D-Lactic Acid Metabolism and Control of Acidosis

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ABSTRACT

D-lactic acidosis (DLA) is a disease associated with D-lactatemia, acidosis and neurological signs. However, these associations are ill-defined. Bacterial fermentation in the intestine causes increasing D-lactic acid concentrations in the body. Therefore, DLA is reported secondary to gastrointestinal diseases, such as short bowel syndrome, gastroenteritis or diarrhea. Despite intestinal origin, sudden D-lactatemia is often a result of impaired D-lactate metabolism in the body.

Aims of this work were to determine: 1) Influence of the presence of D-lactate or acidity on neurological disturbances; 2) Effectiveness of parenteral $NaHCO_3$ therapy in correcting cerebrospinal acidity and DLA; 3) Prevalence of DLA in diarrheic lambs and fecal D-lactate thresholds; 4) Effectiveness of malate in preventing DLA.

The methodological tools consisted of animal models (calves and lambs): 1) Advanced surgical procedure in calves for long-lasting atlanto-occipital catheterizations; 2) Intravenous infusions of acids to experimentally induce acidosis; 3) Intravenous $NaHCO_3$ therapies; 4) Sampling of cerebrospinal fluid (CSF), blood, urine and feces from experimental / treated calves or diarrheic lambs for blood gas analysis, and D-lactate separation by chromatography.

D-lactate entered the central nervous system (> 2 mmol/L) from the circulation following experimentally induced D-lactatemia (> 5 mmol/L) and was responsible for neurological disturbances which correlated ($r = 0.9$, $P < 0.05$) with both CSF and serum D-lactate concentrations. A zenith of neurological disturbances, ataxia was evident when D-lactate concentration exceeded 12 mmol/L (CSF) and 26 mmol/L (serum), however, a nadir of acidosis (pH 6.9) caused by HCl infusions produced only mild neurological disturbances ($P < 0.05$). Therapeutic $NaHCO_3$ infusions did not result paradoxical CSF acidosis, but supportive in correcting ($P < 0.05$) acidosis ($\Delta pH + 0.11$) and D-lactatemia in calves.

In lambs, metabolic acidosis following a range of mild to severe diarrhea was observed with a corresponding range of D-lactate concentrations in both serum (< 0.05−24.0 mmol/L) and feces (< 0.05−31.0 mmol/L). D-lactate was absorbed into the circulation when the fecal D-lactate concentration exceeded 10.2 mmol/L (threshold).

In calves, moderate oral use of malate produced a > 50% ($P < 0.05$) decrease in fecal and serum D-lactate concentrations suggesting prebiotic properties to prevent DLA.

This dissertation answers the critical questions about the onset of neurological signs in D-lactic acidosis, and advances the current knowledge on the metabolism of D-lactate, the prevention and treatment of acidosis.

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LIST OF ABBREVIATIONS AND ACRONYMS

%	Percentage or of each hundred
±	Plus or / and minus
×	Multiplied by
°C	Degrees of Celsius
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine 5'-triphosphate
BBB	Blood brain barrier
BE	Base excess
bid	*bis in die* (twice a day)
BW	Body weight
c	Clear (about the quality of CSF)
$C_3H_4O_3$	Pyruvate
$C_3H_5O_3$	Lactate
$C_4H_6O_5$	Malic acid
Ca^{++}	Calcium cation(s)
CCAC	Canadian Council of Animal Care
cfu	Colony forming units
Cl^-	Chloride anion(s)
CNS	Central nervous system
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
DLA	D-lactic acidosis
D-lactic acid	Dextro (- or R) lactic acid
DL-LA	DL-lactic acid
DL-LA_R	DL-LA followed by rapid $NaHCO_3$ infusion
DL-LA_S	DL-LA followed by slow $NaHCO_3$ infusion
DL-lactic acid	Dextro and levo lactic acid
Ed.	Edition (of a book)
EPI	Exocrine pancreatic insufficiency
et al.	*et alia* (and others)
g	gram
GC	Gas chromatography
h	hour
H^+	Hydrogen ion (proton)
H_2CO_3	Carbonic acid
H_2O	Water
Hb	Hemoglobin
HCl	Hydrochloric acid
HCl_R	HCl followed by rapid $NaHCO_3$ infusion
HCl_S	HCl followed by slow $NaHCO_3$ infusion
HCO_3^-	Bicarbonate

Hg	Mercury
HPLC	High performance liquid chromatography
i.e.	*id est* (that is)
IM	Intra muscular
IV	Intra venous
IU	International unit(s)
K^+	Potassium cation(s)
kg	kilogram
L	Liter
L-LA	L-lactic acid
L-lactic acid	Levo (+ or S) lactic acid
LOQ	Limit of quantitation
m	miter(s)
m^2	square meter(s)
MANOVA	Multivariate analysis of variance
MCT-1	Mono-carboxylic transporters-1
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mL/min	milliliter(s) per minute
mM	millimolar (millimole per liter)
mm	millimeter(s)
mmol	millimole(s)
mmol/h	millimole(s) per liter
mmol/L	millimole(s) per hour
mol	mole
NA	Not available
Na^+	Sodium cation(s)
NaCl	Sodium chloride (saline)
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide-reduced form
$NaHCO_3$	Sodium bicarbonate
nm	nanometer(s)
nq	Not quantified
ODS	Octadecyl silane
P_{CO_2}	Partial pressure of carbon dioxide
pH	negative logarithm of hydrogen ions to the base 10 (measurement of acidity)
Ph Eur U	European Pharmacopoeia Unit
P_{O_2}	Partial pressure of oxygen
P-value	Probability of significance
r	Coefficient of correlation
RCF	Relative centrifugal force
SAS	Statistical Analysis System
SBS	Short bowel syndrome
sd	Standard deviation

se	Standard error
SNK	Student-Newman-Keul's test
SPSS	Statistical Package for Social Sciences
t	Turbid (about the quality of CSF)
TNS	Total neurological score
t-test	Student t-test
UV	Ultra violet
v/v	Volume to volume
v	Version
Vol	Volume (of a book)
wk	week(s)
µ	micro
µg	microgram(s)
µg/kg	microgram(s) per kilogram (body weight)
µL	microliters

