JOURNAL OF MEDICINAL FOOD
J Med Food 19 (5) 2016, 450–456

DOI: 10.1089/jmf.2015.0077

Benefits of Preventive Administration of *Chlorella* sp. on Visceral Pain and Cystitis Induced by a Single Administration of Cyclophosphamide in Female Wistar Rat

Sophie Hidalgo-Lucas,[1] Pascale Rozan,[1] Laetitia Guérin-Deremaux,[2] Blandine Baert,[2] Nicolas Violle,[1] Marie-Hélène Saniez-Degrave,[2] Jean-François Bisson[1]

[1]*ETAP-Lab, Parc Technologique de Nancy-Brabois, Vandœuvre-lès-Nancy, France.*
[2]*ROQUETTE, rue de la Haute Loge, Lestrem, France.*

ABSTRACT *Chlorella* sp. is a green microalgae containing nutrients, vitamins, minerals, and chlorophyll. In some communities, *Chlorella* sp. is a traditional medicinal plant used for the management of inflammation-related diseases. In a rat model, ROQUETTE *Chlorella* sp. (RCs) benefits were investigated on visceral pain and associated inflammatory parameters related to cystitis both induced by cyclophosphamide (CYP). RCs was orally administered every day from day 1–16 (250 and 500 mg/kg body weight). Six hours after an intraperitoneal injection of 200 mg/kg body weight of CYP, body temperature, general behavior, food intake, and body weight were recorded. Twenty-four hours after CYP injection, rats were tested in two behavioral tests, an open field and the aversive light stimulus avoidance conditioning test, to evaluate the influence of pain on general activity and learning ability of rats. After euthanasia, bladders were weighed, their thickness was scored, and the urinary hemoglobin was measured. RCs orally administered at the two dosages significantly reduced visceral pain and associated inflammatory parameters related to cystitis both induced by CYP injection, and improved rat behavior.
To conclude, RCs demonstrated beneficial effects against visceral pain and cystitis.

KEY WORDS: • ***behavior*** • **Chlorella** ***sp.*** • ***cystitis induced by a chemotherapeutic agent*** • ***microalgae*** • ***oral administration*** • ***pain*** • ***rodent***

INTRODUCTION

DIFFERENT AUTHORS HAVE shown that nutrition is one of the most important parameters that is involved in modulating inflammation.[1,2] *Chlorella* sp. is a green unicellular microalgae with a well-balanced content of numerous macro- and micronutrients including carbohydrates, proteins, nucleic acids, essential amino acids, fatty acids, dietary fibers, growth factors, vitamins, minerals, and chlorophyll.[3] In some communities, *Chlorella* sp. has even been cited as a traditional medicinal plant used for the management of inflammation-related diseases.[4] The beneficial effects of *Chlorella* sp. on various inflammation pathologies is of great interest and was recently reported in humans and rodents: on skin damages, chronic inflammation-related vascular diseases, and hyperlipidemia.[5–9] In atlantic salmon, authors have shown that *Chlorella vulgaris* counteracts an intestinal inflammation, the enteropathy in the distal intestine.[10] In a rat model of visceral hypersensitivity induced by colonic instillation of butyrate, ROQUETTE *Chlorella* sp. (RCs) has demonstrated an increase of the pain threshold by more than 30%. To evaluate the effects of RCs on pain, a well-known model inducing pain was used, the cyclophosphamide (CYP) model.[11] CYP is a usual antitumoral agent used to cure cancers as breast cancer and is known to induce visceral pain and cystitis. It is known to produce bladder inflammation in humans and rodents through the action of the toxic metabolite acrolein, which undergoes urinary excretion.[12,13] The urinary bladder is the organ most affected by CYP because of its reservoir function, which leads to relatively long exposure of the mucosa to acrolein. This exposure results in a painful hemorrhagic cystitis in humans and a pain behavior, frequency, and low volume voiding in rat.[11,14,15] It also involves edema, hemorrhage, neutrophil infiltration, urothelial damage, and increased bladder wet weight and cytokine levels in tissue.[16,17]

The aim of this study was to first investigate in rats the preventive effects of oral administration of RCs on visceral pain induced by intraperitoneal injection of cylophosphamide and second its effects on cystitis, the secondary effect observed in this model.

Manuscript received 29 June 2015. Revision accepted 28 February 2016.

Address correspondence to: Pascale Rozan, PhD, ETAP-Lab, 13 rue du Bois de la Champelle, Technopôle de Nancy-Brabois, 54500 Vandœuvre-lès-Nancy, France, E-mail: prozan@etap-lab.com or *Jean-François Bisson, PhD, ETAP-Lab, 13 rue du Bois de la Champelle, Technopôle de Nancy-Brabois, 54500 Vandœuvre-lès-Nancy, France, E-mail:* jfbisson@etap-lab.com

MATERIAL AND METHODS

Plant material

RCs is a *Chlorella sorokiniana* powder produced by ROQUETTE (Lestrem, France) in a closed-controlled environment. RCs contains 55% proteins, 9% carbohydrates, 9% lipids, 10% fibers, 3% pigments, 6% ash, and 8% moisture.

