Effects of the mycelial extract of cultured *Cordyceps sinensis* on *in vivo* hepatic energy metabolism and blood flow in dietary hypoferric anaemic mice

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The beneficial effects of a traditional Chinese medicine, *Cordyceps sinensis* (Cs), on mice with hypoferric anaemia were evaluated by NMR spectroscopy. Experimental hypoferric anaemia was induced in mice by feeding with an Fe-free diet for 6 weeks. They were then given extract from cultured Cs (200 mg/kg body weight daily, orally) and were placed on an Fe-containing recovery diet (35 mg Fe/kg diet) for 4 weeks. *In vivo* ^{31}P and ^{2}H NMR spectra acquired noninvasively and quantitatively at weekly intervals were used to evaluate hepatic energy metabolism and blood flow in the mice. During the 4-week Cs-extract treatment, consistent increases were observed in liver β-ATP : inorganic phosphate value by liver ^{31}P NMR spectroscopy, representing the high energy state, and in blood-flow rate as determined by ^{2}H NMR spectroscopy of deuterated water (D_2O) uptake after intravenous injection of D_2O. The haematological variables (the packed cell volume and the haemoglobin level) and the hepatic intracellular pH, which was determined from the NMR chemical shift difference between the inorganic phosphate peak and the α-phosphate peak of ATP, were not significantly different between Cs-extract-treated and control mice. As blood flow and energy metabolism are thought to be linked, the Cs-extract-increased hepatic energy metabolism in the dietary hypoferric anaemic mice was concluded to be due to increased hepatic blood flow.

Nuclear magnetic resonance: Energy metabolism: Chinese medicine

Cordyceps sinensis (Cs) is a parasitic fungus found on larvae of *Lepidoptera*. The fruiting bodies of Cs together with the larvae have been used for a long time in traditional Chinese medicine. Various pharmacological effects of this medicine, a hot-water extract of fruiting bodies and mycelia of Cs, on human subjects and animals both *in vitro* and *in vivo* have been reported (Chatterjee *et al*. 1957; Furuya *et al*. 1983; Hamada, 1991; Yoshida *et al*. 1992; Tsunoo *et al*. 1995; Manabe *et al*. 1996). These include a cure for tuberculosis and a restorative action after various diseases (Chatterjee *et al*. 1957), *in vivo* inotropic effects on the left atrium (Furuya *et al*. 1983) and anti-tumour activities (Hamada, 1991; Yoshida *et al*. 1992). Previous *in vitro* pharmacological studies have shown that mycelial extract of cultured Cs (Cs extract) produces non-purinergic and non-adrenergic inhibition of tracheal twitch contraction and relaxes persistent contraction of the trachea and the aorta, and the Cs extract was more effective in reducing tracheal activities than other traditional Chinese medicines (Tsunoo *et al*. 1995). Previously, *in vivo* ^{31}P NMR spectroscopic analyses have revealed a consistent increase in the liver β-ATP : inorganic phosphate (Pi) ratio, which represents the high energy state in the liver, in Cs-extract-treated mice (Manabe *et al*. 1996). The liver intracellular pH was not significantly different between Cs-extract-treated and control mice, and no histopathological changes, steatosis, necrosis, inflammation or fibrosis, were observed in the liver specimens from Cs-extract-treated mice. These clinical effects of, and experimental findings about, this traditional Chinese medicine indicated that the Cs extract may increase blood flow in the liver and other organs. The present study was performed to confirm the efficacy, i.e. ability to increase hepatic blood flow, of Cs in anaemia. We evaluated the effects of Cs-extract treatment on hepatic blood flow and energy metabolism in mice with dietary hypoferric anaemia. *In vivo* ^{31}P NMR analysis of the liver was performed on

Abbreviations: Cs, *Cordyceps sinensis*; Pi, inorganic phosphate.

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Fe-deficient anaemic mice administered Cs extract. As blood flow and energy metabolism are thought to be linked (Okunieff *et al*. 1988; Tozer *et al*. 1990), ^{2}H NMR spectroscopy of deuterated water (D_2O) uptake after intravenous injection of D_2O was used to measure hepatic blood flow in the anaemic mice treated with Cs extract (Mattiello & Evelhoch, 1991; Zhao *et al*. 1995). The *in vivo* structure-biological assessment techniques for energy metabolism and blood flow are useful not only for pharmaceutical and toxicological sciences but also for nutritional sciences.