"Not everything that counts can be counted, and not everything that can be counted counts."

~~~ Albert Einstein (1879-1955) ~~~.

1 INTRODUCTION

1.1 Rationale

Bacterial fermentation in the gastro-intestinal system (rumen or colon) produces D-lactic acid which is responsible for D-lactic acidosis (DLA), a disease common in humans and animals. DLA is found secondary to gastrointestinal diseases in monogastrics with short bowel syndrome, gastroenteritis or other diarrheic diseases. Ruminants are also vulnerable to DLA related to the above pathological conditions during calf-hood and with grain over-feeding at any age. DLA is associated with nervous symptoms and the severity of the disease depends on the concentration of D-lactate found in blood. Onset of clinical metabolic DLA occurs when the serum levels of D-lactic acid are greater than 2 mmol/L (Ewaschuk et al. 2002). Biologically significant D-lactate accumulations in the body are often subsequent to a combination of impaired metabolism and excretion. The mechanism(s) for neurological disturbances in DLA are not clear and may be due to direct neurotoxic effects of D-lactate; although other compounds may also be important.

Diarrhea is a common neonatal disease in many animals and human and has strong association with other complications. Viral diarrhea is the main cause of concern in neonatal calves in many countries. Juvenile diarrhea is a significant problem in humans, as well as in animal agriculture, and can cause death in infants. With the occurrence of dehydration due to diarrhea, or any other cause, excretion and elimination of D-lactic acid is impeded and acidosis aggravated. The cause of death may be a result of dehydration, high anion gap, acidosis or D-lactic acid toxicity. Therefore the control of DLA is very important to minimize the lethal metabolic acidosis in infants regardless of the species. Research experiments conducted on DLA seem to be rather limited (Ewaschuk et al. 2005; Petersen 2005).

Since the therapeutic use of antibiotics in diarrhea (and acidosis) has some controversial aspects, a better therapeutic approach could be to use probiotics or prebiotics. These could reduce the risk of development of antibiotic resistant bacteria. One aim in this thesis is to identify probiotic and prebiotic methods of controlling D-lactic acid production in the calf gut, particularly in calves with a damaged gastrointestinal tract. Manipulating the gut microbial flora by introducing competitive inhibitors of D-lactic producers, feeding microbes that utilize lactate or providing compounds such as malic acid ($C_4H_6O_5$) which enhances the removal of lactate are approaches which which could be used to control D-lactic acid production. Consideration of renal clearance is also very important, as it permits the effects of dehydration, which are usually associated with diarrhea, on D-lactic acid clearance and in predisposing to lethal acidosis to be determined. The study findings would inform the treatment of diseased humans and animals, improve the prognosis for cases of diarrhea and gastroenteritis, and prevent the onset of DLA in susceptible humans and animals.

DLA is mainly a consequence of poor gastrointestinal health and dysbiosis. When the rumen or colon microbial environment is disturbed due to the entry of highly fermentable food materials, bacterial fermentation results in excessive production of D-lactic acid. Hence enteropathies, diet, species, and age predispose to the occurrence of DLA. Prevention and treatment approaches are case dependant; history, nutrition, age and the type or nature of

gastrointestinal disease should all be taken into consideration. Dietary management is key to prevention of DLA. Correct, quick diagnostic procedures (serum D-lactate concentrations) and appropriate treatment strategy (depending on the case) to reduce neurological signs will be useful to avoid life threatening sequalae. There are a number of clinically oriented case studies (Lorenz and Vogt 2006; Petersen 2005; Uribarri et al. 1998). However, controlled experimentation is limited. Therefore, further investigations are needed to understand the mechanism of neuropathy, and to investigate novel possibilities for control, prevention and treatment. The mechanisms resulting in neurological signs, metabolism of D-lactate in the body compartments, effective treatment and control measures for DLA are yet to be discovered.

1.2 Hypothesis

This research project tested the following hypotheses.

- **Experimental model studies:**

I. Increasing serum D-lactate (D-lactatemia) increases cerebrospinal fluid (CSF) D-lactate and correlates with neurological depression.
II. Rapid bicarbonate infusion to correct acute acidosis may produce paradoxic CSF acidemia.

- **Clinical studies:**

III. D-Lactate in feces, serum and urine may be increased in lambs with diarrhea.
IV. Malate reduces lactate producing bacteria and increases lactate-utilizing bacteria in the hind gut, and hence reduces D-lactate in feces (gut) and serum, and protects calves against DLA.

1.3 Objectives

The objectives of this research project are mentioned under each study.

- **Experimental model studies:**

I. Mechanistic (metabolism) acid infusion study

This study investigated the effect of different acids including DL-lactic acid (DL-LA), L-lactic acid (L-LA) and hydrochloric acid (HCl) on blood, CSF, and neurological manifestation / depression. The specific objectives were
1) To determine and compare the influence of acidosis and D-lactatemia on blood and CSF parameter changes.

2) To determine whether neurological disturbances are caused by increased acidity or by D-lactate accumulation in serum and CSF.

II. Short term therapeutic infusions of bicarbonate and the effect on CSF acid-base status [Pradoxic bicarbonate therapy trial]

This study investigated paradoxic acidosis in CSF during systemic acid infusions, and paradoxic acidity in CSF with rapid and slow rate bicarbonate treatment of acidemia. The specific objectives were:

1) To determine the effect of rapid versus slow $NaHCO_3$ therapy on CSF acidosis in experimentally induced acidosis by DL-lactic acid (DL-LA) or hydrochloric acid (HCl).

- **Clinical studies:**

III. Incidence of D-lactic acidosis in diarrheic lambs

This study investigated the incidence of DLA in diarrheic lambs in the University Sheep Barn.

1) To determine the incidence of fecal D-lactic acidosis and prevalence of metabolic acidosis secondary to diarrhea in lambs.

IV. Use of sodium malate as a prebiotic to prevent D-lactic acidosis

This study investigated the optimal use of sodium malate (food grade) administered orally to reduce D-lactate concentrations in calf feces, blood and urine. The specific objectives were

1) To determine the ability of malate to reduce D-lactate concentrations in fecal, urine and blood matrices.

2) To determine the effect of malate on blood pH, bicarbonate (HCO_3^-), base excess, gasses, hemoglobin (Hb), chloride (Cl^-) and cations (Na^+, K^+, Ca^{++})

3) To evaluate the calves for signs of toxicity.

4) To determine an appropriate oral dose for beneficial responses in calves.

1.4 Structure of thesis

This dissertation on D-lactate metabolism and control of acidosis is a multifaceted research study that investigates several aspects of DLA using experimental mechanistic model studies and clinical therapeutic studies. The study had many phases including the surgical preparation of experimental animal models, acid infusion experiments, neurological examinations, sample collections from calves, acidosis correction by an alkali, laboratory analyses, a clinical exploration of DLA in lambs, and a therapeutic approach with prebiotic in calves.

Chapter 2 is a review of literature. The review begins with a general overview of acidosis providing a basis for understanding metabolic acidosis, lactic acidosis and DLA. Chemistry of lactic acid, formation, metabolism and elimination are discussed as a

pathophysiological basis for understanding DLA connected to gastrointestinal ailments. Analytical procedures leading to diagnosis are also detailed.

Two experimental model investigations; D-lactate neurotoxicity and effective use of bicarbonate therapy are presented in Chapter 3.

Chapter 4 consisted of clinical studies of the incidence of lamb DLA and prebiotic use in control of DLA.

Chapter 5 contains an overall discussion of the major findings of this research and the future scope for related work.

The conclusions of my overall study are that D-lactate produces neurotoxicity when its concentration increases in the body. The common alkali, $NaHCO_3$ infusion therapy is safe in the treatment of DLA and metabolic acidosis. Short term lamb diarrhea did not cause DLA. An appropriate dose of malate can be fed to avoid the onset of DLA in calves.

2 LITERATURE REVIEW

2.1 Acidosis, lactic acidosis and D-lactic acidosis: An overview

Acidosis is increased H^+ ion (proton) concentration in the body (Evans 1986; Fall and Szerlip 2005; Kreisberg 1980; Owens et al. 1998; Robergs et al. 2004; Stern 1994). It may be a result of excessive production of protons, impaired elimination of protons or excessive loss of bicarbonate. Metabolic acidosis is diagnosed based on an increased base excess, which is accompanied by decreased blood bicarbonate, and possibly an increased anion gap (Berchtold et al. 2005; Bongaerts et al. 1997; Ewaschuk et al. 2005; Fall and Szerlip 2005; Uribarri et al. 1998). Acidemia means abnormal acidity of the blood; it can be measured by analyzing a venous or arterial blood sample for pH and commonly used marker for acidosis. The major parameters used to quantify acidosis are arterial blood pH, bicarbonate, base excess, carbon dioxide (P_{CO_2}), oxygen (P_{O_2}) and anion gap (Astrup et al. 1966; Gossett et al. 1990; Omole et al. 2001; Patra et al. 1993). Blood electrolytes and serum organic acids may be helpful in diagnosing the type of acidosis which may in turn be useful in selection of treatment (Severinghaus 2002; Zwart et al. 1987). Acidosis can be found in many body fluids or tissues. Assessment of acidosis in peritoneal and cerebrospinal fluids is clinically useful. Measuring organic acid concentrations in the blood helps identify the cause of acidosis. Acidosis may cause many deleterious effects on the body tissues and systems.

Acidemia is a common problem in humans and animals (Editorial. 1990; Evans 1985; Ewaschuk et al. 2002; Ewaschuk et al. 2005; Gossett et al. 1990). In diarrheic calves, the severity of acidemia correlates with neurological depression (Gentile et al. 2004; Naylor 1989). Lactic acidosis is a specific form of metabolic acidosis containing elements of acidemia and hyperlactatemia. Lactatemia means high lactate concentrations found in the blood. Propionate concentrations are increased in propionic acidemia and keto acidosis (Benoist et al. 2003; Robergs et al. 2004).

L-lactic acidosis most frequently occurs during strenuous exercises, or due to hypoxia (Robergs et al. 2004; Stern 1994). L-lactate concentrations are increased in lactic acidosis in the body compartments. An impaired activity of lactate dehydrogenase enzyme activity is a rare cause of L-lactic acidosis. L-lactate may also result from intestinal bacterial production (Crichlow and Chaplin 1985; Ewaschuk et al. 2003; Omole et al. 2001).

In contrast to L-lactic acidosis, D-lactic acidosis (DLA) is characterized by D-lactatemia and acidemia (Ewaschuk et al. 2005; Uribarri et al. 1998). D-lactate usually comes from an intestinal source (Bongaerts et al. 1997; Uribarri et al. 1998), but may rarely result from endogenous glyoxalase pathway activity in the body at a sub-clinical level in patients with type II diabetes, ketoacidosis, or genetic metabolic disorder (Ewaschuk et al. 2005; Thornalley 1988). DLA is a cause of an elevated anion gap (Ewaschuk et al. 2003; Ewaschuk et al. 2005; Omole et al. 2001; Uribarri et al. 1998). Within the body D-lactic acid is ionized to protons and D-lactate. Mammals do not metabolize D-lactate as fast as L-lactate (Borba et al. 2004; Cammack 1970; Ewaschuk et al. 2005; Tubbs 1965; Uribarri

et al. 1998). Accumulation of D-lactic acid directly leads to acidification of the blood and other body fluids causing acidemia and acidosis, and is considered a distinct form of metabolic acidosis (Bongaerts et al. 1997; Hove and Mortensen 1995; Petersen 2005). The main clinical characteristic of DLA is neurological disturbances (Dunlop and Hammond 1965; Ewaschuk et al. 2004; Petersen 2005; Uribarri et al. 1998); signs such as slurred speech and incoordination are commonly found (Petersen 2005; Uribarri et al. 1998). DLA may be incompletely diagnosed as acidosis, metabolic acidosis or even lactic acidosis.

2.2 Chemistry of lactic acids

2.2.1 Structure and properties

Lactic acid, or 2 –hydroxypropanoic acid was first reported in 1780 by a Swedish chemist, Scheele who isolated it from milk (Chahal 1990; Ewaschuk et al. 2002). There are two enantiomers (optic isomers or stereoisomer) of lactic acid naturally present due to its asymmetric C2 atom (∝ hydroxy radical). Threfore, Lactic acid is the simplest hydroxycarbnoxylic acid which exists as two enantiomers.

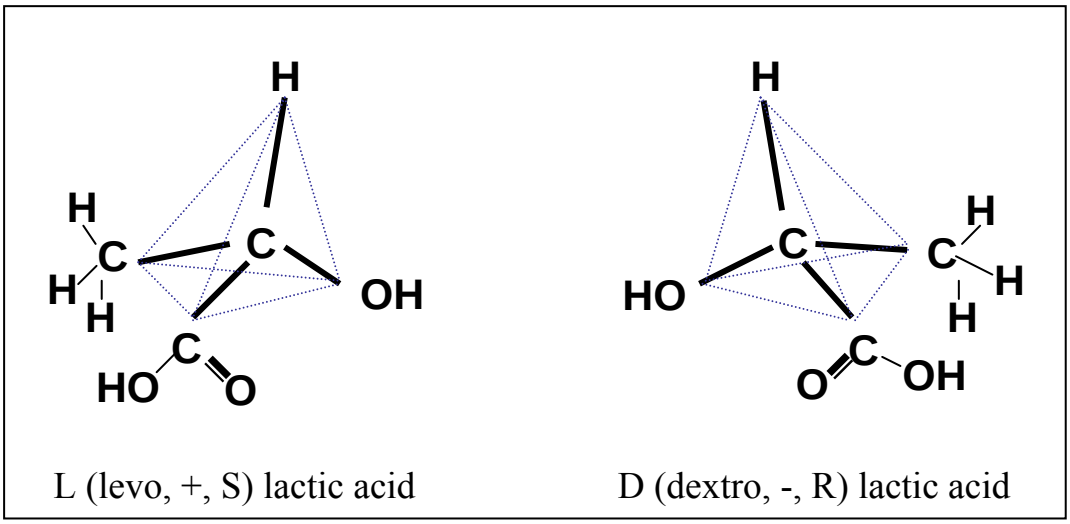


Figure 1. Lactic acid enantiomers; L (+, S)- and D (-, R)- lactic acid optic isomers (Chahal 1990; Chahal and Starr 2006). L-lactic acid is the more common form and is produced by body tissues under hypoxic conditions or by bacterial fermentation mainly in the gastro-intestinal tract. In conary, the D- form is mainly formed by bacterial fermentation hence the main source of D-lactate to the mammalian body is exogenous; from gastro-intestinal tract bacterial fermentation (Editorial. 1990; Haschke-Becher et al. 2000; Petersen 2005; Uribarri et al. 1998).

When a molecule has a hypbridized (SP3) atom with four different chemical moieties attached, it is called a chiral molecule and exists two molecular forms that are non-superimposable mirror images of one another, similar to human hands, and named optical isomers. They have the ability to rotate light in different directions; the clockwise rotation is dextrorotatory (D, −, R) and anticlockwise is levarotory (L, +, S). Therfore, these lactiac acid enantiomers are called the L (+, S) and D (−, R) forms (Figure 1). Both enantiomers indicated similar physical and chemical properties; colorless, odorless, extremely hydroscopic and water soluble with a pK of 3.86 (Borba et al. 2004, Buglass et al. 2003, Chahal et al. 2006, Omole et al. 1999). At physiological pH (7.4), both D- and L-lactic acid are almost completely dissociated into lactate isomers and protons (De Vrese et al. 1990; Ewaschuk et al. 2005; Robergs et al. 2004).

L-lactic acid which is the common form is produced by body tissues under hypoxic conditions or by bacterial fermentation in the body; gastro-intestinal tract, vagina or other infected tissues. The D- form is mainly acquired from exogenous sources and is either ingested or produced by certain bacteria in the gastro-intestinal tact, vagina or other infection sites (Editorial. 1990; Connolly et al. 2005, Haschke-Becher et al. 2000; Hove et al. 1999, Petersen 2005; Uribarri et al. 1998).

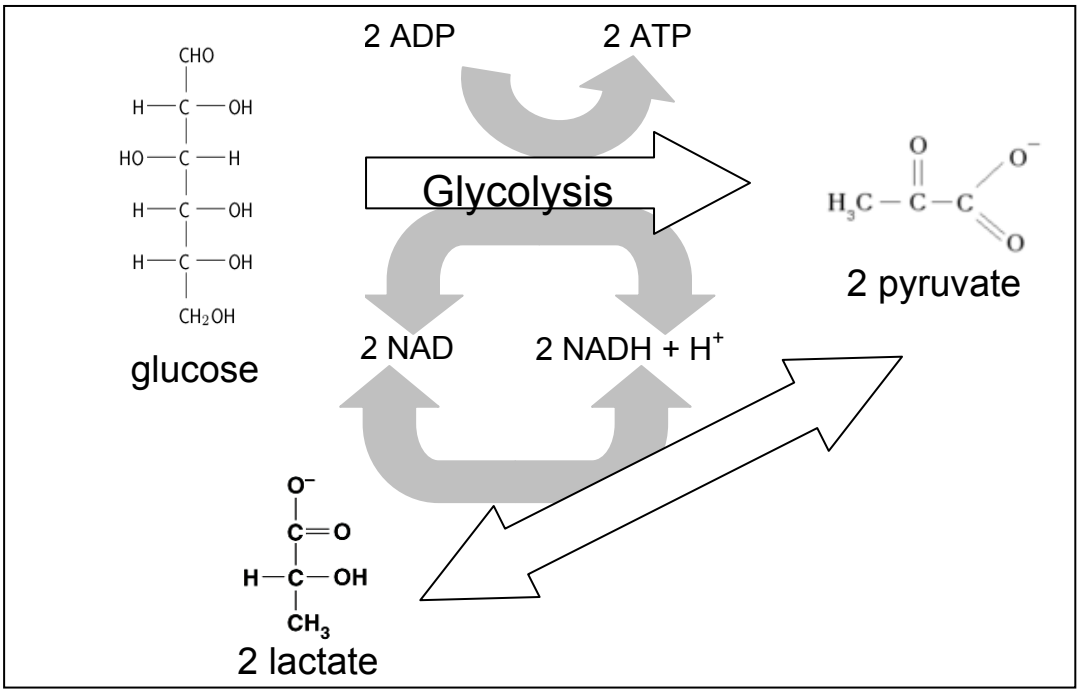


Figure 2. L-lactic acid production (endogenous) in body tissues; the conversion of pyruvate to L-lactate, (a reversible reaction) is catalyzed by L-lactate dehydrogenase enzyme with NADH as a cofactor in cytosol (McClendon et al. 2005).

2.2.2 Endogenous production

Endogenous lactate production is by the conversion of pyruvate ($C_3H_4O_3$) to lactate ($C_3H_5O_3$) catalyzed by lactate dehydrogenase with NADH as a cofactor (Figure 2) (McClendon et al. 2005). This occurs in a variety of tissues including the muscles and erythrocytes, particularly under hypoxic conditions secondary to poor tissue perfusion, to generate NAD and allow glycolysis to continue. This type of endogenous production is exclusively L-lactate.

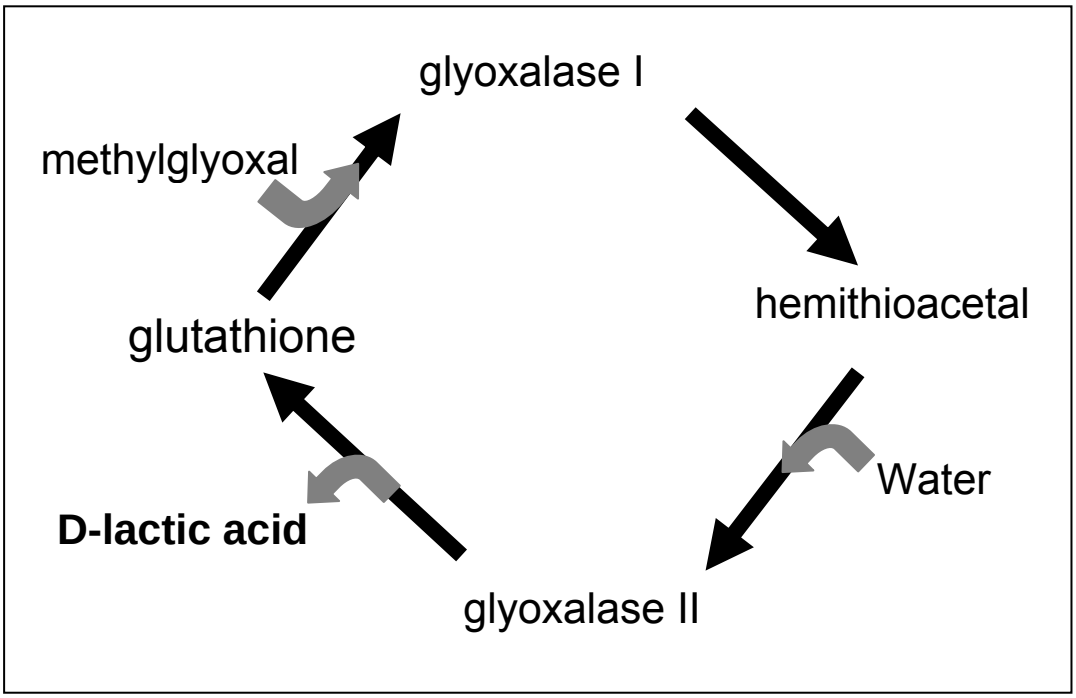


Figure 3. Glyoxalase pathway; the generation of D-lactic acid occurs in mitochondria (Thornalley 1988).

In mammalian cells D-lactate can be formed via the glyoxalase system (Figure 3). It is a ubiquitous reaction, but produces physiologically insignificant quantities of D-lactic acid (Thornalley 1988). Endogenous production of D-lactate from pyruvate by mammalian D-lactate dehydrogenase is yet to be understood (Flick and Konieczny 2002).

2.2.3 Exogenous or gastrointestinal production

Exogenous production of lactic acid is by anaerobic bacterial (mainly gram-positive) fermentation (Figure 4) (Bongaerts et al. 1997). Both D- and L-lactate are found in many fermentation products including pickles, beer, wines and yogurt (Buglass and Lee 2003; Fall and Szerlip 2005; Uribarri et al. 1998). Bacterial fermentation in the

gastrointestinal tract (rumen or colon) is the main cause and source of D-lactic acid (Editorial. 1990; Hove and Mortensen 1995; Omole et al. 2001; Uribarri et al. 1998). Some *Lactobacilli* species (*L. acidophilus, L. buchneri and L. fermenti* and *Escherichia coli*) are capable of producing D-lactic acid (Hove et al. 1999; Ku et al. 2006).

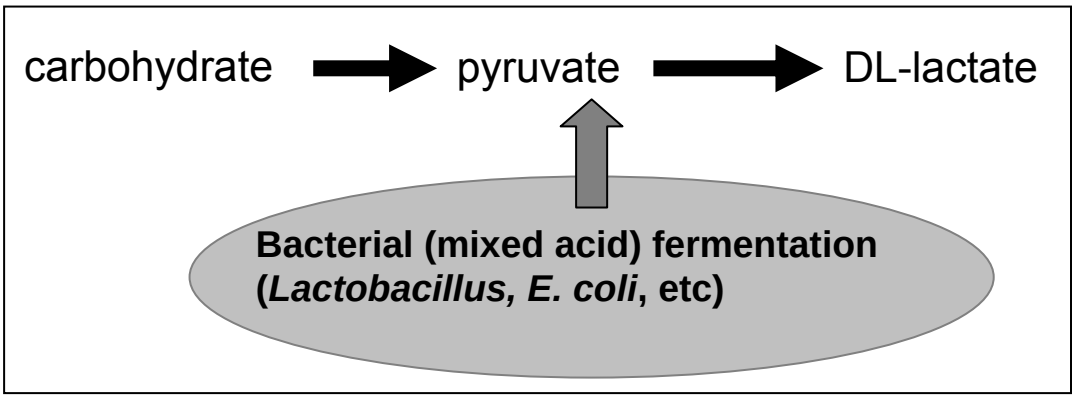


Figure 4. Exogenous DL-lactic acid (DL-LA) generated by bacteria in the gastrointestinal tract by fermenting dietary carbohydrates (Bongaerts et al. 1997; Hove et al. 1999).

The main sites of gastrointestinal production in mammals, are the colon and, in ruminants, the rumen (Dunlop and Hammond 1965; Editorial. 1990; Ewaschuk et al. 2005; Petersen 2005; Uribarri et al. 1998). D-lactate is usually detected at very low levels in the body fluids (Petersen 2005; Uribarri et al. 1998). Mild overproduction of D-lactic acid may not be unusual. In patients with gastrointestinal diseases elevated D-lactate concentrations are frequently present in serum and urine even in the absence of acidosis (Bongaerts et al. 1995; Thurn et al. 1985; Zhang et al. 2003). In some high D-lactatemia cases, alteration of the flora probably occurred following dietary changes (Puwanant et al. 2005) or ingestion of *Lactobacillus acidophilus* (Ku et al. 2006; Mack 2004; Oh et al. 1979) and in others following antibiotic administration (Coronado et al. 1995).

2.2.3.1 Colon

The colon is the main site of fermentation for D-lactic acid production in monogastrics or non-ruminants (human, canine, feline, and swine) and an important site in pre-ruminants (calves, kids and lambs).

The monogastric digestive tract contains over 400 different bacterial species and more than 10^{14} microbes (Luckey 1972). This is approximately 10 times the total number of individual cells in the human body (10^{13}) and approximately 100 times the population of bacteria on human skin (10^{12}). There are more than 200 different species of bacteria in the

colonic flora of a single individual, the total population of which is about $10^{12\text{-}14}$ bacterial cells (Luckey 1987; Simon and Gorbach 1984; Simon and Gorbach 1986).

In pre- and non-ruminants, the maldigestion and malabsorption of carbohydrate in the small intestines leads to an increased passage of carbohydrates to the colon intensifying subsequent fermentation by colonic bacteria to produce L- and D-lactic acid (Editorial. 1990; Hove and Mortensen 1995; Uribarri et al. 1998).

The presence of a dense bacterial flora in the colon facilitates the production of D-lactic acid. Stool cultures from D-lactatemic cases have invariably shown a marked predominance of Gram-positive anaerobes such as species of *lactobacillus* (*Lactobacillus acidophilus* and *Lactobacillus fermentum* > 10^{12} cfu/g feces); these are known to produce D-lactic acid in vitro (Bongaerts et al. 1997; Kaneko et al. 1997). Other potential D-lactic acid producers are *Escherichia coli* (10^{10} cfu/g feces) and *Citrobacter freundii*. *Lactobacilli* produce not only D-lactate dehydrogenase but also DL-lactate racemase (Figure 4), which could potentially explain interconversion of D- and L-lactate (Hove and Mortensen 1995).

2.2.3.2 Rumen

The rumen facilitates the fermentation of crude fiber and is the largest compartment of the fore stomach (complex stomach) of ruminants. It has been referred to as a large fermentation vat containing cud and over 400 microbial species particularly gram negative anaerobic bacteria (Russell and Rychlik 2001). The rumen provides the environment for fermentation and the surface area for absorption of its products. Proper composition of feed (especially crude fiber) is important for the process of ruminal digestion and optimal health of the animal (Crichlow and Chaplin 1985; Kleen et al. 2003; Russell and Rychlik 2001). Ruminal acidosis and lactic acidosis in cattle and other ruminants are most commonly found subsequent to imbalanced feeding, which is unfortunately a too frequent occurrence in modern intensive farming. Specifically when fiber level in the feed is low and soluble nutrients, particularly carbohydrate are high, ruminal fermentation leads to ruminal acidosis. DLA in ruminants has been found after over-feeding of grain or soluble carbohydrate (Crichlow and Chaplin 1985; Dunlop and Hammond 1965; Ewaschuk et al. 2005; Gentile et al. 2004; Kleen et al. 2003). Over-feeding may occur as an accident or deliberate farm practice.

Ruminal D- and L-lactic acid levels were found to be as high as 100 mmol/L in healthy ruminants; presumably half of this racemic lactic acid is D- lactic acid (Dunlop and Hammond 1965; Ewaschuk et al. 2005; Owens et al. 1998). Both D- and L-lactic acid are absorbed effectively by the rumen mucosa (Dunlop and Hammond 1965; Owens et al. 1998). Ruminal lactic acidosis is a common disease in both feedlot and dairy cattle in many parts of the world.

A part of D-lactic acid produced in either rumen or colon is further utilized by ruminal microflora to produce less harmful or beneficial compounds to the host such as short chain fatty acids (Piva et al. 2002; Shimomura and Sato 2006). Propionate produced

from lactate in the presence of malate has been shown by a few workers (Evans and Martin 1977; Martin and Streeter 1995; Martin et al. 1999).

2.2.4 Estimation of D-lactic acid production

Estimation of the likely rate of D-lactic acid production in the average fed human individual has value in helping determine whether a defect in body D-lactate metabolism plays a role in the pathogenesis of DLA. In 1998 Uribarri and coworkers (Uribarri et al. 1998) estimated that D-lactic acid production in healthy humans was 87 mmol/h, applying the following assumptions:

The daily caloric intake is 3,000 calories.

a) 50% of the calories ingested are absorbed in the small intestine and the other 50% is delivered to the colon.

b) 50% of the daily caloric intake comes from 1 big meal such as dinner.

c) 50% of the entire caloric intake is in the form of carbohydrates.

All the carbohydrates delivered to the colon are converted by bacteria to L- and D-lactate in equal proportions.

In this case, if 1,500 calories are ingested during dinner, and 750 calories are delivered to the colon, 375 calories will be as carbohydrates and 375 as some other nutrients. These 375 calories would come from 94 g of carbohydrates, which could then produce 522 mmol of D-lactate and 522 mmol of L-lactate. If we further assume that 522 mmol of D-lactate was produced over a 6 h period, the production rate will be lower than 100 mmol/h (Oh et al. 1985). Soluble and insoluble carbohydrate, fiber (neutral detergent, acid detergent, and acid insoluble) and other nutrients in the diet may have an effect on colonic acid production which may lead to excessive D-lactic acid production (DeGregorio et al. 1982; Editorial. 1990; Ferguson et al. 1981; Hove and Mortensen 1995; Uribarri et al. 1998).

2.2.4.1 Lactate concentrations

D-lactate can be found in many different body fluids. However, the origin of this D-lactate is normally by gastrointestinal fermentation. In general serum D-lactate concentrations are negligible, and sometimes undetectable, because gastrointestinal lactate is utilized by luminal bacteria for many other biochemical pathways such as fatty acid synthesis (Hove et al. 1999; Simon and Gorbach 1984; Uribarri et al. 1998). There may not be excess lactate available for uptake and absorption. Although serum D-lactate levels may be very low (< 2 mmol/L) in healthy individuals under normal physiological status, in certain situations serum D-lactate concentrations can elevate to over 10 mmol/L. In a human case of severe accidental propylene glycol ingestion, the serum D-lactate concentration was found to be 110 mmol/L (Jorens et al. 2004).

Peritoneal and cerebrospinal values are reported in some cases. They are not consistently similar to serum levels (Benoist et al. 2003; Fine 1989; Petersen 2005; Uribarri et al. 1998). In a European study healthy juvenile urinary D-lactate concentrations were shown to be correlated to ethnicity and diet (Haschke-Becher et al. 2000).

Serum L-lactate concentration is the most common and meaningful measurement of L-lactate status. However, (whole) blood L-lactate is reported by many clinical chemistry laboratories. Serum L-lactate increases during strenuous exercise, hypoxic conditions, ischemia, tissue necrosis, metabolic disorders, gastro intestinal diseases, and drastic dietary changes.

Baseline serum L-lactate levels in human as well as in other domestic mammals range from 0.5 to 3 mmol/L (Kreisberg 1980; Minniti et al. 2001; Omole et al. 1999). In other body fluids lactate concentrations may be different (Ewaschuk et al. 2004; Fine 1989; Haschke-Becher et al. 2000; Shimomura and Sato 2006; Yasuda et al. 1993). Cerebrospinal fluid contains similar concentrations of lactate to serum in normal animals, however, the relationship changes in disease. Fecal and urinary D-lactate concentrations (> 10 mmol/L) are always higher than blood concentrations in clinical cases. Urinary values are also typically reported relative to creatinine (mmol of D-lactate per mol of creatinine) because it indicates the renal function and excretion (Connor et al. 1983; Haschke-Becher et al. 2000).

Fermented fruits and vegetables such as pickles, sauerkraut, yogurt, wines and other foodstuff contain both L- and D-lactic acid ranging from 2–15 mmol/L (Buglass and Lee 2003; Fall and Szerlip 2005; Ohmori and Iwamoto 1988). Lactated Ringer's solution and peritoneal dialysate also contain racemic mixtures of both isomers (Anderson et al. 1997; Chan et al. 1994; Fine 1989; Uribarri et al. 1998).

2.2.5 Metabolism of D-lactic acid in the body

High D-lactate uptake and absorption into the circulation were found in many studies (Fine 1989; Flemstrom 1979; Frenning 1972; Owens et al. 1998; Poole and Halestrap 1993). D-lactate can be transported via proton dependent mono-carboxylic transporters-1 (MCT-1) in the colon and rumen (Muller et al. 2002; Poole and Halestrap 1993). Absorbion of large amounts of D-lactate can create a crisis for a normal healthy individual because mammals are not biologically equipped with an efficient mechanism to deal with D-lactate elimination (Giesecke and Von-Wallenberg 1985; Oh et al. 1985).

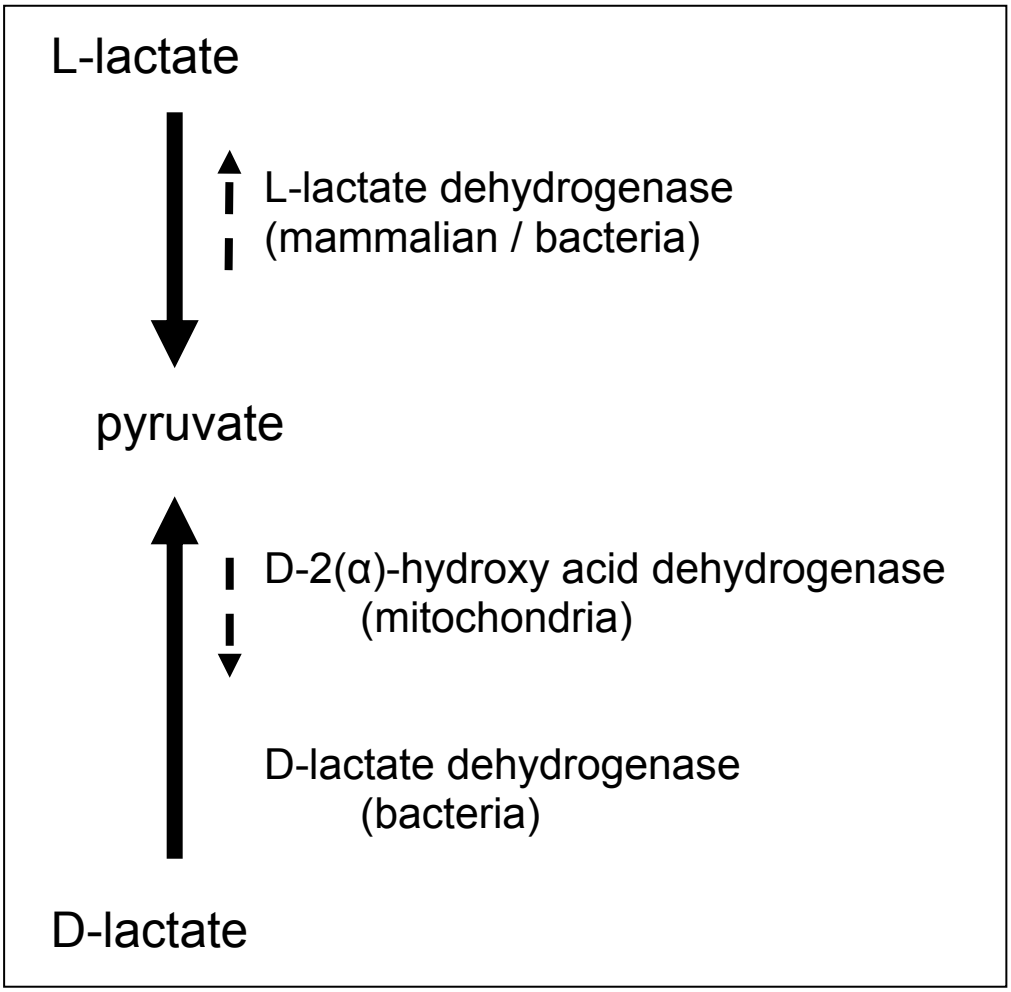


Figure 5. L- and D- lactate metabolism; L- isomer is converted to pyruvate by L-lactate dehydrogenase (v = ~50 μmoles/min per cm^3) enzyme in the cytosol (mainly in the liver) and bacteria (Nakae et al. 1997). D-lactate is converted to pyruvate by D-2 (α)–hydroxy acid dehydrogenase (v = 5-20 μmol/min per mL) in mitochondria (Cammack 1975). D-lactate dehydrogenase (v = 15±17 nmol/min per mg), a peripheral membrane dehydrogenase in bacteria is also capable of this conversion (Cammack 1969; De Bari et al. 2002; Dym et al. 2000). DL-Lactate recemase (v = ~0.1 μmol/min per mL) mainly in bacteria can covert L-lactate to D- isomer and vise versa (Hiyama et al. 1968).

Metabolism of D-lactate in mammals requires D-2 (∝)-hydroxy acid dehydrogenase, which converts D-lactate to pyruvate (Cammack 1969). The conversion of D-lactic acid to pyruvate by D-2 (∝)-hydroxy acid dehydrogenase is slower than the conversion of L-lactic acid to pyruvate by L-lactate dehydrogenase (Figure 5). Vmax of L-lactate dehydrogenase reported to be 5 to 97 μmol/min per cm^3 in different tissues (Nakae et al. 1997). D-2 (∝)-hydroxy acid dehydrogenase (v = 5-20 μmol/min per mL) is present in the mitochondria in the liver and kidney of several animal species including humans, but not in as high quantities as L-lactate dehydrogenase in mammalian cells (Cammack 1969;

Cammack 1970; Cammack 1970; Ewaschuk et al. 2005; Tubbs 1965; Yasuda et al. 1993). Therefore, in normal physiology D-lactate metabolism is several times slower than the L-lactate metabolism. D-2 (∝)-hydroxy acid dehydrogenase is a non-specific flavoprotein and is active over a limited pH range. This enzyme can utilize all D-2 (∝)-hydroxy acids including D-lactate as its substrates (Cammack 1969; De Bari et al. 2002; Tubbs 1965; Uribarri et al. 1998). Mammalian D-lactate dehydrogenase (v = 15±17 nmol/min per mg) was found in the muscle mitochondria recently (De Bari et al. 2002; Flick and Konieczny 2002). However, its impact on D-lactate metabolism in mammalian body is not well understood (Flick and Konieczny 2002). Normal subjects metabolize D-lactate and D-lactic acid equally well despite the fact that the former tends to increase the blood pH and the latter decreases it. Mainly based of renal excretion, healthy humans can metabolize D-lactate at a rate greater than 100 mmol/h with only a modest increase in serum concentration (Oh et al. 1985).

2.2.5.1 Elimination of D-lactate from the body

D-lactate elimination from the blood was rapid in experimental studies but not consistent in clinical case reports (Lorenz et al. 2005; Petersen 2005; Uribarri et al. 1998). Infused or injected D-lactate exhibited an exponential decay (first order kinetics) in calf experiments (Lorenz et al. 2005). D-lactate clearance from blood occurs at least in part through renal excretion (Connor et al. 1983; Ewaschuk et al. 2004; Jørgensen and Sheikh 1984; Oh et al. 1985; Ullrich et al. 1982b). Previous studies show that serum L-lactate is cleared through two independent processes: Hepatic removal follows second order kinetics while extrasplanchnic removal is linearly related to serum concentration (Naylor et al. 1984). D-lactate may also be taken up by hepatic and extrahepatic tissue (Naylor et al. 1984); mitochondrial uptake and utilization is promoted by malate (De Bari et al. 2002). However, this hepatic metabolism is slow and the main route of excretion is renal (Connor et al. 1983; Jørgensen and Sheikh 1984; Oh et al. 1985; Ullrich et al. 1982b).

2.2.5.2 Excretion

The main route of elimination of D-lactate from the mammalian body is urinary excretion (Jørgensen and Sheikh 1984; Oh et al. 1985; Ullrich et al. 1982a; Yasuda 1988). The renal clearance of both D and L-lactic acids are apparently similar (Connor et al. 1983; Jørgensen and Sheikh 1984; Yasuda 1988).

As renal excretion is a major root of elimination of D-lactate, urine contains D-lactate. Urinary concentrations fluctuate; although serum D-lactate levels may be low or undetectable, urine may contain detectable or high levels of D-lactate.

D-lactate transport in kidney tubules occurs via diffusion and excretion via a monocarboxylate transporter 1 (MCT1) (Ewaschuk et al. 2004; Haschke-Becher et al. 2000; Jørgensen and Sheikh 1984; Ullrich et al. 1982a). Since renal excretion of D-lactate

is effective in healthy individuals (Jørgensen and Sheikh 1984; Oh et al. 1985), it is the major factor in reducing serum D-lactate levels (Connor et al. 1983; Ewaschuk et al. 2004; Neale et al. 2005; Ullrich et al. 1982a) in normal physiology.

2.3 D-lactic acidosis; a secondary complication

DLA is a disease condition caused by the accumulation of D-lactic acid in the body (Dunlop and Hammond 1965; Ewaschuk et al. 2004; Petersen 2005; Uribarri et al. 1998). A metabolic crisis starts not only because of increased H^+, but slow metabolism of ionized D-lactate in the mammalian body (Borba et al. 2004; Cammack 1970; Ewaschuk et al. 2005; Tubbs 1965; Uribarri et al. 1998). Therefore, accumulation of D-lactic acid directly leads to D-lactic acidemia and D-lactatemia, and is considered a distinct form of metabolic acidosis (Bongaerts et al. 1997; Hove and Mortensen 1995; Petersen 2005). DLA is associated with gastrointestinal diseases and neurological signs (Hingorani and Chan 2001; Perlmutter et al. 1983; Petersen 2005; Puwanant et al. 2005; Uribarri et al. 1998). However, the mechanism linking neurological signs and DLA is not well understood (Editorial. 1990; Ewaschuk et al. 2005; Lorenz 2004b; Petersen 2005; Uribarri et al. 1998). Therefore, treatment and control are usually focused on the primary gastrointestinal disease.

2.3.1 Clinical manifestation of D-lactic acidosis

2.3.1.1 General clinical signs

The main clinical manifestation of DLA is neurological impairment (Hingorani and Chan 2001; Petersen 2005; Uribarri et al. 1998). General clinical symptoms and signs common to many gastrointestinal diseases can be observed in many DLA cases since DLA is often secondary to gastrointestinal diseases. Some of the observed non specific clinical signs are abdominal pain, flatulence, loss of appetite, dehydration, vomiting, semisolid feces or diarrhea, fever, headache, debilitation, weakness and many others linked to the primary disease (Bongaerts et al. 1995; Hove and Mortensen 1995; Petersen 2005; Puwanant et al. 2005; Uribarri et al. 1998). Further, many of these general clinical signs are similar regardless of the species, sex or age of the patient.

2.3.1.2 Neurological signs

Neurological signs in patients with high serum D-lactate concentrations include weakness, drunken appearance, altered mental status, dizziness, dysarthria, disorientation or confusion, sleepiness, memory loss, behavioral changes, vision disturbances, slurred speech, incoordination and ataxia (Carr et al. 1982a; Catlin 1982; Petersen 2005; Thurn et al. 1985; Uribarri et al. 1998). Comparable neurological signs are observed in continuous ambulatory peritoneal dialysis patients receiving DL-lactate solutions (Anderson et al.

1997; Chan et al. 1994). Many of these signs resemble alcohol intoxication (Handley and Ward-Smith 2005; Hudson et al. 1990; Uribarri et al. 1998). Calves who received intravenous hypertonic sodium DL-lactate infusions also showed neurological symptoms with vision disturbances and abnormal gate (Lorenz 2004b).

In general, neurological signs often appear in the later phase of a primary disease, and are associated with acute increase in blood D-lactate concentration (Petersen 2005; Uribarri et al. 1998).

2.3.2 Prevalence of D-lactic acidosis

DLA is commonly found in humans and cattle. Cat and dog cases are also reported (Fine 1989; Packer et al. 2005). Rodents are also susceptible according to certain studies (Giesecke and Fabritius 1974; Giesecke and Von-Wallenberg 1985; Ohmori and Iwamoto 1988). Other species have yet to be investigated. Under-reporting and inaccurate or premature diagnosis may have played a role in masking certain cases, so current statistics are in doubt (Petersen 2005). North America and Europe share a larger number of case reports than the other regions. Therefore, the current incidence data is indirectly associated with the availability of diagnostic facilities.

In humans, short bowel syndrome (SBS) causes the highest number of DLA case reports (Petersen 2005; Uribarri et al. 1998). Other gastrointestinal diseases and digestive disorders were also responsible for a few cases (Christopher et al. 1990; Jorens et al. 2004; Petersen 2005; Uribarri et al. 1998). DLA was found in cases of calf diarrhea with a relatively high prevalence (Ewaschuk et al. 2003; Lorenz 2004a; Omole et al. 2001). Grain overfeeding and ruminal acidosis is the major cause of DLA in cattle (Dunlop and Hammond 1965).

Human DLA cases over a wide range of ages from a few months to over 60 years old were reported all over the globe beginning in 1970. Young children and neonates were more susceptible (Bongaerts et al. 2000; Perlmutter et al. 1983; Puwanant et al. 2005). Although humans have the highest incidence, the total number of cases in the literature is less than 100. Interestingly, these numbers have more male cases than females (Uribarri et al. 1998).

A large number of DLA cases were diagnosed and reported from the early 1980s to 2000s. Therefore, predisposing factors for DLA have gradually become apparent. Although sex does not have a proven association in causing DLA, more males (> 62%) are affected than females (Neale et al. 2005; Oh et al. 1985; Uribarri et al. 1998).

According to case reports, 99% of DLA cases have serum D-lactate concentrations higher than 2 mmol/L. Over 90% of cases have serum D-lactate higher than 5 mmol/L, and over 20% have serum D-lactate over 10 mmol/L. Therefore, in most cases (70%) serum D-lactate lies between 5 to 10 mmol/L. Few cases have levels higher than 20 mmol/L. Urinary D-lactate values in these cases were inconsistent and fluctuated, however, the values were higher than 20 mmol/L (Bongaerts et al. 1995; Haschke-Becher et al. 2000; Perlmutter et al. 1983; Uribarri et al. 1998; Zhang et al. 2003).

The reported incidence of human DLA is higher than that in animals because human patients are subjected to a more thorough investigation than animals. Even in humans many neurologically affected patients were not correctly diagnosed or perhaps misdiagnosed. Therefore, the possible incidence of DLA may be higher than the present presumption (Petersen 2005). The majority of human cases are reported in young children and some in adults. This statistical observation suggests that the body can improve its capability to metabolize D-lactate with maturity (Cammack 1969; Cammack 1970; Flick and Konieczny 2002; Tubbs 1965).

Lambs and other animal neonates have not been well investigated for the occurrence of DLA.

2.3.3 Etiology of D-lactic acidosis

Overwhelming evidence from humans and many different animal species supports the association between gastrointestinal disease and DLA. Some of these predisposing gastrointestinal diseases are short bowel syndrome (Caldarini et al. 1996; Hudson et al. 1990; Oh et al. 1979; Puwanant et al. 2005; Zhang et al. 2003), active inflammatory bowel disease, enteritis (Hove and Mortensen 1995), diarrhea (exudative, secretary or osmotic) (Ewaschuk et al. 2003; Ewaschuk et al. 2004; Omole et al. 2001) exocrine pancreatic insufficiency (Packer et al. 2005), propylene glycol ingestion (Christopher et al. 1990; Jorens et al. 2004) indiscriminate eternal feeding, indiscriminate oral antibiotic use (Coronado et al. 1995), and lactose intolerance in human and non ruminant animals (Ewaschuk et al. 2005; Petersen 2005; Uribarri et al. 1998).

The mechanism of DLA has been described by many authors in relation to the following areas (Uribarri et al. 1998):

a) Microbial over growth and D-lactic acid production in the gastrointestinal tract,

b) Absorption of D-lactate or D-lactic acid from the intestines (or rumen) into the circulation causing elevation in serum D-lactate levels,

c) Onset of clinical symptoms in relation to serum D-lactate or D-lactic acid and metabolic impairment in elimination.

Although there is evidence associating D-lactatemia and neuropathy, the direct pathways which link D-lactatemia to encephalopathy or neurological disturbances are not well understood.

2.3.3.1 Primary diseases leading to D-lactic acidosis

DLA was respectively found secondary to short bowel syndrome in human (monogastrics), grain over feeding in ruminants, diarrhea in calves (preruminants), pancreatic insufficiency in cats (monogastrics), and other gastrointestinal diseases (Editorial. 1990; Ewaschuk et al. 2005; Petersen 2005; Uribarri et al. 1998).

2.3.3.1.1 Short bowel syndrome

The short bowel syndrome (SBS) is a disorder clinically characterized by malabsorption, diarrhea, steatorrhea, fluid and electrolyte disturbances, and malnutrition (Hudson et al. 1990; Nightingale 1995; Oh et al. 1979). SBS is generally defined as malabsorption resulting from anatomical or functional loss of a significant length of the small intestine. Most commonly this occurs after a surgical resection of the small intestines in the newborn period due to various reasons such as torsion, degeneration, necrosis or cancer (Nightingale 1995; Perlmutter et al. 1983). Massive small intestinal resection compromises digestive and absorptive processes (Bongaerts et al. 2000; Carr et al. 1982b; Nightingale 1995; Perlmutter et al. 1983).

In healthy adults, the small intestine has an average length of approximately 6 meters. SBS usually appears when there is less than 1.8 meters (30%) of the small intestine left to absorb sufficient nutrients. Adequate digestion and absorption cannot take place, and proper nutritional status cannot be maintained without supportive care. The most common causes of short-bowel syndrome in adults include Crohn's disease, radiation enteritis, mesenteric vascular accidents, trauma, and recurrent intestinal obstruction. In the pediatric population, necrotizing enterocolitis, intestinal atresia, and intestinal volvulus are the most common etiologic factors (Nightingale 1995). Other conditions associated with short-bowel syndrome include congenital short small bowel, gastroschisis and meconium peritonitis (Nightingale 1995; Nightingale 2001).

The final common etiologic factor in all causes of short-bowel syndrome is the functional or anatomic loss of extensive segments of small intestine so that absorptive capacity is severely compromised (Nightingale 1995; Nightingale 2001). Although resection of the colon alone typically does not result in short-bowel syndrome, its presence or loss can be a critical factor in the management of patients who lose significant amounts of small intestine (Bongaerts et al. 2000; Hudson et al. 1990; Nightingale 1995; Nightingale 2001).

Patients with intestinal resection or bypass surgery or jejunoileostomy usually develop DLA after a delay of several months to a few years. This suggests that a change in the intestinal flora occurs during this time. Ongoing colon acidosis may allow the over growth of acidophilic D-lactic acid producing bacteria (Kaneko et al. 1997). Studies with stool homogenates have shown that the formation of D-lactic acid is stimulated by low pH and inhibited at pH > 6.5 (Caldarini et al. 1996).

Jejunoileal bypass patients (100%) had plasma D-lactate levels greater than 0.5 mmol/L. Further, more than 90% (25 out of 27) had plasma D-lactate levels greater than 5.0 mmol/L (Uribarri et al. 1998). However, in 282 randomly chosen hospitalized patients with a history of gastrointestinal surgery or disease, plasma D-lactate levels were greater than 0.5 mmol/L only in 13 (5%).

In 2001 Gavazzi and colleagues reported that intestinal resection followed by antibiotic treatment (vancomycin 1 g bid) precipitated DLA with a 9.8 mmol/L serum D-lactate concentration (Gavazzi et al. 2001). Oral antibiotics are one predisposing factor to

the onset of DLA in patients with gastroenteropathies (Coronado et al. 1995; Gavazzi et al. 2001).

The fact that DLA and encephalopathy has not been described with greater frequency in patients with malabsorption syndrome not related to a short bowel may also be partly explained by the limited feed intake that sometimes occurs in these other conditions. In general, malabsorption syndrome is also more severe in the short bowel syndrome (Meier et al. 2003). DLA in dogs was reported secondary to bowel surgeries and experimentally secondary to peritoneal dialysis (Arieff et al. 1982; Fine 1989; Westermarck and Wiberg 2003).

DLA develops intermittently even in SBS patients and does not occur following every meal. If malabsorption with increased delivery of nutrients to the colon led immediately to osmotic diarrhea, there might not be a sufficient amount of substrate in the colon to produce D-lactate (Field 2002; Lorenz and Vogt 2006; Meier et al. 2003). Hence, colonic stagnation might be another important contributing factor.

Over the last 3 decades short bowel syndrome was the most common primary disease predisposing patients to DLA (Carr et al. 1982a; Carr et al. 1982b; Hudson et al. 1990; Petersen 2005; Uribarri et al. 1998). This syndrome has accounted for more than half of the reported human DLA cases.

2.3.3.1.2 Grain over-feeding and ruminal acidosis

The rumen is the major site and reservoir for microbiological colonization and fermentation shortly after weaning of young ruminants. The bacterial population in healthy rumen is over 10^{10} and about 80% is gram negative (Allison et al. 1975; Russell and Rychlik 2001). The major rumen bacteria are *Selenomonas*, *Clostridia*, *Ruminococcus*, *Fibrobacter* and *Megasphaera*. Apart from bacteria, the presence of a large amount of fungi and protozoa adds to the complexity of this biological ecosystem (Allison et al. 1975; Russell and Rychlik 2001).

The consequence of overfeeding high grain or readily fermentable diets to ruminant is ruminal acidosis (Allison et al. 1975; Crichlow and Chaplin 1985; Gentile et al. 2004; Kleen et al. 2003; Owens et al. 1998). An important point is that even slightly overfeeding fermentable carbohydrate can cause microecological changes (Russell and Rychlik 2001) in the rumen leading to sub-acute ruminal acidosis (Crichlow and Chaplin 1985; Kleen et al. 2003). This is in agreement with the colon analogy of bacterial overgrowth in monogastric SBS.

D-lactic acid is a major component in the blend of organic acids produced by bacteria during ruminal acidosis (Dunlop and Hammond 1965; Ewaschuk et al. 2005). The concentration can be high as 100 mmol/L. Ruminal DL-LA levels increased over 300 mmol/L in many ruminal acidosis cases; presumably half of this racemic lactic acid is D-lactic acid (Dunlop and Hammond 1965; Ewaschuk et al. 2005; Owens et al. 1998). Both D- and L-lactic acid are absorbed effectively by the rumen mucosa into the circulation causing systemic acidosis (Dunlop and Hammond 1965; Owens et al. 1998). Ruminal

lactic acidosis is a common disease finding in both feedlot and dairy cattle all over the world. Many times these diseases were imprecisely credited to lactic acidosis because ruminal or serum D-lactic acid concentrations were not measured. Some clinical signs reported after ruminal lactic acidosis in cattle are neurological. They include abnormal gate, limping, drunken appearance, fatigue, depression, loss of appetite and sometimes ataxia.

Many adult cattle DLA cases are associated with the management practice of grain over feeding in modern intensive feedlot and dairy farms (Dunlop and Hammond 1965; Editorial. 1990; Ewaschuk et al. 2005; Owens et al. 1998).

In 2004 Gentile and coworkers also reported significant and rapid elevation of serum D-lactate in calves as a consequence of experimentally induced ruminal acidosis (Gentile et al. 2004).

2.3.3.1.3 Diarrhea

Diarrhea is a condition caused by a variety of gastrointestinal diseases; either it is infectious or non-infectious, the pathology may be malabsorptive, exudative or secretory (Field 2002). During severe, chronic diarrhea the composition of the gastro-intestinal bacterial flora is disrupted, the mucosa damaged, and the immune system over-stimulated. Since pathogens generally have shorter generation times than commensals, there is a higher relative abundance of pathogens during diarrhea and the initial stages of intestinal recovery (Field 2002; Katelaris 1996). The inflammatory reaction during enteritis and colitis can facilitate organic (lactic) acid uptake and absorption from the lumen into the circulation (Field 2002; Meier et al. 2003).

Diarrhea is associated with malabsorption, major metabolic disturbances including dehydration and metabolic acidosis. The passage of fermentable materials to the colon in diarrheal diseases mimics SBS causing DLA. Neonates with diarrhea may be predisposed.

Metabolic acidosis develops in the diarrheic calf as a result of the loss of bicarbonate via the intestinal tract and the high production of acids in the colon (Field 2002; Lorenz 2004a; Meier et al. 2003; Omole et al. 2001). In addition reduced tissue perfusion may result in L-lactic acid production, reduced renal perfusion and reduced excretion of hydrogen ions and acids which can exacerbate the acidosis (Field 2002; Schelcher et al. 1998). Ewaschuk and coworkers in 2004 showed a linear relationship between serum and fecal or rumen D-lactate concentrations in diarrheic calves (Ewaschuk et al. 2004). Many calf cases of DLA had diarrhea (Ewaschuk et al. 2003; Ewaschuk et al. 2004; Lorenz 2004a; Lorenz and Vogt 2006; Omole et al. 2001).

Diarrhea affects 4 billion people yearly worldwide. Juvenile or neonatal diarrhea is a significant problem in humans causing death. Diarrhea causes more than 12,000 deaths per day in children worldwide. In North America, more than 100,000 infants and children are hospitalized per year as a result of diarrhea. Patients with diarrhea and other intestinal diseases from developing countries may not undergo expensive diagnostic procedures, and

hence DLA may be under-investigated (Bhan et al. 1989; Ewaschuk et al. 2005; Field 2002; Katelaris 1996; Ruuska and Vesikari 1991).

Diarrhea is a major cause of morbidity and mortality in livestock. Young calves are susceptible worldwide and severely affected. Among the calf population in North America about 11% are affected by neonatal diarrhea with mortality close to 0.7% (Ewaschuk et al. 2003; Ewaschuk et al. 2004). In 2002 Ewaschuk and coworkers reported over 60% correlation between anion gap and serum D-lactic acid in diarrheic calves (Ewaschuk et al. 2002).

