effects of the different treatments on each of these variables are shown in Table 2 and the Figure.

Table 1. Microclimate conditions in the stable and mean body temperatures (±SE) of cows during the experimental period.

Days	Daily temperature °C	Relative humidity %	THI*	Morning body temperature °C	Afternoon body temperature °C	Mean body temperature °C
–4	34.1	26.7	79.0	38.7 ± 0.09	39.7 ± 0.10	39.2 ± 0.09
–3	38.5	23.2	82.8	38.9 ± 0.11	39.8 ± 0.10	39.5 ± 0.10
–2	36.6	23.5	80.9	38.9 ± 0.09	39.8 ± 0.10	39.3 ± 0.09
–1	36.0	25.0	80.6	38.9 ± 0.09	39.9 ± 0.10	39.4 ± 0.09
0	41.3	25.7	86.3	39.2 ± 0.09	39.9 ± 0.10	39.5 ± 0.09
1	38.5	25.0	83.2	38.9 ± 0.09	39.9 ± 0.10	39.4 ± 0.09
2	38.0	25.5	82.8	39.3 ± 0.09	39.8 ± 0.10	39.6 ± 0.09
3	41.5	23.5	85.9	39.0 ± 0.09	39.8 ± 0.10	39.4 ± 0.09
4	41.5	23.25	85.9	39.1 ± 0.09	40.0 ± 0.10	39.5 ± 0.09
5	40.2	23.0	84.4	39.0 ± 0.09	40.0 ± 0.10	39.5 ± 0.09
6	38.7	28.7	84.4	38.9 ± 0.09	39.7 ± 0.10	39.3 ± 0.09
7	28.8	47.7	76.3	38.8 ± 0.09	39.4 ± 0.10	39.1 ± 0.09

*Temperature humidity index. THI = 0.8 × ambient temperature + [(% relative humidity ÷ 100) × (ambient temperature – 14.4)] + 46.4.

Table 2. Least square means (±SE) for the effects of injecting vitamins C (VCG) and E (VCE) and their combination (VCEG) on follicular and corpus luteum development and estrus presentation in Holstein dairy cows under heat stress conditions.

Variables	Treatment				
	Control	VCG	VEG	VCEG	P-value
Follicular and corpus luteum development					
Diameter of the largest follicle at CIDR removal (mm)	13.0 ± 1.0	15.2 ± 1.1	15.6 ± 1.0	15.0 ± 0.9	0.22
Diameter of the largest follicle at estrus detection (mm)	17.5 ± 1.1	18.0 ± 1.2	18.0 ± 1.1	17.9 ± 1.0	0.99
Growth rate of the largest follicle (mm day^{-1})	2.3 ± 0.4	1.5 ± 0.5	1.6 ± 0.5	1.6 ± 0.4	0.61
Volume of the corpus luteum (cm^3)	10.4 ± 1.6	8.1 ± 1.7	9.6 ± 1.7	9.1 ± 1.4	0.81
Estrus detection					
Time of estrus after CIDR removal (h)	59.2 ± 5.1	50.7 ± 5.6	49.5 ± 5.3	49.3 ± 4.8	0.47

Within rows, means with different superscripts are significantly different (P < 0.05).

The experimental units (cows) behaved similarly in terms of the evaluated variables, regardless of the experimental group. Although cows injected with both vitamins showed estrus 10 h earlier and 10% higher pregnancy rates compared to animals in the control group, these differences were not statistically significant (P > 0.05).

4. Discussion

Heat stress, as a result of high THI values, causes an increase in normal body temperature. The body temperatures of the cows used in the present study were similar to those reported by Srikandakumar and Johnson (15), who studied cows under heat stress conditions. We therefore assume that the animals in our study were subject to heat stress.

The mechanism by which heat stress diminishes fertility in dairy cattle is not fully understood, but abnormal follicle and CL development, low quality oocytes, and high embryo mortality could be considered the leading factors. To the best of our knowledge, there is no information regarding the effects of supplementing vitamin C and E on preovulatory follicle development in cows under heat stress. However, early onset of estrus in cows receiving vitamins has also been reported in ewes supplemented with