Materials and methods

Cs-extract preparation

Hot-water extract from cultured Cs (Cs extract) was prepared according to the method described previously (Tsunoo *et al*. 1995; Manabe *et al*. 1996). Briefly, the Cs strain SMIH8819 was isolated at the Sanming Mycological Institute (Sanming, China). This strain was maintained on malt extract agar slants composed of (g/l): 20 malt extract, 1 peptone and 20 glucose, and then cultured in fermentation medium (g/l: sucrose 40, K_2HPO_4 4, asparagine 4, $(NH_4)_2HPO_4$ 2, $MgSO_4.7H_2O$ 2, $CaCO_3$ 0·25, $CaCl_2$ 0·1 and yeast extract 4). The pH of the medium was adjusted to 5·5 before sterilization. During fermentation, the medium was aerated at a rate of 30 litres/min. After fermentation, the mycelia of cultured Cs were extracted with hot water (90°) for 2 h, and then the extract was filtered through a membrane filter (0·22 μm) and lyophilized.

Experimental protocol

Male ICR mice purchased from SLC (Shizuoka, Japan) weighing an average of 13·9 (SE 0·8) g at the beginning of the experiment were used at 3 weeks of age. During the experiment, the mice were given free access to tap water in an air-conditioned room (22 ± 2°, relative humidity 55 ± 5 %) under a controlled 12 h light–dark cycle. All animals received humane care as outlined in the *Guide for the Care and Use of Laboratory Animals* (Japanese Animal Care Committee, 1981). The mice were given an Fe-free diet (Table 1) for 6 weeks to induce Fe-deficiency anaemia as described previously (Tsuchita *et al*. 1991). Normal mice were given a basal diet (Table 1; containing 35 mg ferric citrate/kg) (Tsuchita *et al*. 1991).

Twelve mice with dietary hypoferric anaemia were divided into two groups, vehicle control and Cs-extract-treated groups, with six mice in each group. Twelve normal mice were also divided into two groups, vehicle control and Cs-extract-treated groups, with six mice in each group. The anaemic and normal Cs-extract-treated groups received Cs extract dissolved in distilled water, orally, at a dose of 200 mg/kg per d in a volume of 5 ml/kg for 4 weeks. The anaemic and normal vehicle control groups were given distilled water orally (5 ml/kg per d) for 4 weeks. During the 4 weeks of Cs-extract treatment, all animals were given a recovery diet (Table 1; containing 35 mg ferric chloride/kg) (Tsuchita *et al*. 1991).

Histological and haematological analyses

As described earlier, six mice in each group (normal and anaemic groups) were killed under diethyl ether anaesthesia at the beginning, 3 weeks after commencement of the experiment and before Cs-extract treatment. After the final NMR experiment, the remaining animals (six mice in each group) were killed under diethyl ether anaesthesia. The livers were removed, weighed and fixed in 100 ml/l buffered formalin solution (pH 7·2). After fixation, the liver samples were embedded in paraffin and cut into sections 3 μm thick which were then stained with haematoxylin and eosin by routine methods. The liver histopathology was examined by light microscopy and scored as indicated by Takahashi *et al*. (1990).

During Cs-extract treatment, collection of blood samples from the tail vein was repeated at intervals of 1 week, and packed cell volumes and haemoglobin levels were measured by conventional methods.

NMR measurements

^{31}P, ^{1}H and ^{2}H NMR spectra were acquired on a JNM-alpha-400 FT-NMR spectrometer (JEOL, Tokyo, Japan). All NMR experiments were repeated at 1-week intervals during Cs administration and were performed at 25 ± 1°. The

Table 1. Composition of mineral mixture (g/kg mixture) contained in the basal, recovery and iron-free diets fed to mice*

Minerals	Basal diet	Recovery diet	Fe-free diet
$CaHPO_4$	500·0	500·0	500·00
NaCl	74·0	74·0	74·0
Tripotassium citrate monohydrate	220·0	220·0	220·0
K_2SO_4	52·0	52·0	52·0
MgO	24·0	24·0	24·0
$MnCO_3$	3·5	3·5	3·5
$ZnCO_3$	1·6	1·6	1·6
$CuCO_3$	0·3	0·3	0·3
KIO_3	0·01	0·01	0·01
$Na_2SeO_3.5H_2O$	0·01	0·01	0·01
$CrK(SO_4)_2.12H_2O$	0·55	0·55	0·55
Ferric citrate	5·63	–	–
$FeCl_3.6H_2O$	–	4·84	–

* Diets contained the following ingredients (g/kg): casein 200, DL-methionine 3, maize starch 350, sucrose 300, cellulose powder 50, soyabean oil 50, vitamin mixture 10, choline bitartrate 2 and mineral mixture 35.

