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Journal of Ethnopharmacology 64 (1999) 141–147

Journal of ETHNO-PHARMACOLOGY

Studies on the preventive effect of *Spirulina maxima* on fatty liver development induced by carbon tetrachloride, in the rat

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Received 20 January 1998; received in revised form 18 June 1998; accepted 29 June 1998

Abstract

The aim of the present work was to assess if the feeding of either the oil extract of *Spirulina maxima* or of its defatted fraction would prevent fatty liver development, induced in rats by a single intraperitoneal dose of carbon tetrachloride (CCl_4). Liver and serum lipids were evaluated 4 days after treatment with this agent. Concentration of liver lipids did not differ in rats fed on a purified diet either without or with one of the fractions of *Spirulina*, except for total cholesterol, which showed a slight increase in the group receiving the oil extract of *Spirulina*. However, after CCl_4 treatment, liver total lipids and triacylglycerols were significantly lower in rats fed on a diet containing any fraction of *Spirulina* (defatted or the oil fraction) than in rats without *Spirulina* in their diet. Furthermore, the increased liver cholesterol values, induced by CCl_4 treatment, were not observed in rats receiving *Spirulina*. In addition, rats receiving whole *Spirulina* in their diet and treated only with the vehicle showed an increase in the percentage of HDL values. The changes in VLDL and LDL induced by CCl_4 treatment were not observed in the whole *Spirulina* group. Furthermore, after CCl_4 treatment the values of the liver microsomal thiobarbituric acid-reactive substances were lower in the whole *Spirulina* group than in the control group. These results support the potential hepatoprotective role of *Spirulina*.

Keywords. *Spirulina maxima*; Fatty liver; Carbon tetrachloride: Triacylglycerol; Cholesterol

1. Introduction

Spirulina maxima is a cyanobacterium belonging to the Oscillatoriaceae family which grows commonly in alkaline waters (Ciferri, 1983). This

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0378-8741/99/$ - see front matter
PII S0378-8741(98)00120-2

cyanobacterium grows naturally in Texcoco lake in Mexico and is consumed by humans, in general as a food supplement.

Toxicological studies of several *Spirulina* species have not revealed any toxic effect during and after different acute, chronic and reproductive tests (Yoshino et al., 1980; Krishnakumari et al., 1982; Chamorro et al., 1988; Chamorro and Salazar, 1990; Chamorro et al., 1996; Salazar et al., 1996).

It has been reported that *Spirulina* sp. reduce body weight in obese humans (Becker et al., 1986) as well as their total cholesterol plasma levels (Iwata et al., 1990). Furthermore, *Spirulina maxima* has been shown to decrease vascular tone of aortic rings from rats fed on a normal purified diet (Paredes-Carbajal et al., 1997) as well as preventing the development of fatty liver induced by a fructose-rich purified diet (González De Rivera et al., 1993), or by carbon tetrachloride treatment (Torres-Durán et al., 1998).

As a first attempt to elucidate the mechanisms by which *Spirulina maxima* prevents fatty liver induced by carbon tetrachloride, in this study the effects of two fractions of *Spirulina* (the oil fraction and its residual defatted fraction) on hepatic and serum lipids were evaluated. In addition, the effects of the whole *Spirulina* on the percentage of the different kinds of serum lipoproteins and the hepatic content of thiobarbituric acid-reactive substances (TBARS) were evaluated.

2. Materials and methods

2.1. Reagents

All reagents and chemicals used were of analytical grade, except corn oil and corn starch, which were commercial products for culinary use. The rest of the diet components were from Sigma (St. Louis, MO). Glucose and organic solvents were from Merck (Mexico).

The spray-dried powder of *Spirulina maxima* (herbarium NSBC no. 12584, authenticated by C.M. González, National School of Biological Sciences) used in these experiments was a generous gift from Spirulina Mexicana, S.A. de C.V. (Mexico).

The oil extract of *Spirulina* was obtained using a Soxhlet extractor and a chloroform–methanol mixture (3:1, v/v) for 24 h. Throughout the procedure special care was taken to avoid light exposure in order to preserve the *Spirulina* constituents. Removal of the solvent mixture was initiated in a vacuum rotavapor and thereafter completed by the use of a nitrogen stream at 50°C (yield, 15%). The remaining *Spirulina* (defatted) extract was dried and pulverized.

The whole or defatted *Spirulina* powder, as well as the oil extract, were mixed to homogeneity with the rest of the respective diet ingredients.

