Responses of Nutrients and Energy Metabolism to Chinese Herbal Medicine in Sheep

2014

The United Graduate School of Agricultural Sciences

Bioproduction Science

Animal Production

(Iwate University)

Xi Liang

Responses of Nutrients and Energy Metabolism to Chinese Herbal Medicine in Sheep

A Thesis

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

March, 2014

by

Xi Liang

Under the supervision of

Professor Dr. Hiroaki SANO

The United Graduate School of Agricultural Sciences

Bioproduction Science

Animal Nutrition and Physiology

Iwate University, Japan

Contents

Chapter-3

Chapter-4

Dedication

I dedicate this thesis to my parents, teachers and wife for their love, support and encouragement throughout my time in Japan.

Xi Liang

March, 2014

Acknowledgements

I am extremely grateful to my reverend supervisor, Professor Dr. Hiroaki Sano, Department of Animal Science, Faculty of Agriculture, Iwate University, Japan for his scholastic guidance, constructive advice, and responsible supervision on this research.

I am also grateful to my co-supervisors, Professor Dr. Kazumi Kita, Department of Animal Science, Faculty of Agriculture, Iwate University, and Professor Dr. Ken-ichi Horiguchi, Faculty of Agriculture, Yamagata University, Japan for their kind support during the research period and valuable comments on this thesis.

I would like to thank all the members of the laboratory of animal nutrition and physiology, Iwate University, for their kind help and effort on carrying out the experiments and performing the chemical analysis of samples.

I also would like to thank Jianjun Liu, Harbin University of Commerce, China for providing Chinese herbal medicine for the experiments.

Abstract

Background: The use of antibiotics in animal diets is facing negative feedback due to the hidden danger of drug residues to human health. Traditional Chinese herbal medicine has been used to replace antibiotics in the past two decades and played an increasingly important role in livestock production. The present study was carried out to assess the feeding effects of a traditional nourishing Chinese herbal medicine mixture (Astragalus root, Angelica root and Atractylodes rhizome) on kinetics of intermediate nutrients and energy metabolism in sheep.

Experiment 1: As a basic investigation, the first experiment was conducted to assess the feeding effects of the Chinese herbal medicine mixture as feed additive on kinetics of plasma glucose, protein and energy metabolism in sheep. Ruminal fermentation characteristics were also determined. Four sheep were fed either mixed hay (MH-diet) or MH-diet supplemented with 2% of Chinese herbal medicine (CHM-diet) over two 35-day periods using a crossover design. The turnover rate of plasma glucose was measured with an isotope dilution method using [U-^{13}C]glucose. The rates of plasma leucine turnover and leucine oxidation, whole body protein synthesis (WBPS) and metabolic heat production were measured using the [1-^{13}C]leucine dilution and open circuit calorimetry. Body weight gain of sheep was higher (*P=0.03*)

for CHM-diet than MH-diet. Rumen pH was lower (*P=0.02*), concentration of rumen total volatile fatty acid tended to be higher (*P=0.05*) and acetate was higher (*P=0.04*) for CHM-diet compared with MH-diet. Turnover rates of plasma glucose and leucine did not differ between diets. Oxidation rate of leucine tended to be higher (*P=0.06*) for CHM-diet than MH-diet, but the WBPS did not differ between diets. Metabolic heat production tended to be greater (*P=0.05*) for CHM-diet compared with MH-diet.

Experiment 2: Livestock animals exposed to cold causes a variety of hormonal and physiological changes, which result in negative effects on growth and production. Based on the findings in the first experiment, the second experiment was conducted to assess the feeding effects of Chinese herbal medicine on kinetics of plasma glucose, protein and energy metabolism in sheep kept at thermoneutral environment (23°C) or cold environment (2-4°C). Four sheep were subjected to either MH-diet or CHM-diet over two 23-day periods using a crossover design. Cold exposure was conducted for 5 days. The dilution of [U-^{13}C]glucose with open-circuit calorimetry was used to determine the turnover and oxidation rates of plasma glucose and metabolic heat production. The dilution of [1-^{13}C]leucine and N balance test were used to determine the turnover rate of plasma leucine, whole body protein synthesis (WBPS) and degradation (WBPD). N intake was higher (*P<0.01*), N excretion through feces was lower (*P=0.04*)

and N digestibility was higher (*P=0.02*) for CHM-diet than MH-diet. Rumen pH was lower (*P=0.03*), concentration of rumen NH_3 was higher (*P=0.04*), concentrations of rumen total VFA and acetate tended to be higher (*P<0.10*) and propionate was higher (*P=0.04*) for CHM-diet compared MH-diet. Turnover rate of plasma glucose was higher (*P=0.02*) for CHM-diet than MH-diet, and increased (*P<0.01*) during cold exposure. Oxidation rate of plasma glucose did not differ between diets and also between environments. Turnover rate of plasma leucine, WBPS and WBPD were higher (*P<0.05*) for CHM-diet than MH-diet but remained similar between environmental temperatures. Metabolic heat production was greater (*P=0.03*) for CHM-diet compared with MH-diet, and increased (*P<0.01*) during cold exposure. No significant interaction was detected in diet and environment.

Experiment 3: It was expected that the processing of Chinese herbal medicine might also be an important factor to enhance the feeding effects due to considering about its importance in human treatment. Therefore, the third experiment was conducted to assess the feeding effects of the extract of Chinese herbal medicine on kinetics of plasma glucose and protein metabolism in sheep. Six sheep were subjected to either MH-diet or ECHM-diet (MH-diet supplemented with 2% of extract of Chinese herbal medicine) over two 21-day periods using a crossover design. The dilution of [U-^{13}C]glucose was used to determine the turnover rate of plasma glucose.

The dilution of [$^{2}H_5$]phenylalanine and [$^{2}H_2$]tyrosine was used to determine the turnover rates of plasma phenylalanine and tyrosine, the rate of phenylalanine hydroxylation to tyrosine as well as calculating the WBPS. Rumen pH was lower (*P=0.04*), concentrations of rumen total VFA, acetate and propionate tended to be higher (*P<0.10*) for ECHM-diet than MH-diet. Turnover rate of plasma glucose was higher (*P=0.04*) for ECHM-diet compared with MH-diet. Turnover rates of plasma phenylalanine and tyrosine tended to be higher (*P<0.10*), and rate of phenylalanine hydroxylation to tyrosine was higher (*P=0.02*) for ECHM-diet than MH-diet. The WBPS was also higher (*P=0.04*) for ECHM-diet compared with MH-diet.

Experiment 4: Microbial protein is the most important source of amino acids for ruminants because it provides 50 to 80% of total absorbable protein to the small intestine. Enhancing microbial protein synthesis in the rumen would be beneficial to protein metabolism in ruminants. It was expected that the Chinese herbal medicine would also enhance microbial protein synthesis as well as improve intestinal amino acid absorption. Therefore, the fourth experiment was conducted to assess the feeding effects of the extract of Chinese herbal medicine on nitrogen balance, microbial nitrogen supply and plasma leucine kinetics in sheep. Six sheep were subjected to either MH-diet or ECHM-diet over two 21-day periods using a crossover design. The dilution of [1-^{13}C]leucine was used to determine the plasma leucine kinetics. N intake

was higher (*P<0.01*), N excretion through feces was lower (*P=0.02*) and N digestibility was higher (*P=0.02*) for ECHM-diet than MH-diet. Concentration of rumen NH_3 tended to be higher (*P=0.08*) for ECHM-diet compared with MH-diet. Microbial N supply was higher (*P<0.01*) for ECHM-diet than MH-diet. Turnover rate of plasma leucine tended to be higher (*P=0.06*) for ECHM-diet compared with MH-diet.

Conclusions: From the present findings, it could be concluded that the supplementation of the Chinese herbal medicine mixture to mixed hay diet could increase rumen VFA concentration as well as enhance plasma glucose, protein and energy metabolism in sheep. However, the responses of these nutrients and energy metabolism to cold exposure were not modified by Chinese herbal medicine. In addition, the extract of Chinese herbal medicine was considered to be more suitable and safer for feeding animals. Therefore, these results suggested that the Chinese herbal medicine mixture should be considered as a potential feed additive for sheep.

ヒツジにおける漢方薬に対する栄養素およびエネルギー代謝の反応

【背景】飼料添加物としての抗生物質の使用はその残留性から人の健康に悪影響を及ぼす危険性が懸念されている。近年、漢方薬は抗生物質の代替として使用されており、家畜生産でもその重要性が増している。本研究はヒツジにおける栄養素およびエネルギー代謝に及ぼす漢方薬添加の影響を評価するために実施した。

【実験Ⅰ】ヒツジの血漿グルコース、タンパク質、エネルギー代謝に及ぼす漢方薬(黄耆 55%、当帰 27%、蒼朮 18%)添加の影響を検討した。ルーメン発酵性状も併せて測定した。ヒツジ４頭を用い、混播牧草(MH 飼料)と混播牧草に２％の漢方薬を添加した CHM 飼料の２飼料区を設定した。実験は１期 35 日間のクロスオーバー法に従って実施した。血漿グルコース代謝は[U-^{13}C]グルコースの同位元素希釈法を用いて測定した。血漿ロイシン代謝回転速度(LeuTR)、酸化率(LeuOX)、全身のタンパク質合成速度(WBPS)、熱生産量は[1-^{13}C]ロイシン(Leu)の同位元素希釈法および呼吸試験装置を用いて測定した。日増体量は CHM 飼料が MH 飼料よりも多かった(P= 0.03)。CHM 飼料のルーメン pH は低く(P= 0.02)、揮発性脂肪酸(VFA)濃度は高い傾向を示し(P = 0.05)、酢酸濃度は高かった(P = 0.04)。血漿グルコース代謝回転速度(GluTR)、LeuTR は両飼料区に差がなかった。LeuOX は CHM 飼料が高い傾向を示したが(P = 0.06)、WBPS に差はなかった。熱生産量は CHM 飼料が高い傾向を示した(P = 0.05)。

【実験２】家畜が寒冷環境に暴露されると様々な生理、内分泌機能が変化し、

結果として成長や生産に負の影響を及ぼす。実験２では常温(23℃)および寒冷環境(2-4℃)に暴露したヒツジの血漿グルコース、タンパク質、エネルギー代謝に及ぼす漢方薬添加の影響を検討した。ヒツジ４頭を１期 23 日間のクロスオーバー法に従い、MH 飼料と CHM 飼料に振り分けた。血漿 GluTR、グルコース酸化率(GluOX)、熱生産量は[U-^{13}C]グルコースの同位元素希釈法および呼吸試験装置を用いて測定した。血漿 LeuTR、WBPS、全身のタンパク質分解速度(WBPD)は[1-^{13}C]Leu の同位元素希釈法、窒素出納試験により測定した。MH 飼料と比較して CHM 飼料の N 摂取量は多く($P < 0.01$)、N 排泄量は低く($P = 0.04$)、N 消化率は高かった($P = 0.02$)。CHM 飼料のルーメン pH は低く($P = 0.03$)、総 VFA、酢酸濃度は高い傾向を示し($P < 0.10$)、プロピオン酸、アンモニア(NH_3)濃度は高かった($P = 0.04$)。血漿 GluTR は CHM 飼料が高く($P = 0.02$)、寒冷暴露時に増加した($P < 0.01$)。GluOX は飼料間および環境温度間で差がなかった。血漿 LeuTR、WBPS、WBPD は CHM 飼料が高かったが($P < 0.05$)、環境温度では差がなかった。熱生産量は CHM 飼料が MH 飼料よりも高く($P = 0.03$)、寒冷暴露時に増加した($P < 0.01$)。飼料と環境温度の交互作用は認められなかった。

【実験３】漢方薬は一般的に煎じて服用することから、漢方薬の服用法も重要であると考えられる。そこで、実験３ではヒツジの血漿グルコース、タンパク質代謝に及ぼす漢方薬の煎じ液(ECHM 飼料)の影響を検討した。ヒツジ６頭を１期 23 日間のクロスオーバー法に従って MH 飼料と ECHM 飼料に振り分けた。血漿グルコース代謝は[U-^{13}C]グルコースの同位元素希釈法を用いて測定した。血漿フェニルアラニン(Phe)、チロシン(Tyr)代謝回転速度(PheTR、TyrTR)および Phe

から Tyr への酸化(PheOX)は[$^{2}H_{5}$]Phe と[$^{2}H_{2}$]Tyr の同位元素希釈法を用いて測定した。MH 飼料と比較して ECHM 飼料のルーメン pH は低く(P = 0.04)、ルーメン総 VFA、酢酸およびプロピオン酸濃度は高い傾向を示した(P< 0.10)。血漿 GluTR は CHM 飼料が高く(P = 0.04)、血漿 PheTR、TyrTR は ECHM 飼料が高い傾向を示し(P < 0.10)、PheOX は高かった(P = 0.02)。WBPS もまた ECHM 飼料が高かった(P = 0.04)。

【実験４】微生物態タンパク質は、小腸に達する吸収可能なタンパク質の 50 から 80%を供給するので、反芻家畜にとって最も重要なアミノ酸源である。ルーメン内における微生物態タンパク質合成の増加はタンパク質代謝にとって有益である。漢方薬は微生物態タンパク質合成を促進すると同時に小腸でのアミノ酸吸収を改善するかもしれない。実験４はヒツジにおける微生物態タンパク質合成および血漿 Leu 代謝に及ぼす漢方薬の煎じ液の影響を検討した。ヒツジ６頭を用い、１期 23 日間のクロスオーバー法に従って MH 飼料と ECHM 飼料に振り分けた。血漿 Leu 代謝は[1-^{13}C]Leu の同位元素希釈法を用いて測定した。N 摂取量は MH 飼料よりも ECHM 飼料が多く(P< 0.01)、糞中 N 排泄量は少なく(P= 0.02)、N 消化率は高かった(P = 0.02)。ルーメン NH_3 濃度は MH 飼料と比較して ECHM 飼料が高い傾向を示した(P = 0.08)。微生物態 N 供給量は MH 飼料よりも ECHM 飼料が多かった(P < 0.01)。血漿 LeuTR は MH 区と比較して ECHM 区が高い傾向を示した(P = 0.06)。

【結論】本研究で得られた知見から、漢方薬はルーメン VFA 産生を増加させ、血漿グルコース、タンパク質、エネルギー代謝を増加させると結論される。さ

らに、漢方薬の煎じ液は効果的であると考えられる。以上の結果から、漢方薬はヒツジにとって有力な飼料添加物になり得ることが示された。

General Introduction

Antibiotics have been widely used as feed additive in livestock production for more than 50 years (Dibner and Richards, 2005). They have played a very important role in helping the animals to prevent diseases and enhance productivity. However, their use in animal diets has also brought along the hidden danger of drug residues to human health (Barton, 2000). At present, the use of antibiotic growth promoters in animal industry is restricted in the European Union. It is likely that the restrictions on the use of antibiotics in animal husbandry will spread to the rest of the world due to the increasing public concern on human health. Therefore, replacing antibiotics with alternative feed additives has become the specific research interest to animal scientists.

Chinese herbs as the traditional medicine have been widely used in East Asian countries. For thousands of years, they have made great contributions to the maintenance of human health. Most of the Chinese herbal medicine comes from the different parts of perennial herbs, such as the leaves, roots and stems. It has been well-known that Chinese herbal medicine contains the bioactive components which have anti-bacterial activity, anti-inflammatory properties and immune enhancing effects (Huang, 1998). It is used not only to treat diseases but also to promote health and wellbeing

(Opara, 2004). Because of the natural origin, Chinese herbal medicine would not cause excessive drug residue or toxicity and thus it could be considered as a safe and suitable substitute for antibiotics in animal feeding. In recent years, a lot of Chinese herbal medicine has already been reported to promote growth and boost immune system in pigs, chickens and other animals (Kong *et al.*, 2004; Lien *et al.*, 2007; Wang & Zhou, 2007). It was considered that such numerous Chinese herbal medicine provides a great potential for practical application and some of them could be used as alternative feed additives for ruminant animals.

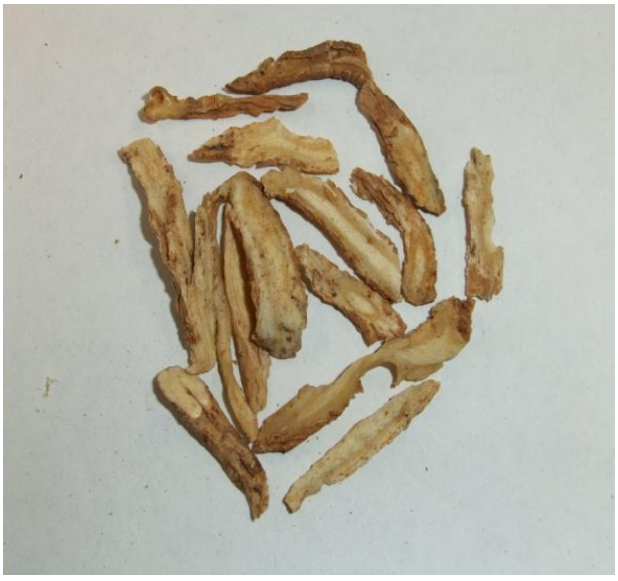
In the traditional system of Chinese herbal medicine, herbs are normally combined in formulas to treat diseases or promote health. The combination of different herbs in a formula creates a new therapeutic agent that can treat much more effectively and completely than a single herb (Li and Wei, 2002). The mixture of Astragalus root (*Astragalus membranaceus*), Angelica root (*Angelica sinensis*) and Atractylodes rhizome (*Atractylodes lancea*) is a classical herbal formula for nourishment purpose in traditional Chinese medicine. The herbs are known to be rich in two major components, polysaccharides (Astragalus root) and essential oils (Angelica root and Atractylodes rhizome). In humans, it is commonly used as a health regulator to remove tiredness and comfort stress by inducing hematopoiesis. Because the sources of these herbs are abundant and inexpensive, it can be expected

from the animal feeding point of view that the mixture of Astragalus root, Angelica root and Atractylodes rhizome is feasible to use as an alternative feed additive to antibiotics in livestock production.

Intermediary metabolism is very important for livestock animals because it contributes to almost all life activities including maintenance, growth and production. Until now, little information is available regarding the performance of Chinese herbal medicine on intermediary metabolism in ruminant animals. We hypothesized that the use of the mixture of Astragalus root, Angelica root and Atractylodes rhizome would be beneficial to nutrients and energy metabolism in ruminants due to its bioactive properties on hematopoiesis. Therefore, the research proposal was designed to investigate the effects of feeding this traditional nourishing Chinese herbal medicine mixture on kinetics of intermediate nutrients and energy metabolism in sheep.

Astragalus root

(*Astragalus membranaceus*)



Angelica root

(*Angelica sinensis*)

Atractylodes rhizome

(*Atractylodes lancea*)

Chapter-1

Effects of Chinese Herbal Medicine on Plasma Glucose, Protein and Energy Metabolism in Sheep

Introduction

In traditional Chinese herbal medicine, the mixture of Astragalus root, Angelica root and Atractylodes rhizome is commonly used for nourishment purpose. It is performed as a health regulator to remove tiredness and comfort stress, which focuses on restoring a balance of energy, body, and spirit to maintain health rather than treating a particular disease or medical condition. Based on the knowledge from human research, it has been well-known that this Chinese herbal medicine mixture is able to induce hematopoiesis by accelerating the generation, growth and maturity of blood cells (Li and Wei, 2002).

It was hypothesized that the use of the Chinese herbal medicine mixture would be beneficial to intermediate nutrients and energy metabolism in ruminant animals due to its bioactive properties on hematopoiesis. As a basic study of the research proposal, the present experiment was carried out to assess the feeding effects of the mixture of Astragalus root, Angelica root and Atractylodes rhizome as feed additive on kinetics of plasma glucose, protein and energy metabolism in sheep.

Materials and methods

1. Animals, diets and management

The handling of animals, including cannulation and blood collection, was reviewed and approved by the Animal Care Committee of Iwate University. All experimental procedures were performed without any noticeable stress to the animals.

The Chinese herbal medicine was purchased from a traditional Chinese drug market in China. Each herb had been cut into small pieces (2-3 cm). According to the formulation of classical Chinese pharmacopoeia, the herbs were mixed in proportion as 55% of Astragalus root, 27% of Angelica root, and 18% of Atractylodes rhizome.

Four crossbred (Corriedale × Suffolk) shorn wethers, aged 7 months on average, weighting 29 ± 2 kg, were used in the experiment. The sheep were assigned to two dietary treatments, including either mixed hay (MH-diet) of orchardgrass (*Dactylis glomerata*) and reed canarygrass (*Phalaris arundinacea*) offered at maintenance level (NRC, 1985) or MH-diet supplemented with 2% of Chinese herbal medicine (CHM-diet). The chemical compositions of mixed hay and Chinese herbal medicine on air dry matter basis are shown in Table 1-1. The sheep received mixed hay at 67 g/kg$^{0.75}$/d for both dietary treatments. The experiment was performed using a

crossover design over two 35-day periods. The sheep were housed in individual pens in an animal room during the first four weeks of the experiment for adaptation. Then animals were moved to metabolic cages in a controlled environment house at an air temperature of 23 ± 1°C, with lighting present from 08:00 h to 22:00 h. Two sheep were fed the MH-diet during the first period and then fed the CHM-diet during the second period. The other two sheep were subjected to the dietary treatments in the reverse order. The animals were given either diet twice a day at 08:30 h and 20:30 h, and they commonly consumed all the diets which were fed within 1 h. Water was available *ad libitum*. The sheep were weighted at the start of experiment, on day 15 and 29, and at the end of each dietary treatment.

