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RESEARCH ARTICLE

The Change in Luteal Blood Flow and Luteal Size after Beta Carotene and GnRH Injections in Early Pregnant Dairy Cows

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Summary

The aim of this study was to determine the effectiveness of intramuscular injections of beta-carotene (βC) and GnRH on luteal size (LS), luteal blood flow (LBF) and serum βC concentrations in early pregnant cows. Twenty-nine Holstein-Friesian cows with a mature corpus luteum (>19mm) were randomly assigned to two groups: βC not received (βC-; n=15) or received (βC+; n=14). All cows were treated with $PGF_2\alpha$ and inseminated twice, 48 and 72h after the treatment. Last AI was considered to be day 0. All cows received GnRH on day inseminations, 7 and 17. Different from the βC-, the βC+ group received βC intramuscularly on day 7 and 17. In both groups, measurement of LS and LBF were performed on days 7, 10, 17, 27 and 37 by transrectal B-mode and colour Doppler ultrasonography. Blood samples were collected on each examination day. Only cows that became pregnant were included in the statistical evaluation. The concentration of βC in the βC+ group was higher than in the βC- at all examination days except day 17 ($P<0.05$). There was no significant difference between groups concerning the progesterone concentrations ($P>0.05$). The LS and LBF of βC+ group on day 7 ($P<0.05$) and 27 ($P<0.01$) was higher than in the βC- group and values increased significantly until day 37 (LS: $P<0.05$, LBF: $P<0.01$). We conclude that βC injections significantly increased serum βC concentrations, as well as LS and LBF.

Keywords: *Beta carotene, Dairy cows, Luteal blood flow, Luteal size*

Erken Gebe Sütçü İneklerde Beta Karoten ve GnRH Enjeksiyonlarından Sonra Luteal Kan Akışı ve Luteal Büyüklükteki Değişiklikler

Özet

Sunulan çalışmada erken gebe ineklere kas içi yapılan beta-karoten (βC) ve GnRH enjeksiyonlarının luteal büyüklük (LS), luteal kan akışkanlığı (LBF) ve serum βC düzeyine etkilerinin belirlenmesi amaçlandı. Olgun korpus luteuma (>19 mm) sahip toplam 29 Holstein-Friesian inek rastgele olarak βC uygulanmayan (βC-; n=15) ve uygulanan (βC+; n=14) olmak üzere iki gruba ayrıldı. İneklerin hepsi $PGF_2\alpha$ uygulamasından 48 ve 72 saat sonra tohumlandı. Son tohumlama günü 0. gün olarak kabul edildi. Tohumlamalar sırasında, ayrıca 7. ve 17. günde tüm hayvanlara GnRH uygulandı. βC- gruptan farklı olarak βC+ gruba 7 ve 17. günde βC kas içi uygulandı. Grupların LS ve LBF ölçümleri 7, 10, 17, 27 ve 37. günlerde rektal yolla B-mod ve renkli Doppler ultasonografi ile gerçekleştirildi. Her bir uygulama gününde kan örnekleri toplandı. Tohumlamalar sonucunda gebe olan inekler istatistiksel değerlendirmede kullanıldı. Serum βC düzeyi βC+ grupta, 17. gün hariç, βC- gruptan yüksek bulundu ($P<0.05$). Grupların progesteron düzeyi arasında ise fark bulunamadı ($P>0.05$). LS ve LBF değerlerinin βC+ grupta 7. ($P<0.05$) ve 27. günlerde ($P<0.01$) βC- gruptan daha yüksek olduğu ve 37. güne kadar önemli düzeyde artarak devam ettiği belirlendi (LS: $P<0.05$, LBF: $P<0.01$). Sonuç olarak βC enjeksiyonlarının serum βC konsantrasyonunu, LS ve LBF'yi önemli derece yükselttiği belirlendi.

Anahtar sözcükler: *Beta karoten, Sütçü inek, Luteal kan akışkanlığı, Luteal büyüklük*

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INTRODUCTION

The corpus luteum (CL) is a transient and steroidogenic organ in the ovary in mammals and its main function is to produce progesterone (P_4), a critical hormone during early pregnancy. It stimulates production of a variety of endometrial secretions that is required for successful embryonic development [1].