2.2. Animals and diets

In a first series of experiments, designed to identify the active substances of *Spirulina* by means of their solubility properties, 30 male Wistar rats, weighing 190–250 g (bred in the animal care and breeding unit of the School of Medicine, UNAM, Mexico City), were randomly allocated to one of three groups (groups A, D and O) of 10 rats. Each group was fed on one of the purified diets (shown in Table 1):

Group A	Control	Purified diet, without *Spirulina* extract
Group D	Experimental	Purified diet, with defatted *Spirulina* (5%) extract
Group O	Experimental	Purified diet, with an oil extract equivalent to 5% *Spirulina*

The diets were prepared according to the guidelines recommended by the American Institute of Nutrition (Bieri et al., 1977) and they were adjusted to have the same protein, carbohydrate and lipid content (Table 1). Animals were housed by lots of two or three in a room with controlled temperature (20–25°C) and light exposure (07:00–19:00 h). Water was supplied ad libitum, and the amount of diet provided was 20

Table 1
Diet composition

Ingredients[a] (%)	Diets			
	A (control)	D (defatted)	O (oil)	S (whole)
Casein	20.0	17.0	20.0	17.0
Corn starch	7.0	5.0	7.0	5.0
Corn oil	5.0	5.0	4.3	5.0
Cellulose	3.0	3.0	3.0	3.0
Vitamin mixture	1.0	1.0	1.0	1.0
Mineral mixture	3.5	3.5	3.5	3.5
Methionine	0.3	0.3	0.3	0.3
Choline chloride	0.2	0.2	0.2	0.2
Glucose	60.0	60.0	60.0	60.0
Spirulina maxima				
Defatted	—	5.0	—	—
Oil extract	—	—	0.7	—
Dried whole	—	—	—	5.0

[a] Composition of diets A, D, O and S was adjusted to the same content of protein, carbohydrate, and oil (total lipids of *Spirulina maxima* were 15%, estimated contents of defatted *Spirulina* were 60 and 40% for protein and carbohydrate, respectively).

g/day per rat. After an acclimatization period of 5 days, during which each group was fed on its respective diet, the animals were treated with a single intraperitoneal injection of either carbon tetrachloride (AC, DC and OC groups: 1.0 ml/kg body weight, diluted 1:2 with corn oil as vehicle,) or an equivalent volume of the vehicle (groups A, D and O). These latter groups were included in order to determine if diets could produce some effects on hepatic and serum lipid levels.

In a second series of experiments, designed to evaluate the effects of the whole *Spirulina* on serum lipoproteins distribution and its antioxidant properties in fatty liver, two groups of 10 rats each were fed on a purified diet with addition or not of 5% whole *Spirulina* (diets S and A, in Table 1) for 5 days, and thereafter treated with either CCl_4 (groups SC and AC) or vehicle (groups S and A).

On the fourth day after the intraperitoneal injection all animals were starved for 12 h and thereafter anesthetized with diethylether and killed by cervical dislocation. The serum was separated by blood centrifugation, and stored at −78°C. Fresh serum was used for lipoprotein analysis. Livers were excised, weighed and stored at −78°C until lipid analyses were performed.

2.3. Preparation of the liver lipid extracts

Total lipids were extracted with chloroform–methanol (3:1, v/v) by a modified version of Folch's method. For liver samples, 1 g of tissue was homogenized in 4 vol of 0.05 M phosphate buffer, pH 7.2. Then the pH was adjusted to 6.0 by the addition of 2 N HCl solution and this suspension was extracted three times with 4 vol each of a chloroform–methanol mixture (3:1, v/v). The extract was washed with 10 ml of water, the organic fraction was evaporated under a nitrogen stream, then weighed (for total lipids), and stored at −78°C until cholesterol and triacylglycerols analyses were performed.

2.4. Analytical methods

Serum and liver concentrations of cholesterol or triacylglycerols were measured using commercial enzymatic–colorimetric kits from Lakeside, Mexico (CHOD-PAP and GPO-PAP, respectively). Total liver lipids were measured gravimetrically.

