METABOLIC PROFILE OF CROSSBRED DAIRY COWS DURING TRANSITION PERIOD

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(15-MVM-009)

THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Metabolic profile of crossbred dairy cows during transition period"** is a bonafide record of research done by me during the course of research and that this thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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EXTERNAL EXAMINER

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Mannuthy ANISHA J. PERUMBILLY

DEDICATED TO

MY PARENTS AND GUIDE

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LIST OF ABBREVIATIONS

%	Per cent
ABTS	2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
APP	Acute phase protein
BHB	β-hydroxybutyrate
CPL	Ceruloplasmin
DLC	Differential Leukocyte Count
DMI	Dry matter intake
h	hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HDL	High density lipoprotein
Hp	Haptoglobin
MDA	Malondialdehyde
min	minute
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide dehydrogenase
NEB	Negative energy balance
NEFA	Non-esterified fatty acid

nM	nanomol/litre
PBS	Phosphate- buffered saline
Sec	Second
SNF	Solid not fat
TG	Triglycerides
TAS	Total antioxidant status
TBA	Thiobarbituric acid
TCA	Tricarboxylic acid
TEAC	Trolox Equivalent Antioxidant Capacity
TMP	1, 1, 3, 3-Tetramethoxypropane
TROLOX	6-hydroxy-2, 5, 7, 8-tetmethychroman-2-carboxylic acid
VFA	Volatile fatty acid
VLDL	Very low density lipoprotein

Introduction

1. INTRODUCTION

The period from three weeks before calving to three weeks after calving referred to as the transition period, is the most stressful period in the lactation cycle of dairy cows. Almost 75 percent of diseases in dairy herds are reported to occur within the first month of lactation (Leblanc *et al*., 2005), the maximum risk being the first ten days following parturition (Ingvartsen *et al*., 2003). During the transition period animals experience a tremendous physiological change along with drastic metabolic and endocrinal adjustments to accommodate the transition from the state of late pregnancy to early lactation.

The most vital changes happening during transition are the reduction in the intake of dry matter around calving and a quick increase in nutrient demand for milk production. This imbalance between reduced feed intake in and around calving and increased postpartum energy demands for colostrogenesis and lactation leads the animal to a state of energy imbalance, especially in high producing dairy cows during the first few weeks after parturition. To overcome this negative energy balance, lipolysis occurs in the adipose tissue with a resultant increase in non-esterified fatty acid and triacylglycerol concentration. Negative energy balance is the leading cause of metabolic alterations and subsequent production cow diseases. The increased metabolic demands associated with pregnancy, parturition and onset of lactation along with the managemental factors make transition dairy animals prone to oxidative stress. A certain degree of immune dysfunction is also experienced by transition dairy cows. These massive adaptive changes can have a negative impact on the health of the animal, especially in high yielding dairy cows causing severe economic loss to farmers. There are reports suggesting that fatty liver develops in the animal far before the occurrence of ketosis and other diseases.

The management of dairy cows during transition period can greatly impact animal health, production potential and animal well-being. A better

understanding of the biology of transition period will go a long way in reducing health problems and increase success of dairying. Metabolic profiling has been reported to provide a valuable method to monitor animal health during this critical period. In metabolic profiling, blood biochemical constituents are evaluated and are used to assess metabolic homeostasis of the cow which in turn can be used to predict the occurrence of diseases associated with economically important herd parameters like milk yield and reproductive performance. An understanding of the limits of the normal metabolic profile is an essential prerequisite for its use as pre-symptomatic diagnostic aid. This information about the dairy cows in Kerala is scanty. Hence the present study was designed and conducted with the following objectives:

1. Assessing the normal profile of various analytes during the transition period of dairy cows

2. Assessing the antioxidant status during transition state

Review of Literature

2. REVIEW OF LITERATURE

2.1 DAIRYING IN KERALA

Kerala accounts for 1.13 per cent of the total cattle population of the country. Of this 78.59 per cent are crossbred cattle. Though crossbred cattle are more susceptible to diseases than the indigenous breeds, they are more popular due to their higher production potential. But the profitability of dairy industry is affected severely by increased incidence of production diseases particularly during transition period (Van Saun, 2006). Also there are reports of lowered fertility rate among high yielding dairy cows. These are often attributed to intense selection for milk production (Oltenacu and Broom, 2010). The ability to undergo extensive metabolic adaptations associated with the transition to lactation is heritable and is a key to ensure transition success. This necessitates selection of dairy animals based on health, fitness and reproduction traits also, in addition to production traits, through genome wide selection techniques. Ensuring transition success is important in ensuring profitability in dairy industry.

2.2 THE METABOLICALLY CHALLENGED TRANSITION PERIOD

2.2.1 **Transition period in dairy cows**

Transition period, the period from a pregnant non-lactating to a non-pregnant lactating state, is the most critical period through which all dairy cows have to pass through in their lactation cycle and was defined by Grummer (1995) as the period extending from three weeks before calving to three weeks after calving.

The tremendous physiological and metabolic changes along with the metabolic stress associated with pregnancy, calving and lactation make the transition period critically important and widely studied among dairy cows (Kara, 2013).

The last three weeks of gestation are characterized by the rapid development of foetus, synthesis of colostrum, development of mammary gland and metabolic adjustments favouring these physiological changes along with reduced dry matter intake. The first three weeks after calving are characterized by onset of lactogenesis and increased nutrient loss through milk (Lean *et al*., 2013).

2.2.2 **Transition cow biology**

Transition cow biology and its association with diseases and reproductive performance are multifactorial and complex. There are several reports suggesting that the transition animal health is influenced by physiological, endocrinal, metabolic and nutritional changes.

According to Goff and Horst (1997) the major endocrine changes that occur during transition period are the drastic reduction in the level of plasma insulin and progesterone and a transient increase in the level of cortisol and estrogen. These changes affect the dry matter intake (DMI) which inturn leads the animal to a state of negative energy balance (NEB).

A reduction in the volume of rumen by the developing fetus also results in a reduction in dry matter intake along with the associated endocrine and environmental changes. The lowest feed intake occurs at the time of calving. During two to five weeks postpartum the milk production typically peaks, but the animal returns to its normal feeding behaviour usually after eight weeks postpartum only creating an imbalance between the energy uptake and utilization (Ingvartsen and Andersen, 2000).

Dry matter intake has been reported to be decreased by 30 per cent during the last three weeks of gestation, limiting the availability of nutrients to support fetal growth and lactogenesis. The demand for glucose, amino acids and fatty acids were increased several fold within four days postpartum (Hayirli *et al*., 2002).

According to Bertoni *et al*. (2009) milk yield and the amount of fat, proteins and lactose secreted through milk rapidly increase and exceed the feed intake within three weeks of the onset of lactation.

The reduced feed intake in and around calving with the increased energy demands for the growing fetus in the late gestation and lactation lead the animal to a state of negative energy balance which was found to be more prominent during the first few weeks after parturition, especially in high producing dairy cows (Drackley, 2011).

Puppel and Kuczyriska (2016) reported that in dairy cattle the NEB had a greater impact on endocrine balance, organ function, the relative ratio of lactose, protein and fat content in milk and the overall health status of the animal. Although NEB during transition period in ruminants is a physiologically normal process, poor adaptation has consequences on the health and productivity of the animal.

2.2.3 **Metabolic adaptations to negative energy balance**

The dairy cattle respond to negative energy balance by rapid lipid mobilization from body fat depots. Lipolysis in transition cows is favoured by reduced glucose and insulin levels and increased levels of catecholamines, growth hormone and glucocorticoids. The altered expression of key hormone and reduced tissue responsiveness increase lipolysis and reduces lipogenesis during the period of negative energy balance (Herdt, 2000).

During lipolysis the stored triglycerides in the adipose tissue are converted into glycerol and non-esterified fatty acids (NEFA), initiated by adipose tissue lipases. NEFA from the adipocytes bind with albumin and reach all body tissues through blood. Mammary gland efficiently takes NEFA and converts them to milk fat. The concentration of NEFA reaching the liver increases around calving (Drackley *et al*., 2001).

There are 3 main fates of NEFA within the liver cells- they undergo beta-oxidation within the mitochondria and peroxisomes generating ATP, are re-esterified to form triacylglycerol in the liver and secreted as VLDL or are partially oxidized to form ketone bodies (Katoh, 2002).

When the level of NEFA incorporated in the liver exceeds its ability to be secreted as very low density lipoproteins (VLDL), excess TG gets deposited in the liver resulting in impaired liver function. Since the VLDL secretion is limited in ruminants, the increased level of NEFA reaching the liver as a sequel of increased lipolysis resulting from NEB makes the animal more susceptible to fatty liver development during the transition period. Thus the increased level of NEFA reflects increased rate of lipolysis and thus the extent of NEB (Drackley, 2007).

Contreras and Sordillo (2011) reviewed the importance of lipolysis during the transition period in dairy cows and defined lipid mobilization as a physiological adaptation acquired by the mammals to survive the critical conditions of reduced nutrient and energy availability. They reported that the increased rate of lipolysis alters the lipid composition of the body which is reflected in the membrane lipid composition of erythrocytes and peripheral blood mononuclear cells which might predispose the animal to infections due to reduced immunity.

According to Tian *et al.* (2015) membrane fluidity, lipid rafts and receptor binding are affected by increased concentration of NEFA which also activates signal transduction cascades, leading to inflammatory responses.

2.2.4 Maintenance of glucose homeostasis in dairy cows during transition period

The requirement of glucose increases with milk production in dairy cows and they are mainly met by gluconeogenesis in the liver (Reynolds, 2005).

In ruminants, unlike in other animals, the demand for glucose for the normal tissue functions is met by gluconeogenesis in the liver; propionate being the major substrate. Reduced dry matter intake (DMI) leads to a decreased level of ruminal propionate which is the major glucogenic volatile fatty acid (VFA) in ruminants (Contreras *et al*., 2010).

During the feed restriction states of transition period, glycerol from lipolysis of adipose tissue, lactate and alanine rather than propionate contribute to gluconeogenesis in liver reflecting NEB. The relative contribution of other amino acids to hepatic gluconeogenesis is minimal redirecting them for milk protein synthesis (Donkin, 2012).

2.3 OXIDATIVE STRESS AND ANTIOXIDANT STATUS IN TRANSITION COWS

Free radicals are usually generated in the body as a result of the various metabolic processes and its effects are usually nullified by the antioxidants such as glutathione in reactions catalyzed by enzymes like glutathione peroxidase, catalase, superoxide dismutase etc., which form the natural oxidative defense mechanism of the body.

During various pathological conditions and also with the increased metabolic demands more number of free radicals are produced which exceeds the antioxidant capacity of the body resulting in oxidative stress (Castillo *et al*., 2005).

According to Sordillo (2005), the number of reactive oxygen species (ROS) produced in the body increased with the increased metabolic demands associated with pregnancy, parturition and initiation of lactation.

Oxidative stress results in changes in many important physiological and metabolic functions (Bernabucci *et al*., 2005).

Lykkesfeldt and Svendsen (2007) defined oxidative stress as an imbalance between oxidants and antioxidants in the body. The farm animals are exposed to both endogenous and exogenous sources of oxidants. The exogenous sources include radiations of ionizing and non-ionizing nature, polluted air and natural toxic gases, chemicals and toxins, including those used as oxidizing disinfectants. The major source of endogenous oxidants is the various metabolic processes occurring in the body.

Oxidative stress in dairy cows is reported to have effects on health status of the animal directly by peroxidation damage to lipids and macromolecules like DNA and proteins and indirectly by altering metabolic pathways resulting in altered physiology (Castillo *et al*., 2006).

The high level of unsaturated fatty acids in the cellular membranes of immune cells makes the cells more prone to peroxidation and production of reactive oxygen species (ROS) in large amounts. Thus these cells are very sensitive to oxidative stress resulting in impaired immunity (Spears and Weiss, 2008).

The exaggerated processes of NEFA oxidation in the hepatocytes as a consequence to negative energy balance results in the generation of ROS and oxidative stress development in dairy cows, more particular during late gestation and early lactation (Turk *et al*., 2008).

Sordillo and Aitken (2009) reported that the genetic, physiological as well as the environmental changes associated with parturition results in a loss in overall antioxidant potential and this could compromise the animal's immunological defenses resulting in increased incidence of diseases during the transition period. This implies that the inflammation and immune dysfunction occurring in dairy cows during transition period is predisposed by oxidative stress.

Dairy cows were reported to be exposed to high oxidative stress and low antioxidant defense during the period of early lactation than that of advanced pregnancy or late lactation as evidenced by the increased level of MDA and various antioxidant enzymes in the plasma of crossbred dairy cows in a study conducted by Sharma *et al*. (2011).

2.4 IMMUNE CHALLENGES IN TRANSITION COWS

Immunosuppression has been reported in dairy cows around calving which lasted several weeks postpartum that predisposed the animal to different health problems associated with altered metabolism and production (Goff and Horst, 1997).

The metabolic stress arising from NEB, shortage of proteins, vitamins and minerals were reported to be the most important factor for reduced immunity in transition dairy cows. The metabolic stress resulted in the synthesis of cortisol and adrenaline both of which impairs the immunity of the animal by decreased proliferation and function of immune cells (Goff, 2006).

Negative energy balance was associated with reduced antibody production and impaired neutrophil function. A compromised immune function was found associated with dairy cows during early lactation (Van Knegsel *et al*., 2007).

High levels of NEFA and BHB were reported to cause impaired lymphocyte and neutrophil function respectively (Contreras and Sordillo, 2011).

Diminished leukocytic activity, effects of metabolism associated with colostrogenesis and lactation, accompanying hypocalcaemia and NEB, moreover, the process of parturition itself contribute towards suboptimal immunity during periparturient period. The sub optimal immune response was reported to be the major contributing factor for the commonly occurring diseases during transition period (Aleri *et al*., 2016).

