Short communication

Effects of supplemental cystine or methionine on growth and lifespan of stroke-prone spontaneously hypertensive rats

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Stroke-prone spontaneously hypertensive (SHRSP) rats are considered a suitable model for studying the effects of dietary and other environmental factors on human essential hypertension and haemorrhagic stroke. To investigate the suitability of a control diet for this strain of rats, we studied the effects of supplementing casein and soya protein isolate (SPI) with two sulphur amino acids (methionine and cystine) on the growth and lifespan of SHRSP rats. The source of dietary protein and the type of supplemental sulphur amino acid had significant ($P < 0.05$) effects on food intake and weight gain measured after 31 d of the feeding study, while only the type of supplemental sulphur amino acid had significant effects on mean survival times and the survival rates. On average, the casein groups had higher food intake and weight gain compared with the SPI groups. The methionine-supplemented groups had lower food intake but higher weight gain than the cystine-supplemented groups. Similarly, the methionine-supplemented groups had higher mean survival times and survival rates compared with the cystine-supplemented groups. The data would suggest that a control diet based on cystine-supplemented casein (as recommended for normal healthy rats by the American Institute of Nutrition), may not meet the sulphur amino acid requirements for SHRSP rats, and that the methionine-supplemented casein would be an appropriate control diet for this animal model.

Diets: Methionine: Cystine: SHRSP rats: Haemorrhagic stroke

In 1993, the American Institute of Nutrition revised Rodent Diets, AIN-76 and AIN-76A, which had been extensively used by researchers around the world (Reeves *et al.* 1993). The revision resulted in the derivation of two new formulations, AIN-93G for growth, pregnancy and lactation, and AIN-93M for adult maintenance. The revision in protein included the supplementation of casein (200 g/kg diet) with L-cystine (3 g/kg diet) instead of L-methionine (3 g/kg diet), as casein is a rich source of methionine (3·03 g/100 g protein) but contains a small amount of cystine (0·41 g/100 g protein) (Sarwar & Peace, 1994).

The cystine-supplemented casein control (200 g casein + 3 g L-cystine/kg diet), as recommended by the American Institute of Nutrition for rodents, has been considered to meet the indispensable amino acid requirements of normal healthy rats (based on growth) in short- and long-term studies with laboratory rodents (Reeves *et al.* 1993).

Stroke-prone spontaneously hypertensive (SHRSP) rats are one of the most suitable animal models for human essential hypertension and haemorrhagic stroke. The amino acid requirements of SHRSP rats are not known but are assumed to be similar to those of normal healthy laboratory rats. When fed a high-methionine control diet, SHRSP rats had delayed onset of hypertension and strokes and prolonged lifespan (Yamori *et al.* 1984). However, the adequacy of the cystine- and methionine-supplemented casein as control diets for SHRSP rats has not been compared. Therefore, the influence of the supplementation of casein with cystine or methionine on the growth and lifespan of SHRSP rats was investigated in the present study. The supplementary effect of the two sulphur amino acids was also studied in diets based on soya protein isolate (SPI), which could potentially be used as a reference protein in animal studies.

Experimental methods

Diets

Casein (90 % protein) was purchased from ICN (Cleveland, OH, USA), while alcohol-washed SPI (Pro-Fam 930, 90 % protein devoid of isoflavones) was obtained from Archer Daniel Midland Company (Decatur, IL, USA). Isoflavone-free SPI was used because isoflavones have been reported to have potential beneficial effects on hypertension. The alcohol treatment has a minimal effect on the protein structure of SPI. Moreover, the amino acid compositions of the alcohol-washed SPI and that containing endogenous isoflavones are known to be similar. L-Cystine and L-methionine were purchased from Sigma Chemicals (St Louis, MO, USA). The four experimental diets (casein + cystine, casein + methionine, SPI + cystine and SPI + methionine) were formulated to contain

(g/kg): casein or SPI, 222 (providing 200 g protein); L-cystine or L-methionine, 3; soyabean oil, 100; sucrose, 100; AIN-93 G mineral mixture (Reeves et al. 1993), 35; AIN-93G vitamin mixture (Reeves et al. 1993), 10 %; choline bitartrate (Sigma Chemicals), 2; cellulose (Alfa-floc; Teklad Diet, Madison, WI, USA), 50; cornstarch, 478. The amino acid compositions of the four experimental diets are shown in Table 1.

