

# Effect of time of eCG administration on the fate of ovarian follicle in Holstein heifers

Hosseini, A.<sup>1</sup>; Niasari-Naslaji, A.<sup>2\*</sup>; Vojgani, M.<sup>2</sup> and Gharagozloo, F.<sup>2</sup>

<sup>1</sup>Resident of Theriogenology, Department of Theriogenology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran;

<sup>2</sup>Department of Theriogenology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

\*Correspondence: A. Niasari-Naslaji, Department of Theriogenology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. E-mail: niasari@ut.ac.ir

(Received 27 Jul 2017; revised version 7 Oct 2017; accepted 2 Dec 2017)

## Summary

The objective of this study was to investigate the effect of equine chorionic gonadotropin (eCG) on ovarian follicles at three stages of development (emergence, dominance and early static phases) during the first follicular wave (FFW) in Holstein heifers. Heifers (n=20) were randomly assigned into four experimental groups (n=5 in each group). Heifers received eCG (500 IU; Folligon<sup>®</sup>; Intervet, Holland; i.m) a) on the day of follicle emergence (day of ovulation; group 1), b) on the dominant phase (dominant follicle (DF): the first day in which follicle was observed at  $\geq 10$  mm; group 2, and c) on the early static phase (group 3) of the FFW. Control group heifers did not receive any treatment. Daily ultrasonography was conducted to monitor ovarian structure throughout estrous cycle. All treatment group heifers, regardless of the stage of follicle development, displayed follicle growth after eCG injection. Administration of eCG, in group 1, hastened DF detection and induced co-dominant follicles; whereas, in groups 2 and 3, it delayed DF regression, and increased cycle length compared to control. In all treatment group heifers, DF was present 84 h after eCG injection. Maximum diameter of corpus luteum was larger in eCG treated groups compared to control ( $P < 0.05$ ). In conclusion, depending on the time of eCG administration throughout the FFW (emergence, dominant and early static phases), co-dominancy, maintenance of DF, enhancement of follicle and corpus luteum growth and increase in estrous cycle length could be observed in Holstein heifers.

**Key words:** Dominant phase, eCG, Emergence, Holstein heifers, Static phase

## Introduction

Follicle growth in cattle occurs in a wave like pattern (Ginther *et al.*, 1989a). Predominantly, two (Ginther *et al.*, 1989a; Knopf *et al.*, 1989; Ahmad *et al.*, 1997; Burke *et al.*, 2000) and three (Savio *et al.*, 1988; Sirois and Fortune, 1988; Ahmad *et al.*, 1997; Burke *et al.*, 2000) follicular waves were observed throughout estrous cycle. Each follicle wave starts with the recruitment of a cohort of follicles followed by the selection of growing follicle as dominant follicle and regression of subordinate follicles (Savio *et al.*, 1988; Ginther *et al.*, 1989b; Adams *et al.*, 1992; Ginther *et al.*, 2000). Then dominant follicle remained at statistic phase followed by its regression in the presence of corpus luteum (Ginther *et al.*, 1989b).

Several hormonal interventions could affect the fate of the ovarian follicle within follicular wave. The ovarian follicle could be luteinized or ovulated following GnRH administration (Macmillan and Thatcher, 1991; Moghaddam *et al.*, 2001; Souza *et al.*, 2009; Niasari-Naslaji *et al.*, 2012) or regressed subsequent to the administration of regressing agents such as estradiol and progesterone (Bo *et al.*, 1995; Niasari-Naslaji *et al.*, 2001). Within follicular wave, a cohort of ovarian follicles could continue to grow in the presence of follicle stimulating hormone (FSH) throughout superovulation (Bever *et al.*, 1989; Dieleman *et al.*,

1989) or a single follicle might become persistent in the presence of high Luteinizing hormone (LH) pulse frequency induced by low progesterone concentrations (Savio *et al.*, 1993; Niasari-Naslaji *et al.*, 2001, Niasari-Naslaji *et al.*, 2012).

