Original Article

Delayed ovarian resumption in Holstein lactating cows: association with elevated beta-hydroxybutyrate levels during early postpartum period

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Abstract

The present study was aimed to investigate body condition, hepatic insulin-like growth factor (IGF)-1, IGF binding protein (BP)-1 and growth hormone receptor (GHR) mRNA abundance and specific energy metabolites in association with delayed ovarian resumption in Holstein lactating cows. Of 10 cows studied, 5 cows had normal ovarian resumption (NR group), whereas the remaining 5 cows had delayed ovarian resumption (AB group) within 7 wks after calving. No significant differences in mean BW and BCS between the two groups were observed ($P > 0.05$). In addition, mean hepatic IGF-1, IGFBP-1 and GHR mRNA abundance of the NR and AB groups was not significantly different ($P > 0.05$). Mean non-esterified fatty acid (NEFA) levels of the two groups were elevated, particularly at 1 wk after calving, but no significant differences between the two groups were observed ($P > 0.05$). In contrast, differences in mean beta-hydroxybutyrate (BHB) levels between the NR and AB groups were significant ($P = 0.047$) and mean BHB levels at 3 and 5 wks after calving of the AB group were higher than those of the NR group ($P < 0.05$). In conclusion, the effects of BW, BCS, hepatic IGF-1, IGFBP-1 and GHR mRNA abundance and NEFA levels were not observed, but a degree of negative energy balance (NEB), indicated by the elevated BHB levels during the early postpartum period, was associated with the occurrence of delayed resumption of ovarian activity in the Holstein lactating cows.

Keywords: ovarian resumption, body condition, insulin-like growth factor-1, growth hormone, energy metabolites, early lactating cows

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Introduction

Under tropical climate, heat stress seriously influences milk production, nutritional status (by reducing dry matter intake) and reproductive efficiency of lactating dairy cows. Lower rates of pregnancy among dairy herds in the tropics have apparently been indicated and alteration in the normal process of ovarian resumption, e.g. delayed ovulation and anovulation, has been proposed as one of the main factors (Kornmatitsuk et al., 2008). Abnormal ovarian cycles during postpartum adversely affect fertility by reducing pregnancy proportions and extending calving to conception interval (Ranasinghe et al., 2011). Previous studies found a close relationship between body condition and ovarian resumption in lactating dairy cows (Shrestha et al., 2005; Kafi and Mirzaei, 2010). It was suggested that cows loosing 1 unit BCS after calving had a prolonged interval to commencement of luteal activity (Shrestha et al., 2005). Kadivar et al. (2014) reported that cows that did not ovulate 45 d after calving also lost more BCS from 2 wks before to 4 wks after calving. In contrast, there was no significant difference in BW loss between cows with and without resumption of ovarian activity within 7 wks postpartum (Konigsson et al., 2008).

During postpartum, cows often go to a state of NEB when the rate of dry matter intake lags behind the high demand in milk production. NEB and changes in some energy metabolites, e.g. elevated NEFA levels at 1 wk after calving and higher BHB levels on day 42 after calving, have been implicated in delayed first ovulation (Kafi and Mirzaei, 2010; Jackson et al., 2011). Free fatty acids (FFA) are elevated while energy demand increases, leading to accumulation of lipids in the liver. This phenomenon affects the function of growth hormone (GH)/IGF-1 axis and release of IGF-1, which is important to follicular growth and development of corpus luteum and its function. IGFBP is necessary for IGF-1 in order to bind to IGF-1 receptor (IGF-1R) of various tissues including follicular and luteal cells. The expression of GHR in the liver positively correlates with plasma IGF-1 and levels of nutrition (Butler et al., 2003). Besides, van Dorland et al. (2009) reported that there were some variations of the hepatic regulation of metabolism in dairy cows with high and low BHB levels. Moreover, elevated levels of NEFA and ketone bodies were responsible for lower levels of IGF-1 and time to first ovulation postpartum (Konigsson et al., 2008). Moreover, cows that became pregnant after artificial insemination (AI) had more GHR and IGFBP mRNA abundance in their liver and these liver indices of metabolic state may be indicative of pregnancy success (Rhoads et al., 2008). Aungier et al. (2014) revealed that increased IGF-1 and lower NEB status gave a higher probability of cows achieving their reproductive targets. Furthermore, greater understanding of the molecular regulation of hepatic expression of growth hormone (GH)/IGF-1 genes is important in elucidating how the system may influence the fertility of cows (McCarthy et al., 2009).