Chemicals

CYP ($C_7H_{15}Cl_2N_2O_2P.H_2O$, CAS Number 6055-19-2, >97% purity) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Physiological sterile saline (NaCl, 0.9%) was purchased from Cordier's pharmacy in Nancy.

Animals

Thirty-two female Wistar rats weighing 225–250 g were purchased from Charles River France (Charles River Breeding Centre, L'Arbresle, France). After 1 week of acclimatization, the rats were housed at two per cage in an air-conditioned room under controlled conditions of temperature (22°C ± 2°C), relative humidity (50% ± 20%), with an inverted 12-h light:dark cycle (light off at 8:00 a.m.) and they had access to standard laboratory chow (2016 diet; Harlan Teklad US, Madison, WI, USA) and tap water *ad libitum*. On day 16, rats were euthanized by intraperitoneal injection of an overdose of Pentobarbital (Ceva Santé Animale, Libourne, France). The study received the approval of the Director's Office of Veterinary Services and the Local Animal Care Committee in Nancy, and was authorized by the French government (Governmental authorization no. A 54-547-1). The experimental procedures were conducted in accordance with the European Communities Council Directive of 22 September, 2010 on the approximation of laws, regulations, and administrative provisions of the Member States regarding the protection of animals used for scientific purposes, the NIH Guide for the Care and Use of Laboratory Animals, and the United Kingdom legislation of *in vivo* aspects in inflammation research.[18–20] All efforts were made to minimize suffering of animals.

Treatments

Oral administrations of RCs or vehicle were performed daily from day 1–16 by intragastric gavage and CYP or vehicle was injected on day 15 by intraperitoneal (i.p.) route. The negative control group (V/V) received spring water (Source Cristaline Aurèle, France) by oral route and an i.p. injection of 0.9% NaCl. The positive control group (V/CYP) received spring water by oral route and CYP by i.p. injection. Two groups were orally treated with RCs at doses of 250 and 500 mg/kg body weight (RCs250/CYP and RCs500/CYP respectively), and received CYP injection. RCs was freshly dissolved each day in spring water before oral administration by intragastric gavage with an administration volume of 10 mL/kg.

Pain induction

On day 15, CYP was prepared with 0.9% NaCl, and was injected i.p. at the dose of 200 mg/kg in V/CYP, RCs250/CYP, and RCs500/CYP groups. Negative control group V/V received an i.p. injection of 0.9% NaCl alone.

Monitoring of rats

Rats were observed daily during the study. Food intake, water consumption, and bodyweight were monitored three times a week before induction of pain with CYP, and also the day of induction and 24 h after. Feces consistency was scored on day 15 before induction of pain and on day 16 (0 = hard feces, 1 = hard and soft feces, 2 = soft feces, and 3 = diarrhea).

Visceral pain evaluation

Global behavior score. Global behavior score (GBS) of rats was calculated on day 15, before pain induction and 24 h after induction. GBS corresponds to the sum of three scores: eyes (0 = opened; 1 = partially opened; 2 = almost closed, and 3 = completely closed); posture (0 = normal, 1 = partially arched, and 2 = completely arched); and fur (0 = normal; 1 = slight piloerection, and 2 = piloerection). Rats were observed individually and the maximal GBS possibly attributed was seven.

Behavioral tests. The two behavioral tests were performed 24 h after CYP injection, recorded, and results were analyzed by videotracking Anymaze Software (Version 4.95, Stoelting, Ireland).

Open field test. To estimate the general motor and exploratory activity after CYP injection, rats were tested on day 16 in a cylindrical open field (OF) (60 cm in diameter with 30 cm high walls). Each rat was placed in the center of the arena and tested for 5 min. Distance covered, rearings (standing on hind legs against the walls), and total duration in rearings were recorded. The floor was washed with a water-alcohol solution and dried before each individual subject to eliminate odor clues.

Aversive light stimulus avoidance conditioning test. The objective of this test was to evaluate the general locomotor activity and the efficiency of learning process of rats 24 h after CYP injection. It was performed immediately after OF by placing the animals in a highly illuminated (1200 lux) cage (50 × 40 × 37 cm) equipped with two levers for a 20-min period. By pressing the active lever the rats switched off the light for 30 sec, whereas the inactive lever had no effect on the environment. The total number of pressings on the two levers was recorded and the learning performance (discrimination between the two levers) was determined by comparing the numbers of active lever and inactive lever pressings recorded during the light period.[21]

Inflammatory parameters related to cystitis induction

Body temperature. The body temperature of each rat was recorded with a rectal thermometer on day 1, before the first oral treatment with RCs, on day 15 before CYP or NaCl 0.9%

injection, on day 15 6 h (day 15 + 6 h) after CYP or NaCl 0.9% injection and on day 16. Except on day 15 + 6 h, the body temperature was recorded each morning at 8:00 a.m.