Table 1-1 Chemical compositions of experimental diets

Items*	MH	CHM
Dry matter (%)	88.3	91.2
CP (%)	11.9	10.8
NDF (%)	65.1	47.2
ME (kcal/g)	1.79	N/A

*Values are presented on air dry matter basis.

MH = mixed hay of orchardgrass and reed canarygrass; CHM = Chinese herbal medicine mixture; CP = crude protein; NDF = neutral detergent fiber; ME = metabolizable energy; N/A = not available.

2. Experimental procedures

On day 33 of each dietary treatment, rumen fluid (30 ml) was collected through a stomach tube before feeding, 3 and 6 h after feeding. After determination of pH values of rumen fluid with a pH meter (HM-20E, Toa Electronics Ltd., Japan), the rumen fluid was centrifuged at 8,000 x *g* for 10 min at 4°C (RS-18IV, Tomy, Japan). Then 1 ml of supernatant was mixed with 1 ml of 0.1 N HCl. The prepared samples and the residuals of rumen fluid were stored at -30°C for the determinations of rumen ammonia (NH_3) and volatile fatty acid (VFA), respectively.

Two sheep were subjected to the experimental procedures to determine plasma glucose, protein and energy metabolism on day 34 and 35, respectively. The other two sheep were determined in the reverse order. The turnover rate of plasma glucose was measured with an isotope dilution method using [U-^{13}C]glucose. The rates of plasma leucine turnover and leucine oxidation, whole body protein synthesis and metabolic heat production were measured using the [1-^{13}C]leucine dilution and open circuit calorimetry. Two catheters, one for isotope infusion and the other for blood collection were inserted into the left and right jugular veins on the morning of day 34 of each dietary treatment. The catheters were filled with sterile solution of 3.8% trisodium citrate.

At 12:00 h on the day of [U-^{13}C]glucose dilution, 3.0 μmol/kg$^{0.75}$ of [U-^{13}C]glucose (D-glucose-^{13}C, 99 atom% excess ^{13}C; Cambridge Isotope Laboratories, USA) dissolved in saline solution (0.9% sodium chloride) was injected into the jugular catheter for infusion as a priming dose. After the priming injection, [U-^{13}C]glucose was then continuously infused by multichannel peristaltic pumps (AC-2120, Atto, Japan) at rate of 3.0 μmol/kg$^{0.75}$/h through the same catheters for 4 h. Blood samples were taken from the sampling catheter immediately before (12 ml) and at 30-min intervals (6 ml) during the last 120 min of isotope infusion. The collected samples were immediately transferred into centrifuge tubes containing heparin sodium and were stored in crushed ice. Plasma was separated from blood samples by means of centrifugation at 10,000 x *g* for 10 min at 4°C and then stored at -30°C for further analysis.

At 11:30 h on the day of [1-^{13}C]leucine dilution, the sheep were fitted with a clear head chamber (approximately 0.2 m^3) to collect the samples of exhaled gas. After collecting gaseous samples in the pre-infusion period, 7.2 μmol/kg$^{0.75}$ of [1-^{13}C]leucine (L-leucine-1-^{13}C, 99 atom% excess ^{13}C; Cambridge Isotope Laboratories, USA) and 3.5 μmol/kg$^{0.75}$ of NaH^{13}CO$_3$ (sodium bicarbonate-^{13}C, 99.2 atom% excess ^{13}C; Cambridge Isotope Laboratories, USA) dissolved in saline solution were injected into the infusion catheter as a priming dose. Then, [1-^{13}C]leucine was continuously

infused by multichannel peristaltic pumps at rates of 7.2 μmol/kg$^{0.75}$/h through the same catheters for 6 h. Blood samples were taken from the sampling catheter immediately before (12 ml) and at 30-min intervals (6 ml) during the last 120 min of isotope infusion. The catheters were removed at the end of isotope infusions on day 35 of each dietary treatment.

Oxygen (O_2) consumption and carbon dioxide (CO_2) production were monitored continuously for 30 min at the end of [1-^{13}C]leucine infusion using a metabolic monitor (Coast Electronics, UK). An aliquot of exhaled CO_2 was collected in 4 ml of 1 N NaOH for 30 min immediately before and three times during the last 90 min of [1-^{13}C]leucine infusion to measure the enrichment of exhaled $^{13}CO_2$ derived from leucine oxidation. The CO_2 samples were stored at -30°C for further analysis.

The schematic layout of experiment drawing sampling protocol is shown in Figure 1-1.

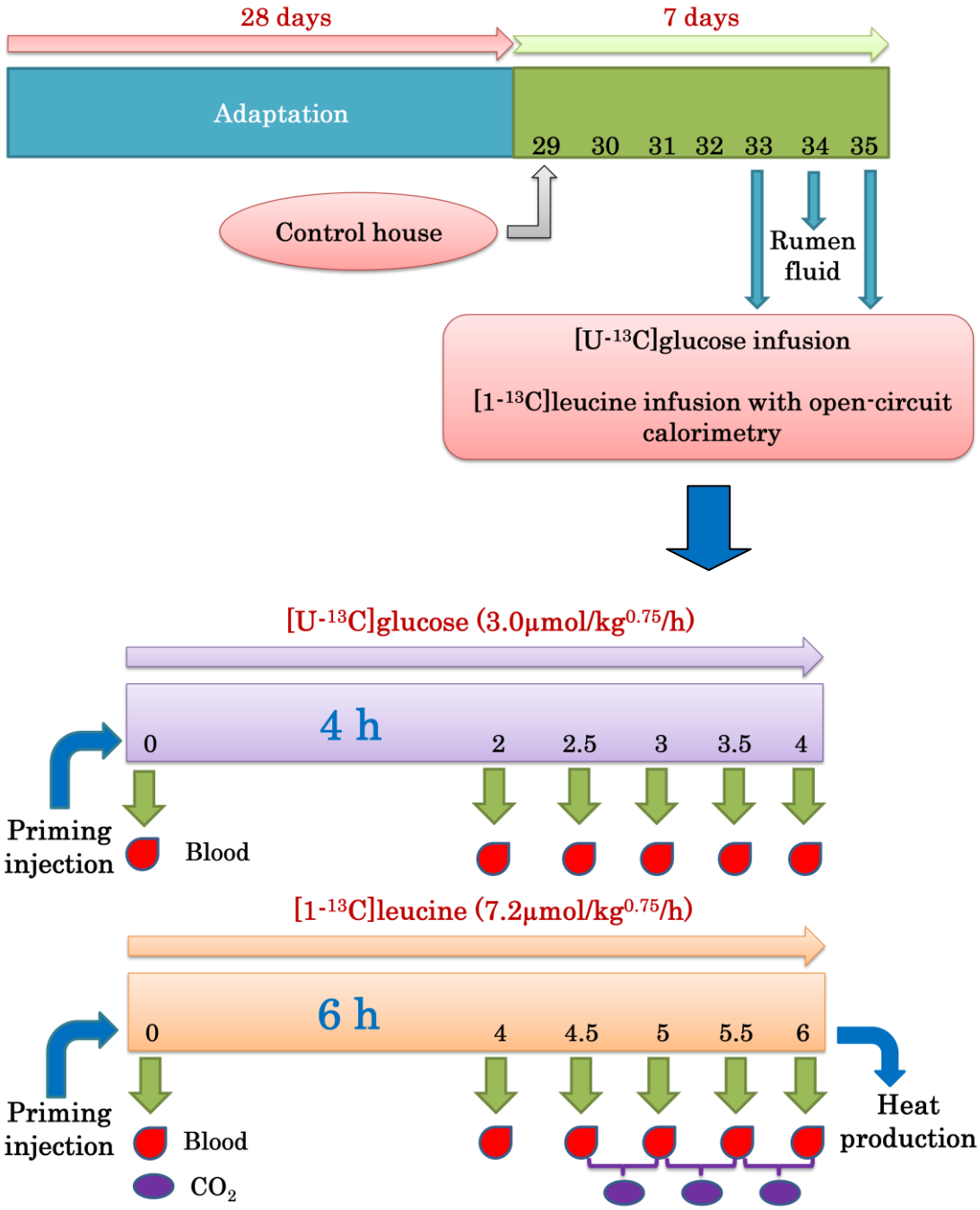


Figure1-1 The experimental layout showing sampling protocol.

3. Chemical analysis

Analysis of chemical components of the diets was performed according to Association of Official Analytical Chemists (AOAC, 1995). Nitrogen contents in diets were analyzed using the Foss Kjeltec System (Kjeltec 2300, Foss, Sweden). The NDF contents in diets were measured according to Van Soest *et al.* (1991) using the Foss Analytical FiberCap™ system (Foss, Sweden).

Concentrations of rumen NH_3 were determined with a colorimetric method (Weatherburn, 1967) using a spectrophotometer (V-630, JASCO, Japan). Concentrations of rumen VFA were measured using a gas chromatography (HP-5890, Hewlett Packard, USA) after steam distillation.

Concentrations of plasma free amino acids and urea were determined using an automatic amino acid analyzer (JLC-500/V, JEOL, Japan). Concentrations of plasma non-esterified fatty acid (NEFA) were enzymatically determined using a diagnostic kit (NEFA C, Wako Pure Chemicals, Japan).

Plasma glucose was derivatized to glucose aldonitrile pentaacetate according to the procedure of Tesrng and Kalhan (1983) with slight modifications by Fujita *et al.* (2006). Enrichments of plasma [U-^{13}C]glucose were measured by gas chromatography mass spectrometry (QP-2010, Shimadzu, Japan). Concentrations of plasma glucose were enzymatically determined with a glucose oxidase method of Huggett and Nixon (1957).

Concentrations of plasma α-ketoisocaproic acid (α-KIC) and enrichments of plasma α-[1-^{13}C]KIC were measured by gas chromatography mass spectrometry according to the procedures of Rocchiccioli *et al.* (1981) and Calder and Smith (1988).

The exhaled CO_2 captured in 1 N NaOH solutions was released by adding 6 N H_2SO_4 into the samples in rubber capped vials under a vacuum condition, and the isotopic abundance of exhaled $^{13}CO_2$ was determined using a gas chromatography-combustion-isotope ratio mass spectrometric system (DELTA^{plus}, Thermo Electron, USA).

4. Calculations

The turnover rates of plasma glucose and leucine (GluTR and LeuTR, respectively) as well as leucine oxidation rate (LeuOX) were calculated using the equations given by Wolfe (1984):

$$TR = I \times (1 / E - 1)$$

Where, I represents the infusion rates of [U-^{13}C]glucose and [1-^{13}C]leucine, and E represents the plasma isotopic enrichments of [U-^{13}C]glucose and α-[1-^{13}C]KIC during the steady state, respectively. In the present study, the enrichment of plasma α-[1-^{13}C]KIC was used to calculate LeuTR instead of plasma [1-^{13}C]leucine, because it is a more useful indicator to determine

leucine metabolism (Magni *et al.*, 1994). The turnover model of plasma leucine is shown in Figure 1-2.

$$\text{LeuOX} = Eco_2 / E_{KIC} / 0.81 \times Vco_2$$

Where, Eco_2 is the isotopic enrichment of exhaled $^{13}CO_2$ and Vco_2 is the CO_2 production rate. The recovery fraction of exhaled CO_2 production in the animal body was estimated to be 0.81 (Allsop *et al.*, 1978). Whole body protein synthesis (WBPS) was calculated using the following equation as described by Wolfe *et al.* (1982):

$$\text{WBPS} = (LeuTR - LeuOX) / \text{leucine concentration in carcass protein}$$

Leucine concentration in carcass protein (66 g/kg) was used as described by Harris *et al.* (1992).

Heat production (HP) was calculated based on the Brouwer's equation (Brouwer, 1965) using O_2 consumption and CO_2 production as described by Young *et al.* (1975):

$$\text{HP} = 3.866 \times Vo_2 + 1.2 \times Vco_2$$

Where, Vo_2 is the O_2 consumption rate and Vco_2 is the CO_2 production rate.

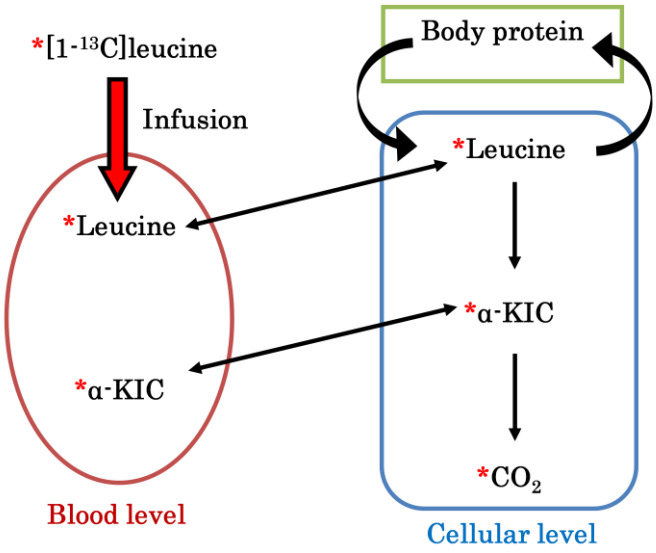


Figure 1-2 Pool model of plasma leucine kinetics (α-KIC = α-ketoisocaproic acid).

5. Statistical analysis

Statistical analysis was conducted according to the MIXED procedure of SAS (1996). The analysis of variance was used to test the effects of period and diet, and sheep were the random effect. Results were considered significant at $P<0.05$ level, and tendency was at $0.05 \leq P<0.10$.

Results

Mean values with standard error of the mean (SEM) were given. Body weight gain of sheep and the ruminal fermentation parameters are shown in Table 1-2. The sheep fed CHM-diet had a greater (*P=0.03*) daily weight gain than those fed MH-diet. Rumen pH was lower (*P=0.02*) for CHM-diet than MH-diet. Concentration of rumen NH_3 remained similar between diets. Concentration of rumen total VFA tended to be higher (*P=0.05*) and acetate was higher (*P=0.04*) for CHM-diet compared with MH-diet.

Plasma free amino acids, urea and NEFA determined in the pre-infusion period of isotope dilution method are presented in Table 1-3. Concentration of plasma aspartic acid was higher (*P=0.02*) and those of threonine, leucine, phenylalanine, alanine, proline and total amino acid tended to be higher (*P<0.10*) for CHM-diet than MH-diet. Concentrations of plasma urea and NEFA did not differ between diets.

Concentration of plasma glucose and enrichment of plasma [U-^{13}C]glucose remained constant during the last 2 h of isotope infusion for each dietary treatment (Figure 1-3). Concentration of plasma glucose and GluTR did not differ between diets (Table 1-4).

Concentration of plasma α-KIC and enrichments of plasma α-[1-^{13}C]KIC and exhaled $^{13}CO_2$ were stable during the latter period of [1-^{13}C]leucine

infusion (Figure 1-4). Concentration of plasma α-KIC remained similar for both dietary treatments. Plasma LeuTR did not differ between diets. The LeuOX tended to be greater (*P=0.06*) for CHM-diet than MH-diet, but the WBPS did not differ between diets. Metabolic heat production tended to be greater (*P=0.05*) for CHM-diet compared with MH-diet (Table 1-4).

Table 1-2. Effects of Chinese herbal medicine on body weight gain and ruminal fermentation characteristics in sheep

Items	MH-diet	CHM-diet	SEM	*P*-value
BW gain (g/day)	16	25	6	0.03
pH	6.99	6.84	0.04	0.02
NH_3 (mmol/L)	10.7	11.2	0.6	0.65
Total VFA (mmol/L)	86.2	92.8	3.2	0.05
Individual VFA concentrations (mmol/L)				
Acetate	60.5	64.5	2.1	0.04
Propionate	17.2	19.1	0.9	0.35
Iso-butyrate	0.6	0.7	0.01	0.15
Butyrate	6.5	7.2	0.3	0.34
Iso-valerate	0.8	0.9	0.02	0.21
Valerate	0.5	0.5	0.01	0.12

MH-diet = mixed hay of orchardgrass and reed canarygrass; CHM-diet = MH-diet supplemented with 2% of Chinese herbal medicine; SEM = standard error of the mean; BW = body weight; VFA = volatile fatty acids.

Table 1-3 Effect of Chinese herbal medicine on concentrations of plasma metabolites at pre-infusion period in sheep

Items	MH-diet	CHM-diet	SEM	*P*-value
Free AA (μmol/L)				
Threonine	258	282	20	0.07
Valine	268	315	17	0.13
Methionine	25	27	2	0.52
Iso-leucine	89	109	7	0.21
Leucine	129	156	9	0.09
Phenylalanine	61	68	4	0.06
Histidine	58	64	3	0.44
Lysine	112	130	9	0.16
Aspartic acid	7.2	8.1	1.1	0.02
Serine	141	159	6	0.30
Asparagine	52	60	3	0.24
Glutamic acid	78	90	4	0.15
Glutamine	311	349	17	0.32
Glycine	467	501	25	0.39
Alanine	190	207	9	0.08
Tyrosine	90	95	7	0.37
Tryptophan	32	47	4	0.34
Arginine	141	149	11	0.19
Proline	107	124	8	0.09
Total AA	2616	2940	268	0.07
Urea (mmol/L)	6.5	7.2	0.8	0.10
NEFA (mEq/L)	0.16	0.14	0.03	0.12

MH-diet = mixed hay of orchardgrass and reed canarygrass; CHM-diet = MH-diet supplemented with 2% of Chinese herbal medicine; SEM = standard error of the mean; AA = amino acid; NEFA = non-esterified fatty acid.

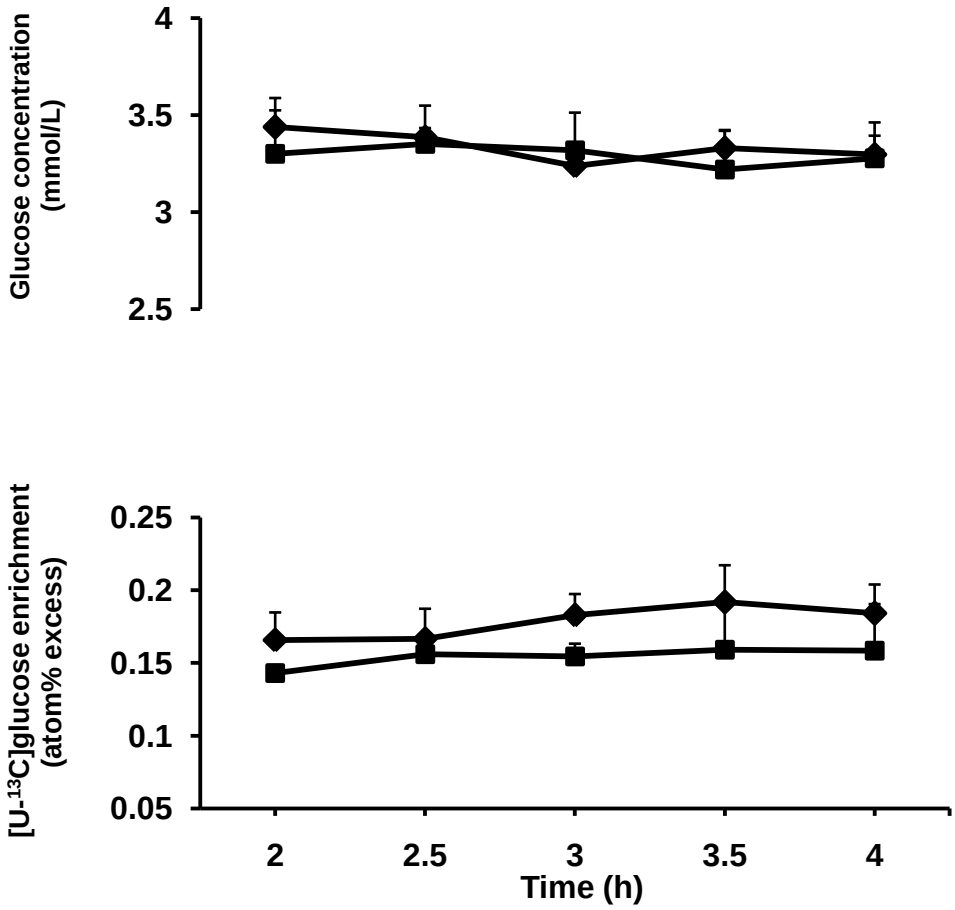


Figure 1-3 Time course of changes in plasma glucose concentration and [U-^{13}C]glucose enrichment during the last 2 h of [U-^{13}C]glucose infusion in sheep fed two different diets (◆ = MH-diet, ■ = CHM-diet).

