Bioactive Constituents, Metabolites, and Functions

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Purified dietary red and white meat proteins show beneficial effects on growth and metabolism of young rats compared to casein and soy protein

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Abstract

This study compared the effects of casein, soy protein (SP), red (RMP) and white meat (WMP) proteins on growth and metabolism of young rats. Compared to casein, the ratio of daily feed intake to daily body weight gain of rats was not changed by meat protein but reduced by SP by 93.3% ($P<0.05$). Feeding RMP and WMP reduced the liver total cholesterol (TC) contents by 24.3% and 17.8% respectively ($P<0.05$). Only RMP increased plasma HDL-cholesterol concentrations (by 12.7%, $P<0.05$), whereas SP increased plasma triacylglycerol, TC and LDL-cholesterol concentrations by 23.7%, 19.5% and 61.5% respectively ($P<0.05$). Plasma essential and total amino acid concentrations were increased by WMP (by 18.8% and 12.4%, $P<0.05$) but reduced by SP (by 28.3 and 37.7%, $P<0.05$). Twenty five liver proteins were differentially expressed in response to different protein sources. Therefore, meat proteins were beneficial for growth and metabolism of young rats compared to casein and SP.

Keywords: red meat; white meat; protein quality; molecular nutrition; proteomics;
Introduction

Meat is a nutrient dense food which contains high quality protein and important micronutrients such as vitamin B12, iron and zinc\(^1\). Mammalian muscle meat such as beef and pork are regarded as red meat\(^2\), whereas chicken and fish\(^3\) are regarded as white meat. Recently, some epidemiologic studies associated high consumption of red or processed meat with several types of cancer\(^2\). In October, 2015, WHO released a report, which classified red and processed meat as “probably carcinogenic to humans” (Group 2A) and “carcinogenic to humans” (Group 1), respectively\(^2\). The publication of the report soon aroused widespread concerns about meat food all over the world. It also sparked heated debate in both academic and meat industrial areas, because the report was produced only based on the review of epidemiologic studies\(^4\). The reported carcinogenic effects of red and processed meat were mainly attributed to heme iron and the carcinogenic chemicals, such as N-nitroso-compounds and polycyclic aromatic hydrocarbons, that can be formed during meat processing and cooking\(^2\).

However, it is unequivocal that lean meat is an important protein source in human diets. It has been acknowledged that meat protein has high biological availability due to its high digestibility and containing all nutritionally essential amino acids (AAs), compared to plant protein\(^1\). Therefore, moderate intake of meat is advised, instead of avoiding meat food.

Under the globally increasing prevalence of obesity and metabolic syndrome in both adult and children\(^5\text{-}^6\), dietary protein is regarded as the most promising macronutrient for improving of body composition and metabolic profile due to its pronounced satiating, thermogenic and lean body mass preserving effects compared to other macronutrients lipid and carbohydrate\(^7\text{-}^9\). Until now, most of the studies on dietary protein have focused on dietary protein levels\(^7\text{-}^10\). However, very few studies forced
on different protein sources. Milk and meat are important animal protein sources whereas soy is an important plant protein source for human health. Considering their profound differences in AA and protein compositions\textsuperscript{1,11-12}, different biological effects were thus anticipated. Our previous study found that soy and meat proteins induce distinct physiological and metabolic responses in rats after a short time intervention (7 days)\textsuperscript{13-15}. It has been acknowledged that the nutritional conditions in early life can profoundly influence human long-term health\textsuperscript{16}. It was recommended by the 2015-2020 Dietary Guidelines for Americans that for children aged 2 and over, a health eating pattern should include a variety of protein foods in nutrient-dense forms from both animal and plant sources, like dairy, seafood, poultry, nuts and soy products, but reduce consumption of red meat and processed meat products\textsuperscript{17}. These guidelines were put forward on the basis of evidence from mostly epidemiologic studies, which have shown that reduced intake of red meat as well as processed meat are associated with reduced risk of cardiovascular disease, obesity, type 2 diabetes, and some types of cancer\textsuperscript{17}. However, there is still lack of sufficient and rigorous animal experiments to compare red meat with other protein sources. The aim of this study was to compare the effects of purified dietary protein sources from red meat, white meat, milk, and soy provided for a longer time (14 days) on growth and metabolism of young rats. To this end, young weaning rats were fed for 14 days the nutritionally balanced semi-synthetic AIN-93G diets with the only differences in protein sources. Growth, body compositions and blood biochemistry profiles were measured. To explore the molecular mechanism that may underlie the changes, liver metabolism in response to different dietary proteins were measured using 2-dimensional gel electrophoresis (2-DE) and mass spectrometry. There are three points to make our study unique. Firstly, to avoid the disturbance of the carcinogenic compounds that may be formed
during meat processing (such as curing, smoking, high cooking temperature), the purified meat protein sources were isolated from the cooked meat that was boiled in a 72°C water bath until the internal temperature reaching 70°C. Secondly, to avoid the disturbance from protein level or other nutrients, all diets in our study were prepared having the same balanced nutritional levels with the only differences in protein sources. Especially, the effects of red and white meat proteins were compared in this study. Our study provided novel evidence and important suggestions for the health effects of different protein sources in children diets.

