Feeding Value Evaluation of Growing and Fattening Diets by *in vivo* and *in vitro* Methods

（ *In vivo* および *in vitro* 法によるメンヨウの育成・肥育期における給与飼料評価 ）

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Thesis submitted to the United Graduate School of Agricultural Sciences, in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy of Tottori University, Japan

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADL	Acid detergent lignin
ANOVA	Analysis of variance
aP2	Fatty-acid-binding protein
BW	Body weight
C/EBP	CCAAT/enhancer binding protein
CP	Crude protein
DCP	Digestible crude protein
DG	Daily gain
DM	Dry matter
DNA	Deoxyribonucleic acid
EBW	Empty body weight
EE	Ether extract
FBS	Fetal bovine serum
GIT	Gastro-intestinal tract
IR	Italian ryegrass straw
IRD	Italian ryegrass straw with concentrate diet
LBW	Live body weight
MBN	Microbial nitrogen
ME	Metabolisable energy
MP	Metabolizable protein
mRNA	Messenger RNA
N	Nitrogen

NDF	Neutral detergent fiber
NEFA	Nonesterified fatty acids
NFC	Non fiber carbohydrates
OM	Organic matter
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPARγ	Peroxisome proliferator-activated receptor gamma
RNA	Ribonucleic acid
SCD	Stearoyl-CoA desaturase
TDN	Total digestible nutrients
TG	Triglycerid
TH	Timothy hay
THD	Timothy hay with concentrate diet

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Declaration

I hereby declare that this thesis is my original work and it has not been presented for the award of any degree elsewhere. All sources of information have been duly acknowledged by means of references.

CHAPTER ONE

General Introduction

1.1 Background Information

Forage is the most important feed to provide a variety of nutrients and fed as a basal diet for ruminant animals. Feeding program has been developed to achieve the most effective productions performance of ruminant with having an appropriate balancing feed ingredients and nutrients composition at the least cost (Chaudhry 2008). In general, evaluation of chemical composition of forage is essential for the nutritive value.

As compared to the nutritive value of diets, feeding value has more valuable meanings for developing animal feeding regimen and agronomic practice. The feeding values of feed allowed to meat producing animal were measured by an amount of feed intake, digestibility, weight gain, feed efficiency and carcass characteristics in a long term period of feeding the animal (Andersen et al. 2005). However, a large scale *in vivo* feeding trial to evaluate the feeding value of forages is hard to carry out because of time needed to accomplish, labor to do and its cost. Conversely, researchers have developed *in vitro* digestion technique as an alternative to laborious or time consuming *in vivo* digestion trial (Goering and Van Soest 1970; Menke and Steingass 1988; Hall and Mertens 2012). A number of *in vitro* analysis have been developed to predict DM digestibility (Tilley and Terry 1963), gas production (Close and Menke 1986) or the artificial fibre bag technique (Erwin and Elliston 1959; Johnson 1966; Rodriguez 1968). Likewise for the *in vitro* digestion technique which enabled to determine feed nutritive value at laboratory level to some extent, simple and rapid measurement to evaluate feeding value of diets with accuracy are required in livestock production section.

Blood metabolite concentrations represent an integrated index of the adequacy of nutrient supply in relation to nutrient utilization (Cronjé and Pambu 1996). Therefore, researchers have analyzed the blood metabolites to determine the

nutritional status in conjunction with the feeding trial. If a novel method for predicting the feeding value of the specific diet by utilizing *in vitro* cell culture system with applying blood serum (*ie*. cell culture substrate), obtained from animal fed with a diet the feeding value of which is being evaluated, is established and applicable to use feed evaluation, it can be used as an alternative to the conventional *in vivo* feeding study and would also be expected to considerably contribute to rapid development of a series of feeding regimens even though the results displayed by the *in vitro* method should not be thought to be quantitative but qualitative. There is little information available about feeding value assessment by using *in vitro* cell culture technique which aiming carcass quality evaluation. Thus, a novel method for *in vitro* feed evaluation system was tried to develop in this study with concomitant conventional *in vivo* feeding value evaluation and quantitative nutrients flow to body tissue of sheep fed on tested diets.

1.2 General Objective

The objective of this experiment was to develop a simple and rapid method for forage feeding value using blood serum in *in vitro* adipocytes culture to determine bio-available nutrients supply to body tissue which closely relating animal production in wethers.

1.3 Specific Objectives

Three experiments were designed as followings:

1. The first study was to evaluate the effects of grass forage species and long-term period of low quality forage diet feeding on growth performance,

nutrient utilization, microbial nitrogen yield and carcass characteristics in growing wether lambs (Chapter2).

2. The second study was to estimate the relationship between nutrient supply to muscle and adipose tissues and nitrogen retention in growing wethers fed forage-based diets (Chapter3).
3. The final experiment was conducted to assess the effects of circulation materials in the blood of sheep fed forage-based diet on adipogenesis of ovine adipocytes for developing new feeding strategies to improve animal performance (Chapter4).

The general conclusion of the three experiments and relevant discussion were described in Chapter 5.

CHAPTER TWO

Effects of Grass Forage Species and Long-Term Period of Low Quality Forage Diet Feeding on Growth Performance, Nutrient Utilization, Microbial Nitrogen Yield and Carcass Characteristics in Growing Wethers

2.1 Summary

Six growing wether lambs were used to evaluate feeding values of two forage-based diets for 12 months period of feeding trial with measuring BW gain, nutrients digestibilities, N retention and MBN yield and carcass characteristics of the animals slaughtered at the conclusion of the feeding trial. The basal forage of each diet was offered at 2% BW as DM basis, and concentrate was fed at 40% of forage intake as fed basis. The BW gain averaged 25.3 to 55.3 kg and 28.5 to 48.7 kg for THD and IRD, respectively. Feed efficiency was significantly greater for THD than for IRD. Digestion coefficients of OM and NFC did not differ significantly between diets or period, while CP and EE digestibility were slightly but significantly higher for THD than for IRD. The DCP intake was significantly greater for THD than for IRD, while NFC intake was significantly greater for IRD than for THD. Although N intake was significantly greater for THD than for IRD, ratio of retained N to absorbed N showed tendency toward significance for IRD as compared to THD. Neither diets nor period had significant effect on MBN supply and efficiency of ruminal MBN synthesis. The EBW and carcass weight were greater for THD than IRD. The lean meat weight was numerically greater for THD as compared to IRD, and the weight of subcutaneous fat and abdominal fat, their weight ratio to EBW were also markedly greater for THD than those for IRD. The results of this study indicated that THD feeding displayed superior growth performance of growing lambs during the 12 months of feeding period and digestible N supply as compared to IRD, but long term period of THD feeding tended to deposit adipose tissue at higher ratio to EBW as compared to IRD. It is also suggested that the relatively higher N assimilation ratio of wether lambs fed with IRD than that fed with THD might have associated with the observed comparable dressing ratio and lean meat ratio to EBW.

2.2 Introduction

Forage based diets are conventionally used for small ruminant feeding for both growing and fattening periods in many parts of the world. Therefore, the feeding program has been developed to achieve the most efficient production performance with consideration of ruminant species (cattle, sheep or goats), breed, production purpose (dairy, hair or meat) and physiological stage of growth or production. Evaluation of chemical composition and nutrients digestibility are essential for assessing the nutritive value of forage diet, but it is not sufficient for demonstrating the feeding value applicable for agronomic practice level. To evaluate a feeding value of animal feed, measurement of amount of feed intake, ease of feed availability or preparation and production performance when fed to the animal should be required in addition to nutritive value score. Regarding the items of feeding value assessment, determination of BW gain and carcass characteristics are thought to be the most important for feeding a meat producing animal. However, it is time consuming and laborious and inaccessible for most field laboratories as well as raise the concerns about animal welfare (Adesogan 2002; Huhtanen et al. 2006).

By using recent feeding standards, nutrients and ME requirements to meet various production levels can be estimated accurately in feeding practices. Moreover, MBN syntheses in the rumen and its supply to the small intestine have been evaluated quantitatively to estimate MP supply to ruminant animals (ARC 1984; AFRC 1993). Ichinohe and Fujihara (2008) suggested that lengthening feeding duration over 30 days might cause ameliorative changes in utilization of both ME and MP, and improvement in MBN synthesis when fed diets having low ruminal degradation synchronicity between OM and N to adult rams at a maintenance level of feeding. Their result might indicate an occurrence of some adaptive response of rumen

fermentation and N utilization with continual feeding of a diet formulated to have an asynchronous degradation of OM and N within a daily feeding regimen by both rumen microbes and host animal. Hovell et al. (1983) and Chowdhury et al. (1995) reported that N accretion rate and ME utilization of lambs varied clearly when responding to the fluctuation of intra-gastric nutrient infusion levels, and suggested a metabolic modulation responding to changes in nutrients supply by the growing ruminant. Recently, effects of diet, level of feeding, supplements feeding, rumen degradation synchronicity and forage quality on MBN supply from the rumen have been reviewed by Dewhurst et al. (2000). However, in their review, the effect of feeding duration of diet on ruminal MBN synthesis with relevant to feed quality was not well addressed. There is still insufficient information available to identify the adaptation response to diets, differing in their nutritive value, along with feeding duration at various physiological stages of ruminant animals (Delavaud et al. 2007). It was expected that the rumen fermentation characteristics, utilization of ruminal fermentation substances by host animal, ruminal MBN yield and MP utilization efficiency for production would change with age of ruminants and also lengthening feeding period of low quality diet; hence, the combination of both microbial and metabolic adaptation by host animal might lead to ameliorating of low quality forage diet by growing ruminant.

The knowledge of feeding values assessed by *in vivo* trail including carcass characteristics evaluation, dietary adaptation status of ruminal fermentation and host animal metabolism during a long term period feeding of forage diet and a mechanism by which dietary adaptation will be applicable to establish feeding regimen for meat producing growing ruminant. Therefore, the present study was conducted to evaluate the effects of forage species of basal diet and 12 months of long-term feeding period on BW gain, nutrients digestibility, N balance, MBN supply and carcass

characteristics of animal slaughtered at the conclusion of 12 months of feeding in wethers. Italian ryegrass (*Lolium multiflorum* Lam.) is being utilized as a major domestic forage source for ruminant production practice in Korea. Two typical cool season grass forage, TH and IR, were chosen as basal diets because they are commonly used in Korea and also Japan.

2.3 Materials and Methods

Animal experiment described in this Chapter was conducted at Shimane University from August 2013 to August 2014. Use and animal care procedures were approved by Animal Care Committee of Shimane University.

2.3.1 Animals and diets

Six spring born (February or March) Suffolk wether lambs, initial mean BW of 26.4±0.7 kg, were used in this study. They were purchased from a commercial sheep farm at 4 to 5 months of age in July, 2013. All lambs were dewormed prior to use in the study. The animals were randomly divided into two groups (n = 3, each group) to be similar mean BW (26.1 vs. 26.7 kg), and were allocated to one of two dietary treatments for 12 months of feeding period. Except for metabolism trials by total collection, the animals were housed in well-ventilated pens under continuous lighting and had free access to water and mineral blocks during the feeding experiment. Imported TH and IR from USA were purchased and used as basal diets. Due to the forage storage space at the animal farm of Shimane University, different lots of imported TH and IR had to use in this study (Table 2.1). The forages were coarsely chopped using an electric forage chopper to about 4 cm in length. Both of the forages were offered to the six wether lambs *ad libitum* in a change-over design to determine voluntary feed intake of TH and IR for at 7 day period, respectively. The

Table 2.1 Chemical composition of feed offered wether lambs for 12 months of feeding period

Item	TH†			IR‡			Concentrate
	0–2	3–8	9–12	0–4	5–8	9–12	
OM	93.2	93.4	92.5	93.8	95.2	95.3	93.1
CP	6.3	8.4	7.4	5.9	6.4	4.7	22.0
EE	1.7	2.4	2.0	1.7	2.0	1.2	4.5
NDF	67.8	65.8	66.0	67.0	68.5	66.7	24.8
ADL	7.1	6.3	6.3	5.4	6.5	3.8	1.2
NFC§	17.4	16.8	17.1	19.2	18.3	22.7	41.8

†Different lots of imported timothy hay (TH) were used. Chemical compositions of TH fed to animals from the beginning of experiment to 2 months (0–2), from 3 to 8 months (3–8) and at 9 to 12 months (9–12) were listed as a dry matter basis (in %).

‡Different lots of imported Italian ryegrass straw (IR) were used. Chemical compositions of IR fed to animals from the beginning of experiment to 4 months (0–4), from 5 to 8 months (5–8) and at 9 to 12 months (9–12) were listed as a dry matter basis (in %).