2.4.1 Inflammation in Transition Cows

According to Trevisi *et al.* (2012), compromised immunity, incidence of diseases, inflammatory changes and metabolic stress experienced during transition period are interrelated. The overt inflammatory response shown by the transition dairy cows compromise the host immune defenses by increasing the metabolic stress. The inflammation and metabolic stress in early lactation is correlated with an increased immunosuppression and reduced immunity observed during the stages of advanced pregnancy.

A high degree of inflammation, immune activation and associated increase in the concentration of plasma proteins are usually associated with days around calving (Huzzey and Overton, 2013). Decreased milk yield during early lactation and longer days to conception are accompanied by higher levels of biomarkers of stress and inflammation.

Most of the dairy cows experience certain degree of inflammation during transition period, but the extent of inflammation varies between animals. Bradford *et al.* (2015) reviewed the role of inflammation during transition period. Although high degree of inflammation is a risk factor for many pathological conditions, a low degree of inflammation during early lactation is an adaptive condition for the animal.

The transition period is also characterized by inflammatory changes which occur due to the release of cytokines consequent to lipid mobilization. The cytokines promote an inflammatory response in the liver and induces an increase in positive acute phase proteins (APP) such as haptoglobin, ceruloplasmin and serum amyloid A and decrease the synthesis of negative APP such as albumin and cholesterol (Montagner *et al*., 2016).

The pro-inflammatory cytokines were found to be higher in dairy cows during late pregnancy and a relationship between pro-inflammatory cytokines with

concentration of plasma metabolites, acute phase reactants, health and reproductive status of the animal was established by Trevisi *et al*. (2015).

2.4.1.1 Acute Phase Proteins

Eckersall (2000) has proposed acute phase proteins (APP) as sensitive and rapid indicators of inflammatory disturbances in ruminants. The most frequently studied acute phase proteins in bovines include haptoglobin (Hp), serum amyloid A, fibrinogen, ceruloplasmin, alpha 1-antitrypsin andalpha1-acid glycoprotein.

Bertoni *et al*. (2008) investigated the association of degree of inflammation with the milk yield by measuring the biomarkers of inflammation throughout the first month of lactation. There was a negative correlation between inflammation and milk yield.

Inflammation and infections in transition dairy cows were associated with the development of acute phase response mediated by cytokines. The plasma concentrations of the acute phase proteins, produced in the liver, are low in healthy animals (Bradford *et al*., 2015).

The severity and consequences of inflammation in transition dairy cows could be characterized by the presence of positive and negative acute phase response and a post-partum decrease in negative APP and increase in positive APP was reported in dairy cows (Boassaert *et al*., 2012; Trevisi *et al*., 2012).

2.5 PRODUCTION DISEASES OF DAIRY COWS

According to Morrow (1976) metabolic, digestive and reproductive disorders as well as infectious diseases are more frequently observed in cows over conditioned during calving. Milk yield was reported to be directly associated with diseases by

Rowland *et al.* (1985). Also, dairy cows during transition were reported to be more susceptible to diseases.

Many dairy cows are reported to suffer from more than one of the production diseases like metritis, mastitis, retained fetal membrane, displaced abomasum; hypocalcaemia and ketosis during transition period and the complications of one become additive to another (Rajala-Schultz *et al.,* 1999; Drackley, 1999).

The NEB and production diseases are directly linked and have considerable impact on herd health (Mullingan and Doherty, 2008).

2.5.1 Diseases Associated with Energy Metabolism

Diseases such as fatty liver, ketosis and sub-acute ruminal acidosis are manifestations of improper energy metabolism and are reported to have increased incidence during transition period.

2.5.1.1 Fatty Liver (Fat Cow Syndrome)

Fatty liver is a major metabolic disease of dairy cows which occurs mainly during early stages of lactation when there is an imbalance between lipid uptake and oxidation by the liver. It is usually preceded by increased rate of lipolysis indicated by increased level of NEFA. The excess fat gets deposited in the liver resulting in altered metabolic processes in the liver. It is reported that fatty liver is more common in over conditioned high producing cows in their second parity or above and is rare in heifers (Reid, 1980).

Reduced dry matter intake, negative energy balance, obesity and elevated estrogen levels are reported to be the cause of fatty liver (Rukkwamsuk *et al*., 1999)

Ureagenesis and the ability of insulin to increase the hepatic synthesis of proteins are decreased in cows with fatty liver and are indicated by the increased level of ammonia (Bobe *et al*., 2004).

Serum level of glucose, cholesterol, total proteins, albumin and urea are considered as markers of hepatic function and a decrease in their levels may suggest hepatic fat infiltration (Djokovic *et al*., 2015).

2.5.1.2 Ketosis

Ketosis is a condition in which the excess acetyl CoA reaches the liver in a level excess than its capacity to oxidize acetyl CoA in TCA cycle resulting in the accumulation of acetyl CoA and increased synthesis of ketone bodies (Wieland *et al.*, 1964).

Over feeding during the dry period is also one of the major contributing factors in the development of ketosis (Markusfeld, 1985).

The β-oxidation of NEFA in the liver cells results in the formation of acetyl CoA which binds with oxaloacetate and gets further oxidized in the TCA cycle. Acetyl CoA is also an intermediate for gluconeogenesis pathway. In dairy cows, since increased rate of gluconeogenesis is triggered by the NEB and increased milk lactose synthesis, the oxaloacetate get depleted and will not be available for complete oxidation of acetyl CoA. The accumulated acetyl CoA which cannot enter TCA cycle enters the ketogenesis pathway. This partial oxidation pathway results in the formation of ketone bodies such as acetone, acetoacetate and β-hydroxybutyrate (BHB), excess of which result in the development of ketosis. The lack of carnitine to transport acyl CoA, insufficient supply of B vitamins, and endocrine factors are the other reported factors for the incomplete oxidation of acetyl CoA in the liver (Goff and Horst, 1997; Katoh, 2002).

It has been reported that the elevated concentration of ketone bodies have a depressive effect on liver gluconeogenesis exaggerating the ketone body development (Schlumbohm and Harmeyer, 2004).

High incidence of clinical and subclinical ketosis causes huge economic loss to the dairy farmers due to decrease in milk production and a sharp drop in the SNF content of milk and failure of affected animals to return to normal production after recovery (Radostits *et al*., 2007).

Acetone is volatile and acetoacetate is unstable. BHB is stable and is considered most suitable for detection of ketone. β-hydroxybutyrate is considered as the golden marker for ketosis (Liu *et al.*, 2011).

Kara (2013) reviewed the effective use of calcium propionate in dairy cattle during transition period. Propionate being the major glucose precursor in ruminants, the reduced dry matter intake and the associated negative energy balance can be met effectively by the administration of calcium propionate and it is reported to have reduced the incidence of ketosis in dairy cows.

Li *et al*. (2016) determined the level of BHB and NEFA in ketotic cows and their association with the level oxidative stress. They have reported a considerable decrease in the level of glucose and cholesterol in ketotic cows and an increase in the level of NEFA and BHB.

A significant increase in the level of MDA and decrease in the level of antioxidants with varying levels of fatty infiltration of liver were reported by Ghanem *et al*. (2016) in ketosis affected dairy cows.

2.5.1.3 Subacute and Acute Ruminal Acidosis (SARA)

The sudden change in rumen pH when the animals is suddenly switched to an early lactation diet, without the rumen papillae being adapted for the energy rich diet, causes improper function of the rumen (Norduland *et al*., 1995).

Esposito *et al*. (2014) also reported that postpartum dairy cows are more confronted with SARA due to the lack of adaptation of rumen to a low fiber high energy lactation diet.

2.5.2 Diseases Associated with Reduced Immunity

2.5.2.1 Retained placenta

Retained placenta or retention of fetal membranes occurs when the animal fails to expel the fetal membranes within 24h after calving. The incidence rate is high in high yielding dairy cows. The occurrence of retained placenta after parturition might be a manifestation of reduced immunity during peri-parturient period. A direct link has been established between retained placenta, reduced dry matter intake and the resulting elevated NEFA level during the period (Le Blanc, 2008).

Dervishi *et al*. (2016) investigated the association of inflammation and energy metabolism in the occurrence of retained placenta in multiparous Holstein dairy cows and reported that animals with retained placenta showed an activated innate immunity eight weeks prior to diagnosis of disease.

2.5.2.2 Left displacement of abomasum

Displaced abomasum is associated with mild to severe fatty infiltration of liver, but if the development of fatty liver induced the displacement of abomasum or *vice versa* is uncertain. Hypocalcaemic cows are reported to have higher risk of developing displaced abomasum (Muylle *et al.*, 1990; Rukkwamsuk *et al*., 1999).

Cows with NEFA concentration greater than 0.5 mEq/L one week before calving and BHBA concentration greater than 800 µmol/L one week after calving are reported to be more likely to develop left displacement of abomasum (LeBlanc *et al*., 2005).

2.5.2.3 Infectious diseases

A high prevalence of infectious diseases, mainly metritis and mastitis are reported to occur during early post-partum period. Impaired immunity resulting from impaired neutrophil function, lymphocyte responsiveness, antibody production and cytokine production by immune cells is considered the major reason for this condition (Esposito *et al*., 2014).

2.5.3 DISEASES ASSOCIATED WITH MINERAL METABOLISM

2.5.3.1 Milk fever

Hypocalcaemia or parturient paresis is a commonly occurring metabolic disorder in dairy cows induced by the sudden increase in calcium demand for the production of colostrum and milk and the incidence was reported to be more pronounced in over conditioned cows when the dietary supply of calcium become limited with the reduced feed intake around calving (Rukkwamsuk *et al*., 1999).

2.6 ECONOMICS OF TRANSITION

The rate of production cow diseases remains the same even in well managed dairy farms inspite of the significant advances in the knowledge about causes and treatment of these diseases (Mullingan and Doherty, 2008).

According to Ingvartsen *et al*. (2003) milk yield is not associated with production cow diseases except for cystic ovarian disease, mastitis and lameness.

The incidence rate of milk fever is reported to be 0-10 % of calving in the field conditions and is reported to be eighty per cent of calving in the research trials conducted (De Garis and Lean, 2008).

2.7 BLOOD METABOLITE ASSESSMENT DURING TRANSITION PERIOD

A metabolic profile test, to indicate whether a herd is liable to production disease or not, was first developed by Payne *et al*. (1970). The metabolic homeostasis of the cow could be assessed by common metabolic parameters like glucose, cholesterol and albumin concentrations (Turk *et al*., 2008).

Blood metabolite levels during transition period could predict the occurrence of diseases and was reported to be associated with economically important herd parameters like milk yield and reproductive performance (Huzzey and Overton, 2013).

2.7.1 Assessment of Energy Balance

2.7.1.1 Glucose

The requirement of glucose was reported to increase considerably in pregnant animals to meet the increased energy demand of the growing fetus and for lactogenesis (Lindsay, 1973).

Based on a study conducted on the effects of pregnancy and lactation on blood composition on 21 Friesian cows by Tainturier *et al*. (1984), the level of blood concentration of glucose was found to be decreased by the end of pregnancy.

Glucose is the major metabolic fuel required for the vital organs, fetal growth and milk production (Leblanc, 2010). A state of a physiological hypoglycemia is experienced by all dairy cows-during early lactation due to the sudden increased demand for glucose for lactose synthesis and also due to a considerable reduction in

gluconeogenesis by the liver induced by the energy imbalance, lipomobilization and increased accumulation of triglycerides (TG) in the liver.

Djokovic *et al.* (2015) compared various metabolic parameters in pre-partum and post-partum dairy cows and found that the level of glucose in pre-partum cows were within physiological limits, while a reduced level of glucose was found in post-partum cows.

The level of blood glucose was reported to be lower in cows which were clinically or subclinically ketotic (Li *et al*., 2015).

2.7.1.2 Non-esterified Fatty Acids: Biomarker of NEB

Adewuyi *et al*. (2005) reported that the level of lipid mobilization from body fats reserves was reflected in the serum concentration of NEFA. Thus NEFA would be a good indicator for the magnitude of NEB and DMI and the extent of lipid mobilization.

The level of DMI before calving and the level of NEFA were reported to be inversely related. The estimation of the level of blood NEFA helps in evaluating the energy balance in peri-parturient cows (Alamouti *et al*., 2009; Roche *et al*., 2009).

Ospina *et al*. (2010) evaluated the effects of NEFA and BHB on milk yield and reproductive performance in 15 pre-partum and post-partum transition cows. High levels of plasma NEFA and BHB were reported to be associated with higher incidence of ketosis, retained fetal membranes and displacement of abomasum and had detrimental effects on the production characteristics of the animals.

According to LeBlanc (2010), the value of NEFA greater than 0.4 mmol/L, during the period from seven to ten days before the day of calving, was reported to be associated with reduced milk yield and a greater risk of postpartum diseases.

Lipid mobilization in transition dairy cows was reviewed by Contreras *et al*. (2010). The level of NEFA usually remained at less than 0.2 mmol/L before the beginning and after the end of the transition period. The level increases from two weeks before calving and peaks about 10 days after parturition, the concentration being greater than 0.75 mmol/L. A value greater than one mmol/L is suggestive of ketosis.

The level of NEFA during the pre-partum period is a strong predictor of post-partum health of the animals. Huzzey and Overton (2013) reported that cows with greater concentration of NEFA particularly during the two weeks prior to calving, develops multiple health disorders, low milk yield and poor reproductive performance post-partum and is more pronounced in multiparous than in primiparous cows and heifers.