Materials and Methods

Chemicals

*Longissimus dorsi* muscle of pigs and cattle and breast muscle of chicken were purchased from Su Shi Company (Nanjing, China). Dorsal muscle of fish were purchased from the local market. Diet ingredients including casein, cornstarch, dyetos, sucrose, soybean oil, cellulose, mineral mix, vitamin mix, L-Cystine and choline bitartrate were from Dyets Inc. (Bethlehem, PA). Food grade soy protein isolates were from Linyi Shansong biological products company (Linyi, China). Tissue triacylglycerol (TAG) and total cholesterol (TC) contents assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Plasma insulin Radioimmunoassay kit were from Beijing North Institute of Biological Technology (Beijing, China). Protease inhibitor cocktail was from Roche Applied Science (Penzberg, Germany). Chemicals used for 2-dimensional gel electrophoresis including RC DC protein assay kit II, ReadyPrep 2-D cleanup kit, bio-lyte 3/10 ampholyte 40%, IPG ReadyStrip/pH3-10/11cm/12, 12% precast gels, XT MOPS running buffer, iodoacetamide were from Bio-Rad (Hercules, CA, USA). The following reagents: Tris–HCl, SDS, urea, thiourea, 3-[(3-cholamidopropyl) dimethyl
ammonio]-1-propane-sulfonate (CHAPS) and DTT were purchased from Sigma (St. Louis, MO, USA).

**Animals and experimental diets**

All animals were handled in accordance with the guidelines for care and use of laboratory animals of the Jiangsu Provincial Academy of Agricultural Sciences (The license number was SCXK (Su) 2002-0029). Male *Sprague Dawley* rats at 3 weeks of age were randomly assigned to 6 groups of 10 rats each. The rats had free access to water and feed through the feeding period. After one-week acclimation, the rats were fed 14 days of one of the six experimental diets that were different only in protein sources (i.e. casein, soy, chicken, fish, beef or pork). The protein sources and diets used in this study were the same with our previous study. Briefly, raw meat materials were cooked in a 72°C water bath to an internal temperature of 70°C. Cooked meat were then freeze-dried and twice defatted with methylene chloride/methanol (2:1, v:v). The residual solvent was removed by evaporation and the resulting protein powder was passed through a 30 Mesh (0.595 mm) sieve. The final protein powders consisted of more than 90% of protein and 6-9% of water. All the diets were prepared according to the recommendations of the nutritionally balanced semisynthetic AIN-93G diet, which contained energy 4056 Kcal/Kg, protein 177 g/Kg, fat 70 g/Kg and carbohydrate 68 g/Kg. See Table 1 for specific diet formulations. To compare red and white meat proteins with casein and soy protein, beef and pork protein groups were combined as single red meat protein group (n=20), whereas chicken and fish protein groups were combined as single white meat protein group (n=20). Therefore, there were finally 4 groups of red meat protein group (n=20), white meat protein group (n=20), casein (n=10), and soy protein group (n=10).

**Sample collection**
During the 14 days’ feeding period, body weights and dietary intakes were measured every 2 days. On the day of sacrifice, rats were deprived of feed for 4 h prior to sacrifice but were given free access to water. Rats were anaesthetized with ether inhalation. Blood was taken by orbital puncture and plasma was isolated. Liver and epididymal adipose tissues were obtained, weighed and snap frozen in liquid nitrogen. All samples were stored at -80 °C until analysis.

**Liver lipid contents and plasma parameters detection**

Triacylglycerol (TAG) and total cholesterol (TC) contents in the liver were determined using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Plasma TAG, TC, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and total protein (TP) concentrations were analyzed using a Hitachi 7180 auto analyzer (Tokyo, Japan).

Plasma insulin concentrations were determined using a radioimmunoassay kit purchased from Beijing North Institute of Biological Technology (Beijing, China). The HOMA-IR was calculated according to the equation $\text{IR} = \frac{\text{fasting insulin in } \text{mU/L} \times \text{fasting glucose in mM}}{22.5}$. Plasma free AA concentrations were determined using a Hitachi L-8900 AA analyzer (Tokyo, Japan).

**Two-dimensional gel electrophoresis**

**Protein extraction and purification.** Protein extraction was performed as reported with some modifications. Livers were weighed and 100 mg tissue was homogenized with 1 ml lysis buffer: 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, wt/vol), 65 mM DTT, 2% biolyte pH 3-10, and 1% protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). Then the sample was centrifuged at 15,000 × g for 30 min at 4 °C and the
supernatant was transferred into new tubes. Protein extract was purified using the trichloroacetic acid (TCA)/acetone precipitation method described by Li et al.\textsuperscript{21} Briefly, protein was precipitated in 9 volumes of 10% TCA/80% acetone solution at -20 °C for 2 h. After centrifugation at 10,000 g for 30 min at 4 °C, the supernatant was discarded and the pellet was resuspended in a rehydration buffer (7 M urea, 2 M thiourea, 1% DTT). The protein contents were determined using RC DC Protein Assay Kit (BioRad, Cat. 500-0122).