NMR spectrometer was equipped with a vertical 9·20 Tesla, 89 mm bore (inside gradient) superconducting magnet (Oxford Instruments, Bedford, MA, USA). During NMR examination, each mouse was anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), immobilized with surgical tape on a probe-bed, and maintained in a probe-chamber at a controlled air temperature of $25 \pm 1°$. As previously reported (Manabe *et al*. 1996), ^{31}P NMR was performed according to a modification of the method of Takahashi *et al*. (1990) to obtain *in vivo* ^{31}P NMR spectra of the steady-state livers in intact animals. Briefly, the mice were mounted on a surface coil probe (two-turns, 20 mm in diameter; JEOL) which was tuned to ^{31}P at 161·7 MHz used in conjunction with a ferrite screen composed of strips of computer magnetic tape (Scotch 700; Three M Co., St Louis, MO, USA) to eliminate ^{31}P NMR signals arising from superficial muscles (Geoffrion *et al*. 1988). Field homogeneity was controlled by adjusting shim parameters using a water ^{1}H signal derived from the liver sample. Acquisition conditions were set as follows to obtain fully relaxed signals: pulse width, 20 μs; pulse repetition time, 3·0 s; 200 scans. Thus, it took 10 min for each measurement. After exponential multiplication, the accumulated free induction decays were subjected to Fourier transformation. Individual spectral peak areas were calculated by computer integration with JEOL software, and the creatine phosphate peak was used as an internal reference (0 ppm). The relative amount of each compound was converted to absolute concentration by comparison with the data of an external reference (20 mM-methylene diphosphonate in water) in a spherical bulb. The relative ATP levels were obtained by taking the ratio β-phosphate peak of ATP (β-ATP) : reference peak (Azuma *et al*. 1994), and then the β-ATP : Pi peak area ratio was calculated to evaluate *in vivo* liver energy metabolism. The intracellular pH was determined from the chemical shift difference between the Pi peak and the α-phosphate peak of ATP (Malloy *et al*. 1986).

In vivo ^{2}H and ^{1}H NMR spectra of the livers were obtained by modification of the method of Zhao *et al*. (1995). Briefly, the ^{2}H and ^{1}H signals were also detected with a coil probe. Spectra were acquired under optimized signal-to-noise conditions where a repetition time : T_1 value of 0·93 was used to acquire both ^{2}H and ^{1}H spectra. ^{2}H spectra were acquired with a 60° flip angle and 0·25 s repetition time (Mattiello & Evelhoch, 1991). For blood flow measurement, 100 μl/mouse of 9 g NaCl/l in D_2O was injected into the tail vein via a catheter. Time domain data (2×256 points) were averaged into ninety consecutive 4 s time blocks. Relative volume average blood flow values were calculated by computer integration with JEOL software using the D_2O uptake integral approach as described by Mattiello & Evelhoch, (1991). Blood flow rates are expressed as a percentage of the mean value for normal controls on day 0 (0 week).

Statistical analysis of data

The significance of differences between Cs-extract-treated and vehicle control mice was assessed by Student's paired *t* test carried out with the StatView IV program (J4.5, 95.8.14; Abacus Concepts Inc., Berkeley, CA, USA) using a Macintosh computer. Differences at a probability of $P < 0·05$ were considered significant. All data are shown as means with their standard errors.

Results

Body and liver weight

As summarized in Table 2, there were no significant differences in body weight, liver weight or relative liver weight (liver weight/body weight × 100) between vehicle control and Cs-extract-treated normal or anaemic mice.

Haematology and liver histopathology

As shown in Table 3, severe Fe-deficiency anaemia was induced by feeding with the Fe-free diet for 6 weeks. During the period of feeding with the Fe-free diet, significant decreases ($P < 0·01$; more than 50 % decrease) were observed

Table 2. Changes in body weight, liver weight and relative liver weight in normal and anaemic mice administered vehicle or an extract from *Cordyceps sinensis* (Cs) for 4 weeks*

(Mean values with their standard errors for six mice)