The four experimental diets were isoenergetic, and the metabolizable energy per kg diet of the four diets was 16·48 MJ. Metabolizable energy was calculated using the Atwater factors of 17, 37 and 17 kJ/g for protein, fat and available carbohydrates respectively.

Animals and experimental design

Young male SHRSP rats (aged 29 d) from the breeding colony of Health Canada in Ottawa were used. The breeding strain was originally obtained from SEAC Yoshiomoti Ltd (Fukuoka-ken, Japan). These animals have been widely used to investigate the influence of environmental factors on hypertension and stroke (Ratnayake & Vavasour, 2004). The animals (with initial body weight of 99 (SD 5) g) were placed into four dietary groups of sixteen rats per group in a completely randomized design. The rats were housed individually in metal cages in a climate-controlled facility maintained at 22 ± 1°C and 60 % relative humidity with a 12 h d/12 h night cycle. Rats had free access to one of the four diets and drinking water. To induce early hypertension, the drinking water contained 10 g/l NaCl. Records of weekly food consumption and body weights, and lifespan were kept. This was a lifespan study and it was expected that the dietary treatments may influence the longevity of animals. Therefore, to include growth data for all sixteen animals per group, food consumption and weight gains were recorded on 31 d of feeding or at the age of 60 d, a time period well before the start of the death of animals on any treatment group. This time period was selected based on our previous experience. Animals were closely observed for abnormal symptoms leading to death. Canadian Council on Animal Care guidelines for the care and use of laboratory animals were followed, and the study protocol was approved by the Animal Care Committee of Health Canada.

Animals were identified for potential slaughter if they showed clinical signs resulting from a severe stroke such as paralysis, tremor and spasms. Animals were killed (deep inhalation anaesthesia and bleeding) if there was no evidence for recovery or if they were in pain. The animals were studied by histopathology. Stroke was either the cause of death or the major contributory factor for euthanasia and confirmed by necropsy. As shown in Fig. 1 (G. S. Gilani, unpublished results), the skull was carefully opened and stroke was diagnosed if intracranial haemorrhage was present in the form of meningeal or intraventricular bleeding or blood clots.

Statistics

Effects of diets on food consumption, weight gain and mean survival time were identified using a two-way ANOVA with the Statistical Systems for Personal Computers (SAS Institute, Cary, NC, USA). Analysis of survival rates was performed by using Wilcoxon's nonparametric test (Lawless, 1982) to compare survival curves for the effects of diets. Factors of interest were two types of protein (casein and SPI) and two types of supplementary sulphur amino acids (methionine and cystine) and protein source × sulphur amino acid source interactions. Post hoc comparisons of means were performed using Tukey's honest significant difference test (Steele & Torrie, 1980). Significance was established at $P < 0.05$.

Results

The data on food consumption and weight gain measured after 31 d of feeding, and mean survival time, are shown in Table 2. The two main factors (protein source and type of supplemental sulphur amino acid) had significant effects ($P < 0.05$) on food intake and weight gain, while only the type of supplemental sulphur amino acid had a significant effect on mean survival time. The effects of protein source × supplemental sulphur amino acid interactions were, however, not significant ($P > 0.05$) for the three variables studied, i.e. food intake, weight gain and mean survival time.

On average, the two casein groups had higher ($P < 0.05$) food intake and weight gain compared with the two SPI groups after being on the test for 31 d (Table 2). The methionine-supplemented groups (casein + methionine and SPI + methionine) had lower ($P < 0.05$) average food intake but higher ($P < 0.05$) weight gain compared with the cystine-supplemented groups (casein + cystine and SPI + cystine).

On average, the methionine-supplemented groups (casein + methionine and SPI + methionine) had longer ($P < 0.05$) survival time compared with the cystine-supplemented groups (casein + cystine and SPI + cystine; Table 2).

There was also a significant effect of supplementary sulphur amino acid on survival rates of SHRSP rats. Survival rate was determined for a group and expressed as the percentage of the original number of animals still alive on a certain day.

Table 1. Dietary indispensable amino acids (mg/MJ metabolizable energy) provided by experimental diets*†

Diet	His	Ile	Leu	Lys	Met	Cys	Met + Cys	Phe + Tyr	Thr	Trp	Val
Casein + cystine	375	661	1194	1025	368	232	600	1364	563	161	794
Casein + methionine	375	661	1194	1025	550	50	600	1364	563	161	794
SPI + cystine	315	595	971	801	158	340	498	1140	461	146	570
SPI + methionine	315	595	971	801	340	158	498	1140	461	146	570

SPI, soya protein isolate.