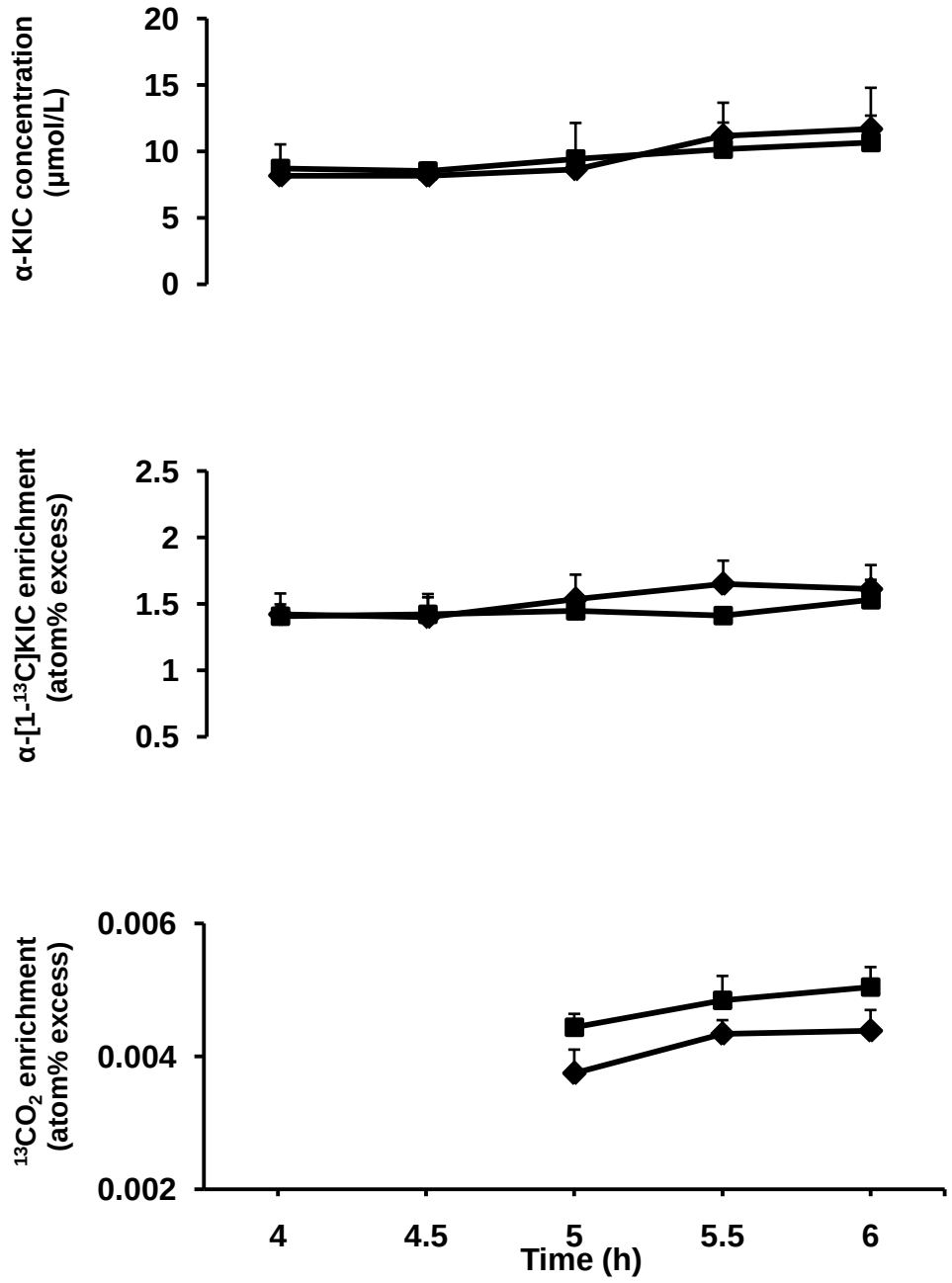


Figure 1-4 Time course of changes in plasma α-ketoisocaproic acid (α-KIC) concentration and enrichments of plasma α-[1-^{13}C]KIC and exhaled $^{13}CO_2$ during the latter period of [1-^{13}C]leucine infusion in sheep fed two different diets (◆ = MH-diet, ■ = CHM-diet).

Table 1-4 Effects of Chinese herbal medicine on plasma glucose, protein and energy metabolism in sheep

Items	MH-diet	CHM-diet	SEM	*P*-value
Glucose concentration (mmol/L)	3.36	3.33	0.15	0.84
GluTR (mmol/kg$^{0.75}$/h)	1.45	1.56	0.04	0.17
α-KIC concentration (μmol/L)	9.5	8.9	1.3	0.42
LeuTR (μmol/kg$^{0.75}$/h)	398	422	23	0.16
LeuOX (μmol/kg$^{0.75}$/h)	102	160	11	0.06
WBPS (g/kg$^{0.75}$/d)	14.1	12.5	1.4	0.15
Heat production (kcal/kg$^{0.75}$/h)	3.94	4.25	0.07	0.05

MH-diet = mixed hay of orchardgrass and reed canarygrass; CHM-diet = MH-diet supplemented with 2% of Chinese herbal medicine; SEM = standard error of the mean; GluTR = turnover rate of plasma glucose; α-KIC = α-ketoisocaproic acid; LeuTR = turnover rate of plasma leucine; LeuOX = oxidation rate of leucine; WBPS = whole body protein synthesis.

Discussion

1. Ruminal fermentation characteristics

In ruminants, the rumen is a large fermentation chamber which plays a very important role in feed digestion. The VFA is produced as the digestive product of dietary carbohydrates through microbial fermentation in the rumen. In the present study, concentration of rumen total VFA tended to be

higher and acetate was higher in sheep fed the CHM-diet than the MH-diet. These results indicated that ruminal fermentation conditions might be improved and the fermentation of dietary carbohydrates was modified by Chinese herbal medicine. Angelica root and Atractylodes rhizome contain considerable amounts of essential oils. Essential oils are numerous and diversified, which are normally defined as the aromatic and volatile compounds derived from herbal plants. Essential oils have been demonstrated to modify rumen fermentation due to their antimicrobial activity selectively against rumen microbes (Patra, 2011). A variety of essential oils have been shown to favorably manipulate rumen fermentation by changing VFA production in both *in vitro* and *in vivo* studies. Castillejos *et al.* (2005; 2007) determined the effect of a blend of essential oils (thymol, limonene and guaiacol) on ruminal fermentation characteristics in continuous culture fermenters and reported that essential oils could increase total VFA concentration and acetate proportion. The feeding effects of essential oils (carvacrol and cinnamaldehyde) in growing lambs were studied by Chaves *et al.* (2008), who observed the significantly higher concentration of rumen total VFA in the experimental groups than in the control group. Similar results can also be found in other reports, regarding the uses of an essential oil mixture (eucalyptus oil, menthol crystal and mint oil) in dairy cows and ropadiar in adult sheep (Soltan *et al.*, 2009; Wang *et al.*, 2009). In

general, most of the essential oils mentioned above are classified into two chemical groups, terpenoids and phenylpropanoids, which are considered as the most important active compounds of essential oils (Patra, 2011). It has been shown that most of the essential oils derived from Angelica root and Atractylodes rhizome are in classifications of terpenoids and phenylpropanoids (Wu *et al.*, 2005; Wang *et al.*, 2008). Therefore, these research findings may clarify the observation of our current experiment, where the changes in rumen VFA concentration might be largely due to the effect of essential oils in CHM-diet.

The NH_3 is produced as the product of dietary nitrogenous substances through microbial fermentation in the rumen. In the present study, although rumen VFA concentration was increased in CHM-diet, rumen NH_3 concentration remained similar between diets. Castillejos *et al.* (2006) reported that essential oils could modify microbial fermentation but would not influence NH_3 concentration of rumen fluid in a long term *in vitro* incubations. Similar responses on rumen NH_3 concentration were also observed in cattle by Devant *et al.* (2007) and Yang *et al.* (2007). These findings are in agreement with the present observation.

Rumen pH values were within the normal range for both dietary treatments. The lower rumen pH for CHM-diet might be associated with the higher rumen VFA concentration, which agrees with Salman *et al.* (2008)

who demonstrated that rumen pH values were contrary to total VFA concentration in goats.

2. Plasma NEFA and free amino acids

Plasma NEFA is an important indicator of nutritional status in farm animals, which can directly indicate the adverse responses to such as fasting, negative energy balance or stress (Sticker *et al.*, 1995; Chelikani *et al.*, 2004; Hristov *et al.*, 2012). In the present study, concentration of plasma NEFA did not differ between diets and the values were presented within normal range for sheep. It indicated that the sheep fed CHM-diet had comparable nutritional status with those fed MH-diet without causing any adverse response or stress.

El-Shafei *et al.* (2013) supplemented Astragalus root to broiler chick diets, and reported that the total protein in serum was significantly increased probably due to a hormonal regulating effect of Astragalus root on protein metabolism. In association with the current experiment, although the level of total protein in blood was not determined, the trend of higher concentrations of certain plasma amino acids and total amino acid might also be related to the bioactivity of herbs in CHM-diet.

3. Plasma glucose and protein kinetics

Although it was expected that plasma glucose metabolism would be enhanced by the Chinese herbal medicine in sheep due to its bioactive properties on hematopoiesis, no positive impact was found on either plasma glucose concentration or plasma GluTR in the current experiment. In ruminants, the supply of glucose precursor is considered as the major factor to influence glucose metabolism (Ortigues-Marty *et al.*, 2003). In the present study, propionate, the major glucogenic substrate produced in the rumen, did not differ between diets. It indicated that glucose precursors from the rumen were similar and thus the gluconeogenesis might be comparable between dietary treatments. Al-Mamun *et al.* (2007) reported that plasma glucose concentration and turnover rate did not differ in sheep fed plantain herb (a perennial herb having anti-bacterial and anti-inflammatory properties) or mixed hay, which is in accordance with the present findings. Moreover, glucose metabolism is regulated by endocrine hormones. Insulin has been reported to be the most important regulator of glucose disposal and production, which inhibits gluconeogenesis and the output of glucose from the liver (McDowell, 1983). In association with the current experiment, Astragalus root has been used as traditional Chinese medicine to treat diabetes, and polysaccharides (the main bioactive component of Astragalus root) have been reported to improve insulin sensitivity (Liu *et al.*, 2010).

Hence the lack of changes in plasma glucose concentration and plasma GluTR might also be partly related to the increased insulin sensitivity in CHM-diet.

Plasma LeuTR and the WBPS did not differ between diets. Nevertheless, it was found that higher numerical values of plasma LeuTR resulted in lower numerical values of the WBPS for CHM-diet compared with MH-diet. This may be largely due to the calculation method as we used plasma LeuTR and LeuOX to calculate the WBPS, where the differences in LeuOX were greater than those in LeuTR. Similar data can also be found in a previous report of Sano *et al.* (2004). So far, little information is available regarding the performance of Chinese herbal medicine on plasma amino acid kinetics and protein synthesis in ruminants. Al-Mamun *et al.* (2008) reported that plasma LeuTR decreased but the WBPS remained unchanged in sheep fed plantain herb compared with fed mixed hay, which partly agrees with the present findings.

Taken together, the sheep fed CHM-diet did not enhance plasma GluTR, LeuTR and WBPS as expected under the conditions of the present study. In the treatment of human diseases, Chinese herbal medicine is normally taken as syrup of its extract. During the feeding periods of the current experiment, the Chinese herbal medicine was directly given to the animals without any treatment, thus the lack of changes in plasma GluTR, LeuTR and WBPS

between dietary treatments might be partly related to the factor that we did not process the Chinese herbal medicine, so that it was not as effective as expected.

4. Heat production

Until now, to our knowledge, the effect of Chinese herbal medicine on heat production has not been investigated either in humans or in animals. Heat is defined as the released energy that is produced from the oxidation process of the nutritive substances such as carbohydrate, protein and fat in the body. In our present study, metabolic heat production tended to be greater for CHM-diet than MH-diet. The trend of increased heat production indicated that the Chinese herbal medicine might play a role in accelerating nutrients oxidation in sheep. It is in good accordance with the result of LeuOX, which also tended to be greater for CHM-diet than MH-diet. Furthermore, the sheep fed CHM-diet had a higher body weight gain than those fed MH-diet. Because energy is essential to animals in almost all life activities including maintenance, growth and production, the trend of increased heat production also demonstrated that the CHM-diet could provide more available energy for growth than MH-diet in sheep.

Chapter-2

Effects of Chinese Herbal Medicine and Cold Exposure on Plasma Glucose, Protein and Energy Metabolism in Sheep

Introduction

Cold exposure is one of the major stresses to livestock animals in winter. Under a cold environment, the animals have to generate more heat from diets and body reserves to maintain a constant body temperature. Cold stress also causes a variety of hormonal and physiological changes, which result in negative effects on growth and production in livestock animals (Young, 1981).

From the results of the past experiment in Chapter-1, the sheep fed mixed hay diet supplemented with 2% of Chinese herbal medicine mixture showed positive impacts on growth and heat production. Because energy requirements are increased for livestock animals during cold exposure (NRC, 1985), it is expected that the use of Chinese herbal medicine mixture would help the animals to improve physiological conditions and enhance intermediary metabolism by reducing stress during cold exposure. Considering the above points, the present experiment was carried out to assess the feeding effects of the mixture of Astragalus root, Angelica root and Atractylodes rhizome on kinetics of plasma glucose, protein and energy metabolism in sheep kept at thermoneutral environment or exposed to cold.

Materials and methods

1. Animals, diets and management

The handling of animals, including cannulation and blood collection, was reviewed and approved by the Animal Care Committee of Iwate University. All experimental procedures were performed without any noticeable stress to the animals.

In this experiment, the Chinese herbal medicine mixture was ground into a fine powder for feeding. Four crossbred (Corriedale × Suffolk) shorn wethers, aged 2 years on average, weighting 41 ± 2 kg, were used in the experiment. The sheep were assigned to two dietary treatments, including either mixed hay (MH-diet) of orchardgrass (*Dactylis glomerata*) and reed canarygrass (*Phalaris arundinacea*) offered at maintenance level (NRC, 1985) or MH-diet supplemented with 2% of Chinese herbal medicine (CHM-diet). The sheep received mixed hay at 67 g/kg$^{0.75}$/d for both dietary treatments. The experiment was performed using a crossover design over two 23-day periods. The sheep were housed in individual pens in an animal room during the first 14 days of the experiment for adaptation. On day 15, the animals were moved to metabolic cages in a controlled environment house at an air temperature of 23 ± 1°C, with lighting present from 08:00 h to 22:00 h for 4 days. Then, the sheep were exposed to a cold environment for 5 days (from

day 19 to 23). The environmental temperature for cold exposure was maintained at 2 ± 1°C from 08:00 h to 22:00 h and at 4 ± 1°C from 22:00 h to 08:00 h. Two sheep were fed the MH-diet during the first period and then fed the CHM-diet during the second period. The other two sheep were subjected to the dietary treatments in the reverse order. The animals were given either diet twice a day at 08:30 h and 20:30 h, and they commonly consumed all the diets which were fed within 1 h. Water was available *ad libitum*. The sheep were weighted at the start of experiment, on day 15 and 19, and at the end of each dietary treatment.

2. Experimental procedures

Nitrogen balance trial was conducted twice over 3 successive days (from day 16 to 18 at thermoneutral temperature and from day 21 to 23 at cold exposure, respectively) of each dietary treatment. Urine and feces samples were collected from each sheep at 24-hour intervals. The urine was collected in a plastic bucket containing 50 ml of 6 N H_2SO_4 solutions to prevent N loss. After recording the total volume, subsamples (50 ml) were stored at -30°C for further analysis. The collected feces were dried at 60°C for 48 h and then placed at room temperature for 5 days. After weighing the air dried samples, subsamples were ground and stored for further analysis.

On day 17 at thermoneutral temperature and on day 22 at cold exposure of each dietary treatment, rumen fluid (30 ml) was collected through a stomach tube before feeding, 3 and 6 h after feeding. After determination of pH values of rumen fluid with a pH meter (F-51, HORIBA Ltd., Japan), the rumen fluid was centrifuged at 8,000 x *g* for 10 min at 4°C (RS-18IV, Tomy, Japan). Then 1 ml of supernatant was mixed with 1 ml of 0.1 N HCl. The prepared samples and the residuals of rumen fluid were stored at -30°C for the determinations of rumen ammonia (NH_3) and volatile fatty acid (VFA), respectively.

Two sheep were subjected to the experimental procedures to determine plasma glucose, protein and energy metabolism either on day 16 and 18 in thermoneutral environment or on day 21 and 23 in cold environment, respectively. The other two sheep were determined in the reverse order. The turnover and oxidation rates of plasma glucose and metabolic heat production were measured using the isotope dilution method of [U-^{13}C]glucose with open circuit calorimetry. The turnover rate of plasma leucine was measured with an isotope dilution method using [1-^{13}C]leucine. Two catheters, one for isotope infusion and the other for blood collection were inserted into the left and right jugular veins on the morning of each isotope dilution method. The catheters were filled with sterile solution of 3.8% trisodium citrate.

At 11:30 h on the respective day of [U-^{13}C]glucose dilution, the sheep were fitted with a clear head chamber (approximately 0.2 m^3) to collect the samples of exhaled gas. After collecting gaseous samples in the pre-infusion period, 3.0 μmol/$kg^{0.75}$ of [U-^{13}C]glucose (D-glucose-^{13}C, 99 atom% excess ^{13}C; Cambridge Isotope Laboratories, USA) and 3.5 μmol/$kg^{0.75}$ of $NaH^{13}CO_3$ (sodium bicarbonate-^{13}C, 99.2 atom% excess ^{13}C; Cambridge Isotope Laboratories, USA) dissolved in saline solution (0.9% sodium chloride) were injected into the jugular catheter as a priming dose for infusion. Then, [U-^{13}C]glucose was continuously infused by multichannel peristaltic pumps (AC-2120, Atto, Japan) at rates of 3.0 μmol/$kg^{0.75}$/h through the same catheters for 6 h. Blood samples were taken from the sampling catheter immediately before (12 ml) and at 30-min intervals (6 ml) during the last 120 min of isotope infusion. The collected samples were immediately transferred into centrifuge tubes containing heparin sodium and were stored in crushed ice. Plasma was separated from blood samples by means of centrifugation at 10,000 x *g* for 10 min at 4°C and then stored at -30°C for further analysis.

Oxygen (O_2) consumption and carbon dioxide (CO_2) production were monitored continuously for 30 min at the end of [U-^{13}C]glucose infusion using a metabolic monitor (Coast Electronics, UK). An aliquot of exhaled CO_2 was collected in 4 ml of 1 N NaOH for 30 min immediately before and three times during the last 90 min of [U-^{13}C]glucose infusion to measure the

enrichment of exhaled $^{13}CO_2$ derived from glucose oxidation. The CO_2 samples were stored at -30°C for further analysis.

At 12:00 h on the respective day of [1-^{13}C]leucine dilution, 7.2 μmol/kg$^{0.75}$ of [1-^{13}C]leucine (L-leucine-1-^{13}C, 99 atom% excess ^{13}C; Cambridge Isotope Laboratories, USA) dissolved in saline solution was injected into the infusion catheter as a priming dose. After the priming injection, [1-^{13}C]leucine was then continuously infused by multichannel peristaltic pumps at rate of 7.2 μmol/kg$^{0.75}$/h through the same catheters for 4 h. Blood samples were taken from the sampling catheter immediately before (12 ml) and at 30-min intervals (6 ml) during the last 120 min of isotope infusion.

The schematic layout of experiment drawing sampling protocol is shown in Figure 2-1.

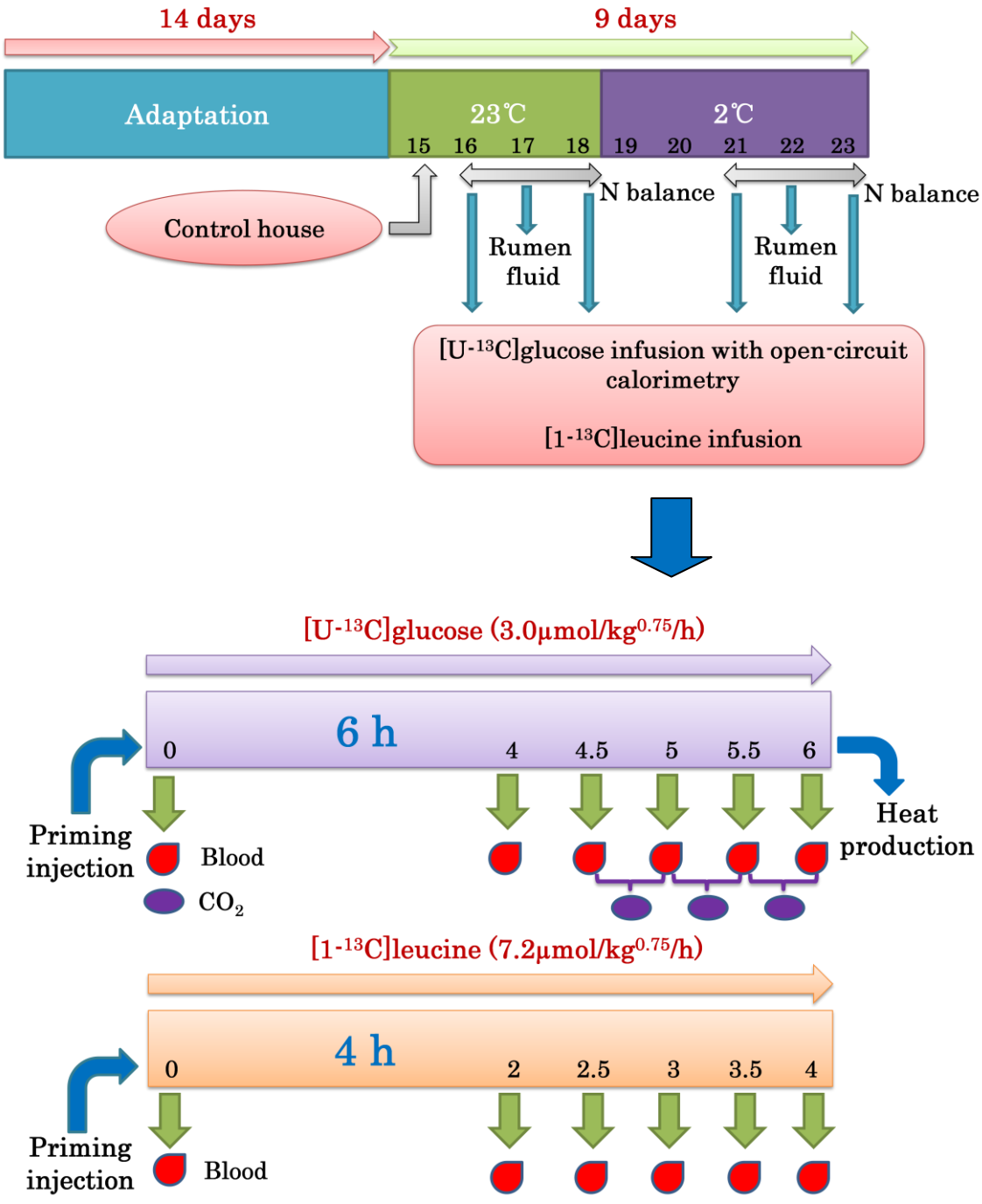


Figure2-1 The experimental layout showing sampling protocol.