Bladder structure. After euthanasia, bladders were collected, weighed, and their thickness was scored (0 = normal; 1 = slightly thick; 2 = moderately thick, and 3 = very thick).

Hemoglobin in urine. Urine was collected after euthanasia by gentle pressure of the abdomen above the bladder in a sterile tube and hemoglobin contained in urine was measured by using a modified spectrophotometric Drabkin method at 540 nm adapted in our laboratory to this model.[22] The maximal detection of hemoglobin was 25 μL/mL of urine.

Statistical analysis

All data are presented in figures and tables as median, first quartile (Q_1), and third quartile (Q_3). Nonparametric tests were applied: one-way ANOVA with Kruskal–Wallis test followed, when significant, by the Mann–Whitney *U*-test for inter-group comparisons. Wilcoxon test was used to compare the results obtained for discrimination in Aversive light stimulus avoidance conditioning test (ALSAT) and the body temperature evolution on day 15 + 6 h and day 16. Statistical difference was defined at $P < .05$ level for all comparisons. All statistical analyses were carried out using the StatView®5 statistical package (SAS', Institute, Inc., Cary, NC, USA).

RESULTS

Effect of RCs on physiological parameters

No death, no signs of suffering, and no toxicity due to treatments were observed in the four experimental groups of rats, throughout the whole experiment. Food intake, water consumption, and body weight were not statistically different between the four experimental groups from day 1–15 (data not shown). Between day 15 and 16 (after CYP injection), the mean body weight change of rats of V/V group was significantly higher than the ones of rats of V/CYP, RCs250/CYP, and RCs500/CYP groups ($P = .01$, $P = .003$, and $P = .002$, respectively) (Table.1). The rats of V/V group gained on average 0.9g, whereas the rats of V/CYP lost 7.9 g and the ones of RCs250/CYP and RCs500/CYP lost 8.0 and 8.4 g, respectively. The food intake of rats of V/V group was significantly higher than that of rats of V/CYP, RCs250/CYP, and RCs500/CYP groups ($P = .02$ for all comparisons) (Table.1). The score of consistency of the feces of the four groups was not statistically different (score = 0 for the four groups, data not shown).

Effect of RCs on visceral pain

On day 15, before injection of CYP, the GBS was not statistically different between all groups (Scores = 0, data not shown). On day 15 + 6 h and 16, the GBS of rats of V/V group was significantly lower than the ones of rats of V/CYP, RCs250/CYP, and RCs500/CYP groups and the GBS of rats of V/CYP group was significantly higher than that of rats of RCs-treated groups at both tested doses (Table.2). The rats of RCs250/CYP and RCs500/CYP groups had a GBS significantly lower on day 16 than on day 15 + 6 h ($P = .03$ and $P = .02$, respectively). The GBS of rats of V/V and V/CYP groups were not statistically different on day 15 + 6 h as compared to the GBS obtained on day 16 (Table. 2).

On day 16 in the OF, the distance covered by rats, the number of rearings, and the total duration in rearings during the 5 min of test for V/V group were significantly higher than the results obtained for rats of V/CYP, RCs250/CYP, and RCs500/CYP groups and the results obtained for rats of V/CYP group were significantly lower than that obtained for RCs-treated groups at both tested doses (Table.2).

In the ALSAT, on day 16, the total lever pressings of rats of V/V, RCs250/CYP, and RCs500/CYP groups were significantly higher than the one of rats of V/CYP group ($P = .006$, $P = .016$, and $P = .004$, respectively) (Table.2).

On day 16, the rats of V/V and RCs250/CYP groups showed a significant discrimination between the two levers of the ALSAT ($P = .026$ and $P = .041$, respectively) and rats of the RCs500/CYP group showed a trend to discriminate between the two levers ($P = .075$). There was no statistical difference for the discrimination between the two levers for rats of the V/CYP group (Fig. 1).

TABLE 1. MBWC (DAY 1–15 AND DAY 15–16) AND FOOD INTAKE (DAY 15–16) [MEDIAN (Q_1; Q_3)]

Group	*MBWC day 1–15 (g)*	*MBWC day 15–16 (g)*	*Food intake day 15–16 (g/kg/day)*
V/V	+6.0 (0.5; 11.5)	+0.5 (–1.5; 3.0)	66.2 (59.3; 73.3)
V/CYP	+9.0 (7.0; 11.0)	–8.5† (–13.0; –3.0)	28.8† (19.3; 34.5)
RCs250/CYP	+6.0 (1.5; 12.5)	–8.0‡ (–8.5; –6.5)	32.1† (25.3; 37.3)
RCs500/CYP	+6.0 (0.5; 13.5)	–8.0‡ (–13.5; –3.5)	38.8† (32.1; 43.3)
Kruskal Wallis	$H_{(ddl = 3)} = 1.04$	$H_{(ddl = 3)} = 13.08$	$H_{(ddl = 3)} = 9.88$
Significance	*NS*	$P = .004$	$P = .020$

†$P < .05$ compared with V/V group (Mann–Whitney *U*-test).

