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Effects of blood β-hydroxybutyric acid levels on the response to Ovsynch in primiparous cows

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Abstract

The aim of this study was to investigate the effects of low and moderate blood β-hydroxybutyric acid (BHBA) levels in primiparous cows during the early postpartum period on follicle development and pregnancy rate after the Ovsynch protocol. Holstein primiparous cows with low (n = 85) and moderate (n = 71) BHBA concentrations were used as experimental animals. At 7-8 weeks postpartum, blood samples were collected and the Ovsynch protocol was performed on the same day. Ultrasonographic examination was performed to determine the dominant follicle (DF) diameter and presence of corpus luteum on the day of the first and second gonadotropin releasing hormone (GnRH1 and GnRH2) injections and prostaglandin F2 alpha (PGF2α) injection as well as 24 and 48 h after the GnRH1 and PGF2α injections. The ovulatory response to GnRH1 and pregnancy rate at day 30 and day 60 post-insemination were also evaluated. No significant differences were observed between groups in the DF diameter on the day of GnRH1 and PGF2α injections, and at 24 and 48 h after these injections, as well as in the ovulation and atresia response to GnRH1 ($P > 0.05$). Significant differences were observed between groups in DF diameter (low-BHBA group, 13.8 ± 0.2 mm; moderate-BHBA group, 13.2 ± 0.2 mm; $P = 0.039$) at the time of insemination, and in pregnancy rates at 30 days (low-BHBA group, 38.82%; moderate-BHBA group, 21.13%, $P = 0.017$) and 60 days after insemination (37.6 and 18.3%, respectively; $P = 0.008$). Our findings suggest that moderate blood levels of BHBA (0.80-1.19 mmol/L) in primiparous cows during the early postpartum period affect the response to the Ovsynch protocol.

Keywords: β-hydroxybutyric acid, cow, follicle diameter, ovsynch, pregnancy rate.

Introduction

The calving interval is a prominent factor in the economic profitability of dairy farms. Therefore, estrus should be exactly determined and inseminations of animals should be performed on time (Ribeiro *et al.*, 2012). However, it has been reported that the majority of cows have low pregnancy rates at various dairy farms due to a less than 50% detection rate of estrus (Homer *et al.*, 2013) or because of mistakes made during the insemination while cows are in estrus (Barrett *et al.*, 2004). Negative energy balance (NEB)/hyperketonemia is the most frequent problem of high-producing dairy cows due to an increase in milk yield, which leads to a decrease in estrus signs and pregnancy rate due to adverse effects on fertility (Gilmore *et al.*, 2011; Hayırlı and Çolak, 2011). Different synchronization programs have been developed to cope with this adverse effect and these methods have recently become very popular. Maximum efficiency is very crucial in synchronization protocols. Therefore, it is very important to determine the factors that influence the success of synchronization programs (Kasimanickam, 2014).

An NEB characterized with lipolysis in fatty tissue and increased blood concentrations of non-esterified fatty acid (NEFA) and β-hydroxybutyric acid (BHBA) lasts until 10-12 weeks postpartum (Butler, 2003). Cows with postpartum BHBA ≥ 10 mg/dl (0.96 mmol/L) had a lower pregnancy risk (Ospina *et al.*, 2010b) and cows with excessive NEB had a lower pregnancy rate at the first insemination (Walsh *et al.*, 2007). Rutherford *et al.* (2016) stated that cows with subclinical ketosis (postpartum BHBA > 1.2 mmol/L) had lower reproductive performance and exhibited a lower activity around postpartum estrus. However, these studies examined higher BHBA concentrations (≥ 1.2 mmol/L).

Although follicular development depends mainly on gonadotropins and local growth factors, some factors such as feeding-energy balance and lactation (Lucy *et al.*, 1992) can also affect follicular development and oocyte quality (Webb *et al.*, 2004). NEB causes disruption of the hypothalamus-pituitary-ovary axis during early lactation (Butler, 2003), and is associated with decreased pulsatile luteinizing hormone (LH) release (Diskin *et al.*, 2003) and disruption of the luteal activity (Wathes *et al.*, 2007). Cows having higher NEFA and BHBA concentrations were unable to ovulate their first postpartum dominant follicle (Marr *et al.*, 2002). Higher NEFA and BHBA levels can inhibit follicular estradiol secretion, and thus ovulation capacity (Butler, 2005; Butler *et al.*, 2006). Therefore, it was thought that the response of the ovary to exogenous hormone treatment can change according to BHBA concentrations depending on the severity of NEB.

