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Original Contribution

SUPEROVULATORY RESPONSE IN DAIRY COWS AFTER APPLICATION OF ECG

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ABSTRACT

PURPOSE: The aim of the present study was to determine the follicular dynamics and superovulatory response in dairy cows after the administration of eCG.

METHODS: The study included 6 Holstein cows bearing CL in the ovaries treated with PGF2α for estrus induction. After estrus detection daily ultrasound scanning to monitor the dominant follicle of the first follicular wave was made. Superovulation treatment (2500 IU eCG and two doses of 500μg PGF2α analog on the 48th and 60th hour after the eCG injection) started at the emergence of the second follicular wave. The number and diameter of the follicles ≥ 5 mm, the number of corpora lutea and anovulatory follicles (AF) and the ovulation rate (OR) were estimated.

RESULTS: At the start of the superovulation treatment (SO) the average number and diameter of the medium and large follicles were 2.7±1.2 and 0.67±0.13 cm and 1.6±0.5 and 1.45±0.28 cm, respectively. There were high positive correlations between the days of the treatment and number of large and medium follicles (r = 0.80, P = 0.018 and r = 0.75, P = 0.03). The average number of large follicles on day 5 was greater than those at the start of SO. On day 8 the OR was 55.9% and the difference between anovulatory follicles and corpora lutea was not significant.

CONCLUSIONS: The used protocol provided acceptable OR, but the average number of AF (44.1%) remained high. Ultrasonography could be used to monitor the first dominant follicle and the emergence of a new follicular wave.

Key words: cattle, eCG, superovulation, follicular dynamics, follicle diameter

INTRODUCTION

Embryo transfer (ET) is an important assisted reproductive procedure that represents the collection of one or more embryos from a valuable female (donor), and transfer to one or more less valuable females (recipients) (1). This technique allows relative faster genetic improvement in comparison with conventional artificial insemination (AI), prevention of inbreeding depression and also preservation of endangered domestic and wild animal species and breeds (2, 3). The procedure is vastly commercially used in cattle (4), buffalo (5), horses (6) and sheep (7), and is getting more popular in other domestic animals like donkeys (8) and camelids (9).

A successful ET procedure requires the selection of superior genetic or phenotypic donor animals, stimulation of follicle growth and development by administration of gonadotropins, estrus induction after application of prostaglandin analog, artificial insemination and collection of embryos 7 days post insemination. The procedure could finish with fresh embryo transfer or freezing of collected embryos (10).

The main purpose of superovulation (SO) is to stimulate development, and ovulation of more than one follicle by administration of hormonal products with follicle-stimulating hormone (FSH) activity (11). The first modern protocols

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for induction of SO response were based on the application of equine chorionic gonadotropin (eCG) while later on porcine FSH (pFSH) was isolated and introduced in more recent protocols (12).

The eCG also known as pregnant mares serum gonadotrophin (PMSG) is a complex glycoprotein heterodimer which consists of conjoined glycosylated α and β chains, with the stronger LH activity in the mare and FSH activity in the other domestic non-equid species. It has a half-life of more than 40 h, could be detected in the body for up to 10 days in cows, and a single application induces effective SO (13).

One of the main problems with gonadotrophin preparations used for superovulation is the high variability and unpredictability of follicular response between individuals (13, 15). Although application of pFSH has resulted in more satisfactory and predictable follicular development in comparison with an eCG, the use of the last-mentioned for SO treatment shouldn't be neglected. The advantages of eCG are that it can be found at a reasonable price relatively easily in countries where pFSH is not provided (16). Also, the eCG can be applied as a single dose compared with the multiple injections normally required when using pituitary preparations (16, 17).

The aim of the present study was to determine the follicular dynamics and superovulatory response in Holstein cows, after application of eCG, at the emergence of the second follicular wave.

MATERIAL AND METHODS

Animals

The study was carried out with 6 Holstein cows, bodyweight 540-620 кg, 4-7 years old, and body condition score between 3 and 3.5 (18), within a period from June 2021 to May 2022. The animals were housed in the Academic technological complex and Biobase, Faculty of Veterinary Medicine, Trakia University, Stara Zagora (42° 25' 31" N, 025° 38' 05" E). The daily mixed ratio consisted of 8 kg of concentrate forage for lactating animals, 5 kg of alfalfa hay, 4 kg of meadow hay and 20 kg of maize silage. Clear drinking water was provided *ad libitum*. All animals were subjected to clinical evaluation and rectal palpation, and those with normal general health conditions and absences of abnormalities of the reproductive organs were included.