‡$P < .01$ compared with V/V group (Mann–Whitney *U*-test).

MBWC, mean body weight change; NS, not significant; Q_1, first quartile; Q_3, third quartile; V, vehicle; CYP, cyclophosphamide; RCs, ROQUETTE *Chlorella species*; 250–500, doses of RCs in mg/kg BW/day.

TABLE 2. COMPARISONS OF GBS ON DAY 15+6 H AND DAY 16 AND RESULTS OF OPEN FIELD AND ALSAT ON DAY 16 [MEDIAN (Q_1; Q_3)]

	Global behavioral score		*Open field*			*ALSAT*
Group	*Day 15+6 h*	*Day 16*	*Distance covered* (m)	*Rearings* (n)	*Total duration of rearings* (s)	*Total lever pressings* (n)
V/V	0.0 (0.0; 0.0)	0.0 (0.0; 0.0)	18.0 (14.4; 19.4)	42.0 (38.0; 48.5)	79.7 (64.6; 94.1)	22.5 (15.0; 34.5)
V/CYP	6.5* (5.5; 7.0)	6.0* (5.0; 6.0)	4.3† (2.3; 9.8)	7.5‡ (3.5; 21.0)	5.6‡ (2.0; 33.5)	7.5‡ (3.5; 10.5)
RCs250/CYP	3.0*$^{\$}$ (3.0; 5.5)	1.5*$^{\epsilon\circ}$ (1.0; 2.0)	11.1†§ (6.3; 16.0)	29.5†§ (20.0; 34.0)	38.5‡§ (29.5; 47.8)	23.5§ (12.0; 43.0)
RCs500/CYP	4.0*$^{\$}$ (3.0; 6.0)	2.5*$^{\epsilon\circ}$ (1.5; 4.0)	10.7†§ (6.9; 13.3)	27.0^{T,§} (18.0; 41.5)	52.6^{T,§} (30.6; 65.1)	24.5$^{\$}$ (18.5; 58.0)
Kruskal Wallis	$H_{(ddl\ =\ 3)}=21.00$	$H_{(ddl\ =\ 3)}=25.02$	$H_{(ddl\ =\ 3)}=15.03$	$H_{(ddl\ =\ 3)}=15.60$	$H_{(ddl\ =\ 3)}=17.66$	$H_{(ddl\ =\ 3)}=9.76$
Significance	$P < .001$	$P < .001$	$P=.002$	$P=.002$	$P < .001$	$P=.021$

Mann–Whitney *U*-test:
T $P<.10$ Trend to significant with V/V group.
$^{\dagger}P<.05$ compared with V/V group.
$^{\ddagger}P<.01$ compared with V/V group.
$^{*}P<.001$ compared with V/V group.
$^{\S}P<.05$ compared with V/CYP group.
$^{\$}P<.01$ compared with V/CYP group.
$^{\epsilon}P<.001$ compared with V/CYP group.
Wilcoxon test: $^{\circ}P<.05$ compared with results obtained on Day 15+6h.
ALSAT, aversive light stimulus avoidance conditioning test; GBS, global behavior score; 250–500: doses of RCs in mg/kg/day.

Effect of RCs on inflammatory parameters related to cystitis induction

No statistical differences were observed between the body temperature of rats of the four treatment groups on day 1 and 15 (data not shown). On day 15+6 h and day 16, the body temperature of rats of V/V group was significantly higher than the one of rats of the three groups injected with CYP and body temperature of rats of V/CYP group was significantly lower than that of rats treated with RCs at both tested doses The body temperatures of rats of RCs250/CYP and RCs500/CYP groups were lower on day 15+6 than on day 16 ($P=.01$ in both cases; Fig. 2).

The bladder weight ratio and the thickness score of bladder of rats of V/V group were significantly lower than that of rats of the three groups injected with CYP. The bladder weight and the thickness score of bladder of rats of V/CYP group were significantly higher than that of rats of RCs-treated groups at both tested doses (Table.3). Figure 3 illustrates the aspect of representative bladders of the four groups of treatment. The concentration of hemoglobin in

