New evaluation markers for energy metabolism in various species

(各種動物のエネルギー代謝評価マーカーの開発と応用)

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CONTENTS

General Introduction

Energy metabolism drives all cellular functions in living cells. Status of energy metabolism is dynamic as all things associated with life are, reflecting, and reflected in, macroscopic and/or microscopic changes occurring in living things. Enzymes, involved in the metabolism of glucose and fatty acids and in energy homeostasis, are crucial in maintaining life, and may be variable among animal species, tissues, and various physiological and nutritional status of a given animal (Washizu et al., 2002, 1998). In this thesis, I focused on the enzymes involved in energy metabolism, in particular, on the enzymes of NADH shuttles, to show whether their activity levels faithfully reflect the variations in nutrient metabolism, metabolic status, and health conditions of different species and individuals. The malate-aspartate (MA) shuttle is one of the NADH shuttles, along with the glycerol-3-phosphate shuttle, which transports cytosolic NADH, the product of glycolysis, into mitochondria (Wang et al., 2014). In the MA shuttle, membrane-impermeable cytosolic NADH is transferred to mitochondria in the form of malate. Once in the mitochondria, malate is converted back to oxaloacetate as it reduces NAD^+ to NADH, which is the key molecule for oxidative

metabolism as it provides electrons that act as the major driving force for mitochondrial ATP production (Styler, 1995). Malate dehydrogenase (MDH) is the rate-limiting enzyme of the MA shuttle that catalyzes the inter-conversion of oxaloacetate to malate and NADH to NAD^+ both within the cytosol and mitochondria.

Cytosolic NADH level is one of the key factors in determining the direction of glucose metabolism as evidenced by the fact that increased cytosolic NADH/NAD^+ ratios inhibit glycolysis and the production of pyruvate (Styler, 1995). Pyruvate, the end product of glycolysis is either converted to lactate via lactate dehydrogenase (LDH)-mediated reaction, or to alanine within the cytosol, or transferred to mitochondria to be converted to oxaloacetate and acetyl CoA, the substrates for citrate production that initiates TCA cycle, which in turn activates subsequent ATP production through the electron transport chain. Therefore cytosolic pyruvate and NADH, the end-products of glycolysis, both contribute largely to ATP production which take place within the mitochondria.

MDH is considered the key enzyme in determining the rate of glucose metabolism and oxidative metabolism, as it is the rate-limiting enzyme of the MA shuttle and contributes to the coupling of glycolysis with the mitochondrial ATP productions. We hypothesized that the elevations in MDH activity likely indicate heightened energy metabolism, whereas depressions in MDH activity would indicate

inefficient or dysregulated glucose energy metabolism. By dividing the cytosolic MDH by LDH, a relatively stable cytosolic marker enzyme for glycolysis, MDH/LDH ratio was analyzed as the parameter for evaluating metabolic status in animal tissues. A better grasp of trends in shifts of the energy metabolism enzymes may assist us to better understand species-species and individual differences in energy production and usage, and early detection and prevention of energy metabolism dysregulations associated with serious disorders such as diabetes, neoplasia, and obesity.

The present thesis consists of five chapters, evaluating the activities of MDH, LDH, other energy metabolism enzymes, and M/L ratio in various physiological and pathological conditions. In Chapter 1, we showed the species-specific differences between dogs and cats in the activities of enzymes in peripheral blood leukocytes. We expected that the differences may reflect how each species metabolizes energy sources such as carbohydrates, lipids, and amino acids more efficiently than the others. Deeper understanding of the unique demands and usages of nutrients in cats, with higher incidence of obesity, insulin resistance, and diabetes mellitus, may elucidate the mechanisms of the risk factors for and the development of these serious pathologies. In Chapter 2, we examined the activities of various enzymes and biochemical markers in dogs with diabetes mellitus to see how reliably the changes in leukocytic MDH activity and M/L ratio reflect the overall metabolic status of diabetic dogs. Deeper

understanding of the correlation of the MA shuttle function with DM risks, prognosis, and response to therapy, may assist in the development of effective preventative and therapeutic measures.

In the first two studies, we focused on the diagnostic values of peripheral blood leukocytes (PBL) as potential markers of physiological changes occurring within animal tissues. The term “sentinel principle” has risen from the idea that peripheral leukocytes travel through the entire body and continuously interact with the cells of each tissue, accurately reflecting the subtle changes occurring in response to internal and external stimuli as they may arise (Liew et al., 2006). Previously, it was shown that PBL shared over 80% of their gene expression with any one of 9 human tissues. In other studies, it was shown that peripheral blood mononuclear cell gene expressions reflect the energy homeostasis adaptations that typically take place in liver and adipose tissue¸ and show a clear association with changes in nutritional status and obesity (de Mello et al., 2008; Oliver et al., 2013). Because PBL express many genes that are involved with response to physiological and environmental changes, and interact with each cell of other organs, and have fast turnover rate, they can potentially serve as the “sentinel” that reflect and represent subtle and early changes in metabolic and physiological state of other tissues and the whole body. Furthermore, PBL are abundant and easily accessible with minimum invasion compared to the traditional tissue

sampling of other organs, they may serve as the ideal, practical diagnostic and prognostic tools in the evaluation of the whole body status. Therefore, there's been an increasing interest in peripheral blood mononuclear cell (PBMC) gene expression studies for the development of diagnostic tools (Burczinsky et al., 2005; Liew, 2007; de Mello et al., 2008). Extrapolating from the usefulness of PBMC gene expression, we focused on the activities of enzymes of energy metabolism in leukocytes as the reflection of changes occurring in gene expression, and as the way to evaluate the metabolic state in the whole body of animals.

In Chapter 3, metabolic enzymes in plasma and leukocytes of the racehorses were compared against those of the riding horses. The aim was to see if adaptation to continuous intense exercise in racehorses would be reflected in the changes seen in MDH activity and M/L ratio in plasma. Because discrepancies in results of leukocytic enzymes were noted in previous horse studies, we sought the usefulness of plasma in this case as less errors and variables are expected in processing and measument. In this study, the use of plasma as opposed to PBL to determine M/L ratio turned out to be more sensitive, since it resulted in significant differences between racehorses and riding horses. The results indicated that plasma M/L ratio may serve as the practical marker to assess the effectiveness of different exercise regimen, diet, and nutritional supplements in performance animals.

In Chapter 4, the final chapter, we analyzed the sensitivity of plasma and leukocytic enzyme activities and M/L ratios to early weight gain. We sought to observe whether the plasma and leukocytic activities of enzymes involved in energy metabolism would serve as the early marker of weight gain before clinical sequela of weight gain is apparent.

Finally, in conclusion, I summarized the results of various studies which analyzed the shifits in MDH, LDH, and M/L ratio in varying species and physiological/pathological condition. The chart may help ease the interpretation/utilization of MDH, LDH, and M/L ratio assay as one of the potential diagnostic parameters.

Chapter 1

Comparison of leukocytic energy metabolism enzymes and plasma metabolites between dogs and cats

Introduction

Glucose serves as the main source of energy in mammalian species. Glucose is converted to pyruvate via glycolysis, and the subsequent oxidation within the TCA cycle provides mitochondrial NADH. Additional mitochondrial NADH is provided by the NADH shuttles, which transfer cytosolic NADH and contribute to oxidative metabolism and ATP production. It has been reported that glucose metabolism in the various cells and tissues of dogs and cats vary greatly (Arai et al., 1998; Washizu et al., 1998). In particular, cats with low glycolytic enzyme activities show significantly lower glucose transport activities in their erythrocytes and hepatocytes than in dogs, which in turn, may reflect great differences in the incidence of obesity and the onset of diabetes mellitus between cats and dogs. Plasma concentrations of glucose and insulin were also found to be different between dairy and beef cattle (Arai et al., 2003a), reflecting their differences in feeding conditions and metabolic states (Landau et al., 2000).

Likewise, the enzymes associated with energy metabolism such as ATP production are variable among animal species (Washizu et al., 1998). Particularly, enzyme activities within the NADH shuttles, such as malate-aspartate shuttle, which contribute largely to the transfer of NADH from the cytosol to mitochondria, and to the coupling of glycolysis to mitochondrial energy production, vary remarkably depending

on the energy metabolism state of the animals, and among animal species (Washizu et al., 1998; Arai et al., 2003c; Hirakawa et al., 2012).

In the present study, the activities of various enzymes, particularly, malate dehydrogenase (MDH), a rate-limiting enzyme for the malate-aspartate shuttle, lactate dehydrogenase (LDH), a cytosolic marker enzyme, their ratio (M/L ratio), and glutamate dehydrogenase (GLDH), a mitochondrial marker enzyme, were measured to analyze the differences in energy metabolism within the peripheral blood leukocytes of dogs and cats.

Materials and Methods

Animals

Six mixed-breed male dogs (4-6 years of age) and 6 mixed-breed male cats (3-7 years of age) from our research laboratory were entered into the study. They were each fed commercially prepared Science diet© (Hill's Pet Products, Topeka, KS, USA) at a regular maintenance amount (1.4–1.8×RER per day, whereby RER means resting energy requirement and equals to ($BW^{0.75}$×70). The hematological and plasma biochemistry analyses revealed no significant values.

Collection and preparation of blood samples

Fasted blood was collected from the jugular vein into the heparinized tubes, and centrifuged at 3,000 rpm for 15min at 4℃. The obtained plasma was stored at -25℃ until use. The leukocytes were collected by gradient centrifugation with a commercial lymphocyte-isolating solution (Lympho-separation medium, ICN Biochemical, Aurora, OH, USA), followed by multiple washings with cold phosphate-buffered saline (0.15 mol/L NaCl, pH 7.2).

Fractionation of cellular components

Washed leukocytes were re-suspended in 1 ml ice-cold STE solution (0.25 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.2, containing 2 mmol/L EDTA), and

homogenized via 3-second ultrasonic processing using VP-5 (Taitek, Koshigaya, Japan). The resultant homogenate was centrifuged at 100g for 1 min to remove nuclei and cell debris. Then the supernatant was centrifuged at 6,000g for 20 min at 4℃, and was further centrifuged 100,000g for 30 minutes at 4℃ in SCP55H (Hitachi, Tokyo, Japan). The supernatant separated was the cytosolic fraction. Separation of plasma membrane and mitochondrial fractions were attained via previously reported method (Belsham et al., 1980) with few modifications as described as follows: The pellet portion was re-suspended in 0.5 ml of 50 mmol/L Tris-HCl, pH 7.5 containing 2 mmol/L 2-mercaptoethanol and 1 mmol/L EDTA, followed by homogenation with an ultrasonic processor for 5 seconds at 20 Hz, then centrifugation at 20,000G for 30 min at 4℃. The resulting membranous bands, one immediately below the surface (plasma membrane) and the other close to the bottom (mitochondria) were each collected into 4 volumes of 10mmol/L Tris-HCl buffer, pH 7.5, containing 0.15 mmol/L NaCl. These two fractions were then re-centrifuged for 10 minutes at 10,000g for washing. The cytosolic and mitochondrial fractions were both stored at -80 ℃ until use.

Enzyme activity assays

The activities of the enzymes in the plasma, cytosol, and mitochondrial fractions were measured via methods previously reported: hexokinase (HK) (Bergmayer, 1974b), fructokinase (FK) (Anderson and Sapico, 1974), pyruvate kinase (PK) (Hess

and Wieker, 1974), glucose-6-phosphate dehydrogenase (G6PD) (Bergmeyer et al., 1974a), lactate dehydrogenase (LDH) (Kaloustian et al., 1969), malate dehydrogenase (MDH) (Bergmeyer and Bernt, 1974c), aspartate amonotransfearse (AST) (Rej and Horder, 1983), ATP-citrate lyase (ACL) (Takeda et al,. 1969), and glutamate dehydrogenase (GLDH) (Schmidt, 1974) (Schmidt, 1974). The cytosolic ratio of MDH/LDH activity (M/L ratio) was calculated from division of MDH specific activity by LDH specific activity. All enzymatic activities measured at 24-26℃ were expressed as mU per mg of protein. The enzyme unit (U) represents 1μmol of substrate degraded per min. The protein concentrations were measured by the Bradford method (1976) with bovine serum albumin as the standard.