§Calculated as 100－(crude ash + crude protein + ether extract + neutral detergent fiber).

determinations of voluntary feed intake of the forages were carried out during 14 day of preliminary period which was set before the commencement of the 12 months period of the feeding study. The voluntary feed intake for TH and IR were determined under group-housed condition (three heads per pen), and the measured values were used to formulate the feeding regimen for the feeding study. Following the 14 days of preliminary period, the animals were kept in individual pens, and fed THD or IRD for 12 months. With both group animals, TH or IR were allowed at 2% BW (as DM basis) and amount of concentrate allowance was set at 40% of forage intake (as fed basis). The animals were fed two equal sized meals at 09.00 and 16.00 hours throughout the experiment. The quantity of concentrate allowance was adjusted daily according to the previous day's forage intake for each animal. In feeding each meal, the forage was supplied in the feed trough after completing concentrate consumption. The THD and IRD were designed to satisfy ME requirements for 100 g DG with a provision of 5% safety margin based on the estimation equations by the AFRC (1993) using the tabular values listed in Standard Tables of Feed Composition in Japan (NARO 2009), TDN concentration of commercial concentrate and voluntary feed intake of TH and IR. Chemical compositions of TH, IR and commercial concentrate are listed in Table 2.1.

2.3.2 Experimental procedures and measurements

Feeding period of both THD and IRD lasted for 12 months, and during which, 5 days of metabolism trials were conducted six times using the six wether lambs at 2 (October 2013), 3 (November 2013), 5 (January 2014), 7 (March 2014) , 9 (May 2014) and 11 (July 2014) months of the experiment period. The BW measurement was conducted weekly at 13.00 hours for each animal throughout the experimental

period. Based on the BW of the animals, ME requirement and feed provision level to each animal were calculated weekly. Feed refusals were weighed and recorded daily for each animal throughout the feeding experiment. During the metabolism trials, animals were transferred from pens and were kept in metabolism crates in a temperature controlled room (20°C) under continuous lighting, and total collections of feces and urine were conducted. Total urine was collected for estimating urinary N excretion and MBN supply to the small intestine. Urine was collected into the container containing 50 mL of 10% (v/v) H_2SO_4 to keep urine pH below 3. Representative samples of feeds, orts, feces and urine were obtained daily during the total collection period.

At the end of 12 months period of the feeding trial, 17 to 18 months old of two wethers were chosen from each diet feeding group and slaughtered to evaluate carcass characteristics. The day before slaughtering, feed was withheld overnight with free access to water. After slaughter, weight of head with tongue, feet, skin with hair, blood, trachea, heart, lung, liver spleen, kidney, GIT and GIT contents, abdominal and subcutaneous fat, lean meat, longissimus and born were recorded for slaughter and carcass evaluation. Carcass and non-carcass components were weighed immediately after slaughter. The EBW was calculated by subtracting the weight of GIT contents from LBW weighed at the slaughter. Dressing percentage was calculated as percentage of carcass weight to LBW or EBW.

2.3.3 Chemical analyses

The fecal samples were dried in a forced air oven at 60^{o}C for more than 48 h. Samples of air dried feces, feeds and orts were ground to pass through a 1 mm screen. Samples of feeds, orts and feces were determined for OM, CP, EE by AOAC

1984). The NDF and ADL were determined by the procedure of Van Soest et al. (1991). Urinary N concentration was determined by Kjeldhal method. The daily excretion of purine derivatives (PD) was determined by a colorimetric method according to Fujihara et al. (1987) and the MBN supply to the small intestine (g/d) was estimated based on the urinary PD as an indicator of intestinal absorbed MBN (Chen and Gomes 1995).

2.3.4 Statistical analysis

Data were analyzed by a two-way repeated measures analysis of variance (ANOVA) to evaluate the effects of diets and feeding period on feed intake, DG, digestibility, N retention, ruminal MBN yield and plasma metabolites concentration. Data for daily feed intake and DG were summarized as mean values for each month of the feeding period. The measured variables in the metabolism trial carried out at 6 months of the feeding period and measurements of feed intake and DG at 12 months were considered as repeated observations on each block. The general linear model included diets, feeding periods and diet × period interaction as fixed effects, where animal within the dietary treatments was specified as a random effect. Effects were deemed to be statistically significant when $P \leq 0.05$, and tendencies were considered to exist when $0.05 < P \leq 0.10$. When feeding period effect was detected by ANOVA, differences between the means were separated using Duncan's multiple range test. All statistical procedures were performed using SPSS 14.0 (SPSS 2006).

2.4 Results and Discussion

2.4.1 Diet characteristics

There were slight variations in the diets chemical compositions due to the difference

in import lots from the USA for both forages (Table 2.1). The CP concentration (DM basis) ranged from 6.3 to 8.4% for TH and from 4.7 to 6.4% for IR. The mean value of CP concentration of the three lots of TH and IR seemed to be similar to the tabular values of imported hay listed in Standard Tables of Feed Composition in Japan (NARO 2009), although the CP concentration of IR fed from 9–12 months was quite lower compared to the tabular value of 6.2% DM (NARO 2009). The NDF concentration of TH and IR were almost similar to the tabled values (NARO 2009). The extent of fiber lignification (ADL/NDF, in %), which indirectly indicated physical coarseness and also rate and extent of ruminal fiber degradability (Ichinohe et al. 1994), averaged 9.9% and 7.7% for TH and IR, respectively, and the value appeared to be lower for IR than that for TH. The commercial concentrate used in this study was more than 70% of TDN concentration for feeding growing beef cattle.

2.4.2 Growth performance and feed intake

The BW of wether lambs fed each diet increased in a linear curve with advancing feeding months and exhibiting a logistic growth pattern (Figure 2.1), and BW changed from 25.3 to 55.3 kg and 28.5 to 48.7 kg for THD and IRD, respectively. Animals were allotted to the two diet groups to yield the similar mean BW, although at the conclusion of the 14 days voluntary feed intake measurement of the basal forages during July in confinement conditions led to the observed difference in BW between the groups. Hence, there was BW difference between the groups at the beginning of the feeding study ($P > 0.05$). Both of the diets were fed to the animals to achieve 100 g DG through the experimental period, however, the months of the feeding period at which animals reached the targeted value was at 7 months onward and 9 months onward for THD and IRD, respectively (Table 2.2). The average DG throughout the

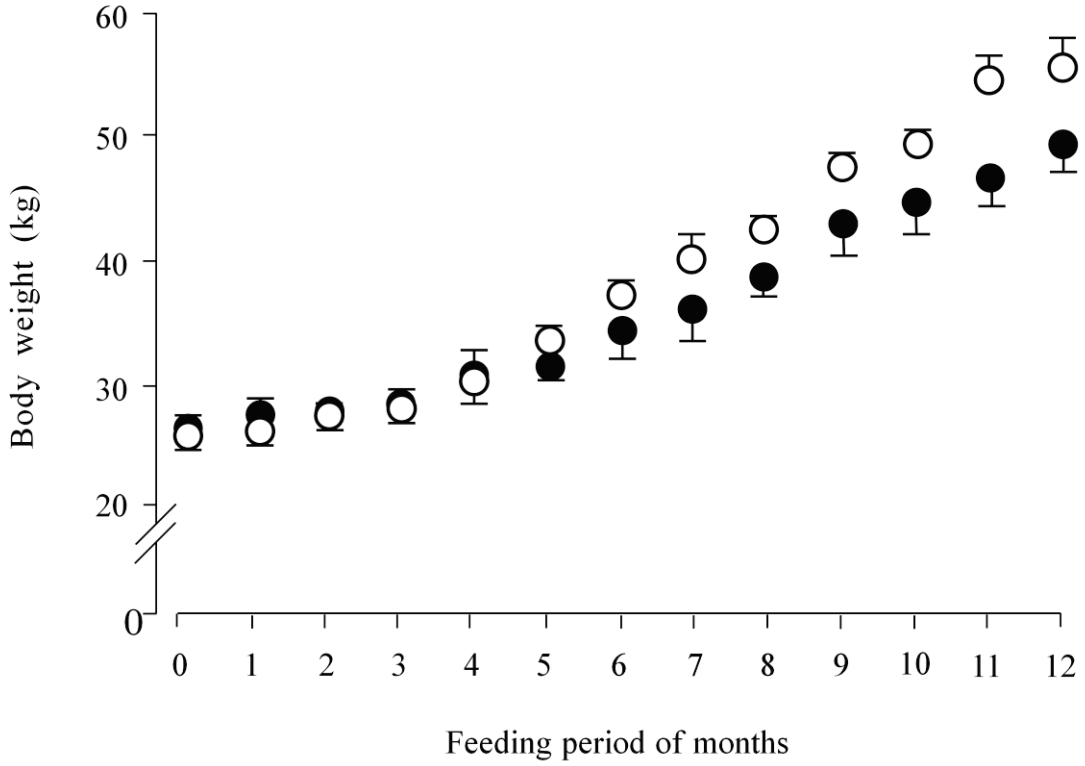

Figure 2.1 Body weight changes of wethers fed diet based on THD or IRD for 12 months of feeding period. ○, means of growing wethers fed THD; ●, means of growing wethers fed IRD. Each symbol with vertical bar shows mean value with the standard deviation. Body weight were measured every week, and summarized as mean value for a month of feeding period for each diet.

12 months period did not reached the targeted value, and was calculated as 60.1 and 81.7 g/d for IRD and THD, respectively, and was significantly higher for THD than for IRD. Both of the forage intakes (g DM/d) increased according to BW gain through the feeding period, and both variables (g DM/d and % BW) appeared to be greater for IR than for TH from 1 to 7 months. In this experiment, feed particle size of machinery-chopped forages seemed to be smaller for IR than that for TH. Even though we did not determine the feed particle size distribution and eating behavior of the animals, it appeared that the difference in physical properties of forages might contribute to the feed acceptability, ease of bolus formation for deglutition in eating (Ichinohe et al. 1994; Ueda et al. 1997, 2001) and hence might be thought to influence eating rates of growing lambs, which might partly explain the reason that lambs ate IR more than TH. The difference in consumption variables of TH and IR did not agree with the study by Assefa and Ledin (2001), in which they indicated that animals ate more high CP concentration forage than lower CP forage. Animals consumed offered concentrate thoroughly during the experimental period. Concentrate intake did not differ between the diets and significantly increased with advancing feeding period. Feed efficiency varied between months of the observation for both diets, and was statistically higher for THD than for IRD with the value at 9 months being greatest for both diets. In the current study, the significant lower values of BW gain for IRD thanfor THD from 1 to 7 months of period have resulted in significant dietary difference.

2.4.3 Apparent digestibility and nutrients intake

The apparent digestibility for OM, NDF and NFC did not differ significantly between the diets, although CP and EE digestibility were 3 percent point higher for THD than

Table 2.2 Body weight gain, feed intake and feed efficiency in wether lambs fed forage based diets for 12 months of feeding period

Items	Feeding period of months						SEM	*P*-value¶		
	1	3	5	7	9	12		D	P	D×P
Daily gain (g/d) †										
THD	37.6^{b}	24.4^{b}	87.2^{a}	111.7^{a}	116.7^{a}	130.6^{a}	15.54	0.06	0.03	0.82
IRD	21.5^{bc}	13.9^{c}	57.8^{bc}	72.2^{ab}	105.0^{a}	100.6^{a}	14.32		0.03	
Forage intake†										
TH (g DM/d)	513.9^{dB}	513.7^{d}	636.6^{c}	749.5^{c}	911.8^{bA}	1229.6^{aA}	24.21	0.06	0.01	0.02
IR (g DM/d)	577.2^{eA}	635.0^{e}	706.9^{d}	779.2^{c}	860.2^{bB}	1081.5^{aB}	19.05		0.01	
TH (% BW/d)	1.9	1.8	1.9	1.8	1.9	2.0	0.01	0.05	0.32	0.89
IR (% BW/d)	2.0^{a}	2.0^{a}	2.0^{a}	2.0^{a}	1.8^{b}	2.0^{a}	0.01		0.01	
Concentrate intake										
THD (g DM/d)	216.6^{e}	237.9^{de}	272.2^{cd}	275.1^{c}	368.8^{b}	491.9^{a}	11.09	0.08	0.01	0.59
IRD (g DM/d)	215.0^{d}	231.9^{d}	253.8^{c}	273.5^{c}	316.6^{b}	432.6^{a}	6.47		0.01	
Feed efficiency§										
THD	36.5	44.6	102.3^{A}	104.7	135.8	86.5	14.90	0.01	0.06	0.33
IRD	24.2^{b}	18.1^{b}	12.1^{bB}	62.6^{b}	134.5^{a}	73.1^{b}	21.64		0.02	

†Animals were weighed every week and feed intake was determined every day through the feeding period; these data were summarized as mean value for a month of feeding period for both diets.

§Expressed as g BW gain/kg DM intake.

¶*P*-value is probability of significant for diet (D), period (P) and diet × period interaction (D×P).

[a, b, c, d, e] Means within a row with different superscripts differ ($P \leq 0.05$).