We hypothesized that BHBA levels below 1.2 mmol/L may affect the success of the Ovsynch protocol in lactating primiparous cows. It was expected that BHBA levels below 1.2 mmol/L would negatively affect ovulation response to first gonadotropin-releasing hormone (GnRH) injection, DF diameter at the time of artificial insemination (AI), and pregnancy rate. The aim of this study was to investigate the effects of blood

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BHBA levels of primiparous cows during the early postpartum period on follicle development and pregnancy rate after the Ovsynch protocol.

Material and Methods

Animals, housing and management

The study was conducted on a commercial dairy farm consisting of approximately 1,850 lactating Holstein dairy cows, and located in the Aegean province of İzmir, Turkey (39°04'06.5" N; 27°00'56.1" E). Records of daily milk yield, reproductive health, feeding, and management were maintained regularly for each cow using herd management software (Alpro Windows® 6.93; DeLaval, Tumba, Sweden). All cows were fed three times daily with total mixed ration (TMR), which was prepared according to the National Research Council (NRC) recommendations (National Research Council - NRC, 2001). About 66.7% of the herd consisted of primiparous cows, while the rest were multiparous cows (second and third lactation). The mean milk production over 305 days of the primiparous cows and of the herd was 9,146.95 ± 97.29 kg and 10,488.95 ± 125.65 kg per cow, respectively. All cows were separated from their calves immediately within 2 h until 30 days postpartum and moved into fresh pens. Cows at 30 days in milk (DIM) were moved to a high-producing stall barn. The study design was approved by the Local Ethical Committee on Animal Experiments of Ankara University in Ankara, Turkey (approval no. 2013-14-96).

Primiparous Holstein cows (n = 156) at 7-8 weeks postpartum (52 ± 3 DIM) that were milked three times daily were used as experimental animals. The cows were chosen randomly among healthy animals.

Monitoring of postpartum reproductive health

Cows with a history of parturition-related disorders such as placental retention, uterine prolapse, and genital laceration were excluded from the study. All fresh cows were checked daily for disorders like mastitis, hypocalcemia, displaced abomasum, ketosis, puerperal metritis, and cystic ovarian follicles. Reproductive health examinations were performed regularly once a week after moving to the fresh pen (>30 DIM).

Puerperal metritis was diagnosed by detecting foul-smelling brownish red discharge, enlarged uterus, signs of systemic illness including fever >39.5°C, toxemia, inappetence, and decreased milk yield at <21 DIM. Endometritis was diagnosed by the presence of vaginal cloudy discharge and enlarged uterus with or without other clinical symptoms. All cows were examined using transrectal ultrasonography to check uterine involution after moving from fresh pen to high-producing barn (>30 DIM). Detection of intrauterine fluid accumulation like pus during transrectal ultrasonographic examination was diagnosed as pyometra.

Cystic ovarian follicles (follicular cyst: <3 mm wall thickness; luteal cyst: >3 mm wall thickness) were defined through the detection of follicular structure, with ≥17 mm diameter and lack of CL and uterine tonicity persisting for over 6 days, using transrectal ultrasonography and rectal palpation (Jeengar *et al.*, 2014). When follicles with diameter ≥17 mm were detected, findings of examination were noted and a second examination was subsequently performed after 7 days.

Blood sampling and determination of blood BHBA levels

In order to determine blood BHBA levels, blood samples were drawn from the coccygeal vein in all cows according to recommendations by Mahrt *et al.* (2014). Blood samples were then evaluated using a hand-held electronic meter (Precision Xtra®, Abbott). Although it was reported that the sampling time did not affect BHBA concentrations in dairy cows feeding with TMR continuously (Mahrt *et al.*, 2014), measurements of blood BHBA levels were taken at the same time (4 h after feeding) throughout the study in order to provide uniformity.

