

## Effects of Dietary Supplementation of Conjugated Linoleic Acid on Pro- and Anti-Inflammatory Cytokines Gene Expression in Uterus of Holstein Dairy Cows

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### ABSTRACT

After parturition, inflammation of the reproductive tract in cows is common because of bacterial contamination of the uterine lumen. To investigate the beneficial effects of dietary Conjugated Linoleic Acid (CLA) supplementation on health status mediated by change in pro- and anti-inflammatory cytokine genes expression, endometrial samples were collected from Holstein cows with similar parturition date and reproductive records. From day 21 before calving to day 42 after calving, cows were fed isonitrogenous, isocaloric, and isolipidic diets that differed only in the source of fats. Cows were fed diets supplemented with palm oil as control group (saturated FA; n= 8), and CLA for the treatments, and the rate of each fat in any diet was 75 g d<sup>-1</sup>. CLA-supplemented diet was provided with a mixture of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA isomer. Rumen protected CLA provided 10 g d<sup>-1</sup> each of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA isomers. Uterine endometrial biopsies were collected at days 21 and 42 after calving and were prepared to determine pro-inflammatory [including Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ), InterLeukins (IL-1, IL-6, IL-8) and InterFeron-gamma (IFN- $\gamma$ )] and anti-inflammatory [interleukin 10 (IL-10)] cytokine genes expression. Results showed that dietary CLA supplementation decreased the expression of IL-1 and IL-8 at days 21 (respectively, 1.8 and 3.9 fold) and 42 (respectively, 4 and 104 fold) postpartum and increased expression of IL-10 at days 21 and 42 (respectively, 9.7 and 2.5 fold). The TNF $\alpha$  expression significantly decreased in day 21 in CLA groups compared with palm (2.5 fold). There was no difference between groups for IL-6 expression. IFN- $\gamma$  expression decreased in day 21 (3.0 fold) and, conversely, increased in day 42 (2.5 fold) in CLA group compared to palm. Our results showed that, during transition period, dietary supplementation with CLA reduced inflammatory processes via inhibiting pro-inflammatory cytokines and stimulating anti-inflammatory cytokines.

**Keywords:** Endometrium, Inflammation, Palm oil, TNF $\alpha$  expression.

### INTRODUCTION

In female genital tract, microbial disease is common and contributes to significant economic losses in humans and cattle among the mammals (Sheldon *et al.*, 2009). After parturition, in dairy cows, uterine function is often compromised by bacterial contamination, and pathogenic bacteria often persist, causing some uterine diseases which are the main factors of infertility in cattle

and affect resumption of ovarian follicular cycle (Sheldon *et al.*, 2006; Sina *et al.*, 2018). Postpartum endometritis, which is caused by bacterial infections, can increase the number of days to conception, services per conception, and risk of culling (Ghasemi *et al.*, 2012), because the presence of bacteria in the uterus causes inflammation and delays uterine involution (Williams *et al.*, 2005).

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The innate immune system is responsible for identifying and responding to the bacterial contamination. In response to pathogen challenge in the uterus, neutrophil cells are the most important phagocytic cell type recruited from the peripheral circulation. After pathogen diagnosis, immune cells release pro-inflammatory molecules including Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ), InterLeukins (IL-1, IL-6, IL-8), and nitric oxide (Herath *et al.*, 2006) and major anti-inflammatory cytokines containing InterLeukin (IL)-1 Receptor Antagonist (IL-1 RA), IL-4, IL-10, IL-11, and IL-13. Furthermore, specific cytokine receptors for IL-1, tumor necrosis factor- $\alpha$ , and IL-18 function as pro-inflammatory cytokine inhibitors (Bonsale *et al.*, 2018).