Equine chorionic gonadotropin (eCG) is a glycoprotein molecule, with FSH and LH like activities in non-equid species (Gonzalez-Menico *et al.*, 1978; Newcomb *et al.*, 1979; Murphy *et al.*, 1991; Murphy *et al.*, 2012) with the half-life of 45.6 h in cattle (Schams *et al.*, 1978). The main objectives of using eCG in cattle could be summarized into three categories: a) supporting the growth of follicles in superovulation programs (Monniaux *et al.*, 1983; Goulding *et al.*, 1996), b) enhancing the growth and ovulation of the existing follicle near the end of estrous synchronization programs (Garcia-Ispierto *et al.*, 2012; Núñez-Olivera *et al.*, 2014; Pessoa *et al.*, 2016), following parturition (Rostami *et al.*, 2011; Voigani *et al.*, 2013) and for resynchronization of non-pregnant cows (Bartolome *et al.*, 2012) and c) increasing the diameter of CL and its progesterone production (Souza *et al.*, 2006; O'Hara *et al.*, 2016). We have hypothesized that the administration of eCG at different stages of follicle development could change the fate of the follicle within follicular wave. This, in turn, will enable us to manage the variation of response following eCG administration. The aim of the present

study was to investigate the effect of eCG administration at emergence, dominance and early static phases of the first follicular wave (FFW) in Holstein heifers.

## Materials and Methods

### Location and animals

This study was carried out, between December and January 2015, at the experimental dairy herd of the Veterinary Research Institute, Faculty of Veterinary Medicine, University of Tehran (latitude: 35°39'8"N; longitude: 51°26'38"E; altitude: 1029 m). Healthy cyclic Holstein heifers (n=20; 14 months of age; 350 ± 20 kg LW) were selected for this study. Heifers were housed in an open shed barn and received total mixed ration according to NRC recommendation (NRC, 2001).

### Experimental design

Heifers were synchronized using two doses of PGF<sub>2</sub>α analogue (Cloprostenol; Estroplan<sup>®</sup>, Parnell, Canada) 14 days apart. Following ovulation (day 0 of experiment), heifers were randomly divided into 4 experimental groups (n=5 in each group) and received eCG (500 IU; Folligon<sup>®</sup>; Intervet, Holland; i.m) at different stages of follicular development. Heifers received eCG during the FFW: at the emergence of follicular wave (group 1), at the detection of morphologically dominant follicle (≥10 mm; group 2) and at the second day of static phase (group 3). Control group heifers did not receive any treatment. Ovarian structures (follicle and CL) were monitored daily using ultrasonography for the completion of one estrous cycle.

### Ultrasonography

Daily ovarian ultrasound examinations were conducted, throughout the experiment, using a real time ultrasound scanner (Emperor, V-9; China) equipped with 7.5 MHz linear array trans-rectal transducer. During each examination, the diameter of follicles (≥4 mm in diameter) and CL were recorded using the internal electronic calipers. The horizontal and vertical diameters of ovarian structures were recorded and the average diameters were used for further analysis. New follicle wave emergence was defined as the day in which the dominant follicle was, retrospectively, identified at a diameter of 4-5 mm (Ginther *et al.*, 1989a; Knopf *et al.*, 1989). Morphologically dominant follicle refers to the presence of at least one follicle of ≥10 mm in diameter, originated from new follicular wave. Initiation of static phase was defined when the detected dominant follicle remained at similar diameter for at least two days followed by follicle regression. The day of ovulation was confirmed by the disappearance of the largest follicle followed by the subsequent formation of corpus luteum.

### Statistical analysis

Data were analyzed using GLM procedure including Tukey statement in the model, if assumptions of parametric tests were in place. Otherwise, Kruskal-Wallis procedure was used to analyze the data. Follicle

growth rate was estimated using Proc Rsreg, including Lackfit in the model. In case the lack of fit was not significant, and the linear relationship between time and follicle size was significant, linear regression was used to estimate follicle growth rate. Data were presented as mean±SE of the mean.

## Results

All treatment group heifers that received eCG, at the emergence of follicular wave (group 1), at the detection of dominant follicle (group 2) and at the early static phase (group 3), have shown follicle growth following eCG administration (Figs. 1A-C). Administration of eCG, concurrent with the emergence of follicular wave, did not affect the length of estrous cycle (treatment: 21.5 ± 0.65 days; control: 20.4 ± 0.24 days; Table 1; P>0.05). However, eCG treatment increased the cycle length when it was administrated at the detection of morphologically dominant follicle (22.8 ± 0.49 days) and at the early static phase (22.8 ± 0.73 days) compared to control (Fig. 1D; 20.4 ± 0.24 days; Table 1; P<0.05). All experimental groups displayed two follicular waves throughout estrous cycle.