* The previously reported amino acid data for casein (Sarwar & Peace, 1994) and that provided by the manufacturer for SPI were used in calculating amino acid compositions of the experimental diets. The two casein diets met or exceeded the indispensable amino acid requirements for growth of normal healthy laboratory rats. The two SPI diets met or exceeded the requirements for all indispensable amino acids except sulphur amino acids for rat growth. These two diets were marginally deficient in methionine + cystine.

† The indispensable amino acid requirements (mg/MJ metabolizable energy) for normal healthy rats were: His, 165; Ile, 365; Leu, 629; Lys, 541; Met + Cys, 576; Phe + Tyr, 619; Thr, 365; Trp, 118; Val, 435 (National Research Council, 1995).
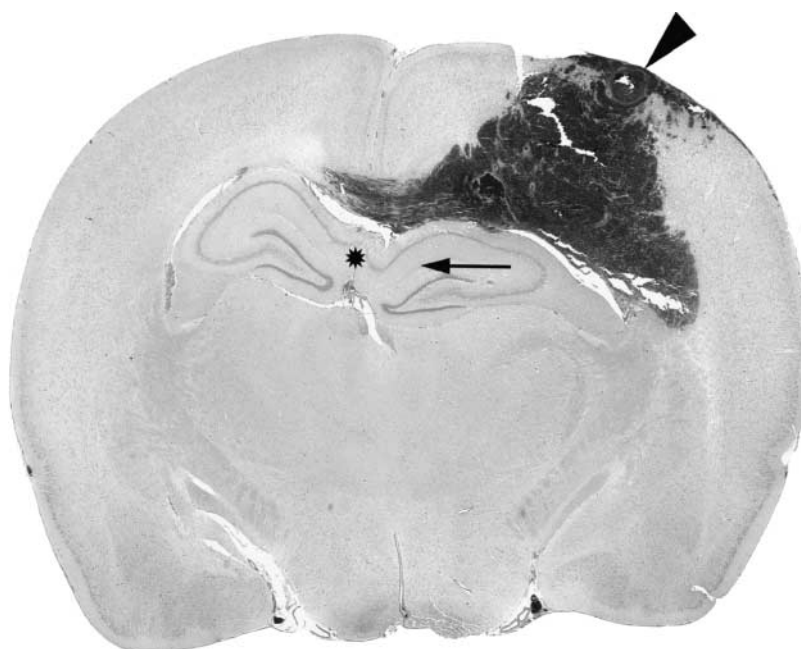
Fig. 1. Brain anatomy of a stroke-prone spontaneously hypertensive (SHRSP) rat. The figure shows a coronal section at approximately 2 mm caudal to optic chiasm in a 135 d old male SHRSP rat (fed methionine-supplemented 40 % casein diet) that showed hind-end paralysis and tremor (G.S. Gilani, unpublished results). The typical haemorrhage (▶) extends from the cortex into the lateral ventricle. There is pressure on the dorsal third ventricle (*) causing a deviation from the midline. The right hippocampus is moved to the left (◀—) because of the pressure from the haemorrhage.

The death rates of rats fed the casein and SPI diets were not different ($P > 0.05$). However, death occurred significantly earlier ($P < 0.05$) in the cystine-supplemented groups compared with the methionine-supplemented groups. The data on survival rates of rats fed the casein and the SPI diets are shown in Figs. 2 and 3, respectively.

Discussion

The cystine- and methionine-supplemented casein diets used in the present study were similar in composition to the AIN-93G and AIN-76A diets, respectively. The AIN diets contained 200 g casein/kg diet, while the casein diets tested in this investigation contained 200 g protein from casein, as casein from different sources is known to contain different amounts of total protein.

The cystine- or methionine-supplemented casein diets tested in this investigation met or exceeded the indispensable amino acid requirements for rat growth as specified by the National Research Council (1995). However, the SPI diets were marginally deficient in sulphur amino acids for rat growth. Therefore, the lower weight gain for the SPI groups compared with those for the casein groups could be due to the deficiency of sulphur amino acids in the SPI diets.