The measurement of NEFA was reported to have higher sensitivity and specificity than BHB and thus provide a more accurate parameter to assess the level of NEB than ketone bodies (McArt *et al*., 2013). The level of pre-partum NEFA for predicting the post-partum health problems in dairy cattle ranges from 0.3 mEq/L to 0.5 mEq/L and 0.70 mEq/L to 1.0 mEq/L for predicting postpartum health problems.

Increased plasma level of NEFA has been reported to have a negative effect on immune cells which might lead to the development of mastitis during transition period. A high level of NEFA was found in ketotic and subclinically ketotic cows than in non ketotic animals (Li *et al*., 2016).

2.7.1.3 β- hydroxybutyrate: A Golden Marker for Ketosis

β-hydroxybutyrate is the most stable ketone body which can be easily measured. Like NEFA, BHB also reflects the level of NEB in transition dairy cows. A rise in the level of BHB in blood to 1400 µmol/L was reported to be associated

with increased risk of metabolic disturbances whereas a level greater than 2000 µmol/L was associated with reduced milk yield (Duffield, 2000).

BHB concentration was suggested to be the golden marker for diagnosis of ketosis in cattle (Kaneko *et al*., 2008).

According to Moghaddam and Hassanpour (2008) ketone bodies are produced by excess breakdown of fat to compensate the NEB. The incomplete oxidation of fatty acids to acetyl CoA results in the production of ketone bodies which include acetone, acetoacetate and BHB. The BHB is the predominant ketone body formed as a final product of fat metabolism.

The amount of ketone bodies produced was increased when the liver is supplied with NEFA in a level greater than which it can completely be oxidized to supply energy (Leblanc, 2010). The increased level of ketone bodies also resulted in decreased dry matter intake.

The elevated ketone bodies are also reported to inhibit hepatic gluconeogenesis, further increasing the hypoglycemic condition of the animal (Duehlmeier *et al*., 2011). Different authors have suggested different concentrations of BHB in determining subclinical ketosis (Anoushepour *et al*., 2014).

The analysis of BHB in which the blood, urine and milk could be used as substrate, was reported to be less expensive and comparatively easy than NEFA (McArt *et al*., 2013).

Li *et al*. (2016) reported that serum BHB concentration between 1.0 - 3.0 mmol/L is usually measured in dairy cows with subclinical ketosis and concentrations less than 1 mmol/L is normal.

2.7.1.4 Cholesterol

Measurement of total cholesterol in the blood was suggested as a good parameter for assessing lipid metabolism. Cholesterolemia can be used as a method to assess energy imbalance and hepatic integrity. The level of cholesterol in the serum or plasma was considered as an indirect index of liver function in the transition cows (Alamouti *et al*., 2009).

In transition cows the fatty acids that reach the liver as a result of lipid mobilization are re-esterifed into TG and subsequently transported as VLDL in which cholesterol forms an important component. Hence the level of blood cholesterol could serve as an indirect measure of liver function in producing VLDL and thus a method to monitor the transition health of dairy cows (Lager and Jordan, 2012).

Djokovic *et al*. (2015) evaluated various metabolic parameters in 15 Simmental dairy cows during late pregnancy, early lactation and late lactation. A lower concentration of cholesterol was found during late pregnant than in other two groups and was suggested to be due to fat infiltration of the liver.

Boassaert *et al*. (2012) reported a lowered cholesterol concentration around calving and an increased trend in concentration thereafter, based on a study conducted on 21 dairy cows. The authors suggested that it might be due to inflammatory changes and associated acute phase response during the period around calving as cholesterol is a negative acute phase protein.

A reduced level of serum cholesterol during the post-partum period compared to that of pre-partum was reported by Djokovic *et al*. (2015) based on a study conducted in transition dairy cows. The level of total cholesterol was reported to be lower both in clinically and sub clinically ketotic cows (Li *et al*., 2016).

2.7.2 Assessment of Protein Status

2.7.2.1 Albumin

Albumin is considered to be a negative acute phase protein and its serum level with infection and inflammation (Kaneko *et al*., 2008).

In a study conducted by Boassaert *et al*. (2012) it was found that the concentration of albumin decreased around calving and increased thereafter.

The level of serum albumin was reported to indicate the synthetic capacity of the liver and its level showed a decreased trend in early lactating animals (Djokovic *et al*., 2015).

Lower serum albumin is indicative of impaired synthetic function of liver (Krause *et al*., 2014). The increased concentration of serum albumin is suggestive of severity of uterine disease and ovulatory potential during early postpartum period. Its concentration is higher in cows which resumed early ovarian activity post-partum.

According to Montagner *et al*. (2016), low albumin levels in transition dairy cows indicated impairment in liver function, higher incidence of fatty liver and negative energy balance. It was also associated with reproductive disorders and infectious diseases like metritis.

2.7.2.2 Urea

An increase in the concentration of urea was found in the first month of calving in a study conducted by Tainturier *et al*. (1984) on the effects of pregnancy and lactation on blood composition on 21 Holstein Friesian cows.

The synthesis of urea was reported to be increased by 60 per cent during pregnancy and the level fell by 36 percent following parturition and lactation (Ramin *et al*., 2007).

An increase in blood urea indicated an accelerated rate of protein catabolism rather than decreased urinary excretion of urea (Kaneko *et al*., 2008).

2.7.3 Evaluation of Oxidative Stress Status

Malondialdehyde and TAS are reported to provide an accurate reflection of the physiological status of the animal and are good parameters for the estimation of oxidative stress in dairy cows during late pregnancy and early lactation (Castillo *et al*., 2005).

2.7.3.1 Malondialdehyde: A Golden Marker of Oxidative Stress

Lipids are prone to oxidation, especially those that are polyunsaturated. The extent of lipid peroxidation can be assessed by MDA assay. MDA is formed *in vivo* by the breakdown of lipids. This is the most useful and widely used measurement of oxidative damage (Lykkesfeldt and Svendsen, 2007).

Turk *et al*. (2008) estimated the level of oxidative stress in dairy cows and found that the level of MDA was higher in early pregnant and mid lactating cows.

Kizil *et al*. (2010) found a higher level of MDA in metritis affected cows than that of healthy normal cows.

According to Contreras and Sordillo (2011) the increased concentration of NEFA and Acyl CoAs increase ROS production by slowing down electron flux within the electron transport chain of mitochondria and also during beta-oxidation when NEFA were utilized as an alternate source of energy. The condition was

reported to be more pronounced in fat cows due to the higher the rate of lipolysis in them.

In a study conducted by Sharma *et al*. (2011) on oxidative stress in twenty dairy cows during transition period, the level of MDA was found to be elevated during early lactation period than during late pregnancy.

Abuelo *et al*. (2015) reviewed the importance of oxidative stress in peri-parturient dairy cows and opined that the oxidative status of the animals can be well established either by calculating the ratio between ROS and TAS which represents the oxidative stress index or by separately calculating the lipid or lipid protein oxidative damage.

A positive correlation between plasma NEFA concentrations and the level of MDA was reported in ketotic dairy cows by Li *et al*. (2016) suggesting the production of excess reactive oxygen species during periods of NEB.

2.7.3.2 Total Antioxidant Status (TAS)

Total antioxidant status was reported to be an integrated parameter which measures the cumulative action of the known and unknown antioxidants present in the blood along with their synergistic action (Ghiselli *et al*., 2000).

An increasing trend of TAS was observed by Castillo *et al*. (2005) in late pregnant cows, the value peaking at one week after calving and declining thereafter.

Total antioxidant status in combination with MDA was suggested to provide a complementary tool in assessing the oxidative status of the animal (Castillo *et al*., 2006).

Spears *et al*. (2008) reported that dietary supplementation of cellular antioxidants like vitamin E and β-carotene and/or Se which has marked antioxidant

property reduced the incidence of metritis, mastitis and retained placenta which are the common diseases in dairy cows during transition period.

2.7.4 Biomarkers of Inflammation

2.7.4.1 Positive Acute Phase Proteins

Haptoglobin (Hp) is one of the major positive APP primarily synthesized in liver. It is a haemoglobin binding protein found in humans and mammals and has antioxidant and angiogenic properties. The level of haptoglobin is found to be increased during inflammation, infection and malignancy. The hepatic synthesis of haptoglobin is stimulated by cytokines and glucocorticoids (Marinkovic and Baumann, 1990).

Haptoglobin was reported to have a bacteriostatic action in cows with fatty liver and associated diseases and has role in liver regeneration by scavenging the TG accumulated hepatic cells (Kim *et al*., 1995).

Kanno and Katoh (2001) determined the presence of Hp in HDL and VLDL fractions of dairy cows with fatty liver suggesting the role of Hp in lipid metabolism.

Katoh (2002) reported that the serum from healthy cattle did not contain Hp, but elevated levels were found during inflammation. Further, the increased level of Hp found in dairy cows around parturition was suggestive of inflammation associated with parturition. Also, higher Hp concentration was reported to be present in sera of cows with naturally occurring fatty liver, ketosis and milk fever, downer cow syndrome and retained placenta.

Haptoglobin was suggested to provide a valuable tool for the diagnosis and prognosis of infectious diseases like mastitis, enteritis, endometritis, hepatic lipidosis and also useful in differentiating acute and chronic inflammation. Ceruloplasmin is a

copper containing APP and has various antioxidant and cytoprotective action. (Murata *et al*., 2004).

Hiss *et al*. (2009) has reported that the negative energy balance in the cows can be related to the increased haptoglobin levels for cows with increased milk Hp also showed increased concentration of NEFA in the serum.

Eckersall and Bell (2010) reviewed the importance of APP in veterinary medicine and reported that the concentration of serum haptoglobin was less than 20µg/L in healthy cattle and increased in case of inflammation to a value greater than 2g/L.

The concentration of ceruloplasmin and haptoglobin was found to be increased at calving and the ceruloplasmin concentration elevated throughout the transition period but the concentration of haptoglobin decreased to a basal level in the days following parturition in a study conducted by Boassaert *et al.* (2012) on transition dairy cows.

Though haptoglobin is a non-specific marker of inflammation, injury and infection, its level after calving was suggested to be an indicator of transition cow disorders like metritis and mastitis (Huzzey and Overton, 2013).

A marked increase in the concentration of haptoglobin and ceruloplasmin was reported by Trevisi *et al.* (2015) after calving especially during first week of lactation suggesting associated inflammatory events mediated by pro-inflammatory cytokines. The study was conducted on 21 multiparous Holstein Friesian cows during transition period.

2.7.4.2 Negative Acute Phase Proteins

The plasma concentration of total cholesterol and albumin was reported to be lower in dairy cows during first month of lactation and were considered as negative APP (Boassaert *et al.,* 2012; Trevisi *et al*., 2015).

2.8 Differential Leukocyte Count

Dairy cows were reported to exhibit leukocytosis along with impaired inflammatory responses during transition. Associated increase in susceptibility to bacterial infections was also noted. Hence these cells were suggested to be an excellent model for studying peri-partal immunosuppression. Increased WBC count as a result of neutrophilia and monocytosis was detected by Meglia *et al*. (2001) in dairy cows at the time of calving. Also, a comparatively decreased level of lymphocytes was also observed at the same period. It was suggested that the elevated level of corticosteroids at the time of calving induces neutrophilia by increasing the synthesis of cells from the bone marrow or an increased demargination of the cells from the endothelium.

Materials and Methods

3. MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

The study was conducted in 15 clinically healthy pregnant crossbreed dairy cows in second to fifth parity maintained at University Livestock Farm and Fodder Research Station, College of Veterinary and Animal Sciences, Mannuthy and Cattle Breeding Farm, Thumburmuzhy during the period from November 2016 to May 2017. The study period started eight weeks before the predicted date of calving until eight weeks after calving.

3.2 COLLECTION OF BLOOD SAMPLES

Blood sample was collected at fortnightly intervals from eight weeks before the predicted calving date until eight weeks after calving. Approximately 10 ml of blood was collected aseptically by jugular venipuncture from each animal and was transferred to a vial without any anticoagulant. Blood was allowed to clot and serum was separated by centrifugation at 3000 rpm for 15 min. Samples were stored at -40^{o}C until further investigations were made.

A thin blood smear was made on a clean glass slide for carrying out the differential leukocyte count (DLC).

3.3 MATERIALS

The measurements of biochemical parameters (glucose, albumin and urea) were done using commercial kits supplied by Labkit, total cholesterol concentration by Cormay diagnostic kit. Serum concentrations of NEFA, BHB, ceruloplasmin and haptoglobin were measured using kits supplied by Randox Laboritories Ltd. All these analysis were done in semiautomatic analyzer (Hospitex Master T) in the Department of Veterinary Biochemistry following the instructions given by the

manufacturer. Malondialdehyde (MDA) and total antioxidant status (TAS) were measured on UV/VIS spectrophotometer (Perkin–Elmer) using the standards 1, 1, 3, 3-Tetramethoxypropane (TMP) manufactured by HiMedia Laboratories Pvt. Ltd. and TROLOX (6-hydroxy-2, 5, 7, 8-tetmethychroman-2-carboxylic acid; Sigma Aldrich Co., USA) respectively.

3.4 ESTIMATION OF BIOCHEMICAL PARAMETERS USING SEMIAUTOMATIC ANALYZER

3.4.1 Assessment of Energy Status

3.4.1.1 Glucose

Glucose concentration was estimated directly in a semiautomatic analyzer (Hospitex Master T) by glucose oxidase method, as suggested by Trinder (1969) using commercial kit. Glucose is oxidized to gluconic acid by the enzyme glucose oxidase. The hydrogen peroxide (H_2O_2) produced during this process is detected by a chromogenic oxygen acceptor. The red colour formed proportional to the glucose concentration in the sample. A volume of ten µL of sample is mixed with 1 mL of the reagent, incubated at 37°C for 10 min and the reading was taken directly in the semiautomatic analyzer.