Administration of eCG on the day of follicle emergence advanced the detection of morphologically dominant follicle (treatment: 3.2 ± 0.25 days vs control: 4.8 ± 0.37 days; P<0.05; Table 1) and induced the growth of two dominant follicles (co-dominance) compared to control group in which single follicle grew and became dominant (Fig. 1A). The follicle growth rate was similar among control (1.2 ± 0.1 mm/day), group 1 (1.6 ± 0.15 mm/day), group 2 (1.6 ± 0.13 mm/day) and group 3 (1.3 ± 0.23 mm/day) heifers (Table 1; P>0.05). Dominant follicle reached at maximum diameter earlier in control (day: 6.4 ± 0.4) and group 1 (day: 6.2 ± 0.48) compared to group 2 (day: 9.8 ± 0.86) and group 3 (day: 9.7 ± 0.85; P<0.05; Table 1). The maximum diameter of the first wave dominant follicle was smaller in control (12.3 ± 0.49 mm) and group 1 (13.7 ± 0.73 mm) than in group 2 (18.3 ± 0.47 mm) and group 3 (17.3 ± 0.65 mm; P<0.05; Table 1).

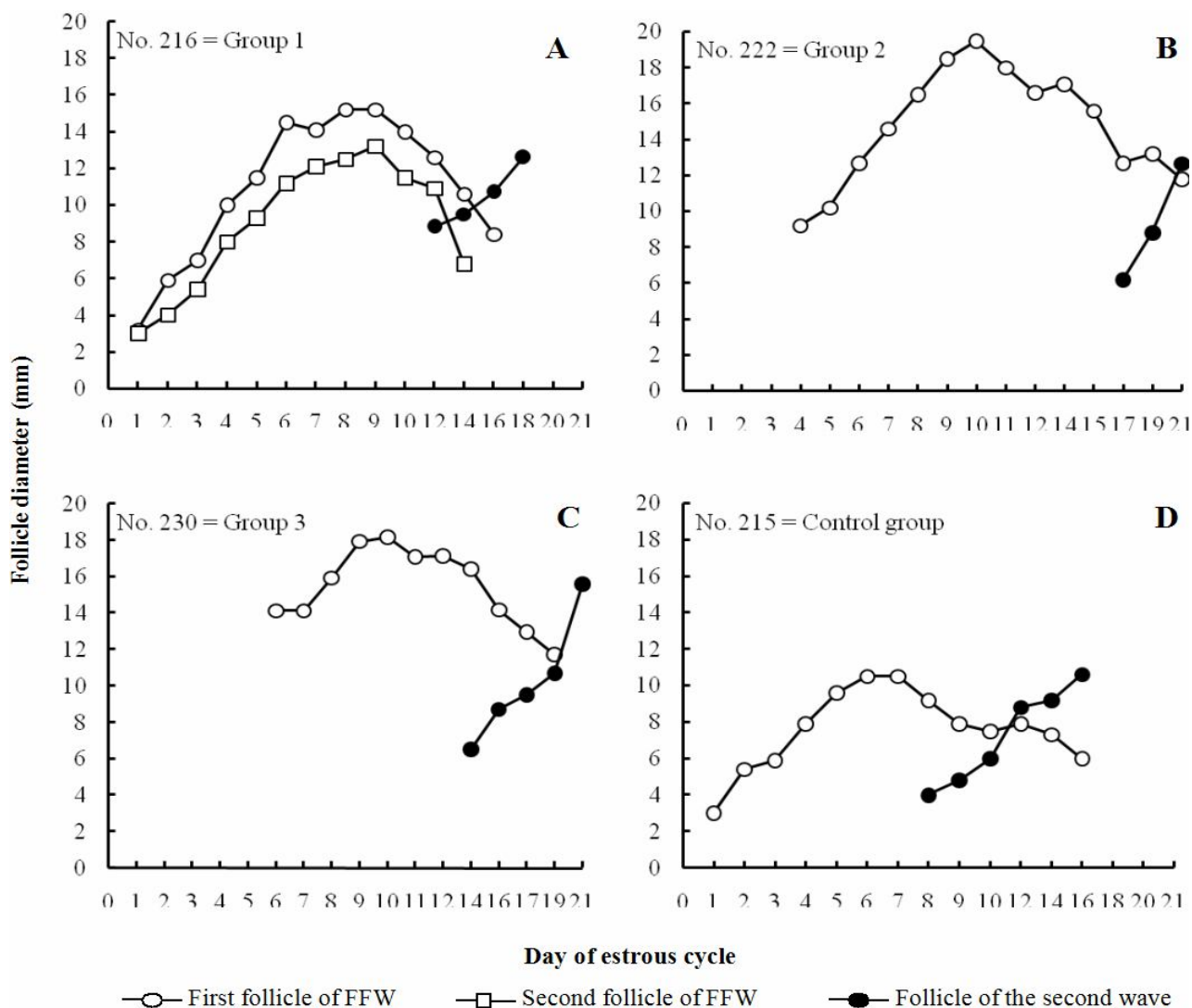
The day of the first wave dominant follicle regression was delayed following administration of eCG on dominant (group 2: 12.4 ± 0.75) and early static (group 3: 13.7 ± 1.31) phases compared to the heifers that received eCG on the day of follicle emergence (group 1: 9.2 ± 0.25) and control group (9.2 ± 0.37; P<0.05; Table 1). The emergence of the second follicular wave was delayed in group 2 (13 ± 0.89) and 3 (13 ± 0.63) heifers compared to control (7.6 ± 0.24) and group 1 (9) heifers (P<0.05; Table 1).