Plasma metabolite assays

Plasma glucose concentrations were determined via the glucose oxidase method described by Huggett and Nixon (1957). And the immunoreactive insulin (IRI) concentrations were measured by the micro ELISA sandwich method of Arai et al. (1989). The triglyceride (TG), non-esterified fatty acids (NFFA), total cholesterol (TC) and high-density lipoprotein (HDL) cholesterol concentrations were measured by commercial kits (Wako Pure Chemical Industries).

Statistical analysis

All values are expressed as mean±SD and the differences between means were analyzed by Student's *t*-test. Statistical significance was set at $P < 0.01$.

Results

As shown in Table 1, there were no significant differences in plasma glucose, IRI, TG, NEFA between the canine and feline groups. TC concentrations were higher and HDL cholesterol concentrations were lower in the feline plasma when compared to those of the canine group, resulting in significantly lower HDL/TC ratio (H/T ratio) in feline plasma. Table 2 shows the enzyme activities in cytosol and mitochondria of peripheral leukocytes of the canine and feline groups. The activities of cytosolic FK, PK, G6PD and LDH in the feline leukocytes were significantly higher than those of the canine leukocytes, while cytosolic MDH, M/L ratio and mitochondrial GLDH activities were significantly lower in feline leukocytes. There were no significant differences in cytosolic HK, AST, ACL, mitochondrial MDH and AST between the two groups.

Discussion

Enzymatic activities of energy metabolism of feline leukocytes were remarkably different compared to those of canine leukocytes. GLDH is a mitochondrial marker enzyme that catalyzes the reversible conversion of glutamate to alpha-ketoglutarate and ammonia, allowing glutamate to fuel the TCA cycle for energy production under low cellular energy status. The enzyme is associated with interconnection of amino acid and carbohydrate metabolism (Spanaki et al., 2012). Feline leukocytes showed considerably lower activity of GLDH, along with the relatively lower activity of the malate-aspartate shuttle, which has an essential role in the production of ATPs via glycolysis. Together, they suggest that the feline cells utilize less glucose as an energy source compared to the canine cells. In the previous studies, glucokinase activities were observed to be absent in feline leukocytes, erythrocytes, and hepatocytes with higher total activities of hexokinases (Pilkis et al., 1968; Arai et. al., 1992, 1995; Washizu et al., 1998). Feline cells may be more efficient in utilizing other hexoses in place of glucose, such as fructose as evidenced by the higher FK activity in this study. Fructose exerts unique effects on carbohydrate and lipid metabolism and it does not directly stimulate insulin secretion. The lack of stimulation by fructose causes disruption in insulin's ability to inhibit food intake and increase energy expenditure.

Furthermore, some fructose metabolites induce PK and G6PD activation and subsequent fatty acid synthesis (Naismith, 1971). G6PD provides cytosolic NADPH for fatty acid synthesis. Additionally, in cats, pyruvate tends to be reduced to lactate by the higher activity of LDH, rather then being utilized for ATP production. Higher activities of FK, PK, LDH and G6PD in feline leukocytes observed in this study may indicate active utilization of fructose, subsequent fatty acid synthesis and storage, and lower utilization of glucose for energy production. Lower MDH activity and M/L ratio, together with higher FK, PK, LDH, and G6PD, may reflect the unique characteristics in nutrient needs and energy metabolism in cats with higher incidence of obesity, insulin resistance, and diabetes mellitus compared to dogs.

Understanding the association of energy metabolism activities to species-specific nutrient requirements and diseases may elucidate the mechanisms of energy metabolism conditions and pathologies, and may help with the future investigation and development of energy metabolism disease therapy. Further studies of such enzymes as diagnostic markers to assess the nutrient metabolism of dogs and cats will be valuable.

Conclusion

In conclusion, our date indicates that differences in various leukocytic enzymes of energy metabolism faithfully reflect differences in how cats and dogs utilize various nutrients. Deeper understanding of enzymes such as MDH, FK, PK and G6PD may be useful in the field of nutraceutical as the way to detect, prevent, and treat various metabolic conditions such as obesity and diabetes in cats and dogs.

Tables

Table 1 Plasma concentrations of glucose, triglyceride, free fatty acid, total cholesterol, HDL cholesterol and immunoreactive insulin in dogs and cats

		Dogs (n=6)	Cats (n=6)
Glucose	mg/dl	85 (6)	78(6)
Triglyceride	mg/dl	64(17)	63(25)
NFFA	mEq/l	0.2(0.1)	0.2(0.1)
TC	mg/dl	149(26)	188(25)
HDL	mg/dl	126(24)	118(25)
H/T	%	85(3)	63(8)*
IRI	μU/ml	17(3)	21(3)

Values are presented as mean (SD) of animals.
H/T (%) is calculated by HDL cholesterol divided byTC×100
* Significantly different ($p < 0:01$) from the values of dogs.

Table 2 PBL enzyme activities of dogs and cats

		Dogs (n=6)	Cats(n=6)
Cytosol			
	HK	12 (3)	11 (2)
	FK	30 (10)	59 (13)*
	PK	48 (13)	94 (17)*
	G6PD	92 (26)	167 (29)*
	MDH	740 (93)	375 (52)*
	LDH	1541 (236)	2711 (365)*
	M/L	0.48 (0.04)	0.14 (0.02)*
	AST	41 (21)	26 (8)
	ACL	3.7 (1.4)	4.7 (0.3)
Mitochondria			
	MDH	783 (109)	667 (159)
	AST	79 (25)	72 (30)
	GLDH	98 (20)	52 (16)*

Values are presented as mean (SD).

Enzyme activity is presented as mU/mg protein.

M/L is calculated as MDH specific activity divided by LDH specific activity.

* Significantly different ($P < 0:01$) from the values of dogs.

Chapter 2

The use of leukocytic MDH and M/L ratio to assess metabolic condition of diabetic dogs

Introduction

Diabetes mellitus is one of the most commonly seen metabolic diseases in small animal practices. Diabetes is a disorder of glucose regulation and is characterized by hyperglycemia due to insulin deficiency, insulin resistance, or both. Diabetes mellitus is categorized into 2 main types: type 1 (insulin-dependent) with an absolute deficiency in insulin secretion, and type 2 (non-insulin dependent) with a combination of resistance to insulin action and insulin secretory defect (American Diabetes Association, 2010). In the case of type 1 diabetes, insulin injection, which supplements the deficiency in insulin secretion, is an essential part of the treatment. In the previous study (Eto et al., 1999), the importance of NADH shuttle function in glucose-induced insulin secretion was proposed. There are two NADH shuttles, the glycerol phosphate and the malate aspartate, which transfer cytosolic NADH into mitochondria for oxidative metabolism and ATP production, thus generating mitochondrial energy. Therefore, NADH shuttles are essential for coupling glycolytic metabolism to the activation of mitochondrial signals, which in turn, triggers glucose-induced insulin secretion.

Glucose and lipids are the main sources of energy in most mammals. Just like aspartate aminotransferase (AST), malate dehydrogenase (MDH), a rate-limiting

enzyme of the malate-aspartate shuttle, is involved in glucose and lipid metabolism (Setoyama et al., 1988) by transporting cytosolic NADH into mitochondria to initiate oxidative ATP production (Hedskov et al., 1987). Alternately, lactate dehydrogenase (LDH) mediates a reaction that converts cytosolic pyruvate to lactate, consuming cytosolic NADH. Theoretically, since cytosolic LDH activity is considered to be relatively stable under various metabolic conditions, a cytosolic MDH/LDH (M/L) ratio may be useful in determining energy expenditure rate in various animal tissues. In this study, MDH, LDH, AST, and M/L ratio were measured and compared in diabetic dogs against normal control dogs. The objective was to evaluate the usefulness of aforementioned parameters as diagnostic/monitoring tools to evaluate the overall condition of the diabetic dogs.

Materials and Methods

Animals

16 mixed-breed dogs were utilized in this study. The subjects were separated into 2 groups: diabetic (4) and control (12). The diabetic group included 3 females and 1 male of ages between 5-7 years old, previously diagnosed of having type 1 diabetes mellitus. The spot plasma glucose (GLU) and immunoreactive insulin (IRI) concentrations, measured without any regard to the time elapsed since the latest meal, were greater than 302.4 mg/dl and 60pmol/l, respectively. These dogs were presented to the Veterinary Medical Teaching Hospital of Nippon Veterinary and Life Science University with uncontrolled diabetes mellitus, despite the previous administrations of insulin therapy at the referring hospitals. The insulin treatment was implemented with the use of 2-3U of injectable insulin (Monotard 40, Novo Nordisk, Denmark) every 12 hours for 2 weeks until the diabetic condition was stabilized with their GLU concentration between 126 and 144 mg/dl at 4 hours after the morning insulin injection. The control group consisted of 6 females and 6 males of ages between 5-9 years. They were laboratory dogs maintained for research in our laboratory and deemed clinically normal.

Collection and preparation of blood samples

Blood samples were collected from the jugular vein of each dog fasted overnight (at least 8 hours after the last meal), into the heparinized tubes. The blood collection was carried out in the morning and each diabetic dog received its morning meal and insulin administration immediately following the blood collection. The blood samples were left at room temperature for 15 to 20 min after the collection, then plasma was recovered by centrifugation at 3,000 rpm for 15min at 4℃ and stored at -80℃ until subsequent use.

Leukocytes were isolated by gradient centrifugation with LSM™ lymphocyte separation isolating solution (ICN Biochemical, Aurora, OH, USA) as instructed by manufacturer`s instructions. Cytosolic and mitochondrial fractions of leukocytes were prepared and isolated via a method previously described (Washizu et al., 1998).

Plasma metabolite assays

Plasma glucose (GLU) concentrations were determined by the glucose oxidase method described by Huggett and Nixon (1957) and immunoreactive insulin (IRI) concentrations were determined by the ELISA method (Arai et al., 1989). Plasma free fatty acids (FFA) and total triglyceride (TG) concentrations were determined using commercial kits (Wako Pure Chemical Industries, Tokyo, Japan).

Enzyme activity assays

The MDH (Bergmeyer and Bernt, 1974c), LDH (Kaloustian et al.,1969), and

AST (Rej and Horder, 1983) activities in both the cytosolic fraction of leukocytes and plasma were measured by previously reported methods. Glutamate dehydrogenase (GLDH) activities in mitochondria were measured by the method described by Schmidt (1974). All enzymatic activities measured at 24-26ºC were expressed as mU per mg of protein. The enzyme unit (U) represents 1μmol of substrate degraded per min. Protein concentration was measured by the Bradford (1976) method. The cytosolic M/L ratio was calculated as MDH specific activity divided by LDH specific activity.

Results

All values of the diabetic group are presented in Table 1, and the control values are also presented as mean (SD) of 12 dogs in Table 1. In Table 2, the percentage differences of parameters of the diabetic group as compared against the healthy normal dog values (control) are listed. Although there is a range due to the individual variation of the diabetic status, plasma GLU, FFA, TG concentrations of the diabetic dogs are much higher, and leukocytic MDH, M/L and AST are much lower than the control dogs.

Mean fasting plasma GLU of the diabetic dogs was 188% greater with its range from 142-244% increase. Mean plasma FFA was 70% greater in the diabetic group with a range of 44-96% increase. All of the individuals within the diabetic group showed higher plasma TG concentrations than the mean control value (7-44% greater) with the mean percentage increase of 24%. Activities of cytosolic MDH (42% lower) and AST (24% lower), and the resultant M/L ratio (33% lower) were noticeably lower in the diabetic dog group. The diabetic dogs demonstrated the average of 32% (range 32-44%) less leukocytic mitochondrial MDH activity as compared to the control group.