3. Chemical analysis

Analysis of chemical components of the diets was performed according to Association of Official Analytical Chemists (AOAC, 1995). Nitrogen contents in diets, urine and feces were analyzed using the Foss Kjeltec System (Kjeltec 2300, Foss, Sweden).

The chemical analysis of rumen fluid and plasma samples was performed with the same procedures as described in Chapter-1.

4. Calculations

The turnover rates of plasma glucose and leucine (GluTR and LeuTR, respectively) as well as heat production were calculated using the same equations as described in Chapter-1.

The glucose oxidation rate (GluOX) was calculated from the following equation given by Wolfe (1984):

$$\text{GluOX} = Eco_2 / E_{Glu} / 0.81 \times Vco_2$$

Where, Eco_2 and E_{Glu} are the isotopic enrichments of exhaled $^{13}CO_2$ and [U-^{13}C]glucose, respectively, and Vco_2 is the CO_2 production rate. The recovery fraction of exhaled CO_2 production in the animal body was estimated to be 0.81 (Allsop *et al.*, 1978).

Whole body protein synthesis (WBPS) and degradation (WBPD) were determined from the relationships among whole body protein flux (WBPF), N

absorption and urinary N excretion using the following equations as described by Schroeder *et al.* (2006):

$$\text{WBPF} = LeuTR / 0.066$$

$$\text{WBPS} = WBPF - (\text{urinary N excretion} \times 6.25)$$

$$\text{WBPD} = WBPF - (\text{N absorption} \times 6.25)$$

Where, the value 0.066 (66 g/kg) was the leucine concentration in carcass protein as described by Harris *et al.* (1992).

5. Statistical analysis

Statistical analysis was conducted according to the MIXED procedure of SAS (1996). The analysis of variance was used to test the effects of period, diet, environment and the interaction of diet and environment. Sheep were the random effect. Results were considered significant at $P<0.05$ level, and tendency was at $0.05 \leq P < 0.10$.

Results

Mean values with standard error of the mean (SEM) were given. The nitrogen balance data are presented in Table 2-1. Nitrogen intake was higher ($P<0.01$) for CHM-diet than MH-diet. Nitrogen excretion through

feces was lower (*P=0.04*) for CHM-diet compared with MH-diet, but N excretion through urine was similar for both diets. Nitrogen retention did not differ between dietary treatments. Nitrogen digestibility was higher (*P=0.02*) for CHM-diet than MH-diet.

The ruminal fermentation parameters are shown in Table 2-2. Rumen pH was lower (*P=0.03*) for CHM-diet than MH-diet. Concentration of rumen NH_3 was higher (*P=0.04*) for CHM-diet compared with MH-diet. Concentrations of rumen total VFA and acetate tended to be higher (*P<0.10*), and propionate was higher (*P=0.04*) for CHM-diet than MH-diet.

Plasma free amino acids, urea and NEFA determined in the pre-infusion period of isotope dilution method are presented in Table 2-3. Concentrations of plasma leucine, phenylalanine, lysine and tyrosine tended to be lower (*P<0.10*) for CHM-diet than MH-diet. Concentration of plasma proline was lower (*P=0.04*) for CHM-diet compared with MH-diet, and decreased (*P=0.03*) during cold exposure. Concentration of plasma NEFA was lower (*P=0.04*) for CHM-diet than MH-diet, and increased (*P=0.01*) during cold exposure.

Concentration of plasma glucose and enrichment of plasma [U-^{13}C]glucose remained constant during the last 2 h of isotope infusion (Figure 2-2). Concentration of plasma glucose did not differ between diets but increased (*P=0.02*) during cold exposure (Table 2-4). The GluOX did not differ between diets and also between environments. Plasma GluTR was

higher (*P=0.02*) for CHM-diet compared with MH-diet, and increased (*P<0.01*) during cold exposure.

Concentration of plasma α-KIC and enrichment of plasma α-[1-^{13}C]KIC were stable during the last 2 h of [1-^{13}C]leucine infusion (Figure 2-3). Concentration of plasma α-KIC did not differ between diets and also between environments. Plasma LeuTR, WBPS and WBPD were higher (*P<0.05*) for CHM-diet compared with MH-diet but remained similar between environmental temperatures. Metabolic heat production was greater (*P=0.03*) for CHM-diet than MH-diet, and increased (*P<0.01*) during cold exposure (Table 2-4).

Table 2-1 Effects of Chinese herbal medicine and cold exposure on nitrogen balance in sheep

Items	MH-diet		CHM-diet		SEM	*P*-value		
	TN	Cold	TN	Cold		Diet	Env	Diet×Env
Parameters of N balance (g/kg$^{0.75}$/d)								
N intake	1.07	1.07	1.09	1.09	0.01	<0.01	0.96	0.89
N in feces	0.44	0.43	0.41	0.39	0.03	0.04	0.78	0.32
N in urine	0.32	0.34	0.35	0.36	0.02	0.23	0.25	0.51
N balance	0.30	0.29	0.32	0.34	0.02	0.44	0.93	0.70
N digestibility (%)	59	60	62	64	1	0.02	0.56	0.47

MH-diet = mixed hay of orchardgrass and reed canarygrass; CHM-diet = MH-diet supplemented with 2% of Chinese herbal medicine; SEM = standard error of the mean; TN = thermoneutral temperature; Env = environment.

Table 2-2 Effects of Chinese herbal medicine and cold exposure on ruminal fermentation characteristics in sheep

Items	MH-diet		CHM-diet		SEM	*P*-value		
	TN	Cold	TN	Cold		Diet	Env	Diet×Env
pH	6.73	6.86	6.65	6.77	0.06	0.03	0.24	0.66
NH_3 (mmol/L)	10.1	9.5	12.7	11.4	1.03	0.04	0.11	0.27
Concentrations of rumen VFA (mmol/L)								
Total VFA	83.1	79.1	89.1	90.2	4.9	0.09	0.42	0.76
Acetate	64.2	60.4	68.9	67.7	2.8	0.07	0.37	0.65
Propionate	13.0	13.7	14.4	15.9	1.2	0.04	0.30	0.25
Iso-butyrate	0.6	0.6	0.5	0.6	0.1	0.12	0.41	0.68
Butyrate	4.3	3.6	4.4	4.9	0.4	0.18	0.59	0.87
Iso-valerate	0.7	0.6	0.6	0.7	0.1	0.26	0.65	0.73
Valerate	0.4	0.3	0.3	0.4	0.1	0.41	0.38	0.50

MH-diet = mixed hay of orchardgrass and reed canarygrass; CHM-diet = MH-diet supplemented with 2% of Chinese herbal medicine; SEM = standard error of the mean; TN = thermoneutral temperature; Env = environment; VFA = volatile fatty acids.

Table 2-3 Effects of Chinese herbal medicine and cold exposure on concentrations of plasma metabolites at pre-infusion period in sheep

Items	MH-diet		CHM-diet		SEM	*P*-value		
	TN	Cold	TN	Cold		Diet	Env	Diet×Env
Free AA (μmol/L)								
Threonine	195	171	183	191	11	0.68	0.40	0.52
Valine	281	244	214	237	12	0.32	0.26	0.35
Methionine	21	21	19	18	2	0.35	0.63	0.47
Iso-leucine	93	95	73	83	6	0.66	0.24	0.68
Leucine	150	129	106	114	6	0.07	0.70	0.56
Phenylalanine	56	56	47	49	3	0.05	0.47	0.81
Histidine	58	52	59	54	5	0.55	0.37	0.46
Lysine	148	146	134	139	10	0.09	0.59	0.53
Serine	142	147	157	158	11	0.10	0.14	0.25
Asparagine	83	86	72	75	8	0.15	0.21	0.42
Glutamic acid	61	51	36	45	4	0.37	0.73	0.91
Glutamine	279	277	301	262	19	0.66	0.54	0.51
Glycine	636	657	748	717	23	0.21	0.31	0.24
Alanine	214	158	179	153	10	0.13	0.16	0.44
Tyrosine	66	69	57	58	3	0.08	0.27	0.19
Arginine	132	138	142	138	7	0.40	0.72	0.74
Proline	112	77	84	70	6	0.04	0.03	0.20
Total AA	2726	2574	2610	2559	232	0.19	0.10	0.33
Urea (mmol/L)	7.1	6.8	7.2	7.4	1.0	0.35	0.82	0.71
NEFA (mEq/L)	0.26	0.38	0.19	0.27	0.04	0.04	0.01	0.40

MH-diet = mixed hay of orchardgrass and reed canarygrass; CHM-diet = MH-diet supplemented with 2% of Chinese herbal medicine; SEM = standard error of the mean; TN = thermoneutral temperature; Env = environment; AA = amino acid; NEFA = non-esterified fatty acid.

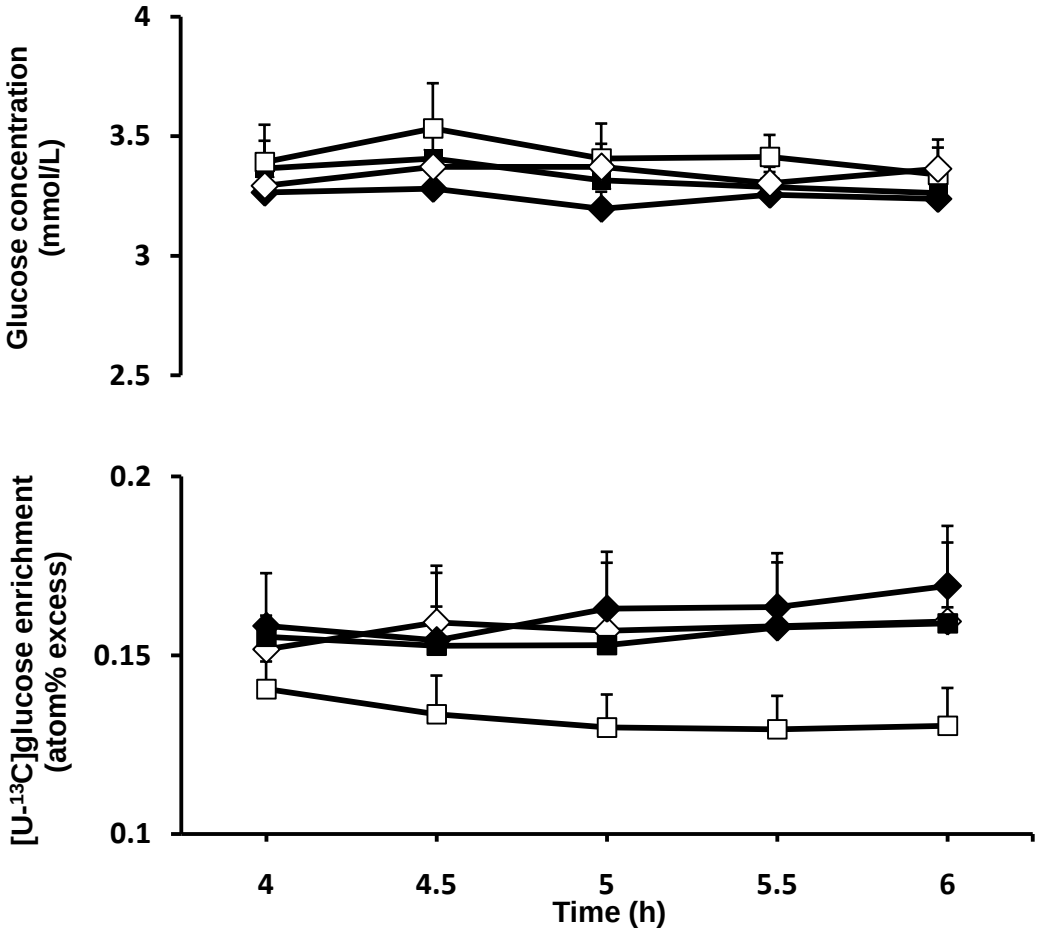


Figure 2-2 Time course of changes in plasma glucose concentration and [U-^{13}C]glucose enrichment during the last 2 h of [U-^{13}C]glucose infusion in sheep fed MH-diet at thermoneutral temperature (◆) and at cold exposure (◇) or fed CHM-diet at thermoneutral temperature (■) and at cold exposure (□).

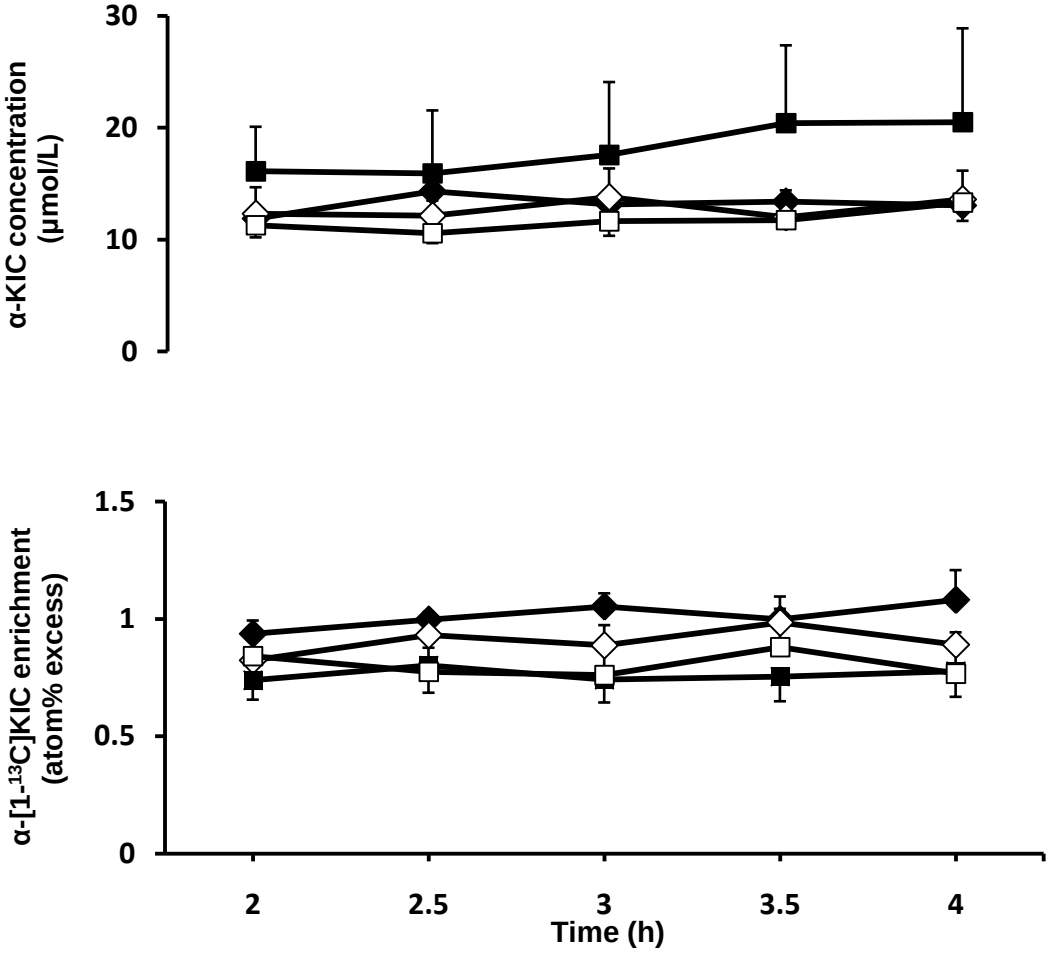


Figure 2-3 Time course of changes in plasma α-ketoisocaproic acid (α-KIC) concentration and plasma α-[1-^{13}C]KIC enrichment during the last 2 h of [1-^{13}C]leucine infusion in sheep fed MH-diet at thermoneutral temperature (◆) and at cold exposure (◇) or fed CHM-diet at thermoneutral temperature (■) and at cold exposure (□).

Table 2-4 Effects of Chinese herbal medicine and cold exposure on plasma glucose, protein and energy metabolism in sheep

Items	MH-diet		CHM-diet		SEM	*P*-value		
	TN	Cold	TN	Cold		Diet	Env	Diet×Env
Glucose conc. (mmol/L)	3.29	3.46	3.38	3.53	0.17	0.24	0.02	0.35
GluOX ($mmol/kg^{0.75}/h$)	0.43	0.50	0.50	0.57	0.1	0.13	0.19	0.47
GluTR ($mmol/kg^{0.75}/h$)	1.33	1.57	1.54	1.70	0.06	0.02	<0.01	0.23
α-KIC conc. (μmol/L)	13.8	12.7	17.2	11.7	2.8	0.07	0.37	0.65
LeuTR ($\mu mol/kg^{0.75}/h$)	310	345	400	380	16	0.03	0.40	0.29
WBPS ($g/kg^{0.75}/d$)	14.9	16.6	19.2	18.7	0.8	0.04	0.41	0.68
WBPD ($g/kg^{0.75}/d$)	13.8	15.4	17.2	16.9	0.9	0.04	0.59	0.77
Heat production ($kcal/kg^{0.75}/h$)	4.49	4.70	4.82	5.04	0.32	0.03	<0.01	0.30

MH-diet = mixed hay of orchardgrass and reed canarygrass; CHM-diet = MH-diet supplemented with 2% of Chinese herbal medicine; SEM = standard error of the mean; TN = thermoneutral temperature; Env = environment; GluOX = oxidation rate of glucose; GluTR = turnover rate of plasma glucose; α-KIC = α-ketoisocaproic acid; LeuTR = turnover rate of plasma leucine; WBPS = whole body protein synthesis; WBPD = whole body protein degradation.

Discussion

1. Nitrogen balance

In the present study, nitrogen intake was higher for CHM-diet than MH-diet. The higher N intake might be due to the CP content of Chinese herbal medicine in CHM-diet (the CP content of Chinese herbal medicine was presented in Chaprer-1). Nitrogen excretion through feces was lower, which resulted in a higher N digestibility for CHM-diet compared with MH-diet. This might be due to the higher dietary CP intake for CHM-diet, because similar results were found in sheep in previous reports (Sano *et al.*, 2004; Al-Mamun *et al.*, 2008; Alam *et al.*, 2010), where increased N excretion and N digestibility were observed with higher dietary CP intake. Moreover, although the N intake was significantly different, the numerical values were very close between diets (1.07 for MH-diet and 1.09 for CHM-diet), which seems insufficient to influence N digestibility. Therefore, the higher N digestibility for CHM-diet might also be partly related to the effect of the bioactive components of Chinese herbal medicine.

Animals exposed to cold is responsive to a variety of physiological changes (Young, 1981). Kennedy *et al.* (1976) reported that in sheep, cold exposure could increase the rate of dry matter passage through the gastrointestinal tract, and the digestibility was reduced as a concomitant

result. However, their findings are inconsistent with the present results, where N digestibility remained unchanged between environmental temperatures. This inconsistency might be related to the short-term of cold exposure, because in their study, cold exposure was conducted for 45 days and it was just 5 days in the current experiment. Al-Mamun *et al.* (2008) deduced that it takes a relatively long period for sheep to get adjusted to new stable conditions in digestive function. Sano *et al.* (1995) also observed an unchanged CP digestibility in sheep exposed to cold for 18 days.

2. Ruminal fermentation characteristics

The results of rumen VFA concentration and pH value were comparable to the first experiment in Chapter-1. The sheep fed CHM-diet had lower rumen pH, higher propionate concentration and tended to have higher acetate and total VFA concentrations than those fed MH-diet. The changes in rumen VFA production might be due to the antimicrobial effect of essential oils (the main bioactive components of Angelica root and Atractylodes rhizome) on rumen fermentation in CHM-diet. The lower rumen pH for CHM-diet might be related to the higher total VFA concentration. The higher rumen NH_3 concentration for CHM-diet might be due to the higher dietary CP intake, because various reports have verified that rumen NH_3 concentration was influenced by dietary CP intake

(Haaland *et al.*, 1982; Freeman *et al.*, 1992; Alam *et al.*, 2010), which is in agreement with the present observation. In addition, the higher rumen NH_3 concentration for CHM-diet in current experiment was in inconsistent with the result of the experiment in Chapter-1, where rumen NH_3 concentration remained similar between dietary treatments. This inconsistency might be largely related to the factor that the Chinese herbal medicine was fed in powder form in current experiment, which is considered to be more easily digestible in the rumen.