Beta-carotene (βC) is a precursor of vitamin A and has a specific effect on reproductive functions independent from vitamin A [2]. It has been reported that supplementation of βC improved fertility [3] and decreased puerperal disorders [4] in cows. Beta-carotene has an antioxidant effect and bovine CL contains up to more than five times βC from other tissues as liver, adipose tissue or plasma [5]. This may be necessary due to the free radicals produced during the hydroxylation reactions involved in steroid biosynthesis [6]. A positive correlation has been reported between βC concentrations of the CL, CL diameter and plasma P_4 concentrations [2,7]. Aslan et al.[8] reported that delayed formation of the CL and low concentrations of P_4 production after estrus are observed in cows with low serum concentrations of βC. It has been reported that βC increased P_4 secretions by stimulating LH secretion from hypophysis [9].

Luteinizing hormone surge was also induced by GnRH treatment [10]. Gümen and Sequin [11] reported a rapid rise in peripheral serum LH levels after the GnRH injections. It is well-known, that the use of GnRH after AI improves pregnancy rates [12]. The use of GnRH at the time of AI or on days 11 to 14 post-AI in cows was found to decrease embryonic loss and to increase pregnancy rate [13].

Blood flow in the CL is one of the most important factors for the development of the CL and maintenance of its function [14], it is also closely associated with the production and release of P_4 [15]. The CL is one of the most highly vascularized organs and receives the greatest rate of blood flow per unit of tissue of any organ in the body [16]. The change in blood flow of CL during the different stages of CL life span was shown by many authors [17-20]. Colour Doppler ultrasonography is a useful, non-invasive technique for the evaluation of ovarian vascular function, allowing visual observation of blood flow in a delimited area in the wall of preovulatory follicles, in the CL [15] or uterus [21] of cows.

The aim of this study was to determine the effectiveness of intramuscular injections of βC and GnRH on luteal size (LS), luteal blood flow (LBF) and serum βC concentrations in early pregnant cows.

MATERIAL and METHODS

Animals

Twenty-nine Holstein-Friesian cows aged 2 to 6 years from a commercial dairy farm were used. All animals were lactating for 50 or 80 days prior to the start of the study. The cows were kept in a half-open barn and fed with a total mix ration twice daily that included corn and grass silage, hay, triticale, canola and a balanced grain ration and water intake was ad libitum.

Study Design

Prior to the start of the applications, all cows were controlled for general health status. The body condition score of animals ranged from 3.0 to 3.5 (five point scale). Only cows that were reproductively healthy and had a mature CL (>19 mm) were chosen for the study. Animals were randomly assigned to two groups: beta-carotene not received (βC-; n=15) and beta-carotene received (βC+; n=14). All cows were treated with one dose of $PGF_2\alpha$ (Dinoprost; Enzaprost®, 25 mg/per cow, IM, Ceva-Dif, Turkey) and AI was performed 48 and 72 h after treatment. Last AI was considered to be day 0. A synthetic GnRH analogue (Buserelin; Receptal®, 10 µg/per cow, IM, Intervet, Turkey) was applied during the AIs, as well as on day 7 and 17 in all cows of the study. The cows in the βC+ group additionally received βC (Carofertin®, 200 mg/per cow, IM, Alvetra&Werfft AG, Austria) on the day of the $PGF_2\alpha$ injection, as well as on day 7 and 17 after the last AI. The βC dose was determined in accordance with both previously described [22] and the manufacture's recommendation. Transrectal B-mode and colour Doppler ultrasonography were used to determine and compare LS and LBF on days 7, 10, 17, 27 and 37 in the βC+ and βC- group *(Fig. 1)*. The pregnancy control was performed by transrectal B-mode ultrasonography on d 27.