The experiment was done according to the recommendations of the Animal Ethics Committee, Faculty of Veterinary Medicine, Trakia University – Stara Zagora and in compliance to the Ordinance of the Ministry of Agriculture and Food on the minimum requirements for the protection and welfare of experimental animals (Ordinance 20/1.11.2012 of the Ministry of Agriculture, Food and Forestry).

Ultrasound examination and superovulation protocol of treatment

The experimental animals were subjected to transrectal ultrasound examination of the uterus and ovaries, with the ultrasound device SonoScape S2Vet (SonoScape Co. LTD, Shenzhen, China), equipped with multifrequency (5-11 MHz) linear transducer. As main selection criteria were used the presence of matured a fully developed corpus luteum with good blood supply on one of the ovaries (**Figure 1A**), and the absence of pathological uterine content. After the formation of the experimental group, all cows were treated with intramuscular (IM) injection of 500μg PGF2α analog (Cloprostenol,Veyx® forte, Veyx-Pharma GmbH Soehreweg, Schwarzenborn, Germany) and subjected to daily observation for expression of estrus behavior and ultrasound scanning of the ovaries. The follicular wave pattern of all cows was determined through the monitoring of cohort development and growth of the dominant follicle of 1st follicular wave. The diameter of the largest and subordinate follicles was measured with the aid of built-in; on-screen calipers and ultrasound machine software. Based on that the phases of a dominant follicle dominance, atresia and emergence of the 2nd follicular wave were determined. As a phase of dominance was accepted moment when two identical diameters of the largest follicle were measured in two successive examinations, and the phase of atresia was determined when the second measurement revealed a smaller diameter in comparison with the previous one. Immediately after the detection of atresia of the dominant follicle of the first follicular wave (day 0) superovulation (SO) treatment protocol was initiated by a single IM application of 2500 IU eCG (Folligon®, MSD, Animal Health), followed by IM injection of two doses 500μg PGF2α analog (Cloprostenol, Veyx® forte,

Veyx-Pharma GmbH Soehreweg, Schwarzenborn, Germany) 12 h. apart (on the 48th and 60th hour after the start of SO protocol) **(Figure 2)** (16). Animals were kept under observation for estrus clinical signs (increased tonicity of the uterus and plentiful, translucent, watery cervical mucus) and were subjected to daily ultrasound monitoring of the follicular dynamics continued up to the 8th day after the start of the protocol. The number and diameter of all follicles larger than 5 mm. were estimated and based on that were categorized as medium size (≥ 5 mm. 9 mm. ≤) and large size (≥ 10 mm.) follicles (**Figure 1B**). Two days after the expression of estrus clinical signs (day 6 of the protocol) the number of ovulations was determined (**Figure 1C**), and the number of corpora lutea, and anovulatory follicles was finally estimated on the 8th day of the treatment protocol (**Figure 1D**). Ovulation rate (OR) was calculated based on the formula: OR = (number of corpora lutea on the 8th day/number of follicles in estrus) x 100.