The activity of microbes in the rumen could be affected by environmental factors (Romero-Perez *et al.*, 2011), and cold exposure could depress rumen VFA concentration in sheep exposed to prolonged periods of cold (Kennedy, 1985). However, the rumen VFA concentration was not affected by cold exposure in the current experiment. Same as discussed on N balance, the lack of change in rumen VFA concentration might also be related to the short-term of cold exposure. The effect of environmental temperature on rumen VFA concentration in cows was determined by Kelley *et al.* (1967), who reported that low temperature (1.6°C) did not affect rumen VFA concentration compared with thermoneutral temperature (18.2°C), where in their experiment, the period of cold exposure was one week. It is in good agreement with the present observation.

3. Plasma NEFA and free amino acids

Concentration of plasma NEFA was lower for CHM-diet than MH-diet and increased during cold exposure. Plasma NEFA is the best indicator of body lipid loss (Chillard *et al.*, 2000). The lower plasma NEFA concentration might be due to a reduction in lipolysis as well as an increase in fatty acid oxidation in sheep fed the CHM-diet. Sano *et al.* (2007; 2010) reported that plasma NFFA concentration increased during cold exposure in sheep, which agrees with the present findings. The increased NEFA concentration indicated that the animals were stressed by cold exposure.

Concentrations of certain plasma amino acids were lower or tended to be lower for CHM-diet than MH-diet. The lower plasma amino acid concentrations for CHM-diet might be related to the higher WBPS, where more plasma free amino acids took part in protein synthesis in CHM-diet than in MH-diet. In the present study, concentrations of almost all plasma amino acids remained unchanged during cold exposure, except proline, which was lower during cold exposure. Miaron and Christopherson (1997) reported that acute cold exposure may exert relatively small effects on whole blood arterial amino acid concentrations in sheep. A previous report of Sano *et al.* (2010) showed that plasma amino acid concentrations were little influenced (only leucine, valine and glutamine changed) by cold exposure in sheep, which is in agreement with the present observation.

4. Plasma glucose and protein kinetics

Concentration of plasma glucose did not differ between diets but increased during cold exposure. Various reports have well verified that plasma glucose concentration was affected by cold exposure in sheep (Weekes *et al.*, 1983; Sano *et al.*, 1999; Sano *et al.*, 2007). Therefore, the increased plasma glucose concentration during cold exposure could be considered as a normal physiological change in sheep in response to cold stress. Plasma GluTR was higher for CHM-diet than MH-diet and increased during cold exposure. In ruminants, because the dietary carbohydrates are fermented to VFA by microbes in the rumen, most of the glucose must be supplied through gluconeogenesis. Propionate, the major glucose precursor produced in the rumen, was higher for CHM-diet than MH-diet in current experiment. Thus the higher plasma GluTR might be largely due to an increased gluconeogenesis in sheep fed the CHM-diet. Moreover, it was considered that the Chinese herbal medicine might also play a positive role to enhance plasma glucose kinetics in sheep due to their bioactive properties on hematopoiesis. Plasma GluTR has been reported to be increased in response to cold stress in sheep (Tsuda *et al.*, 1984; Sano *et al.*, 2007), which is in accordance with the present results. When the animals are exposed to cold, heat production generally increases to maintain a constant body temperature. As a concomitant result, the oxidation of nutritive substances

in the body should increase to meet the increased heat requirement. In the present study, the GluOX remained unchanged in sheep during cold exposure. Tsuda *et al.* (1984) reported that the contribution of plasma glucose oxidation to total heat production did not differ between the thermoneutral temperature (20°C) and cold exposure (0°C) in sheep. Therefore, the lack of change in GluOX in current experiment indicated that the increased heat requirement during cold exposure was supplied by the acceleration of the oxidation of other nutritive substances rather than glucose. Sano *et al.* (1995) observed that heat production and nonprotein substances oxidation increased but carbohydrate oxidation remained unchanged in sheep during cold exposure, which may partly clarify the observation of the present study.

Plasma LeuTR, WBPS and WBPD were higher for CHM-diet than MH-diet but remained unchanged during cold exposure. The higher plasma LeuTR for CHM-diet might be partly due to the higher CP intake, because dietary CP intake is positively correlated with plasma LeuTR in sheep (Al-Mamun *et al.*, 2008). Similarly, the increased WBPS and WBPD might also be related to the higher dietary CP intake in CHM-diet. However, Sano *et al.* (2004) reported that plasma LeuTR and WBPS as well as WBPD changed toward reduction with increased dietary CP intake in sheep. The inconsistency between the present results and their findings might be partly

due to the effect of the bioactive components of Chinese herbal medicine in CHM-diet. Because, in a previous report of human research, Li *et al.* (1995) used [^{15}N]glycine as the tracer to determine the effect of a Chinese herbal medicine mixture (Astragalus root and Angelica root, same as the present study) on protein metabolism in nephrotic patients, and observed that the Chinese herbal medicine could improve the disorder of protein metabolism and increase the level of serum protein by improving the net rate of protein synthesis. It is in good agreement with the observation of the current experiment in sheep. Sano *et al.* (2009) stated that plasma LeuTR and WBPS were influenced by cold exposure in sheep. However, the results observed in current experiment were inconsistent, where cold exposure had no influence on plasma LeuTR and WBPS in sheep. Like WBPS, the WBPD also responded similarly to cold exposure. Compared with plasma glucose kinetics, these results suggested that glucose metabolism was more responsive to cold exposure than protein metabolism in sheep under the conditions of the present study.

In the present experiment, the numerical values of WBPS were higher than those observed in the first experiment in chapter-1, although the same isotope was used. This may be largely due to the different calculation method as we used plasma LeuTR and urinary N excretion to calculate the WBPS in

the present experiment. Similar observation was also reported by Al-Mamun *et al.* (2008).

5. Heat production

The result of heat production was comparable to the first experiment in Chapter-1. The sheep fed CHM-diet had a greater metabolic heat production than those fed MH-diet and increased during cold exposure. As it discussed in chapter-1, the increased heat production for CHM-diet might be due to the effect that the Chinese herbal medicine played a role in accelerating nutrients oxidation in sheep. Heat production generally increases during cold exposure because the animals require more energy to maintain a constant body temperature. Lee *et al.* (1989) and Sano *et al.* (1995) reported that heat production was significantly increased by cold exposure in sheep, which agrees strongly with the present findings. However, it was found that the numerical values of heat production on cold exposure were lower than those observed in sheep in other reports (Lee *et al.*, 1989; Sano *et al.*, 1995). This might be largely related to the factor that we did not shear the sheep in current experiment, thus the stress to cold might be slightly reduced.

In addition, although plasma glucose, protein and energy metabolism were enhanced by CHM-diet and plasma glucose and energy metabolism increased during cold exposure, no significant interaction was detected

between diet and environment. Therefore it could be concluded that the responses of plasma glucose, protein and energy metabolism to cold exposure were not modified by CHM-diet in sheep under the conditions of the present study.

Chapter-3

Effects of Extract of Chinese Herbal Medicine on Plasma Glucose and Protein Metabolism in Sheep

Introduction

During the feeding periods of the first experiment in Chapter-1, the Chinese herbal medicine was directly given to the animals without any treatment, which resulted in lack of changes in plasma glucose and protein metabolism. In the second experiment in Chapter-2, the Chinese herbal medicine was given to the sheep after grinding into powder form, which resulted in significant changes in plasma glucose and protein metabolism.

In the treatment of human diseases, Chinese herbal medicine is normally taken as syrup of its extract. The herbs are processed by boiling in order to remove foreign substances and reduce toxic contents as well as increase therapeutic effects (Li and Wei, 2002). The inconsistency of results between past experiments indicated that the processing of herbs also may be an important factor to enhance the feeding effects in animals. It was expected that the Chinese herbal medicine would be more effective to the sheep after purification. Therefore, the current experiment was carried out to assess the feeding effects of the extract of Astragalus root, Angelica root and Atractylodes rhizome on kinetics of plasma glucose and protein metabolism in sheep.

Materials and methods

1. Animals, diets and management

The handling of animals, including cannulation and blood collection, was reviewed and approved by the Animal Care Committee of Iwate University. All experimental procedures were performed without any noticeable stress to the animals.

The extract of Astragalus root, Angelica root and Atractylodes rhizome was made in liquid form according to the traditional processing method. The herbs were boiled for three times (20 min for each time) and the boiled water was taken as the extract.

Six crossbred (Corriedale × Suffolk) shorn wethers, aged 3 years on average, weighting 46 ± 2 kg, were used in the experiment. The sheep were assigned to two dietary treatments, including either mixed hay (MH-diet) of orchardgrass (*Dactylis glomerata*) and reed canarygrass (*Phalaris arundinacea*) offered at maintenance level (NRC, 1985) or MH-diet supplemented with 2% of extract of Chinese herbal medicine (ECHM-diet). The 2% of extract was calculated according to the ratio between the amount of herbs and the volume of boiled water (approximately per 100 g to 325 ml). The sheep received mixed hay at 67 g/kg$^{0.75}$/d for both dietary treatments. The experiment was performed using a crossover design over two 21-day

periods. The sheep were housed in individual pens in an animal room during the first 14 days of the experiment for adaptation. On day 15, the animals were moved to metabolic cages in a controlled environment house at an air temperature of 23 ± 1°C, with lighting present from 08:00 h to 22:00 h. Two sheep were fed the MH-diet during the first period and then fed the ECHM-diet during the second period. The other two sheep were subjected to the dietary treatments in the reverse order. The animals were given either diet twice a day at 08:30 h and 20:30 h. The extract of Chinese herbal medicine was injected into animal's mouth by a syringe before giving the mixed hay, and they commonly consumed all the diets which were fed within 1 h. Water was available *ad libitum*. The sheep were weighted at the start of experiment, on day 8 and 15, and at the end of each dietary treatment.

2. Experimental procedures

On day 20 of each dietary treatment, rumen fluid (30 ml) was collected through a stomach tube before feeding, 3 and 6 h after feeding. After determination of pH values of rumen fluid with a pH meter (F-51, HORIBA Ltd., Japan), the rumen fluid was centrifuged at 8,000 x *g* for 10 min at 4°C (RS-18IV, Tomy, Japan). Then 1 ml of supernatant was mixed with 1 ml of 0.1 N HCl. The prepared samples and the residuals of rumen fluid were

stored at -30°C for the determinations of rumen ammonia (NH_3) and volatile fatty acid (VFA), respectively.

On day 21 of each dietary treatment, the isotope dilution method of [U-^{13}C]glucose and the dilution of [$^2H_5$]phenylalanine (Phe) and [$^2H_2$]tyrosine (Tyr) were simultaneously conducted to determine the kinetics of plasma glucose, Phe, Tyr and whole body protein synthesis in sheep. Two catheters, one for isotope infusion and the other for blood collection were inserted into the left and right jugular veins on the morning of each determination of the isotope dilution method. The catheters were filled with sterile solution of 3.8% trisodium citrate. At 12:00 h, 3.0 μmol/kg$^{0.75}$ of [U-^{13}C]glucose (D-glucose-^{13}C, 99 atom% excess ^{13}C; Cambridge Isotope Laboratories, USA), 2.5 μmol/kg$^{0.75}$ of [$^2H_5$]Phe (L-phenylalanine, ring-D5, 98%; Cambridge Isotope Laboratories, USA), 1.6 μmol/kg$^{0.75}$ of [$^2H_4$]Tyr (L-4-hydroxypheny1-2,3,4,5,6-D4-alanine, 98 atom%; Isotec Inc., A Matheson, USA Co., USA) and 1.6 μmol/kg$^{0.75}$ of [$^2H_2$]Tyr (L-tyrosine, ring-3,5-D2, 98%; Cambridge Isotope Laboratories, USA) dissolved in saline solution (0.9% sodium chloride) were injected into the jugular catheter as a priming dose for infusion. Then, the isotopes were continuously infused by multichannel peristaltic pumps (AC-2120, Atto, Japan) at rate of 3.0 μmol/kg$^{0.75}$/h for [U-^{13}C]glucose, and at rates of 2.6 and 1.5 μmol/kg$^{0.75}$/h for [$^2H_5$]Phe and [$^2H_2$]Tyr, respectively, through the same catheters for 4 h. Blood samples

were taken from the sampling catheter immediately before (12 ml) and at 30-min intervals (6 ml) during the last 120 min of isotope infusion. The collected samples were immediately transferred into centrifuge tubes containing heparin sodium and were stored in crushed ice. Plasma was separated from blood samples by means of centrifugation at 10,000 x *g* for 10 min at 4°C and then stored at -30°C for further analysis. The catheters were removed at the end of isotope infusion of each dietary treatment.

The schematic layout of experiment drawing sampling protocol is shown in Figure 3-1.

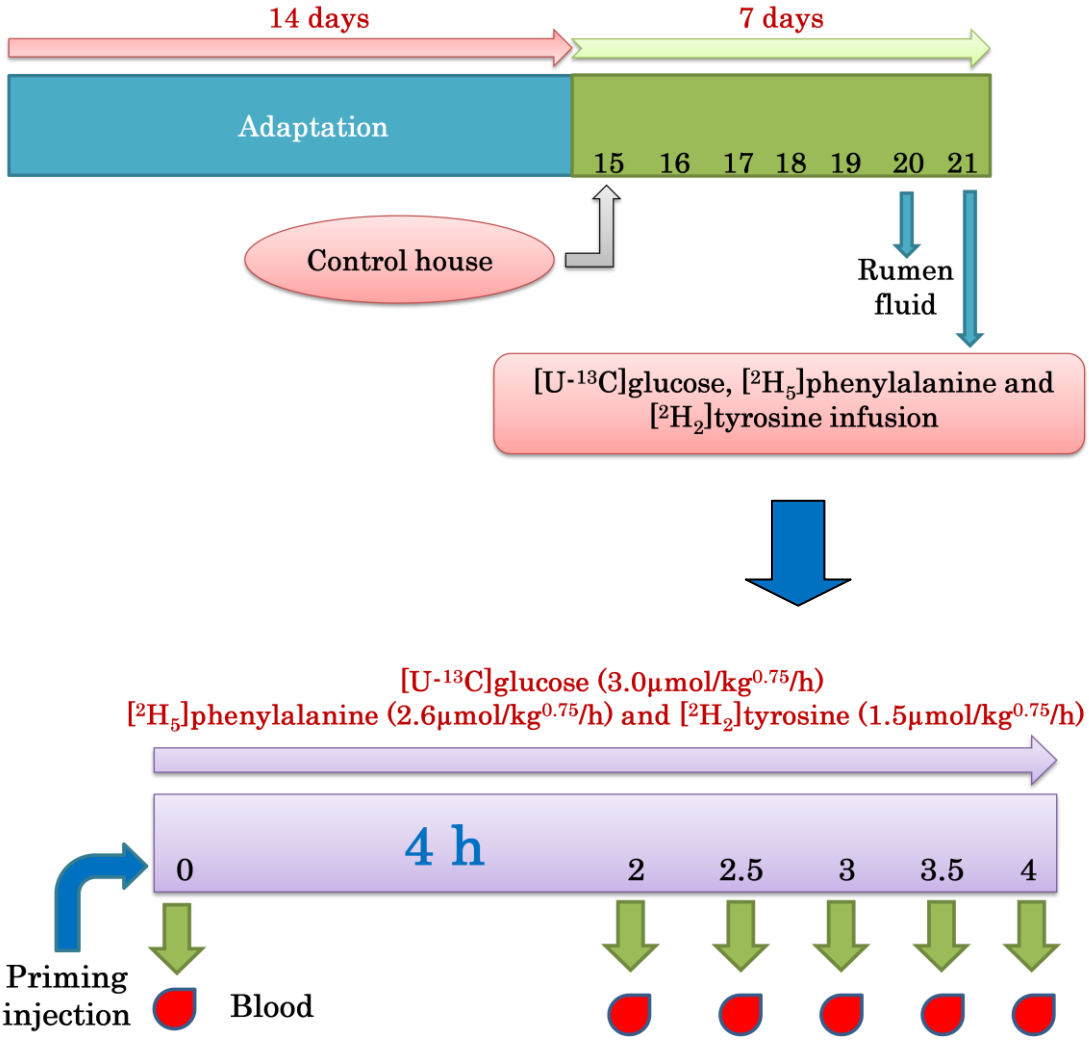


Figure3-1 The experimental layout showing sampling protocol.

3. Chemical analysis

The chemical analysis of rumen fluid and plasma samples (amino acids, urea, NEFA, glucose concentration and [U-^{13}C]glucose enrichment) was performed with the same procedures as described in Chapter-1.

Concentrations of plasma Phe and Tyr, and enrichments of plasma [$^{2}H_5$]Phe, [$^{2}H_4$]Tyr and [$^{2}H_2$]Tyr were measured by gas chromatography mass spectrometry according to the procedures of Rocchiccioli *et al.* (1981) and Calder and Smith (1988).

4. Calculations

The turnover rates of plasma glucose, Phe and Tyr (GluTR, PheTR and TyrTR, respectively) were calculated using the equations given by Wolfe (1984):

$$\text{TR} = I \times (1 / E - 1)$$

Where, I represents the infusion rate of each isotope, and E represents the plasma isotopic enrichment during the steady state. The turnover model of plasma Phe is shown in Figure 3-2. The rate of Phe hydroxylation to Tyr (PheOX) was calculated as described by Thompson *et al.* (1989):

$$\text{PheOX} = TyrTR \times (E_{tyr} / E_{phe}) \times (PheTR / (I_{phe} + PheTR))$$

Where, E_{tyr} and E_{phe} are the plasma isotopic enrichments of [$^2H_5$]Phe and [$^2H_4$]Tyr, respectively, and I_{phe} is the infusion rate of [$^2H_5$]Phe. Whole body protein synthesis (WBPS) was calculated using the following equation:

WBPS = *(PheTR - PheOX) / Phe concentration in carcass protein*

Phe concentration in carcass protein was estimated to be 3.5% (Harris *et al.*, 1992).

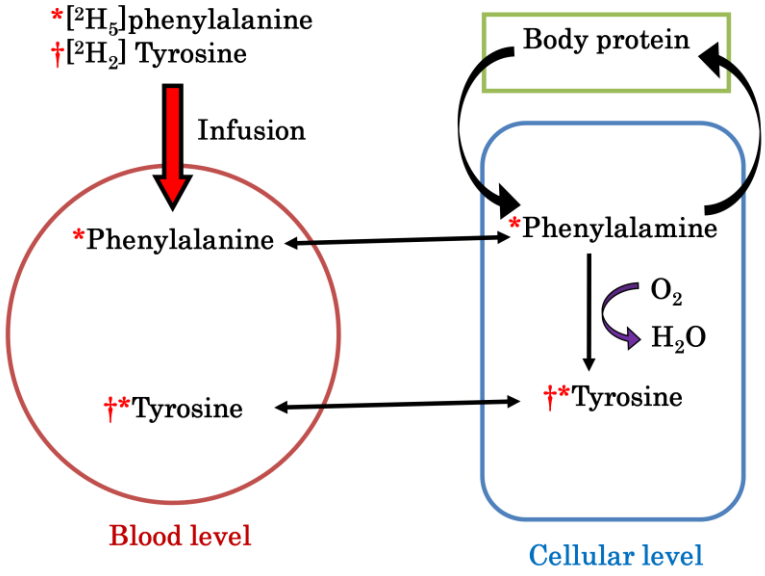


Figure 3-2 Pool model of plasma phenylalanine kinetics.

5. Statistical analysis

Statistical analysis was conducted according to the MIXED procedure of SAS (1996). Results were considered significant at $P<0.05$ level, and tendency was at $0.05 \leq P < 0.10$.

Results

Mean values with standard error of the mean (SEM) were given. The ruminal fermentation parameters are shown in Table 3-1. Rumen pH was lower (*P=0.04*) for ECHM-diet than MH-diet. Concentration of rumen NH_3 did not differ between diets. Concentrations of rumen total VFA, acetate, propionate and iso-valerate tended to be higher (*P<0.10*) for ECHM-diet compared with MH-diet.

Plasma free amino acids, urea and NEFA determined in the pre-infusion period of isotope dilution method are presented in Table 3-2. Concentrations of plasma valine, leucine and glycine were lower (*P<0.05*) for ECHM-diet than MH-diet. Concentrations of plasma threonine, asparagine and alanine tended to be lower (*P<0.10*) for ECHM-diet compared with MH-diet. Concentration of plasma urea was similar between diets. Concentration of plasma NEFA was lower (*P=0.02*) for ECHM-diet than MH-diet.

Concentration of plasma glucose and enrichment of plasma [U-^{13}C]glucose remained constant during the last 2 h of isotope infusion for each dietary treatment (Figure 3-3). Concentration of plasma glucose did not differ between diets. Plasma GluTR was higher (*P=0.04*) for ECHM-diet than MH-diet (Table 3-3).

Concentrations of plasma Phe and Tyr and enrichments of plasma [$^2H_5$]Phe, [$^2H_4$]Tyr and [$^2H_2$]Tyr were stable during the last 2 h of isotope infusion (Figure 3-4). Concentrations of plasma Phe and Tyr did not differ between dietary treatments (Table 3-3). Plasma PheTR and TyrTR tended to be higher ($P<0.10$), and plasma PheOX was higher ($P=0.02$) for ECHM-diet than MH-diet. The WBPS was also higher ($P=0.04$) for ECHM-diet compared with MH-diet.

