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Effects of Feeding Rumen-Protected Choline and Vitamin E on Serum Protein Fractions, Total Thiol Molecules and Total Antioxidant Capacity in Early Lactating Dairy Cows

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ABSTRACT

Twenty four primiparous and multiparous Holstein cows on early lactation, beginning five weeks postpartum, were used for four weeks to investigate the effects of supplementation of rumen-protected choline (RPC) or vitamin E on blood serum protein fractions, plasma total thiol molecules (TTM), and plasma total antioxidant capacity (TAC). Cows were randomly assigned to one of the following treatments: I - no supplement (control), II - 90 g/d of RPC, and III - 4400 IU/d of vitamin E. Serum protein electrophoresis of samples exhibited four main fractions in the blood serum of the cows including: albumin, α, β, and γ. The electrophoresis was carried out by capillary zone electrophoresis (CZE). In this study, feeding RPC or vitamin E affected the blood serum albumin fraction as well as blood plasma TTM ($P<0.05$) but the treatments did not affect the different fractions of globulin as well as plasma TAC ($P>0.05$). The results showed that the increases in serum albumin fractions and TTM which observed in this study, pointed towards a beneficial role of RPC and vitamin E in early lactating dairy cows.

Keywords: Electrophoresis, Serum Proteins, Choline, Vitamin E, Dairy cow.

INTRODUCTION

Normal plasma or serum protein electrophoresis leads to identification of two major protein fractions: albumin and globulin. In humans, sheep, goats, rabbits, dogs, guinea pigs and rats, albumin predominates over globulin while in horses and cows the ratio of albumin and globulin is nearly equal, or globulin predominates (Swenson, 1993). In addition to such species characteristics, there are evidences that some physiological factors, namely: hormones, sexual influences, pregnancy, lactation (Nath *et al.*, 2005; Pourouchottamane *et al.*,

2005; Richard Jagatheesan *et al.*, 2005), nutritional state and many other conditions (dehydration, hemorrhage, liver and kidney dysfunctions, and inflammatory processes) affect serum protein level (Doxey, 1983; Coles, 1986).

Albumin is essential due to its contribution in the maintenance of osmotic pressure of plasma, because it is carrier of many vital substances like steroid hormones, hemin and fatty acids. The albumin value frequently and markedly declines during different diseases. Globulin fraction contains enzymes, hormones and antibodies, which are synthesized at various places in the body (Nicholson *et al.*, 2000). The α-globulin value increases mainly in traumas, and some alterations of the lipoprotein metabolism induce changes in the β-globulin fraction. γ-globulin concentration is a reliable indicator of humoral immunity. Its principal component is IgG but other isotypes of antibodies are also present in this fraction (Goldsby *et al.*, 2001). Blood proteins, with the exception of the γ-globulin fraction, are synthesized in liver (Diehl and Delincee., 1986).

To measure classical protein fractions in serum several electrophoretic techniques are available, like separation on cellulose acetate membrane, agarose gel, etc. but capillary zone electrophoresis (CZE) has been suggested as a useful technique for separation and quantification of serum proteins (Henskens *et al.*, 1998).

Total thiol molecules (TTM) are powerful reducing agents capable of acting antioxidants (Ueland *et al.*, 1996). TTM status is important for normal physiological function. Changes in TTM status have been linked to induction of apoptosis (programmed cell death) (Marchetti *et al.*, 1997), and have been observed in a number of diseases including vascular disease and renal failure (Ueland *et al.*, 1996).

Free radicals can be produced during the respiratory oxidation of different cells. Since free radicals can damage various macromolecules as protein, fat, nucleic acids, etc. they are harmful for body (Jamro and Beltwoski, 2002). The natural defense system, which can prevent the damage of free radicals and neutralize them, has been referred to as total antioxidant capacity (TAC) (Kankfer and Lipko, 2006).

Choline is in the structure of lipoproteins which transport lipids in the blood, thus it is an important factor in preventing fatty liver and ketosis in lactating cows (Cooke *et al.*, 2007). Unfortunately most of dietary choline is degraded by microbial populations in the rumen (Sharma and Erdman, 1989), and not much is available for absorption; therefore, choline must be in rumen-protected form when fed.