Discussion

Enzyme activities within the malate–aspartate shuttle reflect energy metabolism in animal tissues (Arai et al., 1998). An elevated M/L ratio would reflect a heightened level of energy metabolism and ATP production, and a decreasing M/L ratio would indicate a conservation or defect in energy metabolism and ATP production (Washizu et al., 2001). Previously, cytosolic M/L ratios of the canine lymphoma cells were compared against those of the healthy dogs' normal lymphocytes. The elevated M/L ratios faithfully reflected the changes in glucose metabolism and the increased efficiency in energy production, generally expected in neoplastic cells (Washizu et al, 2005). Conversely, in the previous study (Fararh et al., 2010), untreated diabetic rats with glucose metabolism dysfunction showed significantly lower cytosolic MDH activities and M/L ratios in peripheral blood leukocytes than those of the healthy control and the treated diabetic rats, and the depressed activities restored after successful glycemic control.

Glycolysis yields cytosolic NADH and pyruvate for the TCA cycle, which then activate ATP generation in mitochondria. In the study performed by Eto et al. (1999), a blockage of NADH shuttle function in pancreatic ß cells depressed insulin secretion, indicating its crucial role in coupling of glycolysis with insulin secretion. Decrease in MA shuttle enzyme activities may be associated with depressed use of glucose such as

glucose uptake and glycolysis in peripheral tissues, resulting in increased circulating plasma glucose concentrations (Ferrannini et al., 1985). In the present study, type 1 diabetic dogs showed lower levels of MDH, resultant M/L ratio, as well as AST, compared to the healthy control dogs. Decreased activity levels of MA shuttle enzymes may be one of the characteristics of energy metabolism in diabetic dogs, and may be useful as a diagnostic indicator to monitor the overall metabolic condition of diabetic patients. Evaluating MDH activities and ML ratios in various diabetic patients, such as uncontrollable patients vs. controlled, or obese vs. cachectic patient etc., may elucidate the association between NADH shuttle function and diabetes development, and may help identify those at risk and predict the disease progression. Mechanism of decrease in MDH activities and its effect on development of diabetes should be studied further and in other metabolic diseases such as type 2 diabetes mellitus and obesity.

Conclusion

Assays of MDH and M/L ratio on leukocytes may serve as sensitive diagnostic tools for assessing overall conditions of type 1 diabetic dogs. Further investigations elucidating the association between NADH shuttle function and insulin secretion may help identify those at risk and predict the disease progress. The diagnostic significance of these parameters should be further examined on type 2 diabetic animals and various types of metabolic diseases.

Tables

Table 1 Plasma GLU, IRI, FFA, and TG concentrations and leukocytic metabolic enzyme activities and M/L ratio of diabetic and control dogs

	Leukocyte (Cytosol)				Leukocyte (Mitochondria)		
	MDH	LDH	M/L	AST	GLDH	MDH	AST
Diabetic							
1	455	1484	0.31	37	30	396	68
2	484	2035	0.24	41	36	380	52
3	428	1719	0.25	30	28	377	34
4	249	1120	0.22	32	33	323	38
Mean(SD)	404(105.83)	1590(385.9)	0.26 (0.0387)	35(4.966)	31.8(3.5)	369(31.78)	48(15.4)
Control(n=1	700(140)	1740(280)	0.39(0.07)	46(5)	36(8)	580(88)	48(8)

	Age	Sex	Plasma			
			Glu(mg/dl)	IRI(pmol/l)	NEFA(mEq/l)	TG(mg/dl))
Diabetic						
1	6	M	226.8	120	0.72	51.3
2	5	F	286.2	120	0.80	53.10
3	7	F	322.2	144	0.88	63.7
4	5	F	244.8	240	0.98	69
Mean(SD)			270(42.8)	156(57)	0.85(0.11)	59.3(0.84)
Control(n=12)			93.6(12.6)	132(24)	0.50(0.12)	47.8(8.84)

Enzyme activitiy is presented as mU/mg of protein

M/L ratio is calculated by the division of MDH activity by LDH activity.

Control values are presented as mean (SD) of 12 dogs

Table 2 Percentage difference of the diabetic plasma metabolite concentrations and enzyme activities as compared to the control mean values

	Leukocyte (Cytosol)				Leukocyte (Mitochondria)		
	MDH	LDH	M/L	AST	GLDH	MDH	AST
Diabetic							
1	-35%	-15%	-21%	-24%	-17%	-32%	42%
2	-31%	17%	-38%	-11%	0%	-34%	8%
3	-39%	-1%	-36%	-35%	-22%	-35%	-29%
4	-64%	-36%	-44%	-30%	-8%	-44%	-21%
Mean	-42%	-9%	-33%	-24%	-12%	-32%	0%

	Plasma			
	Glu	IR	NEFA	TG
Diabetic				
1	142%	-9%	44%	7%
2	206%	-9%	60%	11%
3	244%	9%	76%	33%
4	162%	82%	96%	44%
Mean	188%	18%	70%	24%

Values represent percentage difference as compared to the control mean values

(-) values indicate the degrees fewer than the control mean values in percentage

Chapter 3

Comparison of plasma malate dehydrogenase, lactate dehydrogenase, and M/L ratio and lipid mobilization rate between racehorses and riding horses

Introduction

Extraneous training can cause mitochondrial functional changes in horses including improved mitochondrial respiration in certain muscle groups (Votion et al., 2010). Increased activities of the malate–aspartate shuttle enzymes indicate improved efficiency in energy metabolism in animal tissues (Arai et al., 1998). Specifically, malate dehydrogenase (MDH) plays a crucial role in metabolism of glucose and lipids (Setoyama et al., 1988) by transporting cytosolic NADH into mitochondria to initiate oxidative ATP production (Fig 1) (Hedskov et al., 1987). Alternately, lactate dehydrogenase (LDH) mediates a reaction that converts cytosolic pyruvate to lactate consuming cytosolic NADH (Fig 1). Pyruvate is the final product of glycolysis, and is converted to oxaloacetate acid, acetyl CoA, and alanine, which is affected by energy metabolic changes in animal tissues. Therefore, pyruvate metabolism is considered to reflect the energy metabolic status within animal tissues. Since cytosolic LDH appears to maintain its stability under various metabolic conditions, MDH/LDH (M/L) ratio may be a useful marker for energy expenditure, and an elevation in M/L ratio would indicate a heightened level of energy metabolism such as increased ATP productions within animal cells and tissues (Washizu et al., 2001).

Previous study (Arai et al., 2002) revealed a significant elevation in the activity of MDH in racehorses undergoing continuous extraneous training, compared to that

of riding horses. An elevated MDH activity may reflect improved efficiency in energy production within the muscle tissues necessary for the maintenance of high performance level. In the previous Thoroughbred racehorse studies, the usefulness of M/L ratio in the peripheral blood leukocytes (PBL) has been assessed (Arai et al., 2002, 2001; Hosoya et al., 2004). However, the use of PBL as a source for determining M/L ratio has resulted in inconsistent results: one study yielded a significantly higher M/L ratio for racehorses (Arai et al., 2002), whereas another study did not (Arai et al., 2001). The use of PBL may not be appropriate to determine M/L ratios in horses with varying activity levels, since the enzymatic activities of PBL malate-aspartate shuttle may not directly reflect the ATP production rate in skeletal muscles, but rather, may reflect the energy metabolism of other tissues in horses (Arai et al., 2003b). This is because of the possible differences in LDH among various tissue sources, as suggested by the difference in LDH isoenzyme patterns between plasma and PBL (Arai et al., 2003a; Hatzipanagiotou et al., 1991). Additionally, because PBL are involved in immune function and host defense, the shifts in immune status are considered to affect energy metabolism and MDH activities.

The goal of this study is twofold. The first is to compare the activities of MDL, LDH, and the MDL ratio between racehorses and riding horses, and to assess

the usefulness of M/L ratio in PBL and plasma as an evaluation marker. Secondly, we profiled plasma lipid metabolism analytes to determine whether the heightened energy metabolism in racehorses is reflected in the rate of lipolysis.

Materials and Methods

Animals

All of the horses studied were Thoroughbreds, and were deemed healthy and non-pregnant (in the case of mares) by the veterinarian on site. Five Thoroughbred racehorses (3 males and 2 females, 3-7 years old) used in this study were held and trained at the Saitama Prefectural Urawa Horse racing Association's Noda Training Center in Saitama, Japan. Five riding horses (1 male, 1 female, and 3 geldings, 6-22 years old) were kept at the Saitama Riding Club Association within the same property as the training center mentioned above. The study duration was 10 weeks. A 2- hour (5:00 to 7:00 am) daily exercise regimen for each racehorse was as follows: a 10-day workout period allotting 3 days to fast galloping (13 to 18 $ms^{-1}$for 1000–1200 m), 6 days to slow walking (6 to 8 ms^{-1} for 1500–2000 m) with each following a warm-up and ending with a cool-down, and 1 day to rest. Each riding horse was exercised 6 days out of one week by walking (2 to 3 ms^{-1} for 5 to 10 min) and trotting (4 to 6 ms^{-1} for 15 to 20 min), and resting on a 7^{th} day, over a 10 week period. Each horse was provided high quality hay and concentrated feed, and reared on a grass field. All racehorses received additional protein supplementation. Daily feeding was from 5 am to 10 am, and 5 pm to 7 pm.

Sample collection

The study subjects belonging to either group were rested on the day of blood collection. Each blood sample was collected from the jugular vein into the heparinized tubes between 12:00 to 14:00 (4 hours postprandial). Each horse was handled with care during the blood collection procedure to avoid fear and excitement. Blood samples were left at room temperature for 15-20 minutes, then centrifuge at 4℃, 3,500rpm, then stored at -25℃, until use. Leukocyte isolation was performed by gradient centrifugation with LSM™ lymphocyte separation isolating solution (MP Biochemicals LLC, Solon, OH, USA) following the manufacturer's instructions. Cytosolic fractions of leukocytes were prepared and isolated according to the method described by Washizu et al (1998).

Plasma metabolites

Plasma glucose (GLU), total cholesterol (TC), total triglyceride (TG) were measured using an autoanalyzer (AU2700, Olympus Corporation, Tokyo, Japan) with the manufacturer's reagents. Plasma non-esterified fatty acid (NEFA) and adiponection concentrations were measured using a Wako NEFA-C test commercial kit (Wako Pure Chemical Industries, Inc., Tokyo, Japan) and a mouse/rat adiponectin kit (Otsuka Pharmaceutical Co., Tokyo, Japan) respectively. Immunoreactive insulin (IRI) concentration was determined by using a commercial porcine insulin ELISA kit (AKRIN-013T, SHIBAYGI Co., Gunma, Japan) and equine insulin standard (AKRIN-019, SHIBAYAGI Co., Gunma, Japan).

Enzyme activity assays

The MDH (Bergmeyer and Bernt, 1974c) and LDH (Kaloustian *et al.*, 1969) activities in both the cytosolic fraction of leukocytes and plasma were measured by previously reported methods. All enzymatic activities measured at 24-26ºC were expressed as U per liter of plasma (volume activity) and mU per mg of protein in cytosolic fractions (specific activity). The enzyme unit (U) represents 1µmol of substrate degraded per min. Protein concentration was measured by the Bradford (1976) method. The cytosolic M/L ratio was calculated via MDH specific activity divided by LDH specific activity.

Statistical analysis

Results are presented as median ± min/max range. Groups were compared using the Mann–Whitney U-Test for data with non-normal distribution. Statistical significance level was set at $P < 0.05$. All tests were conducted using Sigmaplot analysis software (Sigmaplot 11.0, Build 11.0.077; Systat Software Inc., San Jose, CA).

Results

The results are as shown in Table 1. Riding horse group was set as the control as comparatively lower energy metabolism level than racehorses was expected. In PBL, MDL activity was significantly higher (Mann Whitney U-test, $p<0.05$) in racehorses, but no significant difference was seen in LDH activity and the resultant M/L ratio. Meanwhile both MDH and LDH activities were higher in racehorse plasma, and the resultant M/L ratio difference was twice that of the riding horses (2.04 x). Additionally, M/L ratio in plasma compared to that in PBL was 4 times more in racehorses, and about twice in riding horses. In Table 2, the measurements of the plasma metabolites are shown. Compared to the control (riding horses), racehorses showed significantly higher concentrations of most metabolites measured in this study. Particularly, differences in parameters of energy metabolism were remarkable: Racehorses showed 12% more GLU and 50% more NEFA concentrations, twice the amount of TG and adiponectin concentrations than seen in riding horses.

