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Effects of Glutamine on Oxidative Stress and Immune Response and Acute Phase Proteins in Prepartum in Holstein Dry Cows

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Abstract: The objective of this study was to investigate whether consuming of Protected Glutamine (PG) before parturition in close up period would affect biomarkers of oxidative stress, immune system and Haptoglobin (HP) and Serum Amyloid A (SAA). About 36 pregnant Holstein dairy cows were assigned into two treatment groups based on their BCS and expected calving date in at student examination. Treatment groups consisted of glutamine supplementation 100 g day^{-1} per cow before calving (F), glutamine did not supplementation before calving (N). There were not any significantdifferences among treatments in DMI and BCS on 21, 14 and 7 days before parturation. There were not significant differences in the Total Antioxidant Status (TAS), Haptoglobin (HP), Serum Amyloid A (SAA), No Esterified Fatty Acids (NEFA) and blood and immune cells. The plasma Glutathione Peroxidase activity (GPX) was significant difference between two group and it seems that supplementation diets with glutamine on the close up period can enhanceplasma Glutathione Peroxidase activity (GPX).

Key words: Holstein cow, oxidative stress, Serum Amyloid A (SAA), glutamine, haptoglobin, glutathione peroxidase activity

INTRODUCTION

The non-lactating period of the dairy cow is commonly referred to as the dry period. Throughout this period, the mammary gland undergoes remarkable changes in histology and physiology, characterized as involution which is believed to be necessary for maximal milk production in the subsequent lactation (Drackley *et al*., 2001). The high-producing dairy cow experiences huge metabolic changes during the transition from the dry period in late conception to the start of numerous milk production in early lactation (Bell, 1995). The periparturient period is thus associated with an increased risk of metabolic and production related diseases, arise because of inadequate metabolic homeorhetic adaptation (Drackley *et al*., 2001). Approximately, one half of economic losses in treatments of dairy industry assigned to transition period (Grummer, 1993). After calving the intake of NEl and metabolizable protein by healthy dairy cattle are less than requirement, 26 and 25%, respectively on 4th day postpartum (Bell, 1995). Increasing demand of energy and protein for lactation causes animal to be in catabolic situation and catabolic pathways increases the production of Reactive Oxygen Metabolites (ROM) (Bernabucci *et al*., 2005). The considerable increase in oxygen requirement during times of increased metabolic demands results in increased production of Reactive Oxygen Species (ROS). Animbalance between increased production of ROS and the availability of antioxidant defenses needed to reduce ROS accumulation during the periparturient period may expose cows to increased oxidative stress.

There are now several more recent studies to support the concept that oxidative stress is a significant causal factor to dysfunctional host immune and inflammatory responses that can increase the weakness of dairy cattle to a variety of health disorders, particularly during the transition period (Bernabucci *et al*., 2005). It has been hypothesized that an involvement of oxidative stress during transition period is the etiology of some diseases and disorders in dairy cows (Bernabucci *et al*., 2005). During the transition period, immunosuppression commonly occurs and cows exhibit great susceptibility to a number of diseases (Sordillo and Aitken, 2009). A number of components of the host defense system are changed during this period including neutrophil function, lymphocyte responsiveness to mitogen stimulation,

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antibody responses and cytokine production by immune cells (Politis *et al*., 1995). Impaired neutrophil function prior to parturition has been linked to the occurrence of mastitis, metritis and retained placenta in dairy cows (Kimura *et al*., 2002). A relationship between the physiological changes associated with parturition and a loss in overall antioxidant potential was established in both humans and dairy cows (Bernabucci *et al*., 2005). Supplementing dairy cows with sufficient levels of both vitamin E and C was shown to increase the phagocytosis, bacterial killing and oxidative metabolism of peripheral blood and mammary gland neutrophils when compared to cows that were otherwise deficient in this micro-nutrient (Gyang *et al*., 1984; Hogan *et al*., 1992). Some earlier studies presented effective treatment of vitamin A on udder health. Cows with mastitis had lower levels of plasma vitamin A and cows supplemented with b-carotene prior to involution had lower rates of new intramammary infections during the dry period when compared to un-supplemented animals (Chew and Park, 2004). An increased occurrence of mastitis and the severity of clinical symptoms wasassociated with decreased concentrations of plasmatic vitamin C (Weiss and Spears, 2006). Cytosolic Glutathione Peroxidase (GPX) is the selenoenzyme usually associated with antioxidant functions in cattle. By increasing use of NADPH in oxidative stress (for reducing glutathione peroxidas) the ability of neutrophils to destroy microbes will be diminish and immune system will be suppressed (Hammon *et al*., 2006). The *in vitro* studies have shown that glutamine can provide about 38% metabolizable energy for macrophages (Newsholme, 1987). Other evidence suggested that proliferative response of rat, mouse and humans lymphocytes to mitogens are depend upon availability of glutamine (Chuang *et al*., 1990). Considering what was mentioned above, researchers hypothesized that increasing glutamine in prepartum period can decrease oxidative stress and consequently improve immune function performance.