Table 2. Effects of source of dietary protein and of supplemental sulphur amino acid on growth and survival time of SHRSP rats*

(Mean values and standard deviations)

Treatment	Food intake (g/31 d)		Weight gain (g/31 d)		Mean survival time (d)	
	Mean	SD	Mean	SD	Mean	SD
Protein source						
Casein	456[b]	27	133[b]	14	87·0[a]	13·2
SPI	430[a]	44	124[a]	17	88·1[a]	14·1
Sulphur amino acid supplement						
Methionine	437[c]	35	137[d]	14	97·2[d]	18·1
Cystine	449[d]	36	119[c]	15	77·8[c]	10·0
Protein source × sulphur amino acid supplement†						
Casein + methionine	450	30	141	12	96·1	16·0
Casein + cystine	462	20	125	13	78·2	9·8
SPI + methionine	424	39	134	16	98·8	18·2
SPI + cystine	436	48	114	14	77·4	9·0

SPI, soya protein isolate.

[a,b,c,d]Mean values (n 32) within a column for protein source or sulphur amino acid supplement with unlike superscript letters were significantly different ($P < 0.05$).

* For details of procedures and diets, see p. 443 and Table 1.

† Mean values (n16) within a column for protein source × sulphur amino acid supplement interactions were not significantly different: $P > 0.05$.
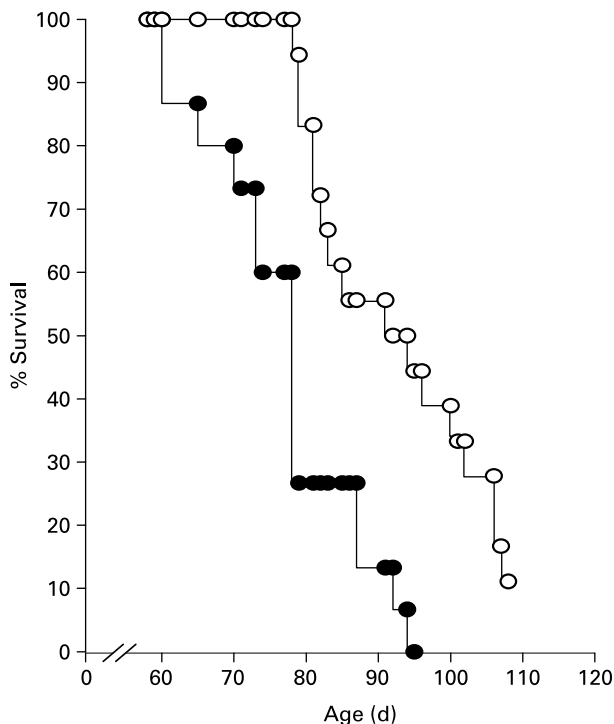
Fig. 2. Effects of cystine-supplemented (●) and methionine-supplemented (○) casein diets on survival of stroke-prone spontaneously hypertensive rats. Each curve represents the proportion surviving to the indicated age in days; each step indicates the death of one or more rats. For details of procedures and diets, see p. 443 and Table 1.
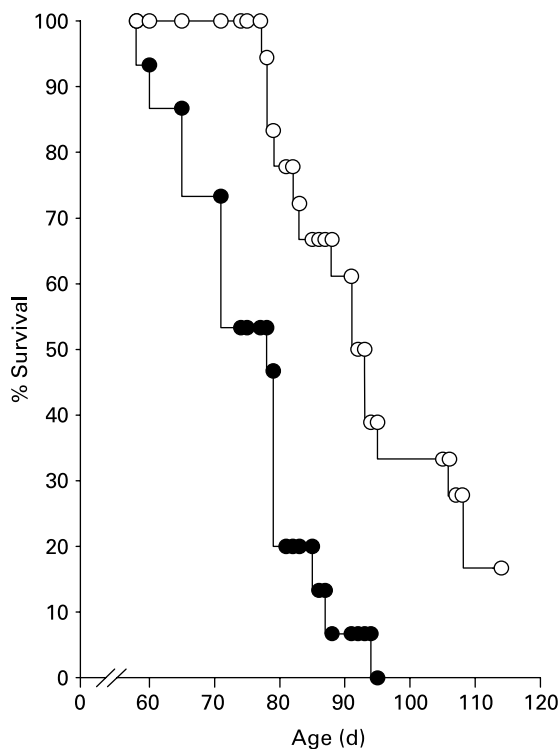

Fig. 3. Effects of cystine-supplemented (●) and methionine-supplemented (○) soya protein isolate diets on survival of stroke-prone spontaneously hypertensive rats. Each curve represents the proportion surviving to the indicated age in days; each step indicates the death of one or more rats. For details of procedures and diets, see p. 443 and Table 1.