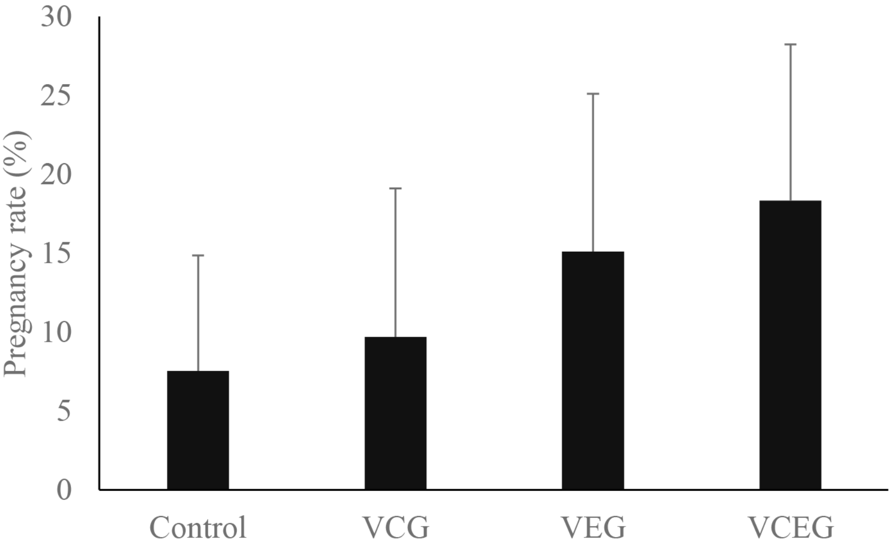

Figure. Influence of injecting vitamins C (VCG) and E (VCE) and their combination (VCEG) on pregnancy rates (33 days after AI) in Holstein dairy cows under heat stress conditions.

vitamin E (16), indicating that vitamin supplementation positively affects some endocrine processes controlling its onset.

The low fertility observed in Holstein dairy cattle under heat stress may be a consequence of ovaries carrying a preovulatory follicle with reduced oxidative status (17). However, this scenario could be reversed by vitamin C and E. On one hand, vitamin C is necessary for normal follicle development (18), and supplementation of this vitamin increases the follicle diameter in a dose-dependent manner (19). However, in this study, vitamin C supplementation did not significantly affect the diameter of the preovulatory follicle. A likely explanation for this may be the small sample size; in addition, the dose of supplemented vitamin C was probably not sufficient to alter the preovulatory follicle diameter.

On the other hand, the effect of vitamin E on fertility is mediated by a direct antioxidant effect on the follicle (20). This is noteworthy, since heat stress impairs fertility by follicle and oocyte disruption (17), probably through inducing oxidative damage (21). Since vitamin C is necessary for reactivating vitamin E functionality (22), these vitamins may act together or separately to improve follicle functionality and oocyte quality.

The suppressed luteal function (low progesterone) caused by heat stress may be responsible for the low fertility in dairy cattle (9). In order to improve fertility in dairy cattle under heat stress, an increase in progesterone production by increasing CL size seems logical. Previous studies have shown a positive correlation between vitamin C supplementation and CL diameter, progesterone concentration (23), and pregnancy rate (24). With regard to vitamin E, there is little information about its effect on CL functionality, but Vierk et al. (25) demonstrated that vitamin E supplementation protects the CL from apoptosis. In the present study, the supplementation of vitamin C and E did not improve the volume of the CL, but it is possible that progesterone production could be affected. However, since we did not measure progesterone concentrations, we cannot confirm such an asseveration.

To the best of our knowledge, there is no previous evidence of the impacts of vitamin C or E supplementation on dairy cattle under heat stress conditions. However, in chickens (26), rabbits (27), and rats (20) under heat stress, supplementation of these vitamins improved reproductive performance.

The results show that vitamin C and E supplementation did not significantly improve pregnancy rates in cows suffering heat stress. The reason for this could be the small sample size used in this study. According to McIntosh (28), several injections of vitamin C are required to improve fertility in cattle. On the other hand, blood concentrations of vitamin E are increased for up to 7 days after parenteral injection (29), and we consider that this period of time is sufficient to affect fertility. The reason for supplementing 3000 IU of vitamin E originates from previous field experience. On the other hand, the dose of vitamin C was chosen based on previous research (30).

The first injections of vitamin C and E were carried out with the objective of protecting both follicle development and oocyte quality. The decision to inject vitamin C at estrus detection was based on results from past research that reported an increase in vitamin C concentrations at this time (30). The third injection of vitamin C was performed

2 days after AI and intended to protect the embryo from heat stress as well as to support CL development.

In conclusion, the supplementation of vitamin C and E before and after synchronized estrus under heat stress conditions did not affect preovulatory follicle or corpus luteum development or pregnancy rates in Holstein cows. However, the reliability of these results is limited by the small sample size. Furthers studies evaluating the effects of vitamin C and E supplementation on reproductive performance in cows under heat stress conditions should therefore include larger numbers of animals to obtain reliable results.

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