The animals were divided into two groups, low-BHBA (n = 85; blood BHBA level 0.40-0.79 mmol/L) and moderate-BHBA (n = 71; blood BHBA level 0.80-1.19 mmol/L) in order to investigate the effects of different BHBA levels on the response to the Ovsynch protocol. Cows having ≥1.2 mmol/L blood BHBA levels (subclinical ketosis) were not included in the study.

The Precision Xtra monitoring system consist of an electronic hand-held meter (Precision Xtra meter, Abbott) and test strip (Precision Xtra β-ketone, Abbott), and is validated to identify ketosis at cow side. A drop of collected blood samples was immediately applied on the end of the test strip. After 10 sec, the BHBA concentration was displayed as mmol/L. The values displayed on the meter were recorded. The ketone test strip contains BHBA dehydrogenase that oxidizes BHBA to acetoacetate. This enzyme induces conversation from oxidized form of nicotinamide adenine dinucleotide (NAD+) to reduced form (NADH), and NADH is then reoxidized to NAD+. These electron transfer reaction is measured by the meter (Iwersen *et al.*, 2009).

Synchronization treatments and timed artificial insemination (TAI)

Healthy cows were selected for the study and treated with the Ovsynch-48 protocol at 7-8 weeks postpartum (52 ± 3 DIM). All cows received an injection of GnRH (Buserelin acetate, intramuscular [i.m.], 10 µg Receptal®, MSD) at a random stage of the estrous cycle without presynchronization, followed 7 days later by PGF2α (Cloprostenol, 500 µg, i.m., Estrumate®, MSD) injection and a second GnRH injection 2 days after the PGF2α injection (GnRH - 7 days - PGF2α - 48 h - GnRH - 16 h - TAI). At the end of the protocol, all cows were inseminated by the same

practitioner at approximately 16 h after the second GnRH injection.

Transrectal ultrasonography

Transrectal ultrasonographic examination was performed using a 7.5-MHz linear probe connected to a portable B-mode ultrasound scanner (Hasvet 838®, Hasvet, Turkey) to determine the DF diameter and the presence of CL throughout the synchronization protocol (Fig. 1).

All cows were examined by using transrectal ultrasonography (7.5 MHz linear probe) for determination of pregnancy rate at 27-30 days (first pregnancy check) and for reconfirmation of pregnancy at 60-70 days (second pregnancy check) after AI.

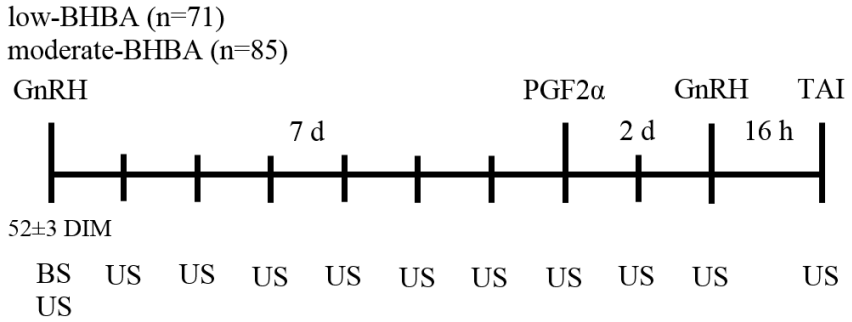

Figure 1. Schematic diagram of blood sampling (BS), ultrasonographic examinations (US) and hormonal treatments in experimental cows.

Statistical analyses

Data of continuous variables, including age, DIM, milk yield on day 0, milk yield in the last 7 days, and DF were checked for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene's tests, respectively. According to the results of the evaluation, the association between groups with continuous variables was analyzed by Student's t-test (using the assumption of parametric test) and the Mann-Whitney U-test. The association between categorical variables (ovulation, pregnancy) and the study groups was analyzed using the Chi-square test. All statistical analyses were performed with a 5% margin of error, and the commercial software package Stata 12.1 MP4 (License number: 50120500264; StataCorp LP, Texas, USA) was used.