Thus, the present study was aimed to investigate changes in body condition (BW and BCS), hepatic IGF-1, IGFBP-1 and GHR mRNA abundance and specific energy metabolites (NEFA and BHB) during the early postpartum period in association with the occurrence of delayed resumption of ovarian activity in Holstein lactating cows.

Materials and Methods

Animals and experimental designs: The present study was carried out at a commercial farm located at Wangmuang, Saraburi (14°50′44″N, 101°8′4″E), a province in the upper central region of Thailand. Holstein (HF ≥ 87.5%) dairy cows (N = 10), parity 2-5, were studied. They were kept in a free-stall barn and fed 2 times a day on total mixed ration (TMR). The TMR contained 12% crude protein, 15% crude fiber and 64% total digestible nutrients with at least 14 kg dry matter intake (DMI) per day according to nutritional requirements for lactating cows (NRC, 2001). Water was offered *ad libitum*. The cows were machine-milked twice daily (0700 and 1500 h) and the average 305-day milk yield per cow was approximately 4,500-5,000 kg. The study protocol (No.1431077) was approved by the local animal ethics committee, Faculty of Veterinary Science, Chulalongkorn University following the Guide for the Care and Use of Experimental Animals, National Research Council of Thailand (NRCT).

Identification of postpartum ovarian activity: The cows' ovaries were bi-weekly examined during 1-7 wks after calving using transrectal-ultrasonical technique. A real-time B-mode ultrasound scanner (Falco Vet®, Esoata-Pie Medical, Italy) was equipped with a 6-/ 8-MHz rectal linear-array transducer. Appearances of the follicles as well as of the corpus luteum (CL) were documented according to Petyim et al. (2000). In addition, identification of postpartum ovarian activity was given in details according to Kornmatitsuk et al. (2008). The first postpartum ovulation was expected if CL was identified. Delayed first ovulation was a condition in which first ovulation occurred over 5 wks after calving.

Measurement of body condition and blood sampling: BW and BCS were recorded at 1, 3, 5 and 7 wks after calving. BW was measured by an electronic scale concurrent with assessment of BCS. BCS was visually determined by the same person according to Edmonson et al. (1989) using a five-point scale with 0.25 increments (BCS, 1 = emaciated to 5 = obese).
Blood samples were collected from the coccygeal vein using 1-inch long, 21-gauge needles into 6-mL plain vacuumed tubes (BD Vacutainer□, Becton Dickinson and Company, NJ, USA). After leaving for 1 h at 4□C, the blood samples were centrifuged at 1000 □ g for 15 min and serum samples were harvested and kept at -20□C for further analyses.

Liver biopsies: Liver biopsies were obtained from all cows at 1 and 5 wks after calving. Liver biopsy technique is described elsewhere. Briefly, local intramuscular infiltration of 5 mL of 2% lidocaine HCl and a 1-cm stab incision were made at the intersection of a line running from the tuber coxae to the shoulder joint with the 9th or 10th intercostal space. A 14-gauge custom biopsy needle was inserted through the intercostal muscle into the liver, and a sample

(approximately 1 g of tissue) was collected. The liver samples were immediately placed in a 2-mL Eppendorf tube with RNAlater® solution (100 mg of tissue/mL; Ambion®, TX, USA) and stored at -80°C until total RNAs were isolated.