3.4.1.2 Non-esterified fatty acids

Non-esterified fatty acid content of the samples was estimated directly in a semiautomatic analyzer based on a colorimetric assay as defined by Hosaka *et al*. (1981) using the Randox NEFA kit from Randox Laboratories Ltd. The assay consisted of the measurement of H_2O_2, produced from free fatty acids by Acyl CoA synthetase and Acyl CoA oxidase and quantitatively determining the absorbance at 550nm in the presence of peroxidase, 4-aminoantipyrine and N-ethyl-N-(2hydroxy-3-sulphopropyl) m-toluidine. The enzyme was reconstituted in the buffer provided

(Reagent 1) and maleimide with the provided enzyme reagent and the enzyme diluent (Reagent 2). Reagent 1 and reagent 2, each of 1 mL was added separately to 25 µL of the sample or standard and the absorbance was measured in a semiautomatic analyzer following the incubation of the mixture for 10 min after the addition of each reagent.

3.4.1.3 β-hydroxybutyrate

The estimation of BHB was done in a semiautomatic analyzer based on a kinetic enzymatic method using RANBUT kit (Randox Laboratories Ltd.). The method was based on the oxidation of D-3-hydroxybutyrate by the enzyme dehydrogenase. The change in absorbance due to reduced NAD^+ with was measured directly at 340 nm. The standard or sample of volume 25 µL was mixed with one mL of the reconstituted reagent (enzyme and buffer) and the absorbance was measured in the semiautomatic analyzer.

3.4.1.4 Total Cholesterol

Total cholesterol was estimated by an enzymatic colorimetric method using cholesterol esterase and cholesterol oxidase with cholesterol kit. One mL of the reagent and 10 µL of the sample or standard were mixed, incubated for five min and the absorbance was measured directly at 500 nm in the semi-automatic analyzer.

3.4.2 Assessment of Protein Status

3.4.2.1 Albumin

The concentration of albumin was estimated directly in a semi-automatic analyzer based on the method suggested by Rodkey (1965) using the Albumin kit (Labkit). One mL of reagent was mixed with five µL of sample or standard and the reading was taken after incubating the mixture at room temperature for 10 min at

630nm against reagent blank. Albumin in the presence of bromocresol green in acidic medium converts the colour of the indicator to green blue, the intensity of which is proportional the concentration of albumin present in the sample.

3.4.2.2 Urea

Urea was estimated using urea kit (Labkit) in a semiautomatic analyzer by mixing one mL of reconstituted reagent (buffer and urease enzyme) with 10 µL of the sample or standard. Urea is hydrolyzed by the enzyme urease to produce ammonia which further gets combined to *a*-ketoglutarate and NADH in the presence of GLDH to yield glutamate and NAD^+. The urea concentration in the sample is proportional to the extinction of NADH in unit time.

3.4.3 Assessment of Biomarkers of Inflammation

3.4.3.1 Haptoglobin

The estimation of haptoglobin was done using haptoglobin (Hp) kit (Randox Laboritories Ltd.) based upon the reaction between anti-haptoglobin antibody and haptoglobin present in the sample and measuring the decrease in the absorbance at 340 nm. Five µL of sample and one ml of reagent 1 (haptoglobin buffer) were mixed and incubated for five min at 25°C. Reading was taken after the addition of 100 µL of Reagent 2 (reconstituted haptoglobin antibody and buffer) and incubation for five min in the semi-automatic analyzer.

3.4.3.2 Ceruloplasmin

Ceruloplasmin was estimated using the ceruloplasmin (CPL) kit (Randox Laboratories Ltd.). Anti-ceruloplasmin antibodies when mixed with samples containing ceruloplasmin formed complexes with concomitant change in absorbance proportional to the concentration in the sample. This was measured directly in the

semi-automatic analyzer at 340 nm. A volume of 460 µL of Reagent 1(buffer) and 80 µL of Reagent 2 (antibody) were mixed separately with five µL of sample. Reading was taken after incubating the sample for five minutes after the addition of reagents.

3.5 ESTIMATION OF PARAMETERS USING SPECTROPHOTOMETER

3.5.1 Assessment of Oxidative Stress Status

3.5.1.1 Plasma malondialdehyde

Malondialdehyde was determined by a spectrophotometric assay described by Yagi (1984). The method was based on the reaction of MDA with thiobarbituric acid (TBA) forming a red coloured adduct. 200 µL of serum was added into the reaction mixture containing four ml N/12 H_2SO_4 and 0.3 ml of 10 per cent of phosphotungstic acid. The reaction mixture was incubated at room temperature for five min, centrifuged and the supernatant discarded. The pellet was resuspended in four ml distilled water and one ml of TBA reagent was added. The mixture was kept in a water bath maintained at a temperature of 95°C for 60 min. After cooling with tap water, 5 ml of n-Butanol was added and centrifuged at 3000 rpm for 15 min after vigorous shaking. The absorbance of the butanol layer was measured at 532 nm by keeping n-Butanol as blank. The results were calculated by preparing the standard 5nm TMP following the same procedure. The concentration of MDA was calculated using the formula

$$\text{Level of MDA (nmol/ml of serum)} = \frac{a}{A} \times \frac{0.5}{0.2}$$

Where a: Absorbance of the sample

A: Absorbance the standard

0.5 nM: Concentration of standard solution

0.2 ml: Volume of sample taken

3.5.1.2 Total Antioxidant Status (TAS)

Total antioxidant status in the serum was measured using a modified decolourising assay developed by Re *et al*. (1998). Seven mM 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, ABTS) and 2.45 mM potassium persulfate in distilled water were mixed in a ratio of 1:1. The reaction mixture was kept overnight in dark for generating monocation radicals of ABTS ($ABTS^{+\cdot}$), which is a blue/green chromophore with absorption maxima at 734 nm. The $ABTS^{\bullet+}$ solution was diluted with PBS, pH 7.4, to an absorbance of 0.70 (±0.02) at 734 nm keeping PBS as blank. After addition of one ml of diluted $ABTS^{+\cdot}$ with 10 µL of sample the reduction in absorbance was measured exactly after 1 min. The method was repeated with distilled water as control and the per cent inhibition was calculated based on the formula

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The method was repeated with trolox as antioxidant standard. Trolox (2.5 mM) was prepared in PBS (pH 7.4) for use as stock standard. Different concentrations of the standard were prepared from the stock by diluting with PBS. The reduction in absorbance was measured and per cent inhibition was calculated. A graph was plotted as a function of trolox concentration against per cent inhibition for generating the standard reference data. The concentration of the antioxidants in the sample was obtained by comparing the values with the standard curve prepared and was expressed as trolox equivalent antioxidant capacity (TEAC).

3.6 DIFFERENTIAL LEUKOCYTE COUNT (DLC)

A thin blood smear was prepared on a clean glass slide at the time of blood collection. The air dried blood smears were stained with a few drops of Leishman stain for 2 min. It was then double diluted with distilled water and kept for 10 min with intermittent blowing. The stain was washed under running tap water, air dried and examined under oil immersion objective for accurate cell identification. A minimum of 100 cells were counted to determine the number of different leukocytes and per cent calculated.

3.7 STATISTICAL ANALYSIS

Data was analysed using the statistical software SPSS version 24.0. Pre-transition, transition and post-transition periods were compared by repeated measure ANOVA. Comparison between transition and outside transition or between pregnancy and lactation was done using paired t test.

Results

4. RESULTS

The present investigation was carried out in 15 crossbred dairy cows, from eight weeks before the predicted calving date till eight weeks after calving. The measurements observed were compared during these time periods. Comparison was also done by dividing these time periods into (i) transition period (-2 wk,0wk, and 2wk) and outside transition periods (-8wk, -6wk, -4wk, 4wk, 6wk, 8wk), (ii) pregnant (-8wk, -6 wk, -4wk, -2wk) and lactating (0wk, 2 wk, 4 wk, 6 wk, 8 wk) (iii) pre-transition period (-8wk,-6wk,-4wk), transition (-2wk, 0wk, 2wk) and post- transition period (4wk, 6wk, 8wk). Day of calving was considered as day one of lactation.

Blood was collected aseptically from jugular vein. Serum was separated by centrifugation and stored at -40^{0}C till further analysis. Metabolic profiling was carried out for glucose, cholesterol, NEFA, BHB, albumin and urea. The acute phase proteins ceruloplasmin and haptoglobin were studied. Oxidative stress status was determined by measuring MDA and TAS. Differential leukocyte count was done to monitor the haematological changes. The results were analyzed and presented here.

4.1 PARAMETERS RELATED TO ASSESSMENT OF ENERGY BALANCE

4.1.1 Glucose

The concentration of glucose during the various points of study is shown in Table 4.1. The concentration of glucose during the study period ranged from 45.06 ± 2.32 mg/ dL to 56.40 ± 3.42 mg/ dL and did not differ significantly between different points of study. A significant decrease ($p<0.05$) in serum glucose concentration was noted during transition period when compared to concentration outside the transition period whereas no significant difference was observed in glucose concentration between pregnancy and lactation (Table 4.4 and Table 4.5).

4.1.2 Non-Esterified Fatty Acids

The results for the concentration of NEFA are given in Table 4.1. The concentration of NEFA showed an increasing trend towards transition period and decreased afterwards, but the difference was not significant during the different observed time periods. The highest value observed was at two weeks before calving (0.702 ± 0.18 mmol/L). A highly significant increase ($p<0.01$) was observed during transition period when compared to pre and post transition period. The increase was significant when concentration during transition period was compared to that outside transition period (Table 4.4). No significant difference was observed when data of pregnancy was compared with that of lactation (Table 4.5).

4.1.3 β- hydroxybutyrate

The experimental results for the level of BHB are shown in Table 4.1. A significant increase ($p<0.05$) was observed in the level of BHB from eight weeks before calving till eight weeks after calving. The highest value observed was at eight weeks after calving (0.762 ± 0.06 mmol/L). The concentration during transition and post-transition periods differed significantly from that of pre-transition period. Also a significant increase ($p<0.05$) was observed in BHB concentration during lactation when compared with that of pregnancy (Table 4.5). No significant difference was observed between the concentration during transition period and outside transition period (Table 4.4).

4.1.4 Total cholesterol

The concentration of cholesterol during the various points of study is presented in Table 4.1. A significant difference in cholesterol concentration was observed during the different time periods observed. A declining trend was noted for the level of cholesterol towards the day of calving. Although the level shows an increasing trend after calving, the increase was significant only from week 6 post-

partum. A highly significant decrease ($p<0.01$) in cholesterol concentration was observed during transition period when compared to outside transition period (Table 4.4). A non-significant decrease was observed during pregnancy period when compared with that of lactation (Table 4.5).

Table 4.1 Concentration (Mean ± SE) of various biochemical parameters for assessing energy balance

Periods	Glucose (mg/dL)	NEFA (mmol/L)	BHB (mmol/L)	Cholesterol (mg/dL)
-8 wk	56.40 ± 3.42	0.351 ± 0.12	0.520 ± 0.03^{b}	109.26 ± 5.22^{ab}
-6 wk	51.00 ± 2.10	0.308 ± 0.05	0.514 ± 0.07^{b}	112.89 ± 5.00^{a}
-4 wk	52.87 ± 3.88	0.368 ± 0.09	0.560 ± 0.06^{b}	112.39 ± 6.72^{a}
-2 wk	49.00 ± 1.85	0.702 ± 0.18	0.617 ± 0.06^{ab}	96.86 ± 5.67^{b}
0 wk	45.06 ± 2.32	0.577 ± 0.14	0.594 ± 0.06^{ab}	88.70 ± 6.94^{b}
2 wk	48.73 ± 2.67	0.382 ± 0.17	0.703 ± 0.10^{a}	101.92 ± 6.06^{ab}
4 wk	51.06 ± 3.09	0.247 ± 0.06	0.707 ± 0.07^{a}	117.40 ± 10.48^{ab}
6 wk	52.06 ± 3.45	0.258 ± 0.08	0.722 ± 0.09^{a}	122.67 ± 8.72^{a}
8 wk	53.00 ± 3.38	0.378 ± 0.12	0.762 ± 0.06^{a}	126.26 ± 9.98^{a}
F value	1.269^{ns}	1.348^{ns}	2.177^{*}	3.139^{*}

ns: Non-significant. Means bearing different superscripts within a column differ significantly ($p<0.05$).

4.2 PARAMETERS RELATED TO ASSESSMENT OF PROTEIN STATUS

4.2.1 Albumin

The albumin concentration during the period of study is shown in Table 4.2. There was no significant difference in albumin concentration during different time periods. Also, the values obtained during transition period did not differ significantly from those obtained during pre and post- transition period. No significant difference was observed in albumin concentration between transition and outside transition periods or between the periods of pregnancy and lactation (Table 4.4 and Table 4.5).

4.2.2 Urea

There was no significant difference in urea concentration between pre-transition, transition and post-transition periods. Also, the concentration did not differ significantly between transition period and outside transition period. The results are shown in Table 4.2. But, the concentration of urea was significantly higher ($p<0.05$) during pregnancy (12.43 ± 1.05 mg/ dL) than that observed during lactation (11.42 ± 0.80 mg/ dL) as shown in and Table 4.5.

4.3 PARAMETERS RELATED TO ASSESSMENT OF INFLAMMATION

4.3.1 Haptoglobin

The mean concentration of haptoglobin during transition period and outside transition period were 5.60 ± 0.54 mg/ dL and 4.80 ± 0.59 mg/ dL respectively and the difference was statistically significant ($p<0.05$) as shown in Table 4.4.

4.3.2 Ceruloplasmin

The level of ceruloplasmin did not show any significant difference between different observed time periods as shown in Table 4.2. The highest mean value was observed at two weeks before calving and the lowest at eight weeks before calving.