Discussion

Previously, we focused on the diagnostic values of peripheral leukocytes as potential markers of physiological changes occurring in animal tissues. The term "sentinel principle" is based on the idea that peripheral leukocytes travel through the entire body and continuously interact with the cells of each tissue, accurately reflecting the subtle changes occurring in response to internal and external stimuli as they may arise (Liew et al., 2006). However, in this case, the use of plasma as opposed to PBL to determine M/L ratio appeared to be more sensitive, since it resulted in significant difference between racehorses and riding horses. In fact, PBL based data indicated no significance in M/L ratio between the groups.

The reasons why plasma may have been more appropriate in this case to determine M/L ratio are as follow. First, the isoenzyme pattern of LDH between plasma and PBL are different: LDH-1, -2, and -3 are dominant in plasma (Arai et al., 2003a; Hatzipanagiotou et al., 1991) while LDH-3 and -4 are dominant in peripheral leukocytes (Arai et al., 2003a) suggesting a possible difference in LDH tissue source representation. LDH is a tetramer, composed of four subunits of the H and M molecules, and its subunit composition determines the kinetic behavior of each isoenzyme (Bishop et al., 1972). Isoezymes, as described by Hunter and Markert (1957), are the different variants of the same enzyme with identical functions within the same individual. LDH-1 are dominant

in heart and RBCs; LDH-2 in monocytes and macrophages; LDH3 in lungs; LDH-4 in kidneys, placenta, and pancreas; and LDH-5 in liver and striated muscle. As such, LDH activity in plasma may be more encompassing of the whole body as opposed to that exhibited by peripheral leukocytes. In racehorses, that face increased energy demands especially in skeletal muscles, plasma M/L ratio may be a better indicator of the whole body energy metabolism than leukocytic M/L ratio, that mainly reflects the energy metabolism of the hepatic cells. Secondly, because leukocytes are involved in immune function and host defense, any stimuli to the immune system may have an effect on energy metabolism, affecting the MDH activity levels. For example, it has been previously documented that dogs and rats suffering from Type 1 DM exhibit lower PBL MDH activity levels than those of the healthy control. (Arai et al. 2003b; Fararh et al., 2010).

The results of plasma metabolites from this study were in accordance with the theory that MDH/LDH (M/L) ratio may be a useful marker for assessing energy expenditure, and elevation in M/L ratio would indicate a heightened level of energy metabolism such as increased ATP productions within animal tissues (Arai et al., 2001, 2002). In this case, racehorses would represent a model for adaptation to a regular extraneous training over a long period, while riding horses would represent a model for adaptation to a suboptimal semi-regular non-extraneous training. A long-term

extraneous training most likely induced heightened state of energy metabolism and was reflected by the elevated levels of plasma MDH and LDH activities in racehorses.

In order to evaluate the association between this heightened overall energy status and metabolism of glucose and lipids, we compared the plasma metabolite values between racehorses and riding horses.

During the long-term extraneous training, glycogenolysis and lipolysis are essential for energy production in muscle tissues (Rose et al., 1980; Essén-Gustavsson and Jensen-Waern, 2002). Significantly higher glucose and insulin in racehorses reflect a higher rate of glycogenolysis to provide free glucose-6-phosphate muscle cells and glucose for other tissues. Additionally, evidence of accelerated lipid mobilization is indicated by significant elevations in NEFA (50% greater), TG (100% greater), adiponectin (100% greater), and TC (20% greater) in racehorses as compared to riding horses. These findings suggest that the muscle tissues of the racehorses have adapted to prolonged extraneous training by increasing fat utilization as an energy source and gaining a higher oxidative capacity. This may support the ideas that the horses adapted to high fat diet may have more efficient metabolism during exercise compared to those provided high carbohydrate diet (Treiber et al., 2006).

The limitations of this study are as follow. First of all, the number of horses in each group was small and a one-time blood sample collection per horse may have resulted in

low statistical power. Secondly, the riding horses used in this study were significantly older compared to the racehorses, with a mean age of 12.8 as opposed to 5.2 years. Aging may have an impact on energy expenditure, and thus matching the age between the two groups may be appropriate for the future. Third, postprandial influence on plasma metabolite values such as glucose, insulin, and triglycerides should be considered since the blood was collected within 4 hours of feeding. In general, 8-12-hour fasting time is recommended to avoid postprandial dynamic changes in plasma concentrations of lipid components, However, since the food formula provided to both groups of horses was fundamentally the same, except for some additional protein supplementations for race horses, any variations can be interpreted as the individual differences.

Conclusion

A long-term extraneous exercise regiment appears to result in increased efficiency in energy production and expenditure, as evidenced by higher plasma M/L ratio and increased lipolysis rate in racehorses as compared to riding horses. This may be due to the adaptation of the racehorses to higher activity levels resulting in mitochondrial functional changes, such as marked increases in muscle mitochondrial respiration, in order to process and consume energy more efficiently. Racehorses also appeared to have an increased rate of lipid mobilization, which would indicate the changes in their skeletal muscles gaining a higher oxidative capacity and increased capacity for fat utilization as an energy source to adapt to prolonged extraneous exercise. The results indicated that M/L ratio may serve as the useful marker to assess the effectiveness of different exercise regimen, diet, and nutritional supplements in performance animals.

Tables and Figures

Table 1 Comparison of MDH, LDH activities, and M/L ratio between leukocytes and plasma in race and riding horses

		Racehorses (n=5)	Riding horses (n=5)
Leukocytes	MDH (U/L)	393.00*	248.58
		(307.88, 425.63)	(235.33, 265.62)
	LDH (U/L)	769.96	622.38
		(553.79, 983.23)	(329.21, 778.99)
	M/L ratio	0.51	0.41
		(0.31, 0.74)	(0.31, 0.71)
Plasma	MDH (U/L)	433.88*	112.60
		(322.12, 536.37)	(80.86, 168.11)
	LDH (U/L)	193.01*	111.21
		(169.39, 279.48)	(80.66, 162.77)
	M/L ratio	2.04*,**	1.00**
		(1.62, 3.17)	(0.87, 1.40)

values are presented as median with (range)

* significant difference as compared to riding horses (Mann Whitney U-test, $p < 0.05$)

** significant difference as compared to PBL (Mann Whitney U-test, $p < 0.05$)

Table 2 Plasma metabolite comparison between race and riding horses

Metabolites	Racehorses (n=5)	Riding horses (n=5)
Glucose (mg/dL）	105.69* (99.36, 161.01)	93.86 (65.78, 99.63)
Insulin (ng/mL)	1.22* (1.19, 1.57)	0.68 (0.49, 0.88)
Triglyceride (mg/dL）	25.15* (14.80, 33.20)	11.35 (11.35, 14.8)
NEFA (mEq/dL）	0.075* (0.067, 0.104)	0.050 (0.02, 0.069)
Total cholesterol (mg/dL）	61.92* (58.96, 80.87)	53.10 (48.13, 57.70)
Adiponectin (μg/ml)	0.77* (0.55, 1.06)	0.39 (0.28, 0.45)

values are presented as median with (range)

* denotes significant difference as compared to riding horses (Mann Whitney U-test, $p < 0.05$)

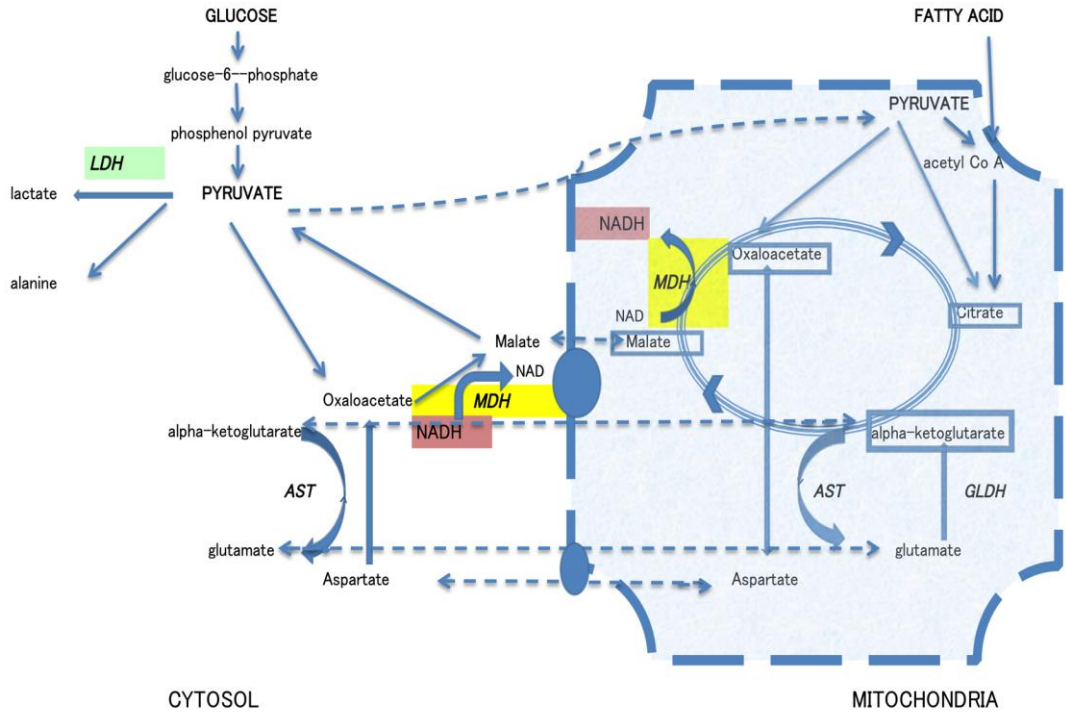

Fig 1. A schematic view of a typical animal cell showing glycolysis, malate aspartate shuttle, and TCA cycle

Figure legend

Fig 1: A schematic view of a typical animal cell showing glycolysis, malate aspartate shuttle (MA shuttle), and TCA cycle.

Note how the enzymes of MA shuttle, MDH (malate dehydrogenase) and AST (aspartate transferase), contribute to coupling of glycolysis and TCA cycle via NADH transfer. Pyruvate, lactate, malate, oxaloacetate, and NADH are closely associated via the activities of LDH and MDH.

Chapter 4

Changes in plasma malate dehydrogenase, lactate dehydrogenase, and M/L ratio as energy metabolism markers of acute weight gain in dogs

Introduction

In recent years, the prevalence of obesity and its associated metabolic diseases in companion animals has been increasing and the awareness of prevention of weight gain/obesity has risen more than ever (Lund et al., 2005, 2006). Obesity is one of the risk factors for the metabolic syndrome (MS), a cluster of the risk factors for heart attack, which include insulin resistance, hyperglycemia, abdominal obesity, high cholesterol, high triglyceride, and high blood pressure (IDF, 2005). Overweight and obese individuals are at increased risk for developing associated diseases such as diabetes mellitus, orthopedic disease, neoplasia, respiratory and urinary disease, and have shorter life span (Burkholder, 2000; German et al., 2010). Additionally, a recent developing concern revolves around the concept that obesity is accompanied by chronic low-grade systemic inflammatory response caused by increased insulin resistance and production of inflammatory mediators, which in turn, may contribute to the onset of obesity-related diseases. An adipocyte is considered not only inert fuel storage, but also an active secretory and endocrine organ (Trayhurn et al., 2005, 2006). Among many secreted substances, protein factors referred to as adipokines, are of particular interest, since they are involved in a wide range of physiological processes such as hemostasis, lipid metabolism, blood pressure regulations, insulin sensitivity, and immune functions

(pro-inflammatory and anti-inflammatory), and may contribute to the development of MS in obese individuals (Laflamme et al., 2012).