Table 3-1 Effect of extract of Chinese herbal medicine on ruminal fermentation characteristics in sheep

Items	MH-diet	ECHM-diet	SEM	*P*-value
pH	6.78	6.51	0.06	0.04
NH_3 (mmol/L)	10.8	11.6	0.4	0.19
Total VFA (mmol/L)	83.2	94.4	5.4	0.08
Individual VFA concentrations (mmol/L)				
Acetate	60.3	67.4	2.3	0.07
Propionate	15.7	18.6	0.5	0.08
Iso-butyrate	0.6	0.6	0.1	0.32
Butyrate	5.3	6.2	0.4	0.21
Iso-valerate	0.8	0.9	0.06	0.09
Valerate	0.5	0.6	0.03	0.17

MH-diet = mixed hay of orchardgrass and reed canarygrass; ECHM-diet = MH-diet supplemented with 2% of extract of Chinese herbal medicine; SEM = standard error of the mean; VFA = volatile fatty acids.

Table 3-2 Effect of extract of Chinese herbal medicine on concentrations of plasma metabolites at pre-infusion period in sheep

Items	MH-diet	ECHM-diet	SEM	*P*-value
Free AA (μmol/L)				
Threonine	274	232	23	0.07
Valine	322	245	21	0.03
Methionine	22	24	2	0.32
Iso-leucine	101	89	5	0.16
Leucine	151	120	11	0.04
Phenylalanine	49	45	4	0.28
Histidine	57	49	7	0.40
Lysine	132	121	20	0.47
Serine	166	172	19	0.38
Asparagine	62	43	9	0.06
Glutamic acid	91	88	11	0.75
Glutamine	302	321	42	0.61
Glycine	569	503	52	0.04
Alanine	263	210	24	0.09
Tyrosine	66	51	8	0.33
Tryptophan	37	39	7	0.84
Arginine	134	152	10	0.29
Proline	130	107	14	0.19
Total AA	2928	2611	268	0.12
Urea (mmol/L)	7.5	6.9	0.4	0.56
NEFA (mEq/L)	0.27	0.19	0.02	0.02

MH-diet = mixed hay of orchardgrass and reed canarygrass; ECHM-diet = MH-diet supplemented with 2% of extract of Chinese herbal medicine; SEM = standard error of the mean; AA = amino acid; NEFA = non-esterified fatty acid.

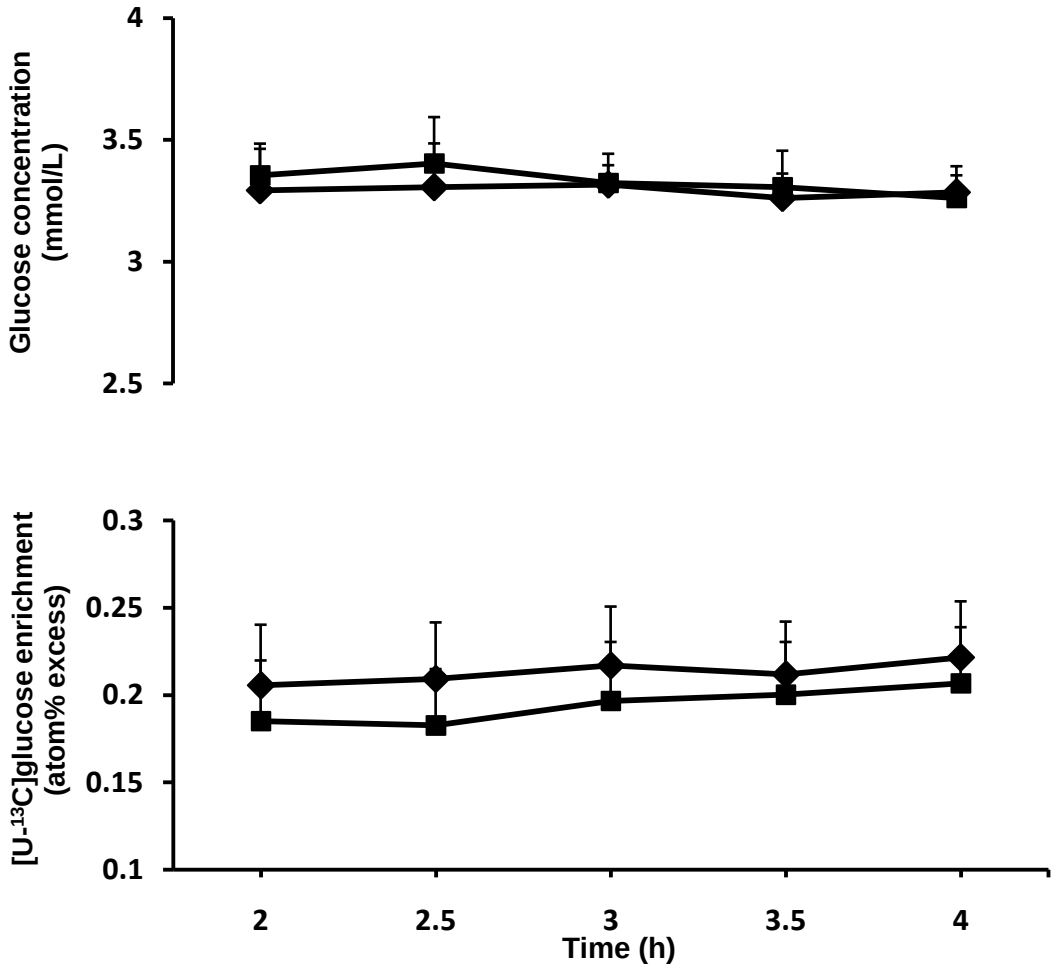


Figure 3-3 Time course of changes in plasma glucose concentration and [U-^{13}C]glucose enrichment during the last 2 h of [U-^{13}C]glucose infusion in sheep fed two different diets (◆ = MH-diet, ■ = ECHM-diet).

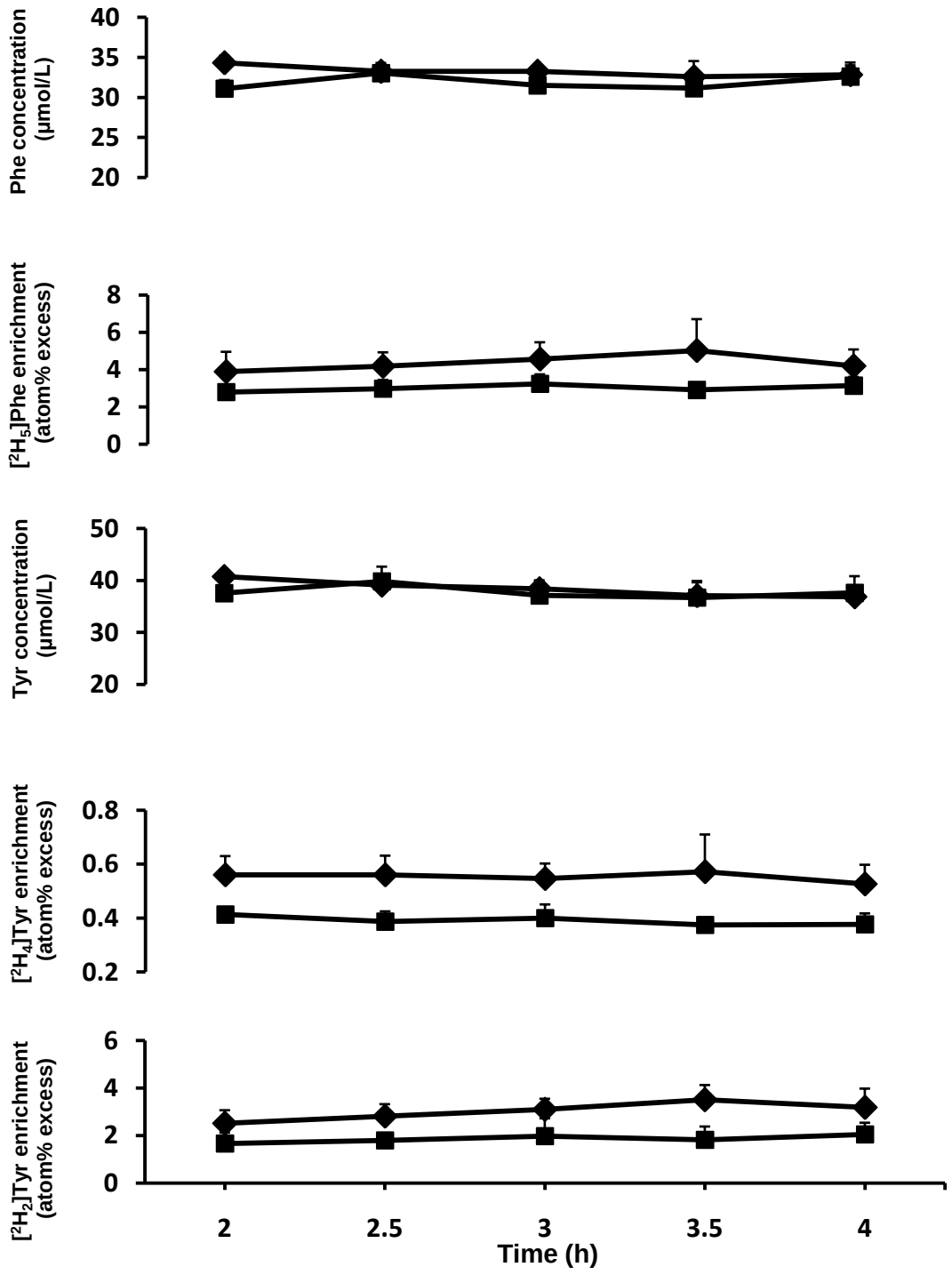


Figure 3-4 Time course of changes in concentrations of plasma Phe and Tyr and enrichments of plasma [$^2H_5$]Phe, [$^2H_4$]Tyr and [$^2H_2$]Tyr during the last 2 h of isotope infusion in sheep fed two different diets (◆ = MH-diet, ■ = ECHM-diet).

Table 3-3 Effects of extract of Chinese herbal medicine on plasma glucose and protein metabolism in sheep

Items	MH-diet	ECHM-diet	SEM	*P*-value
Glucose concentration (mmol/L)	3.30	3.33	0.09	0.71
GluTR (mmol/kg$^{0.75}$/h)	1.29	1.44	0.05	0.04
Phe concentration (μmol/L)	33.2	31.9	2.3	0.41
PheTR (μmol/kg$^{0.75}$/h)	69.0	96.0	12	0.07
PheOX (μmol/kg$^{0.75}$/h)	8.2	11.9	1.2	0.02
Tyr concentration (μmol/L)	38.4	37.8	3.1	0.53
TyrTR (μmol/kg$^{0.75}$/h)	59.4	91.4	14	0.06
WBPS (g/kg$^{0.75}$/d)	7.3	10.1	1.4	0.04

MH-diet = mixed hay of orchardgrass and reed canarygrass; ECHM-diet = MH-diet supplemented with 2% of extract of Chinese herbal medicine; SEM = standard error of the mean; GluTR = turnover rate of plasma glucose; PheTR = turnover rate of plasma phenylalanine; PheOX = rate of phenylalanine hydroxylation to tyrosine; TyrTR = turnover rate of plasma tyrosine; WBPS = whole body protein synthesis.

Discussion

1. Ruminal fermentation characteristics

In the present study, concentrations of rumen total VFA, acetate and propionate tended to be higher in sheep fed ECHM-diet than MH-diet. As a concomitant result, rumen pH was significantly lower for ECHM-diet than

MH-diet. Concentration of rumen NH_3 did not differ between dietary treatments. As discussed in the past chapters, the changes in rumen VFA concentration in ECHM-diet might be largely due to the effect of the bioactive components of Chinese herbal medicine on rumen fermentation. Therefore, in association with the results of the past two experiments in Chapter-1 and Chapter-2, it could be well concluded that the Chinese herbal medicine mixture is able to improve the fermentation of dietary carbohydrates but only slightly influence the fermentation of dietary nitrogenous substances in the rumen in sheep.

2. Plasma NEFA and free amino acids

Concentration of plasma NEFA was lower for ECHM-diet than MH-diet. The lower plasma NEFA concentration indicated a decreased lipolysis in sheep fed the ECHM-diet, which might be largely due to the effect that the Chinese herbal medicine played a role in accelerating fatty acid oxidation.

Concentrations of plasma valine, leucine and glycine were lower, and concentrations of plasma threonine, asparagine and alanine tended to be lower for ECHM-diet compared with MH-diet. Generally, a decrease in plasma amino acid concentrations implies greater utilization for protein synthetic purposes (Wessels *et al.*, 1997). Therefore, the lower concentrations of certain plasma amino acids for ECHM-diet indicated a higher utilization

of plasma free amino acids on protein synthesis in sheep fed ECHM-diet than MH-diet. This deduction is in good agreement with the result of WBPS, which was higher for ECHM-diet compared with MH-diet.

3. Plasma glucose and protein kinetics

Plasma GluTR was higher for ECHM-diet than MH-diet, which agrees with the findings in Chapter-2. The increased plasma GluTR for ECHM-diet might be largely related to the trend of the higher concentration of rumen propionate, where more glucogenic substrates were available for gluconeogenesis in ECHM-diet than in MH-diet. Moreover, the bioactive properties of Chinese herbal medicine on hematopoiesis could also be considered as a factor to enhance the plasma glucose kinetics in sheep.

A number of isotope dilution methods using different amino acid isotopes have been used to estimate the WBPS. Among the isotopes, [1-^{13}C]leucine is the most widely used amino acid isotope (the model as used in Chapter-1). In the current experiment, the isotope dilution method of [$^{2}H_5$]Phe and [$^{2}H_2$]Tyr was applied to determine protein kinetics and the WBPS was calculated from the relationship between PheTR and PheOX, where PheOX is the rate of Phe hydroxylation to Tyr. Compared with the [1-^{13}C]leucine model, the WBPS can be accurately determined within a short time, because it does not need to measure the exhaled CO_2 production that

derived from amino acid oxidation. However, the numerical values of WBPS observed with the [$^{2}H_5$]Phe model in the present study were lower than those observed with the [1-^{13}C]leucine model in the experiment in Chapter-1. These results accord with the observation of Al-Mamun *et al.* (2007), who used both [$^{2}H_5$]Phe and [1-^{13}C]leucine models to determine the WBPS in sheep and reported that the numerical values of WBPS obtained from the [$^{2}H_5$]Phe model were lower than those obtained from the [1-^{13}C]leucine model. As discussed in Chapter-1, the higher PheOX in sheep fed the ECHM-diet in current experiment might be related to a positive impact of Chinese herbal medicine on accelerating nutrients oxidation. In addition, because the nitrogen content was estimated to be higher for ECHM-diet than for MH-diet, the trends of higher plasma PheTR and TyrTR as well as the higher WBPS might be due to a higher dietary CP intake and the effect of the bioactive components of Chinese herbal medicine in ECHM-diet.

In addition, the results of the present study showed that the supplementation of the extract of Chinese herbal medicine could improve rumen fermentation as well as enhance plasma glucose and protein metabolism in sheep. Therefore, compared with the unprocessed form, the extract of Chinese herbal medicine was considered to be more suitable and safer for feeding animals due to the reduced foreign substances and toxic contents.

Chapter-4

Effects of Extract of Chinese Herbal Medicine on Nitrogen Balance, Microbial Nitrogen Supply and Plasma Leucine Kinetics in Sheep

Introduction

In ruminants, amino acids are supplied by three types of protein, the microbial protein, the undegradable protein and the endogenous protein. Among the three types, microbial protein is the most important source of amino acids because it provides 50 to 80% of total absorbable protein to the small intestine (Stern *et al.*, 2006). Microbial protein is also a high quality protein that is easily digestible in the small intestine. Therefore, enhancing microbial protein synthesis in the rumen will be beneficial to protein metabolism in ruminants.

The results of the experiment in Chapter-3 have confirmed that the supplementation of the extract of Chinese herbal medicine could improve the WBPS in sheep. Thus, it can be expected that the extract of Chinese herbal medicine would also improve microbial protein synthesis as well as promote intestinal amino acid absorption in sheep. Considering about this point, the present study was carried out to assess the feeding effects of the extract of Astragalus root, Angelica root and Atractylodes rhizome on nitrogen balance, microbial nitrogen supply and plasma leucine kinetics in sheep.

Materials and methods

1. Animals, diets and management

The handling of animals, including cannulation and blood collection, was reviewed and approved by the Animal Care Committee of Iwate University. All experimental procedures were performed without any noticeable stress to the animals.

The extract of Astragalus root, Angelica root and Atractylodes rhizome was made in liquid form according to the traditional processing method. The herbs were boiled for three times (20 min for each time) and the boiled water was taken as the extract.

Six crossbred (Corriedale × Suffolk) shorn wethers, aged 4 years on average, weighting 51 ± 3 kg, were used in the experiment. The sheep were assigned to two dietary treatments, including either mixed hay (MH-diet) of orchardgrass (*Dactylis glomerata*) and reed canarygrass (*Phalaris arundinacea*) offered at maintenance level (NRC, 1985) or MH-diet supplemented with 2% of extract of Chinese herbal medicine (ECHM-diet). The 2% of extract was calculated according to the ratio between the amount of herbs and the volume of boiled water (approximately per 100 g to 325 ml). The sheep received mixed hay at 67 g/kg$^{0.75}$/d for both dietary treatments. The experiment was performed using a crossover design over two 21-day

periods. The sheep were housed in individual pens in an animal room during the first 14 days of the experiment for adaptation. On day 15, the animals were moved to metabolic cages in a controlled environment house at an air temperature of 23 ± 1°C, with lighting present from 08:00 h to 22:00 h. Two sheep were fed the MH-diet during the first period and then fed the ECHM-diet during the second period. The other two sheep were subjected to the dietary treatments in the reverse order. The animals were given either diet twice a day at 08:30 h and 20:30 h. The extract of Chinese herbal medicine was injected into animal's mouth by a syringe before giving the mixed hay, and they commonly consumed all the diets which were fed within 1 h. Water was available *ad libitum*. The sheep were weighted at the start of experiment, on day 8 and 15, and at the end of each dietary treatment.

2. Experimental procedures

Nitrogen balance trial was conducted over 3 successive days (from day 18 to 20) of each dietary treatment. Urine and feces samples were collected from each sheep at 24-hour intervals. The urine was collected in a plastic bucket containing 50 ml of 6 N H_2SO_4 solutions to prevent N loss. After recording the total volume, subsamples (50 ml) were taken for further processing. From each subsample, 5ml of urine was taken and diluted with water by 5 times to prevent precipitation for determining purine derivatives (PD)

excretion. Then the diluted urine samples and the remaining subsamples were stored at -30°C for further analysis. The collected feces were dried at 60°C for 48 h and then placed at room temperature for 5 days. After weighing the air dried samples, subsamples were ground and stored for further analysis.

On day 20 of each dietary treatment, rumen fluid (30 ml) was collected through a stomach tube before feeding, 3 and 6 h after feeding. The rumen fluid was centrifuged at 8,000 x *g* for 10 min at 4°C (RS-18IV, Tomy, Japan). Then 1 ml of supernatant was mixed with 1 ml of 0.1 N HCl. The prepared samples were stored at -30°C for the determination of rumen ammonia (NH_3).

On day 21 of each dietary treatment, the isotope dilution method of [1-^{13}C]leucine (Leu) was conducted to determine the plasma Leu kinetics in sheep. Two catheters, one for isotope infusion and the other for blood collection were inserted into the left and right jugular veins on the morning of isotope dilution method. The catheters were filled with sterile solution of 3.8% trisodium citrate. At 12:00 h, 7.2 μmol/kg$^{0.75}$ of [1-^{13}C]leucine (L-leucine-1-^{13}C, 99 atom% excess ^{13}C; Cambridge Isotope Laboratories, USA) dissolved in saline solution was injected into the infusion catheter as a priming dose. After the priming injection, [1-^{13}C]leucine was then continuously infused by multichannel peristaltic pumps at rate of 7.2

μmol/kg$^{0.75}$/h through the same catheters for 4 h. Blood samples were taken from the sampling catheter immediately before (12 ml) and at 30-min intervals (6 ml) during the last 120 min of isotope infusion. The collected samples were immediately transferred into centrifuge tubes containing heparin sodium and were stored in crushed ice. Plasma was separated from blood samples by means of centrifugation at 10,000 x *g* for 10 min at 4°C and then stored at -30°C for further analysis. The catheters were removed at the end of isotope infusion of each dietary treatment.

The schematic layout of experiment drawing sampling protocol is shown in Figure 4-1.