Inflammatory and immune cells have PolyUnsaturated Fatty Acids (PUFAs) in their cell membrane, therefore, the dietary fatty acids supplementation can affect fatty acid composition of these cells via changing the proportion of PUFAs in these cells (Calder, 2001). Conjugated Linoleic Acid (CLA) is an intermediate product in biohydrogenation of linoleic acid to stearic acid (Kepler *et al.*, 1966) and is a component of an unsaturated fatty acids group that exist as positional and geometric isomers of octadecadienoate (18:2) (Belury, 2002). CLA is naturally found in many animal products, such as those from ruminant sources where it is synthesized by rumen bacteria from linoleic acid or in ruminant tissues, particularly mammary tissue (Griinari *et al.*, 2000). It has been shown that CLA inhibits carcinogenesis and atherosclerosis, enhances immunologic function while protecting against the catabolic effects of immune stimulation, and affects body composition, and stimulates the growth (Pariza *et al.*, 2000). It has beneficial effect on reproduction (Abolghasemi *et al.*, 2016; Rezaei Roodbari *et al.*, 2016).

As a whole, there are limited studies that consider the effects of CLA on inflammation. However, its beneficial effects on inflammatory responses have been reported in a number of animal models

including decreased antigen induced cytokine production by immune cells, reduced adverse effects of immune challenges, and modulation of inflammatory mediators such as cytokines, prostaglandins, leukotrienes, and immunoglobulins (Viladomiu *et al.*, 2016).

Cook *et al.* (1993) demonstrated that dietary supplementation of conjugated linoleic acid (CLA) in chicks could enhance the lymphocyte proliferation. Turek *et al.* (1998) found that CLA supplement might alter the production of immune elements in rats. Moreover, study on human showed that CLA could beneficially affect immune function in healthy human volunteers by improving volunteer's ability to respond effectively to infectious agents and increasing their resistance to allergic agents (Song *et al.*, 2005).

In the present study, our hypothesis was that CLA has positive effect on health status and followed that reproduction may be mediated via changing inflammatory condition (pro- and anti-inflammatory cytokines). Therefore, this study was designed to investigate the effects of dietary CLA supplementation on the inflammatory status in cows after parturition to find out the effect of CLA on a pathway to decrease the inflammation.

## MATERIALS AND METHODS

The Ethical Commission approved the experiment for Experimental Use of Animals of Sari Agricultural Sciences and Natural Resources University.

### Cows and Treatments

Sixteen multiparous lactating dairy cows were blocked based on expected calving date and parity, and randomly assigned to one of the two dietary treatments. All cows fed by adjusted diet between days -21 to +42 (Calving= Day 0). Neither of the two groups differed in parity ( $3.1 \pm 0.4$ ) and body

condition score at calving ( $3.1 \pm 0.14$ ). All cows checked by expert veterinarian from calving to clean test (at days 7, 14, 21 and 28 postpartum) via measurement of rectal temperature and palpation and only healthy cows were assigned into experiment as described by Dirandeh *et al.* (2013). The uterus was considered fully involutes when the cervix and previous gravid horn diameter after calving decreased to unchanged and was similar to that of a non-gravid uterus. The cervix decreased from approximately 30 cm immediately after parturition to approximately 2 cm by day 7 postpartum.

Both diets were equal in concentration of

dry matter, crude fat, and crude protein, but were different in fat sources. The prepartum and postpartum diets were adjusted based on NRC (2001) (Table 1). The cows were fed on diets that included rumen-protected supplements of palm oil (saturated FA;  $n=8$ ) as the control, or CLA (Lutrell® pure, BASF SE, Ludwigshafen, Germany), and the quantity of each fat in any diet was  $75 \text{ g d}^{-1}$ . The CLA-supplemented diet was prepared by a mixture of *trans*-10, *cis*-12 CLA and *cis*-9, and *trans*-11 CLA isomer. The diets included the rumen-protected supplements of palm oil plus basal diet (saturated FA;  $n=8$ ), and CLA plus basal