Almost 84 h after eCG administration, the diameter of ovarian follicle was significantly different between treatment groups and control counterparts (control: 8.4 ± 0.36 mm vs group 1: 10.6 ± 0.33 mm, P<0.05; control: 11.6 ± 0.98 mm vs group 2: 16.9 ± 0.13 mm, P<0.01; control: 9.1 ± 0.78 mm vs group 3: 17.1 ± 0.58 mm, P<0.001; Fig. 2).

The maximum diameter of corpus luteum was

significantly larger in treated groups (group 1:  $25.3 \pm 1.03$  mm, group 2:  $24.3 \pm 1.19$  mm, group 3:  $25.5 \pm 0.94$  mm) compared to control ( $21.2 \pm 0.081$ ;  $P < 0.05$ ; Table

1; Fig. 3). The day in which corpus luteum reached to the maximum diameter was delayed in treated groups compared with control ( $P < 0.05$ ; Table 1).

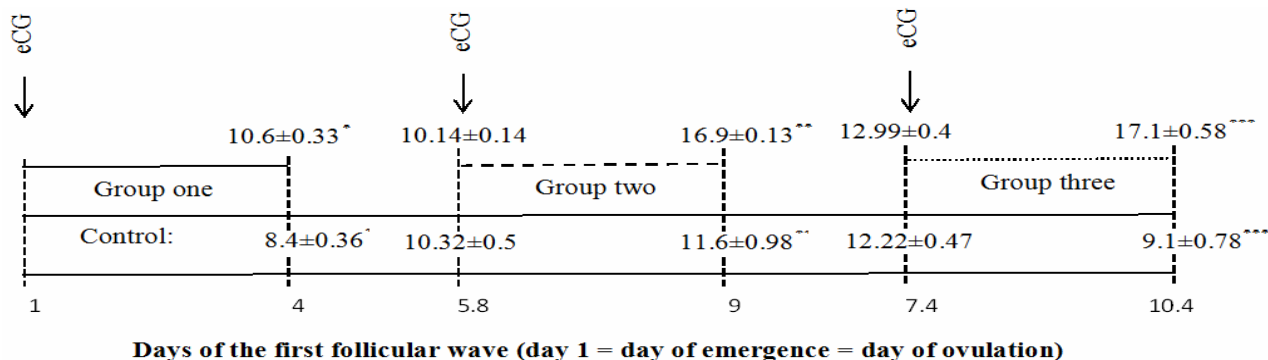


**Fig. 1:** Follicle growth pattern throughout estrous cycle in a representative heifer belonged to the group of Holstein heifers that received eCG (500 IU) throughout the FFW: on the day of follicle emergence (group 1; n=5; **A**), at dominant phase (group 2; n=5; **B**) and at early static phase (group 3; n=5; **C**), or remained untreated as control (n=5; **D**)