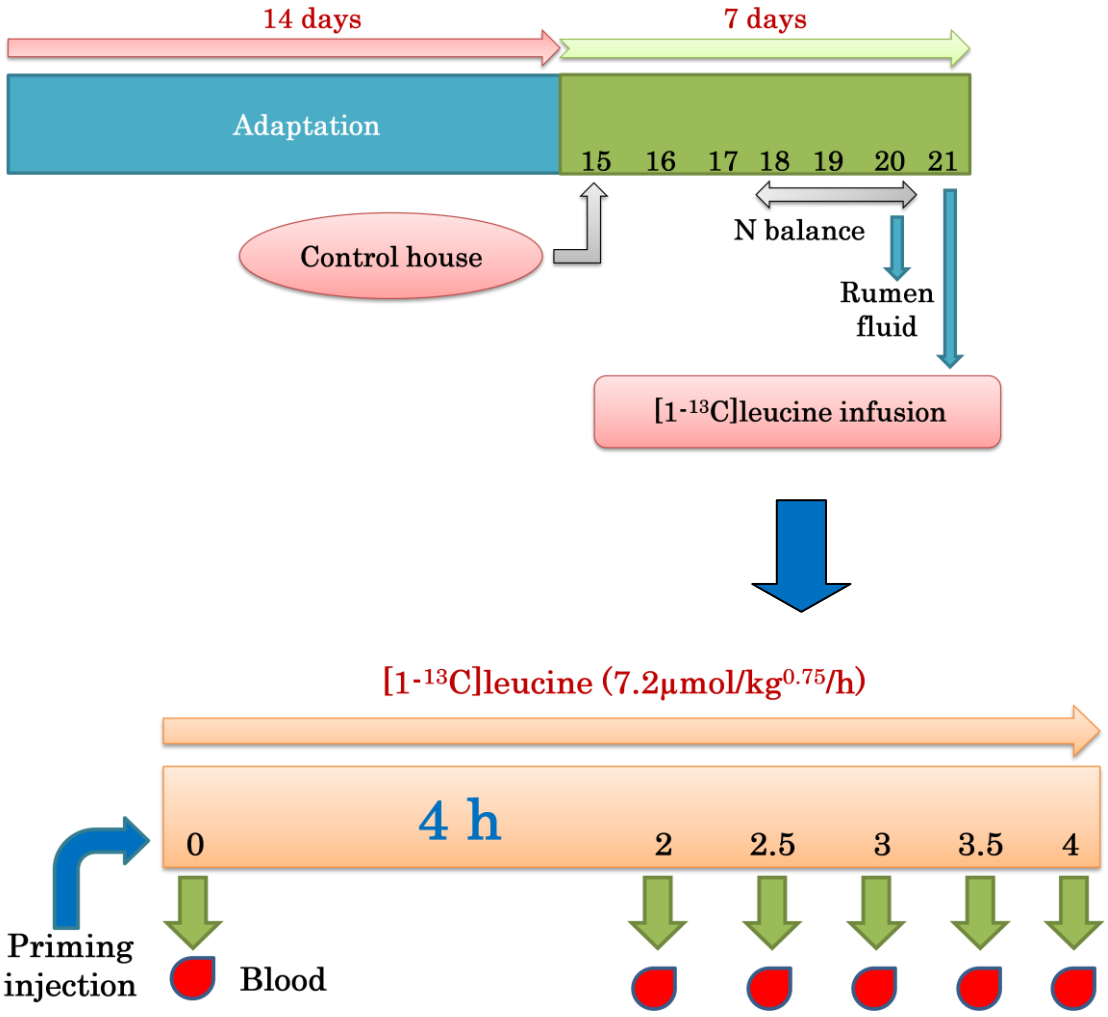


Figure4-1 The experimental layout showing sampling protocol.

3. Chemical analysis

The pool model of PD excretion is shown in Figure 4-2. The PD excretion, including allantoin, uric acid and xanthine plus hypoxanthine from diluted urine samples were determined by spectrophotometer (V-630 BIO, JASCO, Japan) according to the procedure of Chen and Gomes (1992).

The chemical analysis of diets, urine and feces (N contents), rumen fluid (NH_3 concentration) and plasma samples (amino acids, urea, α-KIC concentration and α-[1-^{13}C]KIC enrichment) was performed with the same procedures as described in Chapter-1 and Chapter-2.

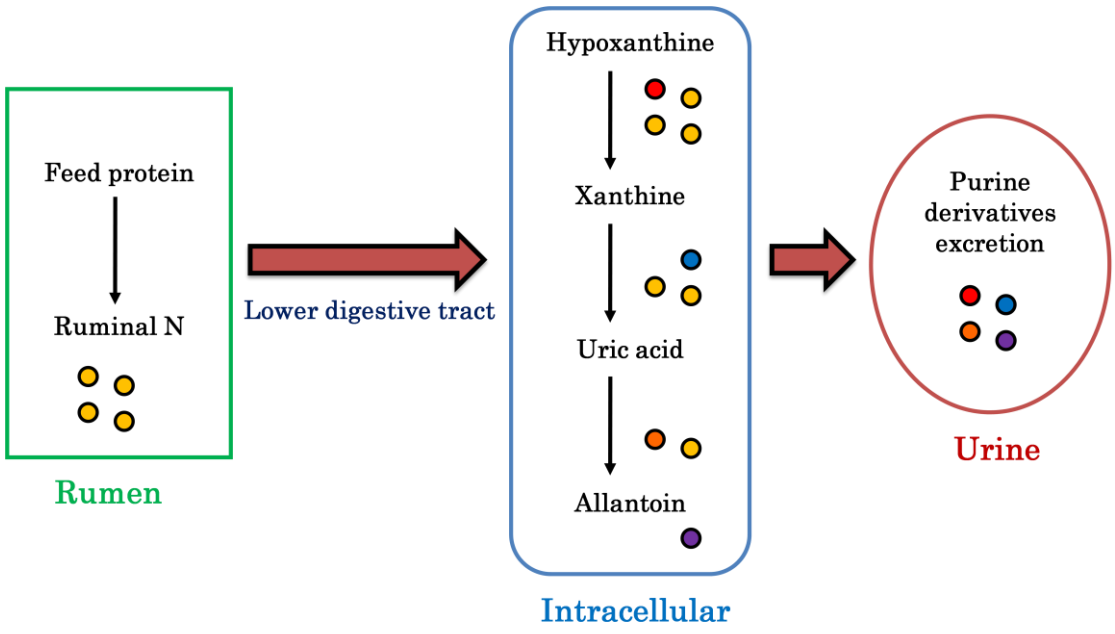


Figure 4-2 Pool model of purine derivatives excretion.

4. Calculations

The amount of microbial purines absorbed (X) corresponding to the PD excreted (Y) was calculated using the following equation as described by Chen *et al.* (1990):

$$Y = 0.84\,X + (0.150BW^{0.75}\,e^{-0.25X})$$

Where, *0.84* is the recovery of absorbed purines as PD in urine and $0.150BW^{0.75}$ (metabolic body size) represents the endogenous contribution of purine excretion. The calculation of X from Y based on the equation was made by means of the Newton's iteration process.

The microbial N supply (MNS) was calculated using the equation given by Chen and Gomes (1992):

$$MNS = 70 \times X / 0.83 \times 0.116 \times 1000$$

Where, *70* is the N content of purines, *0.83* is the digestibility of microbial purines, and *0.116* is the ratio of purine N to total N in mixed microbial biomass (Chen, 1989).

The turnover rate of plasma leucine (LeuTR) was calculated using the same equation as described in Chapter-1.

5. Statistical analysis

Statistical analysis was conducted according to the MIXED procedure of SAS (1996). Results were considered significant at $P<0.05$ level, and tendency was at $0.05 \leq P < 0.10$.

Results

Mean values with standard error of the mean (SEM) were given. The nitrogen contents in experimental diets are shown in Table 4-1. Nitrogen balance and rumen NH_3 concentration data are presented in Table 4-2. Nitrogen intake was higher ($P<0.01$) for ECHM-diet than MH-diet. Nitrogen excretion through feces was lower ($P=0.02$) for ECHM-diet compared with MH-diet. Nitrogen excretion through urine was similar for both diets. Nitrogen retention did not differ between dietary treatments. Nitrogen digestibility was higher ($P=0.02$) for ECHM-diet than MH-diet. Concentration of rumen NH_3 tended to be higher ($P=0.08$) for ECHM-diet than MH-diet.

Plasma amino acids and urea determined in the pre-infusion period of isotope dilution method are presented in Table 4-3. Concentrations of plasma threonine and glycine were lower ($P<0.05$) for ECHM-diet than MH-diet.

Concentrations of plasma iso-leucine, leucine, serine and total amino acid tended to be lower (*P<0.10*) for ECHM-diet compared with MH-diet. Concentration of plasma urea did not differ between diets.

Urinary allantoin, uric acid, xanthine plus hypoxanthine and total PD excretion in the urine were higher (*P<0.01*) for ECHM-diet than MH-diet. The MNS was also higher (*P<0.01*) for ECHM-diet compared with MH-diet (Table 4-4).

Concentration of plasma α-KIC and enrichment of plasma α-[1-^{13}C]KIC were stable during the last 2 h of [1-^{13}C]leucine infusion (Figure 4-3). Concentration of plasma α-KIC tended to be lower (*P=0.09*) for ECHM-diet than MH-diet. Plasma LeuTR tended to be higher (*P=0.06*) for ECHM-diet compared with MH-diet (Table 4-4).

Table 4-1 Nitrogen contents in experimental diets

Item	MH*	ECHM
N content	18.7 mg/g	3.9 mg/ml

*Values are presented on air dry matter basis; MH = mixed hay of orchardgrass and reed canarygrass; ECHM = extract of Chinese herbal medicine.

Table 4-2 Effects of extract of Chinese herbal medicine on nitrogen balance and rumen NH_3 concentration in sheep

Items	MH-diet	ECHM-diet	SEM	*P*value
Parameters of N balance ($g/kg^{0.75}/d$)				
N intake	1.27	1.29	0.01	<0.01
N in feces	0.50	0.46	0.03	0.02
N in urine	0.46	0.47	0.07	0.65
N balance	0.31	0.35	0.04	0.17
N digestibility (%)	61	64	1	0.02
Rumen NH_3 (mmol/L)	10.0	11.9	0.9	0.08

MH-diet = mixed hay of orchardgrass and reed canarygrass; ECHM-diet = MH-diet supplemented with 2% of extract of Chinese herbal medicine; SEM = standard error of the mean.

Table 4-3 Effects of extract of Chinese herbal medicine on plasma amino acid and urea concentrations in sheep

Items	MH-diet	ECHM-diet	SEM	*P*-value
Plasma free AA (μmol/L)				
Threonine	245	210	17	0.03
Valine	297	285	14	0.14
Methionine	29	30	6	0.30
Iso-leucine	116	83	10	0.06
Leucine	160	111	19	0.08
Phenylalanine	60	51	4	0.34
Histidine	68	79	15	0.27
Lysine	95	74	11	0.12
Serine	178	139	25	0.06
Asparagine	50	47	5	0.41
Glutamic acid	91	88	11	0.45
Glutamine	278	259	29	0.19
Glycine	492	422	33	0.04
Alanine	212	189	18	0.17
Tyrosine	74	80	3	0.51
Tryptophan	44	41	2	0.60
Arginine	160	143	13	0.17
Proline	101	84	20	0.33
Total AA	2750	2415	301	0.07
Urea (mmol/L)	6.7	5.8	0.2	0.18

MH-diet = mixed hay of orchardgrass and reed canarygrass; ECHM-diet = MH-diet supplemented with 2% of extract of Chinese herbal medicine; SEM = standard error of the mean; AA = amino acid.

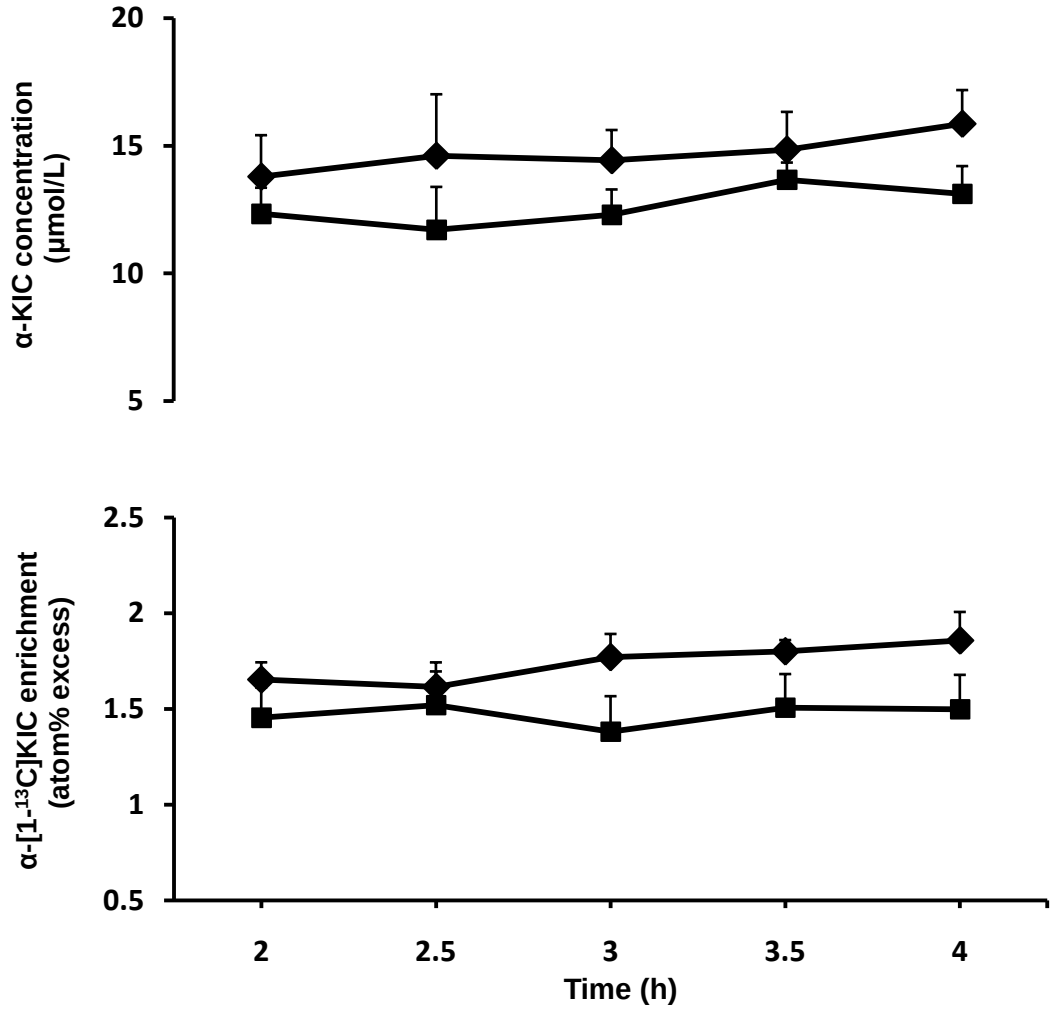


Figure 4-3 Time course of changes in plasma α-ketoisocaproic acid (α-KIC) concentration and plasma α-[1-^{13}C]KIC enrichment during the last 2 h of [1-^{13}C]leucine infusion in sheep fed two different diets (◆ = MH-diet, ■ = ECHM-diet).

Table 4-4 Effects of extract of Chinese herbal medicine on purine derivatives (PD) excretion, microbial nitrogen supply (MNS) and plasma leucine kinetics in sheep

Items	MH-diet	ECHM-diet	SEM	*P*-value
Parameters of PD excretion (mmol/kg$^{0.75}$/d)				
Allantoin	0.34	0.46	0.02	<0.01
Uric acid	0.03	0.05	0.01	<0.01
Xanthine + hypoxanthine	0.05	0.06	0.01	<0.01
Total PD	0.43	0.57	0.02	<0.01
MNS (g/kg$^{0.75}$/d)	0.36	0.49	0.02	<0.01
Plasma leucine kinetics				
α-KIC concentration (μmol/L)	14.7	12.9	1.2	0.09
LeuTR (μmol/kg$^{0.75}$/h)	373	437	16	0.06

MH-diet = mixed hay of orchardgrass and reed canarygrass; ECHM-diet = MH-diet supplemented with 2% of extract of Chinese herbal medicine; SEM = standard error of the mean; α-KIC = α-ketoisocaproic acid; LeuTR = turnover rate of plasma leucine.

Discussion

1. Nitrogen balance and rumen NH_3 concentration

The results of nitrogen balance test were comparable to the experiment in Chapter-2. Lower N excretion through feces resulted in higher N digestibility for ECHM-diet compared with MH-diet, whereas N retention did not differ

between diets. Although N intake was higher for ECHM-diet than MH-diet, the numerical figures were very close (1.27 and 1.29, respectively), which seems insufficient to influence N digestibility. Therefore, these results suggested that the Chinese herbal medicine might play a role to enhance N digestibility but without improving N retention in sheep.

Concentration of rumen NH_3 tended to be higher for ECHM-diet than MH-diet, which might be largely related to the higher dietary CP intake in ECHM-diet. The dietary nitrogenous substances are fermented to NH_3 in the rumen, and then the NH_3 is used as the main N source for microbial protein synthesis in the rumen. Hence, the higher rumen NH_3 concentration indicated that more N source is available for microbial protein synthesis in sheep fed ECHM-diet than MH-diet.

2. Plasma free amino acids

Concentrations of plasma threonine and glycine were lower, and concentrations of plasma iso-leucine, leucine, serine and total amino acid tended to be lower for ECHM-diet than MH-diet. The lower plasma amino acid concentrations indicated a higher protein synthetic state of sheep (Wessels *et al.*, 1997). Moreover, microbial protein is the major source of amino acids in ruminant animals, which contributes about two thirds of the absorbable amino acids (Pathak, 2008). Thus it could be assumed that more

microbial protein is available as the source of absorbable amino acids in sheep fed ECHM-diet than MH-diet.

3. Microbial nitrogen supply

The MNS was higher for ECHM-diet than MH-diet. Ruminal microbes provide the main source of protein to the ruminants. In the present study, the urinary excretion of purine derivatives was used to estimate the MNS because it is an important index of microbial protein status in ruminants (Chen, 1989). The higher MNS for ECHM-diet might be partly related to the higher N digestibility, where more N was absorbed and utilized for microbial protein synthesis in the rumen. Similar data can be found in a previous report of Kamruzzaman *et al.* (2014), where increased MNS was also observed with higher N digestibility in sheep. Furthermore, the findings of the past experiments have well demonstrated that the Chinese herbal medicine could modify rumen fermentation and increase whole body protein synthesis in sheep. Because microbial protein constitutes the major part of protein supply to the ruminants, the higher MNS for ECHM-diet in current experiment also indicated that the Chinese herbal medicine might play a role to improve N utilization in the rumen as well as enhance ruminal N metabolism in sheep.

4. Plasma leucine kinetics

Concentration of plasma α-KIC tended to be lower and plasma LeuTR tended to be higher for ECHM-diet compared with MH-diet. The enrichment of plasma α-[1-^{13}C]KIC was used to calculate plasma LeuTR instead of plasma [1-^{13}C]leucine because it represents the true kinetics of plasma leucine (Magni *et al.*, 1994). The trend of higher plasma LeuTR for ECHM-diet agrees with the findings in Chapter-3, where similar trends on plasma phenylalanine and tyrosine kinetics were also observed in sheep fed the same diets.

In ruminants, microbial protein is the high quality protein that is easily digestible and absorbable in the small intestine (Stern *et al.*, 2006). In the present study, the trend of higher plasma LeuTR in sheep fed the ECHM-diet might be partly caused by a higher supply of microbial protein as the source of absorbable amino acids from the small intestine to the tissues to support amino acid turnover. Fujita *et al.* (2006) reported that the supplementation of starch increased plasma phenylalanine and tyrosine turnover rates in goats through enhancing microbial protein synthesis in the rumen and amino acid absorption. Although the feeding treatment was different, their findings might also clarify the observation of the present study.

Summary and Conclusions

The use of antibiotics in animal diets is facing negative feedback due to the hidden danger of drug residues to human health. At present, the use of antibiotic growth promoters in animal industry is restricted in the European Union. Traditional Chinese herbal medicine has been used to replace antibiotics in the past two decades and played an increasingly important role in livestock production. However, so far, little information is available regarding the performance of Chinese herbal medicine on nutrients and energy metabolism in ruminant animals.

The mixture of Astragalus root, Angelica root and Atractylodes rhizome is a classical herbal formula for nourishment purpose in traditional Chinese medicine. In humans, it is commonly used as a health regulator to remove tiredness and comfort stress by inducing hematopoiesis. It was expected that the use of this Chinese herbal medicine mixture would be beneficial to nutrients and energy metabolism in ruminants due to its bioactive properties on hematopoiesis. Therefore, the present study was carried out to assess the feeding effects of the traditional nourishing Chinese herbal medicine mixture on kinetics of intermediate nutrients and energy metabolism in sheep.

As a basic investigation, the first experiment was conducted to assess the feeding effects of the Chinese herbal medicine mixture as feed additive on kinetics of plasma glucose, protein and energy metabolism in sheep. Ruminal fermentation characteristics were also determined. Four sheep were fed either mixed hay (MH-diet) or MH-diet supplemented with 2% of Chinese herbal medicine (CHM-diet) over two 35-day periods using a crossover design. The isotope dilution methods of [U-^{13}C]glucose and [1-^{13}C]leucine and open circuit calorimetry were used to determine plasma glucose and protein kinetics and heat production. The results showed that body weight gain of sheep was higher (*P=0.03*) for CHM-diet than MH-diet. Rumen pH was lower (*P=0.02*), concentration of rumen total volatile fatty acid tended to be higher (*P=0.05*) and acetate was higher (*P=0.04*) for CHM-diet compared with MH-diet. Turnover rates of plasma glucose and leucine did not differ between diets. Oxidation rate of leucine tended to be higher (*P=0.06*) for CHM-diet than MH-diet, but whole body protein synthesis did not differ between diets. Metabolic heat production tended to be greater (*P=0.05*) for CHM-diet compared with MH-diet. Although no significant effect on plasma glucose and protein metabolism was found, the sheep fed CHM-diet had a higher body weight gain and showed positive impacts on rumen fermentation and energy metabolism without resulting in any adverse response than those fed MH-diet.

