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Research Article

Biomarkers for negative energy balance and fertility in early lactating dairy cows

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Abstract

Most dairy cows suffer from negative energy balance in early lactation that can affect their fertility. Adding fat to the cattle diet increases the diet energy content. In this study, ketone bodies (acetone and acetoacetate), nonesterified fatty acids (NEFAs), and some individual fatty acids in serum and milk were used as biomarkers for negative energy balance and fertility in dairy cows supplemented with two sources of fat. A comparison of calcium salts of palm oil fatty acid versus MixPro-omega-3 (a heat-treated linseed oil) diet on the level of fatty acids in serum and milk of early lactating Holstein cows was assessed in relation to their fertility. The diets were formulated to be iso-nitrogenous and iso-caloric. Two diets were provided from day 0 to 30 days postpartum. Blood and milk samples were collected at 1st, 2nd, and 3rd weeks after calving. Results revealed that the increased serum and milk linoleic and linolenic acid with decreased saturated (palmitic and stearic acid) fatty acids and oleic acid are related to improved energy status with decreasing the ketone bodies and (NEFAs), and enhance the fertility of dairy cows received MixPro-omega-3 when compared with calcium soap fed group. The level of ketone bodies and NEFAs, increased in milk than in serum in the same group. The fertility was enhanced by giving MixPromega-3 diet compared with calcium soap diet.

Keywords: Dairy cattle, Fatty acids, Fat supplement, Ketone bodies

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Introduction

The early lactation period in high-producing dairy cows is markedly affected by negative energy balance and synthesis of large amounts of ketone bodies and nonesterified fatty acids (NEFAs), resulting in many metabolic disorders and impaired fertility (LeBlanc, 2010). Therefore, increasing diet energy content by adding fat to the diet may decrease the length and severity of negative energy balance and improve fertility. Several techniques have been developed to protect the fat especially polyunsaturated fatty acids (PUFA) from rumen biohydrogenation and safeguarded rumen environment, including production of calcium soap of fatty acids (Upritchard et al., 2005) and heat treatment of oil seeds in MixPro-omega-3 (Weiss and Wyatt, 2004). However, the protection of fatty acids from rumen biohydrogenation is only partial (Theurer et al., 2009).

Serum and milk fatty acids are related to the stage of lactation, suggesting that they are related to the energy balance of dairy cows (Dórea et al., 2017). Serum palmitic and stearic acids have unique and specific functions in lactating dairy cows beyond an energy source (Loften et al., 2014; Costa et al., 2020). The increased concentration of oleic acid in milk fat may be used as a biomarker for the early diagnosis of elevated blood levels of ketone bodies and NEFAs in the early stages of lactation in high-yielding cows (Puppel et al., 2017). Linoleic and linolenic acid are considered essential fatty acids to improve fertility by affecting ovarian follicles and improving embryo quality and pregnancy maintenance (Silvestre et al., 2011).

The objective of this study was to analyze the level of some fatty acids in serum and milk in early lactating dairy cows after adding two different sources of fats as early predictors of negative energy balance with relation to elevated NEFAs, ketone bodies, and fertility.

Materials and Methods

Study area and animal population

The study was conducted on a private dairy farm in Alexandria-Cairo desert road, Egypt, from May to September 2020. Twenty multiparous early lactating Holstein cows with almost the same body condition score, parities (3.1±0.2), and milk yield (30±2 Kg) were selected for the study. The animals were randomly divided into two groups and given diets according to Nutrient Requirements of Animals Collection (NRC) recommendations (NRC, 2001).

Dietary supplements and diets analysis

The calcium salts of palm oil (Norii Company, Egypt) contain 48% palmitic acid, 5% stearic acid, 36% oleic acid, 9% linoleic acid, and 2% others acids. The MixPro-omega-3 dietary supplement is a heat-treated linseed oil (Kemit Co., Egypt) that contains 13.36% palmitic acid, 6.07% stearic acid, 25.48% oleic acid, 28.8% linoleic acid, and 27.97% linolenic acid in dry matter. The calcium salt of palmitic acid was added to the ration for the first group, while the MixPro-omega-3 was added to the second group. The two diets were iso-nitrogenous and iso-caloric.

The first diet is composed of 6.75 kg corn, 6 kg soybean, 22.5 kg corn silage, 9 kg Egyptian clover, 3 kg hay, vitamins and minerals to which 0.56 kg Megalac calcium salt of palm oil per head was added daily. The second diet contains the same composition but 2 kg Mixpromega-3 was added instead of the calcium salt per head daily. The chemical composition of the two diets is presented in Table 1. Dietary treatments started from 0 day of calving till 30 days of lactation (first lactation period).