	Time (weeks) …	−6		−3		0		2		4	
Variable	Group	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body weight (g)	Normal, vehicle	13·9	0·8	30·9	1·4	35·4	0·5	36·3	0·5	37·2	1·3
	Normal, Cs extract	–		–		–		36·8	1·0	37·3	1·0
	Anaemic, vehicle	–		29·0	1·2	34·7	1·0	35·8	0·7	37·3	0·9
	Anaemic, Cs extract	–		–		–		35·6	0·7	37·2	0·9
Liver weight (g)	Normal, vehicle	0·61	0·05	1·52	0·08	1·68	0·10	–		1·75	0·07
	Normal, Cs extract	–		–		–		–		1·80	0·04
	Anaemic, vehicle	–		1·44	0·09	1·63	0·13	–		1·77	0·05
	Anaemic, Cs extract	–		–		–		–		1·80	0·04
Relative liver weight (%)	Normal, vehicle	4·51	0·64	4·35	0·26	4·62	0·27	–		4·71	0·03
	Normal, Cs extract	–		–		–		–		4·83	0·05
	Anaemic, vehicle	–		4·10	0·23	4·54	0·35	–		4·74	0·08
	Anaemic, Cs extract	–		–		–		–		4·84	0·09

* For details of diets, extracts and procedures, see Table 1 and pp. 198–199.

Table 3. Changes in packed cell volume and haemoglobin levels in normal and anaemic mice administered vehicle or an extract from *Cordyceps sinensis* (Cs) for 4 weeks*

(Mean values with their standard errors for six mice)

	Time (weeks) …	−6		−3		0		2		4	
Variable	Group	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Packed cell volume (%)	Normal, vehicle	13·6	0·9	13·3[a]	1·0	13·3[a]	1·2	13·9[a]	1·1	13·8[a]	1·1
	Normal, Cs extract	–		–		–		13·7[a]	1·5	13·9[a]	1·4
	Anaemic, vehicle	–		6·0[b]	0·8	4·5[b]	0·7	12·7[a]	0·8	13·7[a]	0·8
	Anaemic, Cs extract	–		–		–		13·1[a]	0·9	13·2[a]	1·0
Haemoglobin (g/l)	Normal, vehicle	525	36	527[a]	47	518[a]	44	527[a]	53	529[a]	33
	Normal, Cs extract	–		–		–		526[a]	38	533[a]	32
	Anaemic, vehicle	–		211[b]	24	174[b]	25	490[a]	31	526[a]	33
	Anaemic, Cs extract	–		–		–		505[a]	35	528[a]	25

[a,b] Mean values within a row not sharing a common superscript letter were significantly different: $P < 0{\cdot}01$.
* For details of diets, extract and procedures, see Table 1 and pp. 198–199.