Table 2
Effects of *Spirulina* on changes induced by carbon tetrachloride on liver lipids

Treatment	Group	Total lipids (mg/g, wet weight)	Total triacylglycerols (mg/g, wet weight)	Total cholesterol (mg/g, wet weight)
Without CCl_4	A	38.7 ± 2.6	1.30 ± 0.47	0.89 ± 0.20
	D	38.8 ± 2.2	1.03 ± 0.27	1.17 ± 0.49
	O	43.2 ± 1.9	1.19 ± 0.19	1.78 ± 0.37*
With CCl_4	AC	50.0 ± 4.6*	2.90 ± 1.05*	1.92 ± 0.40*
	DC	36.2 ± 3.6**	0.49 ± 0.20*,**	0.49 ± 0.14*,**
	OC	34.9 ± 2.5**	1.33 ± 0.10**	0.56 ± 0.08*,**

Animals receiving purified diets, A (control), D (5% defatted *Spirulina*), or O (oil extract equivalent to 5% *Spirulina*) were treated with a single dose of vehicle (groups A, D and O) or carbon tetrachloride (groups AC, DC, and OC). Values are expressed as mean ± S.D. of $n = 5$.
* Significantly different from corresponding value of group A; or ** significantly different from the corresponding value of group AC.

2.5. Lipoprotein analysis

Aliquots of 5 μl from each fresh serum sample were used to estimate the percentage of the serum lipoproteins. The electrophoretic separation of lipoproteins was performed on a Ciba-Corning T-790 clinical electrophoresis unit, equipped with a densitometer. Percentage determination of each lipoprotein was performed at 520 nm.

2.6. Lipid peroxidation

This was measured by determining microsomal liver TBARS as reported by Recknagel and Ghoshal (1966).

2.7. Statistical analysis

Results were evaluated by analysis of variance (ANOVA) and Duncan's test using SPSS software. A P value less than 0.05 was considered significant.

3. Results and discussion

Livers from rats of groups D or O (experimental–purified diet, with either 5% defatted *Spirulina* extract or the oil extract equivalent to 5% *Spirulina*, and treated with vehicle) showed no differences in lipid concentrations relative to livers from rats of group A (Table 2), except that livers from group O contained higher levels of total cholesterol than those of group A. Treatment with carbon tetrachloride increased the levels of total lipids, total triacylglycerols and total cholesterol only in rats from group AC. Whereas animals fed on either defatted *Spirulina* extract or on the oil extract of *Spirulina* (groups DC and OC, respectively) showed either no changes (total lipids) or even a reduction (total triacylglycerols and total cholesterol) in the concentration of liver lipids. The former results are in agreement with those from previous reports indicating that total cholesterol and triacylglycerols are increased in carbon tetrachloride-induced fatty liver (Seakins and Robinson, 1963; Torres-Durán et al., 1998).

Rats fed on a purified diet with or without one of the fractions of *Spirulina* and receiving only the vehicle (i.e. rats from group A, D or O, Table 3), showed only minor differences in serum total cholesterol levels. On the other hand, total triacylglycerols were only increased in serum from rats fed on a diet supplemented with defatted *Spirulina* (group D). These data are in line with those observed previously with whole *Spirulina* (Torres-Durán et al., 1998).

On the other hand, the increased serum levels of triacylglycerols, induced by carbon tetrachloride administration, were not modified by any fraction of *Spirulina* (groups AC, DC or OC). It is possible that changes in blood lipids would require a longer time period or a larger dose of *Spirulina* to become manifest.

Table 3
Effects of *Spirulina maxima* on changes induced by carbon tetrachloride on serum lipids

Treatment	Group	Total triacylglycerols (mg/dl)	Total cholesterol (mg/dl)
Without CCl_4	A	51.1 ± 9.8	68.0 ± 15.4
	D	168.8 ± 58.6*	88.0 ± 9.8
	O	60.7 ± 24.0	56.5 ± 9.4
With CCl_4	AC	110.1 ± 40.1*	78.4 ± 13.8
	DC	77.8 ± 6.0*	73.6 ± 5.5
	OC	103.8 ± 23.9*	79.8 ± 13.6

Animals receiving purified diets, A (control), D (5% defatted *Spirulina* or O (oil extract equivalent to 5% *Spirulina*), were treated with a single dose of vehicle (groups A, D and O) or carbon tetrachloride (groups AC, DC and OC). Values are expressed as mean ± S.D. of $n = 5$.
* Significantly different from corresponding value of group A.

It has been suggested that carbon tetrachloride administration induces an increased synthesis of fatty acids, as well as a decreased release of hepatic lipoproteins (Maling et al., 1962; Seakins and Robinson, 1963; Pencil et al., 1984; Glende and Recknagel, 1991). In preliminary experiments, the qualitative determination of lipoproteins showed that serum HDL values of rats fed on a diet containing whole *Spirulina* (group S) were higher than those observed in group A (Table 4). Furthermore, both the increase of serum VLDL and the decrease of serum LDL percentages induced by carbon tetrachloride administration (group AC) were prevented by the inclusion of whole *Spirulina maxima* in the purified diet (group SC).