Choline can also be used as an antioxidant (Elsawy *et al.*, 2014) because it has significant antioxidant properties that protects cells (Jansen, 2014). In a research, dietary choline decreased the oxidant damage and regulated the antioxidant system in immune organs of fish (Wua *et al.*, 2014).

Vitamin E (*α*-tocopherol) is a powerful antioxidant for body defense against oxidative stress (Burton and Traber, 1990; Ibrahim *et al.*, 1997) and is not degraded in the anaerobic ruminal environment (Burton and Traber, 1990; Leedle *et al.*, 1993).

In peripartum and early lactating cows, lipid peroxidation increases (Castillo *et al.*, 2005) while serum *a*-tocopherol decreases (LeBlanc *et al.*, 2004) indicating a higher level of oxidative stress which subsequently can lead to reduced health in dairy cows (Miller *et al.*, 1993).

The role of vitamin E in recovering from postpartum-related oxidative stress and decrease in lipid peroxidation in liver has been reported in cattle, mice and rats (Ferre *et al.*, 2001; Bouwstra *et al.*, 2008). In a study, supplemental vitamin E could improve liver antioxidant status in mice with fatty liver (Soltys *et al.*, 2001).

The present study was carried out to compare the oxidative status of dairy cows on early lactation which received either supplemental RPC or vitamin E or those unsupplemented, and also to assess the changes in serum protein fractions.

MATERIALS AND METHODS

Cows, treatments and experimental design

Twenty four early lactating primiparous and multiparous Holstein cows beginning five weeks postpartum (BCS = 2.82 ± 0.12; mean ± S.D. and number of lactation = 2.56; mean) were used for four weeks from October 2011 to November 2011. The cows were free from any diseases, with a normal healthy appearance, and were housed in individual tie stalls. All experimental procedures were in accordance with the guidelines for the use and care of experimental animals and approved by the animal ethical committee of Tehran University. Selection of the cows was based on parity, milk yield of previous lactation (milk yield of dams for the cows in their first lactation) and BCS. In this study, there were 3 blocks based on lactation numbers 1, 2, and 3 or greater. Lactation number was indication of age. Eight cows per treatment were randomly assigned to receive one of the following treatments, using block randomization based on parity: I- no supplement (control), II- 90 g/d of RPC and III- 4400 IU/d of vitamin E. The RPC (Reashure Choline, Balchem, USA; 25%) was a rumen protected source of choline chloride, and the vitamin E was the product of Roche Company (Vitamins Ltd; Switzerland). The cows were fed total mixed rations (TMR) *ad libitum*. The diet (Table 1) was formulated to meet the nutritional requirements of dairy cows (NRC, 2001). The RPC and vitamin E were top dressed onto the TMR.

Table 1 - Ingredients and nutrient composition of the diet

Ingredient (g/kg of DM)		(Analyzed or Calculated) Chemical Composition [3]	
Alfalfa hay (medium chopped)	204.7	DM (g/kg) [4]	590
Corn silage	175.8	CP (g/kg) [4]	171.7
Beet pulp	41.3	Ash (g/kg) [4]	57.6
Ground barley grain	198.8	Total fat (g/kg) [4]	43.8
Ground corn grain	58.7	NDF (g/kg) [4]	304.8
Ground wheat grain	28.5	ADF (g/kg) [4]	183.8
Solvent extracted soybean meal	79.9	NFC (g/kg) [4, 6]	382.1
Wheat bran	7.1	Ca (g/kg) [4]	8.1
High lint whole cottonseed	29.5	P (g/kg) [4]	5.0
Canola meal	100.2	NEL (Mcal/kg) [5]	1.66
Corn gluten meal	11.5	RUP (g/kg of CP) [5]	314.5
Minerals and vitamins supplement [1]	6.3	RDP (g/kg of CP) [5]	685.5
Fat supplement (energy booster) [2]	15.9	Met (g/kg MP) [5]	22.1
Salt	2.5	Lys (g/kg MP) [5]	76.9
Calcium carbonate	3.2	Vitamin E (IU/kg) [5]	18.9
Sodium bicarbonate	10.2		
Di calcium phosphate	3.7		
Magnesium oxide	1.9		
Mycosorb	0.6		
Biotin premix	0.7		
Zeolit	19.0		