Results

Descriptive data

Descriptive data such as age, DIM, milk yield on day 0 of Ovsynch, and average milk yield in the last 7 days of all cows are presented in Table 1. Although differences of age and DIM between the groups were not significant ($P > 0.05$), the differences of milk yield on day 0 of Ovsynch and the average milk yield in the last 7 days between groups were significant ($P < 0.001$).

Dominant follicle diameter

The effects of blood BHBA levels on DF diameter during the protocol are presented in Tables 2 and 3. Differences in DF diameter between groups at 0 h, 24 h and 48 h after $GnRH_1$ (Table 2) and PGF2α (Table 3) were not significant ($P > 0.05$), however, significant differences were observed at the time of AI ($P = 0.039$; Table 3).

Table 1. Descriptive data of the cows at the beginning of study (Mean ± SEM).

Variable*	Low-BHBA (n = 85)	Moderate-BHBA (n=71)	P
Age (month)	27.53 ± 0.31	27.66 ± 0.24	0.738
DIM (day)	50.56 ± 0.32	51.14 ± 0.35	0.225
MY (kg)	31.75 ± 0.68	35.17 ± 0.61	<0.001
wMY (kg)	31.76 ± 0.64	35.4 ± 0.62	<0.001

*DIM: days in milk; MY: milk yield per day; wMY; average milk yield at last seven days.

Table 2. Comparison of DF diameter at day of, 24 and 48 hours after $GnRH_1$ (mm; X ± SE).

		BHBA			
Time*	n**	Low-BHBA	n**	Moderate-BHBA	P
Day 0	68	12.2 ± 0.2	53	12.4 ± 0.3	0.489
Day 1	50	12.7 ± 0.2	42	12.9 ± 0.3	0.644
Day 2	11	12.1 ± 0.5	15	12.7 ± 0.5	0.446

All cows were enrolled in the study without presynchronization or detecting of estrus cycle stage. When a cow had a DF (≥10 mm) or more than a DF at the examination, only the diameter of the follicle or the largest follicle was included in the statistical analysis, respectively. *Day 0: At $GnRH_1$ injection; Day 1: 24 h after $GnRH_1$; Day 2: 48 h after $GnRH_1$;** n means that the number of animals that the DF was measured at each specific time point.

Table 3. Comparison of DF diameter from $PGF_{2\alpha}$ treatment to timed-insemination (mm; X ± SE).

		BHBA			
Time*	n**	Low-BHBA	n	Moderate-BHBA	P
Day 7	54	11.5 ± 0.1	40	11.4 ± 0.2	0.683
Day 8	72	12.0 ± 0.2	59	11.8 ± 0.2	0.64
Day 9	74	12.8 ± 0.2	66	12.3 ± 0.2	0.067
Day of AI	76	13.8 ± 0.2	68	13.2 ± 0.2	0.039

All cows were enrolled in the study without presynchronization or detecting of estrus cycle stage. When a cow had a DF (≥10 mm) or more than a DF at the examination, only the diameter of the follicle or the largest follicle was included in the statistical analysis, respectively. Cows without DF were not included in the statistical analysis. *Day 7: At $PGF_{2\alpha}$ injection; Day 8: 24 h after $PGF_{2\alpha}$; Day 9: 48 h after $PGF_{2\alpha}$; Day of AI: At timed-AI. **n means that cumulative number of cows with DF follicle measured up to that specific time point.

Ovulation response

The ovulation response to $GnRH_1$ was 62.4% (53/85) and 50.7% (36/71) in the low-BHBA and moderate-BHBA groups, respectively. The percentage of cows that exhibited ovulation in response to GnRH1 was similar (P > 0.05) between the groups 24 and 48 h after GnRH1 (Table 4).

Table 4. Rate of ovulation response to 1st GnRH treatment.