Isolation and purification of RNA and quantitative real-time RT-PCR: Total RNAs were isolated from the liver tissues using the acid guanidium-phenol-chloroform technique (TRIzol® Reagent, Invitrogen Life Technologies, CA, USA) according to the manufacturer's protocol. Concentration, relative purity and quantity of the isolated RNAs were determined by using Nanodrop® 2000 Spectrophotometer (Thermo Scientific Inc., DE, USA) and the ratio of A260-A280 nm exceeded 1.8 for all preparations. First-strand cDNA synthesis was performed with 1 µg of total RNAs per 60 µL of reaction using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc., CA, USA) following the manufacturer's instructions. The specific forward and reverse primers used in the RT-PCR were according to Fenwick et al. (2008) and Rhoads et al. (2008). The gene used as an internal control was GAPDH. All selected gene expressions were performed by using KAPA SYBR FAST® master mix (KAPA Biosystems, MA, USA) and relative quantification was determined by cycle threshold method. The PCR was conducted with the ABI Prism 7500 Sequence Detection System (Applied Biosystems, CA, USA). PCR reactions were performed in a total of 20 µL volume containing 200 nM of each primer, 1x KAPA SYBR Fast qPCR Master Mix Universal (KAPA Biosystems, MA, USA) and the cDNA template. The amount of cDNA template in the PCR condition was 1 µg per reaction. Thermal cycling conditions were as follows: 1 cycle at 95°C for 2 min followed by 40 cycles at 95°C for 3 s, 60°C for 20 s and 72°C for 1 s. Each reaction was performed in duplicate with 3 independent runs. Data from the FAM/SYBR channel operating at an excitation maximum of 495 nm and an emission maximum of 520 nm were evaluated. Melting curve analysis was used to determine purity of the amplified products. Relative expression levels were analyzed by REST-2009 software. The threshold cycles of all targets in the test samples were normalized to the corresponding GAPDH levels in the control samples.

Analyses of energy metabolites: Serum NEFA and BHB levels were determined at 1, 3, 5 and 7 wks after calving. NEFA levels were analyzed by enzymatic-colorimetric methods (Randox NEFA□, Randox Laboratories Ltd., UK). Linearity of the standard curve was determined with a range of standards: 0.1, 0.5, 1.0 and 1.91 mmol/L. The correlation coefficient (r^2) of the standard curve was 0.99. BHB levels were obtained by kinetic enzymatic method with commercially available kits (Ranbut□ D-3-hydroxybutyrate, Randox Laboratories Ltd., UK). The lower detection limit of the kits was 0.1 mmol/L.

Statistical analysis: Data were presented as mean and pooled standard errors (pooled SE). Statistical analyses were performed with a commercial statistical package (SPSS□ version 19, SPSS Inc., IL, USA). All data were checked for normal distribution by Shapiro Wilk test. With the observed mean, changes in BW, BCS, hepatic IGF-1, IGFBP-1 and GHR mRNA, NEFA and BHB levels in the two groups of postpartum ovarian activity, time and their interaction were analyzed with ANOVA for repeated measures using the general linear model (GLM) procedure. When the effect of group or the interaction between group and time was significant, pairwise comparison of individual means between groups at specific time points after calving was carried out by the Bonferroni's *t*-test. Cross tabulation analysis was obtained by Chi-square test. Probability values of less than 0.05 were considered to be significant.

Results

Characteristics of postpartum ovarian activity: Of the 10 cows studied, 5 cows showed normal ovarian resumption (NR group), whereas the remaining 5 cows had delayed ovarian resumption (AB group). In the NR group, 4 cows had the first appearance of CL within 3 wks after calving and 1 cow had the first appearance of CL within 5 wks after calving. In the AB group, 2 cows had the first appearance of CL at 7 wks after calving and 3 cows did not have the first appearance of CL within 7 wks after calving. There was a significant difference in the first appearance of CL between the groups ($P = 0.019$). In addition, 3 cows in the AB group developed cystic ovaries. Details of the postpartum ovarian activity and uterine characteristics in the cows with normal (NR) and delayed (AB) ovarian resumption are presented in Table 1.

BW and BCS: Mean BW of the NR group decreased from 1 to 5 wks after calving and afterwards it slightly increased closely to the BW at 1 wk after calving, whereas mean BW of the AB group decreased throughout the study period. In contrast, mean BCS of the NR group showed a decrease only from 1 to 3 wks after calving, while of the AB group, it decreased from 1 to 7 wks after calving. No significant differences in the mean BW and BCS between the two groups were observed ($P > 0.05$), even though the mean BW and BCS losses of the AB group were numerically larger than those of the NR group. Details of the mean BW and BCS changes during 1-7 wks after calving in the NR and AB groups are summarized in Table 2.