1 - Contained 190 g Ca/kg, 90 g P/kg, 30 g Mg/kg, 4 g Fe/kg, 0·5 g Cu/kg, 5 g Mn/kg, 4 g Zn/kg, 0·1 g Co/kg, 0·1 g I/kg, 0·03 g Se/kg, 0·4 g antioxidant/kg, 5×10^5 IU vitamin A/kg, 10^5 IU vitamin D/kg and 3×10^3 IU vitamin E/kg.
2 - Rumen protected fat - Energizer RP10.
3 - Analysis conducted with four TMR samples.
4 - The nutrients which were determined by laboratory.
5 - The nutrients which were calculated using the standards (NRC, 2001).
6 - NFC=1000− (CP+ash+total fat+NDF).

Blood Sampling

Blood samples were obtained before morning meal from the coccygeal vein (tail vein) on the last day of the experiment, by using heparinized and non-heparinized Vacutainers tubes (Becton Dickinson, Franklin Lakes, NJ). Blood samples were placed on ice immediately following collection. Then plasma and serum were harvested after centrifugation of the blood at 3000 g for 15 min and were stored at -20 °C until subsequent analyses. The indices of oxidative status including TTM and TAC concentration were analyzed in plasma samples, and the different fractions of blood serum protein were analyzed in serum by CZE.

Capillary Zone Electrophoresis

The Capillary system (Sebia, Issy-les-Moulineaux, France) was operated according to the manufacturer's instructions under software version 1.4.1. The instrument has eight fused silica capillaries (17 cm in length and 25 µm ID). The alkaline buffer (borate and additives) is pH 10 and sample is diluted 1:10. Detection voltage is 9 kV. Separation is carried out at 35°C and takes 2.5 min. Ultraviolet detection at 200 nm is used for direct quantification of the peptide bonds. When the samples are analyzed in batch, capillary has a throughput of 100 samples/h. A typical electrophoretic pattern for blood serum of a cow is shown in Figure. 1. The proteins were measured at 200 nm wavelength. Protein values were expressed as percentages.