**2-D gel electrophoresis.** The 2-D gel was run as reported previously\textsuperscript{21} with some modifications. Firstly, the purified protein samples were mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS (wt/vol), 1% DTT (wt/vol), 0.2% biolyte pH 3-10 (vol/vol), 0.002% bromophenol blue(wt/vol) to a final concentration of 1 mg/mL. Two hundred micrograms of protein (200 µL) was loaded on linear immobilized pH gradient strips (isoelectric point (pI) 3-10, 11 cm, BioRad, Cat. 1632014, Hercules, CA). After rehydrating at 17 °C for 12 h, isoelectric focusing was performed according to the program: 250 V (15 min), 8000 V (2.5 h) and 8000 V (35000 Vh). After finishing isoelectric focusing, the strip was first equilibrated in 5 ml equilibration buffer I (50 mM Tris–HCl, pH 8.8, 6 M urea, 20% glycerol (vol/vol), 2% SDS (wt/vol) and 1% DTT (wt/vol)) for 15 min, and then transferred to 5 ml equilibration buffer II (50 mM Tris-HCl, pH 8.8, 6 M urea, 20% glycerol (vol/vol), 2% SDS (wt/vol) and 4% (wt/vol) iodoacetamide) for 15 min. The equilibrated strip was placed on the top of a SDS-PAGE gel (12%), and then the second dimension electrophoresis was run at 200 V for 2 h at 4 °C. The 2-DE map was visualized by commassie blue staining.

**Image analysis.** Commassie blue stained gels were scanned, and the spots were detected and quantified with PDQuest v8.0.1 software (BioRad, Hercules, CA)
according to the software tutorial and the descriptions in other papers\textsuperscript{22-23}. For spot identifying and gel matching, both automatic and manual editing were performed to improve accuracy. The expression level of protein spot was normalized as a percentage of the total volume of all of the spots in the gel. Statistical analysis were based on the intensities of protein spots in gels (Supplementary Table 2), while protein expression changes were represented as fold changes. The numbers of biological repetitions of 2-DE analysis of casein, soy and red meat and white protein groups were 5, 5, 10 and 10, respectively.

\textit{In-gel trypsin digestion of protein.} The spots of interest were cut from the polyacrylamide gels and were destained with 500 µl of a solution (25 mM NH$_3$HCO$_3$ in 50% ACN) for 3×60 min, and then they were dehydrated using 100% ACN, reduced with 10 mM DTT at 56°C, and alkylated with 55 mM iodoacetamide without light exposure. Afterwards the samples were treated with 50 µl trypsin solution (1 µg trypsin in 100 µl 25 mM ammonium hydrogen carbonate in 25% ACN, pH 8.0) at 37 °C overnight.

\textit{Protein identification by mass spectrometry and functional analysis.} Proteins were identified by MALDI-TOF/TOF. The MS/MS data were searched against Mascot 2.3.02 (Matrix Science) applied to NCBI Rattus 1031(51807 seqs) based on the following search parameters: peptide mass tolerance: 100ppm; fragment mass tolerance, 0.6 Da; fixed modifications: Carbamidomethyl (C); variable modifications: Gln->pyro-Glu (N-term Q), Oxidation (M) and Deamidated (NQ); max missed cleavages: one. Significant scores > 70 and at least five peptide matches for each protein were used as criteria for positive protein identification. The gene ontology (GO) interpretation of proteins was done using PANTHER analysis\textsuperscript{24}.

\textbf{Statistical methods}
The diet effect on measured variables were analyzed by one-way ANOVA and means were compared by least significant difference (LSD) multiple comparison. Statistical significance was set at $P < 0.05$. Values are shown as means ± SD.

**Results**

**Body weight and body adiposity**

Rats in red or white meat protein groups had slightly higher initial body weights (IBWs) than the rats in casein group ($P < 0.05$, Figure 1A), whereas the IBWs of the rats in soy protein group were not different from casein or meat protein groups. Feeding red or white meat protein diets significantly increased the daily feed intakes (DFIs), daily body weight gains (DBWGs) and final body weights (FBWs) of rats. However, the DFI/DBWG ratio was not different between meat proteins and casein groups (Figure 1E). Feeding soy protein diet significantly reduced DBWGs (by 47.7%) and FBWs (by 22.7%) of rats ($P < 0.05$, Figure 1B) without affecting the DFIs compared to casein. As a result, the DFI/DBWG ratio was significantly increased by dietary soy protein compared to casein ($P < 0.05$, Figure 1E).

In order to evaluate the effects of different dietary protein sources on body adiposity, epididymal adipose tissue weight (EATW) and liver lipid contents were measured (Figure 2). Compared to casein, the percentage of EATW to BW was not affected by meat or soy proteins ($P > 0.05$, Figure 2A2). When compared between meat proteins and soy protein, the percentage of EATW to BW was lower for the soy protein group than meat protein groups. Liver TC contents were significantly reduced by red (by 24.3%, $P < 0.05$) or white meat proteins (by 17.8%, $P < 0.05$) but were not affected by soy protein compared to casein. The changes in liver TAG contents did not reach the significant level. Liver weight was reduced by soy, red meat and white meat proteins compared to casein ($P < 0.05$, Figure 2B).
Plasma profiles