Ultrasonographic Examinations

Ultrasonographic examinations and image collection were performed as described previously [23-25]. The B-mode and colour Doppler mode of the portable LOGIQ Book XP machine (General Electric Healtcare, Solingen, Germany) and its equipment with 10 MHz linear probe were used to measure the LS and LBF. The CL was identified by B-mode, and its image was frozen at the maximum cross-sectional area and stored for further off-line measurements. Colour LBF mapping in various transverse sections was conducted using the power Doppler mode as previously. To minimize the variations in recording, the settings of the Power Doppler

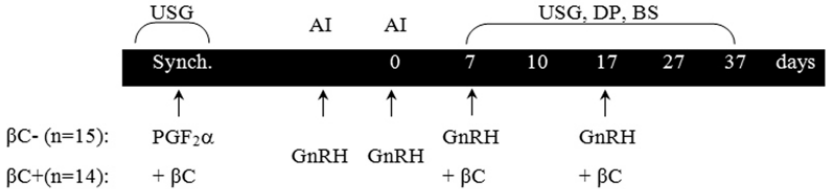

Fig 1. Experimental design of the study (Synch: Synchronization; USG: B-mode Ultrasonography; DP: Colour Doppler Ultrasonography; BS: Blood samples)

Şekil 1. Çalışma planı (Synch: Senkronizasyon; USG: B-mod Ultrasonografi; DP: Renkli Doppler Ultrasonografi; BS: Kan örneği)

system were fixed and used for all examinations. The entire cross-sectional area of the CL was visible within the Power Doppler sample box. After record of at least six video sequences, six images without flash artifacts and with the maximum number of coloured areas were stored from these video sequences. These images were exported in DICOM format into a computer. The CL was measured on B-mode images using a computer-assisted image analysis program (Pixelflux, version 1.0; Chameleon-Software, Leipzig, Germany); the mean value of six pictures was determined. The same software was also used to assess the total area of colour pixels within the luteal tissue. For this purpose the whole luteal structure and its blood flow area were chosen as the region of interest (ROI) and the coloured area within this ROI was calculated. The averages from four of the six images were used for further evaluation of LBF.

Blood Samples and Assays

The blood samples were obtained from the coccygeal vein by use of vacutainer vials before the applications at all days of treatments and controls. Blood samples were centrifuged immediately after collection for 15 min at 2.000 x g at room temperature; serum was stored at -20°C until assayed.

The concentrations of βC were determined by spectrophotometric analysis (Düzen Laboratory Group, TURKAK, Ankara, Turkey) and measured as described previously [26] after extraction using a carotene photometer (Schimadzu UV-mini 1240, Japan). Extraction efficiency was >95%. The inter-assay coefficient of variation averaged 9.7%.

Serum P_4 concentrations were measured by electrochemiluminescence immunoassay as described by Thienpont et al.[27]. The intra- and inter-assay coefficients of variation averaged 1.4% and 2.9%, respectively.

Statistical Analysis

Statistical analyses were carried out using SPSS® Version 16.0 (SPSS Inc, Chicago, Illinois, USA). Descriptive statistics were used for determination of mean and standard error of means. Mann-Whitney U test was used for analysis of LS, LBF and blood serum βC and P_4 concentrations. Repeated Measures Define Factors test were used to determine the difference between days. A P value <0.05 was considered to be significant.

RESULTS

Animals During the Study

A total of 38 animals were synchronized and inseminated. However, only early pregnant cows were considered. Therefore, four cows in the βC- group and five cows in the βC+ group (non-pregnant animals) were withdrawn from the study. At least 15 out of 19 in the βC- group (78.94%) and 14 out of 19 in the βC+ group (73.68%), all early pregnant cows, were included in the statistical evaluation.

Beta-carotene and Progesterone Concentrations

In the βC+ group, a significant increase in serum βC concentration was determined on day 10 of gestation (P<0.05). However, this concentration was not maintained thereafter, especially on day 17 and 37, values were significantly lower (P<0.01 and P<0.05; *Table 1*). On the day of $PGF_2\alpha$ treatment for induction of lutelolysis, the mean serum βC concentrations of βC- and βC+ groups were determined to be 226.3±105.4 µg/dL and 229.1±42.9 µg/dL, respectively (P>0.05). When the mean serum βC concentration of both groups was compared to each other during early pregnancy, it was

Table 1. *The mean beta-carotene concentration (µg/dL) during early pregnancy in groups*
Tablo 1. *Erken gebelik döneminde grupların ortalama beta-karoten konsantrasyonları (µg/dL)*

Groups	Days					P value
	7	10	17	27	37	
βC-	280.8±50.4	289.8±42.1	226.3±43.1	287.9±48.8	272.4±49.7	n.s.
βC+	478.3±43.9^{a}	568.0±72.8^{b*}	390.9±88.8^{b**}	443.9±60.2^{a}	449.8±72.8^{b*}	P*<0.05, P**<0.01

n.s.: *Non-significant (P>0.05), Values with different superscripts (a, b) in the same row are significantly different*