[A, B] Means within a column with different superscripts differ ($P \leq 0.05$).

for IRD (Table 2.3). The effect of feeding period was not detected for digestion coefficients for both diets. The diet × period interaction reached significant levels for digestion coefficient of OM, EE and NDF. The CP concentration of ingested feed (% of DM, data not shown in Table 2.3) for THD and IRD during the 12 months of feeding period was estimated to be 12.0% and 10.5% DM ($P > 0.1$) on average, respectively. The DCP intake ($g/BW^{0.75}$) was significantly greater for THD than for IRD with response to the significantly higher CP digestibility. Moreover, the lower CP concentration of IR offered to animals from 9–11 months (Table 2.1) may be one of the reasons for causing the interaction. Since Loy et al. (2002) indicated that energy provision was the first limiting factor to nursing growing ruminants fed with low–quality forage diet, THD and IRD were designed to suffice ME requirements for 100 g of DG (AFRC 1993) without taking into account rumen degradable N (RDN) requirements (ARC 1980, 1984) or MP requirements (AFRC 1993) in the current study. Reflecting the feeding regimen of this study, neither diets nor feeding period had significant effect on ME intake ($MJ/BW^{0.75}$), and the value was kept around 0.7 for both diets and throughout the feeding periods. The ratio of ME intake to ME requirement of 100 g DG (AFRC 1993) was estimated to be range from 0.86 to 0.96 for THD and from 0.85 to 0.96 for IRD, suggesting estimated ME intake almost met the recommendation by AFRC (1993) for both diets. However, the reason for lacking BW gain response to ME sufficiency, observed from 1 to 3 months, was not found in the current study. The NFC intake ($g/BW^{0.75}/d$) was significantly greater for IRD than for THD, and

Table 2.3 Apparent digestibility and intakes of digestible CP, ME and NFC in wether lambs fed forage based diets for 12 months of feeding period

Items	Diet		SEM	Feeding period (months)						SEM	*P*-value		
	THD	IRD		2	3	5	7	9	11		D	P	D×P
Digestibility (%)													
OM	64.8	63.4	0.97	62.3	64.0	63.2	65.4	64.7	64.7	2.15	0.32	0.37	0.01
CP	67.8	64.2	1.11	63.1	65.7	67.2	67.4	65.6	67.0	2.08	0.03	0.21	0.12
EE	72.1	69.0	0.85	69.7	68.6	70.5	72.1	71.2	71.2	1.86	0.01	0.24	0.01
NDF	54.6	53.1	1.14	52.1	54.3	52.0	53.6	55.7	55.5	2.79	0.35	0.41	0.01
NFC	86.6	85.3	0.68	85.3	85.4	85.8	90.2	83.1	86.0	1.29	0.17	0.21	0.09
DCP intake													
g/ $BW^{0.75}$/d	5.6	5.0	0.09	5.1^{bc}	4.7^{c}	5.6^{ab}	5.4^{b}	5.3^{b}	5.8^{a}	2.02	0.01	0.01	0.01
ME intake§													
MJ/$BW^{0.75}$/d	0.7	0.7	0.01	0.7	0.7	0.7	0.7	0.7	0.7	0.02	0.97	0.15	0.37
NFC intake													
g/ $BW^{0.75}$/d	18.4	20.4	0.19	17.0^{c}	16.5^{c}	17.4^{c}	17.1^{c}	21.6^{b}	26.9^{a}	0.56	0.01	0.01	0.01

§Estimated using the value of total digestible nutrients intake.
[a, b, c]Means within a row with different superscripts differ ($P \leq 0.05$).

showed the effect of feeding period and diet × period interaction.

2.4.4 Nitrogen balance and MBN supply

Variables for N balance, MBN supply and efficiency of ruminal MBN synthesis were shown in Table 2.4. The N intake (g/$BW^{0.75}$) was greater for THD than for IRD, and significant diet × period interaction were found. The greater urinary N excretion (g/$BW^{0.75}$) was also found for THD than for IRD, and effect of feeding period and significant diet × period interaction were found. There were no dietary or period effects on N retention variables (g/$BW^{0.75}$ and % of N intake). The two variables for N retention varied among the six observation periods for THD and IRD, ranging 0.3–0.6, 0.4–0.5 g/$BW^{0.75}$ and 26.7–45.3, 29.6–44.1% of N intake, respectively, and the difference in N retention variables between the diets and feeding period yielded significant or tendency toward significant for diet × period interaction, respectively. However, the numerically greater (P = 0.08) ratio of retained N to absorbed N for IRD (56.2%) than that for THD (49.7%) was prominent in the current study. The N retention variables observed in this study did not agree with the result by Ichinohe and Fujihara (2008) indicating an adaptive increase in N retention with advancing feeding period.

Neither diets nor feeding periods had significant effect on either MBN supply or efficiency of MBN synthesis. Ichinohe and Fujihara (2008) also reported an adaptive improvement in MBN supply with advancing feeding period, while the result of this experiment is contrasted to this. Although there was no period effect, MBN efficiency appeared to be numerically higher for

Table 2.4 Nitrogen balance, microbial nitrogen supply and efficiency of microbial nitrogen synthesis in wether lambs fed forage based diets for 12 months of feeding period

Items	Diet		SEM	Feeding period (months)						SEM	*P*-value		
	THD	IRD		2	3	5	7	9	11		D	P	D×P
N intake (g/$BW^{0.75}$/d)	1.3	1.2	0.02	1.2	1.2	1.3	1.3	1.3	1.3	0.04	0.01	0.34	0.03
Urinary N excretion (g/$BW^{0.75}$/d)	0.5	0.3	0.01	0.3^{b}	0.4^{ab}	0.4^{ab}	0.4^{a}	0.4^{ab}	0.4^{ab}	0.02	0.02	0.01	0.01
N retention (g/$BW^{0.75}$/d)	0.4	0.4	0.02	0.4	0.4	0.5	0.4	0.4	0.4	0.03	0.31	0.35	0.05
(% of N intake)	33.8	35.8	1.51	37.4	34.7	37.4	32.2	35.0	32.2	2.62	0.32	0.44	0.08
(% of absorbed N)	49.7	56.2	1.70	58.8	52.5	55.6	47.7	53.6	49.5	2.93	0.08	0.50	0.05
MBN supply (g/$BW^{0.75}$/d)	0.8	0.6	0.06	0.8	0.9	0.8	0.6	0.4	0.6	0.10	0.55	0.50	0.40
MBN efficiency (g N/kg RDOM‡)	31.1	27.1	2.45	34.0	38.9	36.0	25.6	18.6	22.1	1.25	0.62	0.40	0.49

Means within a row with different superscripts differ ($P \leq 0.05$).
‡Rumen degradable organic matter intake (ARC 1980).

the early stage of feeding period than those observed from 9 to 11 months and from 7 to 11 months for THD and IRD, respectively. These values from 7 to 11 months were relatively lower as compared with tabular values of the ARC (1984) for sheep fed hay supplemented with concentrate (30.8±7.1 g MBN/d of rumen degraded OM (RDOM) intake). The value of g MBN/kg RDOM at 7 and 9 months for THD was calculated to be 16.4 and 23.5, respectively, and at 7, 9 and 11 months for IRD was calculated to be 20.7, 20.8 and 20.8, respectively. The reasons for the depression of the efficiency of MBN synthesis for the late stage of the feeding period than those for the early stage were not clearly elucidated in the current study. However, it might be attributable to an asynchronous supply of RDN and ruminal fermentable energy in a daily feeding regimen (Sinclair et al. 1993), and also owing to lowered RDN and TDN supply or their imbalanced ratio (Bodine et al. 2001) at the late stage of the feeding period as compared to those at the early stage for both THD and IRD. Furthermore, Bruinenberg et al. (2004) reported that low CP diet lowered ruminal ammonia concentration, thus contributing less ammonia loss from the rumen due to an effective capture of ruminal N by rumen microbes.

2.4.5 Carcass characteristics of wethers in hogget production

Since two wethers were chosen for slaughter and carcass characteristics Berge et al. (1993) and Ponnampalam el al. (2002) reported that the dietary intake of protein and energy are not the only factors that control the protein and fat deposition in the carcass. This experiment is contrasted that MacRae and Lobely (1982) reported protein provided in excess in the diet would be

Table 2.5 Carcass characteristics of wethers used in the feeding trial

Items	THD		IRD	
	1	2	1	2
EBW, kg	47.2	42.5	37.0	29.2
Carcass weight, kg †	32.5	28.0	23.9	19.1
Dressing % ‡				
LBW basis	55.7	50.0	50.5	50.1
EBW basis	68.9	65.9	64.6	65.3
Gut fill, % of live weight	25.4	30.4	28.7	28.6
Total non carcass component§				
Weight, kg	25.9	28.0	23.4	19.0
% of EBW	54.9	65.9	63.3	65.0
Lean meat				
Weight, kg	21.0	15.7	15.7	12.4
% of EBW	44.6	37.0	42.4	42.4
Longissimus dorsi				
Weight, kg	1.7	1.2	1.4	1.1
Area, cm^2	32.9	27.5	28.8	17.1
Subcutaneous fat				
Weight, kg	2.5	2.5	0.2	0.5
% of EBW	5.4	5.8	0.6	1.7
Abdominal fat				
Weight, kg	1.5	2.6	0.4	0.8
% of EBW	3.2	6.0	1.1	2.6

† Calculated as LBW – Total non carcass component

‡ Calculated as LBW basis, (Carcass weight / LBW) × 100; EBW basis, (Carcass weight / EBW) × 100

§ Calculated as blood + head + skin with hair + GIT + GIT contents + feet.

utilized for fat synthesis in the animals. Hence, the greater DCP provision for THD than for IRD might be partly associated with the observed increased adipose tissue deposit ratio to EBW in the current study.

The results obtained from feeding trial and slaughter trial indicated that THD feeding led higher growth performance (DG and feed efficiency) of growing wether lambs and greater DCP supply as compared to IRD, but long term period of feeding THD showed greater deposition of both subcutaneousfat and abdominal fat as compared to IRD. The two experimental diets were designed to be iso-energetic and were allowed to the animals at similar ME provision, however, the relatively higher N assimilation ratio (N retention per absorbed N) of wether lambs fed with IRD than that fed with THD might be associated with comparable dressing ratio and lean meat ratio to EBW. From the point of view of lean meat production, IRD can be used for basal diet for growing or finishing purpose as THD. Nevertheless, the reason for lacking BW gain response without mirroring ME sufficiency and N retention observed at early stage of growth of wether lambs are remained to be solved.

CHAPTER THREE

Relationship between Nutrient Supply to Muscle and Adipose Tissues and N Retention in Growing Wethers on Forage Based Diets Fed with Different Forage Sources

3.1 Summary

Effect of forage species comprising basal diets on concentrations of blood plasma metabolites and amount of energy yielding substances supply to both of muscle and adipose tissue concomitant with N retention were examined with three growing wethers formed carotid artery-skin loops and located indwelling catheters in the mesenteric vein and the hepatic portal vein. Wethers were offered two experimental diets (THD and IRD) as used in the animal experiment described in Chapter 2, for 11 days of period for each diet, in a one-way layout design. The experimental diets were designed and allowed to the animals to have 100 g of DG. The glucose concentration of blood plasma sampled at artery and portal vein were higher for THD than for IRD. Available amount of TG in the tissues was estimated to be higher for IRD than for THD. The daily amount of glucose and fatty acid supplied to both muscle tissue and adipose tissue were numerically higher for THD than those for IRD without showing significant difference. The N retention did not differ markedly between the diets, reflecting the observed absence of significant differences between the diets in the amount of energy yielding substances supplied to the muscle tissues. The results suggest that the difference in amount of glucose delivered to muscle tissue might associate with N retention responses to the forage based diets in early growing stage of wether lambs.

3.2 Introduction

In Chapter 2, feeding value of both THD and IRD as growing and fattening diet to ruminant animal by long term *in vivo* study was discussed. The results obtained by the 12 months period of feeding trial and carcass characteristics evaluation showed that the greater intakes of dietary N or DCP for THD as compared to IRD did reflect neither N retention (% of intake N or digested N) (Kim et al. 2015) nor ratio of lean meat weight to EBW (Kim et al. 2014). Furthermore, greater values of weight ratio of adipose tissue to EBW were also evident for THD than for IRD feeding in hogget production performance of Suffolk wethers slaughtered at 17 or 18 months of age (Kim et al. 2014). The differences in N retention values and carcass characteristics between the two diets may be due to the differences in ME intake (Yan and Agnew 2004), differences in MP supply originating rumen microbes (Clark et al. 1992), amino acid sparing (Neale 1971; Neale and Waterlow 1974), gluconeogenesis from the ruminal propionic acid, and urea recycling into the rumen (Sands and Layton 2009). However, the effect of factors above mentioned was not cleared thoroughly in the previous studies (Kim et al. 2014; Kim et al. 2015). The plausible reason for the difference in carcass characteristics might be due to the difference in amount of nutrients absorbed though the GIT or delivered to the liver, rate and extant of nutrients processing in the hepatic tissue, which in turn, resulted in the difference in amount of available nutrients for the development and growth of adipocytes or muscle cells.

The quantification of energy yielding substances supplied to both of muscle and adipose tissue is needed to elucidate the difference in growth performance and carcass characteristics of wethers fed with THD and IRD as described in Chapter 2. In the previous study (Kim et al. 2015, Chapter 2), there were large difference in the DG value from 1 to 3 months of feeding period between the diets, and the value of these

periods for both diets were far below the targeted value (100 g DG) even though ME allowance for 100 g DG requirement was almost sufficed from 1 to 3 months of feeding period (sufficient ratio ranging from 80 to 116%). In this study, calculation of amount of nutrients supplied to both of muscle tissue and adipose tissue were carried out for elucidating the reason for the observed lower DG for IRD group than for THD from 1 to 3 months of feeding period, and for the difference in the ratio of adipose tissue to EBW between the diets. The objective of this experiment was to determine the effect of feeding forage based diets with differing forage species on blood plasma nutrients flow to muscle tissue and adipose tissue in relevant to N retention in wether lambs being early stage of growing period.