Plasma lipid concentrations were significantly changed by different dietary protein sources (Figure 3). Plasma TAG concentrations were significantly increased by soy protein intake (by 23.7%, $P < 0.05$) but were not affected by red or white meat proteins compared to casein (Figure 3A1). When compared between red meat and white meat proteins, the rats fed white meat protein had lower plasma TAG concentration than the rats fed red meat protein (Figure 3A1). The pattern of the plasma TC concentration changes was the same with the plasma TAG concentrations regulated by dietary casein, soy, and meat proteins (Figure 3A2). Only red meat proteins increased the plasma HDL-C concentrations (Figure 3A3, by 12.7%, $P < 0.05$) in rats. Only soy protein increased the plasma LDL-C concentrations in rats (Figure 3A4, by 61.5%, $P < 0.05$). Plasma glucose concentrations, insulin level and HOMA-IR were significantly reduced by soy protein ($P < 0.05$, Figure 3B). Only red meat protein increased the plasma insulin levels and HOMA-IR.

Because that liver weights of rats were reduced by dietary soy and meat proteins, therefore plasma biomarkers for liver health, i.e. AST and ALT$^{25}$, were measured. The ratio of AST to ALT was calculated (Figure 4A). It was showed that plasma AST and ALT concentrations were significantly increased by soy protein (increased by 74.8% and 86.8%, respectively, $P < 0.05$) and white meat protein (increased by 26.2% and 34.2%, respectively, $P < 0.05$) but were not changed by red meat protein compared to casein (Figure 4A1 & A2). Notably, no significant changes were observed in the ratio of AST to ALT in any group (Figure 4A3). Plasma urea and total protein concentrations were measured to indicate the changes of AA degradation$^{26}$ and protein synthesis$^{27}$ in the liver. Only soy protein increased plasma urea concentrations (increased by 32%, $P < 0.05$, Figure 4B2) but reduced plasma total protein.
concentrations (reduced by 6.8%, $P < 0.05$, Figure 4B1). At the same time, plasma total AA concentrations were significantly reduced by soy protein compared to casein (reduced by 28.3%, $P < 0.05$, Table 2), among which the essential AA concentrations were reduced by 37.7% ($P < 0.05$) and non-essential AA concentrations were reduced by 16.3% ($P < 0.05$). In contrast, feeding white meat protein increased plasma essential and total AA concentrations compared to casein (increased by 18.8% and 12.4%, respectively, $P < 0.05$), whereas feeding red meat protein to rats did not affect their plasma essential and total AA concentrations.

Liver protein expression changes

The liver protein expressions were evaluated using 2-DE. Twenty five proteins were identified as differentially expressed in response to different dietary protein sources (Table 3). One liver protein relating to ATP biosynthesis ($\text{Atp5a1, ATP synthase subunit alpha}$) was significantly upregulated by dietary soy, white meat and red meat proteins compared to casein. Several proteins involving in AA metabolism, such as $\text{GOT1 (aspartate aminotransferase, AST)}$, $\text{OTC (ornithine carbamoyltransferase, urea cycle)}$, $\text{ALDH6A1 (methylmalonate-semialdehyde dehydrogenase, valine metabolic process)}$ and $\text{MAT1A (s-adenosylmethionine synthase isoform type-1, methionine metabolic process)}$, protein biosynthesis ($\text{EF1A1, elongation factor 1-alpha 1}$) and gluconeogenesis ($\text{FBP1, fructose-1,6-bisphosphatase 1}$) were significantly upregulated by dietary soy protein only ($P < 0.05$). On the contrary, several proteins relating to proteolysis ($\text{LAP3, cytosol aminopeptidase}$), protein transport ($\text{GCC2, GRIP and coiled-coil domain-containing protein 2}$), glycolysis ($\text{PKLR, Pyruvate kinase PKLR}$), and triacylglycerol biosynthesis ($\text{GPD1, Glycerol-3-phosphate dehydrogenase [NAD(+)]}$) were significantly downregulated by dietary soy protein only. Two liver proteins relating to iron ion transport ($\text{TF, serotransferrin}$) and...
response to oxidative stress (PRDX1, Peroxiredoxin-1) were upregulated by soy and
white meat proteins. In addition, seven liver proteins were found upregulated
specifically by dietary white meat protein, among which four proteins were
dehydrogenases and five proteins were in mitochondrion. These proteins were mainly
related to oxidation reactions in mitochondrion including processes of fatty acid
oxidation and electron transport. Two liver proteins relating to lactate metabolic
process (LDHA, L-lactate dehydrogenase A chain) and glycolysis (PKLR, pyruvate
kinase PKLR) were upregulated only by dietary red meat protein. Two other liver
proteins relating to hydrogen peroxide catabolic process (CAT, catalase) and
tricarboxylic acid cycle (MDH2, malate dehydrogenase) were upregulated and one
liver protein relating to transsulfuration (MPST, 3-mercaptopuruvate sulfurtransferase)
was downregulated by both dietary white and red meat proteins.