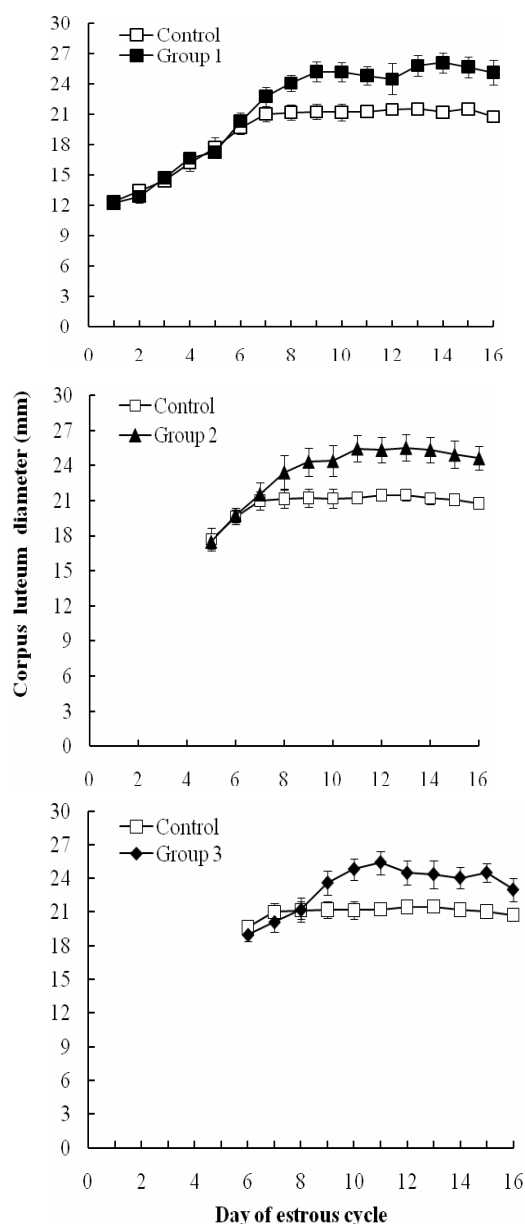
**Table 1:** Follicular dynamics and corpus luteum formation in a group of Holstein heifers that received eCG (500 IU, IM) throughout the FFW: on the day of follicle emergence (group 1; n=5), at the dominant phase (group 2; n=5) and at the early static phase (group 3; n=5) compared to control (n=5). Data were presented as mean±SEM

Parameters	Control	Group 1	Group 2	Group 3
<b>Estrus cycle</b>				
Length	20.4 ± 0.24 <sup>a</sup>	21.5 ± 0.65 <sup>ab</sup>	22.8 ± 0.49 <sup>b</sup>	22.8 ± 0.73 <sup>b</sup>
Number of follicular wave	2	2	2	2
<b>Follicle growth rate (mm/day)</b>				
First wave follicle growth rate	1.2 ± 0.1 <sup>a</sup>	1.6 ± 0.15 <sup>a</sup>	1.6 ± 0.13 <sup>a</sup>	1.3 ± 0.23 <sup>a</sup>
Second wave follicle growth rate	1.1 ± 0.08 <sup>ab</sup>	0.8 ± 0.13 <sup>a</sup>	1.4 ± 0.11 <sup>b</sup>	1.1 ± 0.07 <sup>ab</sup>
<b>Follicle characteristics</b>				
Day of DF detection	4.8 ± 0.37 <sup>a</sup>	3.2 ± 0.25 <sup>b</sup>		
Maximum diameter of the first wave DF	12.3 ± 0.49 <sup>a</sup>	13.7 ± 0.73 <sup>a</sup>	18.3 ± 0.47 <sup>b</sup>	17.3 ± 0.65 <sup>b</sup>
The day in which the first wave DF reached at maximum diameter	6.4 ± 0.4 <sup>a</sup>	6.2 ± 0.48 <sup>a</sup>	9.8 ± 0.86 <sup>b</sup>	9.7 ± 0.85 <sup>b</sup>
Day of the second follicle wave emergence	7.6 ± 0.24 <sup>a</sup>	9 <sup>a</sup>	13 ± 0.89 <sup>b</sup>	13 ± 0.63 <sup>b</sup>
Day of follicle regression	9.2 ± 0.37 <sup>a</sup>	9.2 ± 0.25 <sup>a</sup>	12.4 ± 0.75 <sup>b</sup>	13.7 ± 1.31 <sup>b</sup>
<b>Corpus luteum characteristics</b>				
Maximum CL diameter	21.2 ± 0.81 <sup>a</sup>	25.3 ± 1.03 <sup>b</sup>	24.3 ± 1.19 <sup>b</sup>	25.5 ± 0.94 <sup>b</sup>
The day in which CL reached at maximum diameter	6.8 ± 0.37 <sup>a</sup>	8.2 ± 0.25 <sup>b</sup>	9.2 ± 0.25 <sup>b</sup>	9.2 ± 0.37 <sup>b</sup>