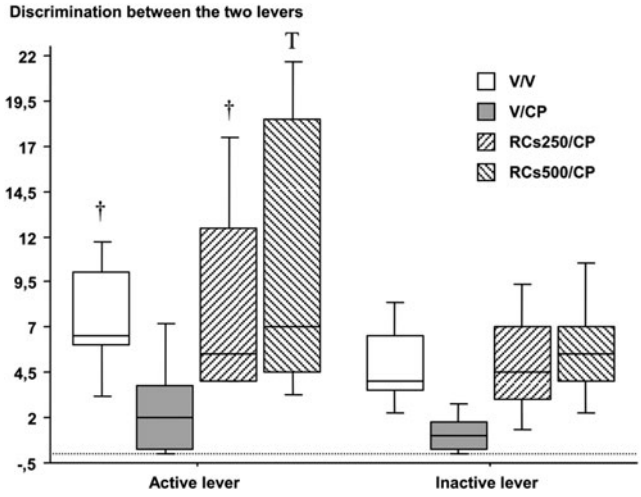

FIG. 1. Discrimination between the two levers in the ALSAT on day 16 [Median (IQ; SQ)] Wilcoxon-test: $^{T}P<.10$ compared with inactive lever of the same group of treatment. $^{\dagger}P<.05$ compared with inactive lever of the same group of treatment. ALSAT, aversive light stimulus avoidance conditioning test; IQ, inferior quartile; SQ, superior quartile; V, vehicle; CYP, cyclophosphamide; RCs, ROQUETTE *Chlorella* sp.; 250–500, doses of RCs in mg/kg BW/day.

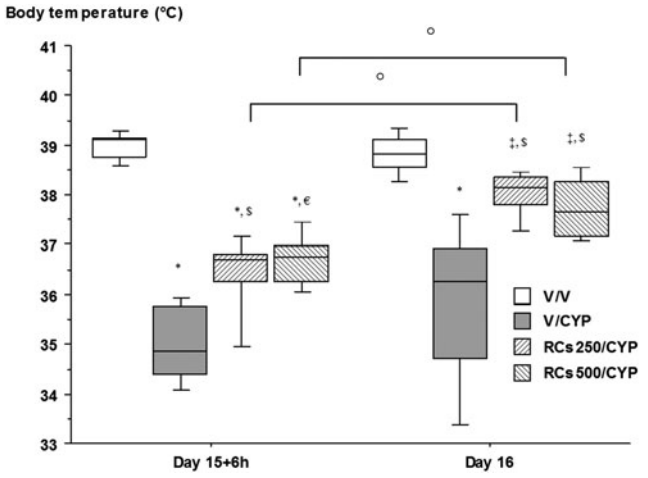

FIG. 2. Body temperature of rats on day 15+6 h and on day 16 [Median (IQ; SQ)] 250–500, doses of RCs in mg/kg BW/day. Mann–Whitney *U*-test: $^{\ddagger}P<.01$ compared with V/V group. $^{*}P<.001$ compared with V/V group. $^{\$}P<.01$ compared with V/CYP group. $^{\epsilon}P<.001$ compared with V/CYP group. Wilcoxon test: $^{\circ}P<.05$.

Table 3. Comparisons of Mean Bladder Weight, Thickness Score, and Hemoglobin Concentration In urine on Day 16 [Median (Q_1; Q_3)]

Group	*Bladder weight (mg/100 g)*	*Bladder thickness score*	*Hemoglobin concentration in urine (μL/mL)*
V/V	21.8 (19.9; 24.9)	0.0 (0.0; 0.0)	1.8 (0.7; 4.9)
V/CYP	66.4* (57.9; 70.8)	2.5* (2.0; 3.0)	13.7‡ (6.3; 22.2)
RCs250/CYP	40.4*§ (34.8; 47.6)	1.0*§ (1.0; 2.0)	2.5$ (0.8; 3.8)
RCs500/CYP	44.2*§ (40.0; 55.8)	1.0‡$ (1.0; 2.0)	6.4 (1.3; 16.2)
Kruskal Wallis	$H_{(ddl\ =\ 3)} = 21.65$	$H_{(ddl\ =\ 3)} = 21.40$	$H_{(ddl\ =\ 3)} = 10.14$
Significance	$P < .001$	$P < .001$	$P = .017$

Bladder thickness score: 0 = normal; 1 = slightly thick; 2 = moderately thick and 3 = very thick.
Mann–Whitney *U*-test:
‡$P < .01$ compared with V/V treated group.
*$P < .001$ compared with V/V group.
§$P < .05$ compared with V/CYP group.
$$P < .01$ compared with V/CYP group.

urine of rats of V/V and RCs250/CYP groups were significantly lower than that of rats of V/CYP group (Table. 3).

DISCUSSION

There is a growing interest in studying and understanding how natural products can contribute to improve health and even treat diseases. Among those products, *Chlorella* sp., a green microalgae, is a popular food supplement in Asia considered as a traditional medicinal plant.[4] However, scientific studies supporting its effectiveness for the prevention or the treatment of human disorders remain scarce.

In this study, preventive administration of RCs did not prevent the immediate effects of CYP as observed with body weight loss and a significant decrease of food intake, principally due to prostration and visceral pain after CYP injection.

After CYP injection, RCs groups had a lower GBS than the V/CYP group from day 15 + 6 h and their behavior improved rapidly on day 16. Rats treated during 2 weeks with RCs recovered more rapidly after induction of pain with CYP as shown by their activity observed.