The mean value ranged from 2.44 ± 0.20 to 3.76 ± 0.68 mg/ dL. Although the concentration obtained during transition period was comparatively higher than that of pre and post- transition period, the difference was not significant. The concentration did not differ significantly between transition and outside transition period and between pregnancy and lactation (Table 4.4 and Table 4.5).

4.4 PARAMETERS RELATED TO ASSESSMENT OF OXIDATIVE STRESS STATUS

4.4.1 Plasma MDA

The mean value for MDA ranged between 4.99 ± 0.72 mmol/mL at eight weeks after calving and 9.61 ± 2.54 mmol/mL at two weeks before calving. The results obtained are shown in Table 2. The level of plasma MDA did not differ significantly during different observed periods. An increased value was observed during transition period than that of pre and post-transition period. The level of MDA did not show any significant difference between transition and outside transition periods or between pregnancy and lactation (Table 4.4 and Table 4.5).

4.4.2 Total Antioxidant Status

The results obtained for the level of TAS are shown in Table 2. The level of TAS did not differ significantly between different observed time periods. No significant difference was observed between transition, pre-transition and post-transition periods. Also, there was no significant difference between transition and outside transition periods or between pregnancy and lactation (Table 4.4 and Table 4.5).

Table 4.2 Concentration (Mean ± SE) of various biochemical parameters for assessing protein status, inflammation and oxidative stress status

Period	Albumin (g/ dL)	Urea (mg/ dL)	Ceruloplasmin (mg/ dL)	Plasma MDA (mmol/mL)	TAS (mM TEAC)
-8 wk	3.43 ± 0.07	11.26 ± 1.13	2.44 ± 0.20	6.63 ± 1.15	0.89 ± 0.06
-6 wk	3.23 ± 0.12	11.84 ± 1.06	3.64 ± 0.59	5.88 ± 1.00	1.02 ± 0.06
-4 wk	3.64 ± 0.16	13.59 ± 1.38	2.67 ± 0.19	5.13 ± 1.00	0.89 ± 0.06
-2 wk	3.40 ± 0.16	13.03 ± 1.47	3.76 ± 0.68	7.30 ± 1.84	0.92 ± 0.06
0 wk	3.48 ± 0.17	12.55 ± 1.05	3.42 ± 0.50	6.75 ± 1.54	0.95 ± 0.07
2 wk	3.29 ± 0.17	11.22 ± 0.88	3.24 ± 0.26	9.61 ± 2.54	0.95 ± 0.05
4 wk	3.44 ± 0.08	11.77 ± 1.06	3.42 ± 0.28	5.38 ± 0.91	0.98 ± 0.06
6 wk	3.49 ± 0.09	11.12 ± 1.00	3.50 ± 0.28	5.10 ± 0.94	1.00 ± 0.04
8 wk	3.42 ± 0.11	10.47 ± 0.98	3.19 ± 0.42	4.99 ± 0.72	0.93 ± 0.06
F-value	0.940^{ns}	1.866^{ns}	1.906^{ns}	1.246^{ns}	1.058^{ns}

ns- Non significant.

Means bearing different superscripts within a column differ significantly ($p<0.05$).

Table 4.3 Concentration (Mean ± SE) of various biochemical parameters during pre-transition, transition and post-transition periods

Parameter	Pre-transition	Transition	Post-transition	F value
Glucose (mg/dL)	53.37 ± 1.18^{a}	47.65 ± 1.32^{b}	52.00 ± 1.86^{ab}	3.266^{*}
Cholesterol (mg/dL)	111.52 ± 3.22^{a}	95.83 ± 3.62^{b}	122.11 ± 5.53^{a}	9.866^{**}
NEFA (mmol/L)	0.372 ± 0.05^{b}	0.576 ± 0.08^{a}	0.279 ± 0.04^{b}	7.216^{**}
BHB (mmol/L)	0.531 ± 0.03^{b}	0.638 ± 0.05^{a}	0.731 ± 0.04^{a}	7.843^{**}
Albumin (g/dL)	3.43 ± 0.07	3.39 ± 0.09	3.45 ± 0.05	0.175^{ns}
Urea (mg/dL)	12.23 ± 0.69	12.26 ± 0.66	11.12 ± 0.58	2.298^{ns}
Ceruloplasmin (mg/dL)	2.92 ± 0.23	3.48 ± 0.29	3.37 ± 0.19	2.543^{ns}
MDA (mmol/ml)	5.88 ± 0.60^{ab}	7.89 ± 1.16^{a}	5.16 ± 0.49^{b}	3.421^{**}
TAS (mM TEAC)	0.94 ± 0.05	0.94 ± 0.05	0.97 ± 0.03	0.669^{ns}

*p< 0.05; **p< 0.01; ns- non significant

Means bearing different superscripts within a row differ significantly

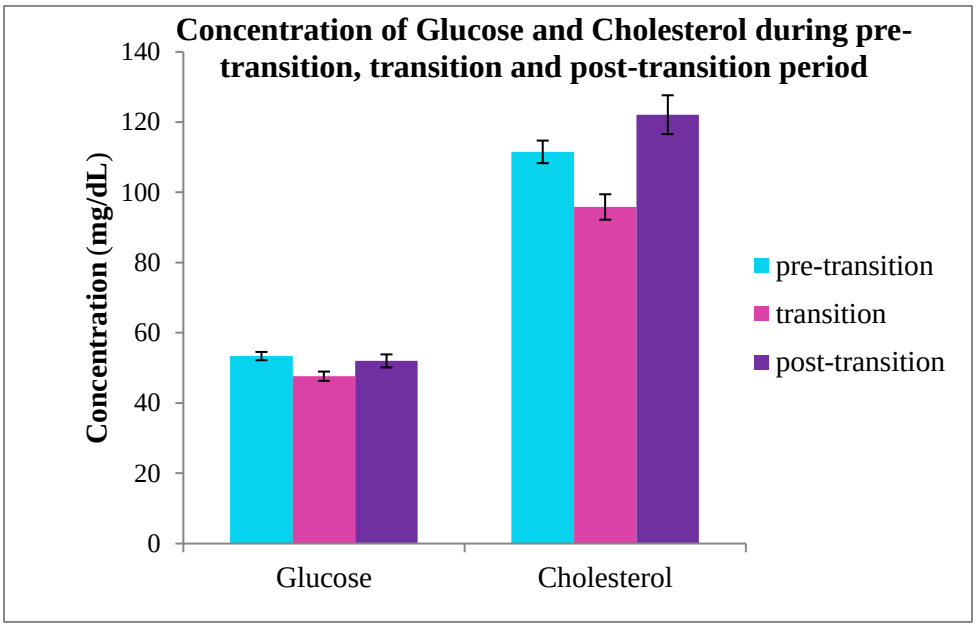

Fig. 4.1 Concentration of Glucose and Cholesterol during pre-transition, transition and post-transition period

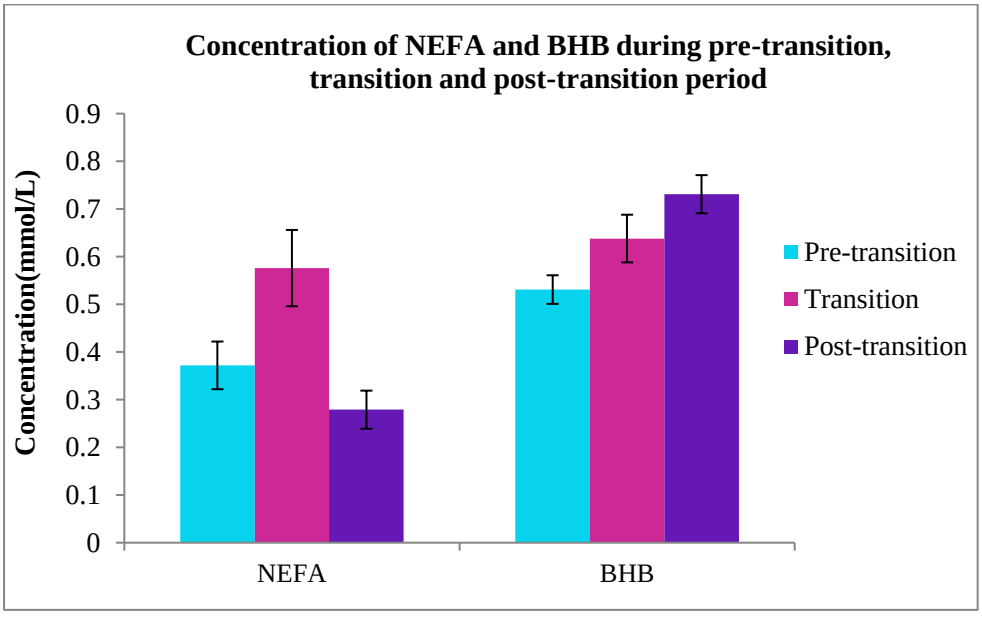

Fig. 4.2 Concentration of NEFA and BHB during pre-transition, transition and post-transition period

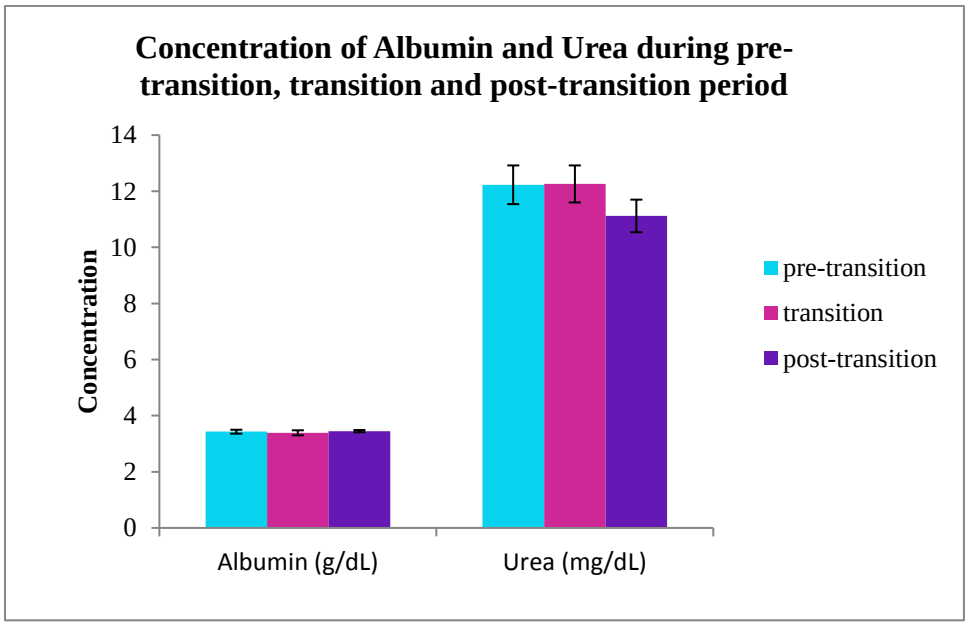

Fig. 4.3 Concentration of Albumin and Urea during pre-transition, transition and post-transition periods

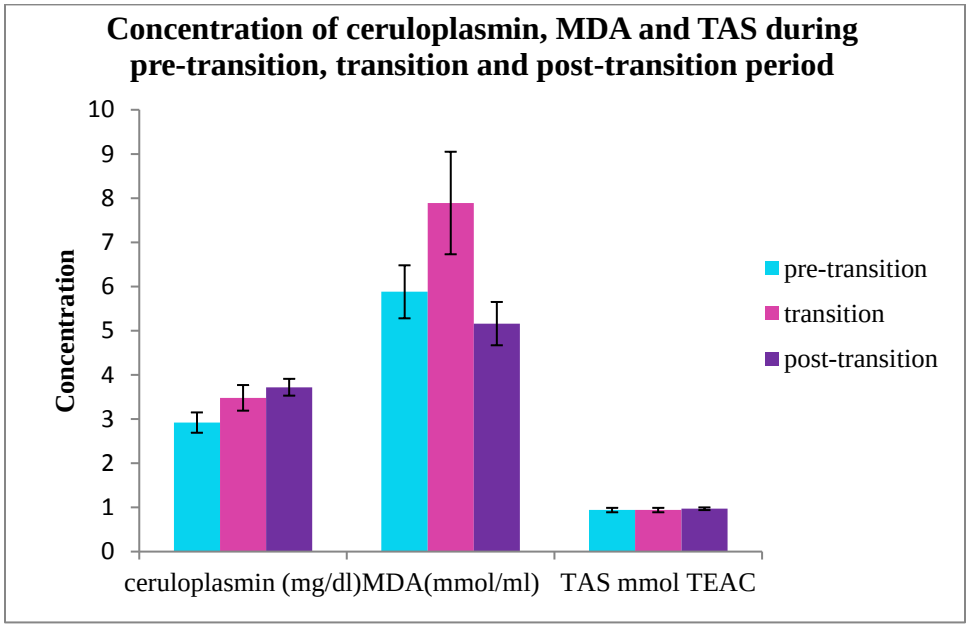

Fig. 4.4 Concentration of ceruloplasmin, MDA and TAS during pre-transition, transition and post-transition periods

Table 4.4 Concentration (Mean ± SE) of various biochemical parameters during transition and outside transition period

Parameters	Transition	Outside	t-value	p-value
Glucose (mg/dL)	47.65 ± 1.47^{b}	52.68 ± 1.73^{a}	2.233^{*}	0.035
Cholesterol (mg/dL)	95.83 ± 4.62^{b}	116.81 ± 4.88^{a}	3.235^{**}	0.006
NEFA (mmol/L)	0.576 ± 0.10^{a}	0.328 ± 0.03^{b}	2.568^{*}	0.022
BHB (mmol/L)	0.638 ± 0.04	0.630 ± 0.047	0.158^{ns}	0.877
Albumin (g/dL)	3.39 ± 0.13	3.44 ± 0.06	0.398^{ns}	0.697
Urea (mg/dL)	12.26 ± 0.99	11.68 ± 0.85	1.837^{ns}	0.088
Haptoglobin (mg/ dL)	5.60 ± 0.54^{a}	4.80 ± 0.59^{b}	2.379^{*}	0.041
Ceruloplasmin (mg/ dL)	3.48 ± 0.42	3.14 ± 0.23	1.622^{ns}	0.129
MDA (mmol/mL)	7.89 ± 1.38	5.52 ± 0.46	1.795^{ns}	0.093
TAS (mMTEAC)	0.94 ± 0.20	0.95 ± 0.16	0.366^{ns}	0.720