	Low-BHBA	Moderate-BHBA	P
Ovulation in 24 h, %, (n/N)	21.2 (18/85)	18.3 (13/71)	0.655
Ovulation in 48 h, %, (n/N)	41.2 (35/85)	32.4 (23/71)	0.159
Total ovulation, %, (n/N)	62.4 (53/85)	50.7 (36/71)	0.143

Differences in DF were not significant between the groups at 24 and 48 h after GnRH1 in all animals with/without ovulations (P > 0.05). DF diameters of cow ovulated and non-ovulated after first GnRH1 with low-BHBA (at 24 h) and moderate-BHBA (at 48 h) were different (P < 0.05; Table 5).

The results of comparison of the DF diameters between groups according to the presence of CL with/without ovulation were shown in Table 6.

The atresia rate of follicles in low-BHBA and moderate-BHBA animals was 17.6 and 21.1%, respectively, at 48 h after GnRH1; the difference between groups was not significant (P > 0.05; Table 7).

Pregnancy rate

Pregnancy rate (P/AI) was 30.77% (48/156) in all cows at 27-30 days post-inseminations. Pregnancy rate was greater in low-BHBA group than moderate-BHBA group at 30 days (38.82 *vs.* 21.13%; P = 0.017, respectively) and 60 days (37.6 *vs.* 18.3%; P = 0.008, respectively) after AI.

Table 5. DF diameters in groups according to ovulation response (mm; X ± SE).

			BHBA			
	Variable	n	Low-BHBA	n	Moderate-BHBA	P
Ovulation in 24 h	Yes	18	13.0 ± 0.5	13	13.3 ± 0.6	0.771
	No	50	11.9 ± 0.2	40	12.2 ± 0.3	0.451
	P		0.016		0.069	
Ovulation in 48 h	Yes	35	12.9 ± 0.2	23	13.6 ± 0.4	0.07
	No	15	12.2 ± 0.3	19	11.9 ± 0.4	0.495
	P		0.09		0.002	

Table 6. Comparison of the diameters of DF between the groups according to the presence of CL with/without ovulation (follicle diameters (mm); X ± SE).

Variables				BHBA			
CL	Time	Ovulation	n	Low-BHBA	n	Moderate-BHBA	P
Yes	Day 1	Yes	11	1.38 ± 0.07	4	1.25 ± 0.12	0.330
		No	36	1.19 ± 0.03	25	1.22 ± 0.03	0.418
	Day 2	Yes	29	1.3 ± 0.02	16	1.33 ± 0.04	0.505
		No	8	1.22 ± 0.04	9	1.18 ± 0.02	0.400
No	Day 1	Yes	7	1.19 ± 0.06	9	1.36 ± 0.06	0.061
		No	13	1.19 ± 0.03	15	1.21 ± 0.05	0.824
	Day 2	Yes	5	1.2 ± 0.04	7	1.45 ± 0.08	0.042
		No	7	1.23 ± 0.05	10	1.2 ± 0.07	0.724

Day 1: 24 h after $GnRH_1$; Day 2: 48 h after $GnRH_1$.

Table 7. Comparison of atresia of follicles between groups.

		Atresia			
		Yes	No	Total	P*
BHBA	low-BHBA, %, n	15 (17.6%)	70 (82.4%)	85 (100%)	>0.05
	moderate-BHBA, %, n	15 (21.1%)	56 (78.9%)	71 (100%)	

*Fisher exact chi-square test.

Discussion

Primiparous and multiparous cows have different responses to synchronization protocols and reproductive performance. These responses still prove to be controversial today (Tenhagen *et al.*, 2004). Although problematic, the possible reason for better responses of primiparous cows to fertility and synchronization protocols is that there is a lower risk of metabolic problems in early lactation (Erb and Grohn, 1988; Gröhn and Rajala-Schultz, 2000). Similarly, Huszenicza *et al.* (1987) showed that reproductive disorders, except for the later onset of the postpartum ovarian activity, were less frequent in primiparous cows compared with others. More recently, Meikle *et al.* (2004) contrarily indicated that the effect of NEB was observed more on primiparous cows than multiparous cows. Additionally, the authors showed that primiparous cows, particularly those with low body weight, had retardation in the postpartum first ovulation time dependent on the NEB. Furthermore, loss of body condition score of primiparous cows was also more severe compared with multiparous cows. However, to date, there is no study addressing the effects of different levels of BHBA, which is a sign of NEB, on the synchronization protocol applied in the early postpartum period in primiparous cows.