Diarrhea in lambs is considered an important constraint in sheep farming. This condition is associated with considerable morbidity (2.8%) and mortality (0.7%) in Canadian lambs for the last few decades. The Prairie Provinces have a greater share of these scouring cases than other lamb diseases (Dohoo et al. 1985). Infectious agents such as bacteria, virus and cryptosporidium are responsible for 99% of lamb dysentery. More than 75% of these scouring cases are due to *Escherichia coli* infection. Some strains are known to be able to produce D-lactic acid (Bongaerts et al. 1997; Connolly et al. 2005; Hove et al. 1999; Petersen 2005; Uribarri et al. 1998). An episode of lamb diarrhea reduces the growth of lambs permanently with about 2 kg weight loss at the age of five weeks (Green et al. 1998). Diarrhea occurs at all ages of lambs and 15% to 40% of the flock may be affected any time, however, peak prevalence is 3 to 5 weeks of age (Green et al. 1998). Lambs affected with diarrhea may also be prone to DLA.

2.3.3.1.4 Exocrine pancreatic insufficiency

Exocrine pancreatic insufficiency (EPI) is the inability to digest food due to a lack of digestive enzymes secreted by the pancreas (DiMagno 1993). EPI is found frequently in dogs, cats and also in humans afflicted with cystic fibrosis (DiMagno 1993; Steiner and Williams 1999; Westermarck and Wiberg 2003). EPI is caused by a progressive loss of the pancreatic cells that make digestive enzymes, most commonly subsequently to pancreatic acinar atrophy. The atrophy in turn can be caused by previous infections, a blocked pancreatic duct, or genetics. The genetic form, which is common in dogs starts at the young age of about two months. Chronic pancreatitis is the most common cause of EPI in humans and cats. A loss of digestive enzymes leads to maldigestion and malabsorption of nutrients, which in turn resembles SBS further causing DLA.

A cat with exocrine pancreatic insufficiency ended up exhibiting neuropathy and was diagnosed with DLA (Packer et al. 2005). A high serum D-lactate (over 5 mmol/L) and loss of reflexes were the main characteristics of this case.

2.3.3.1.5 Other causes of DLA

The other gastrointestinal diseases and conditions that may lead to DLA are active inflammatory bowel disease, malabsorption syndrome, ingestion of propylene glycol,

colonic intubation, oral antibiotics, some probiotics and oral oxalate (Petersen 2005; Uribarri et al. 1998). In 1995 Hove and coworkers (Hove and Mortensen 1995) found high or detectable D-lactate concentrations in patients with gastrointestinal ailments including active inflammatory bowel disease and malabsorption. Patients with metabolic acidosis, high serum anion gap, relatively normal L-lactate concentrations and malabsorption following enteropathy were more likely to suffer from D-lactic academia and acidosis (Azhar and Beach 2002).

The gut metabolism of propylene glycol, an agent frequently used in medications and food, can lead to generation of D-lactate (Christopher et al. 1990). In cats, DLA occurs in experimental propylene glycol intoxication (Christopher et al. 1990) and is associated with central nervous depression, ataxia and weak eye reflexes, and coma.

Floppy kid syndrome, a disease in young goats is proven to be DLA (Bleul et al. 2006). A lack of proper diagnostic facilities, misdiagnosis and under reporting may mask the incidence of DLA in animals. Therefore, lambs and other animal neonates with gastrointestinal or neurological diseases needed to be investigated for DLA.

2.3.4 Clinical pathophysiology

Onset of clinical signs varies with many factors such as the type of primary disease, species, healthiness of the individual, and age of the patient. Other toxins produce by the over growing bacteria may have a role in the initiation of clinical signs. Some of these substances (organic acids, formate, histamine, ethanol, tyramine, and endotoxin) may interfere with the intake absorption, metabolism (Cammack 1969; Cammack 1970; Tubbs 1965), renal clearance or perhaps promote neuropathic actions of D-lactate (Cammack 1969; Cammack 1970; Ewaschuk et al. 2005; Hudson et al. 1990; Tubbs 1965; Uribarri et al. 1998).

Thiamine deficiency has been suggested to play a contributory role in the pathogenesis of DLA because DLA did not recur after initiation of thiamine supplementation. However, thiamine deficiency has not been shown to increase production of D-lactate (Uribarri et al. 1998).

Injection of 2.23 M sodium D-lactate produced ataxia, depression and a reduced palpebral reflex in calves within 6 h of injection (Lorenz et al. 2005). However, the above experiment was criticized since the solution was hyperosmolar; therefore, some of the neurological signs might have been related to fluid shifts (Stampfli 2005). Similarly, disagreement about whether D-lactate is directly responsible for neurological signs in humans exists (Laptook et al. 1988; Petersen 2005; Uribarri et al. 1998). Human patients undergoing Ringer's lactate peritoneal dialysis experienced neurological signs that disappeared with cessation of the lactate infusion (Anderson et al. 1997; Chan et al. 1994). Most of these clinical signs were acute in nature. Researchers have assumed that elevating serum D-lactate levels above 3 mmol/L is the point at which the onset of neurological symptoms occurs (Petersen 2005; Uribarri et al. 1998). Although gastrointestinal fermentation is a major cause of DLA, oral supplementation of D-lactic acid did not cause

clinical signs in many studies (De Vrese et al. 1990; Ding and Xu 2003; Partanen and Mroz 1999).

2.3.4.1 Impairment of D-lactate metabolism in the body

Healthy humans can metabolize D-lactate absorbed at a rate greater than 100 mmol/h with only a modest increase in serum concentration (Oh et al. 1985). Therefore, one might argue that impaired metabolism is a prerequisite for the production of DLA. On the other hand, DLA occurs when gastrointestinal fermentation is grossly excessive; therefore, the rate of D-lactate production could exceed the rate estimated above. It is also possible that the rate of D-lactate metabolism is reduced in patients with such acidosis, not because of any specific metabolic defect but as the result of generalized diminution in metabolism due to chronic malnutrition. If there is a specific mechanism of impaired ability to metabolize D-lactate, it is currently unknown.

As the liver and kidney contain D-2 (∝)-hydroxy acid dehydrogenase, disease of these organs might diminish the ability to metabolize D-lactate (Cammack 1969; De Bari et al. 2002; Uribarri et al. 1998). Loading rats with D-lactate over several days significantly increased the activity of D-2 (∝)-hydroxy acid dehydrogenase in the liver, but this increase was much less in rats and humans with chronic renal failure than in control animals (Anderson et al. 1997; Yasuda 1988; Yasuda et al. 1993). Although renal function has been described in only a few patients with DLA, some degree of renal insufficiency is common because of volume depletion; calcium oxalate kidney stones are frequent complications in patients with the short bowel syndrome (Hudson et al. 1990; Oh et al. 1985; Petersen 2005; Uribarri et al. 1998). Unfortunately, the rate of metabolism of D-lactate in patients with DLA has not been successfully measured.

Oxalate is a powerful competitive inhibitor of D-2 (∝)-hydroxy acid dehydrogenase (Tubbs 1965). Patients with the short bowel syndrome frequently have intestinal hyper absorption of oxalate, perhaps due to low intestinal pH, and it seems possible that high plasma and tissue levels of oxalate could inhibit enzyme activity. Low pH, which also diminishes the dehydrogenase activity, could play a role in the diminished metabolism of D-lactate. The effect of pH on the in vivo metabolism of D-lactate has not been investigated fully (Ullrich et al. 1982b; Uribarri et al. 1998).

Another mechanism by which renal dysfunction might alter D-lactate metabolism is interference with renal excretion. The renal threshold for D-lactate is low because other carboxylic acids including L-lactate may compete for the same transporter in reabsorption (Oh et al. 1985; Ullrich et al. 1982a; Ullrich et al. 1982b; Uribarri et al. 1998). However, renal fractional excretion is very high (Oh et al. 1985). Impaired renal function or volume depletion may decrease renal excretion of D-lactate and thereby increase plasma levels. Urinary secretion of D-lactic acid (5.0-30.0 mmol per creatinine mol) was correlated with age and ethnicity of young children (Haschke-Becher et al. 2000).

A combination of the above evidence suggests that the development of DLA requires the following conditions (Petersen 2005; Uribarri et al. 1998):

a) Ingestion of large amounts of carbohydrate or similar substrate,

b) Carbohydrate malabsorption with increased delivery of nutrients to the colon,

c) Colonic bacterial flora of a type that produces D-lactic acid,

d) Diminished colonic motility, allowing time for nutrients in the colon to undergo bacterial fermentation,

e) Damage to intestinal mucosa, and

f) Impaired D-lactate metabolism or excretion (dehydrated, debilitated or neonates).

2.3.4.2 Neonates at risk

Neonates are more susceptible to DLA. In human, DLA cases were reported in neonates more frequently than adults (Petersen 2005). Neonatal DLA cases exhibited severe neurological symptoms and encephalopathy (Gurevitch et al. 1993). Neonatal calves with diarrhea which had serum D-lactate concentration over 5 mmol/L showed neurological symptoms (Ewaschuk et al. 2005; Lorenz 2004b; Lorenz et al. 2005). Enzymatic activities in neonates are at a developing stage and may be less effective than in adults. The enzyme D-2 (∝)-hydroxy acid dehydrogenase which metabolizes D-lactate does not exist in neonates (Cammack 1969; Cammack 1970; Flick and Konieczny 2002; Tubbs 1965). Functional abilities of liver and kidney are comparatively lower in neonates (Connolly et al. 2005; Haschke-Becher et al. 2000; Mack 2004; Marko et al. 2004; Yasuda et al. 1993). Digestion and absorption in neonates are not as efficient as in adults and neonates are more susceptible to enteric infections which compromise digestion and absorption and hence could result in a high passage of fermentable nutrients to the colon (Bongaerts et al. 2000; Packer et al. 2005; Puwanant et al. 2005; Uribarri et al. 1998). All these factors contribute to put neonates at higher risk for D-lactate production and elevation in blood. Many DLA cases in neonates are reported at the ages of 1 to 2 years. However, there may be cases in infants which may not be diagnosed accurately or may not be available for diagnosis (Petersen 2005).

2.3.4.3 Possible mechanisms of neurological manifestations

Apart from the common clinical symptoms such as drowsiness, lethargy and depressed appearance, there may be specific neurological symptoms such as ataxia, slurred speech, nystagmus, seizures and confusion. Three main hypotheses have been proposed to explain the neurological manifestations of DLA:

a) D-lactate itself is toxic to the brain (neurotoxic),

b) H^+ or increased systemic acidosis, and

c) Unknown compounds produced or released along with D-lactate are toxic to the brain.

Indeed, compounds such as formate, histamine, ethanol, tyramine, and endotoxin have been found in the rumen of cattle with DLA (Dunlop and Hammond 1965), and excessive urinary excretion of hydroxyphenyllactic and phenylacetic acids (Uribarri et al. 1998) has been observed in patients with this condition. The role that each of these compounds may play in causing the neurological abnormalities, is uncertain but it is clear that ethanol is not responsible for the symptoms. Although the patients appear to be drunk, serum levels of ethanol are normal (Hudson et al. 1990). Neonates with DLA are more vulnerable to neurological symptoms and encephalopathy (Gurevitch et al. 1993; Petersen 2005; Uribarri et al. 1998). Neonatal calves with diarrhea had neurological symptoms when serum D-lactate concentration exceeded 5 mmol/L (Ewaschuk et al. 2005; Lorenz 2004b; Lorenz et al. 2005).

D-lactate can penetrate into the central nervous system by simple diffusion and can thus reach high concentrations in the brain (Uribarri et al. 1998). Recent studies also showed that D-lactate can cross the blood brain barrier (Aubert et al. 2005; Martin et al. 2006; Ros et al. 2001; Tekkök et al. 2003). Although it has been hypothesized that D-lactate may accumulate in the brain because of the low local level of metabolizing enzyme (Oh et al. 1985), levels of D-lactate in the cerebrospinal fluid have been found to be similar to those in the blood (Benoist et al. 2003; Karton et al. 1987; Okubo et al. 2000; Uribarri et al. 1998). However, CSF D-lactate levels were not well established in many cases (Benoist et al. 2003; Oh et al. 1985; Okubo et al. 2000; Thurn et al. 1985). Moreover, the correlation between serum D-lactate levels and severity of symptoms is poor (De Vrese et al. 1990; Lorenz 2004b; Thurn et al. 1985). However, Lorenz and coworkers in 2004 and 2005 showed a correlation (70%) between serum D-lactate concentration and the degree of nervous symptoms (ataxia) in diarrheic calves; they also documented that D-lactate concentrations were higher in the less severely dehydrated calves (Lorenz 2004b; Lorenz et al. 2005). They did not measure CSF lactates.

In 1985, Thurn and coworkers (Thurn et al. 1985) found that patients with jejunoileal bypass reported symptoms consistent with D-lactic acid encephalopathy but only a few of them had high plasma levels of D-lactate, while some individuals with high plasma D-lactate levels did not have symptoms. The fact that some patients present with encephalopathy did not have high plasma D-lactate levels strengthens the suggestion that the encephalopathy results from toxins which may be produced simultaneously with D-lactic acid, or may be due to trapped D-lactate in nervous tissue. However, CSF was not available to measure D-lactate levels in any or many of these encephalopathic patients.

Acidosis itself is not likely to be responsible for neurological manifestations since patients with other types of acidosis of comparable or greater severity do not present with neurological signs (Owens et al. 1998; Petersen 2005; Uribarri et al. 1998). Altered pH may have some role in the toxic effect of D-lactate or other chemicals on the brain since patients with DLA usually have significant metabolic acidosis. It is also probable that D-lactate is harmful to the central nervous system only in the presence of another metabolic defect, such as deficiency of certain vitamins, which might only be present in patients who develop the syndrome (Uribarri et al. 1998). Interference of pyruvate metabolism by D-lactate has been postulated based on the observation of clinical similarities between DLA and inherited or acquired abnormalities of pyruvate metabolism (Cross and Callaway 1984).

Excessive production of D-lactate either in the colon or rumen is the beginning of a crisis (Giesecke et al. 1985; Hove and Mortensen 1995; Oh et al. 1985; Petersen 2005; Uribarri et al. 1998). However, if D-lactic acid or D-lactate is not absorbed into the circulatory system, the patient may not exhibit clinical manifestation of D-lactatemia; DLA and neuropathy.

Slow metabolism and the single route of elimination of D-lactate from the body result in accumulation of D-lactate in the blood and other body compartments. Once the serum D-lactate concentration exceeds 3 mmol/L level, patients start exhibiting neurological signs of DLA on top of the signs of the primary disease (Chan et al. 1994; Hingorani and Chan 2001; Petersen 2005; Thurn et al. 1985; Uribarri et al. 1998). In cases of renal and hepatic impairment, elimination of D-lactate from the body is more severely compromised. The result is further accumulation of D-lactate in the body. In extreme cases, with very high D-lactate concentrations (110 mmol/L), ataxia, paralysis and coma are seen (Christopher et al. 1990; Jorens et al. 2004; Petersen 2005; Uribarri et al. 1998). Therefore, impairment in D-lactate metabolism or excretion can lead to a metabolic crisis.

Differences in blood pH in acidosis experiments conducted by few previous workers (De Vrese et al. 1990; Gentile et al. 2004; Laptook et al. 1988; Lorenz et al. 2005; Patra et al. 1993) were quite modest compared with those observed in patients with clinical DLA (Bongaerts et al. 1997; Ewaschuk et al. 2005; Uribarri et al. 1998; Yasuda et al. 1993).

2.3.4.3.1 D-lactate and the central nervous system

Low pH (< 7.2) enhances L-lactate transport across the blood brain barrier (Oldendorf et al. 1979). In addition to lipid mediated penetration (Oldendorf et al. 1979), L-lactate may be transported across the blood brain barrier by mono-carboxylic acid transporters (Gladden 2004; Hertz and Dienel 2005; Schurr 2006) which are reported to be stereo specific (Tekkök et al. 2003; Tekkök et al. 2005). L-lactate is formed within nervous and muscle tissues by metabolism of glucose (Aubert et al. 2005; Gladden 2004).

D-lactate also has the ability to rapidly pass the blood brain barrier though it may not be pH dependent (Oldendorf et al. 1979). L-lactate competes with D-lactate for uptake from blood by cerebrospinal tissue (Tekkök et al. 2003; Tekkök et al. 2005).

As many of the neurological signs are acute and depressive in nature, D-lactate may mediate these by affecting neurotransmitters or receptors. Since glutamate and γ-aminobutyrate (GABA) are important chemical neurotransmitters (Ge et al. 2005; Gjedde et al. 2002; Horikawa et al. 1996; Krnjević 2005) D-lactate may interfere with these transmitters to cause acute neuropathy. D-lactate might interrupt (antagonize) the excitatory activities of glutamate or initiate (agonize) the inhibitory activities of GABA. D-lactate, unlike L-lactate, inhibits the development of an action potential in the optic nerve of rodents in vitro (Tekkök et al. 2003; Tekkök et al. 2005). Neurons are not capable of utilizing D-lactate (Oh et al. 1985; Tekkök et al. 2003). Cassady and coworkers in 2001 (Cassady et al. 2001) reported an acute increase in GABA release in rodent cerebral cortical superfusates following topical application of D-lactate (20 mM). Similarly, D-

lactate in vivo may initiate GABA release which could trigger inhibitory pathways (Krause and Schwarz 2005; Krnjević 2005; Kuffler and Edwards 1958; McCormick and Tunnicliff 1998) causing somnolence, depression and paresis (Waagepetersen et al. 2003). L-lactate can be metabolized by neurons (Aubert et al. 2005; Gladden 2004; Schurr 2006) and can be exchanged with glutamate during baseline or excitation both in neurons and astrocytes (Gjedde et al. 2002; Hertz and Dienel 2005), thereby playing a major role in synaptic activity (Magistretti and Pellerin 1999; Pellerin and Magistretti 1994). D-lactate may interfere with this coupling synaptic activity which is critical for excitatory pathways (Krnjević 2005; Magistretti and Pellerin 1999). L-lactate is thought to be cerebro-protective (Gladden 2004; Medina and Tabernero 2005; Ros et al. 2001; Schurr 2006) and can act as an energy source (Bouzier-Sore et al. 2003; Gladden 2004; Hertz et al. 2007; Schurr 2006). Unlike L-lactate, D-lactate does not interfere with glutamate accumulation (Cassady et al. 2001) or with acetate uptake (Waniewski and Martin 1998) in nervous tissue cultures.

In acute ethanol consumption, neurological depression may be caused by low concentrations of circulating tryptophans (Badawy et al. 1995). However, alcohol is also able to interact with the $GABA_A$ receptor complex and facilitate GABA action (Gonzales and Hoffman 1991; Starke 1991), therefore, D-lactate may have similar actions in similar fashion in those receptors.

2.3.5 Therapies and prevention

Therapeutic measures in DLA vary with the type of primary disease and predisposing causes. Therapeutic and prevention measures can be categorized into immediate (treatment) and long term (control) measures. General therapeutic measures are fluid or bicarbonate therapy, peritoneal dialysis, antibiotic use, dietary management and many others. Treatments basically focus on removal of clinical signs, elimination of D-lactate, correction of acidosis, nourishing the patient and correction of microbial overgrowth and imbalance in the intestines. General control measures are appropriate dietary management, proper use of antibiotics, and use of prebiotics or / and probiotics to maintain a healthy intestinal flora.

Therapeutic management of DLA cases focuses on different strategies; clinical symptomatic treatments, elimination of D-lactate and acidosis, reducing D-lactate production and absorption in the gut, and also on preventive or control measures to avoid recurrence of DLA (Petersen 2005; Puwanant et al. 2005; Uchida et al. 2004; Uribarri et al. 1998). Many strategies are based on the fact that many DLA cases have a gastro intestinal link.

2.3.5.1 Clinical approach to treatment

Common approaches to the treatment of DLA use simple methods to eliminate D-lactate from the body (Anderson et al. 1997; Mathieu et al. 1991; Puwanant et al. 2005;

Uribarri et al. 1998). Bicarbonate is the most commonly used infusion to reduce acidosis and acidemia (Arieff et al. 1982; Booth and Naylor 1987; Cooper et al. 1990; Kasari and Naylor 1985; Mathieu et al. 1991; Puwanant et al. 2005). Doses of bicarbonate, amount of fluid and rate of infusions administered to patients with DLA were case dependant. The results were not consistent and raise many doubts about the effectiveness of these treatments; hence further research is needed. Regular monitoring of renal clearance of D-lactate is important to understand the efficacy of fluid therapy, dehydration status and renal health. Faster removal of D-lactate can be achieved by peritoneal dialysis in weak patients with renal failure (Fine 1989; Petersen 2005). Intermittent or continuous dialysis can be performed with saline.

The next step is to minimize the production and absorption of D-lactate. Some measures are alteration of diet, use of antibiotics and use of probiotics or prebiotics. Many of these measures target intestinal flora to reduce D-lactic acid production (Gurevitch et al. 1993; Narula et al. 2000; Petersen 2005; Uribarri et al. 1998). Neomycin or kanamycin (5-10 mg/kg body weight) and other gut acting antibiotics have been used; however, they were not very successful (Bongaerts et al. 2000; Bongaerts et al. 1997; Gavazzi et al. 2001; Hudson et al. 1990; Uchida et al. 2004). Bongaerts and coworkers recently showed that yeast mediated a suppression of D-lactate production after antibiotic cocktail treatment in a patient with short small bowel (Bongaerts et al. 2005). Other probiotics have also been used in certain cases with limited success (Chermesh and Eliakim 2006; Ewaschuk et al. 2004; Ewaschuk et al. 2006; Katelaris 1996; Uchida et al. 2004). The most commonly administered organisms were *Lactococcus* and *Lactobacillus* species which could compete with and inhibit D-lactic acid producers. Feeding microbes that utilize D-lactate is another approach. Prebiotics seem to be a potential treatment method which has yet to be fully investigated (Evans and Martin 1977; Martin and Streeter 1995; Martin et al. 1999; Passos et al. 2003; Vicini et al. 2003).

2.3.5.2 Dietary management

Dietary management is applied following bowel surgery (Caldarini et al. 1996; Hudson et al. 1990; Puwanant et al. 2005; Puwanant et al. 2005), enteritis(Vella and Farrugia 1998), diarrheic diseases (Ferguson et al. 1981; Pearson et al. 1978), exocrine pancreatic insufficiency (Packer et al. 2005), and lactose intolerance (Hove and Mortensen 1995) to manage excessive organic acid production (DeGregorio et al. 1982) including D-lactic acid production (Editorial. 1990; Kleen et al. 2003; Petersen 2005; Uribarri et al. 1998). Parental or enteral nutrition is the choice of feeding in certain cases for the high risk period. Strict limitation of diet may be beneficial to avoid supplying fermentable compounds to the colon. In cases of exocrine pancreatic insufficiency, the missing enzymes may be supplemented in the diet, or predigested food (ready made nutrients) in the right quantities can be fed enterally (Westermarck and Wiberg 2003). In veterinary practice, such patients (dogs) are treated by supplements such as Lypex pancreatic enzyme capsules (VetPlus International Ltd, Lytham, United Kingdom) which contain lipase 30000, amylase 18750 and protease 1200 Ph Eur U. These patients are fed with low fat diets such as beef liver or pork pancreas extract / soup with an appropriate commercial food

/ products similar to Waltham® Chappie dog food (Waltham® Centre for Pet Nutrition, Waltham, Massachusetts, USA). Chappie consists of protein 5.5%, oil 2.0%, ash 1.5%, fibre 0.4%, moisture 77.0%, copper 2mg/kg (as Cu_2SO_4), vitamin A 500 IU/kg, vitamin D 150 IU/kg and vitamin E 10 mg/kg (Westermarck and Wiberg 2003).

2.3.5.3 Control (and prevention)

Control has two themes; control of recurrence in an affected patient, and control of occurrence (prevention) in a susceptible patient or herd.

Bacterial overgrowth (dysbiosis) (Hove and Mortensen 1995) and decreased gut pH (Caldarini et al. 1996) play a major role in increasing D-lactic acid producing bacteria (Bongaerts et al. 2000; Bongaerts et al. 1997; Connolly et al. 2005; Hove et al. 1999). The two most commonly used control approaches are to supplement the diet with live microbes (probiotics) or with nutritional compounds (prebiotics) that enhance the proliferation of favorable, non-D-lactate producing (gram negative strictly anaerobic) bacteria such as *Selenomonas ruminantium* and *Megasphaera elsdenii* (Martin 1998). Probiotics (such as *Lactobacillus* strains) (Chermesh and Eliakim 2006; Ewaschuk et al. 2004; Ewaschuk et al. 2006; Katelaris 1996; Uchida et al. 2004) and prebiotics (such as malate) (Martin et al. 1999; Montano et al. 1999; Uchida et al. 2004) may be used in the treatment / prevention of DLA.

The use of antibiotics (feed growth additives) reduces the incidence of lactic acidosis. The mechanisms by which growth promoting antibiotics improve growth performance are not known with certainty, but may include:

a) Nutrients are more efficiently absorbed because of a thinner small intestinal epithelium;

b) Nutrients are spared due to a reduction in competing microorganisms;

c) Microorganisms responsible for subclinical infections are reduced or eliminated;

d) Production of growth-depressing toxins or metabolites by the gastrointestinal microbiota is reduced;

e) Microbial deconjugation of bile salts is reduced.

Growth promoting antibiotics accomplish their effect in the small intestine while they have some effect on the microbiota in the large intestine (Hove et al. 1999). The microbiota in the hindgut of the healthy animal has a beneficial effect on the host animal via the fermentation of feed material that escaped digestion in the small intestine and via its stabilizing effects on microbial population and the mucosal structure and function throughout the gut.

Since the use of antibiotics to control D-lactic acid producing bacteria is controversial, a better approach would be use of probiotics (Chermesh and Eliakim 2006; Ewaschuk et al. 2006; Katelaris 1996) or prebiotics (Martin et al. 1999; Montano et al. 1999; Uchida et al. 2004) together with symptomatic remedies (vitamins-thiamine,

electrolytes and fluid therapy) (Booth and Naylor 1987; Lorenz and Vogt 2006; Uribarri et al. 1998). Manipulating the gut microbial flora (by *Lactococcus / Lactobacillus* species), introducing competitive inhibitors to D-lactic producers, feeding microbes that utilize lactate, providing prebiotic compounds such as malic acid ($C_4H_6O_5$) which enhances the removal of lactate are approaches which could be further investigated in order to control D-lactic acid production in the gastrointestinal tract. Malate has the ability to modulate pH in its environment (Passos et al. 2003; Piva et al. 2002; Russell and Rychlik 2001). Malate promotes the growth of gut bacteria that can convert lactate to propionate (Evans and Martin 1977; Martin 1998). Malate has been used to reduce rumen D-lactic acid production in cattle (Martin 1998; Nisbet and Martin 1991; Nisbet and Martin 1994; Russell and Rychlik 2001). Therefore, malate may be a potential candidate in the prevention of DLA. Dietary supplements of $CaCO_3$ and $MgCl_2$ may potentially diminish intestinal production of D-lactic acid (Caldarini et al. 1996) by increasing the luminal rumen and colonic pH. Ca malate may be used to gain both properties.

Thiamine supplementation may reduce recurrence of DLA when given to susceptible individuals (Hudson et al. 1990; Uribarri et al. 1998). Surgical (serial transverse enteroplasty) management enhanced the recovery of refractory DLA in a patient with short-bowel syndrome recently (Modi et al. 2006).

2.4 Analysis of lactic and other organic acids

2.4.1 Diagnosis and confirmatory tests

Biological fluids including blood and urine are commonly analyzed for D- and L-lactate determination (Ewaschuk et al. 2002; Ohmori and Iwamoto 1988; Okubo et al. 2000; Omole et al. 1999; Van-Hee et al. 2004). Blood lactate concentrations are measured by some modern automated analyzers (Braconnier et al. 2003; Zwart et al. 1987). Serum concentrations are measured using either enzymatic assays (Buttery and Pannall 1986; Haschke-Becher et al. 2000; Martí et al. 1997) or chromatographic methods (Ewaschuk et al. 2002; Ohmori and Iwamoto 1988; Okubo et al. 2000; Omole et al. 1999; Van-Hee et al. 2004).

For modern automated analyzers (blood gas machines) only 0.5 ml blood sample is needed, however, the sample needs to be tested soon after collection (immediate fresh sample or analyzed within 2 h kept in ice). Serum separated from blood can be stored at -20 °C for years for chromatography and less than 1 mL blood sample is needed for serum separation.

Stereospecific identification of D-lactate in body fluids (serum and cerebrospinal fluid), urine and feces is important in diagnosis and treating DLA. High performance liquid chromatography (HPLC) is preferred for accurate quantifications (Ewaschuk et al. 2002; Minniti et al. 2001; Okubo et al. 2000; Omole et al. 2001) to the other methods such as enzymatic procedure (Buttery and Pannall 1986; Haschke-Becher et al. 2000; Martí et al. 1997).

2.4.2 Measuring lactates

Modern automated blood analyzers measure blood gases, lactate, electrolytes, hemoglobin, lactate and glucose (Severinghaus 2002). Lactate measurement is based on spectrophotometry (reflective photometry) or enzymatic assay (lactate oxidase) and usually express total lactates in mmol(s) per liter (mmol/L) of blood.
Enzymatic methods are interfered by certain compounds including sodium fluoride, acetaminophen and potassium oxalate. HPLC systems are accurate, precise, repeatable, and have negligible interference from contaminants (Ohmori and Iwamoto 1988; Omole et al. 1999). The HPLC method is also applicable to a variety of biological fluids (and extracts).

2.4.3 Measuring L- and D-lactates using HPLC

Measurement of D-lactate in body fluids using HPLC system was established by Omole and coworkers (Omole et al. 1999) in 1999. Both L- and D-lactates are separately eluted in the chromatogram and can be quantified relative to known standards. Venous blood (3 mL) is collected, allowed to coagulate for 20 min and serum separated by refrigerated centrifugation (IEC Centra-7R Refrigerated Centrifuge; International Equipment Company, Needham Heights, Massachusetts, USA) at 1000 RCF for 30 min. Serum is stored at -20 °C. D- and L-lactate concentrations measured from 0.2 mL serum sample using an HPLC system (Waters 600 HPLC pump, 486 UV detector and 786 Ultra WISP auto-injector, Millenium32 v.4 software; Waters, Mississauga, Ontario, Canada). For the separation of D- and L-lactic acid a stereoselective 3 μm ODS (octadecyl Silane) packed (50 mm × 4.6) analytical ligand exchange column coated with N,N-dioctyl- alanine (ChiralPak MA+; Chiral Technologies Inc., Exton, Pennsylvania, USA) is employed (Omole et al. 1999). A Guard-Pak Precolumn Module containing an ODS (octadecylsilyl) cartridge (Waters Guard-Pak™ precolumn; Waters, Mississauga, Ontario, Canada) insert is placed serially just before the analytical column to reduce the risk of obstruction, to limit interfering substances and enhance elution capability. Internal standard is a 2 mmol/L malonic acid solution. The mobile phase is a 2 mmol/L copper sulfate in 1% acetonitrile. The flow rate for the mobile phase is 0.4 mL/min and the column works at room temperature (25 ºC). UV detection occurs at 236 nm wavelength.

2.4.4 Measuring other organic acids using HPLC

Measurement of organic acids in body fluids is performed using HPLC system (Ewaschuk et al. 2002; Omole et al. 1999) which has a nonstereoselective, reverse phase Shodex RSPAK KC-811 (300 mm × 8) analytical column with a KC-811 precolumn (Showa Denko K.K., Tokyo, Japan). Internal standard is a 7 mmol/L adepate solution. The mobile phase is a 0.1% phosphoric acid in double distilled water. The flow rate for the

mobile phase is 0.7 mL/min and the column temperature is maintained at 50 ºC. UV detection wavelength is at 236 nm. The rest of the procedure and quantifications are similar to the above section (2.4.3).

3 EXPERIMENTAL MODEL STUDIES

3.1 D-lactate induced neurotoxicity in a calf model

3.1.1 Introduction

Acidemia is a common problem in humans and other animals. In diarrheic calves, the severity of acidemia correlates with neurological depression (Naylor 1989). In humans with short bowel syndrome, DLA is suggested as the cause of certain neurological symptoms (Bongaerts et al. 1995; Bongaerts et al. 2000; Bongaerts et al. 1997; Hingorani and Chan 2001; Hudson et al. 1990; Oh et al. 1979; Perlmutter et al. 1983; Uribarri et al. 1998). Similarly, in a variety of ruminant diseases including neonatal diarrhea, ruminal carbohydrate overload and acidosis without dehydration syndromes, DLA occurs in association with functional impairment of central nervous system (CNS) including signs of ataxia and coma (Ewaschuk et al. 2003; Gentile et al. 2004; Jorens et al. 2004; Omole et al. 2001; Schelcher et al. 1998). In cats, DLA occurs in experimental propylene glycol intoxication (Christopher et al. 1990) and in exocrine pancreatic insufficiency (Packer et al. 2005). In both cases DLA is associated with CNS depression, ataxia and in one case, coma. In a recent human case, accidental propylene glycol ingestion was associated with a very high serum D-lactate concentration (110 mmol/L), subsequent loss of CNS function and development of coma (Jorens et al. 2004).

At physiological pH, D-lactic acid is almost completely dissociated (De Vrese et al. 1990; Ewaschuk et al. 2005). Whether or not acidosis per se or the D-lactate anion is responsible for impaired CNS function is not well established. A bolus injection of 223 mmol sodium D-lactate in 100 mL of water produced ataxia, depression and a reduced palpebral reflex in calves (Lorenz et al. 2005). However, the experiment was criticized since the solution was hyperosmolar with an osmotic pressure approximately 15 times that of plasma; therefore, some of the neurological signs might have been related to fluid shifts (Stampfli 2005). Similarly, there is disagreement about whether D-lactate is directly responsible for neurological signs in humans (Uribarri et al. 1998).

The objectives of this study were to compare the influence of different types of acidosis on neurological function, and to determine whether signs are caused by either increased acidity or D- or L-lactate concentrations.

3.1.2 Materials and methods

3.1.2.1 Experimental animals and care

Calves for this study were obtained from the Dairy Barn of the Department of Animal and Poultry Sciences, University of Saskatchewan, Canada (Table 1) which had Holstein breed and only male calves were provided for the experiment. The calves were housed indoors in the Animal Care Facility at the Western College of Veterinary Medicine, University of Saskatchewan. The indoor environment was held at 22 ± 1°C, 65 ± 5% relative humidity, and was lit from 6:00 to 20:00 h. Each calf was housed in a 2.0 × 1.5 m^2 pen. Flooring was a 3 cm thick rubber mat covered with approximately 4 cm of wood shavings.

Clean water was freely available to these calves through automatic water bowls fixed to the walls. The stalls were cleaned twice a day. The calves were offered alfalfa hay and fed milk replacer (20 - 20 - 20 Wet Nurse™, green tag; Prairie Micro-Tech Inc., Regina, Saskatchewan, Canada) twice a day. Milk replacer solution was made on a 20% v/v basis and fed at 2.5 - 3.0 L a meal. The calves were weighed (Norac Instaweigh™ Instrumentation Animal Scale; Norac Weighing and Control Systems, Norac International Inc., Saskatoon, Saskatchewan, Canada) on the arrival to the Animal Care Facility, and prior to the catheterization (Table 1). The latter weight was used for dosage calculations.

Table 1. Calf information and order of infusion of DL-lactic acid (DL-LA), hydrochloric acid (HCl), L-lactic acid (L-LA) and saline

Calf ID	Body weight (kg)	Age (d)	Order of infusions			
			DL-LA	HCl	L-LA	Saline
648588504	62	35	2	NA	1	NA
287348803502	68	41	4	2	3	1
8648585501	64	34	NA	1	NA	NA
8648583499	77	47	1	2	3	4
8648582498	88	37	NA	NA	1	2
277634660	75	30	1	4	3	2
8648573489	55	17	1	4	3	2
8648572488	68	15	1	NA	NA	2
Total number of infusions			6	5	6	6
Mean ± se	69.6 ± 3.6	32.0 ± 3.9				

NA= not available (infusion not performed).

3.1.2.2 Catheterization

Calves were catheterized with indwelling jugular intravenous, ear arterial, and atlanto-occipital CSF catheters (Cox and Littledike 1978; De Craene et al. 1997; Jones and Robinson 1981). All surgeries were performed in the Department of Large Animal Clinical Science, Western College of Veterinary Medicine, University of Saskatchewan. The calves were premedicated with intravenous hydromophone at 0.1 mg/kg, sometimes in combination with metatomidine at 7 µg/kg or medetomidine at 4 µg/kg, induced with a combination of ketamine at 2 to 6 mg/kg and diazepam at 0.1 to 0.5 mg/kg, intubated and maintained under gaseous isoflurane anesthesia. Strict aseptic technique was followed throughout the catheter placement procedure.

3.1.2.2.1 Atlanta-occipital CSF catheter

A spinal Tuohy needle (18 G, 9 cm) from a freshly opened epidural kit (Mila International Inc., Florence, Kentucky, USA) was inserted into the subarachnoid space (cisterna magna, site confirmed by needle 'pop' and aspiration of CSF). The catheter (20G, 9203 wire reinforced, closed end) was threaded 10 to 15 cm into the space, the needle withdrawn, and a sampling adaptor and cap attached to the open end of the tube. The catheter was fixed in place with a butterfly tape and sutures near the skin-catheter interface. The sampling tip was closed with an infusion cap, wrapped in sterile gauze soaked in

chlorhexidine acetate 1% (Hibitane® Veterinary Ointment; Wyeth Animal Health, Division of Wyeth Canada, Guelph, Ontario, Canada) and placed in a sterile plastic bag.

3.1.2.2.2 Jugular intravenous catheters

A sterile catheter was made using sterile vinyl tube (Dural Plastics and Engineering, Auburn, Australia) with an inner diameter of 1 mm and a length of 45 cm. The catheter was inserted through a needle into the jugular vein. The catheter was placed 25-30 cm into the vein (depending on the calf body weight) so that the tip lay in the anterior vena cava. The sampling end was tunneled subcutaneously (5-6 cm) to reduce the possibility of thrombophlebitis. Both jugular veins were catheterized; one catheter was used for infusion and the other for venous blood sampling. The entire neck was covered with sterile dressing to protect the three catheters. Pockets in the dressing allowed access to the catheters.

3.1.2.2.3 Ear arterial catheter

A freshly opened arterial catheter (22-G [0.9 mm] × 25 mm length, BD Insyte™; Becton Dickinson, Infusion Systems Inc., Sandy, Utah, USA) was placed in the ear artery on the dorsal surface of one of the ears for arterial sampling.

3.1.2.3 Post surgical care

The post surgical rest period was a minimum of 4 days during which the catheterized calves were treated with an antibiotic, enrofloxacin, 5 mg/kg (Baytril® 100 injectable solution; Bayer HealthCare, Bayer Inc., Animal Health Division, Toronto, Ontario, Canada) and an analgesic, ketoprofen, 3 mg/kg (Anafen® 100; Merial Canada Inc., 500 boul. Morgan, Baie d'Urfe, Quebec, Canada) intra muscularly. Sixteen calves were surgically catheterized; eight remained healthy with functioning catheters and were used for infusions (Table 1). Following completion of the experiment, two calves were randomly euthanized using IV dose (2 mL/4.5 kg) of Pentobarbital Sodium USP (Euthanyl®, 240 mg/mL, Din 00141704; Bimeda-MTC Animal Health Inc, Vėtoquinol Canada Inc (distributor), Lavaltrie, Quebec, Canada) to confirm the location of the catheters at necropsy. This was carried out to ensure that CSF was being sampled. The placement of CSF catheters in surviving calves was confirmed by contrast radiology.

3.1.2.4 Experimental design

The calves (n = 8) with indwelling cerebrospinal fluid (CSF), venous and arterial blood catheters were experimentally infused for 6 h with solutions of DL-lactic acid (DL-LA), L-lactic acid (L-LA), hydrochloric acid (HCl) or physiological saline (NaCl) as a control. The order of treatments was randomly assigned. CSF fluid, venous and arterial blood were sampled over a 24 h period and concentrations for D- and L-lactic acid and blood gas values were determined. Neurological assessments of the calves were also carried out throughout the experimental period. The study received approval from the Animal Research Ethics Board of the University of Saskatchewan, and was carried out in accordance with the guidelines specified by the Canadian Council on Animal Care (Canadian Council on Animal Care 1993).

3.1.2.5 Infusates and infusion protocol

DL-LA, L-LA (Sigma-Aldrich, St. Louis, Missouri, USA), and HCl (BDH Inc. Toronto, Ontario, Canada) were formulated as 300 mmol/L solutions while saline (NaCl; EMD Chemicals Inc., Gibbstown, New Jersey, USA) was 150 mmol/L; pH values, mean ± se, for the four solutions were 2.5 ± 0.1, 2.6 ± 0.1, 1.2 ± 0.2 and 5.6 ± 0.1, respectively.

By analysis, DL-LA contained 53% D-lactate and 47% L-lactate. DL-LA was the choice of infusion instead of pure D-lactic acid due to financial circumstances. Since bulk quantities (8-10 L of 300 mmol/L) of infusions needed for a calf, purchase of D-lactic acid in such quantities was not affordable. All infusates were autoclaved prior to use. A separate experiment with three sets of solution documented a small loss in weight to 99.93 ± 0.02 % of the original weight and no loss of lactate or its isomers following autoclaving; for DL-lactate solutions, D-lactate concentrations, mmol/L, were 141.5 ± 1.4 and 144.7 ± 1.1, L-lactate 146.5 ± 2.0 and 150.6 ± 1.4 pre and post autoclaving respectively.

The eight calves randomly received intravenous infusions of DL-LA, L-LA, HCl or saline (Table 1) at an infusion rate of 20 mL/kg BW per hour using an automated infusion pump (Imed 980 volumetric infusion pump; Imed Corporation San Diego, California, USA) for 4 h. Infusion was continued for a further 2 h or until venous pH reached 7.05. If venous pH rose above 7.1 which was considered a typical lower point in acidosis, infusion was restarted. After 12 h from the beginning of the infusion, severely acidotic calves were treated with 155 mmol/L $NaHCO_3$ (Sigma-Aldrich, St. Louis, Missouri, USA) solution to correct blood pH and alleviate neurological disturbance. There was a minimum period of 2 days between each infusion.

3.1.2.7 Sampling and measurements

Samples of CSF fluid (~0.7 mL), venous (4 mL) and arterial (1 mL) blood were collected, and neurological assessment was performed at –1 (pre infusion), 0 (start of infusion), 1, 2, 3, 4, 5, 6 (completion of infusion), 7 (post infusion), 8, 10 and 24 h.

Neurological assessment was performed by a veterinarian who was blinded to the type of infusion being administered. Neurological function was scored based on the strength of the suck, menace (response of the integrity of the entire visual pathway), palpebral (sensory component-trigeminal nerve and motor component-facial nerve), tactile (local sensitivity along spine) reflexes, and ability to stand (Kasari and Naylor 1986; Lorenz 2004b; Naylor 1989). Each item was given a score of from 0 (normal) to 2 (severely abnormal) according to a previously published scale (Kasari and Naylor 1986). The values were summated to produce a total neurological score (TNS). Calves with normal CNS function had scores of 0 while severely obtunded calves could have a maximum score of 10.

3.1.2.8 Laboratory analyses

Heparinized arterial and venous blood (1 mL each) and 0.3 mL of CSF fluid were placed on ice and analyzed within 40 minutes of collection for pH, bicarbonate (HCO_3^-), base excess, partial pressure of carbon dioxide and oxygen (P_{CO_2} and P_{O_2}) using a blood gas analyzer (ABL^{TM} 5; Radiometer Medical A/S, Copenhagen, Denmark) (Severinghaus 2002). Hemoglobin and its oxygenation status were measured in a hemoximeter (OSM3; Radiometer Medical A/S, Copenhagen, Denmark) (Zwart et al. 1987).

The remaining venous blood (3 ml) was allowed to coagulate for 20 min, and serum was separated in a refrigerated centrifuge (IEC Centra-7R Refrigerated Centrifuge; International Equipment, Needham Heights, Massachusetts, USA) at 1,000 RCF for 30 min. The serum and remaining CSF fluid were stored at –20°C. D- and L-lactate concentrations were measured from 0.1 ml of serum or CSF sample using an HPLC system (Waters 600 HPLC pump, 486 UV detector, 786 Ultra WISP autoinjector, and Millenium v. 4 software; Waters, Mississauga, Ontario, Canada) as we previously described (Omole et al. 1999). The sample mixed with an internal standard (2 mmol/L malonic acid) in 1:1 vol/vol ratio was centrifuged and filtered to deproteinize via Ultrafree®-MC 5000 NMWL filter units (Millipore Corporation, Bedford, Massachusetts, USA) under 3000 RCF (AccuSpin Micro 17; Fishers Scientific, Schwerte, Germany) for 30 min. For the separation of D- or L-lactate isomer a stereoselective 3 µm ODS-packed (50 mm × 4.6 mm) analytical ligand exchange column coated with *N*,*N*-dioctyl-alanine (ChiralPak MA+; Chiral Technologies, Exton, Pennsylvania, USA) was employed. A Guard-Pak Precolumn Module containing an ODS (octadecylsilyl) cartridge (Waters Guard-Pak™ precolumn; Waters) insert was placed serially ahead the analytical column to reduce the risk of obstruction and to enhance elution capability. The mobile phase was a 2 mmol/L copper sulfate in 1% acetonitrile. The flow rate for the mobile phase was 0.4 mL/min and the column worked at room temperature (25 ºC). UV detection occurred at 236 nm wavelength

(Omole et al. 1999); 20 µl aliquots of the final filtrate were injected into the HPLC system and a sample was run for 30 min (Ewaschuk et al. 2002).

Infusate pH was measured using a pH meter (Beckman Ø 32 pH meter; Beckman Instruments Inc., Fullerton, California, USA).

3.1.2.9 Statistical analysis

Graphs and Figures were created using a spreadsheet (Microsoft Excel; Microsoft Corporation, Redmond, Washington, USA). Descriptive statistics and Pearson Correlation Coefficients were calculated using a statistical analytical software package (SPSS statistical software v.13, 2005; SPSS Inc., Chicago, Illinois, USA). Analysis of Variance (ANOVA) and Multivariate Analysis of Variance (MANOVA) with Repeated Measures was carried out using SAS statistical software (SAS/Stat® statistical software package for Windows, v. 8.02; SAS Institute Inc., Cary, North Carolina, USA). The significance of treatment effects on neurological scores, blood gas values, and serum and CSF lactates was determined by repeated measures analysis of variance using treatment (infusion) as the independent variable (Steel and Torrie 1980). Mean values at individual time points were compared using the Student-Newman-Keul's (SNK) test. Treatment differences were also tested using orthogonal contrasts to compare DL-LA versus HCl, L-LA and saline. Polynomial contrast was also used to deferenciate CSF and serum D-lactate concentrations. A P value of ≤ 0.05 was considered to be statistically significant. Values are reported as mean and standard error (mean ± se).

3.1.3 Results

All infusions were continued for 6 h except for HCl which was given intermittently between hours 4 and 6 to maintain venous pH above 7.0 and below 7.10.

3.1.3.1 Neurological assessment

Saline had no effect on neurological score (Figure 6). L-LA and HCl infusions produced mild impairments in neurological function and had no significant effect on the menace, tactile and palpebral reflexes at any time period (Video clip 1.). DL-LA infused calves had a significantly reduced menace reflex between 1 and 10 h; the menace response was completely absent at 6 h, 7 h and 8 h of infusion. Ability to stand was significantly inhibited by DL-LA infusion between 2 and 10 h; weakness was detected at 2 h, developed into ataxia at 4 h, and progressed to involuntary recumbency at 8h. During infusions other than DL-LA, involuntary recumbency was only seen on one occasion, in a calf receiving L-LA. In DL-LA infused calves significant reductions in the palpebral and tactile reflexes were first detected at 3 h, increased to maximum at 7 or 8 h (Video clip 2) and slightly recovered by 10 h. The suck reflex was unusual in that HCl and DL-LA similarly depressed this reflex; L-LA had a smaller effect (Figure 6).

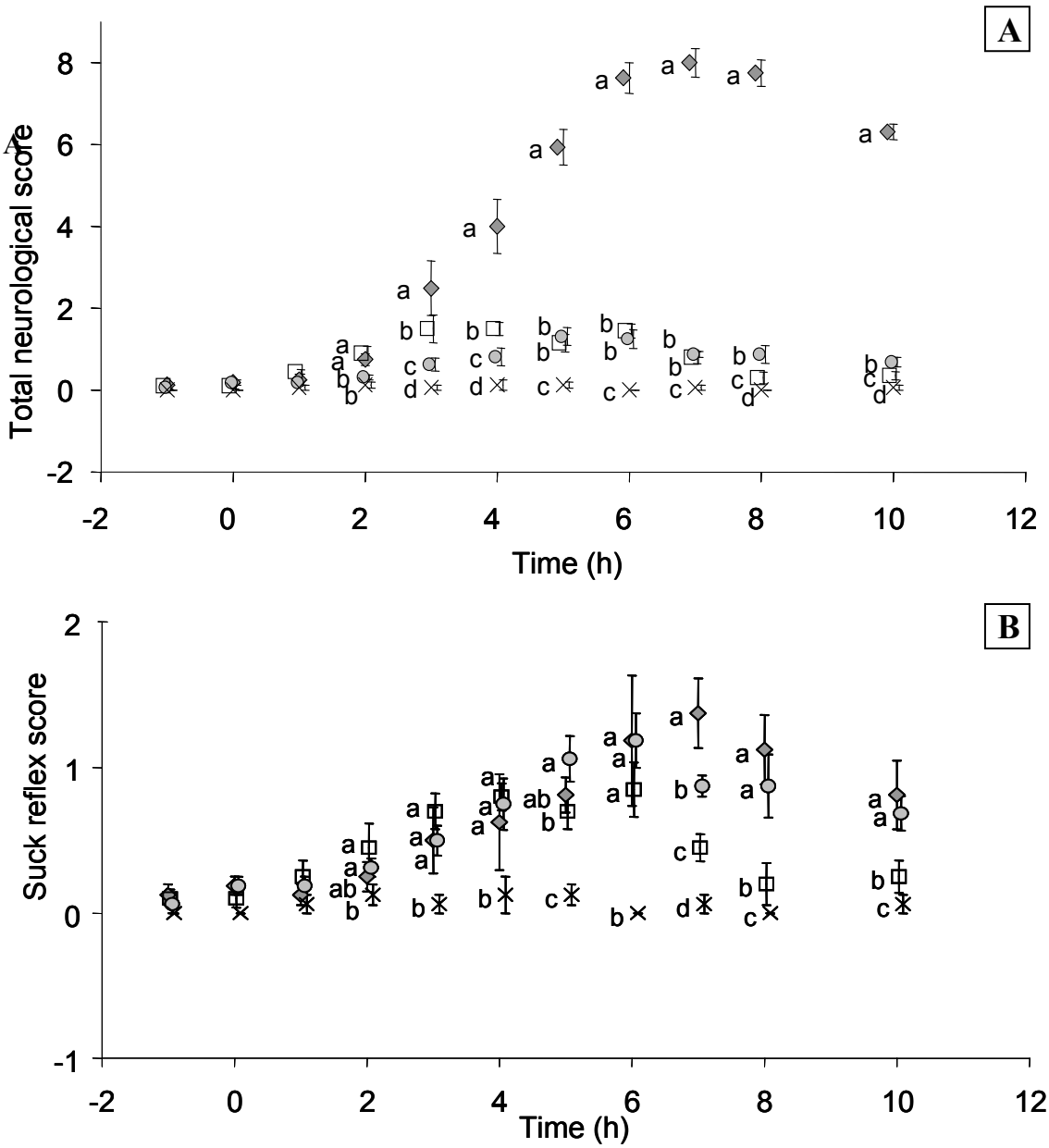
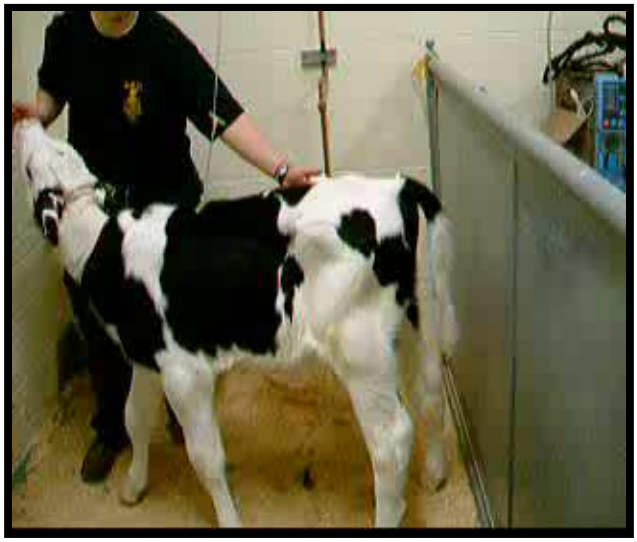


Figure 6. Change in total neurological score [graph **A**] and suck reflex [graph **B**] during (0 to 6 h) and after infusion of DL-lactic acid (DL-LA ◆), L-lactic acid (L-LA □), hydrochloric (HCl ▫) or saline (×). Values at 24 h returned to baseline. Repeated measures analysis showed significant effects of treatment ($P < 0.01$), time ($P < 0.01$), and treatment versus time interaction ($P < 0.01$). Values at each time point that do not share a common letter (a, b, c or d) are statistically significant at $P < 0.05$ by Student-Newman-Keul's test.



Video clip 1. Minimal or no neurological signs at 4 h after infusion of hydrochloric acid (300 mmol/L) in calves. Mean serum D-lactate concentrations were > 0.5 mmol/L at 6 h and the infusion was discontinued. However, mean serum pH was > 7.1. (Please double click on the middle of the media player to see the video).