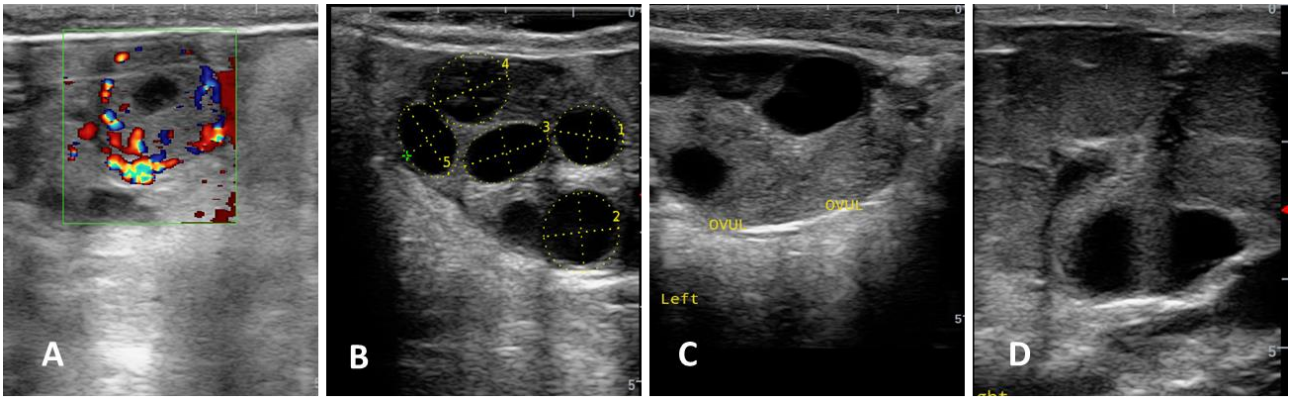

Figure 1. Ovarian ultrasonography. A: Color flow Doppler imaging of a functional corpus luteum with good blood supply. B: Large and medium size follicles on day 4 of the protocol. C. Two detected ovulations and anovulatory follicles on day 6 of the protocol. D: Three corpora lutea and two anovulatory follicles on day 8 of the protocol.

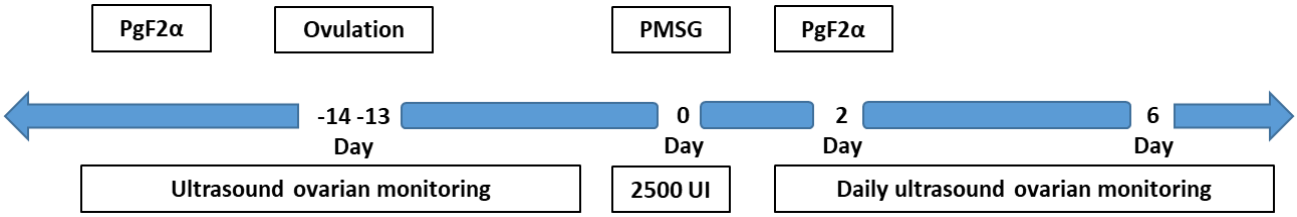

Figure 2. Treatment protocol for superovulation.

Statistical Analysis

The values of different parameters were presented as mean ± standard deviation (mean ± SD). The data were processed by statistical software Statistica version 10.0 (Stat-Soft. Inc. Tulsa, OK, USA) by means of ANOVA and nonparametric analysis for comparison of proportions based on Student's t-criterion. Pearson's correlation method was used to correlate the number of large and medium size follicles with the days of the treatment protocol. Values were considered to be statistically significant at $P < 0.05$.

RESULTS

The SO treatment was initiated on the 12.2±0.8 day after preceding ovulation represented the day of atresia of the dominant follicle of the first and emergence of the second follicular wave. The results of studied parameters after ovarian superstimulation with eCG are summarized in **Table 1**. One day before the start of the treatment protocol average number of large and medium size follicles was 1.3±0.5 and 2.7±1.2 with respective diameters of 1.66±0.33 and 0.67±0.13 cm. On day 0 the size of the largest follicle tended to be smaller (1.45±0.28cm.) although the difference was not significant. There was a very strong positive correlation between the days of the treatment protocol and

the number of large size follicles (r = 0.8, P = 0.018). This resulted in a substantial (P < 0.001) increase in the number of follicles larger than 10 mm on day 4. A similar strong positive correlation was registered between the days of the treatment protocol and the size of medium size follicles (r = 0.75, P = 0.03).

On day 5 all treated cows expressed clinical signs of estrus and had significantly more, large size follicles (5.8±2.3, P < 0.01) in comparison with days -1 and 0, with an average diameter of 1.50±0.16 cm. Although it seemed that the treatment protocol resulted in a higher number of anovulatory follicles (5.2±1.5) and a lower number of corpora lutea (3.8±1.3) the difference was not significant. The overall estimated OR on day 8 was 55.9%.