**Table 1.** Ingredients and nutrient composition (% DM unless otherwise noted) of pre and postpartum diets.

Item	Prepartum	Postpartum
Ingredient		
Alfalfa hay mid	24.4	22.8
Wheat straw	9.6	
Corn silage	28.8	22.0
Beet pulp, dehydrated		4.1
Corn grain, ground	14.1	16.4
Barley grain, rolled	8.5	14.5
Soybean meal, 48%	7.0	14.5
Soybean whole, roast		0.8
Cottonseed whole	1.0	
Wheat	2.1	
Sodium bicarbonate		0.9
Salt		0.4
DCP		0.3
Magnesium oxide		0.3
Glucosa	2.0	1.3
Choline chloride	0.8	0.4
Palm oil <sup>a</sup> /CLA supplement <sup>b</sup>	0.5	0.3
Mineral and vitamin premix <sup>c</sup>	1.2	1.0
Composition		
NEL (MJ kg <sup>-1</sup> DM)	6.7	7.3
Fat	3.3	4.1
Crude protein	13.4	17.1
Neutral detergent fiber	36.8	32.0
Acid detergent fiber	23.1	22.3

<sup>a</sup> Energizer-RP10; Iffco, Johor Bahru Johor, Malaysia. <sup>b</sup> Lutrell Pure, BASF, Ludwigshafen, Germany. <sup>c</sup> Contained (Per kg): 500,000 IU of vitamin A, 100,000 IU of vitamin D, 1,000 of mg vitamin E, 9,000 mg of P, 195,000 mg of Ca, 2,000 mg of Mn, 55,000 mg of Na, 2,000 mg of Zn, 2,000 mg of Fe, 280 mg of Cu, 100 mg of Co, 100 mg of Br, 1 mg of Se, 3,000 mg of anti-oxidant.



diet (Lutrell® pure, BASF SE, Ludwigshafen, Germany), and the amount of each fat in any diet was 75 g d<sup>-1</sup>. Rumen protected CLA provided 10 g d<sup>-1</sup> each of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 isomers.

Feeding time during prepartum was twice a day (8:00 AM and 4:00 PM) and after calving, it changed to four times a day (7:00 and 11:00 AM; 3:00 and 7:00 PM) by TMR formulated based on NRC (2001) to meet all animal requirements. To ensure palatability, fat supplements were manually mixed with 425 g specially-formulated concentrate and were top-dressed on the morning TMR feeding once a day.

### Uterine Biopsy and Tissue Sampling

By passing a biopsy implement through the cervix into the uterine horn ipsilateral to the corpus luteum by means of transrectal manipulation, uterine endometrial biopsies were collected on days 21 and 42 of postpartum (Dirandeh *et al.*, 2015). The open jaws of the biopsy basket (2×1) were compacted against the endometrium and extractions of endometrium samples (approximately 100 mg) were collected. Then, uterine samples were washed by sterile Phosphate-Buffered Saline (PBS), placed in a screw-cap micro centrifuge tube and instantly got snap-frozen in liquid nitrogen, and were stored at -80°C up to RNA extraction.

### RNA Extraction and cDNA Synthesis

Total RNA from the samples was extracted according to the manufacturer's instructions of Total RNA Purification Kit (Jena bioscience, Jena, Germany). Quality of extracted RNA was confirmed by testing them on agarose gel (2%) electrophoresis. To eliminate the DNA contaminations, total RNA (1 mg) was treated with 1 U DNase, and treated RNA was transcribed to cDNA by utilization of Cycle Script RT PreMix

(dN6) cDNA Synthesis kit (Bioneer, Seoul, South Korea) according to the manufacturer's instructions.

### Real-Time PCR

A total volume of 20 µL with 1 µL cDNA (50 ng mL<sup>-1</sup>), 10 µL SYBR Green 1 master mix (QuantiNova™ SYBR® Green PCR Kit, Qiagen Inc., Tehran, Iran land), 1 µL forward and reverse primers (20 ng of each) and 8 µL nuclease free H<sub>2</sub>O were used for Real-time PCR reactions. All primers used in this study were extracted from Salehi *et al.* (2017). After finishing reactions for each gene to make sure of the presence of a single PCR product, dissociation curves were examined.