Sample collection and measurements

Blood and milk samples were collected at the 1^{st}, 2^{nd} and 3^{rd} weeks postpartum. Sera were immediately separated from blood samples and stored at -20°C until analysis. Milk samples were taken from the collected daily milk. Concentrations of ketone bodies (acetone and acetoacetate) in serum and milk were determined spectrophotometrically using Vanillin in an alkaline medium (Nadeau, 1952). The total NEFAs concentration in serum and milk was determined using the microtitration method (Dole, 1956). Lipids in serum were extracted according to Folch et al. (1957). Milk samples were initially centrifuged at 12000 rpm for 30 minutes at 4°C. One gram of the fat cake was centrifuged at 19300 $\times$g for 20 minutes (Feng et al., 2004). Serum and milk fats were saponified with potassium hydroxide. After acidification, fatty acid methyl ester was removed by continuous extraction with hexane (Christie, 1983) and analyzed with gas chromatography to evaluate fatty acids.

The fatty acids were analyzed using Model 5890 gas chromatograph (Hewlett Packard, USA) equipped with an HP-5 capillary column (30 mm $\times$ 0.32 mm $\times$ 0.25 μm film thickness; USA) a flame-ionizing detector. The initial temperature of the oven was set at 140°C for 4 min, increased at 4°C/min to 250°C, and then held for 5 min. The injection system temperature was 250°C, and the detector temperature was 275°C. The carrier gas was hydrogen, flowing at a 1.6 m/min linear velocity and the injection volume was 1µl. The measured reproductive parameters were calving to first estrus interval, the number of services per conception, and open days (days interval between calving and conception confirmed by ultrasound examination).

Statistical analysis

The data obtained were analyzed statistically by T-test (SAS, 2003). Only the results were considered statistically significant on ($p<0.05$).

Results and discussion

The current study results showed significantly decreased serum ketone bodies (acetone and acetoacetate) concentration in the 1^{st} and 3^{rd} week of lactation in the MixPro-omega-3 diet group compared with the calcium soap diet group (Table 2). Enjalbert et al. (2001), reported that the concentration of serum acetone was 356 μmol/l and acetoacetate was 236 μmol/l in the true positive ketotic cows. The ketone bodies serum level of the MixPro-omega-3 diet group was lower than that threshold for clinical and subclinical ketosis definition. In contrast, the serum ketone bodies level of the calcium soap diet group was near that threshold.

The results showed significantly increased NEFAs, in the 3^{rd} week of lactation in the calcium soap diet group compared to the MixPro-omega-3 and near the critical threshold. Serum NEFAs values were >0.7mmol/L, which are considered to be an indicator of severe negative energy balance (Whitaker, 2003). A previous study by Santschi et al. (2009), concomitant with our results, showed that plasma NEFAs levels were higher with cows fed with Megalac (mainly palmitic acid) than with whole flaxseed and micronized soybeans (mainly linoleic and linolenic acid).

Linseeds are an excellent source of linolenic acid, one of the most beneficial fatty acids for the liver. Its addition to the bovine ration resulted in decreased triglyceride concentrations and increased gluconeogenesis rates compared with other long-chain fatty acids (Mashek and Grummer, 2003). Serum fatty acids (FA) can be derived from many major pathways like the diet, the rumen (biohydrogenation, bacterial degradation, and synthesis), and body fat mobilization (Stoop et al., 2009). Our results showed that serum myristic, palmitic, and stearic acids were increased in the calcium soap diet group compared with the MixPro-omega-3 diet group. Myristic acid is found naturally in palm oil which explains the cause of its increase in serum. Similar results obtained by Purushothaman et al. (2008), who showed that saturated fatty acids, especially palmitic acid (C16:0) increased with calcium salt of palm oil fatty acids diet. Also, Tessari et al. (2020), showed an increased concentration of myristic acid in the serum of hyperketonemic cows and identified myristic acid as a new biomarker of lipomobilization.

Meanwhile, Dawod et al. (2020), reported that the extruded linseed diet feeding significantly reduced palmitic and stearic concentrations in postpartum cows. Oleic acid decreased in the serum of MixPro-omega-3 supplemented cows at the 2^{nd} and 3^{rd} week postpartum compared with the other group.

Table 1: Chemical analysis of the dry matter of the calcium soap diet and MixPro-omega-3 diets.