3.3 Materials and Methods

The animal experiment of this study was conducted at Shimane University from May to October 2014. Use and animal care procedures were approved by Animal Care Committee of Shimane University. Surgical preparation, post-surgical care and management were conducted in accordance with the guideline of the animal use regulation of Shimane University and also with the 'Guide for the Care and Use of Agricultural Animals in Agricultural research and Teaching' (Federation of Animal Science Societies, 1999).

3.3.1 Animals and diets

Three spring born (January to February 2014) Suffolk wethers, initial mean BW of 27.3 ± 2.3 kg, were used in this study. The animals were allocated to two dietary treatments at the age of 6 to 7 months. The animals were also kept individually in metabolism crates throughout the experimental period, and fed THD and IRD (Table

3.1) in a one-way layout design. Similar to the animal experiment described in Chapter 2 (Kim et al. 2014; Kim et al. 2015), the forages were allowed at 2% BW (as DM basis) and the amount of concentrate allowance was set at 40% of forage intake (as fed basis) for each animal. The diets were designed to satisfy ME requirements for 100 g daily gain with a provision of 5% safety margin based on the estimation equations by the AFRC (1993). The animals were fed two equal sized meals at 09.00 and 21.00 hours throughout the experiment.

The experimental animals were surgically prepared under general anesthesia for forming skin loops enclosing the right carotid artery and for locating indwelling catheters in the mesenteric vein and the hepatic portal vein at 3 months before the start of the experiment.

3.3.2 Experimental procedures

The animals were housed in an environment controlled room with air temperature being 23^{o}C with free access to water throughout the experimental period. The experiment was carried out in two periods of 11 days, of which first 7 days for adaptation to the diet, followed by 3 days of metabolism trials and the one day of blood sample collections. During the metabolism trials, daily excretion of feces and urine were recorded for 3 consecutive days. Urine was collected into the container containing 50 mL of 10% (v/v) H_2SO_4 to keep urine pH below 3. Representative samples of feeds, orts, feces and urine were obtained daily during the total collection period. On d-11 of the experiment, continuous infusion of para-aminohippuric acid (PAH) solution (1% w/v) was conducted via the mesenteric vein as described by Ortigues et al. (1994). The blood samples at portal vein and artery were simultaneously collected into 10 mL heparinized syringes at every 60 min after

Table 3.1 Chemical compositions of the diets used in the feeding trial

Item	TH	IR	Concentrate
OM	92.5	95.3	93.1
CP	7.4	4.7	22.0
EE	2.0	1.2	4.5
NDF	66.0	66.7	24.8
ADL	6.3	3.8	1.2
NFC	17.1	22.7	41.8

morning feeding. Total 12 sets of blood samples were immediately analyzed for hematocrit value. Whole blood samples were centrifuged for 15 min at 4^{o}C, and the blood plasma samples were stored at –21^{o}C until further analysis.

3.3.3 Chemical analyses

The samples of feeds, orts and feces were dried in a forced air oven at 60 ^{o}C for more than 48 h. Air dried samples were ground to pass through a 1 mm screen. The N in feeds and feces samples was determined by AOAC method (1984). Blood plasma concentrations of glucose, NEFA and TG were determined by commercial kits (Glucose C-test, NEFA C-test and TG E-test, Wako Pure Chemical Industries, Osaka, Japan) and PAH concentrations of blood plasma samples were determined by the method of Huntington (1982).

3.3.4 Calculations

Blood plasma flow rate was calculated using an indicator-dilution technique previously described by Katz and Bergman (1969). In the current study, one-compartmental pharmaco kinetics model (Oriuchi et al. 1995) was introduced to estimate the rate constant of nutrients absorption from the GIT and first stage nutrients elimination in the hepatic tissue. According to the graphical shape of the change in nutrients concentration of arterial blood plasma (glucose, NEFA and TG) postprandial time, analytical equation was generated based on the pharmacokinetics model. In this model, change in specific nutrients concentrations of arterial blood plasma post feeding (t, hr) can be described as:

$$C_{nutr} = [C \cdot k_a/(k_a - k_d)] \times (e^{-kd \times t} - e^{-ka \times t}),$$

where C_{nutr}, nutrient concentration of arterial blood plasma; C, scale parameter; k_a, rate constant of nutrients absorption though the small intestine (fraction per h) and k_d, rate constant of nutrients disappearance at the hepatic tissue by the metabolism of first passage effect (fraction per h). In this model, assumptions, $k_a \neq k_d$ and $k_a > k_d$, were introduced for estimating the parameter relating nutrients absorption, hepatic tissue metabolism and supply to the body tissue. All curves fitted to estimate the parameters were performed using commercial software generated by Daniel G. (Curve Expert 1.4, http://curveexpert.net). The amount of bioavailable nutrients delivered to the tissues the weight of muscle and adipose tissue were calculated using allometric equations listed in by ARC (1980).

3.3.5 Statistical analysis

Effects were deemed to be statistically significant when $P < 0.05$. Data were analyzed by one-way ANOVA and differences between the means were separated using Duncan's multiple range test. All statistical procedures were performed using SPSS 14.0 (SPSS, 2006).

3.4 Results and Discussion

3.4.1 Concentrations of blood plasma metabolites

Blood metabolites are shown in Table 3.2. Blood plasma concentrations of all items investigated were higher for arterial blood than for portal vein blood except that of TG in IRD. Blood plasma concentration of glucose at arterial and portal vein was significantly higher for THD than for IRD. Sletmoen et al. (2000) has suggested that plasma glucose concentration was increased by degraded intake protein based

Table 3.2 Blood plasma concentration of glucose, NEFA and TG at arterial and portal vein in growing wether lambs fed forage based diets

Items	Diet		SEM	*P*-value
	THD	IRD		
Glucose concentration (mg/dL)				
Arterial	80.2	73.9	1.05	0.01
Portal	79.1	72.6	1.05	0.01
NEFA concentration (μEq/L)				
Arterial	258.0	252.3	12.66	0.70
Portal	139.4	184.8	19.38	0.08
TG concentration (mg/dL)				
Arterial	17.2	17.5	4.34	0.99
Portal	16.9	18.7	5.34	0.24

The results are mean value of concentration of diurnal blood plasma.

Means within a row with different superscripts differ ($P \leq 0.05$).

supplements. However, other studies have shown no significant effect of protein supplementation on blood glucose in ruminants (Krysl et al. 1987; Cheema et al. 1991b). In the current study, the slightly difference in N intake between the diets (appeared in Table 3.5) did not affect blood plasma concentration of glucose, and agreed with the results of Krysl et al. (1987) and Cheema et al. (1991b). The plasma concentrations of NEFA of arterial blood did not differ significantly between the diets, although which of portal venous blood was tended to be higher for IRD than for THD (P = 0.08). Higher levels of NEFA at lower intake levels have been also reported in sheep by Hatfield et al. (1999) and indicate body fat mobilization to compensate for low energy intake. In current study, concentrations of low NEFA in blood plasma were decreased by the sufficient energy balance diets designed to satisfy metabolizable energy requirements for 100 g daily gain. Blood plasma concentrations of TG at arterial and portal vein did not differ significantly between the diets.

3.4.2 Plasma flows and nutrients supply to body tissues

The EBW, estimated portal plasma flow rate, absorption rate constant of energy yielding nutrients from the GIT and available nutrients circulations are shown in Table 3.3. The EBW and portal plasma flow rate (L/min) did not differ between the diets. The k_a (fraction/h) variable of glucose and NEFA were not different significantly between the diets, and the k_a for TG was significantly lower for THD than for IRD (P = 0.01). Additionally, available amount of glucose and NEFA were higher for THD than for IRD without showing significant differences between the diets. The available amount of TG was significantly higher for IRD than for THD (P= 0.05). The difference of basal forages might have resulted the changes in nutrients

Table 3.3 The EBW, portal plasma flow, absorption rate constant of nutrients and available nutrient circulation in growing wether lambs fed forage based diets

Items	Diet		SEM	*P*-value
	THD	IRD		
EBW (kg) ‡	19.9	20.5	0.90	0.65
Portal plasma flow (L/min)	1.2	1.3	0.08	0.39
k_a (/h) §				
Glucose	0.04	0.02	0.007	0.15
NEFA	0.003	0.002	0.001	0.37
TG	0.002	0.004	0.000	0.01
Available amount (g/d) ¶				
Glucose	1181.6	846.4	183.69	0.14
NEFA	32.5	22.1	8.93	0.31
TG	11.5	25.4	7.07	0.05

‡ Calculated as LBW × 0.75 (Kim et al. 2014)

§k_a, rate constant of nutrients absorption at the small intestine (fraction/h)

¶ Available amount was calculated as portal plasma flow (L/d) × concentration of blood plasma (g/L) × k_a × 24

absorption rate constant at GIT and portal plasma flow rate.

3.4.3 The proration of nutrients in the body

The daily amount of bioavailable nutrients supply to muscle tissue and adipose tissue in growing wethers were listed in Table 3.4. Bio-available glucose and NEFA supplied to both of tissues were numerically higher for THD than for IRD, however they did not reach the significant difference between the diets. In addition, the amount of TG showed no significant differences between the diets. As shown in our results, supplement of bio-available nutrients in forage diets showed no significantly different in both of muscle and adipose tissue of early stage of growing wether lambs. However, little is known that the roles of bio-available nutrients in the concentration of blood plasma were investigated in the *in vivo* model because of its very complex process. It was noteworthy that the estimated amount of glucose, NEFA and TG to muscle tissue and adipose tissue differed in numerically between THD and IRD, however, the tendencies toward statistical significance were not detected for all items.

3.4.4 Effect of forage based diets feeding on N balance

The N balances in growing wethers fed with the two forage based diets are shown in Table 3.5. The N intake was slightly greater for THD than that for IRD without showing significant difference. Fecal N excretion tended to be higher for IRD than for THD. Hence, the N digestibility was calculated to be higher for THD than for IRD, and accounted for 71.4 and 50.5%, respectively. Urinary N excretion

Table 3.4 The bio-available nutrients flow in muscle tissue and adipose tissue

Items	Diet		SEM	*P*-value
	THD	IRD		
Glucose				
Muscle tissue (g/d)‡	303.5	217.3	49.29	0.16
Adipose tissue (g/d)‡	258.0	185.2	36.72	0.12
NEFA				
Muscle tissue (g/d)	8.3	5.6	2.23	0.29
Adipose tissue (g/d)	7.1	5.0	2.11	0.37
TG				
Muscle tissue (g/d)	3.0	8.1	1.72	0.30
Adipose tissue (g/d)	2.5	7.1	1.79	0.31

‡Muscle tissue, $\log_{10}$Protein mass = –0.6451 + 0.8955 × $\log_{10}$EBW; Adipose tissue, $\log_{10}$fat mass = –1.918 + 1.821 × $\log_{10}$EBW (ARC, 1980)

was numerically greater for THD than for IRD. The N retention (g/d) and its ratio to N intake were calculated to be numerically higher for THD than IRD (P = 0.22, 0.19). However, there were no significant differences between the diets when expressed as proportion to N absorbed (P = 0.97). In the present study, N intake, urinary N, N digestibility and N retention (% of N intake) all were increased in response to dietary CP concentration (Table 3.1). Swanson et al. (2000) and Cheema et al. (1991) reported that CP levels increased N intake, urinary N, N absorption and N retention, and Caton et al. (1988) showed increased N intake, urinary N and N retention in response to CP supplementation to diet. Other studied showed that the N retention increased with an increase in apparent digestible N intake in sheep (Sarraseca et al. 1998; Lobley et al. 2000; Marini et al. 2004; Kamalzadeh and Shabani 2007). In addition, the ratio of N retention to apparent digestible N intake increased with an increase in apparent digestible N intake in 40–50 kg Suffolk wether sheep (Sarraseca et al. 1998). Taking into consideration of the results on N retention, it was suggested that the lack of prominent difference of energy yielding substances (glucose, NEFA and TG) supply to muscle tissue might have partly explained the comparable N assimilation (% of absorbed N) response of early growing stage of wether lambs observed in this study (Table 3.4, 3.5). In the current study, amount of bio-available glucose, NEFA and TG supply to both muscle tissue and adipose tissue were estimated by using one-compartmental pharmaco kinetics model and equations to predict mass of muscle or adipose tissue of growing sheep, and these values exhibited no significant differences between the diets. Our results showed that bio-available glucose supplement to muscle tissue was estimated to be numerically higher for THD than for IRD

Table 3.5 Effect of forage based diets on N balance in wether lambs

Items	Diet		SEM	*P*-value
	THD	IRD		
The N intake (g/d)	14.2	13.5	0.92	0.51
Fecal N excretion (g/d)	4.0	6.7	1.01	0.06
Urinary N excretion (g/d)	5.3	3.6	0.91	0.15
N retention (g/d)	4.9	3.2	1.18	0.22
(% of N intake)	34.1	23.5	6.63	0.19
(% of N absorbed)	47.4	47.1	7.71	0.97

(P = 0.16) and which might to be thought to associate with the relatively greater N retention (g/d or % of N intake) for THD as compared to IRD.

