Original Article

Evaluation of the Protein Requirement in Chinese Young Adults Using the Indicator Amino Acid Oxidation Technique*

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Abstract

Objective To accurately calculate the protein requirements in Chinese young adults using the indicator amino acid oxidation technique.

Methods Nine women and ten men received a restricted daily level of protein intake (0.75, 0.82, 0.89, 0.97, and 1.05 g/kg), along with L-[1-^{13}C]-leucine. Subjects' protein requirement was determined by a biphasic linear regression crossover analysis of $F^{13}CO_2$ data. In doing so, a breakpoint at the minimal rate of appearance of $^{13}CO_2$ expiration specific to each level of dietary protein was identified. This trial was registered with the Chinese clinical trial registry as ChiCTR-ONC-11001407.

Results The Estimated Average Requirement (EAR) and the Recommended Nutrient Intake (RNI) of protein for healthy Chinese young adults were determined to be 0.87 and 0.98 g/(kg·d), respectively, based on the indicator amino acid oxidation technique.

Conclusion The EAR and RNI of mixed protein are 5% and 16% that are lower than the current proposed EAR and RNI (0.92 and 1.16 g/(kg·d), respectively), as determined by the nitrogen balance method. The respective EAR and RNI recommendations of 0.87 and 0.98 g/(kg·d) of mixed protein are estimated to be reasonable and suitable for Chinese young adults.

Key words: Protein requirement; Indicator amino acid oxidation; Adult protein intake; Biphasic linear regression crossover analysis; Mixed diet

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INTRODUCTION

In 2000 the Chinese Nutrition Society proposed respective Estimated Average Requirement (EAR) and Recommended Nutrient Intake (RNI) of 0.92 and 1.16 g/(kg·d) of mixed protein for Chinese adults[1]. The proposal was based on a nitrogen balance study of 16 Chinese adult men in 1984[2] and are now outdated. The 2002 China National Nutrition and Health Survey revealed that only 18.4% of Chinese residents met or exceeded the RNI for protein, although the incidence of protein malnutrition in adults in fact was very low[3-4]. As known to all, dietary reference intakes (DRIs) are the most fundamental scientific basis of nutrition science. Therefore, if the current protein

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requirement is continuously used to evaluate protein nutritional status of the Chinese residents, wrong conclusion may be drawn that people are under protein malnutrition. We believe that the current EAR and RNI for protein are overestimates and need to be re-evaluated.

The nitrogen balance technique was widely used and was generally accepted to be accurate before the isotope technique had been used to measure protein and amino acid requirements, However, this method has substantial practical limitations[5-6] in that it is easy to overestimate nitrogen intake and to underestimate nitrogen excretion, both of which would report a false positive nitrogen balances[7]. The indicator amino acid oxidation technique (IAAO), on the other hand, is a robust method that calculates protein requirements by measuring changes in the oxidation of a labeled amino acid, which is co-administered with a controlled amount of protein. This method has been successfully used to determine protein and amino acid requirements in both adults and children in which purified amino acid mixtures are used for a study diet[8-12]. It is possible, however, that the use of purified amino acid mixtures as protein sources are not reflective of a real-world scenario, in which individuals are consuming mixed diets. Therefore, in the current study, we used the IAAO technique to determine the protein requirements of Chinese young adults who were consuming a mixed study diet containing a known amount of complex proteins. Excess protein in the body can’t be stored and will be oxidated[13]. Protein intake is mainly used for the synthesis of body protein and its oxidation rate remains at a relatively low level when protein intake does not meet the body's physiological needs. The oxidation rate of protein will increase when protein intake meets and exceeds the physiological requirements and there will be a breakpoint (turning point) in the protein oxidation rate of protein intake on the dose-response curve. The protein needs of the human body are essentially the needs for amino acids. The protein physiological requirements are actually the intake of essential amino acids. Protein oxidation and decomposition can be reflected by amino acid oxidation and decomposition. Therefore, the increase of protein oxidation rate will inevitably be reflected in the rate of oxidation of the amino acid when protein intake exceeds the physiological requirements[14-15]. The present study takes ^{13}C-leucine as indicator to reflect the variation of the protein oxidation rate on the basis that breath $^{13}CO_2$ abundance changes reflect the leucine oxidation and decomposition[16-17]. Also, the protein physiological requirement (breakpoint, equal to EAR) is determined by application of the biphase linear regression model of the breath $^{13}CO_2$ production and protein intake data.

