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Changes in Malate Dehydrogenase, Lactate Dehydrogenase and M/L Ratio as Energy Metabolism Markers of Acute Weight Gain

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ABSTRACT

Obesity is associated with the metabolic syndrome, diabetes, hypertension and chronic inflammation and early detection of weight gain and prompt intervention are the keys to promoting increased quality of life and longevity in veterinary animals. We evaluated the changes in Malate Dehydrogenase (MDH), Lactate Dehydrogenase (LDH) and MDH/LDH ratio as energy metabolism markers in dogs before and after the 4-week overfeeding trial. The acute weight gain was attained by overfeeding of 2x Daily Energy Requirement (DER) separated into 3 meals/day (overfed group). The experimentally overfed dogs showed about 28.2% increase in the Body Weight (BW), the increase of Body Condition Score (BCS) from 1.9-3.4 and significant elevations were noted in Triglyceride (TG), total cholesterol (TC), glucose (GLU) alkaline phosphatase (ALP), Blood Urea Nitrogen (BUN), leukocyte MDH and LDH. 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 leucocytic M/L ratios showed mild increase in the over-fed group after the feeding trial. In conclusion, assays of MDH, LDH and M/L ratio on plasma and leukocytes are not sensitive as diagnostic tools for detecting acute weight gain. The diagnostic significance of the above mentioned parameters should be further examined on various types of weight gain and target tissues.

Key words: Malate dehydrogenase, lactate dehydrogenase, M/L ratio, weight gain, obesity, dog

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. 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, elevated plasma glucose, abdominal obesity, high cholesterol, high triglyceride and high blood pressure (IDF., 2005). Overweight and obese individuals have an increased risk of developing associated diseases such as diabetes mellitus, orthopedic disease, neoplasia, respiratory and urinary disease and have shorter life span (Burkholder and Toll, 2000; German *et al.*, 2010). Additionally, a recent developing concern revolves around the concept that obesity is accompanied by a 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 an inert fuel storage, but also an active secretory and endocrine organ (Trayhurn and Wood, 2005; Trayhurn *et al.*, 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, 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 obesity 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 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, 2005) and lipoprotein profiles (Jerico *et al.*, 2009; Mori *et al.*, 2011) and their reference values to distinguish overweight and obese individuals from the normal ones.

In this study, we investigated the changes in 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 in early detection of weight gain and prevention of MS.

MATERIALS AND METHODS

Animals: Sixteen male Beagle dogs of ages 2-4 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). Thirteen dogs in the overfed group were each fed twice the Daily Energy Requirement (DER) as appropriate for its age and current weight ($132\times\text{weight}^{0.75}$) separated into 3 feedings per day. The control dogs were also fed the diet mentioned above, but only given 1×DER separated into 3 meals/day. Each subject was kept in a cage measured 45 cm (width)×55 cm (length)×75 cm (depth) and was given water *ad libitum* and allowed 10 h 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 the Nippon Veterinary and Life Science University Animal Research Committee.

Collection and preparation of blood samples: Five milliliters of postprandial blood samples were collected from the cephalic vein of each dog, fasted overnight (at least 8 h after the last meal),

into the heparinized tubes. The blood samples were left at room temperature for 15-20 min after collection and then plasma was recovered by centrifugation at 3000 rpm for 15 min at 4°C and stored at -80°C 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: The MDH (Bergmeyer and Bernt, 1974) 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 24-26°C were expressed as U L^{-1} of plasma (volume activity) and mU mg^{-1} of protein in cytosolic fractions (specific activity). The enzyme unit (U) represents 1 μmol 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.

Statistical analysis: Results are 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 1.9-3.4, 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, leukocyte 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 leucocytic 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 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 the malate-aspartate shuttle and is involved in metabolism of glucose and lipids (Setoyama *et al*., 1988) by transporting cytosolic NADH into mitochondria to initiate oxidative ATP production (Hedeskov *et al*., 1987). Alternately, lactate dehydrogenase mediates a reaction that

Table 1: Changes in BW, BCS and plasma and leukocytic biomarker levels after 4 week overfeeding of DER×2 (1-13) vs. 4 week feeding of DER×1 (C1-3)