In spite of a lower food intake, the weight gain of the methionine-supplemented groups was significantly higher than of the cystine-supplemented groups, indicating a more efficient food utilization in the case of the methionine-supplemented groups.

The cystine- or methionine-supplemented casein or SPI diets supplied the same amounts of total sulphur amino acids. However, the cystine : methionine ratios in the cystine- and methionine-supplemented casein diets (0·63 *v.* 0·09) or SPI diets (2·15 *v.* 0·46) would be quite different. In normal healthy rats, cystine may supply up to 50 % of the methionine + cystine requirement on a weight basis (National Research Council, 1995). However, information on the extent of conversion of methionine to cystine in SHRSP rats is not available. The significantly lower weight gain and survival time of the cystine-supplemented groups than of the methionine-supplemented groups observed in the present study could be due to the higher cystine : methionine ratios and the resultant inefficient conversion of methionine to cystine in the cystine-supplemented diets. Although a desirable cystine : methionine ratio for SHRSP rats is not known, it appears that a high ratio may be less effective in optimum utilization of sulphur amino acids (methionine + cystine) for growth and development and lifespan in this strain of rats. Further studies are required to optimize amino acid requirements of this animal model.

Dietary proteins (casein or SPI) tested in this investigation had no effect on survival times of SHRSP rats. However, the survival times were significantly shorter in the cystine-supplemented groups than in the methionine-supplemented groups. Since the development of stroke in SHRSP rats is influenced by blood cholesterol (Hamano *et al.* 1995; Watanabe *et al.* 2002), the prolonged longevity of SHRSP rats fed supplemental methionine could be due to its influence on cholesterol metabolism, as methionine is known to be a hypercholesterolaemic amino acid (Sugiyama & Muramatsu, 1990). A diet containing added cholesterol significantly increased blood cholesterol concentration, and this subsequently delayed the onset of stroke and prolonged the lifespan of SHRSP rats compared with SHRSP rats fed diets containing no cholesterol (Hamano *et al.* 1995). As compared with other rat strains, SHRSP rats have defective, less deformable and fragile erythrocyte membranes due to low amounts of cholesterol in cell membranes (Yamori, 1989). The cell membrane abnormalities are of pathogenic importance in hypertensive lesions because the cerebral haemorrhage and infarctions noted in SHRSP rats are commonly caused by damage to walls of small arteries. Thus, the beneficial effects of higher blood cholesterol on stroke prevention and longevity in SHRSP rats could most likely be due to incorporation of exogenous cholesterol into cell membranes, which may lead to improved cell membrane physical characteristics.

Records of water consumption were not kept in the present study. Since the amino acid composition of the experimental diet may influence water (containing NaCl) intake, induction of hypertension might be diet dependent in the present study.

Blood cholesterol concentrations were not determined in the present study. However, the addition of methionine to a 25 % casein diet has been reported to significantly increase plasma cholesterol concentrations in rats (Sugiyama & Muramatsu, 1990). Similarly, in rats fed cholesterol-free diets containing different animal and vegetable proteins, significant positive

correlation between blood cholesterol and dietary methionine, and negative correlation between blood cholesterol and dietary cystine have been reported (Sautier *et al.* 1983, 1986). Therefore, it is possible that feeding the methionine-supplemented diets in the present study may have resulted in higher blood cholesterol, which could have a protective effect on the longevity of SHRSP rats. Further studies are required to investigate the effects of supplemental methionine and cystine on blood and tissue sterols and on cell membrane deformability and longevity in SHRSP rats.

The influence of supplemental amino acids on the incidence of stroke in SHRSP rats was also studied by Yamori (1989). According to their findings, supplemental methionine and taurine attenuated the development of severe hypertension and reduced the incidence of stroke by their central effect on sympathetic blood pressure regulation and also on platelet aggregation. But supplemental cysteine or proline had an adverse effect on blood pressure and stroke incidence. Data on blood pressure and platelet aggregation were not obtained in the present study. Therefore, it was not possible to confirm or dispute the suggested mechanism of supplemental amino acids on the incidence of stroke in SHRSP rats. Further research is needed to study the influence of supplementary methionine and cystine on blood pressure and platelet aggregation in SHRSP rats.

Acknowledgements

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