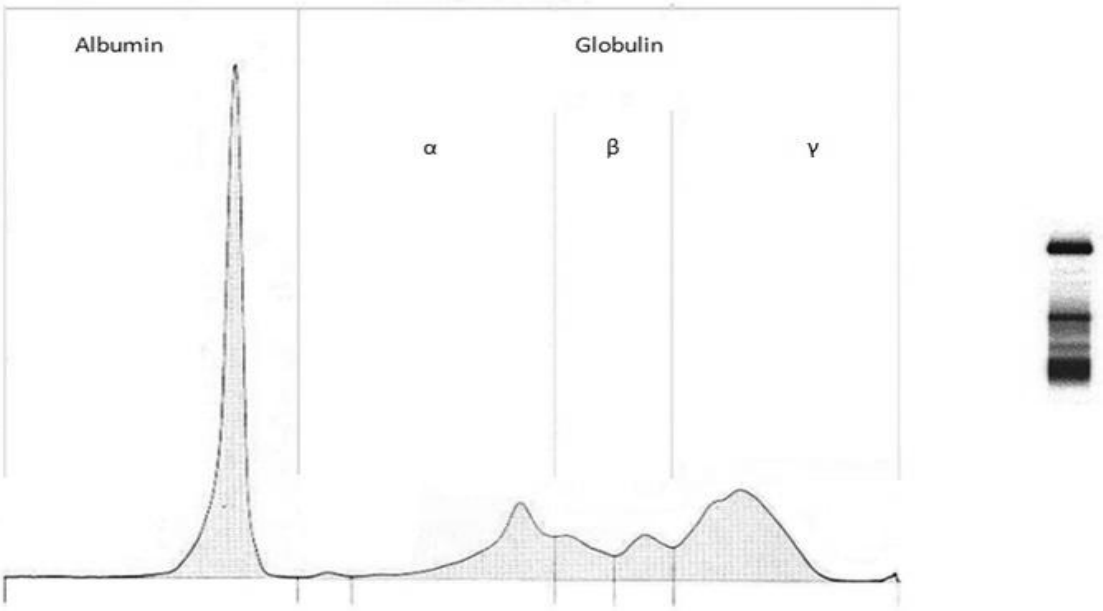

Figure 1- Capillary zone electrophoresis for blood serum of a cow

Assessment of plasma total antioxidant capacity (TAC)

TAC of plasma was evaluated by applying the FRAP assay (ferric reducing antioxidant power or ferric reducing ability of plasma) (Benzie and Strain, 1999). The method is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion at low pH. This causes a formation of blue colored ferroustripyridyltriazine (Fe^{2+}-TPTZ) complex, which absorbs at 593nm. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma (Liu *et al.*, 1982; Benzie and Strain, 1999). Results were expressed as mmol/l (mM). The method could be described in brief as the following: the working FRAP reagent was prepared ex tempore by mixing 300 mmol/l acetate buffer, pH 3.6 with 2,4,6-tripyridyl-striazine (TPTZ) solution (10 mM in 40 mM HCl) and 20 mmol/l $FeCl_3$ solution in ratio10:1:1 respectively, and was pre-tempered at 37°C. The reaction was performed by adding of 100 µl plasma, previously diluted 1:1 with distilled water, to 900 µl FRAP working

reagent and the mixture was incubated for 25 min at 37°C. The absorbance was measured on 593nm compare to a blank mixture where 100 µl water was added to the working FRAP reagent instead of plasma. Aqueous solutions of known Fe^{2+} concentration, in range 0.2 to 1 mmol/l (FeSO4. 7H2O) were used for creating of the standard curve. The results were expressed in mmol/l (mM) Fe^{2+}.

Measurement of Plasma Total Thiol Molecules (TTM)

Total sulfhydryl content was determined in plasma by the method of Hu (Hu and Dillared, 1994). A volume of plasma (0.20 ml) was mixed in a 10 ml test tube with 0.6 ml of Tris–EDTA buffer (Tris base 0.25 M, EDTA 20 mM, pH 8.2) followed by the addition of 40 ml of 10 mM of DTNB (Dithiobis-2-nitrobenzoic acid) in methanol. The final volume of the reaction mixture was made up to 4.0 ml by adding 3.16 ml of methanol. The test tube was capped, and the color was developed for 15–20 min, followed by centrifugation at 3000 g for 10 min at ambient temperature. The absorbance of the supernatant was measured at 412 nm. The TTM capacity was expressed as nmol per mg of protein in samples.

Statistical Analyses

Raw data were transformed to their natural logarithm to achieve a normal distribution for analysis. All transformed data were back-transformed for reporting least squares means. Statistical analyses were performed with SAS (SAS, 2002) using GLM procedure in SAS by inspection of standardized residuals plotted against the predicted residuals. Standardized residuals were also inspected graphically to assess fit to a normal distribution. Differences among means were separated with Duncan multiple range test. Each metabolite was considered as an outcome in separate models over the whole experimental period. Significant levels were declared at $P<0.05$.

RESULTS AND DISCUSSION

Cows undergo a variety of physiological changes during lactation. These occur with respect to the cows' blood composition due to metabolic changes. In fact, the cows make adjustments to provide an adequate supply of nutrients for producing milk while lactation (Drackley, 1999).

In our study, we fed the experimental cows 90 g/d of RPC, because according to some researches feeding 90 g/d of choline would be optimal dose in lactating dairy cows (Sharma and Erdman, 1989; Xu *et al.*, 2006). In an experiment, oral administration of 800 IU/d of vitamin E resulted in significant improvements in liver function in people with non-alcoholic fatty liver disease (Sanyal *et al.*, 2010), thus we decided to feed 4400 IU/d of vitamin E because a cow's body weight is approximately 5.5 times heavier than a human's body weight.