<sup>ab</sup> Values within rows with different superscripts differ ( $P < 0.05$ )



**Fig. 2:** Experimental design and the diameter of ovarian follicle (mean±SEM) for the group of Holstein heifers that received eCG (500 IU): on the day of follicle emergence (group 1; n=5; \* P<0.05), at dominant phase (group 2; n=5; \*\* P<0.01) and at early static phase (group 3; n=5; \*\*\* P<0.01), or remained untreated as control (n=5)



**Fig. 3:** Corpus luteum diameter (mm) throughout estrous cycle in the group of Holstein heifers that received eCG (500 IU) throughout the FFW: on the day of follicle emergence (group 1; n=5), at dominant phase (group 2; n=5) and at early static phase (group 3; n=5) compared to control (n=5)

## Discussion

The objective of the present study was to evaluate the effect of eCG, prescribed at different times within the FFW, on follicle dynamics and corpus luteum formation in Holstein heifers. In the present study, regardless of the stage of follicle development, eCG did not affect the number of follicular wave, but induced follicle growth. The enhancing effect of eCG on follicle growth was evident throughout superovulation (Monniaux *et al.*, 1983; Goulding *et al.*, 1996) and estrous synchronization (Sa Filho *et al.*, 2010; Garcia-Isperto *et al.*, 2012; Pessoa *et al.*, 2016) programs. The FSH and LH like effects of eCG is implemented through its attachment to FSH and LH receptors in theca and granulosa cells (Gonzalez-Menico *et al.*, 1978; Soumano *et al.*, 1998; Murphy, 2012).

In the present study, injection of eCG, concurrent with the emergence of the FFW supported the simultaneous growth of two follicles. This is consistent with the result of the previous study in which eCG administration prior to the follicle selection (day 3 after ovulation) could enhance multiple follicle development in a dose dependent manner (O'Hara *et al.*, 2016). Accordingly, 250 and 500 IU eCG may not be able to induce co-dominancy; whereas, 750 and 1000 IU of eCG could induce twin follicle growth (O'Hara *et al.*, 2016). The result of the present study indicated that 500 IU eCG, administered at the day of follicle emergence, is sufficient to induce co-dominancy. Therefore, the amount and the time of eCG administration are important factors for co-dominancy of ovarian follicles in cattle.

Administration of eCG at dominant (group 2) and early static (group 3) phases of follicular wave delayed follicle regression and emergence of the second follicle wave subsequent with an increase in the length of estrus cycle (group 2:  $22.8 \pm 0.49$ ; group 3:  $22.8 \pm 0.73$  days). This is in agreement with the previous study in which high doses of eCG (750-1000 IU) on day 3 of the estrous cycle could delay the emergence of the second follicular wave and increase estrous cycle length (O'Hara *et al.*, 2016). In contrast, eCG administration concurrent with the emergence of the FFW, did not affect estrous cycle length ( $21.5 \pm 0.65$  days) and was similar to control ( $20.4$