**Discussion**

This study compared the effects of dietary purified protein sources from milk, red
meat, white meat and soy provided at the nutritional recommended level on growth,
body compositions, blood insulin, lipid and AA profiles and live protein expression in
young weaning rats. Casein was chosen as reference protein source because from a
nutritional perspective it is a high-quality protein, and it is therefore used as protein
source in the well-balanced semi-synthetic AIN-93G diet\(^ {18} \). The AIN-93 diet is the
global standard for a purified rodent diets proposed by the American Institute of
Nutrition (AIN), and is considered as ‘golden standard’ in nutrition research. We
therefore used the AIN-93G diet as reference diet. For nutritional studies of
protein/amino acids, laboratory rats have been recommended and are generally
accepted as a valid animal model for predicting protein/amino acid nutrition and
metabolism in humans\(^ {28-29} \). Most of the early work about dietary amino acid tolerance
was done with rats fed casein-based purified diets\textsuperscript{30}. It has been suggested that use of
diets containing mixed ingredients and with normal protein levels is probably more
relevant in terms of extrapolation to humans\textsuperscript{30}. In our study, we used rats as animal
model, and the casein-based semi-synthetic diet (AIN-93G) was used as the reference
diet. All diets used in our study have normal protein levels but different protein
sources. Therefore, we believe the findings in our study might be relevant to humans.
Except for rodent, the farm animals like pigs have also been commonly used in
protein/amino acid studies\textsuperscript{28-29}. Recently, the voice of promoting the use of pigs as
animal model for human nutrition study is increasing\textsuperscript{31-32}. However, the early studies
with pigs (farm animals) were usually oriented to the immediate objective of
improving food production. This is quite different from human nutrition, in which
costs and efficiency of nutrient usage are often not overriding concerns\textsuperscript{28}. Therefore,
compared to studies with rats, the results from studies with pigs are less comparable
to human nutrition.
Our results showed that compared to meat proteins, feeding soy protein diet
significantly reduced the DFI of the rats, which was independent of the IBW of the
rats. These results were consistent with our previous study\textsuperscript{13}, in which the rats were
fed the same diets for a shorter time (7 days). As proved in our previous study, the
feed intake inhibition effects of dietary soy protein to the young rats were attributed to
the AA limitation (methionine) in the soy protein source. This was also found in the
present study from the responses of plasma AA concentrations in young rats. In the
present study the plasma total AA concentrations in the young rats fed soy protein diet
were significantly reduced (by 28.3\%), among which the essential AA concentrations
were especially reduced (by 37.7 \%). Notably, plasma methionine and valine
concentrations was significantly reduced by more than 40\% by dietary soy protein.
This was correlated to the liver proteins expression relating to methionine and valine metabolisms that were significantly upregulated by dietary soy protein only. On the contrary, white meat protein intake increased both essential and total AA concentrations in rats’ plasma, while dietary red meat had similar effects with casein on plasma total AA concentrations. It has been proved that elevated intake of dietary protein can regulate feed intake due to high satiety\textsuperscript{7-9, 33}. The study from Hall et al (2003) showed that whey protein increased the satiety in human subjects compared to casein\textsuperscript{34}, indicating that satiety can be regulated by different protein sources. However, previous studies showed that under the condition of dietary AA limitation, the meal termination is not due to satiety, which was evidenced by the absence of the satiety sequence\textsuperscript{35-36}. The underlying mechanisms of the feed intake depression effects of dietary AA limitation have been well reviewed\textsuperscript{35}. Therefore, we concluded that the feed intake reduction effects of the dietary soy protein was caused by the AA limitation but not by satiety that may affected by dietary soy protein. It is also suggested that when study the effects of different protein sources on satiety, the AA compositions of protein sources should be considered firstly.

In order to evaluate the effects of different protein sources on growth of young rats, the ratio of DFI/DBWG were calculated. Both white and red meat proteins had similar DFI/DBWG ratios with casein indicating that meat proteins had similar effects with milk protein on regulation of growth of young rats. However, compared to casein and meat proteins, dietary soy protein had a significantly higher DFI/DBWG ratio. This indicated that when feeding the same amount of soy protein, casein or meat proteins, the body weight gain of the young rats fed soy protein will be much lower (by about 50%) than the rats fed casein or meat proteins. The body compositions of the young rats after 14 days’ consumption of different protein diets were measured. It was found
that the adipose tissue mass and liver weight of rats were significantly reduced by dietary soy protein. At the same time, the negative body nitrogen and protein balances were observed in the rats fed soy protein diet according to the changes in plasma urea and total protein concentrations, which are biomarkers for body nitrogen and total protein balance. It was showed that plasma urea concentration were significantly increased but plasma total protein concentration were significantly reduced by dietary soy protein intake. Unlike soy protein, plasma urea and total protein concentrations were similar between casein, red meat and white meat protein groups. This indicates that meat proteins are more balanced protein sources than soy protein in term of body protein metabolism. The liver plays an important role in regulating AA and protein metabolism. Since in the present study the liver weights of young rats were significantly reduced by both dietary soy and meat proteins compared to casein. In order to evaluate the health status of the liver, plasma AST/ALT ratio was calculated. It was showed that no significant changes were observed in AST/ALT ratios, indicating that the liver function was not impaired by any dietary protein sources in this study. Only the individual plasma AST or ALT concentrations were increased by dietary soy and white meat proteins. This was consistent with the changes in liver protein expression of GOT1 (i.e. AST), which was significantly upregulated by dietary soy protein only. The increased AST and ALT indicated that the AA metabolism in the liver was activated by soy protein and white meat protein. However, the mechanisms are different between soy and white meat protein. For soy protein, this was caused by AA limitation (low plasma AA concentrations)) and will lead to negative nitrogen balance. For white meat protein, this was caused by AA excess (high plasma AA concentrations) and will lead to AA waste. Although, the plasma total protein concentrations was reduced specifically by dietary soy protein, the liver
protein expression relating to protein biosynthesis was increased but the liver protein expression relating to proteolysis was reduced specifically by dietary soy protein. This was suggested to be a compensatory increase in protein synthesis in response to inadequate in essential AA intake in soy protein group.