In the ALSAT, RCs completely prevented the negative effects induced by CYP injection and rats of RCs250/CYP group discriminated the two levers of the ALSAT expressing an improvement of the learning process under induction of pain and inflammation. In both behavioral tests, rats treated with RCs at both doses had a better locomotor activity than rats of the positive group. Few studies demonstrated the correlation between visceral pain and behavior. Authors have shown the correlation between cognitive impairments and visceral pain using the ALSAT.[21] This observation was confirmed by other authors who demonstrated that a 5% food dextrin diet administered for 2 weeks prevented alcohol and trinitrobenzene sulphonic acid-induced inflammation and by the way the cognitive performances of rats, that is, learning and memory, were not perturbed.[23]

Several authors reported that among potential mediators of inflammation, neurotrophins (*e.g.*, nerve growth factor) have been implicated in the peripheral sensitization of nociceptors.[24,25] Proinflammatory cytokines also cause sensitization of polymodal C-fibers and facilitate A-beta input to the spinal cord.[25–27] Several studies have demonstrated increased expression of cytokines (IL-6, IL-1α, and IL-4, among others) and chemokines and the beneficial effects of receptor blockade in the urinary bladder after CYP-induced bladder inflammation.[17,28] Recently the expression and function of another cytokine, TGF-β, was examined in the inflamed urinary bladder. TGF-β has an extensive role in

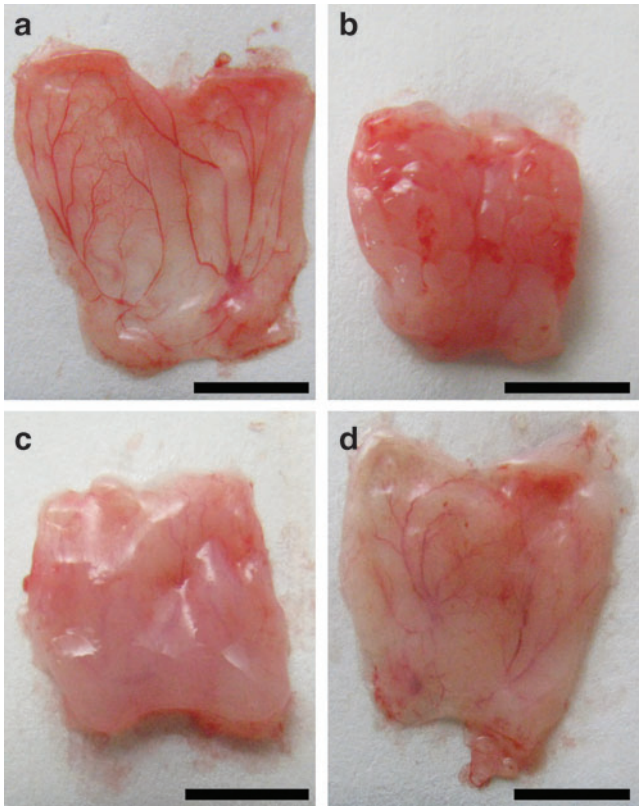

FIG. 3. Representative photographs of bladder of rats of the non-induced group treated with vehicle **(a)**, CYP-induced groups treated with vehicle **(b)**, RCs at 250 mg/kg/day **(c)**, and RCs at 500 mg/kg/day **(d)**, after sacrifice on day 16. Scale bar: 0.5 cm. Color images available online at www.liebertpub.com/jmf

the immune system and has been implicated in nociception and detected in the urine and urothelium of rats treated with CYP-induced cystitis.[29] As a result of its biphasic and modulatory role in the peripheral and central transmission of nociception, TGF-β appears to have a profound impact on the perception of pain and may initiate, in part, pathological pain syndromes.[30]

The expression of cytokines, alone or in combination with other cytokines, growth factors, or other mediators, may form a bidirectional communication network between the nervous system and the immune system.[31]

In vitro and animal studies have shown that *Chlorella* or *Chlorella* extract are involved in the modulation of immune response against tumors along with bacterial and viral infection but there are no studies to demonstrate its effect on visceral pain.[32–35] Few *in vitro* and *in vivo* studies on *Chlorella* extracts have shown anti-inflammatory, immunomodulatory, analgesic, and free radical scavenging activities.[36,37] Authors have reported that *Chlorella* short-term supplementation in uninfected normal people enhanced Natural Killer cell activity and early inflammatory response (serum concentration of interferon-γ and interleukin-1β increased).[38] It is difficult to determine which compounds could be responsible for such immunomodulatory or analgesic effects as the microalgae Chlorella is a mix of many components as various as proteins, lipids, carbohydrates, pigments, and also not so well-characterized compounds yet such as growth factors, the whole probably having a synergistic effect.

In our experimental conditions, RCs limited visceral pain appearance and associated markers, reduced visceral pain significantly 24 h after injection of CYP and allowed normal locomotor and learning activities. The internal temperature dropped after CYP injection because blood converges to the peritoneum close to the injection site of CYP to fight the infection due to CYP. The massive blood influx to the peritoneal cavity decreases the blood flow in some organs, in particular to the colon, leading to the drop of rectal body temperature.