	BW (kg)		BCS		TG (mg dL^{-1})		TC (mg dL^{-1})		TP (g dL^{-1})		GLU (mg dL^{-1})		ALT (IU L^{-1})		AST (IU L^{-1})		ALP (IU L^{-1})	
No.	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	10.0	13.8	3.0	4.0	15.0	47.0	112.0	151.0	7.5	6.5	95.0	111.0	55.0	49.0	51.0	40.0	189.0	204.0
2	7.7	10.2	1.0	3.0	21.0	22.0	101.0	112.0	8.1	8.0	98.0	107.0	32.0	35.0	53.0	49.0	128.0	166.0
3	9.4	11.8	2.0	3.0	21.0	32.0	113.0	143.0	7.2	6.8	103.0	110.0	37.0	38.0	43.0	40.0	135.0	119.0
4	9.1	11.6	2.0	3.0	21.0	30.0	112.0	127.0	6.7	6.5	92.0	110.0	127.0	58.0	63.0	45.0	170.0	170.0
5	9.9	13.0	2.0	4.0	12.0	27.0	94.0	112.0	7.1	6.7	109.0	106.0	41.0	55.0	37.0	53.0	91.0	113.0
6	9.9	11.1	3.0	3.5	15.0	28.0	103.0	119.0	7.1	7.0	97.0	95.0	27.0	23.0	29.0	27.0	105.0	125.0
7	8.6	11.1	2.0	3.5	11.0	20.0	85.0	92.0	6.6	6.8	104.0	105.0	36.0	57.0	43.0	65.0	80.0	111.0
8	7.5	10.4	1.0	3.0	28.0	40.0	82.0	111.0	5.0	5.2	58.0	95.0	24.0	32.0	77.0	73.0	99.0	118.0
9	8.5	11.2	1.0	3.0	15.0	27.0	86.0	96.0	7.8	6.8	98.0	106.0	35.0	43.0	47.0	46.0	130.0	161.0
10	9.2	12.1	2.0	3.5	14.0	27.0	88.0	124.0	6.3	6.4	105.0	109.0	37.0	44.0	55.0	55.0	178.0	174.0
11	9.3	10.7	3.0	3.0	28.0	57.0	164.0	257.0	7.1	6.8	98.0	97.0	36.0	32.0	36.0	29.0	140.0	143.0
12	10.0	12.9	2.0	4.0	15.0	32.0	142.0	184.0	7.5	6.9	98.0	100.0	31.0	38.0	33.0	33.0	95.0	119.0
13	9.8	13.4	1.0	3.3	17.0	25.0	77.0	104.0	7.0	7.1	87.0	105.0	47.0	56.0	56.0	57.0	112.0	114.0
AVE	9.2	11.8	1.9	3.4	17.9	31.8	105.5	135.0	7.0	6.7	95.5	104.3	43.5	43.1	47.9	47.1	126.8	141.3
C1	10.8	11.2	3.0	3.0	26.0	20.0	168.0	187.0	7.2	6.5	114.0	109.0	33.0	37.0	24.0	27.0	172.0	164.0
C2	11.1	11.7	3.0	3.5	15.0	17.0	120.0	173.0	7.0	6.4	110.0	118.0	33.0	37.0	28.0	29.0	143.0	161.0
C3	10.8	11.2	2.0	3.0	22.0	20.0	100.0	125.0	6.9	6.7	99.0	94.0	50.0	59.0	40.0	40.0	107.0	111.0
C AVE	10.9	11.4	2.7	3.2	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

Table 1: Continue

					Plasma						Leukocyte					
	BUN ($mg\ dL^{-1}$)		CRE ($mg\ dL^{-1}$)		MDH ($IU\ L^{-1}$)		LDH ($IU\ L^{-1}$)		$M\ L^{-1}$		MDH ($IU\ L^{-1}$)		LDH ($IU\ L^{-1}$)		M/L	
No.	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	13.0	15.0	0.9	0.8	128.29734	81.78018	86.10188	49.07807	1.49006	1.66633	193.09864	237.29687	827.08866	1130.88156	1.23400	0.20983
2	18.0	16.0	0.8	0.6	60.02215	57.02104	70.60354	24.96955	0.85013	2.28362	179.90653	250.25407	916.55947	1711.23521	0.19628	0.14626
3	16.0	20.0	1.0	0.9	102.87930	71.29630	68.02049	65.43743	1.51113	1.08923	113.40700	219.21378	617.67061	876.20279	0.78360	0.25019
4	13.0	12.0	1.0	0.8	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	12.0	21.0	0.8	0.8	78.28790	72.77685	99.01717	112.79347	0.78803	0.64522	186.12738	239.23346	820.98804	1011.89161	0.22671	0.23642
6	9.0	15.0	0.8	0.7	21.00775	24.00886	55.96622	22.38649	0.37536	1.07247	198.58601	237.32080	821.49913	1191.34136	0.24141	0.19920
7	14.0	22.0	0.6	0.7	31.51384	79.52935	39.60687	91.26800	0.84716	0.87138	192.49347	241.97443	1053.17200	1103.59467	0.18277	0.21926
8	23.0	18.0	0.8	0.7	11.04097	67.52492	70.60354	110.21041	1.57174	0.61269	61.78751	232.38664	N/A*	N/A*	N/A*	N/A*
9	15.0	17.0	0.8	0.7	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	18.0	20.0	0.9	0.7	132.24873	73.52713	80.93577	31.85770	1.63152	2.30799	212.99477	188.80052	974.82727	938.88352	0.21849	0.20109
11	9.0	13.0	0.7	0.7	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	11.0	17.0	0.7	0.7	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	19.0	18.0	0.9	0.8	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	14.6	17.2	0.8	0.7	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	11.0	13.0	0.9	0.9	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	8.0	9.0	0.8	0.7	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	11.0	16.0	0.8	0.8	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	10.0	12.7	0.8	0.8	50.00000	55.30000	51.76357	58.83627	1.00271	1.00526	191.34884	212.92184	894.40000	1069.00294	0.21409	0.19908