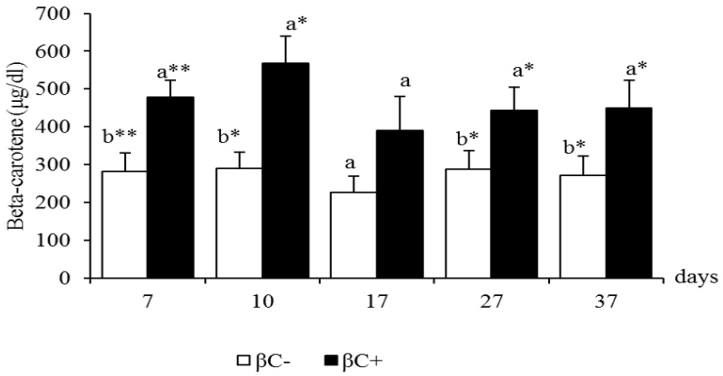

Fig 2. Comparison of mean beta-carotene concentrations (µg/dL) during early pregnancy between groups

Şekil 2. Erken gebelik döneminde ortalama beta-karoten konsantrasyonlarının (µg/dL) gruplar arasında karşılaştırılması

Values with different superscripts (a, b) on the same day are different (P<0.05 and P**<0.01)*

Table 2. *Comparison of mean progesterone concentrations (ng/mL) during early pregnancy between groups*
Tablo 2. *Erken gebelik döneminde grupların ortalama progesteron konsantrasyonları (ng/mL)*

Groups	Days				
	7	10	17	27	37
βC-	2.38±0.49	6.13±1.28	9.78±1.85	13.43±3.27	14.67±3.41
βC+	4.24±2.04	8.97±2.59	7.91±1.62	14.84±1.89	13.46±3.71
P value	n.s.	n.s.	n.s.	n.s.	n.s.

n.s.: *Non-significant (P>0.05)*

found to be significantly higher in the βC+ group at all days investigated *(Fig. 2)*. There was no correlation between βC concentrations, LS and LBF throughout the study.

There was no difference between groups concerning P_4 concentrations (P>0.05; *Table 2*).

Luteal Size

In the βC+ group, the mean LS was 4.82 cm² on day 7 and increased until day 10 (P<0.01), 17 and 37 (P<0.05). A similar increase in LS was found on day 17 and 37 of gestation in the βC- group (P<0.01; *Table 3*).

On the day of $PGF_2\alpha$ treatment, there was no significant difference between the mean LS in the βC- and the βC+ group (4.48±0.99 cm^2 and 4.26±0.74 cm^2, respectively, P>0.05). During early pregnancy, LS was significantly higher in the βC+ group than in βC- group on day 7 (P<0.05) and 27 (P<0.01; *Fig. 3*).

Luteal Blood Flow

In the βC+ group, the mean LBF during pregnancy was significantly higher on day 27 and 37 than on day 7 (P<0.01). In the βC- group, it was only found higher on day 37 than on day 7 (P<0.01; *Table 4*).

On the day of $PGF_2\alpha$ injection, the mean LBF was 1.1±0.73 mm^2 in the βC- group and 0.62±0.27 mm^2 in the βC+ group,

Table 3. *The mean luteal size (cm^2) during early pregnancy in groups*
Tablo 3. *Erken gebelik döneminde gruplardaki ortalama luteal büyüklükler (cm^2)*

Groups	Days					P Value
	7	10	17	27	37	
βC-	4.65±1.41^{a}	4.96±0.75^{a,b}	4.89±0.71^{b**}	4.19±0.99^{b*}	4.68±1.39^{b**}	P*<0.05, P**<0.01
βC+	4.82±1.44^{a}	5.05±0.94^{b**}	4.95±0.96^{b*}	4.73±0.69^{a,b}	4.80±0.88^{b*}	P*<0.05, P**<0.01