Accordingly, not just for adult people, cardiovascular morbidity can now be considered to be, in part, a prenatal and pediatric disease. Blood TG, TC, HDL-C and LDL-C are important biomarkers for lipid homeostasis and thus the cardiovascular diseases. It has been found that soy protein may have beneficial effects on lipid metabolism. However, in this study we found that soy protein had deleterious effects on liver adiposity and blood lipid profiles, whereas both red and white meat proteins showed beneficial effects. Specifically, dietary red and white meat proteins reduced the liver TC contents. Feeding red meat protein increased the plasma HDL-C concentration. When analyzing metabolism in the liver, we found that feeding white meat protein diets increased fatty acid beta-oxidation. Whereas dietary soy protein had no significant effects on liver lipid contents but increased the plasma TAG, TC and LDL-C concentrations.

Insulin resistance is the main mechanism for type 2 diabetes and a main component for metabolic syndrome. Notably, plasma insulin and HOMA-IR levels were significantly higher in the rats fed red meat protein than white meat protein, casein and soy protein groups. This suggest that red meat may increase the risk of type 2 diabetes (T2D). Findings from epidemiologic studies also suggest positive associations of red meat with risk of T2D. However, it is unclear whether it is the protein per se or other components of protein-rich foods in those epidemiologic studies. Energy metabolism in the liver were significantly increased by white meat protein compared to red meat protein. This can be related to the increased blood AA
concentrations after intake of white meat protein. This was supported by other study that rapid increase of AA concentrations after a meal is related to stimulation of oxidation and protein syntheses. The study from Mikkelsen et al (2000) found animal protein in pork meat produced a 2% higher 24-h energy expenditure than did the vegetable protein in soy. Notably, our 2-DE analysis results showed that iron transport protein serotransferrin (short name: transferrin) was significantly upregulated in the liver of rats fed soy protein and white meat protein diets compared to casein and red meat protein groups. This indicated that dietary soy or white meat protein intake increased liver transferrin synthesis. Transferrin is mainly synthesized in the liver. The main role of transferrin is to transport iron from sites of absorption (duodenum) and red blood cell recycling (macrophages) to tissues for storage (liver) and utilization (bone marrow). A high transferrin level may indicate iron deficiency which is often seen in patients suffering from iron deficiency anemia and also in the rats fed a low-iron diet. Therefore, we deduced that the increased liver transferrin level found in the rats fed soy and white meat protein diets in our study can be attributed to the null heme iron (highly bioavailable iron) in the soy protein source and relative low heme iron contents in the white meat protein sources compared to red meat protein sources. Except for the differences in iron content directly, it has been proved that dietary protein can also affect iron absorption. Etcheverry et al (2006) assessed the effects of beef and soy proteins on the bioavailability of non-heme iron in children. Their findings indicated that beef protein increased non-heme iron absorption compared to soy protein. Iron deficiency remain substantial problems in small children in both developed and developing nations. Therefore, when designing diets for children, the effect of protein source on iron absorption should be one of the factors taken into account.
Taken together, dietary soy protein showed deleterious effects on liver adiposity and blood lipid profiles and induced negative nitrogen balance and growth inhibition in young rats due to its limitation in essential AAs. In contrast to soy protein, both red and white meat proteins showed beneficial effects on growth and lipid metabolism of rats. Thus, soy protein is not an optimal protein source for growth and metabolism health of young animals, while meat protein is if not better than but at least as well as milk protein to the growth and metabolism health of young animals.

There were still some limitations in this study. The treatment time was 14 days, which was a single time point and relatively short. To better understand the process and the development of metabolism changes, longer feeding time or different time points could be studied and compared in future studies. The age of the rats could affect some parts of the responses to dietary proteins. Since we did not include rats with different ages in this study, it is difficult, if not impossible to tell which parts. The study investigates the effects of normal meat protein levels. It would be interesting to test the effects of higher levels of meat proteins on metabolism in future. Therefore, more studies are needed to get a comprehensive understanding of health effects of meat proteins and its molecular mechanisms.