*p< 0.05; **p< 0.01; ns- non significant

Means bearing different superscripts within a row differ significantly

Table 4.5 Concentration (Mean ± SE) of various biochemical parameters during pregnancy and lactation

Parameters	Pregnancy	Lactation	t-value	p-value
Glucose (mg/dL)	52.26 ± 1.72	50.00 ± 1.94	0.862^{ns}	0.403
Cholesterol (mg/dL)	107.85 ± 3.11	111.39 ± 5.48	0.647^{ns}	0.528
NEFA (mmol/L)	0.454 ± 0.05	0.308 ± 0.046	1.883^{ns}	0.081
BHB (mmol/L)	0.552 ± 0.03^{b}	0.697 ± 0.05^{a}	2.667^{*}	0.019
Albumin (g/dL)	3.43 ± 0.07	3.42 ± 0.09	0.026^{ns}	0.980
Urea (mg/dL)	12.43 ± 1.05^{a}	11.42 ± 0.80^{b}	2.218^{*}	0.044
Ceruloplasmin (mg/dL	3.13 ± 0.319	3.36 ± 0.273	1.622^{ns}	0.129
MDA (mmol/mL)	6.23 ± 0.80	6.37 ± 0.80	0.123^{ns}	0.904
TAS (mM TEAC)	0.93 ± 0.05	0.97 ± 0.04	1.299^{ns}	0.214

*p< 0.05; ns- non significant

Means bearing different superscripts within a row differ significantly

4.5 DIFFERENTIAL LEUKOCYTE COUNT

The number of neutrophils, lymphocytes ($p<0.01$) and monocytes ($p<0.05$) showed significant difference between different observed time periods. There was no significant difference in the number of eosinophils and basophils (Table 4.6). During transition period the number of neutrophils increased significantly whereas the number of lymphocytes decreased (Table 4.7). A significant increase in the number of neutrophils and monocytes and a highly significant decrease in the number of lymphocytes were observed during transition period when compared to outside transition period (Table 4.8). The number of neutrophils was significantly lower during pregnancy than that during lactation whereas the number of lymphocytes was significantly higher (Table 4.9). No significant difference was observed in the number of eosniophils and basophils during transition and outside transition period or between periods of pregnancy and lactation.

Table 4.6 Differential leukocyte count during different periods (Mean ± SE)

Period	Neutrophil (%)	Eosinophil (%)	Basophil (%)	Lymphocyte (%)	Monocyte (%)
-8 wk	21.73 ± 1.20^{d}	1.80 ± 0.29	0.23 ± 0.11	70.66 ± 1.03^{a}	6.20 ± 0.76^{ab}
-6 wk	23.26 ± 1.70^{d}	3.20 ± 0.92	0.26 ± 0.11	68.20 ± 1.90^{ab}	6.00 ± 0.56^{ab}
-4 wk	28.60 ± 1.81^{c}	3.26 ± 0.90	0.60 ± 0.19	61.86 ± 1.52^{c}	8.26 ± 0.75^{a}
-2 wk	34.06 ± 1.47^{b}	3.73 ± 0.51	0.33 ± 0.12	53.60 ± 1.70^{de}	8.40 ± 0.98^{a}
0 wk	38.20 ± 1.84^{ab}	3.93 ± 0.65	0.20 ± 0.10	52.13 ± 1.71^{e}	6.53 ± 0.60^{ab}
2 wk	39.73 ± 2.24^{a}	3.80 ± 0.59	0.93 ± 0.39	47.33 ± 2.25^{e}	8.00 ± 0.85^{a}
4 wk	35.20 ± 1.61^{ab}	2.53 ± 0.41	0.40 ± 0.16	56.26 ± 1.63^{e}	5.80 ± 0.86^{b}
6 wk	28.66 ± 1.03^{c}	3.20 ± 0.59	0.40 ± 0.13	61.00 ± 0.92^{c}	6.73 ± 0.87^{ab}
8 wk	25.00 ± 1.42^{cd}	2.73 ± 0.50	0.46 ± 0.16	66.46 ± 1.88^{bc}	5.33 ± 0.71^{b}
F value	18.68^{**}	1.586^{ns}	1.573^{ns}	26.020^{**}	2.372^{*}

*p< 0.05; **p< 0.01; ns- non significant
Means bearing different superscripts within a column differ significantly

Table 4.7 Differential leukocyte count during pre-transition, transition and post-transition periods (Mean ± SE)

Leukocyte	Pre-transition	Transition	Post-transition	F value
Neutrophil (%)	24.53 ± 1.04^{c}	37.33 ± 1.12^{a}	29.62 ± 1.03^{b}	36.39^{**}
Eosinophils (%)	2.75 ± 0.44^{ab}	3.82 ± 0.33^{a}	2.82 ± 0.29^{b}	3.239^{*}
Basophil (%)	0.37 ± 0.08	0.48 ± 0.14	0.37 ± 0.08	0.275^{ns}
Lymphocyte (%)	55.86 ± 1.13^{b}	51.02 ± 1.14^{c}	61.24 ± 1.07^{a}	23.348^{**}
Monocyte (%)	6.82 ± 0.42^{ab}	7.64 ± 0.48^{a}	5.95 ± 0.47^{b}	3.711^{*}

*p< 0.05; **p< 0.01; ns- non significant;
Means bearing different superscripts within a row differ significantly

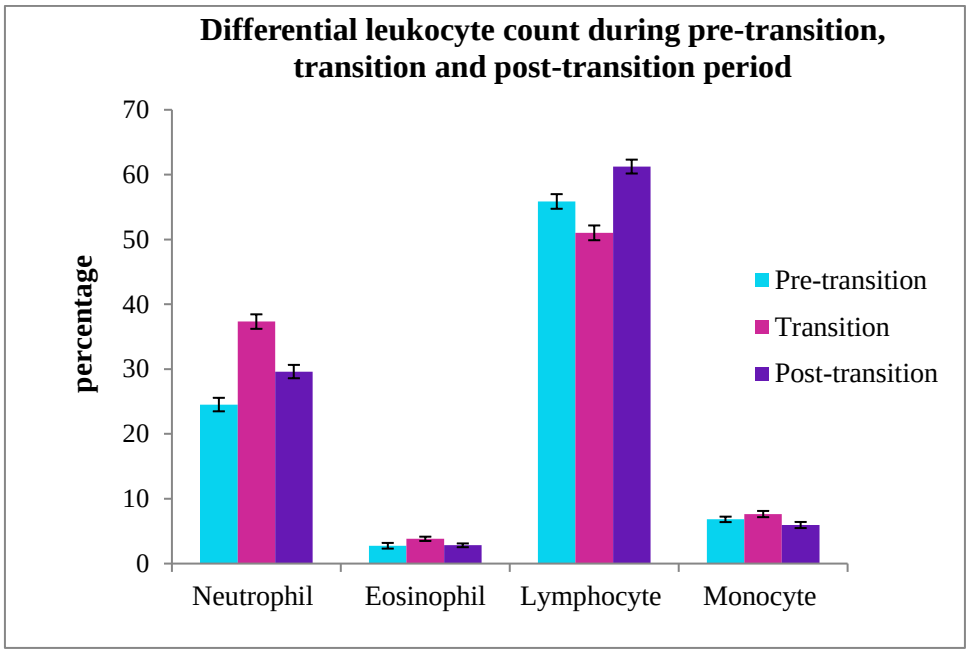

Fig. 4.5 Differential leukocyte count during pre-transition, transition and post-transition periods

Table 4.8 Differential leukocyte count during transition and outside transition periods (Mean ± SE)

Leukocyte	Transition	Outside Transition	t-value	p-value
Neutrophil (%)	37.33 ± 1.39^{a}	27.08 ± 0.72^{b}	7.733^{**}	<0.001
Eosinophils (%)	3.82 ± 0.47	2.78 ± 0.41	2.070^{ns}	0.057
Basophil (%)	0.48 ± 0.14	0.40 ± 0.08	0.576^{ns}	0.573
Lymphocyte (%)	51.02 ± 1.51^{b}	64.07 ± 2.91^{a}	8.571^{**}	<0.001
Monocyte (%)	7.60 ± 2.10^{a}	6.38 ± 0.37^{b}	2.301^{*}	0.037

*p< 0.05; **p< 0.01; ns- non significant

Means bearing different superscripts within a row differ significantly

Table 4.9 Differential leukocyte count during pregnancy and lactation (Mean ± SE)

Type of leukocyte	Pregnancy	Lactation	t-value	p-value
Neutrophil (%)	26.92 ± 0.93^{b}	33.36 ± 0.97^{a}	5.754^{**}	<0.001
Eosinophils (%)	3.00 ± 0.53	3.24 ± 0.35	0.475^{ns}	0.642
Basophil (%)	0.36 ± 0.10	0.48 ± 0.13	0.658^{ns}	0.521
Lymphocyte (%)	63.58 ± 0.88^{a}	56.64 ± 1.06^{b}	5.853**	<0.001
Monocyte (%)	7.21 ± 0.44	6.48 ± 0.41	1.544^{ns}	0.145

*p< 0.05; **p< 0.01; ns- non significant

Means bearing different superscripts within a row differ significantly

Discussion

5. DISCUSSION

Transition state is a critical period in the lactation cycle of all dairy cows which demands high energy for foetal growth and onset of lactation. The maternal body undergoes metabolic adjustments to maintain a homeorhesis which is reflected in the serum biochemical and haematological values. The levels of glucose, NEFA, BHB, cholesterol, albumin, urea, haptoglobin, ceruloplasmin, MDA, TAS and DLC were evaluated to understand their changes during transition period of 15 crossbred dairy cows.

5.1 PARAMETERS RELATED TO ASSESSMENT OF ENERGY BALANCE

Negative energy balance occurs when the energy obtained from dietary sources fail to meet the energy demands of lactation. Although negative energy balance is a physiologically normal phenomenon in transition dairy cows, poor adaptation by animals has consequences on their health and productivity. Relationship has been established between NEB and metabolic disorders in ruminants. NEB is very well reflected directly by the serum concentration of glucose and NEFA and indirectly by that of BHB and total cholesterol and hence these parameters are used as indicators of NEB in dairy cows during transition state.

5.1.1 Glucose

Glucose is the major metabolic fuel utilized by vital organs and is a biochemical parameter defining energy status. It is of utmost importance in foetal growth and lactose synthesis in dairy animals. As per Kaneko *et al.* (2008), the normal physiological concentration of glucose in dairy cows ranges between 45 and 75 mg/dL. The demand for glucose increases with pregnancy and lactation and is met by gluconeogenesis in the liver. In ruminants, VFAs derived from carbohydrates are the precursors for gluconeogenesis.

In the present study, the concentration of glucose observed during different periods was within reported physiological limits (Table 4.1). The mean values obtained for glucose during pre-transition, transition and post-transition period were 53.37 ± 1.18, 47.65 ± 1.32 and 52.00 ± 1.86 mg/dL respectively. Castillo *et al.* (2005) reported glucose concentration between 68.31 ± 5.34 and 73.44 ± 2.18 mg/dL in 39 multiparous Holstein Friesian cows maintained in a commercial dairy farm. The difference between the report and results of the present study might be due to differences in breed and managemental conditions between the two groups.

A significant decrease ($p<0.05$) towards the lower physiological limit in the concentration of glucose was found during transition period when compared with pre-transition and post-transition periods. This finding is in accordance with Djokovic *et al.* (2015) who suggested that hypoglycemia during transition period in Simmental dairy cows could be the result of increased fetal growth during late pregnancy, the sudden activity of mammary gland and lactose synthesis. It is also reported that metabolic and endocrine changes associated with transition reduces feed intake of the animals further limiting precursors of gluconeogenesis, thus creating an energy imbalance. Thus it could be inferred that all dairy cows are exposed to a hypoglycemic condition during transition period (LeBlanc, 2010).

5.1.2 Non-esterified fatty acids

In dairy cows, concentration of NEFA averages to less than 0.2 mM during the stages of early pregnancy and late lactation and peaks to a concentration of above 0.7 mM upto 10 days post-partum (Adewuyi *et al.*, 2005). During transition period the NEB experienced is compensated by the intense lipid mobilization by which the stored TG in the adipose tissue is catabolized to glycerol and NEFAs by adipose tissue lipases. The utilization of the small amount of available glucose can therefore be prioritized for foetal growth and lactation (Contreras and Sordillo, 2011).

In the present study, the mean concentration of NEFA during pre-transition, transition and post-transition periods were 0.372 ± 0.05, 0.576 ± 0.078 and 0.279 ± 0.042 mmol/L respectively. These values fall within the reference interval reported elsewhere (Quiroz-Rocha *et al.*, 2009). The highly significant ($p<0.01$) increase of serum NEFA observed during transition period is an indicator of the increased rate of lipolysis to compensate NEB. The results are in agreement with Castillo *et al.* (2006), Contreras *et al.* (2010) and Li *et al.* (2015).