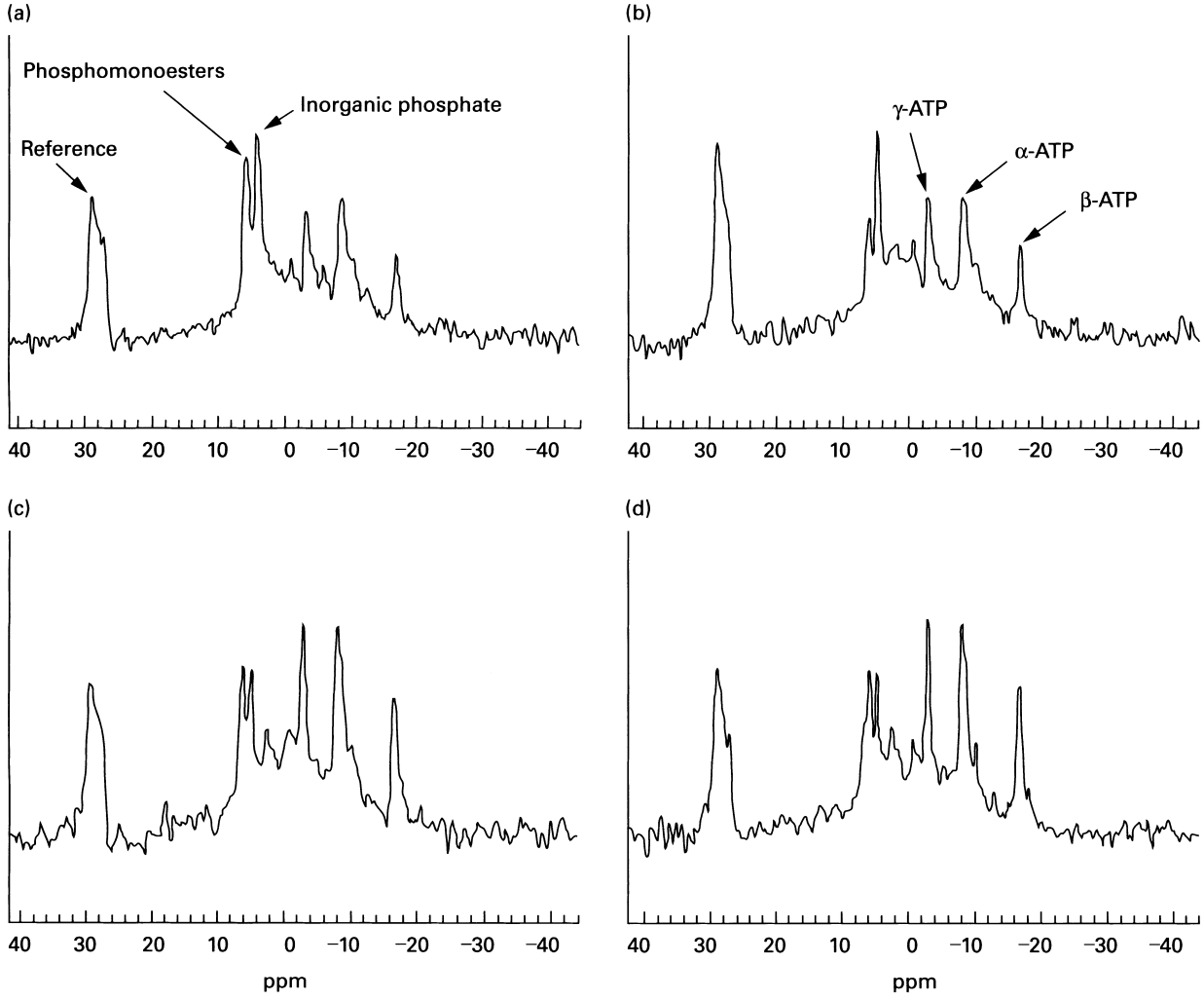

Fig. 1. Representative *in vivo* ^{31}P NMR spectra obtained from normal (a and c) and anaemic (b and d) mice administered vehicle (a and b) or an extract from *Cordyceps sinensis* (c and d) at 4 weeks after the initial administration. The creatine phosphate peak was used as the internal reference (0 ppm), and prominent peaks observed were phosphomonoester, inorganic phosphate and nucleotide triphosphate (mainly γ-ATP, α-ATP and β-ATP). The γ-ATP and α-ATP signals may contain contributions from β- and α-ADP respectively. The signal corresponding to β-ATP that has no overlap with the other phosphate signals was used to quantify ATP, and its spectral peak area was calculated by computer integration. Increases in hepatic ATP level and decreases in the inorganic phosphate levels were demonstrated in both normal and anaemic mice treated with *Cordyceps sinensis* extract.

in both packed cell volumes and blood haemoglobin levels. During Cs-extract administration, a recovery diet was given to the anaemic mice. The Cs extract had no effect on packed cell volumes or blood haemoglobin levels in normal or anaemic mice.

During the experiment, conventional histopathological evaluation of the liver was performed. No liver specimens obtained from either Cs-extract-treated or vehicle control mice in either normal or anaemic groups showed steatosis, necrosis, inflammation or fibrosis (results not shown).

^{31}P NMR spectroscopy for liver ATP measurement

Representative examples of the *in vivo* liver ^{31}P NMR spectra acquired from vehicle control and Cs-extract-treated groups of both normal and anaemic mice at 4 weeks after the initial administration (final NMR measurement) are shown in Fig. 1. Peak assignments are given in the legend.