Values with different superscripts (a, b) in the same row are different

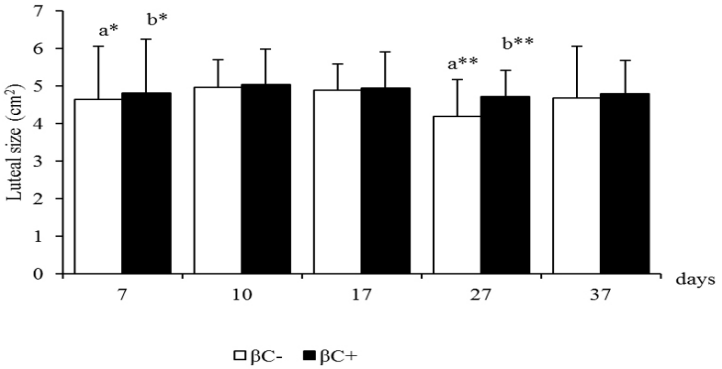

Fig 3. Comparison of mean luteal size (cm^2) during early pregnancy between groups

Şekil 3. Erken gebelik döneminde ortalama luteal büyüklüklerinin (cm^2) gruplar arasında karşılaştırılması

Values with different superscripts (a, b) on the same day are different (P<0.05 and P**<0.01)*

Table 4. *The mean luteal blood flow (mm^2) during early pregnancy in groups*
Tablo 4. *Erken gebelik döneminde grupların ortalama luteal kan akışkanlığı (mm^2)*

Groups	Days					P Value
	7	10	17	27	37	
βC-	0.61±0.32^{a}	0.84±0.42^{a,b}	0.92±0.53^{a,b}	0.83±0.36^{a,b}	1.03±0.38^{b**}	P*<0.05, P**<0.01
βC+	0.88±0.42^{a}	0.74±0.34^{a,b**}	0.78±0.44^{a,b**}	1.21±0.65^{c*,**}	0.98±0.33^{c**}	P*<0.05, P**<0.01

Values with different superscripts (a, b, c) in the same row are different

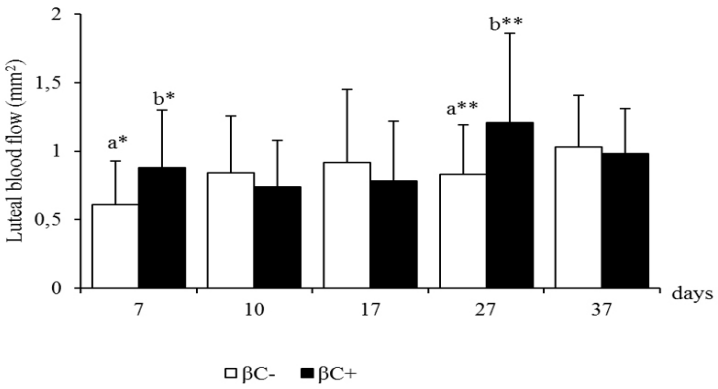

Fig 4. Comparison of luteal blood flow (mm²) during early pregnancy between groups

Şekil 4. Erken gebelik döneminde luteal kan akışkanlığının (mm²) gruplar arasında karşılaştırılması

Values with different superscripts (a, b) on the same day are different (P<0.05 and P**<0.01)*

which was non significant (P>0.05). When the mean LBF was compared between groups, it was determined to be higher in the βC+ group than in the βC- group on day 7 only (P<0.05) and 27 (P<0.01; *Fig. 4*).

DISCUSSION

Corpus luteum is a heterogeneous tissue [28] consisting of steroidogenic cells (large and small luteal cells) and non-steroidogenic cells (endothelial cells, fibroblasts, pericytes and cells) originating from the bloodstream [29]. In a complex tissue like that the various cell types must interact to ensure normal growth and development. Tissue growth depends upon growth of new blood vessels and establishment of a functional blood supply [28]. Twenty-two percent of the mature CL consists of capillary lumina [29] and nearly every parenchymal cell is in contact with at least one capillary [30]. After angiogenesis the CL becomes one of the most highly vascularised organs and receives the greatest rate of blood flow [28].

Local changes in blood flow within the ovary are closely related to changes in the biosynthesis of prostaglandins and steroid hormones [31,32]. Beta-carotene is normally transported to the ovary incorporated in high density lipoprotein in the bovine [33]. It is found in extremely high concentrations in the bovine CL and is responsible for the characteristic bright yellow colour of the CL [34]. The role of βC in the bovine CL could be to support optimal steroid release [35]. However, the relationship between serum βC and P_4 concentrations is still controversial. While many authors report no correlation [36-38], others do so [8,39].