Video clip 2. Severe neurological disturbances at 6 h after infusion of DL-lactic acid (DL-LA, 300 mmol/L) caused paralysis to calves. Mean serum D-lactate concentrations were > 10 mmol/L at 6 h and the infusion was discontinued. However, mean serum pH was > 7.2. (Please double click on the middle of the media player to see the video).

3.1.3.2 Acidosis

HCl infusion produced the most severe metabolic acidosis which reached a nadir at 4 h of infusion: venous blood pH 6.9 ± 0.1, bicarbonate 7.00 ± 1.45 mmol/L and BE -23.3 ± 1.7 mmol/L. DL-LA produced a moderate metabolic acidemia. In contrast, L-LA infusion resulted in little change in acidemia with blood gas values similar to those during saline infusion (Table 2).

Both HCl and DL-LA infusions produced similar decreases in CSF pH, bicarbonate (Figure 7) and P_{CO_2} (Table 2). Arterial blood bicarbonate (r = 0.87), BE (r = 0.84), and P_{CO_2} (r = 0.78) were highly correlated ($P < 0.01$) with the corresponding CSF measurements. However, arterial and CSF pH were only moderately correlated (r = 0.57, $P < 0.05$). The correlations between CSF and venous blood gas measurements were not as strong; correlations for CSF and venous measurements of bicarbonate, P_{CO_2} and pH were 0.85, 0.56 and 0.46 respectively.

3.1.3.3 D- and L-lactate concentrations

Serum and CSF D-lactate were only increased by DL-LA infusion. Venous serum and CSF D-lactate concentrations were highly correlated (r = 0.91, $P < 0.01$). However, changes in CSF D-lactate lagged those in serum (Table 3).

Serum L-lactate concentrations were unaffected by HCl infusion, increased to a moderate extent during DL-LA infusion and were highest during L-LA infusion. CSF L-lactates were also highest with L-LA infusion and moderately increased with DL-LA infusion (Table 2). During L-LA infusion, peak CSF L-lactate concentrations were attained at 6 h (11 ± 1 mmol/L) and were similar to peak serum L-lactate concentration (10 ± 1 mmol/L). During DL-LA infusion, CSF L-lactate concentration (8 ± 1 mmol/L) peaked at 6 hours, and was 60% higher than serum concentration (5 ± 2 mmol/L). During DL-LA infusion peak serum and CSF L-lactate concentrations were considerably lower than those of peak D-lactate concentrations (Table 2).

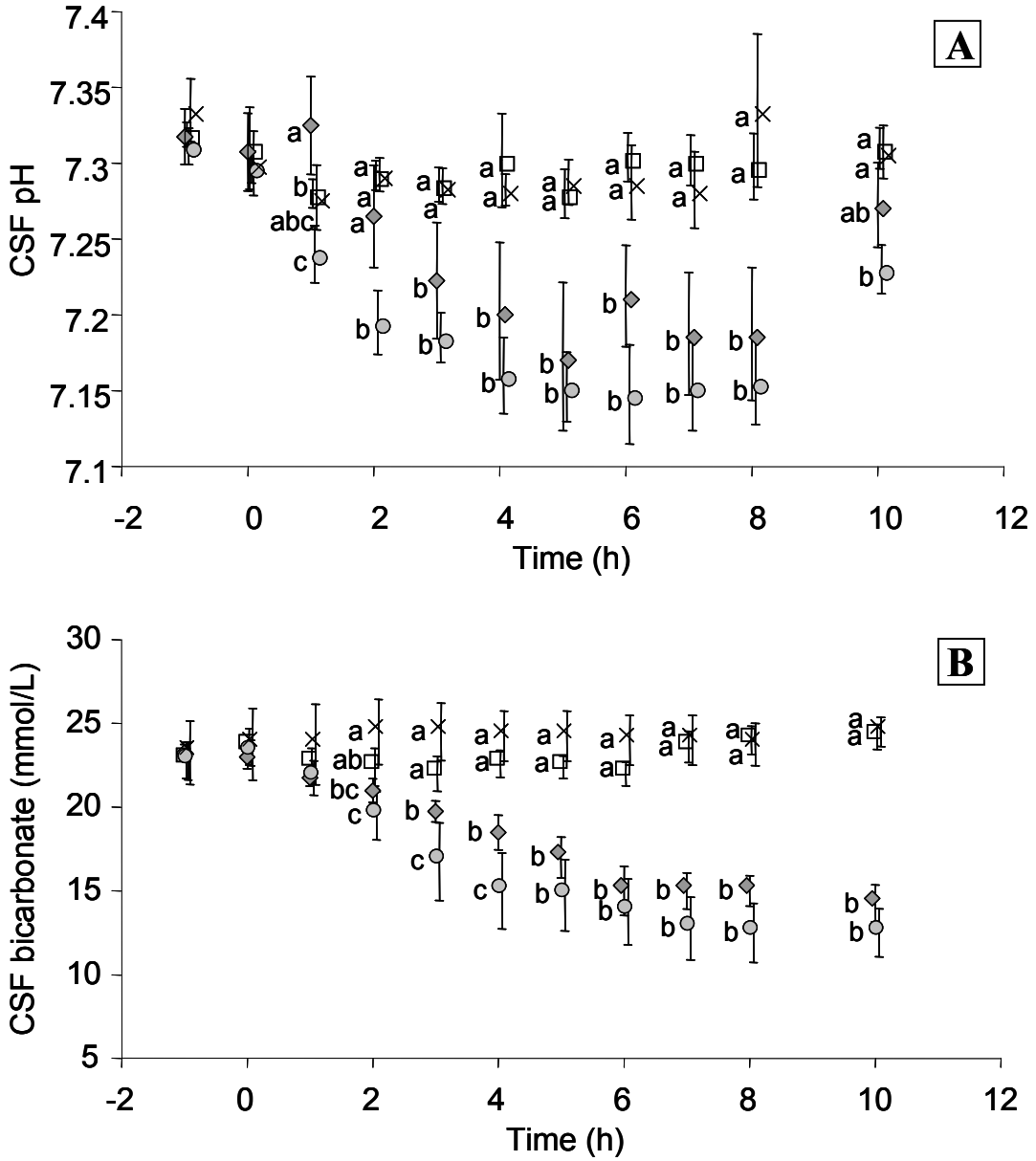


Figure 7. Change in cerebrospinal pH [graph **A**] and bicarbonate concentration [graph **B**] during (0 to 6 h) and after infusion of DL-lactic acid (DL-LA ◆), L-lactic acid (L-LA □), hydrochloric (HCl ▫) or saline (×). Values at 24 h returned to baseline. Repeated measures analysis showed significant effects of treatment ($P < 0.01$), time ($P < 0.01$), and treatment versus time interaction ($P < 0.01$).

Table 2. Effects of DL-LA, L-LA, HCl, or saline infusions on arterial, venous and cerebrospinal fluid blood gas values, and cerebrospinal fluid and venous-serum lactate concentrations

	Infusions				*P*- value
	DL-LA*	L-LA*	HCl	Saline	
Serum					
D-lactate, mmol/L	23.5±0.7	1.1±0.2 (0.01)†	0.9±0.1 (0.01)†	0.6±0.1 (0.01)†	<0.01
L-lactate, mmol/L	4.5±0.4	8.5±0.5 (0.02)	1.7±0.2 (0.12)	1.8±0.1 (0.01)	<0.01
Venous blood					
pH	7.21±0.02	7.33±0.02 (0.01)	7.05±0.02 (0.01)	7.35±0.01 (0.01)	<0.01
BE, mmol/L	-11.1±1.0	0.4±0.9 (0.01)	-18.6±1.0 (0.01)	1.7±1.0 (0.01)	<0.01
HCO_3^-, mmol/L	15.6±0.6	25.3±0.9 (0.01)	10.1±0.5 (0.04)	26.5±0.9 (0.01)	<0.01
P_{CO_2}, mm Hg	40.3±1.4	48.0±1.9 (0.01)	34.2±1.7 (0.75)	49.7±1.5 (0.01)	0.03
P_{O_2}, mm Hg	44.3±1.2	39.5±1.8 (0.69)	41.4±2.3 (0.35)	36.4±1.3 (0.02)	0.32
Hemoglobin, g/L	82.9±9.2	74.1±6.5 (0.54)	85. 7±8.9 (0.80)c	75.6±7.8 (0.39)	0.82
Arterial blood					
pH	7.26±0.02	7.38±0.02 (0.01)	7.08±0.02 (0.01)	7.41±0.01 (0.01)	<0.01
BE, mmol/L	-11.1±1.1	-3.0±0.9 (0.01)	-20.2±0.9 (0.01)	2.8±1.6 (0.01)	<0.01
HCO_3^-, mmol/L	14.3±0.1	20.9±1.1 (0.01)	7.4±0.7 (0.01)	26. 6±1.9 (0.01)	<0.01
P_{CO_2}, mm Hg	32.8±1.7	36.8±1.7 (0.07)	25.2±1.9 (0.01)	44.1±2.9 (0.01)	<0.01
P_{O_2}, mm Hg	107.1±5.3	105.0±7.2 (0.25)	120.2±5.5 (0.68)	95.3±6.9 (0.25)	0.04
Hemoglobin, g/L	71.8±10.0	62.3±6.7 (0.41)	74.5±9.1 (0.69)	61.8±13.3 (0.33)	0.71
CSF					
D-lactate , mmol/L	14.8±3.8	0.2±0.1 (0.01)	0.2±0.1 (0.01)	0.04±0.01 (0.01)	<0.01
L-lactate, mmol/L	6.9±1.2	10.7±0.8 (0.04)	3.8±1.0 (0.08)	3.4±0.5 (0.01)	<0.01
pH	7.24±0.04	7.29±0.02 (0.01)	7.20±0.06 (0.33)	7.29±0.02 (0.01)	0.170
HCO_3^-, mmol/L	18.3±0.9	22.5±0.9 (0.01)	16.3±1.8 (0.55)	24.2±1.7 (0.01)	<0.01
P_{CO_2}, mm Hg	44.9±2.5	49.5±2.5 (0.02)	43.4±3.9 (0.75)	53.3±1.9 (0.05)	0.07
P_{O_2}, mm Hg	80.9±1.6	73.2±3.8 (0.67)	81.7±3.5 (0.51)	83.1±2.9 (0.35)	0.82

* Values are mean ± se for data collected between 1 and 7 h of infusion.
† *P*-values shown in parenthesis were determined for values between 1 and 7 h of infusion inclusive by Repeated Measures MANOVA (over-all *P*-value) and orthogonal contrast comparison for DL-LA versus L-LA, HCl or saline.

3.1.3.4 Removal of D-lactate from the body compartments

Following cessation of infusion, serum D-lactate concentrations declined in an exponential fashion with time ($r = 0.98$, $P < 0.01$). Removal of D-lactate from CSF was slower, and more gradual than from serum (Figure 8). CSF removal was constant over the 12 h post-infusion period while removal from serum declined with time and correlated ($r = 0.73$, $P < 0.01$) with serum D-lactate concentration.

3.1.3.5 Correlation of neurological disturbances with changes in blood and CSF chemistry

CSF and serum D-lactate concentrations strongly correlated ($P < 0.01$) with all neurological scores except for the strength of the suck reflex (Table 3). Although DL-lactate concentrations in the CSF and serum also strongly correlated with neurological signs, L-lactate concentrations correlated weak (Table 3).

The suck reflex was affected similarly by both DL-LA and HCl infusions (Figure 6) and was better correlated with CSF bicarbonate, BE or pH than with CSF D-lactate concentration (Table 3). Arterial or CSF pH or bicarbonate correlated weakly with other reflexes and with total neurological score (Table 3).

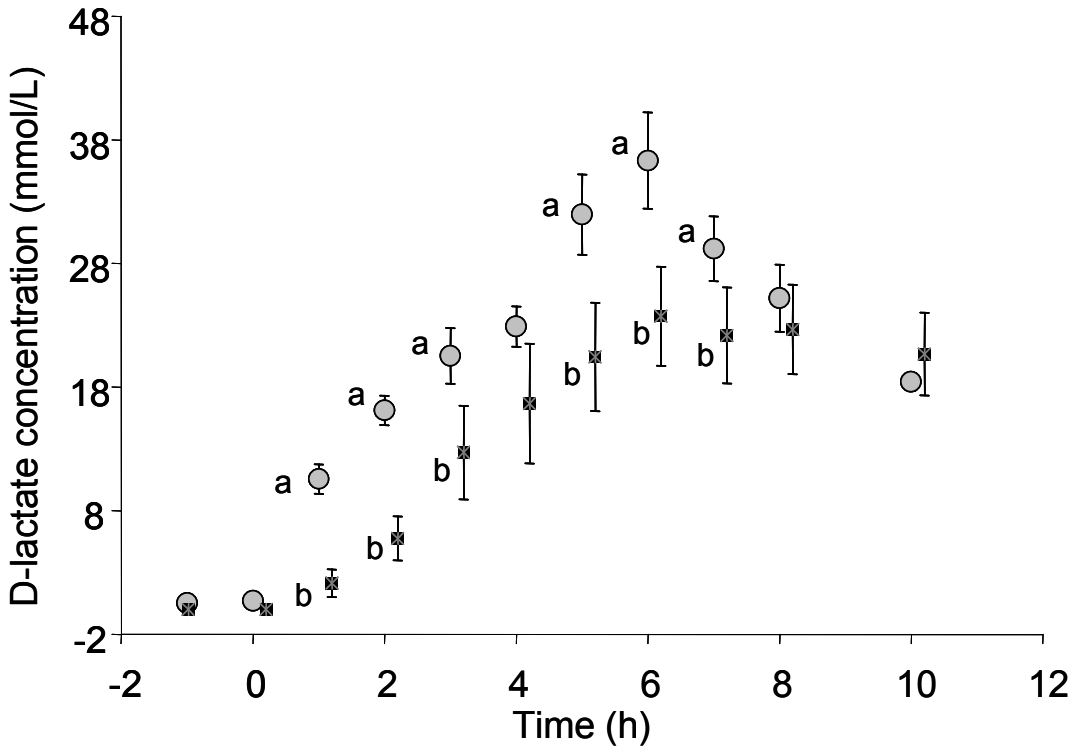


Figure 8. Change in serum (□) and cerebrospinal fluid (■) D-lactate concentration during (0 to 6 h) and after infusion of DL-LA. Values at 24 h returned to baseline. There was no change in serum or CSF D-lactate concentrations from baseline during infusion of saline, HCl or L-LA (data not shown). At all time points from 1 to 10 h inclusive D-lactate concentrations in serum and CSF were significantly higher with DL-LA than saline, HCl or L-LA infusion. Repeated Measures (polynomial contrast) and SNK showed significant statistical differences ($P < 0.05$). Values at each time point that do not share a common letter (a or b) are statistically significant at $P < 0.05$.

Table 3. Correlation of total neurological score (TNS), menace, palpebral, tactile, stand and suck with individual CSF and blood parameters

Parameter	TNS	Menace	Palpebral	Tactile	Stand	Suck
CSF D-lactate	0.86*	0.85*	0.88*	0.81*	0.88*	0.34*
CSF L-lactate	0.22*	0.21*	0.11NS	0.07NS	0.17†	0.38*
CSF D+L-lactate‡	0.89*	0.87*	0.87*	0.79*	0.85*	0.53*
CSF HCO_3^-	-0.48*	-0.35*	-0.41*	-0.39*	-0.43*	-0.57*
CSF pH	-0.37*	-0.30*	-0.33*	-0.28*	-0.30*	-0.44*
CSF P_{CO_2}	-0.25*	-0.13NS	-0.20*	-0.22*	-0.26*	-0.32*
Serum D-lactate	0.83*	0.63*	0.56*	0.66*	0.65*	0.29*
Serum L-lactate	0.13†	-0.02NS	-0.09NS	-0.06NS	-0.03NS	0.12NS
Serum D+L-lactate‡	0.85*	0.83*	0.74*	0.78*	0.81*	0.53*
Venous HCO_3^-	-0.43*	-0.33*	-0.34*	-0.36*	-0.37*	-0.49*
Venous BE	-0.42*	-0.31*	-0.31*	-0.34*	-0.34*	-0.50*
Venous pH	-0.35*	-0.23*	-0.21*	-0.25*	-0.26*	-0.44*
Venous P_{CO_2}	-0.26*	-0.23*	-0.30*	-0.27*	-0.26*	-0.39*
Arterial HCO_3^-	-0.42*	-0.34*	-0.29*	-0.30*	-0.36*	-0.57*
Arterial BE	-0.40*	-0.32*	-0.27*	-0.29*	-0.34*	-0.56*
Arterial pH	-0.33*	-0.26*	-0.19†	-0.24*	-0.29*	-0.49*
Arterial P_{CO_2}	-0.28*	-0.19†	-0.23*	-0.15 NS	-0.21†	-0.48*

Correlations (r) are calculated using all values obtained between from -1 to 24 h inclusive for all four infusions, number of calves = 8. ‡ indicates total of D and L isomer concentration (mmol/L).

* and † indicate statistically significant correlation at $P < 0.01$ and < 0.05 respectively.

NS indicates not significant at $P = 0.05$.

3.1.4 Discussion

D-lactic acidosis has been associated with neurological dysfunction in a variety of species including humans, cattle, cats and dogs (Jorens et al. 2004; Packer et al. 2005; Petersen 2005; Uribarri et al. 1998). The reported signs in humans include slurred speech, loss of reflexes, abnormal gate, ataxia, paresis, and sometimes coma. In calves (Lorenz et al. 2005) and cats (Packer et al. 2005) impairment of palpebral and menace reflexes have been reported in association with other neurological signs. While clinical studies have consistently shown an association between D-lactate concentration and neurological disturbances a causal link has not been established (Hudson et al. 1990; Jorens et al. 2004; Packer et al. 2005; Petersen 2005; Uribarri et al. 1998; Zhang et al. 2003). In many of these studies, other parameters, such as acidemia were also associated with CNS impairment. In this study, severe acidemia and mild impairments of neurological function were induced by HCl infusion alone whereas DL-LA infusion produced severe neurological derangements (Figure 6). These derangements are similar to those reported clinically and include ataxia, decreased palpebral and menace reflexes. These severely affected calves became ataxic, developed involuntary recumbency (Video clip 2) and appeared comatose. This toxicity is independent of changes in blood or CSF pH because HCl infusion produced a more severe metabolic acidosis and a similar CSF acidosis but only small changes in neurological function (Video clip 1). Toxicity is also unrelated to the L-LA component of the DL-LA infusion as infusion of pure L-LA produced a more marked L-lactatemia but had only small effects on neurological function. Thus, D-lactate was the neurotoxic agent in this study. The majority of the infused D-lactic acid would dissociate into D-lactate and hydrogen ions in the blood stream. The conclusion that D-lactate is neurotoxic is supported by the high correlation between CSF D-lactate concentrations and neurological disturbances (Table 3). A secondary role for L-LA cannot be completely excluded, as this was also infused and DL-lactate concentrations also had strong correlations with neurological scores (Table 3). However, this inference that D-lactate is neurotoxic is consistent with earlier work in which signs of ataxia, somnolence and impaired palpebral reflexes developed following hypertonic sodium D-lactate injection in calves (Lorenz et al. 2005). This study also indicates that D-lactate, not hypertonicity as some have proposed (Stampfli 2005), is the toxic agent. D-lactate may be transported into neurons with either sodium or hydrogen ions via Na^+- or H^+-monocarboxylate transporters (Martin et al. 2006; Shimozono et al. 1998). Hence, either D-lactate or D-lactic acid may be the toxic agent intracellularly.

In previous experiments, oral administration of DL-LA did not significantly influence humans physiologically or pathologically (De Vrese et al. 1990). This lack of effect can be explained by the much smaller amounts of DL-LA administered, a maximum of 12.8 mmol/kg$^{0.75}$ of racemic DL-LA per day. The total dosage administered in the present experiment was 8 times higher. Consequently, plasma D-lactate did not exceed 1 mmol/L in the previous experiment whereas we obtained peak serum concentrations of around 36 mmol/L. Clinical reports of neurological dysfunction suggest that D-lactate concentrations have to be at least 3 mmol/L before clinical signs are seen (Uribarri et al. 1998).

In the present study the suck reflex (Figure 6) was correlated more strongly with CSF bicarbonate, base excess and pH than with CSF D-lactate concentration (Table 3). Similarly, clinical studies in calves have noted that ataxia and loss of the palpebral reflex are correlated with D-lactate concentrations whereas the strength of the suck reflex is correlated with acidemia (Lorenz 2004b). Taken together, this work suggests that acidemia depresses the suck reflex but is not responsible for ataxia or changes in the palpebral reflex.

In the present study, neurological depression was first observed at 2 h of DL-LA infusion when mean serum D-lactate was 16 ± 1 mmol/L which is higher than the serum D-lactate concentrations of about 3 mmol/L reported in some clinical cases (Uribarri et al. 1998). Previous studies in this laboratory established that diarrheic calves with neurological depression have serum D-lactate concentrations as high as 26 mmol/L (Ewaschuk et al. 2004; Omole et al. 2001). The serum D-lactate concentrations in this infusion study are well below the serum value (110 mmol/L) reported in a patient intoxicated after over-ingestion of propylene glycol (Jorens et al. 2004). Furthermore, these data show that it takes time for D-lactate to diffuse into the CSF. At 1 h of infusion, mean serum D-lactate was 11 ± 1 mmol/L but CSF D- lactate was 2 ± 1 mmol/L. It is possible that a more gradual infusion would result in greater equilibration between CSF and blood D-lactate concentrations with clinical signs at lower venous serum D-lactate concentrations.

In the brain, astrocytes are net producers of L-lactate, because their thin processes are too small to accommodate mitochondria (Hertz et al. 2007; Hyder et al. 2006). The L-lactate is metabolized by neurons (Aubert et al. 2005; Bouzier-Sore et al. 2006; Hyder et al. 2006; Medina and Tabernero 2005; Schurr 2006). It has been estimated that 75% of neuronal oxygen consumption is accounted for by L-lactate metabolism and 25% by glucose metabolism (Bouzier-Sore et al. 2006). Lactate utilization may be particularly important during acidemia, since low pH can inhibit phospofructokinase activity, a key glycolytic regulatory enzyme (Kreisberg 1980). In neurons, uptake of L-lactate is close to saturation at physiologic L-lactate concentrations (Hertz and Dienel 2005). D-lactate blocks the ability of isolated optic nerves to generate an action potential, probably by competitively blocking L-lactate entry into neurons and limiting neuronal metabolism (Tekkök et al. 2003; Tekkök et al. 2005). L-lactate can act as an energy source during ischemia / reperfusion injury and reduces cerebral injury (Cassady et al. 2001; Ros et al. 2001). In contrast, D-lactate enhances neuronal injury in ischemia-reperfusion models (Cassady et al. 2001). Although, D-lactate dehydrogenase is found in mammalian mitochondria, particularly in liver and kidney, it is poorly expressed in human brain tissue (Flick and Konieczny 2002). It is therefore possible that the neurotoxic effects of D-lactic acid infusion observed in the present study are the result of reduced L-lactate availability within neurons and energy deficit. In support of this, the serum-CSF L-lactate difference was slightly greater during DL-lactate infusion; this may reflect reduced neuronal removal of astrocyte produced L-lactate, Table 2. Similarly, others have shown that blockade of neuronal monocarboxylate transporters responsible for L-lactate entry results in increased neuronal cell death in the developing brain (Adle-Biassette et al. 2007).

Rapid ethanol consumption is another situation where acute neurological signs are observed in humans (Handley and Ward-Smith 2005). We were impressed with the ‘drunken’ appearance of these calves during DL-LA infusion. In acute ethanol

consumption, neurological depression may be caused by low concentrations of circulating tryptophan (Badawy et al. 1995) or interactions with the $GABA_A$ receptor complex and facilitation of GABA action (Gonzales and Hoffman 1991; Starke 1991). However, ethanol also has early effects on energy metabolism (Adle-Biassette et al. 2007; Juhlin-Dannfelt 1977; Volkow et al. 2006) which may be similar to some of the actions of D-lactate.

In addition to lipid mediated penetration (Oldendorf et al. 1979), L-lactate may be transported across the blood brain barrier by mono-carboxylic acid transporters (Gladden 2004; Hertz and Dienel 2005; Schurr 2006) which are stereo specific (Tekkök et al. 2003; Tekkök et al. 2005). During DL-LA infusion, CSF L-lactate concentrations increased steadily and were higher than serum concentrations by a similar amount in all infusions (Table 2). L-lactate was probably being formed within nervous tissue by metabolism of glucose and this likely contributes to the higher concentrations in CSF (Aubert et al. 2005; Gladden 2004). In this study D-lactate rapidly crossed the blood brain barrier, although CSF concentrations were about half those of serum (Table 2). This could be explained by serum L-lactate competing with serum D-lactate for uptake from blood by CSF tissue (Tekkök et al. 2003; Tekkök et al. 2005).

In the present study, and in previous work (Lorenz et al. 2005), serum D-lactate exhibited an exponential decay. However, CSF D-lactate removal was constant over the period studied suggesting that the metabolic process involved in removal was saturated. Mono-carboxylic transporters may remove D-lactate from CSF (Tekkök et al. 2003; Tekkök et al. 2005). In contrast, D-lactate removal from blood occurs at least in part through renal clearance (Connor et al. 1983; Ewaschuk et al. 2004; Ewaschuk et al. 2005; Jørgensen and Sheikh 1984; Oh et al. 1985; Ullrich et al. 1982b). This slow elimination of CSF D-lactate would explain the prolonged neurological disturbances and encephalopathy observed in many clinical DLA cases even after treatment has been initiated (Petersen 2005; Puwanant et al. 2005; Uribarri et al. 1998). Serum L-lactate elimination was not exponential. Previous studies show that serum L-lactate is cleared through two independent processes: Hepatic removal follows second order kinetics while extrasplanchnic removal is linearly related to serum concentration (Naylor et al. 1984). D-lactate may also be taken up by hepatic and extrahepatic tissue (De Bari et al. 2002; Naylor et al. 1984), although some is excreted in urine (Connor et al. 1983; Ewaschuk et al. 2004; Ewaschuk et al. 2005; Jørgensen and Sheikh 1984; Oh et al. 1985; Ullrich et al. 1982b). The current understanding of the distribution of lactate enantiomers supported by this study is summarized in Figure 9.

D-lactate is a potent neurotoxic agent. In acidemic calves, the majority of neurological signs (i.e. ataxia, and depressed menace, palpebral and tactile reflexes) are related to D-lactate accumulation in CSF rather than in blood or to acidosis in CSF. According to our observations and evaluations, this total neurological score (TNS) which was characterized by the above depressive signs was a state of neurological disturbance. Therefore we refer to TNS as neurological disturbance in the future sections of our studies.

HCl infusion has mild depressive effects on neurological function and the suck reflex was similarly depressed by both DL-LA acid and HCl infusions. D-lactate accumulation may be responsible for the majority of clinical signs of depression and loss of

function in a variety of diseases including neonatal calf diarrhea and other animal and human syndromes characterized by DLA. Research needs to be undertaken to determine the precise mechanism of neurological depression caused by D-lactate.

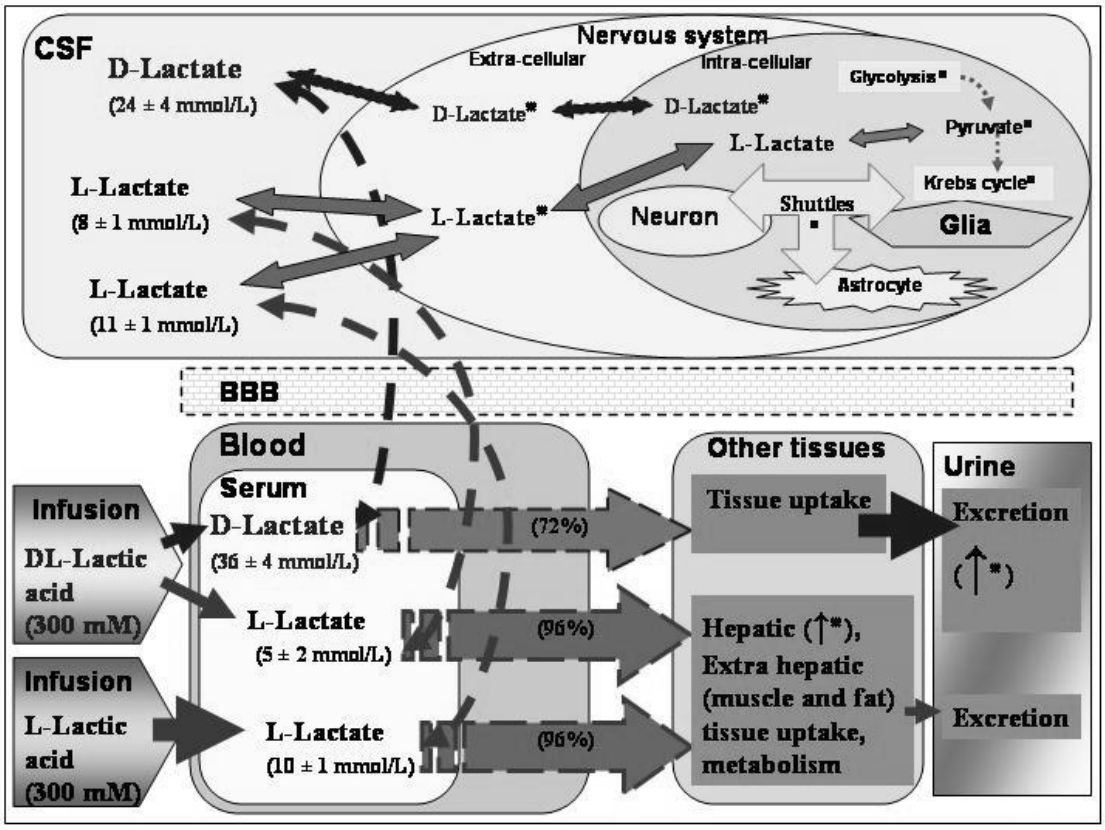


Figure 9. Serum and CSF peak concentrations of lactate at 6 hours of either DL-lactic acid (DL-LA) or L-lactic acid (L-LA) infusions, and possible fate of D and L-lactate in serum and CSF. Peak mean ± se values are reported.

*Adopted from previous workers (Connor et al. 1983; De Bari et al. 2002; Ewaschuk et al. 2004; Ewaschuk et al. 2005; Gladden 2004; Hertz and Dienel 2005; Jørgensen and Sheikh 1984; Magistretti and Pellerin 1999; Medina and Tabernero 2005; Naylor et al. 1984; Oh et al. 1985; Oldendorf et al. 1979; Schurr 2006; Ullrich et al. 1982b).
% removals of L- or D-lactates from serum were calculated using a formula.
% removal = (amount infused – amount remained in serum) / amount infused × 100.
Amount infused = infused volume of infusate × concentration in infusate.
Amount remained in serum = concentration in serum × plasma volume.
Plasma volume = 10% of calf body weight (Quigley et al. 1998)
Infused volume = pump flow rate × time.
(CSF – cerebrospinal fluid, BBB – blood brain barrier, ↑ - higher route).

3.2 Effects of sodium bicarbonate therapy on blood, CSF, and neurological status in experimentally induced metabolic and D-lactic acidosis

3.2.1 Introduction

Intravenous (IV) infusion of sodium bicarbonate ($NaHCO_3$) is the mainstay of therapy for severe acidemia (Charles and Heilman 2005; Narins and Cohen 1988). Treatment with IV $NaHCO_3$ solutions can alleviate potentially dangerous effects of the acidotic state (Narins and Cohen 1987; Naylor et al. 2006; Suzuki et al. 2002a). However, there is concern that rapid $NaHCO_3$ therapy can lead to paradoxic CSF acidosis and other problems (Arieff et al. 1982; Feeney-Stewart 1990; Forsythe and Schmidt 2000; Forsythe and Schmidt 2000; Graf and Arieff 1986). One concern is that increased blood P_{CO_2} can diffuse into the CSF, increasing CSF P_{CO_2}; which can be followed by dissociation into protons and bicarbonate acidifying the CSF (Figure 10) (Astrup et al. 1966; Herrera and Kazemi 1980; Javaheri et al. 1979). The condition is referred to as paradoxic CSF acidosis because CSF pH falls as blood pH rises (Bureau et al. 1980; Javaheri et al. 1983). The extent of this problem is poorly documented (Berchtold et al. 2005; Holander 1987). Changes in CSF pH need to be investigated during $NaHCO_3$ therapy of metabolic acidosis (Berchtold et al. 2005; Herrera and Kazemi 1980; Mathieu et al. 1991) to define the nature and extent of this problem. Delayed recovery of CSF pH in HCl induced acute acidosis regardless of $NaHCO_3$ infusion in our previous work (see the section 3.1, Abeysekara et al. 2007). The clinical observation that calves improve their neurological status at a slower rate than improvements in blood pH during therapy, and a desire to determine if $NaHCO_3$ could be given rapidly, making treatment more convenient were concerns which instigated our investigation of possible paradoxic CSF acidosis during rapid IV $NaHCO_3$ therapy.

The primary objective of this second study was to determine the effect of rapid versus slow $NaHCO_3$ therapy on acidemia and CSF acidosis in experimentally induced DL-LA or hydrochloric acid (HCl) acidosis. A secondary objective was to investigate the effects of chronic exposure to concentrations of DL-LA that are commonly encountered in disease states on neurological function.

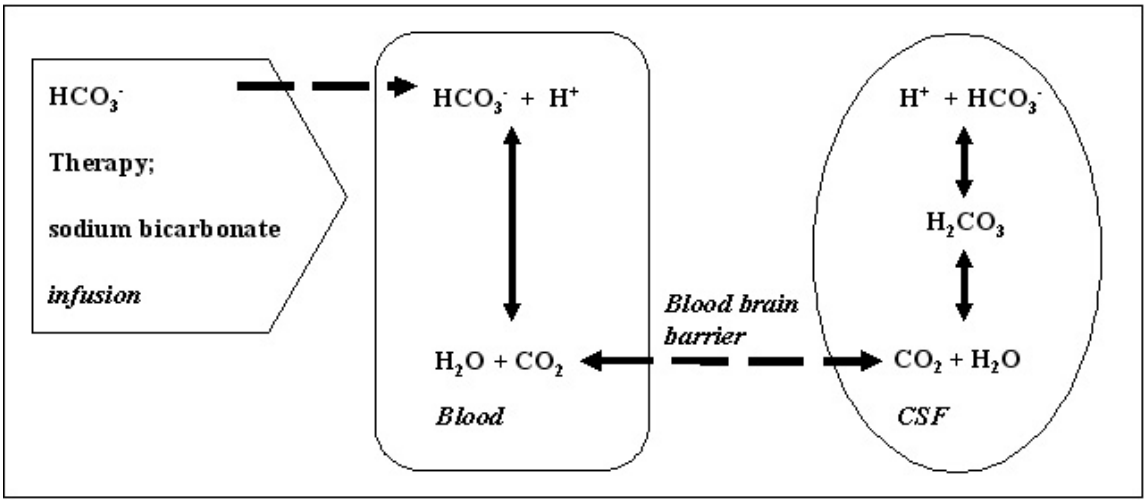


Figure 10. Postulated mechanism of how sodium bicarbonate infusion increases blood P_{CO_2} and then CSF P_{CO_2} to reduce CSF pH and cause paradoxic CSF acidosis (Astrup et al. 1966; Herrera and Kazemi 1980; Javaheri et al. 1979).

3.2.2 Materials and methods

This study with calves was approved by the Animal Research Ethics Board of the University of Saskatchewan, and was carried out in accordance with the guidelines specified by the Canadian Council on Animal Care.

3.2.2.1 Experimental animal model

Calves (n = 8) with indwelling venous and arterial blood and CSF catheters were experimentally infused with solutions of 150 mM DL-LA, or HCl. Then the acidotic calves were treated with equimolar iso-osmotic $NaHCO_3$ infusions for 4 h (rapid) or 24 h (slow). The order of treatments was randomly assigned.

3.2.2.2 Experimental animals and care

Calves for this study were obtained from the Dairy Barn of the Department of Animal and Poultry Sciences, University of Saskatchewan, Canada. Animal care, feeding and management were performed according to our previous work (chapter 3 section 3.1.2.1, page 34) (Abeysekara et al. 2007).

Pages 35, 36 and 36 (section 3.1.2) contain details on atlanto-occipital CSF, jugular (venous), and ear (arterial) catheterization, and on postsurgical care.

3.2.2.5 Experimental design

The calves (n = 8) with indwelling CSF, venous, and arterial blood catheters randomly received 150 mM solutions of DL-LA or HCl intravenously a rate of 20 mL/kg. h^{-1} for a period of 24 h (intermittent infusion > 12 h) or until they became severely acidotic. If hourly venous pH was below 7.05 the infusion was ceased until pH recovered to 7.1. After 25 h of acidosis the calves were intravenously treated with equimolar amounts of iso-osmotic $NaHCO_3$ infused over either 4 h (rapid) or 24 h (slow). The dosage of $NaHCO_3$ was calculated using the venous base excess (BE) value at the end of the acidotic stage (Naylor and Forsyth 1986; Naylor 1987).

Equation 1. Bicarbonate required, mmol = BE × body weight × 0.5 (factor)

CSF, and venous and arterial blood were sampled over a 48 h period and concentrations for D- and L-lactic acid and blood gas values were determined.

Neurological assessments of the calves were also carried out throughout the experimental period.

We ended up with four treatments; HCl followed by rapid $NaHCO_3$ infusion (HCl_R), HCl followed by slow $NaHCO_3$ infusion (HCl_S), DL-LA followed by rapid $NaHCO_3$ infusion (DL-LA_R), and DL-LA followed by slow $NaHCO_3$ infusion (DL-LA_S).

3.2.2.6 Infusates and infusion protocol

DL-LA (Sigma-Aldrich, St. Louis, Missouri, USA), HCl (BDH, Toronto, Ontario, Canada) infusions, and $NaHCO_3$ (Sigma-Aldrich, St. Louis, Missouri, USA) treatment were formulated as 150 mM; pH values (means ± se) for the three solutions were 2.8 ± 0.1, 1.4 ± 0.2, and 8.6 ± 0.1, respectively. By analysis, DL-LA solution contained 51% D-lactate and 49% L-lactate. All infusates were autoclaved prior to use. A separate experiment with three sets of solutions documented a small loss in weight to 99.95 ± 0.02% of the original weight and no loss of lactate or its isomers following autoclaving; for DL-LA solutions, D-lactate concentrations were 77.5 ± 1.1 and 77.6 ± 1.1 mmol/L, L-lactate 72.4 ± 1.3 and 72.5 ± 1.4 mmol/L pre- and post-autoclaving, respectively.

The eight calves randomly received intravenous infusions of HCl, or DL-LA at an infusion rate of 20 mL·kg body $wt^{-1}·h^{-1}$ using an automated infusion pump (Imed 980 volumetric infusion pump; Imed, San Diego, California, USA) starting at 0h for 4-8 h until venous pH reached 7.05; if venous pH dropped below 7.05 acid infusions were ceased until pH rose back to 7.1. Once pH rose above 7.1 acid infusion was restarted and continued intermittently until 24 h to maintain venous pH below 7.1. The calves were then rested for an hour. At 25 h from the beginning of the acid infusion, calves were treated with equimolar amounts of iso-osmotic (150 mM) $NaHCO_3$ solution in two regimens; slow, 24 h or rapid, 4 h to correct acidosis. $NaHCO_3$ infusion rates were established using volume/24 h or volume/4 h accordingly as below.

Equation 2. Slow rate = [(base excess × body weight × 0.5) ÷ 150] ÷ 24 $l·h^{-1}$

Equation 3. Rapid rate = [(base excess × body weight × 0.5) ÷ 150] ÷ 4 $l·h^{-1}$.

There was a minimum of 2 days rest between each infusion protocol.

3.2.2.7 Sampling and measurements

Samples of CSF fluid (~0.7 ml) and venous (~4 ml) and arterial (~1 ml) blood were collected, and neurological assessment was performed at acid infusion hours -25 (pre acid infusion), -24, -22, -20, -18, -16, -1 h (completion of acid infusion). During $NaHCO_3$ therapies, the sampling and assessments were performed at 0 (start of rapid or slow

$NaHCO_3$ therapy), 1, 2, 4 (end of rapid $NaHCO_3$ therapy), 6, 8, 24 h (end of slow $NaHCO_3$ therapy).

Neurological assessment was performed by a veterinarian who was blinded to the type of infusion being administered. Neurological function was scored on the basis of the strength of the suck, menace, palpebral, and tactile reflexes and ability to stand (Abeysekara et al. 2007; Kasari and Naylor 1986; Lorenz et al. 2005; Naylor 1989). Each item was given a score of from 0 (normal) to 2 (severely abnormal) according to a previously published scale (see the section 3.1.2.7). The values were summated to produce a total neurological disturbance score. Calves with normal CNS function had scores of 0, whereas severely obtunded calves could have a maximum score of 10.

3.2.2.8 Laboratory Analyses

See section 3.1.2.8, page 38 for details.

3.2.2.9 Statistical Analysis

Descriptive statistics, ANOVA and MNOVA with repeated measures were carried out using a statistical analytical software package (SPSS statistical software v. 15, 2006; SPSS, Chicago, Illinois, USA). The significance of treatment effects on neurological scores, blood gas values, and serum and CSF lactates which were reported in Table 4, was determined by repeated-measures ANOVA / MANOVA with treatment (infusion and therapies) as the independent variable (Steel and Torrie 1980) using SAS statistical software (SAS/STAT statistical software package for Windows, v. 8.02; SAS Institute, Cary, North Carolina, USA). Mean values at individual time points were compared using the Student-Newman-Keul's (SNK) test. Treatment differences were also tested using orthogonal contrasts to compare slow vs. rapid $NaHCO_3$ therapies. A P value of < 0.05 was considered to be statistically significant. Values are reported as means ± se.

3.2.3 Results

3.2.3.1 Neurological assessment and recovery

Severe depression was found in calves with DL-LA induced acidosis (Figure 11 and Figure 12, $P < 0.05$). DL-LA infusion induced complete ataxia, recumbency and paralysis when serum D-lactate concentrations were > 10 mmol/L. HCl produced mild impairments in neurological function (Figure 11 and Figure 12); only the suck reflex was affected. Serum (Figure 12, r = 0.95) and CSF D-lactate (Figure 11 and Figure 12, r = 0.98) concentrations strongly correlated ($P < 0.05$) with neurological disturbance scores. The suck reflex was affected by both DL-LA and HCl infusions. The suck reflex had stronger correlation with bicarbonate, base excess or pH than with D-lactate concentration. Alleviation of neurological disturbances was slow. The suck reflex recovered faster with rapid $NaHCO_3$ therapy and both regiments had a greater recovery in sucking than the other reflexes.

Only, DL-LA calves showed marked neurological disturbances, (Figure 11 and Figure 12). Both $NaHCO_3$ therapies alleviated neurological disturbances by only 10% at 4 h of correction despite the fact that blood pH was close to normal in calves rapidly corrected with bicarbonate at this time; however, a 33% improvement (Δ neurological score 2.0) was seen at 8h (Figure 12). At 4, 6 and 8 h, rapid $NaHCO_3$ therapy had a non significant, but numerically faster recovery from neurological disturbances compared to slow therapy. Neurological disturbances recovered back to base line level by 24 h in both $NaHCO_3$ therapy groups.

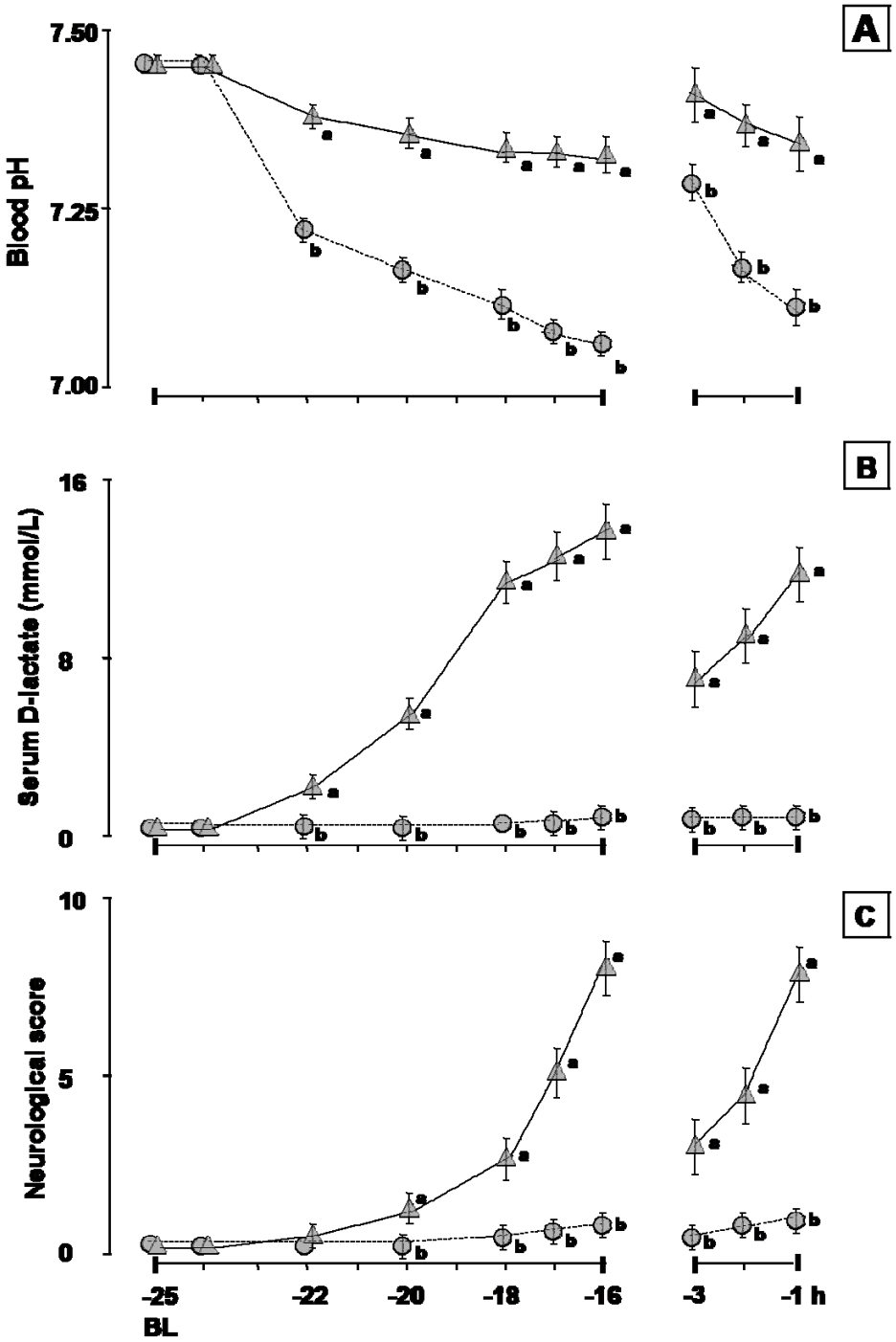


Figure 11. Blood (venous) pH [graph A], serum D-lactate concentration (mmol/l) [graph B], Neurological disturbance score [graph C] as affected by 150 mM hydrochloric (HCl, ●) or DL-lactic acid (DL-LA, ▲) infusions. The X axis represents the time course of the experiment where BL is a healthy baseline sample, followed by a period where acidosis was induced for 24 h intermittently to produce blood pH <7.30 and >7.05. Values at each time point that do not share a common letter (a or b) are statistically significant at $P < 0.05$.

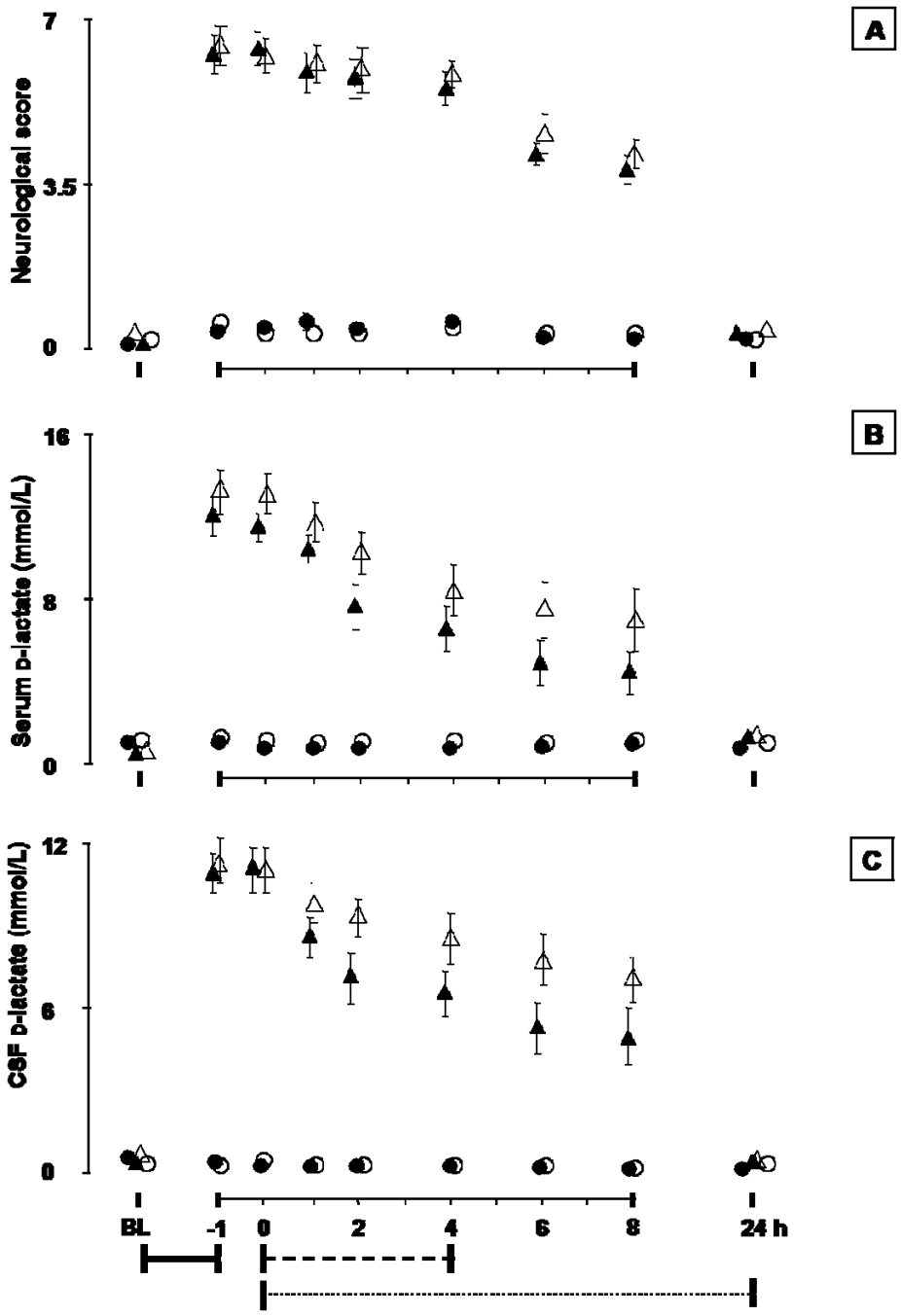


Figure 12. Neurological disturbance score [graph A], serum D-lactate concentration (mmol/L) [graph B], CSF D-lactate concentration (mmol/L) [graph C] as affected by rapid or slow equimolar $NaHCO_3$ intravenous therapies in severe acidosis induced by HCl or DL-lactic acid infusions respectively. Data are presented as ▲ for DL-LA_R, △ for DL-LA_S, ● for HCl_R, and ○ for HCl_S. The X axis represents the time course of the experiment where BL is a pre-acid infusion baseline sample, followed by a period (24 h) where acidosis was induced (_), and then either rapid, 4 h (----) or slow, 24 h (….) $NaHCO_3$ infusion.

3.2.3.2 Acidosis and recovery

HCl infused calves had the most severe metabolic acidosis (Figure 11A, $P < 0.05$) which reached a nadir after 24 h of acid infusion (Figure 13), comprising venous blood pH 7.0 ± 0.1, bicarbonate 7.0 ± 1.5 mmol/L, P_{CO_2} 32 ± 2 mmHg and BE -24 ± 2 mmol/L. The lowest arterial blood values were recorded with HCl acidosis and had pH 7.05 ± 0.06, bicarbonate 6.8 ± 1.1 mmol/L, P_{CO_2} 26 ± 3 mmHg, P_{O_2} 86 ± 30 mmHg and base excess -20 ± 3 mmol/L. The lowest hematocrit and hemoglobin concentrations (25 ± 6% and 76 ± 17 g/L) also appeared with HCl induced acidosis. HCl infusion ($P < 0.05$) caused a drop in body temperature (36 ± 1 °C). DL-LA also produced a metabolic acidemia which was moderate compared to the HCl infusion and had had less severe ($P < 0.05$) acidosis parameters.

Both $NaHCO_3$ therapies increased ($P < 0.05$) blood bicarbonate, pH and P_{CO_2} (Figure 13). However, rapid $NaHCO_3$ therapy corrected acidemia faster ($P < 0.05$) than slow therapy (Figure 13). HCl acidotic calves with slow therapy had the slowest recovery for all blood acidosis parameters. Arterial pH, P_{CO_2} and other parameters were recovered faster ($P <0.05$) by rapid $NaHCO_3$ therapy than slow therapy (Table 4). However, no significant changes were found in hemoglobin concentrations or P_{O_2} levels with time.

After 24 h of both $NaHCO_3$ therapies, blood pH had recovered to baseline values; however, blood bicarbonate and P_{CO_2} did not recover completely (Figure 13).

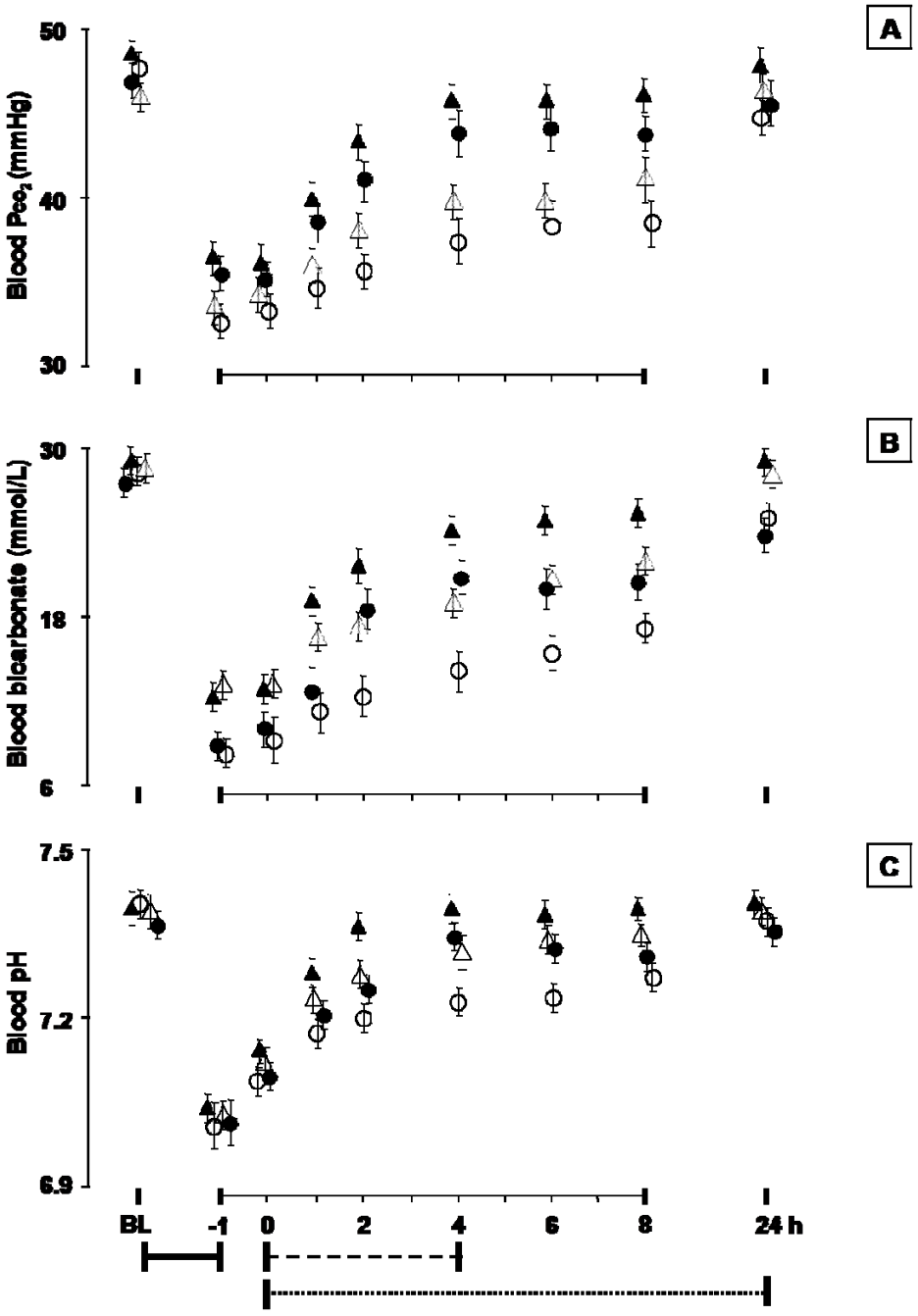


Figure 13. Blood P_{CO_2} (mm Hg) [graph A], bicarbonate concentration (mmol/L) [graph B], pH [graph C] as affected by rapid or slow equimolar $NaHCO_3$ intravenous therapies in severe acidosis induced by HCl or DL-lactic acid infusions respectively. Data are presented as ▲ for DL-LA_R, △ for DL-LA_S, ● for HCl_R, and ○ for HCl_S. The X axis represents the time course of the experiment where BL is a pre-acid infusion baseline sample, followed by a period (24 h) where acidosis was induced (_), and then either rapid, 4 h (---) or slow, 24 h (….) $NaHCO_3$ infusion.

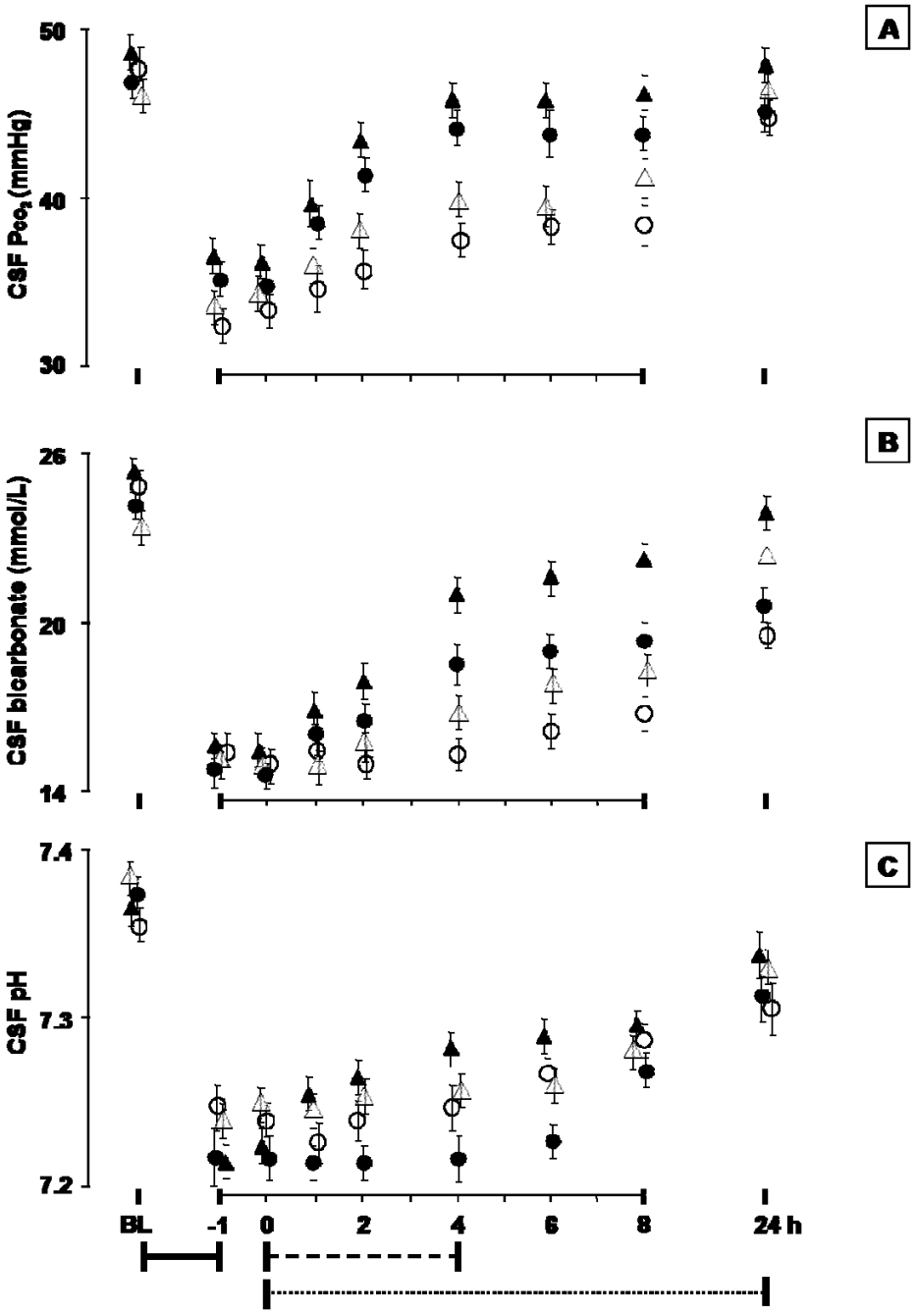


Figure 14. CSF P_{CO_2} (mm Hg) [graph A], CSF bicarbonate concentration (mmol/L) [graph B], CSF pH [graph C] as affected by rapid or slow equimolar $NaHCO_3$ intravenous therapies in severe acidosis induced by HCl or DL-lactic acid infusions respectively. Data are presented as ▲ for DL-LA_R, △ for DL-LA_S, ● for HCl_R, and ○ for HCl_S. The X axis represents the time course of the experiment where BL is a pre-acid infusion baseline sample, followed by a period (24 h) where acidosis was induced (_), and then either rapid, 4 h (---) or slow, 24 h (….) $NaHCO_3$ infusion.

Both HCl and DL-LA infusion produced decreases in CSF pH, bicarbonate and P_{CO_2} (Figure 14). Rapid $NaHCO_3$ therapy produced a greater ($P < 0.05$) increase in CSF P_{CO_2} (Figure 14A) and bicarbonate (Figure 14B) at 4 h in both DL-LA and HCl induced acidosis. Following rapid $NaHCO_3$ therapy in HCl acidotic calves a further decrease (Δ pH 0.03) in CSF pH occurred at 4 h of correction. However, DL-LA induced acidosis calves receiving rapid $NaHCO_3$ therapy (Figure 14) had a faster recovery in CSF pH ($P < 0.05$). The lowest CSF pH (7.20 ± 0.02) was found when calves with HCl induced acidosis received rapid $NaHCO_3$ therapy (Figure 14). However, all acidotic calves responded to either $NaHCO_3$ therapy with a ~50% pH recovery (Δ pH -0.11) at 8 h of correction (Figure 14). Calves with DL-LA induced acidosis had a faster recovery in CSF bicarbonate and pH when they received rapid $NaHCO_3$ therapy (Figure 13, $P < 0.05$). After 24 h of correction, CSF pH and P_{CO_2} recovered to near baseline values. However, CSF bicarbonate in HCl acidotic calves did not fully recover to the base line values (Figure 14). All acidotic parameters had returned to the base line values by 48 h of correction.

3.2.3.3 D and L-lactate concentrations

Serum and CSF D-lactate (Figure 11 and Figure 12) were only increased by DL-LA infusion. Peak CSF D-lactate concentration (11 ± 1 mmol/L) after > 12 hours of DL-LA infusion was 15 % lower than the peak serum D-lactate concentration (13 ± 2 mmol/L). Venous serum D-lactate concentration was correlated ($r = 0.91$, $P < 0.05$) with CSF D-lactate concentration. Serum and CSF L-lactate concentrations were unaffected by HCl infusion (2 ± 1 and 3 ± 1 mmol/L). CSF L-lactates (6 ± 2 mmol/L) were increased with DL-LA infusion and were 20% higher than the peak serum L-lactate concentration. However, peak serum and CSF L-lactate concentrations were lower than peak D-lactate concentrations (Table 4).

Table 4. Effects of rapid or slow $NaHCO_3$ therapy on neurological disturbance, arterial, venous and cerebrospinal fluid blood gas values, and cerebrospinal fluid and venous serum lactate concentrations in calves with induced acidosis either by HCl or DL-lactic acid (DL-LA)

	DL-LA*		HCl*		Significance (*P*-value) †		
	Fast	Slow	Fast	Slow	Therapy and time	DL-LA versus HCl	Fast versus Slow
Neurological score	4.4±1.2	4.4±1.3	1.0±0.5	1.2±0.6	<0.05	<0.05	0.18
Serum							
D-lactate, mmol/L	6.7±1.4	7.9±1.3	0.08±0.1	0.09±0.1	<0.05	<0.05	0.07
L-lactate, mmol/L	3.5±0.4	3.7±0.5	1.8±0.2	1.9±0.1	<0.05	0.12	0.10
Venous blood							
pH	7.38±0.01	7.34±0.01	7.32±0.03	7.24±0.02	<0.05	<0.05	<0.05
BE, mmol/L	2.0±0.5	-3.8±0.08	-4.0±1.0	-9.8±2.2	<0.05	<0.05	<0.05
HCO_3^-, mmol/L	25.5±1.0	21.5±1.0	21.0±1.0	14.8±1.4	<0.05	0.06	<0.05
pCO_2, mm Hg	45.0±2.0	40.0±1.5	42.2±2.5	35.2±3.9	<0.05	0.16	<0.05
pO_2, mm Hg	34.3±1.8	34.0±2.0	36.2±1.5	34.3±3.3	0.06	0.81	0.72
Hemoglobin, g/L	69.8±4.8	71.0±7.5	71.5±7.3	72.0±4.8	0.81	0.72	0.39
Arterial blood							
pH	7.41±0.03	7.28±0.05	7.40±0.02	7.27±0.04	<0.05	0.34	<0.05
BE, mmol/L	1.0±2.0	-7.3±3.4	-4.8±0.8	-11.3±2.4	<0.05	0.07	<0.05
HCO_3^-, mmol/L	23.0±2.5	16.0±1.5	18.0±1.5	12.4±1.5	<0.05	0.05	<0.05
pCO_2, mm Hg	40.8±3.3	35.3±2.8	35.0±2.5	29.6±3.3	<0.05	<0.06	<0.05
pO_2, mm Hg	90.8±9.3	103.5±22.0	101.0±5.5	99.8±15.8	0.07	0.87	0.56
CSF							
D-lactate, mmol/L	6.7±2.3	7.9±2.1	0.06±0.1	0.07±0.01	<0.05	<0.05	0.09
L-lactate, mmol/L	6.9±1.2	10.7±0.8	3.8±1.0	3.4±0.5	<0.05	0.82	0.04
pH	7.29±0.08	7.25±0.07	7.20±0.06	7.29±0.02	<0.05	0.06	0.08
HCO_3^-, mmol/L	21.8±1.4	17.3±1.6	18.5±1.6	15.7±1.7	<0.05	0.11	<0.05
pCO_2, mm Hg	46.3±1.8	39.7±2.1	44.0±1.5	37.3±1.5	<0.05	0.13	<0.05
pO_2, mm Hg	106±17.5	90.6±5.9	89.8±3.5	83.1±6.25	0.23	0.36	0.18

* Values are mean ± se for data collected at 6 h from 1 and 24 h of $NaHCO_3$ therapy.
†Statistically significance was determined for values between 1 and 24 h of $NaHCO_3$ therapy inclusive by repeated measures MANOVA for $NaHCO_3$ therapy and time interaction effects, and orthogonal contrast comparison for DL-LA versus HCl infusions and fast versus slow $NaHCO_3$ therapy.

3.2.3.4 D-lactate removal from the body

Serum D-lactate concentrations declined following the cessation of infusion and the start of correction of acidemia. Serum D-lactate removal was concentration dependant and had an exponential decrease. At 10 mmol/L concentration, the rate was ~ 1.5 mmol/L· h^{-1}, and at 5 mmol/L it was 0.5 mmol/L· h^{-1}. Rapid $NaHCO_3$ therapy had a faster serum D-lactate decline (Figure 12, ~57%, Δ D-lactate concentration 7.5 mmol/L in 8 h, $P < 0.05$). Slow $NaHCO_3$ therapy had a steady serum D-lactate decline (Figure 12, ~47%, Δ D-lactate concentration 6.1 mmol/L in 8 h).

CSF D-lactate concentrations did not change following cessation of infusion (Figure 12C, -1 to 0 h, $P > 0.05$) and only started to decline when bicarbonate therapy commenced. Rapid $NaHCO_3$ therapy had a faster CSF D-lactate decline (Figure 12C, ~50%, Δ D-lactate concentration 5.5 mmol/L in 8 h, $P < 0.05$). Slow $NaHCO_3$ therapy had a steady CSF D-lactate decline (Figure 12, ~32%, Δ D-lactate concentration 3.5 mmol/L in 8 h). Regardless of the type of correction, removal of D-lactate from CSF was slower, and more gradual than from serum. Although CSF D-lactate removal was not concentration dependant and had a constant decrease (0.1−0.3 mmol/L· h^{-1}), $NaHCO_3$ therapy promoted the decline (Table 4). All D-lactate concentrations returned to baseline values in 24 h of correction.

3.2.4 Discussion

Infusion of DL-LA or HCl produced profound acidemia (pH close to 7.0), a less severe CSF acidosis and marked clinical signs of neurological disturbance only in calves receiving DL-LA. $NaHCO_3$ therapy relieves neurological disturbances; with a trend for faster alleviation of neurological disturbances with rapid therapy.

DL-LA infusion caused neurological dysfunction with severe ataxia, even though peak blood and CSF concentrations were lower than in our previous study (see section 3.1.3). Longer exposure to D-lactate in this study is a better approximation to natural DLA and may have allowed greater penetration of D-lactate into the neurons (Lorenz 2007; Petersen 2005). The serum concentrations in the present experiment (10 mmol/L) are comparable to those in naturally occurring DLA in calves and humans with gastrointestinal diseases (Ewaschuk et al. 2004; Uribarri et al. 1998). Deteriorating acid base parameters from both HCl and DL-LA infusions were once again associated with a weakened suck reflex (Abeysekara et al. 2007; Lorenz 2004b). The suck reflex recovered quickly with both $NaHCO_3$ therapies and rapid regimen produced a faster recovery. In previous studies of calves, acidemia has been associated with a weak suck reflex (Kasari and Naylor 1986; Lofstedt et al. 1999; Lorenz 2004b).

Rapid $NaHCO_3$ therapy caused paradoxic CSF acidosis in HCl acidotic calves (HCl_R) where the lowest CSF pH (~7.2) was found after 4 h of rapid $NaHCO_3$ therapy (Table 4 and Figure 14). However, neurological scores were not affected by this paradoxical acidosis. Rapid $NaHCO_3$ therapy caused increase in CSF P_{CO_2} and bicarbonate (Figure 14) subsequent to similar increases in blood. In agreement with Figure 10, excess CSF P_{CO_2} may dissolve to form carbonic acid (H_2CO_3) further lowering CSF pH. This would explain the drop in CSF pH in the HCl_R group. Anions may also play a role in retention or elimination of H^+ in CSF (Berchtold et al. 2005; Gowrishankar et al. 2007). It is possible that the lack of paradoxic CSF acidosis in DL-LA calves, and the apparent enhancement of CSF D-lactate removal with rapid bicarbonate therapy are both the result of the transport or diffusion of D-lactic acid carrying D-lactate and a proton out of the CSF in response to therapy

The mechanism that enhances D-lactate removal by $NaHCO_3$ is not well understood. Lactate uptake (100 $\mu mol/kg \cdot min^{-1}$) is increased by 1 mol/L $NaHCO_3$ infusions in rat liver (Beech et al. 1993). Although D-lactate may not be transported the same way, permeation across transporters and membranes may be enhanced (De Bari et al. 2002; Hertz and Dienel 2005; Poole and Halestrap 1993; Ullrich et al. 1982b). Rising alkalinity of blood with bicarbonate in blood does not modulate D-lactate transport via kidneys. However, Na^+ and P_{CO_2} have a positive effect on D-lactate transport (Ullrich et al. 1982b).

In conclusion in the models of severe prolonged acidosis that we used in calves with HCl acidemia, rapid correction of blood pH with bicarbonate induced paradoxic CSF acidosis but there were no adverse clinical signs. There appears to be no danger of inducing paradoxic CSF acidosis by IV $NaHCO_3$ therapy in calves with DL-LA acidosis. Rapid correction of DL-LA acidosis was exceptional in that DL-lactate concentrations

decreased faster. This is presumably the explanation for the absence of paradoxical CSF acidosis in this group. However, these results certainly do not exclude the possibility that the effect of $NaHCO_3$ therapy on cellular pH in other critical organs such as the heart may be deleterious. Further research studies in animals and humans will be necessary with therapies focused to the type and cause of acidosis.

4 CLINICAL STUDIES

4.1 Incidence of D-lactic acidosis in diarrheic lambs

4.1.1 Introduction

D-Lactic acidosis (DLA) is common in cases of calf diarrhea (Ewaschuk et al. 2003; Omole et al. 2001). It has also been described in humans, secondary to short bowel syndrome, propylene glycol ingestion (Christopher et al. 1990; Jorens et al. 2004) and other digestive disorders (Uribarri et al. 1998). The common feature in all these cases is that D-lactic acid originates in the gut (Uribarri et al. 1998) and is absorbed into the blood circulation resulting increased serum D-lactate (D-lactatemia) (Lorenz et al. 2005). Neurological signs (Hingorani and Chan 2001), a drop in blood pH, bicarbonate, base excess, and increase in anion gap (Lorenz 2004a; Omole et al. 2001) are all characteristics of the clinical stage of DLA (Ewaschuk et al. 2005; Uribarri et al. 1998).

Lamb diarrhea is considered an important constraint in sheep farming. Lamb diarrhea has caused considerable morbidity (2.8%) and mortality (0.7%) rates in Canada for last few decades; the Prairie has a greater share of these cases (Dohoo et al. 1985). Infectious agents such as bacteria, viruses and cryptosporidia are responsible for 99% of lamb diarrhea. More than 75% of cases are due to *Escherichia coli* infection; a bacterium that can produce D-lactic acid. An episode of lamb diarrhea reduces the growth of lambs permanently yielding about 2 kg weight loss at the age of five weeks (Green et al. 1998). Diarrhea occurs at all ages of lambs and 15% to 40% of the flock may be affected in lamb at any age, however, peak prevalence is 3 to 5 weeks of age (Green et al. 1998).

Lambs affected with diarrhea may also be prone to DLA. The objective of this study was to determine the incidence of DLA and prevalence of metabolic acidosis secondary to lamb diarrhea / dysentery / scours.

4.1.2 Materials and methods

Diarrheic lambs at the University sheep barn were monitored in this study from January 2004 to June 2005.

4.1.2.1 Severity of diarrhea and fecal score

Diarrhea was determined and assessed by a fecal score system. Healthy lambs with solid fees had a score of 0, and diarrheic lambs with watery feces had a maximum score of 4 (Lofstedt et al. 1999; Tzipori et al. 1981).

4.1.2.2 Sampling and analyses

Samples collected for analysis were blood, feces and urine from 27 diarrheic and 4 healthy lambs (31 ± 12 days of age, 16 ± 8 kg). Blood (1 mL) was drawn to a heparinized syringe and kept in ice (less than 1 h) until used for blood gas analyses. Venous blood (4 mL) from the jugular vein was collected into a serum tube; the blood was allowed to coagulate for 20 min, spun in a refrigerated centrifuge (IEC Centra-7R Refrigerated Centrifuge; International Equipment Company, Needham Heights, Massachusetts, USA) at 1000 RCF for 30 min and the serum harvested. Serum samples were stored in a freezer (-20 °C) until analyzed for lactates.

Fresh urine (~10 mL) and feces (~50 g) were collected in the morning from the reported diarrheic lambs. Urination and defecation was stimulated manually by rubbing the groin and perineum. Urine samples were stored in a freezer (-20 °C) until analyzed for lactates and creatinine. Feces were dissolved 1 to 1 in 1% sodium ethylmercuric thiosalicylate (thiomerosal; ICN Biomedicals Inc., Aurora, Ohio, USA) solution (a bacteriostatic agent) to minimize bacterial growth and stored at -20 °C.

4.1.2.2.1 Quantification of acidemia and serum electrolytes

Blood was immediately analyzed for pH, HCO_3^-, base excess, hemoglobin (Hb), Na^+, K^+, Ca^{++}, Cl^-, anion gap, glucose using an ABL5 Blood Gas Analyzer (Radiometer Medical A/S, Copenhagen, Denmark) and an electrolyte analyzer (Braconnier et al. 2003). Hemoglobin was measured in an OSM3 Hemoximeter (Zwart et al. 1987).

4.1.2.2.2 Lactate measurements

D- and L-lactate concentrations in serum, urine and feces were measured using an HPLC system (see the section 3.1.2.8, Omole et al. 1999; Ewaschuk et al. 2002). Serum

could be analyzed without dilution. In order to achieve accurate measurement within a detection range (0.05–10 mmol/L), fecal and urines samples were appropriately diluted after identifying they contained higher concentrations of lactate. All dilutions were performed using deionized water from a Milli-Q synthesis A10 (Millipore Corporation, Bedford, Massachusetts, USA). The lactate concentrations which were lower than 0.05 mmol/L were considered 0 or not quantified (nq). However, an arbitrary value of 0.04 mmol/L was used in statistical calculations and drawings.

Fecal sample preparation and analysis: Samples were thawed at 4 °C and then shaken for 20 min on an automatic shaker (VWR DS-500E Orbital Shaker). One gram of feces (or 1 mL of semisolid feces) was added to 9 mL of deionized water from a Milli-Q synthesis A10 (Millipore Corporation, Bedford, Massachusetts, USA) and homogenized for 1 min, and centrifuged at 20,000×g for 30 min. The supernatant was removed and syringe filtered through an Acrodisc PF (0.8/0.2 μm) filter. Then 100 μL of filtrate were added to 50 μL of internal standard (2 mmol/L malonic acid for stereoselective assay) and 50 μL of deionized water in an Ultrafree®-MC 5000 NMWL filter units (Millipore Corporation, Bedford, Massachusetts, USA) under 3000 RCF (AccuSpin Micro 17; Fishers Scientific, Schwerte, Germany) for 30 min; 20 μL aliquots of the final filtrate were injected into the HPLC system (Ewaschuk et al. 2002).

Urine sample preparation and analysis: Urine samples were diluted 1:5 (or appropriately) with deionized water. Then 100 μL of diluted urine were mixed with 50 μl of internal standard and 50 μL of deionized water in Ultrafree®-MC 5000 NMWL filter units (Millipore Corporation, Bedford, Massachusetts, USA) under 3000 RCF (AccuSpin Micro 17; Fishers Scientific, Schwerte, Germany) for 30 min; 20 μL aliquots of the final filtrate were injected into the HPLC system. Urine samples were run for 40 min because of late eluting peaks (Ewaschuk et al. 2002).

4.1.2.2.3 Urine creatinine measurement

