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

Seckin Salar¹, Ayhan Bastan

Ankara University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Ankara, Turkey.

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 = 0.039). 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
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, hypocalcaemia, 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 conversion 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., Estramate®, 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 GnRH₁ (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).

<table>
<thead>
<tr>
<th>Variable*</th>
<th>Low-BHBA (n = 85)</th>
<th>Moderate-BHBA (n=71)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (month)</td>
<td>27.53 ± 0.31</td>
<td>27.66 ± 0.24</td>
<td>0.738</td>
</tr>
<tr>
<td>DIM (day)</td>
<td>50.56 ± 0.32</td>
<td>51.14 ± 0.35</td>
<td>0.225</td>
</tr>
<tr>
<td>MY (kg)</td>
<td>31.75 ± 0.68</td>
<td>35.17 ± 0.61</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>wMY (kg)</td>
<td>31.76 ± 0.64</td>
<td>35.4 ± 0.62</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*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 GnRH₁ (mm; X ± SE).

<table>
<thead>
<tr>
<th>Time*</th>
<th>BHBA</th>
<th>n**</th>
<th>Low-BHBA</th>
<th>n**</th>
<th>Moderate-BHBA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>68</td>
<td>12.2 ± 0.2</td>
<td>53</td>
<td>12.4 ± 0.3</td>
<td>0.489</td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>50</td>
<td>12.7 ± 0.2</td>
<td>42</td>
<td>12.9 ± 0.3</td>
<td>0.644</td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>11</td>
<td>12.1 ± 0.5</td>
<td>15</td>
<td>12.7 ± 0.5</td>
<td>0.446</td>
<td></td>
</tr>
</tbody>
</table>

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 GnRH₁ injection; Day 1: 24 h after GnRH₁; Day 2: 48 h after GnRH₁; ** 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).

<table>
<thead>
<tr>
<th>Time*</th>
<th>BHBA</th>
<th>Low-BHBA n</th>
<th>Moderate-BHBA n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>n**</td>
<td>54</td>
<td>40</td>
<td>0.683</td>
</tr>
<tr>
<td>Day 8</td>
<td>11.5 ± 0.1</td>
<td>72</td>
<td>12.0 ± 0.2</td>
<td>0.64</td>
</tr>
<tr>
<td>Day 9</td>
<td>12.8 ± 0.2</td>
<td>74</td>
<td>12.3 ± 0.2</td>
<td>0.067</td>
</tr>
<tr>
<td>Day of AI</td>
<td>13.8 ± 0.2</td>
<td>76</td>
<td>13.2 ± 0.2</td>
<td>0.039</td>
</tr>
</tbody>
</table>

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 GnRH$_1$ was similar (P > 0.05) between the groups 24 and 48 h after GnRH$_1$ (Table 4).

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

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

Differences in DF were not significant between the groups at 24 and 48 h after GnRH$_1$ in all animals with/without ovulations (P > 0.05). DF diameters of cow ovulated and non-ovulated after first GnRH$_1$ 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 GnRH$_1$; 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).

<table>
<thead>
<tr>
<th>Variable</th>
<th>BHBA</th>
<th>Low-BHBA n</th>
<th>Moderate-BHBA n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulation in 24 h</td>
<td>Yes</td>
<td>18</td>
<td>13.0 ± 0.5</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>50</td>
<td>11.9 ± 0.2</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.016</td>
<td>0.069</td>
<td></td>
</tr>
<tr>
<td>Ovulation in 48 h</td>
<td>Yes</td>
<td>35</td>
<td>12.9 ± 0.2</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>15</td>
<td>12.2 ± 0.3</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.09</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Variables</th>
<th>BHBA</th>
<th>Low-BHBA n</th>
<th>Moderate-BHBA n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>Time</td>
<td>Ovulation</td>
<td>n</td>
<td>Low-BHBA n</td>
</tr>
<tr>
<td>Yes</td>
<td>Day 1</td>
<td>Yes</td>
<td>11</td>
<td>1.38 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>36</td>
<td>1.19 ± 0.03</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>29</td>
<td>1.3 ± 0.02</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>8</td>
<td>1.22 ± 0.04</td>
<td>9</td>
</tr>
<tr>
<td>No</td>
<td>Day 1</td>
<td>Yes</td>
<td>7</td>
<td>1.19 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>13</td>
<td>1.19 ± 0.03</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>5</td>
<td>1.2 ± 0.04</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>7</td>
<td>1.23 ± 0.05</td>
<td>10</td>
</tr>
</tbody>
</table>

Day 1: 24 h after GnRH$_1$; Day 2: 48 h after GnRH$_1$. 
## 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 GnRH1 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; Sangsriratvong 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

### Table 7. Comparison of atresia of follicles between groups.

<table>
<thead>
<tr>
<th>BHBA</th>
<th>Atresia</th>
<th>No</th>
<th>Total</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>low-BHBA, %, n</td>
<td>15 (17.6%)</td>
<td>70 (82.4%)</td>
<td>85 (100%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>moderate-BHBA, %, n</td>
<td>15 (21.1%)</td>
<td>56 (78.9%)</td>
<td>71 (100%)</td>
<td>*Fisher exact chi-square test.</td>
</tr>
</tbody>
</table>
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 GnRH1 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 GnRH1 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 estrus 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 70-day postpartum after the voluntary waiting period was 13% lower in cows with blood BHBA level≥10 mg/dl (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 GnRH1 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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