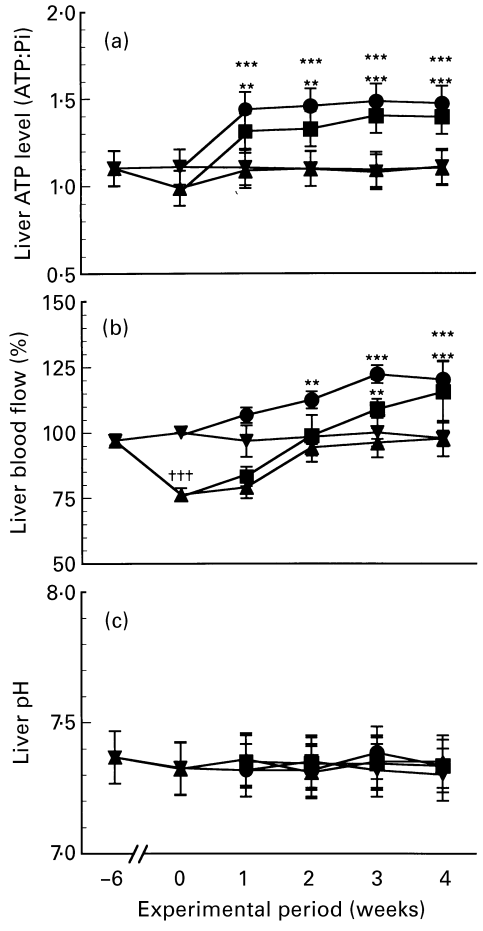

Fig. 2. (a) Hepatic β-ATP : inorganic phosphate (Pi), (b) liver blood flow and (c) intracellular pH in the livers of normal (▼, ●) and anaemic (▲, ■) mice receiving vehicle (▼, ▲) or an extract from *Cordyceps sinensis* (200 mg/kg per d) (●, ■) for 4 weeks. For details of diets, extract and procedures, see Table 1 and pp. 198–199. Blood-flow rates are expressed as a percentage of the mean value for normal controls on day 0 (week 0). Values are means for six mice, with their standard errors represented by vertical bars. Mean values were significantly different from those for the corresponding vehicle control group: $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. Mean value was significantly different from normal control on day 0: ††† $P < 0{\cdot}001$.

Prominent peaks for phosphomonoester, Pi, and nucleotide triphosphate (mainly γ-ATP, α-ATP and β-ATP) were observed. The γ-ATP and α-ATP signals may have included contributions from β- and α-ADP respectively. The signal corresponding to β-ATP that had no overlap with other phosphate signals was used to quantify ATP (Takahashi *et al*. 1990; Manabe *et al*. 1996). The spectral resolution was sufficient to measure individual peak areas using a digital integration technique. The resolution and the signal : noise ratio of the ^{31}P spectra obtained by the present *in vivo* method were sufficient for the quantitative evaluation of peak area ratios. These representative figures from both normal and anaemic mice indicated that the peak intensity of the β-ATP resonance was higher, but the peak intensity of the Pi resonance was lower in the Cs-extract-treated (Fig. 1(c and d)) than in the vehicle control mice (Fig. 1(a and b)).

Fig. 2(a) shows the time courses of the β-ATP : Pi peak area ratios obtained from the liver in the Cs-extract-treated and the vehicle control groups of both normal and anaemic mice, as determined by ^{31}P NMR spectroscopy. Slightly lower ATP : Pi values were observed in anaemic mice in comparison with those of normal controls (at 0 week). During the Cs-extract administration period (at 1, 2, 3 and 4 weeks), the ATP : Pi values of the Cs-extract-treated normal mice were significantly higher than those of the vehicle control normal mice ($P < 0{\cdot}001$). In anaemic groups, the ATP : Pi values were also significantly higher in the Cs-extract-treated than in the vehicle control mice during the Cs-extract administration period (at 1, 2, 3 and 4 weeks; $P < 0{\cdot}01$, 0·01, 0·001 and 0·001 respectively).

The changes in liver blood flow measured by the D_2O uptake ^{2}H NMR spectroscopy method in both normal and anaemic mice are summarized in Fig. 2(b). Each blood-flow rate was expressed as a percentage of the mean value of the normal control on day 0 (0 week). A significantly decreased hepatic blood-flow rate in anaemic mice was noted when compared with that in the normal control mice (at 0 week; $P < 0{\cdot}001$). During the Cs-extract administration period (at 2, 3 and 4 weeks), the hepatic blood-flow rates of the Cs-extract-treated normal mice were significantly higher than those in the vehicle-treated controls ($P < 0{\cdot}01$, 0·001 and 0·001 respectively). In anaemic groups, the liver blood-flow rates were also significantly higher in the Cs-extract-treated than in the vehicle control mice (at 3 and 4 weeks; $P < 0{\cdot}01$ and 0·001).

The time courses of changes in the intracellular pH of the liver (liver pH) measured by the chemical shift difference between the Pi and α-ATP peaks in the ^{31}P NMR spectra are shown in Fig. 2(c). In both normal and anaemic groups, the liver pH in the Cs-extract-treated mice was not significantly lower than that in the vehicle-treated controls throughout the experiment.

Discussion

As described earlier, the fruiting bodies of Cs (parasitic on larvae of *Lepidoptera*) have long been used in traditional Chinese medicine and have various pharmacological effects on human subjects and animals both *in vitro* and *in vivo* (Tsunoo *et al*. 1995). In a recent study, the extract of cultured Cs was given to male long-distance runners over a