The concentration of urinary creatinine was determined by high-performance liquid chromatography with 3-mm Chromsep ODS-2 column (Varian Inc., Mississauga, Ontario, Canada) and with a detection wavelength 220 of the same 486 UV detector and HPLC system (Waters, Mississauga, Ontario, Canada) (Smith-Palmer 2002; Yang 1998). Then 0.2 mL of urine was deproteinized with 1 mL acetonitrile (EMD Chemicals Inc., Gibbstown, New Jersey, USA), and vacuum centrifuged in an Eppendorf concentrator 5301 (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) according to MacNeil et al. 2005 ((MacNeil et al. 2005). This dried sample was mixed with 1.2 mL of deionized water, and then 0.2 mL of mixed sample was used analysis in the HPLC system. Subsequent to calibration and quantification using Waters' Millenium32 v 4 software, creatinine concentrations were determined from their peak heights in comparison to a standard linear curve ranging from 0.5 to 10 mmol/L creatinine (Sigma-Aldrich, St. Louis, Missouri, USA). Mobile phase solution was 20 mM potassium hydrogen phosphate (Sigma-Aldrich, St. Louis, Missouri, USA) buffer, adjusted to a pH 5.0 with 1 M potassium hydroxide. The flow rate and temperature were maintained at 0.7 mL/min and 25 ºC.

4.1.2.3 Lamb care

The lambs monitored in this study were Suffolk lambs taken from the Sheep Barn, Department of Animal and Poultry Sciences, University of Saskatchewan, Canada. They were selected on the basis of age (0-6 weeks) and health or signs of diarrhea for the control or diarrheic samples respectively. All lamb handlings and management were performed according to the guidelines of Canadian Council on Animal Care (Canadian Council on Animal Care 1993). The lambs were either housed indoors at 15 ± 5 °C room temperature and 55 ± 5% relative humidity in 3.0 × 2.5 m^2 mobile pens made up of metal on a concrete floor. The floor of these pens was bedded with 10 cm thick layer of straw. Some lambs were kept outside in straw bedded yards.

Clean water was freely available from water bowls fixed to the fence of these pens. Regular cleaning, watering and feeding were performed twice a day at 8.00 and 17.00 hours. Visual inspections for any ailments were performed at the same time by an animal technician

Feeds for lambs consisted of fresh milk and alfalfa hay. Twice a day, the lambs were fed 0.2-0.8 L of the milk depending on the body weight, using a bottle feeder or a plastic bucket. Approximately 1 kg of second cut alfalfa hay was provided in the feeder in the pen for lambs to eat. However, new born lambs were allowed to be with their mothers and allowed to suckle for a week before introducing them to the lamb pens. Only severe cases of diarrhea were treated. Once the animal technicians observed diarrhea samples and an examination were collected the next morning.

4.1.2.4 Statistical analysis

Data were analyzed using SPSS v14, 2006 (SPSS Inc. 2005) and Figures were created using SPSS and Microsoft Excel (Microsoft Corporation Inc. 2004). One way ANOVA, Student t test, frequency distributions and Pearson correlation procedures were performed (Steel and Torrie 1980). Biphasic Regression (Break Point) Analysis was performed using SAS statistical software (SAS/STAT statistical software package for Windows, v. 8.02; SAS Institute Inc., Cary, North Carolina, USA. 2001). For statistical purposes of Regression Analysis, parameters that were not detectable by the method used were assigned a value of 0, and parameters that were detectable but below the limit of quantitation (LOQ, 0.05 mmol/L) were assigned 0.04 mmol/L. A $P < 0.05$ was considered statistically significant.

4.1.3 Results

4.1.3.1 Diarrhea and fecal score

Severe to moderate diarrhea was observed, however, the scouring lambs were not severely debilitated. Many were active and alert and needed to be restrained for sampling. Fecal scores indicated 26% of mild, 67% of moderate and 9% severe diarrhea among the scouring lambs (Figure 15). Fecal color changed from creamy (7%), yellow (62 %), brown (19%) to chocolate (2%). Most of the moderate cases had yellow diarrhea. Mild diarrhea was often chocolate or the feces were semisolid with misshapen pellets.

4.1.3.2 Lactate concentrations

The distribution of fecal D-lactate appeared normal (Figure 16), but the distribution of serum D-lactate concentration was skewed with many values below 0.1 mmol/L (Figure 17). 74% of diarrheic lambs had < 0.05 mmol/L, the lowest level of detection with our analytical method. In the 26% of scouring lambs with quatifiable serum D-lactate, mean D-lactate concentration was 0.3 ± 0.2 mmol/L. Serum D-lactate (> 0.05 mmol/L) was not quantified in any healthy lamb (Table 5).

Fecal D-lactate concentrations were greater ($P < 0.05$) in diarrheic than in healthy lambs, while L- lactate concentrations were marginally higher. The D- and L- lactate concentrations were 9.5 ± 3.7 and 8.9 ± 2.5 mmol/L respectively (Table 5). Diarrheic lambs also had higher ($P < 0.05$) urine D-lactate concentrations and higher serum L-lactate concentrations ($P < 0.05$).

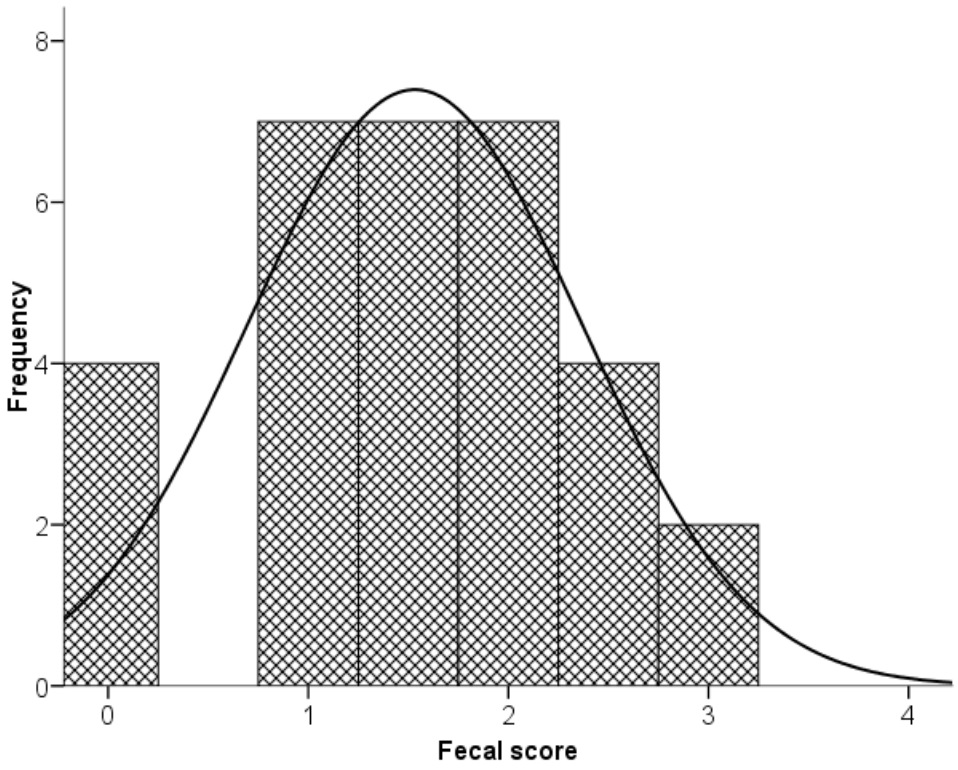


Figure 15. Distribution of fecal score (severity of diarrhea, 0 to 4 score) among diarrheic lambs (n = 31, mean ± sd = 1.5 ± 0.8, normal distribution —).

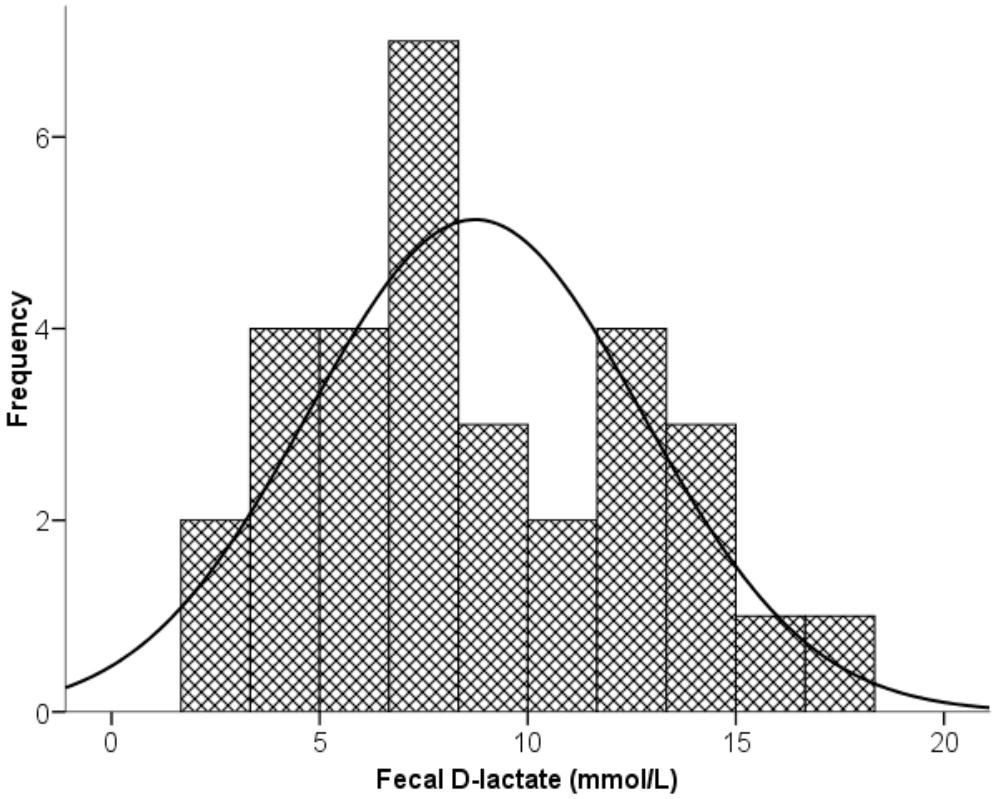


Figure 16. Distribution of fecal D-lactate concentration among diarrheic lambs (n = 31, mean ± sd = 8.7 ± 4.0, normal distribution —).

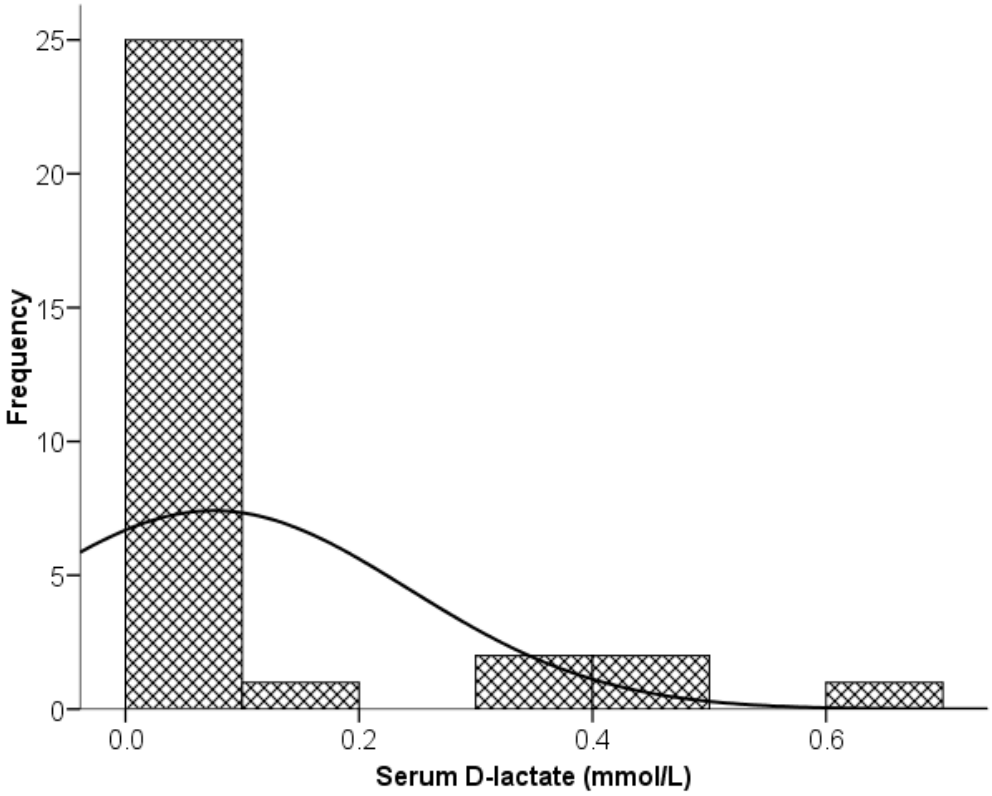


Figure 17. Distribution of serum D-lactate concentration among diarrheic lambs (n = 31, mean ± sd = 0.08 ± 0.17, normal distribution —).

Table 5. Mean fecal and blood indices in diarrheic and healthy lambs

	Diarrheic lamb (n = 27)	Healthy lamb (n = 4)
Feces		
Fecal Score	1.8 ± 0.6*	0
D-lactate, mmol/L	9.5 ± 3.7*	3.9 ± 0.8
L-lactate, mmol/L	8.9 ± 2.5	6.1 ± 1.7
Urine		
D-lactate, mmol/mol creatinine	27.3 ± 5.2*	9.8 ± 3.4
L-lactate, mmol/mol creatinine	3.3 ± 1.7	1.5 ± 1.1
Serum		
D-lactate, mmol/L	0.3 ± 0.2	nq
L-lactate, mmol/L	7.2 ± 4.9*	3.7 ± 1.0
Blood		
pH	7.33 ± 0.03	7.38 ± 0.02
P_{CO_2}, mmHg	49.5 ± 3.6	50.49 ± 1.3
P_{O_2}, mmHg	45.2 ± 4.8	42.2 ± 0.8
Bicarbonate, mmol/L	23.0 ± 3.1	27.1 ± 0.8
Base excess, mmol/L	-2.8 ± 3.8	0. 3 ± 0.5
Hemoglobin, g/L	130.3 ± 10.1	128.0 ± 1.6
Saturation of O_2, %	61.5 ± 6.1	67.2 ± 2.2
Na^+, mmol/L	144.0 ± 2.1	147.3 ± 1.0
K^+, mmol/L	4.9 ± 0.4	4.6 ± 0.1
Ca^{++}, mmol/L	1.4 ± 0.1	1.4 ± 0.1
Cl^-, mmol/L	109.2 ± 2.7	109.5 ± 1.3
Anion gap, mmol/L	17.3 ± 3.0	11.3 ± 1.0
Glucose, mmol/L	5.4 ± 0.9	5.6 ± 0.1

*Statistically significant difference between diarrheic and healthy lambs at $P < 0.05$.
nq = not quantified.

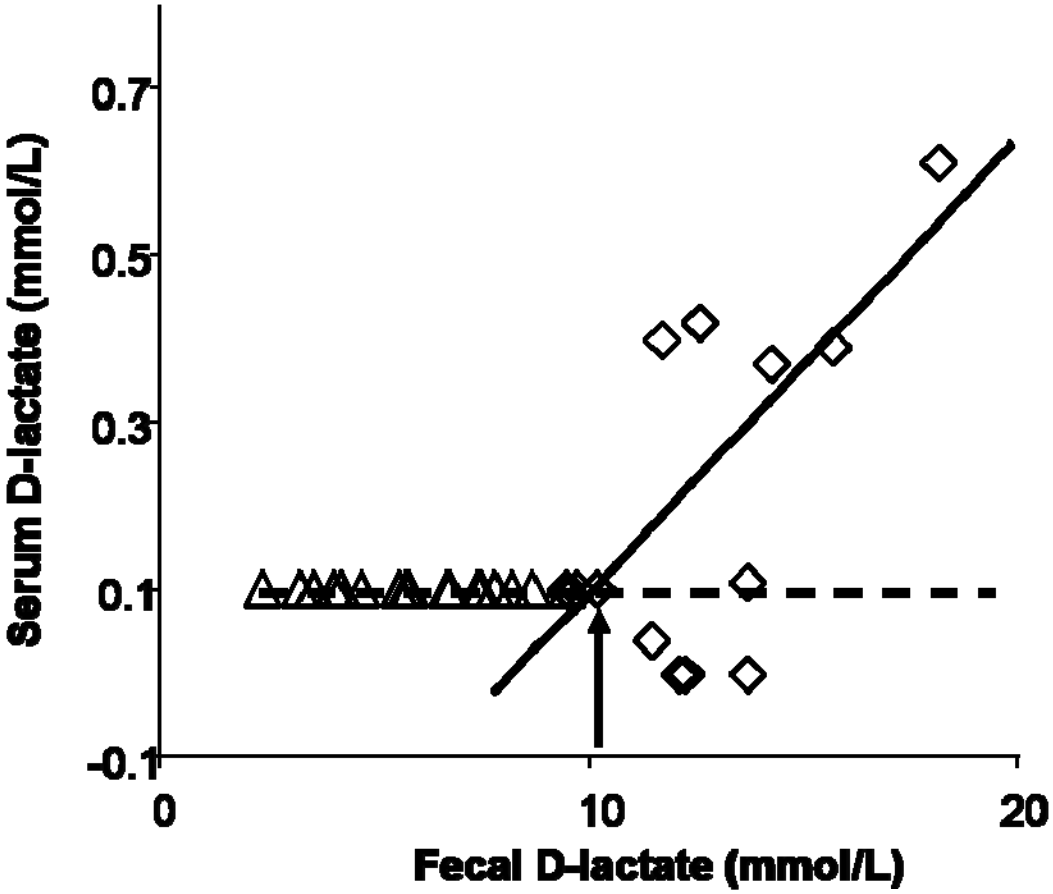


Figure 18. Break Point Analysis showing a threshold concentration for absorption when fecal D-lactate exceeded 10.18 mmol/L (↑) in lambs. The regression equation for Break Point Analysis consists of serum D-lactate concentration (y), fecal D-lactate concentration (x) and a dummy variable (z), n = 31. The equation for the estimated threshold is 'y = -0.69952 + 0.06873 x + 0.69952 z - 0.06873 (x*z)' where, z = 0 for the first line and z = 1 for the second line. First regresssion line (—, strait) was associated with square shape (◇) data points and the second regression line (----, broken) was associated with triangular (Δ) data points. Linear Correlation between fecal and serum D-lactate concentrations (r = 0.75).

4.1.3.2.1 Fecal D-lactate threshold concentrations

Fecal D-lactate threshold is the new finding we came across with the lamb study and then our previous calf data were also analyzed to find species wise associations (Appendix C: Diarrheic calf fecal D-lactate threshold).

In diarrheic and healthy lambs (n = 31), threshold concentration for fecal D-lactate absorption was 10.18 mmol/L which was estimated using Biphasic (Break Point) Regression Analysis (Figure 18). The regression equation for Break Point Analysis consists of serum D-lactate concentration (y), fecal D-lactate concentration (x) and a dummy variable (z). Therefore, the regression equation for the estimated threshold is shown below.

Equation 4. $y = - 0.69952 + 0.06873\ x + 0.69952\ z - 0.06873\ (x*z)$

Dummy variable codes applied in Equation 2 are z = 0 for the fist line and z = 1 for the second line. Linear Correlation between fecal and serum D-lactate concentrations (r = 0.71).

4.1.3.3 Metabolic acidosis

Two diarrheic lambs (7.4%) had moderate metabolic acidosis, blood pH (7.18, 7.25), HCO_3^- (18, 20 mmol/L), base excess (-9, -8 mmol/L) and anion gap (21, 19 mmol/L). Three diarrheic lambs (11%) had mild metabolic acidosis with a pH of 7.29 and improved other parameters. The remainder of the diarrheic lambs (81%) had pH < 7.30 with no signs of metabolic acidosis. However, comparisons between diarrheic and healthy lambs showed a trend for numerical decrease in mean blood pH, HCO_3^- and Na^+. Indicating an opposite trend, methemoglobin was slightly increased with diarrhea (Table 5). Weak correlations ($r \geq 0.45$, $P < 0.05$) were also observed between several parameters. The only significant linear correlations are serum lactate concentration vs. blood pH, fecal D- and L-lactate vs. serum D-lactate (Figure 18), and fecal score vs. blood Na^+ (Figure 19).

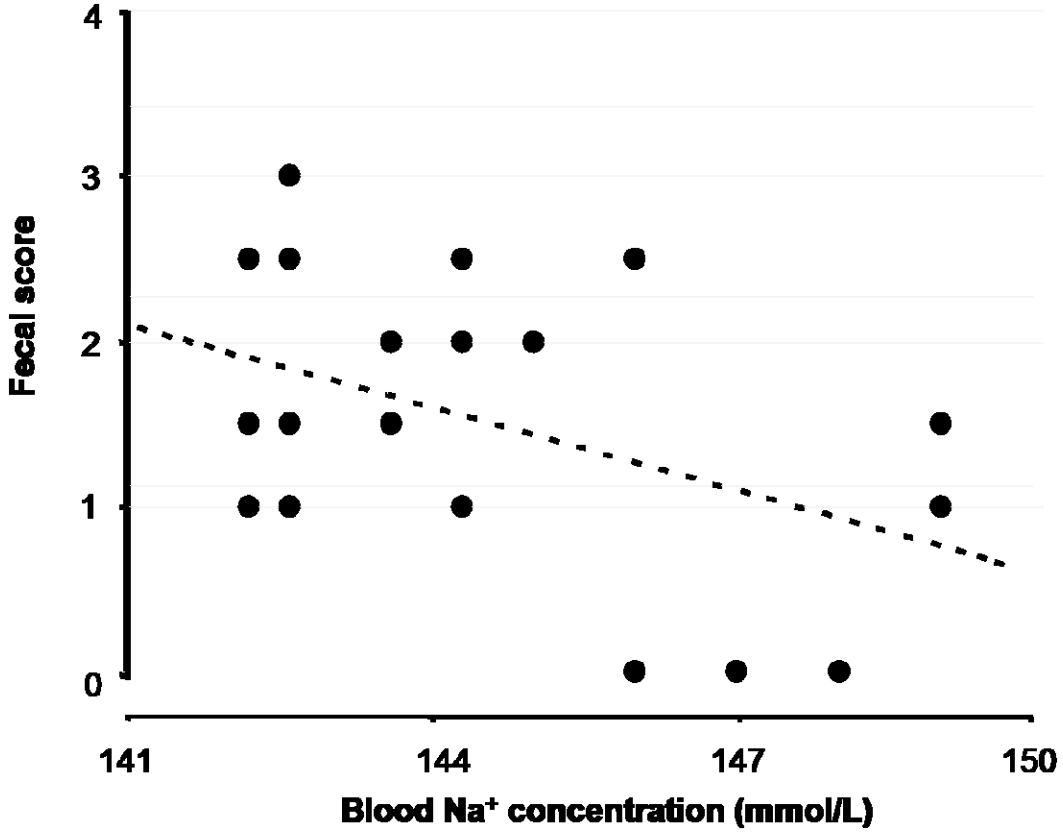


Figure 19. Blood sodium concentration correlated with fecal score (r = 0.51, ----regression line).

4.1.3 Discussion

Severe calf diarrhea has resulted in mean fecal and serum D-lactate concentrations of 25 and 14 mmol/L respectively (Ewaschuk et al. 2003; Ewaschuk et al. 2004). Serum D-lactate values reported by Uribarri et al. 1998 were over 3 mmol/L generally in human DLA cases (Uribarri et al. 1998). However, the present lamb data shows that mean serum D-lactate concentrations did not exceed 0.5 mmol/L in diarrheic lambs although fecal D-lactate concentrations averaged 14 mmol/L. In the following sections (4.2.3.1), we found similar low, but measurable serum D-lactate values in healthy, bucket fed calves prior the malate treatment. However, these diarrheic lamb serum D-lactate concentrations are clinically non-significant (< 2 mmol/L). This may be due to a pathophysiological difference (Field 2002) in the processing of D-lactate absorption in upper intestines of sheep (Ding and Xu 2003). Ding et al (2003) indicated that lactic acid absorption (~0.1 μmol/cm^2.min) in sheep intestine was affected by pH, osmotic pressure and lactic acid concentration (Ding and Xu 2003).

Our findings show that fecal D-lactate should be higher than ~10 mmol/L (fecal D-lactate threshold), to reach a measurable serum concentration (Figure 18). In case of calves, the severity of diarrhea may have resulted to produce more lactates (~23 mmol/L) in colon hence high levels (~13 mmol/L) in serum (Ewaschuk et al. 2003; Ewaschuk et al. 2004). The calf fecal D-lactate threshold was calculated using our laboratory data from 2000 to 2004 and was shown in Appendix D. Lactate transporters (monocarboxylic, MCT) in the gut allow the stereoselective uptake of D-lactate to a greater extent than L-lactate (Ogihara et al. 2000). Once the lumen D-lactate concentration increases, MCT may start transport of D-lactate into the circulation. This gut-originated D-lactate would then appear as increased serum concentration resulting in D-lactatemia (Ewaschuk et al. 2005; Hove and Mortensen 1995). However, the excretion of D-lactate from blood may be rapid if the kidneys are healthy and functionally efficient (Jørgensen and Sheikh 1984; Ullrich et al. 1982b). Our lambs were in the early stages of diarrhea; D-lactatemia was low, probably because of reduced production and more efficient renal clearance than in severely compromised calves (Ewaschuk et al. 2003; Ewaschuk et al. 2004). Serum L-lactate concentrations were higher in diarrheic lambs. Even in healthy lambs values were high, possibly due to acute stress and exercise from the process of catching and restraining for sampling (Arola et al. 1980).

Intestinal infection, which may be the primary cause of diarrhea, has a negative impact on enzymatic digestion in the affected portion of the intestines (Field 2002; Tzipori et al. 1981) and passage of excess nutrients to the colon may transpire (Bongaerts et al. 1997; Field 2002; Luckey 1987; Meier et al. 2003). *Escherichia coli* and *cryptosporidium* greatly reduced lactase enzymatic activity with severe intestinal lesions in the infected lamb (Tzipori et al. 1981). Lactose tolerance in diarrheic lambs infected with rotavirus was not affected (Ferguson et al. 1981) in contrast to calves infected with reo-like virus (Pearson et al. 1978). Therefore, we assume that colonic bacterial over growth may occur secondary to lamb diarrhea (Ewaschuk et al. 2004; Hove and Mortensen 1995).

Urine D-lactate concentrations were high. However, these values were not comparable with the concentrations in clinical DLA cases reported by previous authors

(Haschke-Becher et al. 2000; Inoue et al. 2007; Petersen 2005). Urine D-lactate content, expressed as a creatinine ratio (27 mmol/mol of creatinine) appeared moderate. Since the majority of our lambs had moderate diarrhea and were undergoing only a mild metabolic acidosis (Omole et al. 2001), it is likely that absorbed D-lactate was being effectively excreted by the kidneys (Jørgensen and Sheikh 1984; Ullrich et al. 1982b).

The correlation between serum total lactate and blood pH indicated that lactate contributed to metabolic acidosis. However, serum D-lactate concentration, were often too low to measure and so did not correlate with blood pH. Loss of Na^+ with diarrhea may be the reason for the negative correlation between fecal score and blood Na^+ concentration. We did not find a linear correlation between anion gap and serum D-lactate concentration. Again, this is likely because the majority of lambs had little measurable D-lactate. Overall, our data shows that short term diarrhea rarely causes severe metabolic acidosis in lambs.

In general environment, feeding and nutrition management have an impact on diarrhea syndromes (Meier et al. 2003). Over 80% of cases in this study had mild to moderate diarrhea which may not have been pathologically severe enough to extensively increase production and membrane permeability to intestinal (lumen) D-lactic acid. The barn where these lambs were raised was comparatively clean and routine management practices such as feeding, watering, and cleaning were well maintained. Therefore, these diarrheic lambs had less chance of having cross contamination, dehydration and malnutrition. This situation would minimize the severity of disease. In addition, the stage of diarrhea of these lambs was early. Under worse sanitary conditions and persistent diarrhea, the colonic response might be different and bacterial overgrowth in colon with the production of more D-lactate might be possible (Hove and Mortensen 1995; Vella and Farrugia 1998).

We conclude that DLA may not arise secondary to lamb diarrhea as commonly as in calf diarrhea, although diarrheic lambs had mesurable fecal D-lactate concentrations. Our discovery of lumen threshold for D-lactate absorption in lambs would contribute in achieving the prevention of DLA in general. These results may reflect the mild stage of diarrhea in these lambs as well as the sanitary environment of the experimental barn. Therefore, further investigations on a broader scale are suggested.

4.2 Use of malate to prevent D-lactic acidosis

4.2.1 Introduction

D-lactic acidosis is a common problem in humans and animals. In diarrheic calves, DLA is correlated with neurological depression and mortality (Ewaschuk et al. 2003; Ewaschuk et al. 2004; Omole et al. 2001). Similarly, DLA has been blamed for neurological impairments in humans secondary to short bowel syndrome, propylene glycol ingestion and other digestive disorders (Hudson et al. 1990; Jorens et al. 2004; Uribarri et al. 1998). A common feature of these cases is that D-lactic acid originates from the gut and is absorbed into the blood circulation and other body compartments (Bongaerts et al. 1997; Ding and Xu 2003; Hove and Mortensen 1995; Jorens et al. 2004; Omole et al. 2001; Packer et al. 2005; Petersen 2005). Intestinal bacterial over growth (dysbiosis) and decrease in the gut pH play a major role to increase D-lactic acid producing bacteria (Ewaschuk et al. 2005; Hove and Mortensen 1995; Hudson et al. 1990; Uribarri et al. 1998). The persistence of dysbiosis further amplifies rapid fermentation of simple carbohydrates and over growth of lactic acid forming bacteria, which subsequently leads to an excessive and overwhelming production of D- and L-lactic acid. Both D- and L-lactic acids may be rapidly absorbed in to the circulation (Ewaschuk et al. 2005; Ogihara et al. 2000; Poole and Halestrap 1993; Uribarri et al. 1998). Prevention of DLA involves modulating the gut environment and targeting gut bacteria to minimize D-lactic acid production. Probiotics (such as Lactobacillus strains) (Ewaschuk et al. 2004) and prebiotics (such as malate) could be used in the treatment / prevention of DLA.

Malic acid has been used as a common food flavor (Elkins and Heuser 1994; Nielsen and Richelieu 1999). Malic acid is a major pH modulator in many commonly consumed fruits and vegetables (Lobit et al. 2006; Romero Rodriguez et al. 1992; Velterop and Vos 2001; Vorarat et al. 2002); it has also been used in the fermentation of wine and pickles (Passos et al. 2003). Malate in the form of oral medicinal tablets is a proven treatment (Abraham and Flechas 1992; Russell et al. 1995) for some systemic diseases (fibromyalgia). Malate has the ability to modulate pH (Passos et al. 2003; Piva et al. 2002; Russell and Rychlik 2001), and promotes the growth of gut bacteria that can convert lactate to propionate (Evans and Martin 1977; Martin 1998). Malate has been used to reduce rumen D-lactic acid production in cattle (Martin 1998; Nisbet and Martin 1991; Nisbet and Martin 1994; Russell and Rychlik 2001). Therefore, malate may be a potential candidate in the prevention of DLA. We hypothesized that malate has the ability to reduce body D-lactate by converting lactate to propionate, hence reducing gut pH, and eventually promoting a gut environment for favorable bacteria. The primary objective of the present study was to determine the ability of oral malate to reduce D-lactate concentrations in fecal, urine and blood matrices. Additional objectives were to determine an appropriate oral dose and regimen for this beneficial response in calves.

4.2.2 Materials and methods

Use of calves in this study was approved by the Animal Research Ethics Board of the University of Saskatchewan, and was carried out in accordance with the guidelines specified by the Canadian Council on Animal Care (Canadian Council on Animal Care 1993). The calves for this study were obtained from the Dairy Barn, Department of Animal and Poultry Sciences, University of Saskatchewan, Canada. Calves were selected on the basis of breed (Holstein), age (1-6 weeks), and good health in order to assure a uniform sample.

4.2.2.1 Experimental design and treatment regimen

Sixteen healthy calves (27 ± 2 days of age, 61 ± 2 kg) were randomly (n = 4) fed 4 different doses of sodium malate ($NaC_4H_6O_5$; Fuso Chemicals Company Limited, Osaka, Japan); 1000, 500, 50 and 0 (control) mg/kg body weight (BW) daily in milk for a 5 day period. The calf weight was measured (Norac Instaweigh™ Instrumentation Animal Scale; Norac Weighing and Control Systems, Norac International Inc., Saskatoon, Saskatchewan, Canada) a day prior to the start of treatment.

4.2.2.2 Calf care

The calves were housed in the Stone-Barn Calf Unit of Dairy Barn, Department of Animal and Poultry Sciences, University of Saskatchewan, Canada; this environment provided 20 ± 5 °C room temperature, 65 ± 5% relative humidity throughout day, and automated light system from 06.00 to 20.00 h. Each calf was housed in a pen which was made up of wood and concrete with approximate dimensions of 2.0 × 1.5 m^2. The floor of these pens was bedded with 10 cm thick layer of straw. Clean water was provided to these calves in 4 L plastic buckets fixed to the pen wall. Animal technicians performed regular pen cleaning at 08.00 and 17.00 h. The calves were fed after pen cleaning with fresh milk and alfalfa hay. The calves received a bucket of milk (2.5-3.0 L) depending on the body weight. Approximately 1 kg of second cut alfalfa hay was provided on feeder in the pen for consumption by the calves per day. Once calves were moved into this calf unit, the pre-experimental supervisory period was 5 days where the calves were monitored every day for ailments.

4.2.2.3 Sample collection

Feces, urine and jugular blood samples were collected pre-prandially at 08.00 on 0, 3, 5 and 7 days. Venous blood (4 mL) was aspirated for serum separation in order to conduct analysis of lactate isomers and 1 mL of blood was drawn to heparinized syringes

and kept in ice (less than 1 h) until used for blood gas analyses. Serum separation was performed allowing non-heparinized venous blood to coagulate for 20 min, and spinning in a refrigerated centrifuge (IEC Centra-7R Refrigerated Centrifuge; International Equipment Company, Needham Heights, Massachusetts, USA) at 1000 RCF for 30 min. Serum and urine samples were stored in a freezer (-20 °C) until analyzed for lactate. Feces were dissolved 1 to 1 in 1% sodium ethylmercuric thiosalicylate (thiomerosal; ICN Biomedicals Inc., Aurora, Ohio, USA) solution to minimize bacterial growth and stored at -20 °C.

4.2.2.4 Laboratory analyses

Heparinized blood samples were analyzed for pH, HCO_3^- (mmol/L), base excess, (mmol/L), gasses (P_{CO_2} and P_{O_2} , mm Hg), hemoglobin (Hb, g/L), Na^+ (mmol/L), Ca^{++} (mmol/L), Cl^- (mmol/L) and glucose (mmol/L) using an ABL^{TM} 5 Blood Gas Analyzer (Severinghaus 2002) (Radiometer Medical A/S, Copenhagen, Denmark) and an OSM3 Hemoximeter (Haughton and Radcliff 1987; Zwart et al. 1987) (Radiometer Medical A/S, Copenhagen, Denmark).

Fecal, urinary and serum lactate (D and L isomer) concentrations were measured using an HPLC system with a Waters 600 HPLC pump (see the sections 3.1.2.8 and 4.1.2.2).

4.2.2.5 Statistical analysis

Preliminary data on calf characteristics (health, body weight and age), blood gas values and HPLC readings were managed, and graphs and Figures were created using a spreadsheet (Microsoft Excel, Microsoft Corporation, Redmond, Washington, USA). Lactates and blood gas data were analyzed using a statistical analytical software package (SPSS v13, 2005; SPSS Inc., Chicago, Illinois, USA). General Linear Model ANOVA and repeated measures analysis and Student-Newman-Keul's (SNK) test (Steel and Torrie 1980) were performed to differentiate the treatment as well as time effects. Calf body weight was accommodated as the covariate for Repeated Measures analysis. A $P < 0.05$ was considered as evidence of statistical difference. Results were reported as mean and standard error (mean ± se).

4.2.3 Results

4.2.3.1 Fecal and serum lactates

Malate had a significant influence in reducing fecal and serum lactate concentrations after 5 days of treatment. Malate doses from low to high respectively caused a 55, 80 and 60% decrease in fecal D-lactate concentrations (~36 to ~11 mmol/L on average, Figure 20, $P < 0.05$) at 7 days. Fecal L-lactate concentration was decreased by 20–60% (Figure 21, $P < 0.05$) over the same period. At 7 days, serum total lactate declined by 45–59% (Figure 22, $P < 0.05$), D-lactate by 40–62% (~0.5 to ~0.3 on average, Figure 20, $P < 0.05$) and L-lactate by 45-60% (Figure 21, $P < 0.05$). Urinary D-lactate (27 ± 19 mmol/L, Figure 22) and L-lactate (11 ± 15 mmol/L) were not affected by malate, however, values varied with treatment and time.

D-lactate reduction correlated with malate dosage (r = 0.63). Although malate was fed only for 5 days, the greatest drop in lactate (fecal and serum) was reported on the 7th day. None of the doses caused major adverse clinical reactions; however, a drop in milk palatability was apparent at the 1000 mg/kg BW dose. The 500 mg/kg BW dose provided the most consistent decrease in serum D-lactate over time with no feeding difficulties.

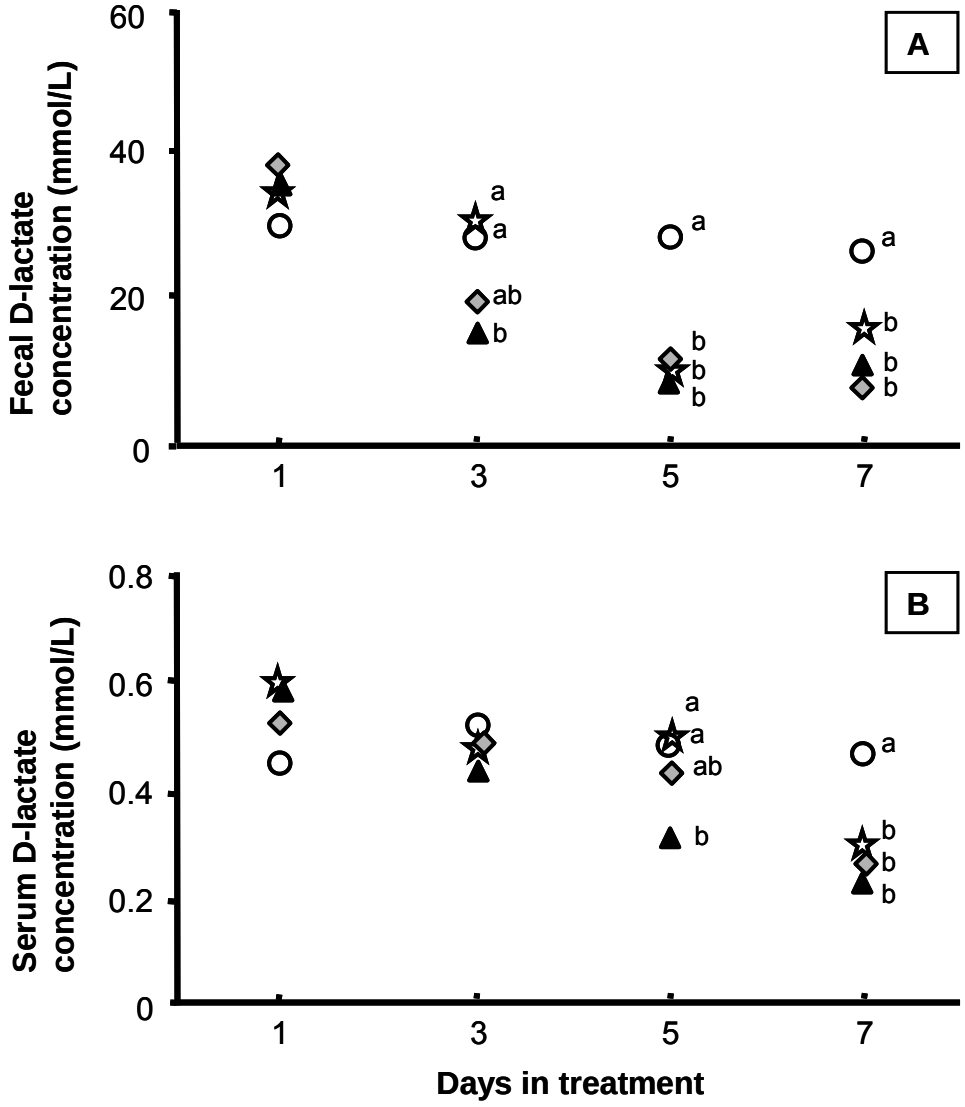


Figure 20. Fecal [graph A] and serum [graph B] D-lactate concentrations (mmol/L) as affected by Na malate doses; 1000 mg/kg BW ▲, 500 mg/kg BW ◆, 50 mg/kg BW ☆ and 0 mg/kg BW ○ (control). Values that do not share a common subscript (a or b) in a day (time point) are statistically significant at $P < 0.05$ by Student-Newman-Keul's test. Treatment, time, and treatment versus time effects indicated by Repeated Measures ANOVA were statistically significant at $P < 0.05$ (n = 4 per treatment group).

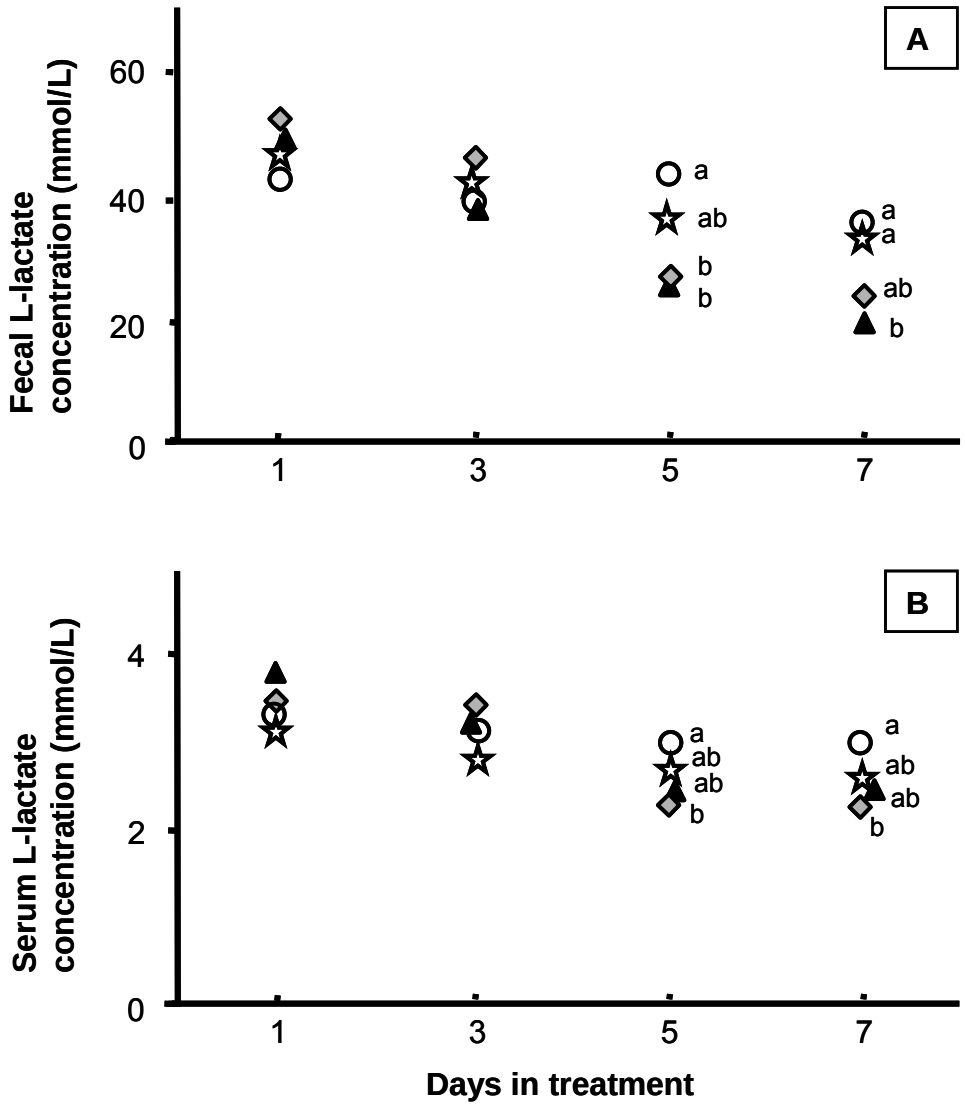


Figure 21. Fecal [graph A] and serum [graph B] L-lactate concentrations (mmol/L) as affected by Na malate doses; 1000 mg/kg BW ▲, 500 mg/kg BW ◆, 50 mg/kg BW ★ and 0 mg/kg BW ○ (control). Values that do not share a common subscript (a or b) in a day (time point) are statistically significant at $P < 0.05$ by Student-Newman-Keul's test. Treatment, time, and treatment versus time effects indicated by Repeated Measures ANOVA were statistically significant at $P < 0.05$ (n = 4 per treatment group).

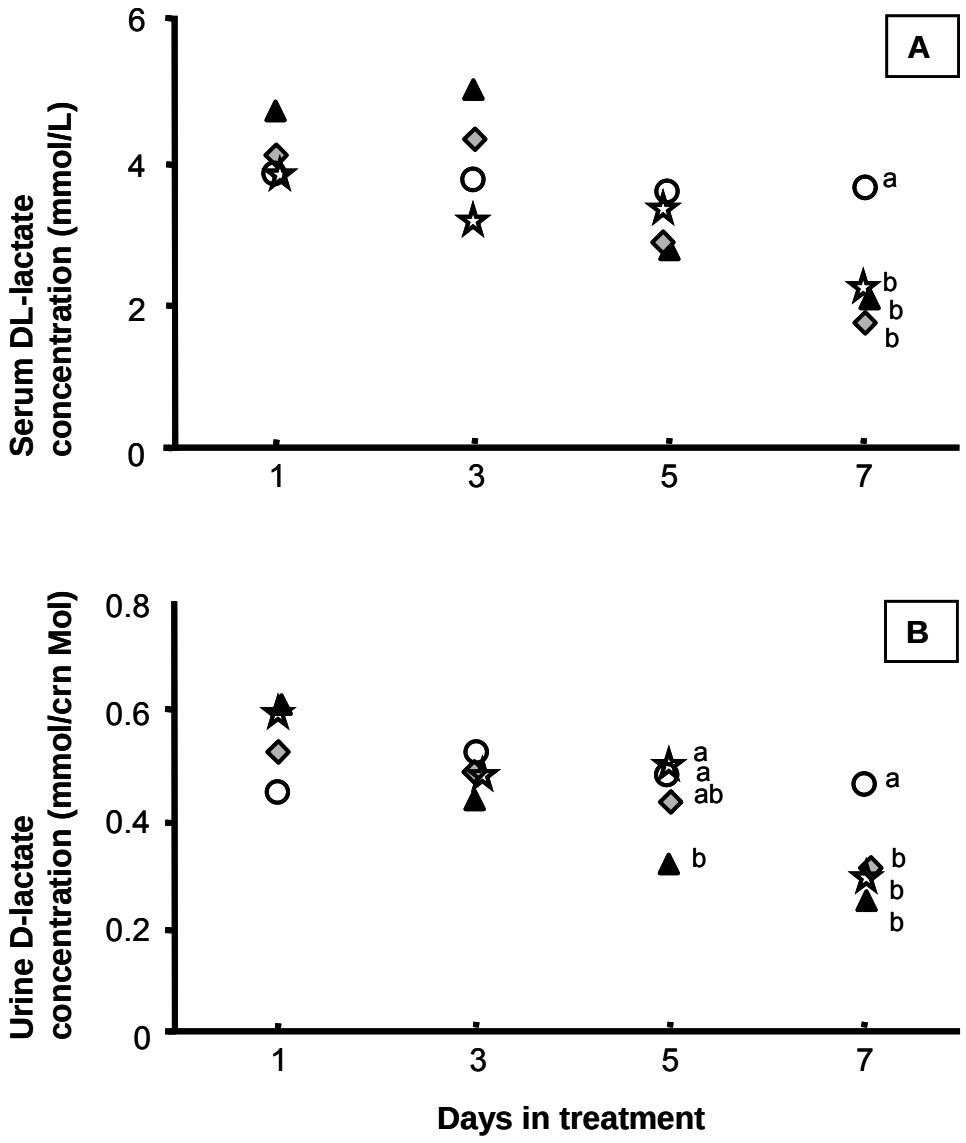


Figure 22. Serum DL-lactate [graph A] concentration (mmol/L) and urine D-lactate [graph B] concentration (mmol/mol of creatinine) as affected by Na malate doses; 1000 mg/kg BW ▲, 500 mg/kg BW ◆, 50 mg/kg BW ★ and 0 mg/kg BW ○ (control). Values that do not share a common subscript (a or b) in a day (time point) are statistically significant at $P < 0.05$ by Student-Newman-Keul's test. Treatment, time, and treatment versus time effects indicated by Repeated Measures ANOVA were statistically significant at $P < 0.05$ (n = 4 per treatment group).

4.2.3.2 Bicarbonate, base excess and other blood values

Blood glucose (4.8 ± 0.3), pH (7.35 ± 0.06), Hb (108 ± 9), Na^+ (133 ± 4), Ca^{++} (1.2 ± 0.2) and Cl^- (99 ± 4) were not affected by malate. A marginal drop in bicarbonate and noticeable drop in BE occurred (Figure 23, $P < 0.05$) at the 1000 mg/kg dose after 5 days of treatment. However, bicarbonate concentrations never dropped below 24 ± 1 mmol/L value (Figure 23). Venous blood gases (P_{CO_2} and P_{O_2}, mm Hg) were not changed statistically although P_{CO_2} ranged 50-53 mm Hg and P_{O_2} ranged 20-35 mm Hg among calves along the course regardless of the treatment.

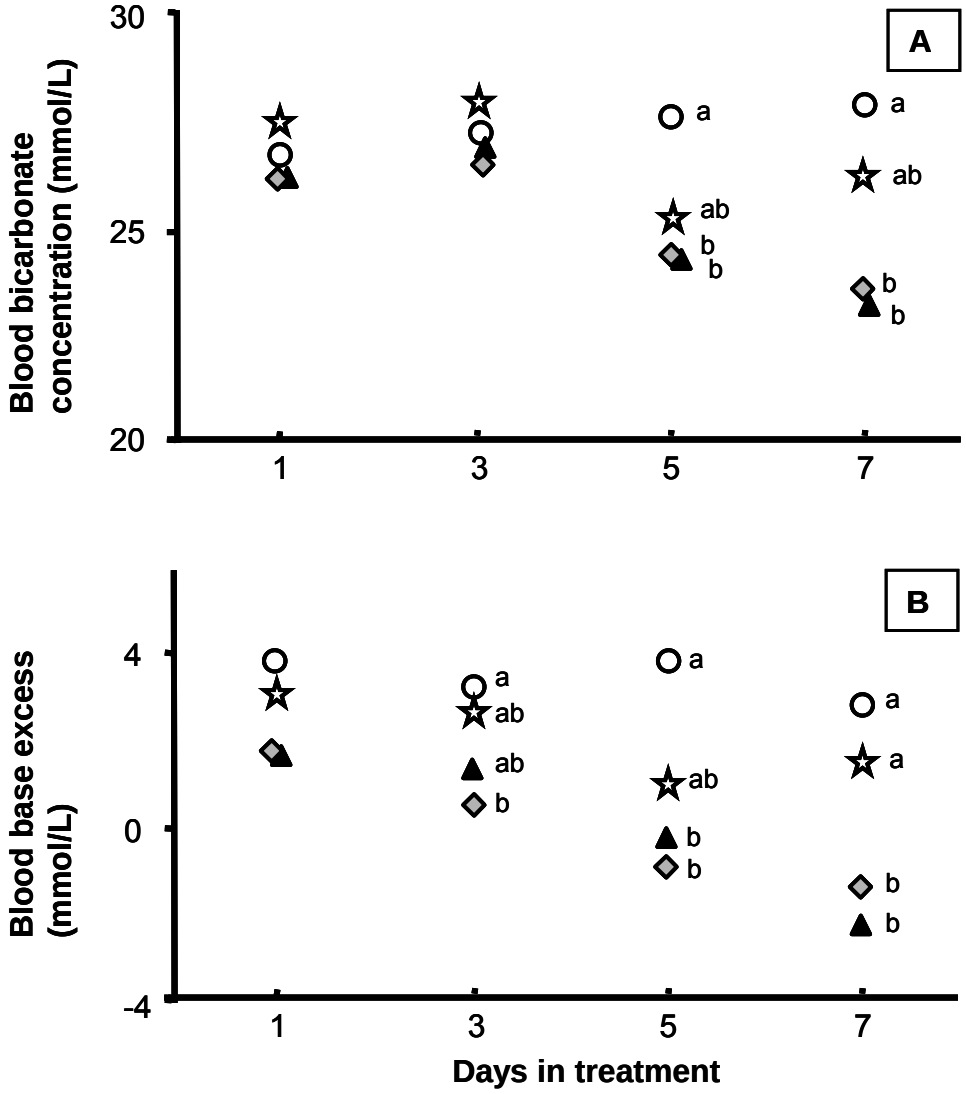


Figure 23. Blood bicarbonate concentration [graph A] and base excess [graph B] as affected by Na malate doses; 1000 mg/kg BW ▲, 500 mg/kg BW ◆, 50 mg/kg BW ☆ and 0 mg/kg BW ○ (control). Values that do not share a common subscript (a or b) in a day (time point) are statistically significant at $P < 0.05$ by Student-Newman-Keul's test. Treatment, time, and treatment versus time effects indicated by Repeated Measures ANOVA were statistically significant at $P < 0.05$ (n = 4 per treatment group).

4.2.4 Discussion

Serum D-lactate concentrations were in measurable quantities (> 0.05 mmol/L) and fecal D-lactates were high in healthy calves at the start of malate treatment. Milk (bucket) feeding may be a reason for these high concentrations of D-lactate. The milk (bucket) fed calves usually retain high colonic fermentation. Colonic lactate production is high even in healthy young calves (Shimomura and Sato 2006) thus both D- and L- isomers are high in feces. Therefore, using healthy calves to understand the effect of malate on lactate reduction is justifiable.

Previously reported DLA cases had serum D-lactate concentration over 2 mmol/L (Ewaschuk et al. 2005; Uribarri et al. 1998). This study showed the effectiveness of Na malate in reducing fecal and serum D-lactate concentrations (clinically non-significant concentrations). Similar effectiveness of malic acid on plasma D-lactate levels was reported by Montano et al. 1999 (Montano et al. 1999) in high-concentrate fed healthy steers after 13 days of malic acid treatment. In an intra ruminal challenge with a high dose of glucose, malic acid did not reduce either ruminal or plasma lactates within 7 h of the challenge. However, malic acid promoted a higher rumenal pH than that in control calves (Montano et al. 1999). In comparison, we evaluated the effectiveness of malate for a longer period (0 to 7 days). We found malate was effective in reducing lactates after 5 days of treatment.

Possible reasons for serum D-lactate reduction include low production of lactates in the intestinal lumen. As affected by malate, fecal D-lactate concentration reduced (from ~36 mmol/L) to ~11 mmol/L. This concentration is closer to our previous finding of lumen (fecal) D-lactate threshold 10 mmol/L for lambs (Section 4.1.3.2) and 9 mmol/L for calves (Apendix C, Ewaschuk et al. 2004). Therfore, D-lactate absorption to the circulation may be minimal. Alternatively, malate may enhance D-lactate utilization in the body tissue. Hepatic uptake of lactate is the major route of serum lactate removal (Ewaschuk et al. 2005; Naylor et al. 1984; Poole and Halestrap 1993; Prior 1983). According to De Beri et al. 2002, rat liver mitochondria can uptake D-lactate rapidly via D-lactate-malate antipoter and D-lactate may be metabolized by D-lactate dehydrogenase present in the inner mitochondrial respiratory chain (De Bari et al. 2002). V_{max} of D-lactate dehydrogenase was reported 15±17 nmol/min per mg (De Bari et al. 2002). Modulation of pH by malate is also a possible benefit for D-lactate transport across plasma membranes (De Bari et al. 2002; Poole and Halestrap 1993). Figure 24 summarizes the possible pathways in serum D-lactate reduction in the gut and the liver.

The decrease in serum bicarbonate in this experiment may not be clinically harmful because the lowest values (24 ± 1 mmol/L) did not drop below clinically critical levels (Robinson and Huffman 1925; Stocker et al. 1999). However, this slight decrease in bicarbonate may be important to follow up in the clinical setting. The drop in base excess caused by high doses of malate may be due the direct absorption and systemic metabolism of malate during the treatment period (5 days). It may also indicate a prebiotic activity with changes in the gut flora and production of different loads of organic acids (Shimomura and Sato 2006). Although venous blood gasses (P_{CO_2} and P_{O_2}, mm Hg) were not changed statistically, there were fluctuations in P_{CO_2} within a range of 50-53 mm Hg, and P_{O_2} within

a range of 20−35 mm Hg among. This indicates the buffering ability in healthy calves and the possible production of different organic acids instead of D-lactic acid in the gastrointestinal tract with malate effect (Graf et al. 1986; Shimomura and Sato 2006; Stocker et al. 1999).

Since malate treatment was more effective after 5 days, prolonged use of malate in potential DLA cases may yield better results. Furthermore, it is reasonable to expect that Na malate may be effective in calves with clinically significant serum D-lactate concentrations (> 2 mmol/L). Malate has been used in monogastric nutrition to improve digestive efficacy and growth promotion by being a buffer or modulator (Partanen and Mroz 1999; Piva et al. 2002). In our experiment, a moderate dose of malate (500 mg/kg BW) was more consistently effective. Dosages used by other workers (Martin et al. 1999; Montano et al. 1999; Vicini et al. 2003) for digestive efficacy and growth promotions ranged from 40 or 80 g per day which is approximately 50 or 100 mg/kg BW.

The use of probiotics and nutraceuticals in clinical diseases has given mixed results (Chermesh and Eliakim 2006). Probiotics played a positive role in rotavirus diarrhea, post antibiotic diarrhea and pouchitis (Chermesh and Eliakim 2006). Probiotics also reduced fecal water content in diarrheic calves (Ewaschuk et al. 2004). Similarly, there was a long debate in the use of organic acids as feed additives (Callaway and Martin 1996; Martin 1998; Partanen and Mroz 1999; Piva et al. 2002) in both monogastric and ruminant nutrition. It is not different when it comes to the use of malate or malic acid (Evans and Martin 1977; Martin and Streeter 1995; Martin and Streeter 1995; Martin et al. 1999; Montano et al. 1999; Russell et al. 1995; Vicini et al. 2003). Therefore, the effectiveness of Na malate in clinical cases needed to be investigated because even the probiotic (*Lactobacillus GG*) did not reduce D-lactate in diarrheic calves in a clinical setting (Ewaschuk et al. 2006). Feeding lower doses of Na malate twice a day may resolve the problem of low palatability that we experienced with higher doses.

We conclude that oral administration of Na malate dissolved in milk at a rate of 50-500 mg/kg BW daily for more than a period of 5 days has the ability to reduce fecal and serum D-lactate. Use of malate dissolved in milk would be readily applicable for on-farm scenarios. However, based on these findings, clinical research investigating the preventative and treatment potential of malate in metabolic acidosis is obligatory.

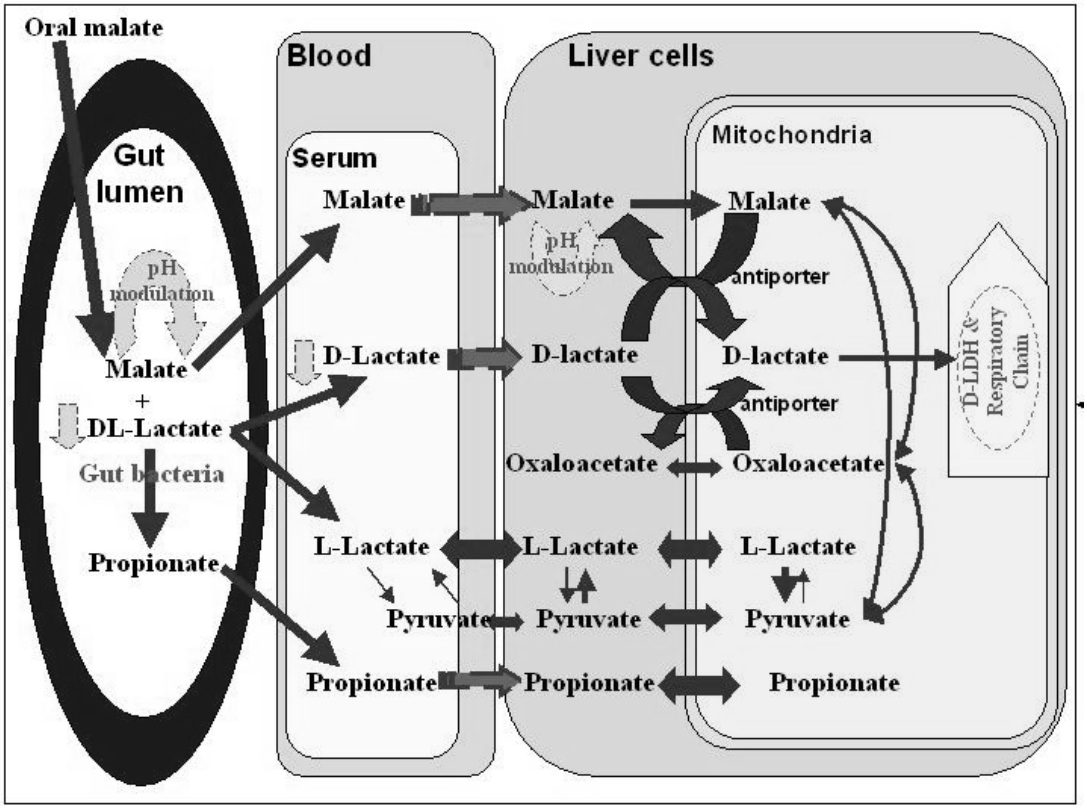


Figure 24. Possible pathways involved in D-lactate reduction with the support of malate in the gut lumen and in the liver cells (De Bari et al. 2002; Evans and Martin 1977; Poole and Halestrap 1993).

5 GENERAL DISCUSSION AND CONCLUSIONS

DLA is associated with gastrointestinal diseases complicated by neurological signs (Jorens et al. 2004; Packer et al. 2005; Petersen 2005; Uribarri et al. 1998). Billions of cases of gastrointestinal diseases, usually including diarrhea occur worldwide, in humans and other species (Bhan et al. 1989; Ewaschuk et al. 2005; Field 2002; Katelaris 1996; Ruuska and Vesikari 1991). The reported neurological signs associated with DLA in humans and animals include slurred speech, loss of reflexes (impairment of palpebral and menace reflexes), abnormal gate, ataxia, paresis and coma (Lorenz et al. 2005; Packer et al. 2005; Petersen 2005; Uribarri et al. 1998). Although clinical studies have consistently shown an association between D-lactate concentration and neurological signs (Hudson et al. 1990; Jorens et al. 2004; Packer et al. 2005; Petersen 2005; Uribarri et al. 1998; Zhang et al. 2003) this thesis is the first to establish a causal link. In many previous clinical studies, understanding the pathophysiology was complicated because other parameters, such as acidemia, deficiencies and dehydration were also associated with neurological impairment. In the controlled studies reported in this thesis severe acidemia and mild impairments of neurological function were induced by HCl infusion alone. DL-LA infusion produced severe neurological dysfunction or disturbance with signs identical to those clinically observed in cases of DLA. This neurotoxicity is independent of acidosis. Toxicity is also unrelated to L-lactatemia. Our studies also rule out hypertonicity as the pathological mechanism for neurological signs, as was proposed recently (Lorenz et al. 2005; Stampfli 2005). Thus, D-lactate is a neurotoxic agent. Since D-lactate can move into neurons with either sodium or hydrogen ions via Na^+- or H^+-monocarboxylate transporters (Martin et al. 2006; Shimozono et al. 1998) either D-lactate or D-lactic acid may be the toxic agent in intracellular environment. The intracellular or interneuronal mechanism that underlies this toxicity needs to be investigated.

The absence of neurological effects following oral administration of DL-LA in a previous experiment (De Vrese et al. 1990) can be explained by the relatively smaller amounts of DL-LA administered per day, and possibly reduced oral absorption, in those experiments with lower serum D-lactate concentrations (< 1 mmol/L). In comparison, eight times higher quantities were delivered by IV infusion to the body in our research study. Therefore, a peak serum concentration of around 36 mmol/L was reached. It is commonly understood that serum D-lactate concentrations have to be at least 3 mmol/L before clinical signs are observed (Uribarri et al. 1998).

In agreement with Lorenz's findings in 2004 we noted that ataxia and loss of the palpebral reflex are correlated with D-lactate concentrations whereas the strength of the suck reflex is correlated with acidemia (Lorenz 2004b). Therefore, we conclude that acidemia depresses the suck reflex but is not responsible for ataxia or changes in the palpebral reflex.

Previous studies in our laboratory established that diarrheic calves with neurological depression have serum D-lactate concentrations as high as 26 mmol/L (Ewaschuk et al. 2004; Omole et al. 2001). The serum D-lactate concentrations (36 mmol/L) in this infusion study are well below the serum value (110 mmol/L) reported in a

patient intoxicated after over-ingestion of propylene glycol (Jorens et al. 2004). Furthermore, these data show that it takes time for D-lactate to diffuse into the CSF. A more gradual and slower infusion could result in greater equilibration between CSF and blood D-lactate concentrations and clinical signs at lower venous serum D-lactate concentrations.

In the nervous tissue, astrocytes are net producers of L-lactate, because their thin processes are too small to accommodate mitochondria (Hertz et al. 2007; Hyder et al. 2006). Neurons metabolize L-lactate (Aubert et al. 2005; Bouzier-Sore et al. 2006; Hyder et al. 2006; Medina and Tabernero 2005; Schurr 2006). The majority (75%) of neuronal oxygen consumption is accounted for by L-lactate metabolism and only 25% by glucose metabolism (Bouzier-Sore et al. 2006). Lactate utilization may be particularly important during acidemia, since low pH can inhibit phospofructokinase activity, a key glycolytic regulatory enzyme (Kreisberg 1980). In neurons, uptake of L-lactate is close to saturation at physiologic L-lactate concentrations (Hertz and Dienel 2005). D-lactate inhibits the ability of isolated optic nerves to generate an action potential, probably by competitively blocking L-lactate entry into neurons and limiting neuronal metabolism (Tekkök et al. 2003; Tekkök et al. 2005). L-lactate can act as an energy substance during ischemia / reperfusion injury and reduces cerebral injury (Cassady et al. 2001; Ros et al. 2001). In contrast, D-lactate enhances neuronal injury in ischemia-reperfusion models (Cassady et al. 2001). D-lactate dehydrogenase is poorly expressed in mammalian nervous tissue, although it is found in mitochondria, particularly in liver and kidney (Flick and Konieczny 2002). We speculate that the D-lactate neurotoxicity observed in this study is the result of reduced L-lactate availability within neurons and energy deficit. In support of this, the serum-CSF L-lactate difference was slightly greater during DL-LA infusion; this may reflect reduced neuronal removal of astrocyte produced L-lactate. Similarly, others have shown that blockade of neuronal monocarboxylate transporters responsible for L-lactate entry results in increased neuronal cell death in the developing brain (Adle-Biassette et al. 2007).

Ethanol also has early effects on energy metabolism (Adle-Biassette et al. 2007; Juhlin-Dannfelt 1977; Volkow et al. 2006) which may be similar to some of the actions of D-lactate. However, in acute ethanol consumption, neurological depression is perhaps caused by low concentrations of circulating tryptophan (Badawy et al. 1995) or interactions with the $GABA_A$ receptor complex and facilitation of GABA action (Gonzales and Hoffman 1991; Starke 1991).

L-lactate can be transported across the blood brain barrier by mono-carboxylic acid transporters (Gladden 2004; Hertz and Dienel 2005; Schurr 2006) which are stereo specific (Tekkök et al. 2003; Tekkök et al. 2005). However, lactate is mainly transported across the blood brain barrier by lipid mediated penetration (Oldendorf et al. 1979). During DL-LA infusion, CSF L-lactate concentrations increased steadily and were higher than serum concentrations by a similar amount in all infusions. L-lactate was probably being formed within nervous tissue by metabolism of glucose and this likely contributes to the higher concentrations in CSF (Aubert et al. 2005; Gladden 2004). In this study D-lactate rapidly crossed the blood brain barrier.

Following the cessation of infusion, serum D-lactate exhibited an exponential decay in this study, and also in previous work (Lorenz et al. 2005). However, CSF D-lactate removal was constant over the period studied suggesting that the metabolic process involved in removal was saturated. Mono-carboxylic transporters may remove D-lactate from CSF (Tekkök et al. 2003; Tekkök et al. 2005). In contrast, D-lactate elimination from blood occurs at least in part through renal clearance (Connor et al. 1983; Ewaschuk et al. 2004; Ewaschuk et al. 2005; Jørgensen and Sheikh 1984; Oh et al. 1985; Ullrich et al. 1982b). Slow elimination of CSF D-lactate would prolong the effects of neurological disturbance and encephalopathy observed in many clinical DLA cases even after treatment has been initiated (Petersen 2005; Puwanant et al. 2005; Uribarri et al. 1998). Serum L-lactate decline was not exponential and the relatively lower concentrations may be partially accountable for our finding of slow removal. Previous studies show that serum L-lactate is cleared through two independent processes, hepatic removal follows second order kinetics while extrasplanchnic removal is linearly related to serum concentration (Naylor et al. 1984). D-lactate may also be up taken by hepatic and extrahepatic tissue (De Bari et al. 2002; Naylor et al. 1984), although some is excreted in urine (Connor et al. 1983; Ewaschuk et al. 2004; Ewaschuk et al. 2005; Jørgensen and Sheikh 1984; Oh et al. 1985; Ullrich et al. 1982b).

D-lactate is a potent neurotoxic agent. According to these findings the neurological disturbance is strongly related to D-lactate accumulation in CSF rather than in blood or to acidosis in CSF. The D-lactate isomer is neurotoxic and may deplete cellular energy metabolism in the nervous tissue. D-lactate accumulation causes the majority of clinical signs of depression and loss of function in a variety of diseases including neonatal calf diarrhea and other animal and human syndromes characterized by DLA. Research needs to be undertaken to determine the precise mechanism of neurological depression caused by D-lactate.

In our second research study IV $NaHCO_3$ therapy eased neurological disturbances. $NaHCO_3$ infusion did not produce paradoxical CSF acidosis in calves made acidotic with DL-lactic acid. Rapid $NaHCO_3$ infusion exhibited a trend for more rapidly correcting CSF acidosis. In agreement with the first infusion study, DL-LA infusion caused neurological dysfunction with severe ataxia. The onset of neurological signs was delayed, however, signs appeared at lower (~40%) serum and CSF D-lactate concentrations. These lower values are compatible with natural DLA found in gastrointestinal diseases (Ewaschuk et al. 2004; Uribarri et al. 1998). However, our study values are higher than the serum D-lactate concentrations of about 3 mmol/L reported in some clinical cases (Uribarri et al. 1998). The suck reflex correlated with acidemia and recovered quickly with $NaHCO_3$ therapy (Kasari and Naylor 1986; Lofstedt et al. 1999; Lorenz 2004b).

The slight acidic tide in the CSF during first few hours of $NaHCO_3$ therapy had no clinical impact in the paradoxic acidosis experiment. The rapid increase in blood pH may have allowed a rapid flow of CO_2 across blood brain barrier from the blood and a paradoxical CSF pH decrease (Herrera and Kazemi 1980; Javaheri et al. 1979; Javaheri et al. 1983; Kraut and Kurtz 2001).

An important finding in the paradoxic acidosis study is that the elimination of D-lactate from CSF and blood was promoted by rapid $NaHCO_3$ therapy. Therefore, the value of $NaHCO_3$ therapy is confirmed as a quick remedy for DLA (Lorenz and Vogt 2006; Lorenz 2007; Petersen 2005; Puwanant et al. 2005). The mechanism that enhances serum D-lactate decrease by $NaHCO_3$ is not well understood. In a previous study hepatic lactate uptake in rat liver (100 μmol/kg $\cdot$ min^{-1}) was increased by 1 mol/L $NaHCO_3$ infusions (Beech et al. 1993). Although D-lactate may not be metabolized by the same enzymes, permeation across transporters and membranes may be enhanced (De Bari et al. 2002; Hertz and Dienel 2005; Poole and Halestrap 1993; Ullrich et al. 1982b). Increasing the alkalinity of blood with bicarbonate does not modulate D-lactate transport via kidneys, however, Na^+ and P_{CO_2} have a positive effect on D-lactate transport (Ullrich et al. 1982b).

Long term exposure (24 h plus) of cells to D-lactate is more comparable to natural DLA than short term exposure. Long term elevations of D-lactate concentrations in blood may allow greater penetration of D-lactate into cells and explain the onset of signs of neurological disturbance at lower serum D-lactate concentrations than in our more acute experiments (Lorenz 2007; Petersen 2005).

In conclusion for the second research study, in the models of severe prolonged acidosis that we used, there appears to be no danger of inducing paradoxical CSF acidosis with IV $NaHCO_3$ therapy at commonly used therapeutic infusion rates. Rapid correction of DL-LA-induced acidosis was exceptional in that DL-lactate concentrations decreased fast with rapid $NaHCO_3$ therapy and this is presumably the explanation for the absence of paradoxical CSF acidosis in this group. However, our results certainly do not exclude the possibility that the effect of $NaHCO_3$ therapy on cellular pH in other critical organs such as the heart exist. Further research studies in animals and humans will be necessary with therapies focused to the type and cause of acidosis.

In our third (clinical research) study diarrheic lambs did not experience DLA within the first few days of onset of diarrhea. Calf diarrhea has resulted in fecal and serum D-lactate concentrations of 25 and 14 mmol/L respectively (Ewaschuk et al. 2003; Ewaschuk et al. 2004). In contrast our diarrheic lamb data showed increased serum D-lactate concentrations but the mean concentrations did not exceed 0.5 mmol/L though fecal D-lactate concentrations averaged 14 mmol/L. Therefore, serum D-lactate concentrations were clinically non-significant (< 2 mmol/L) in those lambs. This may be due to a pathophysiological difference (Field 2002) in the process of D-lactate absorption in upper intestines of sheep (Ding and Xu 2003). Lactic acid absorption (~0.1 μmol/cm^2.min) in sheep intestine was affected by pH, osmotic pressure and lactic acid concentration (Ding and Xu 2003). We found that the threshold level of fecal D-lactate for absorption appeared ~10 mmol/L or above to reach a measurable serum concentration. Lactate transporters (~MCT) in the gut allow the stereoselective uptake of D-lactate greater than L-lactate (Ogihara et al. 2000). Once the lumen D-lactate concentration increases, MCT may start to transport D-lactate into the circulation. This gut-originated D-lactate would produce measurable serum D-lactate concentrations (Ewaschuk et al. 2005; Hove and Mortensen 1995). According to current understanding, the excretion of D-lactate from blood may be fairly rapid if kidneys are healthy and functionally efficient (Jørgensen and Sheikh 1984; Ullrich et al. 1982b). During the early stages of diarrhea D-lactatemia may not arise as fast as in chronic and profound diarrhea cases (Ewaschuk et al. 2003; Ewaschuk et al. 2004)

because of efficient urinary excretion (Jørgensen and Sheikh 1984; Ullrich et al. 1982b). Serum L-lactate concentrations were high in both healthy and diarrheic lambs. The reason may be acute stress and muscular activity resulting from restraint and sampling (Arola et al. 1980); the higher values and greater variability in diarrheic lambs may be the result of dehydration and poor tissue perfusion in some lambs.

Lamb diarrhea primarily caused by intestinal infections negatively affects enzymatic digestion in the affected portion of the intestines (Field 2002; Tzipori et al. 1981). Depending on the type of infection the degree of indigestion and passage of nutrients to the colon is altered (Bongaerts et al. 1997; Field 2002; Luckey 1987; Meier et al. 2003). Therefore, we assume that colonic bacterial over-growth may occur secondary to lamb diarrhea as observed in calves (Ewaschuk et al. 2004; Hove and Mortensen 1995).

Urine D-lactate increase in diarrheic lambs does not indicate DLA and is a weak marker for clinical DLA as reported by previous authors (Haschke-Becher et al. 2000; Inoue et al. 2007; Petersen 2005). The majority of lambs had moderate diarrhea with only minor pathological consequences such as mild metabolic acidosis (Omole et al. 2001). Therefore, D-lactate excretion via the kidneys was sufficient to avoid onset of DLA (Jørgensen and Sheikh 1984; Ullrich et al. 1982b).

A linear correlation between anion gap and serum D-lactate concentration was not found as in calf diarrhea (Ewaschuk et al. 2004; Omole et al. 1999) hence in early stages of lamb diarrhea, D-lactate is not an important contributor to the anion gap in diarrheic lambs. Short term or acute lamb diarrhea may be insufficient to cause severe metabolic acidosis. Farm management practices have an impact on diarrhea syndromes (Meier et al. 2003). Many cases in this study had mild to moderate diarrhea because of better farm conditions. Good management was likely the explanation for the mild to moderate diarrhea. Mild enteritis is probably insufficient for marked intestinal D-lactic acid production. This situation would minimize weight loss and the severity of disease consequences. Therefore, further research is needed using lambs with severe and chronic diarrhea to determine if DLA exists under these circumstances.

Our last DLA prevention study with malate supported the effectiveness of Na malate to reduce fecal and serum D-lactate concentrations. In a previous study an effect of malic acid to decrease plasma D-lactate was reported (Montano et al. 1999). We also found malate was effective in reducing lactates after 5 days of treatment in a standardized setup. Therefore, malate administration may be an effective long term method of preventing DLA.