MATERIALS AND METHODS

Materials

Twelve healthy adult men and twelve healthy adult women, all from Bethune Military Medical College, were recruited with only two men and three women dropping out before the study ended. The cohort characteristics are described in Table 1. All subjects were initially subjected to a routine physical check-up including complete blood count, blood chemistry workup, and hepatic and renal functions.

Table 1. Characteristics of the Study Cohort (Mean±SD)

Characteristics	Men (*n*=10)	Women (*n*=9)
Age (yr)	21.1±1.1	21.3±1.1
Weight (kg)	69.8±7.5	58.6±6.3
Height (cm)	1.8±0.0	1.7±0.0
BMI (kg/m^2)	22.4±2.1	21.0±2.0

The present study was conducted according to the guidelines in the Declaration of Helsinki and was approved by the Ethical Committee of the Institute of Nutrition and Food Safety, Chinese Center for Disease Control and Prevention. Informed written consent was obtained from the participants prior to the initiation of the study. This trial was registered at Chinese clinical trial registry as ChiCTR-ONC-11001407.

Study Diets

All subjects received five dietary protein intakes (0.75, 0.82, 0.89, 0.97, and 1.05 g/(kg·d)) as part of a standard Chinese diet (including rice, wheat flour, meat, shrimp, egg, tofu, vegetables, and fruit). The design of five dietary protein intakes was based on the previous study[18]. The daily protein intake of each subject was calculated according to his/her body weight and was distributed into three meals with a ratio of 3:4:3. Each meal contained one kind of staple food (rice or steamed roll or steamed bread), one kind of high-quality protein food (pork, chicken, shrimp, egg, tofu), one kind of vegetable, and one kind of fruit (except breakfast). The food was separately cooked. As the meat, vegetables, and fruit are not easy to store, before the start of the experiment, we only purchased enough rice and wheat flour for making rice, steamed rolls, or steamed bread for the duration of the study. For the staple, the level of protein was calculated according to the measured protein value of rice and wheat flour, as well as the water content of each staple food. For meat and vegetables, protein contents were calculated based on the China food composition for food quantity. The level of high-quality protein was controlled at 35% of total daily protein. The dietary types of each protein level were the same, but the amount varied. The individual foods consumed were weighed and the subjects were required to consume all foods. Moreover, very low and non-protein foods, such as fried prawn slices or starchy noodles, were provided in case the meal was not sufficient to meet the subjects' energy requirement, especially those consuming the low protein level diets. Subjects could choose these low- and non-protein foods to consume without any restriction. Before and after taking the meal, each food was weighted and recorded in order to determine the actual intake of each food. All subjects' consumed foods were collected and tested for the concentration of main macronutrients (including protein, fat, and carbohydrate) and energy, in order to ensure that the actual protein intakes of the subjects were close to their pre-designed doses.

Experimental Design and Tracer Protocol

All subjects received protein intakes in the following order: 0.75, 1.05, 0.82, 0.97, and 0.89 g/(kg·d). A break of three days was arranged among different protein levels. And it has been shown that a suitable adaptation period of approximately five days is sufficient among different protein intake levels[19]. In the present study, each protein level lasted for six days, which is five days of adaptation and the last day for isotope study. During the entire study, subjects were instructed to maintain light physical activity and wear pedometers. Body weight and body composition were tested at the beginning of each protein period. The body composition was determined by bioelectrical impedance analysis (DF50, Impedimed, Australia).

The isotope study was performed on the protocol previously used by Tian et al.[18] (Figure 1). Diets on the isotope study days were similar to the diets on the adaptation days. Supper was divided into four isocaloric and isonitrogenous meals and was taken by the subjects every hour for three hours. On the isotope day, the tracer protocol was consumed with the supper to measure leucine kinetics, using L-[1-^{13}C]-leucine [99 atom% excess (APE); Cambridge Isotope laboratories, Woburn, MA]. Oral priming doses of 0.11 mg/kg NaH^{13}CO$_3$ (99 APE; Cambridge Isotope laboratories, Woburn, MA) and 0.56 mg/kg L-[1-^{13}C]-leucine were taken with the supper meal. A 20-minute oral dosing protocol of L-[1-^{13}C]-leucine (0.19 mg/kg) was taken as biscuit and was continued for the remaining 4 h of the study. One baseline breath and one baseline blood sample were collected 30 min before the tracer protocol began. Breath samples were collected in a 10 mL glass tube at 60, 120, 180, 195, 210, 225, 240, 255, 270, and