Real-time RT PCR was performed using a Corbett Rotor-Gene™ 3000 Quantitative PCR System (Corbett Life Sciences, Sydney, Australia) with the following cycling programme: 95°C for 15 minutes and 40 cycles of 95°C for 15 seconds, 60°C for 20 seconds, 72°C for 20 seconds, followed by amplicon dissociation (95°C for 1 minute, 50°C for 45 seconds, increasing 0.58/cycle until 95°C was reached).

The  $\Delta\Delta C_t$  method was used for calculating the gene expression results and the correction for amplification efficiency was used for data normalizing to a calibrator sample (Pfaffl, 2001), then the Fold changes in gene expression between the control (palm) and the CLA groups were determined. As  $\beta$ -Actin (ACTB) was in a stable gene under diverse conditions, it was picked out as a housekeeping gene and samples were run in duplicate and were expressed comparative to that.

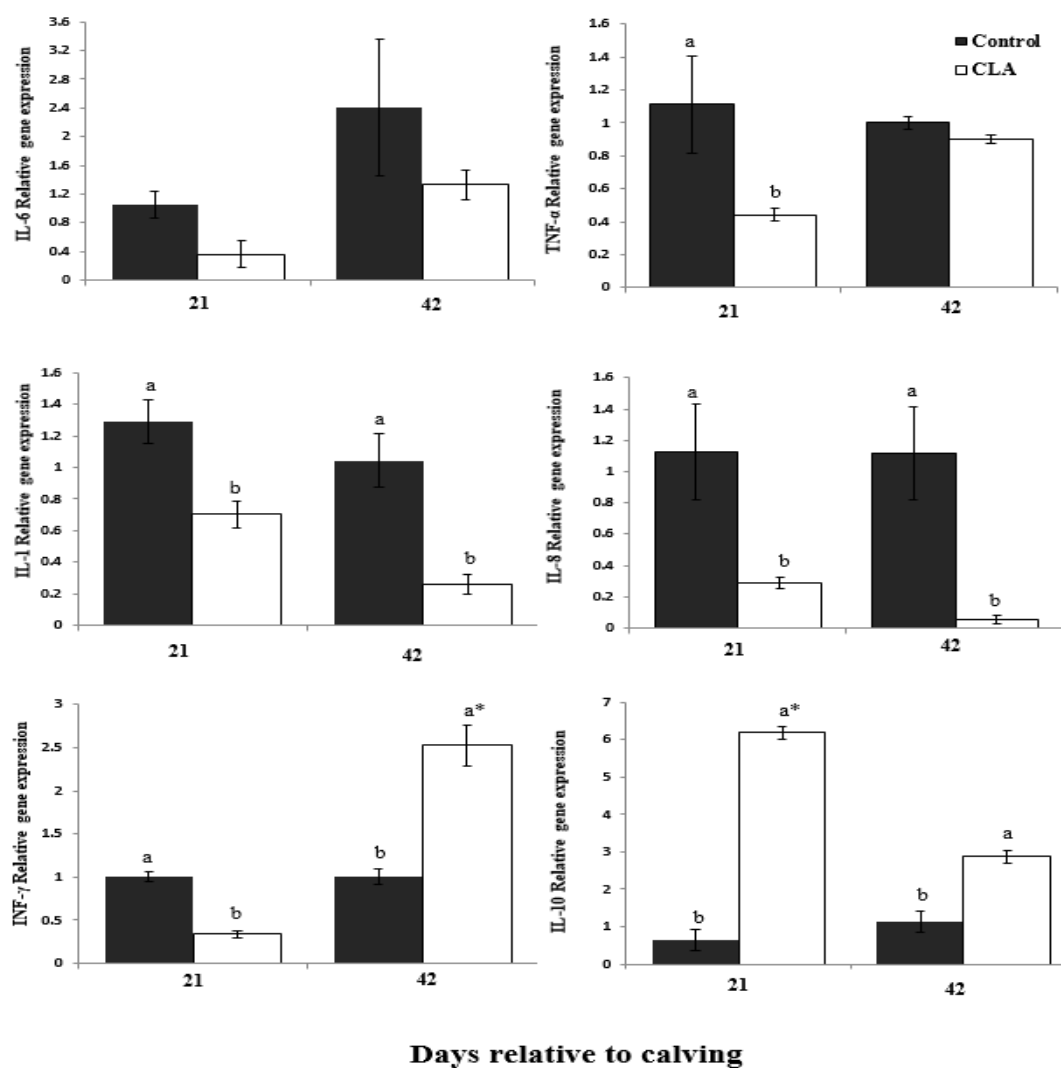
### Statistical Analyses

Data that were not normally distributed as tested with Shapiro-Wilk test were logarithmically transformed. Homogeneity of variance was tested with O'Brien and Brown-Forsythe tests. Analysis of variance

was performed using SAS software with treatments as the main effect and experiment replicates as the random variable in the *F*-test. The differences among means for multiple comparisons were examined by Tukey–Kramer honest significant difference test and significance and tendencies were declared at  $P < 0.05$  and  $P < 0.10$ , respectively, unless otherwise indicated.

## RESULTS

Uterine endometrial pro- and anti-inflammatory genes including IL-8, TNF- $\alpha$ , INF- $\gamma$  and IL-10 were found to be differentially ( $P < 0.05$ ) expressed between the control and CLA-fed animals, and there was no difference in IL-6 gene expression between cows treated with CLA and the control diet in days 21 and 42 ( $P > 0.05$ ) (Figure 1).