In the study, DF diameter between groups at 0, 24 and 48 h after GnRH1 and PGF2α were similar. It has been known that NEB/hyperketonemia decreased GnRH and LH release frequency from the hypothalamus, and subsequently adversely affected the DF diameter (Roche *et al.*, 2000; Butler, 2003; Walsh *et al.*, 2007). However, in this study, it has been also indicated that the DF diameter was similar at the time of and in the following days after GnRH1 and PGF2α application. This can be explained with the exogenous GnRH that can induce similar LH pulse patterns in animals, although these animals have different blood BHBA levels.

The difference between the DF diameters in cows with/without ovulation after GnRH1 in low-BHBA (at 24 h) and moderate-BHBA (at 48 h) groups was significant ($P < 0.05$). This may have been the result of the low diameter of non-ovulatory follicles (11.9 ± 0.2 in low-BHBA *vs.* 11.9 ± 0.4 in moderate-BHBA), which resulted in low ovulation capacity. Sartori *et al.* (2001) also supported this finding, by stating that a follicle gains ovulation capacity when its diameter reaches about 10 mm in dairy cows but follicles of 10 mm need 40 mg LH and large follicles≥(12 mm diameter) need 4 mg LH dose to ovulate. According to these data, it has been asserted that the ovulation capacity of follicles with <13 mm diameter is probably less compared to others. It is possible that high doses of GnRH administration can be required in order to ensure follicular ovulation with DF diameter <12 mm.

DF diameters of low-BHBA and moderate-BHBA groups at the time of insemination were 13.8 ± 0.2 mm and 13.2 ± 0.2 mm; respectively. In our study, at the time of insemination, the DF diameter was smaller than the DF diameter, which was calculated by Keskin *et al.* (2011). Keskin *et al.* (2011) specified that the DF diameter at the time of insemination was 15.6 ± 2.1 mm in primiparous cows. Further, they stated that the DF diameter at the time of insemination was affected by parity, breed and ovulation response obtained in the $GnRH_1$ injection, however, it was not influenced by the season, body condition score (BCS) and DIM. Researchers specified that various factors that had an impact on fertility also influenced the ovulatory follicle diameter after Ovsynch. Cummins *et al.* (2012) reported that genetic merit of animals affected the pre-ovulatory follicle diameter in Holstein cows. In addition to the findings of the researchers mentioned above, it was also thought that factors such as milk yield and feeding could lead to this difference as well.

DF diameters at the time of insemination were different in the low-BHBA group and the moderate-BHBA group (13.8 ± 0.2 mm and 13.2 ± 0.2 mm; respectively; $P = 0.039$). This can be explained by the endocrine changes caused by elevated BHBA levels. NEB elevates blood NEFA and BHBA levels and decreases insulin-like growth factor 1 (IGF-1) and insulin concentrations. These changes cause a decrease in the sensitivity of ovaries to gonadotropins, and therefore growth of follicles is negatively affected (Lucy, 2008). Additionally, decrease in GnRH and LH release frequencies is dependent on the duration and the severity of NEB/hyperketonemia. Therefore, this leads to a decrease in the DF diameter (Roche *et al.*, 2000; Butler, 2003; Walsh *et al.*, 2007).

In our study, it was determined that cows in the moderate-BHBA group yielded 3.5 kg per day more milk compared to cows in the low-BHBA group. In lactating cows, nutritional requirements and extensive feeding due to high milk yield increase hepatic blood flow and metabolizing of steroid hormones (especially estrogen and progesterone; Sangsritavong *et al.*, 2002). Lopez *et al.* (2004) determined that the diameter of DF at natural estrus was different between high and low milk producing cows. The researchers stated that the differences might have been associated with the need for a long period for follicular growth owing to increased steroid metabolism in high-yielding cows. In our study, DF diameters in the moderate-BHBA group

were lower than those in the low-BHBA group. It was thought that this difference might have been associated with DF, which was grown in new follicular wave after the GnRH1 treatment could have been grown slowly in moderate-BHBA groups because of increased metabolic activity caused by high milk yield.