So far the results of the present study show that dietary *Spirulina maxima* is able to prevent the changes induced by carbon tetrachloride on liver lipids. Since the hepatotoxic effect of carbon tetrachloride is related to free radical generation (Glende and Recknagel, 1991; González Padrón et al., 1993), it is reasonable to assume that the potential hepatoprotective role of *Spirulina maxima* may be associated with its antioxidant constituents, such as selenium, chlorophyll, carotene, γ-linolenic acid, and vitamins E and C (Kay, 1991). In order to test this hypothesis, TBARS were determined in liver microsomal lipids from rats fed on the purified diet supplemented or not with whole *Spirulina maxima* (Table 5). The results of these experiments showed no differences in TBARS values between groups treated with vehicle (groups A and S). However, while carbon tetrachloride administration induced a significant

Table 4
Effects of *Spirulina maxima* on changes induced by carbon tetrachloride in the percentage of serum lipoproteins in the rat

Treatment	Group	HDL	LDL	VLDL
Without CCl_4	A	34.4 ± 4.1*	12.4 ± 6.2	48.1 ± 4.1
	S	43.3 ± 5.6*	13.5 ± 0.8	43.9 ± 5.4
With CCl_4	AC	33.8 ± 6.5	7.4 ± 1.1*	57.2 ± 7.0*
	SC	40.8 ± 7.2	13.3 ± 0.9*	45.8 ± 7.1*

Animals receiving purified diets, A (control), S (5% whole *Spirulina*), were treated with a single dose of vehicle (groups A and S) or carbon tetrachloride (groups AC, and SC). Values are expressed as mean ± S.D. of $n = 5$.
* Significantly different from each other for the same kind of lipoprotein after the same treatment.

Table 5
Effect of *Spirulina maxima* on microsomal TBARS values

Treatment	Group	TBARS (μg/g liver, wet weight)
Without CCl_4	A	12.27 ± 3.31
	S	12.05 ± 1.99
With CCl_4	AC	14.25 ± 0.10
	SC	10.93 ± 1.41*

TBARS, thiobarbituric acid reactive substances. Animals receiving purified diets, A (control), S (5% whole *Spirulina*), were treated with a single dose of vehicle (groups A and S) or carbon tetrachloride (groups AC, and SC). Values are expressed as mean ± S.D. of $n = 5$.
* Significantly different from corresponding value of group AC.

increase on TBARS values in group AC, this increase was not observed in rats receiving the purified diet supplemented with whole *Spirulina* (group SC). In a previous report (Mitchell et al., 1988) it was observed that the basal levels of TBARS were lower in rats fed on a purified diet than in rats fed on a *Spirulina*-supplemented diet. Nevertheless, in that study *Spirulina* was employed as a protein source (18%) instead of casein. Since in the experiments reported herein the experimental design is different, further studies are required to confirm the above hypothesis.

4. Conclusions

The addition of defatted *Spirulina maxima* extract (5%) to the purified diet did not modify liver lipid concentrations in animals that received only the vehicle. In contrast, an increase in the liver cholesterol concentration was observed in the group fed on a diet supplemented with the oil extract of *Spirulina maxima*. On the other hand, the increase of liver triacylglycerol and cholesterol concentrations, induced by carbon tetrachloride treatment, was not only prevented but triacylglycerols and cholesterol decreased by the inclusion of any of the fractions of *Spirulina maxima* in the purified diet.

Except in group D, treated with vehicle (i.e. A vs D), no differences were observed in serum total cholesterol or triacylglycerol concentrations between groups receiving the same treatment (either with or without CCl_4).

Since the hepatoprotective effect of *Spirulina maxima* was observed with either the defatted or the oil extract, it may be assumed that both fractions contain active substances for this effect.

The fact that serum lipoprotein changes induced by carbon tetrachloride were prevented by the inclusion of whole *Spirulina maxima* in the diet suggests that either their hepatic synthesis is not affected or that its peripheral metabolism is preserved. This hypothesis would explain the lower accumulation of fatty acids in rat livers in rats receiving *Spirulina* in their diet. Furthermore, the lower liver values of lipoperoxide products found in the whole *Spirulina* group, as compared to those without *Spirulina*, suggest an antioxidant role of the *Spirulina* constituents, and could explain the attenuation of the hepatotoxic effect of carbon tetrachloride.

Taken together, the results described above support the potential hepatoprotective role of *Spirulina maxima*.

Acknowledgements

This work was supported in part by grant IN-201296 from DGAPA-UNAM, Mexico.

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