**Figure 1.** Relative mRNA of pro- inflammatory including Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ), InterLeukins (IL-1, IL-6, IL-8) and InterFeron-  $\gamma$  (INF- $\gamma$ ), and anti-inflammatory interleukin 10 (IL-10) cytokines during days 21 and 42 postpartum. a, b indicates differences due to the main effect of treatment ( $P < 0.05$ ); \* Indicates effect of the same treatment at different times ( $P < 0.05$ ).



Mean relative expression for TNF- $\alpha$  decreased by 2.5 fold in CLA fed cows compared with the controls on day 21 ( $P < 0.05$ ), but there was no significant difference between the two treatments in day 42 ( $P > 0.05$ ).

Relative IL-1 mRNA levels decreased by 1.82 and 3.97 fold in the endometrium of CLA-fed animals compared to those fed the control diets at days 21 and 42 postpartum, respectively ( $P < 0.05$ ). The levels of IL-8 mRNA in the CLA-fed animals were approximately four fold (day 21 postpartum) and hundred fold (day 42 postpartum) lower than those fed with the control diets ( $P < 0.05$ , Figure 1). The mean of IL-8 genes expression was higher in days 42 to 21 in CLA-fed cows ( $P < 0.05$ ).

The mean relative expression of INF- $\gamma$  mRNA was about 3.0 fold lower in animals fed CLA diet than in those fed the control diet at d 21 postpartum ( $P < 0.05$ ). There was a strong tendency towards an approximately 2.5-fold increase in mean INF- $\gamma$  gene expression in animals fed CLA diet compared with the control diet at d 42 postpartum ( $P < 0.05$ ). In addition, the expression of INF- $\gamma$  mRNA showed a 7.5-fold increase in CLA treatment over time ( $P < 0.05$ ).

Mean gene expression for IL-10 increased ( $P < 0.05$ ) by 9.68 fold in CLA fed cows than in the controls at day 21 postpartum and by 2.5 fold at day 42 postpartum ( $P < 0.05$ ). Mean gene expression for IL-10 was lower at day 42 postpartum compared with d 21 postpartum ( $P < 0.05$ ).

## DISCUSSION

In the present study, we investigated the effects of dietary CLA supplementation pro-inflammatory and anti-inflammatory cytokines at gene levels. The results showed that CLA supplementation reduced some key genes related to inflammation, therefore, it can help cow to combat with negative effect of inflammation on health status and reproductive performance, thus it would be a

good strategy to use CLA during transition period to improve the uterus conditions for establishment and maintenance of pregnancy.