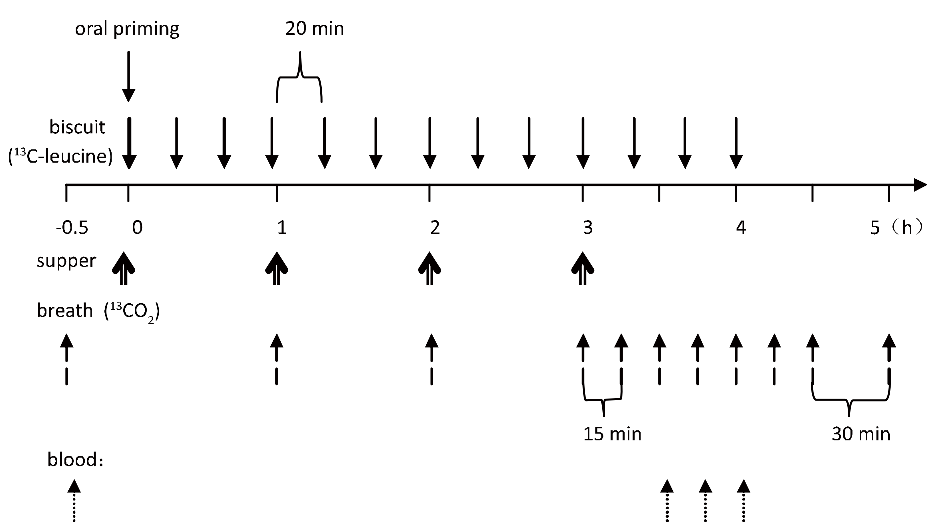

Figure 1. The isotope study protocol.

300 min post-tracer challenge. Three plateau blood samples were collected at isotopic steady state at 210, 225, 240 min post-tracer challenge. When the isotope intakes ended (4 h after tracer challenge), a 3-min breath sample was collected into a 100 L Douglas Bag (Harvard Apparatus) and the percentage of CO_2 was measured with an infrared monitoring device (GXH-3010E, Hua Yun, Beijing). A vacuum extraction system (Schlumberger, Netherland) was then used to determine the volume of the 3-min breath sample.

Sample Collection and Analysis

Diet samples were collected and analyzed for the contents of protein (Chinese standard GB 5009.5-2010), fat (Chinese standard GB 5009.6-2003), ash (Chinese standard GB 5009.4-2010), and moisture (Chinese standard GB 5009.3-2010). Total carbohydrate was equal to the calculation of: [100 - (% protein + % fat + % ash + % moisture)]. Energy was calculated according to the energy coefficient:

Energy (kcal) = protein content (g) × 4 (kcal/g) + fat content (g) ×9 (kcal/g) + carbohydrate content (g) ×4 (kcal/g)

Breath samples were stored at room temperature for the onwards analysis. Blood samples were stored at -20 °C. The enrichment of $^{13}CO_2$ in breath samples was analyzed using a ^{13}C breath analyzer (Helivew, Medichems, Korea). L-[1-^{13}C]-leucine enrichment in blood samples was measured using a triple quadrupole mass analyzer (API 4000, Shimadzu, Japan) coupled to an HPLC system (LC20A, Shimadzu, Japan), as described previously[18]. Isotopic enrichment was expressed as atom percent excess and was calculated from peak area ratios at the isotopic steady state at plateau and at baseline.

Estimation of Lsotope Kinetics

The rate of appearance of $^{13}CO_2$ in breath ($F^{13}CO_2$, μmol/kg/h) after the oxidation of ingested L-[1-^{13}C]-leucine was calculated according to the model of Matthews et al.[20]. $F^{13}CO_2$ was calculated as follows:

$$F^{13}CO_2=[(FCO_2)(ECO_2)(44.6)(60)]/[(W)(100)(0.82)]$$

Where FCO_2 is the carbon dioxide production rate (mL/min), ECO_2 is the $^{13}CO_2$ enrichment in expired breath at the isotopic steady state (atoms percent excess), and W is the weight (kg) of the subject. The constants 44.6 (μmol/mL) and 60 (min/h) are used to convert FCO_2 to μmol/h; 0.82 is the factor to account for the retention of $^{13}CO_2$ in the bicarbonate pool of the body in the fed state[21], and the factor 100 changes the atom percent excess to a fraction.