Cystitis induced by CYP is usually associated with hemorrhagia, and this study showed that RCs prevented the increase in urinary hemoglobin concentrations observed in the positive group.[16] These beneficial effects could be attributed to the stimulation of the immune system with production of cytokines, limiting the inflammation and permitting animals to recover rapidly with less suffering.

RCs could be developed in a new field of application. This microalgae administered to patients could permit to accelerate chemotherapy frequency by avoiding the side effects, pain and cystitis. The locomotor activities, the explorative capacities, and the cognitive performances could also be maintained. Further studies are needed to evaluate the effect of RCs on anti-inflammatory response at cytokine levels and after repeated injection of CYP to mimic chronic chemotherapeutic treatments.

To conclude, RCs tested at doses of 250 and 500 mg/kg body weight in rat significantly reduced visceral pain and associated inflammatory parameters related to cystitis both induced by CYP injection.

ACKNOWLEDGEMENT

We thank Miss Valérie Dernoncourt (ETAP) for her technical support for hemoglobin measurement in urine.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Sarno Ryan A, Goldsmith LA: Nutrition and the skin. *Clin Dermatol* 1996;14:389–406.
2. Rushton D: Nutritional factors and hair loss. *Clin Exp Dermatol* 2002;27:396–404.
3. Panahi Y, Tavana S, Sahebkar A, Masoudi H, Madanchi N: Impact of adjunctive therapy with *Chlorella vulgaris* extract on antioxidant status, pulmonary function, and clinical symptoms of patients with obstructive pulmonary diseases. *Sci Pharm* 2012;80:719–730.
4. Namsa ND, Tag H, Mandal M, Kalita P, Das AK: An ethnobotanical study of traditional anti-inflammatory plants used by the Lohit community of Arunachal Pradesh. *J Ethnopharmacol* 2009;125: 234–245.
5. Hidalgo-Lucas S, Bisson JF, Duffaud A, Nejdi A, Guérin-Deremaux L, Baert B, Saniez-Degrave MH, Rozan P: Benefits of oral and topical administration of ROQUETTE *Chlorella sp.* on skin inflammation and wound healing in mice. *Antiinflamm Antiallergy Agents Med Chem* 2014;13:93–102.
6. Maeda M, Kasuya Y, Yuasa K, Hiranaka T, Chubachi H, Saito M, Takegoshi H: *Chlorella's* anti-inflammatory activity in beagles with skin disorders. The Annual Meeting of the Japan Society for Bioscience. Congress Proceedings. *Biotechnol Agrochem* 2008;10: 142–146.
7. Salman B, Kerem M, Bedirli A, Katircioglu H, Ofluoglu E, Akin O, Onbasilar I, Ozsoy S, Haziroglu R: Effects of *Chorella sp. microalgae* extract on colonic anastomosis in rats with protein–energy malnutrition. *Colorectal Dis* 2008;10:469–478.
8. Shih MF, Chen LC, Cherng JY: *Chlorella* 11-peptide inhibits the production of macrophage-induced adhesion molecules and reduces endothelin-1 expression and endothelial permeability. *Mar Drugs* 2013;11:3861–3874.
9. Renju GL, Kurup GM, Saritha Kumari CH: Effect of lycopene from *Chlorella marina* on high cholesterol-induced oxidative damage and inflammation in rats. *Inflammopharmacology* 2014; 22:45–54.
10. Grammes F, Reveco FE, Romarheim OH, Landsverk T, Mydland LT, Overland M: *Candida utilis* and *Chlorella vulgaris* counteract intestinal inflammation in atlantic salmon (Salmo salar L.). *PLoS One* (Online). Published December 27, 2013, 8:e83213, PMID 24386162.
11. Lantéri-Minet M, Bon K, de Pommery J, Michiels JF, Menétrey D: Cyclophosphamide cystitis as a model of visceral pain in rats: Model elaboration and spinal structures involved as reaveled by the expression of c-Fos and Krox-24 proteins. *Exp Brain Res* 1995;105:220–232.
12. Brock N, Pohl J: The development of mesna for regional detoxification. *Cancer Treat Rev* 1983;10:33–43.
13. Cox PJ: Cyclophosphamide cystitis-identification of acrolein as the causative agent. *Biochem Pharmacol* 1979;28:2045–2049.
14. Foad BS, Hess EV: Urinary bladder complications with cyclophosphamide therapy. *Arch Intern Med* 1976;136:616–619.