Nutrient[1]	**Dry matter basis % of the calcium soap diet**	**Dry matter basis % of Mixpro-omega-3 diet**
Dry matter	100	100
Moisture	0	0
Crude protein	16.5	16.63
NDF	39.56	41.72
ADF	20.18	20.32
Hemicellulose	19.38	21.4
Cellulose	16.08	16.22
Lignin	4.10	3.8
Ash	5.3	5.88
NFC	36.14	33.27
Ether extract	2.5	2.97
TDNix	68.98	68.83
DE(Mcal/Kg)	3.11	3.11
ME(M cal/Kg)	2.69	2.69
NEI(Mcal/Kg)	1.57	1.57

[1]Abbreviations: NDF, neutral detergent fiber; ADF, acid detergent fiber; NFC, non-fiber carbohydrate; TDN, total digestible nutrient; DE, Digestible energy; ME, metabolizable energy; NEI, net energy per lactation.

Table 2: Ketone bodies (mmol/L), nonesterified fatty acids (NEFAs) (mmol/L), and some fatty acid (g/100g fat) analysis in serum of dairy cows given calcium soap diet or MixPro-omega-3 diet.

Item	**First week postpartum**		**Second week postpartum**		**Third week postpartum**	
	Calcium soap	MixPro-omega-3	Calcium soap	MixPro-omega-3	Calcium soap	MixPro-omega-3
Ketone bodies	0.53±0.03	0.24±0.08*	0.63±0.1	0.55±0.17	0.62±0.02	0.47±0.07*
NEFAs	0.53±0.2	0.67±0.12	0.63±0.2	0.61±0.18	0.81±0.02	0.66±0.01*
Myristic acid (C14 :0)	4.8±0.2	3.1±0.11*	5.1±0.18	3.57±0.1*	5.3±0.14	4.58±0.24*
Palmitic acid (C16:0)	18.97±1.55	15.5±1.4	18.09±0.42	14.8±0.8*	17.6±0.8	15.5±0.67*
Stearic acid (C18:0)	17.5±0.81	15.4±1.5	19.53±1.02	15.9±0.5*	19.9±1.1	18.8±1.2
Oleic acid (C18:1cis9)	17±0.9	17.2±1.06	19.2± 0.7	15.76±1.0*	23±0.9	18.3±0.5*
Linoleic acid (C18:2)	1.94±0.2	4.2±0.3*	2.8±0.2	4.6±0.6*	2±0.65	4.2±0.7*
Linolenic acid (C18:3)	0.13±0.06	0.45±0.05*	0.15±0.03	0.66±0.03*	0.15±0.03	0.76±0.02*

*Significant difference at ($p<0.05$).

Oleic acid mainly originates from body reserves during energy deficits (Choi et al., 2013). Serum linoleic and linolenic acid significantly increased in the 1st and 2nd week of lactation in the MixPro-omega-3 diet group. This is probably due to their escape from the rumen biohydrogenation and being absorbed from the gastrointestinal tract providing the blood and mammary gland with linoleic and linolenic acid (Li et al., 2016). Oliveira et al. (2018), suggested that dietary linseed oil is a possible strategy to decrease negative energy balance and improve postpartum metabolic health.

The milk analyses showed a significant increase in the concentration of milk acetone level in the 3rd week of lactation in the calcium soap diet group compared to the MixPro-omega-3 diet group (Table 3). Milk acetone concentrations at levels >0.40 mmol/L were associated with a 3.2 times higher risk for endometritis (Vanholder et al., 2015). In another study, no significant effects on reproductive efficiency were found in cows with milk acetone concentration at a level ⩾0.7 mmol/, but impaired reproduction in cows with milk acetone concentration >1.4 mmol/L was reported (Reist et al., 2003). In the present study, NEFAs were significantly increased in the 1st, 2nd, and 3rd week of lactation in the calcium soap diet group compared to the other group. However, the concentration of total free fatty acids (FFAs) in milk that affects the NEFAs level is an indicator of dairy cow nutrition and the milk straining, bacterial contamination of milk, and storage quality (Dórea et al., 2017).

Milk fatty acids are derived from the same pathways of serum besides de-novo synthesis in the mammary gland (Hanuš et al., 2016). In the present study,

Table 3: Ketone bodies (mmol/L), nonesterified fatty acids (NEFAs) (mmol/L), and some fatty acids (%) analysis in the milk of Holstein dairy cows given calcium soap or MixPro-omega-3 diet.