In veterinary medicine, there is no consensus on quantitative mechanical and biochemical parameters and their reference ranges as potential indicators to gauge the stages of weight gain, and to confirm the presence of pathological weight gain. One commonly accepted evaluation method of weight status is body condition score (BCS), a semi-quantitative assessment with a range of categories from cachectic to severely obese (Laflamme, 1997). However, a classification of BCS may be subjective since it employs visual observation and palpation of an observer. Interobserver variation is inevitable and it can be problematic when a borderline overweight/obesity is being evaluated since it may confound the point where early medical and/or environmental intervention is warranted. Previously, many researchers have introduced various quantitative parameters such as lipid concentrations (Watson and Barrie 1993; Johnson et al., 2005) and lipoprotein profiles (Jericó et al., 2009; Mori et al., 2011), and their reference values to distinguish overweight and obese individuals from the normal ones.

In this chapter, we investigated the changes in plasma biochemical and metabolite concentrations, which had been previously studied as potential diagnostic indicators of pathological weight gain, in experimentally overfed dogs. More importantly, we studied the energy metabolism enzyme markers, malate dehydrogenase

(MDH), lactate dehydrogenase (LDH), and MDH-LDH (M/L) ratio, in experimentally overfed dogs to determine their correlations with body weight (BW) and BCS changes, and to evaluate their potential diagnostic significance as one of the parameters in early detection of weight gain and prevention of MS.

Materials and Methods

Animals

16 male Beagle dogs of ages 2 to 4 years were utilized in this study. The subjects were separated into 2 groups: overfed (13) and control (3). The study duration was 4 weeks. The diet given was BEAUTY pro® (Nihon Pet Food Inc., Tokyo) and its nutrition composition was stated as follow: Crude protein (25.0% minimum), crude fat (12.0% minimum), crude fiber (3.0% maximum), ash (8.5% maximum), moisture (10.0% minimum), calcium (1.0% minimum), phosphorus (0.8% minimum). 13 dogs in the overfed group were each fed twice the daily energy requirement (DER) as appropriate for its age and current weight (1.4×70 x $Weight^{0.75}$), separated into 3 feedings per day. As a reference, the control dogs were also fed the diet mentioned above, but only given 1 x DER separated into 3 meals/day. Each subject was kept in a cage measured 45cm (width) x 55 cm (length) x 75 cm (depth), and was given water ad libitum and allowed 10 hours of day light (8 am to 6 pm), and minimal daily activity within the cage. Each subject was evaluated by the same veterinarian on-site each time and deemed clinically healthy, and was classified by BCS of 1-5 based on palpation and visual inspection. The referred BCS in this study was a 5-point scale with: 1) very thin, 2) underweight, 3) ideal, 4) overweight, and 5) obese. Ethical approval was obtained

from Institutional Animal Care and Use Committee in Nippon Veterinary and Life Science University.

Collection and preparation of blood samples

5ml of postprandial blood samples were collected from the cephalic vein of each dog, fasted overnight (at least 8 hours after the last meal), into the heparinized tubes. The blood samples were left at room temperature for 15 to 20 min after collection, and then plasma was recovered by centrifugation at 3,000 rpm for 15min at 4℃ and stored at -80℃ until subsequent use.

Leukocytes were isolated by gradient centrifugation with LSM™ lymphocyte separation isolating solution (MP Biochemicals LLC, Solon, OH, USA) as instructed by manufacturer`s instructions. Cytosolic fractions of leukocytes were prepared and isolated via a method previously described (Washizu et al., 1998).

Plasma metabolite assays

Plasma glucose (GLU), total cholesterol (TC), total triglyceride (TG), total protein (TP), blood urea nitrogen (BUN) and creatinine (CRE) concentrations, and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities were measured using an autoanalyzer (JCA-BM2250, JEOL Ltd., Tokyo, Japan) with the manufacture’s reagents at Monolis Inc. (Tokyo, Japan).

Enzyme activity assays

MDH (Bergmeyer and Bernt, 1974c) and LDH (Kaloustian et al., 1969) activities in both the cytosolic fraction of leukocytes and plasma were measured by previously reported methods. All enzymatic activities measured at 24-26ºC were expressed as U per liter of plasma (volume activity) and mU per mg of protein in cytosolic fractions (specific activity). The enzyme unit (U) represents 1μmol of substrate degraded per min. Protein concentration was measured by the Bradford (1976) method. M/L ratio was calculated as MDH activity divided by LDH activity.

Statistical analysis

The comparison was made between pre- and post-overfeeding samples of the same group. Results were presented as mean ± SD. Statistical significance was determined by paired Student's *t*-test. The significance level was set at $p < 0.05$.

Results

Table 1 shows the changes in BW, BCS, plasma metabolites, and enzyme activities in both plasma and peripheral leukocytes, and M/L ratios of the overfed and control individuals comparing pre- and post-4-week diet trial period. Table 2 shows the comparison of pre- and post-feeding trial mean values of each group (overfed and control groups). The overfed dogs showed about 28.2% increase in the BW and the increase of BCS from 1.9 to 3.4, whereas the control group showed only a 4.6% BW increase and the BCS increase of 0.5 points. When the plasma and leukocytic parameters of pre- and post-feeding periods were compared, the significant elevations were noted in TG, TC, GLU ALP, BUN, leukocytic MDH and LDH of the overfed group. Although not significant, both the plasma MDH and LDH activities decreased, whereas leukocytic MDH and LDH activities increased in the overfed group after the feeding trial. Both the resultant plasma and leukocytic M/L ratios showed mild increase in the overfed group after the feeding trial.

Discussion

Enzyme activities within the malate–aspartate shuttle reflect energy metabolism in animal tissues (Arai et al., 1998). Glucose and lipids, as well as amino acids, are the main sources of energy in most mammals. Malate dehydrogenase (MDH), a rate-limiting enzyme of the malate-aspartate shuttle, plays a crucial role in metabolism of glucose and lipids (Setoyama et al., 1988) by transporting cytosolic NADH into mitochondria to initiate oxidative ATP production (Hedskov et al., 1987). Alternately, LDH mediates a reaction that converts cytosolic pyruvate to lactate consuming cytosolic NADH. Theoretically, since cytosolic LDH activity is considered to be relatively stable under various metabolic conditions, a cytosolic MDH/LDH (M/L) ratio may be useful in determining energy usage in various animal tissues. An elevated M/L ratio would reflect a level of energy metabolism and ATP production, and a decreasing M/L ratio would indicate a conservation or defect in ATP production (Washizu et al., 2001).

Glycolysis yields cytosolic NADH, and pyruvate for the TCA cycle. The malate-aspartate shuttle plays a crucial role in insulin secretion by coupling glycolysis with the activation of ATP generation in mitochondria (Eto et al., 1999). In the study performed by Eto et al. (1999), blockage of NADH shuttle function in pancreatic ß cells depressed insulin secretion. In other studies, MDH activity levels and M/L ratios in

dogs and cats suffering from spontaneous diabetes were lower in peripheral leucocytes than those of the control animals (Arai et al., 2005; Magori et al., 2005). As presented in Chapter 1, cats that show decreased ability to use glucose as energy and increased tendency to store energy as fat had lower MDH activity and M/L ratio. In the diabetic cats, intrinsically lower activities of MDH in leucocytes decreased even further (Magori et al., 2005). Furthermore, our team previously showed that the changes in MDH activity in leucocytes of experimentally induced diabetic dogs faithfully reflected the changes in metabolic condition, as its depressed activity improved after the successful glycemic control with intensive insulin treatments (Arai et al., 2002).

Since the changes in M/L ratio reflect the energy metabolism and health status in animals, we sought a diagnostic potential in M/L ratio as a marker for confirming early weight gain in conjunction with BCS changes, in apparently healthy animals exhibiting no overt clinical sequelae of weight gain.

In this chapter, we focused on dogs with experimentally induced acute weight gain. The weight gain in our experimentally overfed group was 28.2% over a 4-week period with a BCS increase of 2.5 points. However the resultant BCS was 3.5, and did not quite reach the overweight/obese categories, but rather, it resulted in an improvement of the weight status from cachectic/thin to ideal/mildly overweight.

Concomitantly, significant elevations in GLU, TG, TC were also noted, although the values weren't high enough to reach the levels of hyperglycemia and hyperlipidemia set by the new MS diagnosis criteria (Kawasumi et al., 2012) or the hypertriglyceridemia and hypercholesterolemia levels used as the common signs of obesity (Watson and Barrie, 1993; Johnson, 2005). Although not significant, leukocytic and plasma M/L ratios of the overfed group showed mild increasing trends which may reflect improved energy metabolism status with a better nutritional status and positive energy balance. As a future study, we plan to investigate whether the various types of weight gain (i.e. acute weight gain, chronic, mild, severe, and visceral, or subcutaneous obesity) can influence or induce changes in biochemical metabolite concentrations and energy metabolism markers differently.

Changes in energy metabolism may be reflected more faithfully in tissues, such as muscle, liver, or adipose tissues, that directly require, generate, and process energy in the forms of NADH, NADPH, ATP, lipid and glucose precursors. Higher enzymatic activities in these tissues may reflect elevated energy metabolism, indicating more ATP production, energy generation, and mitochondrial respiration. Conversely, lower activities of energy metabolism enzymes may indicate depressed, conserved, inefficient energy metabolism in these tissues. In the study of Macková et al. (1982),

skeletal muscles of high-performing skiers showed elevated activities of TCA (MDH etc.) and glycolytic (LDH etc.) enzymes during the pre-competition training and post-competition periods, compared to those of the recreational skiers, and the elevation faithfully reflected the increase in physical exercise. The activity of MDH, involved in generation of glucose-derived fatty acid precursors and NADPH production required for fatty acid synthesis, was also shown to be higher in active adipose tissues of the obese swine compared to that of the lean swine (Hood and Allen, 1973).

In general, leykocytes and plasma are considered to reflect subtle physiological changes occurring in animal tissues (de Mello et al., 2008; Oliver et al., 2013), and the enzyme activity of leukocytic and plasma malate-aspartate shuttle could be an indicator for changes in energy metabolism of the whole body (Arai et al., 2003c). In this chapter, we monitored the changes in MDH, LDH activities, and M/L ratios of leukocytes and plasma to assess their usefulness in clinically more accessible forms. However the sensitivity of this method seems to be low in detecting early acute weight gain.

In the future, it will be ideal to measure and compared the changes in various cells/tissues such as leukocytes, plasma, muscle, liver, and adipose tissues in order to follow the trends in energy usage efficiency associated with changes in weight status.

Conclusion

Assays of MDH, LDH, and M/L ratio on plasma and leukocytes are not sensitive as diagnostic tools for detecting acute weight gain, but may reflect energy metabolism and nutritional status of individuals. The diagnostic significance of the above mentioned parameters should be further examined on various types of weight gain and target tissues in order to identify pathological weight gain that results in MS and decreased life span.