Dietary FAs are known as major biologic regulators and have properties that are related to health and disease (Dirandeh *et al.*, 2013; 2015). Dietary CLA supplementation has positive effects on some immune parameters, and enhances the host defenses against invading organisms (Corino *et al.*, 2001). Consistent with earlier findings, analysis of our data illustrated that most pro-inflammatory cytokines cause inflammation decrease during both days 21 and 42, in cows fed with CLA, in contrast to the control group. Other long-chain unsaturated FAs such as linoleic acid and n-3 FAs decrease or inhibit the pro-inflammatory cytokines production (Calder, 2001; Håversen *et al.*, 2009).

The high concentrations of TNF- $\alpha$  and IL-1 are destructive and are implicated in some of the pathologic responses (Calder, 2006). Since, PolyUnsaturated Fatty Acids (PUFA) modulate immune responses and inflammation, n-3 FAs block TNF- $\alpha$  and IL-1 $\beta$  synthesis pathway (Endres *et al.*, 1989; Loscher *et al.*, 2005). In addition, some eicosanoids such as PGE2 and PGE3 prostaglandins inhibit the production of these two cytokines (Miles *et al.*, 2002). CLA by effects on COX-2 may cause an increase in the prostaglandins production, so, it affects the inhabitation of pro-inflammatory cytokines by enhancing prostaglandins (Abolghasemi *et al.*, 2016). Dirandeh and Ghaffari (2018) reported that positive effect of omega-3 on reproduction may act through a mechanism involving the endocannabinoid system.

Another cytokine produced by different types of cells upon stimulation with inflammatory stimuli and exerts a variety of functions on leukocytes is IL-8. There is no definitive evidence presented on its role in activating neutrophils in the lesions of various types of inflammatory reactions, but IL-8 plays a causative role in acute inflammation by recruiting and activating

the neutrophils (Harada *et al.*, 1994). Evidences of a study on the effects of palmitic acid, the common plasma free FA, on IL-8 demonstrated that this FA stimulates the production of IL-8 (Joshi-Barve *et al.*, 2007). On the other hand, the *n*-3 FA DocosaHexaenoic Acid (DHA) consumption that is one of the major components of fish-oil diets, results in the inhibition of some pro-inflammatory cytokines like IL-6 and IL-8. Comparing different FAs reveals that increase in the number of double band in the structure of FAs may increase their anti-inflammatory effects (De Caterina *et al.*, 1994; 2000). In addition, DHA has an inhibitory effect on the IFN- $\gamma$  cytokine (Khair-el-Din *et al.*, 1995).

In contrast to other cytokines, little is known about the effect of FAs on IFN- $\gamma$  production. Although, CLA consumption in healthy young volunteers showed significant decrease in IFN- $\gamma$  during the time (Song *et al.*, 2005), our results proposed that, following the CLA feeding and after reduction of IFN- $\gamma$  in day 21, there is a great increment in uterus by passing days to day 42. Long-term *n*-3 and *n*-6 FAs consumption resulted in a reduction of circulating the key pro-inflammatory cytokines including interleukin-1, 6, TNF- $\alpha$  and IFN- $\gamma$  (Purasiri *et al.*, 1994). The results from the present study suggest that the CLA supplement has no significant effects on IL-6, but other FAs like EicosaPentaenoic Acid (EPA) cause decrease in IL-6 secretion after UVB-irradiation (Pupe *et al.*, 2002).

It is believed that IL-10 confers protection against an overwhelming inflammatory response. An experiment on adipocytes incubated with DHA showed that the expression of genes IL-10 was increased to two fold (Bradley *et al.*, 2008). In other study on blood samples, the *n*-3 fatty acid did not have any significant effect on IL-10 secretion (Vedin *et al.*, 2008). Same as our results about IL-10, Loscher and colleagues (2005) have shown that the *cis*-9, *trans*-11 isomer of CLA may enhance the production of IL-10 in murine dendritic cells (DC).