Decreased production of lactates in the gut lumen may explain the serum D-lactate decrease. Other mechanisms, which enhance D-lactate utilization in the body tissue in the presence of malate, may also be responsible. Hepatic uptake of lactate is the major route of serum lactate removal (Ewaschuk et al. 2005; Naylor et al. 1984; Poole and Halestrap 1993; Prior 1983). Rat liver mitochondria can uptake D-lactate rapidly via D-lactate-malate antipoter and D-lactate may metabolized by D-lactate dehydrogenase present in the inner mitochondrial respiratory chain (De Bari et al. 2002). Modulation of pH is an inherent property of malate which is also supportive for D-lactate transport via plasma membranes (De Bari et al. 2002; Poole and Halestrap 1993).

Colonic lactate production is high even in healthy young calves (Shimomura and Sato 2006) thus both D- and L- isomers are high in feces. Therefore, this study using healthy calves to understand the effect of malate on lactate reduction is justified.

We suggest that prolonged use of malate may be more effective in control of potential DLA cases. Malate can effectively reduce lumen (fecal) D-lactate concentration to the threshold (~10 mmol/L) needed for absorption. This may be possible because malate has been used effectively in monogastric nutrition to increase digestive efficacy and promote growth through its actions as a buffer or pH modulator (Partanen and Mroz 1999; Piva et al. 2002). In our studies, a moderate malate dose (500 mg/kg BW) was more appropriate and consistent than higher or lower dosages. This dosage is consistent with previous work which describes digestive efficacy and growth promotion properties of malate (Martin et al. 1999; Montano et al. 1999; Vicini et al. 2003)

The use of probiotics in calf diarrhea is controversial. In other species they have a positive impact on relieving rotavirus diarrhea, post antibiotic diarrhea and pouchitis (Chermesh and Eliakim 2006). Probiotics are also effective on diarrhea in calves by reducing fecal water content (Ewaschuk et al. 2004). A long debate on the use of organic acids as feed additives was also common in nutrition literature (Callaway and Martin 1996; Martin 1998; Partanen and Mroz 1999; Piva et al. 2002). A similar debate surrounds the use of malate or malic acid (Evans and Martin 1977; Martin and Streeter 1995; Martin and Streeter 1995; Martin et al. 1999; Montano et al. 1999; Russell et al. 1995; Vicini et al. 2003) in different nutritional or clinical perspectives. The effectiveness of Na malate in clinical cases needed to be further investigated.

We conclude that oral administration of Na malate dissolved in milk at a rate of 0.05−0.5 g/kg BW daily for more than a period of 5 days has the ability to reduce fecal and serum D-lactate. Use of malate dissolved in milk would be a worthy, preventive measure for on-farm practice. However, based on our findings, clinical research investigating the preventative and treatment potential of malate in metabolic acidosis is obligatory.

In conclusion, we proved that D-lactate is a potent neurotoxic agent and that clinical signs occur experimentally at concentrations similar to these seen in disease. The common alkali treatment, $NaHCO_3$ infusion therapy is safe and effective in the treatment of DLA and metabolic acidosis. Mild to moderately diarrheic lambs do not develop DLA. Malate can be used as a prebiotic (or premixer with animal feed) to prevent the onset of DLA. Our research studies shed a new light on understanding DLA and D-lactate metabolism and address the prevention, control and treatment of acidosis.

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APPENDICES

A: Measurement of high and low concentrations of D-lactate: An alternate quantification (analytical) method

D- and L-lactate concentrations in biological samples (matrices) were measured using an HPLC system (see the section 3.1.2.8 Laboratory analyses).

A1: Measurement of high D-lactate concentrations

Samples could be analyzed without dilution when concentrations are expected to be within 0.5-40 mmol/L in order to achieve accurate measurement within our (normal standard curve) detection range (0.5–10 mmol/L). Otherwise, samples are appropriately diluted after identifying they contain higher concentrations (> 40 mmol/L) to gain accurate detection (Buglass and Lee 2003; Ohmori and Iwamoto 1988; Okubo et al. 2000; Tormo and Izco 2004). In our model experiments we applied this dilution procedure. All dilutions are performed using deionized water from a Milli-Q synthesis A10 (Millipore Corporation, Bedford, Massachusetts, USA).

A2: Measurement of low D-lactate concentrations

Low lactate concentrations (<0.5 mmol/L) are run with a low concentration linear standard curve (0.05-1.0 mmol/L). By this way we can eliminate curving error effect of very low to very high concentrations (large range).

We also workout to use high sample volume (> 100 μL) in sample preparation (substituting water volume) or high injection volume (> 10 μL) in the autosampler for dispatching higher D-lactate quantity to the column for a countable separation to achieve a visible and quantifiable detection (Buglass and Lee 2003; Ohmori and Iwamoto 1988; Okubo et al. 2000; Tormo and Izco 2004). A standard curve equation ($y = mx + c$) supports quantifiable concentrations $\geq$ 0.05 mmol/L. However, values < 0.04 mmol/L is not used in statistical calculations and 0.04 mmol/L was used as the lowest limit for statistical purposes.

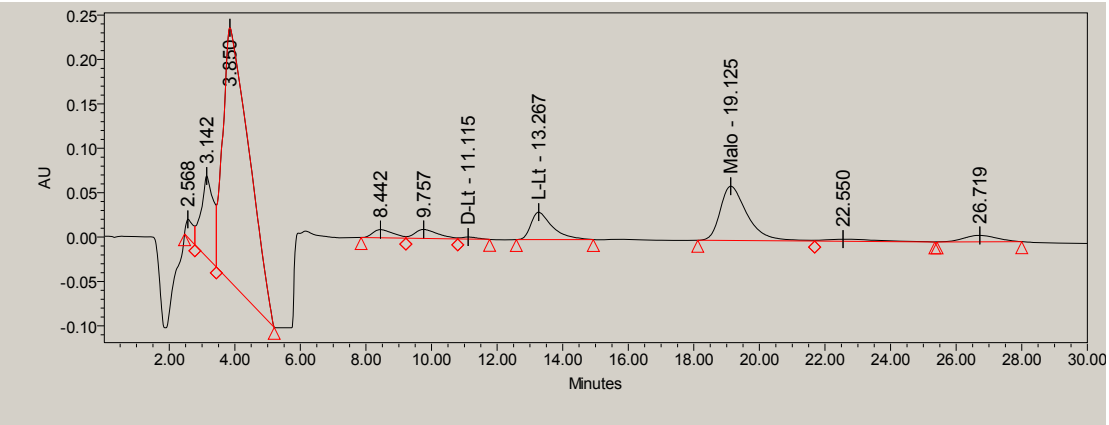

Figure 25 (Appendix A). Appearance of D- and L- isomer peaks at the concentration of 0.05 mmol/L DL-lactic acid in a volume of 100 µL sample following an injection of 10 µL to HPLC system. D-isomer appears at 11.115 min and L-isomer appears at 13.267 min. L-isomer is taller than D-isomer because of the interference of L-lactates from adding standard (healthy) serum.

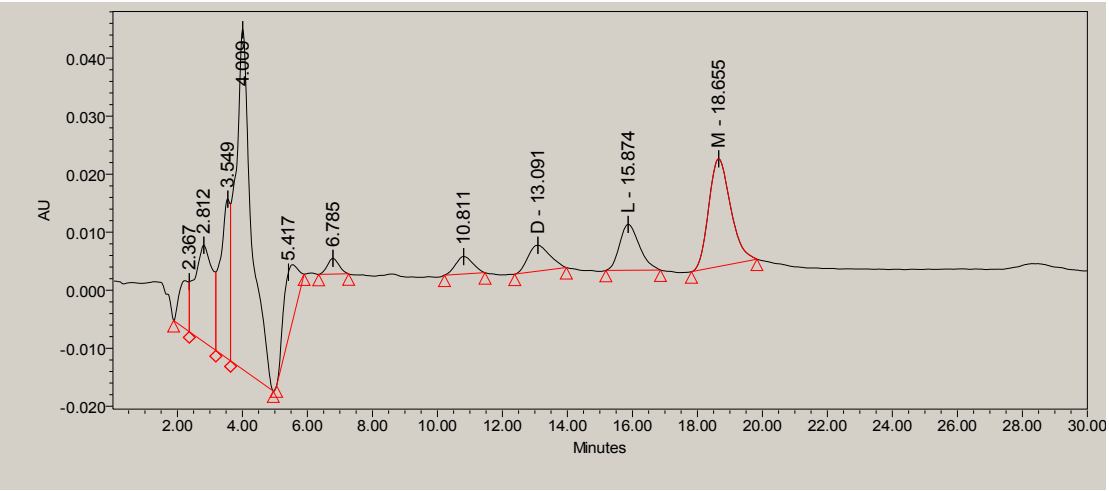

Figure 26 (Appendix A). After increasing injection volume to 20 µL, D- and L- isomer peaks at the concentration of 0.05 mmol/L DL-lactic acid in a volume of 100 µL sample appeared taller. D-isomer appears at 13.091 min and L-isomer appears at 15.874 min. L-isomer is taller than D-isomer because of the interference of standard serum L-lactates.

A3: Reference

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B: Surgical procedures; a calf model for repeated sampling of CSF and blood

B1: Introduction

Repeated sampling of CSF is a requirement in many neurological research studies. However, in common practice the placement and existence of CSF catheter in many species are not satisfactorily attainable or successful. A main constraint with CSF epidural catheter, reported by many authors is the rapid development of fibrous tissue around the catheter, which quickly limits the usefulness of the epidural catheter (Iwase et al. 2002).

The main sites of CSF sampling were the lumba-sacral and atlanto-occipital (Dyce et al. 1996). The atlanto-occipital site reaches cisterna magna (cerebellomedullary cistern) which seems to be a more convenient site for calves and other animals such as rodents. A prerequisite for accurate studies on cisternal CSF is that its composition is not significantly influenced by the sampling technique per se (Huang et al. 1995).

It is important to perform the experiment under least injurious conditions (atraumatic or normal and undistorted) a much as possible (Cox and Littledike 1978; De Craene et al. 1997). In order to over come those mishap, we develop a calf model with indwelling CSF catheter, two venous catheters, and arterial catheter to ensure frequent sampling (every 1-2 h), and infusions to last more than two weeks. In the process of developing a surgically catheterized model, 24 calves were subjected. Calves are fixed with atlanto-occipital CSF catheter, 2 jugular (left and right) venous catheters and ear arterial catheter, infusing stressful acids and repeatedly sampling CSF, venous and arterial blood for 2 weeks.

B2: Materials and Methods

B2.1: Surgery

The surgery is performed in the Department of Large Animal Clinical Science, Western College of Veterinary Medicine, University of Saskatchewan. The calves were premedicated with intravenous hydromophone at 0.1 mg/kg, sometimes in combination with metatomidine at 7 µg/kg or medetomidine at 4 µg/kg, induced with a combination of ketamine at 2 to 6 mg/kg and diazepam at 0.1 to 0.5 mg/kg, intubated and maintained under gaseous isoflurane anesthesia. Strict aseptic technique was followed throughout the catheter placement procedure.

B2.1.1: Atlantooccipital catheter

The calf is appropriately anesthetized and kept in lateral recumbence. A 12 by 12 cm area of the skin, centered on the atlanto-occipital space is surgically prepared (Bouman and Van Wimersma Greidanus, T.B. 1979; Cox and Littledike 1978; Gordh and Wiklund 1986; Jones and Robinson 1981). The calf head is flexed in 90 degree angle by the assistant. A stab incision 3 mm wide and 7 mm deep is made with a #15 scalpel blade through the skin over the atlanto-occipital space (2/3 from the posterior end of atlas towards the level of lateral ridges of occipital vertebra). A spinal (Tuohy) needle (18 G, 9 cm) from a freshly opened epidural kit (Mila International Inc., Kentucky, USA) is inserted (controlled jabbing, 3-5 cm from the skin depending on the body size of the calf) into the subarachnoid space (cisterna magna, site confirmed by needle ‘pop’ and aspiration / flow of CSF fluid; several 'pops' may be felt as the needle passes through various tissue planes; Figure 27 and Figure 28). It is vital to ensure that the needle is parallel to the floor (or surgery table) and is being advanced perpendicularly to the patient's spine exactly on the patient's midline and that the open bevel of the needle tip is facing caudally. The stylet of the Tuohy needle is then removed and carefully wiped on a sterile portion of the gloved hand to ensure no cerebrospinal fluid is visible. Accurate placement of the needle into the epidural / subarachnoid space is then verified using the loss of resistance, and free flow of clear cerebrospinal fluid. The catheter (20G, 9203 wire reinforced, closed end) is threaded 10 to 15 cm into the space, the needle withdrawn, a sampling adaptor and 0.22 µ bacterial filter device attached to the open end of the tube. A clean tape butterfly or clamp style rubber butterfly from the kit fixed the catheter near the skin-catheter interface is sutured to ensure that the catheter is rigid, but, flexible and unlikely to kink. The sampling tip (infusion cap) of CSF catheter is placed in a sterile plastic bag with an antiseptic such as hibitane.

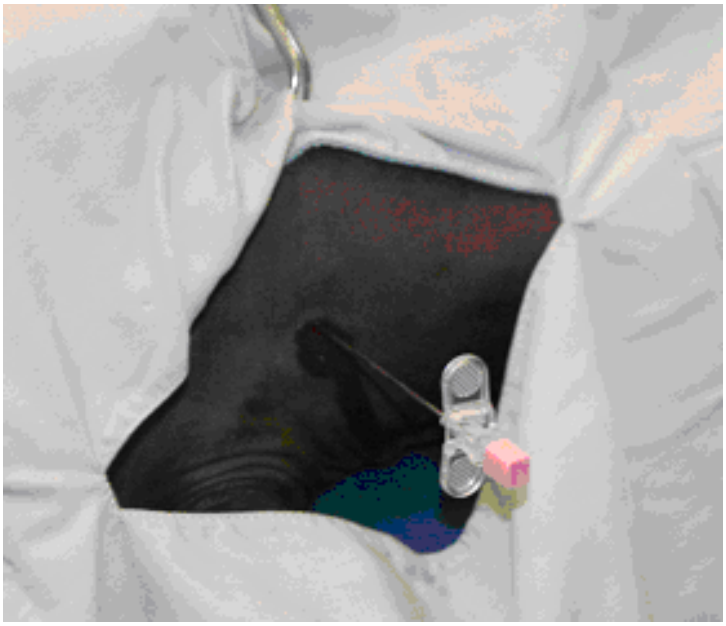

Figure 27 (Appendix B). CSF / epidural needle (Tuohy) is inserted through atlanto occipital junction of a calf.

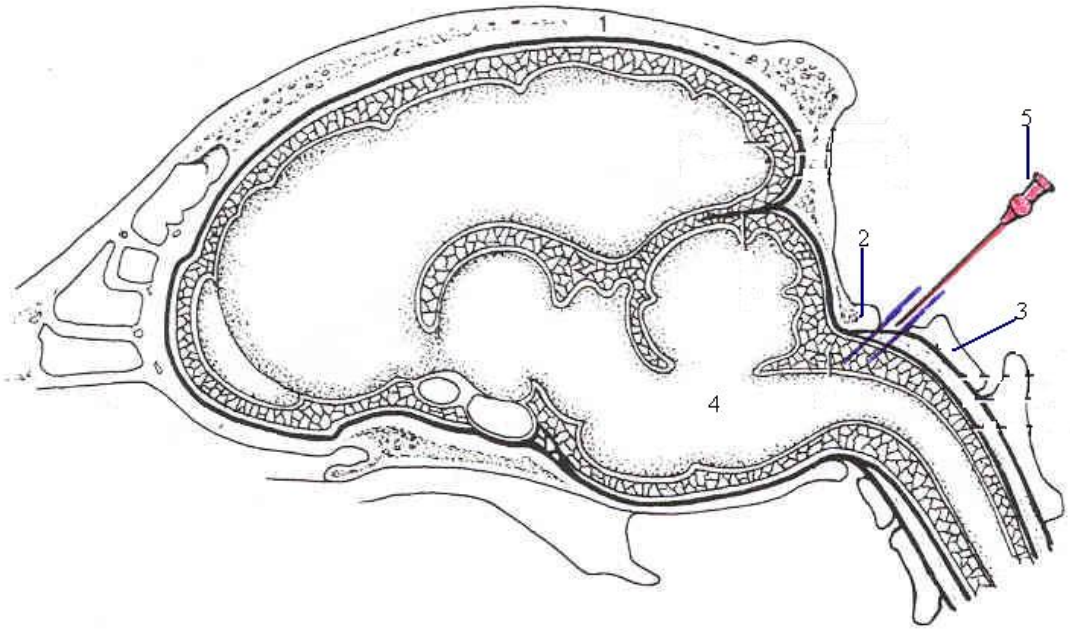

Figure 28 (Appendix B). CSF catheter placement through atlanto-occipital joint: 1, calvaria; 2, occipital; 3, atlas; 4, cerebellum + colliculus + medulla oblongata area; 5, Tuohy needle pointed to duramater + epidural space + subarachnoid space in cerebellomedullary cistern (adopted from Dyce et al.1996).

B2.1.2: Intravenous catheter

An incision is made with a #15 scalpel blade on a side of the calves' neck adjacent to the jugular vein after this area has been shaved and appropriately disinfected (surgically prepared). A catheter is made using clean vinyl tube (Dural Plastics & Engineering, Auburn, Australia) with an inner diameter of 1 mm and a length of 45 cm. The catheter is placed through a needle into the jugular veins. The catheter is placed 25-30 cm into the vein (depending on the calf body weight), and tunneled subcutaneously (5-6 cm) to maximize the durability. Then it is sutured to the skin with sutures using a piece of tape folded in butterfly shape. Both jugulars are catheterized; one catheter will be used for infusion and the other for blood sampling.

B2.1.3: Arterial catheter

Dorsal surfaces of ears are shaved and surgically prepared. A freshly opened arterial catheter (BD Insyte^{TM}, Becton Dickinson, Infusion Systems Inc., Sandy, Utah, USA) was placed in (22 G [0.9 mm] × 25 mm of length) ear (auricular) artery on the dorsal surface of one of the ears. The catheter is fixed and glued or sutured with a clean tape. The entire neck is covered with sterile dressing to protect the three catheters. Pockets are designed in the dressing to allow easy access to the catheters, and endured maximum security to all catheters from later animal accidents and aberrations.
Calves are clinically evaluated everyday for change in body temperature or other discomforts. Daily body temperature is recorded.

B2.2: Justification

Catheterization of the atlantooccipital space is chosen as it was deemed more successful than catheterization of the lumbosacral space in previous attempts. Compared to the lumbosacral space, catheters in the atlantooccipital space were placed easier and lasted longer. The reason may be the space in cisterna magna being larger. An iso-osmotic infusate is used, as it is least likely to cause hemolysis or other complications.

B2.3: Statistical analysis

Data were analyzed using SPSS v13, 2003. SNK (mean comparison) and Pearson correlation procedures were also performed. A $P < 0.05$ was considered as evidence of statistical difference.

Table 6 (Appendix B). Viability of catheters placed in calves

Calf	CSF catheter > 1 or 2 wk	CSF quality (1 wk)				Venous catheter > 1 or 2 wk	Arterial catheter > 1 or 2 wk
		Color	Protein g/L	RBC 10_6/L	Nucleated cells 10_6/L		
488	1	c	0.2	1000	3	2	1
498	1	c	0.3	10	16	2	1
499	2	c	0.2	1	3	2	1
501	1	c	0.3	1	5	2	1
502	2	c	0.2	1	1	1	1
504	1	c	0.2	1	8	2	2
526	2	c	0.3	1	10	2	1
527	2	c	0.2	10	6	1	1
530	2	c	4	99	187	2	2
534	2	t	5	1	7000	2	2
560	2	c	0.2	74	1	2	1
572	1	t	5	1	7000	2	1
610	2	c	0.3	44	16	2	1
660	2	c				2	2
528	2	c				2	1
541	2	c				2	2
546	2	c				2	2
319	1	t				1	1
489	2	c				2	2
536	2	c				2	1
573	2	c				2	1
580	2	c				2	2
529	2	c				2	1
556	2	c				2	1
549	2	c				2	2

Visual quality of CSF (color): c for clear and t for turbid.

RBC, red blood cells

wk, weeks.

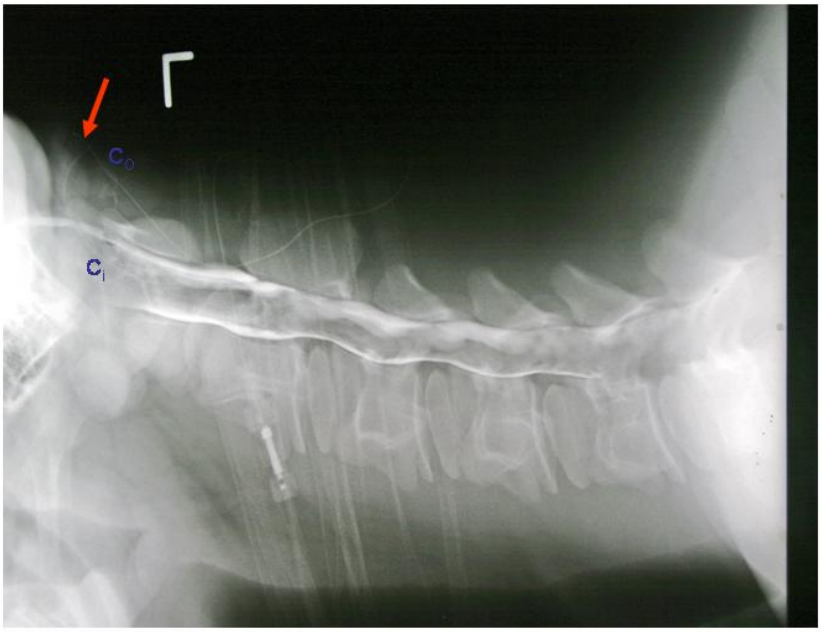

Figure 29 (Appendix B). X-ray picture after surgery; Ci, catheter inside portion in subarachnoid space; Co, catheter outside portion on skin; red arrow showed the site of insertion.

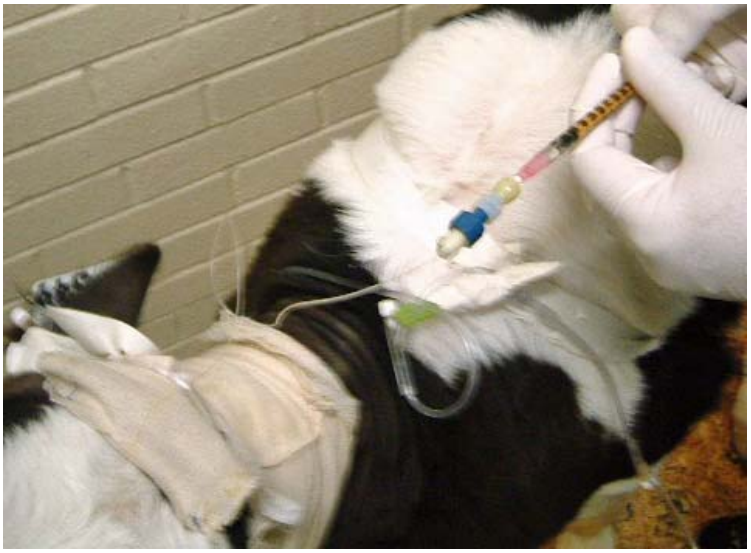

Figure 30 (Appendix B). Post surgical rapping around cervical area is applied for securing the site of catheter. CSF sample (~0.7 mL) is carefully aspirated after removing a 0.2 mL dead volume.

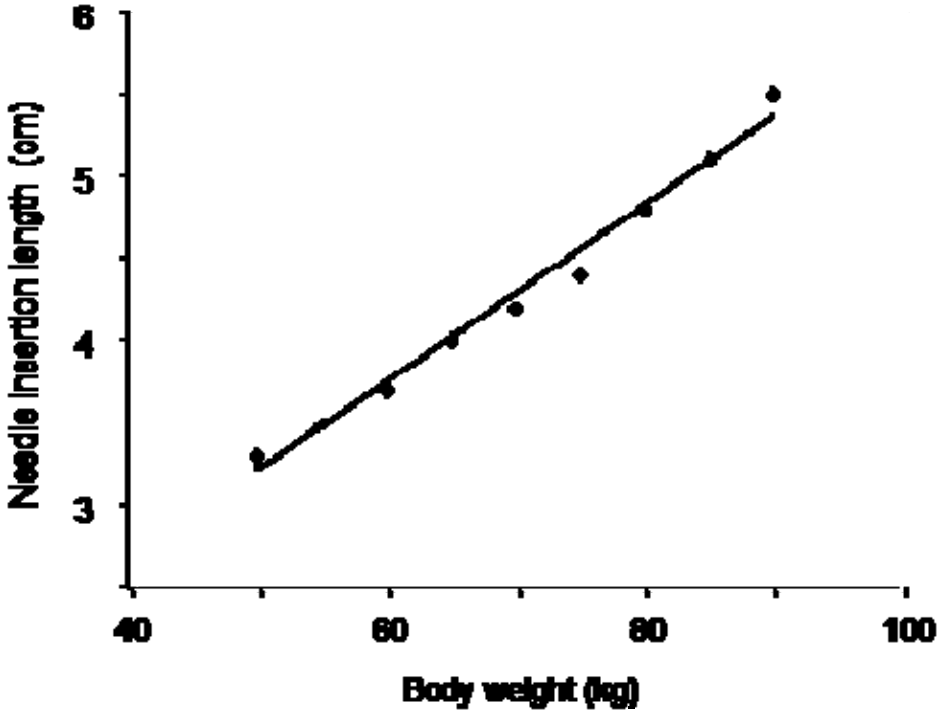

Figure 31 (Appendix B). Calf's body weight correlates with CSF catheter needle insertion length (skin to epidura). The needle insertion length (y) can be calculated using the equation, $y = 0.5x + 0.5$ where x is calf's body weight (kg). Correlation coefficient (r) is 0.9 ($P > 0.05$).

B3: Results and discussion

B3.1: CSF catheter

Accurate placement of CSF catheter radio-graphically confirmed (Figure 3). Safe sampling could be carried out (Figure 30) with minimal animal restraining by a single technician. There as no any noticeable discomfort either to animal or sampler. The existence of indwelling CSF catheter over 2 weeks was successful (90%, Table 6) with stressful infusions. Free CSF flow and clear fluid were found (> 75%) over 2 week period. However, CSF quality was deteriorating with time (50%) indicating higher cell counts and protein content. The difficulty in maintaining an entirely aseptic environment around the catheter seemed to be a main reason to provoke signs of cell reaction (infection and inflammation). No microbes were found except single case (> 5%) in the CSF samples. There was a strong correlation ($r = 0.9$) between calf body weight and the length of CSF catheter needed to penetrate into cisterna magna for free flow of CSF (Figure 31). This relationship which could be estimated with this simple formula, $y = 0.05x + 0.5$, may torch guidance in future CSF catheterizations.

B3.2: Implications

This cauterization model would be useful to future neuro-metabolism studies. These procedures may be applicable to other species such as canine, feline and caprine.

B4: Reference

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C: Diarrheic calf fecal D-lactate threshold

Data from diarrheic calves collected in 2001 to 2004 by our research group was analyzed to find fecal D-lactate threshold.

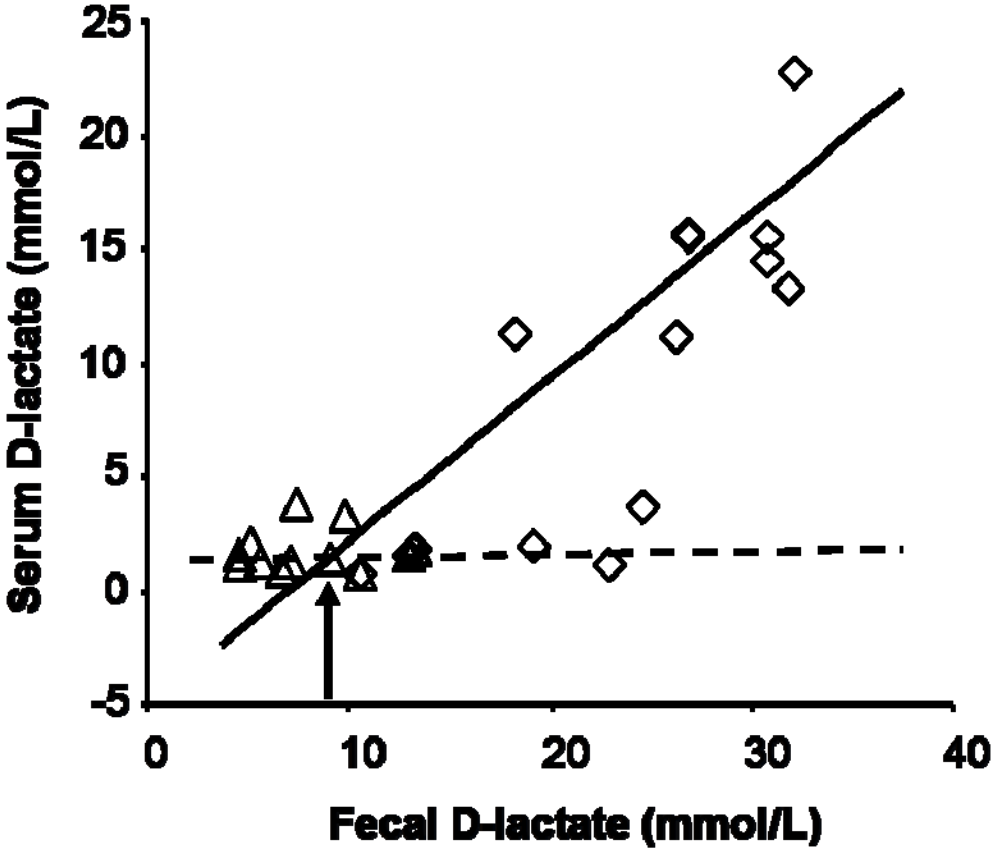


Figure 32 (Appendix C). Break Point Analysis showing a threshold concentration for absorption when fecal D-lactate exceeded 8.82 mmol/L (↑) in diarrheic calves. The regression equation for Break Point Analysis consists of serum D-lactate concentration (y), fecal D-lactate concentration (x) and a dummy variable (z), n = 23. The equation for the estimated thresh hold is shown below.

Equation 5. $y = -9.45119 + 0.80199\ x + 9.70740\ z - 0.56156\ (x*z)$

This equation definitions indicate, z = 0 for the fist line and z = 1 for the second line. First regresssion line (—, strait) was associated with square shape (◇) data points and the second regression line (----, broken) was associated with triangular (Δ) data points. Linear Correlation between fecal and serum D-lactate concentrations (r = 0.75). Pease see the section 4.1.3.2.1 (Figure 18) for diarrheic lamb's fecal D-lactate threshold.

D: Ethics certificates

Copies of Ethics Certificates for both model and clinical experiments are shown below.

Memorandum

TO: Dr. Naylor, Large Animal Clinical Sciences
FROM: UCACS Protocol Review Committee, Animal Resources Centre
DATE: 27-May-04
RE: Animal Care Committee Review of Your Protocol - Effect of organic acid infusion on blood and CSF pH: correlations with demeanor

PROTOCOL ID: 20040046

The Protocol Review Committee of the University Committee on Animal Care and Supply recently reviewed and approved the above-noted protocol for the next twelve months.

The Biosafety Office of the Department of Health Safety and Environment (DHSE) requires that all university principal investigators / laboratories working with animals, animal blood, tissues or excreta must be registered with the Biosafety Office. Working with blood, tissues or excreta from healthy animals are considered level 1 biosafety activies. Please contact Ms Corrine Harris, Biosafety Manager (telephone 8496, email: corrine.harris@usask.ca.

Thank you.
Sincerely,

Chair, UCACS Protocol Review Committee
cmm

MEMORANDUM

TO: Dr. J. Naylor, Large Animal Clinical Sciences

FROM: Colette Wheler, UCACS Protocol Review Committee, Animal Resources Centre

DATE: June 23, 2005

SUBJECT: Animal Care Committee Review of Your Protocol #20050064 – Does quick correction of acidosis produce paradoxical acidosis?

The Protocol Review Committee of the University Committee on Animal Care and Supply recently reviewed the above-noted protocol and approved it for the next twelve months, with the following conditions:

1. Re: Defining an Appropriate Endpoint in Invasive Studies. Please forward an endpoint checklist for your study. See the attached examples for more information. You can view the entire document: "Guidelines on choosing an appropriate endpoint in experiments using animals for research, teaching and testing" at:
http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/ENDPTS/APPOPEN.HTM

Thank you for assisting the PRC in this matter.

Sincerely,

Colette L. Wheler, DVM, MVetSc
for Protocol Review Committee

cmm

Memorandum

TO: Dr. Lohmann, Large Animal Clinical Sciences

FROM: UCACS Protocol Review Committee, Animal Resources Centre

DATE: 30-Aug-06

RE: Animal Care Committee Review of Your Protocol - Does quick correction of acidosis produce paradoxical acidosis?

PROTOCOL ID: 20060047

The Protocol Review Committee of the University Committee on Animal Care and Supply recently reviewed and approved the above-noted protocol for the next twelve months.

Thank you.
Sincerely,

Chair, UCACS Protocol Review Committee
cmm

Memorandum

TO: Dr. Naylor, Large Animal Clinical Sciences

FROM: UCACS Protocol Review Committee, Animal Resources Centre

DATE: 26-Feb-04

RE: Animal Care Committee Review of Your Protocol - Determination of serum, fecal and urinary organic acids in diarrheic calves, lambs and kids, and the effects of the oral administration of sodium malate

PROTOCOL ID: 20040014

The Protocol Review Committee of the University Committee on Animal Care and Supply recently reviewed and approved the above-noted protocol for the next twelve months.

Thank you.
Sincerely,

Chair, UCACS Protocol Review Committee
cmm

E: Study Protocols

Protocols and Tables for both model and clinical experiments are shown below.

Infusion Protocol

Sample collection takes place at -1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 hours. During these times, samples are collected, analyzed, and stored; as well the calf is assessed.

At each sample collection time the following happens:

- Two blood (3mL and 1mL) and one CSF (1mL) sample are taken.
- Urine is collected in a urine tube, and the total urine volume is measured.
- The calf is assessed for its suck, tactile and menace reflex, and ability to stand.
- The samples are taken to the lab to be analyzed and stored.

Materials:

- 12cc syringes (box)
- 3cc syringes (box)
- 20g, 1" needles (box)
- 1cc syringes with needles (box)
- Test tubes
- Sharp's container
- Pump
- Extension sets
- Assembly sets
- Infusate and tubing
- Ziplock bags
- A-cups (labeled)
- Ice box
- Hepernized saline
- Alcohol swabs
- Lubricant
- Thermometer
- Intermittent infusion plug
- Tape
- Elastoplast
- Scissors

Blood Samples:

First extract about 10cc's of blood from the jugular catheter. Then, take a 3cc syringe to extract blood into a test tube. A hepernized 1cc syringe is used to extract blood from the catheter. This syringe is rolled and then placed in ice. Inject the 10cc's of blood back into the catheter followed by 10cc's of hepernized saline to flush the catheter. This syringe can stay attached to the catheter to act as a plug, and to be used to draw out blood during the next sampling, but if the catheter has an intermittent infusion plug on it, this wont be necessary.

CSF samples:

A 1cc, non-hepernized syringe is used to extract CSF fluid. The CSF catheter should have an intermittent infusion plug on it. About a half cc is drawn then thrown out then a full cc is removed and kept in ice.

Urine:

A pan is kept under the calves' crate to collect urine. Rubbing the peritenum of the calf aids in his urination. Once the calf is finished urinating, the pan can be removed and emptied into a measuring cylinder to record the volume of urine. Some urine can

collected into a urine tube from the measuring cylinder or directly from the calf if he is standing. The urine is kept in ice.

Calf Assessment:

A lubricated thermometer is placed in the calves' rectum to measure the body temperature. Menace, tactile and suck reflexes are tested. The calf is lifted and then tested to see if he can stand properly. For these assessments, a '0' is normal, and a '2' is the worst score. These assessments, as well as the bodily temperature and urine volume are recorded.

Lab Work:

When going to the lab, the student should take the 1mL blood and CSF samples in ice along with the urine sample. Also, the test tube with the 3mL blood sample should be taken to the lab, but not placed in ice.

When the samples are taken to the lab, the 1mL blood and CSF samples are put through the blood gas machine. The results are printed, labeled with the calf ID, date and sample (blood or CSF) and time of sampling. They are then placed in order with the results of the other times. These results should also be placed onto a results sheet that will have the results for the entire infusion.

The CSF fluid can be placed directly into A-cups and then frozen in correctly marked zip-lock bags. The blood sample should used to measure PCV and total hemoglobin, and then disposed of. Urine should also be put into A-cups, but the remaining urine should be kept for other testing.

The test tube can then be centrifuged, and the serum collected into A-cups.

Make sure all A-cups and zip-lock bags are correctly labeled.

Preparation of Materials for Infusion

During infusion, there should be a box of 12cc, 3cc and 1cc syringes ready. Also, a box of needles and several urine collection tubes should be there.

Before the Infusion prepare two sets of 13 1cc needles. Label one as CSF, and the other as blood. Also add the hour (-1, 0, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24) to the label. Prepare 13 test tubes and add the hour to their labels as well. Allocate two or three test tubes to take samples from the infusate. Take these to site of infusion.

In the lab keep three sets of 13 A-cups. Label one set as CSF, another set as Serum, and the third as Urine. Add the hours to the labels.

Table 7 (Appendix E). Infusate composition; pre versus post autoclaved DL-lactic acid, L-lactic acid and HCl.

Infusate	Measurement	Pre-autoclaved	Post-autoclaved
DL-lactic acid	Weight, %	100	99.93 ± 0.02
	Total lactate, mmol/L	292.9 ± 4.2	302.7 ± 4.6
	D-lactate, mmol/L	141.5 ± 1.4	144.7 ± 1.1
	L-lactate, mmol/L	146.5 ± 2.0	150.6 ± 1.4
L-lactic acid	Weight, %	100	99.92 ± 0.02
	Total lactate, mmol/L	295.5 ± 3.9	299.8 ± 4.1
	D-lactate, mmol/L	nq	nq
	L-lactate, mmol/L	293.6 ± 3.7	299.1 ± 4.3
HCl	Weight (%)	100	99.92 ± 0.03
	Chloride*, mmol/L	358.6 ± 5.7	364.7 ± 4.1
	Chloride†, mmol/L	304.5 ± 2.0	313.1 ± 2.6

* Hitachi blood gas machine
† Digital chloride meter
nq, not quantified (concentrations were lower than our detection limit, 0.05 mmol/L).

Revised Protocol for Creation of Lactic Acid

DL-Lactic acid:

Amount needed: 4L
Concentration: 0.3M

4L x 0.3M x 90g/mol x 100g(solution) / 85g (lactic acid) = 127g (solution)

This amount of solution can be diluted with distilled water until the total volume is 0.400L*. The solution should be autoclaved. Before infusion, this volume can be added to distilled water in a 3600mL brown bottle. The new solution will therefore be a 0.3M, 4L solution.

L-Lactic acid:

Amount needed: 4L
Concentration: 0.3M

4L x 0.3M x 90g/mol x 100g(solution) / 30g (lactic acid) = 360g (solution)

This amount of solution can be diluted with distilled water until the total volume is 0.400L*. The solution should be autoclaved. Before infusion, this volume can be added to distilled water in a 3600mL brown bottle. The new solution will therefore be a 0.3M, 4L solution.

HCl:

Amount needed: 4L
Concentration: 0.3M

100g (solution)/37g (HCl) x 36.5g/1 mol x 0.3mol/L x 4L = 120g (solution)

This amount of solution can be diluted with distilled water until the total volume is 0.400L*. The solution should be autoclaved. Before infusion, this volume can be added to distilled water in a 3600mL brown bottle. The new solution will therefore be a 0.3M, 4L solution.

*The solution can be kept in 1L bottles, wrapped in tin foil, and kept under the autoclave.

An empty 3.6L brown bottle weighs 1.33Kg. With this information, and the weight of each full bottle, the amount of distilled water already in the brown bottle can be determined.

A sample Table for recording basic information on infusions and calves is shown below.

Infusion Sheet

	Date:	Infusate Code	Infusion	Concentration	Calf
1	21/06/04	A1, A2, A3, A4	DL-lactic acid	1·00 M	8648572
2	23/06/04	B1, B2, B3	Saline	0·300M	"
3	24/06/04	C1, C2	L-lactic acid	1·00M	"
4	29/06/04	D1, D2	DL-lactic acid	0·300M	8648573
5	05/07/04	E1, E2	Saline	"	"
6	07/07/04	F1, F2	L-lactic acid	"	"
7	09/07/04	G1	Hydrochloric acid	"	"
8	23/07/04	H1, H2	DL-lactic acid	"	319
9	18/08/04	I1, I2	DL-lactic acid	"	277-634-660
10	18/08/04	J1, J2, J3	Saline	"	"
11	20/08/04	K1	L-lactic acid	"	"
12	24/08/04	L1, L2	L-lactic acid	"	"
	26/08/04	M1, M2	HCl	"	"
	8/10/04	N	L-Lactic Acid	"	8648582-498
	8/10/04	O	SALINE	0·3 M	"
	26/10/04	P	DL-LACTIC ACID	"	8648583-499
	27/10/04	Q	HCl	0·3M	"
	29/10/04	R	L Lactic Acid	0·3M	"
	1/11/04	S	Saline	"	"
	22/11/04	T	HCl	"	8648585 501
	29/11/04	U	Saline	0·3M	8648585 502
	30/11/2004	V	HCl	0·3M	"
	2/12/2004	X	L-Lactic Acid	0·3M	"
	3/12/2004	Y	DL-Lactic Acid	0·3M	"
	9/12/2004	Z	L-Lactic Acid	0·3M	8648585504
	10/12/2004	1	DL-Lactic Acid	"	"

Experiment completed

DL DY H I P

L F L N R

HCl C M Q T V

Saline E H O S U

A sample Table used for recording blood gas results during the experiment (24 h) is shown below.

Blood Gas Results

Calf ID: 864 8582-498 Date: 06/10/2004 Experiment:

Actual Time	Time (hrs)	Sample	pH	pCO_2	pO_2	sO_2	$HCO3^-$	ABE	PCV	tHb	T
	-1	Blood	7.36	49	30	47	26	1	.34L	10	39.1
		CSF	7.33	47	84	94	23	-2			
		Urine									
	0	Blood	7.36	49	38	62	27	2	.27	9	39.3
		CSF	7.35	47	80	93	25	0			
		Urine									
	1	Blood	7.18	66	40	53	23	-5	.25	8.3	38.8
		CSF	7.32	50	76	92	24	-1			
		Urine									
	2	Blood	7.13	77	41	52	24	-5	.27	9	38.8
		CSF	7.30	53	20	89	25	-2			
		Urine									
	3	Blood	7.32	51	33	54	26	-0	.26	8.7	38.8 CHECKED
		CSF	7.31	51	80	93	24	-1			
		Urine									
	4	Blood	7.32	53	33	80	26	0	.27	9	38.8
		CSF	7.31	50	94	95	24	-2			
		Urine									
	5	Blood	7.13	76	21	60	25	-4	.27	9	38.9
		CSF	7.33	49	81	92	24	-1			
		Urine									
	6	Blood	7.33	47	31	47	24	-1	.26	8.7	39
		CSF	7.29	51	74	91	23	-3			
		Urine									
	7	Blood	7.36	47	38	61	25	1	.28	9.3	
		CSF	7.31	49	84	93	23	-2			
		Urine									
	8	Blood	7.37	38	46	58	26	2	.27	9.0	39.2
		CSF	7.30	53	75	91	24	-2			
		Urine									
	10	Blood	7.39	50	88	95	29	4	.27	9.0	38.9 *
		CSF	7.36	45	105	97	25	0			
		Urine									
	12	Blood	7.38	51	30	48	28	4	.27	9.0	39.2
		CSF	7.34	50	83	94	25	0			
		Urine									
	24	Blood	7.38	51	32	53	29	5	0.26	8.6	
		CSF	7.34	49	79	93	25	0			
		Urine									

pCO_2,pO_2 (mmHg); sO_2 (%); $HCO3^-$, ABE (mmol/L)

* Serum sample heat block for 1.5 hours
Gases on ice for 1.5 h.

A sample Table for recording neurological examination results and body temperature during the experiment (24 h) is shown below.

Calf ID: 864 8582-498 Date: 6-OCT-04 Experiment:

Actual Time	Time (hrs):	Suck	Menace*	Palpebral**	Tactile	Stand	Total	Temperature(° C)
8:00	-1	0	0	0	0	0	0	39.1°
9:00	0	0	0	0	0	0	0	39.3
10:00	1	0	½	0	0	0	½	38.8
11:00	2	0	½	0	0	½	1.0	38.8
12:00	3	¼	1	¼	0	1	2.5	38.8
1:00	4	½	½	0	0	½	1.5	38.8
2:05	5	½	0	0	0	0	½	39.1°
3:05	6	½	0	0	0	½	1	39.0
4:05	7	¼	0	0	0	½	¾	39.2
5:05	8	0	0	0	0	0	0	39.2°
7:05	10	0	0	0	0	0	0	38.9
9:05	12	0	0	0	0	0	0	39.2
9:15	24	0	0	0	0	0	0	38.8

chewing

* Do not touch eye lashes
** Touch third eyelid

A sample Table for recording acid infusion information and progress during the experiment (24 h) is shown below.

Date: Dec 09, 2004 Calf Id: 8648588-504 Calf Weight: 62 kg

Infusion: L-lactic acid

Infusion Started: 7.40 a.m

Starting Infusion Rate: 1240 ml / hr

Weight of Bottle:	Bottle 1	Bottle 2	Bottle 3	Bottle 4
Beginning:	5·325	5,255		
End:	1·335	1·365		
Mass Used:	3·990	3 890		

Actual Time	Time (hrs):	Rate of Infusion(mL/hr):	Volume (mL):	Sample Taken:
6·40	-1			
7·40	0	1240	3900	✓
8·40	1			
9·40	2			
10·40	3			
10·45		1240	3900	✓
11·40	4			
12·40	5			
1·40	6		132 END	
	7			
	8			
	10			
	12			
	24			

Does quick correction of acidosis produce paradoxical acidosis?
Principle Investigator: Dr. JM Naylor

Observational Checklist Used to Determine Endpoints

General
Dr. Naylor, DVM, will be present at the beginning of each experiment and will check on the calves regularly. He will be contacted if there are any unforeseen changes in the condition of the calves. All changes in the condition of the calves will be recorded. Calves will be euthanized if diagnosed with meningitis.

Response Variables and Scoring System

Suck

0	Normal (Strong suck, can feel tongue exerting pressure)
0.5	Mildly Weak Suck (Slightly weaker suck)
1	Weak Suck (Can feel very little pressure inside mouth, calf not eager to suck)
1.5	Very Weak Suck (Calf is only barely exerting pressure on hand)
2	No Suck (Cannot detect any movement in the calf's mouth)

Menace
Menace refers to the calf's reaction (blinking) when a researchers hand moves quickly and stops abruptly in front of calf's eye.

0	Normal (Calf blinks strongly and quickly)
0.5	Mildly Weak (blinking is slightly delayed)
1	Weak (blinking is clearly delayed and calf may not respond to all hand movements)
1.5	Very Weak (Calf barely responds to any hand movements)
2.0	No Menace (Calf won't blink in response to hand movement)

Palpebral
Palpebral refers to the calf's reaction (blinking) when a researcher touches the calf's third eye lid). Scoring is similar to menace.

0	Normal (Calf blinks strongly and quickly)
0.5	Mildly Weak (blinking is slightly delayed)
1	Weak (blinking is clearly delayed and calf may not respond to all hand movements)
1.5	Very Weak (Calf barely responds to any hand movements)
2.0	No Menace (Calf won't blink in response to hand movement)

Tactile: A pen is used to poke the calf on the back. A healthy calf twitches after each poke. A depressed calf may twitch less, or with less strength.

0	Normal (Calf twitches strongly and quickly after each poke)

0.5 Mildly Weak Tactile Response (twitching seems weaker and slightly delayed)
1 Weak Tactile Response (Calf won't respond to all pokes and twitches are weak)
1.5 Very Weak Tactile Response (Calf only rarely twitches after a poke)
2 No Tactile Response (No detectable to response to a pen poke)

Stand
0 Normal (Calf can easily stand on its own)
0.5 Mildly Depressed Ability to Stand (Calf has to be lifted slightly in order to stand)
1 Depressed Ability to Stand (Calf has to be lifted up completely in order to stand)
1.5 Very Depressed Ability to Stand (Calf is wobbling after being lifted up completely)
2 Recumbent (Calf cannot stand)

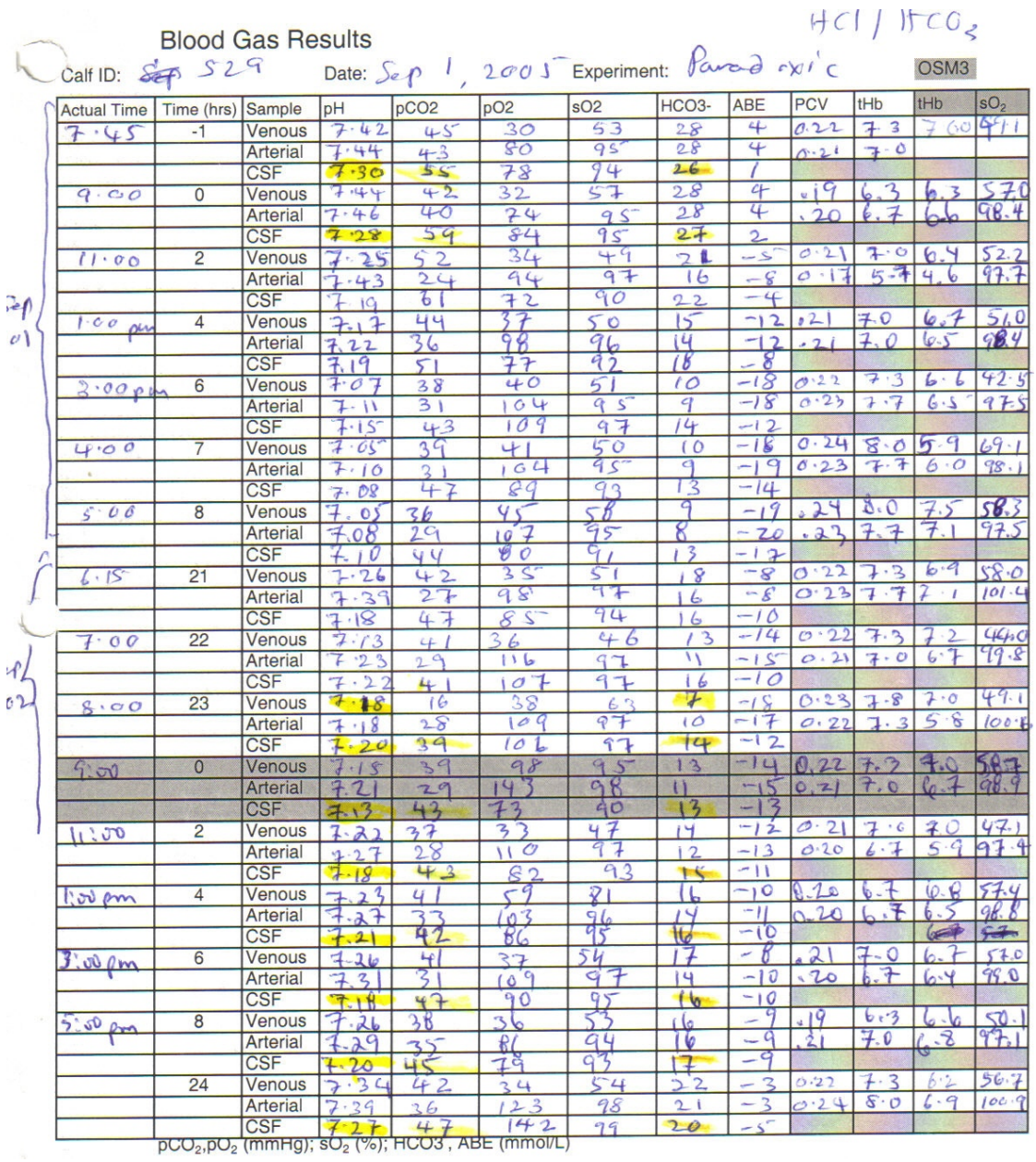
The response variables will add up to a maximum of 10. If the calf has a score of 7 or greater, sodium bicarbonate treatment will commence immediately. If the calf's score stays over 7 for 24 hours, then the calf will be euthanized.

Sickness

Diarrhea
0 Normal (Hard, solid feces)
0.5 Slightly Diarrheic (Feces appears soft)
1 Diarrheic (Feces has no distinct shape, calf appears slightly dehydrated. Electrolytes will be given in its regular feeding.)
1.5 Very Diarrheic (Feces is watery and calf is dehydrated. Blood may be seen in feces. Electrolytes are given to calf.)
2 Extremely Diarrheic Calf (Profuse, watery feces with blood. Calf will be euthanized).

Diarrhea is not an expected part of this experiment, but calves may get diarrhea from an unrelated infection.

A sample Table for recording blood gas results during the experiment (48 h) is shown below



Blood Gas Results

HCl / HCO_3

Calf ID: ~~Sep~~ S29 Date: Sep 1, 2005 Experiment: Paired oxi'c OSM3

Actual Time	Time (hrs)	Sample	pH	pCO2	pO2	sO2	HCO3-	ABE	PCV	tHb	tHb	sO_2
7·45	-1	Venous	7·42	45	30	53	28	4	0.22	7.3	7.60	94.1
		Arterial	7·44	43	80	95	28	4	0.21	7.0		
		CSF	7·30	55	78	94	26	1				
9·00	0	Venous	7·44	42	32	57	28	4	.19	6.3	6.3	57.0
		Arterial	7·46	40	74	95	28	4	.20	6.7	6.6	98.4
		CSF	7·28	54	84	95	27	2				
11·00	2	Venous	7·25	52	34	49	21	−5	0.21	7.0	6.4	52.2
		Arterial	7·43	24	94	97	16	−8	0.17	5.7	4.6	97.7
		CSF	7·19	61	72	90	22	−4				
1·00 pm	4	Venous	7·17	44	37	50	15	−12	.21	7.0	6.7	51.0
		Arterial	7.22	36	98	96	14	−12	.21	7.0	6.5	98.4
		CSF	7.19	51	77	92	18	−8				
3·00 pm	6	Venous	7·07	38	40	51	10	−18	0.22	7.3	6.6	42.5
		Arterial	7·11	31	104	95	9	−18	0.23	7.7	6.5	97.5
		CSF	7·15	43	109	97	14	−12				
4·00	7	Venous	7·05	39	41	50	10	−18	0.24	8.0	5.9	69.1
		Arterial	7·10	31	104	95	9	−19	0.23	7.7	6.0	98.1
		CSF	7·08	47	89	93	13	−14				
5·00	8	Venous	7·05	36	45	58	9	−19	.24	8.0	7.5	58.3
		Arterial	7.08	29	107	95	8	−20	.23	7.7	7.1	97.5
		CSF	7·10	44	80	91	13	−17				
6·15	21	Venous	7·26	42	35	51	18	−8	0.22	7.3	6.9	58.0
		Arterial	7·39	27	98	97	16	−8	0.23	7.7	7.1	101.4
		CSF	7·18	47	85	94	16	−10				
7·00	22	Venous	7·13	41	36	46	13	−14	0.22	7.3	7.2	44.0
		Arterial	7·23	29	116	97	11	−15	0.21	7.0	6.7	99.8
		CSF	7·22	41	107	97	16	−10				
8·00	23	Venous	7·18	16	38	63	7	−18	0.23	7.8	7.0	49.1
		Arterial	7·18	28	109	97	10	−17	0.22	7.3	5.8	100.6
		CSF	7·20	39	106	97	14	−12				
9:00	0	Venous	7·15	39	98	95	13	−14	0.22	7.3	7.0	58.7
		Arterial	7.21	29	143	98	11	−15	0.21	7.0	6.7	98.9
		CSF	7.13	43	73	90	13	−13				
11:00	2	Venous	7·22	37	33	47	14	−12	0.21	7.0	7.0	47.1
		Arterial	7·27	28	110	97	12	−13	0.20	6.7	5.9	97.4
		CSF	7·18	43	82	93	15	−11				
1:00 pm	4	Venous	7·23	41	59	81	16	−10	0.20	6.7	10.8	57.4
		Arterial	7·27	33	103	96	14	−11	0.20	6.7	6.5	98.8
		CSF	7·21	42	86	95	16	−10				
3:00 pm	6	Venous	7·26	41	37	54	17	−8	.21	7.0	6.7	57.0
		Arterial	7·31	31	109	97	14	−10	.20	6.7	6.4	98.0
		CSF	7·14	47	90	95	16	−10				
5:00 pm	8	Venous	7·26	38	36	53	16	−9	.19	6.3	6.6	50.1
		Arterial	7·29	35	86	94	16	−9	.21	7.0	6.8	97.1
		CSF	7·20	45	79	93	17	−9				
	24	Venous	7·34	42	34	54	22	−3	0.22	7.3	6.2	56.7
		Arterial	7·39	36	123	98	21	−3	0.24	8.0	6.9	100.9
		CSF	7·27	47	142	99	20	−5				

pCO_2,pO_2 (mmHg); sO_2 (%); HCO_3^-, ABE (mmol/L)

A sample Table for recording changes of neurological signs and body temperature during the experiment period (48 h) is shown below.

Calf ID: 549 Date: March 21 2006 Experiment: Paradoxic (HCl) $itCO_3^-$ slow correction

Actual Time	Time (hrs):	Suck	Menace*	Palpebral**	Tactile	Stand	Total	Temperature(° C)	Comments
10.00	-1	0	0	0	0	0	0	38.6°C	
11.00	0	0	0	0	0	0	0	38.4	
1.00	2	0	0	0	0	0	0	38.4	
3.00	4	1/4	0	0	0	0	1/4	38.4	
5.00	6	1/4	0	0	0	0	1/4	38.4	
6.00	7	1/4	0	0	0	0	0	38.2	
7.00	8	1/2	0	0	0	0	0	38.2	
6.30	21	0	0	0	0	0	0	38.2	
7.30	22	1/4	0	0	0	0	1/4	38.2	
8.30	23	1/2	0	0	0	1/4	3/4	37.6	
10.30	1	1/4	0	0	0	0	1/4	37.9	
9.30	0	1/4	0	0	0	1/4	1/2	37.8	
11.30	2	1/4	0	0/1	0	0	1/4	37.9	
1.30	4	1/4	0	0	0	0	1/4	38.1	
3.30	6	0	0	0	0	0	0	38.2	
5.30	8	0	0	0	0	0	0	38.7	
8.30	24	0	0	0	0	0	0	38.7	

* Do not touch eye lashes

** Touch third eyelid

A sample Table for recording acid infusion or bicarbonate therapy information and progress is shown below.

Date: Mar 21, 2006 Calf Weight: 60 Calf Id: 549 Infusion: HCl

Infusion Started: 1200 mL/h

Starting Infusion Rate: 11.00 am

Weight of Bottle:	Bottle 1	Bottle 2	Bottle 3	Bottle 4
Beginning:	5.370	5.380	5.375	5.360
End:	1.350	1.360	1.300	1.330
Mass Used:	4.020	4.020	4.075	4.030

Actual Time	Time (hrs):	Rate of Infusion(mL/hr):	Volume (mL):	Sample Taken:
10.00	-1	0		
11.00	0	1200		✓
1.00	2	1200	4800	
3.00	4	1200	9200	✓
5.00	6		10000	
6.00	7		12600	
7.00	8			
6.30	21	1200		
7.30	22		15000	✓
8.30	23		15800	
9.30	0	192 mL/h		
10.30	1	〃	192	
11.30	2	〃	384	
1.30	4	〃	768	
3.30	6	〃		
5.30	8	〃	1536	
9.30	24	〃	4830	

Protocol for Lamb Diarrhea and Acidosis Trial

.

·**Investigators**

Principal Investigator: Dr. Jonathan M. Naylor
Western College of Veterinary Medicine

Co-investigator: Dr. Gordon A. Zello
College of Pharmacy and Nutrition

Grad student : Saman Abeysekara (Sam)
College of Pharmacy and Nutrition

Contact : 966-5831/6913 or 373-4107 (Sam)
Saman.abeysekara@usask.ca

Title: D-lactic acid levels in diarrheic lambs

Objective

1. To determine if D-lactic acid contents increase in diarrheic lambs, and supports acidosis.

Methodology

Study Design

Sampling (serum, feces and urine) of diarrheic lambs the University sheep barn will be conducted throughout the year. We hope to ensure the coverage of at least 15 cases per year.

Blood (10 mL), fecal (100 g) and urine (10 mL) samples will be collected from scouring lambs. In order to have a control, whenever samples are collected from diarrheic cases, a sample will be taken from a healthy lamb. All samples will be stored at -20°C until analysis. Blood serum will be analyzed for lactate, other organic acids, electrolytes and blood gases. Feces will be analyzed for lactates. Urine will be analyzed for lactates, other organic acids and electrolytes. Data will be statistically analyzed by Proc t-test with unequal variance using SAS.

Sample Collection

1. Blood

A blood (5 mL) sample will be collected from anerobically from the jugular vain of lambs in a preheparinized plastic syringe (Smooth; Radiometer America, Westlake, Ohio, USA) for blood gas measurements. A second blood sample (5 mL) for the determination of serum organic acid concentration will also be collected into a tube containing no anticoagulant (Vacutainer; Becton Dickinson, Rutherford,

New Jersey, USA). Blood sampling would be conducted with the help of the Department of Large Animal Clinical Sciences.

2. Feces

Fecal samples (50 g) will be collected into a cream color plastic container which contains a preservative (thiomersal). Thiomersal is a toxic substance, containing a small amount of mercury. Therefore, you should not spill or contact thiomersal bear hand. Always use gloves when you are sampling or handling. Scouring/diarrhea will be determined in lambs depending on fecal scoring for clinical diarrhea.

***Fecal score is based on the consistency and volume of faces.

- Volume: filling of tray (rank 1-4)
- Consistency

1.	Firm	0
2.	Pasty	1
3.	Loose	2
4.	Watery	3

- Fecal score will be the sum of these two ranks

(Volume + consistency = 0→7), Maximum fecal score is 7.

- Clinical diarrhea (scouring) is defined as a score over 2.5.

3. Urine

Urine (5-10 ml) will be collected into a urine collecting tube. Urine collection will be performed at micturition or using a urinary catheter.

Procedure:

- When you find a scouring lamb:

1. Record the lamb number, body (rectal) temperature and the scouring number on the given record sheet
2. Take initial samples from the lamb

✓ Feces – cream color containers

✓ Blood – red top tubes and green top tubes

✓ Urine – given tubes

3. Take samples from a healthy lamb, record lamb number, and body temperature as a control
4. All the collected samples should be well covered in the containers, (should be refrigerated within a half an hour of collection)
5. Avoid sampling the same lamb next time (either a scouring or a control)

Regularly / everyday we may visit the barn to look for new scouring lambs. Or you can call us (Sam) at anytime.

Tel – day time/office : 966-5831
- cell : 261-0248
– night/home : 373-4107
E-mail : saman.abeysekara@usask.ca

A sample Table for recording lamb information.

Lamb Diarrhea and Acidosis Trial

Lamb Information **Date:**

ID Number	Date of Birth	Sex	Remarks

Protocol for Prebiotic (Malate) Trial

· **Investigators**

Principal Investigator: Dr. Jonathan M. Naylor
Western College of Veterinary Medicine

Co-investigator: Dr. Gordon A. Zello
College of Pharmacy and Nutrition

Grad student : Saman Abeysekara
College of Pharmacy and Nutrition

Contact : 966-5831/6913 or 373-4107 (Sam)

· **Project Title**

Oral use of malate, as a prebiotic, to manipulate gut flora and to reduce lactic acid production in calves

· **Project Outline**

Objectives

4. To determine the effective dose of malate (a prebiotic) to observe a biological response in calves.
5. To determine if malate reduces lactic acid production in calves' colon.

Methodology

Study Design

Twelve Holstein calves (age below 2 months) from the Dairy Barn, University of Saskatchewan will be selected and randomly allocated in to 3 groups; G1, G2, G3 where 4 calves would be in each group. After an adaptation period of 5 days for a relatively uniform diet these calves will be given sodium malate in three different dosages for consecutive 5 days. The groups; G1, G2 and G3 will receive daily dose of 50, 500 and 1000 mg kg^{-1} respectively. Malate can be mixed with feed / milk given to calves in the morning. Malate daily treatment doses would be pre-weighed for each calf, labeled and made available accordingly. The guidelines of the Canadian Council on Animal Care (CCAC, 1993) will be followed in dealing with the calves.

Sample Collection

Fecal and jugular blood (serum) samples will be taken at the date of start (day 0), day 4 and day 7 (at 8.00–9.00 h just prior to feeding). Blood sampling would be conducted with help of the Dept. of Large Animal Clinical Sciences.

Sample Analysis

Serum will be analyzed for

i. Organic acids (D-lactic acid and L-lactic acid)
ii. Electrolytes (HCO_3^-, Cl^-, Na^+, K^+, Ca^{++})
iii. Blood gases (base excess, bicarbonate, pCO_2, pO_2)
iv. pH, anion gap
v. Hemoglobin
vi. Glucose

Feces will be analyzed for organic acids. The high performance liquid chromatography (HPLC) would be preferred methods of sample analysis as required.

Data Analysis

Values for the samples of day 0 will be the control for each variable. Data will be statistically analyzed using appropriate statistical techniques.

Summary work-plan for the experiment

Item/event	Group		
	G1	G2	G3
Number of calves	4	4	4
Dosage (mg kg^{-1})	50	500	1000
Adaptation period (d)	5	5	5
Treatment period (d)	5	5	5
Sampling dates (d)	0, 4 & 7	0, 4 & 7	0, 4 & 7
Sampling time (h)	8.00-9.00	8.00-9.00	8.00-9.00
Total days for trial	12	12	12

Farm level work

1. Adaptation of calves to a uniform dietary schedule - *5 days*

2. Oral administration of malate dosages (50 – 1000 mg kg^{-1}) and sample collections - *7 days*

- **The treatment (group) of calf is marked on his collar (such as G1, G2, and G3), and Calf numbers belonging to these G1, G2, and G3 groups are displayed on the black board.**

- **We hope we may have a calf for each group at a time.**

- **Labelled Treatment (G1, G2 and G3) vials are kept in 3 separate zip-lock bags in the refrigerator.**

- **One vial contains a single dose per day per calf. Content in one vial should be mixed in morning milk –oral administration of the treatment. (Gx calf should get Gx vial dose).**

- **Treatment time and sampling time would be 8 – 9 am.**

- **Calf No: _____ Calf wt: _____** kg

➢ **Starting date: _____ (day 0 + 1)**

- **Samples**
  - **Jugular blood (~5 ml) _____**
  - **Urine (~10 ml) _____**
  - **Feces (~ 50 g) _____**
  - **Then treatment would be given.**

➢ **Day 2: _____- Treatment only _____**

➢ **Day 3: _____- Treatment only _____**

➢ **Day 4: _____- Treatment and sampling _____**

➢ **Day 5: _____- Treatment and sampling _____**

➢ **Day 6: _____ - No Treatment _____**

➢ **Day 7: _____ - Sampling only _____, and then it is done with this calf.**

A sample Table for recording information on calf and treatment in malate prebiotic experiment is shown below.

Dairy Barn Copy (from Sam)

Calf Prebiotic Study -Preliminary Trail

(Grad student: Sam, Supervisors: Naylor/Zello)

Date: Mar. 25, 2005 (day 1) Step: 2

Calf ID	Birth date	Calf wt (kg)	Treatment group	Treated/not	Sampling
510 ♂	Feb 24, 2005	72	G1	Y	Y
511 ♂	Mar 02, 2005	72	G2	Y	Y
512 ♂	Mar 06, 2005	59	G3	Y	Y

Date: Mar. 26, 2005 (day 2)

Calf ID	Birth date	Calf wt (kg)	Treatment group	Treated/not	Sampling
510 ♂	"	72	G1	✓	No
511 ♂	"	72	G2	✓	No
512	"	59	G3	✓	No

Date: Mar. 27, 2005 (day 3)

Calf ID	Birth date	Calf wt (kg)	Treatment group	Treated/not	Sampling
510 ♂	"	72	G1	✓	No
511 ♂	"	72	G2	✓	No
512 ♂	"	59	G3	✓	No

Date: Mar. 28, 2005 (day 4)

Calf ID	Birth date	Calf wt (kg)	Treatment group	Treated/not	Sampling
510 ♂	"	72	G1	✓	✓
511 ♂	"	72	G2	✓	✓
512 ♂	"	59	G3	✓	✓

Date: Mar. 29, 2005 (day 5)

Calf ID	Birth date	Calf wt (kg)	Treatment group	Treated/not	Sampling
510 ♂	"	72	G1	✓	✓
511 ♂	"	72	G2	✓	✓
512 ♂	"	59	G3	✓	✓

Date: Mar. 30 2005 (day 6)

Calf ID	Birth date	Calf wt (kg)	Treatment group	Treated/not	Sampling
510 ♂	"	72	G1	No	No
511 ♂	"	72	G2	No	No
512 ♂	"	59	G3	No	No

Date: Mar. 31, 2005 (day 7)

Calf ID	Birth date	Calf wt (kg)	Treatment group	Treated/not	Sampling
510 ♂	"	72	G1	No	✓
511 ♂	"	72	G2	No	✓
512 ♂	"	59	G3	No	✓

A sample Table for recording laboratory results of blood samples in malate prebiotic experiment is shown below.

Prebiotic Study -Preliminary Trail Data 1

Date: Mar. 25, 2005 (day 0) **Step:** (2)

Calf ID	Birth date	Calf wt (kg)	Temp ©	pH	pCO2	pO2	sO2	HCO3-	ABE	tHb	PCV
510	Feb 24, 2005	72	39	7.35	49	32	50	26	1	9.3	28
511	Mar. 02, 2005	72	39	7.33	52	22	26	26	1	7.5	23
512	Mar. 06, 2005	59	39	7.23	72	23	23	28	0	10.3	31

Date: Mar. 28, 2005 (day 4)

Calf ID	Birth date	Calf wt (kg)	Temp	pH	pCO2	pO2	sO2	HCO3-	ABE	tHb	PCV
510	"	72	39	7.35	49	29	44	26	1	9	27
511	"	72	39	7.36	44	20	23	26	2	8.7	26
512	"	59	39	7.26	60	18	16	25	-1	[illegible]	30

Date: Mar. 29, 2005 (day 5)

Calf ID	Birth date	Calf wt (kg)	Temp	pH	pCO2	pO2	sO2	HCO3-	ABE	tHb	PCV
510	"	72	39	7.33	46	29	44	23	-2	8.0	24
511	"	72	39	7.30	45	19	19	21	-4	8.0	24
512	"	59	39	7.32	51	21	25	25	-1	9.3	28

Date: Mar. 31, 2005 (day 7)

Calf ID	Birth date	Calf wt (kg)	Temp	pH	pCO2	pO2	sO2	HCO3-	ABE	tHb	PCV
510	"	72	39	7.33	50	25	33	25	0	8.5	24.5
511	"	72	39	7.32	43	18	19	21	-4	8.0	24
512	"	59	39	7.32	56	21	25	27	1	9.0	27

A sample Table for recording the detailed laboratory results of malate experiment is shown below.

Prebiotic Study -Preliminary Trail Data 2

Date: Mar 25, 2005 (day 0) Step: (2)

Calf I	Birth date	Na+	K+	Ca++	Glu	Lac	Hct	pH'	pCO2'	pO2'	HCO3-	BEec	SO2c	tHbc	PCV	Cl⁻
510	Feb 24, 2005	125	4.2	0.93	4.3		24	7.35	50	33	27.3	2.3	65.7	8.3	24	100
511	Mar 02, 2005	127	3.8	1.14	4.9		20	7.34	54.6	21.8	27.9	2.6	39.5	6.7	20	98
512	Mar 06, 2005	127.3	3.63	0.87	4.3			7.18	69.7	20.9	24.9	-3.0	27.8	8.1		98

Date: Mar 28, 2005 (day 4) Step: (2)

Calf I	Birth date	Na+	K+	Ca++	Glu	Lac	Hct	pH'	pCO2'	pO2'	HCO3-	BEec	SO2c	tHbc	PCV	Cl⁻
510	"	126.4	3.96	1.05	4.6		24	7.365	48.1	28.9	26.3	1.4	56.7	8.2	27	100
511	"	126.6	3.54	1.02	4.4		22	7.358	54.6	19.0	29.4	4.4	32.9	7.5	26	95
512	"	126.4	4.16	0.99	4.4		24	7.275	56.3	16.8	25.0	-1.4	48.8	8.2	30	98

Date: Mar 29, 2005 (day 5) Step: (2)

Calf I	Birth date	Na+	K+	Ca++	Glu	Lac	Hct	pH'	pCO2'	pO2'	HCO3-	BEec	SO2c	tHbc	PCV	Cl⁻
510	"	116.5	3.71	0.77	4.7		21	7.364	42.1	23.8	23.5	-1.9	53.5	8.1	24	99
511	"	123.3	3.77	1.07	4.0		19	7.322	45.4	17.3	23.0	-3.1	37.0	7.3	24	100
512	"	122.6	3.90	0.95	3.6		24	7.372	42.2	22.8	24.0	-1.3	52.3	6.6	28	100

Date: Mar 31, 2005 (day 7) Step: (2)

Calf I	Birth date	Na+	K+	Ca++	Glu	Lac	Hct	pH'	pCO2'	pO2'	HCO3-	BEec	SO2c	tHbc	PCV	Cl⁻
510	"	130.9	4.21	1.23	4.3		23	7.368	49.2	32.0	27.1	2.3	62.7	7.8	24.5	99
511	"	122.8	3.76	1.00	4.2		20	7.304	49.9	17.6	23.7	-2.2	23.3	6.7	24	100
512	"	119.7	3.97	0.88	4.2		24	7.321	46.2	25.8	22.8	-2.8	51.3	8.0	27	98

F: Program for SAS

SAS for Windows v.8 was used to perform repeated measures / polynomial analysis, MANOVA and contrast of experimental model studies data. Breakpoint regression analysis of clinical studies data were performed using SAS.

F1: A sample SAS program for repeated measures analysis used in experimental model studies