period of 12 weeks (Hiyoshi *et al*. 1996). Echocardiography revealed a 6·5 % increase in the ejection fraction and five of seven runners recorded their personal best times during the trial period. The Cs extract evaluated by conventional *in vitro* pharmacological tests showed non-purinergic and non-adrenergic inhibition of tracheal twitch contractions, and relaxation of persistent contractions of the trachea and the aorta (Tsunoo *et al*. 1995). Our previous examination of *in vivo* ^{31}P NMR liver energy levels revealed a consistent increase in high energy state in the liver of normal healthy mice administered Cs extract (Manabe *et al*. 1996). In the normal mice, moreover, no significant differences were observed in the hepatic intracellular pH between Cs-extract-treated and control mice, and histopathological evaluation revealed no steatosis, necrosis, inflammation or fibrosis of the liver in Cs-extract-treated mice. In the present study, we investigated the hepatic activation effects of Cs extract on mice with anaemia induced by an Fe-free diet. In general, growing animals require more than 5 mg Fe/kg per d from food (Wickramasinghe, 1988). We previously reported that an Fe-free diet induced severe anaemia in rats (Tsuhichita *et al*. 1991). As summarized in Table 3, severe anaemia was also induced in mice (more than 50 % reductions in packed cell volume and blood haemoglobin level) by feeding on an Fe-free diet for 6 weeks. In the present study, the dietary anaemic mice were given a recovery diet during Cs-extract administration for a period of 4 weeks. Our findings confirmed that the Cs extract had no effect on these haematological variables in either normal or anaemic mice. Moreover, histopathological evaluation demonstrated no pathological changes (steatosis, necrosis, inflammation or fibrosis) in anaemic or control groups during the experiment in the liver sections prepared from either Cs-extract-treated or vehicle control mice. We concluded that the reciprocal administration of Cs extract, at a high dose of 200 mg/kg per d, has no toxic effect on the liver of either normal or anaemic mice.

The use of *in vivo* ^{31}P NMR spectroscopy made it possible to overcome the sampling problems associated with conventional biochemical analysis, i.e. HPLC analysis of liver extract, although we measured only free and not total ATP. Moreover, the NMR technique used in the present study is the only non-invasive quantitative method available. Previous studies showed that *in vivo* serial ^{31}P NMR spectroscopy is a useful technique to detect the changes in liver energy state induced by CCl_4 administration in mice (Geoffrion *et al*. 1988) and rats (Bates *et al*. 1988; Sandhu *et al*. 1991), and chronic ethanol feeding in rats (Takahashi *et al*. 1990; Brauer & Ling, 1991). Bowers *et al*. (1992) used ^{31}P NMR to evaluate the liver energy state in rats following orthotopic liver transplantation. The relationship between NMR-visible high-energy phosphate, i.e. ATP, and transplant outcome in the case of liver damage by ischaemia was examined. *In vivo* ^{31}P NMR spectroscopy was used to analyse the pre-transplant liver of donor rats and the post-transplant liver of recipient rats. They confirmed that recovery of NMR-visible high-energy phosphates 20 min after re-establishment of portal blood flow was a good indicator of transplant outcome in cases of rat liver damage by ischaemia. Yang *et al*. (1995) also reported that liver energy metabolism assessed by ^{31}P-NMR spectroscopy is a good indicator of the viability of graft livers in rats. Recently, Takahashi *et al*. (1997) evaluated the effects of anaesthetic agents, halothane and isoflurane, on the hepatic phosphor-energetic state by *in vivo* ^{31}P NMR. They compared the effects of halothane *v*. isoflurane on the phosphor-energetic state, which was evaluated from the changes in β-ATP : Pi levels, and intracellular pH of the rat liver using *in vivo* ^{31}P NMR spectroscopy during and after haemorrhagic shock, and collected consecutive *in vivo* ^{31}P NMR spectra throughout the study. Their results indicated that intracellular acidosis was more severe in the halothane group and that recovery of β-ATP : Pi level was better in the isoflurane group. Thus, *in vivo* ^{31}P NMR spectroscopy, which is the only non-invasive quantitative method available, is useful for consecutive evaluation of both liver energy metabolism and intracellular pH.

In the present study, the ^{31}P NMR spectral resolution and signal : noise ratio were sufficient to measure individual peak areas as shown in Fig. 1. In both normal and anaemic mice, the peak intensity of the β-ATP resonance was higher, but the peak intensity of the Pi resonance was lower in the Cs-extract-treated mice than in the vehicle-treated controls. During the Cs-extract administration period, the β-ATP : Pi values of the Cs-extract-treated groups were higher than those in the vehicle-treated controls. Such an increase in the β-ATP : Pi value strongly suggests that there is a higher energy state in the liver of the Cs-extract-treated mice than in vehicle-treated controls. Interestingly, no significant changes were observed in hepatic intracellular pH, which is determined by the chemical shift difference between the Pi and α-ATP peaks in the ^{31}P NMR spectra, in any group, indicating that the Cs extract had no severe hepatotoxicity and maintained normoxic conditions. In general, intracellular acidosis can be induced by hepatotoxic reagents and hypoxic conditions (Desmoulin *et al*. 1987; Takahashi *et al*. 1990). Moreover, histopathological findings have revealed that Cs extract does not induce severe hepatotoxic changes in the liver, i.e. no steatosis, necrosis, inflammation or fibrosis.