The results obtained from the *in vivo* study described in this Chapter suggested that the observed lacking significant dietary difference in the amount of energy yielding substances which supplied to the muscle tissues might have reflected the identical rate of N retention (% of absorbed N from GIT) between the diets.

CHAPTER FOUR

Effects of Forage Based Diet on Adipogenesis of Ovine Adipocytes

4.1 Summary

The objective of this study was to conduct an adipogenic evaluation of the effect of difference in forage sources and feeding levels during adipocyte differentiation of ruminant animal in *in vitro* assessment. Six wether sheep were divided into two dietary group of THD and IRD (n=3, each). Sheep serum samples collected on the last day of each dietary treatment adaptation period were added to an adipogenic induction medium for differentiation of preadipocytes which derived from subcutaneous adipose tissue of sheep used for this study. Three experimental treatments, fed HR, MR, and LR were designed and fed to sheep in a one-way layout design in which a 6-day period of feeding was made for each treatment. The cytoplasmic lipid accumulations in the THD serum-treated preadipocytes were significantly higher than those of the IRD serum-treated preadipocytes on day 12 of cell culture. The mRNA expression of C/EBPα, C/EBPβ, C/EBPδ, aP2 and SCD were regulated by each serum treatment. These results suggest that serum treatment culture method is able to elucidate the effects of circulation materials in the blood on adipocyte differentiation. Also, the results of this study show that different forage source of diets and forage to concentrate ratio diets can regulate adipogenic genes during adipocyte differentiation *via* ruminant blood.

4.2 Introduction

In the long term period of *in vivo* studies described in Chapter 2 and Chapter 3, both of nutritive value and feeding value of forage based diets (THD and IRD) were evaluated concomitantly with carcass characteristics evaluation for meat production performance. By the animal experiments aforementioned, the evidence of relationship between amount of energy yielding nutrients supply to both of muscle tissue and adipose tissue and N assimilation rate was shown to some extent be allowed. However, the *in vivo* feeding trials is time consuming and laborious measure, additionally it is costly to carry out. Although the *in vivo* feeding trial should be needed to show the actual feeding value to the animal, alternative method being relatively simple and rapidly is required to clarify the feeding value or novel functional property of tested diet as *in situ* nylon bag technique, *in vitro* digestion test or *in vitro* gas production test are now becoming as routine work for preceding *in vivo* feeding evaluation of diets (Blümmel and Ørskov 1993).

Soliman et al. (2007) and Dervishi et al. (2011) reported the method of forage evaluation using adipocyte, and they investigated the expression of some adipogenic, lipogenic genes and transcription factor using mRNA extracted from adipose tissue of slaughtered sheep fed with different types of forage diet for long term period. In the previous research (Arasu et al. 2014; Ilavenil et al. 2014), different concentration of forage extracts were treat on 3T3-L1 mouse preadipocytes to investigate expression of viability, lipid accumulation, lipolysis, cell cycle progression, and mRNA synthesis in the cells. However, it seemed not to be sufficient to detect a biological response for nutrient substance synthesis and degradation which being occurring in *in vivo*. The

differentiation of adipocytes is induced by the expression of the adipogenic transcription factor C/EBP family and PPARγ in rodents and ruminants (Christy et al. 1989; Tontonoz et al. 1994; Rosen and Spiegelman 2001; Roh et al. 2006). In addition, SCD and aP2 are involved in the synthesis of fatty acid in adipocytes (Ntambi et al. 1988; Amri et al. 1991; Taniguchi et al. 2003; Pannier et al. 2010). It is reported that expressions of adipogenic genes are controlled by various factors such as fatty acid, glucose, TG and insulin (Carlson et al. 1993; Schoonjans et al. 1996; Kim et al. 1998; Bajaj et al. 2007). It was also reported that the nutrient and hormone compositions of blood were affected by differences in forage source and the forage to concentrate ratio in some *in vivo* experiments of targeted ruminants (Bassett et al. 1971; Evans et al. 1975; Wang et al. 2003). In addition, some adipokines such as adiponectin, leptin and chemerin, released by adipocytes play a role in the control of adipocyte differentiation via various metabolic pathways. (Suzuki et al. 2012a, b). These results suggest that the absorbed ingredients from the digestive system of ruminants were able to regulate nutritional and physiological factors, which are involved, either directly or indirectly, in the differentiation of adipocytes.

Blood metabolite concentrations represent an integrated index of the adequacy of nutrient supply in relation to nutrient utilization that is independent of physiological state and give an immediate indication of nutritional status at that point in time (Cronjé and Pambu 1996). However, the relationship between variations of nutrients derived by serum and differentiation status of adipocyte is thought to be needed. Therefore, in this study, a new method for *in vitro* feed evaluation instead of *in vivo* study was

aimed. The blood samples were collected from sheep fed with forage based diets differing forage to concentrate ratio, and blood serum were supplemented to ovine adipocyte in *in vitro* to observe adipocyte differentiation.

4.3 Materials and Methods

Animal experiment described in this Chapter was conducted at Shimane University from January 2014 to February 2014. Use and animal care procedures was approved by Animal Care Committee of Shimane University.

4.3.1 Animals and diet

Seven Suffolk wether sheep were allocated to two dietary treatments at the age of 12 months. The six wethers were also kept individually in metabolism crates throughout the experimental period each diet group, and fed THD and IRD in one-way layout design each over a 6-d period. The complete diets of each feeding group were made up of varying concentrations of forage as follows: 80:20 (w:w) (high forage; HR), 60:40 (w:w) (medium forage; MR), and 40:60 (w:w) (low forage; LR). Intake compositions of each complete diet were shown in Table 4.1. The diets were designed to satisfy ME requirements for 100 g DG with a provision of 5% safety margin based on the estimation equations by the AFRC (1993). The animals were fed two equal sized meals at 09.00 and 21.00 hours throughout the experiment. Animals had free access to water and a mineral block during the experimental period. A single sheep was used for preadipocyte isolation.

Table 4.1 Chemical composition intake of experimental diets in whether sheep (g /day)

Item	THD			IRD		
	HR	MR	LR	HR	MR	LR
OM	999.8±38.8	865.5±24.9	800.2±55.3	845.5±77.5	745.9±59.9	680.1±83.6
EE	30.4±1.1	29.9±0.6	30.4±2.0	22.0±2.1	23.4±2.1	25.0±3.0
CP	118.5±4.3	126.3±1.8	135.6±8.9	84.4±8.5	99.4±9.4	113.4±13.7
NDF	617.7±24.8	464.0±18.9	372.8±27.0	535.0±47.0	405.1±28.9	308.4±38.6
ADL	379.1±15.3	272.7±12.2	208.2±15.4	300.0±81.9	238.0±15.7	193.6±27.0
NFC	240.9±8.9	252.3±3.8	268.0±17.7	240.0±23.0	243.6±21.3	251.1±30.5

4.3.2 Blood sampling and serum fractionation

Blood sampling was performed *via* jugular vein catheter on day 6 of each dietary feeding period. Jugular vein catheters were fitted to each sheep 3 h before blood sampling. Approximately 5–6 mL of blood was collected every 30 min for 24 h from the start of the morning feeding. The jugular vein catheters were filled with heparinized saline (200 IU/mL) before and after each blood sampling. After blood collection, the blood samples were incubated at room temperature for 1 h and centrifuged at 1700 × g for 30 min to separate the serum in the blood samples. Each serum sample, collected by blood sampling over 24 h, was mixed with same amount. The mixed serum samples were divided by forage types and feeding levels. Each group of the mixed serum samples was sterilized by filtration through a Millipore filter and stored at –30°C until cell culture.

4.3.3 Preparation of sheep preadipocytes and cell culture

The stromal vascular cells, including preadipocytes, were collected from the subcutaneous white adipose tissues of a wether sheep. The collected tissue was immersed in Dulbecco's modified Eagle's medium (DMEM: Wako Pure Chem., Osaka, Japan) containing 10% fetal bovine serum (FBS) and carefully separated from the other connective tissues. The adipose tissue was minced and digested with DMEM medium, which included 0.5 mg/mL of Type I collagenase, for 70 min at 37°C. The digested tissue was filtered through a stainless steel mesh to separate the cell suspension from the undigested tissue fragments. The filtrate was centrifuged at 800 × g for 2 min at room temperature. The discarded supernatant pellet was resuspended in DMEM medium supplemented with a 10% FBS and 1% antibiotic mixture. The resuspended cells were seeded on 100-mm diameter dishes containing a cell culture

medium (DMEM including 10% FBS and 1% antibiotic mixture) at a final density of approximately 2.0×10^5 cells per dish. The cells were trypsinized with a trypsin solution once every 5 day thereafter. Preadipocytes were cloned using a limiting dilution on the second day of trypsinization. Each cloned cell was proliferated on 24-well plates and transferred to six-well plates after 8 days (some of the proliferated cells were placed in a cell stock solution and maintained at −80°C). After the cells reached confluence, the medium was replaced with a differentiation medium, including DMEM supplemented with the 20% calf serum, 1% antibiotic mixture, 50 ng/mL insulin, 0.25 μmol/L dexamethazone, and 5 mmol/L acetic acid. After 12 days, one clone from each well was selected based on the amount of lipid accumulation, which was assessed via oil red O staining. The selected preadipocytes were taken out of the frozen stock and seeded on 100-mm diameter dishes. This stage was defined as the first passage of a cloned preadipocyte. At the fourth passaging, the cloned preadipocytes were seeded on six-well plates that included a cell culture medium at a final density of approximately 1.5×10^5 cells per dish. After the cells reached confluence, the medium was replaced with a differentiation medium (DMEM supplemented with the 20% sheep serum (obtained from the sheep fed the different diets), 1% antibiotic mixture, 50 ng/mL of insulin, 0.25 μmol/L dexamethazone and 5 mmol/L acetic acid). The differentiation medium was replaced with fresh differentiation medium once every 2 days. Total RNA extraction and oil red O staining were performed after 12 days of differentiation induction.

4.3.4 Total RNA extraction and real-time PCR

Total RNA was extracted from culture cells using RNAiso Plus (Takara Bio, Inc., Otsu, Japan). The concentration of total RNA was determined with a NanoDrop. For

cDNA preparation, total RNA was reverse-transcribed using Prime Script RT reagent (Takara Bio, Inc., Otsu, Japan). Real-time polymerase chain reaction (PCR) was performed with the Thermal Cycler Dice Real Time System (Takara-Bio, Shiga, Japan) using SYBR Premix Ex Taq 2 (Takara-Bio, Shiga, Japan) according to the manufacturer's instructions. The mRNA expression levels of the genes of interest were normalized with the beta-actin. Primers specific for sheep adipogenic factor genes and beta-actin are shown in Table 4.2.

4.3.5 Oil red O staining

Lipid droplets in differentiated adipocytes were stained with oil red O. The cells were washed with PBS (Phosphate-buffered saline) and then fixed in 10% formaldehyde for 1 h. After two washings with PBS, cells were stained with an oil red O solution (six parts oil red O stock and four parts H_2O; oil red O stock solution was 0.5% oil red O in isopropanol), followed by further washing with PBS. Cell images were taken using an EVOS® Cell Imaging Station (Life Technologies, Grand Island, NY, USA). Oil red O was eluted by 60% isopropanol for 5 min, and optical density (OD) was measured at 520 nm.

4.3.6 Statistical analysis

All data in each figure are presented as the means ± SEM. The differences among each of the different serum sample treatments for sheep preadipocyte differentiation were statistically analyzed using one-way ANOVA, followed by Duncan's multiple range test ($P < 0.05$). All statistical procedures were performed using SPSS 14.0 (SPSS 2006).

Table 4.2 Primers used for real-time PCR

Gene name		Primer	Length (bp)
C/EBPα	forward	5’- cgacctgttccaacacagc -3’	85
	reverse	5’- cgggtagtcaaagtcgttgc -3’	
C/EBPβ	forward	5’- gacaagcacagcgacgagta -3’	158
	reverse	5’- agctgctccaccttcttctg -3’	
C/EBPδ	forward	5’- aatgtgacggcagcttctct-3’	280
	reverse	5’- ggcctgtttgagaagcaaag-3’	
PPARγ2	forward	5’- gataaagcgtcagggttcca -3’	54
	reverse	5’- acccttgcatccttcacaag -3’	
aP2	forward	5’- gccaggaatttgatgaagtc -3’	190
	reverse	5’- ctctcgtaaactctggtagc -3’	
SCD	forward	5’- gctgtcagagaaaagggtgc -3’	193
	reverse	5’- cagcgtaacggagaaaggtg -3’	
β-actin	forward	5’- gcattcacgaaactaccttc -3’	169
	reverse	5’- atcttgatcttcatcgtgct -3’	

4.4 Results and Discussion

4.4.1 Nutrients intake

There were large variations of the nutrients intake between THD and IRD and between forage ratios within the diet (Table 4.1). Nutrients consumptions were greater for THD than for IRD. The consumption of OM, NDF and ADF increased with increasing ratio of forage to concentrate, conversely consumption of EE and CP decreased with increasing of forage ratio. All diets were designed to satisfy ME allowance for 100 g DG requirement.