15. Boucher M, Meen M, Codron JP, Coudore F, Kemeny JL, Eschalier A: Cyclophosphamide-induces cytitis in freely-moving conscious rats: Behavioral approach to a new model of visceral pain. *J Urol* 2000;164:203–208.
16. Dornelles FN, Andrade EL, Campos MM, Calixto JB: Role of CXCR2 and TRPV1 in functional, inflammatory and behavioural changes in the rat model of cyclophosphamide-induced haemorragic cystitis. *Br J Pharmacol* 2014;171:452–467.
17. Smaldone MC, Vodovotz Y, Tyagi V, Barclay D, Philips BJ, Yoshimura. N, Chancellor MB, Tyagi P: Multiplex analysis of urinary cytokine levels in rat model of cyclophosphamide induced cystitis. *Urology* 2009;73:421–426.
18. Brain S: UK Legislation of in vivo aspects in inflammation research. In: *In vivo Models of Inflammation*, Vol. 1, 2nd edn (Stevenson CS, Marshall LA, Morgan DW, eds.). Birkhaüser Verlag, Basel, 2006, pp. 177–186.
19. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal L 276, 20/10/2010 p. 0033–0079.
20. Committee for the update of the guide for the care and use of laboratory animals; National Research Council. *Guide for the care and use of laboratory animals*, 8th edn (The National Academies Press) Washington DC, 2011.
21. Messaoudi M, Desor D, Grasmück V, Joyeux M, Langlois A, Roman FJ: Behavioral evaluation of visceral pain in a rat model of chronic inflammation. *NeuroReport* 1999;10:1137–1141.
22. Cook JD: Measurement of iron status. A report of the International Nutritional Anaemia Consultive Group (INACG), New York, Washington DC, 1985, Ch.II.
23. Rozan P, Deremaux L, Hidalgo S, Wils D, Bisson JF, Messaoudi M, Saniez MH: Food dextrin protects against colonic inflammation and prevents cognitive impairments. *Curr Topics Nutr Res* 2009;7:141–148.
24. Dinarello CA: Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. *Chest* 1997; 112:321S–329S.
25. Dray A: Inflammatory mediators of pain. *Br J Anaesth* 1995; 75:125–131.
26. Baba H, Doubell TP, Woolf CJ: Peripheral inflammation facilitates Aβ fiber-mediated synaptic input to the substantia gelatinosa of the adult rat spinal cord. *J Neurosci.* 1999;19:859–867.
27. Woolf CJ, Doubell TP: The pathophysiology of chronic pain-increased sensitivity to low threshold A??-fibre inputs. *Curr Opin Neurobiol* 1994;4:525–534.
28. Malley SE, Vizzard MA: Changes in urinary bladder cytokine mRNA and protein after cyclophosphamide-induced cystitis. *Physiol Genomics* 2002;9:5–13.
29. Tyagi P, Tyagi V, Yoshimura N, Witteemer E, Barclay D, Loughran PA, Zamora R, Vodovotz Y: Gender-based reciprocal expression of transforming growth factor-ß1 and the inducible nitric oxide synthase in a rat model of cyclophosphamide induced cystitis. *J Inflamm* 2009;6:23.
30. Gonzalez EJ, Arms L, Vizzard MA: The role(s) of cytokines/chemokines in urinary bladder inflammation and dysfunction. *Biomed Res Int* 2014;2014:120525.
31. Watkins LR, Maier SF: Implications of immune-tobrain communication for sickness and pain. *Proc Natl Acad Sci U S A* 1999;96:7710–7713.
32. Konishi F, Tanaka K, Himeno K, Taniguchi K, Nomoto K: Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): Resistance to Meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. *Cancer Immunol Immunother* 1985;19:73–78.
33. Tanaka K, Tomita Y, Tsuruta M, Konishi F, Okuda M, Himeno K, Nomoto K: Oral administration of *Chlorella vulgaris* augments concomitant antitumor immunity. *Immunopharmacol Immunotoxicol* 1990;12:277–21.
34. Hasegawa T, Okuda M, Nomoto K, Yoshikai Y: Augmentation of the resistance against *Listeria* monocytogenes by oral administration of a hot water extract of *Chlorella vulgaris* in mice. *Immunopharmacol Immunotoxicol* 1994;16:191–202.
35. Hasegawa T, Okuda M, Makino M, Hiromatsu K, Nomoto K, Yoshikai Y: Hot water extracts of *Chlorella vulgaris* reduce opportunistic infection with *Listeria* monocytogenes in C57BL/6 mice infected with LP-BM5 murine leukemia viruses. *Int J Immunopharmacol* 1995;17:505–512.
36. Guzman S, Gato A, Calleja JM: Antiinflammatory, analgesic and free radical scavenging activities of the marine microalgae *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. *Phytother Res* 2001;15:224–230.
37. Guzman S, Gato A, Freire-Garabal M, Calleja JM: Anti-inflammatory and immunomodulatory activities of polysaccharide from *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. *Phytother Res* 2003;17:665–670.
38. Kwak JH, Baek SH, Woo Y, Han JK, Kim BG, Lee JH: Beneficial immunostimulatory effect of short-term *Chlorella* supplementation: Enhancement of natural killer cell activity and early inflammatory response (randomized, double-blind, placebo-controlled trial). *Nutr J* 2012;11:53–60.