Table 1 Changes in BW, BCS and plasma and leukocytic biomarker levels after 4-wk overfeeding of DERx2(1-13) vs 4-wk feeding of DERx1(C1-3)

					Plasma						Leukocyte					
No.	BW(kg)		BCS		MDH(IU/L)		LDH(IU/L)		M/L		MDH(IU/L)		LDH(IU/L)		M/L	
	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post
1	10	13.8	3.0	4.0	128.29734	81.78018	86.10188	49.07807	1.49006	1.66633	193.03864	237.29687	827.08866	1130.88156	0.23340	0.20983
2	7.7	10.2	1.0	3.0	60.02215	57.02104	70.60354	24.96955	0.85013	2.28362	179.90653	250.28407	916.55947	1711.23521	0.19628	0.14626
3	9.4	11.8	2.0	3.0	102.78793	71.27630	68.02049	65.43743	1.51113	1.08923	113.40700	219.21378	617.67061	876.20279	0.18360	0.25019
4	9.1	11.6	2.0	3.0	116.29291	96.78571	60.27132	48.21705	1.92949	2.00729	252.17515	201.96456	1043.27040	930.31818	0.24172	0.21709
5	9.9	13.0	2.0	4.0	78.02879	72.77685	99.01717	112.79347	0.78803	0.64522	186.12738	239.23346	820.98804	1011.89161	0.22671	0.23642
6	9.9	11.1	3.0	3.5	21.00775	24.00886	55.96622	22.38649	0.37536	1.07247	198.58601	237.32080	821.49913	1191.34136	0.24174	0.19920
7	8.6	11.1	2.0	3.5	37.51384	79.52935	39.60687	91.26800	0.94716	0.87138	192.49347	241.97443	1053.17200	1103.59467	0.18277	0.21926
8	7.5	10.4	1.0	3.0	111.04097	67.52492	70.60354	110.21041	1.57274	0.61269	61.78751	232.38664	N/A *	N/A *	N/A *	N/A *
9	8.5	11.2	1.0	3.0	57.77132	50.26855	70.60354	32.71872	0.81825	1.53639	110.54236	252.35934	487.58579	894.14499	0.22671	0.28224
10	9.2	12.1	2.0	3.5	132.04873	73.52713	80.93577	31.85770	1.63152	2.30799	212.99477	188.80052	974.82727	938.88352	0.21849	0.20109
11	9.3	10.7	3.0	3.0	72.77685	49.51827	58.54928	42.18992	1.24300	1.17370	254.96764	280.35714	1065.73102	1089.18734	0.23924	0.25740
12	10.1	12.9	2.0	4.0	42.01550	48.01772	37.02381	43.05094	1.13482	1.11537	143.57604	264.96766	578.13504	1578.77883	0.24834	0.16783
13	9.8	13.4	1.0	3.5	118.54374	98.28627	90.63123	74.90864	1.30798	1.31208	206.76921	367.78277	949.19821	1327.07153	0.21784	0.27714
AVE	9.2	11.8	1.9	3.4	82.93445	66.94778	68.30267	57.62203	1.19998	1.40000	177.41321	247.22631	846.31047	1148.62763	0.22140	0.22200
C1	10.8	11.2	3.0	3.0	41.26523	50.26855	37.88483	37.02381	1.08923	1.35774	173.18180	233.78192	805.37649	1193.34104	0.21503	0.19591
C2	11.1	11.7	3.0	3.5	42.01550	49.51827	64.88372	59.41030	0.64755	0.83350	197.23572	231.05412	906.72511	1131.17751	0.21753	0.20426
C3	10.8	11.2	2.0	3.0	66.77464	66.02436	52.52215	80.07475	1.27136	0.82453	203.62901	173.92948	970.95439	882.49028	0.20972	0.19709
C AVE	10.9	11.4	2.7	3.2	50.00000	55.30000	51.76357	58.83629	1.00271	1.00526	191.34884	212.92184	894.40000	1069.00294	0.21409	0.19908

continued

No.	TG(mg/dL)		TC(mg/dL)		TP(g/dL)		GLU(mg/dL)		ALT(IU/L)		AST(IU/L)		ALP(IU/L)		BUN(mg/dL)		CRE(mg/dL)	
	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post
1	15	47	112	151	7.5	6.5	95	111	55	49	51	40	189	204	13	15	0.9	0.8
2	21	22	101	112	8.1	8	98	107	32	35	53	49	128	166	18	16	0.8	0.6
3	21	32	113	143	7.2	6.8	103	110	37	38	43	40	132	119	16	20	1	0.9
4	21	30	112	127	6.7	6.5	92	110	127	58	63	45	170	170	13	12	1	0.8
5	12	27	94	112	7.1	6.7	109	106	41	55	37	53	91	113	12	21	0.8	0.8
6	15	28	103	119	7.1	7	97	95	27	23	29	27	105	125	9	15	0.8	0.7
7	11	20	85	92	6.6	6.8	104	105	36	57	43	65	80	111	14	22	0.6	0.7
8	28	40	82	111	5.0	5.2	58	95	24	32	77	73	99	118	23	18	0.8	0.7
9	15	27	86	96	7.8	6.8	98	106	35	43	47	46	130	161	15	17	0.8	0.7
10	14	27	88	124	6.3	6.4	105	109	37	44	55	55	178	174	18	20	0.9	0.7
11	28	57	164	257	7.1	6.8	98	97	36	32	36	29	140	143	9	13	0.7	0.7
12	15	32	142	194	7.5	6.9	98	100	31	38	33	33	95	119	11	17	0.7	0.7
13	17	25	77	104	7.0	7.1	87	105	47	56	56	57	112	114	19	18	0.9	0.8
AVE	17.9	31.8	104.5	134.0	7.0	6.7	95.5	104.3	43.5	43.1	47.9	47.1	126.8	141.3	14.6	17.2	0.8	0.7
C1	26.0	20.0	168.0	187.0	7.2	6.5	114.0	109.0	33.0	37.0	24	27.0	172.0	164.0	11.0	13.0	0.9	0.9
C2	15.0	17.0	120.0	173.0	7.0	6.4	110.0	118.0	33.0	37.0	28	29.0	143.0	161.0	8.0	9.0	0.8	0.7
C3	22.0	20.0	100.0	125.0	6.9	6.7	99.0	94.0	50.0	59.0	40	40.0	107.0	111.0	11.0	16.0	0.8	0.8
C AVE	21.0	19.0	129.3	161.7	7.0	6.5	107.7	107.0	38.7	44.3	30.7	32.0	140.7	145.3	10.0	12.7	0.8	0.8

Table 2 Mean BW, BCS, and plasma and leukocytic biomarker levels comparing pre- and post feeding trial of DERx2 (over-fed) and DERx1 (control)

			Pre	4-wk post over feeding
BW (kg)	over-fed	(13)	9.15 ± 0.24	11.79 ± 0.32*
	control	(3)	10.9 ± 0.1	11.4 ± 0.2*
BCS	over-fed	(13)	1.9 ± 0.2	3.4 ± 0.1*
	control	(3)	2.7 ± 0.3	3.2 ± 0.2
TG (mg/dl)	over-fed	(13)	17.9 ± 1.5	31.8 ± 2.9*
	control	(3)	21.0 ± 3.2	19.0 ± 1.0
TC (mg/dl)	over-fed	(13)	104.5 ± 6.9	134.0 ± 12.7*
	control	(3)	129.3 ± 20.2	161.7 ± 18.8
TP (mg/dl)	over-fed	(13)	7.0 ± 0.2	6.7 ± 0.2*
	control	(3)	7.0 ± 0.1	6.5 ± 0.1
GLU (mg/dl)	over-fed	(13)	95.5 ± 3.5	104.3 ± 1.6*
	control	(3)	107.7 ± 4.5	107.0 ± 7.0
BUN (mg/dl)	over-fed	(13)	14.6 ± 1.1	17.2 ± 0.8*
	control	(3)	10.0 ± 1.0	12.7 ± 2.0
CRE (mg/dl)	over-fed	(13)	0.8 ± 0.0	0.7 ± 0.0*
	control	(3)	0.8 ± 0.0	0.8 ± 0.1
ALT	over-fed	(13)	43.5 ± 7.3	43.1 ± 3.1
	control	(3)	38.7 ± 5.7	44.3 ± 7.3
AST	over-fed	(13)	47.9 ± 3.7	47.1 ± 3.8
	control	(3)	30.7 ± 4.8	32.0 ± 4.0
ALP	over-fed	(13)	126.8 ± 9.6	141.3 ± 8.4*
	control	(3)	140.7 ± 18.8	145.3 ± 17.2
Plasma MDH	over-fed	(13)	82.9 ± 10.4	66.9 ± 5.8*
	control	(3)	50.0 ± 8.4	55.3 ± 5.4
Pasma LDH	over-fed	(13)	68.3 ± 5.1	57.6 ± 8.6
	control	(3)	51.8 ± 7.8	58.8 ± 12.4
Plasma M/L	over-fed	(13)	1.2000 ± 0.1198	1.3611 ± 0.1572
	control	(3)	1.0027 ± 0.1852	1.0053 ± 0.1763
Leukocyte MDH	over-fed	(13)	177.4 ± 15.6	247.2 ± 12.1*
	control	(3)	191.3 ± 9.3	212.9 ± 19.5
Leukocyte LDH	over-fed	(12)	846.3 ± 56.2	1148.6 ± 77.4*
	control	(3)	894.4 ± 48.2	1069.0 ± 95.0
Leukocyte M/L	over-fed	(12)	0.2214 ± 0.0065	0.2220 ± 0.0012
	control	(3)	0.2141 ± 0.0023	0.1991 ± 0.0026*

Leukocytic enzyme activity is presented as mU/mg of protein

Plasma enzyme activity is presented as IU/L

Data ae presented as mean ±SE.

The numbers in parentheses indicate the number of animals examined.

*Significant ($p<0.05$) when compared against each starting level (paired *t*-test).

General Conclusion

Energy metabolism drives all cellular functions in living cells. Status of energy metabolism is dynamic as all things associated with life are, reflecting, and reflected in, macroscopic and/or microscopic changes occurring in living things. In this thesis, I focused on enzymes involved in energy metabolism, in particular, the enzymes of NADH shuttles, to show whether their activity changes faithfully reflect the variations in nutrient metabolism, metabolic status, and health conditions of various species and individuals. Specifically, malate dehydrogenase (MDH) is a rate-limiting enzyme of the malate-aspartate shuttle that contributes to the transfer of cytosolic NADH into mitochondria, coupling glycolysis with mitochondrial ATP productions. By dividing the cytosolic MDH by lactic dehydrogenase (LDH), a relatively stable cytosolic marker enzyme, MDH/LDH ratio was analyzed as the parameter for evaluating metabolic status in animal tissues. A better grasp of trends in shifts of the energy metabolism enzymes may assist us to better understand species-species differences in energy production and usage, and early detection and prevention of energy metabolism dysregulation associated with medical conditions such as diabetes, neoplasia, and obesity.

In Chapter 1, enzyme activities of energy metabolism, MDH, LDH, M/L ratio,

and glutamate dehydrogenase (GLDH), of the feline leukocytes were compared against those of the canine leukocytes. GLDH is associated with the interconnection of amino acid and carbohydrate metabolism, and catalyzes the reversible conversion of glutamate to alpha-ketoglutarate and ammonia, allowing glutamate to fuel the TCA cycle for energy production under low cellular energy status (Spanaki et al., 2012). Significantly lower cytosolic MDH, M/L ratio and mitochondrial GLDH activities in feline leukocytes suggest that the feline cells utilize less glucose as energy source compared to the canine cells. Furthermore, higher activity levels of FK in feline leukocytes indicates active utilization of fructose, and subsequent activation of PK and G6PD, which in turn, contribute to fatty acid synthesis (Naismith, 1971). Lower MDH, M/L ratio, GLDH, and higher FK, PK, and G6PD may reflect the unique demands and usages of nutrients and energy sources in cats with higher incidence of obesity, insulin resistance, and diabetes mellitus compared to dogs.

Fig 1 is a summary chart of MDH, LDH, M/L ratios and their relative changes in various physiological and pathological states of dogs, cats, rats and horses. The arrows denote the direction of relative levels compared against the normal control or paired starting level. Elevations in cytosolic and mitochondrial MDH and the resultant cytosolic M/L ratio are noted in cells that may be experiencing increased mitochondrial ATP productions to provide for increased energy demands (intense exercise, neoplastic

cell growth, acute weight gain). Diabetes mellitus, which causes dysregulation of glucose metabolism, is associated with depressions in leukocytic MDH activity and resultant M/L ratio. These changes reflect the defect in glucose usage and uptake as energy source in peripheral tissues, resulting in increased circulating plasma glucose concentrations (Ferrannini et al., 1985).

This may explain why type 1 diabetic dogs with higher plasma glucose concentrations, showed lower levels leukocytic MDH, resultant M/L ratio, as well as AST, as compared to the healthy control dogs, as shown in Chapter 2. Decreased activity levels of MA shuttle enzymes may be one of the characteristics of energy metabolism in diabetic dogs, and may be useful as a diagnostic indicator to monitor overall metabolic condition of the diabetic patients.