4.4.2 Fat accumulation of cloned sheep preadipocytes during treatment with an FBS-free induction medium

In this study established cloned preadipocytes derived from the subcutaneous adipose tissue of wether sheep. To investigate the ability of the cells to accumulate fat in the absence of FBS, they were treated with an adipogenic induction medium containing 20% bovine calf serum (FBS-free induction medium) for 12 days. According to morphological observations via oil red O staining, the cells were fibroblast-like, and lipid droplets were not observed in confluent cells. However, the first lipid droplets were observed on day 3, and the amount of the lipid droplets increased on days 6, 9 and 12 during the FBS-free induction medium treatment (Figure 4.1A). In addition, the oil red O elution analysis showed that the amount of cytoplasmic fat accumulation significantly increased during treatment with the FBS-free induction medium (Figure 4.1B). The FBS is widely used for *in vitro* culture studies of eukaryotic cells as a supplement because FBS facilitates cell growth and survival. However, the objective of this experiment was to evaluate the effects of different dietary conditions on adipocyte differentiation using sheep serum as absorbed ingredients from the

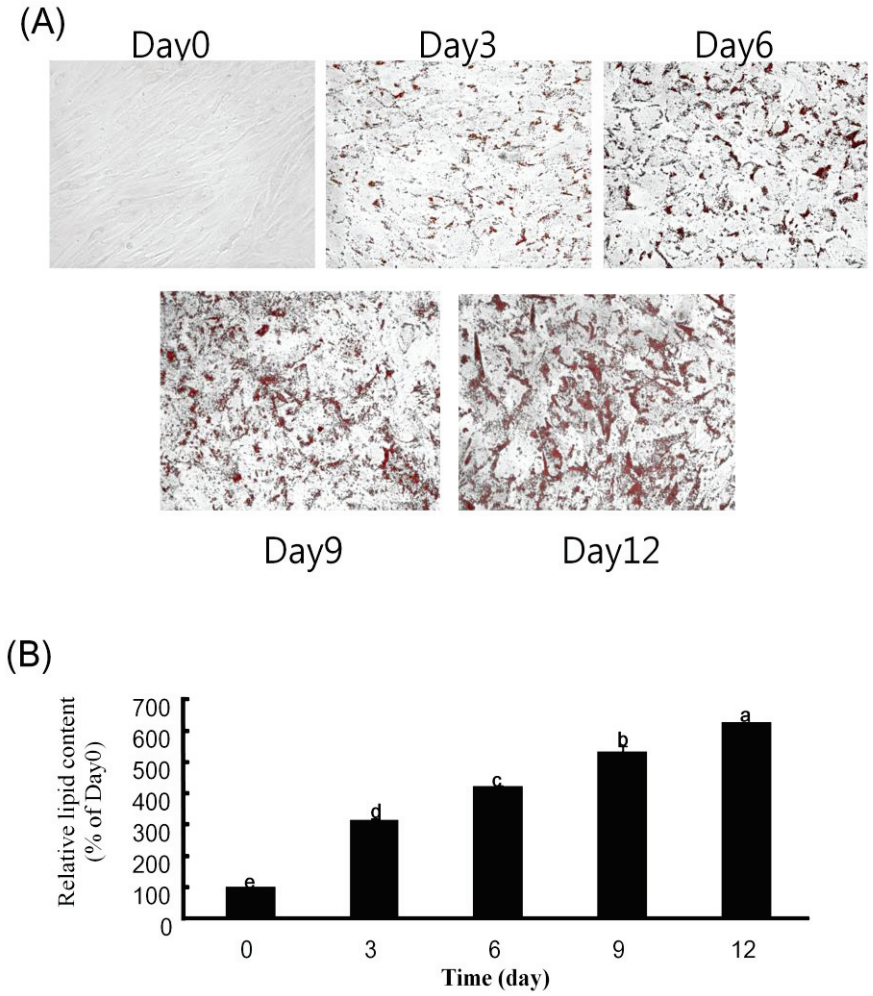

Figure 4.1 Fat accumulation during adipocyte differentiation of preadipocytes prepared from subcutaneous adipose tissues of sheep. (A) Cell images of oil red O staining during adipocyte differentiation in sheep preadipocytes. Each image was taken using an EVOS® Cell Imaging Station (200-fold). (B) Oil red O eluting analysis was performed on confluent preadipocytes (day 0) and adipocytes that had differentiated for 12 days.

The data represent the means ± SEM of the three experiments.

[a, b, c, d, e] Values marked with different letters within each day differ significantly ($P < 0.05$).

digestive system of ruminants. Therefore, the result of this study decided to establish the FBS-independent culture method. In the present study was established cloned preadipocytes derived from the subcutaneous adipose tissue of wether sheep, and cloned preadipocytes could differentiate and accumulate fat when treated with an induction medium containing insulin, acetic acid, dexamethazone and calf serum.

4.4.3 The relationship between adipogenic gene mRNA expression levels and fat accumulation

In order to identify the relationship between the adipogenic gene mRNA expression patterns and fat accumulation during adipogenic differentiation, real-time PCR analysis was performed using the cell culture samples during treatment with the FBS-free adipogenic induction medium on days 0, 3, 6, 9 and 12. Figure 4.2 shows the relationship between the mRNA expression patterns of six adipogenic genes and fat accumulation during adipogenic differentiation of cloned sheep preadipocytes. The expression of C/EBPα mRNA was rapidly up-regulated until day 6, when it was maximally expressed, but decreased thereafter (Figure 4.2A). The expression of C/EBPβ was down-regulated until day 3, however, it was smoothly up-regulated from days 4 to 9 when it was maximally expressed, but rapidly decreased thereafter (Figure 4.2B). In addition, the expression of C/EBP δ was down regulated until day 3, then rapidly up-regulated until day 6, decreased until day 9, and maximally up-regulated on day 12 (Figure 4.2C). The expression pattern of PPARγ2 mRNA was similar to that of C/EBPα mRNA (Figure 4.2D). The expression of aP2 mRNA was rapidly up-regulated until day 6, when it was maximally expressed, but rapidly decreased thereafter (Figure 4.2E). The expression of SCD mRNA was down-

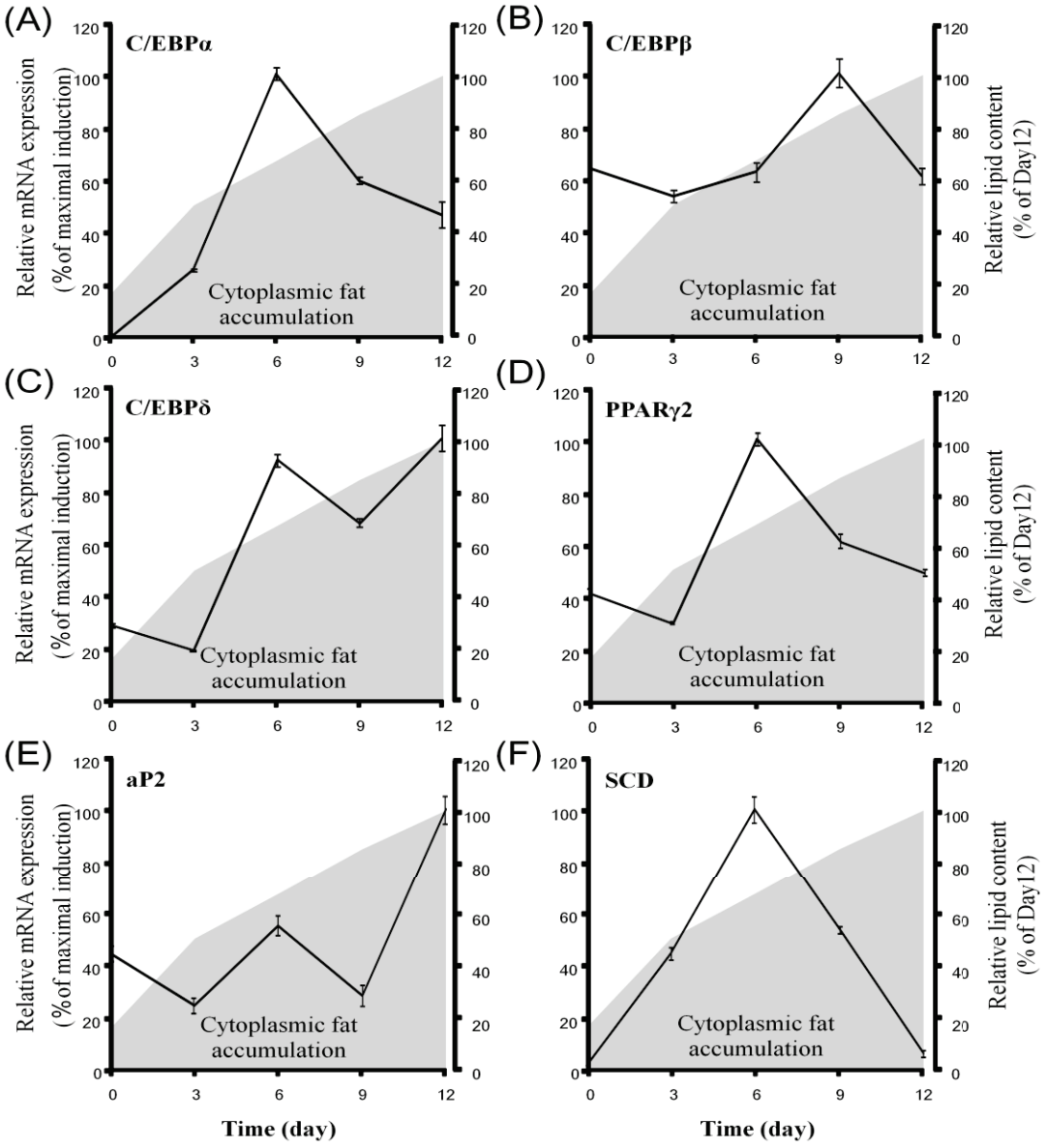

Figure 4.2 The relationship between the six adipogenic genes and fat accumulation amount. C/EBPα (A), C/EBPβ (B), C/EBPδ (C), PPARγ2 (D), aP2 (E) and SCD (E) mRNA expression during adipocyte differentiation of preadipocytes prepared from subcutaneous adipose tissues of sheep. Real-time PCR analysis was performed on confluent preadipocytes (day 0) and adipocytes that had differentiated for 12 days. The data represent the means ± SEM of the three experiments. Gray color means oil red O eluting analysis resulting from Figure 4.1B.

regulated until day 3, then up-regulated until day 6, down-regulated until day 9, and maximally up-regulated on day 12 (Figure 4.2F). Meanwhile, fat accumulation increased steadily until day 12 and was maximally accumulated on day 12 (Figure 4.1 A and B). In this study was investigated the relationship between the mRNA expression patterns of six adipogenic genes and the amount of cytoplasmic fat accumulation during cell differentiation. The C/EBP family, PPARγ2, aP2 and SCD mRNA expression were typically up-regulated or down regulated during cell differentiation. These results were consistent with those of a previous study by Yamamoto et al. (2010). This suggests that adipocytes are able to obtain the ability to differentiate in the absence of FBS. However, these gene expression levels did not vary in direct proportion to the amount of fat accumulation.

4.4.4 Treatment of serum samples collected from sheep fed different diets and proportion of forage and concentrate on adipocyte differentiation

To examine the effects of the absorbed ingredients from the digestive system of ruminants fed different diets on adipogenesis, serum samples were collected from sheep fed different diet (THD and IRD) to concentrate ratios (HR, MR and LR). The serum samples were added to the adipogenic induction medium and then used to treat sheep preadipocytes. Figure 4.3 shows the results of the oil red O staining, indicating the different effects of the serum samples collected from sheep fed different diets on the fat accumulation of preadipocytes. The effect of THD serum samples on fat accumulation was stronger than that of the IRD serum samples across all dietary conditions (HR, MR and LR; Figure 4.3A). In addition, the oil red O elution analysis showed that the amount of cytoplasmic fat accumulation significantly increased with the THD serum-treated preadipocytes greater than that of the IRD serum-treated

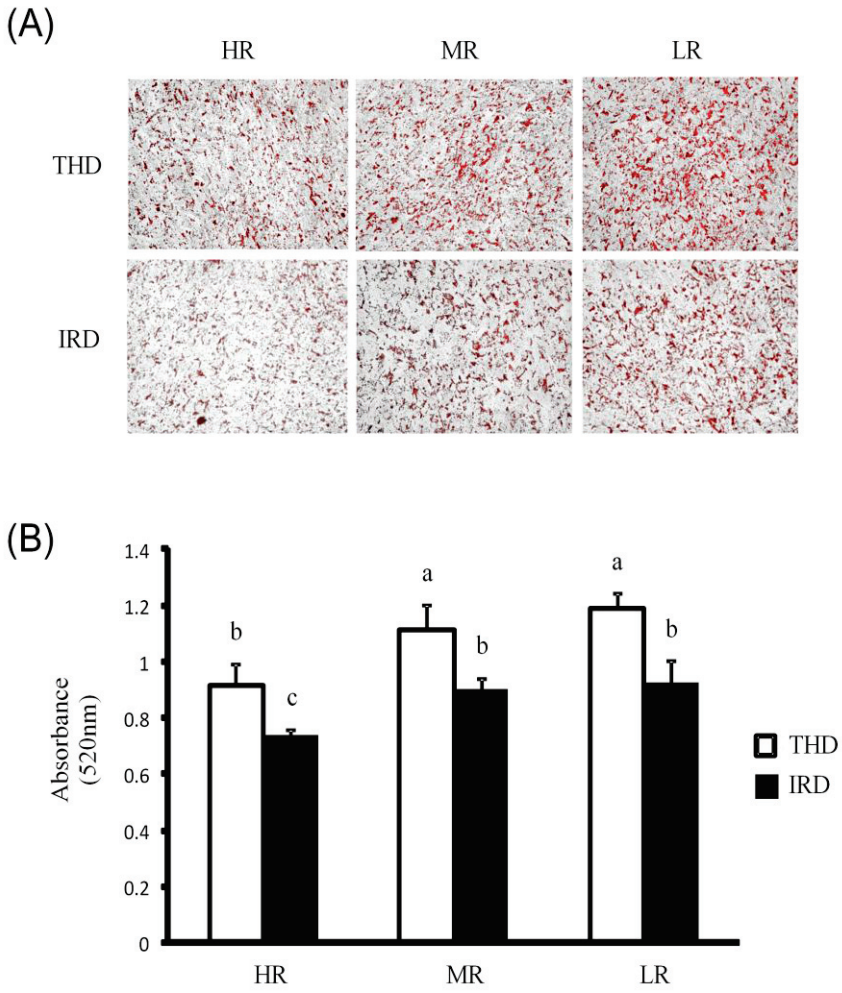

Figure 4.3 Fat accumulation of preadipocytes treated with serum collected from sheep fed different diets (A) Cell images of oil red O stained adipocyte treated with serum collected from sheep in each of the different diets. Each image was taken using an EVOS® Cell Imaging Station (100-fold) at day 12. (B) Oil red O eluting analysis was performed at day 12. The data represent the means ± SEM of the three experiments.