Many authors suppose that serum βC concentrations are affected by the nutrients taken up by cows [40-42]. This study demonstrates that intramuscular βC treatments increase serum βC concentrations. In the control group, there was no significant increase in serum βC concentrations during pregnancy, and values were significantly lower than in the treatment group on days 7, 10, 27 and 37 of gestation. These findings are similar to those of others [23,37]. On the other hand, Gossen and Hoedemaker [43] found that only some cows with low initial concentrations of βC (<200 µg/dL) showed a significant increase in serum βC concentrations after intramuscular application of βC.

However, in the present study, in the βC+ group, a significant decrease in serum βC concentrations was measured on day 17 after the last AI which resembles the findings of Ataman et al.[44] who found a significant decrease in plasma βC concentrations between day 12 and 21 of pregnancy. During early pregnancy, the up-regulation of oxytocin receptors and consecutive luteolysis is inhibited by secretion of interferon tau from the tropho-ectoderm between days 12 and 25 in cattle [45]. Embryos may be transferred, and pregnancies established, as late as day 16 or 17 after estrus [46]. The decrease of serum βC concentrations despite βC application might be explained by this mechanism. Eisele [47] measured high serum βC concentrations after injections of βC. Chew et al.[48] obtained high serum βC concentrations in cows after oral administration of βC. However, in the above cited publications it was reported that βC concentration significantly decreased within 10 to 14 days after the application. In our study we observed a significant decrease in serum βC concentrations 10 days (day 17 of pregnancy) after the last βC administration on day 7. On the other hand βC concentrations were maintained high for 20 days between day 17 and 37 of pregnancy, which made us suppose that the decrease prior to it might be associated with the time and period of administration.

In the present study, there were no significant differences in P_4 concentrations between groups. Some authors reported that supplementation of βC had no effect on P_4 concentration [3,49], however, others reported the contrary [8,9]. It was found that P_4 concentrations in luteal tissue increased after supplementation of βC [50]. However, many fold factors such as season [7,50] and free radicals during steroidogenesis are supposed to influence these results. In the present study, we could not assess any effect of βC treatment on serum P_4 concentrations.

Studies describing the positive effect of βC on fertility have been reported previously [8,49,51]. In the present study, investigation of the effect of βC treatment on LS and LBF was aimed. Although a significant increase in LS during early pregnancy was observed in both groups, it was found

that the CL diameter increased more markedly in the βC+ group. On the same pregnancy days, we detected higher blood flow levels in the βC+ groups and therefore suppose that βC administration not only affects LS but in addition the LBF. It was shown previously that increasing plasma βC concentrations caused an increase in LS [2], and higher βC concentrations were determined in the ovaries and especially in CL by others [2,6], which supports our findings. The developing CL is characterized by highly active vascularisation and repeated mitoses of steroidogenic cells in parallel [52]. Vascular endothelial growth factor seems to be a major angiogenic factor responsible for vascularisation of the developing CL [28,38]. Meanwhile, abundance of reports using colour Doppler ultrasonography for the monitoring of blood flow within the ovary and CL in cows can be found [17,33,53]. These studies mostly revealed that there is no relation between blood flow and change in LS during the estrous cycle [20,54], which resembles our findings. The physiological process of CL growth and formation is probably regulated by many different factors; the mechanism is not yet completely understood [55]. Recently, intraluteal mediators including cytokines and nitric oxide are thought to play some roles as pro-apoptotic and anti-apoptotic factors in the bovine CL, respectively [56,57]. We therefore believe that βC treatment affects angiogenic factors thereby promoting vascularisation and causing the increase in LBF measured in this study.

There are several studies reporting about positive effects of βC on uterine involution and reproductive parameters [49], follicular ovulation after first postpartum follicular wave [58], embryo quality [23] and pregnancy rate [37]. With the results we obtained in this study results we conclude that βC treatment increases serum βC concentrations, LS and LBF. The effect of βC treatment after estrus synchronisation with $PGF_2\alpha$ and GnRH on fertility should be investigated in a follow up study.

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