In Chapter 3, racehorses were studied against riding horses to show whether the long-term extraneous exercise regiment induces increased efficiency in energy production and expenditure. As evidenced by a higher plasma M/L ratio, and increased lipolysis rate in racehorses, racehorses may have adapted to the demands of higher activity levels by increasing muscle mitochondrial respiration, oxidative capacity, and fat utilization of the skeletal muscles as energy source in order to process and consume energy more efficiently.

Acute weight gain in response to increased positive energy balance, although

not significant, did show relative increases in leukocytic and plasma M/L ratios to reflect improved energy metabolism status with a better nutritional status. The aim of the study was to seek a diagnostic potential in M/L ratio as a marker for confirming early weight gain in conjunction with BCS changes, in apparently healthy animals exhibiting no overt clinical sequelae of weight gain. Although future studies on various types of weight gain (i.e. acute weight gain, chronic, mild, severe, and visceral, or subcutaneous obesity), and tissue types (leukocytes, plasma, muscle, and liver) are needed in order to follow the trends in energy usage associated with changes in weight status, M/L ratio may be a good indicator for detecting early weight gain that may result in MS and decreased life span, if used appropriately.

As the life spans of companion animals are extending, the prevalence of metabolic syndrome such as obesity and DM, as well as neoplasia and other serious diseases, is increasing. Plasma metabolites are easy to collect and measure, and are highly accessible in clinical settings. The development of useful plasma markers, that can identify the risk factors and diagnose such life-threatening diseases early, is in need to extend the “health span” of companion animals.

The MA shuttle enzyme activities, in particular, MDH, are dynamic, and faithfully reflect glucose metabolism and ATP production. When combined with common biochemical parameters, M/L ratio may be used as potential

diagnostic/monitoring parameter in determining the metabolic status of each individual, and for various health conditions. Further studies on various disease models and metabolic states in different species may assist in a better understanding of their clinical usage.

Figure

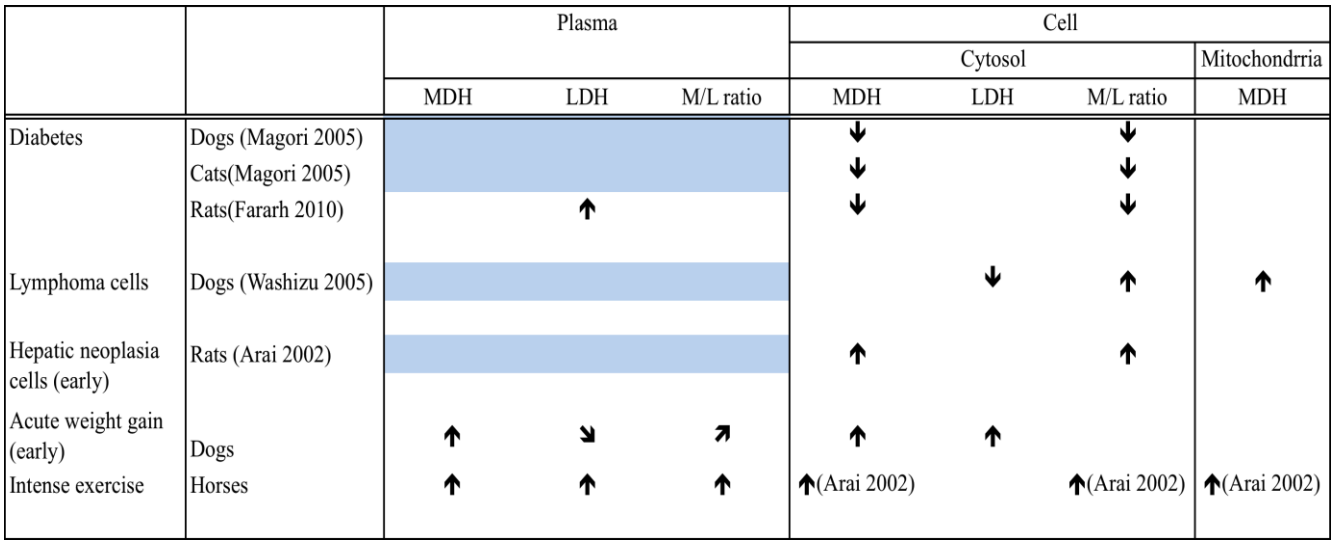

Fig 1: Summary chart MDH, LDH, M/L changes in various conditions

Figure legend

Fig. 1 Summary chart MDH, LDH, M/L changes in various conditions

Shifts in enzyme activities of energy metabolism faithfully reflect metabolic changes in various health conditions.Arrow directions indicate significantly increased (↑) and decreased (↓) levels compared to the normal control (healthy individuals or starting levels in the case of weight gain). “Cells” represent peripheral blood leukocytes unless otherwise indicated. Slanted arrows indicate increasing (↗) and decreasing (↘) tendencies although not statistically significant.

References

American Diabetes Association, 2010. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 33(1), S62-69.

Anderson, R.L. and Sapico, V.L., 1975. D-fructose (D-mannose) kinase. In: Wood, W.A. (ed.) Methods of Enzymology, vol.42, Academic Press, New York, 39-43.

Arai, T., Machida, Y., Sasaki, M., Oki, Y., 1989. Hepatic enzymes activities and plasma insulin concentrations in diabetic voles. Veterinary Research Communications 13(6), 421-426.

Arai, T., Washizu, T., Sako, T., Motoyoshi, S., 1992. D-glucose transport activities in erythrocytes and hepatocytes of dogs, cats and cattle. Comparative biochemistry and physiology A 102, 285-287.

Arai, T., Washizu, T., Sagara, M., Sako, T., Nigi, H., Matsumoto, H., Sasaki, M., Tomoda, I.,1995. D-glucose transport and glycolytic enzyme activities in erythrocytes of dogs, pigs, cats, horses, cattle and sheep, Research in Veterinary Science 58, 195– 196.

Arai, T., Kawaue, T., Abe, M., Kuramoto, E., Kawakami E, Sako T, Washizu T., 1998. Comparison of glucokinase activities in the peripheral leukocytes between dogs and cats. Comparative Biochemistry and Physiolology-Part C: Pharmacology, Toxicology and Endocrinology 120(1), 53-56.

Arai, T., Nakamura, M., Magori, E., Fukuda, H, Mizutani, H., Kawakami, E., Sako, T., 2001. Changes in activities of enzymes related to energy metabolism in peripheral leukocytes of diabetic dogs with glycemic control by intensive insulin treatment. Research in Veterinary Science 73(2), 182-186.

Arai T., Hosoya M., Nakamura M., Magoori E., Uematsu Y., Sako T., 2002. Cytosolic ratio of malate dehydrogenase/lactate dehydrogenase activity in peripheral

leukocytes of race horses with training. Research in Veterinary Science 72, 241-244.

Arai, T., Inoue, A., Takeguchi, A., Mizutani, H., Shimoo, M., Sako, T., Yoshimura, I., Kimura, N., 2003a. Comparison of enzyme activities in plasma and leukocytes in dairy and beef cattle. Journal of Veterinary Medical Science 65(11), 1241-1243.

Arai, T., Nakamura, M., Magori, E., Fukuda, H., Sako, T., 2003b. Decrease in malate dehydrogenase activities in peripheral leucocytes of type 1 diabetic dogs. Research in Veterinary Science 74, 183-185.

Arai T., Inoue A., Uematsu Y., Sako T., Kimura N., 2003c. Activities of enzymes in the malate-aspartate shuttle and the isoenzyme pattern of lactate dehydrogenase in plasma and peripheral leukocytes of lactating Holstein cows and riding horses. Research in Veterinary Science 75, 15-19.

Belsham, G.J., Denton, R.M., Tanner, M.J., 1980. Use of a novel rapid preparation of fat-cell plasma membranes employing Percoll to investigate the effects of insulin and adrenaline on membrane protein phosphorylation within intact fat-cells. Biochemical Journal 192, 457-467.

Bergmeyer, H.U., Gawehn, K., Grassl, M., 1974a. Glucose-6-phosphate dehydrogenase from yeast. In: Bergmeyer, H.U. (ed.) Methods of Enzymatic Analysis, vol. 1, Academic Press, New York, 458-459.

Bergmeyer, H.U., Gawehn, K., Grassl, M., 1974b. Hexokinase. In: Bergmeyer, H.U. (ed.) Methods of Enzymatic Analysis, vol. 1, Academic Press, New York, 473–474.

Bergmeyer, H.U. and Bernt, E, 1974c. Malate dehydrogenase UV-assay. In: Bergmeyer, H.U. (ed.) Methods of Enzymatic Analysis, vol. 2, Acadeimic Press, New York, 613-617.

Bishop, M.J., Everse, J., Kaplan, N.O., 1972. Identification of lactate dehydrogenase isoenzymes by rapid kinetics. Proceedings of National Academy of Sciences 69(7), 1761-1765.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-54.

Burkholder, W.J. and Toll, P.W., 2000. Obesity. In: Hand, M.S., Thatcher, C.D., Remillard, R.L., Roudebush, P., and Lewis, LD. (eds.) Small Animal Clinical Nutrition, 4th edn. Mark Morris Institute, Topeka, 401-430.

Burczynski, M.E., Twine, N.C., Dukart, G., Marshall, B., Hidalgo, M., Stadler, W.M., 2005. Transcriptional profiles in peripheral blood mononuclear cells prognostic of clinical outcomes in patients with advanced renal cell carcinoma. Clinical Cancer Research 11(3), 1181-1189.

Carlson, L.A., Ekelund, L.G., Fröberg, S.O., 1971. Concentration of triglycerides, phospholipids and glycogen in skeletal muscle and of free fatty acids and β-hydroxybutyric acid in blood in man in response to exercise. European Journal of Clinical Investigation 1, 248–254.

de Mello, V.D., Kolehmainen, M., Schwab, U., Mager, U., Laaksonen, D.E., Pulkkinen, L., Niskanen, L., Gylling, H., Atalay, M., Rauramaa, R., and Uusitupa, M., 2008. Effect of weight loss on cytokine messenger RNA expression in peripheral blood mononuclear cells of obese subjects with the metabolic syndrome. Metabolism 57(2), 192-199.

Essén-Gustavsson, B., Jensen-Waern, M., 2002. Effect of anendurance race on muscle amino acids, pro- and macroglycogen and triglycerides. Equine Veterinary Journal Supplement 34, 209-213.

Eto, K., Tsubamoto, Y., Terauchi, Y., Sugiyama, T., Kishimoto, T., Takahashi, N., Yamauchi, N., Kubota, N., Murayama, S., Aizawa, T., Akanuma, Y., Aizawa, S., Kasai, H., Yazaki, Y., and Kadowaki, T., 1999. Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. Science 283(5404), 981-5.

Fararh, K.M, Ibrahim, A.K., Elsonosy, Y.A., 2010. Thymoquinone enhances the activities of enzymes related to energy metabolism in peripheral leukocytes of diabetic rats. Research in Veterinary Science 88(3), 400-404.

Ferrannini, E., Smith, J.D., Cobelli, C., Toffolo, G., Pilo, A., Defronzo, R.A., 1985. Effect of insulin on the distribution and disposition of glucose in man. Journal of Clinical Investigation 76, 357-364.

German, A.J., Ryan, A.C., German, A.C., Wood, I.S., Trayhurn, P., 2010. Obesity, its associated disorders and the role of inflammatory adipokines in companion animals. Veterinary Journal 185, 4-9.

Hambitzer, R., Bent, E., 1988. Effect of long distance exercise on free fatty acids in blood plasma of Arab horses. Zentralblatt fur Veterinarmedizin A 35, 622-625.

Hatzipanagiotou, A., Lindner, A., Sommer, H., 1991. LDH and CK isoenzyme patterns in the blood plasma of horses with elevated CK, LDH and AST activities. Deutsche Tierarztliche Wochenschrift 98, 284-286.