In this research, serum protein electrophoresis of the samples separated into four major fractions: albumin, α-globulin, β-globulin, and γ-globulin (Table 2). The treatments affected albumin fraction ($P<0.05$), but not different fractions of globulin ($P>0.05$).

Albumin is associated with postpartum diseases and can be used to predict disease risks in early lactation period (Saun, 2004). In spite of concerns about variables confounding albumin interpretation, it seems to be a good disease risk indicator possibly reflecting availability of amino acids from the labile protein pool. In our study, feeding RPC or vitamin E affected albumin fractions ($P<0.05$). Therefore, due to increases in albumin fractions of the treated cows we could speculate that the treated cows might be more resistant against various diseases compared with the control group.

Generally at the beginning of lactation cycle, the blood level of NEFA, are elevated mainly due to negative energy balance which would result in a reduced performance of the liver (Overton and Waldron, 2004). Choline reduces NEFA in blood stream due to donation of methyl groups which may lead to improving liver function (Cooke *et al.*, 2007; Soltan *et al.*,

2012). Some researchers have reported that the blood metabolites, like total protein, albumin and globulin, in cows and goats were not affected by choline supplementation (Ambrosio *et al.*, 2007; Toghdory *et al.*, 2007; Mohsen *et al.*, 2011).

Table 2: percentages of serum protein fractions through CZE, and the amount of TTM and TAC in supplemented cows with RPC or vitamin E

Item	Control	Choline	Vitamin E	P- Value
Albumin	36.69±0.98[b]	39.70±0.83[a]	40.21±1.09[a]	0.0486
α-globulin	16.21±1.16	15.34±1.45	14.60±1.30	0.7413
β-globulin	11.45±0.75	9.10±0.76	8.55±0.65	0.4252
γ-globulin	35.65±1.68	35.86±0.67	36.64±0.80	0.8343
TTM (nmol/mg)	1.44±0.07[b]	1.66±0.06[a]	1.64±0.04[a]	0.0362
TAC (mmol/l)	0.12±0.01	0.13±0.01	0.14±0.01	0.5519

[ab] different superscripts are indicating significant differences (*P<0.05*) between the various study groups

In an experiment on ewes, the data demonstrated that receiving vitamin E, starting two weeks before mating and extending through pregnancy till occurrence of lambing, improved levels of albumin, globulin and total serum protein in treated ewes (El-Shahat and Monem, 2011). Similar finding was obtained by other researches in buffaloes (Helal *et al.*, 2009). In a study on vitamin E deficient rabbits, total plasma protein concentration was not significantly affected by vitamin E deficiency, but albumin levels were lower and globulin levels were higher in deficient animals (Diehl and Delincee., 1986).

In this research, supplementation of RPC or vitamin E affected the concentrations of TTM (*P<0.05*), but the treatments did not affect TAC (*P>0.05*; Table 2).

TTM are organic compounds that contain a sulphydryl group. Among all the antioxidants that are available in the body, thiols constitute the major portion of the total body antioxidants and play a significant role in defense against reactive oxygen species. Albumin is exclusively synthesized by the liver, and it is the main source of plasma thiols. Glutathione is mainly synthesized *de novo* within the liver (Jefferies *et al.*, 2003). The reduction of liver function that is usually observed in the early lactating cows might explain lower plasma thiol levels (Bernabucci *et al.*, 2005).

Considering additional data from literature (Goff and Stabel, 1990; Goff and Horst, 1997), the reduction of vitamins E and A in plasma might help to explain the alteration of the oxidative status after calving. In this regard, some studies have demonstrated that, besides enhancing plasma level of fast-acting antioxidants, the supplementation of vitamin E can be useful against oxidative stress in early lactating dairy cows (Weiss *et al.*, 1990; Brzezinska-Slebodzinka *et al.*, 1994).

CONCLUSION

From the results of the present study, we can conclude that the increases in serum albumin fraction and TTM which observed in both RPC and vitamin E groups pointed towards a beneficial role of RPC and vitamin E.

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