**Abbreviations Used**

2-DE: two dimensional gel electrophoresis; AA: amino acid; DBWG: daily body weight gain; DFI: daily feed intake; DFI/DBWG: ratio of daily feed intake to daily body weight gain; EATW: absolute weight of epididymal adipose tissue; EATW/BW: relative weight of epididymal adipose tissue to body weight; FBW: final body weight; HDL-C: high density lipoprotein-cholesterol; IBW: initial body weight; LDL-C: low density lipoprotein-cholesterol; LW: absolute weight of liver; LW/BW: relative weight of liver to body weight; T2D: type 2 diabetes; TAG: triacylglycerol; TAG-L:
triacylglycerol in the liver; TC: total cholesterol; TCA: trichloroacetic acid; TC-L: total cholesterol in the liver; TP: total protein

**Funding Sources**

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**References**


15. Song, S.; Hooiveld, G. J.; Li, M.; Zhao, F.; Zhang, W.; Xu, X.; Muller, M.; Li, C.;


39. Mikkelsen, P. B.; Toubro, S.; Astrup, A., Effect of fat-reduced diets on 24-h energy expenditure: comparisons between animal protein, vegetable protein, and


Figure Captions

Figure 1. Growth performance of rats fed casein, soy, red meat and white meat protein diets.


Values are shown as means ± SD. The numbers of biological repetitions of casein, soy and red meat and white protein groups were 10, 10, 20 and 20, respectively. Different letters above bars indicate significant difference at $P < 0.05$ analyzed by one-way ANOVA and LSD multiple comparisons.

Figure 2. Adipose tissue weight, liver weight, liver TC and TAG content of rats fed casein, soy, red meat and white meat protein diets.


Values are shown as means ± SD. The numbers of biological repetitions of casein, soy and red meat and white protein groups were 10, 10, 20 and 20, respectively. Different letters above bars indicate significant difference at $P < 0.05$ tested by one-way ANOVA and LSD multiple comparisons.

Figure 3. Plasma triacylglycerol, cholesterol, glucose and insulin concentrations of rats fed casein, soy, red meat or white meat protein diets.


Values are shown as means ± SD. The numbers of biological repetitions of casein, soy and red meat and white protein groups were 10, 10, 20 and 20, respectively. Different letters above bars indicate significant difference at $P < 0.05$ tested by one-way ANOVA and LSD multiple comparisons.

**Figure 4. Plasma transaminase, total protein and urea concentrations of rats fed casein, soy, red meat or white meat protein diets.**


Values are shown as means ± SD. The numbers of biological repetitions of casein, soy and red meat and white protein groups were 10, 10, 20 and 20, respectively. Different letters above bars indicate significant difference at $P < 0.05$ tested by one-way ANOVA and LSD multiple comparisons.
### Table 1. Ingredient composition and nutritional content of diets

<table>
<thead>
<tr>
<th>g/Kg diet</th>
<th>Casein</th>
<th>Soy</th>
<th>Pork</th>
<th>Beef</th>
<th>Chicken</th>
<th>Fish</th>
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<tr>
<td>diet composition, g/Kg diet</td>
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<td>190</td>
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<tr>
<td>Dyetros</td>
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<td>132</td>
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<td>Sucrose</td>
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<td>Soybean oil</td>
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<td>L-Cystine(^4)</td>
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<td>0</td>
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<td>Choline Bitartrate</td>
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<td>2.5</td>
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<table>
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<tr>
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<td>Protein, g</td>
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<td>177</td>
<td>177</td>
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<td>Fat, g</td>
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<td>Carbohydrate, g</td>
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</tbody>
</table>

Protein\(^1\): the amount of protein powder was adjusted and balanced according to the protein content in soy and meat protein powder. Mineral mix\(^2\): the formulation of mineral mixes for the six diets was listed in the Supplemental Table 1 online. Vitamin mix\(^3\): the formulation of vitamin mix was referenced to the paper\(^4\). L-Cystine\(^4\): the amino acid composition of soy and meat protein diets were not modified.
Table 2. Plasma amino acid concentrations of rats fed casein, soy, red meat or white meat protein diets.

<table>
<thead>
<tr>
<th></th>
<th>casein (μmol/L) n=10</th>
<th>soy (μmol/L) n=10</th>
<th>white meat (μmol/L) n=20</th>
<th>red meat (μmol/L) n=20</th>
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</thead>
<tbody>
<tr>
<td>TAA</td>
<td>3609±349&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2586±220&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4058±416&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3527±615&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>EAA</td>
<td>2030±255&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1265±129&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2412±332&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2102±381&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NEAA</td>
<td>1579±102&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1321±110&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1646±151&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1424±261&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Val</td>
<td>207±38.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>121±18.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>189±25.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>160±29.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ile</td>
<td>99±15.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67±15.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>95.4±14.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>82.0±15.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Leu</td>
<td>148±24.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88.7±17.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>135±22.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>113±24.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lys</td>
<td>581±92.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>355±81.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>576±102&lt;sup&gt;a&lt;/sup&gt;</td>
<td>466±86.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Met</td>
<td>82.0±8.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.9±9.85&lt;sup&gt;c&lt;/sup&gt;</td>
<td>76.9±11.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.8±9.77&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Phe</td>
<td>56.9±5.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36.0±8.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>66.7±7.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.1±14.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Thr</td>
<td>653±133&lt;sup&gt;b&lt;/sup&gt;</td>
<td>370±34.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1037±194&lt;sup&gt;a&lt;/sup&gt;</td>
<td>963±208&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>His</td>
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<td>64.8±6.89&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>73.5±9.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.1±11.20&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Arg</td>
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<td>115±14.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>162±28.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>138±30.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pro</td>
<td>318±42.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>328±29.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>374±39.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>334±55.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Tyr</td>
<td>99.1±11.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.7±9.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>101±20.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.9±21.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Asp</td>
<td>21.5±4.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.8±4.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.2±5.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.5±7.55&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Glu</td>
<td>127±25.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>75.1±15.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87.4±13.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85.4±24.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ala</td>
<td>466±59.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>264±42.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>400±97.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>326±7.19&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Ser</td>
<td>256±30.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>269±21.5&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Gly</td>
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<td>294±30.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>335±38.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Cys</td>
<td>14.3±3.2</td>
<td>18.1±6.4</td>
<td>16.2±2.57</td>
<td>16.7±6.5</td>
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</table>