The mechanism underlying the high hepatic energy state brought about during Cs-extract administration, however, remains unclear. The present study revealed that Cs extract treatment in both normal and anaemic groups increased liver blood flow, as determined by D_2O uptake-^{2}H NMR spectroscopy performed according to a modification of the methods of Mattiello *et al*. (1991) and Zhao *et al*. (1995), which were suitable for rats. We established a technique appropriate for mice and estimated the mouse liver blood flow by *in vivo* ^{2}H NMR spectroscopy of D_2O uptake after intravenous injection of D_2O. Okunieff *et al*. (1988) and Tozer *et al*. (1990) reported that hepatic blood flow and energy metabolism are closely linked. In a recent study, Whalen & Shapiro (1991) measured liver blood flow using a D_2O washout technique, using ^{2}H NMR and liver concentration of ATP and intracellular pH determined by ^{31}P NMR techniques in normal rats, and confirmed that liver blood flow rates are linked to liver ATP levels. Zhao *et al*. (1995) acquired *in vivo* ^{31}P and ^{2}H NMR spectra to evaluate the blood flow, intracellular pH and energy metabolism (β-ATP : Pi) in mice with implanted tumours and normal livers. They reported that the anaesthetic halothane decreased tumour blood flow, β-ATP : Pi value and intracellular pH. *In vivo* ^{31}P NMR measurements of normal mouse liver

indicated that halothane had a similar effect in the liver. The induction of ATP synthesis has been suggested to be caused by activated adenine translocase activity and/or mitochondrial respiratory-chain function in the livers of mice receiving Cs extract. The decrease in ATP consumption attributed to depressed Na pump function can theoretically produce a high energy state in the liver (Takahashi *et al*. 1990). When Na pump function is depressed by toxic agents and hypoxic conditions, severe intracellular acidosis is induced (Desmoulin *et al*. 1987; Takahashi *et al*. 1990). However, no such decrease in ATP consumption occurred in the liver of Cs-extract-treated mice, because no severe changes were observed in liver intracellular pH. The hepatotrophic synthetic reagent malotilate, which induces liver hypertrophy, activates hepatic mitochondrial function and increases ATP levels in the liver of normal and partially hepatectomized rats (Niwano *et al*. 1986). However, Cs extract has no such hypertrophic activity in the liver, and so the mechanism of the increase in hepatic ATP level must be different from that mechanism for the synthetic reagent malotilate. Natural substances also increase hepatic blood flow and ATP levels. For example, Walsh *et al*. (1993) measured blood flow and high-energy phosphates in control rat livers and in those damaged by ischaemia using *in vivo* NMR spectroscopy to determine the effects of glucagon on these variables. Ischaemia led to a loss of liver ATP, and then reperfusion showed recovery of ATP. Glucagon treatment before induction of ischaemia accelerated the recovery of hepatic ATP level. Ischaemia-reperfusion decreased hepatic blood flow, and glucagon treatment stimulated liver blood flow in a dose-dependent manner. These results indicated that ischaemia followed by reperfusion leads to a decrease in hepatic blood flow before alterations in ATP and that the response of the liver to glucagon was altered in the reperfused liver. Further investigations to determine the mechanism underlying the high hepatic energy state induced by Cs-extract administration are currently in progress in our laboratory.

The present findings indicate that the traditional Chinese medicine Cs extract increases hepatic blood flow in mice with dietary hypoferric anaemia, a condition associated with reduced blood flow. The Cs extract is considered to relax contractions in the liver blood vessel system and to increase hepatic blood flow, causing liver ATP levels to increase. Such hepatic ATP augmentation may contribute to the acceleration of recovery and improvement of liver function in patients with anaemia. Moreover, the present study showed that *in vivo* ^{31}P NMR and ^{2}H NMR analyses of the liver, measurement of hepatic energy state and blood flow respectively, were useful tools for evaluation of efficacy of the various nutritional elements, crude drugs, orthodox medicines, traditional herbal remedies and health foods, which improve, recover and/or maintain health. Thus, *in vivo* assessment methods for energy metabolism and blood flow are useful tools for nutritional sciences.

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