Whole-body leucine flux was calculated from the dilution of L-[1-^{13}C]-leucine in the whole-body amino acid pool at the isotopic steady state, using the following equation[18,20]:

$$Q = i[E_i/E_u-1]$$

Where Q is leucine flux (μmol/kg/h), i is the rate of L-[1-^{13}C]-leucine infused (μmol/kg/h), and E_i and E_u are the isotopic enrichments as mole fractions (atom percent excess) of the infusate and blood leucine, respectively, at the isotopic plateau.

The rate of leucine oxidation was calculated as follows[18,20]:

$$O = F^{13}CO_2\,(1 / E_u -1/ E_i) \times 100$$

Where O represents leucine oxidation (μmol/kg/h).

Statistical Analysis

Study results were expressed as means±standard deviation (SD). Student Newman-Keuls (SNK) tests were performed for the comparisons of body weight and compositions among different protein periods. The protein requirement (breakpoint, equivalent to the EAR) was determined by a biphasic linear regression crossover model on the measure of $F^{13}CO_2$. In our model, the first regression line was horizontal, with minimal slope or without slope, and the second line had a slope. The safe intake (equivalent to the RNI) was equal to EAR + 2SD. All statistical analyses were performed using the Statistical Analysis Systems 9.1 software. A value of $P<0.05$ was considered statistically significant.

RESULTS

Subjects Body Weight and Composition

At the different dietary protein levels, subjects' body weight and body composition (Table 2) were not significantly different ($P>0.05$), indicating that the energy intake of subjects was enough. Daily steps were between 8000 and 10 000 and subjects maintained a light physical activity level.

Dietary Structures

The subjects' intakes of main macronutrients and energy, among five dietary protein levels, are shown in Table 3. The energy proportions from protein, fat, and carbohydrate were reasonable according to Dietary Guidelines for Chinese

Residents[22]. The study diet well represented the traditional dietary pattern of Chinese adult residents.

Table 2. Subjects' Body Weight and Body Composition (Mean±SD)

Characteristics	Protein Intake [g/(kg·d)]				
	0.75	0.82	0.89	0.97	1.05
Female					
Body wt (kg)	58.6±6.3	58.3±6.5	58.9±6.5	58.4±6.2	58.3±6.5
FFM (kg)†	44.1±3.9	43.3±4.0	44.0±4.2	44.3±3.9	44.1±4.4
FM (kg)‡	14.5±2.9	15.0±2.8	14.9±3.0	14.1±2.6	14.2±3.0
Male					
Body wt (kg)	69.8±7.5	70.4±8.5	71.3±8.7	70.4±8.1	70.3±8.2
FFM (kg)†	59.7±4.1	58.8±4.3	60.1±4.4	60.2±4.0	59.8±4.2
FM (kg)‡	10.1±4.4	11.6±5.2	11.3±5.4	10.3±5.3	10.5±5.1

Note. †FFM: Fat Free Mass, as determined by bioelectrical impedance analysis; ‡FM: Fat Mass, equal to body weight minus FFM.

Leucine Flux, Oxidation, and Breath $^{13}CO_2$ Excretion

Leucine flux was not affected by protein intake (Table 4), indicating that the size of the precursor pool for indicator oxidation remained unchanged in response to the test protein intake. Leucine oxidation and breath $^{13}CO_2$ excretion were not significantly different among subjects who ingested 0.75 to 0.89 g/(kg·d) of protein (P>0.05), but was increased slightly among subjects who ingested 0.89 to 1.05 g/(kg·d) of protein (P<0.05).

Subjects' protein requirement was determined by a biphasic linear regression crossover analysis on the $F^{13}CO_2$ data (Table 5). Analysis of $F^{13}CO_2$ data resulted in the identification of a breakpoint protein intake value (estimate of mean protein requirement, equivalent to the EAR) of 0.85 g/(kg·d) for females and 0.88 g/(kg·d) for males and an RNI measurement of 0.97 g/(kg·d) for females and 0.98 g/(kg·d) for males. The comparison of EAR values between female and male subjects were not significantly different (P>0.05), and a mean EAR and RNI value was calculated therefore for Chinese young adults as 0.87 g/(kg·d) and 0.98 g/(kg·d), respectively.