Abundance of hepatic IGF-1, IGFBP-1 and GHR mRNA: Mean hepatic IGF-1 and GHR mRNA abundance of the two examined groups increased from 1 to 5 wks after calving, while mean hepatic IGFBP-1 mRNA abundance decreased. However, the mean hepatic IGF-1, IGFBP-1 and GHR mRNA abundance between the two groups was not significantly different ($P > 0.05$). Details of the mean hepatic IGF-1, IGFBP-1 and GHR mRNA abundance at 1 and 5 wks after calving in the NR and AB groups are presented in Table 3.

NEFA and BHB levels: Mean NEFA levels of the NR group were elevated at 1 and 3 wks after calving and decreased afterwards, while mean NEFA levels of the AB group were elevated at 1, 3 and 5 wks after calving and dropped at 7 wks after calving. No significant

differences in the mean NEFA levels between the two examined groups were recorded (P > 0.05).

Mean BHB levels of the AB group were higher than those of the NR group throughout the study period. Differences in the mean BHB levels between the two groups were significant (P = 0.047) and the mean BHB levels of the AB group at 3 and 5 wks after calving were significantly higher than those of the NR group (P < 0.05). Details of the mean NEFA and BHB levels during 1-7 wks after calving in the NR and AB groups are presented in Table 4.

Table 1 Postpartum ovarian activity and uterine characteristics in cows with normal and delayed ovarian resumption

Group	N	1st CL (wks after calving)*				Total DFs[1] (n)	COD[2] (n)	DUI[3] (n)	UI[4] (n)
		3	5	7	>7				
Normal (NR)	5	4	1	0	0	10	0	0	0
Delayed (AB)	5	0	0	3	2	20	3	2	1

* P-value = 0.019, [1] = a total number of dominant follicles, [2] = cystic ovarian disorder, [3] = delayed uterine involution, [4] = uterine infection

Table 2 Mean BW and BCS during 1-7 wks after calving and differences (diff.) from 1-3, 3-5 and 5-7 wks after calving in cows with normal and delayed ovarian resumption

Variables	Group	Wks after calving				Pooled SE	P-value		
		1	3	5	7		Group	Time	Group × Time
BW (diff.)	NR	449.8	429.4	434.2	443.2	5.8	NS	**	NS
(kg)		(n/a)	(-17.9)	(-7.7)	(9.0)			(0.004)	
	AB	466.2	436.8	424.8	422.0	15.0			
		(n/a)	(-25.1)	(-12.0)	(-2.8)				
BCS (diff.)	NR	3.00	2.60	2.85	2.85	0.08	NS	**	NS
(unit)		(n/a)	(-0.40)	(0.25)	(0.00)			(0.007)	
	AB	3.20	2.95	2.90	2.70	0.08			
		(n/a)	(-0.25)	(-0.05)	(-0.20)				

NR = normal ovarian resumption, AB = delayed ovarian resumption
** P-value < 0.01 and NS = not significant

Table 3 Mean hepatic IGF-1, IGFBP-1 and GHR mRNA abundance at 1 and 5 wks after calving in cows with normal and delayed ovarian resumption

Variables	Group	Wks after calving		Pooled SE	P-value		
		1	5		Group	Time	Group × Time
IGF-1 mRNA	NR	20.03	21.23	0.67	NS	NS	NS
	AB	19.72	20.76	0.45		(0.100)	
IGFBP-1 mRNA	NR	18.65	17.98	0.77	NS	NS	NS
	AB	19.29	18.03	0.77			
GHR mRNA	NR	20.32	20.89	0.56	NS	NS	NS
	AB	19.01	19.49	0.51	(0.175)		

NR = normal ovarian resumption, AB = delayed ovarian resumption
NS = not significant

Table 4 Mean NEFA and BHB at 1, 3, 5 and 7 wks after calving in cows with normal and delayed ovarian resumption

Variables	Group	Wks after calving				Pooled SE	P-value		
		1	3	5	7		Group	Time	Group × Time
NEFA	NR	0.49	0.39	0.20	0.21	0.04	NS	***	*
(mmol/L)	AB	0.64	0.54	0.38	0.17	0.06		(0.000)	(0.038)
BHB	NR	0.52	0.53[a]	0.47[a]	0.54	0.03	*	NS	NS
(mmol/L)	AB	0.62	0.78[b]	0.78[b]	0.55	0.05	(0.047)		(0.056)

NR = normal ovarian resumption, AB = delayed ovarian resumption
* P-value < 0.05, *** P-value < 0.001 and NS = not significant
[a-b] Different superscript letters between columns denote significant differences (P < 0.05).