The study was initiated without applying any pre-synchronization protocol and without determining the stage of the estrus cycle. The cumulative ovulation rate of all animals was 57.1% at the beginning of the synchronization protocol and 48 h after the GnRH1 injection. Vasconcelos *et al.* (1999) classified 156 Holstein cows according to the stages of their estrous cycle and they applied the Ovsynch protocol in the 56 ± 16 days after parturition. As a result of the study, it was found that the total ovulation rate in the $GnRH_1$ injection was 64% and this rate changed according to the stages of the estrous cycle. Pursley *et al.* (1995) defined the Ovsynch protocol and they determined the ovulation response to GnRH1 injection as 90% in cows and 54.17% in heifers. In other studies (Gümen *et al.*, 2003; Souza *et al.*, 2008; Galvão and Santos, 2010), it was similarly specified that the total ovulation rate in the $GnRH_1$ injection was around 60%. Keskin *et al.* (2011) compared the response of Holstein and Swedish red cows to the Ovsynch protocol and reported ovulation responses of 60.2 and 62.2%, respectively. They showed that racial differences did not affect the ovulation rate after the GnRH1 injection. Other studies also indicated similar results. In this present study, when we compared the total ovulation response according to the low and moderate BHBA groups, we ascertained that these rates were 62.4 and 50.7%; respectively; however, the difference was not significant ($P > 0.05$). It was thought that this could be due to the blood BHBA level differences as well as the application of the synchronization protocol without the determination of the stage of the estrous cycles.

We found a total pregnancy rate of 30.77% at the 27-30 days after insemination in all animals. These results were in line with previous findings reporting a range of 28.3-42.4% (Moreira *et al.*, 2000; Cartmill *et al.*, 2001; Williams *et al.*, 2002). However, when we compared the low and moderate BHBA groups, P/AI were 38.82% (33/85) and 21.13% (15/71) at day 30 post-AI ($P = 0.017$) *vs.* 37.6% (32/85) and 18.3% (13/71) at day 60 post-AI ($P = 0.008$), respectively. Walsh *et al.* (2007) specified that each 0.1 mmol/L (100 µmol/L) increment in the BHBA concentration in the first and second week postpartum led to a 2 and 3% decrease in the pregnancy rate, respectively. Some studies (Ospina *et al.*, 2010a; Garverick *et al.*, 2013) stated that the increment in the NEFA and BHBA levels adversely affected pregnancy rate in dairy cows. Ospina *et al.* (2010b) detected that the pregnancy rate in the 70th-day postpartum after the voluntary waiting period was 13% lower in cows with blood BHBA level≥10 mg/d l (0.96 mmol/L) compared with cows with blood BHBA level <10 mg/dl (0.96 mmol/L). Various reasons may account for the effects of blood BHBA levels on pregnancy rates in the Ovsynch protocol in primiparous cows, as evidenced by other studies. Indeed, previous studies have shown that elevated BHBA levels have toxic effects on oocyte quality, maturation, and subsequent early embryonic development *in vitro* (Leroy *et al.*, 2006). It has also been shown that BHBA negatively affects the follicular steroidogenesis in granulosa cells (Vanholder *et al.*, 2006). As a consequence, alteration of progesterone production occurs, resulting in increased risk of embryo survival and decreased fertility (Santos *et al.*, 2004).

In conclusion, our results suggest that BHBA concentrations lower than 1.2 mmol/L can affect the success of synchronization protocols. The present study demonstrated that 1) BHBA levels below 1.2 mmol/L have no effect on the ovulation response to $GnRH_1$ treatment in Ovsynch, 2) concentrations of moderate-BHBA negatively affect the DF diameter at the time of AI, and 3) P/AI was higher in cows with low-BHBA concentrations. BHBA measurements may be used as a management tool to maximize the success of synchronization in lactating primiparous dairy cows. Further work is required to determine the threshold value for the synchronization protocol.

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