Table 3. Intakes of Main Macronutrients and Energy of Five Dietary Protein Levels (Mean±SD)

Intended Protein Intake [g/(k·d)]	Protein			Fat		Carbohydrate		Energy
	Actual Intake [g/(kg·d)]	Protein† (%)	Energy from Protein (%)	Actual Intake [g/(kg·d)]	Energy from Fat(%)	Actual Intake [g/(kg·d)]	Energy from Carbohydrate (%)	Intake [kcal/(kg·d)]
Female								
0.75	0.74±0.02	0.33±0.01	9.1±0.5	1.1±0.1	29.3±2.1	5.2±0.4	61.7±2.2	32.3±2.1
0.82	0.79±0.02	0.35±0.00	9.9±1.2	1.1±0.3	29.4±3.6	5.0±0.5	60.6±2.6	32.9±4.2
0.89	0.87±0.07	0.34±0.00	9.7±1.0	1.4±0.3	32.9±3.2	5.3±0.5	57.4±2.3	37.7±3.8
0.97	0.95±0.01	0.34±0.00	10.3±1.0	1.3±0.2	30.3±3.1	5.6±0.2	59.4±2.4	37.8±3.0
1.05	0.99±0.02	0.37±0.01	13.3±0.4	0.9±0.0	27.2±1.1	4.5±0.2	59.6±1.4	31.1±1.1
Male								
0.75	0.76±0.02	0.32±0.00	8.2±1.1	1.3±0.2	29.4±1.6	6.1±0.9	62.4±1.5	39.0±5.1
0.82	0.83±0.01	0.33±0.01	9.6±1.2	1.1±0.2	28.2±2.1	5.6±0.8	62.2±1.8	35.9±4.8
0.89	0.91±0.02	0.33±0.01	9.6±1.2	1.5±0.3	33.5±2.6	5.7±0.8	56.9±1.8	40.1±5.6
0.97	0.97±0.01	0.34±0.01	10.3±0.9	1.2±0.2	28.4±2.9	5.9±0.5	61.3±2.3	38.3±3.6
1.05	1.02±0.02	0.35±0.01	11.2±1.1	1.1±0.1	27.3±1.0	5.7±0.7	61.4±1.1	37.1±4.3

Note. †Expressed as percentage of high-quality protein to total protein.

Table 4. Leucine Flux, Oxidation, and Breath $^{13}CO_2$ Excretion for Five Levels of Protein Intake in the Study Subjects (Mean±SD)

Protein Intake [g/(kg·d)]	Leucine Flux [μmol/(kg·h)]	Leucine Oxidation [μmol/(kg·h)]	$F^{13}CO_2$ [μmol/(kg·h)]
Female			
0.75	116.26±50.13	28.16±10.29	0.74±0.15
0.82	120.26±57.19	33.20±16.24	0.71±0.16
0.89	112.46±22.01	32.72±7.34	0.74±0.15
0.97	134.61±23.35	54.14±10.54	1.04±0.10
1.05	123.69±29.67	58.87±19.92	1.03±0.29
Male			
0.75	113.87±27.59	29.19±7.95	0.66±0.15
0.82	135.92±52.99	32.88±10.22	0.64±0.15
0.89	126.82±52.22	36.96±17.52	0.67±0.22
0.97	138.43±30.02	62.96±23.67	1.10±0.35
1.05	128.56±35.04	61.12±20.55	1.10±0.25

Table 5. Protein Requirement Derived from Breath $^{13}CO_2$ Expiration [g/(kg·d)]

Female Subject	Protein Requirement	Male Subject	Protein Requirement
1	0.82	1	0.76
2	0.88	2	0.89
3	0.84	3	0.86
4	0.87	4	0.91
5	0.85	5	0.89
6	0.92	6	0.93
7	0.80	7	0.93
8	0.91	8	0.88
9	0.74	9	0.91
		10	0.87
Mean (EAR)	0.85	Mean (EAR)	0.88
SD	0.06	SD	0.05
RNI†	0.97	RNI†	0.98

Note. †RNI = EAR + 2SD.

DISCUSSION

There are disagreements about the link of the onset of certain disorders and diseases (such as obesity, osteoporosis, kidney stones, etc.) with increasing protein intake. Taking obesity as an example, some epidemiological studies have shown a positive correlation between protein intake and body fatness, body mass index, and subscapular skinfold[23-24]. In contrast, a 6-month randomized trial demonstrated that the replacement of some dietary carbohydrates by protein actually improved weight loss, as part of a reduced fat diet[25]. Yet, there are no conclusions on the diseases associated with high protein diets, and it is generally accepted that individuals should avoid a long-term high-protein diet, especially those containing high proteins of meat or animal origin, primarily because high-protein diets are often accompanied with other high-fat and high-energy dietary components.