Discussion

The findings of a number of delayed ovarian resumption in Holstein lactating cows (5 out of 10) during the early postpartum period in the present study are similar to our previous report (Kornmatitsuk et al., 2008). It has been suggested that the differential types of abnormal ovarian cycles, particularly delayed ovulation and anovulation in Holstein lactating cows, could lead to lower AI submission rates and pregnancy outcomes (Kornmatitsuk et al., 2008). In the NR group, a number of total dominant follicles (DFs) was larger than those in the AB group, which might indicate that the ovarian activity of the NR group was likely to be more active. In addition, the incidence of cystic ovaries could also be associated with the lower recruitment rates of DFs in the AB group. However, the formation of cystic ovaries in dairy cows could normally occur and simultaneously recover during early lactating period (Roth et al., 2012).

The losses of BW and BCS in both groups were markedly recorded during 1 to 3 wks postpartum, but continuous losses of BW and BCS

were observed in the AB group. Additionally, no differences in BW loss between the NR and AB groups were recorded in the present study and the average loss of BCS was lesser than the cut-off level of 1 unit as suggested by Shrestha et al. (2005). Samarutel et al. (2008) mentioned that cows with delayed start of cyclicity lost more BCS (1.2 units) during 40 d after calving than normal resumption cows (0.75 units). Similar findings to the present results were reported by Konigsson et al. (2008), who found that there was no significant difference in BW loss (<10%) between cows with and without resumption of ovarian activity within 7 wks postpartum. Therefore, it could be suggested that BW or BCS loss (<10% or 1 unit) during the early lactating period was not directly related to delayed resumption of ovarian activity in dairy cows.

The GH/IGF-1 system, consisting of GH, IGF-1, and IGF binding proteins (IGFBP), is dynamically regulated during the periparturient period and during periods of high milk production or undernutrition (Rhoads et al., 2008). In the present study, no differences in the hepatic IGF-1, IGFBP-1 and GHR mRNA abundance between the groups were observed. This might indicate that changes in the hepatic IGF-1, IGFBP-1 and GHR mRNA abundance were relatively minor in early lactating cows within the present results. Meikle et al. (2004) also reported that a larger decrease in IGF-I plasma levels was found in primiparous compared to pluriparous cows. Furthermore, the presence of other factors, e.g. breed, stage of lactation, subclinical mastitis and lameness, are involved, in some degree, in GH/IGF-1 axis and the onset of the luteal phase in postpartum dairy cows (McCarthy et al., 2009; van Dorland et al., 2009; Peake et al., 2011).

Although no differences in the NEFA levels between the groups were recorded, the NEFA levels of the AB group were clearly elevated, particularly at 1 wk after calving. Similar changes in NEFA levels between cows with and without ovarian resumption were found by previous studies (Shrestha et al., 2005; Konigsson et al., 2008). Jackson et al. (2011) also reported that cows with extreme values of energy metabolites were likely to be in severe NEB and these cows would be expected to be at a higher risk of developing abnormal ovarian resumption. Therefore, it could suggest that the cows in the AB group, with the higher elevated NEFA levels, underwent increased mobilization of body fat, accumulated more triacylglycerols in the liver and had more NEB than the cows in the NR group. The stage of NEB in the AB group was also confirmed by the higher elevated BHB levels during the study period. In another term, the BHB levels in the present study proved to be a more reliable predictive variable of the severity of NEB than the NEFA levels during early lactating period in Holstein dairy cows. Aungier et al. (2014) also revealed that a lower energy balance status (decrease in NEFA concentration in wk 1, decrease in BHB concentration in wk 2, BCS loss between calving and d 28 pp < 0.5) was one of the key risk factors in the early postpartum period that gave a higher probability of cows achieving their reproductive targets and having a first AI pregnancy.