MATERIALS AND METHODS

Animals and feeding: The experiment was carried out in a commercial dairy herd. The average milk yield per lactation (305-DIM) of the herd was >9100 kg. The period of trial was between 12th September to 1st November. Total 36 pregnant Holstein cows multiparous were assigned into two group based on their BCS and expected calving date. The 25 days before expected parturition, cows were assigned to one of two dietary treatments arrangement; glutamine supplementation 100 g day^{-1} per cow before calving (F), glutamine did not supplementation before calving (N). The two groups received a ration as TMR for based on their requirement to supply their need in close up period based on NRC (2001) recommendations. Experimental diet has been shown in Table 1. The diets administered throughout the trail consisted of a basal ration given *ad libitum* to achieve 5-10% orts as a daily TMR that offered at 0830 until calving. Dry matters of feeds were measured weekly by drying in a oven at 105°C for 48 h.

Table 1: Experimental diet for cows

	Treatment		
Parameters (days)	F	N	p-values
TAS (mmol L^{-1})			
7	0.311	0.327	0.2900
14	0.255	0.276	0.2500
21	0.215	0.227	0.5100
GPX (mg mL^{-1} PCV)			
7	57.440	47.940	<0.0001
14	45.870	41.720	0.0400
21	30.870	33.730	0.2700
BUN (mg dL^{-1})			
10	13.640	13.820	0.9800
21	13.570	13.470	0.7700
DMI (kg day^{-1})			
10	13.290	13.400	0.2200
21	13.200	13.360	0.1200
BCS			
10	3.560	3.660	0.7500
21	3.250	3.340	0.8900

Measurements and sampling: In the trail period, dry matter of diets was determined by forced air oven drying at 55°C to static weight. Samples of feeds were analyzed for CP (AOAC, 2000; ID 984), ether extract (AOAC, 2000; ID 920.39) and ash (AOAC, 2000; ID 942.05), ADF and NDF. Body Condition Score (BCS) was scored (5-point scale where 1 = emaciated and 5 = obese) by three skilled individuals in 0, +10 and +21. Feed intake was determined daily by measuring supplied feed and refusals for TMR and was averaged per week. Dry Matter Intake (DMI) in calving day measured individually and reported but DMI in 7, 14 and 21 days after parturition was the average of DMI in days (0-7), (7-14) and (14-21). The refusals were monitored to avoid selection in rations by cows. NRC (2001) requirements were used for diet formulation. Blood samples spontaneously were obtained by using evacuated tubes from coccygel vein in two distinct tubes, one containing Li-heparin to separate plasma to assay plasma Glutathione activity (GPX), Total Antioxidant Status in plasma (TAS), Blood Urea Nitrogen (BUN), HP, SAA and NEFA and the other containing anticoagulant to assay Packed Cell Volume (PCV) and blood cells specially RBC, neutrophils and lymphocyte at 4 h after morning feeding. BUN was analysed by manual colorimetric method (Evans, 1968). GPX activities were

determined by a kinetic method with a commercial kit (RANSEL by Randox laboratories Ltd.). The method was based on Paglia and Valentine (1967). Glutathione peroxidase catalyzes the oxidation of glutathione by cumenehydroperoxde.

In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was followed at 340 nm for 3 min. Enzyme activity was reported in units per milliliter in plasma. TAS in plasma was measured by using the kit supplied by Randox laboratories Ltd. based on the incubation of ABTS (2, 2′-azino-di-[3-ethylbnzthiazoline sulphonate], Boehringer Mannheim) with a peroxides (metamyoglobin) and H_2O_2 to produce the radical cation $ABTS^+$ (Ghiselli *et al*., 2000). This has a relatively stable blue-green colour which is measured at 600 nm. HP, SAA and NEFA were determined by a kinetic method with a commercial kit (RANSEL by Randox laboratories Ltd.). For accuracy and reproducibility control, a commercial kit (Randox TAS kit, Randox laboratories Ltd.) was used.