*No data available

Table 2: Mean BW, BCS and plasma and leukocytic biomarker levels comparing pre and post overfeeding of DER×2 (overfed) and DER×1 (control)

Parameters	No. of animals examined	Pre feeding	4 week post feeding
BW			
Overfed	13	9.15±0.24	11.79±0.32*
Control	3	10.90±0.10	11.40±0.2*
BCS			
Overfed	13	1.90±0.2	3.40±0.1*
Control	3	2.70±0.3	3.20±0.2
TG			
Overfed	13	17.90±1.5	31.80±2.9*
Control	3	21.00±3.2	19.00±1.0
TC			
Overfed	13	104.50±6.9	134.00±12.7*
Control	3	129.30±20.2	161.70±18.8
TP			
Overfed	13	7.00±0.2	6.70±0.2*
Control	3	7.00±0.1	6.50±0.1
GLU			
Overfed	13	95.50±3.5	104.30±1.6*
Control	3	107.70±4.5	107.00±7.0
ALT			
Overfed	13	43.55±7.3	43.10±3.1
Control	3	38.70±5.7	44.30±7.3
AST			
Overfed	13	47.90±3.7	47.10±3.8
Control	3	30.70±4.8	32.00±4.0
ALP			
Overfed	13	126.80±9.6	141.30±8.4*
Control	3	140.70±18.8	145.30±17.2
BUN			
Overfed	13	14.60±1.1	17.20±0.8*
Control	3	10.00±1.0	12.70±2.0*
CRE			
Overfed	13	0.80±0.0	0.70±0.0*
Control	3	0.80±0.0	0.80±0.1
Plasma MDH			
Overfed	13	82.90±10.4	66.90±5.8*
Control	3	50.00±8.4	55.30±5.4
Pasma LDH			
Overfed	13	68.30±5.1	57.60±8.6
Control	3	58.10±7.8	58.80±12.4
Plasma M/L			
Overfed	13	1.2000±0.1198	1.3611±0.1572
Control	3	1.0027±0.1852	1.0053±0.1763
Leukocyte MDH			
Overfed	13	177.4±15.6	247.20±12.1
Control	3	191.3±9.3	212.90±19.5
Leukocyte LDH			
Overfed	12	846.3±56.2	1148.60±77.4
Control	3	894.4±48.2	1069.00±95.0
Leukocyte M/L			
Overfed	12	0.2214±0.0065	0.2220±0.0012
Control	3	0.2141±0.0023	0.1991±0.0026*

Data is presented as Mean±SE. *Significant ($p<0.05$) when compared against each starting level (paired t-test)

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 an increased 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 β 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 (Magori *et al.*, 2005). Interestingly, cytosolic MDH activity and M/L ratio in feline leucocytes were significantly lower than those in canine leucocytes which may reflect the differences in carbohydrate and lipid metabolism between the two species. 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 study, we focused on 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/mild overweight. Concomitantly, significant elevations in GLU, TG and 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 a 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 and 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 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 Mackova *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, PBL and plasma are considered to reflect subtle physiological changes occurring in animal tissues (Oliver *et al.*, 2013; De Mello *et al.*, 2008) and the enzyme activity of PBL and plasma malate-aspartate shuttle could be an indicator for changes in energy metabolism of the whole body (Arai *et al.*, 2003). In this study, 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. The diagnostic significance of the above mentioned parameters should be further examined on various types of weight gain and target tissues.

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