In conclusion, the effects of BW, BCS, hepatic IGF-1, IGFBP-1 and GHR mRNA abundance and NEFA levels were not observed, but a degree of NEB, indicated by the elevated BHB levels during the early postpartum period, was associated with the occurrence of delayed resumption of ovarian activity in Holstein lactating cows. Therefore, improvement in the energy status of early postpartum cows through nutritional and health management is needed to ensure and optimize reproductive efficiency of dairy herds.

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บทคัดย่อ

ภาวะรังไข่ทำงานล่าช้าหลังคลอดในแม่โครีดนมพันธุ์โฮลสไตน์: ความเกี่ยวข้องกับการเพิ่มขึ้นของเบตาไฮดรอกซีบิวทีเรตในระยะต้นหลังคลอด

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งานวิจัยชิ้นนี้มีวัตถุประสงค์เพื่อศึกษาความสมบูรณ์ของร่างกาย IGF-1 IGFBP-1 และ GHR mRNA จากเซลล์ตับและเมแทบอไลต์ของพลังงานที่จำเพาะ และความเกี่ยวข้องกับภาวะรังไข่ทำงานล่าช้าหลังคลอดในแม่โครีดนมพันธุ์โฮลสไตน์ จากแม่โครีดนมจำนวน 10 ตัวที่ศึกษา พบว่า 5 ตัวมีภาวะรังไข่ทำงานปกติหลังคลอด และ 5 ตัวมีภาวะรังไข่ทำงานหลังคลอดล่าช้าในระยะเวลา 7 สัปดาห์หลังคลอด ค่าเฉลี่ยน้ำหนักตัวและความสมบูรณ์ของร่างกายของทั้งสองกลุ่มไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($P > 0.05$) เช่นเดียวกันกับค่าเฉลี่ยปริมาณของ IGF-1 IGFBP-1 และ GHR mRNA จากเซลล์ตับของแม่โคทั้งสองกลุ่มซึ่งไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P > 0.05$) ค่าเฉลี่ยกรดไขมันอิสระ (non-esterified fatty acids) ของทั้งสองกลุ่มสูงขึ้น โดยเฉพาะในสัปดาห์ที่ 1 หลังคลอด แต่ไม่พบความแตกต่างระหว่างกลุ่มอย่างมีนัยสำคัญทางสถิติ ($P > 0.05$) ขณะที่ค่าเฉลี่ยเบตาไฮดรอกซีบิวทีเรตระหว่างกลุ่มแม่โคที่มีภาวะรังไข่ทำงานปกติหลังคลอดและกลุ่มแม่โคที่มีภาวะรังไข่ทำงานล่าช้าหลังคลอดแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P = 0.047$) และค่าเฉลี่ยเบตาไฮดรอกซีบิวทีเรตในสัปดาห์ที่ 3 และ 5 หลังคลอดของกลุ่มแม่โคที่มีภาวะรังไข่ทำงานหลังคลอดล่าช้าสูงกว่าในกลุ่มแม่โคที่มีภาวะรังไข่ทำงานหลังคลอดปกติ ($P < 0.05$) สรุปได้ว่า น้ำหนักตัว ความสมบูรณ์ของร่างกาย ปริมาณ IGF-1 IGFBP-1 และ GHR mRNA จากเซลล์ตับ และกรดไขมันอิสระนั้นไม่แตกต่างกันระหว่างแม่โคทั้งสองกลุ่ม ขณะที่ภาวะขาดสมดุลพลังงาน ซึ่งบ่งชี้จากค่าเบตาไฮดรอกซีบิวทีเรตที่เพิ่มสูงขึ้นในระยะต้นหลังคลอดนั้น มีความเกี่ยวข้องกับภาวะรังไข่ทำงานล่าช้าหลังคลอดในแม่โครีดนมพันธุ์โฮลสไตน์

คำสำคัญ: การทำงานของรังไข่หลังคลอด ความสมบูรณ์ของร่างกาย อินซูลินไลค์โกรทแฟคเตอร์-1 โกรทฮอร์โมน เมแทบอไลต์ของพลังงาน แม่โครีดนมระยะต้น

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