The *cis*-9, *trans*-11 isomer of CLA may enhance the production of IL-10 in murine dendritic cells (Loscher *et al.*, 2005). Similarly, we found a greater genes expression of IL-10 in cows fed by CLA compared to control cows in both days 21 and 42. The IL-10 confers protection against an overwhelming inflammatory responses, the expression of IL-10 gene, in adipocytes incubated with DHA, increased to two fold (Bradley *et al.*, 2008), but in another study on blood samples, the *n*-3 FAs didn't have any significant effects on IL-10 levels (Vedin *et al.*, 2008).

The success of early pregnancy in the mated cow is dependent on the successful maternal recognition of pregnancy (Mann *et al.*, 1999). To achieve this embryo must prevent the demise of the corpus luteum by the timely production of InterFeron tau (IFN- $\tau$ ), the embryonic signal that acts to inhibit the development of the maternal luteolytic mechanism. IFN- $\tau$  acts locally in the uterus to suppress the development of oxytocin receptors in the endometrium and thereby suppresses the secretion of luteolytic episodes of PGF $_{2\alpha}$  generated by the binding of oxytocin to its receptors (Mann *et al.*, 1999). Supplementation with inhibitory fatty acids such as EPA during early pregnancy by dietary or parenteral means may further enhance the suppression of PGF $_{2\alpha}$  secretion in concert with the action of embryonic IFN- $\tau$  (Dirandeh *et al.*, 2015). Dynamics of maternal progesterone secretion also appear important for conceptus development and secretion of IFN- $\tau$ , which is secreted by the embryo for gestation recognition by the mother. Dirandeh *et al.* (2015) reported *n*-3 fatty acids prevent PGF $_{2\alpha}$  secretion from bovine endometrium and increased progesterone concentrations. Abolghasemi *et al.* (2016) reported that CLA supplementation during transition period increased progesterone concentrations in dairy cows.

Results of the present study showed that the production levels of pro and anti-inflammatory cytokines were regulated by CLA in the bovine endometrium tissue. We



suggested that the CLA has inhibitory effect on pro-inflammatory cytokines and enhances the anti-inflammatory cytokine IL-10, leading to reduction in inflammation of endometrium during early lactation.

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### تأثیر مکمل سازی جیره با اسید لینولئیک کنژوگه بر بیان ژن های سایتوکین های پیش و ضد التهابی در رحم گاوهای شیری هلشتاین

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#### چکیده

برای بررسی اثرات اسید لینولئیک مزدوج (CLA) خوراکی بر بیان ژن های سایتوکین های پیش التهابی و ضد التهابی در اندومتریم، از گاوهای هلشتاین که از نظر تاریخ زایش، سوابق تولید مثلی و رکورد پیشین مشابه بودند نمونه برداری شد. گاوها از ۲۱ روز پیش از زایش تا ۴۲ روز پس از زایش با جیره ی ایزونیتروژنوس، ایزوکالریک و ایزولیپیدیک که تنها از لحاظ منبع چربی متفاوت بود تغذیه شدند. از نظر چربی جیره ی تیمار شاهد دارای ۷۵ گرم روغن عبوری پالم و جیره ی آزمایشی دارای ۷۵ گرم CLA بود. مکمل CLA دارای ۱۰ درصد از هر یک از ایزومر های ۱۰-ترانس، ۱۲ سیس و ۹ سیس، ۱۱ ترانس بود. نمونه های رحم که در روزهای ۲۱ و ۴۲ پس از زایش به روش بیوپسی جمع آوری شدند، برای ارزیابی ژن های سایتوکین پیش التهابی از جمله: اینترلوکین ها (IL-1، IL-6، IL-8، IL-10، اینترفرون گاما (INF- $\gamma$ ) و سایتوکین ضد التهابی اینترلوکین ۱۰ (IL-10) مورد بررسی قرار گرفتند. نتایج نشان دادند که تغذیه با CLA موجب کاهش ژن های IL-1 و IL-6 و افزایش بیان ژن IL-10 در هر دو روز ۲۱ و ۴۲ پس از زایش شد. با توجه به نتایج به دست آمده، تغذیه CLA در دوره ی پس از زایمان با کاهش سایتوکین های پیش التهابی، در پی کاهش التهاب، موجب بهبود عملکرد رحم می شوند.