The concentration of NEFA was found to decrease during post-transition period. The probable reasons for this decreased NEFA concentration during post-transition period could be its increased uptake by mammary gland for milk fat synthesis or the improvement in dry matter intake by the animal and consequent improvement in energy balance (McArt *et al.*, 2013). A significant positive correlation ($r=0.183$) of NEFA with MDA could be obtained in the study which suggests that the animals could probably be in an imbalanced redox state leading to increased peroxidation of lipids and as the concentration of non-esterified fatty acids increases the products of oxidative damage of fatty acids also increases. It is probable that with increase in NEFA concentration peroxisomal oxidation increases leading to increased lipid peroxidation. Li *et al.* (2016) reported a positive correlation between NEFA and MDA in a group of 10 ketotic dairy cows.

5.1.3 β- hydroxybutyrate

Non-esterified fatty acids reaching the liver in excess of its capacity to re-esterify into TG result in its partial oxidation and formation of ketone bodies (Leblanc, 2010). A certain level of BHB in the blood of transition dairy cows is an adaptive measure to counteract the NEB in peripheral tissues. Certain tissues like brain and heart can efficiently utilize the ketone bodies, thus redirecting the available glucose for lactose synthesis. But the increased concentration of BHB exacerbates the reduced feed intake and also exerts an inhibitory effect on gluconeogenesis by the

liver. In dairy cows, a BHB concentration of less than 1mmol/L is reported normal, between 1-1.4 mmol/L is reported to indicate subclinical ketosis with increased risk of displacement of abomasum and more than 1.5mmol/l is reported to indicate clinical ketosis (Li *et al.*, 2016). The BHB is the final product of excess fat metabolism and is the most stable and easily measurable ketone body (McArt *et al.*, 2013).

In the current study, the concentration of BHB ranged between 0.514 and 0.762 mmol/L during the different weeks of study. The mean concentration of BHB during pre-transition, transition and post-transition periods were 0.531 ± 0.03, 0.638 ± 0.05, 0.731 ± 0.04 mmol/L respectively. The highly significant increase observed during transition and post-transition periods when compared to pre-transition period is indicative of excess lipolysis and consequent ketogenesis. The results are in agreement with Quiroz-Rocha *et al.* (2009), Trevisi *et al.* (2012), Djokovic *et al.* (2015). Post-transition period is marked by progressive increase in milk yield and increased concentration of BHB could be attributed to the increased energy demand of lactation (Li *et al.*, 2016). The level of BHB was found negatively correlated (r = -0.195) with TAS. Increased oxidative stress in cattle with increased BHB concentration has been reported previously by Li *et al.* (2016) and Bernabucci *et al.* (2005).

5.1.4 Cholesterol

The concentration of cholesterol at different points of study from eight weeks pre-partum to eight weeks postpartum is given in Table 4.1. It falls within the reference limits of 80-120 mg/dL in adult healthy cattle reported by Kaneko *et al.* (2008). Castillo *et al.* (2005) had estimated the level of various metabolic parameters in dairy cows during late pregnancy and early lactation and found a declining trend in level of serum cholesterol between six weeks before calving and two weeks after calving which is similar to the results obtained in the present study. Bossaert *et al.*

(2012) and Trevisi *et al.* (2012) also reported reduced cholesterol level during late pregnancy which attained minimum level at calving and increased thereafter.

In the present study, the mean concentration of cholesterol during pre-transition, transition and post-transition periods were 111.52 ± 3.22, 95.83 ± 3.62 and 122.11 ± 5.53 mg/dL respectively. A significant lowering of cholesterol concentration could be observed during transition period. It could be inferred from the parameters assessing energy status that the animals were in a low energy level indicating decreased ATP concentration. In animals with NEB, pentose phosphate pathway will not be operational leading to depletion of NADPH. This causes HMG CoA, common to both ketone body and cholesterol synthesis pathways, to be directed towards ketogenesis rather than cholesterogenesis. Also the activity of HMG CoA reductase, the key regulatory enzyme in cholesterol synthesis, decreases with decrease in ATP concentration due to phosphorylation by cAMP dependent protein kinase. This enzyme is activated by increased cAMP level elicited by increased glucagon, which in turn is produced during hypoglycemia. Thus, the observed decrease in concentration of cholesterol could be attributed to the lowered energy status of animals during transition. Also, during periods of intense negative energy acetyl CoA becomes restrictive as it gets directed towards ketone body synthesis.

In a study conducted on 25 dairy cows one week post-partum, Gross *et al.* (2015) reported a decrease in cholesterol concentration and concluded that metabolism of cholesterol in dairy animals is affected by nutrient and energy deficiency and depends on the stage of lactation. Sepulveda-Varas (2015) also reported a lower post-partum cholesterol level in dairy cows which was associated with development of metritis or other clinical diseases after calving. It was also reported that for every 0.4 mmol/L (15.5 mg/dL) decrease in serum cholesterol, the chances of contracting multiple clinical diseases after parturition increased by two times. Usually inflammatory events are associated with a decrease in cholesterol level and hence cholesterol is reckoned as a negative acute phase protein (Bertoni *et*

al., 2008; Li *et al.,* 2016). The decrease in concentration during transition could also be due to the transient inflammatory events associated with parturition.

The decreased level of cholesterol during pregnancy is associated with the increased energy requirement during lactation. The increasing trend in concentration of cholesterol with progression of lactation could be due to the animal's adaptation towards the requirements of lactation and regaining of energy balance (Ashmawy, 2015)

5.2 PARAMETERS RELATED TO ASSESSMENT OF PROTEIN STATUS

5.2.1 Albumin

The mean concentration of albumin obtained in the present study was 3.43 ± 0.07, 3.39 ± 0.09 and 3.45 ± 0.04 g/dL respectively during pre-transition, transition and post-transition periods. The concentration lies within the physiological limit of 2.1-3.6 g/dL as suggested by Radostits *et al.* (2007). No significant difference could be observed for the concentration of albumin during pre-transition, transition and post transition period. The results are in accordance with those reported by Castillo *et al.* (2006) and Piccione *et al.* (2012). On the contrary, Trevisi *et al.* (2012), Djokovic *et al.* (2015) and Trevisi *et al.* (2015) have reported decreased concentration of albumin during transition period. Albumin is an indicator for the synthetic capacity of liver. In the present study, it appears that the liver function of the animals was not compromised.

5.2.2 Urea

Dietary protein of ruminants can have multiple fates. It is either degraded by ruminal microbes or moves into the anterior chambers and small intestine where it gets broken down into amino acids and smaller peptides and gets absorbed. The nitrogen obtained from the ruminal protein break down is used by microbes for

synthesis of protein by incorporating free amino acids/ peptides into the nascent protein. The ammonia produced from amino acid deamination will also be incorporated into nascent protein. Urea, which is a non-protein nitrogenous substance, can also be converted into microbial protein in rumen following enzymatic conversion or its breakdown into ammonia. Optimum ratio of energy to nitrogen ensures maximum microbial protein synthesis. Increased nitrogen to energy ratio in the rumen, leads to increased ammonia in rumen which will be absorbed through rumen wall and transported to the liver for detoxification. Amino acids produced from abomassal digestion will be deaminated in the liver leading to formation of ammonia and consequently urea. This urea gets excreted in urine or diffuses back from blood into the rumen directly or through saliva.

In the present study, the mean concentration of urea ranged from 10.47 ± 0.98 to 13.59 ± 1.38 mg/dL during the study period. The mean concentrations were 12.23 ± 0.69, 12.23 ± 0.66 and 11.12 ± 0.58 mg/dL respectively during pre-transition, transition and post-transition periods. In a field study conducted among 1072 animals, from one week before calving to one week after calving, the concentration of urea ranged between 11.41 and 48.04 mg/dL (Quiroz-Rocha *et al.*, 2009). The concentration of urea during pre-transition, transition and post transition periods fell within the range reported. But Castillo *et al.* (2006) reported urea concentration of 24.25 ± 2.43 mg/dL in a group of ten Holstein cows between one and eight weeks of calving. The difference might be due to difference in the breed and consequent difference in size, feeding, management and protein turn over.

There was no significant difference ($p>0.05$) in urea concentration during pre-transition, transition and post-transition period. This is in agreement with Castillo *et al.* (2006), but contrary to Djokovic *et al.* (2015) who reported decrease in urea concentration during transition period.

A significant decrease in the concentration of urea was noted during lactation when compared to that of pregnancy. This suggested decreased synthesis of urea consequent to increased incorporation of amino acids into milk protein and increased utilization of urea for amino acid synthesis. The results are in accordance with the findings of Castillo *et al.* (2005) who observed reduced urea concentration during early lactation period than that during pregnancy. According to Piccione *et al.* (2012) the reduced level of urea during post-partum period might also be due to the decreased level of ammonia in the rumen due to reduced dry matter intake.

5.3 PARAMETERS RELATED TO ASSESSMENT OF INFLAMMATION

Transient inflammation and changes in the concentration of acute phase proteins during peri-parturient period have been reported. Some of these acute phase proteins could likely be biomarkers of inflammation associated with pregnancy, parturition and onset of lactation.

5.3.1 Haptoglobin

In the present study, a significant increase ($p<0.05$) in haptoglobin concentration was observed during transition period (5.60 ± 0.54 mg/dL) when compared to that outside transition period (4.80 ± 0.59 mg/dL). The concentration of haptoglobin obtained during the entire study period was higher than the reported concentration of less than 2mg/dL in healthy ruminants (Kaneko *et al.*, 2008). The increase in haptoglobin might reflect inflammation associated with parturition and onset of lactation. It could also be that crossbred cows in tropical countries possess higher haptoglobin level which needs to be verified in a larger population. The increase in concentration during transition might be due to transient inflammation associated with transition. Widely varying concentration of haptoglobin has been reported in apparently healthy dairy cattle by several authors (Chan *et al.*, 2004; Trevisi *et al.*, 2012). A transient immune dysfunction and inflammation during

transition has been reported in dairy cows by Aleri *et al.* (2016) and Bertoni *et al.* (2008). An elevated concentration of haptoglobin at calving which decreased to basal levels during later stages of lactation was observed by Bionaz *et al.* (2007). Bossaert *et al.* (2012) reported an increase in the level of positive acute phase proteins in transition Holstein Friesian cows.

5.3.2 Ceruloplasmin

The mean concentration of ceruloplasmin during pre-transition, transition and post-transition periods were 2.92 ± 0.23, 3.48 ± 0.19 and 3.37 ± 0.19 mg/dL respectively. Hussein *et al.* (2012) reported serum ceruloplasmin concentration of 8.8 to 33 mg/dL in a group of 300 Holstein Friesian cows. Though non-significant, an increase in ceruloplasmin was noted which might be due to the temporary inflammatory changes in the peri-parturient period. Bossaert *et al.* (2012) and Trevisi *et al.* (2012) reported a continuous and significant increase in concentration of ceruloplasmin in Holstein Friesian cows after calving. Higher inter-individual variability for ceruloplasmin has been reported by Trevisi *et al.* (2012).

5.4 PARAMETERS RELATED TO ASSESSMENT OF OXIDATIVE STRESS

There are different methods for assessing the oxidative stress status, electron spin resonance being considered the gold standard. Determination of plasma reactive oxygen species have been used successfully by several authors for determining the redox status of animals (Abuelo *et al.*, 2015). In addition, products of free radical attack on macromolecules, such as advanced oxidation protein products and malondialdehyde (lipid peroxidation product) have been employed as biomarkers of oxidative stress. Organisms are armed with several antioxidant substances to deal with oxidant attacks. Assessment of total antioxidant activity gives a better reflection of antioxidant status than estimation of individual antioxidants as many of the antioxidants act synergistically and are often reciprocally compensated.

5.4.1 Plasma Malondialdehyde

In transition dairy cows, the increased rate of metabolism results in production of free radicals at a level greater than that can be counteracted by the body's defense mechanism and these results in oxidative stress (Sordillo, 2005). The concentration of plasma MDA in the present study ranged between 4.99 ± 0.72 and 9.61 ± 2.54 mmoles/mL during the study period. The concentration of MDA during transition period (7.89 ± 1.16 mmoles/mL) was significantly higher than that of post-transition period. This might be the result of increased oxidants generated by hypermetabolic (catabolic) response to the changes in homeostasis evoked by parturition and lactation. The results are in agreement with that of Castillo *et al.* (2005) who reported increased peroxidation of lipids in the peri-parturient period. They have reported high variation between individual cows. High variation in MDA between individual animals could be observed in this study also. Sharma *et al.* (2011) reported significant increase in the concentration of MDA in early lactating cows compared to the late pregnant ones when a group of pregnant animals were compared to a group of early lactating animals. Castillo *et al.* (2006) reported that there was no significant difference in plasma MDA concentration between animals in different stages of lactation.

The decreasing trend in the concentration of MDA that could be noted during the post-transition period might be due to the gradual adaptation of the animal's body to the metabolic alterations leading to the animal regaining homeorhesis (Abuelo *et al.*, 2015). Malondialdehyde shows a positive correlation with NEFA suggesting that increased lipolysis is associated with increased rate of lipid peroxidation. Thus, adopting practices to alleviate negative energy balance during transition would contribute to better oxidative status also.

5.4.2 Total Antioxidant Status

Antioxidants are body's natural defence mechanisms against the generation of free radicals. The changes associated with parturition results in a loss in overall antioxidant potential and this could compromise the animal's immunological defences also resulting in increased incidence of diseases during the transition period (Sordillo, 2005). The total antioxidant activity remained relatively constant throughout the study period without any significant difference. The mean values observed during pre-transition, transition and post transition periods were 0.94± 0.05, 0.94 ± 0.05, 0.97 ± 0.03 mM TEAC respectively. According to Castillo *et al*. (2005) and Turk *et al.* (2013) a decreased level of TAS were observed during transition period when compared to that of early pregnancy and late lactation.