```
data VenpO2;
input CalfNo Aged Treatment$ t1-t12;
cards;
504     36     D-Lactic     30     33     31     37     38     42     47     40
 51     35     34     32
504     35     L-Lactic     28     31     28     49     31     30     31     30
 34     25     36     34
502     45     D-Lactic     33     34     43     34     38     39     41     41
 41     39     44     30
502     44     L-Lactic     29     36     33     38     35     34     37     39
 32     32     36     34
502     42     HCL    29     33     34     37     38     41     38     41     40
 41     34     33
502     41     Saline       34     31     37     36     35     33     34     33
 33     32     30     32
501     34     HCL    35     35     51     52     50     49     31     39     35
 31     33     32
499     52     Saline       44     35     35     40     34     37     56     35
 38     39     43     36
499     50     L-Lactic     42     32     36     44     41     35     36     38
 34     41     41     92
499     48     HCL    30     38     40     49     48     51     60     49     56
 44     45     37
499     47     D-Lactic     40     36     93     36     31     46     52     59
 45     41     51     40
498     39     Saline       32     35     33     34     36     35     31     36
 31     35     38     36
498     37     L-Lactic     30     38     40     41     33     53     45     31
 38     46     88     32
660     40     HCL    37     41     40     43     46     41     39     40     40
 28     46     49
660     38     L-Lactic     37     38     35     38     35     36     57     34
 133    37     34     39
660     32     Saline       38     38     46     43     39     40     41     39
 38     39     39     41
660     30     D-Lactic     40     41     46     35     40     39     48     44
 53     37     53     43
489     27     HCL               31       27     29     38     35     28     33
 33     42     29     67     37
489     25     L-Lactic     33     41     36     35     42     40     58     37
 35     40     36     36
489     23     Saline              65     31     37     35     38     31     32
 40     56     37     35     42
```