The 2002 China National Nutrition and Health Survey, a nationally representative cross-sectional survey covering 31 provinces, autonomous regions, and municipalities, showed that the current dietary protein intakes among the Chinese adults were 65.9 g/d[3], being equivalent to 1.01 g/(kg·d) (reference weight was 65 kg). The current proposed RNI value of protein was therefore higher than the findings in the present survey and needed to be reassessed. In our present study, the EAR and RNI measurements for protein in the study subjects were 0.87 and 0.98 g/(kg·d), respectively, which were much lower than those of the current EAR and RNI values (0.92 and 1.16 g/(kg·d), respectively). The IAAO method has been widely used to determine amino acids requirements, protein requirements as well. Humayun and Elango previously used IAAO to estimate protein requirements for young men[8], using amino acid mixtures in their study diet. Previous studies have also suggested that protein requirements can be estimated by measuring amino acid catabolism over periods as short as several minutes, without longer periods of adaptation[26-31]. Contrary to this, we hypothesize that this short length of study and the use of simple amino acids, which are actually digested and absorbed more rapidly than complex proteins, are not sufficient to estimate the real protein requirement of subjects.

To test this hypothesis, a traditional Chinese diet was employed in our current study. The protein content of the staple is of great importance for controlling the actual protein intakes of subjects at their desired pre-designed doses, because protein intake from the staple accounts for a large portion of the daily total protein intake. Therefore, at the initiation of the experiment, we purchased enough rice and wheat flour to prepare rice, steamed rolls, or steamed bread for the subjects in order to have a consistent amount of protein from these sources. Before and after each meal, the food was weighed to determine the actual intake for each food. All consumed foods were collected and the main macronutrient levels (including protein, fat and carbohydrate) were tested. From the dietary macronutrients analysis, we observed that the actual protein intakes of subjects were very close to their pre-designed doses.

The EAR and RNI values for protein in the present study were higher than those currently proposed in most countries. One possible reason is that the method for the determination of the protein requirement is nitrogen balance method. This classical method has faced technical challenges of easily under-calculating the protein requirement[7]. The other possible reason is that some other studies used different sources of dietary protein, which was found to have a significant effect on the protein requirement[32-35], as higher-quality protein (e.g., protein from meat or bean products) is better utilized than lower-quality protein (e.g., protein from vegetables). Diet used in our current study was a mixed one, with high-quality protein accounting for about 35% of total protein. We chose this diet because the 2002 China National Nutrition and Health Survey revealed that the percentage of high-quality protein for residents was actually 33% of their total protein intake[3]. Suppose we increase high-quality protein content in the diet used for the study, the EAR for protein may be reduced.

Protein utilization and deposition are energy dependent at all stages of amino acid transport, interconversion, protein synthesis, and proteolysis. If energy intake is not sufficient to meet the energy need, the evaluation of protein requirements will be inaccurate. Therefore, one of the premises of accurately evaluating protein requirements is to account for the subjects' energy balance. When consuming the low-protein diets, energy intake is easily to be deficient, especially for a complex diet model. Therefore, very low-and non-protein-containing foods, containing higher energy, were provided to the subjects without restriction. Body weight is a sensitive indicator of energy balance and expenditure. During our entire study, the subjects' body weight and body composition were monitored and did not change significantly, allowing us to assume that the subjects maintained a consistent energy balance during the study period.

Compared to the nitrogen balance method, the IAAO technique has many advantages[8]. First, it does not determine nitrogen balance, the determination of which is technically demanding. Second, it allows the study on same individual over an entire range of protein intakes, thus decreasing inter-subject variability. Third, it is non-invasive and can be used to determine accurate protein requirements in healthy individuals and in patients with different diseases and health problems.

In conclusion, findings of the present study suggest that the current recommendations for Chinese adult protein intake are very high and therefore require a reassessment. We estimate that the respective EAR and RNI values of 0.87 and 0.98 g/(kg·d) of mixed protein are reasonable and suitable for Chinese adults.

COMPETING INTERESTS

All authors declared there were no conflict interests involved.

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