[a, b, c]Values marked with different letters within each day differ significantly ($P < 0.05$).

preadipocytes. There was no significant difference between MR and LR, but the amount of fat accumulation with MR and LR was significantly higher than that of HR treatment cells in each of the two groups (THD and IRD; Figure 4.3B). To investigate the effect of each serum (THD-HR, THD-MR, THD-LR, IRD-HR, IRD-MR and IRD-LR) on the mRNA expression levels of adipogenic genes during adipocyte differentiation, total RNA was extracted at 12 days after the adipogenic induction of each sheep serum. Figure 4.4A shows the C/EBPα mRNA expression level at day 12. C/EBPα mRNA expression of IRD-HR was significantly higher than that of THD-HR. Moreover, C/EBPα mRNA expressions of THD-HR and IRD-HR were significantly higher than those of MR and LR for each of the respective forage groups. Figure 4.4B shows the C/EBPβ mRNA expression level at day 12. There were no significant differences among forages or varying forage-to-concentrate ratios. Figure 4.4C shows the C/EBPδ mRNA expression level at day 12. The C/EBPδ mRNA expressions of all THD treatment cells were significantly higher than those of C/EBPδ mRNA expressions across all IRD treatment cells. In addition, there was no significant difference across all THD. However, in IRD, the C/EBPδ mRNA expression level of IRD-MR was higher than that of the C/EBPδ mRNA expression level of IRD-HR. Figure 4.4D shows the PPARγ2 mRNA expression level at day 12. The PPARγ2 mRNA expression level of THD-MR was lower than the PPARγ2 mRNA expression level of IRD-MR. Figure 4.4E shows the aP2 mRNA expression at day 12. The aP2 mRNA expression levels of all THD samples were significantly lower than the aP2 mRNA expression levels of all IRD samples. However, there were no significant differences among HR, MR and LR in each of the different forage feeding groups. Figure 4F shows the SCD mRNA expression level at day 12. The

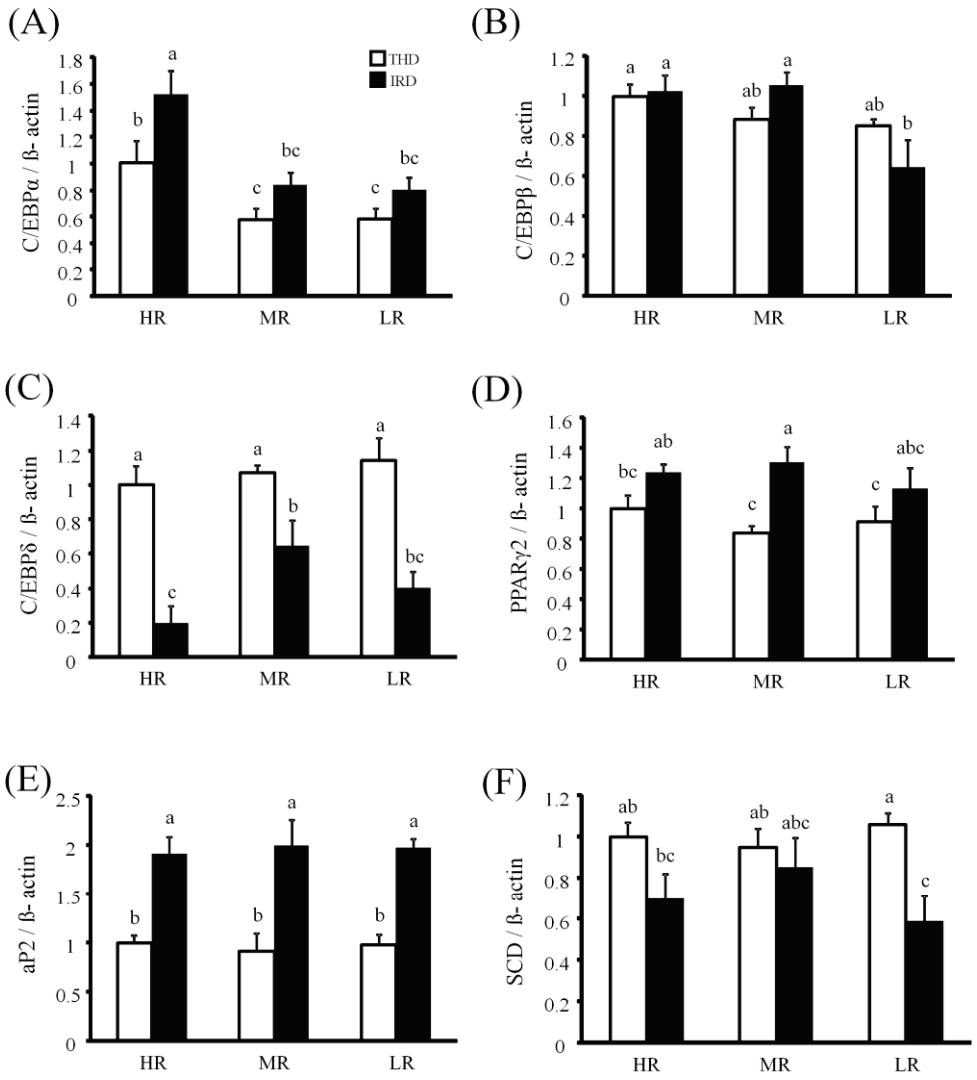

Figure 4.4 The expression of six adipogenic genes mRNA of differentiated adipocytes treated with serum collected from sheep in each of the different diets. C/EBPα (A), C/EBPβ (B), C/EBPδ (C), PPARγ2 (D), aP2 (E) and SCD (E) mRNA expression in preadipocytes treated with serum collected from sheep in each of the different diets. Real-time PCR analysis was performed at day 12. The data represent the means ± SEM of the three experiments.

[a, b, c]Values marked with different letters within mRNA expression levels of each serum-treated cells differ significantly ($P < 0.05$).

SCD mRNA expression levels of THD-HR and THD-LR were significantly higher no significant differences between the different forage-to-concentrate ratios. As a consequence of the induction of differentiation with a medium containing serum collected from sheep fed different diets, the THD serum treatment up-regulated fat accumulation to a greater extent than the IRD serum treatment. Our result shows that timothy hay contains higher concentrations of EE, CP, NDF and NFC compared to IR (Table 4.1). It is reported that high levels diet of fat, protein and carbohydrate up-regulate body fat deposition in goats, cattle and sheep (Ballard et al. 1972; Waghorn et al. 1987; Chilliard 1993; Atti et al. 2004). In addition, the LR and MR serum treatment up-regulated fat accumulation to a greater extent than the HR serum treatment. However, our results of the mRNA expression analysis show that gene levels did not vary in direct proportion to the amount of fat accumulation. Therefore, we considered about the relationship of fat accumulation amount and adipogenic genes expression patterns. Consequently, IRD and HR treated cell mRNA expression patterns were similar to expression levels of days 6–9 stage in Figure 4.2. These results also suggest that gene expression is not an absolute indicator that can be used to evaluate the development of adipose tissue *in vivo* because the adipose tissue includes adipocytes at various developmental stages. Furthermore, in adipose tissue, there are many other types of cells. Our studies to investigate the effect of serum collected from sheep fed different forage types and varying forage to concentrate ratios show that the TH and a more concentrated diet increased the development of adipocytes in comparison to the IR and high forage diets.

It suggests that the *in vitro* culture forage evaluation method has high reproducibility. Yamada et al. (2009) and Yamada and Nakanishi (2012) reported that different forage feeding levels and different forage-to-concentrate ratio diets were

regulate adipogenic genes and growth factors in adipose tissue of Wagyu steers. Adipocytes and fat in ruminants play an important role as a reserve of energy and the regulation of fattening cycles. The changes in adipose tissue metabolism and its acute regulatory mechanisms are regulated by growth hormone and steroid hormones (Bauman and Currie 1980). These results suggested that serum treatment culture method is able to realize the effects of circulation materials in the blood on adipocyte differentiation. Also, this study shows that different forage source diets and forage-to-concentrate ratio diets can regulate adipogenic genes during adipocyte differentiation via ruminant blood.

CHAPTER FIVE

General Conclusions

The feeding values of diet allowed to ruminant animals were evaluated based on the concentration of nutrients and anti-nutritional components, digestibility of nutrients, feed intake and production performance when fed the tested feed to animals. However, physiological mechanisms by which rate and extent of digested and absorbed nutrients utilization for the body tissue assimilation are not well understood; hence establishment of more detailed approaches from the stand point of ruminant physiology and molecular biology are required. In this study, the conventional feeding trial (Chapter 2), quantitative evaluation of nutrients supply to adipose tissue and muscle tissue by physiological and pharmaco kinetics approaches (Chapter 3) and a novel feeding value evaluation by utilizing adipocytes culture system applied blood serum which obtained from animals being fed with test feeds as substrates of cell culture (Chapter 4) were carried out.

In the *in vivo* experiment describe in Chapter 2, feeding value of two growing and fattening purpose diets based on THD and IRD were evaluated by 12 months of long term feeding trial and carcass characteristics measurement. Six growing wether lambs were used for feeding trial and four animals of them were chosen to evaluate carcass characteristics at the conclusion of the feeding experiment. The basal forage of each diet was offered at 2% BW as DM basis, and concentrate was fed at 40% of forage intake as fed basis. Feed efficiency was significantly greater for THD than that for IRD, although it was not affected by feeding periods. Digestion coefficients of OM and fiber did not differ significantly between diets or feeding period, while CP digestibility was slightly higher for THD than for IRD. The digestible CP intake was estimated to be significantly greater for THD than for IRD. Although the N intake was

significantly greater for THD than for IRD, relatively greater ratio of retained N to absorbed N for IRD was prominent as compared to THD. The EBW and carcass weight at 12 months of the feeding period were greater for THD than IRD. The lean meat weight was numerically greater for THD as compared to IRD, and the weight of subcutaneous fat and abdominal fat, their weight ratio to EBW were also markedly greater for THD than those for IRD. The results of this experiment indicated that THD feeding had superior growth performance of growing lambs and digestible N supply as compared to IRD, but long term period of THD feeding tended to deposit adipose tissue at higher ratio to EBW as compared to IRD. It is also suggested that the relatively higher N assimilation ratio of wether lambs fed with IRD than that fed with THD might have associated with the observed comparable dressing ratio and lean meat ratio to EBW.

In the *in vivo* experiment describe in Chapter 3, quantitative estimation of energy yielding nutrients delivered to both of muscle tissue and adipose tissue *via* blood plasma flow, in relevant to N retention in wether lambs being early stage of growing period, were carried out for elucidating the reason for the observed animal response in the experiment described in Chapter 2, indicating lower DG for IRD group than for THD at early stage of growth period even though ME requirement for growth requirement was almost sufficed identically for both diets and the marked difference in ratio of adipose tissue to EBW of carcass between the diets. Three growing wethers formed carotid artery-skin loops and located indwelling catheters in the mesenteric vein and the hepatic portal vein were used. Wethers were offered THD and IRD as used in the experiment described in Chapter 2 for 11 days of period for

each diet. The daily amount of glucose supplied to both muscle tissue and adipose tissue were numerically higher for THD than those for IRD without showing significant difference. The N retention did not differ markedly between the diets, and which was thought to be associated with the observed lacking differences between the diets in the amount of daily energy yielding substances supplied to the muscle tissues. The results in this experiment suggested that the difference in amount of glucose delivered to muscle tissue might reflect N retention responses to the forage based diets in early stage growing lambs.