Values are shown as means ± SD. The different superscript letters within the same column mean statistical significant difference at $P < 0.05$ analyzed by one-way ANOVA and LSD multiple test. TAA: the sum of 17 kinds of amino acids in plasma including Arg, Pro, Met, Val, Ser, Gly, Lys, Thr, Phe, Asp, Ile, Leu, Cys, Glu, Ala, Tyr, His. EAA: the sum of 9 kinds of essential amino acids in plasma including Arg, Met, Val, Lys, Thr, Phe, Ile, Leu, His. NEAA: the sum of 8 kinds of non-essential amino acids in plasma including Pro, Ser, Gly, Asp, Cys, Glu, Ala, Tyr.
Table 3. Liver protein expression changes of rats fed casein, soy, red meat or white meat protein diets.

<table>
<thead>
<tr>
<th>ID</th>
<th>symbol</th>
<th>protein name</th>
<th>casein</th>
<th>soy</th>
<th>white meat</th>
<th>red meat</th>
<th>GO BP</th>
<th>GO MF</th>
<th>GO CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>P15999</td>
<td>ATP5A1</td>
<td>ATP synthase subunit alpha</td>
<td>1.00(^b)</td>
<td>1.54(^a)</td>
<td>1.55(^a)</td>
<td>1.45(^a)</td>
<td>ATP synthesis</td>
<td>ATPase activity</td>
<td>mitochondrion</td>
</tr>
<tr>
<td>P13221</td>
<td>GOT1</td>
<td>Aspartate aminotransferase</td>
<td>1.00(^b)</td>
<td>2.22(^a)</td>
<td>1.73(^b)</td>
<td>1.06(^b)</td>
<td>amino-acid biosynthesis</td>
<td>aminotransferase</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>P00481</td>
<td>OTC</td>
<td>Ornithine carbamoyltransferase</td>
<td>1.00(^b)</td>
<td>1.83(^b)</td>
<td>0.78(^b)</td>
<td>0.71(^b)</td>
<td>urea cycle</td>
<td>transferase</td>
<td>mitochondrion</td>
</tr>
<tr>
<td>Q02253</td>
<td>ALDH6A1</td>
<td>Methylmalonate-semialdehyde dehydrogenase [acylating]</td>
<td>1.00(^ab)</td>
<td>1.32(^b)</td>
<td>0.87(^b)</td>
<td>1.06(^ab)</td>
<td>valine metabolic process</td>
<td>oxidoreductase</td>
<td>mitochondrion</td>
</tr>
<tr>
<td>P13444</td>
<td>MAT1A</td>
<td>S-adenosylmethionine synthase isoform type-1</td>
<td>1.00(^ab)</td>
<td>1.26(^b)</td>
<td>0.82(^b)</td>
<td>0.80(^b)</td>
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<tr>
<td>P62630</td>
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<td>1.45(^a)</td>
<td>1.07(^b)</td>
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<td>1.86(^a)</td>
<td>1.27(^b)</td>
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<td>Peroxiredoxin-1</td>
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<td>1.41(^b)</td>
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<td>ligase</td>
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<td>1.07(^b)</td>
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<td>Alcohol dehydrogenase 1</td>
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<td>1.05(^b)</td>
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<td>GO-MF</td>
<td>GO-CC</td>
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Protein expression changes were represented as fold changes. The different superscript letters within the same column mean statistical significant difference at $P<0.05$ analyzed by one-way ANOVA and LSD multiple comparison of protein spots intensities (Supplementary Table 1). The numbers of biological repetitions of 2-DE analysis of casein, soy and red meat and white protein groups were 5, 5, 10 and 10, respectively.

GO-BP: Gene Ontology-biological process; GO-MF: Gene Ontology-molecular function; GO-CC: Gene Ontology-cellular component.
Figure 1
Figure 2

A1. EATW (g)

A2. EATW/BW (%)

B1. LW (g)

B2. LW/BW (%)

C1. TAG-L (mg/g)

C2. TC-L (mg/g)
Figure 3

A1. TAG (mmol/L)

A2. TC (mmol/L)

A3. HDL-C (mmol/L)

A4. LDL-C (mmol/L)

B1. Glucose (mmol/L)

B2. Insulin (mIU/L)

B3. HOMA-IR
Figure 4
Graphic for table of contents

Rats were fed diets containing different protein sources for 14 days

Meat proteins were beneficial for growth and metabolism of young rats compared to casein and soy protein.