Livestock animals exposed to cold causes a variety of hormonal and physiological changes, which result in negative effects on growth and production. In the first experiment, the CHM-diet showed positive impacts on growth and energy metabolism in sheep. Based on these findings, the second experiment was conducted to assess the feeding effects of Chinese herbal medicine on kinetics of plasma glucose, protein and energy metabolism in sheep kept at thermoneutral environment (23°C) or cold environment (2-4°C). The herbs were ground into a fine powder for feeding. Four sheep were subjected to either MH-diet or CHM-diet over two 23-day periods using a crossover design. Cold exposure was conducted for 5 days. The dilutions of [U-^{13}C]glucose and [1-^{13}C]leucine, N balance test and open-circuit calorimetry were carried out for determinations. The results showed that N intake was higher ($P<0.01$), N excretion through feces was lower ($P=0.04$) and N digestibility was higher ($P=0.02$) for CHM-diet than MH-diet. Rumen pH was lower ($P=0.03$), concentration of rumen NH_3 was higher ($P=0.04$), concentrations of rumen total VFA and acetate tended to be higher ($P<0.10$) and propionate was higher ($P=0.04$) for CHM-diet compared MH-diet. Turnover rate of plasma glucose was higher ($P=0.02$) for CHM-diet than MH-diet, and increased ($P<0.01$) during cold exposure. Oxidation rate of plasma glucose did not differ between diets and also between environments. Turnover rate of plasma leucine, whole body protein synthesis

and degradation were higher ($P<0.05$) for CHM-diet than MH-diet but remained similar between environmental temperatures. Metabolic heat production was greater ($P=0.03$) for CHM-diet compared with MH-diet, and increased ($P<0.01$) during cold exposure. No significant interaction was detected in diet and environment. It could be concluded that rumen fermentation as well as plasma glucose, protein and energy metabolism were enhanced by CHM-diet, but their responses to cold exposure were not modified by CHM-diet in sheep. Moreover, these results also suggested that plasma glucose and energy metabolism were more responsive to cold exposure than protein metabolism in sheep under the conditions of the present experiment.

The Chinese herbal medicine was directly given to the animals without any treatment in experiment 1, but was given in powder form in experiment 2. In the treatment of human diseases, Chinese herbal medicine is normally taken as syrup of its extract. The inconsistency of results between past experiments indicated that the processing of herbs also may be an important factor to enhance the feeding effects in animals. Therefore, the third experiment was conducted to assess the feeding effects of the extract of Chinese herbal medicine on kinetics of plasma glucose and protein metabolism in sheep. According to the traditional processing method, the herbs were boiled for three times and the boiled water was taken as the

extract. Six sheep were subjected to either MH-diet or ECHM-diet (MH-diet supplemented with 2% of extract of Chinese herbal medicine) over two 21-day periods using a crossover design. Plasma glucose kinetics was measured with the [U-^{13}C]glucose dilution. The dilution of [$^{2}H_5$]phenylalanine and [$^{2}H_2$]tyrosine was used to determine protein kinetics in this experiment. Rumen pH was lower (*P=0.04*), concentrations of rumen total VFA, acetate and propionate tended to be higher (*P<0.10*) for ECHM-diet than MH-diet. Turnover rate of plasma glucose was higher (*P=0.04*) for ECHM-diet compared with MH-diet. Turnover rates of plasma phenylalanine and tyrosine tended to be higher (*P<0.10*), and rate of phenylalanine hydroxylation to tyrosine was higher (*P=0.02*) for ECHM-diet than MH-diet. Whole body protein synthesis was also higher (*P=0.04*) for ECHM-diet compared with MH-diet. The results of the present experiment verified that the extract of Chinese herbal medicine could improve rumen fermentation as well as enhance plasma glucose and protein metabolism in sheep. Thus the extract was considered to be more suitable and safer than the unprocessed form for animals due to the reduced foreign substances and toxic contents.

Microbial protein is the most important source of amino acids for ruminants because it provides 50 to 80% of total absorbable protein to the small intestine. Enhancing microbial protein synthesis in the rumen will be

beneficial to protein metabolism in ruminants. It was expected that the Chinese herbal medicine would also enhance microbial protein synthesis as well as improve intestinal amino acid absorption. Therefore, the fourth experiment was conducted to assess the feeding effects of the extract of Chinese herbal medicine on nitrogen balance, microbial nitrogen supply and plasma leucine kinetics in sheep. Six sheep were subjected to either MH-diet or ECHM-diet over two 21-day periods using a crossover design. The dilution of [1-^{13}C]leucine was used to determine the plasma leucine kinetics. N intake was higher (*P<0.01*), N excretion through feces was lower (*P=0.02*) and N digestibility was higher (*P=0.02*) for ECHM-diet than MH-diet. Concentration of rumen NH_3 tended to be higher (*P=0.08*) for ECHM-diet compared with MH-diet. Microbial N supply was higher (*P<0.01*) for ECHM-diet than MH-diet. Turnover rate of plasma leucine tended to be higher (*P=0.06*) for ECHM-diet compared with MH-diet. From the results, the increased N digestibility and higher microbial N supply in sheep fed ECHM-diet suggested that the Chinese herbal medicine might play a role to improve N digestibility and might produce positive impact on ruminal N metabolism in sheep.

From the present findings, it could be concluded that the supplementation of the Chinese herbal medicine mixture to mixed hay diet could increase rumen VFA concentration as well as enhance plasma glucose,

protein and energy metabolism in sheep. However, the responses of these nutrients and energy metabolism to cold exposure were not modified by Chinese herbal medicine. In addition, the extract of Chinese herbal medicine was considered to be more suitable and safer for feeding animals due to the reduced foreign substances and toxic contents. Therefore, these results suggested that the Chinese herbal medicine mixture should be considered as a potential feed additive for sheep.

References

Al-Mamun M, Hanai Y, Tanaka C, Tamura Y, Sano H. 2008. Responses of whole body protein synthesis and degradation to plantain herb in sheep exposed to heat. *Archives of Animal Nutrition* **62,** 219-229.

Al-Mamun M, Ito C, Sato A, Fujita T, Sano H. 2007. Comparison of the [$^{2}H_{5}$]phenylalanine model with the [1-^{13}C]leucine method to determine whole body protein synthesis and degradation in sheep fed at two levels. *Asian-Australasian Journal of Animal Sciences* **20,** 1517-1524.

Al-Mamun M, Tanaka C, Hanai Y, Tamura Y, Sano H. 2007. Effects of plantain (*Plantago lanceolata* L.) herb and heat exposure on plasma glucose metabolism in sheep. *Asian-Australasian Journal of Animal Sciences* **20,** 894-899.

Alam MK, Ogata Y, Sako Y, Al-Mamun M, Sano H. 2010. Intermediary metabolism of plasma acetic acid, glucose and protein in sheep fed a rice straw-based diet. *Asian-Australasian Journal of Animal Sciences* **23,** 1333-1339.

Allsop JR, Wolfe RR, Burke JF. 1978. Tracer priming the bicarbonate pool. *Journal of Applied Physiology* **45,** 137-139.

AOAC. 1995. *Official Methods of Analysis* 16th edn. Association of Official Analytical Chemists, Arlington, VA.

Barton MD. 2000. Antibiotic use in animal feed and its impact on human health. *Nutrition Research Reviews* **13,** 279-299.

Brouwer E. 1965. Report of sub-committee on constants and factors. In: KL Blaxter (ed), *Energy Metabolism*, pp. 302-304. Academic Press, London.

Calder AG, Smith A. 1988. Stable isotope ratio analysis of leucine and ketoisocaproic acid in blood plasma by gas chromatography/mass spectrometry. Use of tertiary butyldimenthylsilyl derivatives. *Rapid Communications in Mass Spectrometry* **2,** 14-16.

Castillejos L, Calsamiglia S, Ferret A, Losa R. 2007. Effects of dose and adaptation time of a specific blend of essential oil compounds on rumen fermentation. *Animal Feed Science and Technology* **132,** 186-201.

Castillejos L, Calsamiglia S, Ferret A. 2006. Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in in vitro systems. *Journal of Dairy Science* **89,** 2649-2658.

Castillejos L, Calsamiglia S, Ferret A, Losa R. 2005. Effects of a specific blend of essential oil compounds and the type of diet of on rumen microbial fermentation and nutrient flow from continuous culture systems. *Animal Feed Science and Technology* **119,** 29-41.

Chaves AV, Stanford K, Gibson LL, McAllister TA, Benchaar C. Effects of carvacrol and cinnamaldehyde on intake, rumen fermentation, growth performance, and carcass characteristics of growing lambs. *Animal Feed*

Science and Technology **145,** 396-408.

Chelikani PK, Ambrose JD, Keisler DH, Kennelly JJ. 2004. Effect of short-term fasting on plasma concentrations of leptin and other hormones and metabolites in dairy cattle. *Domestic Animal Endocrinology* **26,** 33-48.

Chen XB. 1989. Excretion of purine derivatives by cattle and sheep and its use for the estimation of absorbed microbial protein. [Ph.D. thesis], University of Aberdeen.

Chen XB, Gomes MJ. 1992. Estimation of microbial protein supply to sheep and cattle based on urinary excretion of purine derivatives - an overview of the technical details. International Feed Resources Unit, Rowett Research Institute, Bucksburn Aberdeen AB2 9SB, UK Occasional Publication.

Chen XB, Hovell FD, 0rskov ER, Brown DS. 1990. Excretion of purine derivatives by ruminants: effect of exogenous nucleic acid supply on purine derivative excretion by sheep. *British Journal of Nutrition* **63,** 131-142.

Chilliard Y, Ferlay A, Faulconnier Y, Bonnet M, Rouel J, Bocquier F. 2000. Adipose tissue metabolism and its role in adaptations to undernutrition in ruminants. *Proceedings of the Nutrition Society* **59,** 127-134.

Devant M, Anglada A, Bach A. 2007. Effects of plant extract

supplementation on rumen fermentation and metabolism in young Holstein bulls consuming high levels of concentrate. *Animal Feed Science and Technology* **137,** 46-57.

Dibner JJ, Richards JD. 2005. Antibiotics growth promoter in agriculture: history and mode of action. *Poultry Science* **84,** 634-643.

El-Shafei AA, Al-Gamal MA, Abdelrahman AS, Arafa MM. 2013. Influence of different levels of Astragalus root powder in broiler chick diets on the physiological and biochemical changes. *Journal of Applied Sciences Research* **9,** 2104-2118.

Freeman AS, Galyean ML, Caton JS. 1992. Effects of supplemental protein percentage and feeding level on intake, ruminal fermentation, and digesta passage in beef steers fed prairie hay. *Journal of Animal Science* **70,** 1562-1572.

Fujita T, Kajita M, Sano H. 2006. Responses of whole body protein synthesis, nitrogen retention and glucose kinetics to supplemental starch in goats. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **144,** 180-187.

Fujita T, Majima H, Itoh T, Sano H. 2006. Combined effect of salinomycin and feeding on whole body glucose kinetics in sheep fed a high-concentrate diet. *Reproduction, Nutrition, Development* **46,** 503-514.

Haaland GL, Tyrrell HF, Moe PW, Wheeler WE. 1982. Effect of crude protein level and limestone buffer in diets fed at two levels of intake on rumen pH, ammonia-nitrogen, buffering capacity and volatile fatty acid concentration of cattle. *Journal of Animal Science* **55,** 943-950.

Harris PM, Skene PA, Buchan V, Milne E, Calder AG, Anderson SE, Connell A, Lobley GE. 1992. Effect of food intake on hind-limb and whole-body protein metabolism in young growing sheep: chronic studies based on arterio-venous techniques. *British Journal of Nutrition* **68,** 389-407.

Hristov S, Maksimovic N, Stankovic B, Zujovic M, Pantelic V, Stanisic N, Zlatanovic Z. 2012. The most significant stressors in intensive sheep production. *Biotechnology in Animal Husbandry* **28,** 649-658.

Huang KC. 1998. *The Pharmacology of Chinese Herbs* 2nd edn. CRC Press, Boca Raton, Florida.

Huggett AG, Nixon DA. 1957. Enzymatic determination of blood glucose. *Biochemical Journal* **66,** 12.

Kamruzzaman M, Liang X, Sekiguchi N, Sano H. 2014. Effect of feeding garlic leaf on microbial nitrogen supply, kinetics of plasma phenylalanine, tyrosine and protein synthesis in sheep. *Animal Science Journal*. In press.

Kelley RO, Martz FA, Johnson HD. 1967. Effect of environmental temperature on ruminal volatile fatty acid levels with controlled feed

intake. *Journal of Dairy Science* **50,** 531-533.

Kennedy PM, Christopherson RJ, Milligan LP. 1976. The effect of cold exposure of sheep on digestion, rumen turnover time and efficiency of microbial synthesis. *British Journal of Nutrition* **36,** 231-242.

Kong X, Hu Y, Rui R, Wang D, Li X. 2004. Effects of Chinese herbal medicinal ingredients on peripheral lymphocyte proliferation and serum antibody titer after vaccination in chicken. *International Immunopharmacology* **4,** 975-982.

Lee SR, Sato H, Sasaki Y. 1989. Body temperature and heat production in sheep exposed to intermittent cold. *Asian-Australasian Journal of Animal Sciences* **2,** 214-215.

Li L, Yu H, Pan J. 1995. A study on protein metabolism in nephrotic patients treated with Chinese herbs. *Chinese Journal of Internal Medicine* **34,** 670-672.

Li X, Wei W. 2002. *Chinese Materia Medica: Combinations and Applications*. Donica Publishing, Potters Bar, Herts.

Lien TF, Horng YM, Wu CP. 2007. Feasibility of replacing antibiotic feed promoters with the Chinese traditional herbal medicine Bazhen in weaned piglets. *Livestock Production Science* **107,** 97-102.

Liu M, Wu K, Mao X, Wu Y, Ouyang J. 2010. Astragalus polysaccharide improves insulin sensitivity in KKAy mice: regulation of PKB/GLUT4

signaling in skeletal muscle. *Journal of Ethnopharmacology* **127,** 32-37.

Magni F, Arnoldi L, Galati G, Kienle MG. 1994. Simultaneous determination of plasma levels of α-ketoisocaproic acid and leucine and evaluation of α-[1-^{13}C]ketoisocaproic acid and [1-^{13}C]leucine enrichment by gas chromatography-mass spectrometry. *Analytical Biochemistry* **220,** 308-314.

McDowell GH. 1983. Hormonal control of glucose homoeostasis in ruminants. *Proceedings of the Nutrition Society* **42,** 149-167.

Miaron JOO, Christopherson RJ. 1997. Metabolic responses of the whole body, portal-drained viscera and hind quarter to acute cold exposure and feeding in sheep: Effects of nonselective and selective ß-adrenoceptor blockade. *Canadian Journal of Animal Science* **77,** 707-714.

NRC. 1985. *Nutrient requirement of sheep* 6th edn. National Academy Press, Washington, DC.

Opara EI. 2004. The efficacy and safety of Chinese herbal medicines. *British Journal of Nutrition* **91,** 171-173.

Ortigues-Marty I, Vernet J, Majdoub L. 2003. Whole body glucose turnover in growing and non-productive adult ruminants: meta-analysis and review. *Reproduction, Nutrition, Development* **43,** 371-383.

Patra AK. 2011. Effects of essential oils on rumen fermentation, microbial ecology and ruminant production. *Asian Journal of Animal and*

Veterinary Advances **6,** 416-428.

Pathak AK. 2008. Various factors affecting microbial protein synthesis in the rumen. *Veterinary World* **1,** 186-189.

Rocchiccioli F, Leroux JP, Cartier P. 1981. Quantitation of 2-ketoacids in biological fluids by gas chromatography chemical ionization mass spectrometry of o-trimethylsilyl-quinoxalinol derivatives. *Biomedical Mass Spectrometry* **8,** 160-164.

Romero-Perez GA, Ominski KH, McAllister TA, Krause DO. 2011. Effect of environmental factors and influence of rumen and hindgut biogeography on bacterial communities in steers. *Applied and Environmental Microbiology* **77,** 258-268.

Salman FM, EI-Kadi RI, Abdel-Rahman H, Ahmed SM, Mohammad MI, Shoukry MM. 2008. Biologically treated sugar beet pulp as a supplement in goat rations. *International Journal of Agriculture and Biology* **10,** 412-416.

Sano H, Kajita M, Fujita T. 2004. Effect of dietary protein intake on plasma leucine flux, protein synthesis, and degradation in sheep. *Comparative Biochemistry and Physiology* **B139,** 163-168.

Sano H, Murakami S, Sasaki S, Al-Mamun M. 2010. Effects of dietary energy intake and cold exposure on kinetics of plasma phenylalanine, tyrosine and protein synthesis in sheep. *Archives of Animal Nutrition* **64,** 47-55.

Sano H, Nakamura S, Kobayashi S, Takahashi H, Terashima Y. 1995. Effect of cold exposure on profiles of metabolic and endocrine responses and on responses to feeding and arginine injection in sheep. *Journal of Animal Science* **73,** 2054-2062.

Sano H, Sawada H, Takenami A, Al-Mamun M. 2009. Effects of diet and cold exposure on rates of plasma leucine turnover and protein synthesis in sheep. *Journal of Agricultural Science (Cambridge)* **147,** 91-97.

Sano H, Sawada H, Takenami A, Oda S, Al-Mamun M. 2007. Effects of dietary energy intake and cold exposure on kinetics of plasma glucose metabolism in sheep. *Journal of Animal Physiology and Animal Nutrition* **91,** 1-5.

Sano H, Takebayashi A, Kodama Y, Nakamura K, Ito H, Arino Y, Fujita T, Takahashi H, Ambo K. 1999. Effects of feed restriction and cold exposure on glucose metabolism in response to feeding and insulin in sheep. *Journal of Animal Science* **77,** 2564-2573.

SAS. 1996. SAS/STAT® *Software: Changes and Enhancements through Release* Version 6.11. SAS Institute, Cary, NC.

Schroeder GF, Titgemeyer EC, Awadeh MS, Smith JS, Gnad DP. 2006. Effects of energy level on methionine utilization by growing steers. *Journal of Animal Science* **84,** 1497-1504.

Soltan MAE, Shewita RS, Al-Sultan SI. 2009. Influence of essential oils

supplementation on digestion, rumen fermentation, rumen microbial populations and productive performance of dairy cows. *Asian Journal of Animal Sciences* **3,** 1-12.

Stern MD, Bach A, Calsamiglia S. 2006. New concepts in protein nutrition of ruminants. *21st Annual Southwest Nutrition & Management Conference* **AZ,** 45-66.

Sticker LS, Thompson DLJ, Bunting LD, Fernandez JM, DePew CL. 1995. Dietary protein and (or) energy restriction in mares: plasma glucose, insulin, nonesterified fatty acid, and urea nitrogen responses to feeding, glucose, and epinephrine. *Journal of Animal Science* **73,** 136-144.

Thompson GN, Pacy PJ, Merritt H, Ford GC, Read MA, Cheng KN, Halliday D. 1989. Rapid measurement of whole body and forearm protein turnover using a [$^{2}H_{5}$]phenylalanine model. *American Journal of Physiology* **256,** E631-639.

Tserng KY, Kalhan SC. 1983. Estimation of glucose cabron recycling and glucose turnover with [U-^{13}C]glucose. *American Journal of Physiology* **245,** E476-E482.

Tsuda T, Ambo K, Fujita M, Sunagawa K, Shoji Y. 1984. Distribution of energy source expenditure in warm- and cold-exposed sheep. *Canadian Journal of Animal Science* **64,** 265-266.

Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for dietary fiber,

neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* **74,** 3583-3597.

Wang CJ, Wang SP, Zhou H. 2009. Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. *Animal Feed Science and Technology* **148,** 157-166.

Wang HX, Liu CM, Liu Q, Gao K. 2008. Three types of sesquiterpenes from rhizomes of *Atractylodes lancea*. *Phytochemistry* **69,** 2088-2094.

Wang J, Zhou H. 2007. Comparison of the effects of Chinese herbs, probiotics and prebiotics with those of antibiotics in diets on the performance of meat ducks. *Journal of Animal and Feed Sciences* **16,** 96-103.

Weatherburn MW. 1967. Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry* **39,** 971-974.

Weekes TEC, Sasaki Y, Tsuda T. 1983. Enhanced responsiveness to insulin in sheep exposed to cold. *American Journal of Physiology* **244,** E335-345.

Wessels RH, Titgemeyer EC, St Jean G. 1997. Effect of amino acid supplementation on whole-body protein turnover in Holstein steers. *Journal of Animal Science* **75,** 3066-3073.

Wolfe RR. 1984. *Tracers in metabolic research: radioisotope and stable isotope/mass spectrometry methods*. Alan R. Liss, New York.

Wolfe RR, Goodenough RD, Wolfe MH, Royle GT, Nadel ER. 1982. Isotopic analysis of leucine and urea metabolism in exercising humans. *Journal*

of Applied Physiology **52,** 458-466.

Wu MJ, Sun XJ, Dai YH, Guo FQ, Huang LF, Liang YZ. 2005. Determination of constituents of essential oil from *Angelica sinensis* by gas chromatography-mass spectrometry. *Journal of Central South University* **12,** 430-436.

Yang WZ, Benchaar C, Ametaj BN, Chaves AV, He ML, McAllister TA. 2007. Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. *Journal of Dairy Science* **90,** 5671-5681.

Young BA. 1981. Cold stress as it affects animal production. *Journal of Animal Science* **52,** 154-163.

Young BA, Kerrigan B, Christopherson RJ. 1975. A versatile respiratory pattern analyzer for studies of energy metabolism of livestock. *Canadian Journal of Animal Science* **55,** 17-22.