Comparison of the feeding value between THD and IRD were carried out as whole animal level or tissue level in Chapter 2 and Chapter 3, respectively. In the *in vitro* experiment describe in Chapter 4, the effect of forage species of basal diet and difference in forage to concentrate ratio on differentiation characteristics of adipocytes were investigated. Six wether were divided into THD (n = 3) and IRD (n = 3). The sheep were fed HR, MR and LR diets in a one-way layout design for 6-day of period. Sheep serum samples were collected on the last day of each dietary treatment, and were added to an adipogenic induction medium for differentiation of preadipocytes derived from sheep subcutaneous adipose tissue. The cytoplasmic lipid accumulations in the THD serum-treated preadipocytes were significantly higher than those of the IRD serum-treated preadipocytes on day 12. The mRNA expression of C/EBP-α, C/EBP-β, C/EBP-δ, aP2 and SCD were regulated by each serum treatment. This study shows that different forage source of basal diet and forage-to-concentrate ratio of diets can regulate adipocytes differentiation in relevant to the variation of blood components composition.

From the results obtained in this studies, the differences in characteristics of animal production responses when fed the tested diet to growing wethers in terms of growth performance, N balance and carcass component yield were shown clearly. Feed efficiency value of IR was lower as compared to TH; while lean meat production property of low quality IR appeared to be superior to that of TH when fed them for both growing and fattening stage of production period. The effect of ME intake on lean meat production was masked by some factors, although the effect of glucose supply to muscle tissue on N retention and lean meat proportion of carcass was suggested. Furthermore, it was also suggested that the novel feeding value evaluation method utilizing cell culture system can be used to elucidate the superiority of TH for adipocytes differentiation and fat accumulation in the adipocytes in terms of adipogenic gene expression.

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Summary (Japanese)

摘　要

反芻家畜に給与する飼料価値の評価は、飼料中の栄養素含量、栄養素消化率、飼料摂取量および飼料給与によって得られた生産成績を主体として行われてきた。しかし、消化・吸収された飼料由来の各種栄養素が体組織同化調節についての生理的な機能は解明されていないのが現状であり、栄養生理学、分子生物学的手法を用いた詳細な検討が必要とされている。本研究では、従来の手法による飼料価値の査定（試験 1）に加え、消化・吸収された飼料成分が反芻家畜の体組織蓄積に影響を及ぼす生理的機能の解明（試験 2）および反芻家畜生産に関連する因子を制御する方策を追究することを目的とし、供試飼料の給与後に採取した反芻家畜の血液を培地基質として利用した反芻家畜体組織由来細胞の *in vitro* 培養法を用い、反芻家畜の生産成績に関連する遺伝・生理的因子の寄与率の調査を実施し、新規の飼料価値査定法についての検討（試験 3）を実施した。

試験1において、*in vivo*試験によりチモシー乾草を主体とし配合飼料を添加した飼料（THD）およびイタリアンライグラスストローを主体とし配合飼料を添加した飼料（IRD）の飼料価値の査定を行った。6頭のサフォーク種去勢育成メンヨウを供試して3頭ずつTHD区およびIRD区に割り当て、12ヵ月の育成・肥育試験を実施すると共に、各区の供試動物のうち2頭を屠殺・解体して産肉成績を評価した。両飼料区とも日増体量を100 gに設定し、粗飼料給与量は体重の2%相当量（乾物ベース）を給与し、配合飼料は粗飼料摂取量の40%（原物ベース）を給与した。試験

期間において供試動物の体重は、THD区で25.3から55.3 kgへ、IRD区では28.5から48.7 kg へ増加した。飼料効率はTHD区がIRD区より高く、給与期間による効果はみられなかった。有機物、繊維成分および非構造性炭水化物の消化率は飼料間、給与期間で差はなかったが、粗タンパク質と粗脂肪消化率はTHD区がIRD区より高かった。代謝体重あたりの可消化粗タンパク質摂取量はTHD区がIRD区に比べて多かったが、非構造性炭水化物摂取量はIRD区がTHD区より多かった。窒素（N）摂取量はTHD区がIRD区より多かったが、吸収Nの蓄積割合はIRD区がTHD区より多い傾向を示したのが特徴的であった。反芻胃内微生物体N合成量と合成効率に、飼料間差と給与期間は影響を及ぼさなかった。空体重、枝肉重および赤肉重量はTHD区がIRD区より重かった。皮下脂肪および腹腔内脂肪重量およびそれらの空体重に対する割合はTHD区がIRD区に比べてはるかに高い値を示した。試験1より、去勢育成メンヨウの増体成績と可消化N供給率はTHD給与がIRD給与より良好な値を示したが、長期間にわたるTHD給与は過剰な脂肪組織の蓄積も促進することが示された。また、吸収N蓄積率がIRD区で高い傾向を示したことが、枝肉重に占める赤肉割合に飼料間差がみられなかったことの一因であることが示唆された。

試験2において、試験1で解明されなかった家畜の生産応答、すなわち代謝エネルギー摂取量が同一であったが過剰な体脂肪蓄積がTHD区で明瞭であったこと、肥育初期（試験1での給与試験の1－3ヵ月時に相当）の設定日増体量(100 g)に要する代謝エネルギー要求量は概ね充足していたが、両区とも設定値より低く、IRD区がTHD区より低かったことの原因について、栄養生理面から追究した。右体側に頸動脈ループを形成し、空腸部位腸間膜静脈および肝門脈にカテーテルを挿入した6－7ヵ月齢のサフォーク種去勢育成メンヨウを3頭用いて、基礎飼料となる粗飼料の

違いが血中栄養素濃度、筋および脂肪組織へのエネルギー生産物質の供給量に及ぼす影響について、窒素出納成績とあわせて検討した。試験1と同様に、日増体量を100 g に設定し、THDおよびIRDを一元配置法によって供試動物に11日間給与した。7日間の予備期の後、3日の窒素出納試験を実施し、試験最終日では腸間膜静脈へのパラアミノ馬尿酸マーカーの連続・定速注入および頸動脈血と肝門脈血サンプルの経時的採取を行った。血液サンプルのグルコース、遊離脂肪酸およびトリグリセライド濃度を測定し、薬理動態モデルおよび筋組織・脂肪組織重量の推定式を用いて、各組織へのエネルギー生産物質の供給量を推定した。動脈血および肝門脈血中のグルコース濃度はTHD区がIRD区より高かった。体組織が利用可能なトリグリセライド供給量はIRD区がTHD区より多いと推定された。脂肪組織および筋組織へのグルコース供給量はいずれもTHD区がIRD区より多いと推定されたが、推定値に有意差はみられなかった。試験2では両試験区のN蓄積率に明瞭な差はなく、このことは、筋組織へのエネルギー生産物質の供給量に有意差がなかったことを反映したものと解された。試験2の*in vivo*実験結果より、早期育成期のメンヨウにおいて、エネルギー供給物質のうちでグルコースの筋組織供給量の多寡が吸収窒素の体蓄積率に影響を及ぼすことが示唆された。

試験1では個体レベル、試験2では組織レベルで2種類の粗飼料主体飼料の飼料価値について*in vivo*法による査定を行った。試験3では、*in vitro*細胞培養の手法を用い、細胞レベルで供試粗飼料の種類および粗飼料給与水準（粗濃比）の違いが反芻家畜の脂肪細胞分化に及ぼす影響について検討した。6頭のメンヨウをTHD区およびIRD区に3頭ずつ振り分け、さらに給与飼料中の粗飼料割合を高水準区（HR）、中程度区（MR）および低水準区（LR）の3区を設定し、各設計飼料を給与した。給与開

始6日目で採取した血清を脂肪細胞分化誘導培地と混合し、12日間メンヨウ脂肪組織を由来とする培養前駆脂肪細胞に処理した結果、細胞内の脂質蓄積量はTHD血清処理区がIRD血清処理区に比べ有意に高かった。さらに、異なる飼料給与条件下において供試動物から採取した血清の添加処理は脂肪細胞の分化関連遺伝子である C/EBPα, C/EBPβ, C/EBPδ, aP2 および SCDのmRNAの発現を特異的に制御した。これらの結果から、血清を用いた培養手法は、反芻動物の脂肪細胞における体内循環物質の分化および遺伝子調節作用の再現が可能な方法であり、脂質蓄積能に関する飼料評価に応用できることが示唆された。

試験 1、2 および 3 で得られた知見より、本供試飼料を肉用反芻家畜に給与した場合の増体成績、窒素蓄積成績、枝肉成績についての特徴的差異を明確にすることが可能であった。一般的に チモシー乾草と比較して低品質とされるイタリアンライグラスストローの飼料効率はチモシー乾草に比べて劣るが、その一方で、赤肉生産成績については長期間のチモシー乾草を給与した場合に比べて優位であることが示された。赤肉生産量に及ぼす代謝エネルギー摂取量の影響は明確ではなかったが、筋肉組織へのグルコース供給量が関与することが薬理動態モデルを用いた解析によって示唆された。また、本研究で主課題とした細胞培養法による飼料価値の検討により、反芻家畜の肉質評価で重要とされる脂肪交雑値、すなわち脂肪細胞の分化と脂肪細胞内の脂質蓄積に対するチモシー乾草の優位性を脂肪細胞の分化関連遺伝子発現の面から明らかにすることが可能であり、肉用反芻家畜へ給与する飼料価値の新たな査定法として有用であることが示された。

Summary

In this study, the author evaluated feeding values of two types of growing and fattening sheep diets by both of *in vivo* and *in vitro* methods in three experiments.

In the first experiment, feeding value of two growing and fattening purpose diets based on timothy hay (TH) supplemented with commercial concentrate (THD) and Italian ryegrass straw (IR) supplemented with commercial concentrate (IRD) were evaluated conventionally by long term period of feeding trial in addition with carcass characteristics measurement. Six growing wether lambs were used for feeding trial and four animals within them were chosen to evaluate carcass characteristics. The results of the first experiment indicated that THD feeding had superior growth performance of growing lambs and digestible nitrogen (N) supply as compared to IRD, but long term period of THD feeding tended to lead greater adipose tissue deposition compared to IRD. It is also suggested that the relatively higher N assimilation ratio of wether lambs fed with IRD than that fed with THD might have associated with the observed comparable lean meat ratio to empty bodyweight.

In the second experiment, quantitative estimation of energy yielding nutrients supply to muscle tissue and adipose tissue and N retention were studied using wether lambs being early stage of growth period. Three growing wethers formed carotid artery-skin loops and located indwelling catheters in the mesenteric vein and the hepatic portal vein were used. Wethers were

offered THD and IRD as used in the first experiment. The results in the second experiment suggested that the difference in the amount of glucose supplied to muscle tissue might reflect N retention responses to the forage based diets in early stage of growing wether lambs.

In the third experiment, the effect of forage species and forage to concentrate ratio on differentiation characteristics of adipocytes were studied by means of *in vitro* cell culture system. Six wether sheep were divided into two groups and fed THD or IRD. Within the two dietary treatments, high, medium and low forage treatments were introduced. Each diet was offered to the animals for 6-day of period. Sheep serum samples were collected on the last day of each diet feeding period, and were added to an adipogenic induction medium for differentiation of preadipocytes derived from sheep subcutaneous adipose tissue. The cytoplasmic lipid accumulations in the THD serum-treated preadipocytes were significantly higher than those of the IRD serum-treated preadipocytes on day 12. The mRNA expression of CCAAT/enhancer-binding protein (C/EBP)α, C/EBPβ, C/EBPδ, fatty-acid-binding protein and stearoyl-coenzyme A desaturase were regulated by each serum treatment.

The results of the three experiments cleared the differences in characteristics of animal production responses. Feed efficiency value of IR was lower as compared to TH, although lean meat production property of low quality IR was superior to TH feeding. The effect of glucose supply to muscle tissue on N retention was suggested in growing lambs. Furthermore, it was also suggested that the new *in vitro* method utilizing cell culture system can be used to evaluate feeding value of diet enhancing adipocytes deposition of meat producing ruminant.

List of Publications

Chapter – 2

Da-Hye KIM, Ki-Choon CHOI, Sang-Houn SONG and Toshiyoshi ICHINOHE.

Effects of grass forage species and long-term period of low quality forage diet feeding on growth performance, nutrient utilization and microbial nitrogen yield in growing wether lambs. Animal Science Journal 87(2): 202-208.

Da Hye KIM, Ki Choon CHOI, Toshiyoshi ICHINOHE and. Sang Houn SONG.

Hogget production of Suffolk fed with forage based diets. Japanese Journal of Sheep Science. 51: 9–14.

Chapter – 3

Da Hye Kim, Toshiyoshi Ichinohe, Ki Choon Choi, Shinichi Oda, Akihiko Hagino and Sang Houn Song

Relationship Between Nutrient Supply to Muscle and Adipose Tissues and Nitrogen Retention in Growing Wethers on Forage Based Diets Fed with Different Forage Sources. Journal of the Korean Society of Glassland and Forage Science 35 (3): 238–244.

Chapter – 4

Da-Hye KIM, Ki-Choon CHOI, Toshiyoshi ICHINOHE and Sang-Houn SONG

Effects of different roughage sources and feeding levels on adipogenesis of ovine adipocytes. Animal Science Journal. 86 (11): 943–951