Amino acid protection method and feeding: Glutamine amino acid was provided amount 80 kg in powder form. In order to protect, researchers used formaldehyde (Davies *et al*., 1993). To ensure and apply the best level of formaldehyde, they designed an experiment by the use of fresh rumen liquid. They prepared cultures that the only source of nitrogen was protected glutamine by formaldehyde. Before initiation of experiment, add 0.5, 1, 1.5 and 2% (w/w) formaldehyde solution by spring on the 5 g glutamine and after reaction dried them in an oven at 40°C at 24 h (Davies *et al*., 1993). After preparation of cultures in bottles that have not any source of nitrogen the protected glutamine with different levels of formaldehyde and rumen liquid from dairy cattle injected to bottles (any level protection in 3 bottles). After 24 h holding in 39°C, researchers repeated and continued inoculation 3rd sub-culture. It was clear that the amount of bacteria growth depend on glutamine availability. Therefore, researchers measured the amount of offence (representation of bacteria population) in bottles by use of a spectrophotometer and then compared them with t-student. Then, observed that the best level of formaldehyde is 1% and there was not significant between levels >1% but protection with 1% was better than 0.5%.

Statistical analysis: The means of two group comparated by using t-student between groups. Data measured over time (DMI and other parameters) within the period of interest were subjected to procedure of SAS. In this study, differences among treatments were considered significant if $p<0.05$ whereas when $0.05<p<0.15$, differences were considered to indicate a trend towards significant.

RESULTS AND DISCUSSION

DMI, TAS, GPX, BUN and BCS: The two ration were iso-nitrogenous and iso-energetic except adding 100 g Protected Glutamine (PG) per cow day^{-1}. The Dry Matter Intake (DMI), BUN, BCS changes, TAS and GPX have been shown in Table 2. There was no significant difference in DMI among groups in 10 and 21 days before parturition and this finding is in agreement with previous studies that abomasal infusion of glutamine did not affect DMI (Plaizier *et al*., 2001). To investigate the redox conditions of plasma dynamically and biologically, measuring TAS is an effective method that provides valuable information (Castillo *et al*., 2006). There were not any significant difference in TAS at calving day among treatments. Miller *et al*. (1993) reported that the decrease of oxidative stress before parturition might be ascribed to the increase of antioxidant protection that occurs in that particular physiological stage. This means that when the risk of oxidative damage increases, endogenous antioxidant protection increases too.

Therefore, researchers suppose that the increasing antioxidant capacity just before calving could have a confusing effect on the responses. There were significant differences among treatments at 7 and 14th day before parturition in GPX condition but there was not significant

Table 2: The Dry Matter Intake (DMI), BUN, BCS, TAS and GPX changes

	Treatment		
Parameters (days)	F	N	p-values
HP (g L^{-1})			
21	0.36	0.37	0.23
0	0.50	0.49	0.15
SAA (g L^{-1})			
21	0.12	0.13	0.25
0	0.15	0.14	0.33
NEFA (µEq L^{-1})			
21	234.00	243.00	0.19
0	276.00	265.00	0.22
Blood cells			
RBC ($\times 10^6$ µL^{-1})			
21	6.54	6.74	0.54
0	6.03	6.05	0.14
Neutrophill (%)			
21	44.35	42.33	0.19
0	45.29	44.16	0.12
Lymphocyte (%)			
21	54.50	56.79	0.45
0	53.50	52.80	0.35
PCV (%)			
21	30.20	29.90	0.94
0	31.50	30.00	0.85

differences between treatments in GPX condition at 21st day before calving. In high producing dairy cows, especially in the transition period with increasing milk production, >1 kg of milk protein is secreted daily, ≥30% of plasma protein flux (Bequette *et al*., 1996). Glutamine is the most abundant amino acids in the plasma and milk and during early lactation a decline of 25-30 and 75% was reported for the plasma (Meijer *et al*., 1995) and free pool of GLU in the muscle (Palmer *et al*., 1996).