Hedeskov, C.J., Capito, K., Thams, P., 1987. Cytosolic ratios of free [NADPH]/[NADP+] and [NADH]/[NAD+] in mouse pancreatic islets, and nutrient-induced insulin secretion. Biochemical Journal 241, 161-167.

Hess, B., Wieker, H.J., 1974. Pyruvate kinase from yeast. In: Bergmeyer, H.U. (ed.) Methods of Enzymatic Analysis, vol. 2, Academic Press, New York, 778-783.

Hirakawa, Y., Kawasumi, K, Lee, P., Mori, N., Yamamoto, I., Terasawa, F., 2012. Determination of Oxidative Energy Metabolism and Plasma LDH Isoenzyme Patterns of Dolphins. The Open Veterinary Science Journal 12, 6(1), 30-36.

Hood, R.L. and Allen, C.E., 1973) Lipogenic enzyme activity in adipose tissue during growth of swine with different propensities to fatten. Journal of Nutrition 103, 353.

Hosoya, M., Inoue, A., Kimura, N., Arai, T., 2004. Enzyme activities in some types of peripheral leukocytes of thoroughbred race horses before and after the races. Research in Veterinary Science 77, 101-104.

Huggett, A.G., Nixon,D.A., 1957. Use of glucose oxidase, peroxidase, and O-dianisidine in determination of blood and urinary glucose. Lancet 2, 368-370.

IDF, 2005. The IDF consensus world wide definition of the metabolic syndrome. http://www.idf.org/webdata/docs/Meta_syndrome_def.pdf .

Jericó, M.M., De Camargo, C.F., Kajihara, K., Moreira, M.A., Gonzales, R., Machado, F.L., Nunes, V.S., Catanozi, S., and Nakandakare, E.R., 2009. Chromatographic analysis of lipid fractions in healthy dogs and dogs with obesity or hyperadrenocorticism. Journal of Veterinary Diagnostic Investigation 21, 203–207.

Johnson, M.C., 2005. Hyperlipidemia disorders in dogs. Compendendium of Continuing Education in Dentistry 27, 361–364.

Kadowaki, T., Yamauchi, T., 2005. Adiponectin and adiponectin receptors. Endocrine Reviews 26, 439-51.

Kaloustian, H.D., Stolzenbach, F.E., Everse, J., Kaplan, N.O., 1969. Lactate Dehydrogenase of Lobster (Homarus americanus) Tail Muscle: I. PHYSICAL AND CHEMICAL PROPERTIES. Journal of Biological Chemistry 244, 2891-2901.

Kawasumi, K., Suzuki, T., Fujiwara, M., Mori, N., Yamamoto, I., Arai, T., 2012. New Criteria for Canine Metabolic Syndrome in Japan. Journal of Animal and Veterinary Advances 11(21), 4005-4007.

Kearns, C.F., McKeever, K.H., Roegner, V., Brady, S.M., Malinowski, K., 2006. Adiponectin and leptin are related to fat mass in horses. Veterinary Journals 172, 460-465.

Laflamme, D.P., 1997. Development and validation of a body condition score system for dogs. Canine Practice 22, 10–15.

Laflamme, D.P., 2006. Understanding and managing obesity in dogs and cats. Veterinary Clinics of North America: Small Animal Practice 36, 1283–1295.

Laflamme, D.P., 2012. Companion Animals Symposium: Obeisty in dogs and cats: What is wrong with being fat? Journal of Animal Science 90, 1653-1662.

Landau, S., Braw-Tal, R., Kaim, M., Bor, A., Bruckental, I., 2000. Preovulatory follicular status and diet affect the insulin and glucose content of follicles in high-yielding dairy cows. Animal Reproduction Science 64, 181-197.

Liew, C.C., Mam J., Tang, H.C., Run, Z., Dempsey, A.A., 2006. The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. Journal of Laboratory and Clininical Medicine 147(3), 126-32

Lucke, J.N., Hall, G.M., 1980. Long distance exercise in the horse: Golden Horseshoe Ride 1978. Veterinary Record 106, 405-407.

Lund, E., Armstrong, P., Kirk, C., 2006. Prevalence and risk factors for obesity in adult dogs from private US veterinary practices. International Journal of Applied Resarch in Veterinary Medicine 4, 177–186.

MacDonald, M.J., 1995. Feasibility of a mitochondrial pyruvate malate shuttle in pancreatic islets. Further implication of cytosolic NADPH in insulin secretion**.** Journal of Biological Chemistry 270**,** 20051–20058.

Macková, E.V., Bass, A., Šprynarová, Š., Teisinger, J., Vondra, K., and Bojanovský, I., 1982. Enzyme Activity Patterns of Energy Metabolism in Skiers of Different Performance Levels (M. Quadricepa femoris). European Journal Applied Physiology 48, 315-322.

Magori, E, Nakamura, M., Inoue, A., Tanaka, A., Sasaki, N., Fukuda, H., Mizutani, H., Sako, T., Kimura, N., Arai, T., 2005. Malate dehydrogenase activities are lower in some types of peripheral leucocytes of dogs and cats with type 1 diabetes mellitus. Research in Veterinary Science 78(1), 39-44.

Markert, C., Moller, L.F., 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. Proceedings of the National Academy of Sciences of the USA 45, 753-763.

Mori, N., Lee, P., Kondo, K., Kido, T., Saito, T., Arai, T., 2011. Potential use of cholesterol lipoprotein profile to confirm obesity status in dogs. Veterinary Research Communications 35, 223-235.

Naismith, D.J., 1971. Differences in the metabolism of dietary carbohydrates studied in the rat. The Proceedings of the Nutrition Society 30, 259-265.

Oliver, P., Reynés, B., Caimari, A., Palou, A., 2013. Peripheral blood mononuclear cells: a potential source of homeostatic imbalance markers associated with obesity development. European Journal of Physiology 465, 459-468.

Olsson, A.G., Eklund, B., Kaijser, L., Carlson ,L.A., 1975. Extraction of exogenous plasma triglycerides by the working human forearm muscle in the fasting state. Scandinavian Journal of Clinical Laboratory Investigation 35, 231-236.

Pilkis, S.J., Hansen, R.J., Krahl, M.E., 1968. Hexose-ATP phosphotransferases: comparative aspects. Comparative Biochemistry and Physiology 25, 903-912.

Prince, A., Geor, R., Harris, P., Hoekstra, K., Gardner, S., Hudson, C., Pagan, J., 2002. Comparison of the metabolic responses of trained Arabians and Thoroughbreds during high-and low-intensity exercise. Equine Veterinary Journal Supplement 34, 95-99.

Pösö, A.R., Viljanen-Tarifa, E., Soveri, T., Oksanen, H.E., 1989. Exercise-induced transient hyperlipidemia in the racehorse. Zentralblatt fur Veterinarmedizin A 36, 603-611.

Romijn, J.A., Coyle, E.F., Sidossis, L., Gastaldelli, A., Horowitz, J.F., Endert, E., Wolfe, R.R., 1993. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity. American Journal of Physiology-Endocrinology-Metatabolism 265, 380-391.

Rose, R.J., lkiw, J.E., Arnold, K.S, Backhouse, J.W., Sampson, D., 1980. Plasma biochemistry in the horse during 3-day event competition. EquineVeterinary Journal 12, 132-136.

Rej, R., Horder, M. Aspartate aminotransferase. In: Bergmeyer, H.U. (ed.) Methods of enzymatic analysis, 3rd edn, vol. ,Verlag Chemie, Weinhein, 416–444.

Schmidt, E., 1974. Glutamate dehydrogenase UV assay. In: Bergmeyer, H.U. (ed.) Methods of Enzymatic Analysis. Academic Press, New York, 650-656.

Setoyama, C., Joh, T., Tsuzuki, T., Shimada, K.C., 1998. Structural organization of the mouse cytosolic malate dehydrogenase gene: Comparison with that of the mouse mitochondrial malate dehydrogenase gene. Journal of Molecular Biology 202(3), 355-364.

Sloet van Oldruitenborgh-Oosterbaan, M.M., Wensing, T., Barneveld, A., Breukink, H.J., 1991. Heart rate, blood biochemistry and performance of horses competing in a 100km endurance ride. Veterinary Record 128, 175-179.

Spanaki, C., Zaganas, I., Kounoupa, Z., Plaitakis, A., 2012. The complex regulation of human glud1 and glud2 glutamate dehydrogenases and its implications in nerve tissue biology. Neurochemistry International 61, 470-481.

Styler, L., 1995. Biochemistry. W.H. Freeman and Company, New York.

Suominen, H., Heikkinen, E., 1975. Enzyme Activities in Muscle and Connective Tissue of M. vastuslateralis in Habitually Training and Sedentary 33 to 70-Year-Old Men. European Journal of Applied Physiology 34, 249-254.

Takeda, Y., Suzuki, F., Inoue, H., 1969. ATP-citrate lyase (Citrate-cleavage enzyme). In: Lowenstein, J.M., Methods in Enzymology, vol. 13, Academic Press, New York, 153-160.

Tanaka A., Urabe S., Takeguchi A., Mizutani H., Sako T., Imai S., Yoshimura I., Kimura N., Arai T., 2006. Comparison of activities of enzymes related to energy metabolism in peripheral leukocytes and livers between Holstein dairy cows and ICR mice. Vet Research Communications 30:29-38

Trayhurn, P. and Wood, I.S., 2005. Signaling role of adipose tissue: Adipokines and inflammation in obesity. Biochemical Society Transactions 33, 1078–1081.

Trayhurn, P., Bing, C., and Wood, I.S., 2006. Adipose tissue and adipokines—Energy regulation from the human perspective. Journal of Nutrition 136, 1935S–1939S.

Treiber, K.H., Hess, T.M., Kronfeld, D.S., Boston, R.C., Geor, R.J., Friere, M., Silva, A.M., Harris, P.A., 2006. Glucose dynamics during exercise: dietary energy sources affect minimal model parameters in trained Arabian geldings during

endurance exercise. Equine Veterinary Journal S36, 631-636.

Treiber, K.H., Geor, R.J., Boston, R.C., Hess, T.M., Harris, P.A., Kronfeld, D.S., 2008. Dietary energy source affects glucose kinetics in trained Arabian geldings at rest and during endurance exercise. Journal of Nutrition 138, 964-970.

Vachharajani, V. and Granger, D.N., 2009. Adipose tissue: a motor for the inflammation associated with obesity. IUBMB Life 61, 424–430

Votion, D.M., Fraipont, A., Goachet, A.G., Robert, C., van Erck, E., Amory, H., Ceusters, J., de la Rebière de Pouyade, G., Franck, T., Mouithys-Mickalad, A., Niesten, A., Serteyn, D., 2010. Alterations in mitochondrial respiratory function in response to endurance training and endurance racing. Equine Veterinary Journal 42(38), 268-274.

Wang, C., Chen, H., Zhang, J., Hong, Y., Ding, X, Ying, W, 2014. Malate-aspartate shuttle mediates the intracellular ATP levels, antioxidation capacity and survival of differentiated PC12 cells. Int J Physiol Pathophysiol Pharmacol 6(2), 109-114.

Washizu, T., Kuramoto, E., Abe, M., Sako, T., Arai, 1998. A comparison of the activities of certain enzymes related to energy metabolism in leukocytes in dogs and cats. Veterinary Research Communications 22(3), 187-192.

Washizu, T., Takahashi, M., Azakami, D., Ikeda, M., Arai, T., 2001. Activities of enzymes in the malate-aspartate shuttle in the peripheral leukocytes of dogs and cats. Vet Researh Communications 25(8), 623-629.

Washizu, T., Azakami, D., Bonkobara, M., Washizu, M., Arai, T., 2005. Changes in Activities of Enzymes Related to Energy Metabolism in Canine Lymphoma Cells. Journal of Veterinary Medical Science, 615-616.

Watson, T.D.G. and Barrie, J., 1993. Lipoprotein metabolism and hyperlipidemia in the dog and cat-a review. Journal of Small Animal Practice 34, 479–487.

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