Maintenance of optimum antioxidant status is essential as the undesired products of pro-oxidant action if not eliminated by antioxidants, can enter into a vicious cycle that results in membrane damage and even cell death if the cycle continues without intervention. The lack of significant difference in total antioxidant status in the present study might be due to the inability of the animal to respond to the increasing demands imposed on it. Castillo *et al.* (2006) reported that it is not necessarily a desirable condition to have an increase in TAS value due to adaptive oxidative stress response and also it is not undesirable to have a decreased value of TAS if the production of reactive oxygen species is less. However, in the present study it appeared that the total antioxidant activity was not optimum as eleven out of the 15 animals studied suffered from ketosis, mastitis or hypocalcaemia during the period and remains to be ascertained if supplementation of antioxidants could help in improving the total antioxidant status.

The negative correlation shown by TAS (-0.168) with BHB reiterates the oxidative stress suffered by the animal during periods of negative energy balance and consequent lipolysis.

5.5 DIFFERENTIAL LEUKOCYTE COUNT

A significant increase in neutrophils and monocytes and a highly significant decrease in the number of lymphocytes were observed during transition period when compared with that outside transition period. In transition dairy cows, the corticosteroids released during calving induce neutophilia by increasing the synthesis of cells from the bone marrow or by an increased demargination of the cells from the endothelium. In a study involving 10 animals of Swedish Red and White breed, Meglia *et al.* (2001) reported a significant increase in the number of neutrophils and monocytes with concomitant decrease in the number of lymphocytes during transition period. The results are also in accordance with the findings of Patel *et al.* (2017) who conducted study in a herd of 18 Kara Fries animals. In the present study, a significant increase in number was observed for eosinophils also which is contrary to the findings of the Meglia *et al.* (2001). But, eosinophilia was reported in ketotic buffaloes during transition period by Kumar *et al.* (2015).

Elevated levels of NEFA and BHB are reported to cause impairment in lymphocyte and neutrophil function respectively (Sordillo and Aitken, 2009). The neutrophilia observed during the transition period might be an adaptive mechanism of the animal to compensate the reduced functionality of the cells.

Various metabolites analysed during the study period showed significant difference in their concentration during transition period. An elaborate study involving a larger population is essential to establish accurate reference values of these analytes during transition in dairy cows.

Summary

6. SUMMARY

Dairy cows are most prone to diseases during transition period, i.e. during the period from three weeks before calving to three weeks after calving. This period is characterized by rapid alterations in endocrine and immune systems and consequent adaptive changes by the animal, the efficiency of which determines the post-parturient health of the animal. Knowledge about the normal physiological alterations during the period might help in controlling the incidence of the diseases and increase the profitability of dairying.

With the objectives of determining the reference values for various metabolites and for evaluating the antioxidant status during transition period 15 crossbred dairy cows were studied from 8 weeks before the predicted date of calving till 8 weeks after it. Blood was collected at fortnightly intervals; serum was separated and stored at -40^{o}C until analysis. Concentrations of glucose, NEFA, BHB and cholesterol were determined for assessing the energy status of the animal, urea and albumin to assess protein status, ceruloplasmin and haptoglobin to assess inflammatory changes using commercially available kits in a semi-automatic analyzer. Oxidative stress of the animals was determined by measuring the concentration of MDA and assessing TAS by spectrophotometric assays. Differential leukocyte count was also done at fortnightly intervals during the period. The results obtained were analysed for statistical significance by using repeated measures ANOVA.

The mean concentration of glucose (47.35±1.32 mg/dL) and cholesterol (95.83± 3.62 mg/dL) during transition period was significantly lower than pre- and post-transition period. NEFA (0.576 ± 0.08 mmol/L) and BHB (0.638 ± 0.05 mmol/l) concentrations reported significant increase during transition when compared to pre-transition period. Concentration of indicators of protein status *viz.* albumin and urea were 3.39 ± 0.09 g/dL and 12.26 ± 0.66 mg/dL respectively during transition

period and did not differ significantly from pre and post-transition periods. Out of the two acute phase proteins measured, ceruloplasmin did not show significant difference during the study periods, but a significant increase was shown by haptoglobin during transition period when compared to outside transition period. The level of MDA, a lipid peroxidation product and indicator of oxidative stress was higher during transition period when compared to post-transition, indicating significant oxidative stress during the period. TAS did not show significant changes, which could be the reason for eleven out of fifteen animals exhibiting diseases albeit transient during the period. A significant increase in the number of neutrophils and monocytes and a highly significant decrease in the number of lymphocytes were observed during transition period when compared to that outside transition period. The conditions of energy imbalance, increased lipid peroxidation and inflammation associated with the transition from the physiological state of pregnancy to that of calving could be appreciated in the present study. The concentration of oxidative stress indicators was unfavourable, indicating a state of oxidative stress for the animals under study.

Metabolic profiling of a larger number of transition cows comprising of cows from organized farms and small holder livestock units, maintained at different managemental conditions is necessary to establish reliable reference limits for various analytes. It would also help to identify potential biomarkers of metabolic and infectious diseases occurring during transition.

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METABOLIC PROFILE OF CROSSBRED DAIRY COWS DURING TRANSITION PERIOD

ANISHA J. PERUMBILLY

(15-MVM-009)

ABSTRACT

Submitted in partial fulfillment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

(Veterinary Biochemistry)

2017

Faculty of Veterinary and Animal Sciences

Kerala Veterinary and Animal Sciences University

DEPARTMENT OF VETERINARY BIOCHEMISTRY

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR-680 651

KERALA, INDIA

ABSTRACT

Dairy cows are considered to be most prone to diseases during the period from late pregnancy to onset of lactation, i.e. during the transition period. The present investigation was carried out in blood/serum collected at fortnightly intervals from 15 crossbred dairy cows, from eight weeks before the predicted date of calving till eight weeks after calving, with the objectives of generating a metabolic profile and for evaluating the antioxidant status. Concentrations of glucose, NEFA, BHB, cholesterol, urea, albumin, ceruloplasmin and haptoglobin were determined; differential leukocyte count was done; and assessment of oxidative stress was also performed. The mean concentration of glucose (47.35±1.32 mg/dL) and cholesterol (95.83± 3.62 mg/dL) during transition period was significantly lower than pre and post-transition period. NEFA (0.576 ± 0.08 mmol/L) and BHB (0.638 ± 0.05 mmol/L) concentrations reported significant increase during transition when compared to pre-transition period. Concentration of indicators of protein status *viz.* albumin and urea were 3.39 ± 0.09 g/dL and 12.26 ± 0.66 mg/dL respectively during transition period and did not differ significantly from pre or post-transition period. Out of the two acute phase proteins measured, ceruloplasmin did not show significant variation during the study period, but a significant increase was shown by haptoglobin during transition period. The level of MDA, an indicator of oxidative stress was higher during transition period, indicating significant oxidative stress during the period. TAS did not show significant change but the antioxidant status of the animals could not be considered optimum, as eleven out of fifteen animals exhibited diseases albeit transient during the period. A significant increase in the number of neutrophils and monocytes and a highly significant decrease in the number of lymphocytes observed during transition period could be due to the influence of corticosteroids. A comprehensive study involving more number of transition animals, maintained under different managemental conditions shall help in establishing reference intervals for various analytes during period.

Academic form no.19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY

Faculty of Veterinary and Animal Sciences

PROGRAMME OF RESEARCH WORK FOR MASTER'S DEGREE THESIS

1. Title of the thesis

Metabolic profile of crossbred dairy cows during transition period

2. a) Title of department/KVASU research project to which this forms a part

Not applicable

b) Code No. if any and order by which departmental/KVASU research Project is approved

Not applicable

3. a) Name of the student

Anisha J Perumbilly

b) Admission number

15-MVM-009

4. a) Name of the major advisor

Dr. Shynu M.

b) Designation

Assistant Professor,
Department of Veterinary Biochemistry,
College of Veterinary and Animal Sciences, Mannuthy,
Thrissur-680 651

5. Objectives

1. Assessing the normal profile of various analytes during the transition period of crossbred dairy cows
2. Assessing the antioxidant status during transition state

6. Practical/Scientific utility

The period from 3 weeks prior to calving to 3 weeks after calving called the transition period, is the most stressful period of dairy cattle during the lactation. It is estimated that, in dairy cows 75 per cent of all diseases occur during this period. Increased understanding of the biology of transition period should decrease health problems and increase profitability of dairying. Metabolic profiling has been reported to provide a method to monitor animal health during this critical period. An understanding of the limits of the normal profile is an essential

prerequisite for its use as a presymptomatic diagnostic aid. This information regarding dairy cows in Kerala is scanty. This study aims to bridge this gap in knowledge.

7. Important publications on which the study is based

A Metabolic Profile Test, to indicate whether a herd is liable to production disease, was developed first by Payne et al. (1970).

Detection of oxidative stress and body's defences against it has become complementary tools in the evaluation of metabolic status. Malondialdehyde (MDA) and total antioxidant status (TAS) are reported to provide an accurate reflection of the physiological status of the animal with respect to oxidative stress (Castillo *et al*., 2006).

Contreras and Sordillo (2011) have reported that metabolic adaptations to negative energy balance (NEB) have profound effect on inflammatory responses and immune function.

During the transition period, unfavourable metabolic status makes dairy cattle vulnerable to infectious and inflammatory challenges leading to an acute phase response. Strong associations have been established between concentration of acute phase proteins and post-partum disease incidence (Bossaert *et al*., 2012).

It has been reported that blood metabolite concentrations around calving can predict disease and are associated with economically important herd parameters including milk yield and reproductive performance (Huzzey and Overton, 2013).

Studies have suggested association between resumption of postpartum ovarian activity, uterine health and metabolic/or inflammatory biomarkers around parturition (Krause *et al*., 2014).

8. Outline of the technical programme

Study will be conducted in animals maintained at University farms.

1. Blood will be collected at fortnightly intervals from a minimum of 15 healthy cows two months prior to calving

and two months after calving. Animals in parity two or three, in the age group of four to seven with an average production of seven to nine kilograms in the previous calving will be selected to have a homogenous group.

2. Serum/Plasma will be separated and stored at appropriate temperature until analysis is done.
3. Glucose, blood urea nitrogen, cholesterol, albumin, non-esterified fatty acids (NEFA), beta hydroxy butyric acid (BHB), ceruloplasmin, and haptoglobin, will be assayed using commercially available kit
4. Plasma Malondialdehyde (MDA) and Total Antioxidant Status (TAS) will be assayed following the method of Castillo *et al.* (2006)
5. Differential count of leukocytes in blood will be ascertained using hematology analyzer.

9. Main items of observation to be made

a. Glucose
b. Blood urea nitrogen
c. Cholesterol
d. Albumin
e. NEFA
f. BHBA
g. Ceruloplasmin
h. Haptoglobin
i. Plasma MDA
j. TAS
k. Differential leukocyte count

10. Facilities

Existing facilities in the Department of Veterinary Biochemistry, Mannuthy and Pookode will be utilized for the study

11. Duration of the study

Four semesters

12. Financial estimate

Chemicals including kits	: Rs.20, 000
Contingencies including	: Rs.5, 000
Total	**: Rs.25, 000**

Signature of the student

Signature of Major Advisor

Place: Mannuthy

Date: 27.06.2016

Name and signature of members of the Advisory Committee

Chairperson

Dr. Shynu M.

Assistant Professor,

Department of Veterinary Biochemistry,

College of Veterinary and Animal Sciences, Mannuthy,

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Members

1. Dr. Jayavardhanan K. K.
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   Assistant Professor
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3. Dr. Raji K.
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   Department of Veterinary Physiology, CVAS, Mannuthy, Thrissur-680 651

Appendix I

Reference:

Bossaert, P., Trevisi, E., Opsomer, G., Bertoni, G., De Vliegher, S. and Leroy, J. L .M. R. 2012. The association between indicators of inflammation and liver variables during the transition period in high-yielding dairy cows: An observational study. *Vet. J*. **192**: 222-225.

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Appendix II

Time frame of work

Semester 1

1. Collection of literature
2 Planning and preparation of project proposal

Semester 2

1. Standardization of procedures
2. Collection of samples

Semester 3

1. Collection of samples
2. Analysis of various parameters

Semester 4

1. Analysis of results
2. Preparation and submission of thesis

CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to Animals Act (Amendment, 1998)

Mannuthy **Dr. Shynu M.**

27.06.2016 Major Advisor

CURRICULUM VITAE

Name : Anisha J. Perumbilly

Date of Birth : 28/12/ 1991

Place of Birth : Ollur

Marital Status : Unmarried

Permanent address : Perumbilly House
Chevoor P. O.
Thrissur-680 027

Major field of specialization : Veterinary Biochemistry

Educational Qualifications:

Programme	**Board/University**	**Period**	**% of Marks**
B.V. Sc. & AH	Pondicherry University	2010 - 2015	70.1 %
Plus Two	Board of Higher Secondary Examination Government of Kerala	2009	87.33%
SSLC	Secondary School Leaving Certificate Examination Government of Kerala	2007	88%

Membership in professional societies: Member, Indian Veterinary Association

Annexure

ANNEXURE

I. Reagents for assay of MDA

1. N/12 H_2SO_4

   2.666 mL concentrated H_2SO_4 in 1000 mL distilled water

2. 10% phosphotungstic acid

   10 g phosphotungstic acid powder in 100 ml distilled water

3. TBA reagent (0.67% solution)

   0.67 g of TBA powder in 100 mL distilled water and add equal volume of glacial acetic acid

   4. 0.5nM TMP

   Stock: 8.21µL in 100 mL distilled water

   Working standard: 1µL of stock diluted to 1 L.

II. Reagents for assay of TAS

1. 7mM ABTS

   0.38 g in 100 mL distilled water

2. 2.45 mM potassium persulfate

   0.66 g in 1000 mL distilled water

3. PBS, pH 7.4

   Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4$and 0.2 g of KH_2PO_4 in 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Make up the volume to 1 L.