```
489     17     D-Lactic     27     45     33     44     39     34     93     36
  36     45     26     34
488     17     Saline               35     32     40     45     42     33     60
  32     27     37     31     39
488     15     D-Lactic     31     34     40     47     38     43     47     46
  52     56     45     42
;
run;
proc print data=VenpO2;
run;


proc sort data=VenpO2;
by Treatment;
run;

proc means data=VenpO2 mean std stderr LCLM UCLM;
var t1-t12;
by Treatment;
run;


proc glm;
class Treatment;
model t1-t12=Treatment;
repeated time 12(-1 0 1 2 3 4 5 6 7 8 10 24) polynomial/ summary;
means Treatment/SNK;
run;
```

F2: A SAS program for breakpoint regression analysis

```
data ex;
input Fecal_DL      Serum_DL;
cards;
4.1             0
5.6             0
10.2    0
8.7             0
12.3    0
7.4             0
11.5    0.04
14.3    0.37
9.7             0
6.8             0
5.7             0
8.2             0
5.8             0
18.2    0.61
15.7    0.39
13.7    0
9.5             0
6.7             0
12.1    0
7.5             0
5.7             0
4.7             0
7.8             0
13.7    0.11
7.4             0
12.6    0.42
11.7    0.4
4.2             0
3.3             0
3.6             0
2.4             0
;
run;
proc sort;
by Fecal_DL;
run;
data ex1;
set ex;
D=0;
if Fecal_DL <10 then D=1;
FD=Fecal_DL*D;
run;
proc reg data=ex1;
model Serum_DL=Fecal_DL D FD;
run;
```