Table 1. *Superovulatory response and ovarian follicular dynamics (large (LF), medium (MF) and total number of follicles (TNF), ovulation and anovulatory follicles) after application of eCG.*

Day of treatment	Number of follicles			Diameter of follicles (cm)		Ovulation	Anovulatory follicles
	Large	Medium	Total	Large	Medium		
Day - 1	1.3±0.5[a]	2.7±1.2	3.0±1.4	1.66±0.33	0.67±0.13		
Day 0	1.6±0.5[a]	2.7±0.6	3.2±1.6	1.45±0.28	0.71±0.14		
Day 1	2.4±0.5[a]	3.0±2.3	5.0±2.0	1.35±0.26	0.65±0.13		
Day 2	2.6±0.5[a]	4.0±1.9	6.6±1.5	1.39±0.09	0.75±0.10		
Day 3	4.0±2.0[a]	4.3±2.2	7.4±2.5	1.42±0.09	0.78±0.15		
Day 4	7.0±2.9[b]	2.0±1.2	8.6±3.4[*]	1.46±0.19	0.95±0.04		
Day 5	5.8±2.3[b]	1.3±0.5	6.8±2.8	1.50±0.16	0.92±0.04		
Day 6	4.0±2.2[a]	1.7±0.6	5.0±1.4	1.39±0.13	0.79±0.16		
Day 8						3.8±1.3	5.2±1.5

[ab]Means within columns with no common superscripts are different (p < 0.05).

[*] TNF on day 4 differs significantly with TNF on day -1 and day 0 (p < 0.01).

DISCUSSION

The individual variability and unpredictability in the number of ovulations, that follow a superovulatiory treatment in cattle, is one of the important limits for the vast application of embryo transfer techniques (19). To be successful conventional protocol for ovarian SO should be initiated between days 8 and 12 of the cycle, which is the period of regression of the dominant follicle of the first and emergence of the second follicular wave (11, 20). The dominant follicle of the first follicular wave reaches its maximal diameter between 6th and 8th day after preceding ovulation, maintains the size for a certain period of time, and undergoes regression at mid-cycle, which is represented by the reduction in size (21). By means of the ultrasonography, we determined the static and regressing phases of the dominant follicle of the first follicular wave, proved by the decreased diameter of the largest follicle on day 0 (1.45±0.28 cm) in comparison with day – 1 (1.66±0.33 cm). Based on that, the SO treatment in our study was initiated on the 12.2±0.8 days after the preceding estrus. Cows injected with 3000 IU eCG on day 9, 12 or 14 of the oestrous cycle and 500 μg PGF2α 48 h later showed the highest SO response in animals treated on day 12 and the lowest in these treated on day 14 of the estrous cycle (22). Initiation of SO treatment on the day of follicular wave emergence is another prerequisite to achieve better SO response (11, 23). Follicular wave emergence in cattle is characterized by the sudden growth of three to six follicles with a diameter of 4 to 5 mm that could be initially detected by ultrasonography (24, 25). According to ultrasound findings in our study regression of the first dominant follicle coincided with the time of emergence of the second follicular wave. This was evidenced by the increased total number of follicles ≥ 5 mm. at the start of SO treatment compared with the previous day. The positive reaction of the experimental animals was evidenced by the registered very strong positive correlation between the days of the treatment protocol and the number of large and medium size follicles on day 4. Although nonsignificant, the decreased number of medium and increased number of large size follicles on day 4 is indicative of the continuous stimulatory effect of eCG on follicle development (13). On day 5 all treated cows expressed clinical signs of estrus and had

significantly more large size follicles (5.8±2.3, $P < 0.01$) in comparison with days -1 and 0, but less large size follicles in comparison with day 4. This decreased number of the large size follicles was a result of ovulations that occurred between ultrasound examinations. The detected ovulations on the 8th day after SO treatment in our study coincided with the results of some authors (16), who found that induced SO with eCG resulted in 2 to 5 ovulations per cow, and differed from results of others (26–29), who reported between 10 and 23 ovulations per cow. This discrepancy could be due to the tendency of eCG to overstimulate the ovary resulting in multiple, unovulated follicles. In cattle, the half-life of eCG was determined to be 45.6 h which allows a single application for induction of SO, but because of long half-life its actions on the ovary continue after superstimulation has been achieved (30, 31). A strategy to overcome that problem is the application of antiserum at 60 h after eCG injection which increases double fold both the number of ovulations and the number of transferable embryos (32). The absence of such antiserum in our SO protocol could explain the relatively higher number of anovulatory follicles and lower number of corpora lutea on the 8th day.

CONCLUSION

The analysis of the obtained data showed that the application of 2500 IU eCG in Holstein cows at the emergence of the second follicular wave induced a good superovulatory follicular response. Ultrasonography could be used to determine the phases of dominance and atresia of the dominant follicle, as well as the emergence of a new follicular wave.

Conflict of Interest

The authors have no conflicts of interest to declare.

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