Halliwell and Chirico (1993) reported that plasma glutathione peroxidase could be related to plasma lipid peroxidation and content. Yang *et al*. (2000) showed that mitochondria from the fatty livers produce more superoxide anion (O_2) and H_2O_2 compared other cows and possibly supplementation with PG reduced lipid peroxidation due to alleviated negative energy balance and enhanced GPX plasma activity. Glutathione is mainly synthesized *de novo* from glutamate, cysteine and glycine within the liver and reduction of liver function that is usually observed in the early lactation might have deleterious effect on this pathway (Castillo *et al*., 2006). Cysteine required for glutathione synthesis and liver has the unique and predominantly ability to convert the sulphor amino acids methionine to cysteine (Kaplowitz *et al*., 1985). Glutathione biosynthesis is strictly dependent on precursors amino acids concentration and competes with albumin synthesis for the available cysteine (Droge *et al*., 1994). It is very important to know that the kinetic characteristics expressed by the kilometer rate for amino acids activating enzymes (the rate limiting enzymes for protein synthesis) is 0.003 mmol L^{-1} while that for gamma glutamyle cysteine synthase (the rate limiting enzymes for glutathione synthesis) is 0.035 mmol L^{-1}. This means that the biosynthesis pathways for protein works maximally at concentration approximately 166-fold lower than for glutathione synthesis whose production is subsequently impaired in greatly amounts than that for protein at low cysteine availability (Grimble, 2001). Glutamine has defiantly effects on glutamate availability for glutathione synthesis and in addition by means of save methionine from oxidation on cysteine availability (Blarzino *et al*., 1994). Providing glutamine with effects on saving methionine and providing glutamate can have increasing effects on glutathione synthesis and GPX activity that can be seen at 7 and 14th day before calving.

Haptoglobin, serumamyloid A, NEFA, blood cells and immune cells: Results of measuring amount of Haptogolbin (HP) and SAA on calving day and 21 before calving are shown in Table 2, there is no significant difference between treatments. Regarding to increasing amount of the acute phase proteins such as HP and C-reactive Protein (CRP) in inflammation and infection that are induced by Interleukin-1 (IL-1) and Interleukin-6 (IL-6) (Gyang *et al*., 1984). Probably, not suffering cattle's from inflammations and infections in this study that is required to increasing amount of HP caused to nothing significant difference in the amount of haptoglobin in treatments. By looking at proteins structure of the acute phase responses, it is obvious that these proteins compare to muscles proteins have more aromatic amino acids (phenylalanine, tyrosine and tryptophan). Protective effects of glutamine amino acid on re-amination can influence on amount of the proteins particularly, HP (Blarzino *et al*., 1994). Zebeli and Ametaj (2009) showed that increasing amount of LPS in rumen of the dairy cattle after calving by increasing the amount of barely grain in the ration increased the amount of (CRP). On the other hand, Uchida *et al*. (1993) demonstrate that amount of haptoglobin increases at the time of calving and is more than its amount before and after calving and this results support findings of present research. Also, Ametaj *et al*. (2005) indicate that immediately after calving amount of HP and Serum Amyloid A (SAA) will increase. However, it should be kept in mind that most important stimulations of the acute phase proteins are inflammations and infections.

Since, available cattle in this study do not have any symptoms of fever or infections, it can be justified the reason of no significant difference. To support the issue, some researchers indicate that increase in amount of cortisol in the dairy cattle increase amount of HP (Alsemgeest *et al*., 1996). Also, there was not significant differences among two groups at 7 and 14th day before calving on Not Esterified Fatty Acids (NEFA). But it should be noted that energy metabolism in transition period can effect on APPs. Kushibiki *et al*. (2002) demonstrate that increase of an amount of NEFA after calving may increase APPs and injection of TNFa leads to decrease appetite and increase NEFA and subsequently APPs. Therefore, increase in the amount of NEFA due to increase of negative balance of energy can be considered as a stimulation of the immune system.

Accordingly, Ametaj *et al*. (2005) reported that there is a positive correlation between increase of the amount of SAA and HP after calving and total amount of lipids in liver. Probably, increasing of PG reduces negative energy balance and therefore, reduced NEFA production. There were not significant differences among two group on RBC, neutrophill and lymphocytes. The possibility that oxidative stress during the transition period may be a main causal cause of inflammatory and immune dysfunction in dairy cattle is supported by both *in vivo* and *in vitro*

studies (Bernabucci *et al*., 2005). Tocopherols, ascorbic acid, carotenoids, lipoic acid and GSH (Glutathione peroxidase) are the main non enzymatic antioxidant (Halliwell, 2007). Hogan *et al*. (1992) showed that administration of vitamin E in periparturient dairy cows generally has not affected neutrophil phagocytic activity but has improved the ability of blood neutrophils to kill ingested bacteria. Politis *et al*. (1995) showed dairy cows that supplemented with vitamin E had more production of macrophage-derived interleukin-1 and MHC class II expression when compared to unsupplemented cows. Because short time of experiment and nothing any infection and disease in available cows on the study, there is not comprehensive jugging about effects of glutamine on immune function.

CONCLUSION

The results of this study shows that increasing the amount of glutamine in transition period has effective effects on increasing antioxidant capacity and immune system in dry cattle by means of providing NADPH for antioxidant system and providing energy sources for immune system. More researches is needed to investigation of effects of glutamine on immune system antioxidant capacity.

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