Item	First week postpartum		Second week postpartum		Third week postpartum	
	Calcium soap	MixPro-omega-3	Calcium soap	MixPro-omega-3	Calcium soap	MixPro-omega-3
Ketone bodies	0.89±0.05	0.64±0.04*	0.88±0.02	0.82±0.08	0.82±0.07	0.54±0.06*
NEFAs	1.03±0.07	0.79±0.04*	1.2±0.08	0.65±0.04*	1.25±0.06	0.7±0.04*
Myristic acid (C14:0)	8±0.08	6.2±0.09*	8.25±0.08	6.6±1.2	9.1±0.1	7.3±0.3*
Palmitic acid (C16:0)	27.85±1.2	22.83±1.3*	31.09±1.53	28.43±2.3	33±1.8	28.0±0.4*
Stearic acid (C18:0)	24.53±1.55	21.53±0.81	23.00±0.76	19.83±0.98*	20.1±0.8	20.53±0.62
Oleic acid (C18:1cis9)	19.9±1.04	17.22±1.1	23.11±0.9	16.43±1.36*	22.5±0.73	17.5±0.87*
Linoleic acid (C18:2)	2.1±0.1	2.4±0.2	1.8±0.32	2.87±0.33*	1.4±0.2	2.6±0.3*
Linolenic acid(C18:3)	0.5±0.01	0.9±0.03*	0.42±0.05	0.74±0.03*	0.41±0.03	0.74±0.03*

*Significant difference at ($p<0.05$).

Table 4: Some Fertility parameters in Holstein dairy cows given calcium soap diet or MixPro-omega-3 diet.

Fertility parameters	Calcium soap diet group	MixPro-omega-3 diet group
First postpartum estrus	43±3.4	32 ±1.2*
Services/conception	3.4±0.9	2.7±0.7
Calving interval	430±8.3	405±5.9*

*Results presented as mean± standard error and the star denotes a significant difference at ($p<0.05$).

myristic, palmitic, and stearic acids were significantly decreased in early lactated dairy cows fed MixPro-omega-3 compared with the calcium soap supplemented group. A previous study by Zachut et al. (2010) showed that the high uptake of long-chain fatty acids by the mammary gland tissue inhibits *de novo* synthesis of fatty acids. Compared with the calcium soap diet group, milk oleic acid was significantly decreased in MixPro-omega-3 in the 1st and 2nd weeks of lactation. Accordingly, milk fat C18:1 cis-9 was identified to be useful for diagnosis and early warning of cows suffering from a severe negative energy balance when the content of oleic acid in milk exceeds 24g per 100g fat (Jorjong et al., 2014; Puppel et al., 2017). Linoleic and linolenic acid levels were increased in the milk of cows given the MixPro-omega-3 diet. The same results were previously reported (Vanbergue et al., 2018).

Reproductive response results are affected by the supply of some fatty acids rather than an effect of the energy supply (Ducháček et al., 2015). The palmitic and myristic acids were adversely associated with most cardiovascular diseases in humans (Ebbesson et al., 2015). It is possible that saturated fatty acids induce an increase in systemic oxidative stress (McFadden, 2020) or cellular endoplasmic reticulum stress damaging reproduction (Yang et al., 2016). Higher levels of oleic acid down-regulated the expression of some reproductive genes, reduced estrogen concentration, and reduced the ovulation rate of the oocyte (Sharma et al., 2019).

Saturated fatty acid and palmitic acid in high concentration impaired maturation, viability, and embryo production rates. In contrast, unsaturated fatty acid oleic acid at the physiological level promotes the maturation, cleavage, and embryo production rates of ovine oocyte compensated for the adverse effects of palmitic and stearic acids (Tripathi et al., 2015). Furthermore, supplementation of linseed as a source of polyunsaturated fatty acids positively affects oocyte metabolism, increases progesterone concentration, and improves the growth of ovarian follicles and larger corpus luteum (de Souza et al., 2018). Also, supplementation of dairy cows with extruded linseed aid the production of prostaglandins (eicosanoids) that help establish new estrous cycles, enhance visible signs of heat and increase blood flow to the ovaries (Santos et al., 2008; Zachut et al., 2010).

In conclusion, this study aimed to analyze the level of fatty acids in serum and milk as biomarkers for elevated ketone bodies and NEFAs in early lactated dairy cows in relation to fertility. The study results revealed that decreased myristic, palmitic, stearic, oleic, and increased linoleic and linolenic acid levels are related to improved negative energy balance and fertility.

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