$\pm 0.24$  days). The estrous cycle length of the latter two groups was similar to the mean length of estrous cycle in heifers with two dominant follicles ( $20.5 \pm 1.3$  days; Savio *et al.*, 1988). Several factors could affect the length of estrous cycle such as number of follicular waves (Ginther *et al.*, 1989a) and life span of the CL (Ginther, 1970; Sirois and Fortune, 1990; Wiltbank *et al.*, 2002; Niasari-Naslaji *et al.*, 2012; O'Hara *et al.*, 2016). Heifers with three follicular waves have longer estrous cycle compared to those with two waves (Ginther *et al.*, 1989a; Taylor and Rajamahendran, 1991; Adams, 1999). Heifers at the first estrus after reaching puberty (Gonzalez-Padilla *et al.*, 1975) and in dairy cows at the first ovulation following parturition (Odde *et al.*, 1980) are more likely to have short estrous cycle due to the lack of progesterone priming (Inskeep *et al.*, 1988). It is possible to induce short estrous cycle in cattle, by injecting prostaglandin  $F_2\alpha$  analogues (Taponen *et al.*, 2002). In the present study, injection of eCG, at dominant or early static phases, increased cycle length. Similar phenomena could occur when progesterone concentrations are maintained between 1 and 2 ng/ml, resulting in the prolongation of follicular dominance and increase in cycle length (Sirois and Fortune, 1990).

The growth rate of follicle within the FFW was recorded as  $1.2 \pm 0.1$ ,  $1.6 \pm 0.15$ ,  $1.6 \pm 0.13$  and  $1.3 \pm 0.23$  in control, groups 1, 2 and 3, respectively ( $P > 0.05$ ). These follicle growth rates in Holstein heifers are within the range reported in the previous studies (Ginther *et al.*, 1989b; Sirois and Fortune, 1990).

The maximum diameter of dominant follicle (DF) was greater and the day in which DF was detected was delayed when eCG was administered at dominant ( $18.3 \pm 0.47$  mm;  $9.8 \pm 0.86$  days) and early static ( $17.3 \pm 0.65$  mm;  $9.7 \pm 0.85$ ) phases compared to eCG administration at follicle emergence ( $13.7 \pm 0.73$ ;  $6.2 \pm 0.48$  days) and control ( $12.3 \pm 0.49$  mm;  $6.4 \pm 0.4$  days;  $P < 0.05$ ). The maximum diameter of the first wave ovarian follicle in Holstein heifers was reported to be  $13.6 \pm 0.8$  mm (Sirois and Fortune, 1988) and  $10.78 \pm 0.55$  mm (Niasari-Naslaji *et al.*, 2012), which is similar to the diameter of follicle in group 1 and control but is less than those in group 2 and 3 in the present study. In many studies, the day in which DF reached at the maximum diameter was around day 6 and 7 (Ginther *et al.*, 1989b; Sirois and Fortune, 1990), which is similar to control and group 1 but is less than in groups 2 and 3.

In this study, eCG administration on the day of follicle emergence hastened early detection of DF (day:  $3.2 \pm 0.25$ ) compared with control (day:  $4.8 \pm 0.37$ ). This clearly indicates that the effect of eCG on follicle dynamics depends on the stage of follicle development within follicular wave. Equine chorionic gonadotropin could maintain the growth of the DF for a longer time even in the presence of functional CL; whereas, it is well documented that the growth rate and maximum diameter of ovarian follicle is suppressed via the progesterone negative feedback (Sirois *et al.*, 1988, 1990).

Present results showed that maximum CL diameter in treatment groups was significantly larger than that in

control group. In addition, in all treatment groups the day in which maximum diameter of CL appeared was later than control. Conclusively, administration of eCG at different stages of the FFW could participate in further luteal development. This finding is in accordance with the previous study in which the administration of eCG on day 3 resulted in dose-dependent rise in CL diameter and progesterone production (O'Hara *et al.*, 2016). Equine chorionic gonadotropin might exert the direct effect on theca and granulosa cells (Pulley *et al.*, 2013) and/or gene expression regulation of prolactin receptors, leading to CL volume expansion and progesterone synthesis (Fatima *et al.*, 2012; Rigoglio *et al.*, 2013).

In conclusion, administration of eCG on various stages of the follicle development within the FFW could result in different outcomes including: co-dominancy, when it is administered on the day of follicle emergence, persistence of follicle growth, delayed follicle regression and increased cycle length, when it is administered at dominant and early static phases. Moreover, eCG could increase CL diameter, when it is administered at any stage of follicle development. Furthermore, morphologically dominant follicle could be present 84 h following eCG administration. Accordingly, further research is underway in our research group to incorporate eCG throughout pre-synchronization programs in cattle.

## Acknowledgements

The authors express their sincere appreciation for the financial support of the Deputy for Research of University of Tehran and the kind assistance of the staff of the dairy farm within the Institute of Veterinary Research, at the Faculty of Veterinary Medicine, University of Tehran.

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